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Juan A. Rosado *Editor*

Calcium Entry Pathways in Non-excitable Cells

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Dedicated to Marcos and Maria

Preface

Cytosolic calcium homeostasis is a crucial event in cell biology as this ion acts as a second messenger for a large number of cellular functions. The observations of Ringer, published in the *Journal of Physiology* in the early 1880s, revealed the importance of calcium in cell physiology but specially focused the attention of the researchers on the essential role of extracellular calcium entry. This issue has attracted the attention of an increasing number of basic scientists interested in cellular biology as well as biomedical researchers and clinicians interested in the pathophysiological implications of calcium entry. Since this is a highly dynamic topic, recapitulation of the historic and current knowledge is usually helpful to understand the concepts and stimulate new approaches and directions that allow significant advances in various aspects of calcium influx.

After the assumption that Ca^{2+} influx was a major event in cell biology, voltage-dependent calcium currents were identified, and, later on, a mechanism for calcium influx operated by the occupation of membrane receptors was reported both in electrically excitable and non-excitable cells. Soon after the identification of the so-called receptor-operated calcium entry, the experimental observations indicated that this process was not a unique mechanism, at least concerning the signaling pathway underlying calcium channel gating. Despite that the occupation of membrane receptors by physiological agonists is a common feature for all the mechanisms of receptor-operated calcium entry, the subsequent activation of calcium-permeable channels involves three different pathways. The simplest mechanism involves direct opening of a receptor-operated channel where agonist-receptor binding leads to a conformational change that results in calcium influx. The other two signaling routes involve indirect channel gating either via generation of a diffusible second messenger that is able to activate channel opening by itself (second messenger-operated calcium entry) or by the discharge of the intracellular calcium stores (store-operated or capacitative calcium entry). The term capacitative calcium entry was proposed by James Putney Jr. to refer to a process whereby the discharge of the intracellular calcium stores within a cell secondarily activates calcium entry into the cell across the plasma membrane. During the past decades, a number of research groups have explored the mechanisms underlying the activation, maintenance, and termination

of the different routes for calcium entry in electrically excitable and non-excitable cells, as well as the identity of the molecular components. Concerning the latter, the different members of the Orai and TRPC families of channels have been found to play a relevant role both for capacitative calcium entry and for other calcium influx pathways. Given the importance of calcium influx in cell biology, one would expect that dysfunction of this process might underlie serious pathological consequences. In fact, a number of human disorders have been reported to involve Orai- as well as TRPC-associated channelopathies.

Finally I would like to express my sincere thanks and appreciation to all contributors for their kindly dedicated collaboration in the elaboration of this book and also to Ginés M. Salido for his support editing this book. I would also like to thank Thijs van Vlijmen, from Springer, for his encouraging support in the production of this book.

I hope that the content of this book will be helpful for both PhD students and advanced researchers interested in this fascinating field as well as inspiring for basic and biomedical scientists working in the pathophysiological implications of the calcium entry pathways.

Cáceres, Spain Juan A. Rosado
October 2015

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Part I
Store-Operated Calcium Entry

Chapter 1
Historical Overview of Store-Operated Ca^{2+} Entry

Letizia Albarran, Jose J. Lopez, Ginés M. Salido, and Juan A. Rosado

Abstract Calcium influx is an essential mechanism for the activation of cellular functions both in excitable and non-excitable cells. In non-excitable cells, activation of phospholipase C by occupation of G protein-coupled receptors leads to the generation of inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG), which, in turn, initiate two Ca^{2+} entry pathways: Ca^{2+} release from intracellular Ca^{2+} stores, signaled by IP_3, leads to the activation of store-operated Ca^{2+} entry (SOCE); on the other hand, DAG activates a distinct second messenger-operated pathway. SOCE is regulated by the filling state of the intracellular calcium stores. The search for the molecular components of SOCE has identified the stromal interaction molecule 1 (STIM1) as the Ca^{2+} sensor in the endoplasmic reticulum and Orai1 as a store-operated channel (SOC) subunit. Furthermore, a number of reports have revealed that several members of the TRPC family of channels also take part of the SOC macromolecular complex. This introductory chapter summarizes the early pieces of evidence that led to the concept of SOCE and the components of the store-operated signaling pathway.

Keywords SOCE • STIM1 • Orai1 • TRPC channels

1.1 The Concept of Store-Operated Ca^{2+}Entry

A number of physiological agonists activate cellular functions by inducing changes in cytosolic Ca^{2+} concentration ($[Ca^{2+}]_c$). In general, Ca^{2+} mobilization consist of the release of Ca^{2+} from intracellular stores, as well as Ca^{2+} influx from the extracellular medium through Ca^{2+}-permeable channels. Despite the identification of Ca^{2+}as a

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second messenger in the excitation-contraction coupling took place in the mid-twentieth century [1], the relevance of extracellular Ca^{2+}in cellular physiology was already highlighted in the publications of Ringer in the Journal of Physiology in the early 1880s [2, 3]. Throughout the 1960s and 1970s, different laboratories determined Ca^{2+} signals induced by physiological agonists, first using luminescent photoproteins and later on through the use of fluorescent probes, until Ca^{2+} was recognized as a *bona fide* second messenger [4].

After Ringer's findings, the studies concerning the functional role of Ca^{2+} entry were mainly focused on muscle contraction, especially in the heart and the sartorius muscle, where $^{45}Ca^{2+}$ uptake (influx) by the cell was analyzed under different experimental conditions [5, 6]. Among the first analysis of Ca^{2+} entry in non-electrically excitable cells were performed in HeLa cells in culture, where Borle identified two exchangeable pools, assumed as the extracellular Ca^{2+} and Ca^{2+} stored in intracellular compartments, and two unexchangeable Ca^{2+} pools, corresponding to extracellular and intracellular bound Ca^{2+} [7]. A year earlier, the same author reported that the extracellular medium was a major source of Ca^{2+} [8]. Borle defined Ca^{2+} influx in these cells as a facilitated diffusion process that can be affected by increasing the membrane permeability to this ion and not by stimulating an active or metabolically dependent process [7].

Voltage-dependent Ca^{2+}currents independent of metabolic events were described in the early 70s and a mechanism for Ca^{2+} influx operated by the occupation of membrane receptors was also soon reported in electrically excitable and non-excitable cells [9–11]. But, it was in 1986, when a mechanism for receptor-operated Ca^{2+} influx regulated by the filling state of the intracellular Ca^{2+} stores was proposed [12]. The term capacitative Ca^{2+}entry was coined by James Putney to refer to a process whereby the discharge of Ca^{2+} stores within a cell secondarily activates Ca^{2+}entry into the cell across the plasma membrane [12], a mechanism analog to the function of a capacitor in an electrical circuit, since this process is characterized by the fact that charged or full intracellular Ca^{2+} stores prevent Ca^{2+} current through the plasma membrane, while discharge of the intracellular stores is followed by rapid entry of Ca^{2+} into the store and, in the continued presence of inositol (1,4,5) trisphosphate (IP_3), into the cytosol [12–14].

Analysis of the kinetics of the rises in $[Ca^{2+}]_c$ using the intracellular fluorescent probe fura-2 in human platelets revealed that stimulation with the physiological agonist thrombin, which induces active Ca^{2+} release from the stores via generation of IP_3, resulted in Mn^{2+} entry that starts a few tenths of second after discharge of the intracellular Ca^{2+}stores [15]. This finding was consistent with a mechanism controlled by the agonist-sensitive intracellular Ca^{2+} stores. Further studies revealed that cell treatment with thapsigargin, a sesquiterpene lactone obtained from *Thapsia garganica* [16] that inactivates the sarco-endoplasmic reticulum Ca^{2+}-ATPase (SERCA) [17] and leads to passive store depletion via Ca^{2+} leak, resulted in Ca^{2+} influx across the plasma membrane independently of IP_3 generation [18]. Soon after this publication, it was found that Ca^{2+} influx evoked either by agonist stimulation or by treatment with thapsigargin was mediated via the same mechanism, thus concluding that the mechanism underlying the activation of capacitative Ca^{2+} entry was

the discharge of the intracellular Ca^{2+} stores per se and not the cellular levels of inositol phosphates [19]. The concept of a Ca^{2+} entry mechanism regulated by the stores was further confirmed by electrophysiological studies that reported that Ca^{2+} stores discharge activated a Ca^{2+} current in mast cells called Ca^{2+} release-activated Ca^{2+} current, I_{CRAC}, the first store-operated Ca^{2+} current identified [20]. In fact, in addition to the physiological pathway to activate capacitative Ca^{2+} entry via generation of IP_3, this mechanism for Ca^{2+} influx has been reported to be activated by a number of experimental maneuvers leading to a reduction in the amount of free Ca^{2+} in the intracellular stores. These procedures include treatment with thapsigargin, as well as other SERCA inhibitors, such as cyclopiazonic acid or di-*tert*-butylhydroquinone [21, 22] (agents that prevent Ca^{2+} store refilling), loading of Ca^{2+} stores with the metal Ca^{2+} chelator N,N,N,N-tetrakis(2-pyridylmethyl)ethylene diamine (which reduces free intraluminal Ca^{2+} concentration without altering $[Ca^{2+}]_c$ [23, 24]), or dialyzing the cytoplasm with the Ca^{2+} chelators EGTA or BAPTA, which bind Ca^{2+} leaking from the stores, thus preventing store refilling (see [25]). From that point capacitative Ca^{2+}entry was also known as store-operated Ca^{2+} entry (SOCE) [26] or store-mediated Ca^{2+}entry [27]. Since SOCE is the most extended term we will use this denomination throughout the chapter.

The manuscript by Kwan and coworkers [19] also raised a key conceptual feature of SOCE. The initial observations had suggested that the transport of extracellular Ca^{2+} into the cell involved a direct movement of Ca^{2+} into the intracellular stores, since refilling of the Ca^{2+} stores were found to occur with no substantial elevation in $[Ca^{2+}]_c$ [12, 28]. However, this hypothesis somehow limited the role of Ca^{2+} entry to the refilling of the intracellular Ca^{2+} stores. Using the trivalent cation lanthanum, which induces a concentration-dependent inactivation of Ca^{2+} extrusion through the plasma membrane Ca^{2+}-ATPase, and impairs Ca^{2+} entry [29, 30], Kwan and coworkers observed that, in the absence of Ca^{2+} extrusion and entry, Ca^{2+} stores were refilled by Ca^{2+} released into the cytoplasm upon agonist stimulation, providing evidence that store refilling did not involve a direct route into the intracellular Ca^{2+} pools, but rather is the result of a sequential Ca^{2+} entry into the cytoplasm and subsequent reuptake into the Ca^{2+}stores by active SERCA pumping [19, 31].

SOCE has also been described in a number of excitable cells, including neurons [32], cardiomyocytes [33], smooth muscle cells [34] and both endocrine [35–37] and neuroendocrine cells [38, 39]. In these cells, the functional role of SOCE is not limited to store refilling but it plays an important functional role as revealed by a number of disorders observed in the presence of defective SOCE [40–44].

SOCE activation in electrically excitable cells could trigger Ca^{2+} entry through voltage-operated channels by causing membrane depolarization [45, 46], which might result directly from the activation of store-operated Ca^{2+} (SOC) channels, which can be permeable to Ca^{2+}, as well as to other cations, such as Na^+, and have reversal potentials near 0 mV. In addition, indirect mechanisms for membrane depolarization associated to rises in $[Ca^{2+}]_c$ have been reported, such as a Ca^{2+}-dependent inhibition of voltage-dependent K^+ channels [47] reported in pulmonary arterial smooth muscle cells [48]. In contrast to these observations, current evidence supports that the SOCE element STIM1 interacts with the voltage-gated $Ca_v1.2$ chan-

nels and suppresses its activity [49, 50]. Since functional expression of voltage-operated Ca^{2+} channels, including $Ca_v1.2$, has been reported not only in excitable cells but also in non-electrically excitable cells, such as T and B lymphocytes and mast cells [51–54], these findings further confirm the role of STIM1 as an essential element in the modulation of agonist-induced Ca^{2+} signals.

The electrophysiological analysis of store-operated currents has revealed the presence of two types of currents: the Ca^{2+} release-activated Ca^{2+} current (I_{CRAC}) and a heterogeneous set of non-CRAC currents, described in different cell types with diverse biophysical properties grouped under the denomination of store-operated currents (I_{SOC}) [25]. The I_{CRAC} current was the first store-operated Ca^{2+} current identified using combined patch-clamp and fura-2 measurements to monitor membrane currents in mast cells [20]. I_{CRAC} is a non-voltage-operated current, unlike Ca^{2+} currents through the Ca_v family of channels, that exhibits large current amplitude at negative potentials and approaches the zero current level at very positive potentials. In addition, the current-voltage relationship for I_{CRAC} reveals a significant inward rectification at negative voltages [20, 25]. The channels conducting I_{CRAC} (CRAC channels) show a single channel conductance <1pS and are highly selective for Ca^{2+} over monovalent cations. The Ca^{2+}:Na^+ permeability ratio has been estimated around 1,000:1. Removal of extracellular Ca^{2+} in the presence of external Na^+ and Mg^{2+} has been reported to abolish this current [55, 56]. However, CRAC channels lose this selectivity in divalent-free solutions, which allow Na^+ to permeate the channels, leading to whole cell currents that are five to eightfold larger than the Ca^{2+} currents [57, 58].

The I_{SOC} currents are mediated by poorly selective cation channels that exhibit a significantly greater conductance than CRAC channels and show different biophysical properties. I_{SOC} currents have been described in different cell types, including vascular endothelial cells [59, 60], pancreatic acinar cells [61], human A431 carcinoma cells [62, 63], smooth muscle cells [64, 65], submandibular and parotid gland cells [66], liver cells [67], skeletal muscle cells [68], neurons [69] and adrenal chromaffin cells [70]. The Ca^{2+}:Na^+ permeability ratio of the channels conducting this currents is very heterogeneous, ranging from 1:0.07 to 50:1.

SOCE has been reported to play a number of important roles in cell physiology, including, among others, Ca^{2+} store refilling upon agonist stimulation, support for the sustained elevations in $[Ca^{2+}]_c$ required for a number of cellular functions and maintenance of the amplitude of Ca^{2+} oscillations [71, 72]. In addition, a variety of functions have been reported to be regulated by SOCE in a number of cell types, including endothelial cell permeability [73], vascular smooth muscle cell proliferation and contractility [34, 74], platelet function [75, 76], immunological response [77] or exocytosis [70, 78, 79], among many others.

1.2 Activation Mechanisms: Initial Studies

Since the discovery of SOCE, the mechanism underlying the regulation of SOC channels in the plasma membrane by distantly located intracellular Ca^{2+} stores has been intensely investigated and debated. The description that STIM1 is the Ca^{2+}

sensor of the intracellular Ca^{2+} stores in 2005 [80, 81] was a milestone in the identification of the events that regulate SOC channels following a reduction of the intraluminal Ca^{2+} concentration and marked an inflection point between the initial models and the current one. The initial studies can be grouped into those supporting the indirect coupling between the stores and SOC channels, those that propose a direct or conformational coupling between SOC channels and elements in the stores, and those reporting the insertion of preformed channels in the plasma membrane [25, 82].

The indirect coupling hypothesis assumes that Ca^{2+} store depletion results in the generation or activation of diffusible molecules that participate in SOC channel gating. This model includes roles for cGMP [83, 84], a product of cytochrome P450 [85], probably the metabolite 5,6-epoxyeicosatrienoic acid [86], tyrosine kinases [87–89], monomeric GTP-binding proteins [90–92], calmodulin [93] and a still uncharacterized Ca^{2+}-influx factor (CIF) [94, 95].

The conformational coupling hypothesis proposes a physical and constitutive interaction between elements in the membrane of the Ca^{2+} stores and SOC channels in the plasma membrane. This model was originally proposed by Irvine as a mechanism for the activation of SOCE involving IP_3 receptors in the endoplasmic reticulum (ER) and inositol 1,3,4,5-tetrakisphosphate (IP_4) receptors in the plasma membrane [96, 97]. Later on, Birnbaumer and colleagues demonstrated that the association of the IP_3 receptor with transiently expressed TRPC1, TRPC3, and TRPC6 plays a relevant role in the activation of SOCE [98]. This hypothesis received support from studies providing evidence for a role in the activation of SOCE of the protein junctate, an ER Ca^{2+}-binding protein that induces and/or stabilize the interaction between the ER and the plasma membrane [99]. Junctate has been proposed to play an important role in the association of the IP_3 receptors and TRPC3 [99]. Consistent with the conformational coupling are a number of studies reporting that TRPC3 channels present in excised patches can be activated by IP_3 [100]. Furthermore, the N-terminus of IP_3 receptors, containing the IP_3-binding domain, is essential for the activation of plasma membrane TRPC3 channels [101].

In addition to the indirect and conformational coupling hypotheses, a number of studies also supported the activation of SOCE by the translocation and insertion of preformed channels, initially located in intracellular vesicles, into the plasma membrane. This model, that was proposed by Penner and colleagues [90], requires the participation of the synaptosome associated protein SNAP-25 [102]. In hippocampal neurons, HEK 293 cells stably expressing TRPC6 and neuronal and epithelial cells expressing TRPC3 depletion of the intracellular Ca^{2+} stores results in the expression of TRPC channels in the plasma membrane [103–105].

The conformational coupling model assumed that the IP_3 receptor in the stores and SOC channels are permanently associated, so that a decrease in the intraluminal Ca^{2+} concentration is communicated to the SOC channels via a conformational change in the IP_3 receptor. Analyzing the role of the actin cytoskeleton in the activation of SOCE in different cell types, including smooth muscle cell lines, human platelets and other non-excitable cells, such as pancreatic acinar cells and the human hepatocellular carcinoma cell line HepG2, an alternative to the constitutive conformational coupling model called secretion-like coupling or de novo conforma-

tional coupling was described. This model assumes a reversible physical coupling between the ER and the plasma membrane where the actin cytoskeleton plays an essential role [106–109]. The de novo coupling between elements in the ER and the plasma membrane was found to be strongly dependent on actin filament reorganization. Thus, the actin network located underneath the plasma membrane acts as a negative clamp that prevents constitutive coupling [106, 109, 110]. According to this, stabilization of the cortical actin network has been reported to impair the activation of SOCE in a variety of cell types, including smooth muscle cell lines, human platelets, corneal endothelial cells, the human prostate adenocarcinoma cell line LNCaP, pancreatic acinar cells, smooth muscle cells or neutrophils [39, 45, 106, 107, 109, 111, 112]; however, in pancreatic β cells, thyroid FRTL-5 cells and *Aplisia* bag cell neurons, SOCE has been reported to be insensitive to stabilization of the cortical actin cytoskeleton by treatment with jasplakinolide [113–115], and stabilization of the peripheral cytoskeleton and disassembly of actin microfilaments have been shown to fail to alter the rate or extent of activation of I_{CRAC} in the RBL-1 rat basophilic cell line [116]. This discrepancies might be attributed to the different idiosyncrasy of the cells investigated. The role of the cortical cytoskeleton in the modulation of SOCE has been further supported by analysis of the actin polymerization and Ca^{2+} mobilization on a subsecond time scale. In human platelets stimulated with the physiological agonist thrombin we detected an initial decrease in the actin filament content within 0.1 s after stimulation that reached a minimum 0.9 s after the addition of thrombin. Actin depolymerization, involving the actin-binding protein cofilin, was observed before the initiation of SOCE, which occurred with a latency of 2.1 s after agonist stimulation [117], which is consistent with a role for the actin cortical cytoskeleton in the modulation of SOCE.

After the identification of STIM1 as the ER Ca^{2+} sensor [80, 118] and Orai1 as the prototypical CRAC/SOC channel [77, 119–122] the initial hypotheses had to be necessarily reconsidered and/or reinterpreted. Therefore, as described in a number of cells for SOCE, is the interaction between STIM1 and Orai1 modulated by the cortical actin cytoskeleton? There is a growing body of evidence supporting a role for the cytoskeleton in the interaction between STIM1 and Orai1. In HEK-293 cells and platelets, we have demonstrated that stabilization of the cortical actin cytoskeleton impairs de novo association between the ER Ca^{2+} sensor STIM1 and the SOC channel Orai1 [123, 124], without altering the coupling between plasma membrane-resident STIM1 and Orai1 [125]. These findings might explain why, in the presence of extracellular Ca^{2+}, treatment with jasplakinolide prevented Ca^{2+} entry but not the influx of other cations, such as Mn^{2+} or Na^{+} [126] that might permeate through non-selective cation channels containing TRP subunits. More recently, it has been reported in HeLa cells that calcineurin impairs cytoskeleton remodeling and STIM1/Orai1 puncta-like formation in a KSR-2-dependent manner [127]. Furthermore, in activated T cells, the Rac1 effector protein WAVE2 has been reported to modulate SOCE and STIM1/Orai1 interaction by regulation of actin filament reorganization [128]. Therefore, the participation of the cortical actin cytoskeleton in the modulation of the de novo interaction between STIM1 and Orai1 and, subsequently SOCE, might still be a potential model.

1.3 STIM1, Orai and TRPCs

As we discussed in the previous section, STIM1, originally named GOK, was presented in 2005 as an essential protein involved in the activation of SOCE [80, 81]. The initial studies on STIM1 were published in the mid-1990s, where STIM1 was described as a type-1 transmembrane glycoprotein of 685 amino acids involved in cell-cell interactions in hematopoietic cells [129] and as a cell growth suppressor that plays a pivotal role in the establishment and progression of rhabdomyosarcomas and rhabdoid tumors [130, 131]. Since then, several studies were focused on the study of the structure and function of STIM1 and the determination of the signaling pathways involving STIM1. In 2000, immunofluorescence and cell surface biotinylation analysis revealed that STIM1 is a 90-kDa integral transmembrane phosphoprotein ubiquitously expressed both in the plasma membrane and intracellular membranes of a variety human primary cells, including neonatal foreskin fibroblasts and MG63 osteoblast-like cells, and established tumor cell lines, such as the human leukemic K562, HL60 and U937 cell lines [132]. Later on, the same group demonstrated that the luminal N-terminal region of STIM1 includes an ER signal peptide, EF-hand Ca^{2+}-binding motif, and a single sterile alpha motif (SAM) involved in protein-protein interaction, whereas the cytosolic region consists of two coiled-coil domains, a proline/serine-rich region, and a lysine-rich region [132–134]. These studies also showed that STIM1 is subjected to different post-translational modifications, including phosphorylation on serine residues [133], and N-linked glycosylation in two sites within in the SAM domain [134].

The role of STIM1 in the regulation of SOCE was initially discovered in 2005 by Roos and coworkers [80]. In this study, RNA interference (RNAi)-based screen of more than 170 proteins with known signaling motifs, was designed to identify genes that alter SOCE and the signal pathways controlling them in *Drosophila* S2 cells. With this approach, the *stim* gene and its product, STIM1, were identified as essential regulators of SOCE and CRAC channel activity since RNAi-mediated knockdown of the *stim* gene significantly blocked thapsigargin-induced Ca^{2+} influx in S2 cells. This new promising role of STIM1 was also confirmed in human cells. Suppression of STIM1 expression also significantly reduced thapsigargin-induced SOCE in Jurkat T cells expressing a short RNA hairpin loop (shRNA) targeting human STIM1. Moreover, Ca^{2+} influx was also diminished when STIM1 expression was down-regulated in HEK-293 and SH-SY5Y human cells transfected with STIM1 siRNA [80].

Since STIM1 overexpression in HEK-293 cells was not associated with an increased Ca^{2+} influx and detectable SOCE current, it was proposed that STIM1 could not function as SOC channel itself, and the intraluminal location of the EF-hand motif suggested that STIM1 might act as an ER Ca^{2+} sensor [80]. The report by Roos and coworkers was followed shortly thereafter by two studies from Liou and coworkers [81] and Zhang and coworkers [118]. Cell transfection with a mutated EF-hand motif of STIM1 or *Drosophila* Stim induced a constitutive Ca^{2+} influx mediated by CRAC channels and independent of the filling state of the Ca^{2+}

stores, and treatment with thapsigargin failed to further promote SOCE in HeLa [81], Jurkat and S2 cells [118]. Using different techniques, from immunofluorescence and electron microscopy to surface biotinylation, both studies also demonstrated that, once Ca^{2+} stores are depleted, STIM1 migrates from the ER to the proximity of the plasma membrane. Liou and coworkers first reported that upon store depletion STIM1 redistributes into *punctae* structures, ER regions enriched in STIM1 oligomers and located in close proximity to the plasma membrane [81]. Later on, it was demonstrated that SAM is an essential STIM1 domain for oligomerization and *punctae* formation, while the coiled-coil domains are involved in further stabilization of the STIM1 oligomers and also in functional protein-protein interactions of STIM1 with Ca^{2+} channels located in the plasma membrane [135].

The identification of STIM1 as the intraluminal Ca^{2+} sensor provided an explanation for the communication between the intracellular Ca^{2+} stores and the CRAC/SOC channels located in the plasma membrane, whose identity still remained elusive. In 2006, Orai1 was identified as the pore subunit of the CRAC channels [77, 121, 136–138]. Initially Feske and coworkers [77] and soon thereafter Vig et al. [136] presented Orai1, also named as CRAC modulator 1 (CRACM1), as an essential component regulator of the CRAC channel. Both studies identified *Drosophila* gene *olf186-F* as an essential gene in the activation of thapsigargin-induced SOCE by using a genome-wide RNAi screen in *Drosophila* S2 cells to identify the genes encoding the CRAC channel or other proteins involved in its regulation [77, 136]. The *Drosophila olf186-F* gene has three human homologues that encode the proteins named, for the first time, as Orai1, Orai2 and Orai3 [77]. The term "orai" is referred to the three keepers of heaven's gate in Greek mythology. The gene encoding human Orai1 was mapped on chromosome region 12q24 and was shown to be mutated in the hereditary severe combined immune deficiency (SCID) syndrome patients. Collected T cells from these patients are characterized by an impaired SOCE and CRAC channel activity. This mutation consists in a C→T transition at position 271 of the coding sequence of the human *orai1* gene, leading to replacement of a highly conserved arginine residue by tryptophan at position 91 of the protein (R91W). Expression of an exogenous wild-type Orai1 in SCID T-cells restored store depletion-evoked Ca^{2+} influx, suggesting that this single point mutation R91W in Orai1 sequence was responsible of the defective I_{CRAC}. However, expression of wild-type Orai1 in SCID T-cells did not promote Ca^{2+} influx per se in resting conditions, suggesting that Orai1 is not a Ca^{2+} channel constitutively activated. Furthermore, electrophysiological and pharmacological properties of the restored Ca^{2+} influx current were fully consistent with those observed in I_{CRAC} in normal T cells [77]. According to these results, the selective small interfering RNA (SiRNA)-mediated knockdown of Orai1 reduced both Ca^{2+} influx and I_{CRAC} in response to thapsigargin in human HEK-293 and Jurkat T cells [136]. In addition to the demonstration that Orai1 plays a pivotal role in CRAC current generation, both studies also predicted the topology Orai1 as a membrane protein with four transmembrane domains and cytosolic N- and C- termini, which shows no structural homology to other known ion-channels [77, 136].

Soon after the identification of Orai1 as a the pore forming subunit of the CRAC channel published studies demonstrated the earliest pieces of evidence for the functional interaction between STIM1 and Orai1 in the activation of CRAC currents were reported. Co-expression of STIM1 and Orai1 resulted in a significantly enhanced CRAC current in HEK-293, Jurkat T cells [119, 120, 122] and *Drosophila* S2 cells [139]. The generated current showed Ca^{2+} selectivity, development and inactivation kinetics, pharmacological profiles and biophysical properties indistinguishable from native CRAC current [120, 139].

According to the idea that conserved acidic residues are essential for ion permeation through known Ca^{2+} channels, three simultaneous studies analyzed highly conserved glutamate residues across species and the most interesting results come from the residues E178 and E262 in *Drosophila* and their corresponding human homologues E106 and E190. Mutation of E106 to alanine or glutamine greatly impaired channel function. However, the conservative mutations of E106 to an aspartate (E106D) resulted in a channel with reduced selectivity for Ca^{2+}, thus indicating that this residue functions as part of the selectivity filter, and confirms that Orai1 is a pore forming subunit of the CRAC channel [121, 137, 138]. While mutation of E190 to aspartate or alanine had no significant effect on channel function, the substitution by glutamine (E190Q) resulted in diminished Ca^{2+} selectivity [121, 138]. These studies also demonstrated, for the first time, the STIM1-Orai1 protein interaction upon Ca^{2+} store depletion to initiate CRAC channel activation, by using co-immunoprecipitation in co-transfected HEK-293 cells [138] and *Drosophila* S2 cells [137]. The STIM1/Orai1 complex was reported to be located in the *punctae* region on the plasma membrane after CRAC current activation by fluorescence microscopy [140, 141].

The identification of Orai1 as the pore forming region of the CRAC channel raised the question concerning the architecture of the channel, with reports proposing tetrameric and even higher-order multimeric structures [142–144]. Hou and coworkers [145] reported in 2012 the crystal structure of *Drosophila* Orai channel, which shares 70 % homology with human Orai1. The crystal structure indicates that the pore of *Drosophila* Orai is formed by six subunits, each containing four transmembrane regions (M1 to M4). The transmembrane helices are arranged in three concentric circles around the pore, where the inner ring is formed of six M1 helices, the middle ring consists of M2 and M3 helices and the outer ring is formed of M4 helices located at the periphery of the channel. The side chains of M1 line the pore of Orai, which is composed of four different sections: a selectivity filter formed by a ring of glutamates at the extracellular end of the channel, followed by a hydrophobic section, a basic region spanning near the intracellular side of the channel, which might contribute to the stabilization of the closed state of the channel, and a wider section that extends into the cytosol. The pore region of Orai shows biochemical differences with the known K^+ channels, which emphasizes the distinct mechanisms of ion selectivity and permeation between Orai and K^+ channels.

In addition to the small families of STIM and Orai, another somewhat larger family of ion channels are also linked to SOCE. These are a subfamily of the larger family of TRP channels, called TRPC for classical or canonical TRPs, because it is

comprised of those proteins most highly related to the *Drosophila* TRP channel [146].

The origin of the *Drosophila* TRP goes back to 1969, when Cosens and coworkers identified a spontaneously formed mutant on the basis of a behavioral phenotype [147], but it was not until 1975 that Minke described and named it as "Transient Receptor Potential" (TRP) because of the transient depolarization of the photoreceptors due to Na^+ and Ca^{2+} entry [148]. The identity of the mutated protein was reported in 1989 by Montell and coworkers who cloned, sequenced and presented a molecular characterization of the *Drosophila trp* gen and highlighted its similarity with the Ca^{2+} channels [149]. Later studies characterized TRP and its homologue TRPL as Ca^{2+} permeable channels activated downstream of phospholipase C [150].

After the initial identification of the *Drosophila* TRPs, all the attempts were focused on finding its counterpart in mammals. In 1995, regarding its high homology to the TRP protein sequence, an expressed sequence from a human fetal brain cDNA library was reported by two groups as the first homologue in human (TRPC1) [151, 152]. Since the identification of TRPC1, several mammalian homologues have been described and are classified into six subfamilies: TRPC (canonical), TRPV (vanilloid), TRPM (melastatin), TRPA (ankyrin), TRPP (polycystin), TRPML (mucolipin). In addition, a less related TRPN has been reported in worms and fish and TRPY in fungi [153].

The DNA and corresponding amino acid sequence of the previously called TRPC1, the expression pattern as well as another general features of the novel protein were described by Wes and coworkers and Zhu and coworkers, who characterized it as a polypeptide of 793 amino acids expressed at the highest levels in the fetal brain and in the adult heart, brain, testis and ovaries [151, 152]. The primary structure was supposed to present six transmembrane domains with a pore region between the fifth (S5) and sixth (S6) transmembrane segments and both C and N termini located intracellularly [154]. This structure is shared by all the members of the TRP family. Furthermore, Wes also described that the most highly conserved domain among all TRP family members contains three ankyrin repeats which are thought to play key roles in protein-protein interactions [151, 155]. Later on the whole structure of the protein was reported, with the N-terminus containing three to four ankyrin repeats, a predicted coiled-coil motif and a caveolin binding region. By the way, the citoplasmic C-terminus includes an EWKFAR TRP box, a highly conserved proline rich domain, a coiled-coil motif and a *C*aM/*I*P_3 *r*eceptor *b*inding (CIRB) domain [98, 156]. The presence of coiled-coil domains, commonly involved in subunit oligomerization suggest that they are probably involved in homo-and heteromerization of TRPCs or in linking TRPCs to other proteins also containing coiled-coil domains [157, 158].

Despite the key role of TRPs in receptor-operated Ca^{2+} entry (ROCE) and in SOCE has been reported in different cell types, the latter has been a matter of intense debate, and the role of TRPCs in SOCE has not been demonstrated in all the cellular models investigated. Among the first pieces of evidence for a role of TRPC1 in SOCE comes from a study made in Chinese hamster ovary cells (CHO), where expression of TRPC1A, a splice variant of TRPC1, resulted in a linear nonselective

cation current with similar permeabilities for Na^+, Ca^{2+}, and Cs^+, activated by intracellular infusion of either IP_3 or thapsigargin to deplete intracellular Ca^{2+} stores [159]. At the same time, Zhu and coworkers demonstrated that expression in COS cells of full-length cDNA encoding human TRPC1 increased SOCE while expression of antisense sequences suppressed it [160]. Further evidence supporting the role of TRPC1 in SOCE comes from studies based on overexpression of TRPC1 proteins or knockdown of the endogenous TRPC1 channels in several cell types, including human cells. In human submandibular gland cells and vascular endothelial cells, the overexpression of TRPC1 increased SOCE while the silencing of the endogenous protein by using antisense oligonucleotides reduced thapsigargin-evoked influx [161, 162]. In general, the key role of TRPC1 in SOCE has been supported by several research groups by means of different experimental maneuvers in many cell types, such as DT40-B-lymphocytes, endothelial cells, rat cardiac myocytes, the human megakaryoblastic cell line MEG01, the C2C12 mouse myoblastic cell line, rat kidney fibroblasts or human platelets [162–166]. Other members of the TRPC family have also been reported to be activated by store depletion or to be involved in SOCE both in excitable and non-excitable cells. For instance, the transient expression of the full length cDNA of mouse TRPC2, the homologue of the human *trp2* pseudogene, was reported to evoke a Ca^{2+} entry that was shown to be readily activated not only after agonist stimulation but also by store depletion in the absence of an agonist [167]. In addition, in 1996 Zhu and coworkers observed that the heterologous expression of the human TRPC3 in COS cells enhanced SOCE [160] and similar results were obtained by Boulay and coworkers by expressing the murine TRPC6 in this cells [168]. Over the years, some studies have highlighted that there is also a growing body of evidence supporting a role of TRPC6 in the conduction of SOCE in different cells [169, 170].

Nevertheless, several studies have failed to observe store-operated behavior of exogenously expressed TRPC channels in different cell types, thus demonstrating that TRPCs did not account for SOCE in all cellular models investigated [171]. Moreover, the mode of expression of TRPCs might determine its involvement in SOCE or ROCE in the same cell type, as it was reported by Lievremont and coworkers in 2004, who described that TRPC7 activation mode was different in cells transiently or stably expressing this channel [172].

With the identification of STIM1 and Orai1 as the key molecular players of CRAC currents, most studies focused on the possible interaction between these proteins and TRPC channels. In 2006, after Spassova and coworkers confirmed the crucial functional role of STIM1 over the regulation of SOC channels by using electrophysiological analysis [173], two independent studies demonstrated for the first time that STIM1 is associated with TRPC1, in the plasma membrane upon Ca^{2+} store depletion, and that the STIM1-TRPC1 interaction promotes channel activation and SOCE [174, 175]. In HEK-293 cells expressing exogenous STIM1, Ca^{2+} store depletion increased the association between STIM1 and TRPC1, while the expression of the EF-hand mutant STIM1 yield a constitutive STIM1-TRPC1 complex formation and subsequent TRPC1 activation and Ca^{2+} influx [174]. According to this, electrotransjection of human platelets with anti-STIM1 antibody, directed

toward the EF-hand Ca^{2+}-binding motif, prevented the migration of STIM1 toward the plasma membrane, the interaction between endogenously expressed STIM1 and TRPC1 and reduced SOCE [175]. Functional interaction between STIM1 and TRPC1 has also been reported, among others cellular types, in intestinal epithelial cells [176], salivary and pancreatic acinar cells [79, 177], pulmonary artery cells [178] or messanglial cells [179].

STIM1 also interacts directly with other members of TRPC family involved in SOCE, such as TRPC4 and TRPC5 in HEK293 cells [180] and TRPC6 in human platelets [24], regulating their activation. STIM1 also indirectly regulates TRPC3 and TRPC6 by mediating the heteromultimerization of TRPC3 with TRPC1 and TRPC4 with TRPC6 [180]. Direct STIM1/TRPC interaction has been reported to require interaction of the STIM1 SOAR region with the TRPC C-terminal coiled-coil domains, as well as electrostatic interactions involving the lysine-rich domain of STIM1 [181, 182]. This association is supported by the actin cytoskeleton [175] and plasma membrane lipid rafts domains [183–185].

In addition to the STIM1-TRPC coupling previously described, there is a body of evidence supporting the formation of ternary complexes among the three main elements involved in SOCE, STIM, Orai and TRPCs. In HEK293 cells, SOCs were reported to be built by TRPC pore-forming subunits and Orai regulatory subunits that transduce the Ca^{2+} store depletion signal from STIM1 to TRPCs [186]. Supporting this hypothesis, a dynamic assembly among STIM1, Orai1 and TRPC1 has been proposed to be essential for SOCE in human salivary gland cells [187] and human platelets [123]. Interestingly, the association of these proteins has been reported to occur in specific plasma membrane microdomains known as lipid rafts which provide the adequate environment for the formation of the store-operated Ca^{2+} influx complex (SOCIC) or receptor-operated Ca^{2+} entry complexes [184, 185]. The following chapters will highlight recent advances in the Ca^{2+} entry mechanisms, involving store-operated and receptor-operated signaling pathways, in non-excitable cells.

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Chapter 2
The STIM1: Orai Interaction

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Abstract Ca^{2+} influx via store-operated Ca^{2+} release activated Ca^{2+} (CRAC) channels represents a main signalling pathway for a variety of cell functions, including T-cell activation as well as mast-cell degranulation. Depletion of $[Ca^{2+}]_{ER}$ results in activation of Ca^{2+} channels within the plasmamembrane that mediate sustained Ca^{2+} influx which is required for refilling Ca^{2+} stores and down-stream Ca^{2+} signalling. The CRAC channel is the best characterized store-operated channel (SOC) with well-defined electrophysiological properties. In recent years, the molecular components of the CRAC channel have been defined. The ER – located Ca^{2+}-sensor, STIM1 and the Ca^{2+}-selective ion pore, Orai1 in the membrane are sufficient to fully reconstitute CRAC currents. Stromal interaction molecule (STIM) 1 is localized in the ER, senses $[Ca^{2+}]_{ER}$ and activates the CRAC channel upon store depletion by direct binding to Orai1 in the plasmamembrane. The identification of STIM1 and Orai1 and recently the structural resolution of both proteins by X-ray crystallography and nuclear magnetic resonance substantiated many findings from structure-function studies which has substantially improved the understanding of CRAC channel activation. Within this review, we summarize the functional and structural mechanisms of CRAC channel regulation, present a detailed overview of the STIM1/Orai1 signalling pathway where we focus on the critical domains mediating interactions and on the ion permeation pathway. We portray a mechanistic view of the steps in the dynamics of CRAC channel signalling ranging from STIM1 oligomerization over STIM1-Orai1 coupling to CRAC channel activation and permeation.

Keywords SOCE • STIM1 • Orai1 • CRAC channels

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Abbreviations

2-APB	2-Aminoethoxydiphenyl borate
Å	Angstrom
Ca^{2+}	Calcium
CAD	CRAC activating domain
CC	Coiled coil
CCb9	Coiled coil containing region b9
CCE	Capacitative calcium entry
CDI	Calcium dependent inactivation
CFP	Cyan fluorescent protein
CMD	CRAC modulatory domain
CRAC	Calcium release activated calcium
Cs^{+}	Caesium
dOrai	Drosophila melanogaster Orai
ER	Endoplasmic reticulum
Et al	Et alii
ETON	Extended transmembrane Orai1 N-terminal region
FRET	Förster resonance energy transfer
IH	Inhibitory helix
IP_3	Inositol(1,4,5)triphosphate
K-rich	Lysine rich
Na^{+}	Sodium
NMR	Nuclear magnetic resonance
OASF	Orai activating small fragment
PLC	Phospholipase C
PM	Plasmamembrane
RNAi	Interference ribonucleic acid
SAM	Sterile alpha motif
SCID	Severe combined immunodeficiency
SERCA	Sarcoplasmic/endoplasmic reticulum calcium ATPase
SHD	STIM1 homomerization domain
SOAP	STIM/Orai association pocket
SOAR	STIM/Orai activating region
SOCE	Store operated calcium entry
STIM	Stromal interaction molecule
TG	Thapsigargin
TM	Transmembrane
TRP	Transient receptor potential
YFP	Yellow fluorescent protein

2.1 Introduction

The discovery of store-operated Ca^{2+} entry has a relatively long history. The concept of a calcium entry pathway that is activated upon store depletion of internal Ca^{2+} reservoirs was first proposed by Putney nearly 30 years ago and initially termed capacitative Ca^{2+} entry (CCE) [1]. This Ca^{2+} influx pathway is now referred to as store-operated Ca^{2+} entry (SOCE). SOCE has been observed in a variety of non-excitable and excitable cells including lymphocytes, pancreatic acinar, vascular endothelial and smooth and skeletal muscle cells [2–7]. A few years later, the biophysical properties of the underlying calcium-release-activated-Ca^{2+} (CRAC) – channel conductance were characterized [5, 8–10] and at the molecular level, store-operated Ca^{2+} channels have only been investigated within the past decade.

Physiologically, SOCE is initiated by Ca^{2+} release from the ER lumen into the cytoplasm upon receptor activation. Once activated, the production of the second messenger inositol-1,4,5-triphosphate (IP_3) is initiated which subsequently binds to its receptors in the ER membrane and activates Ca^{2+} release [11, 12]. In vitro, passive depletion of intracellular Ca^{2+} stores is often used to initiate SOCE by preventing Ca^{2+} re-uptake into the ER using sarcoplasmic/endoplasmic reticulum ATPase (SERCA) pump inhibitors such as thapsigargin (TG). The CRAC channel represents one of the most Ca^{2+} selective channels and currents are defined by a set of characteristic features. It is over 1000 times more permeant for Ca^{2+} than Na^+ at physiological conditions [5] and has a conductance that is among the smallest of any ion channel [10]. CRAC currents exhibit strong inward rectification and essentially positive reversal potentials >60 mV [5]. This unique fingerprint has given rise to years of search for candidate genes involved in CRAC channel function. Most prominently, members of the large family of transient receptor potential (TRP) channels have been proposed [13]. Now, two decades after Putney's concept of SOCE, STIM1 and Orai1, the two key-players of the CRAC channel complex, have finally been identified in parallel by RNAi screens in 2005 and 2006, respectively [14–17]. STIM (stromal interaction molecule) proteins have first been described as regulators of SOCE. In this study, Zhang et al. screened 170 candidates genes in *Drosophila* S2 cells and proved the role of STIM1 in SOC and CRAC channel activity whereby STIM1 controls SOCE by sensing $[Ca^{2+}]_{ER}$ and by translocating to the plasmamembrane (PM) where it activates Orai1 [15]. Orai1 was identified in 2006 by Feske et al. [18] through RNAi screens of patients which exhibited a form of hereditary severe combined immunodeficiency (SCID). They have shown that a single missense mutation in Orai1 (R91W) abrogates CRAC channel function and impacts T-cell effector function leading to a severe immunodeficient phenotype. Altogether, RNAi knockdown of either STIM1 or Orai1 completely suppressed a functional CRAC current, pointing to the indispensable requirement of both proteins in store-operated Ca^{2+} entry. Nowadays, the CRAC channel is the best characterized store-operated channel and is endogenously expressed in T-lymphocytes and other cells of the immune system. CRAC channels in the PM are formed by members of the Orai protein family that are activated upon physical interaction with the

ER-resident STIM1 protein. Subsequently, extracellular Ca^{2+} ions can enter the cell, where they act as second messenger to sustain Ca^{2+} oscillations, to maintain Ca^{2+} homeostasis and to provide long-term (in the range of hours) Ca^{2+} signals. These long-term Ca^{2+} signals regulate a plethora of signalling cascades including mast cell degranulation, cytokine secretion, T-cell differentiation into effector subsets, B-cell differentiation into plasma cells, lysis of infected or cancerous target cells by cytolytic T-cells, gene transcription or cell proliferation [16–28].

2.2 CRAC Activation and Key Players

When ER stores are full, the EF hand of STIM1 is bound with Ca^{2+}, which leads to a uniform distribution of STIM1 in the ER. Store-depletion triggers STIM1 oligomerization and translocation to ER-PM junctions, leading to STIM1 puncta [20, 29–31]. Orai1 accumulates at sites opposite to STIM1. This high proximity of the two proteins allows for direct interaction and leads to channel activation [20, 31, 32]. Details of STIM1-Orai1 interaction will be discussed further below.

There are two mammalian homologs of STIM, i.e. STIM1 and STIM2; and three mammalian homologs of Orai, i.e. Orai1-3. Since their discovery, these molecules have been investigated in great detail on the molecular level for their role in CRAC channel function and in vivo as essential components of SOCE in a variety of tissues. They are distributed widely throughout many tissues in various species, consistent with the widespread prevalence of SOCE and CRAC channel currents in many cell types [21, 33].

2.2.1 STIM Proteins

STIM proteins are type I, single-pass ER transmembrane proteins, primarily localized in the ER membrane, which link Ca^{2+} store-depletion and Ca^{2+} influx through the plasmamembrane. They sense $[Ca^{2+}]_{ER}$ via a tandem EF hand like sequence on their luminal side and physically relay this message to Orai channels in the plasmamembrane by translocating into defined ER-PM junctions [14, 15, 29, 34, 35]. By neutralization of acidic residues within the EF hand structure, STIM1 was no longer able to bind Ca^{2+} and yielded constitutive Ca^{2+} entry, mimicking the store-depleted state [14, 29].

2.2.1.1 Ca^{2+} Sensing, STIM1 Activation and Translocation

In case of full ER Ca^{2+} stores (resting cell conditions), STIM1 is homogenously distributed in the cell interior and rapidly moves as an ER membrane anchored protein along microtubules [36]. Decrease of ER luminal Ca^{2+} concentration (termed store

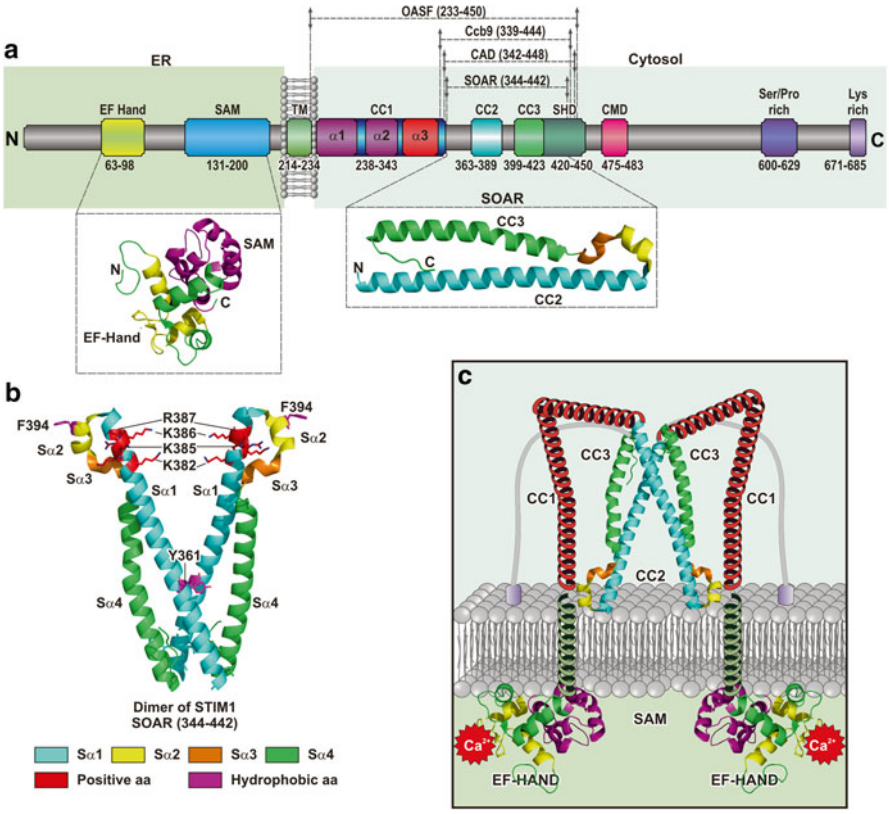

Fig. 2.1 (**a**) Schematic representation of human, full-length STIM1 depicting essential regions for the STIM1/Orai1 signaling cascade. The structure of the EF-SAM domain as well as the STIM1 SOAR (344–442) fragment is shown in detail. (**b**) Cartoon representation of a STIM1 SOAR (344–442) – dimer including coiled-coil domain 2 (CC2) and 3 (CC3) exhibiting a V-shaped structure. Residues mediating dimer interaction and those involved in coupling to Orai1 (positively charged residues) are highlighted. (**c**) Hypothetical model of a STIM1 dimer in the resting state

depletion) leads to STIM1 oligomerization which slows down the movement along microtubules [37]. A former study by Baba et al. has revealed constitutive, dynamic STIM1 movement in full store conditions, while store depletion consequently induced a dramatic redistribution of oligomerized STIM1 forming so called puncta, located at ER – PM junctions [30, 34, 38]. Investigation of STIM1 C-terminal truncation mutants (deletions within the cytosolic strand of STIM1) has shown that STIM1 coiled-coil (CC) domains and the serine-proline rich region (Fig. 2.1a) play a major role for the constitutive movement of STIM1 within the ER membrane under resting cell conditions, whereas STIM1 activation leading to puncta formation involves luminal as well as cytoplasmic STIM1 regions [30]. In line, independent FRET studies revealed that store depletion consequently leads to STIM1 oligomerization and cluster formation [39–41]. Additional experiments with artificially crosslinked

STIM1 at its luminal side revealed luminal STIM-STIM interaction as major signal for STIM1 oligomerization and cluster/puncta formation at ER-PM junctions where it interacts with and activates Orai1 channels [20]. In summary, these results point to the fact that store depletion induces STIM1-STIM1 interaction on the luminal side which finally triggers the STIM1 activation process [14, 15, 34, 38, 42].

2.2.1.2 Domains Within the ER Luminal Part of STIM1

The STIM1 N-terminus resides within the ER lumen followed by a single TM spanning domain and a long C-terminus located in the cytosol (Fig. 2.1a). Highly important domains of the STIM1 ER luminal part are the canonical and hidden EF-hands as well as the sterile-alpha motif (SAM) [35, 43, 44]. The EF-hand structurally reveals a helix-loop-helix motif with negatively charged aspartates and glutamates interacting with Ca^{2+} as long as ER Ca^{2+} concentration is high (full stores). As initial signal for STIM1 oligomerization, Ca^{2+} store depletion leads to Ca^{2+} dissociation from the EF hand (K_d ~200–600 μM) consequently destabilizing the entire EF-SAM domain [43]. The EF-hands Ca^{2+} binding affinity is perfectly adequate to the ER Ca^{2+} concentration range (~400–800 μM) to ensure an accurate response to variable ER Ca^{2+} concentrations. The detailed examination of the EF-SAM complex by Stathopulos et al. [35] (Fig. 2.1a) has revealed that the holo EF-Ca^{2+}- SAM domain contains high α-helicity in contrast to apo EF-SAM (absence of Ca^{2+}) which proved to be less compact. The EF-Ca^{2+}- SAM holoform is monomeric whereas in contrast, the EF-SAM apoform has been proven to be at least a dimer [35, 43]. Furthermore, FRET experiments by Covington et al. [45] using a STIM1 deletion mutant lacking the whole, cytosolic C-terminus result in store depletion mediated di-/oligomerization of the STIM1 luminal parts. The STIM1 homolog STIM2 also contains an ER luminal EF-SAM domain, however, it is shown to have different cellular functions [46]. CRAC currents activated by STIM2 appear upon smaller changes in ER – Ca^{2+} concentrations, suggesting that STIM2 is part of a feedback system keeping ER – Ca^{2+} concentrations within tight limits [46]. The different behaviour of EF-SAM of STIM1 and STIM2 is due to a difference in the N-terminal structural stability of both proteins [47, 48]. Adjacent to the SAM domain, STIM proteins contain a single transmembrane (TM) segment spanning the ER membrane (Fig. 2.1a). Whether the TM domain acts passively or plays an active role in the STIM activation process remains to be resolved.

2.2.1.3 STIM1 C-Terminal, Cytosolic Domains

STIM1 contains a long, cytosolic strand including three coiled-coil (CC1, CC2, CC3) regions, the CRAC modulatory domain CMD, a serine/proline- and a lysine-rich region (Fig. 2.1a). Independent studies performed by Huang et al. [49] and Muik et al. [50] revealed that the STIM1 C-terminus is sufficient to activate CRAC currents through Orai1. In a very close time period, several groups have analyzed

short cytosolic STIM1 fragments to identify the Orai channel activating key domains: OASF (233–450), CAD (342–448), SOAR (344–442) and Ccb9 (339–444) [50–53] (Fig. 2.1a). All Orai activating STIM1 fragments have the CC2 (363–389) and CC3 (399–423) regions with additional 19 residues (424–442) in common (Fig. 2.1a) including the STIM1 homomerization domain (SHD), an Orai coupling and activating domain. The SHD corresponds to the segment ~421–450 and plays a major role in the cytosolic STIM1 homomerization process [50]. Its deletion within OASF dramatically decreases FRET signals in homomerization studies and furthermore abrogates Orai1 activation as measured by patch clamp [50].

2.2.1.4 Crystal Structure of the Orai1 Activating Portion of STIM1

The long awaited first crystal structure of a cytosolic portion of STIM1 has finally been published in 2012. It revealed a crystallized hSOAR protein (345–444 $_{L374M, V419A, C437T}$) forming a dimeric assembly with intra- as well as intermolecular interactions [54] (Fig. 2.1b). The "R" shaped structure of the monomeric SOAR protein consists of an antiparallel arrangement of CC2 and CC3 which are linked by two short α-helices. The dimeric assembly is generated by CC interactions as C-terminal residues (R429, W430, I433, L436) from one monomer interact with N-terminal residues (T354, L351, W350, L347) of the other monomer, finally forming an overall V-shaped structure of the SOAR dimer (Fig. 2.1b). Both CC2 cross each other at amino acid Tyr361 forming a stacked interaction at this position. Mutations within the dimeric binding interface in full-length STIM1 as well as SOAR abolished colocalization with and activation of Orai1 channels [54].

2.2.1.5 CC1 Is Highly Involved in Controlling the Activation State of STIM1

SOAR, the Orai1 activating part of STIM1, is under control of the CC1 domain (aa 238–343, Fig. 2.1a, b) as CC1 is proven to play a key role in transducing luminal di-/oligo-merization to the cytosolic part of STIM1 resulting in homomerization and SOAR exposure [54–57]. Covington et al. [45] have examined homomerization using STIM1 C-terminal truncation mutants (STIM1-CC1 and STIM1-CC1-CAD) to study the impact of the respective cytosolic CC domains. Finally, they concluded that the mere presence of CC1 leads to store independent, yet unstable oligomerization, while the larger construct including CC3+SHD (see Fig. 2.1a) enables strong and store dependent oligomerization. Bioinformatic secondary structure predictions of CC1 suggest the presence of three alpha helical parts (α1, α2 and α3) within CC1 [23]. Helix α1 comprises aa 238–271, helix α2 aa 278–304 and helix α3 aa 308–337 (also known as inhibitory helix IH [54]). The structural resolution of STIM1 CC1 proved to be highly challenging [54], however, Cui et al. [58] have presented a crystal structure of STIM1 CC1 revealing a long helix which dimerizes in an antiparallel conformation. The full length CC1 in context with CAD has still not been shown.

Several studies have been performed to elucidate the role of CC1 in the control of STIM1 activation whereas different hypothetical models have been proposed (Fig. 2.1c). Korzeniowski et al. [59] suggested that an autoinhibitory, intramolecular electrostatic interaction between a glutamate-rich segment within α3 of CC1 and a lysine-rich region (aa382–387) within SOAR keeps STIM1-C terminus inactive. However, $\alpha3_{CC1}$ (the so called inhibitory helix IH) and the K-rich region are not in the close proximity to form the suggested electrostatic interaction, as evident from the CC1+SOAR crystal structure of *C. elegans* (Fig. 2.1b) [54]. Based on this structure, Yang et al. [54] propose that residues within $\alpha3_{CC1}$ interact with amino acids at the beginning of CC2 as well as the end of CC3 (Fig. 2.1c). Finally, they suggest that STIM1 forms a dimer in the resting state with the SOAR domain responsible for dimerization and $\alpha3_{CC1}$ stabilizing the inactive state of STIM1. Another hypothesis by Yu et al. [60] presents an intramolecular shielding model keeping STIM1 inactive. They suggest that the acidic region within $\alpha3_{CC1}$ is not involved in electrostatic interactions. However, they show that multiple mutations within $\alpha3_{CC1}$ affecting the amphipathic character of the helix have an impact. Therefore, they propose a hypothetical model in which the amphipathic nature of the $\alpha3_{CC1}$ regulates the STIM1 activation state [60]. To monitor STIM1 C-terminal conformational rearrangements, a double-labeled conformational sensor construct (YFP-OASF-CFP) has been developed [55] to examine STIM1 aa233–474 (OASF), comprising both CC1 and SOAR. FRET results show that OASF folds into a rather compact structure which likely corresponds to the quiescent state of STIM1 when Ca^{2+} stores are full. In line, Tb^{3+}-acceptor energy transfer measurements performed by Zhou et al. [61] revealed a similar compact structure of STIM1 C-terminus (233–685) where the K-rich segment at the C-terminal end is close to residue 233. To elucidate the molecular processes that trigger the cytosolic part of STIM1 from the inactive into the active form, both artificial crosslinking and mutations have been performed. By substituting amino acids in $\alpha1_{CC1}$ (L251S) or CC3 (L416S L423S) (Fig. 2.1a) within the OASF conformational sensor, FRET measurements have revealed an extended conformation [55]. By mutating full length STIM1 with these CC1 or CC3 point mutations, constitutive CRAC currents were obtained despite resting ER Ca^{2+} store conditions. Hence, the existence of intramolecular CC interactions within the quiescent STIM1 protein has been suggested. Those CC interactions are most likely released by specific $\alpha1_{CC1}$ or CC3 mutations or physiologically upon store depletion [55]. In line, Zhou et al. [61] have observed an extended conformation of STIM1 C-terminus induced by artificial crosslinking of the CC1 domains to promote CC1 dimerization. Furthermore, STIM1-CT with the "activating" mutation L251S also results in conformational extension [57] which is in accordance with FRET experiments using YFP-OASF_L251S-CFP. Together, this reveals the impact of $\alpha1_{CC1}$ on the transition of STIM1 from a quiescent into an active state. Furthermore, Zhou et al. have shown the interaction between monomeric CC1 and SOAR suggesting that store depletion induces CC1 dimerization, consequently leading to the extended conformation due to release of the CC1 interaction with SOAR. Finally, Fahrner et al. have identified, by the use of a FRET based technique, the direct CC1α1 – CC3 interaction as a key molecular determinant in setting the quiescent state of

STIM1 [56]. Release of that interaction is a necessary process for STIM1 activation. In summary, CC1 including α1 and α3 fulfils an important mechanistic role in keeping STIM1 in a quiescent, tight structure (see Fig. 2.1c) [54–57]. Additionally, Fahrner et al. have revealed a slight, destabilizing impact of CC1α2 which is apparently drastically enhanced by a R304W CC1α2 mutation that fully activates STIM1 without store depletion and is associated with the Stormorken Syndrome [62–64].

2.2.1.6 STIM1 Activation Model

Based on functional and structural data of human STIM1, a hypothetical STIM1 activation model [56] has been proposed depicting STIM1 conformations as pivotal function of CRAC activation. The model includes interaction of individual C-terminal domains showing the key steps of STIM1 C-terminal CAD exposure as sequential, molecular mechanism integrating helical rearrangement and clustering (Fig.2.1c). The inactive state of STIM1 is represented by a predominant CC1α1-CC3 clamp controlling the tight-structured quiescent STIM1 state. Additionally, CC1α3 supports this clamp, however the presence of CC1α1 is most essential [56]. Combining the recent functional data, the crystal structure by Yang et al. [54] and the NMR structure by Stathopulos et al. [65] suggest a STIM1 activation model where CC1α1 as well as CC1α3 directly interact with CC3/CC2 to stabilize the quiescent, tight conformation. In a first step, Ca^{2+}-depletion resulting in luminal EF-SAM domain destabilization and conformational changes, yields the driving force which induces the structural changes of the cytosolic STIM1 domains [43, 56, 57, 66]. Consequently, the dominant CC1α1 – CC3 clamp is released, triggering the C-terminal STIM1 extended conformation. Release of CAD induces a rearrangement of CC2 and CC3 resulting in formation of SOAP (STIM Orai activation pocket) [65] and exposure of CC3 for higher-order oligomerization and clustering. In this model, CC1α2 does not markedly contribute to the CC1-CC3 clamp, however, a pathophysiological mutation (R304W) has been reported yielding constitutive STIM1 activity associated with the Stormorken syndrome [62–64]. How this mutation affects the overall CC1 structure and how it induces the activated state of STIM1 still remains to be solved. It is tempting to speculate that the R304W mutation promotes the active, extended state of STIM1 thereby leading to constitutive Orai1 activation. STIM1 has also been reported to be a sensor of oxidative stress [67] and temperature variations stress [68] which indicates that STIM1 may generally act as a stress sensor that initiates Ca^{2+} signalling [69].

The second member of this protein family, STIM2, is also widely expressed and differs from STIM1 mainly in its N-terminal region. Differences affect luminal Ca^{2+} sensitivity and self-activation [46, 48, 70, 71]. Coupling of STIM2 to Orai1 is significantly reduced compared to STIM1 and it is assumed that STIM2 functions as competitive inhibitor of STIM1 mediated Ca^{2+} entry [72–74]. Furthermore, it has been shown that distinct Orai1 coupling domains in STIM1 and STIM2 define the Orai activating site [75]. Residue F394 in STIM1, which is substituted by a leucine (F394L) in STIM2 seems to be highly relevant for gating the Orai1 channel. Very

recently, Ong et al. [76] have demonstrated that STIM2 promotes recruitment of STIM1 to ER-PM junctions when ER stores are mildly depleted.

2.2.2 Orai

Named after the keepers of heaven's gate, Orai proteins represent the pore-forming subunit of the CRAC channel that opens upon physical interaction with STIM1 to permit Ca^{2+} influx [73, 77, 78]. There are three members of the Orai family expressed in mammals (Orai1-3) within selected tissues exhibiting elevated expression [21, 33]. All three Orai isoforms are tetra-spanning membrane proteins and exhibit the characteristic features of CRAC channels. Nevertheless, they behave distinct in their inactivation profiles and 2-aminoethyldiphenyl-borate (2-APB) sensitivity [79–82]. Orai proteins share no significant sequence homology to other previously identified ion channels and their activation by STIM1 proteins in the ER is highly unique. Members of the Orai family are ~30 kDa cell surface proteins that have four TM-spanning helices, with N- and C-termini located intracellularly. One intracellular and two extracellular loops connect the TM-spanning helices (Fig. 2.2a, c). The CRAC channel itself is composed of an oligomeric assembly of Orai1 subunits, potentially forming homo- as well as heteromeric Orai channels [80, 83–85]. Multimerization of Orai1 subunits is assumed to be established by their transmembrane regions as deletion of the C-terminal strands does not affect aggregation [39, 86]. A cluster of positively charged amino acids within the N-terminal first TM domain is fully conserved among the three Orai isoforms, while only Orai1 contains an N-terminal proline-arginine rich region [87]. Each of the Orai family member contains a coiled-coil domain within their C-terminus, essential for physical interaction with STIM1 [39, 88–90]. Ca^{2+} dependent fast inactivation (CDI) occurs in all three Orai homologs, is supposed to be mediated by cytosolic domains of both STIM1 and Orai1 [91–93], whereas Orai3 exhibits the highest degree of CDI [8, 80, 94]. Orai1 remains the best studied CRAC channel protein and appears to be the predominant isoform mediating SOCE in most cells. In the ensuing years after the identification of Orai1, cysteine-scanning studies first identified the pore-lining residues of the CRAC channel [95, 96] and in agreement with these studies, the crystal structure of the *Drosophila* Orai protein was determined recently, proposing – against all expectations – a hexameric assembly of Orai subunits around a central ion pore [90] (Fig. 2.2c–e). Although preliminary and recent biochemical and fluorescence studies pointed to a tetrameric assembly of Orai subunits [97–103], the evidence of a hexameric dOrai channel complex highly challenged this assumption. Although this is the best structural representation available, it is of note that the closed-state dOrai1 crystal structure, in 3.35 Å resolution, lacks substantial parts of the N- and C-termini, the extracellular TM1-TM2 loop, the intracellular TM2-TM3 loop and is furthermore mutated at positions C224S, P276R, P277R and C283T [90]. Atomic structures of human Orai channels will give further insight into channel architecture.

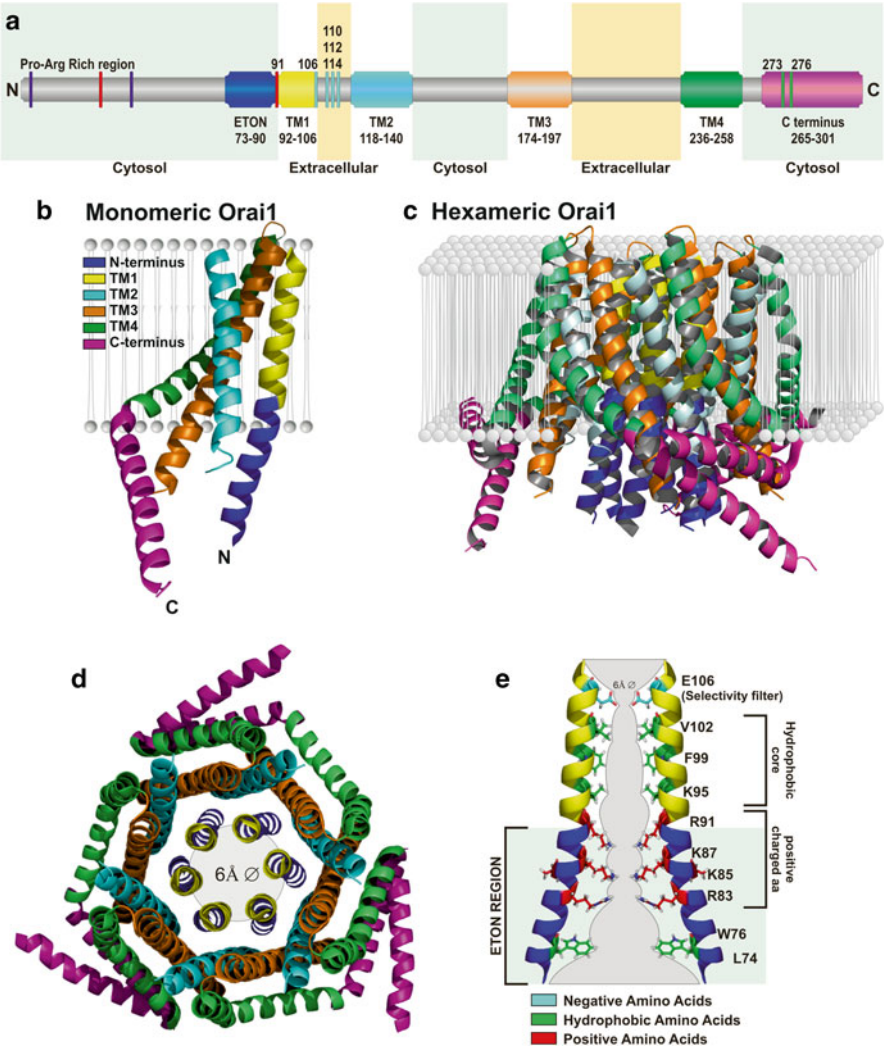

Fig. 2.2 (**a**) Schematic representation of human, full-length Orai1 depicting the overall structure and residues indispensable for Orai1 function. (**b**) Cartoon representing a single Orai1 subunit with the four transmembrane (TM) regions, the N-terminal as well as the C-terminal elongated helices, shown in different colors used throughout (**a**–**d**). (**c**) Cartoon showing the assembly of six Orai subunits based on the X-ray crystal structure of *Drosophila* Orai. Transmembrane domains of the six subunits are arranged as concentric rings around the ion pore, with TM1 forming the inner ring surrounding the ion pore. (**d**) Assembly of the Orai1 pore seen from the *top*. (**e**) Cartoon representing the human Orai1 pore showing 2 TM1 strands together with the cytosolic, helical extensions including the conserved ETON region. Relevant pore-lining amino acids, especially the selectivity filter, the hydrophobic core as well as residues within the ETON region are highlighted

The central architecture of dOrai shows that six TM1 helices compose the funnel structured pore that extends into the cytosol, surrounded by TM2 to TM4 whereby TM2 and TM3 form the second layer, shielding TM1 from the surrounding lipid bilayer and therefore likely provide structural support [90] (Fig. 2.2c, d). The cytoplasmic ends of the N- and C-terminal helices extend into the cytosol and are in close proximity of each other [90, 104] (Fig. 2.2b, c). Both of these regions have been shown to be necessary for interaction with STIM1 and both are essential for channel activation [39, 59, 105–108]. The pore-lining TM1 helix is identical in all three Orai proteins (Fig. 2.2e), with the extracellular loop between TM3 and TM4 being the most divergent among isoforms. It has been shown that a glutamate residue situated at the extracellular end of TM1 conveys Ca^{2+} selectivity (E106). A mutation to either glutamine or aspartate (E106Q/D in the human Orai1 isoform) drastically alters ion selectivity [109–111]. Mutation to aspartate (E106D) is supposed to widen the pore and therefore increases permeability to larger monovalent cations like Cs^{+}. Substitution of this critical glutamate by an uncharged residue results in non-functional channels and in line suppresses endogenous CRAC currents. The selectivity filter is the narrowest part of the pore with a diameter around 6 Å and it is surrounded by a ring of TM3 glutamate residues (E190) (Fig. 2.2e). The selectivity filter at position E106 may be fine-tuned by the help of negatively charged aspartates in the first extracellular loop (D110, D112, D114) that also alter selectivity upon a triple mutation to alanines [110]. However, single point mutations of these aspartates in the first loop retain Ca^{2+} selectivity but affect inhibition by Gadolinium [111]. Several cysteine loop1-mutants are able to form disulphide bonds and dimerize which suggests a close proximity of adjacent first loops within an Orai channel complex [95]. This suggests that the first loop of Orai channels is a flexible segment that can undergo conformational changes [112]. Overall, the currently proposed model is that Ca^{2+} ions are guided via E106 through the pore thereby maintaining the high Ca^{2+} selectivity of CRAC channels. Similarly to E106 in Orai1, it has been shown that E81 in Orai3 (equivalent to E106 in Orai1) also controls Ca^{2+} selectivity [82, 113]. One helix turn more central to the selectivity filter in Orai1, valine at position 102 (V102), has been proposed to function as a gate [114] and to constitute a barrier to ion flux in the closed Orai channel [115] (Fig. 2.2e). A constitutively active V102C/A channel displays changes in selectivity which leads to a reduced Ca^{2+} selectivity due to an increased pore size. Upon coexpression with STIM1 these mutants regain Ca^{2+} selectivity [114, 116]. Recently, it was shown that STIM1 dependent gating entails a structural change in the vicinity of residue E106 and V102 near the external entrance of the Orai1 pore [114, 115]. The Orai1 pore architecture thus changes upon interaction with STIM1 which underlines the close coupling of gating and ion selectivity in CRAC channels. Glycine at position 98 (G98) has been proposed to function as a gating hinge [116]. While mutations to aspartate or proline (G98D/P) lead to constitutively active channels, substitution to alanine completely abrogated channel function. Residue R91, originally discovered in a human Orai1 channelopathy, as R91W leads to non-functional channels [18],

seems to collectively form an electrostatic gate towards the cytosolic portion of TM1. Mutation of position 91 to hydrophilic side chains allowed the channel to function normally [91, 116] while hydrophobic substitution completely abolished channel function. Therefore, arginine at position 91 plays a critical role in channel gating. The constriction site of R91W is bypassed with an additional G98D mutation. The mutation in the gating hinge is able to restore the function of the R91W SCID mutant and seems to extend the channel gate more efficiently than in the non-functional R91W and V102A/C mutant [114, 116]. Residues within TM3 have also been shown to alter gating and permeation of Orai1 channels. Although TM3s do not line the pore they have been shown to modulate both selectivity and gating. E190 in Orai1 contributes to Ca^{2+} selectivity and permeation whereby single point mutations increased the pore diameter to 7 Å [82, 109, 110, 117]. E190 is supposed to affect pore properties by altering intramolecular TM interactions [95, 96]. Residue G183 is also involved in channel gating as a G183A mutant abolished store-operated activation [112, 118]. In summary, the side chains of the following residues protrude into the pore: E106, V102, F99, L95, R91, K87, R83, Y80 and W76 (Fig. 2.2e). This is in accordance with their ability to dimerize upon cysteine substitution in crosslinking experiments except residue F99 [95, 96]. The picture that can be drawn from all the preliminary studies is that the CRAC channel pore can be subdivided into four defined regions (Fig. 2.2e): (1) the selectivity filter that is formed by a ring of glutamates that confer the pore a negative electrostatic potential; (2) a hydrophobic region comprising three α-helical turns with amino acids V102, F99 and L95 that are expected to establish Van der Waals interactions with each other; (3) a basic region comprising three α-helical turns with amino acids R91, K87, R83 that is suggested to coordinate anions. The basic region may be a vital part of the gating mechanism through anion binding that keeps the pore closed in the resting state [90]; and (4) a cytosolic region spanning two α-helical turns. This extension into the cytosol is about 20 Å in length and is called extended TM1 Orai1 N-terminal (ETON) region [119]. Three positively charged residues (R91, K87, R83) directly line the pore at the cytosolic side which creates an unusual environment for a cation channel and they have been suggested to provide both barrier as well as electrostatic stabilization [119]. The ETON region additionally contains two serines (S90, S89) embedded between R91 and K87 which may contribute to the flexibility of the successive N-terminal and TM1 segments [104]. Overall, the inner portion of TM1 (aa91–98) seems to possess more flexibility than the outer region (aa99–104) which forms a more rigid structure [96]. The resolved *Drosophila* crystal structure provides a framework for investigating permeation and selectivity in human CRAC channels. Clearly, it takes further analysis to finally incorporate that knowledge into plausible models for human Orai1 channels. What conformational changes occur during channel opening, closing and inactivation? Channel structures of both closed and open state are needed to provide insight into CRAC channel activation pathways and for drug design of channel blockers and activators.

2.3 STIM1-Orai1 Coupling and CRAC Activation

STIM1 coupling to the Orai1 channel involves the cytosolic strand of STIM1 and both the Orai1 C-terminus as well as the N-terminus (Fig. 2.3). In three independent studies [39, 51, 120] Orai1 C-terminal truncation mutants have for the first time allowed an insight into the STIM1 Orai1 interaction process as it has been shown that Orai1 C-terminus is necessary for the direct coupling to STIM1 C-terminus using co-localization and FRET experiments. As clearly seen in the dOrai crystal structure, the Orai1 TM4 extends into the cytosol (cytosolic part named Orai1 C-terminus) [90] and is therefore accessible to STIM1 C-terminus for coupling. Consequently and based on these data, the focus has been set to elucidate specific residues within Orai C-terminus responsible for coupling to STIM1 C-terminus. Frischauf et al. [89] have suggested a weak coiled-coil probability of Orai1 C-terminus using bioinformatic predictions, while the coiled-coil probability of Orai2 and Orai3 is shown to be 15–17-fold higher suggesting that the affinity for STIM1 increases with the higher probability of Orai C-terminal CC domains. Vice versa, reducing the probability of STIM1 CC2 by an amino acid substitution L373S interferes with Orai1 activation whereas Orai2 and Orai3 still show partial activation [89]. In line, a double mutation in STIM1 CC2 fully disrupts coupling to and activation of all three Orai channels, suggesting that STIM1 – Orai coupling is dependent on these CC domains [89]. The hydrophobic residues L273 and L276 within Orai1 CC domain play an important role in coupling to STIM1. In addition, these positions have been shown to play a role in Orai dimerization within the hexameric Orai structure [90]. Besides coiled coil interactions based on hydrophobic

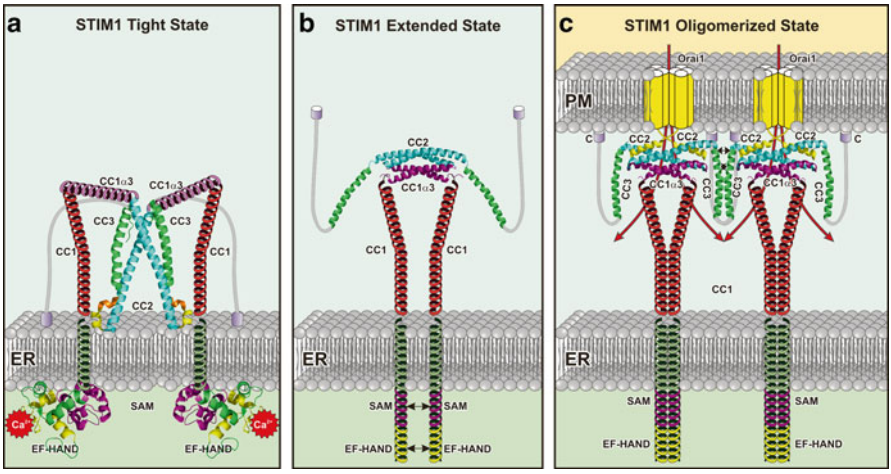

Fig. 2.3 Cartoons representing a model for the oligomerization process of STIM1 (**a**, **b**) and Orai1 coupling (**c**). Upon store depletion, STIM1 proteins adopt an extended conformation (**b**) and oligomerize, processes that are controlled by the CC1 domain. (**c**) Activated Orai1 channel with CC2 involved in the coupling to Orai1 C – terminus

residues, Calloway et al. have proposed electrostatic interactions between STIM1 and Orai1 [105, 121], hypothesizing formation of salt bridges between acidic residues within Orai1 C-terminus and positively charged amino acids in STIM1 (KIKKKR – aa 382–387). Deletion of this basic cluster within STIM1 disrupts store dependent association with Orai1, however, mutating the acidic residues within Orai1 C-terminus still allows for coupling and retained channel function suggesting that the coupling process involves other structural components [65, 105]. Recently, Stathopulos et al. presented two NMR solution structures of a human cytosolic STIM1 fragment (aa 312–387) comprising a part of CC1 and the whole CC2. One structure is presented without Orai1CT and the second structure is shown together with Orai1CT (Orai1 aa272–292) [122]. The STIM1 CC1-CC2 subunits form an antiparallel U-shaped homodimer. Furthermore, the dimeric STIM1 CC2 helix clampes two Orai1 C-terminal fragments in a hydrophobic/basic STIM-Orai association pocket called SOAP. STIM1 mutations interfering with CC1 dimeric interactions inhibit, while substitutions enhancing CC1 stability spontaneously activate Orai1 channels. On the other hand, CC2 mutations have revealed variability in Orai1 activation due to binding Orai1 as well as autoinhibiting STIM1 oligomerization [122]. In summary, STIM1 – Orai1 coupling mediated between Orai1 C-terminus and CC2 of STIM1 predominantly involves hydrophobic interactions as well as ionic bond formation. In another recent paper Wang et al. [75] have studied the differences of SOAR of STIM1 and STIM2 in coupling to and activating Orai1. Herein, they show that SOAR1 and SOAR2 have similar sequences and predicted secondary structures but behave differently in functional coupling to Orai1. By use of SOAR1/SOAR2 chimeras Wang et al. have been able to identify a crucial residue that determines the differences in binding and activation of Orai1. SOAR contains four α-helical parts (see Fig. 2.1b) that are systematically switched and analyzed in these chimeras. Helix Sα4 has been shown to behave similarly in SOAR1 and SOAR2, whereas the residue F394 in Sα2 of SOAR1 seems to be highly crucial for gating Orai1. Introduction of F394 point mutations to L, A or H in full length STIM1 decreases or fully abolishes SOCE, respectively, when coexpressed with Orai1 in HEK293 cells. Patch clamp and FRET analysis finally have revealed that F394 in STIM1 is highly important for both coupling to and gating of Orai1 channels [75]. Park et al. elucidated that additionally to Orai1 C-terminus, the N-terminal strand acts as binding partner for STIM1, however, to a weaker extent [51]. All Orai proteins contain a highly conserved region in the N-terminal part (aa73–90) termed ETON [119] which corresponds to the elongated extension of TM1 into the cytosol. Orai1 deletion mutants lacking ETON or the full N-terminal strand completely lose function. In line, N-terminal truncations of Orai1 (Orai1 Δ1-74 or Δ1-75) still yield store dependent activation whereas the deletion mutant Orai1 Δ1-76 results in loss of function [86, 119]. By introducing point mutations within the ETON region the hydrophobic residues L74 and W76 seem to be part of the Orai1 N-terminal STIM1 binding interface as the double mutant results in highly reduced STIM1 interaction [119]. Furthermore, the positively charged amino acids R83 and K87 have been shown to contribute to the STIM1 binding interface and additionally provide a stabilizing effect to the elongated pore [119]. Another study by McNally et al. [107]

reveals that drastic deletions in the Orai1 N-terminus (Orai1 Δ1-85 and Orai1 Δ73-85) abolished binding to STIM1 as shown with the Orai1-CAD-CAD multiple fusion protein. However, restoration of channel function of Orai1 N-terminal point mutations have been analyzed using the fusion protein but are not visible using the coexpression of Orai1 mutant + STIM1. Additionally, Zheng et al. [108] have analyzed progressive N-terminal truncations yielding the result that Orai1 N-terminus is essential for binding STIM1. However, the presence of both Orai1 N- and C-terminus is crucial for correct STIM1 coupling as they suggest that the STIM1 first binds to Orai1 C-terminus followed by STIM1 – Orai1 N-terminus interaction. In summary, the ETON-region functions as binding partner for STIM1 and provides electrostatic components having an impact on the structure of the elongated pore. ETON is fully conserved between Orai1 and Orai3, however, extensive Orai3 N-terminal truncations still retain STIM1 dependent activation whereas equivalent Orai1 deletion mutants result in loss of function [119, 123]. Therefore, Orai3 may contain additional residues/domains which contribute to STIM1 coupling and Orai3 activation, despite more extensive deletions at the N-terminal strand. Overall, the SOAR domain of STIM1, which is sufficient for Orai1 coupling and activation, most probably involves binding of both Orai1 C- as well as N-terminus. The resulting bridge probably generates a force inducing a conformational change of the ETON and TM1 regions resulting in Ca^{2+} influx probably due to movement of the hydrophobic gate at TM1 V102 as well as removal of the electrostatic hindrance in the ETON region. The mechanistic steps in this process clearly require further analysis.

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Chapter 3
The TRPCs, Orais and STIMs in ER/PM Junctions

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Abstract The Ca^{2+} second messenger is initiated at ER/PM junctions and propagates into the cell interior to convey the receptor information. The signal is maintained by Ca^{2+} influx across the plasma membrane through the Orai and TRPC channels. These Ca^{2+} influx channels form complexes at ER/PM junctions with the ER Ca^{2+} sensor STIM1, which activates the channels. The function of STIM1 is modulated by other STIM isoforms like STIM1L, STIM2 and STIM2.1/STIM2β and by SARAF, which mediates the Ca^{2+}-dependent inhibition of Orai channels. The ER/PM junctions are formed at membrane contact sites by tethering proteins that generate several types of ER/PM junctions, such as PI(4,5)P_2-poor and PI(4,5)P_2-rich domains. This chapter discusses several properties of the TRPC channels, the Orai channels and the STIMs, their key interacting proteins and how interaction of the STIMs with the channels gates their activity. The chapter closes by highlighting open questions and potential future directions in this field.

Keywords TRPC channels • STIM1 • STIM2 • Orai • ER/PM junction

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3.1 Introduction

Ca^{2+} influx across the plasma membrane (PM) is a central component of the receptor-evoked Ca^{2+} signal. Receptor-stimulated Ca^{2+} influx is activated shortly after and in response to IP_3-mediated Ca^{2+} release from the endoplasmic reticulum (ER) [1] and Ca^{2+} release from acidic compartments [2]. Stored Ca^{2+} is limited and cannot sustain the receptor-evoked Ca^{2+} signal without constant replenishment by Ca^{2+} influx from the vast extracellular pool, both during the physiological Ca^{2+} oscillations and at the end of the stimulation period [3, 4]. As such, Ca^{2+} influx across the PM participates in all Ca^{2+}-mediated cellular functions [4, 5]. At the same time excessive Ca^{2+} influx by the PM channels is highly toxic and causes most of the pathology of Ca^{2+}-related diseases [6, 7].

The receptor-stimulated Ca^{2+} influx is mediated by two families of PM channels, the TRPC and Orai channels [6]. It is now firmly established that the ER Ca^{2+} sensor STIM1 gates these channels, although there are significant differences in the way STIM1 gates the TRPC and Orai1 channels. The properties, regulation and role of the TRPC [8–10] and Orai channels [11, 12] and their interaction with and gating by STIM1 have been extensively discussed and reviewed [13]. In this chapter, we briefly summarize the key features of this topic. Rather, we focus on new information on the regulation of Ca^{2+} influx channels emerging in the last few years and how it may guide future studies of the TRPC channels and their interaction and regulation by STIM1 and STIM1-associated proteins and regulators.

3.2 The SOCs Components

3.2.1 The TRPC Channels

The TRPC channels were discovered as the homologues of the drosophila TRP channel that mediate at least part of the receptor-stimulated Ca^{2+} influx [14, 15]. Their discovery lead to the discovery of all 28 members of the TRP channels superfamily and their role in incredibly diverse physiological roles [16]. Like all other TRP channels, TRPC channels are tetrameric, polymodal channels with each subunit having six transmembrane domains. The TRPCs function as non-selective cation permeable channels with significant Ca^{2+} permeability. No information is available on the structure of the TRPC channels, but recent work used electron cryo-microscopy to report the nearly atomic resolution of the structure of the TRPV1 [17, 18] and TRPA1 channels [19] that highlight several general features applicable to other TRP channels. The channels display tetrameric architecture with the subunits arranged in fourfold symmetry around a central ion permeation path that is formed by transmembrane domains S5 and S6 and the pore helix. The C terminus TRP domain that is found in most TRP channels interacts with both the S4–S5 linker and an N terminus helix located prior to transmembrane domain S1

[17]. In TRPC channels the TRP domain is followed by a coiled-coil domain. Interestingly, many channel modulators and activators interact with or in the region between these domains (see [20] and below). The importance of this region and interaction of the TRP domain with the S4–S5 linker and pre-S1 in TRP channels gating is suggested by the gain of function mutations in TRPV3 and TRPV4 that may disrupt these interactions and lead to Olmsted syndrome [21] and skeletal dysplasia syndromes [22], respectively.

The TRPV1 pore has two, upper and lower gates that restrict conduction. The channel can be in conformations with one or both gates open [18] to allow dynamic regulation of channel conductance with the pore helix coupling the upper and lower gates and perhaps their closing and opening. The dynamic nature of the TRPV1 pore is also suggested by pore dilation in response to agonist stimulation [23]. Similar two gates arrangement may exist in other channels, including TRPML3 [24] and TRPC4/TRPC5 [25], that also undergo pore dilation. Pore opening also involves major conformational changes in the S3–S4 helices, S4–S5 linker and S6 helix [18]. The effect of interaction of TRP domain with S4–S5 linker on these conformational changes remains to be determined.

TRPC Channel Interactors TRPC channels have several domains that interact with other proteins, which keep the channels in defined membrane compartments and regulate channel activities. Comprehensive list of TRPC channels interactors can be found in http://www.trpchannel.org/. Here, we only discuss several interactors pertinent to the topic of this review. All TRPC channels except TRPC7 interact with the scaffolding/adaptor Homer proteins [26]. The Homers have an N terminus EVH domain that mediates protein-protein interaction, and a C-terminus coiled-coil domain that serves to multimerize the Homers into large scaffolds. The EVH domain interacts with proteins having the ligands PPXXF, PPXF and/or LPSSP [27, 28]. The Homers bind to the Homer binding ligands in the N and C terminus of the TRPC channels to ligand the PM TRPC channels with the IP_3 receptors at ER/PM junctions and keep the channels in a close state [26, 29].

An interesting set of TRPC channels interactors are Caveolin, Junctate and Junctophilin. Caveolin interacts with many TRPC channels to recruit and stabilize them in PM lipid rafts [30–32]. This interaction is essential for assembly of TRPC1 into complexes within ER/PM junctions to mediate key function of the channels that are both cell specific and channel specific. This is exemplified well in the action of TRPC1 is salivary glands. Ca^{2+} influx by TRPC1 specifically stimulates salivary glands fluid secretion [33] and gene activation [34]. Other examples include the roles of TRPC1 and TRPC4 in endothelial cells function [35], control of myotube size [36] and axonal guidance [37]. Junctate is a widely expressed integral ER/SR Ca^{2+} binding protein that affects Ca^{2+} signaling in both muscle and non-muscle cells [38]. Notably, junctate expression increases and knockdown decreases the ER/PM junctions [39]. Junctate forms a signaling complex with the IP_3Rs and TRPC3 [39] to regulate both the IP_3-mediated Ca^{2+} release and TRPC3-mediated Ca^{2+} influx. Interestingly, expression of TRPC3 with junctate increases the number and size of the ER/PM junctions [40]. Other TRPC channels shown to interact with Junctate are

TRPC2 and TRPC5, all of which localize in the acrosomal crescent of the sperm head [41]. Junctate also interacts with and regulate Orai1 [42]. This topic is addressed further below.

The four Junctophilins (JPs) are also present at ER/PM junctions, but their expression appears to be more restricted, with JP1 and JP2 found mainly in muscles and JP3 and JP4 in brain, although JP3 and JP4 are likely expressed in other tissues as well [43, 44]. The JPs participate in the formation of ER/PM junctions with the eight conserved C terminus JP-specific MORN motifs tightly interacting with $PI(4,5)P_2$ at the PM [44]. Knockout of JP1 and JP2 in mice disrupts the ER/PM junctions in muscles and the crosstalk between PM Ca^{2+} channels and the ER/SR Ryanodine receptors and E-C coupling. Accordingly, mutations in these JPs are associated with several cardiac and other muscle myopathies in humans [43, 44]. Individual deletion of JP3 and JP4 in mice had no obvious phenotype, but deletion of both JP3 and LP4 inhibited salivary secretion and neuronal control of feeding [45], suggesting a role of these JPs in peripheral tissues.

3.2.2 The Orai Channels

The Orai channels were discovered as the proteins that mediate the highly Ca^{2+} selective Ca^{2+} release activated current (CRAC) [46–48]. Numerous studies used mutations analysis with biochemical, biophysical and electrophysiological studies to determine several key features of the Orai channels and these have been extensively reviewed (for several latest reviews see [11, 12, 49]). Each subunit of the Orai channels has four transmembrane spans with cytoplasmic N and C termini. Understanding the Orai structure-function was dramatically increased with the description of the crystal structure of drosophila Orai channel that is homologous with mammalian Orai1 [50]. The structure revealed that the channel is a hexamer, rather than a tetramer as suggested by biochemical and single molecule photobleaching studies [51–53]. The crystal structure revealed several key features of the Orai channels. It confirmed that the pore is formed by the first transmembrane domain [54] and further showed that the selectivity filter is composed of a ring of glutamates [50]. Proline residue in the middle of the fourth transmembrane domain caused a bend resulting in part of the domain lining perpendicular to the membrane. As a result, the fourth transmembrane domains cytoplasmic extensions of each of two subunits fold into coiled-coil domains. Part of this coiled-coil domain interacts with a STIM1 SOAR dimer to participate in channel opening [55, 56] that is driven by STIM1-mediated conformation transition [57].

Orai Channels Interactors The Orai channels, in particular Orai1, interact with several scaffolding and regulatory proteins. A particularly interesting interaction is heterodimerization of Orai1 with Orai3 that generates STIM1-regulated, but store-independent channel that is activated by receptor-generated arachidonic acid [58–60]. Large number of studies reported that the Orai channels are present in complexes

with TRPC channels. However, these interactions are indirect and are largely mediated by STIM1 [9, 13, 30]. A protein that binds both TRPC (see above) and Orai1 channels is caveolin, which interacts with the N terminus of Orai1 to mediate Orai1 endocytosis and recycling [61]. Caveolin is also required for recruitment of the Orai1-STIM1 complex to a $PI(4,5)P_2$-rich domain [62]. The Orai1 cytoplasmic N terminal domain binds calmodulin [63, 64], which was suggested to mediate the fast Ca^{2+}-dependent inactivation (FCDI) of Orai1 [65]. However, the discovery that SARAF mediates the SCDI of Orai1 [66], and that SARAF interacts with the STIM1 SOAR domain [67] open the question of the role of calmodulin in the regulation of Orai1 function. Another protein containing EF-hand that interacts with the N terminus of Orai1 is CRACR2A (for CRAC Regulator 2A) [68]. How CRACR2A regulates channel activity remains to be determined.

3.2.3 The STIMs

A search for the factor that couples Ca^{2+} release from the ER to activation of Ca^{2+} influx channels led to the discovery of ER Ca^{2+} content sensor STIM1 [69, 70]. The original studies noted the presence of STIM2, but its role in Ca^{2+} signaling remains significantly less understood as compared to STIM1. The potential functions of STIM2 are discussed in some details at the end of this review. Extensive deletions and mutations analysis over the last 10 years defined several STIM1 domains, while STIM2 domains are largely inferred based on their homology with STIM1. The STIMs have a single transmembrane domain with N terminus in the ER lumen and C terminus in the cytosol. The STIMs ER lumen has Ca^{2+} binding EF hand and SAM domain. The STIM1 and STIM2 EF-hands are the Ca^{2+} sensors with the STIM1 EF-hand binding Ca^{2+} at higher apparent affinity than the STIM2 EF-hand. The SAM domain participates in STIM1 clustering [71, 72]. Binding of Ca^{2+} to the EF hand keeps STIM1 in a non-clustered form and away from the ER/PM junctions.

The cytosolic domain of STIM1 opens both the TRPC and Orai channels [73]. It starts with the first coiled-coil domain (CC1) that is divided to several sub coiled-coil domains [74] and includes a C terminus short helix that functions as an inhibitory helix [75, 76]. CC1 is followed by the SOAR domain [77], which is also known as CAD [63] or CCb9 [78]. SOAR is the minimal STIM1 domain needed to fully activate Orai1 [63, 77, 78]. The crystal structure of SOAR indicates R-shaped monomers that assemble into a V-shaped dimer [76]. STIM2 splice variants at the tip of the SOAR R curve generates two STIM2 isoforms that can either inhibit or facilitate Orai1 activation [79, 80]. STIM1 SOAR is followed by a C Terminus Inhibitory Domain (CTID) [67] that is followed by a long sequence that may participate in occluding SOAR in a close state [75]. The most C terminal STIM1 and STIM2 sequences are polybasic with multiple lysine residues (K-domains). The STIM1 and likely STIM2 K-domains interact with $PI(4,5)P_2$ at the ER/PM junctions [62, 81] to determine gating of Orai1 by Ca^{2+} [62]. Activation of the Orai and

TRPC channels by STIM1 involves major conformational changes to unocclude the SOAR domain. When STIM1 in not at the ER/PM junctions the SOAR domain is occluded in a sandwich formed by part of CC1 at the N terminus of SOAR and by CTID at the C terminus of SOAR. Ca^{2+} store depletion unwinds STIM1 to an extended conformation to release SOAR and allows its interaction with and activation of the Orai and TRPC channels [67, 75, 82, 83].

STIM Interactors The function of the STIMs is regulated by Ca^{2+} binding and unbinding to the ER hand and by interaction of the open STIMs with plasma membrane $PI(4,5)P_2$. STIM1 also interacts with other proteins that stabilize the open or closed conformations or mediate Ca^{2+}-independent STIM1-dependent activity. An example of the later is interaction of STIM1 with the microtubule-plus-end-tracking protein end binding (EB1) [84]. Interaction of STIM1 with EB1 occurs at the sites of microtubules interaction with the ER and is regulated by phosphorylation of STIM1 in serine/threonine residues [85] and by ERK1/2-mediated phosphorylation at S575, S608 and S621 [86]. The mode of interaction of STIM1 with EB1 that does not affect Ca^{2+} influx appears to participate in ER remodeling [84, 85], while phosphorylation by ERK1/2 leads to dissociation of EB1 to facilitate STIM1 clustering and activation of Ca^{2+} influx channels [86]. The EB1 interaction with STIM1 appears to be regulated by the microtubule binding protein adenomatous polyposis coli (APC) [87]. APC interacts with the STIM1 C terminus (STIM1(650–685)) to dissociate the EB1-STIM1 complex, promotes STIM1 clustering and activation of Orai1 [87]. Another STIM1 function independent of Ca^{2+} influx is the regulation of endothelial barrier function by thrombin stimulated STIM1-mediated activation of RhoA, formation of actin stress fibers and loss of cell-cell adhesion [88].

An interesting regulation of STIM1 is by the ER redox potential. It was reported that oxidative stress leads to STIM1 S-glutathionylation at C56 to promote STIM1 translocation to ER/PM junctions and activation of Orai1 [89]. On the other hand, another study reported that the ER-resident oxidoreductase ERp57 interacts with the ER luminal domain of STIM1 to modify C49 and C56 and inhibits Ca^{2+} influx by reducing STIM1-Orai1 interaction. Mutation of C49 and C56 did not result in spontaneous activation of STIM1 and Ca^{2+} influx [90] as would be predicted by the findings of [89]. Regulation of Ca^{2+} influx by ER redox state has many physiological implications and thus this issue deserves clarifications and further in depth exploration.

A negative regulator of Ca^{2+} influx that interacts with STIM1 is SARAF [66]. SARAF is a single transmembrane span, ER resident protein with the N terminus in the ER lumen and the C terminus in the cytoplasm. A prominent regulation of Ca^{2+} influx by Orai1 is Ca^{2+} itself, which causes two form of inhibition, fast (FCDI) and slow Ca^{2+}-dependent inactivation (SCDI) with time courses of msec and min [4]. Before the discovery of SARAF the only information on the mechanism for Ca^{2+}-dependent inactivation was that a negatively charged STIM1 sequence (^{475}DDVDDMDEE^{483}) mediates the FCDI [65, 91, 92]. However, this may change with the discovery that SARAF mediates SCDI [66] and likely FCDI [62]. Systematic domain analysis showed that SARAF interacts with SOAR and the

interaction is regulated by the STIM1 CTID domain [67]. Furthermore, CTID appears to have two lobes, an N terminal lobe (STIM1(447–490)) that restricts access of SARAF to SOAR and a C terminal lobe (STIM1(491–530)) that facilitate interaction of SARAF with SOAR [67]. Significantly, CTID also regulates FCDI of Orai1 since inhibition of SARAF interaction with STIM1 prevents FCDI [67]. Notably, interaction of SARAF with STIM1 take place only when the STIM1-Orai1 complex is in a ER/PM junctions $PI(4,5)P_2$-rich domain [62].

3.3 Gating by STIMs

3.3.1 Gating TRPC Channels by STIM1

Comprehensive reviews of this topic can be found in [9, 10, 13, 30]. A large body of biochemical and functional evidence indicates direct or indirect interaction between TRPC channels and STIM1 in many cell types. However, unlike the Orai channels, the TRPC channels can function both in a STIM1-dependent and STIM1-independent modes and some TRPC channels are gated by STIM1 only when they heteromultemerize with other TRPC channels that directly bind STIM1. This contributed to the confusion and the misunderstanding of how STIM1 gates the TRPC channels. For example, TRPC1, TRPC2, TRPC4 and TRPC5 directly interact with STIM1, while TRPC3, TRPC6 and TRPC7 do not [73]. The STIM1 SOAR domain mediated the interaction with TRPC channels and similar pattern is observed when interaction of TRPC channels with SOAR is examined [93]. However, when TRPC1-TRPC3 [94, 95] and TRPC4-TRPC6 heteromultimerize TRPC3 and TRPC6 gain access to STIM1 and their function changes from STIM1-independent to STIM1-dependent [94]. Functioning of the channels as a heterodimers also occurs in vivo. This is shown in Fig. 3.1 in pancreatic acini taken from $Trpc1^{-/-}$ $Trpc3^{-/-}$ mice [93]. Knockout of TRPC1 or TRPC3 in mice and inhibition of TRPC3 by Pyrazole 10 reduced receptor-stimulated and store depleted Ca^{2+} influx by about 50 % (Fig. 3.1a, b) and inhibition of TRPC3 in $TRPC1^{-/-}$ acini has no further effect. This indicates that the two TRPC channels cooperate to mediate the same Ca^{2+} influx pathway and Ca^{2+} influx by the channels is interdependent. For this the channels and STIM1 have to be present in the same Ca^{2+} signaling complex. This is indeed shown in Fig. 3.1d, which shows that cell stimulation markedly enhances the interaction between STIM1, TRPC1 and TRPC3.

Exploring the molecular mechanism of turning the TRPC channels from STIM1-independent to STIM1-dependent channels revealed that TRPC3 does have a STIM1/SOAR interacting domain within its C terminus coiled-coil domain (CCD). However, this site is hidden by the N terminus CCD that interacts with the C terminus CCD to mask the STIM1/SOAR binding site. Interaction of the TRPC1 CCD with the TRPC3 N terminus CCD exposed the TRPC3 STIM1/SOAR interaction site at the C terminus CCD. In this open conformation the function of TRPC3 is

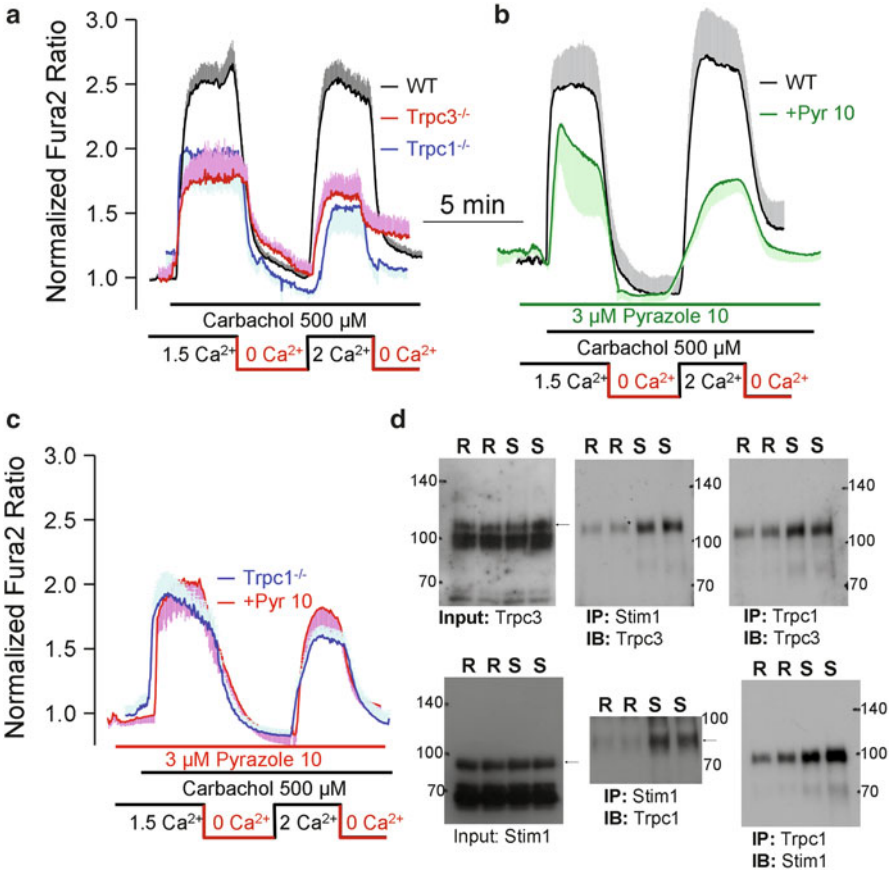

Fig. 3.1 The TRPC1-TRPC3 heterodimer acts to mediate part of the SOCs in vivo. Ca^{2+} was measured in Fura2-loaded salivary gland ducts isolated from wild-type, $Trpc1^{-/-}$ and $Trpc3^{-/-}$ mice. The ducts were perfused with solutions containing 1.5 mM Ca^{2+} or 0.2 mM EGTA (Ca^{2+} free conditions) as indicated by the *bars*. **b**, **c** Ca^{2+} was measured in salivary gland ducts from wild-type **b** and $Trpc1^{-/-}$ mice **c** treated with 3 μM of the Trpc3 inhibitor Pyrazole 10 (see [132]). Note that TRPC1 and TRPC3 mediate the same Ca^{2+} influx pathway and both are needed to keep the pathway active. **d** Mutual co-immunoprecipitation (Co-IP) of Trpc1/Stim1/Trpc3 in resting and stimulated salivary gland cells. Co-IP was tested in resting cells (marked with *R*) and cells stimulated with 100 μM carbachol and 25 μM CPA (marked with *S*) (The results were reproduced from [93])

strictly STIM1-dependent [93]. The two modes of the activity of TRPC1 and TRPC3 channels with respect to STIM1 dependence and independence and how these modes are formed is illustrated in Fig. 3.2.

The STIM1 SOAR domain mediates interaction with the TRPC channels CCDs but the STIM1 polybasic K-domain is essential for the gating [96]. The K-domain is essential for recruiting the STIM1-Orai1 complex to a $PI(4,5)P_2$-rich ER/PM junctions [62]. It may have the same function in TRPC channels (see below-open questions), but in the case of TRPC channels the K-domain appears to have

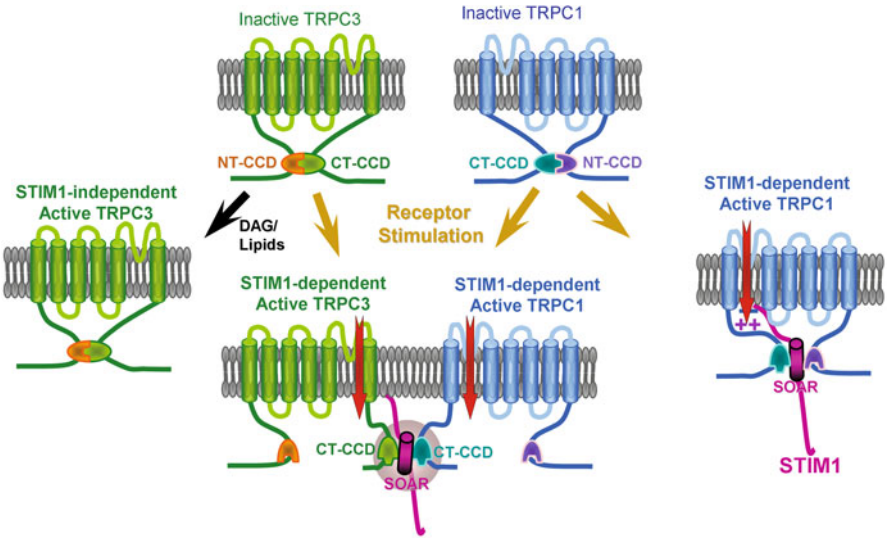

Fig. 3.2 Modes of STIM1-dependent and STIM1-independent functions of TRPC1 and TRPC3. In the inactive state the N and C termini CCDs interact to keep the channel in the inactive state. In cell that express TRPC3 and not TRPC1, TRPC3 functions as a STIM1-independent channel that can be activated by diacylglycerol, other lipids or activators. TRPC1 C terminal CCD has a SOAR binding site and upon cell stimulation and store depletion, this site becomes available for interaction of STIM1 to allow the STIM1 K-domain to facilitate channel activation. In cells expressing TRPC3 and TRPC1, cell stimulation results in formation of TRPC1-STIM1-TRPC3 complexes (see Fig. 3.1). In the complexes, the TRPC1 C terminus CCD interacts with the TRPC3 C terminus CCD to dissociates the interaction between the N and C terminus CCDs of TRPC3, allow binding of SOAR to the TRPC3 C terminus CCD and channel activation. In this mode TRPC3 functions as STIM1-dependent channel

additional function since deletion of the STIM1 K-domain prevents activation of TRPC1 by STIM1 [73] but not activation of Orai1 by STIM1 [62, 96]. The last two lysines (K^{684} and K^{685}) of STIM1 communicate with two negatively charged conserved residues (DD/DE/EE) in the C terminus of TRPC channels [29, 96]. The interaction appears to be electrostatic rather than affecting the channel pore since several combinations of negative-positive charges irrespective of the presence on TRPC channels or STIM1 resulted in active channel [96]. Gating by electrostatic interaction could be observed with TRPC3, TRPC4, TRPC5 and TRPC6 [29].

3.3.2 Gating Orai Channels by STIM1

This topic is reviewed elsewhere in this book and in [11, 12, 97, 98] and is only briefly mentioned here to highlight several points. The first is that physical and functional CCDs interactions of SOAR and the C terminal CCDs of TRPC channels [93] has some similarities with the interaction between STIM1 SOAR and Orai1 C

terminus CCD functionally [99, 100] and structurally [49, 50, 74]. SOAR has a second interacting site within the N terminus of Orai1 [63]. The interaction of STIM1 with both the C and N termini of the Orai1 participates in channel gating [56, 101] and is required for the effect of STIM1 on Orai1 channel selectivity [101, 102]. It will be important to find out whether STIM1 affects TRPC channels ion selectivity and the channels have different properties when functioning as STIM1-dependent and STIM1-independent channels.

Ca^{2+} inactivates/inhibits several TRPC channels like TRPC3 [103]. The three Orai channels also show isoform specific fast and slow Ca^{2+}-dependent inactivation [104]. In the case of Orai1 FCDI and SCDI are mediated by SARAF and regulated by CTID [66, 67]. Whether SARAF also mediate the FCDI of Orai2 and Orai3 and how or whether SARAF affects TRPC channels activity is not known. TRPC and Orai1 channels are present at the same ER/PM junctions where they likely affect the activity of each other [30]. How cellular processes and factors that regulate and modulate Orai1 function and gating by STIM1 affects TRPC channels function is not well understood. Two of these modes of Orai1 regulations that are drawing attention in recent studies are regulation by STIM2 and regulation by localization at specific ER/PM junctions. These are discussed below with respect to questions and future directions in research of TRPC channels as seen by the authors.

3.4 Open Questions and Future Directions

3.4.1 STIM1L Function in Orai and TRPC Channels

STIM1 has two isoforms, the one usually refer to as STIM1 and a long isoform STIM1L that has an additional C terminus actin binding domain of 105 residues [105]. The differential/complementary roles of the two STIM1 isoform have been examined to a limited extent with Orai1 and not at all with respect to TRPC channels. Moreover, the results of the regulation of Orai1 by STIM1L are not consistent. The original work reported that STIM1L preform clusters with Orai1 at the ER/PM junctions allowing robust and very rapid activation of Ca^{2+} influx by store depletion and enhances Ca^{2+} influx more than STIM1. Significantly, repeated activation of myotubes by depolarization with KCl resulted in rundown of the signal in myotubes with knockdown of STIM1L but not with knockdown of STIM1 [105]. Enhancement of Ca^{2+} influx by STIM1L and its interaction with Orai1 was subsequently observed in transfected HEK cells [106]. A more recent study confirmed enhanced pre-clustering of and enhanced Ca^{2+} influx by STIM1L, however, these authors concluded these clusters do not reflect co-clusters of STIM1L with Orai1. The puncta formed by STIM1L appear smaller than those formed by STIM1 and their size does not increase upon store depletion [107]. Finally the recent study concluded that STIM1L slows down Orai1 recruitment to the ER/PM junctions and the rate of

activation of Ca^{2+} influx [107], the opposite of the findings of using virtually the same assay and approach [105].

There is no obvious explanation for the opposite observations with respect to activation of Orai1 and Ca^{2+} influx by STIM1L indicated above. In addition, both studies used Mn^{2+} quench rather than current measurements, so that the exact effect of STIM1L on Orai1 current properties is not known at this time. This is particularly important since STIM1 regulates Orai1 selectivity [102]. Although it was reported that STIM1L interacts with TRPC3 and TRPC6 [106], STIM1L interaction with other TRPC channels is not known, let alone the effect of STIM1L on TRPC channels activation and activity. The role of STIM1L in Ca^{2+} signaling and activation/regulation of the Orai1 and TRPC channels deserves further and thorough examination.

3.4.2 STIM2 Function in Orai and TRPC Channels

Another STIM isoform is STIM2. Originally, it was suggested that the primary role of STIM2 is to set resting Ca^{2+} levels [108]. However, the role of STIM2 in Ca^{2+} signaling is more complex and recent work started to explore the role of STIM2 in Ca^{2+} signaling obtaining very interesting results. STIM2 on its own is not as potent as STIM1 in activation of Orai1 channels or Ca^{2+} influx. However, it has profound effect on Ca^{2+} influx through regulation of STIM1 function. The most interesting finding is that STIM2 controls the recruitment of STIM1 to ER/PM junctions by responding to minimal Ca^{2+} depletion from the ER [109]. Clustering of STIM1 and activation of Ca^{2+} influx in the absence of STIM2 required massive pathological stimulation and ER Ca^{2+} store depletion. STIM2 shifts to the left the dose response to receptor stimulation and store depletion dependent STIM1-Orai1 co-clustering and activation of Ca^{2+} influx. Hence, the main function of STIM2 is to be a better sensor of ER Ca^{2+} than STIM1 and to escort STIM1 to the ER/PM junctions at physiological stimulus intensity [109].

Another mode of regulation of STIM1 function by STIM2 was discovered recently with the identification of two STIM2 splice variants [79, 80]. STIM2.1/STIM2β has a modified SOAR domain that reduces its interaction with Orai1. However, STIM2.1/STIM2β binds strongly to STIM1 and is targeted to the puncta together with STIM1. When in the puncta, STIM2.1/STIM2β strongly inhibits Orai1 activation by STIM1 and Ca^{2+} influx [79, 80]. No information is available on the potential interaction and regulation of TRPC channels by STIM2 or by STIM2.1/STIM2β. However, such a potential regulation is suggested by a recent study reporting that depending on the ration of STIM1/STIM2 in a given cell STIM1 and STIM2 may activate two different channels, possibly Orai1 and TRPC-dependent channels [110]. It is clear that much more work is needed to understand the role of STIM2 in Ca^{2+} signaling, in particular its role in regulation TRPC channels activity.

3.4.3 Orai and TRPC Channels in the ER/PM Junctions

The interaction of STIM1-Orai1, STIM2-Orai1 and TRPC channels-STIM1 to activate Ca^{2+} influx take place at the ER/PM junctions. This topic is being reviewed extensively with several recent reviews on Ca^{2+} signaling in membrane contact sites [12, 30, 98, 111, 112] and on the formation and properties of ER/PM junctions [113–115]. In specialized cells like muscle, neurons and epithelia, the junctions are formed at distinct plasma membrane domains. For example, in polarized acinar cells the Ca^{2+} signal initiates at the apical pole and propagates to the basal pole in the form of Ca^{2+} waves [116, 117]. All Ca^{2+} signaling proteins are clustered at the apical pole including G proteins-coupled receptors, the IP_3Rs, TRPC channels, Orai1, STIM1, PMCA and homer proteins [98]. How the proteins are targeted to these domains is not known at present. However, this problem may be resolved in the near future as proteins that tether, stabilize and regulate the function of the ER/PM junctions are being discovered. One set of proteins are the three Extended synaptotagmins (E-Syts) [118] and their yeast homologues the tricalbins [119]. All three E-Syts can tether the ER and the PM to form ER/PM junctions [120]. Another protein that is at the ER/PM junctions in yeast is Ist2 [121]. The mammalian homologue of Ist2 is not known at this time, but it can be one of the ANO proteins [122]. Septins 4 and 5 were shown to localize at the junctions and regulate $PI(4,5)P_2$ around Orai1 [123], and likely control the size of the ER/PM junctions [124]. Nir2 is an exchange protein that exchanges phosphatidic acid with PI(4)P [125, 126] to maintains plasma membrane $PI(4,5)P_2$ at rest and during stimulation [126, 127]. Junctophilins participate in tethering the ER to the PM [44, 45]. SARAF mediates the slow Ca^{2+}-dependent inactivation of Orai1 only when it is at the $PI(4,5)P_2$-rich ER/PM junctions [62]. A model of the proteins that tether the ER/PM junctions and localization of STIM1, the Orai and TRPC channels at the $PI(4,5)P_2$-poor and $PI(4,5)P_2$-rich junctions is illustrated in Fig. 3.3.

The function of proteins in Ca^{2+} signaling and its related lipids highlight the important of the junctions in the fine-tuning of Ca^{2+} influx. As indicated above, septin 4 and 5 control the $PI(4,5)P_2$ environment around Orai1 [123]. Further studies reveal that the STIM1-Orai1 complex is formed first in a domain that is independent of E-Syt1, Septins and $PI(4,5)P_2$. Then, within a minute of channel activation either $PI(4,5)P_2$ moves to the domain or the Orai1-STIM1 complex moves to an E-Syt1-tethered $PI(4,5)P_2$-rich domain that now allows the recruitment of SARAF and initiation of slow Ca^{2+}-dependent inactivation of Orai1 [62]. Interestingly, the ER/PM junctions formed by STIM1 alone in the absence of Orai1 are different from those formed by E-Syt1 [128]. This supports the notion of more than one ER/PM junctions in which the STIM1-Orai1 complex can exist. Furthermore, the ER/PM junctions formed by the different E-Syts are not the same and show significant functional specificity. For example, the Orai1-STIM1 complex is formed only in E-Syt1 junctions while E-Syt2 and E-Syt3 have no role in controlling the function of STIM1-Orai1 [62]. The ER/PM junctions formed by E-Syt2 mediate EGR receptors

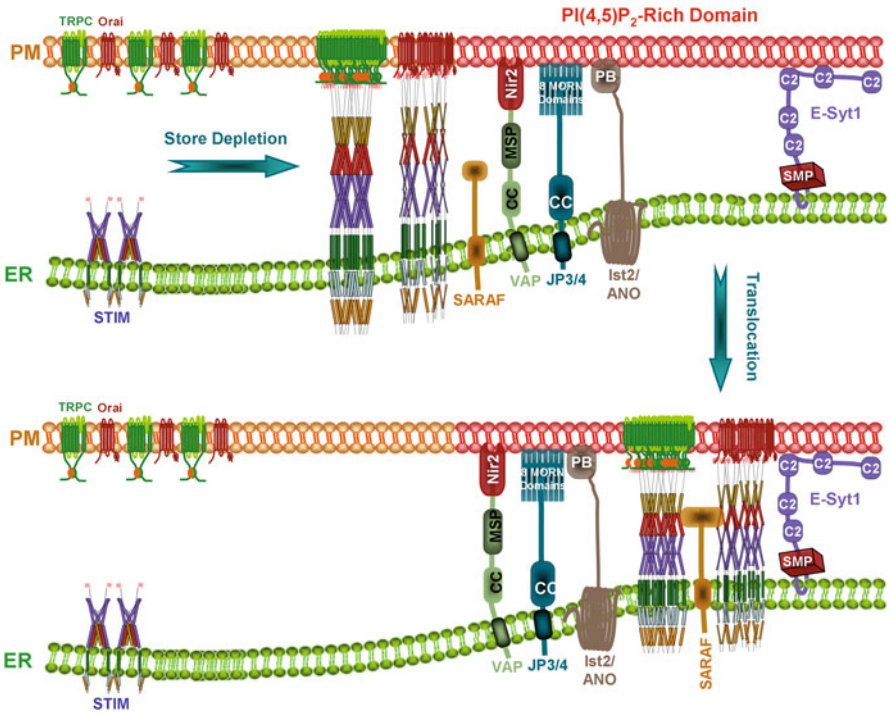

Fig. 3.3 Orai1 and TRPC channels at the ER/PM junctions. The ER/PM junctions are tethered by several proteins that are anchored in the ER and have a phosphatidylinositide (most commonly $PI(4,5)P_2$) binding domain. These include the Extended Synaptotagmins (E-Syt1), the Ist2/ANO, the VAPB-Nir2 complex and likely Junctophilin 3 and or 4 isoforms (JP3/4). At this time only the role of E-Syt1 in regulation of Orai1-STIM1 activity is established. The model shows that in the resting state the distance between the PM and the ER is larger, STIM1 does not access the PM and the TRPC and Orai channels are in a $PI(4,5)P_2$-poor domain and are not clustered (*upper image*). SARAF is in the ER away from STIM1. Upon cell stimulation and store depletion STIM1 unwind and STIM1, Orai1 and the TRPC channels co-cluster at the $PI(4,5)P_2$-poor domain adjacent to the domain formed by the tether proteins and where the channels are fully active but are not inhibited by SARAF. Subsequently the STIM1-Channels complex translocates to the tethers-formed $PI(4,5)P_2$-rich domain (or $PI(4,5)P_2$ coalesces and the tethers around the channels) (*lower image*), allowing access of SARAF to STIM1 to mediate the Ca^{2+}-dependent inhibition of the channels

endocytosis (and likely other forms of endocytosis) and ERK activation [129]. Similar function is observed with E-Syt3 but not with E-Syt1 [130].

Although it is clear that TRPC channels localize to the ER/PM junctions in native cells [131] and expression systems [30] and this is where they interact with STIM1 and Orai1, we know virtually nothing on the role of the proteins that form and regulate the junctions in modulating TRPC channels activity. Experiments similar to those performed with Orai1-STIM1 are very much needed to address this topic. It is certain that this topic will continue to attract much attention in the coming years to further explore the role of the ER/PM junctions in Ca^{2+} signaling and cell function and in the regulation of both the Orai and TRPC channels.

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Chapter 4
TRP-Na^+/Ca^{2+} Exchanger Coupling

Alan G.S. Harper and Stewart O. Sage

Abstract Na^+/Ca^{2+} exchangers (NCXs) have traditionally been viewed principally as a means of Ca^{2+} removal from non-excitable cells. However there has recently been increasing interest in the operation of NCXs in reverse mode acting as a means of eliciting Ca^{2+} entry into these cells. Reverse mode exchange requires a significant change in the normal resting transmembrane ion gradients and membrane potential, which has been suggested to occur principally via the coupling of NCXs to localised Na^+ entry through non-selective cation channels such as canonical transient receptor potential (TRPC) channels. Here we review evidence for functional or physical coupling of NCXs to non-selective cation channels, and how this affects NCX activity in non-excitable cells. In particular we focus on the potential role of nanojunctions, where the close apposition of plasma and intracellular membranes may help create the conditions needed for the generation of localised rises in Na^+ concentration that would be required to trigger reverse mode exchange.

Keywords Na^+/Ca^{2+} exchangers • TRPC channels • Sodium entry • Reverse mode NCX activity • Calcium entry

4.1 Introduction

Animal cells maintain a low cytosolic Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$) in the range of 50–100 nM. To maintain this resting $[Ca^{2+}]_{cyt}$ against leakage of Ca^{2+} from intracellular stores or across the plasma membrane, or to restore resting $[Ca^{2+}]_{cyt}$ after the generation of a Ca^{2+} signal, several Ca^{2+} removal mechanisms are used. These include sequestration of Ca^{2+} into organelles including the endoplasmic reticulum

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or sarcoplasmic reticulum by several isoforms of sarco-endoplasmic reticulum Ca^{2+}-ATPase (SERCA), into mitochondria by the mitochondrial Ca^{2+} uniporter and into acidic organelles by the combined actions of a vacuolar H^+-ATPase (vH^+-ATPase) and a H^+/Ca^{2+} exchanger. Ca^{2+} is also be removed across the plasma membrane either by primary active transport via a plasma membrane Ca^{2+}-ATPase (PMCA), of which four isoforms have been identified [1], or by secondary active transport via Na^+/Ca^{2+} exchange. Two types of Na^+/Ca^{2+} exchangers are found in the plasma membrane: those that exchange Na^+ for Ca^{2+} (NCXs) and those that exchange Na^+ for Ca^{2+} and K^+ (NCKXs) [2]. PMCAs are high affinity Ca^{2+} transporters but have relatively low turnover rates, whilst Na^+/Ca^{2+} exchangers have a lower Ca^{2+} affinity but higher turnover rates than PMCAs [3]. Consequently, PMCAs are important in the maintenance of resting $[Ca^{2+}]_{cyt}$ whilst Na^+/Ca^{2+} exchangers are more important in the restoration of $[Ca^{2+}]_{cyt}$ after it has been elevated during Ca^{2+} signalling. Na^+/Ca^{2+} exchangers influence Ca^{2+} signalling in excitable and non-excitable cells, not just as a means of Ca^{2+} removal, but also as a means of Ca^{2+} entry, since the exchangers can reverse in response to changes in the concentrations of the transported ions and in membrane potential.

4.1.1 Na^+/Ca^{2+} Exchange

Several observations made during the first half of the twentieth century of the effects on cardiac muscle of manipulation of the extracellular Na^+ concentration ($[Na^+]_o$) pointed towards the existence of a Na^+/Ca^{2+} exchange mechanism. During the 1960s several laboratories studying different tissues concluded that a coupled counter transport system for Na^+ and Ca^{2+} existed in the plasma membrane [4–6]. Although earlier studies were generally interpreted in terms of the exchange of two Na^+ ions for one Ca^{2+} ion, evidence suggesting a coupling ratio of >2Na^+:1Ca^{2+} started to emerge in the mid 1970s and over the following decade or so an exchange ratio of 3Na^+:1Ca^{2+} was established in several different excitable tissues [e.g. 7, 8]. The NCX first studied in cardiac muscle is now designated NCX1. Two other NCX isoforms, NCX2 and NCX3 are also expressed in some mammalian cells. Given the similar sequence identity in the transport domains of the proteins, NCX2 and NCX3 are also believed to mediate exchange of 3Na^+ ions for one Ca^{2+} ion [2]. However, there is some uncertainty over the transport ratio, with some electrophysiological experiments suggesting exchange ratios of 4Na^+:1Ca^{2+} [9, 10] or even 1Na^+:1Ca^{2+} [11].

In the 1980s several groups identified K^+-dependent Na^+/Ca^{2+} exchange in rod outer segments and two groups reported an exchange ratio of 4Na^+:(1Ca^{2+} + 1 K^+) [12, 13]. The K^+-dependent Na^+/Ca^{2+} exchanger first identified in rods is now designated NCKX1. Four related exchangers, NCKX2, NCKX3, NCKX4 and NCKX5 have been identified [2]. The stoichiometry of transport by NCKX2 has been determined to be the same as for NCKX1 [14]. Sequence identity suggests the other NCKXs are likely to also exhibit exchange ratios of 4Na^+:(1Ca^{2+} + 1 K^+).

For a detailed historical account of early work on Na^+/Ca^{2+} exchange see [3].

4.1.2 Na^+/Ca^{2+} Exchanger Families

Na^+/Ca^{2+} exchangers form part of a superfamily of Ca^{2+}/cation antiporters (CaCAs) with almost 150 identified members [15]. Three of the five branches of the CaCA superfamily include Na^+/Ca^{2+} exchangers that are expressed in mammalian cells. As indicated above (Sect. 4.1.1), three isoforms of the NCX family and five of the NCKX family have been identified. A third branch, the Ca^{2+}/cation exchangers (CCXs), includes only one identified mammalian member, rather confusingly known after earlier misidentification as NCKX6 or NCLX ($Na^+/Ca^{2+}/Li^+$ exchanger) as, unlike NCXs or NCKXs, it can transport Na^+ or Li^+ in exchange for Ca^{2+}. The NCLX is now recognised as the Na^+/Ca^{2+} exchanger expressed in the inner mitochondrial membrane [16].

The NCXs form the SLC8 (solute carrier 8) family, with NCX1, NCX2 and NCX3 being coded for by the genes SLC8A1, SLC8A2 and SLC8A3 respectively [2, 17]. NCX1 is widely expressed and found in most tissues whereas NCX2 and NCX3 show a more restricted expression [2, 17]. The transcription products of SLC8A1 and SLCA3 but not SLCA2 are subject to alternative splicing which may be tissue specific [2, 17, 18].

The NCKXs form the SLC24 (solute carrier 24) family, with the five members being coded for by the genes SLC24A1- SLC24A5 and some splice variants exist [19]. All five of the NCKX isoforms appear to be expressed in several tissues with NCKX1-NCKX4 being found in the plasma membrane whilst NCKX5 is only reported to be present in internal membranes [19].

4.1.3 Modes of Exchange

Transport by NCXs and NCKXs is fully reversible, with forward mode exchange removing Ca^{2+} from the cytosol across the plasma membrane and reverse mode exchange transporting Ca^{2+} into the cytosol. The direction of exchange is influenced by the prevailing Ca^{2+} and Na^+ concentrations and, since the transporters are electrogenic, by membrane potential. High $[Ca^{2+}]_{cyt}$ favours forward mode exchange whilst high $[Na^+]$ and more depolarised membrane potentials favour reverse mode exchange. Physiologically $[Ca^{2+}]_{cyt}$ and $[Na^+]_{cyt}$ may change in response to increased activity of ion channels permeable to these ions, and there may be marked local effects in sub-plasma membrane microdomains and in areas of restricted diffusion such as nanojunctions formed by closely apposing membranes. The mode of exchange may be influenced by molecular complexes in which exchangers are functionally coupled to ion channels or other transporters (See Sect. 4.2.1).

Reversal of the direction of exchange by NCX1 in cardiac muscle has long been recognised. Under conditions of resting ionic concentrations and membrane potential, NCX1, with a $3Na^+$:$1Ca^{2+}$ stoichiometry, operates in forward mode, but early in the cardiac action potential following Na^+ entry through voltage-gated Na^+ channels

and with membrane depolarisation, the exchanger switches to reverse mode to mediate Ca^{2+} influx [3, 20]. Similarly, Na^+ entry via non-selective receptor- or store-operated channels can lead to reverse mode exchange in non-excitable cells [21]. Reverse mode exchange by NCKX3 or NCKX4 has been suggested to contribute to stimulated Ca^{2+} entry in arterial smooth muscle.

4.1.4 Ionic Regulation of Exchanger Activity

Transport by NCXs involves alternate occupation of an ion binding site by three Na^+ ions or one Ca^{2+} ion that is sequentially exposed at the two faces of the plasma membrane [2]. The intracellular ion transport site of NCX1 has an affinity for Ca^{2+} in the low micromolar range and an affinity for Na^+ in the order of 10 mM. The externally facing site has Ca^{2+} and Na^+ affinities around 1 mM and 60–80 mM respectively [2]. Activity of the exchanger under resting conditions is thus mainly influenced by occupancy of the binding site for transported Ca^{2+} on the cytosolic face. Transport by NCKXs is believed to occur by an alternate access model as for the NCX. The dissociation constants at the external facing binding sites have been estimated at around 1–3 μM for Ca^{2+}, 2–10 mM for K^+ and 25–45 mM for Na^+. The dissociation constants for Ca^+ and K^+ appear to be similar at the internal facing binding sites [22].

In addition to occupancy of the ion transport sites, NCX activity is allosterically regulated by the binding of Ca^{2+} and Na^+ and by other factors. Ca^{2+} binding at the cytosolic face of NCX1 activates the exchanger [23, 24] whilst Na^+ binding at the cytosolic face is inhibitory [25, 26]. Inactivation of NCKX2 by cytosolic Na^+ has also been reported [27].

4.1.5 Additional Regulation of Exchanger Activity

The activity of Na^+/Ca^{2+} exchangers can be modified through their ability to integrate a wide range of cellular signals other than changes in ion concentrations that alter the rate and directionality of transport. These include factors such as cytosolic pH, nitric oxide, reactive oxygen species, ATP and phosphatidylinositol 4,5-bisphosphate (PIP_2) [28–34]. NCX1 activity is also influenced by the binding of a number of other proteins, including phospholemman, calcineurin and 14-3-3 proteins, the binding of which are all inhibitory [2]. NCX1 function may also be influenced by phosphorylation by protein kinase A and protein kinase C, although this is controversial, particularly for PKA, where a number of conflicting reports exist [2, 35]. NCX activity might also be influenced by phosphorylation of binding partners such as phospholemman. The activity of NCKX2, but not of NCKX3 or NCKX4, is reported to be increased by PKC-dependent phosphorylation [36]. Lastly, the activity of NCX1 and NCX3 may also be influenced by calpain-mediated proteolysis,

which acts to favour increased cytosolic calcium concentrations by either down-regulating forward mode exchange or upregulating greater reverse mode exchange [37–39].

These regulatory mechanisms allow the NCX to act as a tunable Ca^{2+} transport system which has the capacity to momentarily switch from conducting Ca^{2+} removal through forward mode exchange to generating Ca^{2+} entry through reverse mode exchange. The NCX can potentially act as a mediator of both Ca^{2+} removal and entry within a single cell, and as such can either facilitate or inhibit Ca^{2+}-regulated processes [40, 41]. The ability of the NCX to function in reverse mode and act as a Ca^{2+} entry mechanism has led to investigations of the relative physiological importance of the NCX acting in forward and reverse mode in a range of different cell types.

4.2 Coupling of Localised Na^+ Increases Generated by Activation of Non-selective Cation Channels with Na^+/Ca^{2+} Exchangers Creates Hybrid Ca^{2+} Entry Systems

Reverse mode exchange requires a significant change in the normal resting transmembrane ion gradients and membrane potential, which normally favour exchangers working to remove Ca^{2+} from cells. In non-excitable cells, a shift into reverse mode activity has been suggested to occur principally via the coupling of a localised Na^+ entry through non-selective cation channels that result in local change in the Na^+ gradient and membrane potential. Most reports have suggested members of the Canonical Transient Receptor Potential (TRPC) family as a potential partner for Na^+/Ca^{2+} exchangers. TRPC isoforms are all non-selective cation channels that facilitate significant Na^+ and Ca^{2+} entry into cells [42], and as such could provide a source of Na^+ to allow the reversal potential of the exchanger to be reached resulting in reverse mode exchange and so Ca^{2+} entry. The possibility of spatial coupling of NCX1 and TRPCs has been supported by immunolocalisation studies in which the two proteins have been found to colocalised in specific domains of the plasma membrane of some cells [43–45]. Previous work has suggest that this colocalisation may be the result of direct interaction of the TRPC and NCX as part of a macromolecular complex [43, 46], but interaction may also be indirect through the recruitment of both proteins to lipid rafts or caveolae in some systems [46–51].

Other Na^+ entry routes have also been implicated in the reversal of Na^+/Ca^{2+} exchangers. These include P2X receptors and Na^+/H^+ exchangers in non-excitable cells [52, 53], whilst NMDA and AMPA receptors, as well as glutamate and glycine transporters have additionally been reported to play a role in the reversal of Na^+/Ca^{2+} exchangers in excitable tissues [54–59]. These results suggest that Na^+/Ca^{2+} exchangers can promiscuously couple with a range of different Na^+ transporting proteins to elicit Ca^{2+} entry. Thus when co-expressed, the dissociated Na^+/Ca^{2+} exchangers and Na^+ transporting proteins can work independently to elicit their

standard cellular activities, yet when coupled together they can generate a hybrid response and potentiate cytosolic Ca^{2+} signalling. This raises the possibility that subpopulations of Na^+/Ca^{2+} exchangers may exist that can locally modulate the Ca^{2+} signalling system of some cells.

4.2.1 *Evidence for Functional Coupling of Na^+ Entry Mechanisms with Reverse Mode Na^+/Ca^{2+} Exchanger Activity*

To ensure effective reverse mode exchange, the membrane potential needs to exceed the reversal potential of the exchanger, which is dependent upon both the transmembrane Na^+ and Ca^{2+} gradients [3]. Na^+ and Ca^{2+} entry through non-selective cation channels will alter the reversal potential of the exchanger, as well as favour depolarisation of the membrane potential of a stimulated cell. Thus it would only be possible to accurately predict the effect of Na^+ entry though non-selective cation channels on the rate and mode of Na^+/Ca^{2+} exchanger activity if simultaneous real-time measurements of all three of these parameters in the local vicinity of the protein were performed. In non-excitable cells, where membrane potential changes will be highly-dependent on the proximity of the open channels, this is a near-intractable task and as such investigators will often have to rely on estimates of some of these parameters in order to estimate the change in $[Na^+]_{cyt}$ needed to trigger Ca^{2+} entry by reverse mode exchange. Such an estimate was performed in aortic smooth muscle cells [60], where it was calculated that the NCX would require $[Na^+]_{cyt}$ to be raised to between 16 and 25 mM depending on the extent that the cell is also depolarised by the cation entry. The same group went on to subsequently demonstrate that agonist-evoked opening of a TRPC6-containing receptor operated channel generated a localised subplasmalemmal Na^+ microdomain around the channel, which caused the local $[Na^+]$ to rise to 30 mM [61]. In contrast, the bulk cytosolic $[Na^+]$ rose to only 14 mM, suggesting that close spatial coupling of the TRPC6-containing channel to the NCX would be required to elicit significant Ca^{2+} entry by reverse mode exchange. This possibility was further supported by the observation that pre-treatment with the NCX inhibitor KB-R7943 potentiated the local Na^+ rises but had no significant effect on the global Na^+ signal.

4.2.2 *Nanojunctions: A Cellular Architecture to Facilitate Reverse Mode NCX Activity*

Whilst the close coupling of NCXs and TRPC channels form primary molecular complexes for eliciting reverse mode exchange, it is becoming clear that the activity of NCX-TRPC complexes may be fine-tuned by their localisation within cellular nanojunctions [62]. Nanojunctions formed by the tight apposition of the membrane of the endoplasmic/sarcoplasmic reticulum with the plasma membrane provide a

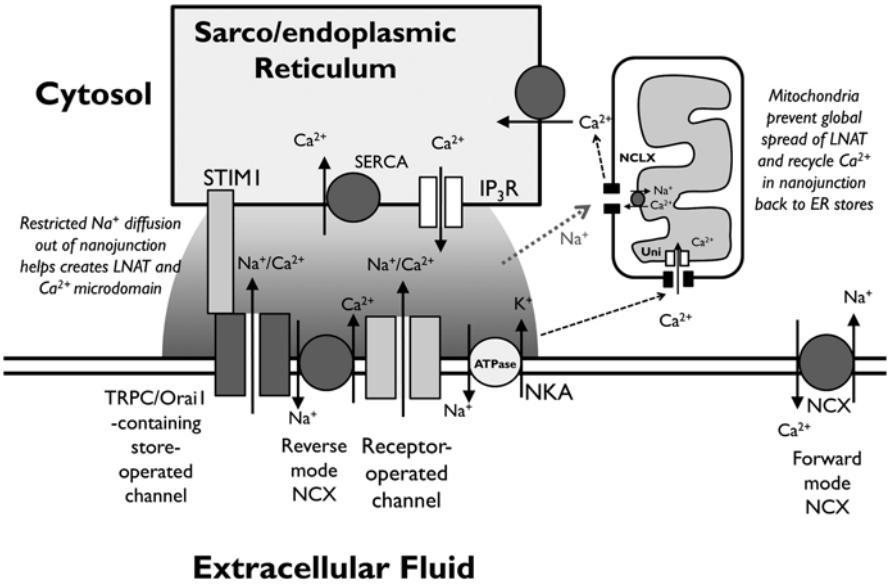

Fig. 4.1 Nanojunctions provide a subcellular environment to favour reverse mode NCX activity. The localisation of non-selective cation channels with the NCX in the restricted environment of the nanojunction creates a highly-concentrated rise in local [Na^+] (Localised Na^+ Transient; LNAT) which can facilitate reversal of the NCX. Through localisation of other cation-transporting proteins such as the Na^+/K^+-ATPase (NKA), IP_3 receptor (IP_3R) and sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA), the local levels of Ca^{2+} and Na^+ can be tightly controlled to facilitate both refilling of the ER Ca^{2+} store via reverse mode exchange, or Ca^{2+} unloading through forward mode exchange (not shown). These localised rises in Na^+ and Ca^{2+} can be isolated from the bulk cytosol by the geometry of the nanojunction restricting diffusion of these cations, the actions of the Ca^{2+} and Na^+ transporting proteins and through the actions of nearby mitochondria which can buffer these local cation rises. The isolation of these localised cation transients from the bulk cytosol allows other NCX proteins in the rest of the plasma membrane to continue to act in forward mode as a high-capacity Ca^{2+} removal system

cellular architecture which may facilitate reverse mode Na^+/Ca^{2+} exchange. The ≈20 nm wide cleft creates a tiny isolated volume of cytosol in which Na^+ and Ca^{2+} gradients can be significantly altered in relative isolation from changes in the bulk cytosol [63, 64]. Thus by recruiting the TRPC-NCX complex into nanojunctions, the opening of the non-selective cation channel can markedly raise the local [Na^+] to allow the exchanger to switch into reverse mode exchange (Fig. 4.1).

In addition to the coupled NCX and non-selective cation channels, nanojunctions are also thought to contain a number of other regulators of local Ca^{2+} and Na^+ concentrations. These include the low affinity α2/α3 isoforms of the Na^+/K^+-ATPase, SERCAs, inositol 1,4,5-trisphosphate (IP_3) receptors and ryanodine receptors [65–69]. The functional interactions of these transporters within the enclosed cytosolic domain at the nanojunction can bias the ionic conditions surrounding the NCX and therefore can fine tune the rate and directionality of NCX transport depending upon their activity (Fig. 4.1).

Moreover, the close interaction of NCXs with the Ca^{2+} release and sequestration mechanisms in the sarco/endoplasmic reticulum can create a form of linked Ca^{2+}

transport which allows the NCX to be able to either unload or load Ca^{2+} into these intracellular Ca^{2+} stores [69, 70]. The effect ultimately depends on the underlying mode of NCX activity. Reverse mode exchange might facilitate store refilling by providing a Ca^{2+} supply to SERCAs [63, 70] or facilitate store unloading via Ca^{2+} induced Ca^{2+} release [71], whilst forward mode exchange would facilitate store-unloading [69, 72]. Therefore the regulation of NCX activity within nanojunctions can affect Ca^{2+} signalling not just via the direct effect of the exchanger on Ca^{2+} flux across the plasma membrane, but also through the ability of the exchanger to control Ca^{2+} release from intracellular stores.

Beyond the edges of the nanojunction, the movement of Ca^{2+} and Na^{+} from the nanojunction into the bulk cytosol are further restricted by the presence of subplasmalemmal mitochondria, which take up these cations through the action of the mitochondrial uniporter and NCLX [60, 73]. This activity is likely not just to act as a way of helping to create spatially-defined ionic subdomains in the cell, but also to control the filling state of the intracellular Ca^{2+} stores, both by providing a recycling pathway for Ca^{2+} from the mitochondria back to the SR [73] and by facilitating store-operated calcium entry [74–76].

4.2.3 A Role for NCX in Store-Operated Calcium Entry?

In addition to providing an optimal environment for eliciting reverse mode NCX activity, nanojunctions also provide an ideal subcellular location for the activation of store-operated channels through their coupling to the endoplasmic reticulum Ca^{2+} sensor STIM1 [77, 78]. Indeed previous work has demonstrated that store-operated Ca^{2+} entry may refill Ca^{2+} stores by allowing Ca^{2+} entry into a restricted portion of the cytoplasm, as would be provided for by a nanojunction [79, 80]. This begs the question as to whether TRPC-containing store-operated channels could also be coupled to the NCX to elicit reverse mode exchange. In support of this possibility there are some reports that the Ca^{2+} signalling caused by the opening of store-operated channels is further supported by triggering reverse mode NCX activity [81, 82]. This further begs the question as to whether store-operated- and receptor-operated channels could both tune NCX activity within the same cell, either by acting independently in distinct nanojunctions, or by working cooperatively to facilitate reverse mode NCX activity within the same nanojunction.

4.3 Characterising the Physiological Important of Reverse Mode NCX Activity: The Potential Pitfalls of Pharmacological Characterisation

There is a growing appreciation of the ability of reverse mode Na^{+}/Ca^{2+} exchange to mediate Ca^{2+} entry into non-excitable cells. This has been reported under both physiological and pathological conditions in a wide variety of cells including endothelial

cells, renal proximal tubule cells, pancreatic duct cells, macrophages, monocytes, neutrophils, platelets and smooth muscle cells of the airways, vasculature, urethra, bladder and gastrointestinal tract [30, 81–90].

4.3.1 Use of Inhibitors of Na^+/Ca^{2+} Exchange

Whilst there have been many reports of the contribution of reverse mode NCX activity in controlling Ca^{2+} signalling and cellular activity in a wide range of excitable and non-excitable cells, many of these studies have been pharmacological and have not provided evidence of the co-localisation of proteins suggested to be involved nor have the parameters which alter the mode of exchange been examined. This leaves some of these reports open to question as teasing apart the forward and reverse modes of exchange experimentally is fraught with pitfalls. Examining the effects of inhibition of Na^+/Ca^{2+} exchangers on agonist-evoked Ca^{2+} signals will not unambiguously establish the role of the exchanger because Na^+ entry through a channel could potentiate a rise in $[Ca^{2+}]_{cyt}$ either by triggering Ca^{2+} entry via reverse mode exchange, or by reducing Ca^{2+} removal by forward mode exchange [81, 91, 92]. In addition, inhibitors of Na^+/Ca^{2+} exchangers may have additional effects on Ca^{2+} release from intracellular stores (See Sect. 4.2.2). Most traditional methods for blocking Na^+/Ca^{2+} exchangers are not mode selective and as such it is difficult to tease apart the relative contribution of forward and reverse mode exchange in intact cells. For instance, removing Na^+ from the extracellular medium can provide a physiological blockade of Na^+/Ca^{2+} exchanger function, yet this experimental manipulation is non-selective and will likely impede both forward mode activity (by removing the transmembrane Na^+ gradient) and reverse mode exchange (by preventing Na^+ entry and cellular depolarisation upon activation). Even genetic knockout of Na^+/Ca^{2+} exchangers will not provide a solution to this conundrum as it will remove all the functions of the exchanger.

4.3.2 Mode-Selective Inhibitors of Na^+/Ca^{2+} Exchange

The introduction of inhibitors which can more potently block reverse mode exchange has provided a potential mechanism to experimentally determine the role of reverse mode Ca^{2+} exchange in a variety of cell types. However, there is still a need for caution in the interpretation of results obtained with mode selective inhibitors without additional supporting data. For instance, the widely-used inhibitor KB-R7943 is often described as being reverse mode selective based on the initial reports of a significant difference in potency against the two modes of NCX1 operation studied electrophysiologically when ionic conditions were set to favour unidirectional exchange in either forward or reverse mode (IC_{50} = 0.3 μM or 17 μM for reverse or forward mode respectively) [93]. Yet when the ionic conditions are set more

physiologically and exchange mode is switched by altering the holding potential above or below the reversal potential, the mode selectivity of this inhibitor against NCX1 was not observable [94].

Further examination of the effect of KB-R7943 on net NCX transport at the cellular level have reported that the relative potency of the drug can vary widely depending on the cellular system studied, with IC_{50} values of 1.6 μM and >30 μM being reported for the inhibition of reverse and forward mode exchange in NCX1-transfected CCL39 cells whilst in cardiac sarcolemmal vesicles the IC_{50} values were much less distinct (5 μM and 11 μM respectively) [95]. The cell-dependence of these findings may, in part, reflect the expression of different NCX isoforms and their splice variants as this appears to significantly alter the effectiveness of the different reverse mode inhibitors [96]. For instance, KB-R7943 and YM-244769 are reported to be more a potent inhibitor of NCX3 compared to NCX1 or NCX2 [97, 98], whilst SN-6 and SEA-0400 are better inhibitors of NCX1 [99, 100]. Additionally, due to the NCX sensitivity to metabolic conditions [28, 101–103], differences in the basic cytosolic conditions and activity of different cell types may alter the activity of the NCX and so affect the sensitivity of the exchanger to reverse mode inhibitors. Whilst we have focussed here on the widely-used KB-R7943, it is important to note that reports of minimal mode selectivity have also been demonstrated in studies of other reverse mode selective inhibitors, such as SN-6 [104] and SEA0400 [105]. The potential pitfalls of relying on the reported mode selectivity of drugs have been demonstrated in our work in human platelets, in which we initially utilised a pharmacological methodology to characterise a role for reverse mode exchange in potentiating the Ca^{2+} signal elicited by the opening of store-operated channels (SOCs) [106], yet later reanalysis demonstrated that this potentiation was actually elicited by forward mode NCX activity regulating autocrine potentiation of the initial Ca^{2+} entry through the SOC [92]. These data therefore suggest that when using NCX inhibitors in cellular systems in which the action of these drugs have not been previously characterised, it is important to determine the effects of the doses of inhibitor applied on reverse and forward mode exchange to ensure mode selectivity [e.g. 107].

4.3.3 Selectivity of Na^+/Ca^{2+} Exchange Inhibitors

When assessing inhibitor function in new cell system, consideration needs to be paid to the many reported non-selective effects of these drugs on other components of the Ca^{2+} signalling apparatus and non-specific effects should be ruled out [108–115]. However, when assessing potential non-specific effects of inhibitors of Na^+/Ca^{2+} exchangers it is worth considering that the coupling of the exchanger with a cation conductance creates not only a hybrid protein complex which has altered Ca^{2+} signalling properties, but also a widened pharmacological profile which will encompass inhibitors of either component. This includes the potential of loss of forward mode activity enhancing Ca^{2+}-dependent feedback on the associated

channel, as shown with the loss of L-type Ca^{2+} channel activity in cardiac and smooth muscle from NCX1 knockout mice [116, 117].

4.3.4 *Targeting the NCX-TRPC Interaction as a Novel Method for Blocking Reverse Mode Exchange*

Increased reverse mode Na^+/Ca^{2+} exchanger activity has been implicated in a number of pathological conditions including systemic and pulmonary arterial hypertension [45, 117–119], overactive bladder [90], cardiac arrhythmias [120, 121], malignant hyperthermia [122], and cerebral, renal and cardiac reperfusion injury [123–125]. This link to a wide range of pathologies suggests the possibility that a selective reverse-mode inhibitor of Na^+/Ca^{2+} exchange may provide significant therapeutic benefits. However, as described above (Sects. 4.3.2 and 4.3.3), current inhibitors lack the mode- and target-selectivity desired to provide an effective treatment. Recent work has demonstrated promising selectivity of a novel mode-independent NCX inhibitor, ORM-1013, in isolated cell systems [126], yet further work will need to ensure that this drug does not have unintended effects on other important functions for which forward mode activity has been suggested to be essential, including pacemaker activity of the sino-atrial node [127], insulin secretion [91, 128], renal Ca^{2+} and Na^+ retention [129] and platelet activation [72].

As detailed above, the tight interaction of Na^+/Ca^{2+} exchangers with non-selective cation channels appears to be essential for creating the local ionic conditions needed to elicit Ca^{2+} entry by reverse mode exchange in non-excitable cells. By breaking the close apposition of this complex, the Na^+/Ca^{2+} exchanger will likely not be exposed to the local rises in cytosolic $[Na^+]$ required to elicit Ca^{2+} entry by reverse mode exchange, without preventing the exchanger from operating in forward mode. This suggests that selectively blocking reverse mode exchanger function may be possible through disrupting the normal interaction of the exchanger with TRPCs (or other associated Na^+-permeable channels). However, whilst this might provide a promising route for future drug development, there is currently no significant information on the proteins involved in the subcellular targeting of Na^+/Ca^{2+} exchangers, or the proteins scaffolding it with TRPC channels in non-excitable cells. All previous work on scaffolding proteins that interact with Na^+/Ca^{2+} exchangers has come from studies in neuronal or cardiac tissues, with proteins such as caveolin-3 [50], CRMP2 [57], α-fodrin and β-spectrin [68], ankyrin B [130], junctophilin 2 [131] and EHD3 [132] being identified as either binding proteins or critical regulators of the subcellular localisation of Na^+/Ca^{2+} exchangers. Future work defining Na^+/Ca^{2+} exchanger binding proteins and their roles in controlling the interaction with TRPC channels may help identify novel pharmacological targets for blocking reverse mode activity selectively in the cells associated with pathologies of abnormal Ca^{2+} entry via Na^+/Ca^{2+} exchangers.

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Chapter 5
Role of TRPC Channels in Store-Operated Calcium Entry

Hwei Ling Ong, Lorena Brito de Souza, and Indu S. Ambudkar

Abstract Store-operated calcium entry (SOCE) is a ubiquitous Ca^{2+} entry pathway that is activated in response to depletion of Ca^{2+} stores within the endoplasmic reticulum (ER) and contributes to the control of various physiological functions in a wide variety of cell types. The transient receptor potential canonical (TRPC) channels (TRPCs 1–7), that are activated by stimuli leading to PIP_2 hydrolysis, were first identified as molecular components of SOCE channels. TRPC channels show a miscellany of tissue expression, physiological functions and channel properties. However, none of the TRPC members display currents that resemble I_{CRAC}. Intensive search for the CRAC channel component led to identification of Orai1 and STIM1, now established as being the primary constituents of the CRAC channel. There is now considerable evidence that STIM1 activates both Orai1 and TRPC1 via distinct domains in its C-terminus. Intriguingly, TRPC1 function is not only dependent on STIM1 but also requires Orai1. The critical functional interaction between TRPC1 and Orai1, which determines the activation of TRPC1, has also been identified. In this review, we will discuss current concepts regarding the role of TRPC channels in SOCE, the physiological functions regulated by TRPC-mediated SOCE, and the complex mechanisms underlying the regulation of TRPCs, including the functional interactions with Orai1 and STIM1.

Keywords TRPC channels • SOCE • Trafficking • Function

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5.1 Introduction

Elevation of cytosolic calcium levels ($[Ca^{2+}]_i$) in response to neurotransmitter stimulation of cells acts as a trigger for activation of many physiological processes, including cell proliferation and differentiation, cell migration, lymphocyte activation, endothelial cell function, as well as protein and fluid secretion from exocrine gland cells. Plasma membrane Ca^{2+} entry channels contribute to $[Ca^{2+}]_i$ elevation and provide critical Ca^{2+} signals that are utilized for regulation of different cell functions. Store-operated calcium entry (SOCE) is a major ubiquitous Ca^{2+} entry pathway that contributes to the control of various physiological functions in a wide variety of cell types. SOCE is a unique mechanism in that it is activated in response to depletion of Ca^{2+} stores within the endoplasmic reticulum (ER). Physiologically, this occurs following stimulation of plasma membrane receptors that lead to phosphatidylinositol 4,5-bisphosphate (PIP_2) hydrolysis and generation of inositol 1,4,5-triphosphate (IP_3). IP_3 binds to its receptor (IP_3R) in the ER membrane and induces Ca^{2+} release from the ER, resulting in a decrease in ER-$[Ca^{2+}]$ and activation of SOCE. This Ca^{2+} entry can also be activated by treating cells with sarco-endoplasmic reticulum (SERCA) pump blockers like thapsigargin or cyclopiazonic acid that inhibit Ca^{2+} uptake into ER, unmasking a passive Ca^{2+} leak pathway that has not yet been clearly elucidated. Importantly, this leads to loss of ER-Ca^{2+} and triggers activation of SOCE in the absence of receptor-stimulated signaling. SOCE is inactivated by refilling the Ca^{2+} stores within the ER, providing further evidence that the activity of channels mediating SOCE are governed by the status of $[Ca^{2+}]$ in the ER [1–3]. The first channel current associated with SOCE, Ca^{2+}-release activated calcium current (I_{CRAC}), was measured in mast cells and T lymphocytes. This highly Ca^{2+}-selective current has a characteristic inward rectification with reversal potential >+40 mV [4–7]. Under the same experimental conditions, currents with varying characteristics and ionic selectivities, ranging from relatively Ca^{2+}-selective to non-selective, have been described in different cell types. These currents have been generally referred to as store-operated calcium current (I_{SOC}) to distinguish them from I_{CRAC} [2, 8].

Search for the molecular components of SOCE, led to the identification of the transient receptor potential canonical (TRPC) channels, part of the superfamily of TRP channels. Mammalian TRPC channels were cloned based on the *Drosophila* TRP channel, which functions as a light-sensitive Ca^{2+}-permeable channel involved in phototransduction. The *Drosophila* phototransduction process, a phospholipase C-mediated pathway [9–11], provided further impetus to the search for mammalian TRP channels. The TRPC subfamily consists of seven members (TRPCs 1–7) that are divided into four subsets based on their amino acid (aa) homology: TRPC1, TRPC2, TRPC3/TRPC6/TRPC7 and TRPC4/TRPC5. All TRPC channels display activation in response to receptor-stimulated PIP_2 hydrolysis and have six transmembrane domains with a pore-forming domain localized between the fifth and sixth domains. The channels contain N-terminal ankyrin repeats, a highly conserved TRP domain in the C-terminus, several calmodulin (CaM)-binding domains and a

putative IP_3R binding site [9–11]. TRPC channels show diverse tissue expression, physiological functions and channel properties. Recent reviews have presented a general overview of the molecular components and mechanisms regulating SOCE [1, 12] as well as overviews of the individual TRPC channels: TRPC1 [13], TRPC2 [14], TRPC3 [15], TRPC4 [16], TRPC5 [17], TRPC6 [18], and TRPC7 [19]. We will not be discussing TRPC2, which is a pseudogene in humans [20, 21], in this review. While the discovery of TRPC channels spurred a large number of studies, none of the TRPC family of Ca^{2+}-permeable cation channels generated currents that resembled I_{CRAC}. Thus, identity of the components for this channel, as well as the regulatory proteins in SOCE continued to be a major focus in the field. Intensive search for these finally led to the identification of the CRAC channel component, Orai1, a four-transmembrane domain protein which is assembled as a hexamer to form the pore of the CRAC channel. Two other Orai proteins, Orai2 and Orai3, were also identified and reported to have some similarity with Orai1 and display store-dependent activation. However, since they also contribute to other non-SOCE mechanisms, such as the arachidonic acid-activated channels, further studies are required to fully understand their physiological function. Importantly, the main components involved in sensing ER-$[Ca^{2+}]$ and activating SOCE were also identified. The STIM family of proteins includes two members, STIM1 and STIM2, both of which are Ca^{2+}-sensing proteins that are localized in the ER membrane and sense $[Ca^{2+}]$ within the ER lumen to regulate SOCE. Of the two, STIM1 has been more extensively studied and is now well established as the critical and indispensable regulatory component of SOCE [22]. Furthermore, there is now considerable evidence that STIM1 can activate both Orai1 and TRPC1. The domains of STIM1 involved in gating of these channels are also known. Intriguingly, TRPC1 function is not only dependent on STIM1 but also requires Orai1. The critical functional interaction between TRPC1 and Orai1, which determines the activation of TRPC1, has also been resolved. In the following sections of this review, we will discuss current concepts regarding the role of TRPC channels in SOCE, the physiological functions regulated by TRPC-mediated SOCE, and the complex mechanisms underlying the regulation of TRPCs, including the functional interactions with Orai1 and STIM1.

5.2 Contribution of TRPC Channels to SOCE

All seven TRPC channels have been implicated as components of SOCE. Furthermore, a variety of physiological functions have been associated with TRPC-mediated SOCE. Recent studies also demonstrate that some human diseases are linked to either loss or gain of function of TRPC channels [23–25]. However, not all the TRPC channels consistently display the hallmarks of SOCE, namely (i) activation by store depletion in response to stimulation with an agonist or treatment with SERCA pump blockers, and (ii) inhibition by Gd^{3+} (1 μM) and 2-aminophenyl borate (2-APB; ≤10 μM). A large number of the studies assessing the role of TRPCs

have used heterologous expression systems where the channels are relatively overexpressed. This does not always result in generation of a functional channel in cells. Some studies have also demonstrated that the mode of regulation of the channels appears to differ depending on the level of their expression. This led to the suggestion that channel overexpression likely results in an unbalanced stoichiometry between TRPCs and the endogenous accessory proteins that regulate and/or modulate their activities. In contrast, more consistent and conclusive data have been provided by studies which assess the function of endogenous TRPCs in SOCE by modulating their expression and/or function in cell lines, primary cell preparations, as well as animal models. So far, the strongest evidence for the contribution of TRPC channels to SOCE has been provided for TRPC1 and TRPC4, whereas the contribution of TRPC3 to SOCE appears to be dependent on cell type and level of expression. TRPCs 5, 6 and 7 have been generally described to be store-independent, with a few exceptions. Note that unlike with Orai1 or STIM1, TRPC channel contribution to SOCE is not seen in all cell types.

TRPC1 was the first mammalian TRPC channel to be cloned and reported to have a role in SOCE [20, 21, 26, 27]. Among the many studies reported, exogenous expression of TRPC1 did not consistently increase SOCE while knockdown of endogenous TRPC1 significantly decreased SOCE (e.g. in HSG, smooth muscle and endothelial cells, as well as platelets) [26–32]. Further conclusive evidence was provided by studies with mice lacking TRPC1 ($TRPC1^{-/-}$), which despite having normal viability, development, and behavior [33], showed reduced SOCE in cell preparations from several tissues. Among these, salivary gland and pancreatic acinar cells and aortic endothelial cells from $TRPC1^{-/-}$ mice displayed significant reductions in SOCE as well as attenuation in Ca^{2+}-dependent physiological functions [34–36]. SOCE is fundamentally important for fluid secretion in salivary glands and for protein secretion in the exocrine pancreas. $TRPC1^{-/-}$ mice displayed reduction in salivary gland fluid secretion which was associated with a decrease in SOCE and K_{Ca} activity in acinar cells from the mice [36, 37]. Similarly defects in Ca^{2+}-activated Cl^- channel activity and protein secretion, as a consequence of reduced SOCE, were reported in pancreatic acinar cells [34]. Notably while there is no change in Orai1 in salivary gland and pancreatic acinar cells from $TRPC1^{-/-}$ mice, the channel does not appear to compensate for the lack of TRPC1 or support cell function on its own. Hence, decreased secretory function in these exocrine glands is primarily due to the loss of TRPC1-mediated SOCE. In endothelial cells, TRPC1 forms a heteromeric channel with TRP vanilloid 4 (TRPV4) to mediate SOCE. This Ca^{2+} entry was significantly reduced in cells from $TRPC1^{-/-}$ mice which adversely impacted vasorelaxation [35].

The caveolae-residing protein, caveolin-1 (Cav-1), is an important modulator of TRPC1 activity and functions as a plasma membrane scaffold for the channel. In the absence of Cav-1, TRPC1 is mislocalized and is unable to interact with STIM1, which is a requirement for TRPC1 activation [38]. Consistent with this, localization of TRPC1, its interaction with STIM1, as well as SOCE were disrupted in salivary gland acinar cells from $Cav\text{-}1^{-/-}$ mice [39]. Together, these findings further establish a role for TRPC1 in mediating SOCE in salivary gland cells. Other physiological

functions that are dependent on TRPC1-mediated SOCE are the contractile function of glomerular mesangial cells [40, 41] and osteoclast formation and function [42]. Loss of TRPC1 has also been implicated in aberrant vasorelaxation [43], muscle fatigue and slower regeneration after muscle injury [44, 45], whereas elevated TRPC1 expression has been linked to myopathies such as those observed in patients with Duchenne's Muscular Dystrophy and *mdx* mice lacking dystrophin [46–48]. However, it remains to be established whether these effects are due to changes in TRPC1-SOCE.

TRPC3 is reported to contribute to both the store-operated and receptor-activated calcium entry pathways. Loss of endogenous TRPC3 in cell lines and tissue preparations (pancreatic acinar and submandibular gland cells) from $TRPC3^{-/-}$ mice led to significant reductions in SOCE [49, 50]. In contrast, overexpression of TRPC3 increased SOCE in COS, HEK293 and HEK293T cells, as well as DT40 chicken B-lymphocytes. However, when the channel was expressed to very high levels, the regulatory mode was switched from store-operated to receptor-activated. Cells with relatively lower levels of TRPC3 expression displayed Gd^{3+} (1 μM)-sensitive Ca^{2+} entry, while those with higher levels of channel expression required higher [Gd^{3+}]. Hence, the mechanism by which TRPC3 is regulated appears to be determined by the level of channel expression in the cells [51–54]. TRPC3-mediated Ca^{2+} entry can also contribute to pathology and tissue damage. Pancreatic acini from $TRPC3^{-/-}$ mice showed significant protection from acute pancreatitis induced by hyperactivation of SOCE. Similar effects were seen by blocking channel function in $TRPC3^{+/+}$ mice by treatment with pyrazole 3, a TRPC3 inhibitor [55, 56]. Unlike TRPC3, TRPC6 and TRPC7 channels are largely believed be receptor-activated as both channels are consistently activated by the second messenger, diacylglycerol and its analogs [11, 18, 19, 57].

Both TRPC4 and TRPC5 have been suggested to contribute to SOCE, although there are very few studies reported for either channel. Moreover, TRPC5 can also be directly activated by Ca^{2+}, which makes it difficult to establish conclusively whether TRPC5 is directly regulated by store depletion [58, 59]. Exogenous expression of TRPC4 in HEK293 cells increased SOCE and generated a relatively Ca^{2+}-selective, inwardly rectifying current [60]. Similar results were obtained by overexpression TRPC4 in CHO, RBL cells [61] and *Xenopus laevis* oocytes [62]. Further evidence for TRPC4-mediated SOCE was provided by studies where TRPC4 expression was suppressed in several cell lines or by knockout of the channel in mice ($TRPC4^{-/-}$ mice). Following siRNA treatment, TRPC4-mediated SOCE was diminished in mouse mesangial cells [63], human adrenal cells [64], both mouse and human endothelial cells [65], human gingival keratinocytes [66], human corneal epithelial cells [67] and human pulmonary artery smooth muscle cells [68]. Additionally, TRPC4 forms a heteromeric channel complex with TRPC1 in human mesangial cells [69] as well as human and mouse endothelial cells [65]. Similar to what has been reported for $TRPC1^{-/-}$, knockout of TRPC4 did not adversely impact mortality and fertility of the mice. Nonetheless, $TRPC4^{-/-}$ mice show significantly reduced TRPC4-mediated SOCE in aortic [70] and lung endothelial cells [65, 71], resulting in defective regulation of vascular tone and endothelial permeability, respectively.

TRPC channels interact with numerous proteins which can underlie the diversity of calcium channel activity, their regulation, and specificity of downstream signaling events in the cells (recently reviewed in [1]). Not only do TRPC channels have the ability to undergo homomeric interactions to form functional channels, they also interact with other TRPCs to generate functional heteromeric channels. Most of the available data in this regard comes from studies with exogenously expressed channels. TRPC1 interacts with TRPC4 and TRPC5, whereas TRPC3 interacts with TRPC6 and TRPC7 [72–74]. It is presently not clear whether the resulting heteromeric channels have distinct properties and functions as compared to those of the individual channels. Very few studies have elucidated the status of endogenous store-dependent heteromeric TRPC channels and their physiological function. The contribution of endogenous heteromeric channel complexes to SOCE have been reported for TRPC1/TRPC3 in a human parotid gland ductal cell line [75] and rat H19-7 hippocampal cell lines [76]; TRPC1/TRPC5 in vascular smooth muscle [77]; TRPC1/TRPC4 in endothelial cells [65], and TRPC1/TRPC3/TRPC7 in HEK293 cells [50]. Given the overlapping expression of more than one TRPC channel in different cells and tissues, some physiological functions may involve multiple channels. For example, SOCE mediated by both TRPC1 and TRPC4 has been proposed to control endothelial cell permeability [65] and myogenesis [78, 79]. Multiple TRPC channels have been implicated in cardiac hypertrophy [80, 81], but it is not clear whether aberrant TRPC-mediated SOCE underlies this phenomena. A few studies have utilized double TRPC knockout mouse models to determine the role of heteromeric TRPC channels. Knockout of both TRPC1 and TRPC4 in mice severely impaired neuronal burst firing and caused neurodegeneration [82], whereas loss of both TRPC3 and TRPC6 impaired sensitivity to mechanical pressure and hearing [83]. The underlying basis for creating these double knockout mice models was the preponderance of co-expressed TRPC1 and TRPC4 in the brain [82] and TRPC3 and TRPC6 in sensory neurons and cochlear hair cells [83]. Whether the pathophysiological effects observed from double knockouts of endogenous TRPC heteromeric channel complexes can be conclusively linked to impaired regulation of SOCE also remains to be shown.

TRPC channels have also been found to associate with other TRP channels, including TRPV6, TRPV4, although in most of these cases it is not clear whether the associating channels form a single channel pore and/or contribute to SOCE. Co-expression of TRPC1 and TRPV4 resulted in formation of a heteromeric channel complex that is activated in response to store depletion in HEK293, vascular smooth muscle and endothelial cells. Moreover, the TRPC1/TRPV4 heteromeric channel exhibited distinct current characteristics when compared to currents mediated by either TRPV4 or TRPC1 alone [35, 84, 85]. TRPC1 was reported to interact with TRPV6 and exert negative regulation of TRPV6 function [86]. A critical heteromeric interaction involving TRPC channels is the TRPC-Orai1 interaction. TRPC1, TRPC3 and TRPC6 functionally interact with Orai1. TRPC1-Orai1 interaction has been confirmed by co-immunoprecipation data as well as TIRFM measurements, where store depletion-dependent clustering of the two channels has been observed in several cell types [87–92]. Further, and more importantly, Orai1 is

required for TRPC function, as knockdown of endogenous Orai1 abolished TRPC1 channel activation [88, 92]. A similar requirement for Orai1 has been reported for activation of TRPC3 and TRPC6 in response to ER-Ca^{2+} store depletion [93–95]. The mechanism underlying the Orai1-dependent regulation of TRPC1 has now been resolved (more details will be presented below). Importantly, TRPC1 and Orai1 have been shown to generate two distinct channels that appear to contribute to specific cellular functions [88, 96]. Interestingly, a recent study reported that a splice variant of TRPC1 interacts with and positively regulates Orai1 channel activity in HEK293 cells. This splice variant, TRPC1ε, was first identified in early pre-osteoclasts and together with I-mfa (an inhibitor of MyoD family), has been proposed to function antagonistically to decrease Orai1 channel activity, fine tuning the Ca^{2+} signaling process that regulates osteoclastogenesis [42]. The multiplicity of interactions between various TRPC channels, as well as between TRPC and other channels or regulatory proteins, lead to the generation of a plethora of signaling complexes that can regulate a wide variety of cellular functions. It is possible that the composition of these heteromeric channels as well as the interacting signaling proteins depends on the type of cell and the particular physiological function to be regulated. There are an increasing number of studies that highlight the importance of spatial and temporal aspects as well as the magnitude of Ca^{2+} signals as major determining factors in the regulation of cellular responses to different physiological stimuli. It is important that these should be taken into account when the physiological functions of TRPC channels are being assessed.

5.3 Role of STIM1 and STIM2 in SOCE and TRPC Channel Regulation

STIM1 and STIM2 were discovered in studies using siRNA screening to identify proteins required for SOCE. Both proteins reside within the ER and during resting (unstimulated) conditions, have Ca^{2+} bound to the luminal N-terminal EF hand domains. Following store depletion, Ca^{2+} is released from the EF hand which leads to multimerization of the protein and translocation to the peripheral region of the cells where it concentrates in the form of puncta within distinct ER-plasma membrane (ER-PM) junctions. Within this microdomain, the ER membrane and plasma membrane are in close apposition to each other [22, 97, 98]. More importantly, the proximity between the two membranes allows STIM1 in the ER to interact with and gate both TRPC and Orai1 channels. Different aa regions in the cytosolic C-terminus of STIM1 are involved in activating Orai1 and TRPC1. Orai1 activation is mediated by the STIM1 Orai1 Activating Region (SOAR; aa 344–442) [99], whereas TRPC channel gating occurs via the polybasic domain (aa 672–685) [91, 100]. It has been suggested that TRPC1 and TRPC4 are the main TRPC channels that can interact with and be gated by STIM1. However, if other TRPC channels are assembled in a heteromeric channel complex with either TRPC1 or TRPC4, they appear to become store-dependent due to the activation of TRPC1 and TRPC4 by STIM1. For

example, although STIM1 does not interact with TRPC3 and TRPC6, STIM1 can activate TRPC1/TRPC3 or TRPC4/TRPC6 channels [101].

Strong evidence for STIM1 in gating TRPC channels comes from studies showing an effect on channel activity of either knockdown or overexpression of STIM1 or STIM1 with mutations that impair STIM1-TRPC channel interactions. TRPC1 was the first TRPC channel shown to be regulated by STIM1. Knockdown of endogenous STIM1 severely reduced endogenous TRPC1-mediated SOCE and Ca^{2+} currents, whereas co-expression of TRPC1 and STIM1 increased SOCE [87, 88, 91]. Store depletion induced interaction between TRPC1 and STIM1, shown by co-immunoprecipitation experiments as well as FRET and TIRFM measurements. Conversely, store refilling terminated TRPC1 function as well as STIM1-TRPC1 association [91, 100–104]. Thus the TRPC1-STIM1 interaction is dictated by the ER-[Ca^{2+}] status. Conclusive studies by Muallem and co-workers resolved the mechanism by which STIM1 gates TRPC1. Their findings demonstrated that gating of TRPC1 involves electrostatic interactions between the negatively charged aspartate residues in TRPC1 (^{639}DD^{640}) with the positively charged lysines in the STIM1 polybasic domain (^{684}KK^{685}) [100]. Further studies showed that these negatively charged residues are conserved in TRPC3, TRPC4, TRPC5, TRPC6 and TRPC7, suggesting the possibility that STIM1 can also gate these other TRPC channels [100]. Consistent with this study, TRPC4-mediated SOCE in murine and human endothelial cells was suppressed by knockdown of STIM1 or expression of a charge-swap mutant of STIM1 (KK684,685EE). Similarly, disrupting the electrostatic interaction between STIM1 and TRPC4 by mutation of the conserved negatively charged residues on TRPC4 (EE647,648KK) significantly reduced SOCE and STIM1-TRPC4 interactions [65]. Co-immunoprecipitation of STIM1 and TRPC3 was also increased following store depletion in salivary gland duct cells, although this could be due to TRPC1-TRPC3 interaction in these cells [105]. In yet another study, mutations in the conserved negative residues in the C-terminus of TRPC3 (DD697,698KK), TRPC4 (EE648,649KK), TRPC5 (DE651,652KK) and TRPC6 (EE755,756KK) prevented electrostatic interactions with and gating by STIM1. While these mutants did not respond to store depletion induced by cyclopiazonic acid, they could still be activated by muscarinic receptor stimulation in a STIM1-independent manner. However, co-expression of the charge-swap STIM1 mutant (KK684,685EE) restored store responsiveness to these TRPC mutants [106].

Collectively, these data demonstrate that STIM1 has the ability to gate all TRPC channels via similar electrostatic interactions, even though not all channels appear to interact directly with STIM1. Since TRPC channels are widely expressed in tissues and species, it is not yet clear what determines the interaction of any particular TRPC channel with STIM1 and thus their mode of activation. In neuroblastoma cells, STIM1 promoted SOCE mediated via TRPC1 and TRPC6, while inhibiting TRPC6-mediated store-independent Ca^{2+} entry [107]. TRPC5 was also reported to contribute to SOCE in RBL cells. In these cells co-expression of STIM1 with TRPC5 increased, while knockdown of STIM1 abolished, thapsigargin-induced cation entry [108]. Thus, STIM1 might not only be involved in gating some TRPC channels but also determine their recruitment into a store-dependent mode. However,

further studies are required to establish this role of STIM1 on the mode of activation of TRPC channels. Involvement of other factors that might contribute to this switch in the mode of regulation also needs further detailed studies. Since several TRP channels show polymodal regulation of their function, possible activation of TRPC channels by mechanisms other than store depletion appears to be quite feasible.

There is considerable information regarding the exact intramolecular rearrangements and molecular domains involved in activation of STIM1. Further, a large number of structure-function studies have now been reported describing the configuration of STIM1 required for binding and activation of Orai1 (recently reviewed in [109]). However, a similar detailed understanding of STIM1-TRPC channel interaction is currently lacking. An ezrin/radixin/moesin (ERM) domain (aa 251–535) was shown to mediate the binding of STIM1 to TRPC channels [91]. However, since the SOAR domain resides within this ERM region of STIM1 it was suggested that the SOAR domain might also be involved in mediating STIM1 binding to TRPC channels [12, 105]. The coiled-coil (CC) motif within the C-terminus of Orai1 is proposed to interact with STIM1 [110]. TRPC channels also have CC domains in both their N- and C-termini. Further, co-immunoprecipitation studies have revealed strong interactions between exogenously expressed SOAR and endogenous TRPC1, TRPC4 and TRPC5, minimal interactions with endogenous TRPC3 and TRPC6, and no interactions with TRPC7 [101, 105]. Mutation of residues in the N-terminal CC domains severely weakened SOAR interactions with TRPC1, TRPC4 and TRPC5 but enhanced association of TRPC3 and TRPC6 with SOAR. Based on this data, it was proposed that interaction of TRPC1 with TRPC3 induces a structural change which exposes a domain in TRPC3 that promotes its binding to STIM1 [105]. A recent study investigating the stoichiometry of TRPC, STIM1, and CaM assembly in a signaling complex reported TRPC channel activation using recombinantly purified SOAR [111]. This study demonstrated that only TRPC channel complexes containing TRPC1, TRPC4 and TRPC5 could be activated by SOAR. Each TRPC tetrameric complex required two SOAR domains for activation and four CaMs for inactivation. SOAR and CaM appeared to reciprocally regulate TRPC channel activity when co-expressed in HEK293 cells. Following application of tenfold higher amounts of CaM, TRPC1 channel activity was reduced, even though SOAR was still bound to the tetramers at the initial stages of inhibition. SOAR eventually detached from TRPC1, which led to further CaM-dependent decline in channel activity [111]. However, to conclusively establish that SOAR directly affects TRPC channel activity, data need to be provided to exclude SOAR-Orai1 effects in the same cell since SOAR domain will also gate Orai1 and Orai1-mediated Ca^{2+} entry will lead to TRPC channel activation. It is worth noting that some studies suggest that Orai1 binding to STIM1 might limit availability of STIM1 for TRPC channels. However, these studies have yet to be confirmed at the level of endogenous proteins. Further studies are required to establish whether STIM1 is indeed a limiting factor for channels contributing to SOCE and whether different physiological conditions favor binding of STIM1 to one type of channel vs the other.

STIM1 shares significant homology with another family member, STIM2. While functional domains such as the EF hand, CC domains, SOAR and polybasic domain are conserved between STIM1 and STIM2 there are several key differences which determine their diverse physiological function and role in SOCE. For example, STIM2 can interact with Orai1 but is a poor activator of the channel compared to STIM1. The difference in the gating efficiency of STIM1 and STIM2 was shown to be due to a single aa difference in their respective SOAR domains; F394 in SOAR1 vs L485 in SOAR2 [112]. The EF hand of STIM2 has a lower affinity for Ca^{2+} than STIM1. Thus, STIM2 can sense and respond to small changes in ER-[Ca^{2+}]. The triggering threshold level of $[Ca^{2+}]_{ER}$ for STIM2 is >400 μM, while STIM1 responds when ER-[Ca^{2+}] is around 200 μM. Based on this, STIM2 has been suggested to aggregate and translocate to ER-PM junction under conditions when there is minimal depletion ER-Ca^{2+} [113] One reported function for STIM2 is the regulation of Orai1 in resting cells to maintain $[Ca^{2+}]_i$ [114]. Another study suggests that STIM2 gates Orai1 in cells stimulated with low agonist where there is less depletion of ER-Ca^{2+} stores while STIM1 is involved in gating Orai1 at high agonist concentration when there is greater depletion [115]. A recent study provides a novel role for STIM2 showing that STIM2 associates with STIM1 and promotes the clustering of STIM1 in ER-PM junctions in cells stimulated with low [agonist]. STIM2 co-clusters with Orai1 and promotes STIM1-Orai1 interactions at low levels of stimulation while STIM1 aggregates efficiently, in a STIM2-independent manner, and interacts with Orai1 in cells stimulated with high [agonist] [116]. Knockdown of STIM2 in HEK293 cells or targeted knockout of STIM2 in mouse salivary glands attenuated STIM1-mediated activation of Orai1 and decreased the agonist sensitivity of SOCE activation. This was especially prominent at lower levels of agonist. On the other hand, knockdown of STIM1 completely eliminated SOCE at low and high levels of stimulus. Hence, STIM2 appears to tune the agonist-sensitivity of the STIM1-Orai1 interactions and associated Ca^{2+} signals [116]. Few studies have investigated the direct contribution of STIM2 to TRPC-mediated SOCE. STIM1 has been proposed to regulate TRPC1 and TRPC3 channel function, whereas STIM2 regulated only TRPC1 function in HEK293 cells [117]. Modulation of the STIM1:STIM2 ratio appears to determine the store responsiveness of TRPC1 channel function in intestinal epithelial cells [118]. Thus, the exact role of STIM2 in TRPC channel function and regulation remains to be determined.

5.4 Orai1-TRPC Channel Interactions in SOCE

The pore-forming component of CRAC channels, Orai1, is indispensable for SOCE. A naturally occurring mutation of the channel, (R91W), which leads to loss of channel function, has been linked to severe combined immune deficiency (SCID) [119–121]. Orai1 has two closely related family members, Orai2 and Orai3, although there is a paucity of data regarding their contribution to SOCE when compared to Orai1 [122]. While endogenous Orai1 function is supported by endogenous

STIM1, exogenously expressed Orai1 does not by itself increase SOCE in cells unless STIM1 is co-expressed with it. The reason for this is not yet clear as cells appear to express STIM1 in excess of Orai1. Subsequent studies identified the pore region of Orai1 by showing that E106Q mutation generates a channel with a non-functional pore, while E106D changes Ca^{2+} selectivity [22, 110, 123, 124]. An additional interesting observation that has been reported is that STIM1 increases the Ca^{2+} selectivity of Orai1 [125]. Again the latter study was carried out with overexpressed protein and needs to be more fully examined using the endogenous channel.

Intriguingly, a number of studies demonstrate that Orai1 is also required for TRPC1 function. Knockdown of endogenous Orai1 abolished SOCE, despite the presence of endogenous or exogenously expressed STIM1 and TRPC1. Further, it was reported that Orai1 and STIM1 form a complex with TRPC1 in response to ER-Ca^{2+} store depletion in HSG cells [103], mouse pulmonary arterial smooth muscle cells [126], human parathyroid cells [127], human liver cells [128] rat kidney fibroblast [129], pancreatic acinar cells and salivary gland acinar cells [34, 39]. Notably assembly of the TRPC1-Orai1 complex requires STIM1 which also gates both channels. Co-localization of the three proteins in ER-PM junctions, as detected by TIRFM, suggests that TRPC1 is also localized in same ER-PM junctions where Orai1-STIM1 complex is assembled. The requirement of Orai1 in TRPC1 function was further revealed by data showing that non-functional Orai1 mutants, either Orai1E106Q or Orai1R91W, abrogated store-dependent activation of TRPC1 [87, 88, 92, 103]. Based on this, it was first proposed that TRPC1 and Orai1 assemble into a heteromeric channel where both proteins contribute to the channel pore. There was also the suggestion that TRPC channel forms the pore while Orai1 serves as a regulator. While this led to an extensive debate regarding the assembly of these putative channels, neither of these proposals was supported by conclusive data. Finally, the mechanism underlying the requirement of Orai1 in TRPC1 function was demonstrated in a study where Ca^{2+} influx mediated by Orai1 triggers plasma membrane insertion of TRPC1 [88]. The insertion presumably occurs within the same ER-PM junctions where the Orai1-STIM1 complex is assembled, to allow for TRPC1 gating by STIM1. Moreover, recruitment of TRPC1 into these junctions brings TRPC1 in close proximity to Orai1, such that Ca^{2+} entry via Orai1 can be sensed locally to trigger plasma membrane recruitment of TRPC1. This requirement of Orai1-mediated Ca^{2+} entry for TRPC1 insertion into the plasma membrane also accounts for the lack of TRPC1 activity when non-functional mutants of Orai1 are expressed. Importantly, Ca^{2+} entry mediated by TRPC1 and Orai1 are utilized by cells to regulate separate functions. Orai1-mediated SOCE is sufficient for activation of NFAT, whereas Ca^{2+} entry via both Orai1 and TRPC1 are required for NFκB expression and function, with TRPC1 contribution being predominant [88, 96]. Thus, Orai1 and TRPC1 form two separate STIM1-regulated channel complexes (Fig. 5.1). TRPC1 and STIM1 form a SOC channel that generates I_{SOC} while Orai1 and STIM1 form the highly Ca^{2+}-selective CRAC channel mediating I_{CRAC}. The smaller I_{CRAC} is masked by the larger I_{SOC} current and unmasked when TRPC1 function is suppressed. It should be noted that true TRPC1 currents

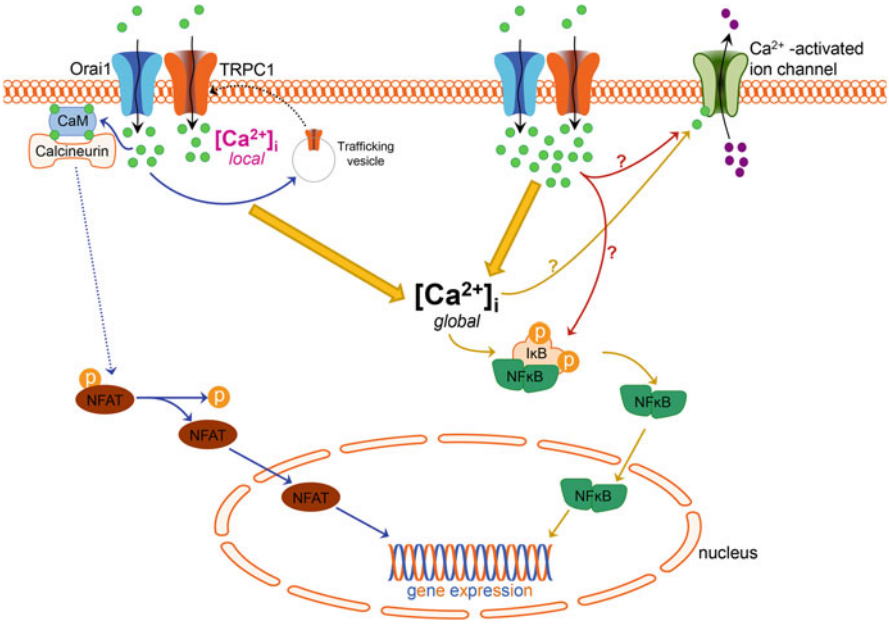

Fig. 5.1 Physiological function of Orai1 and TRPC1 in SOCE. Stimulation with agonists generates $[Ca^{2+}]_i$ changes that occur locally (i.e. close to the channel pore) and globally (i.e. throughout the cell cytosol). Local SOCE mediated by Orai1 has been shown to activate calcineurin, which subsequently induces NFAT translocation into the nucleus to drive gene expression. Local Orai1-SOCE also promotes insertion of TRPC1 into the plasma membrane. Ca^{2+} entry via both Orai1 and TRPC1 contribute to increase in global $[Ca^{2+}]_i$, which has been shown to activate NFκB and NFκB-driven gene expression. While the Ca^{2+}-activated ion channels in the plasma membrane are also activated by global $[Ca^{2+}]_i$, it is not clear whether the activating Ca^{2+} comes from those situated in the vicinity of neighboring Orai1 and TRPC1 channels and/or from the deeper regions of the cell cytosol

have not yet been described as most reported measurements of I_{SOC} include currents generated by both TRPC1+STIM1 and Orai1+STIM1 channels [88]. A requirement for Orai1 in other TRPC channels such as TRPC3 and TRPC6 [93], as well as heteromeric TRPC channels TRPC1/TRPC4 [130], have been reported. Whether TRPC channel trafficking is involved in these cases is not yet known. The exact proteins involved in regulating and mediating exocytosis of TRPC1 have not yet been elucidated.

One interesting suggestion which has been made is that Orai1 can regulate TRPC channels by determining their recruitment into specialized microdomains in the plasma membrane, such as the lipid raft domains (LRDs). This suggestion is consistent with previous studies showing that SOCE requires intact LRDs [39, 88, 90, 104, 131, 132]. Further, STIM1-TRPC1 interaction also takes place within LRDs as disruption of LRD leads to abrogation of STIM1-TRPC1 interaction and loss of SOCE [104]. Similarly, disruption of LRD in human platelets and HEK293 cells reduced interactions between Orai1, TRPC1, TRPC6 and STIM1 [90, 132, 133]. Orai1

interacts with the cytosolic termini of TRPC1 and TRPC6 to modulate their sensitivity to store depletion and STIM1 [93, 134, 135]. Thus, Lutz Birnbaumer and co-workers suggested the hypothesis that recruitment of Orai1, TRPC and STIM1 into LRD confers store-responsiveness to the channels [135]. At this stage, it remains unclear whether Orai1-STIM1 and TRPC-STIM1 complexes are initially formed outside the lipid rafts and subsequently recruited into these microdomains following store depletion or are maintained within this domain by interactions with Orai1 and STIM1. Presence of PIP_2-interacting domains in the C-terminus of STIM1 and STIM2 [132], which are proposed to enable anchoring of the proteins to the plasma membrane within ER-PM junctions, led to several studies examining the role of PIP_2 in SOCE. Effects of PIP_2 depletion on SOCE were inconsistent with some studies showing no effect on SOCE mediated by Orai1 while others demonstrated decreased function and STIM1 clustering [136–139]. Nevertheless, STIM1 or STIM2 lacking the polybasic tail domain do not form puncta within ER-PM junctional domains. However, when Orai1 is expressed with this mutant of STIM1, it rescues STIM1 clustering and CRAC channel activity. It has been suggested that the Orai1-STIM1ΔK complexes might be localized outside the ER-PM junctions, suggesting that the PIP_2 is not required for STIM1-dependent gating of Orai1 [140]. It is unclear whether TRPC-STIM1 interactions can also take place outside the junctional domains. A recent study suggests that dynamic changes in PIP_2 levels within ER-PM junctions mediated by proteins such as septin, impact not only assembly of the Orai1-STIM1 complexes but also regulation of CRAC channel activity [141–143]. It has also been recently reported that the ER-PM junctions might contain different PIP_2 microdomains. This study showed that Orai1-STIM1 complex assembled in ER-PM junctions is transferred from relatively PIP_2-poor to a PIP_2-rich microdomain which dictates the Ca^{2+}-dependent regulation of the channel. Interestingly, this recruitment is determined by Cav-1 and septin [143]. Further studies will be required to fully elucidate the dynamic lipid and protein remodeling that occurs with the ER-PM junctions that critically impact Orai1 and TRPC1 interaction with STIM1 and their function.

Trafficking of TRPC channels has been proposed as a major mode of regulation of their function in the plasma membrane. In addition to the trafficking proteins, scaffolding and regulatory proteins also modulate the magnitude and duration of TRPC-mediated SOCE. The main regulatory pathways that modulate surface expression and function of TRPC channels comprise of constitutive and regulated intracellular trafficking mechanisms. The enhancement of Ca^{2+} influx through TRPC channels can be due to increased exocytosis, retention via interaction with scaffolding proteins, and/or decreased channel endocytosis. As discussed above, TRPC1 function is dependent on LRD [144]. The cholesterol-binding LRD protein Cav-1 is reported to play a pivotal role in plasma membrane localization and activity of TRPC1. TRPC1 interacts with Cav-1 through binding sites located in its N- and C-terminal domains. The N-terminal Cav-1 binding site is involved in scaffolding and localization of TRPC1 in the plasma membrane while the C-terminal domain has been proposed to control channel function and/or inactivation. Knockdown of Cav-1, and mutations in Cav-1 or Cav-1 binding sites in TRPC1

resulted in mislocalization of TRPC1 and impairment of channel activity. Hence, Cav-1 is suggested as an LRD scaffolding protein for TRPC1 that determines plasma membrane localization [38, 39, 131, 145–149]. The current model proposes that in resting cells, constitutive trafficking mechanisms target TRPC1 to cellular regions close to the plasma membrane where the inactive channel interacts with Cav-1 and is retained at that location intracellularly. Following store depletion, STIM1 translocates to ER-PM junctions and activates Orai1. Ca^{2+} entry mediated by Orai1 is a pivotal step in TRPC1 insertion into the plasma membrane and channel activation [88], as it triggers the insertion of TRPC1 into the plasma membrane. Under these conditions, TRPC1 dissociates from Cav-1, interacts with and is gated by STIM1. Following ER-Ca^{2+} store refilling, SOCE is inactivated and TRPC1 disassembles from STIM1. LRDs are essential for STIM1 translocation to the ER-PM junctions as deleting the C-terminal lysine-rich region of STIM1, which contains a PIP_2-binding sequence, impairs puncta formation in these junctions and also alters partitioning of STIM1 into detergent insoluble fractions from cells. In addition, disruption of lipid rafts by cholesterol depletion also affects the ability of STIM1 to interact with TRPC1 [104]. These data demonstrate the importance of structural integrity for caveolar lipid rafts to act as scaffolding platforms for TRPC1-mediated SOCE. Based on the recent study that showed Cav-1 is required for recruitment of Orai1-STIM1 channel to a PIP_2-rich domain [143], it is possible that this event can bring TRPC1 in close proximity to Orai1 such that it can sense local $[Ca^{2+}]_i$ elevation due to Orai1-mediated Ca^{2+} entry. Indeed, Cav-1 might be of utmost importance in Orai1-dependent activation of TRPCs as almost all TRPC channels have once or more Cav-1 binding domains, some of which are fairly well conserved. Nonetheless, Ca^{2+} sensor proteins, as well as the identity of vesicles and intracellular compartments related to TRPC1 trafficking, remain to be determined.

In addition to Cav-1, another scaffolding protein that regulates TRPC1 function is Homer1. The C-terminus of TRPC1 (aa 644–650) forms a complex with Homer1 and IP_3R in resting cells. However, following store depletion, this complex dissociates to enable subsequent TRPC1 interaction with and gating by STIM1 [150, 151]. Further evidence for the contribution of Homer1 came from a study with knockout mice (Homer1$^{-/-}$) that reported impaired SOCE in skeletal muscle cells [47]. Soluble N-ethylmaleimide-sensitive factor attachment receptor (SNARE) proteins, such as synaptosome-associated protein (SNAP-25), are involved in membrane fusion within intracellular compartments or between vesicles and plasma membrane. Interaction of SNAP-25 with TRPC1 is vital for channel function as botulinum toxin treatment, which cleaves and inactivates SNAP-25, decreased SOCE in platelets [152]. Several cytoskeletal and microtubule proteins have also been shown to modulate the TRPC1 channel trafficking and activity. The monomeric GTPase protein, RhoA, regulates TRPC1 translocation to the plasma membrane in endothelial cells [29]. Interaction of β-tubulin with TRPC1 determines surface expression of TRPC1 retinal epithelial cells [153]. Disrupting TRPC1 interaction with either RhoA or β-tubulin significantly decreased SOCE. In aggregate, the data show that proper localization of TRPC1 in the plasma membrane, as well as trafficking to the

specific domains where SOCE is regulated, are vital for its interaction with Orai1-STIM1 and its activation.

5.5 Conclusions

The mechanism(s) underlying SOCE involves multiple interactions that allow cells to display dynamic regulatory modes for each physiological stimulus. The multiplicity of channel-protein and protein-protein interactions underscores the variety of signaling complexes that can be generated within a subregion of the cell. Indeed, TRPC channels interact with a wide range of channels and proteins involved in Ca^{2+} signaling, as well as scaffolding and trafficking processes. Such complexity underlies the physiological functions that have been ascribed to TRPC channels. Many studies have investigated the contributions of STIM1 and Orai1 to TRPC channel function. The functional relevance of STIM2, as well as Orai2 and Orai3, in SOCE remains to be resolved. It is worth noting that many cells and tissues express both STIM proteins and more than one Orai protein. Therefore, depending on the type and intensity of the cell stimulus, TRPC channels may also form dynamic signaling complexes with these STIMs and Orais to generate SOCE. Nonetheless, much remains to be elucidated to expand our current understanding of the exact sequence of molecular events involved in the regulation and function of TRPC channels in response to ER-Ca^{2+} depletion. As TRPC channels have been implicated in a number of human diseases, understanding the mechanism(s) involved in regulating and modulating channel function will provide potentially important information and lead to novel targets for the development of effective therapeutic interventions.

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Chapter 6
Phospholipase A_2 as a Molecular Determinant of Store-Operated Calcium Entry

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Abstract Activation of phospholipases A_2 (PLA_2) leads to the generation of biologically active lipid products that can affect numerous cellular events. Ca^{2+}-independent PLA_2 ($iPLA_2$), also called group VI phospholipase A_2, is one of the main types forming the superfamily of PLA_2. Beside of its role in phospholipid remodeling, $iPLA_2$ has been involved in intracellular Ca^{2+} homeostasis regulation. Several studies proposed $iPLA_2$ as an essential molecular player of store operated Ca^{2+} entry (SOCE) in a large number of excitable and non-excitable cells. $iPLA_2$ activation releases lysophosphatidyl products, which were suggested as agonists of store operated calcium channels (SOCC) and other TRP channels. Herein, we will review the important role of $iPLA_2$ on the intracellular Ca^{2+} handling focusing on its role in SOCE regulation and its implication in physiological and/or pathological processes.

Keywords Phospholipases A2 • SOCE • TRP channels • Lysophospholipids • STIM1 • Orai1

Abbreviations

AA	Arachidonic acid
$AdPLA_2$	Adipose-specific PLA_2
ARC	Arachidonic acid-regulated calcium channels
BEL	Bromoenol lacotone
CaM	Calmodulin
$cPLA_2$	Cytosolic PLA_2

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DAG	Diacylglycerol
ER	Endoplasmic reticulum
$iPLA_2$	Calcium-independent PLA_2
LA	Lysophasphatidyl acid
$LyPLA_2$	Lysosomal PLA_2
OAG	1-oleoyl-2-acetyl-sn-glycerol
PAF-AH	Platelet-activating factor acetylhydrolases
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PG	Phosphatidylglycerol
PS	Phosphatidylserine
ROC	Receptor operated channels
SMC	Smooth muscle cell
$sPLA_2$	Secretory PLA_2
SOCC/SOCE	Store operated Ca^{2+} channels/entry

6.1 Classification of Phospholipase A_2

The phospholipase A_2 superfamily enzymes are characterized by their ability to catalyze the hydrolysis of glycerophospholipids at the sn-2 position and generate several classes of bioactive lipids, fatty acids and lysophospholipids [1]. Six main families of phospholipases have defined physiological implications. They comprise secretory PLA_2 ($sPLA_2$), cytosolic PLA_2 ($cPLA_2$), lysosomal PLA_2, adipose-specific PLA_2 ($AdPLA_2$); and two major Ca^{2+}-independent groups, calcium-independent PLA_2 ($iPLA_2$) and platelet-activating factor acetylhydrolases (PAF-AH). This subdivision was based on their structures, catalytic mechanisms, localizations and evolutionary relationships, and they are collectively identified as groups, using roman numerals (i.e. Group I to Group XVI), with capital letters to distinguish individual sub-families [2]. Many of PLA_2 have contrasted role in cell signaling that involve intracellular Ca^{2+} homeostasis regulation.

6.1.1 Secretory PLA_2 ($sPLA_2$)

The secretory PLA_2s (belonging to Groups I, II, III, V, IX, X and XII in mammalians) were the first type of PLA_2 enzymes discovered. They were identified in organisms such as snakes and scorpions; in components of pancreatic juices; arthritic synovial fluid; and in many different mammalian tissues [3]. Most $sPLA_2$ isoforms are calcium-dependent, and require millimolar concentrations of the ion to function optimally [2, 4, 5]. Consequently, $sPLA_2$s typically function at the external side of the cell hydrolyzing a wide variety of phospholipids [2, 6]. $sPLA_2$ hydrolyzes the sn-2 ester bond in the glyceroacyl phospholipids presents in lipoproteins and cell

membranes, inducing structural and functional changes and forming arachidonic acid (AA), lysopholipids and non-esterified fatty acids with direct proinflammatory effects [7, 8]. In general, $sPLA_2$ isoforms have solid preference for negatively charged phospholipid head groups, in particular phosphatidylserine (PS), phosphatidylglycerol (PG) and phosphatidylethanolamine (PE) [9]. Recent studies have suggested that some $sPLA_2$ isoforms can modify cell functions by binding to receptors and other proteins [5].

6.1.2 *Cytosolic PLA_2 ($cPLA_2$)*

The $cPLA_2$ family (also named Group IVA–F) is one of the major PLA_2 that contains six isoforms, ranging in size from 60 to 85 kDa, which are generally localized in the cytosol. They are active in the presence of mM levels of Ca^{2+} and, with the exception of $cPLA_2\gamma$ (Group IVC), contains in their N-terminals a C2 domain for the binding of two Ca^{2+} ions as well as two conserved phosphorylation sites. $cPLA_2$ family members have a catalytic domain characterized by a three-layer architecture employing a conserved Ser/Asp catalytic dyad, instead of the classical catalytic triad, that is similar in structure to that of $iPLA_2$ [10, 11]. The first group IV $cPLA_2$ (Group IVA) was firstly identified in human platelets in 1986 [12] and was cloned and sequenced 5 years later [13, 14]. $cPLA_2$ is perhaps the far most widely studied cytosolic enzyme and, besides transacylase activity, is also known to have PLA_2 and lysophospholipase activities [15]. $cPLA_2$ is activated by several different mechanisms, and is recruited to the membrane by a Ca^{2+} dependent translocation of the C2 domain. A recent work has localized the lipid binding surface of the enzyme in the presence of Ca^{2+} [16].

From the different PLA_2s, $cPLA_2$ is the only one described to have a preference for AA in the sn-2 position of phospholipids [10, 14]. Upon activation and translocation to intracellular membranes, $cPLA_2$ generates and releases AA from membrane phospholipids leading to an active lipoxygenase and cyclooxygenase metabolism [17]. AA, which acts as precursor for the generation of eicosanoids, is a key player in the prostanoid signaling cascades and therefore its activation is important for regulating various physiological and pathological processes including immune and inflammatory-related processes [2, 18, 19]. Furthermore, AA is also considered as an agonist that induces cytosolic Ca^{2+} entry through cationic channels called arachidonic acid-regulated calcium channels (ARC) [20, 21].

6.1.3 *PAF Acetyl Hydrolase/Oxidized Lipid ($PAF\text{-}AH/LpPLA_2$)*

Platelet activating factor (PAF) acetylhydrolases (AH) (PAF-AH, Group VIIA and B, and VIIIA and B) have low molecular weight (26–45 kDa) and represent a unique group of acyl hydrolases with a catalytic serine that is capable of releasing acetate

from the sn-2 position of PAF, a 1-*O*-alkyl-PC [22]. However, they can also catalyze the release of oxidized acyl groups from the sn-2 position of PC and PE, not just PAF [2, 4, 23]. Its active site is composed of a serine, histidine, and aspartic acid hydrolase triad, unlike all other PLA_2s, which have dyads [24]. There are four members of this family that specifically catalyze these reactions; one of them is a secreted protein (GVIIA PLA_2), known as plasma-type PAF-AH or "lipoproteinassociated PLA_2" ($LpPLA_2$), that has generated interest as a therapeutic target for atherosclerosis [22, 26–29]. On the other hand, $LpPLA_2$ is a potent phospholipid activator that is secreted by multiple inflammatory cells including monocytes/macrophages, T lymphocytes and mast cells [30, 31]. This enzyme was cloned from human plasma in 1995 and was shown to have anti-inflammatory activity in vivo [25]. The $LpPLA_2$ role in cytosolic Ca^{2+} regulation is still unknown.

6.1.4 Lysosomal PLA_2 ($LyPLA_2$)

Lysosomal PLA_2 was purified from bovine brain as an enzyme that esterifies an acyl group with the hydroxyl group in the C-1 position of ceramide using phospholipids as the acyl group donor, so the enzyme was first named 1-O-acylceramide synthase (ACS). The protein possesses Ca^{2+} independent PLA_2 and transacylase activities. Hiraoka et al. [32] proposed that the hydrolyzed acyl group is transferred through an enzyme-acyl intermediate to ceramide or water, resulting either in the production of either 1-Oacyl- ceramide (ACS activity) or the release of free fatty acids (PLA_2 activity). In terms of catalytic activity, Ly-PLA_2 specifically prefers PC and PE head groups at pH 4.5 in a Ca^{2+}-independent manner. Ly-PLA_2 is ubiquitously expressed in diverse cell types, but highly expressed in alveolar macrophages. In fact, it plays a role in surfactant metabolism, and specifically in the phospholipid catabolism of pulmonary surfactant [33, 34].

6.1.5 Adipose Specific PLA_2 ($AdPLA_2$)

Duncan et al. [35] discovered recently a novel intracellular PLA_2, highly and differentially expressed only in adipocytes and induced during preadipocyte differentiation, that releases sn-2 fatty acid from phospholipids in a Ca^{2+}-dependent manner. This recently discovered enzyme named adipose-specific PLA_2 ($AdPLA_2$, Group XVI), has a molecular weight of 18 KDa. It is found abundantly in white adipose tissue, 40–150 times higher that found in liver. The enzyme is not an acyltransferase, but it functions entirely as a phospholipase, producing lysophosphatidylcholine and AA from the phospholipids. In addition, Duncan and colleagues studied the properties of AdPLA and found its optimal pH was 8.0, requiring cysteine and histidine residues at the active site, with maximal enzymatic activity in the presence of 1.0 mM Ca^{2+} [35]. $AdPLA_2$ have been also implicated in energy regulation as it modultes the release of fatty acids, from stored triglycerides in white adipose

Table 6.1 Isoforms of calcium-independent (Group VI) PLA2

PLA_2 family	Group	Source	MW (KDa)	Alternate name
$iPLA_2$	VIA-1	Human/murine	84–85	$iPLA_2\alpha$
	VIA-2	Human/murine	88–90	$iPLA_2\beta$
	VIB	Human/murine	88–91	$iPLA_2\gamma$
	VIC	Human/murine	146	$iPLA_2\delta$, neuropathy target esterase (NTE)
	VID	Human	53	$iPLA_2\varepsilon$, adiponutrin
	VIE	Human	57	$iPLA_2\zeta$, TTS-2.2
	VIF	Human	28	$iPLA_2\eta$, GS2

tissue, which will be later used as energy source by other tissues. $AdPLA_2$ has been also proposed to play a major role in the supply of AA for prostaglandin E2 (PGE2) synthesis in white adipose tissue [36]. Thus, AdPLA is considered a major regulator of adipocyte lipolysis and is crucial for the development of obesity, although it seems possible that AdPLA could promote obesity through a mechanism distinct from PGE2 signaling [37].

6.1.6 Calcium Independent PLA_2 ($iPLA_2$)

The Ca^{2+} independent PLA_2s are members of the GVI family of PLA_2 enzymes. Currently, six isoforms of $iPLA_2$ (Group VIA–F) have been identified as shown in Table 6.1. While their catalytic sites are similar to that of $cPLA_2$, they do not require Ca^{2+} for catalytic activity and they are generally larger in size, with moleculear weights ranging from 55 to 146 kDa except for Group VIF PLA_2 (~28 kDa). $iPLA_2$s are localized either in the cytosol, the endoplasmic reticulum (ER) or in the mitochondrial membrane [38]. $iPLA_2$ are entirely involved in lipid remodeling, in the Land's Cycle, and also mediate cell growth signaling [2, 4]. Members of this family share a protein domain initially discovered in patatin, the most abundant protein of the potato tuber.

In the next part of this chapter, we will go through $iPLA_2$ classification, regulation, and its role in intracellular Ca^{2+} regulation.

6.2 Sub-classification of $iPLA_2$

6.2.1 GVIA PLA_2 ($iPLA_2\alpha$ and $iPLA_2\beta$)

Many new $iPLA_2$ (GVI PLA_2) members have been identified in the last years, but the first member and the best characterized of this family is the GVIA PLA_2, which was purified from macrophages in 1994 [39, 40]. GVIA PLA_2 is expressed in

multiple different splice variants [41] and, similar to cPLA$_2$ (GIV PLA$_2$), it catalyzes the cleavage of the sn-2 ester bond. However, it does not show specificity for AA in the sn-2 position and is fully active in the absence of Ca^{2+}. The GVIA PLA$_2$ also possesses sn-1 lysophospholipase and transacylase activity [41]. The enzyme has a conserved glycine-rich nucleotide-binding motif (GXGXXG) proximal to the catalytic site and it is activated several-fold by ATP [42]. The N-terminal domain of GIVA PLA$_2$ is composed of seven to eight ankyrin repeats, which are responsible for protein-protein interaction between monomers [43]. It is thought that ankyrin repeats enable the oligomeration of Group VIA monomers required for catalytic activity [39]. In fact, the active form of Group VIA PLA$_2$ is a tetramer [39].

Several splice variants of GVIA PLA$_2$ have been identified [39, 44]. Group VIA-1 or iPLA$_2$α, and Group VIA-2 or iPLA$_2$β [44–48], for example, comprise two catalytically active forms of this enzyme [44–48]. Both isoforms are similar in size, 85 and 88 KDa respectively, and contain eight N-terminal ankyrin repeats and a consensus lipase motif (GXS^{465}XG), whereas in GVIA-2 PLA$_2$ the 8 ankyrin repeats are interrupted by an insertion of 54 amino acids and they exhibit a glutamate residue at position 450, while the corresponding position in Group VIA-1 is glutamine.

Three additional splice variants of GVIA iPLA$_2$ have been identified: Group VIA-3 (also known as iPLA$_2$-2); Group VIA Ankyrin-1 (or Ankyrin-iPLA$_2$-1), and Group VIA Ankyrin-2 (or Ankyrin-iPLA$_2$-2). The GroupVIA-3 splice variant encodes an iPLA$_2$ that is identical to Group VIA-2 PLA$_2$ (iPLA$_2$β) at the N-terminus, that retains the GTS^{519}TG active site and that has a truncated C-terminus. However, it is not known whether Group VIA-3 encodes a functional phospholipase A$_2$. Group VIA Ankyrin-1 is identical to Group VIA-2 at the N-terminus but it ends prior to the GTS^{519}TG active site with a three amino acid modification at the C-terminus; it does not encode a functional PLA$_2$ enzyme [46]. Similar to Group VIA Ankyrin-1, Group VIA Ankyrin-2 also lacks the GTS519TG active site and additionally present with a 73 amino-acids shorter N-terminus and a 50-amino-acid variation at the C-terminus. Group VIA ankyrin-1 and Group VIA ankyrin-2 may act as negative regulators of Group VIA-1 and Group VIA-2 by precluding catalytically active tetramer aggregation [39, 46]. Processes in which GVIA PLA$_2$ has been implicated include phospholipids remodeling, AA release causing eicosanoid formation, protein expression, acetylcholine-mediated endothelium-dependent relaxation of the vasculature, secretion, and apoptosis. iPLA$_2$ plays also an important role in lymphocyte proliferation and in Ca^{2+} signaling regulated by calmodulin (CaM) and by a Ca^{2+} influx factor as detailed below [41, 49–52].

6.2.2 GVIB PLA$_2$ or iPLA$_2$ɣ

The iPLA$_2$ɣ called also GVIB PLA$_2$ have been less studied. It has been involved in the release of AA that leads to eicosanoid formation [53, 54]. iPLA$_2$ɣ contains the consensus lipase motif (GXSXG), a C-terminal peroxisome localization

signal (SKL), and a glycine-rich nucleotide binding loop motif (GXGXXG). Interestingly, the nucleotide-binding motif commences 34 amino acids upstream of the putative active Ser, which is closely identical to the location of the nucleotide binding loop motif of Group VIA (35 amino acids upstream) [53]. A recent study demonstrated that $iPLA_2\gamma$ is responsible for the release of AA and prostaglandin E2 (PGE2) and inflammatory mediators in cardiac myocytes infected by Chagas' disease parasite [55]. Previously, $iPLA_2\gamma$ was also suggested as a critical participant in the Ca^{2+}-induced opening of the mitochondrial permeability transition pore (mPTP) in Liver [56].

6.2.3 GVIC, GVID, GVIE, GIVF PLA_2s

Different Ca^{2+}-independent lipases have been identified newly, and classified according to the terminology of the Group system GVIC, GVID, GVIE and GIVF PLA_2s. The GVIC PLA_2 enzyme has some sequence similarity to GVIA PLA_2 and might play a role in membrane homeostasis. This enzyme was previously known as NEST, the recombinantly expressed esterase domain of NTE (neuropathy target esterase), a membrane protein expressed in neurons of human and mice with physiological function elusive [57, 58] that possesses PLA_2 and lysophospholipase activities [59]. NEST might slowly hydrolyze the fatty acid in the sn-2 position of PC and subsequently, in a fast reaction, release the fatty acid in the sn-1 position.

The genes for the three other enzymes have also been identified before. Although, there was no catalytic activity attributed to corresponding proteins. The enzymes were shown to hydrolyze both LA and AA at the sn-2 position in the absence of free Ca^{2+} [60], thus these three enzymes might play a role in the regulation of triacylglycerol homeostasis which implicates the control of energy metabolism in adipocytes. Besides, PLA_2 activity, these enzymes possess high triacylglycerol lipase and acylglycerol transacylase activities and all of them were inhibited by bromoenol lacotone (BEL) at sub-micromolar levels [60].

6.3 Regulation of $iPLA_2$

6.3.1 ATP and PKC

The $iPLA_2$ protein contains a lipase consensus sequence and a putative ATP-binding motif. ATP has been reported to stimulate $iPLA_2$ activity in rat islets [61], murine P388D1 cells [45], but not to affect the $iPLA_2$ activity of Chinese Hamster Ovary cells [44]. In an early study, Ackerman et al. discovered that both Triton X-100 and ATP enhanced the activity of $iPLA_2$ in P388D1 cells [39]. The enzyme activity was 1.2–6 fold higher in mixed micelles when assayed in the presence of ATP and other

di- or triphosphate nucleotides [39]. In other study, ATP stimulation of an $iPLA_2$ isoform was demonstrated in human pancreatic islet [42]. Interestingly, this same group demonstrated that ATP does not directly activate but rather protects $iPLA_2$ from a loss of its activity [61]. On the other hand, there is no consensus regarding the role of PKC in $iPLA_2$ activation [62]. An early study showed that the activation of PKCα ultimately provoked AA release via $iPLA_2$. This AA release was markedly inhibited by BEL or $iPLA_2$ antisense oligonucleotide [63]. Interestingly, we demonstrated that both diacylglycerol analog 1-oleoyl-2-acetyl-sn-glycerol (OAG) and store depletion with thapsigargin produced a PKCε-dependent activation of $iPLA_2\beta$ in proliferating but not in confluent aortic SMC [64].

6.3.2 Ca^{2+}/Calmodulin Regulation of $iPLA_2$

The first evidence of $iPLA_2$ modulation by CaM came from the observation that Ca^{2+} addition to the cytosol of cardiac myocytes inhibited $iPLA_2$ activity induced by ischemia. This inhibition was demonstrated to be due to CaM [65]. In fact, molecular and structural studies showed that in the absence of CaM, the active site of $iPLA_2$ interacts with the CaM-binding domain, resulting in a catalytically competent enzyme, whereas reversible disruption of this interaction through the binding of CaM abrogates this interaction, resulting in a loss of $iPLA_2$ activity [65–67]. $iPLA_2$ was shown to form a catalytically inactive ternary complex with CaM-Ca^{2+} that could be reversibly dissociated by chelation of Ca^{2+} ion with EGTA to regain full catalytic activity. Although $iPLA_2$ activity is independent of Ca^{2+}, it is able to inhibit the $iPLA_2$ activity by Ca^{2+}-activated CaM and this inhibition is apparently due to the binding to the IQ motif. In fact, the dissociation of CaM from $iPLA_2$ is the main mechanism that changes the Ca^{2+}-independent enzyme into an enzyme that is sensitive to modification in intracellular Ca^{2+} ion homeostasis. Moreover, conformational changes provoked in CaM using agents that inhibited the interaction of CaM with its target proteins resulted in $iPLA_2$ activation. Wolf et al. in 1997 have shown that W7, CaM antagonist, activated $iPLA_2$ in A-10 smooth muscle cells (SMC) [68]. Smani and colleagues also demonstrated that CaM inhibition with calmidazolium and a membrane-impermeable CaM inhibitory peptide, promoted $iPLA_2$ activation in SMC and RBL cell line [69]. Later on, compelling evidences have shown that store depletion with thapsigargin or cyclopiazonic acid stimulated $iPLA_2$ activation through displacement of inhibitory CaM [68–70].

6.3.3 Chemical Inhibition of $iPLA_2$

The most important inhibitor for $iPLA_2$ is BEL, which has specificity 1,000 times higher for $iPLA_2$ over other PLA_2 isoforms [41]. BEL is a suicidal substrate for $iPLA_2$ that is widely used as an irreversible mechanism-based, time- and temperature-dependent, inhibitor. For cell-based studies, it has been described previously that

high concentrations of BEL (25 μM) partially inhibit the magnesium-dependent phosphatidate phosphohydrolase (PAP-1), which converts phosphatidic acid to diacylglycerol (DAG) [71, 72]. To some extent it is possible to identify promiscuous effects of BEL on $iPLA_2$ and PAP-1 by performing experiments with BEL and propranolol in parallel [71, 73]. The latter compound inhibits PAP-1 and not $iPLA_2$. Others and we confirmed that $iPLA_2$ activation induced by Ca^{2+} release from the store is inhibited by BEL [68, 70, 73, 74]. Importantly, Jenkins et al. [75] demonstrated that the commonly used BEL is composed of two enantiomers with different specificity for $iPLA_2$ isoforms. S-BEL has higher specificity to $iPLA_2\beta$, and R-BEL is more specific to $iPLA_2\gamma$, which allowed identifying the type of $iPLA_2$ involved in several different cellular processes. Indeed, we confirmed that S-BEL, but not R-BEL, selectively inhibited $iPLA_2\beta$ activity stimulated by intracellular store depletion in SMC and RBL, indicating that S-BEL is a valuable tool to determine the role of $iPLA_2\beta$ in intracellular signaling processes [64, 76].

6.4 $iPLA_2$ Role in the Ca^{2+} Signaling Network

As described above, for long time $iPLA_2$'s main role was especially related to cellular phospholipids remodeling [41]. However, different reports have demonstrated that the specific beta isoform of $iPLA_2$ ($iPLA_2\beta$) is involved in several agonist-stimulated signaling cascades. $iPLA_2$ has several unique features which confused researchers for many years. One of them relies on its activation independently of the presence or absence of Ca^{2+}. $iPLA_2$ is able to function in the presence of strong Ca^{2+} chelators as BAPTA. At the same time $iPLA_2$ is able to bind the Ca^{2+}–CaM complex. Interestingly, conditions for $iPLA_2$ activation are similar to those described for store operated calcium entry (SOCE). In fact, $iPLA_2$ can be activated by depletion of intracellular Ca^{2+} stores caused by vasopressin or by thapsigargin, an inhibitor of Sarco/Endoplamic reticulum Ca^{2+}-ATPase pump [68, 77, 78].

6.4.1 Overview of the Store Operated Ca^{2+} Channels Signaling Pathway

To increase cytoplasmic Ca^{2+} concentration, Ca^{2+} is either released from intracellular stores or enters into the cell by crossing the plasma membrane through ion channels. Store operated Ca^{2+} channels (SOCC) and receptor operated channels (ROC) are considered the main route for Ca^{2+} entry in non-excitable cells, but they also exist in excitable cells such as skeletal muscle, neurons or smooth muscle [79]. In excitable cells, Ca^{2+} entry is achieved largely through opening of voltage and/or voltage independent channels ROC or SOCC that are responsible of SOCE [80]. The concept of SOCE activation seems to be simple: basically upon depletion of ER

stores, a signal is produced that activates specific Ca^{2+}-conducting channels SOCC, in plasma membrane that allows Ca^{2+} entry into the cell. SOCC role was originally linked only to refilling the intracellular store. However, now it's widely agreed that these channels provide a sustained Ca^{2+} influx for a variety of important functions in eukaryotic cells. Among those functions are exocytosis, vascular contraction and relaxation, Ca^{2+} oscillations, gene transcription, regulation of enzymatic activity, cell proliferation and apoptosis [80, 81].

6.4.1.1 Mechanism of SOCE Activation: Emerging Role of STIM1 and Orai1

One of the most intriguing mysteries of the store-operated pathway is the mechanism of its activation. Questions of how do the stores communicate with the plasma membrane channels and which is the signal produced by the stores upon their depletion, have been a matter of intense investigation for long time. Hypotheses presented can be mainly grouped into two main categories: those that propose the generation of a diffusible molecule with ability to induce SOCC opening, and those that assume a physical interaction between channel subunit and an element of the ER (for review see [80, 82]). Soon after the identification of SOCE Robin Irvine proposed a physical or conformational coupling between elements in the ER and SOCC in the plasma membrane [83], as a mechanism that resembles the classical excitation-contraction coupling between ryanodine receptors and dihydropyridine receptors in the skeletal muscle [84]. Consequently, most of the early studies focused on the association between inositol-triphosphate receptors (IP_3R) and the subunit channel suggested to form SOCC. This hypothesis received support from studies demonstrating that, under resting conditions; TRPC1, TRPC3 and TRPC6 can be co-immunoprecipitated with IP_3R [85, 86]. However, the major challenge for this model came from the studies in triple IP_3R knockout DT40 cells, in which SOCE seemed completely normal [87–89]. Importantly, in 2005 and 2006 the Ca^{2+} sensor of the ER was identified as the STIM1 (Stromal Interaction Molecule-1) protein, and Orai1 was identified as the structural subunit of the channel conducting the Ca^{2+} selective CRAC [90–92]. Several reports have showed that upon Ca^{2+} depletion, STIM1 lose Ca^{2+} from its EF hand, oligomerize and accumulate into punctate structures in the ER membrane located in close proximity (10–25 nm) to the plasma membrane. Furthermore, STIM1 and Orai1 have been reported to accumulate and colocalize in punctate structures along the plasma membrane and to associate by a reversible and physical coupling mechanism upon depletion of the intracellular Ca^{2+} stores which support the conformational coupling model (for review see [93]). While direct coupling of ER-resident STIM1 to PM-resident Orai1 is considered as the most straightforward mechanism for signal transduction, there is a growing body of evidence for the presence of additional structural and/or functional linker(s) between STIM1 and Orai1. Indeed, Balla's group suggested the presence of additional molecular components within the STIM1-Orai1 complex [94]; meanwhile Rosado and colleagues nicely showed that both STIM1 and Orai1

also co-immunoprecipitate with other TRPC channels when stores are depleted [95–97]. Recently, we have demonstrated that store depletion stimulated STIM1 and $iPLA_2\beta$ colocalization required for SOCE in coronary artery [98].

6.4.1.2 Calcium Influx Factor and SOCE Activation

The other hypothesis focuses on diffusible messengers generated upon intracellular stores depletion. Different signaling molecules have been reported to play an essential role in the activation of SOCE in different cell types, including cGMP [99], tyrosine kinases [100], and small GTP-binding proteins [101], among others. However, special efforts were dedicated to the still uncharacterized molecule known as Ca^{2+} influx factor (CIF) by Victoria Bolotina's group. Refined CIF extract was obtained from different cell lines, including human platelets, which stimulated an extracellular Ca^{2+} influx and I_{CRAC} (CRAC current) sensitive to the well-known SOCC inhibitors [76]. Interestingly, soon after STIM1 discovery, Bolotina and coworkers presented compelling evidences demonstrating that CIF production is tightly linked with STIM1 expression and requires the functional integrity of glycosylation sites in its intraluminal SAM domain [102]. In this study, authors demonstrated that upon store depletion, CIF is produced before STIM1 accumulation in puncta and activation of SOCE. Authors showed that lack of STIM1 in the rare neuronal cell line (NG115-401L), which features virtually no SOCE responses [103], or STIM1 downregulation in cells transfected with siRNAs, dramatically impaired active CIF production confirming CIF and STIM1 relationship [102]. Unfortunately, the molecular identity of CIF is still unknown, although its presence and its biological activity were detected by numerous groups in a wide variety of cell types ranging from yeast to human (for review see [51, 79]). Previously, we have characterized in our earliest studies that $iPLA_2\beta$ is the physiological target of CIF and the mechanism of CIF-induced activation of SOCE was depicted as illustrated in Fig. 6.1 [69, 76, 104].

6.4.2 Essential Role of $iPLA_2$ in Store Operated Calcium Entry

In the last 1990s, several works established an interesting scenario for $iPLA_2$ activation, showing that it could be activated by depletion of Ca^{2+} stores caused by vasopressin or by thapsigargin in A10 SMC line [68, 77]. A10 cells stimulation with thapsigargin induced release of AA that was directly correlated to thapsigargin-induced depletion of intracellular Ca^{2+} stores [68]. Next, $iPLA_2\beta$, and not $iPLA_2\gamma$, was identified as the mediator of vasopressin-induced AA release in SMC [75]. Therefore, the role of $iPLA_2\beta$ in SOCE activation was explored and the first evidence of $iPLA_2$ requirement for SOCE activation was obtained by studying SOCE in primary culture of SMC as a model for excitable cells, and RBL cells as a model for non-excitable cells. The functional inhibition of $iPLA_2$ with BEL prevented the

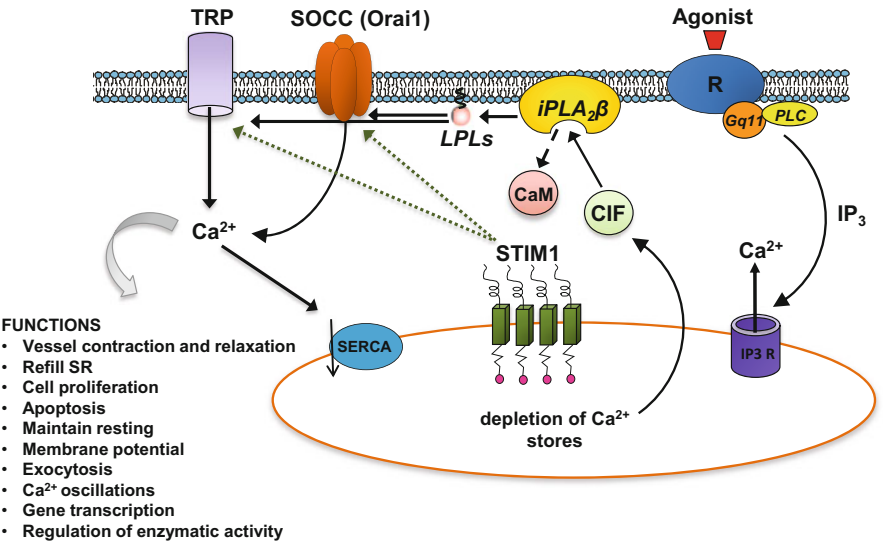

Fig. 6.1 Mechanism suggested for store operated Ca^{2+} entry (*SOCE*), involving Ca^{2+} independent phospholipase $A_2\beta$ (*iPLA*$_2$) pathway. We propose that agonist binding to R, promotes IP_3-induced Ca^{2+} release, activation/translocation of STIM1, Calcium Influx Factor (*CIF*) release, displacement of calmodulin (*CaM*), activation of $iPLA_2$, lysophospholipids (*LPLs*) production, and activation of store-operated Ca^{2+} channels (*SOCC*) (Orai1) and other transient receptor potential channels (*TRP*). The subsequent Ca^{2+} entry is responsible for a large number of functions as indicated

activation of single SOCC in SMC, and whole cell CRAC currents in RBL induced by TG and/or BAPTA-induced depletion of intracellular stores. In addition, molecular inhibition using antisense against $iPLA_2$, or its functional blocking with BEL impaired dramatically SOCE, while Ca^{2+} release from the stores was not affected, which confirmed the novel role of $iPLA_2$ in SOCE pathway [78]. Furthermore, the use of S-BEL enantiomer confirmed that $iPLA_2\beta$ is the isoform responsible of SOCE in RBL [76] and SMC [64]. Interestingly, cell dialysis with recombinant $iPLA_2\beta$ could substitute the endogenous $iPLA_2\beta$ and rescue activation of I_{CRAC} in the cells in which endogenous $iPLA_2\beta$ was knocked down [76]. One of the most important features of $iPLA_2$ is that it exists in a complex with CaM, which keeps it in a catalytically inactive state; and removal of CaM results in $iPLA_2$ activation [66]. Therefore, the inhibition of CaM was found to mimic the effects of thapsigargin-induced SOCE as it activated $iPLA_2$; it evoked a 2APB and BEL-sensitive Ca^{2+} influx; and finally it stimulated single SOCC in SMC [69]. Similar effect of CaM inhibition was also observed in astrocytes [70] and in rat cerebellar granule [105].

The role of $iPLA_2\beta$ in SOCE was further confirmed by us and by many other investigators in a growing number of cell types, including platelets, Jurkat T lymphocytes [69, 78], RBL-2H3 [104], neuroblastoma/glioma [70], keratinocytes [106], skeletal muscle [107], fibroblasts [108], prostate cancer cells [109] and others. In all these studies molecular knock-down and/or functional inhibition of $iPLA_2\beta$ caused full impairment of SOCE. Strikingly, genetic screening of Drosophila

melanogaster performed by Vig et al. indicated that not only STIM1 and Orai1, but also an orthologue of iPLA$_2$β encoded by the CG6718 gene, are gene products with a great impact on SOCE activation [110]. Recently, we have demonstrated that agonist-induced coronary artery contraction involved the activation of SOCE by STIM1, Orai1 and iPLA$_2$ [98]. We have shown that on cells stimulation, STIM1 colocalized with iPLA$_2$β in submembrane compartments suggesting their functional communication and we confirmed that lysophopholipids, product of iPLA$_2$, stimulated an Orai1- but not STIM1- dependent SOCE, suggesting that the functional role of iPLA$_2$β is downstream of STIM1 and upstream of Orai1 in coronary SMC. The complex relationships between the components of the CRAC channel, namely Orai1, STIM1, and iPLA$_2$β in the SOCE pathway have been detailed in a previous review [79].

6.4.3 *iPLA$_2$ and Lysophospholipids Activation of Store Operated Calcium Entry*

Afterwards, numerous studies focused on the molecular mechanism of iPLA$_2$-dependent signal transduction. Several works from Bolotina's lab in the first decade of this centry, provided compelling evidences demonstrating that SOCC can be activated by CIF produced upon depletion of Ca^{2+} stores in the ER, and it in turn, can displace the inhibitory CaM from iPLA$_2$β. The early studies have shown that CIF activated single SOCC in inside-out membrane patches [111], and the channels remained active even after the membrane patches were excised and CIF was washed away [112], indicating the presence of an additional cascade of plasma membrane-delimited reactions that might be involved in CIF-induced activation of SOCC. In 2004, a major finding has been described by Smani et al. demonstrating that CIF extract can displace inhibitory CaM from iPLA$_2$β leading to lysophopholipids production and the activation of SOCC in membrane-delimited manner in SMC [69]. By contrast, CIF dialysis of RBL cells transfected with antisense to iPLA$_2$β failed to activate I_{CRAC}, confirming the need of functional iPLA$_2$β to stimulate SOCE [76]. Furthermore, the exogenous application of lysophopholipids but not AA, products of iPLA$_2$β activation, were able to stimulate SOCE in intact cells and single SOCC in inside-out membrane patches in SMC [69, 74, 98]. Further studies have confirmed that lysophospholids evoked SOCE in different cell lines such as astrocyte [70], rat cerebellar granule neurons [105], skeletal muscle [113], and keratinocytes [114], among others cells. Thus, several independent works established the need of active iPLA$_2$β, and lysophospholipids to stimulate SOCE in a wide range of cells.

However and independently of its role in SOCE signaling, few reports have shown that iPLA$_2$ might activate some TRP channels. Works from Prevarskaya's lab demonstrated that iPLA$_2$β activated both SOCC and TRPM8 channels [109, 115], and AL-Shawaf and colleagues showed recently that lysophosphatidylcholine and AA generated by iPLA$_2$ are involved in TRPC5 activation by sphingosine-1-phosphate [116].

6.5 Significant Potential as Targets for Novel Therapeutics Strategy

The role of iPLA$_2$β, and the consequent activation of SOCE in several physio- and pathological processes have been largely studied. For example, iPLA$_2$β-dependent activation of vascular reactivity was demonstrated in aorta, cerebral, mesenteric, carotid and coronary arteries [98, 117, 118]. Furthermore, iPLA$_2$β-induced SOCE seems involved in SMC proliferation [119] and in HEK cells migration [120]. Molecular knockdown of Orai1, STIM1 or iPLA$_2$β caused a similar reduction in velocity and distance in migrating HEK cells. Previously, Vanden Abeele et al. demonstrated that iPLA$_2$β activated SOCE in LNCaP prostate cancer proliferative cells [109], and they further showed that iPLA$_2$β is also implicated in the lysophospholipid-dependent gating of TRPM8, a cold sensor [115]. On the other hand, Boittin and Reugg published several interesting studies highlighting the involvement of iPLA$_2$-dependent activation of SOCC in dystrophic muscle fibers [109]. They found that iPLA$_2$ is mainly localized in the vicinity of the sarcolemma, suggesting a close proximity with SOCC, which may be located on the sarcolemma and/or in the T-tubular membranes. These authors have also demonstrated that lysophosphatidylcholine acts downstream of iPLA$_2$ and directly activates SOCC in dystrophic fibers [107, 113]. Interestingly, recent studies have determined that iPLA$_2$ can be targeted by secondary signaling pathway to potentiate or inhibit SOCE such as PKCε [64], and Urocortin through cyclic AMP in SMC [98], in skeletal muscle [121], and in hepatoma carcinoma cell lines [122]. These few examples confirm the important role of iPLA$_2$ and SOCE in several physiological and pathological processes and confirm it as a valuable therapeutic target.

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Chapter 7
Extracellular Calcium Has Multiple Targets to Control Cell Proliferation

Thierry Capiod

Abstract Calcium channels and the two G-protein coupled receptors sensing extracellular calcium, calcium-sensing receptor (CaSR) and GPRC6a, are the two main means by which extracellular calcium can signal to cells and regulate many cellular processes including cell proliferation, migration and invasion of tumoral cells. Many intracellular signaling pathways are sensitive to cytosolic calcium rises and conversely intracellular signaling pathways can modulate calcium channel expression and activity. Calcium channels are undoubtedly involved in the former while the CaSR and GPRC6a are most likely to interfere with the latter. As for neurotransmitters, calcium ions use plasma membrane channels and GPCR to trigger cytosolic free calcium concentration rises and intracellular signaling and regulatory pathways activation. Calcium sensing GPCR, CaSR and GPRC6a, allow a supplemental degree of control and as for metabotropic receptors, they not only modulate calcium channel expression but they may also control calcium-dependent K+ channels. The multiplicity of intracellular signaling pathways involved, their sensitivity to local and global intracellular calcium increase and to CaSR and GPRC6a stimulation, the presence of membrane signalplex, all this confers the cells the plasticity they need to convert the effects of extracellular calcium into complex physiological responses and therefore determine their fate.

Keywords Calcium channels • Calcium sensing receptors • GPRC6a • Cell proliferation • Orai • TRPC

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7.1 Introduction

Calcium channels and the two G-protein coupled receptors sensing extracellular calcium, calcium-sensing receptor (CaSR) and GPRC6a, are the two main means by which extracellular calcium can signal to cells and regulate many cellular processes including cell proliferation, migration and invasion of tumoral cells [1–7]. Many intracellular signaling pathways are sensitive to cytosolic calcium rises and conversely intracellular signaling pathways can modulate calcium channel expression and activity. Calcium channels are undoubtedly involved in the former while the CaSR and GPRC6a are most likely to interfere with the latter. Hence it is postulated that extracellular calcium ions may use an ionotropic and metabotropic scheme to modulate calcium fate. Indeed, calcium channels are active at physiological extracellular calcium concentration whereas CaSR and GPRC6a need a rise in extracellular calcium concentration to be activated [8, 9]. Calcium entering the cell may either directly trigger intracellular signaling pathways or modulate the temporal and spatial nature of the cytosolic Ca^{2+} transients enable a cell to tailor its response to a given stimuli [2, 10–12].

Although several calcium entry pathways have been linked to cell proliferation [13, 14], this review is focused on two specific ones, namely the store-operated calcium entry (SOCE) resulting from intracellular calcium store depletion and the store-independent calcium entry (SICE) mainly relying on intracellular second messenger production independently of calcium store depletion [15, 16]. Calcium store depletion is sensed by two proteins, STIM1 and STIM2 (Stromal Interaction Molecule 1 and 2), located on the endoplasmic reticulum membrane [17–19]. The molecular nature of the calcium channels is still a matter of debate but it is clear that SOCE involves a complex of Orai proteins on the plasma membrane and the luminal calcium sensors STIM1 and STIM2 on the endoplasmic reticulum membrane [17–19]. However, transient receptor potential canonical (TRPC) channels are most likely to interfere with this complex [20–22]. SICE was clearly described as a heteropentameric assembly of three Orai1 subunits and two Orai3 subunits [23] coupled to plasma membrane STIM1 [24] and activated by arachidonic acid (AA) [25] but it has also been shown that TRPC channels can be open by another intracellular messenger such as di-acyl-glycerol (DAG) [26]. Although SICE should not be restricted to these two calcium entries only, the few remaining ones were mentioned in a previous review [14].

CaSR and GPRC6a are seven transmembrane domains GPCR sensing increases in extracellular calcium. When activated, they trigger large increase in intracellular signaling pathways and also in cytosolic free calcium concentration [27, 28]. They have been linked to cell proliferation and cancer development [29, 30] and, therefore, they provide another mean for extracellular calcium to modify cell fate.

How extracellular calcium ion rise, calcium channels and CaSR expression and activity are translated by the cell to allow physiological and pathophysiological proliferation will be discussed in this review. The uncoupling of the relation between calcium entry, calcium channel expression and cell proliferation will be the second point addressed here.

7.2 Calcium Influx and Cell Proliferation

Ca^{2+} requirement for cell growth and division has already been addressed in a previous review [14]. It becomes clear that, even if the great majority of cells needs extracellular calcium to grow and proliferate, this requirement may be limited by the degree of cellular transformation and it was suggested that the need for extracellular Ca^{2+} was ruled out during neoplastic transformation. Indeed, we recently confirmed demonstrated that cell proliferation rate was not affected even at very low extracellular calcium concentration (below 10 μM) in human hepatoma cell line Huh-7, in HEK293 and HeLa cells [31]. It was then suggested that intracellular calcium stores were more important than calcium influx to preserve this physiological function. However, one of the strong arguments in favor of a role of calcium influx in cell proliferation still comes from the use of calcium channels blockers. A special effort was made to identify selective I_{CRAC} blockers and several new molecules have now emerged as recently reviewed [14, 32, 33]. Further studies have been performed since to test and characterize the effectiveness of some of these molecules. Two selective small molecules developed by GlaxoSmithKline, GSK-5498A and GSK-7975A, tested for their abilities to inhibit mediator release from mast cells and pro-inflammatory cytokine release from T cells completely inhibited I_{CRAC} currents with an IC_{50} of about 3 μM [34, 35]. Recent studies showed that these two drugs may have potential therapeutic applications in the treatment of asthma and related allergic diseases as well as pancreatitis [35, 36]. Although 2-APB has a wide spectrum of effectors and targets several types of calcium channels [37–43], its structure is still widely used as a platform to design new CRAC blockers. Chemical analogs of 2-APB have proved to be very effective in blocking CRAC currents. The dimeric derivative of 2-aminoethoxydiphenyl borinate (2-APB), DPB162-AE, blocks functional coupling between STIM1 and Orai1 with an IC_{50} (200 nM) 100-fold lower than 2-APB [44]. DPB162-AE does not prevent the SOAR-Orai1 interaction but potently blocks SOAR-mediated Orai1 channel activation, yet its action is not as an Orai1 channel pore blocker [45]. In an effort to discover new inhibitors of TRPV6, 34 novel 2-APB analogs were synthesized. Although several analogs that were more potent inhibitors of TRPV6 than 2-APB, they also inhibited SOCE with comparable IC_{50} [46]. Modifying the two phenyl groups of 2-APB proved to be another way to design more effective CRAC channels blocker. Two molecules, cyclic-APB and dibenzothienyl-APB, inhibited calcium entry with higher sensitivity (IC_{50} of 3 and 0.4 μM, respectively) than 2-APB [47]. This is indeed a notable improvement but there is still a long way to go before any of these drugs based on this molecular structure could be used in clinical trials. Another blocker has been recently identified. SAR7334 is derived from the previous non selective SOCE blocker SKF96365. SAR7334 is rather promising as it blocks TRPC6, TRPC3 and TRPC7 DAG-mediated calcium entry with IC_{50}s of 9.5 nM, 282 nM and 226 nM respectively [48]. SAR7334 has no effect on TRPC4 and TRPC5 mediated calcium entry. Interestingly, this compound was also tested in patch-clamp experiments and application during the response rapidly and totally blocked OAG-induced calcium currents, suggesting

a direct effect on the channel itself. Investigating the effect of SAR7334 on hypoxic vasoconstriction in the perfused isolated mouse lung model showed that this drug was able to completely suppress hypoxia-induced increases in pulmonary arterial pressure. Half maximal inhibition was obtained at about 100 nM strongly suggesting that SAR7334 is able to efficiently block native TRPC6 channels in vivo [48]. As TRPC6 is linked to cancer and cell proliferation, this makes SAR7334 a potential drug to block this disease.

A few clinical trials involving calcium influx blockers have already been launched. Carboxyamidotriazole (CAI) was patented almost 20 years ago [49] and is currently in phase III clinical trials in non-small cell lung cancer [50]. However, CAI has potential side effects including inhibition of mitochondrial calcium import [51] and both cAMP-phosphodiesterases and cGMP-phosphodiesterases activity [52]. CalciMedica (La Jolla, CA, USA) has identified multiple structurally-distinct selective CRAC channel inhibitors including CM2489 and CM3457. CM2489 is the first of these compounds to reach the clinic in a Phase 1 clinical study and is being developed for the treatment of moderate-to-severe plaque psoriasis. CM3457, another selective CRAC channel inhibitor that is structurally distinct from CM2489, inhibits CRAC channels, T cell proliferation, and mast cell degranulation and may be used for autoimmune disorders and asthma treatment. Finally, a Phase I clinical trial (Safety and Tolerability Study of SOR-C13 in Subjects With Advanced Cancers Commonly Known to Express the TRPV6 Channel) to test the effect of SOR-C13, an inhibitor of TRPV6 [53], was recently launched. The purpose of this phase I is to determine the safety and tolerability of the drug SOR-C13 when given as an intravenous infusion in patients with ovarian cancer or other cancers known to over express the TRPV6 calcium channel. However, it appears that this drug is not very effective in blocking TRPV6 as calcium entry was only reduced by 25 % at 25 μM SOR-C13 [53].

A new and original approach was based on monoclonal antibodies directly targeting the second extracellular loop of Orai1 channel [54, 55]. They showed in the first study that Anti-Orai1 mAB binds to Orai1 protein leading to its internalization [54]. This mAB was more effective at blocking human peripheral blood mononuclear cells (PBMC) proliferation (80 % at 200 nM) than calcium fluxes in Jurkat cells (60 % at 2.5 μM). However, time scale for both measurements was radically different as calcium entry was monitored 1 h after mAB addition while cell proliferation was assessed after 3 days. The second study [55] refers to another mAB against human Orai1 (2C1.1) with a high efficiency. 2C1.1 (10 nM) was able to reduce calcium entry by 70 % and NFAT activity by 40 % in Jurkat cells.

Although several small molecules have been shown to be potent CRAC channel inhibitors, their selectivity remains questionable. The immunologic approach to CRAC channel inhibition through the development of fully human monoclonal antibodies to Orai1 that antagonize CRAC channel function may represent the best opportunity to overcome this selectivity matter and could be applied to other calcium channels.

7.3 Calcium Influx and Intracellular Signaling Pathways

Calcium influx, once the channels are open, has to be translated into physiological responses and several intracellular signaling pathways are sensitive to cytosolic calcium concentration increases.

Blocking SOCE or silencing TRPC or Orai channels often resulted in a modification of intracellular signaling pathways linked to cell proliferation. Several examples focusing on Orai and TRPC channels are listed below. Knocking down Orai3 or TRPC3 decreased Akt phosphorylation and cell proliferation in non small cell lung adenocarcinoma cells [56] and FSH-stimulated ovarian cancer cells [57] respectively. Down-regulation of this pathway by TRPC silencing was also observed in other physiological processes such as CXCR2-mediated chemotaxis in murine neutrophils (TRPC6) [58], neurite outgrowth in PC-12 cells (TRPC6) [59], myoblast differentiation and muscle regeneration in TRPC1 −/− mice [60]. However, cardiac stress induced by transverse aortic constriction was abolished in TRPC1 −/− mice and associated with an increase in Akt and phospho-mTOR (mammalian target of rapacycin) signaling which are also linked to cell survival [61]. ERK/p-ERK pathway is also controlled by calcium channel expression and calcium influx. SOCE inhibition using YM58483 (BTP-2) [62] reduced CaMKII/Raf-1/ERK signaling pathway and cell proliferation in human metastatic melanoma cell lines [63]. Interestingly, silencing TRPC5 or TRPC6 but not TRPC1 resulted in a decreased ERK/CREB pathway but not Akt in PFGF-mediated neuroprotection [64]. Phospho-CREB was also downregulated in the presence of Sirolimus in human arterial smooth muscle [65]. Sirolimus, a structural derivate of FK506 that reduces cell proliferation rates, inhibits Orai1-mediated SOCE [65] as well as a NFAT dephosphorylation and mTOR phosphorylation [66]. By blocking calcium entry, Sirolimus reduces calmodulin kinase II (CaMKII) activity, hence CREB phosphorylation and gene transcription. STAT5 (Signal Transducer and Activator of Transcription 5) and c-fos expression are also activated by calcium entry in rat mast cells through a tyrosine kinase Syk-dependent pathway [67]. Phosphorylation of AMPK (AMP-activated potein kinase) by CaMKKβ (calmodulin kinase kinase β) is abolished when TRPC1 expression is reduced or in and in TRPC4 −/− mice [68]. Hence, intracellular signaling pathway activation is dependent on various types of calcium channels, suggesting that local increases in cytosolic free calcium concentration or well-defined membrane complexes enable a cell to selectively respond to one stimulus.

7.4 Local and Global Cytosolic Calcium Increase

If calcium entry is important to refill intracellular calcium stores and/or to feed the oscillations observed in stimulated cells, local calcium concentration increases at the mouth of the channel is also important to specifically trigger one signaling

pathway. Calcium microdomains arising from the diffusion of calcium through an open channel can easily reach several tens of micromolars, several fold higher than average cytosolic calcium concentration [69]. A first evidence arised from the observation in neurons where L-type calcium channels were able to trigger sustained CREB phosphorylation following KCl-induced membrane depolarization, while other voltage-dependent calcium channels were only able to transiently activate this transcription factor [70]. Since then, several other studies have clearly demonstrated that local calcium signaling allows selective calcium activation of NFAT [67, 71, 72], another calcium sensitive transcription factor. Pro-inflammatory molecule leukotriene C4 (LTC4) production measured following an application of the SERCA inhibitor thapsigargin was larger when external calcium was present despite similar increase in global cytosolic calcium concentration in RBL-1 cells [73]. The same group published a year after another study where they showed that calcium microdomains at the mouth of the channel were activating tyrosine kinase syk which in turn activated STAT5 and c-fos while, again, global increase in cytosolic calcium was unable to do so [67].

This then raised a new question concerning the existence of membrane signaling complexes to account for the selective activation of intracellular signaling pathways and transfection factors by calcium influx. For instances, Orai1 but not Orai3 was inducing NFAT dephosphorylation while an Orai3 chimera with Orai1 N-terminal was able to restore NFAT translocation [72]. The scaffolding protein AKAP79 (A-kinase anchoring protein 79) is a member of a large family of proteins known to foster signal transduction by connecting signaling enzymes with their substrate with well-defined membrane localization. AKAP79 connects with several proteins [74] including Cav1.2 L-type voltage-dependent calcium channels [75]. It was later shown that NFAT signaling was connected to these Cav1.2 channels through AKAP79 [76], and that NFAT nuclear translocation was enabled by AKAP79-Orai1 interaction [72]. Iarc, the major component of SICE, is also regulated by AKAP79 [77] through a PKA mediated phosphorylation of T389 residue of the minor pool of STIM1 located on the plasma membrane whilst phosphorylation of the same residue actually inhibits the ability of STIM1 to activate the CRAC channels [78].

7.5 Plasma Membrane Signalplex

It is indeed trivial to say that the multiplicity of calcium-mediated intracellular outputs and the corresponding diversity of calcium channels make the presence of such dynamic structural membrane signaling complexes. Interactions between Orai, STIM and TRPC proteins and between these proteins and other partners have been thoroughly investigated over the last few years [79, 80]. This first list comprises SPCA2, CRACR2A, calmodulin, caveolin, adenylyl cyclase 8, EBp57, junctate, EB1, Golli MBP, SARAF, polycystin1-P100, stanniocalcin 2, POST, PMCA, Cav1.2, and septin. Additional partners have since been identified. Immunoprecipitation experiments showed that DOCK10, thrombospondin-1,

myosin and bestrophin-1 are associated with STIM1 [81, 82], ubiquilin-1 with Orai1 [83] and AKAP79 with Orai1 and Orai3 [72, 78].

In order to get maximal efficiency and specificity, plasma membrane must have a high degree of organization and this could be achieved in specific regions such as lipid raft domains [84]. Early experiments in the 1970s suggested a role of caveolae in regulating Ca^{2+} entry [85]. Later, it was demonstrated that caveolae are preferred sites for SOCE [86, 87]. Interestingly, receptors for two potent mitogens, epidermal growth factors (EGF) and hepatocyte growth factor (HGF), which are known to stimulate TRPC6 expression and cell proliferation in human hepatoma cells [88] are also localized in caveloae [89, 90]. Other growth factors receptors have been found in caveolae [91] and this is known to control and organize Ca^{2+}-dependent signal transduction [92]. Several calcium channels and calcium signaling proteins are found in caveolae [92] and, for example, caveolin has been implicated in TRPC1 targeting the plasma membrane and its association with STIM1 [93–95].

Hence, these complexes may account for the differential activation of intracellular signaling pathways and transcription factor activation following calcium entry. SICE but not SOCE activated by norepinephrine and P2Y-purinergic receptors respectively in primary culture of human prostate cancer epithelial cells, was responsible for nuclear NFAT translocation [96] while it was tightly linked to I_{CRAC} but not to I_{arc} in HEK293 cells [97]. Furthermore, it was recently demonstrated that scaffolding protein caveolin-1 was giving the cell the ability to distinguish between two transcription factors, namely c-fos and NFAT, in response to the same local calcium signal [98]. It was shown in this paper that tyrosine 14 residue of caveolin-1 is the locus for c-fos transcription inhibition. Phosphorylation of Tyr14, which can be triggered by the Src family tyrosine kinases Src, Abl or Fyn [99], unable calcium entry to activate STAT5, hence c-fos expression in RBL cells, while overexpression of the caveolin-1 Y14F mutant oversteps c-fos transcription inhibition [99]. In both conditions, NFAT translocation was not affected. Cells express four members of NFAT family all depending on cytosolic calcium increase to move to the nucleus [100]. Dynamics of nuclear translocation for NFAT1 and NFAT4 are rather different, NFAT4 import and export being almost ten times than for NFAT1 [101]. It was recently demonstrated that Orai1-associated calcium microdomains directly activated NFAT1 while NFAT4 required both local calcium influx and global and nuclear calcium increases allowing a cell to recruit combination of these two isoforms as stimulation strength increases [71]. Altogether, these data emphasize the complex role for local and size in calcium signals in controlling intracellular signaling pathways and, eventually, cell fate (Fig. 7.1).

SOCE and SICE amplitudes increase with membrane potential hyperpolarization and concomitant opening of K+ channels is likely to result in larger rise in calcium microdomains or bulk calcium. It was demonstrated that intracellular signaling pathway activation could depend on how much was entering the cell in membrane microdomains [67]. The presence of K+ channels nearby could therefore increase the driving force for calcium ions. Membrane structural complexes of Orai or TRPC channels and K+ channels precisely exist to modulate calcium entry. There are now evidences that they can be related to cancer development [102].

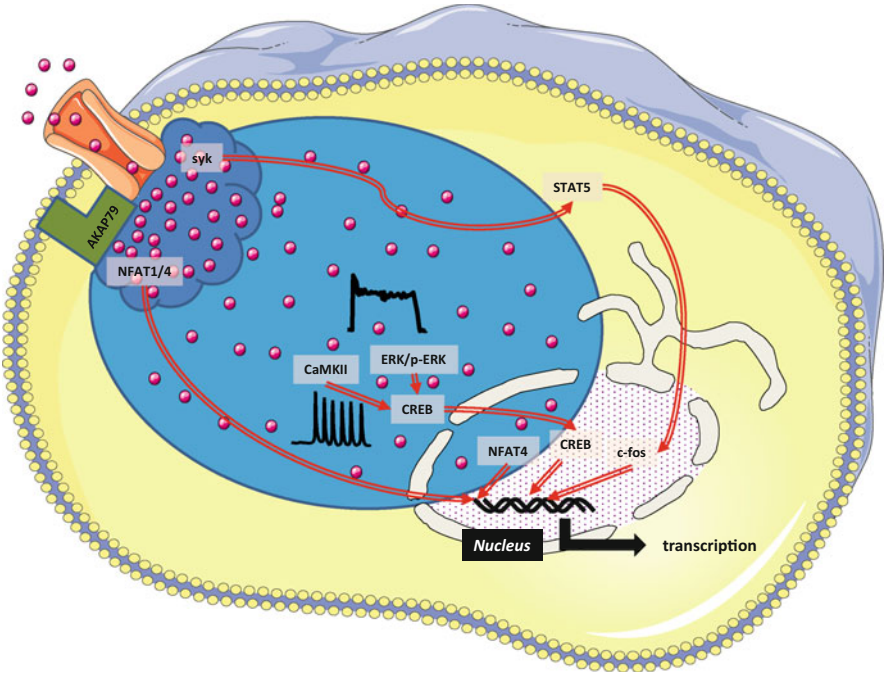

Fig. 7.1 Local and global intracellular calcium increase activates different intracellular signaling pathways to trigger transcription and cell proliferation. NFAT4 activation requires both local and nuclear calcium increase while NFAT1 or syk are sensitive to local rise at the mouth of the channel. Transient or sustained cytosolic increases are likely to act on other signaling pathways

Seventy eight genes over more than 400 for all types of channels, encode for ion channels selectively permeable to potassium [103]. This superfamily of potassium channels can be further divided into four groups based on their mode of gating and the number of structural transmembrane domains. Among them, the complex calcium-dependent K+ channels [104] are the most described as partners for SOCE and SICE channels. SK3 channels are not expressed in normal human prostate and breast epithelial cells as opposed to Orai1 but they appear in tumoral cells. They then form a SK3–Orai1 complex with an essential role in cancer cell migration and bone metastasis development [105]. It was also worth noting that BKCa–Cav3.2 complex also play a major role in the proliferation of LNCaP prostate cancer cells by controlling a constitutive Ca^{2+} entry [106]. BKCa–Cav3.2 complex could maintain membrane potentials within a window of membrane potentials over which a fraction of Cav3.2 are activated. At low voltages, T-type Ca^{2+} channels are known to mediate a phenomenon known as "window current" [107]. The term "window" refers to the voltage overlap between the activation and steady state inactivation at low or resting membrane potentials. As a result, there is a sustained inward calcium current carried by a small portion of channels that are not completely inactivated. Window current allows T-type Ca^{2+} channels to regulate Ca^{2+} homeostasis under

non-stimulated or resting membrane conditions [108]. This means that for these membrane potentials which are controlled by BKCa, Cav3.2 channels are open and support constitutive Ca^{2+} entry. Furthermore, this constitutive Ca^{2+} entry would activate BKCa channels that regulate membrane potential working as a positive feedback loop.

In LNCaP prostate cancer cells IKCa channel regulates calcium entry through TRPV6 by controlling the electrochemical gradient for calcium ions [109]. Immunoprecipitation experiments showed a close physical interaction between the IKCa and TRPV6 in these cells. In the same manner, the human ether a′-gogo potassium Channel 1 (hEag1) has been shown to regulate breast cancer cell migration through Orai1-dependent calcium entry [110].

If links between chloride channel expression, cell proliferation and tumorigenesis are now well demonstrated [111, 112], evidence for a role of chloride channels in the control of SOCE or SICE and cell proliferation is sparse. Concavalanin (ConA) induced calcium entry and cell proliferation of human T lymphocytes were reduced in the presence of 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid (DIDS), a known chloride blocker. When combined with calcium channel blocker SKF96365, DIDS enhances the inhibitory effect of SKF96365 on ConA-induced IL-2 T cell proliferation [113].

Hence, the mechanisms relaying calcium influx, intracellular signaling pathways and cell proliferation are dramatically tangled. Differential expression of partners, local versus global increase in cytosolic calcium, direct and rapid regulation by PKA through anchoring proteins, involvement of other intracellular second messengers via PKC [114–118] or PKG [119–123] activation, all this makes the ground for a very tight and selective activation of calcium entry -dependent physiological responses.

7.6 Calcium Channel Expression and Cell Proliferation

The relation between calcium channel expression and cell proliferation was already emphasized in recent reviews [13, 14]. Another interesting aspect is the uncoupling of these two events.

If there are numerous examples of an effect of calcium channel expression and/or calcium entry related to cell proliferation, in some cases this relation is not observed. Our own work unexpectedly demonstrated that HEK293 cells, human hepatoma cell line Huh-7 and Hela cells were not sensitive to external calcium even at concentration as low as a few micromolars [31]. Cell proliferation was however sensitive to Orai1 and Orai3 expression levels and moreover, overexpression of non permeant mutants, E106Q-Orai1 and E81Q-Orai3, resulted in an increase in cell proliferation in HEK293 cells. Expression of these two mutants in the same cell type totally blocked SOCE [124] and SICE currents [125], strongly suggesting that calcium channel expression was more important than calcium entry to control cell proliferation in these cells. In a similar way, it was recently shown that knocking

down Orai3 in breast cancer cell line MCF-7 was more effective that withdrawing extracellular calcium on cell proliferation [126]. Although extracellular calcium concentration lower than 200 μM were not tested in their experimental conditions, this was clearly in favor of our hypothesis. It is therefore possible that new functions for TRPC and Orai channels could be uncovered in near future and that a direct link between the channel structure and transcription factors would explain why some cells need the channel more than calcium influx to proliferate.

Knocking down TRPC1 and TRPC4 decreased cell proliferation with no effect on SOCE or I_{CRAC} activity in endothelial cells [127]. This is rather unexpected and could suggest an additional role for these channels. In addition, knocking down STIM1, the ER plasma membrane counterpart for SOCE channels [128, 129], has no effect on cell proliferation in vascular smooth muscle cells [130], human myoblasts [131], HEK293 cells [132] and human umbilical vein endothelial cells (HUVEC) derived cell line EA.hy926 [133]. At the same time I_{CRAC} is greatly reduced, which suggested strongly that, in these cells, this current and cell proliferation is uncoupled. This decoupling between STIM1 expression, calcium entry and cell proliferation was also observed in more recent publications. Silencing STIM1 indeed resulted in a dramatic decrease in SOCE/Icrac while cell proliferation rate and cell cycle distribution were not at all affected in human metastatic renal cellular carcinoma and LNCaP [134, 135]. To conclude here, an event that looked exceptional now seems to run through several cell models. Unfortunately, most experiments using siRNA to silence Orai or STIM proteins are not duplicated in the absence of extracellular calcium to validate this interesting observation.

7.7 Calcium Channels and Cell Cycle

The cell cycle consists of four primary phases: G1, the first gap phase; S phase, in which DNA synthesis occurs; G2, the second gap phase; and M phase, or mitosis, in which the chromosomes and cytoplasmic components are divided between two daughter cells. The transitions between the four cell cycle phases are tightly regulated. Checkpoints during cell cycle allow the cell to determine whether all is within the normal limits before proceeding to the next cell cycle phase [136]. We have discussed the role of calcium channels during cell cycle in a previous review [14]. Briefly, knocking down Orai or TRPC channels resulted in a cell cycle arrest preferably in G1 for TRPC1 and Orai3 and G_2/M phase for Orai1 and TRPC6. Recent literature check confirmed the prevalence of these channels in cell cycle control. Silencing TRPC6 in bone marrow stromal cells induced a significant decrease in G_0/G_1 and increase in S and G_2/M phases [137]. Interestingly, this was associated with 10 mV negative shift in membrane potential and a 50 % increase in CPA-mediated SOCE activation. Silencing TRPC6 in a human renal adenocarcinoma cell line also resulted in an increase in G2/M phase with a concomitant decrease in G0/G1 [138].

A reduction in Orai1, Orai2 and Orai3 expression in LNCaP prostate cancer cells evoked a large increase in G_0/G_1 and a parallel decrease in S and G2/M phase [135].

Silencing Orai2 also induces a small (5 %) but significant decrease in G_0/G_1 phase and cell proliferation in brain capillary endothelial cells [139]. Knocking down Orai3 in lung and breast cancer cells confirmed the previous observations concerning this channel with an increase in G_0/G_1 with a decrease in S and G_2/M phases [56, 140]. Finally, silencing TRPC2, a channel that is not expressed in human, induced an increase in G_0/G_1 phase with a parallel decrease in S and G_2/M, resulting in a reduction in rat thyroid FRTL-5 proliferation [141].

The block in G_2/M observed in Orai1 and TRPC6 knock down experiments is interesting as there is evidence that SOCE inactivates during M phase. The first suggestion that calcium influx is inhibited during cell division was reported more than 20 years ago in a study of HeLa cells [142], probably through uncoupling of store depletion from SOCE [143]. This was confirmed recently in HEK293, Cos-7, RBL-2H3 and HeLa cells where SOCE is dramatically reduced during mitosis [132, 144–146]. STIM1 phosphorylation [145], decrease in Orai1 expression [132] and internalization of Orai1 channel [147, 148] during mitosis were recently suggested to be the reasons for the observed decrease in SOCE. The significance of this has already been discussed [149] and involves microtubule-network remodeling [144] as perturbation of the actin cytoskeleton resulted in a decrease in SOCE amplitude [150].

Cell cycle is regulated by cyclins and cyclin dependent kinases (cdk) successively expressed from the onset of G_1 phase up to mitosis. Briefly, cyclin D is present in G_1 phase coupled to cdk1, cdk4 and cdk6, cyclin E with cdk2 at late G_1 and the transition G_1-S, cyclin A with cdk2 during S and cdk1 at the transition S-G_2, and finally cyclin B with cdk1 during mitosis. Cdk activity is down regulated by selective cyclin dependent kinase inhibitors (cdki) and among them P21 and P27 have been associated with calcium channels. The p21 protein binds to and inhibits the activity of cyclin-cdk1, -cdk2, and – cdk4/6 complexes, and thus functions as a regulator of cell cycle progression at G_1 and S phase while P27 inhibitory activity seems to be restricted to the couple cyclinE/cdk2, hence regulating the decision of cell cycle to enter into S-phase or arrested in G_1-phase by blocking G_1 cyclin dependent kinase activities.

A relation between cyclin D1 and D3, calcium channels and calcium influx has been demonstrated in several studies. Cyclin D1 and D3 expression are linked to Orai1 channels [88, 135, 151, 152], Orai3 channels [56, 126, 135], TRPC1 [153] and TRPC6 channels [88] as their expression decreases when these channels are silenced. Knocking down calcium channels often resulted in an increase in P21, P27 and also in tumor suppressor P53. The trend shows an inverse relationship between calcium channel and cdki and P53 expression. TRPC1 silencing is linked to a decrease in cell proliferation, G_1 arrest and increase in P21 expression in endothelial progenitor cells [154], TRPC2 to an increase in P21, P27 and P53 tumor suppressor in rat thyroid FRTL-5 cells [141]. Finally, overexpression of STIM1/Orai1 has been unexpectedly linked to a decrease in SOCE a G_0/G_1 arrest, cyclin D3 expression decrease and P21 increase in A549 lung cancer cells [152].

To summarize here, these data illustrate the strong link between calcium channels, calcium entry and cell proliferation. It makes these channels perfect targets to stop cancer development.

7.8 Extracellular Calcium Sensing G Protein Coupled Receptors

CaSR was identified as the first seven transmembrane domains G protein coupled receptor (GPCR) able to sense extracellular calcium concentration [8]. GPCR are subdivided in three categories, A, B and C, CaSR being part of group C also including, among others, metabotropic glutamate and GABA receptors and basic amino acid receptors such as GPRC6a [4, 5, 155]. This explains that, although a distinct molecular structure, their pharmacology profiles overlap. CaSR can be stimulated by several other cations and modulated by various physiological stimuli including extracellular pH, L-aromatic amino acids and ionic strength [28]. CaSR maintains calcemia through the control of PTH (parathyroid hormone) secretion by parathyroid glands and through PTH-dependent and PTH-independent regulation of the main target organs, kidney and bone, involved in calcium homeostasis. When it comes to cell proliferation and cancer, CaSR activation causes radically different effects depending on whether one is to the breast and prostate or colon and parathydoid [30, 156–159]. One tempting explanation for this apparent contradiction came from experiments in breast cell lines in response to PTHrP (PTH related protein) stimulation. In normal breast cells, PTHrP secretion is inhibited by extracellular calcium concentration increases while, in breast and prostate cancer cells, PTHrP release is augmented following CaSR activation. A recent study demonstrated that the change between inhibition and stimulation of PTHrP release by extracellular calcium ions occured as a result of a switch in G protein activation by the cancerous cells [160]. In normal breast cells, it was shown that the CaSR couples to Gαi, leading to inhibition of cAMP formation and, consequently, PTHrP release, whereas in MCF-7 cancerous breast cells, the CaSR was shown to activate Gαs, thereby promoting PTHrP release.

GPRC6a receptors were cloned and sequenced a little more than 10 years ago [6] and later identified as a calcium-sensing receptor [161]. GPRC6a is activated by calcium, L-arginine, testosterone and osteocalcin [162] and has a strong link with cell proliferation and cancer development. GPRC6a increases proliferation of human dermal fibroblasts stimulated by 6 mM arginine [163] and of osteocalcin stimulated mouse β-cell proliferation [164]. GPRC6a is tightly involved in prostate cancer development and human prostate cancer cell proliferation [162]. Rare variation in protein coding sequence is badly captured by genome-wide association studies arrays and has been hypothesized to contribute to disease heritability. In a large cohort of more than 4000 prostate cancer cases and 7500 control patient, one of the most associated single nucleotide polymorphism (SNP) was in the gene

coding for GPRC6a near to known locus for prostate cancer [165]. The functional consequences for Pro91Ser substitution has yet to be investigated.

Regarding cancer, it has been suggested that calcium supplemented diets may increase the progression of prostate cancer. Our own results on two genetically transformed mouse models to develop prostate cancer strongly confirmed this observation (unpublished data). Calcemia is indeed tightly regulated but is has been shown that calcium supplementation in diet results in transient rise of plasma free calcium concentration [166]. The rise lasted for 8 h with a peak amplitude of about 70 μM from a basal level of 1.25 mM 4 h after ingestion of 1 g of calcium citrate or calcium carbonate. Three questions arise from this observation. Is such a small increase in calcemia able to trigger CaSR or GPRC6a activation? Is this transient elevation in calcemia long enough to induce cell proliferation? Do the downstream events remain activated over a long period of calcemia elevation to trigger cell proliferation? Answering these questions may explain how calcium supplementation can be linked to prostate cancer.

All the major intracellular signaling pathways have been at some point associated to CaSR activation [28] while, to date, GPRC6a are connected to ERK and the cAMP-PKA-CREB trio [162, 163, 167]. Interestingly, Orai1, Orai3, TRPC3 and TRPC6 promotor region have CREB binding sites making their expression likely under the control of these two calcium sensing receptors.

There is a clear connection between CaSR stimulation, calcium influx and calcium channel expression. TRPC1 and mainly TRPC3 and TRPC6 are the identified targets identified so far. In breast cancer cell line MCF-7, a 24-h incubation in the presence of 5 mM external calcium induced a clear increase in TRPC1 channel expression which was prevented by the addition of U1026 to block ERK pathway [168]. Other studies have emphasized an effect of CaSR stimulation of calcium channel expression. In human aortic smooth muscle cells, extracellular calcium increased TRPC6 with an EC_{50} of about 0.5 mM [169]. A rise in cytosolic free calcium concentration was also observed when extracellular was increased. The question here is to know whether a pre-incubation in a calcium-free solution containing 500 μM EGTA induced a partial ER calcium store depletion, hence SOCE activation. A measure of ER calcium contents in their conditions or shortly after the switch in calcium-free solution should discriminate between a real CaSR-induced cytosolic calcium rise and SOCE activation. Elevating extracellular calcium concentration from 1 to 5 mM increased TRPC3 and TRPC6 expression but not TRPC1 and TRPC4 in human mesangial cells [170].

To date, there is no evidence for calcium channel enhanced expression following GPRC6a activation but as they increase the same intracellular signaling pathways as CaSR, it is probably just a matter of time before such a link is highlighted.

Finally, CaSR are also known to trigger intracellular calcium increase via the phospholipase C/InsP3 pathway [171] with a threshold concentration for between 1.5 and 2 mM external calcium concentration [27], just above the physiological levels for plasmatic free calcium concentration [172].

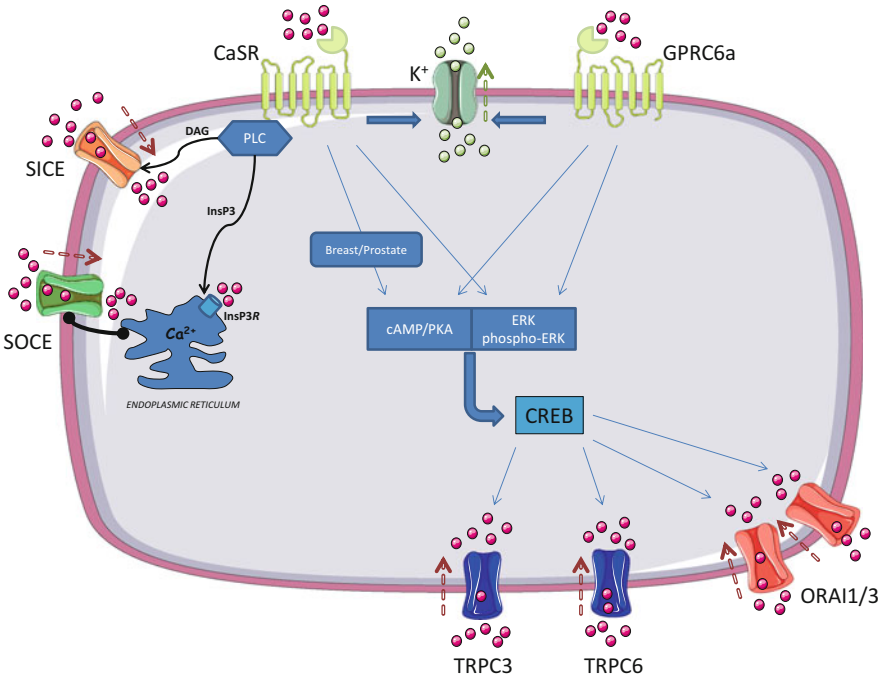

Fig. 7.2 Ionotropic and metabotropic regulation of calcium signaling by CaSR and GPRC6a. CaSR and GPRC6a trigger cAMP/PKA (only in breast and prostate cancer for CaSR) and ERK/phospho-ERK pathways which in turn increase CREB activity. TRPC3, TRPC6, Orai1 and Orai3 have CREB response element resulting in an increase in expression. CaSR also directly increase cytosolic calcium through PLC activation, DAG and InsP3 production, and SOCE and SICE opening. Finally, CaSR and GPRC6a increase K^+ channels activity, hence membrane potential and calcium ions driving force. The *arrows* next to the channel indicate ion flux direction (*red* for calcium and *light green* for potassium ions)

7.9 Conclusion

The data discussed here emphasize the complex relationship between extracellular calcium and cell proliferation. As for neurotransmitters, calcium ions use plasma membrane channels and GPCR to trigger cytosolic free calcium concentration rises and intracellular signaling and regulatory pathways activation (Fig. 7.2). Calcium sensing GPCR, CaSR and GPRC6a, allow a supplemental degree of control and as for metabotropic receptors, they not only modulate calcium channel expression but they may also control calcium-dependent K+ channels [173–175]. The multiplicity of intracellular signaling pathways involved, their sensitivity to local and global intracellular calcium increase and to CaSR and GPRC6a stimulation, the presence of membrane signalplex, all this confers the cells the plasticity they need to convert the effects of extracellular calcium into complex physiological responses and therefore determine their fate.

Conflict of Interest The author declares no potential conflict of interest.

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Chapter 8
Regulation of Platelet Function by Orai, STIM and TRP

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Abstract Agonist-induced changes in cytosolic Ca^{2+} concentration ($[Ca^{2+}]_c$) are central events in platelet physiology. A major mechanism supporting agonist-induced Ca^{2+} signals is store-operated Ca^{2+} entry (SOCE), where the Ca^{2+} sensor STIM1 and the channels of the Orai family, as well as TRPC members are the key elements. STIM1-dependent SOCE plays a major role in collagen-stimulated Ca^{2+} signaling, phosphatidylserine exposure and thrombin generation. Furthermore, studies involving Orai1 gain-of-function mutants and platelets from Orai1-deficient mice have revealed the importance of this channel in thrombosis and hemostasis to those found in STIM1-deficient mice indicating that SOCE might play a prominent role in thrombus formation. Moreover, increase in TRPC6 expression might lead to thrombosis in humans. The role of STIM1, Orai1 and TRPCs, and thus SOCE, in thrombus formation, suggests that therapies directed against SOCE and targeting these molecules during cardiovascular and cerebrovascular events could significantly improve traditional anti-thrombotic treatments.

Keywords Orai1 • TRPC1 • STIM1 • Calcium entry • Human platelets • TRPC6

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8.1 Molecular Basis of Platelet Ca^{2+} Influx: Store-Operated Ca^{2+} Entry

8.1.1 Ca^{2+}-Handling Proteins in Platelets

Platelets are anucleated cell fragments derived from bone marrow megakaryocytes that play an important role in hemostasis. Resting platelets are biconvex discoid structures with 0.5–2 μm diameter. In platelets, Ca^{2+}signaling has mostly been investigated in human and mouse, where these cells exhibit a complex machinery for Ca^{2+} homeostasis. In human platelets, Ca^{2+} entry occurs via both store-dependent (store operated or capacitative) and store-independent (or non-capacitative) mechanisms. Among the proteins and channels involved in the mechanisms underlying Ca^{2+} entry, human platelets express the intraluminal Ca^{2+} sensors STIM1 and STIM2 [1, 2], which have been found both in the dense tubular system (the analog of the endoplasmic reticulum (ER) in platelets) and the acidic stores [2] (agonist-releasable Ca^{2+} compartments that maintain a proton gradient across their membranes mostly identified as lysosomal-like organelles [3]). Moreover, human platelets contain significant levels of Orai1 [4–6] and its two identified homologs, Orai2 and Orai3 [7]. In addition, human platelets express different TRPC subfamily members, including TRPC1, TRPC3, TRPC4, TRPC5 and TRPC6 [7, 8], as well as TRPV1 [9]. We have not detected expression of TRPA1 at the protein level in these cells [10] and the expression of other TRP channels is uncertain. Agonist-stimulated Ca^{2+} release from the intracellular stores is mediated by the generation of inositol 1,4,5-trisphosphate (IP_3) or nicotinic acid-adenine dinucleotide phosphate (NAADP) [11, 12] (Fig. 8.1). Human platelets are reported to express three isoforms of the IP_3 receptor (IP_3R): IP_3R types I, II and III [13] and, although there is no direct evidence for the expression of two-pore channels (TPC) in human platelets, RT-PCR analysis has revealed the expression of TPC1 and TPC2 in the human megakaryoblastic cell line MEG01 [14, 15]. By contrast, ryanodine receptors have not been described in these cells. Ca^{2+} clearance from the cytosol has been reported to occur through the activation of a battery of mechanisms, including Ca^{2+} sequestration into the agonist-sensitive Ca^{2+} stores and extrusion to the extracellular medium. Ca^{2+} reuptake into the stores is mediated by different isoforms of the sarco-endoplasmic reticulum Ca^{2+} ATPase (SERCA), including SERCA2b and SERCA3 [16]. Ca^{2+} extrusion might occur either by active pumping by the two isoforms of the plasma membrane Ca^{2+} ATPase (PMCA) identified in human platelets, PMCA1b and PMCA4b [17–19] or by exchange with Na^+ through the Na^+/Ca^{2+} exchangers [20]. Human platelets exhibit Na^+/Ca^{2+}exchange, as well as, K^+-dependent Na^+/Ca^{2+}exchange activity [21].

Significant inherent differences between mouse and human platelets have been described both at the level of platelet count and size and at the expression of certain Ca^{2+}-handling proteins. While these differences do not preclude the use of mouse models to investigate platelet Ca^{2+} homeostasis, the potential limitations suggest that caution must be exercised in the extrapolation of the data from mouse to human

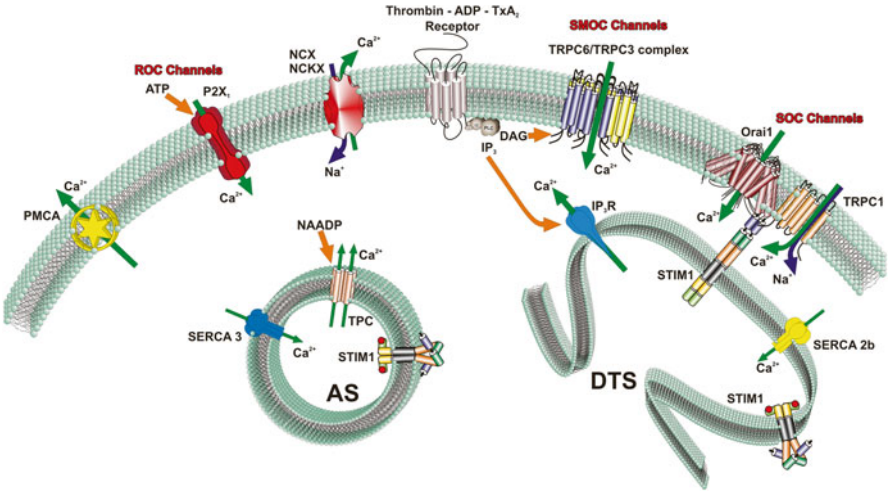

Fig. 8.1 Calcium tuning in human platelets: Cytosolic Ca^{2+} may be increased by three different main routes, which work together under physiological stimuli. **Receptor Operated Calcium** (*ROC*) channels, as the $P2X_1$ receptor: its activation by an agonist induces conformational changes allowing the pass of Ca^{2+} ions through them. **Second Messenger Operated Calcium** (*SMOC*) channels, like the complex *TRPC6/TRPC3*: as its name indicates, these channels are activated by second messengers, for instance diacylglycerol (*DAG*). **Store Operated Calcium** (*SOC*) **channels**, this calcium entry is activated by the depletion of the dense tubular system (*DTS*) and the acidic stores (*AS*), the intracellular calcium stores in human platelets, by the production of inositol triphosphate (IP_3) and nicotinic acid adenine dinucleotide phosphate (*NAADP*) upon agonist stimulation. Intraluminal calcium depletion is sensed by STIM1, located in both stores, and activates Orai and TRPC1 channels in the plasma membrane allowing calcium entry. Platelets present as well mechanisms to return the $[Ca^{2+}]_c$ to basal levels, which will prepare the cell for the response to new stimuli. Two members of the Sarcoplasmic Reticulum Ca^{2+} ATPase (*SERCA*) are expressed in human platelets, SERCA2b in the DTS and SERCA3 in the AS, accumulating high levels of Ca^{2+} in the stores. On the other hand, located on the plasma membrane, human platelets express proteins that extrude Ca^{2+} to the extracellular medium: the Plasma Membrane Calcium ATPase (*PMCA*), the Sodium Calcium Exchanger (*NCX*) and the Potassium dependent Sodium Calcium Exchanger (*NCKX*). *Abbreviations*: *ADP* Adenosine diphosphate, *AS* Acidic Stores, *ATP* Adenosine triphosphate, *Ca^{2+}* Calcium, *DAG* Diacylglycerol, *DTS* Dense tubular system, *IP_3* Inositol 1,4,5-trisphosphate, *IP_3R* Inositol 1,4,5-trisphosphate Receptor, *NAADP* Nicotinic acid adenine dinucleotide phosphate, *NCKX* Potassium-dependent Sodium/Calcium Exchanger, *NCX* Sodium/Calcium Exchanger, *$P2X_1$* ATP receptor, *PMCA* Plasma Membrane Calcium ATPase, *ROC Channels* Receptor Operated Ca^{2+} Channels, *SERCA 2b* Sarco Endoplasmic Calcium ATPase 2b, *SERCA3* Sarco Endoplasmic Calcium ATPase 3, *SMOC Channels* Second Messenger Operated Ca^{2+} Channels, *SOC Channels* Store Operated Ca^{2+} Channels, *TPC* Two Pore Channels, *TxA2* Tromboxane A2

platelets. Concerning Ca^{2+} entry, mouse platelets have been reported to express STIM1 [22] and STIM2 [23], as well as Orai1 [4], which is the predominant Orai member since low levels of *Orai2* and *Orai3* transcripts have been detected in these cells [24]. Mouse platelets express TRPC1, TRPC3 and TRPC6 [25–27], while other members of the TRPC subfamily have not been detected either at the transcript or protein level. Despite TRPM1, TRPM2 and TRPM7 have been reported in

primary murine megakaryocytes, their expression in mouse platelets has not been demonstrated [28], and, in contrast to human platelets, the TRPV1 is not expressed in mouse platelets [29].

Ca^{2+} signaling has also been investigated in platelets from other species, including horses [30], rabbits [31], and rats [32], the latter mostly associated to several pathologies [33], where the mechanisms regulating intracellular Ca^{2+} homeostasis are common to human and mouse platelets. Therefore, we will refer to the cells of these two species hereinafter.

8.1.2 Ca^{2+} Entry Mechanisms in Platelets

The nature of the mechanisms by which agonists induce Ca^{2+} entry from the extracellular medium in human platelets, as well as other non-excitable cells, has been a matter of intense investigation in the last decades due to its relevance in cell physiology. In 1983, De Clerck and Van Nueten reported that flunarizine, a Ca^{2+} entry blocker, attenuated the release of thromboxane B2 and serotonin, a relevant mechanism for platelet-platelet interaction as well as for the communication of platelets with vascular cells [34]. Platelet stimulation with agonists results in an increase in the cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_c$), which mainly consists of two components: the release of Ca^{2+} from intracellular stores, and Ca^{2+} entry across the plasma membrane [35]. Ca^{2+} release from finite intracellular stores induces a transient increase in $[Ca^{2+}]_c$; however, for full activation of platelet processes, as well as the refilling of the Ca^{2+} stores, Ca^{2+} entry plays a relevant role.

The mechanisms by which agonists induce Ca^{2+} entry in human platelets is not fully understood yet but three general routes for Ca^{2+} influx have been described, named receptor-operated Ca^{2+} entry (ROCE), second messenger-operated Ca^{2+} entry (SMOCE) and store-operated Ca^{2+} entry (SOCE) (Fig. 8.1). ROCE is the mechanism for Ca^{2+} influx directly activated by receptor occupation. This Ca^{2+} influx pathway occurs through receptor-operated channels (ROCs) gated by agonist-receptor binding. A good example for ROCE in platelets is the activation of the purinergic $P2X_1$ receptor, a ligand-gated non-selective cation channel activated by ATP [36] that induces a rapid and transient increase in $[Ca^{2+}]_c$. Despite this receptor is rapidly desensitized by purines in vitro studies, experimental maneuvers using high concentrations of apyrase to scavenge the ADP and ATP released have revealed that the activation of $P2X_1$ receptors is involved in a number of platelet functions, including reversible shape change [37], modulation of collagen, adrenaline, thromboxane A2 and thrombin responses [38–41]. In addition, activation of $P2X_1$ receptors in human platelets potentiates the responses induced by occupation of P2Y receptors by ADP [42]. Furthermore, in vivo studies using $P2X_1$ deficient mice have revealed that the $P2X_1$ receptor is involved in thrombus formation although deficient animals display no significant changes in tail bleeding, platelet count or the surface expression of adhesion receptors (see [43]).

SMOCE, also known as non-capacitative Ca^{2+} entry or store-independent Ca^{2+} entry, is a mechanism for Ca^{2+} entry described in platelets and other cells and activated by second messengers generated following G protein-mediated activation of phospholipase C (PLC). PLC hydrolyzes the membrane constituent phosphatidylinositol 4,5-bisphosphate (PIP_2) into IP_3, which, in turn, releases Ca^{2+} from the intracellular stores, and diacylglycerol (DAG). Besides activating protein kinase C (PKC), DAG is a well known direct activator of non-capacitative Ca^{2+} channels. In human and mouse platelets Ca^{2+} entry induced by DAG has been demonstrated using the membrane-permeable analog 1-oleoyl-2-acetyl-sn-glycerol (OAG), which revealed the presence of DAG-sensitive Ca^{2+} permeable channels in these cells [44–46]. In addition, a PKC-dependent DAG-induced route for Ca^{2+} influx has been described in human platelets, which plays a relevant role in thrombin-induced platelet Ca^{2+} signals [47]. This mechanism has been revealed to involve the activation of the isoform 3 of the Na^{+}-Ca^{2+} exchanger followed by the release of dense granule content, including ATP, and subsequent $P2X_1$ receptor activation [48].

Among the second messenger-operated channels (SMOCs) there is a body of evidence supporting the role of TRPC6 in platelets. Platelets from TRPC6-deficient mice show a reduced or undetectable (depending on the mouse strain) Ca^{2+} influx in response to OAG [27, 45, 46]. The involvement of TRPC6 in store-independent Ca^{2+} entry in human platelets was highlighted by Hassock et al. in 2002 [49]. Furthermore, platelet treatment with neutralizing antibodies against TRPC6 resulted in attenuated OAG-evoked Ca^{2+} influx [44]. In the plasma membrane, TRPC6 has been found associated with TRPC3 in membrane microdomains independent of the lipid rafts [8]. Gating of TRPC6 has been found to require a tyrosine phosphorylation step since it necessitates the activation of members of the Src family of tyrosine kinases and is enhanced by inhibitors of tyrosine phosphatases [50]. We have also observed that TRPC6 channels might also been involved in the conduction of SOCE in human platelets; thus, platelet stimulation with thrombin or pharmacological induction of extensive Ca^{2+} store depletion results in the interaction between TRPC6, TRPC1 and the SOCE proteins Orai1 and STIM1, while, cell stimulation with OAG displaces TRPC6 from the SOCE signalplex and enhances Ca^{2+}-dependent association between TRPC6 and TRPC3 [51]. These findings are consistent with a more recent analysis of the protein complexes generated upon the activation of SMOCE and SOCE, which highlighted the dynamism of the Ca^{2+} entry complexes upon Ca^{2+} store depletion or the activation by OAG. In human platelets, Ca^{2+} store discharge leads to rapid and Ca^{2+} independent association of STIM1, STIM2, Orai1, Orai2, TRPC6 and TRPC1. In contrast, platelet treatment with OAG results in the association of Orai3 with TRPC3 [7].

Recent studies have reported the expression of TMEM16F in platelets, a Ca^{2+}-activated nonselective cation channel permeable to monovalent and divalent cations that plays an important role in the regulation of lipid scrambling and pro-coagulant activity of platelets [52, 53]. TMEM16F has been identified as the gene altered in patients with Scott Syndrome, a rare congenital bleeding disorder caused by impaired surface exposure of phosphatidylserine and platelet pro-coagulant activity [54].

In addition to Ca^{2+} influx conducted by ROCs and SMOCs, a mechanism for Ca^{2+} entry regulated by the intracellular Ca^{2+} stores has been described in platelets, as well as in other cells investigated. The so called store-operated Ca^{2+} entry (SOCE), also known as capacitative Ca^{2+} entry or store-mediated Ca^{2+} entry, was first described in platelets soon after the discovery of this mechanism by Putney [55]. In 1989, Sage and coworkers demonstrated the presence of SOCE in human platelets using Mn^{2+} as a surrogate for Ca^{2+} and analyzing its quenching effect of Fura-2 at the Ca^{2+} isosbestic point (360 nM), an excitation wavelength at which Fura-2 fluorescence properties are independent of changes in $[Ca^{2+}]_c$. The authors used Mn^{2+} to avoid interference with other Ca^{2+} transport mechanisms [56]. SOCE has been reported to be induced in platelets by physiological agonists, such as thrombin [56] or ADP [57], as well as by pharmacological agents that induce a decrease in the intraluminal Ca^{2+} concentration, such as the SERCA inhibitors, thapsigargin, 2,5-di-(tert-butyl)-1,4-benzohydroquinone and cyclopiazonic acid [58, 59], or the intraluminal Ca^{2+} chelator N,N,N′,N′-tetrakis(2-pyridylmethyl)ethylenediamine (TPEN) [51].

The initial studies of the mechanism underlying the communication between the filling state of the Ca^{2+} stores and the plasma membrane Ca^{2+} channels in human platelets revealed the involvement of different intracellular messengers and signaling pathways including a protein tyrosine phosphorylation-dependent step [60, 61], an arachidonic acid derivative [62], Ca^{2+}-independent phospholipase A2 [63] or the participation of cyclic nucleotides [32, 64]. The activation of SOCE in human platelets was found to be finely regulated by the remodeling of the actin cytoskeleton, which was suggested as a target of the previously mentioned signaling pathways. The cytosolic actin network supports the intracellular trafficking that allows the approach of portions of the Ca^{2+} stores to the cell membrane, while the cortical actin filament network acts as a negative clamp preventing constitutive activation of SOCE [65, 66] in a model that allows reversible interaction between elements in the membrane of the Ca^{2+} stores and the plasma membrane. According to this model, we have found in human platelets that the SNARE protein SNAP-25 is required for full SOCE activation, probably by leading the establishment of a tight contact between the plasma membrane and the Ca^{2+} stores [67]. According to this, a role for tyrosine kinases, Ras family proteins, cAMP as well as cGMP-dependent protein kinases as well as the cytochrome P450 metabolite 5,6-epoxyeicosatrienoic acid in the regulation of platelet actin cytoskeleton, and, subsequently SOCE, has been described, revealing the regulatory role of the actin cytoskeleton in the activation and maintenance of Ca^{2+} entry [68–74]. In addition, in human platelets, where the existence of two separate agonist-sensitive Ca^{2+} stores have been described: the dense tubular system and the acidic organelles [2, 3], two pathways for SOCE, activated by each Ca^{2+} store and differentially modulated by the actin cytoskeleton have been identified. First, the actin network plays a dual role in the regulation of SOCE mediated by depletion of the dense tubular system, as described above, both supporting the transport of portions of the Ca^{2+} store to the proximity of the plasma membrane to allow the interaction to occur, but preventing constitutive activation of Ca^{2+} entry in the absence of store depletion through the cortical cytoskeleton. On the

other hand, SOCE mediated by depletion of the acidic stores is solely regulated by the cortical cytoskeleton, which acts as a physical barrier to prevent coupling between the Ca^{2+} stores and the plasma membrane under resting conditions. For both pathways, the cortical actin cytoskeleton must be reorganized when the stores are depleted to facilitate the interaction between the Ca^{2+} sensor in the stores and the store-operated channels (SOCs). By contrast, only the pathway activated by the dense tubular system requires reorganization of the cytosolic actin network [75]. The reason of this difference might be attributed to the cellular location of the Ca^{2+} stores in relation to the plasma membrane or the open canalicular system.

Following the identification of STIM1 as the ER Ca^{2+} sensor and a key player for the activation of SOCE in *Drosophila* S2 and different mammalian cell types [76–79], in 2006 we revealed the expression and involvement of the protein STIM1 in the activation of SOCE in human platelets [1]. The identification of STIM proteins, as well as the Ca^{2+}-permeable channels of the Orai and TRPC families represented a significant advance in the characterization of the mechanism underlying SOCE and raised the necessary reinterpretation of the previous data.

8.1.3 Role of STIM, Orai and TRPC Channels

STIM1 has been recognized as the Ca^{2+} sensor in the intracellular Ca^{2+} stores [76–78, 80]. Discharge of the Ca^{2+} stores by agonists via the generation of diffusible messengers, such as IP_3, is sensed by STIM1 via its EF-hand domain. STIM1 becomes activated as the intraluminal Ca^{2+} concentration drops, resulting in the formation of clusters of STIM1-SOCs complexes called "puncta" in regions where the membrane of the Ca^{2+} stores is in close contact with the plasma membrane. As described in detail in Chaps. 2 and 3, STIM1 is a type I transmembrane protein that exhibits intraluminal EF-hand and sterile α-motif (SAM) domains that, in response to a decrease in the intraluminal Ca^{2+} concentration, undergo oligomerization and conformational change. The cytosolic structure of STIM1 is characterized by the presence of three putative coiled-coil segments and a C-terminal lysine-rich region. The coiled-coil segments contain the region of STIM1 that is necessary for the activation of Orai1, identified as STIM–Orai-activating region (SOAR; residues 344–442) [81], Orai-activating STIM fragment (OASF; residues 233–450/474) [82], CRAC-activating domain (CAD, residues 342–448) [83] and CC boundary nine fragment (residues 339–446) [84]. A modulatory domain between amino acids 474 and 485 has been reported to provide a negative feedback signal to Ca^{2+} entry by triggering fast Ca^{2+}-dependent inactivation of Orai channels [85]. Finally, a more distal region between amino acids 448 and 530 has been identified as a C-terminal inhibitory domain (CTID), which shows two lobes, the STIM1(448–490) lobe restricted the association of the regulatory protein SARAF to the STIM1 SOAR region, whereas the STIM1(490–530) lobe directed this association, thus modulating the slow Ca^{2+} -dependent inactivation of Orai channels [86].

Although the role of STIM1 as the intraluminal Ca^{2+} sensor in platelets and other cells is widely accepted, the nature and configuration of the SOC channels is still a matter of intense investigation. Current evidence in different cell types indicates that the channel Orai1 is involved in the conduction of the store-operated Ca^{2+} selective I_{CRAC} [87–89], the first identified store operated Ca^{2+} current [90]. In addition, there is a body of evidence supporting a role for different members of the TRPC subfamily in the conduction of the store-operated cationic current I_{SOC} [6, 91–95]. In 2006, Orai1 was identified as a SOC channel through whole-genome screening of *Drosophila* S2 cells and gene mapping in patients with hereditary severe combined immune deficiency (SCID) syndrome attributed to I_{CRAC} dysfunction [87, 96, 97]. The Orai family includes three human homologs, Orai1, Orai2 and Orai3 (see previous chapters for an extensive review of the architecture of the channel). The crystal structure of Orai from *Drosophila melanogaster* has revealed that the Ca^{2+} channel is composed of an hexameric assembly of Orai subunits arranged around a central ion pore delimited by the first transmembrane region (M1). According to this model, STIM1 interacts with the first and fourth transmembrane domains widening the pore by outward dilation of the M1 region and allowing Ca^{2+} to move [98].

The second type of channels that has been found to be involved in SOCE belongs to the TRPC subfamily. TRP proteins form cation channels identified in 1989 in the *trp* mutant of *Drosophila* [99], which exhibits a transient, rather than sustained, light-sensitive photoreceptor potential due to Na^+ and Ca^{2+} influx through the trp and trpl channels [100]. In 1995, the first mammalian TRP protein was identified, the canonical transient receptor potential protein-1 (TRPC1), both in human [101, 102] and mouse [103]. TRP proteins are classified into seven subfamilies, four groups closely related to *Drosophila* TRP (TRPC, TRPV, TRPA and TRPM), two subfamilies more distantly related (TRPP and TRPML), and the TRPN group that is only expressed in fish, flies and worms [104]. The TRPC subfamily comprises seven members (TRPC1-TRPC7) and, since the identification of mammalian TRPC1, the TRPC channels have been presented as SOC candidates in a variety of cell types, including human platelets [6, 91–95, 105], although their role in SOCE has not been demonstrated in all the cell types and cellular models investigated [106]. The general structure of TRPC channels has been thoroughly described in previous chapters of the present book. Among other mechanisms of gating, TRPC channels have been shown to be activated by store-depletion via STIM1. Mutational analyses have revealed that the C-terminal STIM1 lysine-rich domain directly gates TRPC channels by interacting with two conserved C-terminal negative charged residues [107]. Neutralization or reversing the negative charges D639 and D640 in TRPC1 and D697 and D698 in TRPC3 resulted in inactive channels. Similarly, STIM1 mutants where any positive charge of the polylysine domain, especially K684 and K685, were neutralized inhibited the activity of TRPC1 [108]. Further studies have revealed that the interaction of STIM1 with TRPC1 and TRPC3 also involves the interaction between the SOAR region of STIM1 and the N- and C-terminal coiled-coil domains of the TRPC channels [109]. Therefore, both STIM1 regions, the SOAR domain and the polylysine-rich motif, are required for the interaction and gating of TRPC channels by STIM1.

As mentioned above, STIM1 was described in human platelets in 2006 [1] and, 1 year later, its murine homolog was reported in mouse platelets [22]. By contrast, STIM2 was first reported in mouse platelets in 2010 [23] and the first analysis of the expression of STIM2 in human platelets was reported in 2011 [2]. The functional role of STIM1 in platelet SOCE has been demonstrated by different means. In mice with STIM1-deficient platelets a marked defect in SOCE and agonist-stimulated Ca^{2+} mobilization has been described, together with an impaired platelet activation and thrombus formation under flow in vitro [110]. The evidence supporting a role for STIM1 in SOCE in human platelets are more indirect mostly due to obvious technical limitations. STIM1 has been found to be highly expressed in human platelets and their precursors [22]. Furthermore, interference with STIM1 function by introduction of an antibody directed toward the N-termini of STIM1 has been found to attenuate SOCE in these cells [1]. These findings cannot be attributed to side effects due to the preservatives present in the antibody solution since introduction of a non-specific IgG of the same origin of the anti-STIM1 antibody and containing the same preservatives did not reproduce the effects observed with the anti-STIM1 antibody [111].

In human platelets, STIM1 has been found to be expressed both in the membranes of the dense tubular system and the acidic Ca^{2+} stores [1, 2] as well as in the plasma membrane [112]. STIM1 associates with endogenously expressed Orai1 as well as TRPC1 and TRPC6 upon Ca^{2+} store depletion in human platelets in a Ca^{2+}-independent manner [1, 6, 51]. A major role for Orai1 in SOCE has been reported in human platelets, as well as human megakaryocytes and human megakaryoblastic cell lines on the base of the expression level, as compared to other Ca^{2+}-permeable channels such as TRPC1 or TRPC6, and the electrophysiological recordings of I_{CRAC} in megakaryocytes [4]. Orai1 is also strongly expressed in mouse platelets and further studies in Orai1-deficient mice reported that platelets from Orai1$^{-/-}$ mice show impaired SOCE and defective thrombus formation [24]. In addition, a knock-in mice expressing a loss-of-function mutant of Orai1 (Orai1^{R93W}) revealed the functional role of Orai1 in the activation of SOCE and agonist-induced Ca^{2+} entry in mouse platelets [113].

As reported in different cell types [114–116], in human platelets we have found that STIM1 interacts with Orai1 and members of the TRPC subfamily, such as TRPC1 and TRPC3, forming a SOCE signalplex associated to lipid raft domains [6, 51, 105]. Thus, disruption of the association between Orai1 and TRPC1 results in displacement of TRPC1 from the STIM1-Orai1 complex and loss of responsiveness to store depletion [6].

The involvement of TRPC1 in SOCE was first suggested in human platelets in 2000, when we found that endogenously expressed TRPC1 co-immunoprecipitates with the type II IP_3R upon Ca^{2+} store depletion, but not in resting cells. The role of TRPC1 in SOCE in human platelets was supported by studies where interference with TRPC1 function by treatment with a specific antibody which recognizes the sequence TRPC1$^{557–571}$, located extracellularly in the pore-forming region, significantly inhibits SOCE in human platelets [91]. The role of TRPC1 in SOCE and platelet function has been challenged by studies reporting that platelets from

TRPC1$^{-/-}$ mice show fully intact SOCE compared to wild-type animals, and normal platelet function in vitro and in vivo [25]. The controversy between the data observed in human and mouse platelets deserves further studies. This might be attributed to interspecific differences or to a redundant function of TRPC channels in platelet physiology. More recently, we have reported that TRPC1 plays an important role in the activation of SOCE in these cells in the human megakaryoblastic cell line MEG-01, where TRPC1 expression was silenced using shRNA, an approach that avoids the development of compensatory mechanisms that might interfere with the observations in knock-out animal models [117].

The putative role of TRPC1 in SOCE in human platelets still remains to be clarified. Following the identification of the coupling between TRPC1 and the type II IP_3R, we found many characteristics of the coupling that shows parallelism with SOCE: (1) the interaction between both proteins was activated by depletion of the intracellular Ca^{2+} stores, and was reversed by refilling of the stores [118]; (2) both SOCE and the coupling between TRPC1 and the type II IP_3R were impaired by treatment of platelets with the IP_3R antagonist xestospongin C [118]; (3) stabilization of the membrane cytoskeleton using jasplakinolide prevented the coupling and impaired SOCE, indicating, as mentioned above, that the actin filaments at the cell periphery act as a negative clamp for both events. In addition, the cytosolic actin network plays a positive role, since disruption of the actin network inhibited the coupling and SOCE [65, 118]; and (4) immunophilins, such as FKBP52, are required both for the coupling between TRPC1 and the type II IP_3R and the activation of SOCE in human platelets. The parallelism between both phenomena suggested that a de novo association between TRPC1 in the plasma membrane and the IP_3R type II in the intracellular stores might play a role in SOCE in these cells. To further test this hypothesis we introduced into platelets a peptide corresponding to type II $IP_3R^{317–334}$, which impaired the association of the IP_3R with TRPC1. This maneuver revealed that the association between TRPC1 and the type II IP_3R is important for the maintenance, but not the initial stage, of SOCE [119]. Whether TRPC1, through its association with STIM1 and Orai1, is also involved in early stages of the activation of SOCE in human platelets deserves further analysis.

The role of other TRPC proteins, such as TRPC6, in platelet SOCE, has also been demonstrated. TRPC6 has been reported to participate in SOCE in human platelets by using a neutralizing antibody against the C-terminal region of TRPC6, which reduced both TG-induced Ca^{2+} and Mn^{2+} influx, as well as OAG-stimulated Ca^{2+} entry [44]. The role of TRPC6 in Ca^{2+} homeostasis in platelets is described in more detail in Chap. 10.

It is noteworthy to mention that a pathway for Ca^{2+} entry secondary to agonist stimulation has been reported in human platelets. Harper and coworkers have found that Na^+ entry through TRPC channels results in the activation of the Na^+/Ca^{2+} exchanger working in reverse mode [120]. The Na^+/Ca^{2+} exchanger-3 has been shown to be the predominant isoform in human platelets by using a proteomic screen of the platelet membrane [121] and Western blotting [48]. Ca^{2+} entry via Na^+/Ca^{2+} exchange has been found to potentiate both SOCE and $P2X_1$-mediated Ca^{2+} entry in human platelets [48, 120, 122], a mechanism that involves the increase in

the concentration of Ca^{2+} in the pericellular region, which is recycled back into the cytosol to support cellular events such as dense granule secretion [122, 123].

8.2 Role of SOCE in Thrombosis and Hemostasis

8.2.1 The Importance of Ca^{2+} in Thrombosis and Hemostasis

Hemostasis refers to the property of the vascular system to stop blood loss after vascular damage. Besides platelet function, the early phase comprises the activation of the vascular endothelial cell layer located at sites of injury, local vessel wall contraction, the aggregation of platelets and other blood cells to form a blood clot and seal the broken area, and the participation of many plasma proteins known as the coagulation cascade, which act as thrombotic factors to stimulate and synergize the process (Fig. 8.2). The blood clot formed inside a vessel during this process is known as thrombus [124]. Once sealed the broken area, later phases of hemostasis take place to reestablish the initial conditions, for instance wound healing, thrombus clearance and the final reestablishment of the blood flow at the repaired area [125]. Non-mechanical agents such as chemical, pathological and inflammation are also able to initiate hemostasis and therefore thrombus formation without compromising the integrity of the vessel [126]. Interestingly, the early phase of hemostasis is constantly counter-regulated by many inhibitory agents and anti-thrombotic factors in order to avoid the aberrant and uncontrolled development of the process [127].

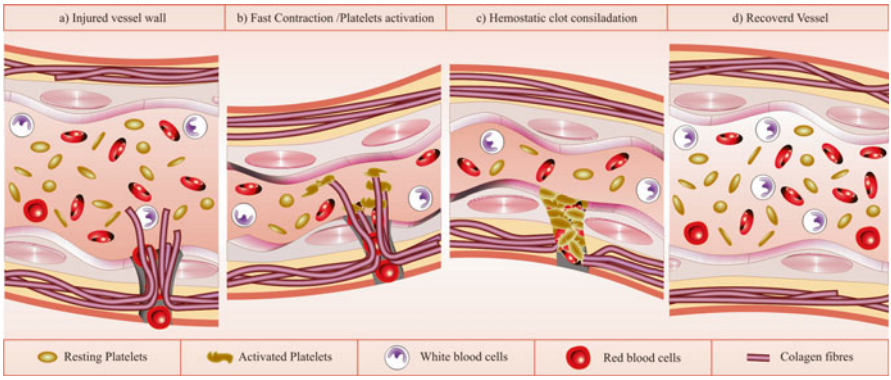

Fig. 8.2 Role of platelets in hemostasis: (**a**) Injuries in the walls of blood vessels lead to the exposition of collagen fibres in the exposed wall and the release of thromboregulatory molecules from the vascular cells, which will start platelets activation. (**b**) Fast contraction of the damaged vessel reduces the blood flow, thus, blood loss. Collagen-activated platelets will recruit and trigger new platelet activation to form, in addition to red blood cells and collagen fibres, a network, the clot, whose function is to stop bleeding. (**c**) When the hemostatic clot is consolidated and the bleeding is stopped, new vascular endothelial cells will be formed, and the clot components are reabsorbed. (**d**) The vessel recovers its original diameter and the normal blood flow is restored

These anti-thrombotic factors are also constantly released to the blood stream to prevent an unwanted spontaneous initiation of the hemostatic process in the intact vascular system. However, an abnormal uncontrolled thrombus formation can lead to a pathologic condition known as thrombosis. Thrombosis (-osis [-ō-sis], from greek, "pathologic process") is medically defined as the partial or complete occlusion of a blood vessel by a thrombus generated at the site of injury, also referred to as thromboembolism when the thrombus was generated elsewhere. Thus, the concept of thrombosis also extends to the pathologic initiation of the otherwise normal processes triggered during the early phase of hemostasis. The importance of thrombosis lies in how frequent it appears in many cardiovascular diseases, and depending on the occluded vessel and the affected organ or area, how easily it spontaneously develops into a life-threatening situation due to a dangerous reduction of the blood flow [126].

Blood platelets are considered the central cellular player of arterial thrombosis and hemostasis. Their decreased number or absence in the circulation leads to uncontrolled hemorrhage [128, 129], while abnormal hyperaggregability observed in many diseases, for instance diabetes mellitus, often leads to thrombosis [18, 126, 130]. In contrast, they seem to be less important in cases of deep venous thrombosis [131]. Circulating small rounded platelets are "inactive" and do not show adherent properties in the absence of damage. They circulate freely along the vascular system, detecting spontaneously released thrombotic and anti-thrombotic factors present in the blood plasma, and analyzing their activatory and inhibitory signals. Thus, platelets integrate these signals and initiate or not their activation if the balance of thrombotic signals exceeds the anti-thrombotic ones [132–134]. Platelet activation comprises cell shape changes and increased adhesive properties, promoting their attachment to the active endothelial surfaces near the damaged vessel wall and to other blood cells to form a thrombus. Platelet activation is also accompanied by the release of additional thrombotic factors stored in cytosolic organelles (α- and dense-granules) and by the exposure of phosphatidylserine (PS) on their outer cell surface, a crucial step in hemostasis [135]. PS-rich platelet membranes act as catalytic surfaces that speed up the activation of the coagulation cascade to promote mainly the conversion of inactive pro-thrombin to active thrombin near platelets, one of the most important thrombotic factor in hemostasis. This process is referred to as procoagulant activity. Platelet stimulation through the glycoprotein (GP)VI collagen receptor (GPVI) with physiologic agonists such as collagen and collagen-related peptides (CRP) leads to a higher PS exposure than for instance other signaling pathways triggered by physiologic agonists such as thrombin or ADP [135–137]. One of the multiple functions of thrombin is to catalyze the polymerization of soluble fibrinogen to insoluble fibrin fibers and thus stabilizing the growing thrombus [135].

New platelet functions in physiology are constantly reported [125, 138], and different platelet subpopulations displaying different specialized functions have been described [139]. However, the function of SOCE in platelets has mainly been studied during hemostasis so far.

The cellular machinery involved in platelet activation comprises Ca^{2+}-dependent and Ca^{2+}-independent signaling cascades. Raises in $[Ca^{2+}]_c$ are considered a central

event in platelet physiology [140–142] as they experiment changes in $[Ca^{2+}]_c$ after attachment to the injured area and during the process of aggregation and thrombus formation [143]. In fact, removal of extracellular Ca^{2+} by chelating agents such as sodium citrate, potassium oxalate or ethylenediaminetetraacetic acid (EDTA) is a classical way to impair platelet function and prevent spontaneous aggregation [144, 145]. A growing body of evidence sustains the idea that SOCE and ROCE are the main pathways of Ca^{2+} entry into platelets, and that they might have a prominent function in their procoagulant activity during thrombosis and hemostasis [23, 24, 26, 45, 110, 113, 146, 147].

8.2.2 Role of STIM and Orai in Thrombosis and Hemostasis

As mentioned above, members of the STIM and Orai family are considered the main molecular components of SOCE. In the case of STIM proteins, there is no available data about STIM2 function for instance in humans, but the analysis of genetically modified murine models suggested a redundant or unclear role in platelet physiology and hemostasis [23, 148]. In contrast, recent evidences revealed that STIM1 plays a determinant role in human platelet physiology and hemostasis when STIM1 function is abnormally increased, while its absence has only a mild impact in hemostasis. Thus, it has been reported that STIM1 mutant proteins exhibiting constitutive or enhanced activity are probably responsible of the Stormorken syndrome in humans, causing an excessive Ca^{2+} entry into platelets and leading to serious problems, such as premature platelet activation, constitutive PS exposure in their outer cell surface, thrombocytopenia and bleeding disorders [149–151]. The heterozygous expression of similar STIM1 mutated forms has also been associated to the York Platelet syndrome (YPS) in humans, characterized by thrombocytopenia, defective Ca^{2+} storage inside platelet granules and aberrant morphology of their intracellular organelles [152]. In contrast, the lack of STIM1 in humans leads to a mild bleeding prolongation despite the decreased SOCE observed in their platelets, or their defect in α- and dense-granule secretion upon thrombin stimulation, or the slightly impaired platelet aggregation in the presence of epinephrine [153]. Interestingly, the analysis of mice lacking STIM1 only in blood cells suggested a more prominent role in arterial thrombosis than in hemostasis, since these animals do not develop vessel occlusion in the absence of STIM1 when they are challenged to develop thrombosis [110, 147]. As in humans, these animals show mild, almost unaltered bleeding times after mechanical injury, indicative of a redundant role in hemostasis. However, the induction of collagen- or thrombin-dependent thrombosis by $FeCl_3^-$ or laser-induced arterial damage in these animals generates unstable micro-thrombi, which are unable to firmly aggregate and form a single stable occluding thrombus. As a consequence, these genetically modified mice are protected from arterial thrombosis and ischemic brain infarction when STIM1 function is absent despite their normal hemostasis [110, 147]. Similar resistance to thrombosis has been reported in mouse bearing a constitutively active STIM1 mutant [22].

What is therefore the molecular mechanism underlying such different roles of STIM1 in thrombosis or hemostasis? Unfortunately, it is controversial, in part for the not completely understood models of thrombosis used in these studies [154, 155], or the genetic variability existing between all mouse strains used [110, 147]. What is clear is that most of the important physiological agonists, such as thrombin, ADP and collagen, trigger SOCE to mediate platelet function and aggregation [142]. STIM1-deficient platelets isolated from plasma show abrogated SOCE in fact, but surprisingly they respond normally to most physiologic agonists except to collagen [110, 147], probably due to a defective GPVI-dependent Ca^{2+} signaling [22, 113]. The reason of these different platelet responses to agonists in the absence of STIM1 is not clear, but might be explained in part by the different PLC isoforms activated by them [156, 157]. As a consequence to their defective collagen responses, STIM1-deficient platelets show diminished GPVI-induced activation of the fibrinogen and fibrin receptor $\alpha_{IIb}\beta_3$ integrin, defective PS exposure on their outer cell surface as well as in GPVI-induced procoagulant activity (thrombin generation near platelets). As a result, isolated platelets lacking STIM1 do not properly aggregate in response to collagen or CRP. In contrast, it is not clear whether they are able to attach and form stable thrombi in response to collagen [23, 110]. Conditional transgenic mice lacking STIM1 in platelets also display a delayed and reduced fibrin generation in a laser injury thrombosis model, suggesting also a defective fibrin generation as a consequence of their defective PS exposure [147]. In summary, STIM1-dependent SOCE seems to play a major role in GPVI/collagen-induced Ca^{2+} signaling, collagen-induced PS exposure and GPVI-dependent thrombin generation. But, if STIM1-deficient platelets are defective in GPVI-dependent signaling and do not respond properly to collagen, why do mice display normal hemostasis in the absence of STIM1? Interestingly, studies carried by Gilio et al. suggested that the presence of the coagulation cascade or thrombin compensates these GPVI-dependent signaling deficiencies observed in STIM1-deficient platelets and restores platelet function [23], probably by the compensation of their deficient SOCE through an activation of additional members of the TRPC family [23, 26, 158]. Therefore, the combined activity of both thrombin and collagen might compensate the defective function observed in STIM1-deficient platelets challenged only with collagen. Since tissue factor is one of the main initiators of the coagulation cascade [135], Gilio et al. suggested a mechanism that might explain the phenotype observed in mice lacking STIM1 in blood cells. These mice are protected from collagen-induced arterial thrombosis because the absence of STIM1 abrogates platelet adhesion to collagen, PS exposure and GPVI-induced thrombin generation, but cannot be compensated by the coagulation due to the limited tissue factor present in these vessels and the subsequent minimal amount of thrombin generated. However, tissue factor is abundantly exposed in wounds, and can compensate the deficient GPVI-induced signaling observed in STIM1-deficient platelets leading to an almost normal hemostasis [23].

However, a more recent study carried by Ahmad et al. in mice lacking STIM1 in platelets argued against this hypothesis, since they observed impaired thrombi formation and vessel occlusion also in a model of thrombin-dependent thrombosis by laser injury [147], in the presence of tissue factor-dependent thrombin generation

[159]. They also observed deficient PS exposure, but normal thrombus formation in response to collagen, in contrast to Gilio et al. Thus, Ahmad et al. concluded that their reported phenotype cannot be explained by a defective collagen adhesion, and it must be attributable to a defective PS exposure and limited thrombin generation in response to collagen, even in the presence of coagulation. Certainly, Ahmad et al. showed that the presence of an specific agonist of the thrombin PAR4 receptor, PAR4p, cannot compensate the defective PS exposure observed in STIM1-deficient platelets challenged by collagen [147], concluding that coagulation might not compensate the absence of STIM1 in platelet function as Gilio et al. proposed [23]. However, Ahmad et al. used a specific agonist of PAR4 [160] for that study, in contrast to the thrombin used by Gilio et al., which has a broader activity than PAR4p. Thus, the comparability between both assays and the conclusion taken must be carefully considered. In summary, Ahmad et al. proposed a more prominent function of STIM1 in PS exposure and procoagulant activity in platelets than in platelet adhesion as Gilio et al. suggested [23]. The appearance of later studies questioning the idea of $FeCl_3$-induced arterial injury as a collagen-induced model of thrombosis increases this discrepancy [154, 155]. Certainly, these data obtained in mice must be carefully considered due to the differences observed, as compared to humans or among mouse strains, in platelet aggregation in response to collagen or granule secretion in response to thrombin [23, 110, 147, 153], indicating possible interspecific differences in certain aspects of platelet function and hemostasis.

In the case of members of the Orai family, it has been reported that Orai1 mutant proteins displaying enhanced activity lead to Stormorken-like phenotype in humans, but interestingly they lack thrombosis or altered hemostasis [150]. Humans carrying inactive Orai1 mutants suffer from thrombocytopenia, but it seems to be more an autoimmune problem than a defect in hemostatic mechanisms, indicating the overall data a minor function of Orai1 in human hemostasis [161]. In contrast, similar alterations in thrombosis and hemostasis to those found in STIM1-deficient mice have been observed in mice lacking Orai1 in blood cells, indicating that those molecules work together in same signaling pathways as expected, and suggesting that SOCE might play a more prominent role in thrombosis than in hemostasis [23, 24, 113]. The role of other Orai isoforms expressed in platelets [7, 24] in thrombosis and hemostasis has not been addressed so far. Interestingly, the fact that both STIM1 and Orai1, and thus SOCE, display such prominent function in thrombus formation and stabilization but not in hemostasis after hemorrhage in mice, suggests that therapies directed against SOCE and these molecules during ischemic cardiovascular and cerebrovascular events could significantly improve traditional anti-thrombotic therapies [162].

8.2.3 Role of TRPC Channels in Thrombosis and Hemostasis

The available data about TRPC function has been obtained from murine models lacking these proteins, since there is no study until date about thrombosis and hemostasis performed in human patients carrying mutated versions of TRPC proteins.

However, there is evidence that genetically-based increase in TRPC6 expression is associated to idiopathic pulmonary arterial hypertension, a disease that is caused by thrombosis among other factors [163]. Thus, increase in TRPC6 expression might be associated to thrombosis in humans. In mice, while the function of TRPC1 seems to be redundant [25], the role of TRPC6 in thrombosis and hemostasis is controversial. Studies reported both normal [45] or prolonged bleedings after mechanical vessel injury and altered thrombus formation and hemostasis in TRPC6-deficient mice [164]. A recent report suggested that, in contrast to previously thought, TRPC3 is expressed in murine platelets and cooperates synergistically with TRPC6 to regulate PS exposure in platelets, and thus their procoagulant activity [26]. The role of TRPC6 in platelet Ca^{2+} homeostasis is also unclear, since it has been reported none, mild or severe reduction of ROCE in platelets isolated from different mouse strains lacking TRPC6 and stimulated with OAG [26, 45, 46]. On the other hand, the sensitivity of TRPC6 [46] and Orai1 [165] to pH might explain the decreased SOCE and the impaired platelet aggregation observed in acidic conditions [166] and in pathological situations where the local pH is very low, for instance during internal hemorrhages along the digestive tract [167] or in cases of massive prolonged bleedings [168]. Finally, it has been suggested that the increased activity of TRPC-dependent ROCE could be an important mechanism for the hyperreactive platelet response and the enhanced thrombosis observed in type 2 diabetic patients. Increased TRPC6-dependent ROCE but decreased Orai1/STIM1-dependent SOCE has been found in platelets isolated from these patients, despite the increased expression of Orai1/STIM1 [169–171], which might be explained because the TRPC6/phosphatidylinositol 3-kinase interaction seems to be favored, while STIM1/Orai1 or STIM1/TRPC interaction seems to be decreased under this pathologic condition. As a consequence of these interesting results, some research groups are considering the importance of TRPC agonists and blockers as useful pharmacological tools for antithrombotic therapies [172].

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Part II
Non-capacitative Ca^{2+} Entry Pathways

Chapter 9
On the Roles of the Transient Receptor Potential Canonical 3 (TRPC3) Channel in Endothelium and Macrophages: Implications in Atherosclerosis

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Abstract In the cardiovascular and hematopoietic systems the Transient Receptor Potential Canonical 3 (TRPC3) channel has a well-recognized role in a number of signaling mechanisms that impact the function of diverse cells and tissues in physiology and disease. The latter includes, but is not limited to, molecular and cellular mechanisms associated to the pathogenesis of cardiac hypertrophy, hypertension and endothelial dysfunction. Despite several of these functions being closely related to atherorelevant mechanisms, the potential roles of TRPC3 in atherosclerosis, the major cause of coronary artery disease, have remained largely unexplored. Over recent years, a series of studies from the authors' laboratory revealed novel functions of TRPC3 in mechanisms related to endothelial inflammation, monocyte adhesion to endothelium and survival and apoptosis of macrophages. The relevance of these new TRPC3 functions to atherogenesis has recently began to receive validation through studies in mouse models of atherosclerosis with conditional gain or loss of TRPC3 function. This chapter summarizes these novel findings and provides a discussion of their impact in the context of atherosclerosis, in an attempt to delineate a framework for further exploration of this *terra incognita* in the TRPC field.

Keywords TRPC3 • Macrophages • Atherosclerosis • Endothelial cells • Calcium entry

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9.1 Introduction

In western societies, coronary artery disease (CAD) is the leading cause of morbidity and mortality due to cardiovascular diseases [1]. The major causative pathological entity behind CAD is atherosclerosis, a disease that affects medium- and large-sized arteries and whose initiation and progression have all the features of a chronic disease ruled by a maladaptive inflammatory response. Typical clinical manifestations of atherosclerosis are the appearance of ischemic symptoms subsequent to lesions causing critical stenosis of the arterial lumen, and/or acute thromboembolic events that often follow the rupture of unstable plaques [2, 3]. Atherosclerotic lesions develop through a complex series of interactions between pro-atherogenic humoral factors – v.g., apo-B containing lipoproteins, inflammatory cytokines–, cellular components of the arterial wall – i.e., endothelial and smooth muscle cells, macrophages-, and immune cells recruited from circulation – v.g., monocytes, lymphocytes–. The earliest molecular and cellular events in atherogenesis are dominated by endothelial cell activation, inflammation and recruitment of inflammatory monocytes to the arterial subintima. As the disease progresses, the added effect of cytokines and pro-inflammatory mediators that coexist in the lesion environment, as well as the critical impact of macrophage death on plaque composition and stability, complete a maladaptive inflammatory vicious cycle that drives the lesion towards critically advanced and clinically relevant stages. A key therapeutic goal in atherosclerosis is to identify molecular targets that can be used to prevent or reduce endothelial inflammation and monocyte recruitment, and/or to improve plaque stability to reduce the risk of acute thromboembolic events. This goal has, so far, been unsatisfactorily met. Therefore, improving current knowledge of signaling mechanisms that mediate endothelial activation and macrophage functions in the plaque is an ongoing effort in the field and key to reach the above mentioned therapeutic objectives.

Transient Receptor Potential Canonical (TRPC) channels (TRPC1-7) are non-voltage gated, non-selective cation channels whose diverse functions in the cardiovascular and hematopoietic systems are now well-recognized (reviewed by us in [4]; see also [5]). And it is by virtue of their diverse functions in molecular and cellular mechanisms associated to the pathogenesis of cardiovascular and hematopoietic diseases that most TRPC proteins have been postulated as potential therapeutic targets [4, 5]. In the particular case of TRPC3, its expression and function have been shown to have implications in hypertension, endothelial dysfunction and, with solid support from in vivo studies, in cardiac hypertrophy [6–8]. Curiously, the potential roles of TRPC3 – and of any other TRPC for that matter – in atherosclerosis have remained largely unexplored. A series of recent studies from our laboratory have pioneered the field by providing experimental evidence revealing previously unforeseen roles of endothelial and macrophage TRPC3 in molecular and cellular mechanisms of relevance to the development of atherosclerotic lesions. The mode of regulation of TRPC3 in these and in most other cells where this channel is expressed, is still incompletely defined (for discussions on this topic, see [9–12]).

Nevertheless, understanding the functions of this and other TRPCs in cardiovascular pathology demands at the very least identification of cellular/molecular events that may be affected by channel expression/function, regardless of what the channel's activating/regulatory mechanism is. Most importantly, the generation of mouse models of atherosclerosis with conditional gain or loss of TRPC3 function has been instrumental in validating these findings and in paving the road for further exploration of this *terra incognita*. The sections below are aimed at providing a compilation of these novel findings with discussions of their implications in the context of atherosclerosis.

9.2 Structural and Topological Features of TRPC3

TRPC3 belongs to the TRPC family of channel forming proteins, which are part of the larger TRP superfamily [12, 13]. The TRPC group is subdivided into TRPC1, TRPC2 – a pseudogene in humans, old monkeys and apes, but a functional channel in rodents-, TRPC3/6/7 and TRPC 4/5, mostly on the basis of structural, functional and pharmacological properties [12]. All TRPC proteins form non-voltage gated, Ca^{2+}-permeable channels that are activated, at least under physiological conditions, downstream of receptor-stimulated phospholipases [4, 11]. Besides this receptor-regulated mode, TRPC3 channels exhibit significant constitutive function in both overexpression and native systems [10, 14]. Similar to the other members of the TRPC group, TRPC3 most salient topological features include six transmembrane domains (TM1-TM6) that separate cytoplasmic N- and C-ends, with a pore region framed in between TM5 and TM6 [15, 16]. The N-terminus contains three to four ankyrin repeats, a coiled-coil region and a caveolin binding domain. The ankyrin repeats form a protein binding interface that allows TRPC3 to interact with other proteins and it also affects trafficking of the assembled channel to the plasma membrane [17]. In fact, deletion of the ankyrin repeats leads to accumulation of TRPC3 in intracellular compartments [17]. The C-terminus contains the typical TRP signature motif (EWKFAR), a highly conserved proline rich motif, the CIRB (calmodulin/IP_3 receptor binding) domain and a predicted coiled-coil region. The coiled-coil regions participate in oligomerization of the channel subunits and contribute not only to the final assembly of a functional channel but also to the association of TRPC3 with other proteins [12]. The proline-rich motif and CIRB region have also been found to interact with a number of signaling molecules [12]. In both native and overexpression systems functional TRPC3 channels are made of homo- or hetero-tetrameric arrangements of four TRPC subunits [12, 15, 16]. When heteromerization occurs, a preference exists for association with members within the TRPC3/6/7 group, although interactions with other members of the TRPC family do take place [4, 12, 18]. The lack of correlation between some functional and pharmacological properties of overexpressed vs. native TRPC3 channels – v.g., sensitivity to low micromolar concentrations of gadolinium, responsiveness to synthetic diacylglycerols- have been traditionally attributed to differences in the final

tetrameric buildup of the channel. However, definitive conclusions on this matter have not been reached.

9.3 Atherorelevant Functions of Endothelial TRPC3

9.3.1 TRPC3 and Regulation of Vascular Cell Adhesion Molecule-1 Expression in Coronary Artery Endothelial Cells

Endothelial inflammatory signaling is a major determinant of monocyte recruitment to the arterial subintima, and although present throughout all stages of atherosclerotic lesion development, it has a particular impact in the early phases of plaque formation (see [19] and references therein). Whereas several endothelial cell adhesion molecules (CAMs) participate in this process, intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1), which belong to the immunoglobulin superfamily [20, 21], define the firm attachment of the monocyte to the endothelium and its subsequent transmigration to the subintima. Perhaps the most important characteristic of VCAM-1 in early atherogenesis is the fact that whereas most other CAMs are constitutively expressed in the non-activated endothelium, VCAM-1 is nearly absent, but its expression increases dramatically when the endothelial cell is exposed to pro-inflammatory stimuli [21]. It is thus not surprising that a significant amount of research effort has been devoted at elucidating the mechanisms underlying VCAM-1 expression, with the expectation of identifying molecular targets to abrogate or at least reduce the recruitment of monocytes mediated by this CAM. Studies from different laboratories coincided in the observation that calcium, at least from intracellular sources, was a common factor in the mechanism by which a number of inflammatory mediators – v.g., substance P, ATP, lipoprotein a – induced VCAM-1 expression in the endothelium [22–24]. Until the publication of our first series of studies on the roles of TRPC3 in regulated expression of VCAM-1 in human coronary artery endothelial cells (HCAECs) [25], it was uncertain whether calcium influx had any specific role in regulation of VCAM-1 expression. Using a combined pharmacological and molecular approach, we found that, despite HCAECs expressing all members of the TRPC family, only TRPC3 formed endogenous calcium permeable channels in these cells [25]. Moreover, TRPC3 contributed, to a great extent, to the ATP-induced calcium influx and, most remarkably, accounted for most of the constitutive calcium influx in these cells. Notably, TRPC3 expression and function were found to be required for maximal induction of VCAM-1 and monocyte adhesion to HCAECs [25]. In most endothelial cells, regardless of their location in the vascular tree, VCAM-1 expression is regulated by the Nuclear Factor kappa B (NFkB) pathway [26]. In a follow up study, it was found that in HCAECs the constitutive function of TRPC3 had an

obligatory role within the inflammatory signaling that drives VCAM-1 expression under pro-inflammatory/pro-atherogenic conditions [27]. In this mechanism TRPC3-mediated constitutive calcium entry was coupled to activation of an IKKβ/IkBα axis in a calmodulin-dependent protein kinase II (CAMKII) manner [27]. Perhaps the most remarkable observation was that the same pro-inflammatory/pro-atherogenic factors that induce VCAM-1 in HCAECs, are also strong inducers of TRPC3 expression in these cells, and this results in enhanced inflammatory signaling due to the augmented number of channels operating in constitutive mode [27]. These findings represented the first piece of experimental evidence suggesting a role of TRPC3 in molecular and cellular events that are critical in atherosclerosis. The in vivo relevance of these in vitro findings has been validated by our recent studies in Apoe knockout mice with endothelial-specific overexpression of TRPC3 (see Sect. 5.2 below).

9.3.2 TRPC3 and Endoplasmic Reticulum Stress-Induced Apoptosis in Endothelial Cells

Endoplasmic reticulum (ER) stress has emerged as a major mechanism of cell apoptosis in atherosclerotic plaques throughout all stages of the disease [28, 29]. A number of circulating atherorelevant stressors – v.g., low density lipoprotein, cytokines-can disturb endothelial ER homeostasis and trigger the unfolded protein response (UPR) in an attempt to restore normal ER function; however, if ER stress is sustained the UPR leads to activation of pro-apoptotic mechanisms [28, 30]. In a number of cell types persistent and/or exacerbated calcium influx can promote or contribute to mechanisms linked to ER stress [31, 32]. It is also known that in some cells and tissues a crosstalk exists between NFkB-dependent inflammatory signaling and the UPR, where ER stress can either activate or enhance NFkB signaling [33, 34]. In HCAECs augmented TRPC3 expression in response to pro-inflammatory factors results in augmented NFkB-signaling ([27] and Sect. 3.1 above). Interestingly, TRPC3 function has been linked to activation of the UPR in epithelial cells [31]. The question then raises whether in endothelial cells TRPC3 can also contribute to initiation and/or maintenance of UPR signaling, integrating the UPR response and inflammation. Recent work in our laboratory shows that in HCAECs inhibition of native TRPC3 with the selective TRPC3 blocker pyrazole-10 [35] results in a marked reduction in both UPR activation and the susceptibility of these cells to ER stress-induced apoptosis (Ampem, Smedlund and Vazquez, unpublished observations). Notably, the ER stress conditions that promote apoptosis of HCAECs also induce activation of NFkB in these cells. These findings do indeed suggest that TRPC3 may couple mechanisms of ER stress-induced apoptosis to inflammatory signaling in endothelial cells.

9.4 Atherorelevant Functions of Macrophage TRPC3

9.4.1 TRPC3 and Survival of Non-polarized Macrophages

Macrophage apoptosis has a prominent effect on the characteristics of atherosclerotic lesions [36]. One of the major pro-apoptotic mechanisms in plaque macrophages is mediated by activation of the UPR that follows chronic, unresolved, ER stress [29, 37]. Accumulation of apoptotic macrophages has a differential effect on early vs. advanced lesions [29, 38]. In early atherosclerotic plaques the clearance of apoptotic macrophages or efferocytosis, is efficient, and apoptotic cells barely accumulate, thus contributing minimally to lesion cellularity [38, 39]. However, in the more advanced lesion setting, increased rates of macrophage apoptosis combined with deficient clearance, lead to in situ cell death and secondary necrosis, major contributors to the enlargement of the plaque necrotic core [38, 40]. There is significant effort in the field to identify molecular components of mechanisms governing macrophage survival/apoptosis in the hope that this can lead to discovery of potential therapeutic targets. The phosphatidylinositol-3-kinase (PI3K)/AKT and NFkB pathways are typical mediators of survival mechanisms in macrophages. For instance, AKT-mediated phosphorylation of the pro-apoptotic Bcl-2 family member BAD prevents the association of BAD with the anti-apoptotic Bcl-2 family members Bcl-2 and Bcl-Xl, reducing apoptosis [41]. In addition, the expression level of these pro-survival genes is to some extent modulated by NFkB. Both pathways depend, directly or indirectly, upon calcium influx into the cell. Studies from our laboratory in both human THP-1-derived macrophages and mouse bone marrow-derived macrophages showed that constitutive calcium influx is required for optimal operation of AKT and NFkB dependent survival signaling [42, 43]. In fact, pharmacological maneuvers that lead to abrogation of constitutive calcium influx or of CAMKII function, impaired the activation of AKT or NFkB and a drastic increase in macrophage apoptosis [42, 43]. These findings indicated that the CAM/CAMKII/AKT and CAM/CAMKII/NFkB axes are common elements in coupling constitutive calcium influx to survival in both human and murine macrophages. The molecular nature of the channels mediating such constitutive calcium influx was not known at the time. In a follow up study we showed that bone marrow-derived macrophages prepared from mice with global deficiency of TRPC3 exhibited an almost complete loss of constitutive calcium influx, impaired survival signaling and increased apoptosis, compared to wild-type cells [44]. These findings represented the first experimental evidence identifying the molecular nature of a channel whose expression and constitutive function had an obligatory role in macrophage survival. Most importantly, the above observations led to the speculation that in the setting of atherosclerosis macrophage deficiency of TRPC3 may lead to accumulation of apoptotic cells and increased necrosis in advanced plaques. Contrarily to this prediction, studies using a bone marrow transplantation strategy in which Apoe knockout mice received TRPC3 deficient bone marrow, revealed unexpected findings ([45] and see Sect. 5.1 below for details). Most strikingly,

advanced lesions in the aortic root of these mice showed a significant reduction in the number of apoptotic macrophages [45]. The speculation was then made that the characteristics of advanced plaques from mice with bone marrow deletion of TRPC3 may be due to the fact that polarized, rather than non-polarized macrophages predominate, and that the contribution of TRPC3 to mechanisms of macrophage apoptosis may depend on the differentiation status.

9.4.2 TRPC3 and Apoptosis of Polarized Macrophages

In lesions from both human and animal models of atherosclerosis, macrophages constitute a heterogenous population that includes a few distinguishable and relatively well-characterized phenotypes [46–48]. In particular, M1 or inflammatory or classically activated macrophages, and M2 or anti-inflammatory or alternatively activated macrophages, probably representing extremes of a wide spectrum of phenotypes, dominate in the setting of the atherosclerotic plaque. The relative abundance of these two subsets – the M1/M2 ratio – varies with lesion stage, and whereas this ratio at a given time point is influenced by a number of factors – i.e., lesion complexity, genetic background of the animal model – in general tends to increase as the lesion progresses. Nevertheless, both M1 and M2 types co-exist throughout plaque progression altogether with intermediate, ill-defined phenotypes [49, 50]. In line with the interpretation above that the effects of TRPC3 on macrophage apoptosis may be different in non-polarized vs. polarized macrophages, M1 cells prepared from mice with TRPC3 deficient bone marrow are notoriously less sensitive to ER stress-induced apoptosis than TRPC3 expressing M1 macrophages [45]. Notably, deletion of TRPC3 does not alter the apoptotic rates of M2 macrophages. In macrophages as in other eukaryotic cells, ER stress leads to activation of the UPR, which signals through the UPR sensors inositol requiring enzyme-1α (IRE-1α), protein kinase R-like endoplasmic reticulum kinase (PERK) and activating transcription factor-6 (ATF6) [51]. The observation that M1, but not M2 macrophages with deletion of TRPC3 had a reduced susceptibility to ER stress-induced apoptosis raised the question whether this was due to a specific effect of TRPC3 on the UPR signaling. This was addressed in a recent follow up study where the effect of TRPC3 on UPR and ER stress-induced apoptosis was examined in polarized M1 and M2 macrophages prepared from wild-type mice or from mice with macrophage-specific deletion of TRPC3 [52]. A number of interesting observations were made and are summarized as follows. Compared to controls, apoptosis induced by thapsigargin, tunicamycin or free-cholesterol loading – conditions that promote sustained, irreversible ER stress – was markedly reduced in TRPC3 deficient M1, but not M2 macrophages. In line with this, expression levels of CCAAT/enhancer binding protein homologous protein (CHOP), spliced X-box binding protein 1 – a proximal indicator of IRE1α activation-, PERK and ER oxidoreductase-1α – a transcriptional target of CHOP – were all decreased in TRPC3 deficient M1, but not M2 macrophages, evidencing impaired UPR activation in these cells [52]. ER

stress-dependent activation of CAMKII and STAT1, mediators of UPR-dependent apoptosis in macrophages [53, 54], was severely impaired again in TRPC3 deficient M1 but not in M2 macrophages. Notably, in wild-type M1 macrophages basal activation of CAMKII was abrogated by genetic or pharmacological inhibition of TRPC3, suggesting that in M1 macrophages, similar to the findings in human and mouse non-polarized macrophages (Sect. 4.1 above), TRPC3 function is coupled to activation of this kinase [52]. This is interesting, as it suggests that despite naïve and M1 macrophages showing similar coupling between TRPC3 and CAMKII, modulation of downstream signaling pathways is distinctively affected by the macrophage phenotype.

Overall, the findings discussed in this section indicate that TRPC3 exerts a differential effect on apoptosis of M1 vs. M2 macrophages, with a clear contribution to mechanisms of ER stress-induced apoptosis in the M1, but not in the M2 cells. At this time, the nature of such selectivity remains unknown. It is possible that differences exist between M1 and M2 macrophages in terms of channel arrangement (i.e., homo- vs. hetero-tetrameric) or in their specific repertoire of potential signaling or adaptor proteins coupling TRPC3 to intracellular pathways.

9.4.3 Impact of TRPC3 Expression/Function on Efferocytic Properties of Macrophages

As described above (Sect. 4.1) the clearance of apoptotic macrophages from the lesion site by resident phagocytes – efferocytosis – is a process that shapes the lesion and has key consequences on its cellularity and volume. The observation that TRPC3 deficiency impairs survival and increases apoptosis of non-polarized macrophages led to the question whether the phagocytic capacity of these cells would also be affected by their TRPC3 expression status. By means of an in vitro efferocytosis assay in which both TRPC3 expressing and TRPC3 deficient macrophages were used either as phagocytes or apoptotic cells, it was found that phagocytes lacking TRPC3 had impaired efferocytic function when compared to wild-type cells [44]. In addition, deletion of TRPC3 resulted in apoptotic macrophages being poor substrates for efferocytosis, regardless of the TRPC3 expression status of the acting phagocyte. These findings suggested a mandatory requirement for TRPC3 both in phagocytosis mechanisms and in cell-cell recognition processes. Similar to the distinctive impact of TRPC3 on apoptosis of non-polarized vs. polarized macrophages (Sects. 4.1 and 4.2 above), the behavior of M1 and M2 phagocytes with deletion of TRPC3 was opposite to that of naïve phagocytes. In fact, TRPC3 deficient M1 and M2 macrophages exhibited higher efferocytic capacity compared to TRPC3 expressing cells [45]. Interestingly, protein levels of MERTK were higher in macrophages lacking TRPC3 compared to control cells [45], which can account, at least in part, for the higher efferocytic capacity of the TRPC3 deficient macrophages. The in vivo relevance of these findings has not yet been assessed.

9.5 Mouse Models of Atherosclerosis with Conditional Gain or Loss of TRPC3 Function

The in vitro findings described in Sects. 3 and 4 above, provided important evidence supporting a role of TRPC3 in molecular and cellular mechanisms of relevance to atherosclerosis. However, definitive associations between changes in TRPC3 expression and/or function and atherogenesis could not be established on the sole basis of in vitro studies. In order to evaluate the contribution of endothelial or macrophage TRPC3 to mechanisms of plaque development in vivo it was thus necessary to generate mouse models of atherosclerosis in which TRPC3 expression could be manipulated – i.e., conditional transgenic or knockout animals for TRPC3-. The sections below discuss some of the most recent findings in mice with hematopoietic deficiency of TRPC3 and mice with endothelial-specific overexpression of TRPC3.

9.5.1 Effects of Bone Marrow-Specific Deletion of TRPC3

The findings discussed in Sect. 4.1 regarding the impaired survival of TRPC3-deficient non-polarized macrophages led to the speculation that, in the setting of the advanced atherosclerotic plaque, macrophage deficiency of TRPC3 would result in accumulation of apoptotic cells and increased necrosis. This prediction was based on the fact that apoptotic macrophages contribute to expansion of the necrotic core in the advanced lesion (discussed in Sect. 4.1). The use of bone marrow transplantation models in gene-targeted mice is a powerful technique to evaluate the contribution to atherogenesis of a particular gene expressed by macrophages [39, 55, 56]. As a first approach to reveal the true impact of macrophage deficiency of TRPC3 in atherosclerotic lesions, we generated Apoe knockout mice chimeric for macrophage TRPC3 expression by means of bone marrow transplantation [45]. Contrarily to our predictions, we found that some typical undesirable features of advanced plaques were actually alleviated by the bone marrow deficiency of TRPC3 [45]. Compared to control mice, the advanced lesions in the aortic root of mice with bone marrow deletion of TRPC3 showed a marked reduction in necrosis, higher collagen content and larger fibrous cap thickness. In addition, despite no changes in lesion size or macrophage content, plaques from these mice had a twofold reduction in the number of apoptotic macrophages [45]. In the light of the previous in vitro findings in non-polarized macrophages, the phenotypic features of these plaques led us to speculate that the contribution of TRPC3 to mechanisms of macrophage apoptosis may actually depend on the differentiation status of these cells. This interpretation was later on supported by in vitro studies in M1 and M2 macrophages (discussed in Sect. 3.2 above).

Among the immune cells found in mouse lesions, lymphocytes, neutrophils and dendritic cells do not express TRPC3 [57–61], favoring the notion that the plaque phenotype in the bone marrow transplantation model presumably reflects the effects

of TRPC3 on macrophage functions. However, TRPC3 is also expressed in the other two major plaque cell types namely, smooth muscle (SMC) and endothelial (ECs) cells [4, 5]. Atherorelevant functions of TRPC3 in SMCs, if any, are not known and recent work from our laboratory shows that in vivo, TRPC3 supports inflammation in endothelial cells ([62] and Sect. 5.2 below). Bone marrow-derived EC and SMC progenitors can contribute to replenishment of lesional cells [63, 64]. Therefore, it remains uncertain if and to what extent this could have contributed to the phenotype of mice with bone marrow deletion of TRPC3. Hence, establishing a more definitive relationship between macrophage TRPC3 and atherosclerosis awaits the characterization of plaques in the recently generated mice with macrophage-specific loss of TRPC3 function [52].

9.5.2 Effects of Endothelial-Specific Overexpression of TRPC3

In Sect. 3.1 above, we discussed the evidence that demonstrated that in HCAECs the endogenous TRPC3 channels mediate constitutive calcium influx and are obligatory for proper operation of inflammatory signaling through the NFkB pathway [25, 27]. The in vivo relevance of these findings was validated in recent studies using Apoe knockout mice with endothelial-specific overexpression of human TRPC3 [62]. Whereas early lesions – 10 weeks on high fat diet- in the aortic root were not different from control animals, advanced plaques – 16 weeks on high fat diet- in the transgenic mice were larger and of increased cellularity compared to non-transgenic mice. As predicted by the in vitro studies, there was increased endothelial inflammation in lesions of the TRPC3 transgenic mice, and this was already evident in the early lesions, long before differences in plaque size and cellularity became evident.

9.6 Targeting TRPC3: Myth or Reality?

The studies discussed in the preceding sections on the effects of TRPC3 on endothelial inflammation [25, 27] and macrophage apoptosis [45, 52], as well as the validation of these findings in mouse models of atherosclerosis with conditional gain or loss of TRPC3 function [45, 62], constitute the first available evidence demonstrating a pro-atherogenic role of a member of the TRPC family in atherosclerosis. The distinctive effects of TRPC3 on the characteristics of atherosclerotic plaques are consequence of the channel's unique functions in these two atherorelevant cell types. These observations are endowed with a therapeutic timbre that merits few considerations. First, the selective contribution of TRPC3 to mechanisms of UPR-related apoptosis in M1 macrophages with no effect on M2 cells provokes the notion of TRPC3 as a potential target for manipulation of macrophage functions in a phenotype selective manner. This is of most importance, as the identification of

signaling molecules that may specifically affect a particular macrophage type but not others is, as of this writing, an as yet unmet goal. As discussed above ER stress is a major pro-apoptotic mechanism in lesional macrophages. Notably, macrophage differentiation towards the M2 phenotype seems to benefit from ER stress, to the extent that ameliorating ER stress shifts M2 differentiation towards the M1 type [65]. This suggests that targeting molecular components of the UPR as a strategy to decrease macrophage apoptosis with no discernment of phenotypes, may ultimately result in a deleterious effect on necrosis of the advanced plaque, as such maneuver may favor M2-to-M1 conversion. In this context, the findings discussed in Sects. 4.2 and 5.1 above suggest that targeting TRPC3, rather than its downstream effectors, may be a better strategy to reduce apoptosis of M1 macrophages and prevent or delay necrotic core enlargement in the advanced lesion. Second, the pro-atherogenic effect of endothelial TRPC3 suggests that molecular or pharmacological targeting of this channel at the endothelial level could also be beneficial in ameliorating endothelial cell activation, inflammation and recruitment of circulating inflammatory cells to the subintima.

Historically, L-type channels have been the only type of calcium permeable channels whose pharmacological targeting seems to be of some beneficial effect in atherosclerosis (discussed by us in [66]). However, these anti-atherogenic effects are for the most part inconsistent, mechanistically uncertain and often unrelated to the channel blocking properties of CCBs, raising the question whether these are manifestations of off-target effects or secondary to their beneficial impact on atherosclerosis risk factors. In this context, TRPC3 targeting is more likely to efficiently suppress signaling effectors downstream TRPC3 without interfering with pathways often affected by non-channel related actions of traditional CCBs [66]. Importantly, whereas the ubiquitous expression of TRPC3 may be seen as a limitation for selective targeting – a problem with most signaling molecules so far postulated as potential targets in atherosclerosis – endothelial – or macrophage-targeted drug delivery is now possible through nanoparticle delivery systems [67, 68]. It is our hope that the novel discoveries on the roles of TRPC3 in atherosclerosis will provoke additional efforts in the field to further our understanding of the functions of this and other TRPC proteins in atherorelevant cells and processes. With no doubt, these efforts should lead to development of a new dimension in experimental therapeutics of this disease.

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Chapter 10
Second Messenger-Operated Calcium Entry Through TRPC6

Alexandre Bouron, Sylvain Chauvet, Stuart Dryer, and Juan A. Rosado

Abstract Canonical transient receptor potential 6 (TRPC6) proteins assemble into heteromultimeric structures forming non-selective cation channels. In addition, many TRPC6-interacting proteins have been identified like some enzymes, channels, pumps, cytoskeleton-associated proteins, immunophilins, or cholesterol-binding proteins, indicating that TRPC6 are engaged into macromolecular complexes. Depending on the cell type and the experimental conditions used, TRPC6 activity has been reported to be controlled by diverse modalities. For instance, the second messenger diacylglycerol, store-depletion, the plant extract hyperforin or H_2O_2 have all been shown to trigger the opening of TRPC6 channels. A well-characterized consequence of TRPC6 activation is the elevation of the cytosolic concentration of Ca^{2+}. This latter response can reflect the entry of Ca^{2+} through open TRPC6 channels but it can also be due to the Na^+/Ca^{2+} exchanger (operating in its reverse mode) or voltage-gated Ca^{2+} channels (recruited in response to a TRPC6-mediated depolarization). Although TRPC6 controls a diverse array of biological functions in many tissues and cell types, its pathophysiological functions are far from being fully understood. This chapter covers some key features of TRPC6, with a special emphasis on their biological significance in kidney and blood cells.

Keywords TRPC6 • Ca^{2+} entry • DAG • Kidney • Blood cells

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10.1 Introduction

In mammals, the super-family of transient receptor potential (TRP) ion channels gathers six families named TRPA, TRPC, TRPM, TRPML, TRPP, and TRPV [1]. Among these, members of the TRPC family, where C stands for "*classical*" or "*canonical*", have the closest homology with TRP and TRPL, the founding TRP members, which were identified in Drosophila [2]. The first TRPC was cloned in 1990s [3]. Since this pioneering work, seven members (TRPC1-TRPC7) are known to date, making the TRPC family an important class of cation channels [4] playing multiple roles in health and disease [5]. Within the TRPC family several groups have been identified: TRPC1, TRPC2, TRPC4/5, and TRPC3/TRPC6/TRPC7 [6]. Depending on the authors, TRPC1 is included into the TRPC4/5 subfamily [4].

In mice, the gene encoding TRPC6 (mTRPC6) has 13 exons and is located on chromosome 9 and full-length cDNA of mTRPC6 was isolated from the brain [7]. In human, the gene encoding TRPC6 (hTRPC6) is localized on chromosome 11q21–q22 and has 13 exons [8], and the cDNA was cloned from the placenta [9]. The mTRPC6 cDNA exhibits a similarity (and identity) of 85.1 % (and 74.1 %) with the mTRPC3 cDNA [7]. Mouse TRPC7 has 75 % identity with mTRPC6 and 84 % similarities [10]. Overall, TRPC6 has 70–80 % amino acid (a.a.) identity with TRPC3 and TRPC7.

The murine and human TRPC6 proteins have 930 and 931 amino acids, respectively, and their amino acid identity is 93 % [7, 9]. Several TRPC6 isoforms are known [11]. They result from the alternative splicing of the TRPC6 gene. However, some of these variants are not functional [12]. For instance, in the rat 3 isoforms were found: rTRPC6A, rTRPC6B, and rTRPC6C, having 930, 876 and 808 a.a., respectively [11]. rTRPC6B has 54 a.a. less than the A isoform, these missing a.a. are in the N-terminus whereas the C isoform lacks 68 a.a. near its C-terminus. rTRPC6A has 88.38 % and 94.41 % nucleic acid identity with the human and mouse TRPC6. Only the isoforms A and B are functional [11].

Nearly 20 years after the cloning of TRPC6, its expression and functional properties are much better characterized. Some of its hallmark features is the fact that TRPC6 is a lipid-dependent membrane protein forming non-selective cation channels conducting Na^+ and Ca^{2+}, but the functional significance of this Ca^{2+}-dependent transport system in health and disease is not yet completely elucidated.

10.2 Structure

Voltage-gated K^+, Na^+ and Ca^{2+} channels have served as structural models to establish the putative molecular architecture of all TRPC channels. They contain six transmembrane domains (named S1–S6), and intracellular N- and C-termini [13, 14]. The current model, which also applies to TRPC6, proposes that a TRPC channel would result from the association of four TRPC proteins. TRPC channels are

thus regarded as tetrameric structures where S5 and S6 contribute to the pore function [15]. TRPC6 subunits can associate with TRPC6 and non-TRPC6 subunits like TRPC1, TRPC3 and TRPC7 subunits (see next Chapter), forming homo- or hetero-tetrameric structures. Within the large intracellular NH_2- and COOH-terminals, several important domains have been identified.

In the NH_2-Terminal

- The ankyrin (ANK) repeat, a relatively conserved structural motif of 33 residues, is a protein module with high affinity for other ANK repeats. These modules are present abundantly in a multitude of proteins and are regarded as ubiquitous scaffolds mediating protein-protein interactions [16] and involved in many processes like signal transduction, cell cycle regulation, vesicular trafficking and cytoskeleton integrity [17]. Members of the super-family of TRP proteins, including TRPCs, possess ANK repeats [18]. For instance, three ANK-like repeats located in the N-terminus were identified in the three rat TRPC6 isoforms [11].
- A coiled-coil-domain (close to the intracellular side of the transmembrane domain 1). This type of domain, thought to control oligomerisation, could thus participate in the oligo- or heteromerization of TRPC6 channels. It could also participate in the association of TRPC6 with other proteins.

In the COOH-Terminal

- TRP domain: it is a motif of 25 a.a. that is highly conserved throughout TRPC channels. This domain is located on the COOH-terminal part, near the sixth transmembrane domain. It contains a group of 6 invariant a.a. that are forming the TRP box (though to be involved in channel gating). This domain would be necessary for the binding of phosphoinositol phosphates (like PIP_2) (see later, section "Activation").
- Prolin-rich motif: a prolin-rich motif (found in all TRPCs) is present downstream to the TRP domain; it would participate in the interaction with immunophilins.
- A part of the COOH-terminal domain of TRPC6, containing several consensus calmodulin (CaM) binding motifs and also interacting with inositol 1,4,5-trisphosphate (IP_3) receptors (IP_3R), is described as the CaM and IP_3R binding domain (CIRB) which is conserved among all TRPC members [19]. The CaM-binding sequences of all TRPCs are known [19]. For mTRPC6 a motif lying from a.a. 838 to 872 has been identified. The binding of CaM to the CIRB motif of TRPCs inhibits their activity. The CIRB motif of mTRPC6 interacts with the COOH-terminal lobe of CaM [19].
- A coiled-coil-domain is present in the COOH-terminal domain.

Two sites participating in TRPC interaction are named *assembly domains* AD1 and AD2 [20]. They would stabilize the interactions between TRPCs. AD1 is located within the NH_2-terminus and overlaps the ankyrin and the coiled-coil domains. It is necessary for self-association of the NH_2-termini of adjacent TRPCs. AD2, which overlaps the pore region and the NH_2-terminal tail, is involved in the interaction with the NH_2- and COOH-terminal parts of adjacent TRPCs. Based on

these results, a model was proposed by [20] with interactions between (i) the NH_2-termini and involving two regions (ANK repeats and coiled-coil), and (ii) between the NH_2- and the COOH-termini of an adjacent TRPC.

10.3 Activation

Understanding the physiological mechanism of activation of TRPCs, including TRPC6, has always been a difficult task. This part of the literature has generated discordant findings [21]. Indeed, multiple modes of TRPC activation have been documented and it has been proposed that their native environment would be essential in defining their recruitment under physiological conditions [21]. Similar remarks apply to TRPC6 which were reported to respond to different types of stimuli (chemical and mechanical) (see below). After its cloning, the analysis of the functional properties of mTRPC6 indicated that it is a non-selective Ca^{2+}-conducting channel blocked by La^{3+} and SKF-96365 and activated downstream to G-protein coupled receptors (GPCR), and not responding to Ca^{2+} store depletion [7]. A subsequent work showed that the gating of TRPC6 (and also TRPC3) channels can be directly controlled by diacylglycerol (DAG) without the need to deplete intracellular Ca^{2+} stores. TRPC6 channels were thus described as *second-messenger operated channels* that open downstream to receptors coupled to the hydrolysis of phosphatidyl inositol 4,5-bisphosphate (PIP_2) [9]. According to a generally held opinion, TRPC6 channels are activated downstream to GPCR or receptor tyrosine kinases via a PLC-dependent signaling process involving PLCβ or PLCγ and leading to the production of DAG.

10.3.1 DAG, Phosphoinositides

There is ample evidence that TRPC6 channels can function as DAG-activated channels and it is now widely accepted that DAG is a physiological activator, at least in some cell types. Experimentally, DAG analogues such as OAG (1-oleoyl-2-acetyl-*sn*-glycerol) are commonly used to activate TRPC6. [11] identified a stretch of a.a. (3–56) located in the NH_2-terminal part of TRPC6 that seemed to confer DAG sensitivity. However, conflicting data have been reported concerning the contribution of this motif of 54 a.a. on conferring the sensitivity to DAG-analogues like OAG. For instance, other OAG-sensitive channels like TRPC3 and TRPC7 lack this motif. In addition, an electrophysiological study conducted on the rTRPC6B isoform (which does not have this motif) and expressed in the T-REx-r6 cell line showed that it could be activated by OAG [22]. A similar finding has been reported for truncated TRPC3 channels, further questioning the role of this 54-a.a. motif [23]. Although, the exact mechanism of action of DAG on TRPC6 is still unresolved, a dissociation constant of DAG binding to TRPC6 of 20–40 μM has recently been calculated [24].

This latter study showed a parallel response between DAG production and TRPC6 activation downstream to receptor stimulation, supporting the view that the channel is DAG-sensitive.

Besides DAG, TRPC6 can be activated by DAG-containing arachidonic acids like SAG (1-stearoyl-2-arachidonyl-*sn*-glycerol) and PAG (1-palmytoyl-2-arachidonic acid-*sn*-glycerol), and by DAG-containing docosahexaenoic acid like SDG (1-stearoyl-2-docosahexaenoyl-*sn*-glycerol) and DOG (1,2-dioctanoyl-*sn*-glycerol) [25]. It was proposed that the arachidonic acid present at the *sn*-2 position is an important molecular determinant controlling TRPC6 activation [25]. By using DAG lipase inhibitors, these authors revealed that the activation of the channels was not due to the metabolism of SAG. Thus, the action of SAG is not mediated by free arachidonic acid or phosphatidic acid. Overall, SAG is the most effective DAG analogue in activating TRPC6 when compared to OAG, DOG and SLG (1-stearoyl-*sn*-glycerol). The SAG-dependent activation of TRPC6 channels requires the integrity of lipid rafts because their disruption with methyl-β-cyclodextrin (MβCD) prevents TRPC6 activation. This is also seen after knockdown of certain raft organizing proteins [26]. In addition, tyrosine phosphorylation would be a necessary step for the activation of the channels [25]. On the other hand, TRPC6 channels do not respond to the application of monoacylglycerols (e.g. 1-oleoyl-glycerol and 2-oleoyl-glycerol) [9]. Besides DAG and DAG analogues, TRPC6 can open in response to the application of DAG lipase inhibitors, which elevate DAG levels [9, 27, 28].

20-HETE (20-hydroxyeicosatetraenoic acid) is a metabolite of arachidonic acid. The external application of 20-HETE on HEK cells over-expressing mTRPC6 generates non-selective inward currents [29]. Arachidonic acid was also able to activate mTRPC6 channels but not the non metabolizable arachidonic acid analog ETYA, suggesting that TRPC6 channels are sensitive to metabolites of arachidonic acid. The authors propose that 20-HETE would be this natural activator [29].

The influence of lipids on TRPC6 activity has been further documented by [30] who showed that the lipid lysophosphatidylcholine is able to activate hTRPC6 channels over-expressed in HEK cells and native TRPC6 channels from bovine aortic endothelial cells in culture. A parallel increase in tyrosine phosphorylation of TRPC6 was noted after a 1 h treatment with lysophosphatidylcholine [30].

As mentioned above, TRPC6 are described as channels activated by the second messenger DAG which is produced in response to the recruitment of receptors coupled to PLCβ or PLCγ. Several phosphoinositides (PIs), like PIP_2, were shown to regulate TRPC6 activity. PIP_2 is more than a precursor of second messengers, it is itself a signaling molecule regulating the activity of many target proteins including ion channels [31, 32]. Contrasting results have been collected regarding the effect of PIP_2 on TRPC6. PIP_2 was shown to exert a positive effect on the activity of hTRPC6 channels [33]. Indeed, the COOH-terminal region of TRPC6 can bind to various PIs such as phosphatidylinositol-3,4,5-tisphosphate (PIP_3) and PIP_2. In fact, the COOH-terminal part of TRPC6 directly binds PIs, its affinity being higher for PIP_3 (compared to PIP_2) [34]. These PIP_3-activated TRPC6 Ca^{2+} responses were sensitive to Gd^{3+} (50 μM) and SKF-96365 (10 μM) but exposure of cells to other phosphoinositides like PI and PIP_2 failed to generate any Ca^{2+} signal [34]. The PI-binding site

overlaps with the CaM-binding site but is not identical to it. It stretches from the residues 842 and 868, with three a.a. (arginine 853, lysine 860, and arginine 861) playing important roles in the binding of CaM and PI. The PI-dependent displacement of the CaM binding causes an enhancement of TRPC6-mediated currents [33]. TRPC6 channels (and also its close relatives TRPC3 and TRPC7) stably overexpressed in HEK cells were shown to be directly activated by PIP_2 [35]. However, in vascular smooth muscle cells the DAG-dependent activation of endogenous TRPC6 channels is antagonized by PIP_2, which is regarded as a physiological TRPC6 antagonist [36]. Based on results obtained with FRET experiments, it was concluded that PIP_2 controls TRPC6 inactivation [24].

Two important intracellular messengers are produced downstream to PIP_2: IP_3 and DAG. If the effect of the later one on TRPC6 activity has been well documented, much less is known about IP_3. It seems however that TRPC6 is not activated by IP_3 [9, 27, 37–39] that could act in a synergistic manner with DAG [39].

10.3.2 Store Depletion

The release of Ca^{2+} from the endoplasmic reticulum (ER) activates a plasma membrane conductance allowing the entry of Ca^{2+} into cells. This Ca^{2+} pathway, also named *capacitative Ca^{2+}entry* (CCE) [40], would reflect a *store-operated Ca^{2+}entry* (SOCE) via *store-operated channels* (SOCs). The activation of a PIP_2-dependent signaling pathway leading to DAG production also generates IP_3, which is followed by the recruitment of IP_3-sensitive intracellular channels (IP_3R) permitting the release of Ca^{2+} into the cytosolic compartment. Therefore, by depleting intracellular Ca^{2+} pools, IP_3 production can indirectly activate SOCs.

So far, TRPC6 channels have been predominantly described as contributing to a Ca^{2+} pathway not operated by Ca^{2+} stores. TRPC6 was indeed originally defined as non-activated by store depletion when over-expressed in HEK or CHO cells [7, 9, 27, 41]. In cardiac fibroblasts [42] and human platelets [43], TRPC6 was also reported to function as receptor-activated channels but not as store-operated channels. However, although the vast majority of studies published so far indicate that TRPC6 is a DAG-sensitive channel, several groups have challenged this view by showing that TRPC6 can be store-operated. This is for instance the case of rTRPC6 where its over-expression in COS cells enhances the thapsigargin (TG)-stimulated Ca^{2+} entry, indicating that it would function as a SOC [15]. A similar conclusion has been reached in pulmonary smooth muscle cells [44] and in the hepatoma cell line Huh-7 where TRPC6 contributes to the SOCE but not to the OAG- or receptor-operated Ca^{2+} entry [45].

Deciphering the molecular identity of SOCs has been a major challenge that generated controversies and lively debates [46]. TRPC proteins have long been thought to be the components of SOCs. However, a series of seminal articles published in 2006 provided strong experimental evidence for a central role of Stim and Orai proteins as important components of SOCs [47–50]. It is now well accepted

that the release of Ca^{2+} from the ER influences the spatial distribution of Stim, a ER Ca^{2+} sensor, that relocalizes to regions near the plasma membrane [51]. If the contribution of Stim proteins in the control of SOCs seems consensual, the next step (namely, how Stim activates a plasma membrane conductance allowing the entry of Ca^{2+}?) is more disputed. Schematically, two models emerge. According to the first one, the translocation of Stim that follows the emptying of the Ca^{2+} stores permits the recruitment of Orai proteins. These are plasma membrane Ca^{2+} channels for which three isoforms are known (Orai1-3). This scenario implies that Stim and Orai are the central molecular components of SOCs able to generate an influx of Ca^{2+} (so-called SOCE) without the need of TRPC channels. This picture has however been challenged. Indeed, Stim but also Orai can interact with TRPC [52]. The second model thus proposes the existence of a more complex and dynamic picture with a tripartite complex involving Stim/Orai/TRPC as components of SOCs [53]. HL-60 cells expressed various TRPCs, with TRPC3 and TRPC6 being the predominant isoforms found [54]. By means of pharmacological agents acting on Ca^{2+}-conducting channels, it was concluded that TRPC6 would contribute (with TRPC1 and Orai1) to SOC in HL-60 cells whereas TRPC3 would contribute to receptor-operated channels [54].

In platelets, an even more dynamic picture has been proposed. Indeed, in these cells TRPC6 seem to be engaged in distinct macromolecular complexes and participate in two different Ca^{2+} routes: one (a SOCE) is under the control of intracellular Ca^{2+} stores whereas the second (non SOCE) functions independently of the Ca^{2+} stores [55]. According to these authors, TRPC6 could form a complex with Orai1 and Stim1 responding to Ca^{2+} store-depletion whereas applications of DAG analogues displaces TRPC6 from this complex, favoring its association with TRPC3. In this latter case, TRPC6 would function as a store-independent channel [55]. This view has however been recently challenged: Ca^{2+} imaging experiments on platelets from WT and $TRPC6^{-/-}$ mice indicated that in these cells TRPC6 does not contribute to SOCE but function as a receptor-operated channel [56].

10.3.3 Mechano-Sensation (or Stretch Activation)

Studies conducted on hTRPC6 channels expressed in HEK and CHO cells concluded that they could be sensors of mechanical stress: application of a hypo-osmotic medium activates TRPC6 in a PLC-independent manner [57]. The biophysical properties of this stretch-activated current are quite similar to the ones previously reported for TRPC6 with a single-channel conductance of ~25 pS. Furthermore, OAG and mechanical stress generate non additive responses. The stretch-induced TRPC6 activation is sensitive to the peptide GsMTx-4, an inhibitor of mechano-sensitive channels. GsMTx-4 also blocks OAG-activated TRPC6 currents further suggesting that stretch and OAG recruit the same cation channel. In their work [57], present TRPC6 channels as sensors of membrane stretch (whether the tension is induced physically or osmotically), and playing a role in regulating

vascular tone. This conclusion was questioned in a subsequent study on hTRPC6 channels transiently over-expressed in CHO and COS-7 cells [58]. In this work, the authors argue against the idea that TRPC6 is a mechano-sensitive protein. Indeed, they found little or even no evidence for a role of TRPC6 as a mechano-sensitive protein. According to [58] analyzing the mechano-sensitivity of a channel over-expressed in a heterologous system is problematic and is not necessarily the best way to precise its mechano-sensitivity. They suggest that is it not possible to conclude from studies performed on heterologous systems that TRPC6 is a stretch-activated channel. A similar conclusion was reached by [59] who found that TRPC6 cannot be regarded as a mechano-sensitive channel. In fact, a mechanical stimulus seems capable of activating Gq-coupled GPCR. According to this scenario, TRPC6 channels are activated downstream to GqPCR that would be the real sensors of a mechanical stress in smooth muscle cells [59]. More recently, [60] show that in mesenteric and renal arteries the angiotensin II receptor of type 1a is able to function as a stretch-activated receptor without the need of its ligand. Furthermore, they showed that TRPC6 plays no role in the downstream response. These conclusions are not supported by all of the studies on vascular smooth muscle [61] or heterologous expression systems [62, 63]. In dorsal root ganglion neurons, TRPC6 channels may closely interact with TRPV4 channels to form a larger complex that can detect mechanical stimuli [64]. Similarly, recent studies of cochlear hair cells and sensory neuron cell lines have suggested that TRPC3/TRPC6 heteromers contribute to low-sensitivity and very short latency mechano-sensitivity [63]. These workers also found that the presence of TRPC3 subunits markedly enhances mechano-sensitivity of TRPC6, although it is significant that this was only observed in certain cell lines [63]. TRPC6 channels in ventricular myocytes [65] and podocytes are activated by a variety of mechanical stimuli, and these responses are also blocked by GsMTx4. Mechanical activation in podocytes persists when G protein signaling is completely inhibited [66] and clearly cannot be attributed to stretch activation of any GPCR in this cell type. Moreover, mechanical activation of TRPC6 in podocytes is enhanced by podocin knockdown, whereas responses to OAG or various GPCRs are enhanced by podocin knockdown [66–68]. It is possible that mechanical activation in this cell type is membrane delimited and requires other proteins to be present (e.g. TRPC3 or TRPC1), but the responses are completely abolished by TRPC6 knockdown. In summary, mechano-sensitivity of TRPC6-containing channels can occur through G protein mediated signaling, may be conferred by other proteins within a larger complex, but it also remains possible that intrinsic or membrane-delimited mechano-sensitivity of TRPC6 is suppressed by interacting proteins in certain cellular contexts.

10.3.4 Hyperforin

It is a molecule extracted from the medicinal plant *Hypericum perforatum*. It exhibits the interesting property to activate TRPC6 channels without influencing the activity of any other TRPC [69]. There is a growing interest for this

pharmacological tool because it can activate native [70–72] and heterologously expressed TRPC6 channels [69]. Leuner and colleagues tried to gain a better molecular understanding of the effects of hyperforin by carrying out a structure-function study of various hyperforin derivatives. They concluded that 2,4-diacylphloroglucinol is a likely important structural part bearing the TRPC6 selectivity [73]. Of note, conflicting results have recently been published and showing that hyperforin controls a proton-conducting pathway unrelated to TRPC6 [74].

10.3.5 Flufenamic Acid (FFA)

FFA is a non-steroidal anti-inflammatory agent that blocks TRPC3- and TRPC7-mediated responses whereas it potentiates TRPC6-mediated currents [27, 75]. It was subsequently shown to directly activate native and heterogously expressed TRPC6 channels [76]. It was proposed to use this agent as a TRPC6 agonist in human podocytes [76]. However, caution is needed when using FFA. Indeed, it depresses TRPC6-mediated Ca^{2+} responses in PC12 cells [77]. In addition, it collapses the mitochondrial membrane potential and releases Ca^{2+} from mitochondria [78]. Investigations by one of us (Stuart Dryer unpublished observation) suggest that FFA acts as a partial agonist of TRPC6.

10.3.6 AlF_4- and Trimeric G Proteins

AlF4- has been shown to activate trimeric G proteins. To verify the role of these signaling molecules in TRPC6 activation, AlF4- was employed in some electrophysiological studies. For instance, AlF_4- (30 μM) was added to the pipette solution to trigger hTRPC6-mediated currents in HEK cells [9, 79]. Application of GTPγS can directly trigger the opening of TRPC6 channels [27]. Conversely application of GDPβS can prevent TRPC6 activation by GPCR [67, 68].

10.3.7 H_2O_2

A role of reactive oxygen species (ROS) in TRPC6 activation has been provided by [72, 80]. In these papers, the authors showed that a low concentration of H_2O_2 (e.g. 10 μM) can directly activate TRPC6 channels expressed in HEK cells and native TRPC6 channels in vascular smooth muscle cells. This H_2O_2-dependent TRPC6 activation does not occur through direct oxidation of the channel but requires thiol oxidation of a cytosolic component. In addition, OAG and H_2O_2 have synergistic effects [80]. This also occurs in a podocyte cell line [81]. Of note, when chronically applied to human mesangial cells for 2–24 h, H_2O_2 down-regulates TRPC6 expression in a concentration-dependent manner via a PKC mechanism [82].

10.3.8 Plasma Membrane Insertion of TRPC6 Upon Activation

The regulation of the plasma membrane abundance of TRPC6 is an important parameter influencing its activity. Experiments conducted on HEK cells over-expressing mTRPC6 revealed that the control of the trafficking of the channel and its insertion into the plasma membrane is a crucial regulatory mechanism. This can be induced by OAG and also by depletion of Ca^{2+} stores via a process that does not require any increase in cytosolic Ca^{2+}. This translocation of the channels leads to larger TRPC6-mediated currents [83]. A similar observation was made in PC12 cells where the application of an agonist recruiting GPCRs is accompanied by the insertion of TRPC6 channels into the plasma membrane; a process associated with an enhanced Ca^{2+} influx [84]. Interestingly, the trafficking of TRPC6 is negatively regulated by the membrane protein Klotho which prevents the exocytosis of TRPC6 by blocking the phosphoinositide-3-kinase. The Klotho-dependent regulation of TRPC6 is associated with depressed TRPC6-mediated Ca^{2+} responses [85]. H_2O_2 can stimulate TRPC6 translocation and its insertion into the plasma membrane but H_2O_2 is also able to influence TRPC6 activity even after blocking its trafficking process indicating that H_2O_2 may act at multiple sites [80]. Lysophosphatidylcholine triggers the translocation of TRPC6 and its insertion into the plasma membrane. This response was observed on hTRPC6 expressed in HEK cells as well as on native TRPC6 from bovine aortic endothelial cells [30]. The TRPC6 trafficking elicited by lysophosphatidylcholine occurs even when chelating intracellular Ca^{2+} or when incubating cells in a Ca^{2+}-free medium. Interestingly, this lysophosphatidylcholine-induced translocation of TRPC6 triggers the plasma membrane insertion of TRPC5 [30].

10.4 TRPC6 Interacting Partners

Many TRPC6-interacting partners have been identified (see for instance the TRIP Database: http://www.trpchannel.org [86]). Schematically, they can be classified as belonging to: channels and pumps, enzymes and intracellular signaling molecules, cytoskeleton-associated proteins, proteins involved in the trafficking, immunophilins, and Stim/Orai proteins (Fig. 10.1).

10.4.1 Interactions with Channels and Pumps

TRP Channels although TRPC6 can form homomeric channels, at least in some cell types like mesenteric artery myocytes [87], it is however able to interact with other TRPCs like TRPC1 [88], TRPC2 [89], TRPC3 [90–93], or TRPC7 [91, 92]. On the other hand, it does not seem to associate with TRPC4 [20, 88] or TRPC5 [30, 94].

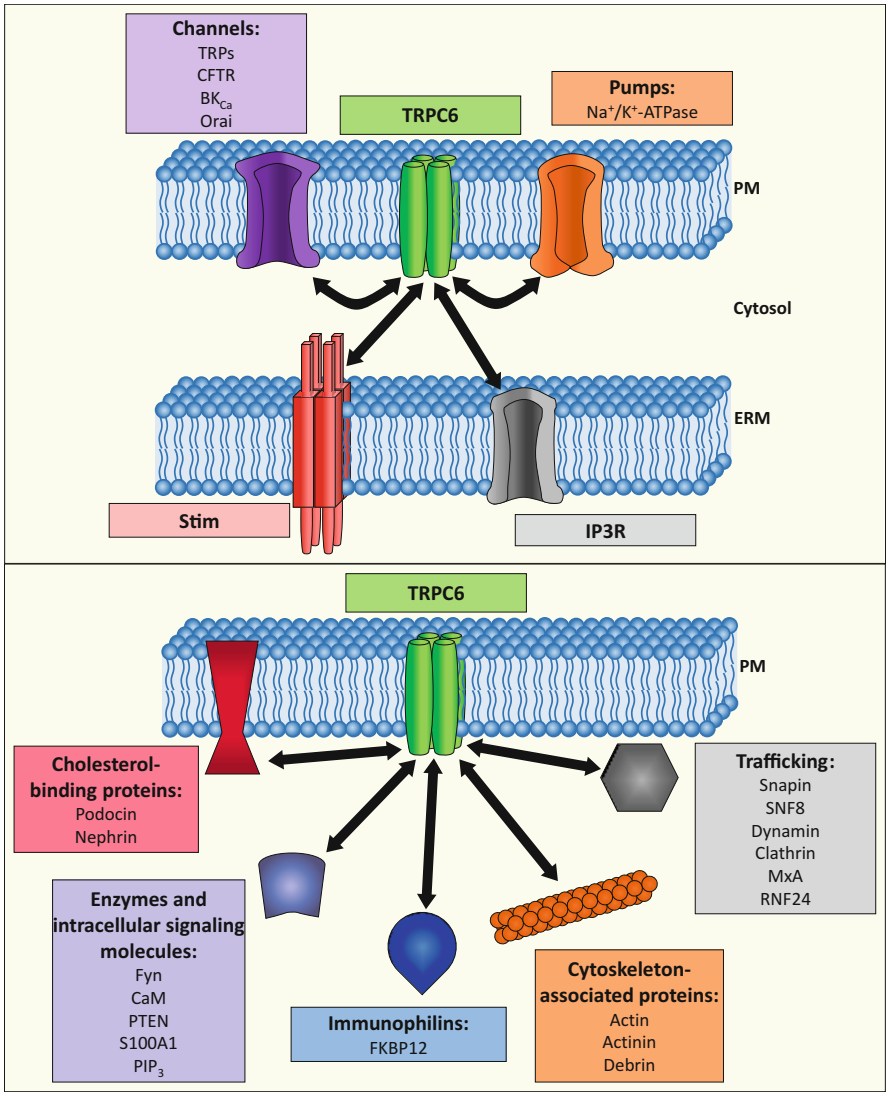

Fig. 10.1 TRPC6-interacting partners. Schematic representation of TRPC6 and its interacting partners. *Top panel*: TRPC6 has been shown to interact with channels and pumps of the plasma membrane (PM) as well as with actors located in the membrane of the endoplasmic reticulum (ERM). *Lower panel*: various groups of proteins have been proposed to interact with TRPC6 like cholesterol-binding proteins, enzymes and intracellular signaling molecules, cytoskeleton-associated proteins, and proteins involved in intracellular trafficking. See text for further details. TRPC6 is represented as a tetrameric structure, and for the sake of clarity the C- and N-termini have been omitted

Interestingly, TRPC6 can also interact with TRPA1, a member of another family of TRP [95]. In the kidney, in the glomerulus and along the collecting duct TRPC6 is detected in heteromeric complexes with TRPC3 [96] and co-localizes with aquaporin 2 [97]. Moreover, native TRPC3/6 heteromers have also been identified in MDCK cells [90]. However, in polarized cultures of M1 and IMCD-3 collecting duct cells, TRPC3 localizes exclusively to the apical domain, whereas TRPC6 is found on basolateral and apical membranes [98].

In human mesangial cells in culture, the results of coimmunoprecipitation experiments indicate that TRPC6 selectively interacts with TRPC1 but not with TRPC3 nor TRPC4 [88]. A lack of interaction with TRPC4 was also reported by [20]. In murine erythroid cells, TRPC6 would co-associate with TRPC2, an interaction that depresses TRPC2-mediated Ca^{2+} responses [89]. However, this report does not provide a molecular description of the TRPC2-TRPC6 interaction. And it is not clear whether the two proteins associate to form a channel. The interaction with TRPC3 and TRPC7 has been shown in a heterologous expression system (HEK cells) and in tissue (rat cerebral cortex) [92]. TRPC6 would interact with TRPC3 in erythroid cells, where erythropoietin stimulates the plasma membrane insertion of TRPC3. This process is associated with increased TRPC3-mediated Ca^{2+} responses. However, TRPC6 exerts a negative effect on this response, thus modulating negatively TRPC3 activity [93].

Na^+/K^+ Pump Immunofluorescence studies showed that hTRPC6 channels over-expressed in HEK-293 cells co-localize with the endogenous α1-subunit of the Na^+/K^+-ATPase [94]. In addition, co-immunoprecipitation and mass spectrometry experiments conducted on rat cerebral lysates, rat kidney lysates, and on lysates of HEK-293 cells over-expressing hTRPC6 revealed that this channel associates with the α1-subunit of the Na^+/K^+-ATPase without the need of additional proteins like other Na^+/K^+-ATPase subunits [94]. In the brain, the interaction involves the α3 isoform of the Na/K pump, the predominant α subunit in this organ.

IP3R TRPC6 can bind to IP3R via a NH_2-terminal sequence that is well conserved between TRPC3, TRPC6 and TRPC7 [99]. This complex is found under basal conditions and does not require cell activation [83].

CFTR or cystic fibrosis transmembrane conductance regulator, is a membrane protein controlling fluxes of chloride ions that plays a central role in the pathogenesis of cystic fibrosis. Experiments conducted on various epithelial cell lines and on freshly ciliated human bronchial epithelial cells showed that CFTR and TRPC6 interact each other and form a multiprotein complex [100].

BK_{Ca} TRPC6 binds directly to large-conductance Ca^{2+}-activated K^+ channels in podocytes, which are non-excitable visceral epithelial cells in kidney glomeruli [101]. This interaction may help to maintain driving force for Ca^{2+} influx during sustained TRPC6 activation in non-excitable cells [28].

Interactions with Stim/Orai Proteins

– Stim1: TRPC6 channels over-expressed in HEK cells and endogenous TRPC6 channels of the A10 smooth muscle cell line are not regulated by Stim1 [102]. These authors found no evidence for any functional regulation. TRPC6 is unable to directly interact with STIM proteins, but can indirectly interact with them in heterotrimeric complexes with TRPC1 [103]. STIM1 regulates the expression of TRPC6 in the plasma membrane and evokes its translocation to the ER [104].
– Interaction with Orai proteins: The question of the contribution of TRPC proteins to the entry of Ca^{2+} through SOCs is still under current investigation and this issue has been the subject of contrasting results. [52] found that COOH- and NH_2-terminal parts of TRPC6 (but also TRPC3) can interact with Orai proteins (Orai 1, 2 and 3). Two reports suggested a TRPC6-Orai interaction in a heterologous expression system (HEK293 cells) stably expressing TRPC6 by observing increased SOC currents [105, 106]. This Orai-TRPC interaction confers the sensitivity to Ca^{2+} store depletion via a Stim1-dependent process. In the hepatoma cell line Huh-7, TRPC6 would be engaged in a complex comprising Stim1 and Orai1 where they would participate in the SOC pathway [45]. A store-operated TRPC6-Stim1-Orai complex might exist in human platelets [55, 107].

10.4.2 Interactions with Enzymes and Intracellular Signaling Molecules

Calmodulin (CaM) TRPC members seem to possess several CaM binding sites on their COOH-terminal part but only one has been identified in the COOH-terminal part of TRPC6 [33, 108]. This CaM-binding site overlaps with the IP_3-binding site [109]. The interaction CaM-mTRPC6 is abolished in the presence of CaM inhibitors like calmidazolium or trifluoperazine [110].

The Tyrosine Kinase Fyn a binding site for Fyn located in the NH_2-terminal of mTRPC6 channels has been identified by [111]. Furthermore, mTRPC6 channels can be tyrosine phosphorylated by Fyn and Src. The Fyn-mTRPC6 interaction is not dependent on the tyrosine phosphorylation status of mTRPC6 [111].

PTEN is a phosphatase that exerts a positive role on TRPC6 activity. It interacts with TRPC6 but not with TRPC1, TRPC3 or TRPC4. This interaction facilitates the plasma membrane insertion of TRPC6 channels but the enzymatic activity of PTEN is not required for this regulatory process to develop [112].

A complex molecular rearrangement leading to the formation of a multiprotein complex centered on TRPC6 has been uncovered by [113]. They carried out their work on endogenous TRPC6 of PC12D cells (which express the TRPC6B isoform). In this cell model, the activation of muscarinic receptors triggers the association between these receptors and TRPC6 channels, a process that induces the recruit-

ment of PKC. This causes the phosphorylation of TRPC6 in a PKC-dependent manner. This phosphorylation depends upon the activation of the muscarinic receptors. Two serine residues (ser768/714) seem to play a central role in this PKC-dependent phosphorylation. The formation of the tripartite complex muscarinic receptor-TRPC6-PKC allows the recruitment of the immunophilin FKBP12. Finally CaM and calcineurin are recruited to this complex, leading to the dephosphorylation of TRPC6 which correlates with the dissociation of the muscarinic receptor from TRPC6 [113].

S100A1 S100 proteins form a large family of Ca^{2+}-binding proteins participating in many cellular functions [114]. S100A, a protein mainly present in the heart where it controls cardiac performance, can interact with TRPC6 via its COOH-tail (801–878) [115].

PIP_3 Binding of PIP_3 was demonstrated to be at the CaM binding site [33] originally identified as CIRB site in TRPC3 [116]. Mutations decreasing the affinity of PIP_3 in this binding site enhanced CaM binding and reduced TRPC6-dependent current. Vice versa, mutations resulting in increased PIP_3 binding led to a reduced affinity of calmodulin and enhanced TRPC6 currents, while a triple amino acid substitution (R853Q/K860Q/R861Q in human TRPC6) resulted in reduced binding of both interaction partners [33].

10.4.3 Interactions with Cytoskeleton-Associated Proteins

Actin, Actinin and Debrin TRPC6 interacting partners like actin, actinin and debrin were found in rat brain lysates [94]. In HEK cells, the over-expression of mTRPC6 enhances the protein levels of ezrin and cofilin-1, two actin-associated proteins, and affects the actin cytoskeleton [117].

10.4.4 Interactions with Proteins Involved in the Trafficking

Snapin is a synaptic vesicle associated protein also known as a SNAP-25 binding protein of the SNARE complex. A motif of 24 a.a. located in the COOH-terminal part of the alpha1A-adrenergic receptor interacts with Snapin [84]. This interaction promotes the association with TRPC6. Furthermore, in the rat cortex, native TRPC6 associates with Snapin. The activation of alpha1A-adrenergic receptor promotes the plasma membrane insertion of TRPC6 via the formation of tripartite complex involving alpha1A-adrenergic receptor/Snapin/TRPC6 [84].

SNF8 a component of the ESCRT-II endosomal trafficking complex, interacts with TRPC6. The a.a. sequence 1–107 seems necessary and sufficient for this interaction to occur [118]. Co-expressing SNF8 and TRPC6 increases the current through TRPC6 without changing its surface expression. However, the mechanism by which SNF8 influences TRPC6 activity is not completely understood. SNF8 could regulate TRPC6 activity by influencing a TRPC6-associated protein [118].

Dynamin, Clathrin co-immunoprecipitation experiments indicate that dynamin and clathrin interact with TRPC6 (in rat brain) [94].

MxA is a member of the dynamin GTPase superfamily. Its COOH-terminal part interacts with the second ankyrin repeat of TRPC6 [41]. Furthermore, MxA upregulates TRPC6 activity without changing its amount at the plasma membrane, a regulatory process that requires the binding of GTP to MxA [41].

RNF24 or ring finger protein 24 is a zinc-binding membrane protein. It is a TRPC-interacting protein that controls the intracellular retention of TRPC6. The ANK-like repeats 1 and 2 of TRPC6 are important in mediating the interaction with RNF24 that seems to occur in the Golgi apparatus. It controls the intracellular trafficking of TRPC6 and its insertion into the plasma membrane but has no effect on the activity of the channel [119].

10.4.5 Immunophilins

FKBP12 or FK505-binding protein of 12 kDa is one of the best characterized immunophilins. These targets of immunosuppressant drugs have the ability to influence cellular Ca^{2+} signaling by altering the activity of Ca^{2+} channels. For instance the drosophila TRPL channels are regulated by the immunophilin FKBP59 [120]. Co-immunoprecipitation experiments indicated the existence of a constitutive association between FKBP12 and TRPC6 involving a stretch of 9 a.a (from a.a. 759 to a.a. 767) at the COOH-terminal of TRPC6A. The association could be disrupted by a low concentration of FK506, a drug that displaces immunophilins from their target proteins. In addition, FK506 inhibits TRPC6-mediated currents [121]. This observation stands in contrast with another report conducted with PC12D cells, and where FKBP12 does not associate with TRPC6 under basal (unstimulated) conditions. In this cell model, the FKBP12-TRPC6 association was only seen after the activation of muscarinic cholinergic receptors [113].

In addition to all these distinct types of TRPC6-interacting partners, TRPC6 seem able to interact with the cholesterol-binding proteins podocin and nephrin [122]. These interactions will be discussed separately further below.

10.5 Expression/Distribution

TRPC6 proteins are found throughout the body [5] (see Table 10.1), but they are mainly expressed in the pulmonary system, lung and brain. The expression of murine TRP channels was studied in various tissues by [123]. They found a special pattern of expression of TRPC6 in adult C57Bl/10SC mice with a high mRNA expression level in the brain and in tissues like the lung and ovary [123]. Of interest, they noted differences between mouse strains with, for instance, a higher mRNA TRPC6 expression in the heart of NOD mice when compared to the heart of C57Bl/10SC or Balbc mice [123]. When analyzing the relative mRNA expression of 22 TRP channels in various mouse organs, Jang et al. also found that mTRPC6 was predominantly expressed in the lung and, to a lower degree, in the brain [124]. TRPC6 expression has also been noted in various cell lines (See Table 10.2 for a non-exhaustive list of TRPC6-expressing cell lines).

At the cellular level, TRPC6 is predominantly found at the plasma membrane. Indeed, biochemical studies indicated the presence of two putative glycosylation sites (a.a. 472 and 560) on TRPC6, suggesting that it is a plasma membrane protein [7]. Since this initial report, many groups have also found that native and heterologously expressed TRPC6 proteins are glycosylated [44, 80, 89, 111, 119].

In polarized epithelial cells, TRPC6 is found in the luminal and basolateral regions [90]. In human platelets [43] found a specific location at the plasma membrane but no TRPC6 expression in inner membranes. On the other hand, in human

Table 10.1 TRPC6 expression in the body

Tissue	References
Adrenal	[15, 258]
Brain	[7, 15, 258]
Epididymis	[123]
Heart	[15, 42, 123, 258]
Intestine	[9, 259]
kidney	[15, 94, 124, 260]
Liver	[45, 124, 260]
Lung	[7, 9, 15, 123, 124, 258, 259]
Lymph nodes	[260]
Ovary	[9, 123, 258]
Pituitary gland	[259]
Placenta	[9, 259]
Prostate	[44]
Skeletal muscles	[259]
Skin	[260]
Stomach	[259]
Spleen	[9, 56, 123, 259, 260]
Testis	[123, 124, 258]
Thymus	[260]
Uterus	[261]

Table 10.2 Expression of TRPC6 in cell lines

Cell type	References
C2C12	[123]
CF-KM4	[100]
CHRF-288	[129]
ciPod	[76]
COS-1	[259]
COS-7	[259]
Dami	[129]
DU145	[26]
HaCaT	[70]
HEK	
HeLa	[259]
Hep G2	[45]
HL-60	[54]
Huh-7	[45]
J82	[26]
LD611	[26]
MCF-7	[262]
MEG01	[95]
MM39	[100]
MPC-5	[81]
PB-3c	[176]
PC3	[26]
RT4	[26]
SHSY5Y	[259]
V2D1	[176]

This table provides a non-exhaustive list of cell lines expressing TRPC6 channels. It is worth adding that conflicting results have been published regarding the expression of TRPC6 in some cell lines since for instance [139] and [263] failed to detect this channel in HaCaT and HL-60 cells, respectively

mesangial cells in culture, TRPC6 expression was primarily found to be localized to the nucleus [88].

Of particular interest is the association of TRPC6 with lipid rafts/caveolae, which are cholesterol-rich specialized microdomains located underneath the plasma membrane and playing crucial roles in calcium signaling [125]. Although one report found no evidence for the presence of TRPC6 in lipid rafts/caveolae in human platelets [126], these channels are commonly described as present in such microdomains [127, 128]. For instance, mTRPC6 expressed in HEK cells were shown to be located in domains of the plasma membrane enriched in caveolin-1/2 [83].

Like any other protein, TRPC6 mRNA and protein levels are subject to regulation. For instance, in human megakaryocytes, there is an up-regulation of the

expression of the TRPC6 mRNA during the maturation process [129]. In pulmonary artery smooth muscle cells, PDGF and c-Jun increased mRNA and protein levels of TRPC6 [44]. In rat cardiac fibroblasts, TRPC6 expression is stimulated by $G\alpha_{12/13}$ [42] whereas forskolin, a PKA activator, inhibits it. A similar up-regulation was seen with angiotensin-II and endothelin-1. This ET-1-dependent regulation of TRPC6 expression is mediated via a signaling pathway involving $G\alpha_{12/13}$, the production of ROS and JNK activation [42]. This influences the transformation of cardiac fibroblasts into myofibroblasts. In Huh-7 cells, a hepatoma cell line, TRPC6 expression is strongly augmented by growth factors such as endothelial growth factor and hepatocyte growth factor [45]. In HL-60 cells, a human promyelocytic cell line, mTRPC6 expression is strongly up-regulated during the differentiation [54]. In human primary keratinocytes (hPK), Ca^{2+} and hyperforin, which induce the differentiation of the cells, stimulate the expression of TRPC6 [70]. A similar observation was made on cultured human skin explants.

TRPC6 seem to play a role in the control of the proliferation of cells, including tumor cells. Of interest, the gallium complex GaQ3 is a new anti-neoplastic agent regulating TRPC6 expression in cancer cells via a p53-dependent process. The trpc6 gene would be a transcriptional target of p53 [130]. Other metals influence TRPC6 expression like Co^{2+} [131] and Gd^{3+} [132]. In fact, many factors can positively or negatively affect TRPC6 expression like hypoxic conditions [133], the mammalian target of rapamycin (mTOR) complex 2 (mTORC2) [134, 135], a chronic treatment of mice with hyperforin [136], the bone morphogenetic protein 4 (BMP4) [137], the tumor necrosis factor alpha (TNF-α), ROS via an intracellular signaling cascade involving PKC [82] and the transcription factor NF-κB [138]. This ROS-dependent regulation of TRPC6 expression controlled by NF-κB [138] recruits the histone deacetylase HDAC2. Of interest, a recent work further highlighted the importance of deacetylases in regulating trpc6 gene expression. Indeed, the histone variant macroH2A negatively regulates the expression of TRPC6 (and TRPC3) without influencing the other TRPC channels. In bladder cells, the expression of macroH2A and TRPC6 is inversely correlated: cells with low levels of macroH2A expression have a high expression level of TRPC6 whereas in cells exhibiting higher expressions levels of macroH2A, TRPC6 was found less abundantly. In fact, macroH2A physically interacts with the histone deacetylases HDAC1 and HDAC2 which, in turns, control TRPC6 (and TRPC3) expression by silencing their genes. MacroH2A appears as a transcriptional regulator suppressing *trpc6* gene expression via a chromatin remodeling process involving deacetylases [26].

The presence of TRPC6 in HaCaT cells has been controversial: the TRPC6 mRNA was not found by [139] whereas [70] found evidence for the presence of TRPC6 mRNA in these cells. In human gingival keratinocytes, external Ca^{2+}, which stimulates keratinocyte differentiation, also stimulated the expression of TRPC6 mRNA [140]. Western blots show the presence of TRPC6 proteins and immunohistochemical experiments revealed that TRPC6 was present in all layers of the oral gingival epithelium [140]. The authors conclude that TRPC6 is present in undifferentiated and differentiated keratinocytes and TRPC6 may participate in the Ca^{2+}-dependent differentiation of these cells. In erythroid cells, TRPC6 expression

decreases during erythroid differentiation [93]. In idiopathic pulmonary arterial hypertension (IPAH) patients, there is an up-regulation of the expression TRPC6 mRNA and protein in pulmonary artery smooth muscle cells, when compared to normal subjects. This indicates that TRPC6 may participate in the development of this disease.

Glucose seems to regulate TRPC6 expression but contrasting results have so far been reported. In glomerular mesengial cells high glucose diminishes TRPC6 expression [141] whereas in podocytes and platelets, high glucose enhances TRPC6 expression [142]. These effects are likely secondary to generation of ROS. Similar effects occur in response to hyperhomocysteinemia [143].

The promoter of the TRPC6 gene contains two conserved sites for the transcription factor NFAT. Furthermore, the calcineurin-dependent activation of NFAT enhances the transcription of the trpc6 gene. Additionally, increasing TRPC6 expression levels activates the calcineurin/NFAT-dependent pathway [144]. This dual regulation has been shown to be involved in the remodeling of cardiac cells under pathologic conditions [144]. A similar mode of regulation also occurs in podocytes [145, 146].

10.6 Regulation and Pharmacological Properties

TRPC6 form channels permeable to a wide range of cations like Na^+, Ca^{2+}, Cs^+, K^+, Ba^{2+} [9, 28]. But they are also permeable to non-canonical cations like iron [147], zinc [117, 148], and even N-methyl D glucamine [28]. TRPC6 opening generates inward and outward rectifying currents (dual rectification) with a reversal potential close to 0 mV [22]. The unitary conductance is in the order of 28–35 pS [9, 27]. Depending on the cell type, the relative permeability ratio is in the order of 5–6 [9, 38, 149]. A well-established consequence of TRPC6 activation is the elevation of the cytosolic concentration of Ca^{2+}. However, the mechanism by which this response occurs is not as trivial at it seems. The TRPC6-dependent Ca^{2+} rise could in fact have three origins: (i) a direct Ca^{2+} entry into cells via opened TRPC6 channels; (ii) it can reflect the contribution of voltage-gated Ca^{2+} channels recruited in response to a TRPC6-mediated depolarization; (iii) the elevation of Ca^{2+} could reflect the contribution of the Na^+/Ca^{2+} exchanger operating in its reverse mode. These pathways are not mutually exclusive. hTRPC6 over-expressed in HEK cells were found to have a low conductance for Ca^{2+}. Under physiological condition, it would rather be a Na^+ conducting channel [28]. Consequently, a Ca^{2+} rise would only be seen when the membrane potential is negative, indicating that TRPC6 functions primarily as a monovalent channel. According to this model, the TRPC6-dependent Ca^{2+} rise is the consequence of the depolarization and does not reflect the influx of Ca^{2+} through TRPC6 channels. The work of van Bremen et al. provided experimental evidence for the existence of a functional coupling between TRPC6 channels and the Na^+/Ca^{2+} exchanger: the entry of Na^+ through TRPC6 channels would promote the entry of Ca^{2+} by reversal of the Na^+/Ca^{2+} exchanger [150–152]. A similar finding has been

noted in cultured cortical neurons where OAG stimulation provokes an intracellular Na^+ rise promoting the entry of Ca^{2+} [153]. The existence of such a functional coupling between the Na^+/Ca^{2+} exchanger and TRPC6 channels has also been suggested in the neuroblastoma cell line IMR-32 [154].

Like any other channel, the activity of TRPC6 can be regulated by various means. For instance, TRPC6-mediated responses can be finely tuned by phosphorylation/dephosphorylation reactions. Consensus sequences for various kinases such as PKA, PKC, PKG, casein kinase II and tyrosine kinases are known [43]. The following section will address the regulatory processes influencing TRPC6 activity.

CaM is generally viewed as a negative regulator of TRPCs, but it seems to exert opposite effects on TRPC6. CaM binding to mTRPC6 regulates its activity because inhibitors of CaM (calmidazolium, trifluoperazine) depress Ca^{2+} entry by abolishing the interaction CaM-mTRPC6 [110]. [38] reported that calmidazolium or an inhibitor of CaMK-II depresses mTRPC6 currents, indicating that phosphorylation of the channel by CaMKII is necessary for its activity.

Ca^{2+} the activity of TRPC6 channels is regulated by Ca^{2+} in a complex manner: chelating $Ca^{2+}{}_{in}$ potentiates TRPC6-mediated currents whereas elevating $Ca^{2+}{}_{in}$ has an opposite effect [37]. A role of external Ca^{2+} was documented for mTRPC6 channels over-expressed in HEK cells [38]: in this case, external Ca^{2+} exerts a complex regulatory action on TRPC6 with positive and negative effects on TPC6 activity. Maintaining human mesangial cells in a Ca^{2+}-enriched (5 mM) culture medium for 24 h augments their TRPC6 protein levels (when compared to cells kept in a culture medium containing 1 mM Ca^{2+}). This response, which is thought to involve the Ca^{2+}-sensing receptor, stimulates their proliferation [155].

Phosphorylation by Tyrosine Kinases Contradictory findings have been published regarding the regulation of TRPC6 channels by tyrosine phosphorylation. In human platelets, Hassock et al. found that TRPC6 does not undergo tyrosine phosphorylation [43]. However, in HEK cells, TRPC6 was reported to be tyrosine phosphorylated in a *Fyn*-dependent manner [111]. *Fyn*, a *src*-family tyrosine kinase, is a TRPC6-interacting protein (binding on the NH_2-terminal part of the channel). This Fyn-dependent phosphorylation of TRPC6 enhances TRPC6 channel activity. Experiments conducted on mTRPC6 expressed in embryonic fibroblasts gave contrasting results [156]: it was first shown that, in GST pull-down tests, *src* interacts with the NH_2-terminal of TRPC6. However, expressing TRPC6 in fibroblasts lacking *src*, *Fyn* and *yes* tyrosine kinases failed to affect TRPC6-mediated Ca^{2+} signals (when activated in response to the activation of GPCR with arginine vasopressin or carbachol). Furthermore, TRPC6-medicated Ca^{2+} signals are insensitive to genistein, a non-selective tyrosine kinase inhibitor. According to [156], tyrosine kinases are not necessary for the activity of TRPC6 channels. Experiments conducted with inhibitors of tyrosine kinases (PP2, SU6656) on mTRPC6 over expressed in HEK cells showed that tyrosine phosphorylation would be a necessary step for the activation of the channels [25]. An indirect evidence for the involvement of *src* in the

modulation of TRPC6 activity in human leukocytes was provided by [157]. Of note, lysophosphatidylcholine, which induces the externalization of native and over-expressed TRPC6 channels, stimulates its tyrosine phosphorylation. But it is unclear whether this phosphorylation process is required to control the trafficking of TRPC6 [30].

Phosphorylation by PKC TRPC6 do not seem to open in response to PKC activation [27, 37] but they can be phosphorylated upon the application of the phorbol ester PMA, a well-known PKC activator, indicating that these channels can be directly phosphorylated by PKC [113]. In HEK cells expressing mTRPC6, TRPC6-mediated currents are inhibited by PKC agonists, indicating that this kinase regulates negatively TRPC6 channel activity [38]. However, in HEK cells expressing hTRPC6, PKC activation with PMA has no effect on OAG-activated TRPC6 channels whereas this protocol strongly depresses carbachol-activated TRPC6-mediated currents [28]. Biochemical studies found that mTRPC6 expressed in HEK cells are weakly phosphorylated under basal conditions but application of carbachol stimulates TRPC6 phosphorylation via a PKC-dependent process [158]. Furthermore, PKC stimulation depresses TRPC6-mediated Ca^{2+} responses. Mutagenesis studies indicated that none of the 12 putative PKC phosphorylation site seems to be involved. Instead, the authors identified a non-canonical phosphorylation site (Ser448) as a target for PKCδ. [158] propose that PKC acts as a negative feedback regulator of TRPC6 activity.

Regulation by PKG Protein sequence alignments revealed the existence of two putative PKG phosphorylation sites on TRPC6 [159]. Studies conducted on mouse TRPC6 channels expressed in HEK cells concluded that PKG activation by means of the NO donor SNAP or the PKG activator 8Br-cGMP depressed TRPC6-mediated currents [160]. This occurs without affecting the cell surface expression of TRPC6, indicating that PKG influences the gating of the channels rather than their plasma membrane abundance. In addition, this PKG-dependent depression of TRPC6-mediated responses critically depends on a phosphorylation site (Thr69) located in the COOH-terminal part of TRPC6 [160].

Regulation by PKA Studies focusing on the role of the cAMP-dependent protein kinase A (PKA) on TRPC6 activity have led to conflicting results. mTRPC6 and hTRPC6 can be phosphorylated by PKA, a process associated with reduced TRPC6-mediated Ca^{2+} responses [161, 162] that does not influence the cellular distribution of the channel [162]. This conclusion stands in contrast with other reports who observed that PKA exerts no modulatory action on TRPC6 activity [43, 163]. In glomerular mesangial cells, cAMP regulates TRPC6 activity but in a PKA-independent manner via a signaling cascade leading to the sequential activation of the following kinases: PIK3, MEK, and ERK1/2 kinase [163]. The existence of an interplay between TRPC6 and ERK has been recently confirmed in a study where the over-expression in HEK cells of gain-of-function TRPC6 mutants activates ERK [161].

Regulation by MLCK Based on their results [164] conclude that MLCK plays no (or very little) regulatory role on mTRPC6 channel activity.

Regulation by Podocin Podocin is a cholesterol-binding protein of the stomatin family expressed primarily in podocytes. The COOH-terminus of podocin interacts with the carboxyl-terminus of TRPC6 [66]. It regulates TRPC6 activity in a cholesterol-dependent manner [128]. Podocin enhances TRPC6 activation by DAG and through GPCRs [66–68] but reduces TRPC6 activation in podocytes by mechanical stimuli [66]. A chronic treatment of human B lymphoma Daudi cells with cholesterol or lovastatin, a statin known to inhibit cholesterol synthesis, respectively up-regulates or depresses TRPC6 expression and activity [165].

Glycosylation the original work on mTRPC6 proposed the presence of two putative glycosylation sites (a.a. 472 and 560) [7]. In the rat, the isoform A was mainly found in the (mature) glycosylated form whereas rTRPC6B was found in the (mature) glycosylated and (immature) non glycosylated forms. On the other hand, rTRPC6C is not glycosylated [11]. Since then, the glycosylation status of TRPC6 has been noted by several groups [44, 89]. This glycosylation has a strong impact on the basal (or constitutive) activity of TRPC6. By studying two related TRPC channels (TRPC3 and TRPC6) [149] could show that hTRPC3 and hTRPC6 channels over-expressed in HEK cells exhibit distinct features: TRPC3 is a mono-glycosylated channel displaying considerable basal activity in contrast to TRPC6, dually glycosylated (in the first and second extracellular loops), that has a low level of basal activity [37, 149]. It appears that the glycosylation status of TRPC6 plays important role in controlling its constitutive activity: the removal of one of these two sites strongly augments TRPC6 basal activity. On the other hand, mutant TRPC3 channels with two glycosylation sites become quiescent. The pattern of glycosylation has thus a strong effect on the basal activity of TRPC channels [149].

Regulation of TRPC6 Activity by Interacting Partners As pointed out, CFTR and TRPC6 are thought to be engaged in a macromolecular complex [100]. These two proteins seem to be functionally coupled. Indeed, suppression of CFTR expression up-regulates TRPC6 mediated Ca^{2+} responses. CFTR has been proposed to be a negative regulator of TRPC6 channels in bronchial cells [100].

Pharmacological Inhibitors Various compounds have been used to block the transport of ions through TRPC6 channels. One major challenge when studying the properties of TRPC channels is the lack of selective inhibitors. Therefore, most (if not all) compounds used to depress TRPC6-mediated responses are also inhibiting other members of the TRPC family. The blockers of cation channels 2-APB, SKF-96365, La^{3+} and Gd^{3+} are the more commonly used TRPC6 antagonists. For instance, the inhibitory effect of La^{3+} and Gd^{3+} on rTRPC6B channels has been defined [22]. These two cations are amongst the most potent TRPC6 antagonists since mTRPC6 expressed in HEK cells are blocked by La^{3+} with an IC_{50} of 4–6 μM and by Gd^{3+} with an IC_{50} of 1.9 μM [27]. These authors determined the IC_{50} values of various

Table 10.3 Pharmacological inhibition of TRPC6 channels

Blockers	Cd^{2+}	La^{3+}	Gd^{3+}	SKF-96365	Amiloride
IC_{50} (μM)	253	4.0	1.9	4.2	129

The table gives the IC_{50} values of several TRPC6 channel inhibitors (Data from Inoue et al. [27])

inhibitors (see Table 10.3). Besides, several other molecules were shown to affect TRPC6 channels like clotrimazole, an azole compound clinically used as antifungal drug, that was first described as a specific TRPM2 blocker [166] but in fact blocks TRPC6, TRPM3 and TRPV4 [167]. Pyrazole compounds like Pyr2 exert inhibitory properties on over-expressed TRPC6 channels [168]. In addition, U73122, an inhibitor of phospholipase C, has been widely used to analyze the contribution of this enzyme in the signaling pathway leading to TRPC6 activation. However, U73122 should not be used for this purpose because it exerts a direct inhibitory effect on TRPC6 channels [164, 169]. For instance, in HEK cells over-expressing mTRPC6, TRPC6-mediated currents activated by carbachol are inhibited by U73122 and by its inactive analogue U73343 [28]. Activation of native TRPC6 channels in podocytes by DAG analogs is also blocked by U73122 [68]. These observations call into question a fairly substantial literature in which these drugs have been used to probe Ca^{2+} dynamics under the supposition that they are specific inhibitors of phospholipases. When analyzing the putative regulatory action of the myosin light chain kinase (MLCK) [164] found that several MLCK inhibitors like ML-9, ML-7, and W7 reversibly depressed in a dose-dependent manner hTRPC6-mediated Ca^{2+} currents recorded in HEK cells. This occurs independently of their action on MLCK. They found IC_{50s} of 7.8 μM for ML-9, 10 μM for ML-7 and 50 μM for W7 [164]. ACA (N-(p-amylcinnamoyl) anthranilic acid) is a broad spectrum PLA2 inhibitor. It blocks various TRP channels, including hTRPC6 (expressed in HEK cells) with an IC_{50} of 2.3 μM [79]. It also blocks native TRPC6 channels of podocytes at micromolar concentrations (SED unpublished observations). On the other hand, another PLA2 inhibitor, p-bromophenacyl bromide (BPB) has no effect on hTRPC6-dependent currents when tested at the concentration of 100 μM [79]. In some studies LOE908 has been presented as a non-specific inhibitor of TRPC6 and receptor-operated non-capacitative calcium entry [170, 171]. In addition to these a number of proprietary inhibitors have been developed by pharmaceutical companies, although to date their specificity is not known.

10.7 Roles of TRPC6 Channels in Non-excitable Cells in Health and Disease

TRPC6 channels support many key physiological functions like the control of the vascular smooth muscle as revealed by experiments conducted on *Trpc6*$^{-/-}$ mice [172]. One key important TRPC6 contribution is its involvement in the cellular Ca^{2+} signaling. By allowing intracellular Ca^{2+} rises, changes in TRPC6 activity most likely influences a wide number of Ca^{2+}-dependent processes like the proliferation

or differentiation of cells. For instance, in human keratinocytes, TRPC6 is sufficient to induce differentiation. Thus, TRPC6 activation by hyperforin triggers, while TRPC6-specific siRNAs inhibit, keratinocyte differentiation [70]. Moreover, triterpenes which also promote keratinocyte differentiation are able to increase TRPC6 expression in human keratinocytes [173]. Fibroblast transdifferentiation into myofibroblasts is an essential step in wound healing and tissue remodeling. In a genome-wide screen, TRPC6 was identified as sufficient for myofibroblast transformation. *Trpc6*$^{-/-}$ mice showed impaired dermal and cardiac wound healing because their fibroblasts were not able to initiate TGFβ- and AngII-induced transdifferentiation [174].

Another important physiological function of TRPC6 is its contribution to the control of the proliferation of normal and malignant cells. Indeed, a positive correlation between TRPC6 expression and the proliferation of tumor cells has been reported in cancers like lung, breast, prostate, ovarian, gastric, liver cancers and also in glioblastoma [175]. On the other hand, mTRPC6 expression was found to be down-regulated in a tumor cell line (mast cells) [176]. In addition, a role of TRPC6 in VEGF-induced angiogenesis has also been documented [177].

The above reported properties may be linked to the fact that TRPC6 channels influence the actin-based cytoskeleton and its dynamics [117, 169, 178, 179]. For instance, TRPC6 is involved in cytoskeletal rearrangements during neutrophil migration, and migration of *Trpc6*$^{-/-}$ neutrophils in response to Macrophage Inflammatory Protein-2 (MIP2 also known as CXCL2) is reduced compared to WT neutrophils [180]. TRPC6 channels are essential for CXR2-mediated intermediary chemotaxis but not for fMLP receptor-mediated end-target chemotaxis of neutrophils, indicating that not all receptors in neutrophils are in signaling complexes with TRPC6 [181].

More recently, some studies unveil additional TRPC6 functions like its contribution in the host-parasite response. Parasites like *Plasmodium falciparum* and *Toxoplasma gondii* use cellular signal transduction cascades for egress from the host cell. It was demonstrated that Ca^{2+} influx through TRPC6 activates host calpain to proteolyse the host cytoskeleton, allowing the release of parasites [182]. In addition, its involvement in the allergic response has been highlighted since ovalbumin-challenged *Trpc6*$^{-/-}$ mice exhibited reduced allergic responses as evidenced by a decrease in airway eosinophilia and blood IgE levels as well as levels of T-helper type 2 cytokines (IL-5, IL-13) in the bronchoalveolar lavage [183]. TRPC6 was also identified in other immune cells of the lungs like alveolar macrophages, and TRPC6 mRNA expression was significantly increased in macrophages obtained from COPD patients compared to healthy controls, while TRPC3 and TRPC7 levels remained unchanged [184]. Cell shrinkage and phospholipid scrambling, two hallmarks of eryptosis (erythrocytes programmed cell death triggered by Ca^{2+} influx), were significantly lower in Cl^--depleted erythrocytes from *Trpc6*$^{-/-}$ mice than from WT mice [185]. Moreover, Ca^{2+} entry in these cells was inhibited after pre-incubation with specific TRPC6 antibodies, but not with antibodies directed against TRPC3 or TRPM2 [185].

Below, the following chapters address the patho-physiological significance of TRPC6 in renal and blood cells where its expression and functions have been particularly studied.

10.7.1 TRPC6 in the Kidney

10.7.1.1 TRPC6 Dysfunction in Chronic Kidney Disease

In 2005 Michelle Winn and her co-workers documented a mutation (P112Q) in the first ANK repeat domain of TRPC6 in a large extended family in which several members comprising at least three generations had an aggressive form of focal and segmental glomerulosclerosis (FSGS) and high-grade proteinuria [186]. The disease in this family exhibited an autosomal dominant form of inheritance and occurred in adulthood (at a mean age of 33 years). The affected members of this family progressed to end-stage renal disease (ESRD) in 10 years or less after initial presentation [187]. The P112Q mutation causes a gain of function when these channels are expressed in HEK293 cells, resulting in markedly increased cationic currents and Ca^{2+} influx after application of DAG analogs or activation of GPCRs [186]. The biophysical basis for the gain of function in this allele is not entirely understood, but the initial report presented evidence for altered trafficking resulting in increased steady-state expression on the cell surface [186]. A number of other TRPC6 mutations were quickly identified in familial forms of FSGS [122]. These alleles also caused an aggressive disease with an adult onset and autosomal mode of inheritance, and most of the alleles encoded channels with at least a modest gain of function when expressed in heterologous expression systems. We should note that some of these mutations were identified from a limited number of patients, and the evidence is not equally strong in all cases that the TRPC6 allele is the cause of the disease, such as for the highly conservative S270T missense mutation, which does not appear to show any gain of function [122, 145]. Since these original reports, several more mutant alleles have been found in affected families [188–194]. At present, 21 different alleles have been reported. Among these, there is at least some evidence suggesting a gain of function for ten, whereas two do not appear to have a gain of function, and nine have not been characterized functionally. A mutation of particular interest (M132T) exhibited the largest gain of function seen to date, with current densities six to sevenfold greater than wild-type channels [195]. Notably, the M132T mutation was identified in a 9-year old girl with a severe nephrotic syndrome. It has been hypothesized on this basis that the age of onset of the disease is related to the extent of the overall gain of function as a result of a long-term "Ca^{2+} dose" effect. More recently, novel TRPC6 mutations have been observed in a 2-year old child (R895L) and in a 4-year old child (N125S) with severe steroid-resistant nephrotic syndrome [189]. Unfortunately, these later alleles have not been characterized using voltage-clamp methods. Most of the TRPC6 mutations identified in patients with FSGS have

been observed in ankyrin repeat domains (such as P112Q and M132T) or close to the carboxyl terminus (such as R895C or E897K).

Nephrotic syndromes caused by TRPC6 mutations are relatively rare. By contrast, non-genetic "acquired" forms of FSGS are quite common, as are other severe glomerular diseases such as diabetic nephropathy, minimal change disease (MCD), and various inflammatory conditions associated with localized fixation of complement within glomerular capillaries. Overall TRPC6 expression appears to be increased in several of these conditions, as well as in rodent models of chronic kidney disease [196]. Moreover, selective over-expression of TRPC6 in podocytes in vivo induces modest proteinuria and glomerulosclerosis [197]. Therefore, it is possible that over-expression of TRPC6 could contribute to several forms of kidney disease, although this may serve initially as a mechanism to compensate for increased pressure within remaining glomerular capillaries that occurs whenever there is a loss of functional nephrons [81, 198].

10.7.1.2 Distribution of TRPC6 in the Kidney

TRPC6 channels, expressed by renal cells (see Table 10.4), are found at highest density within glomeruli, where they are expressed in podocytes and mesangial cells [97, 122, 186]. This distribution can explain why TRPC6 mutations induce glomerular diseases. Within podocytes, TRPC6 channels appear to be expressed at a high density within the slit diaphragm domains of foot processes [128], where they probably form complexes with signaling and scaffolding proteins including nephrin [122] and podocin [66, 128], and NADPH oxidases [199]. These membrane domains are characterized by unusually high cholesterol concentration [200]. TRPC6 can also be found along the major processes of podocytes and within its cell body [201] where nephrin and podocin are generally not expressed on the cell surface. While TRPC6 channels within glomeruli have been the most extensively studied, TRPC6 channels are also detected in aquaporin-2-containing collecting duct cells in cortex and medulla [97]. The function of those channels is not known, but based on their location, it is possible that they contribute to the formation of a concentrated urine. Moreover, based on molecular weight, it is possible that different splice variants of TRPC6 predominate in glomeruli compared to tubules [97]. TRPC3 is detected in the same renal cells as TRPC6, and at least some of the renal TRPC6 protein occurs in heteromers that contain TRPC3 subunits. However, in collecting duct, the TRPC6 appears to be preferentially localized in apical membranes, whereas TRPC3 is in the basolateral membrane [97].

10.7.1.3 Regulation of TRPC6 Gating and Trafficking in Podocytes and Mesangial Cells

The TRPC6 channels in podocytes are of particular interest since those cells are principally affected in patients with TRPC6 mutations, as well as in the early stages of primary FSGS. The TRPC6 channels of podocytes become active in response to

Table 10.4 TRPC6 expression in kidney

	References
Glomeruli	[88, 97, 122, 186]
Mesangial cells	[88, 122, 141]
Podocytes	[68, 122, 128, 264]
Tubules	[97, 122, 186]

three main classes of stimuli: (1) Canonical lipid activators such as DAG and 20-HETE, and their mimics such as hyperforin. (2) Stimuli that liberate ROS. (3) Mechanical deformation of the plasma membrane. Thus, simply perfusing 100 μM OAG, a membrane permeable analog of DAG causes a relatively rapid activation of TRPC6 channels in immortalized mouse podocyte cell lines [66, 81]. A similar effect is seen in response to 10 μM 20-HETE (SED unpublished data). Oxidative activation occurs with many members of the TRP family of channels, but was first observed for TRPC6 in HEK-293 cells, where H_2O_2 evoked increases in surface expression as well as direct activation of gating [80]. Similar effects are seen for native TRPC6 channels in podocytes, as application of 500 μM H_2O_2 causes rapid activation of podocyte TRPC6 accompanied by an increase in steady-state surface expression of these channels [81]. Native podocyte TRPC6 channels co-immunoprecipitate with NADPH oxidases, including NOX2 [199] and NOX4 (SED, unpublished data), whose primary function is to produce the ROS superoxide and H_2O_2 for use in normal physiological processes. Formation of NOX2-TRPC6 complexes only occurs in the presence of podocin, and therefore the interaction between these proteins is indirect [199]. Notably, ROS pathways appear to amplify responses to canonical lipids in podocytes, as TRPC6 activation by DAG analogs is markedly attenuated in the presence of ROS quenchers such as TEMPOL or MnTBAP. Moreover, DAG stimulates assembly of active NOX2 complexes on the cell surface [199]. Disruption of these complexes may explain why loss of podocin markedly decreases or eliminates responses to canonical lipids in podocytes. In this regard, inhibition or knockout of NOX enzymes, and ROS quenching, eliminates TRPC6 activation and mobilization evoked by angiotensin II (Ang II) [67, 202], P2Y receptor agonists [68], and insulin [81]. ROS generation also underlies increased TRPC6 activation in the presence of high glucose [203]. TRPC6 channels are also upregulated by other signals such as VEGF [204] but are down regulated by vitamin D [205]. Finally, we note that expression of syndecan-4 can lead to up-regulation of TRPC6 and an increase in their basal activation in podocytes [206]. In this regard, syndecan-4 knockout mice do not have detectable TRPC6 within glomeruli or in podocyte cell lines, although TRPC3 continues to be present [206].

The TRPC6 channels of mouse and rat podocytes are activated by mechanical stimuli that cause deformation of the plasma membrane in either direction [66]. This can be seen in response to hypo-osmotic stretch, inward indentation of the plasma membrane using a fire-polished glass probe, or by using hydrostatic pressure pulses delivered from a micropipette placed close to the cell surface [67]. These responses are seen in immortalized podocyte cell lines, as well as in podocytes in

acutely isolated glomeruli still attached to the GBM [66]. These currents are not observed after TRPC6 knockdown, in the presence of 50 μM La^{3+} or SKF-96365, when the recording pipettes contain GDP-βS [66], by manipulations that eliminate responses to Ang II, ATP or DAG analogs in the same cells [66–68]. In addition, mechanical activation of podocyte TRPC6 channels is blocked by GsMTx4 [66] and also by proprietary TRPC6 inhibitors (SED, unpublished observations). The observation with GDP-βS suggests that the mechanical activation of TRPC6 occurs by signaling pathways independent of any G proteins, in marked contrast to the mechanical activation of TRPC6 in smooth muscle. In addition, knockdown of podocin markedly increases mechanical activation of podocyte TRPC6 channels, while simultaneously reducing or eliminating responses to canonical lipid signals [66] or responses mediated by G protein-dependent signaling pathways [67, 68]. This is of interest given that mutations in the *NPHS2* gene encoding podocin give rise to a common autosomal recessive form of FSGS primarily seen in children [207].

TRPC6 channels are also expressed in mesangial cells, along with TRPC3 and TRPC1 [97]. These channels have been less extensively studied than their counterparts in podocytes, and it appears that they are regulated differently in certain respects. TRPC6 channels in mesangial cells can be activated by signals through Ca^{2+}-sensing receptors [155] and by glucagon [163] through G protein-coupled pathways that may entail Erk and phosphatidyl inositol-3 kinase signaling. It is especially interesting that ROS and high glucose suppress TRPC6 activation in mesangial cells [82, 141, 208], because the same stimuli increase TRPC6 activation in podocytes [81, 203, 209]. Mesangial cells are excitable contractile cells located on the interior of the glomerular capillary. They make numerous connections with the inner face of the glomerular basement membrane (GBM), and contraction of mesangial cells is thought to prevent excessive expansion of the glomerular capillary in response to elevated transmural pressures [210]. By contrast, podocyte foot processes line the entire outer face of the GBM. Specialized connections between adjacent foot processes, known as slit diaphragms, form a porous matrix that plays a major role in determining the overall permselectivity of the glomerular filter [211]. The arrangement of mesangial cells and podocytes suggests that a modest mesangial relaxation accompanied by contraction of podocyte foot processes could serve to protect delicate slit diaphragms from sustained expansile forces caused by glomerular capillary pressures. Increased transmural glomerular capillary pressures occur, for example, in response to transient elevations in blood glucose [212] and also whenever there is substantial loss in the number of functional nephrons [213]. However, it is likely that a more severe abrogation of mesangial cell contractility would compromise glomerular function [208].

An important feature of TRPC6 channels in podocytes is that their sustained activation can activate a positive feedback loop through calcineurin-NFATc pathways that lead to increased transcription of the trpc6 gene [145, 146]. A similar pathway had earlier been described in the heart, where it is thought to contribute to cardiac hypertrophy [144]. This feedback loop may explain the increase in TRPC6 expression observed in chronic kidney diseases, in which there is an increase in

glomerular capillary pressure [196]. In this regard, TRPC6 knockout mice or mice with reduced TRPC6 expression are protected in certain models of kidney disease, including proteinuria evoked by chronic Ang II infusion [214] or by intraperitoneal injection of high doses of bovine albumin [206]. At the same time, at least in the initial stages of glomerular disease, TRPC6 could play a protective role, as has been suggested in mouse models of inflammatory kidney diseases in which there is complement fixation within the kidney [198].

10.7.1.4 TRPC6-Interacting Proteins in Renal Cells

TRPC6 proteins are concentrated in the slit diaphragm domains of the podocyte foot process plasma membrane, and therefore it is not surprising that they interact with other proteins concentrated there. It bears noting that the slit diaphragm domain has a high concentration of sterols than other regions of the foot process plasma membrane [200]. Because proteins that partition within the same raft domain can co-immunoprecipitate, care has to be used in interpreting results. The strongest evidence for a direct interaction with podocyte TRPC6 channels is with podocin, because it can be detected by co-immunoprecipitation [66, 122, 128], and also using GST pull-down assays using soluble fragments of the proteins [66]. Podocin is a hairpin-loop intrinsic membrane protein in a family that also contains stomatin-like proteins, prohibitins, and flotillins, as well as the MEC-2 protein of *C. elegans* [215]. All of these proteins share a so-called PHB domain that binds cholesterol and usually localizes these proteins to lipid rafts. Proteins in this family also tend to form higher-order oligomers, and it is worth noting that several, including the stomatin-like proteins [216, 217] and MEC-2 [218], regulate (and usually facilitate) the gating of mechanosensitive channels. As already noted, podocin suppresses mechanically-induced activation of podocyte TRPC6 channels [66]. It also enhances activation by lipids or through GPCRs [66–68, 128] at least in part by facilitating co-localization with NOX2 [199]. The interactions between TRPC6 and podocin occur between their respective intracellular carboxyl terminals and do not require the transmembrane domains of either protein [66]. Podocyte TRPC6 channels also co-immunoprecipitate with nephrin [122], although it is not known if this interaction is direct. A wide range of proteins interact with the intracellular domains of nephrin forming so-called pleomorphic ensembles [219]. Among these protein are protein kinases such as *Fyn*, phosphoinositide 3-kinase, and podocin [220], which may therefore allow formation of signaling complexes that induce tyrosine phosphorylation of TRPC6 [221].

The striking deviation of TRPC6 channels from constant-field behavior does not allow for efficient Ca^{2+} flux as cells depolarize, and this effect is even greater than one would expect simply from a reduction in driving force [28]. In excitable cells, the TRPC6-dependent depolarization is sufficient to cause activation of voltage-dependent Ca^{2+} channels, which then allow for substantial Ca^{2+} flux. However, podocytes are non-excitable cells and do not express voltage-activated Ca^{2+} channels. Since TRPC6 activation should cause marked depolarization, the question

arises as to how they can mediate sustained Ca^{2+} flux. One possibility is that TRPC6 channels in podocytes interact with large-conductance (BK-type) Ca^{2+}-activated K^+ channels, which raises the possibility that they are activated coordinately to prevent excessive depolarization in the face of continued TRPC6 activation [101].

10.7.2 TRPC6 in the Blood

10.7.2.1 TRPC6 in the Megakaryoblastic Lineage

The first evidence of the expression of TRPC6 in platelets was provided by Authi and coworkers in 2002 [43], reporting the expression of TRPC6 in the plasma membrane of human platelets and suggesting a role for TRPC6 in a non-capacitative, cAMP-dependent protein kinase-regulated, Ca^{2+} entry pathway. The location of TRPC6 in the plasma membrane of human platelets has been confirmed by a number of studies, i.e. [126, 222], and this channel has been reported to be mostly associated to membrane domains independent of lipid rafts [126], which is consistent with its initial putative role as a non-capacitative channel. Recent studies have provided evidence for a role of the protein STIM1 in the cellular location of TRPC6. Thus, in the NG115-401L cell line, expression of STIM1 results in translocation of a pool of TRPC6 from the plasma membrane to the endoplasmic reticulum, presumably to act as a Ca^{2+} leak channel [104]. The expression of TRPC6 has also been confirmed in murine platelets and megakaryocytes [129, 223, 224] and the megakaryoblastic cell line MEG01 [95] (see also Tables 10.2 and 10.5).

10.7.2.2 TRPC6-Interacting Proteins in Blood Cells

TRPC6 has been reported to interact with a number of intracellular proteins in platelets, including kinases, such as the cAMP-dependent protein kinase [43] and *Src* family tyrosine kinases [225], TRP channels like TRPC3 [55, 126], TRPC1 [226] and TRPA1 [95], the store-operated Orai1/STIM1 complex [55, 227], the Ca^{2+} channel Orai2 [107], and CaM as well as the type II IP_3R, through the CIRB site [228]. The association of TRPC6 with these proteins plays an important regulatory role on the function and behavior of the TRPC6-forming channels. For instance, the CIRB site allows a dual regulation of TRPC6 channel function by Ca^{2+} and IP_3 levels. Hence, elevation of cytosolic Ca^{2+} concentration in human platelets either by stimulation with physiological agonists, such as thrombin, or by treatment with pharmacological compounds, results in Ca^{2+}-dependent binding of CaM to TRPC6 channels and, thus, displacement of IP_3 receptors. Conversely, IP_3 generation increases the affinity of the IP_3R for the CIRB site of TRPC6, attenuating the displacement of IP_3R from this site and, as a result, attenuating the regulatory effect of CaM. Therefore, the CIRB site of TRPC6 dynamically regulates both Ca^{2+} influx and function stimulated by physiological agonists in human platelets [228].

Table 10.5 TRPC6 expression in blood cells

Cell type	References
Platelets	[43, 56, 129]
Megakaryocytes	[129, 223]
Polymorphonuclear leukocytes	[157]
Neutrophils	[54, 263]
T lymphocytes	[260, 265]
B lymphocytes	[260, 266]
Jurkat T cells	[34, 258, 265]
Erythroid cells	[89, 93]
Erythroid progenitors	[93, 185]
Erythrocytes	[185]

Furthermore, TRPC6 tyrosine phosphorylation by *Fyn*, a member of the Src family of protein tyrosine kinases, results in an increase in the channel activity [111], which is consistent with the finding that *Src* family kinase activation is required for store-independent Ca^{2+} entry in human platelets [225]. More interesting is the functional role of the association of TRPC6 with other channels also described in platelets, such as TRPC3, TRPC1 or Orai family members, which is associated to the behavior of TRPC6 channels as store-dependent or -independent channels.

10.7.2.3 Involvement of TRPC6 in Ca^{2+} Influx in Human Platelets

TRPC6 channels have been classically associated to store-independent, or non-capacitative, Ca^{2+} entry in a number of cell types as described above, activated by receptor occupation via DAG or changes in the phosphoinositide composition, or by physical stimuli, such as mechanical stretch. Activation of TRPC6 by DAG, or its membrane permeable analog OAG, has long been demonstrated in human [222] and mouse platelets [56, 227, 229]. In addition, there is a body of evidence supporting that TRPC6 might be sensitive to PIP_3, a substrate of phosphatidylinositol 3-kinase (PI3K), in platelets, Jurkat T cells, and RBL-2H3 mast cells. First of all, fluorometric analysis has revealed the ability of PIP_3 to selectively stimulate Ca^{2+} entry in TRPC6-expressing cells, a response that was altered by TRPC6 overexpression or down-regulation in Jurkat T cells; and, second, specific association of PIP_3 with TRPC6 has been demonstrated by pull-down studies. The activation of TRPC6-dependent non-capacitative Ca^{2+} entry by PIP_3 was found to be reminiscent to that evoked by OAG. However, other phosphoinositides failed to induce Ca^{2+} influx [34]. Furthermore, TRPC6 activation in human platelets has been found to be sensitive to the cellular level of PIP_2 [222], which might interact with the C-terminal region of TRPC6 stabilizing the channel in an open channel configuration, as well as disrupting the interaction of inhibitory molecules with the channel [230]. In human platelets, we hypothesize that activation of membrane receptors by physiological agonists might play a dual role on TRPC6 channel activity. First, PLC

activation would result in IP_3 and DAG generation, leading to the activation of Ca^{2+} entry via TRPC6 and other channels, a process that is positively regulated by PIP_2, but, soon after receptor occupation, PLC-dependent PIP_2 hydrolysis would lead to inactivation of TRPC6, thereby limiting its activity to the initial step of Ca^{2+} influx as described for other PIP_2-regulated TRP channels [231].

In addition to the role of TRPC6 in the store-independent Ca^{2+} pathway, a role for TRPC6 in the conduction of SOCE has also been suggested in human platelets. SOCE was first described in platelets by Sage and coworkers in 1989 by using Mn^{2+} as a surrogate for Ca^{2+} influx, and analyzing its quenching effect on the Ca^{2+}-insensitive fura-2 fluorescence, to avoid contamination with other Ca^{2+} entry routes [232]. The mechanism underlying the communication between the filling state of the agonist-sensitive Ca^{2+} stores to the plasma membrane Ca^{2+} channels in these cells was revealed to involve the protein STIM1 [233], following its identification as the ER Ca^{2+} sensor for the activation of SOCE in *Drosophila* S2 and different mammalian cell types [234–237]. STIM1 and its homologue STIM2 are expressed both in the dense tubular system, the analog of the ER in platelets, and the agonist-releasable acidic Ca^{2+} stores, mostly including lysosomes and lysosome-like organelles [238]. Although the role of STIM1 as the intraluminal Ca^{2+} sensor is widely accepted, the nature of the SOC channels still remains as a matter of intense debate and investigation. Current evidence supports the role of the Ca^{2+} selective channel Orai1 in the conduction of the Ca^{2+} selective I_{CRAC}, the store operated Ca^{2+} release activated Ca^{2+} (CRAC) current, as well as TRPC proteins as candidates for the conduction of the non-selective and store-dependent I_{SOC} current [49, 239–241]. Since the identification of mammalian TRPC1, this channel has been proposed as a SOC candidate in different cell types, including human platelets [242, 243]. The role of TRPC6 in SOCE in human platelets was first suggested in 2008 by using a neutralizing antibody against the C-terminal region of TRPC6, which reduced both thapsigargin (TG)-induced and OAG-evoked Ca^{2+} influx, as well as TG-stimulated Mn^{2+} entry [222]. In addition, treatment with the PLA_2 and TRPC6 inhibitor ACA [244] attenuated TG-induced cation entry in human platelets [222]. Further evidence supporting a role for TRPC6 in SOCE in human platelets comes from studies performed using TPEN [N,N,N′,N′-tetrakis-(2-pyridylmethyl)-ethylenediamine] to decrease the intraluminal Ca^{2+} concentration avoiding the indirect effect induced by the amount of Ca^{2+} released into the cytosol. Introduction of a specific anti-TRPC6 antibody into platelets by electrotransjection resulted in attenuation of SOCE induced by treatment with TPEN [55]. The effect observed after incorporation of the antibody was not due to side-effects of the preservatives present in the antibody solution, which was tested in every set of experiments performed, for a reference see [245]. To further support the role of TRPC6 in SOCE (in platelets?), analysis of the signaling complexes generated upon induction of store-operated and store-independent Ca^{2+} influx revealed that Ca^{2+} store discharge results in the formation of a protein complex involving, among others, STIM1, STIM2, Orai1, TRPC6 and TRPC1 [107, 226], by contrast, stimulation of store-independent Ca^{2+} entry by OAG results in the association of TRPC3 with Orai3 [107]. Cell stimulation with thrombin or Ca^{2+}-store depletion using TG enhanced the interaction between TRPC6

with Orai1 and STIM1. In contrast, stimulation with OAG displaces TRPC6 from the Orai1/STIM1 complex and promotes the association of TRPC6 with the non-capacitative channel TRPC3. The interaction between TRPC6 and TRPC3 was abolished by cell loading with the intracellular Ca^{2+} chelator dimethyl-BAPTA, thus indicating that this phenomenon is Ca^{2+}-dependent [55]. Altogether, these findings support the hypothesis that TRPC6 might be a point of convergence between store-operated and store-independent Ca^{2+} influx pathways, participating in both mechanisms through its interaction with the Orai1/STIM1 complex or TRPC3/Orai3, respectively.

10.7.2.4 Functional Role of TRPC6 in Platelets

The role of TRPC6 in platelet function has been investigated using different murine transgenic models lacking TRPC6 [56, 246–248]. However, the observations reported have been contradictory and the function of TRPC6 in platelets is still uncertain. Ramanathan and coworkers found that platelets from TRPC6 deficient mice were insensitive to DAG while SOCE and agonist-induced Ca^{2+} entry were unaltered, as well as platelet function in vivo and in vitro [56]. Furthermore, this study showed a non-statistically significant decrease in the resting cytosolic Ca^{2+} concentration in platelets isolated from TRPC6-deficient mouse [56]. Paez-Espinosa and coworkers reported that TRPC6 knockout mice show prolonged bleeding time, detected using the tail bleeding time test, as well as an increased time for occlusion of the injured carotid artery as compared to wild type mice [246]. Harper and collaborators reported that Na^{+} entry via TRPC6 is required for full activation of phosphatidylserine exposure in mice platelets, a phenomenon that is essential for the generation of additional thrombin, thus supporting the mechanism of thrombosis and haemostasis [247]. Finally, we have recently found a significant reduction in the resting cytosolic Ca^{2+} concentration in platelets from mice lacking TRPC6 [248], despite we did not detect any significant alteration in thrombin-stimulated Ca^{2+} mobilization or platelet aggregation [229]. The reason of the discrepancies reported by the different groups that explored the function of TRPC6 in mouse platelets might be attributed to the different function in vivo protocols used, the distinct procedure used for platelet isolation and analysis, and/or the intrinsic variability generated by the different genetic background of the murine strains used. For instance, models of TRPC6-deficient mice were generated by inserting a selection cassette that replaced exon 4 or exon 7.

10.7.2.5 TRPC6 and Diabetes Mellitus

Finally, in the last part of this chapter, we would like to discuss the relationships between TRPC6 and diabetes mellitus. Cardiovascular complications are a frequent cause of morbidity and mortality in patients with diabetes mellitus and platelets have been proposed to contribute to diabetic micro and macroangiopathy [249,

250]. Platelet hyperactivity and hyper-aggregability has been associated to abnormal intracellular Ca^{2+} homeostasis, which includes enhanced Ca^{2+} influx and impairment of Ca^{2+} clearance mechanisms probably due to the altered redox state [251–255]. We have analyzed the expression of different Ca^{2+}-permeable channels in platelets from type 2 diabetic patients, including TRPC1, TRPC3, TRPC6 and Orai1, as well as that of STIM1 by Western blotting and found that the expression of TRPC1 in platelets from diabetics was similar to that in healthy donors, while the expression of TRPC3, Orai1 and STIM1 was found to be enhanced in platelets from diabetic patients as compared to controls and the expression of TRPC6 is reduced in diabetics [256]. Further studies by Liu and coworkers reported that direct platelet stimulation with glucose rapidly enhanced TRPC6 expression in the plasma membrane [142], thus suggesting that the reduced expression of TRPC6 at the protein level might be compensated by the increased surface expression of the channel. These observations are consistent with more recent findings from our laboratory testing the ability of platelets from type 2 diabetic donors to activate store-operated and store-independent Ca^{2+} influx mechanisms. For this set of experiments SOCE was stimulated by TG in the presence of specific purinergic and serotoninergic receptor antagonists, to avoid secondary activation of these receptors due to the increase in cytosolic Ca^{2+} concentration. In addition, SOCE was detected using Mn^{2+} as a surrogate for Ca^{2+} to prevent interference with other Ca^{2+} influx mechanisms. We found that store-operated Mn^{2+} entry induced by TG was reduced in platelets from diabetic donors as compared to healthy controls, probably as a result of the impaired association between STIM1 and Orai1, TRPC1 and TRPC6. These findings suggest that store-independent Ca^{2+} entry mediated by TRPC3, TRPC6 and/or Orai3, might be responsible for the enhanced Ca^{2+} influx observed in platelets from diabetic patients upon stimulation with physiological agonists [257].

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Chapter 11
Transient Receptor Potential Canonical 7 (TRPC7), a Calcium (Ca^{2+}) Permeable Non-selective Cation Channel

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Abstract Transient receptor potential canonical subfamily, member 7 (TRPC7) is the most recently identified member of the TRPC family of Ca^{2+}-permeable non-selective cation channels. The gene encoding the TRPC7 channel plasma membrane protein was first cloned from mouse brain. TRPC7 mRNA and protein have been detected in cell types derived from multiple organ systems from various species including humans. G_q-coupled protein receptor activation is the predominant mode of TRPC7 activation. Lipid metabolites involved in the phospholipase C (PLC) signaling pathway, including diacylglycerol (DAG) and its precursor the phosphatidylinositol-4,5-bisphosphate ($PIP_{2)}$, have been shown to be direct regulators of TRPC7 channel. TRPC7 channels have been linked to the regulation of various cellular functions however, the depth of our understanding of TRPC7 channel function and regulation is limited in comparison to other TRP channel family members. This review takes a historical look at our current knowledge of TRPC7 mechanisms of activation and its role in cellular physiology and pathophysiology.

Keywords TRPC7 • Non-selective cation channel • Diacylglycerol • PIP_2 • Phospholipase C • Ca^{2+} signaling • Non-excitable cells

11.1 Gene and Expression

Transient receptor potential canonical subfamily (TRPC) channels are a family of non-selective cation plasma membrane channel proteins. Seven members have currently been identified in mammals (TRPC1-7) [1] while 6 TRPC channel proteins have been identified in humans (TRPC1, TRPC3-7) [2]. The most recently identified member of this family is TRPC7. Below is a brief timeline and discussion of the species specific characterization of the TRPC7 gene and expression patterns of this cation channel (Table 11.1) [3].

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Table 11.1 Species specific TRPC7 expression

Species	mRNA/protein	Date of characterization
Mouse	**mRNA**:	
	Heart, lung, eye, brain, spleen, and testis (1)	1999
	Smooth muscle cells (2)	2001
	MC3T3 osteoblasts (3)	2009
	Embryonic brain and cortex of E13 C57BL6/J mice (4)	2009
	Melanopsin-expressing ganglion cells (5)	2011
	Brain, testis, lung, liver, heart, kidney, and DRG (6)	2012
	MnPO Glutamatergic Neurons (7)	2012
	Protein:	
	Retina (8)	2007
	Cerebellum and ventral respiratory group island (9)	2010
Rat	**mRNA**:	
	Hippocampal H19-7 cells (10, 11)	2004
	A7r5 vascular smooth muscle cells (12)	2006
	Failing myocardium of Dahl salt-sensitive rats (13)	2007
	Cultured rat microglia (14)	2009
	Hypocretin/orexin neurons (15)	2010
	Protein:	
	Brain synaptosomes (16)	2002
	Ganglion neurons (17)	2003
	H19-7 cells (10, 11)	2004
	GH4C1 pituitary cells (18)	2008
	A7r5 vascular smooth muscle cells (12)	2006
Human	**mRNA**:	
	Central nervous system and pituitary gland, kidney, intestine, prostate and cartilage (19)	2002
	Pregnant women myometrium and myometrial cell lines (20, 21)	2002
	Coronary artery endothelial cells (22)	2004
	HEK293 cells (23)	2005
	Undifferentiated gingival keratinocytes (24)	2005
	Differentiated IMR-32 neuroblastoma cells (25)	2006
	HaCaT keratinocyte (26)	2006
	Human breast cancer cell line (ZR-75-1, MCF7 and MDA-MB-231) and human breast epithelial cell line (hTERT-HME1) (27)	2014
	Lung tissue (28)	2010
	Protein:	
	HEK293 cells (29)	2005

(1) Okada et al. [4], (2) Walker et al. [5], (3) Abed et al. [10], (4) Boisseau et al. [7], (5) Perez-Leighton et al. [11], (6) Jang et al. [9], (7) Tabarean [12], (8) Sekaran et al. [6], (9) Ben-Mabrouk and Tryba [13], (10) Wu et al. [22], (11) Numaga et al. [21], (12) Maruyama et al. [23], (13) Satoh et al. [18], (14) Ohana et al. [19], (15) Cvetkovic-Lopes et al. [20], (16) Goel et al. [14], (17) Buniel et al. [15], (18) Lavender et al. [24], (19) Riccio et al. [29], (20) Yang et al. [31], (21) Dalrymple et al. [30], (22) Yip et al. [32], (23) Zagranichnaya et al. [34], (24) Cai et al. [33], (25) Nasman et al. [36], (26) Beck et al. [35], (27) Gogebakan et al. [38], (28) Finney-Hayward et al. [37], (29) Zagranichnaya et al. [34]

Mouse The gene encoding the TRPC7 channel protein was first isolated from mouse brain by Okada et al. in 1999. This gene is made up of 12 exons, and is located at chromosomal region 13 B2. TRPC7 mRNA is highly expressed in mouse eye, lung, and heart; expression was also detected in the brain, spleen and testis, although to a lesser extent [4]. In 2001, TRPC7 transcripts and splice variants were detected in smooth muscle derived from murine colon and jejunum [5]. In 2007, TRPC7 was identified in the mouse retina [6]. In 2009, Boisseau et al. examined the expression patterns of TRPC channels in the embryonic forebrain of C57BL6/J mice; at E13 TRPC7 mRNA expression was detected although to a lesser extent than other TRPC family members [7]. No TRPC7 mRNA expression was detected in skeletal muscle and inner ear organs from embryonic and early postnatal Swiss Webster mice [8, 9]; these studies highlight a development-dependent pattern of expression. TRPC7 mRNA expression was also observed in the mouse osteoblast cell line, MC3T3 in a 2009 study [10]. Recently major focus has centered on characterizing TRPC7 in neuronal cells after TRPC7 was identified in photosensitive retinal ganglion cells in 2011 [11] and glutamatergic preoptic neurons in 2012 [12]. In 2010, examination of mouse brain stem slices containing the respiratory-regulating neuronal bundle, the pre-Bötzinger complex, detected TRPC7 protein expression localized to this regulatory region [13].

Rat Rat TRPC7 genes are also made up of 12 exons, and have been mapped to chromosomal region 17p14. In rat, TRPC7 is predominantly expressed in the nervous system. In 2002, TRPC7 protein was isolated from brain synaptosomes [14]. In 2003, TRPC7 mRNA expression was detected in neurons throughout the ganglia [15]. TRPC7 protein was observed in rat striatal cholinergic interneurons [16], and had a strikingly high level of expression in the neuropil in the rat globus pallidus [17]. In 2007, TRPC7 expression was found to be up-regulated in the failing myocardium of Dahl salt-sensitive rats [18]. Characterization of TRPC7 mRNA expression has also been analyzed in various rat cell lines, including cultured rat microglia where expression was detected in 2009 [19] and in hypocretin/orexin neurons in 2010 [20]. In rat hippocampal H19-7 cells, the levels of mRNA and protein for TRPC7 are high in proliferating cells and decline upon differentiation [21, 22]. In rat vascular smooth muscle cell line A7r5 [23] and pituitary cell line GH_4C_1 [24] TRPC7 transcripts were also detected. TRPC7 mRNA was not detected in freshly isolated rat renal resistance vessels, glomeruli, and aorta [25], nor was it found in rat distal pulmonary arterial smooth muscle [26], and rat dorsal root ganglia (DRG) neurons [27].

Human In 2002, Riccio et al. cloned human TRPC7 gene from brain [28]. The gene encoding human TRPC7 is located at the chromosomal region 5q31.1, and similarly to the mouse and rat gene, has 12 exons. Both human and mouse TRPC7 genes consist of an open reading frame of 2,589 bp yielding a protein of 862 amino acids [4, 28]. Human TRPC7 has 98 % sequence homology with mouse TRPC7 [21]. Human TRPC7 mRNA expression is mostly restricted to the central nervous system however expression has been detected in some peripheral tissues including

cartilage, intestine, kidney, pituitary gland and prostate [29]. TRPC7 expression has also been examined in various human cell lines. In 2002, human myometrial cells were found to express TRPC7 mRNA, and its expression was upregulated in myometrium obtained during active labor from full term pregnant women [30, 31]. In 2004, TRPC7 expression was found in human coronary artery endothelial cells, although not in vascular smooth muscle cells [32]. In 2005, TRPC7 mRNA was also detected in undifferentiated human gingival keratinocytes [33] and protein expression was observed in the human embryonic kidney (HEK) 293 cell line [34]. In 2006, detection was observed in human differentiated keratinocytes [35] and differentiated IMR-32 neuroblastoma cells [36]. In 2009, studies failed to detect TRPC7 mRNA in human osteoblast cell lines MG-63, SaOS, and U2 OS [10], while in 2010 expression was observed in lung tissue [37]. Recently TRPC7 expression was characterized in various breast cancer cell lines including ZR-75-1, MCF7 and MDA-MB-231 and the human breast epithelial cell line hTERT-HME1 [38].

11.2 Protein Structure and Channel Configuration

TRPC7 is composed of 862 amino acid residues [4]. TRPC channel proteins including TRPC7 possess a similar basic domain structures. These defining structures include a cytosolic N-terminal domain containing four ankyrin repeats, six transmembrane spanning domains, within transmembrane domain 5 and 6 lies a highly conserved hydrophobic α helix pore-loop motif and finally a cytosolic C-terminal region [39]. This carboxy terminal region is made up of the TRP domain containing an EWKFAR and proline rich domain [40]. In 2001, Tang et al. further characterized the TRPC family C- terminal domain by identifying a calmodulin and Inositol1,4,5-Trisphosphate (IP_3) receptor binding (CIRB) region [41]. Variable regions between TRPC family members exist within this C-terminal domain contributing to their differential modes of regulation [42].

TRPC family proteins are believed to assemble into tetramers. The stoichiometry of this assembly varies depending on both TRPC family member and cell type, as homo- and heterotetrameric assembly has been proposed [43]. Specifically, TRPC7 has been shown to selectively interact with TRPC3 and TRPC6. TRPC3/6/7 channels share 70–80 % structural homology and are members of a sub-family within the TRPC family [4, 39, 43]. Co-expression of these channels has been observed in various smooth muscle tissues [44] suggesting possible heterotertrameric assemblies. However, existence of cell specific expression of different TRPC channel isoforms does not necessarily mean heteromultimeric assembly.

A study examined native TRPC7 and TRPC6 function highlighting that both the assembly and function of these channels is cell type specific. Biochemical analysis in rabbit portal vein myocytes determined strong association between TRPC6 and TRPC7 and single channel patch recordings in cells treated with anti-TRPC6 and anti-TRPC7 antibodies concluded both of these subunits were necessary for channel activity. However, examination of myocytes from rabbit mesenteric arteries revealed

that the biochemical and functional characteristics of these channel proteins were not the same [45]. More studies assessing native TRPC7 expression and function and the use of TRPC7 deficient animals are necessary to further understand in vivo the native assembly and function of these tetrameric channels [39]. While TRPC7 has been well characterized as a plasma membrane channel, one overexpression study examining localization of this channel protein in COS-7 cells observed a pool of intracellular TRPC7 protein that co-localized with the golgi network. Similar patterns of endogenous TRPC7 distribution were observed in the rat pituitary cell line, GH_4C_1. This localization within the golgi stack was thought to enhance cellular secretion either by mediating cargo transport or plasma membrane fusion [24].

11.3 Biophysical Properties of TRPC7 Channel

Okada et al. studied the permeability of TRPC7 channel using patch clamp recordings from TRPC7-expressing cells and showed constitutively activated and ATP-enhanced inward cation currents mediated by TRPC7 with permeability ratios P_{Cs}:P_{Na}:P_{Ca}:P_{Ba} of 1:1:1.9:3.5 and 1:1.1:5.9:5.0, respectively [4]. Lievremont et al. used Fura2 imaging and observed that diacylglycerol analog OAG- or muscarinic receptor stimulation-induced Ba^{2+} entry that was significantly reduced in TRPC7-deficient DT40 cell, further confirming that TRPC7 channel permeates Ba^{2+} ions [46]. Shi et al. reported that the current-voltage (I-V) relationship obtained from HEK293 cells transfected with mouse or human TRPC7 gene is almost linear with a slight flattening around the reversal potentials; mouse TRPC7 single-channel conductance is 24.3 pS and 24.8 pS under bath solutions containing 10 mM Ca^{2+} and 1 mM Ca^{2+}, respectively. However, when cells were bathed in Ca^{2+} free solution, single-channel conductance was increased to 49.3 pS [42]. Okada et al. also showed that heterologously expressed mouse TRPC7 behaves as receptor-operated DAG-activated cation channel that is insensitive to store depletion. Subsequent studies on native TRPC7 in DT40 cells and on TRPC7 ectopically expressed in HEK293 cells further suggested that TRPC7 forms a store-independent receptor-operated channel [4, 46, 47]. Thus, TRPC7 is a receptor-regulated, store-independent, non-selective cation channel activated through phospholipase C (PLC)-mediated metabolism of phosphatidylinositol 4,5-bisphosphate (PIP_2) and production of DAG [4, 48]. Regarding the pharmacological profile of TRPC7, few inhibitors are known to interfere with TRPC7 activation and none of them are specific to TRPC7. Okada et al. showed that ATP-activated mouse TRPC7 ectopically expressed HEK293 cells is more efficiently inhibited with 100 μM lanthanum (La^{3+}) and 25 μM SKF96365 than 100 μM gadolinium (Gd^{3+}) [4]. Riccio et al. used 250 μM Gd^{3+}, 250 μM La^{3+}, and 25 μM SKF96365 to completely block the Ca^{2+} entry in HEK293 cells expressing human TRPC7 [28]. 300 μM Amiloride also inhibited constitutively activated TRPC7 currents, and 2-aminoethoxydiphenyl borate (2-APB) at concentrations of 10–100 μM partially inhibited TRPC3, TRPC6, and TRPC7 channels by a mechanism not involving the known inhibitory effects of 2-APB on IP_3 receptors [4, 49].

The Schaefer group has screened a chembionet library and found three compounds, 6228-0353, 6228-0473 and 2910-0498 showing strong inhibition towards TRPC7 channels [50].

11.4 TRPC7 Channel Regulation and Function

The exact regulatory mechanisms that control TRPC7 channel activity have been the subject of contention since TRPC7 was first cloned in 1999 with many discrepancies between different groups. TRPC7 channel activity has been shown to vary depending on the molecular reagents used and the expression levels of this channel protein in heterologous model systems [51]. The use of cell specific TRPC7 gene deficient transgenic animals will likely generate more accurate and reproducible characterization of TRPC7 mediated channel activity. The general consensus is that TRPC7 is a receptor-activated channel that depends on DAG production through the catalytic activity of PLC. Below is a brief description of the current understanding of the mediators that regulate TRPC7 channel activity.

11.4.1 Metabolites of the PLC Pathway as Regulators of TRPC7 Channel

G_q-coupled receptor mediated activation is the predominant mode of regulation of the TRPC7 channel. Receptor mediated activation of PLC leads to hydrolysis of PIP_2 and the production of the signaling molecules IP_3 and DAG. TRPC7 channel function was first characterized by ectopically expressing this protein in cultured HEK293 cells [4]; this study proposed DAG as the activator of TRPC7 channels based in the use of exogenous OAG. Addition of OAG to the bath solution during whole-cell patch clamp recordings activated TRPC7 currents in a PKC-independent manner. Subsequently, Beck et al. reported that in human keratinocytes, OAG evoked a TRPC7 channel cation current, and this OAG-induced current was decreased by TRPC7 knock down [35]. Itsuki et al. identified TRPC7 activity being mediated by DAG produced through the hydrolysis of PIP_2 by PLC and reported a requirement for PIP_2 in channel activation as TRPC7 current subsequently inactivated upon hydrolysis of PIP_2. Further, PIP_2 depletion by a heterologously expressed phosphatase inhibited TRPC7 activity independently of DAG [52]. Patch clamp recordings on TRPC7-expressing HEK293 cells using the cell-attached mode detected TRPC7 currents after addition of OAG, but OAG failed to activate TRPC7 channels in excised patches. This finding suggests that DAG does activate TRPC7 channels rather indirectly perhaps through cytosolic proteins or factors that are lost in excised patches [53].

PIP_2 has been shown to directly regulate several ion channels, including the broader TRP family of channels [3, 53]. The ability of PIP_2 to directly activate TRPC7 as well as members of its subfamily TRPC3 and TRPC6 was first observed in 1998 using the phosphoinositide lipid kinase inhibitor, LY294002 which causes PIP_2 depletion [54]. Treatment of HEK293 cells overexpressing TRPC7 with this drug prevented receptor- and OAG-mediated TRPC7 channel activation. In excised inside – out patches, direct application of either PIP_2 or ATP activated TRPC7 single channels while IP_3 application had no effect [53]. Imai et al. [55] used an ectopic expression system of the voltage-sensing PIP phosphatase (DrVSP) and showed that Carbachol, OAG or RHC80267 (a DAG lipase inhibitor that enhances endogenous DAG and thus activates TRPC7) -mediated channel activation was inhibited by DrVSP activation. The extent of phosphatase inhibition was TRPC isoform specific as TRPC7 currents were attenuated to a larger extent than either TRPC6 or TRPC3 currents. Ju et al. used antibodies to inhibit TRPC isoforms in inside-out patches and concluded that noradrenaline-activated native cationic currents in portal vein myocytes are mediated by TRPC6/TRPC7 heteromultimers. These authors reported that PIP_2 inhibited this OAG-activated TRPC6/TRPC7 channel, and that this PIP_2 inhibition is rescued by IP_3 through an IP_3 receptor-independent manner [45]. The reasons for the discrepancy between this study and those reporting PIP_2 requirement for TRPC7 channel activation are unclear.

11.4.2 TRPC7 and Store Operated Ca^{2+} Entry

TRPC7 along with other TRPC channels have been proposed to constitute the pore forming unit of store-operated calcium entry (SOCE) channels [56]; this notion however remains highly controversial. In stably expressing TRPC7 HEK-293 cells either pharmacological Ca^{2+} store depletion with thapsigargin or activation of the PLC signaling pathway was able to promote TRPC7 channel activation [57]. However, in a transient expression system only stimulation of PLC-coupled receptors induced TRPC7 activation. STIM1, the ER residing Ca^{2+} sensor [58, 59] and Orai1, the highly Ca^{2+}-selective plasma membrane ion channel subunit have been established as the *bona fide* molecular components of SOCE and the calcium release-activated calcium (CRAC) current [60]. A number of studies have examined the role of STIM1 and Orai1 in regulating TRPC7-mediated calcium entry. In stably expressing TRPC7 HEK293 cells, silencing of STIM1 and Orai1 proteins by RNA interference did not affect TRPC7 activity nor did the expression of a constitutively active STIM1 mutant [47]. Studies from one group however suggested that STIM1 is capable of regulating specific isoforms of TRPC channels. Huang et al. studied the interaction between STIM1 and TRPC channels, concluding that STIM1 ERM domain mediates the selective binding of STIM1 to TRPC1, TRPC2 and TRPC4, but not to TRPC3, TRPC6 or TRPC7 [61]. Subsequently, the same group reported that STIM1 binds TRPC1, TRPC4 and TRPC5 allowing them to function as

store-operated channels. The authors also demonstrated that STIM1 indirectly regulates TRPC3 and TRPC6 channels by mediating the heteromultimerization of TRPC3 with TRPC1 and TRPC6 with TRPC4. In these studies, TRPC7 was the only TRPC family member that was not regulated by STIM1 [62].

11.4.3 Ca^{2+} and Calmodulin

Most Ca^{2+}-permeable channels including TRPC7 channels are themselves regulated by Ca^{2+}. Specifically studies have shown that TRPC7 channels can be negatively regulated by Ca^{2+} both directly and indirectly. Lemonnier et al. identified a negative feedback mechanism of TRPC7 channels in HEK293 cells, whereby Ca^{2+} entry through TRPC7 channel negatively regulates its own activity [63]. This Ca^{2+}-mediated negative feedback at the mouth of TRPC7 channels is more pronounced when the SERCA pump is inhibited by thapsigargin, suggesting that physiologically SERCA pumps that are closely associated with TRPC7 channels attenuate this negative feedback by buffering calcium into the endoplasmic reticulum. Indeed, application of the SERCA pump inhibitor thapsigargin or CPA prevented OAG-activated TRPC7 channel activation. The inhibitory effect of thapsigargin was reversed by inhibition of calmodulin and was recapitulated by pharmacological disruption of the actin cytoskeleton. Shi et al. used whole-cell and single-channel recordings to reveal that voltage-dependent inhibitory actions of extracellular Ca^{2+} on TRPC7 channel currents are likely mediated through Ca^{2+} interaction with an extracellular site capable of sensing the membrane potential. This group also reported a concentration-dependent inhibitory effect of intracellular Ca^{2+} on TRPC7 channel currents, which they suggested was mediated by calmodulin [42].

The variable C-terminal domain distinguishes members of the TRPC family from one another [42]. TRPC7 has the CIRB domain within this variable region that contains binding sequences for both calmodulin and the IP_3R. Direct interactions between these proteins and TRPC7 have been shown to regulate TRPC7 channel activity. As discussed above, Shi et al. used cell-attached mode single-channel recordings to show that cytosolic Ca^{2+}-mediated inhibition of TRPC7 channel activity was strongly attenuated by pretreatment with pharmacological agent calmidazolium (CMZ), a potent calmodulin (CaM) antagonist or coexpression of the calmodulin mutant, mutCaM. These findings suggests binding of calmodulin to the TRPC7 C-terminal CIRB domain inhibits channel activity [42]. In the avian B-cell line, DT40, using the cell-attached mode of patch clamp, OAG activated single channel activity was not observed in $TRPC7^{-/-}$ cells, nor in $IP_3R^{-/-}$ cells. Interestingly, exogenous expression of either TRPC7 in the $TRPC7^{-/-}$ cells or IP_3R in the $IP_3R^{-/-}$ cells rescued OAG-activated single channel activity to the same extent as wild type cells [51]. These findings support a native role for the TRPC7 channel protein in mediating DAG-activated currents that are dependent on IP_3R.

11.4.4 N-Terminal Binding Proteins

The N-terminal domain of TRPC7 is made up of four ankyrin-like repeats. These protein-protein interacting domains have been shown to regulate TRPC7 channel activity and localization. Co-immunoprecipitation studies revealed that the cytosolic cGMP/cGMP-dependent protein kinase (cGK) isoform, cGK-I α binds to the N-terminal ankyrin-like repeat domain of TRPC7. Yuasa et al. also observed cGK-I α phosphorylates TRPC7 on its threonine 15 site without any effect on TRPC3. This TRPC7 specific phosphorylation reduced carbachol-activated calcium signaling and phosphorylation of the transcription factor, CREB [64]. Lussier and colleagues used protein-protein interaction assays, including yeast two-hybrid screen, GST pull-down, and co-immunoprecipitation and demonstrated that MxA which is a member of the dynamin GTPase superfamily that interacts with the N-terminal second ankyrin-like repeat domain of TRPC1, TRPC3, TRPC4, TRPC5, TRPC6, and TRPC7 [65]. It was proposed that MxA enhances channel activity by regulating trafficking of the channel proteins to the plasma membrane. Although this functional role has not been tested for TRPC7, mutation studies involving subfamily members TRPC3 and TRPC6 have shown deletion of the ankyrin-like repeat domain leads to intracellular retention of channel protein, inhibition of plasma membrane localization and overall channel dysfunction [43, 66]. Future studies looking at TRPC7 channel specific protein trafficking are necessary to gain insights into TRPC7 regulation through membrane trafficking.

11.5 TRPC7 in Pathophysiology of Disease

Altered TRPC7 channel expression and activity are observed in various diseases including malignant breast cancer tumors. Regulation of multiple TRPC genes has been shown to be mediated by the Rho kinase pathway [67]. Rho kinase activity has been associated with metastasis of esophageal cancer cell lines and inhibition of this pathway using the pharmacological inhibitor Y-27632 prevented the growth and invasiveness of these cancer cells [68]. In human breast cancer cell lines ZR-75-1, MCF7, and MDA-MB-231 and the human breast cancer epithelial cell line hTERT-HME1 inhibition of the Rho-kinase pathway with Y-27632 increased the expression of TRPC7 in all cell lines as detected by quantitative real time PCR [38]. Interestingly the Rho-kinase inhibitors attenuated expression of TRPC1 and another TRP family member TRPV2. This study suggests a potential protective role of TRPC7 in cancer cell growth and progression.

TRPC7 expression has also been shown to be up-regulated in animal models of disease. In Dahl salt sensitive rats, TRPC7 mRNA was increased in the failing myocardium of these animals. This increased expression correlated with an increased level of apoptosis as assessed by TUNEL staining. Heart failure in these mice was

suggested to be contributed by angiotensin II-induced Ca^{2+} entry through activation of TRPC7 and subsequent myocardial apoptosis [18].

In a mouse model of pilocarpine induced status epilepticus, TRPC7 was shown to mediate the initiation of acute seizures, as observed by the reduction in pilocarpine-induced gamma wave activity in TRPC7 knockout animals [69]. The mechanism by which TRPC7 mediates seizure induction was characterized in brain slices derived from the hippocampal region cornu ammonis CA3, a central region involved in seizure generation. TRPC7 was shown to generate spontaneous epileptiform burst firing, these signals of seizure induction were initiated at the synaptic level where TRPC7 was shown to be involved in the potentiation of these signals in the CA3 synapses and Schaffer collateral-CA1 synapses. This study using TRPC7 knockout animals was one of the first studies to provide insight into the in vivo function of this enigmatic channel protein.

11.6 Conclusion

Since the initial characterization of the mouse TRPC7 gene in 1999 [4], the following two decades of research studying this channel protein have shown that: (i) TRPC7 expression is present in both excitable and non-excitable cells; (ii) Biophysical and functional studies have described TRPC7 as a store-independent DAG-activated Ca^{2+}-permeable non-selective cation channels [47, 49]; (iii) The mode of TRPC7 channel regulation is likely complex, attracting much debate, including the role of PIP_2 in regulating TRPC7 function. Channel heteromultimerization with other TRPC isoforms and isoforms of the broader TRP superfamily are likely to enhance the diversity of signaling mechanisms through TRPC7. Thus far, TRPC7-associated diseases have rarely been reported. With increasing number of studies employing transgenic TRPC7 knockout animals, understanding of the various physiological functions controlled by this dynamic Ca^{2+}-permeable non-selective cation channel is likely increase.

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Chapter 12
Calcium Entry Through Thermosensory Channels

Francisco J. Taberner, Isabel Devesa, and Antonio Ferrer-Montiel

Abstract ThermoTRPs are unique channels that mediate Na^+ and Ca^{2+} currents in response to changes in ambient temperature. In combination with their activation by other physical and chemical stimuli, they are considered key integrators of environmental cues into neuronal excitability. Furthermore, roles of thermoTRPs in non-neuronal tissues are currently emerging such as insulin secretion in pancreatic β-cells, and links to cancer. Calcium permeability through thermoTRPs appears a central hallmark for their physiological and pathological activities. Moreover, it is currently being proposed that beyond working as a second messenger, Ca^{2+} can function locally by acting on protein complexes near the membrane. Interestingly, thermoTRPs can enhance and expand the inherent plasticity of signalplexes by conferring them temperature, pH and lipid regulation through Ca^{2+} signalling. Thus, unveiling the local role of Ca^{2+} fluxes induced by thermoTRPs on the dynamics of membrane-attached signalling complexes as well as their significance in cellular processes, are central issues that will expand the opportunities for therapeutic intervention in disorders involving dysfunction of thermoTRP channels.

Keywords TRP • ThermoTRP • Structure-function • Signaling • Pathology • Targets

12.1 Introduction

Transient receptor potential (TRP) channels comprehend a large family of ion channels that play a broad diversity of physiological functions [1]. These channels are widely expressed in a large number of tissues and genetic studies have linked mutations in them to human diseases [2, 3]. The majority of TRP channels are non-selective cation channels that permeate Na^+ and K^+, and most of them display significant Ca^{2+} permeability. Ca^{2+}-influx through TRP channels is physiologically

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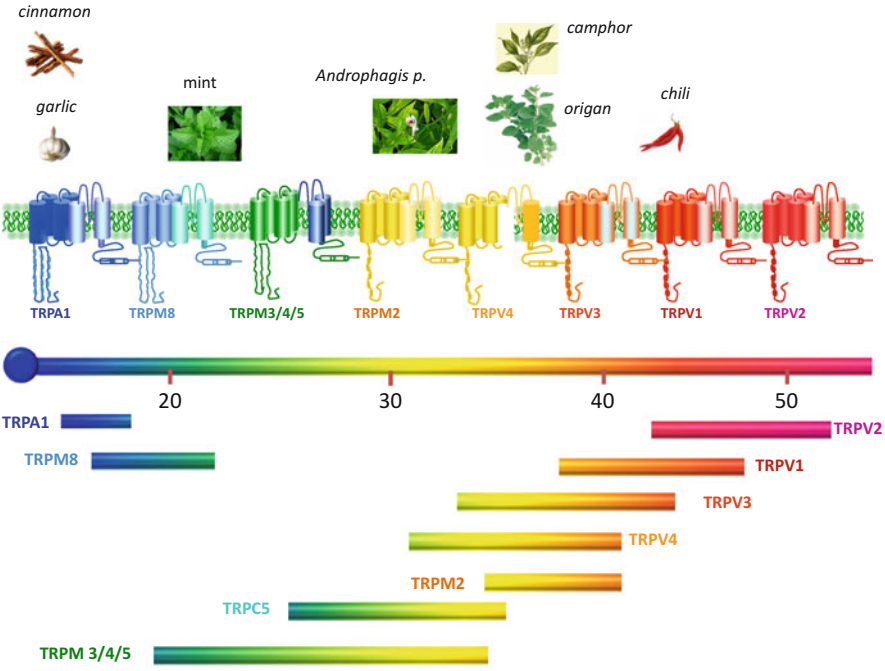

Fig. 12.1 ThermoTRP channels. Structurally thermoTRP tetramers and each subunit contains six transmembrane domains (S1–S6), a hydrophobic pore loop linking transmembrane S5 and S6, and large cytoplasmic *N*- and *C*-terminals (NB not drawn to scale). All thermoTRPs have a variable number of ankyrin repeat domains in the *N*-terminus, except *TRPM8* which has none and instead contains TRPM homology region. ThermoTRPs display distinct thermal thresholds from very noxious cold (TRPA1) to harmful hot (TRPV2). Each thermoTRP is also activated by specific natural or synthetic compounds, known to induce the relevant thermal and pain sensations in humans (Figure adapted from [4])

and pathophysiologically important due to the critical role regulating diverse cellular functions. In mammals, the TRP family consists of 28 different members grouped in 7 subfamilies: TRPC (classical or canonical), TRPV (vanilloid), TRPM (melastatin), TRPA (ankyrin-like), TRPP (polycysteine), TRPML (mucolipin) and the TRPN (no mechanoreceptor potential C; NOMPC), while the TRPY is present in yeast. For a detailed review, see the excellent book edited in 2014 by Nilius and Flockerzi.

Thermosensory channels, also named "thermoTRPs", define a subfamily of the TRP channels that are activated by changes in the environmental temperature, from noxious cold (<15 °C) to injurious heat (>42 °C) (Fig. 12.1). However, these channels are also activated by a wide range of other physical (voltage, pressure) and chemical stimuli [1]. Acting as integrators of several stimuli and signaling pathways, dysfunction of these channels contributes to diverse pathophysiological conditions such as chronic inflammation, neurological disorders, pain or cancer among others [2, 5–7]. For this reason, thermoTRPs have become promising drug targets,

and the development of therapeutic compounds for pharmacological intervention is actively pursued by the academy and the industry [8, 9].

ThermoTRPs participate in Ca^{2+} homeostasis and signaling through different mechanisms: (i) most of them are Ca^{2+} permeable channels allowing extracellular Ca^{2+} entry; (ii) channel gating can be directly modulated by Ca^{2+}, but can also be indirectly regulated by Ca^{2+}-dependent proteins or signaling pathways; (iii) an increase of Ca^{2+} influx through TRP induces Ca^{2+}-dependent exocytosis promoting the release of mediators as well as trafficking of new thermoTRP receptors to the plasma membrane.

Here, we will describe the current knowledge in Ca^{2+}-dependent signaling and modulation of the classical thermosensory channels, namely TRPV1, TRPV2, TRPV4, TRPM8, and TRPA1, as well as the recently included TRPM2, TRPM3, TRPM4, TRPM5 and TRPC5. This book chapter will focus on the direct and indirect effects of Ca^{2+} on/ or through the thermoTRP channels (Table 12.1). The important role of these receptors has opened exciting research lines driven to the development of novel therapeutic approaches with successful clinical results in the treatment of several pathologies.

12.2 Structural Insights into Ion Selectivity and Permeation Through ThermoTRPs

The generally accepted functional TRP channel is an ensemble of four identical subunits. Akin to voltage gated potassium channels each subunit is composed of six membrane-spanning alpha-helices (S1–S6) flanked by the cytosolic N and C-termini [10–13]. By folding together, transmembrane helices S5 and S6 from each subunit delineate the ion-conducting pore whose opening is influenced by agonist-sensing modules in other regions of the protein [6, 12, 14]. Except for the TRPM family, the N-terminus of the channels contains a varying number of ankyrin repeat domains (ARDs) which are involved in channel regulation [15]. Agonist-driven activation requires the TRP domain in the C-terminus [16–18], a 25 amino acids region that is hallmark in TRPC, TRPV and TRPM ion channels. The region contiguous to the S6 plays a central role in the allosteric regulation of TRPV1 and TRPM8 by coupling the stimuli-induced change to open the channel's gate [14, 19, 20].

ThermoTRP channels permeate non-selectively mono and divalent cations, with a discrimination principally dictated by the selectivity filter (SF) located in the loop bridging S5–S6 transmembrane helices. The largest fractional current is due to Na^+ permeation with Ca^{2+} playing a minor contribution. Among the different TRPs, the sequence homology at the SF is limited. However, for members of the TRPV family, the SF signature GM(L)GD has been proposed. The range of selectivity for Ca^{2+} ions varies from channels impermeable to Ca^{2+} (TRPM4 and TRPM5) to channels highly selective (TRPV1, $P_{Ca^{2+}}/P_{Na^+} \sim 10$).

Table 12.1 Ca^{+2} -permeability and -modulation of thermoTRP

Channel	Temperature	Ca^{2+} permeability (estimated P_{Ca}/P_{Na})	Ca^{2+} dependent modulation
TRPV1	>42 °C	10	Desensitization by:
			Calcineurin activation
			High PLC activation
			Sensitization by:
			CaMKII phosphorylation
			Mild PLC activation
			Regulated exocytosis
TRPV2	>52 °C	1–3	Desensitization by:
			PLC activation
			Sensitization by:
			Membrane mobilization
TRPV3	>33 °C	10–11	Rectification due to extracellular Ca^{2+}
			Desensitization by:
			CaM binding
			Sensitization by:
			PLC activation
TRPV4	24–38 °C	6	Rectification due to extracellular Ca^{2+}
			Desensitization by:
			CaM competing with ATP potentiation*
			Potentiation by:
			CaM Binding*
			*Depends on $[Ca^{2+}]$
TRPM2	>35 °C	0.4–1.8	Potentiation depends on CaM-Ca^{2+}
TRPM3	>35 °C	1.6–10	CaM-Ca^{2+} isoform dependent regulation
TRPM4	15–35 °C	Non permeable	Activation by:
			Direct activation Ca^{2+}
			CaM-Ca^{2+}
			Desensitization by:
			Ca^{2+} dependent PIP2 depletion
TRPM5	>15 °C	Non permeable	Activation by:
			Ca^{2+} or unknown Ca^{2+} interacting protein
TRPM8	<22 °C	1–3.3	Inhibition by:
			Extracellular Ca^{2+}
			Desensitization by:
			Ca^{2+} dependent PLC
			CaM-Ca^{2+}

(continued)

Table 12.1 (continued)

Channel	Temperature	Ca^{2+} permeability (estimated P_{Ca}/P_{Na})	Ca^{2+} dependent modulation
TRPC5	25–37 °C	9–10	Inhibition by interaction with:
			CaM-Ca^{2+}
			CaBP1-Ca^{2+}
TRPA1	<10 °C species dependent	0.8–6	Potentiation by:
			Ca^{2+} entry
			Ca^{2+} dependent membrane recruitment of channels
			Desensitization by:
			Sustained Ca^{2+} entry

The major insights into the structural determinants of Ca^{2+} permeability derive from structure-function studies guided by sequence homology and aided by molecular dynamic simulations. Studies in TRPV5, a highly Ca^{2+} selective channel, indicate that selectivity and permeation properties are mainly determined by a ring of Asp residues within the pore [21], reminiscent to the ring of four negative residues present in voltage gated Ca^{2+} channels [21–24]. Exchange of residues in the S5-S6 loop of the warm-temperature activated Ca^{2+} impermeable TRPM4, with amino acids of the Ca^{2+} selective TRPV6, yielded a functional Ca^{2+} permeable channel that recapitulated the TRPV6 sensitivity to extracellular Ca^{2+} and Mg^{2+} blockade [25], substantiating the role of Asp residues in Ca^{2+} permeability. Studies in thermoTRPs further support the requirement of negatively charged residues in ion selectivity. Substitutions of Gln981 and Pro983 of the low Ca^{2+} selective human TRPM2 with equivalent residues Glu and Tyr present in more Ca^{2+} permeable TRPM2 channels from other organisms, increased the Ca^{2+} permeability by fourfold [26]. Conversely, neutralization of the negatively charged Asp546 and Glu682 in the TRPV1 and TRPV4 selectivity filter reduced the divalent permeability of the channels [27, 28]. Noteworthy, evidences in TRPM2 and TRPV4 indicate that residues outside the SF can also contribute to Ca^{2+} selectivity [28, 29].

12.2.1 Pore Dilation

The high resolution TRPV1 structure unveiled that, in contrast to the SF of Nav channels, TRPV1 lacks hydrogen bonding between adjacent pore helices. This feature results in a more flexible SF [12]. Molecular dynamic simulations further support the tenet of a TRPV1 SF highly dynamic, whose conformation is greatly influenced by the permeating ion [30]. The plausible dynamic nature of the SF of thermoTRP channels could underlie the pore dilation phenomenon by which some of them can permeate large organic cations [12]. For instance, long exposure of TRPV1 to capsaicin results in induced permeability to the large monovalent cation

N-methyl-D-glutamine accompanied with major changes in Ca^{2+} permeability [31]. Noteworthy, TRPV1 pore dilation can be influenced from the intracellular side of the channels through signaling cascades that tune channel function [12]. Analogously to the vanilloid receptor, TRPA1 exposure to agonists resulted in a 3 Å channel dilation with a concomitant increase in Ca^{2+} permeability and the fraction of current mediated by Ca^{2+} ions [32, 33]. Pore dilation also allows cells to uptake the dye Yo-Pro, a convenient tool to assess increased permeability to large cations. The inability of TRPM8 to support Yo-Pro entry [34], highlights significant differences in SF flexibility among different TRP channels.

The dynamic nature of the thermoTRP channel pore suggests that modifications of residues near the SF can greatly influence channels permeability. Indeed amino acid modification in the loop S5-S6 also influences cation permeation. Glycosylation of Asp604 in TRPV1, despite not impeding membrane trafficking of the channel, is necessary for sustained Ca^{2+} entry and for Yo-Pro uptake [35].

12.2.2 TRP Heteromers

Although not frequent, thermoTRP channels also form heteromers additionally contributing to modulate the Ca^{2+} permeability spectrum of TRP channels. The cold-activated TRPC5, form heterotetramers with TRPC1, TRPC4 and other TRPC members resulting in permeation properties differing from those of the homotetramers [36–39]. Similarly, TRPV1 has been proposed to form heteromers with TRPV2 and TRPV3 which varies their electrophysiological properties [40, 41]. The use of TRPV1-TRPA1 concatemers has evidenced the compatibility of TRPV1 and TRPA1 monomers to form functional channels with different regulation [42]. The heteromerization capability also extends to TRPV4, which functional heteromers with TRPP2 and TRPC1 are involved in thermal and mechanosensation in kidney cilium and in the store operated Ca^{2+} entry in endothelial cells [43–46].

12.3 ThermoTRP Regulation by Ca^{2+}

In addition to permeate through thermoTRP channels, Ca^{2+} can greatly affect channel function by directly or indirectly influencing posttranslational modifications or protein/lipid interactions. The reports describing a direct effect of Ca^{2+} ions in thermoTRP channel regulation are scarce. However, its action is determinant for TRPM4 and TRPM5 activity, which are impermeable to this cation. Ca^{2+} released from the internal stores activates TRPM4 and TRPM5 [47, 48]. Calmodulin (CaM) can greatly influence TRPM4 functions through its binding in the C-terminus. When CaM interaction is impaired, high concentrations of Ca^{2+} still activate the channel [49], suggesting a direct effect of Ca^{2+} on channel gating. Indeed, negative residues

Asp1049 and Glu1062 within the TRP domain are proposed to form a Ca^{2+} binding site that is essential for the normal TRPM4 Ca^{2+} response [50].

Other thermoTRP channels regulated by Ca^{2+} are TRPC5 and TRPM8. Intracellular increase of Ca^{2+} concentration also activates murine TRPC5 heterologously expressed in HEK cells independently from other regulators and agonists [51, 52]. In TRPM8, extracellular Ca^{2+} opposes its activation by agonists. High amounts of Ca^{2+} (or other divalent cations) shield the effective electrical field detected by the TRPM8 voltage sensor by neutralizing negative charges in the extracellular leaflet of the membrane therefore reducing channel response [53]. TRPA1 is also directly gated by Ca^{2+}. The ability to respond to intracellular Ca^{2+} elevations resides in the EF-Hand Ca^{+2}-binding domain at the N-terminus [54, 55]. Ca^{2+} also appears to modulate TRPV1 desensitization and tachyphylaxia through Ca^{2+}-dependent covalent modification.

By acting on signaling pathways or reshaping channel interactions, Ca^{2+} indirectly regulates thermoTRP function. This regulation is complex and in most cases channel dependent as variations in Ca^{2+} concentration can result in channel activation, potentiation of the basal current, or desensitization. Therefore, the understanding of this layer of regulation deserves a closer look into specific channel biology. We next expose the specific information accrued for thermoTRPs.

12.3.1 TRPV Family

All mammalian homologues of TRPVs are Ca^{2+}-permeable channels, with the thermosensors TRPV1–4 characterized as moderately Ca^{2+}-selective cationic channels [56–58], and the non-thermally activated TRPV5 and TRPV6 as highly Ca^{2+}-selective channels.

12.3.1.1 TRPV1

TRPV1, the founder member of the TRPV1 subfamily, is the most extensively studied thermoTRP channel. Since its cloning and description as the vanilloid receptor [59], TRPV1 has been involved in central processes in different organisms. Its ability to sense different pungent compounds and noxious heat plus its regulation by inflammatory mediators has entitled it as a central player in temperature sensing, body temperature regulation or pain transduction. Besides being activated by capsaicin, is also gated by other pungent compounds such as resiniferatoxin, gingerol or piperine [60]. Remarkably, venoms from different organism such as spiders or scorpions target TRPV1 contributing to the pain sensation [61]. Additionally, TRPV1 also responds to physical stimuli including highly depolarizing voltages and noxious heat [59, 62]. TRPV1 activity is highly temperature dependent (Q10 around 25 versus 1–4 from non-thermal-activated channels) and temperatures over 42 °C readily evoke inward ionic currents [59]. Conversely, it can be pharmacologically

blocked by capsazepine, BCTC, TRPducins and other synthetic compounds [63–65].

Structure-function studies have isolated the binding sites of vanilloids as well as key determinants temperature sensing and function in TRPV1. Domain swapping among capsaicin sensitive and insensitive TRPV1 orthologues, unveiled the role of S2–S3 region in capsaicin and resiniferatoxin activation [66, 67]. Nonetheless, the region accounting for temperature sensing is not well delimited. Indeed, several regions of the protein may take part. In the N-terminus, the region adjacent to the S1 plays a critical role [68]. Mutations in the pore region enhance [69] or reduce [70] channel sensibility to heat and affect proton sensing and capsacin response as well. Transferring the C-terminus of the cold receptor TRPM8 into TRPV1, results in a chimera that responds to cooling [71]. Additionally, mutations in the TRP domain also affect heat, capsaicin and voltage activation [18] by affecting the allosteric coupling of the sensors and the gate [19]. All these findings ought to be explained by the recent high-resolution cryoelectron microscopy model of TRPV1.

The regulation of TRPV1 is complex. Long or repeated agonist stimulation desensitizes the channel, a process completely dependent on Ca^{2+}. In fact, Ca^{2+} dependent phosphatase calcineurin activation results in channel desensitization [72, 73]. Similarly, CaM appears to compete with ATP for binding to the ARD decreasing the channel response. More controversial is the role of phosphoinositides (PIs) on TRPV1. Some findings indicate that PIP_2 has an inhibitory role, and that the activation of phospholipase C (PLC) through G protein coupled receptor (GPCR) increases channel response. Other findings indicate that PIs are central for channel function and Ca^{2+} dependent PLC activity inhibits channel responsiveness [74]. On the other hand, TRPV1 response to heat or chemicals can be potentiated by pro-algesic agents through activation of different signaling cascades promoting post-traductional modification, usually through phosphorylation/dephosphorylation mechanisms by protein kinase A (PKA), protein kinase C (PKC), Src or Ca^{2+}-CaM kinase II (CaMKII). PKC phosphorylation increases the likelihood of channel opening at resting membrane potential. PKA and CaMKII phosphorylation contributes to recovery of desensitization [75, 76]. In addition to these mechanisms, pro-inflammatory mediators also promote the rapid exocytosis of a vesicle reservoir located near the plasma membrane. These vesicles contain new TRPV1 channels that are transported to the membrane through SNARE-dependent mechanism, which is highly sensitive to Ca^{2+} and sensible to botulinotoxin or to a botulinomimetic peptide treatment [77, 78].

In the peripheral nervous system, TRPV1 is highly expressed in dorsal root, trigeminal and nodose ganglia [59], mainly in small and medium peptidergic neurons, and to a lesser extent in non-peptidergic nociceptors [62, 79]. Gating of TRPV1 allows Ca^{2+} entry eliciting pain signaling, promotes Ca^{2+}-dependent release of pro-inflammatory neuropeptides calcitonin-gene related peptide (CGRP) and Substance P, and increases TRPV1 plasma membrane translocation. The enhancement of TRPV1 trafficking from large-dense core vesicles in response to exogenous stimuli facilitates rapid modulation of neuronal excitability [80]. The presence of TRPV1 in sensory nerves and in areas involved in detection, transmission and regulation of

pain revealed its potential key role in nociception. Actually, TRPV1 turned to be a thermosensor able to transduce physical, chemical and thermal noxious stimuli. Pharmacological and knockout studies have demonstrated TRPV1 as an essential channel to pain signaling during inflammation, being responsible of the development and maintenance of thermal hyperalgesia. Indeed, TRPV1 is overexpressed in several chronic painful pathologies such as rheumatoid arthritis [81, 82], osteoarthritis [83], bone cancer-induced pain [84] and several neuropathies [85, 86] among others [87]. All these evidences rapidly promoted TRPV1 as an interesting pharmacological target to develop new analgesic treatments especially in diseases with an inflammatory component.

In the central nervous system, although with some initial controversy on its brain distribution, the role of TRPV1 in synaptic transmission, neurotransmitter release and plasticity is proposed by several groups [88–90]. Prolonged activation of TRPV1 allows Ca^{2+} overload inducing Ca^{2+}-dependent programmed cell death in cortical or hippocampal neurons [91–94]. A potential contribution in cognition, perception and neuropsychiatric disorders is also suggested [95]. Nevertheless, more studies are needed before the relevance of TRPV1 in brain activity can be clearly stated.

There is an extensive list of non-neuronal tissues expressing TRPV1 channel [96, 97], with a diverse range of functions. For instance, TRPV1 participates in many aspects of skin biology [97, 98]. In keratinocytes, TRPV1- mediated Ca^{2+} influx promotes the release of extracellular molecules that may affect the activity of surrounding cells, but also regulates directional migration [99] or contributes to metalloproteinase expression, which is critical for skin inflammation and aging [100]. TRPV1 function is necessary to keep skin homeostasis, as absence of TRPV1 function results in a striking increase in skin carcinogenesis [101]. Ca^{2+} entry through this thermoTRP channel participates in bone homeostasis as well as pathophysiology. In addition to its contributions in joint pain [102], TRPV1 promotes the differentiation of osteoblasts and osteoclasts, and its suppression protects against ovariectomy-induced bone loss [103, 104].

12.3.1.2 TRPV2

The second member of the TRPV family is also gated by heat. Actually, TRPV2 presents the highest temperature activation threshold requiring thermal stimuli over 52 °C. This high temperature requirement questions its relevance as a physiological thermosensor. Indeed, TRPV2 deficient mice do not show abnormalities in thermal sensing [105]. Additionally, TRPV2 responds to mechanical stress and to various ligands as for instance 2-aminoethoxydiphenyl borate (APB), probenecid, lysophospholypids and cannabinoids. Nevertheless, most of them are not specific and act in the micromolar range. These circumstances severely hamper the study of TRPV2, further hindered by the fact that some agonists show species dependent effect. Human TRPV2 insensitivity to 2-APB is a case in point. The pharmacological toolbox for channel blockade is scarce. Only three antagonists have been

described so far, namely ruthenium red, SKF96365 and tranilast, and all of them are non-selective.

Findings on TRPV2 regulation are limited. PIP_2 interaction through the TRP domain is central for channel function since TRPV2 currents show desensitization by Ca^{2+}-dependent PIP_2 depletion [106]. In marked contrast to TRP members with ARDs, TRPV2 ARDs do not bind CaM. Indeed, it has been reported that CaM directly binds to TRPV2 C-terminus through a motif that overlaps the TRP domain. Strikingly, despite the plausible PIP_2/CaM competition, CaM addition does not result in channel desensitization. Consistent with the absence of ATP binding sites in the ARDs, the channel does not respond to intracellular changes in ATP concentration [106, 107]. Membrane mobilization of TRPV2 channels from intracellular membranes after PI3K stimulation (mainly mediated by IGF-1) has been considered an important part of the channel regulation [108]; however, this regulatory step is controversial [109].

In the peripheral nervous system, TRPV2 is expressed in many peptidergic neurons, and similar to TRPV1, TRPV2 activation allows Ca^{2+} entry and CGRP release contributing to the development of inflammatory pain [110]. Cytosolic Ca^{2+} increase through TRPV2 is important for axonal outgrowth during development [111]; while in intestinal sensory neurons regulates the intestinal motility [112].

Most of the thermosensitive TRP channels can function as multimodal receptors in pancreatic β-cells causing Ca^{2+} influx and membrane depolarization at physiological body temperature. In this context, glucose-induced insulin secretion is dependent of TRPV2-mediated intracellular Ca^{2+} increase [113], and intriguingly, insulin promotes a Ca^{2+}-dependent translocation of TRPV2 channel to the plasma membrane [114].

TRPV2 is involved in several physiological as well as pathological conditions in the immune system, where Ca^{2+} signaling through this channel plays an important role [115–117]. In response to high temperatures, as well as other physical and chemical stimuli, TRPV2 allows Ca^{2+} entry inducing pro-inflammatory degranulation of mast cells [118, 119]. In macrophages, sustained elevation of cytosolic Ca^{2+} by activation of PI3kinase pathway allows translocation of TRPV2 from the endoplasmic reticulum to the plasma membrane promoting macrophage migration [120]. In fact, disruption of TRPV2 membrane insertion reduces cytosolic Ca^{2+} increase, which critically regulates assembly of the podosome [120]. In this line, absence of TRPV2 expression reduces the recruitment of macrophages in experimental colitis [121], migration towards injured cardiomyocytes [122] and, attenuates phagocytosis [123]. Interestingly, TRPV2 shows a restricted expression in normal immune cells, in contrast, TRPV2 is widely expressed in the myeloid and lymphoid leukemias, with a very peculiar expression of this channel in different types of B cell lymphomas and multiple myeloma [124].

TRPV2 is a very promising target for early diagnosis or therapy of different human cancers [125]. For instance, TRPV2 negatively controls cell survival and proliferation of human glioma [126, 127] and bladder cancer [128, 129] by induction of apoptotic cell death. Remarkably, activation of TRPV2 synergizes cytotoxic effects of chemotherapies in human gliomas [130]. In contrast, TRPV2 might be a

novel prognostic marker of patients with hepatocarcinogenesis [131], resected esophageal squamous carcinoma [132] or prostate cancer, where TRPV2 overexpression is present [133]. In particular, TRPV2 promotes prostate cancer cell migration and invasive phenotype influencing resting intracellular Ca^{2+} levels [133, 134].

Critical for the maintenance of cardiac structure and function [135], TRPV2 seems a potential target to treat cardiovascular diseases. Under physiological conditions, TRPV2 modulates Ca^{2+} transients and sarcoplasmic reticulum Ca^{2+} loading on myocytes that regulates cardiac contractility, while in smooth muscle contributes to global Ca^{2+} entry helping constriction [136]. TRPV2 deletion results in an impairment in Ca^{2+} handling together with defects on myocardial conduction, among other cardiac features [137]. In muscular dystrophic animals, TRPV2 cell surface expression is increased causing Ca^{2+} overload and cell dead [138]. Noteworthy, attenuation of TRPV2 translocation to the plasma membrane reduces Ca^{2+} increase in muscle fibers attenuating the progression of dystrophic pathology [139]. In this line, abrogation of TRPV2 plasma membrane accumulation, inhibition of TRPV2-mediated Ca^{2+} entry or removal of sarcolemmal TRPV2 ameliorates contractile dysfunction [140, 141]. Consistently, TRPV2 is accumulated in sarcolemma of dilated cardiomyopathy patients and appears to have pathological impact on disease progression through excessive Ca^{2+} influx. In conclusion, TRPV2 contributes to defective cellular Ca^{2+} handling in dystrophic cardiomyopathy [142].

12.3.1.3 TRPV3

TRPV3 is a heat-activated receptor that gates in response to moderate heat (>33 °C). Temperature sensitivity is influenced by voltage as well as the rate at which heat is applied [143]. TRPV3 is also potently and specifically activated by the endogenous molecule farnesyl pyrophosphate from the biosynthetic pathway of steroid hormones [144]. The most used agonist for studying TRPV3 is 2-APB. This molecule as well as other activators such as carvacrol, eugenol, thymol, citral or camphor are not specific and require high concentrations.

A unique feature of TRPV3 is that contrary to other TRPs, currents are potentiated by repeated stimuli application. Noticeably, this property derives from Ca^{2+} regulation of channel activity at both inside and outside domains [145]. By interacting with acidic residues in the pore, extracellular Ca^{2+} channel inhibition is the origin on the double rectifying I–V curves. In the cytosol, CaM-Ca^{2+} complexes have an inhibitory action that is released with repeated application of 2-APB [145]. A CaM binding motif in the N-terminus mediates this inhibitory effect. Lysine residues within this domain are critical for Ca^{2+} dependent regulation as well as for ATP inhibition of TRPV3 currents [145, 146]. Interestingly, CaM and ATP modulatory effects are opposite to those described for TRPV1. On the contrary, TRPV3 regulation by PIP_2 mimics TRPV1 since PLC activation releases PIP_2 tonic inhibition through the TRP domain [54]. Less studied is the channel regulation by phosphorylation. Activation of PKC with a phorbol ester enhances channel function; however, there is no evidence that the potentiation arises by direct phosphorylation.

As TRPV3 is activated by innocuous warm and noxious hot temperatures, this channel was immediately proposed as thermosensor. Interestingly, in humans is activated by heat, but in lower vertebrates by cold [147]. Although the expression of TRPV3 in sensory neurons is not really high in rodents [148], first reports showed that TRPV3 deficiency was critical for thermal sensing of noxious and innocuous heat [149], but later studies restricted the role of TRPV3 on thermoregulation [150].

TRPV3 is a therapeutic target in dermatology, and TRPV3 antagonists might be useful tools in the management of a wide array of skin diseases. TRPV3 is mostly expressed in keratinocytes and it is involved in numerous cutaneous regulatory mechanisms [151, 152]. Increase in Ca^{2+} influx through TRPV3 activation directly inhibits keratinocyte proliferation, decreases hair loss, regulates differentiation, induces apoptosis and maintains cutaneous barrier function. Indirectly, TRPV3 function results in the release of numerous agents that can activate the surrounding cells, such as nociceptors, other keratinocytes or immune cells, inducing pain, itch or skin inflammation. In this regard, activation of TRPV3 upon thermal stimulation promotes a concomitant cytosolic Ca^{2+} increase in keratinocytes leading to the release of pro-inflammatory agents such as ATP, prostaglandin E_2, IL-1b, bradykinin or histamine that are able to activate and sensitize nociceptors [146, 153, 154]. Therefore, epidermal keratinocytes are indirect nociceptor transducers, since via TRPV3-coupled signal mechanism participate in thermal pain signaling. Consequently, molecules able to inhibit TRPV3 channel activity are promising analgesic therapies [9].

Genetic deletion of TRPV3 impairs epidermal barrier structure and hair morphology [155]. In contrast, the gain-of- function Glu573Ser mutation results in a sever dermatitis and itching in mice. Interestingly, identical mutation is described in Olmsted syndrome in humans, together with other two point mutations on TRPV3, Gly573Cys and Trp692Gly, causing constitutive activation of TRPV3 [156]. The enhanced basal activity is accompanied with an increase in Ca^{2+} entry diminishing cell growth and inducing cell death. This channelopathy characterized by a gain of function of the TRPV3 phenotype, causes dramatic effects on skin including hair loss, dermatitis, skin inflammation, keratoderma and deformity of digits. In fact, gain-of-function mutations of TRPV3 result in multiple problems of skin health, which further supports the potential value of TRPV3 antagonist in the treatment of skin diseases [151].

12.3.1.4 TRPV4

TRPV4 is activated by various stimuli ranging from physical stimuli to chemical activators, being considered as a mechano- or osmo-sensor and a moderate heat sensor [157, 158], with higher permeability to Ca^{2+} and Mg^{2+} than Na^{+}. TRPV4 responds to temperature in the range from 24 to 38 °C with strong temperature dependence as indicated by a Q10 between 10 and 20. Initial studies showed that TRPV4 only was heat sensitive in intact cells [159, 160], suggesting that the temperature sensing was dependent on an accessory factor. This tenet has been recently rebated with findings

that demonstrate that TRPV4-PIP_2 interaction, lost in excised patches, is mandatory for temperature and hyposomotic activation [161]. Mutations in the PIP_2 binding domain [161] or in Tyr556 [162, 163] impaired activation by heat. The most extensively used chemical agonist is 4αPDD although it also responds to bisandrographolidea in the mid nanomolar range. The non-specific TRP channel blockers ruthenium red, gadolinium and lanthanum, inhibit TRPV4 currents. In a more targeted manner, the insect repellent citral and the synthetic compounds HC-067047 and GKS block the channel [161].

TRPV4 is subjected to dual Ca^{2+}-dependent regulation, with channel activity potentiated or inactivated during agonist-mediated activation [164]. Ca^{2+} regulation of TRPV4 is intricate. Ca^{2+} affects the channel from both sides of the plasma membrane. Outside the cell, Ca^{2+} accounts for the observed rectification in whole cell currents. Inside the cell, depending on the concentration, it can inhibit or potentiate the channel by a mechanism not yet understood. CaM also regulates TRPV4; while some findings indicate that CaM is a positive modulator [165] previous results show an inhibitory effect [146]. It has been reported a CaM binding site in the N-terminus, which, when complexed with ATP, results in channel potentiation [146]. A second CaM interacting motif also accounts for IP_3R potentiation. TRPV4 is desensitized by PIP_2 depletion since the extended conformation that enables channel response to temperature and hypotonicity is lost. Conversely, TRPV4 currents are potentiated by PKC, PKA and SRC [160, 166, 167].

TRPV4 gating can transduce external mechanical, thermal or osmotic stimuli into the form of elevated intracellular Ca^{2+} concentration, initiating multiple cellular responses [168]. Medical interest in TRPV4 has recently risen since mutations in TRPV4 gene results in several genetic disorders affecting the peripheral nervous and osteoarticular system [157, 169, 170]. Although, the knowledge about the exact underling mechanism is still limited, Ca^{2+}-influx mediated through TRPV4 is critical.

TRPV4 is essential for joint homeostasis and maintenance of musculoskeletal tissues normal function. Expressed in cartilage, bone, and synovium [171], TRPV4 mutation results in the development of a spectrum of skeletal dysplasias and arthropathies. Interestingly, skeletal dysplasias are mainly due to TRPV4 gain-of-function mutations, increasing channel permeability to resting Ca^{2+} as well as Ca^{2+} influx in response to a stimulus [172–174]. The increased TRPV4-mediated Ca^{2+} entry into the chondrocytes results in an improper endochondral ossification [175]. In contrast, arthropathy related TRPV4 mutations cause a decrease in channel function by preventing normal trafficking of TRPV4 channels to the membrane [176]. Actually, TRPV4 knockout mice present an age and sex-dependent development of osteoarthritis and a decreased osteoclast function [177, 178]. As a result, TRPV4 is a possible therapeutic target for osteoarthritis treatment and recently also a valuable tool for the development of tissue-engineered constructs promoting extracellular matrix biosynthesis [179].

TRPV4 is also a critical key player in hereditary neuropathies. TRPV4-axonal neuropathies present a predominantly motor axonal peripheral neuropathy or can be associated with sensory disturbances [157]. A proposed mechanism for motor

neuron degeneration in TRPV4-dependent neuropathies is cell toxicity and increased cell death through Ca^{2+}-overload TRPV4 mutants [180, 181]; however, it is unlikely that Ca^{2+}-induced cell toxicity is a general pathophysiological mechanism. Other potential mechanisms that are altered are neuritogenesis or dysregulation of protein–protein interactions.

TRPV4 has been involved in vascular function, nociception, metabolism or bladder function among others. Abnormal TRPV4 function has been associated with chronic obstructive pulmonary disease, pancreatitis or high risk of hyponatremia [158].

12.3.2 TRPM Family

The TRPM family can be subdivided in different cation channels subgroups that are either highly permeable for Ca^{2+} (TRPM3/6/7), non-selective (TRPM2/8), or Ca^{2+} impermeable (TRPM4/5) [182].

12.3.2.1 TRPM2

TRPM2 is the closest phylogenetic channel to the TRPM8 receptor. Notwithstanding, it responds to warm temperatures (>35 °C) in heterologous expression systems and in pancreatic β-cells. Temperature-elicited currents are synergistically increased by the application of other agonists [183]. It also responds to ADP-ribose/Ca^{2+}, H_2O_2 as well as reactive oxygen and nitrogen species (ROS/NOS). Moreover, channel function is enhanced by NAADP and cyclic ADP-ribose. On the contrary, acidic pH and AMP attenuates its activity [184]. TRPM2 characteristics suggest a major role as cellular metabolic and oxidative stress detector. Noteworthy, several splicing variants of TRPM2 have been documented and some of them are physiologically relevant for tuning channel function.

Ca^{2+} is a central regulator of TRPM2, and this modulation is CaM-dependent. Indeed, different studies indicate that CaM interacts with the N-terminus of the channel [185, 186]. Particularly, the IQ-like CaM binding motif mediates Ca^{2+} activated TRPM2 currents [185]. This interaction is deemed to account for part of the Ca^{2+} dependent activation of the channel. In β-pancreatic cells, TRPM2 currents are potentiated by PKA activation [183]. Physiologically, incretin contribution to insulin secretion is believed to result from potentiation of TRPM2 currents through receptor mediated activation of PKA [187]. Silencing or inhibiting PKC activity reduces Ca^{2+} influx via TRPM2. Interestingly, PKC phosphorylation of the splicing variant TRPM2-S releases TRPM2 from the isoform negative-dominant effect [188]. Some reports evidence that Ca^{2+} entry can directly gates TRPM2 in a dose-dependent manner, although other studies have challenged this statement [185, 189].

As other thermoTRP channels, Ca^{2+} entry through TRPM2 promotes different intracellular signaling cascades and physiological processes. TRPM2 activation by H_2O_2 is being implicated in stress-related inflammatory, vascular and neurodegenerative conditions. The physiological and pathophysiological context of ROS-mediated events makes TRPM2 a promising target for the development of therapeutic tools of inflammatory and degenerative diseases. During inflammation, TRPM2 regulates innate and adaptive immunity, acting as an oxidative stress and metabolic sensor [190]. Through lysosomal Ca^{2+} release, TRPM2 participates in maturation of dendritic cells [191]. In contrast, in macrophages, TRPM2 in the cell surface mediates Ca^{2+} -dependent chemokine and cytokine release, inflammasome formation, cell infiltration and cell death [192, 193]. In addition, TRPM2 contributes to antigen-stimulated Ca^{2+} influx in mucosal mast cells and induces degranulation [194].

TRPM2 is expressed in numerous cell types [195] being embedded in the plasma membrane and in lysosomal compartments. This channel is highly expressed in the central as well as the peripheral nervous system. Due to its high permeability to Ca^{2+}, it exerts a crucial regulatory function, most notably in modulation of DRG neurons to painful signals [196, 197]. Altered intracellular Ca^{2+} homeostasis and oxidative stress are involved in the pathophysiology of bipolar disorder, and TRPM2 genetic variants are associated with this pathogenesis [198]. TRPM2 also spotlights as possible therapeutic target for neurodegenerative diseases, as TRPM2 mediated Ca^{2+}-influx drives neuronal death [199, 200]. Although with still controversy, targeting TRPM2 for potential stroke therapy requires a balance between effects on microglia and neurons, but further investigation is required in this area [201].

Interestingly, temperatures above 35 °C can directly activate TRPM2 and potentiate Ca^{2+} entry upon ADP-ribose stimulation of neuronal cells [202], as well as in pancreatic cells [183]. Here, TRPM2 function acts as lysosomal Ca^{2+} release channel [203], inducing insulin secretion through modulation of intracellular Ca^{2+} influx. Lack of TRPM2 impairs insulin secretion and glucose metabolisms showing lower Ca^{2+} signals in response to glucose [187] and prevents diet-induce obesity [204]. Therefore, TRPM2 also represent a potential new target for diabetes therapy [205].

12.3.2.2 TRPM3

TRPM3 channels are non-selective cationic channels activated by heat, but also by voltage, hypotonic cell swelling and chemical stimuli (pregnenolone sulfate, D-erythro-sphingosine). Nevertheless, less is known about the post-translational mechanisms regulating TRPM3 channel activity. There are only limited studies reporting some insights into the features and/or regulation of Ca^{2+}. Important domains for protein-protein interaction such as CaM and PIP_2 binding domains are present in the N-terminus [206, 207], but there is limited information on the functional implication of this association.

Several splice variants of TRPM3 are known which are divided in three groups depending on their first exon. TRPM3α (α1–α5) isoforms start with exon 1 and lack exon 2, while TRPM3β isoforms (β1–β17) start with exon 2. A third group is

composed of isoforms starting with exon 3 [208]. This plethora of splice variants together with the microRNA seems to facilitate a fine tuning of the channel expression level. The TRPM3β variants seem to display a different kind of Ca^{2+}–dependent regulation [207]. Alternative splicing in exon 24, leads to two different pores and influences cation selectivity [209].

The physiological and/or pathological role of TRPM3 still remains to be deeply elucidated, and only the endogenously Ca^{2+}-permeable variants have been studied. TRPM3 expression is found in the brain, eyes, reproductive system, pituitary gland, and adipose tissue. In pancreatic β-cells, TRPM3 activation induces an intracellular signaling cascade, with a concomitant rise in cytosolic Ca^{2+} followed by an increase in insulin secretion [210]. Therefore, as other thermoTRPs, TRPM3 may influence main functions of pancreatic β-cells using a similar signaling cascade induced by glucose. The functional relevance of TRPM3 in contractile and proliferating vascular smooth muscle cells is via a Ca^{2+}-dependent mechanism [211]. However, TRPM3 has recently drawn much attention since it has been described as a heat sensing channel expressed in nociceptors [212]. Present in a large number of small-diameter DRG and TG neurons, TRPM3 detects noxious heat, is involved in heat-, but not cold-mediated thermal hyperalgesia, but does not regulate homeostasis of body temperature.

12.3.2.3 TRPM4

In marked contrast to the other TRP members, TRPM4 and TRPM5 do not permeate Ca^{2+}. Nevertheless, Ca^{2+} activates their currents [213], foretelling important Ca^{2+} regulation of channel function. Despite Ca^{2+} being able to directly activate TRPM4, it requires high intracellular Ca^{2+} levels. At more physiological Ca^{2+} concentrations, TRPM4 currents are likely to be triggered by CaM interaction with CaM-binding sequences. Actually, mutations on the three putative binding sites impaired current activation [214]. TRPM4 behaves as a heat receptor in the temperature range between 15 and 35 °C [215] in which, the temperature dependency Q10 value is 8.5. In addition, it shows weak voltage sensitivity. Pharmacologically, it can be activated by vanadate and BTP2 and blocked by compounds such as 9-phenanthrol, flufenamic acid, quinine and quinidine [216].

The activity of TRPM4 is dependent on PIP_2 as shown by the inhibition of the currents following PIP_2 depletion. Neutralization of Lys residues within two putative Pleckstrin homology domains (located in the C-terminus), results in low sensitivity to PIP_2 and rapid desensitization [217]. In addition to CaM, and PIP_2 binding motifs, TRPM4 also contains multiple ATP biding sites that greatly influence Ca^{2+} sensitivity. ATP modulation seems to depend on the binding site; it is considered that interaction with one ATP binding site has an inhibitory role while interaction with the other site facilitates Ca^{2+} activation [216]. The mechanism underlying this bimodal regulation is not yet understood.

Under certain conditions, TRPM4 function is also potentiated, by the activity of PKC on Ser1145 and Ser1152 in the C-terminus [49]. Activation of PKCdelta contributes to channel potentiation through increasing its surface expression [218].

Physiologically, TRPM4 is a key regulator of the driving force for Ca^{2+} entry being widely expressed in the body [216]. This thermoTRP channel plays an important role in the immune, cardiovascular and central nervous system. For instance, in dendritic cells, the absence of TRPM4 leads to a Ca^{2+} overload which impairs chemokine–dependent migration [219]; while in mast cells, decreases migration and increases histamine release aggravating anaphylactic reactions [220]. In both cases, due to the absence of the Ca^{2+}-activated depolarizing current mediated by TRPM4. Additionally, deletion of TRPM4 expression, impairs phagocytosis in macrophages [221], and increases Ca^{2+} influx in lymphocytes Th2 [222].

Excessive Na^{+} influx through TRPM4 induced by Ca^{2+} overload is a critical mechanism in experimental autoimmune encephalomyelitis, multiple sclerosis or spinal cord injury. Rise of cytosolic Ca^{2+} activates TRPM4 in neurons contributing to neuronal cell death, which turned to aggravate autoimmune encephalomyelitis [223]. TRPM4 expression is upregulated in spinal cord injury and absence of TRPM4 function reduces secondary hemorrhages and improves behavioral performance [224].

Another example is how Ca^{2+}-dependent modulation of TRPM4 increases osteogenesis or decreases adipogenesis [225]. In fact, blockade of TRPM4 activity reduces glucose-induced Ca^{2+} signal and insulin secretion in β-pancreatic cells, while in α-cells knockdown of TRPM4 decreases Ca^{2+} responses and glucagon secretion [40, 226].

TRPM4 is involved in several aspects of cardiac rhythmicity, including cardiac conduction, pacemaking and action-potential repolarization [227]. The regulation of TRPM4 function in arterial smooth muscle cells is complex and involves release of Ca^{2+} from the sarcoplasmic reticulum through IP_3 receptors and translocation of TRPM4 channels to the plasma membrane [213, 220, 228] and is critical for pressure-induced cerebral arterial myocyte depolarization and myogenic vasoconstriction [229]. A specific mutation in the TRPM4 gene leads to gain-of function of the channel in a progressive Familial Heart Block Type I pathology [230, 231], but this mutation is not determinant as additional factors are required [232].

12.3.2.4 TRPM5

TRPM5 is activated by warm temperatures (>15 °C). Akin to TRPM4, TRPM5 channel does not permeate Ca^{2+} ions; nevertheless, it is activated by Ca^{2+} [233]. The binding of bitter, sweet or umami taste molecules to their cognate receptors activates the PLCβ2 signalling, that ultimately evokes Ca^{2+} release from intracellular stores and activation of TRPM5 [233]. A comparison with TRPM4 reveals that TRPM5 opening requires less Ca^{2+} [234, 235]. Remarkably, TRPM5 seems to lack the CaM-facilitated activation; therefore, it is proposed that either another Ca^{2+} binding protein or Ca^{2+} itself gates the channel [48]. With a total identity of 40 %,

both channels share similar temperature dependence (potentiation over 15 °C) and PLC-mediated desensitization. However, ATP regulation is absent as a consequence of the lack of functional nucleotide binding sites. Pharmacologically, TRPM5 is blocked by quinine, a general inhibitor of ion channels, and triphenylphosphine oxide [236].

As mentioned, TRPM5 channel plays an important role in taste and thermal sensitivity and is highly expressed in the subset of taste receptor cells. TRPM5 contributes to temperature dependence of sweet sensation [215]. Heat is not sufficient to directly activate TRPM5, which at warm temperatures requires an increase of intracellular Ca^{2+}. Because of its role in normal taste signaling, drugs designed to modulate TRPM5 could be useful in controlling taste sensory signals and potentially developed as the anti-obesity therapies with limited drug distribution to the site of action [236]. In small intestine and stomach, as well as respiratory and olfactory epithelium, TRPM5 appears to be specialized in chemosensation [237, 238]. Recently, TRPM5 channels have been shown essential for normal insulin secretion and glucose tolerance [239, 240]. Noteworthy, a genetic variation of TRPM5 gene could contribute to propensity to develop diabetes [241], highlighting TRPM5 as a potential target to control diabetes.

12.3.2.5 TRPM8

In contrast to the above-described thermoTRPs, TRPM8 is considered as the main detector of cold temperatures in mammals. Indeed knock-out mouse lines lose the cold induced responses [242–244]. At the protein level, the channel not only responds to temperatures below 22 °C but it is also active after membrane depolarization and in the presence of compounds such as menthol or icilin [245, 246]. TRPM8 has multiple physiological roles such as cool temperature discrimination, noxious cold sensing, thermoregulation, cold-induced analgesia and an anti-inflammatory effect [242–244].

TRPM8 can be regulated by various intracellular secondary messengers and signalling pathways that allow a fine tuning of channel activity by the cellular environment [247]. Presence of external Ca^{2+} markedly desensitizes TRPM8-dependent currents [246], and recent evidence shows that promotes a displacement of voltage dependent activation of TRPM8 towards more positive potentials [53]. Additionally, TRPM8 acute desensitization during a single agonist application is a Ca^{2+}-dependent mechanism triggered by CaM [248].

Besides, Ca^{2+} influx through TRPM8 activates PLC isoforms which hydrolyze PIP_2 and decreases channel activity, being responsible of Ca^{2+}-dependent tachyphylaxia [249–252]. PIP_2 is a necessary cofactor for TRPM8 activity. The fast run down of the currents is reversed upon addition of PIP_2 analogues to the bath solution [250]. Furthermore, in the presence of extracellular Ca^{2+}, TRPM8 is desensitized as a result of the PIP_2 hydrolysis by a Ca^{2+} dependent PLC pathways [253].

TRPM8 is also positively regulated by PKA through phosphorylation of Ser9 and Thr17 in the N-terminus [254]. However, PKA-mediated phosphorylation has

also been described to desensitize the channel [255]. In heterologous systems and in cold sensitive neurons, chemical activation of PKC inhibits TRPM8 currents [256–258]. Interestingly, inhibition does not result from PKC phosphorylation of the channel but from activation of calcineurin and subsequent dephosphorylation in a Ca^{2+} dependent manner [256].

TRPM8 has a relatively restricted expression in normal tissues, and its overexpression or increased activity in tumors offers an interesting tool for diagnosis or treatment [259]. TRPM8 has emerged as a putative therapeutic target for prostate cancer, as TRPM8 localization and activity are regulated depending on the differentiation and oncogenic status of prostate cancer cells [260]. Expressed in the plasma membrane, TRPM8 seems important for Ca^{2+} signaling involved in proliferation through a short and repeated Ca^{2+} entry. In contrast, activation of TRPM8 in the endoplasmic reticulum, acting as a Ca^{2+} release channel [261, 262], promotes Ca^{2+} stored depletion inducing growth arrest and apoptosis in prostate epithelial cells in advanced prostate cancer [260, 263]. Similarly, in melanoma cells, activation of TRPM8 induces a transient and sustained rise in intracellular free Ca^{2+} concentration that reduces cell viability [264, 265], while in neuroendocrine pancreatic tumor cells Ca^{2+} increase stimulates neurotensin secretion [266]. TRPM8 activation stimulates Ca^{2+} influx membrane current, and migration of human glioblastoma cells [267], but inhibites cell motility in pancreatic ductal adenocarcinoma [268]. Nowadays, an oral active TRPM8 agonist is on clinical evaluation for the treatment of refractory solid tumors (D-3263, Dendreon). The hypothesis is that TRPM8 activation will disrupt Ca^{2+} and Na^{+} homeostasis allowing entry of both ions, which will specifically induce cell death only in TRPM8- expressing neurons.

For a long time, cooling or topical application of menthol has been used for their analgesic effects. This evidence, together with TRPM8 presence in sensory neurons [269] and fibers innervating the skin, mucosa or visceral organs, suggests a potential role of TRPM8 in thermal sensing and nociception [9, 246, 270–272]. In contrast to the pro-algesic activity of other thermoTRPs, TRPM8 mediates analgesia in neuropathic and inflammatory pain models. TRPM8 is a major integrator of cold allodynia, and it is required for behavioral responses to injury-evoked cold hypersensitivity, cooling-mediated analgesia, and oxaliplatin-induced cold pain [273–276].

Recent observations of TRPM8 expression in vagal neurons innervating bronchopulmonary tissue have brought up TRPM8 as a drug target for various respiratory disorders. Other diseases where TRPM8 is involved include dry-eye-syndrome, amyloidotic polyneuropathy, and diseases of the urogenital tract like overactive bladder syndrome and pain bladder syndrome [277–280].

12.3.3 TRPC5

TRPC5 is principally a receptor-operated channel depending on the activation of Gq, phospholipase C and/or Gi receptor to gate the channel. Nonetheless, it is also directly activated by Ca^{2+} [51], nitric oxide [281] and lysophospholipids [282]. Its

role in temperature sensing involves channel activation at innocuous temperatures ranging from 25 to 37 °C [36]. The cytosolic C-terminus contains interaction sites for Ca^{2+} binding proteins that notably modulate channels function [52]. CaM can interact with TRPC5 at different sites [283]. One of these sites, namely CIRB, is additionally targeted by IP_3 receptors that are proposed to displace CaM, therefore releasing the inhibition [283]. Furthermore, the C-terminus can also accommodate Ca^{2+} binding protein 1 that inhibits intracellular Ca^{2+} activation of the channel [284]. In addition, TRPC5 is phosphorylated by PKA (Gs/cAMP/PKA) at residues Ser794, Ser796 resulting in an inhibition of the currents through the channel. Mechanistically, this phosphorylation in the PDZ domain may regulate functionally relevant interactions in a Ca^{2+} dependent manner [285]. The channel is also regulated by PKC phosphorylation that results in desensitization of the currents [283].

TRPC5 is broadly expressed acting as an integrator of extracellular and intracellular signals at the level of Ca^{2+} entry. TRPC5 expressed as homotetramer allows Ca^{2+} entry and causes an intracellular Ca^{2+} increase underlying specific cell functions; however, in complexes with other TRPC proteins can acts as a store operated channel. TRPC5 is involved in many physiological and pathological processes [286]. For instance, TRPC5 is predominantly expressed in central nervous system, participating in brain synapsis [287]. Here, this channel is mainly present in dendrites as well as cell nuclei, suggesting a role of TRPC5 in the nuclear Ca^{2+} signaling [288, 289]. TRPC5 participates in electrogenesis in neurons with a key role in axon guidance during brain development. In this context, TRPC5 is carried to newly forming growth cones and synapses where acts as regulator of hippocampal neurite length and growth cone morphology mediating Ca^{2+} influx and signaling [290]. This receptor is also essential in dendrite regulation through Ca^{2+}/CaM interaction [291]. In mouse sensory neurons, TRPC5 expression is progressively increased during embriogenesis, and may play a role in sensory physiology [36, 292]. In the peripheral organs, TRPC5 is present in liver, heart, vasculature, adipocytes, and kidney among others [286].

12.3.4 TRPA1

Besides being activated by noxious cold temperatures (<10 °C) and Ca^{2+}, TRPA1 is gated by a vast and ever-growing array of compounds among which irritants take the lead. Mustard oil, cinamaldehyde, allylisothiocyanate and icilin are classical TRPA1 activators [293]. Most of them share an electrophilic nature and activate the channel by covalently modifying Cys residues in the N-terminus [109, 294]. Noteworthy, the precise temperature of TRPA1 activation is a hot topic since several findings indicate that it is organism dependent. For instance, while mouse TRPA1 responds to low temperatures, TRPA1 from snakes and Drosophila detects warmth [295, 296]. Initial findings in human TRPA1 was not gated by cold [297]. However, it has been recently shown that human TRPA1 is intrinsically cold sensitive [298].

The activity of TRPA1 is potentiated by PLC and PKA pathways [299, 300]. Interestingly, the response of TRPA1 is also regulated by Ca^{2+}. Findings in the $TRPA1^{Glu918Ala}$ mutant, whose Ca^{2+} permeability is reduced, indicates that the Ca^{2+} entry through the channel initially potentiates the currents and secondly mediates its inactivation [301]. Similar to TRPV1, TRPA1 activity is also potentiated by the recruitment of new channels to the cell membrane through vesicle fusion. The receptor increase on the cell surface requires Ca^{2+} influx through TRPA1 [302], and is at least partially dependent on SNARE-mediated vesicle-fusion [303]. Intriguingly, in sensory neurons TRPA1 is desensitized by the increase in intracellular Ca^{2+} through TRPV1 [304] pointing to a regulatory crosstalk among TRP channels.

TRPA1-positive C-fibers densely innervate the skin, airways and gastrointestinal tract. This location, along with the robust activation of TRPA1 by a long list of exogenous environmental compounds, chemicals as well as endogenous inflammatory mediators, has implicated TRPA1 in pain perception [305, 306]. Pharmacological as well as knockout studies have evidenced the functional role of TRPA1 as chemo-, mechano- and cold sensor in the peripheral nervous system [294]. Although with still some controversy [299], TRPA1 participates in cold allodynia induced by inflammation, nerve injury, diabetic neuropathy or chemotherapy-induced neuropathies; however, it seems that cold responses are independent of Ca^{2+} [307, 308], although Ca^{2+} is an important regulator of TRPA1-mediated responses to cold. The essential role of TRPA1 in pain perception is evidenced by the TRPA1 associated channelopathy. A gain-of-function mutation in TRPA1 (Asn885Ser) is related to an autosomal dominant familial episodic pain syndrome [309]. This mutation renders the channel hyperresponsive to stimuli altering its voltage dependence. Patients are in general healthy but suffer hyperalgesia upon application of TRPA1 agonists or cold. Hence, TRPA1 has become a potential pharmacological target for the treatment of chronic pain [310].

TRPA1 is expressed in both peptidergic and non-peptidergic sensory neurons [311, 312], and mostly found, but not exclusively, in a subpopulation of TRPV1-positive neurons. In addition to pain transduction, Ca^{2+}-entry through TRPA1 causes the release of the pro-inflammatory neuropeptides αCGRP and/or SP from sensory neurons, which enhances neurogenic inflammation, pain signaling, cerebral blood flow [313], cutaneous vasodilatation [314], or promotes and maintains colitis in mice [315]. For instance, TRPA1 acts as a primary vascular cold sensor, through an initially vasoconstriction followed by a subsequent restorative blood flow dependent on αCGRP release [316]. Interestingly, TRPA1 activators are well-known environmental migraine triggers able to induce the release of the pro-migraine peptide αCGRP [305, 317].

The functional relevance of TRPA1 in the central nervous system is still not deeply understood. Recent findings further support the role of TRPA1 on mechanical allodynia central sensitization in the spinal cord [318–320], but also disrupts spinal nociceptive transmission through a Ca^{2+}-dependent mechanism [321]. In astrocytes, TRPA1 channel-mediated fluxes contribute to basal Ca^{2+} levels and neuronal function via constitutive D-serine release [322].

In non-neuronal tissues, TRPA1 seems to trigger airway inflammation as part of the lung defense, but overstimulation leads to pathological states. TRPA1 participates in cough, bronchoconstriction, asthma or chronic obstructive pulmonary diseases [323–325]. Exposure to environmental and occupational respiratory irritants activates TRPA1 on vagal sensory afferents contributing to the pathophysiology of respiratory diseases [326]. Notably, exogenous activators of TRPA1 are able to promote cell survival of small cell lung cancer cells [327]. Therefore, TRPA1 antagonists are actively pursued as therapeutic agents for airway diseases.

Finally, in skin, TRPA1 is also involved in non-histaminergic itch [328], and participates in the integration of immune and neuronal mechanisms leading to chronic inflammatory responses and pruritus associated with contact dermatitis [329].

12.4 Conclusions

ThermoTRP channels enclose a group of structural related receptors activated by different temperatures with a wide range of physiological functions; however, in all of them, directly or indirectly, Ca^{2+} is a fundamental element on their function. Most of the thermoTRP channels permeate Ca^{2+} upon activation leading to different cellular responses such as action potentials, proliferation or cell migration, among others. Additionally, an increase of cytosolic Ca^{2+} through some thermoTRP promotes Ca^{2+}-dependent exocytosis allowing the release of mediators as well as trafficking of new thermoTRP receptors to the plasma membrane. Furthermore, Ca^{2+} also plays a critical role on the regulation of thermoTRP gating through a direct binding or indirectly through Ca^{2+}-dependent proteins or signaling pathways. However, while some of these receptors are desensitized by Ca^{2+} or Ca^{2+} signaling others are potentiated. The contribution of each thermoTRP channel to Ca^{2+} homeostasis and its physiological consequence is completely dependent on the subtype of receptor and the cellular context where it is expressed. The essential role of thermoTRP in maintenance of cellular homeostasis is evidenced by the dramatic consequences observed in patients suffering thermoTRP-associated channelopathies. Moreover, these receptors are also involved in the maintenance and development of numerous pathological conditions such as chronic pain, inflammation, cancer, diabetes, or cardiac disorders. In both cases, there is an unbalance of the cytosolic Ca^{2+} levels resulting in numerous and diverse pathological symptoms. Noticeable, in the last years, there is an increasing progress carried out to fully understand pathophysiological modulation of thermoTRP channels by Ca^{2+} and the implication of these receptors on Ca^{2+} homeostasis. Notwithstanding, although further research is still required, novel therapies are currently under evaluation targeting thermoTRPs with the aim to control their dysfunction.

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Chapter 13
Calcium Signalling through Ligand-Gated Ion Channels such as P2X1 Receptors in the Platelet and other Non-Excitable Cells

Martyn P. Mahaut-Smith, Kirk A. Taylor, and Richard J. Evans

Abstract Ligand-gated ion channels on the cell surface are directly activated by the binding of an agonist to their extracellular domain and often referred to as ionotropic receptors. P2X receptors are ligand-gated non-selective cation channels with significant permeability to Ca^{2+} whose principal physiological agonist is ATP. This chapter focuses on the mechanisms by which P2X1 receptors, a ubiquitously expressed member of the family of ATP-gated channels, can contribute to cellular responses in non-excitable cells. Much of the detailed information on the contribution of P2X1 to Ca^{2+} signalling and downstream functional events has been derived from the platelet. The underlying primary P2X1-generated signalling event in non-excitable cells is principally due to Ca^{2+} influx, although Na^{+} entry will also occur along with membrane depolarization. P2X1 receptor stimulation can lead to additional Ca^{2+} mobilization via a range of routes such as amplification of G-protein-coupled receptor-dependent Ca^{2+} responses. This chapter also considers the mechanism by which cells generate extracellular ATP for autocrine or paracrine activation of P2X1 receptors. For example cytosolic ATP efflux can result from opening of pannexin anion-permeable channels or following damage to the cell membrane. Alternatively, ATP stored in specialised secretory vesicles can undergo quantal release via the process of exocytosis. Examples of physiological or pathophysiological roles of P2X1-dependent signalling in non-excitable cells are also discussed, such as thrombosis and immune responses.

Keywords P2X receptors • Ca^{2+} entry • P2X1 • Ligand-gated ion channels • ATP • Leukocyte • Platelets • Exocytosis • Pannexin • Ionotropic receptors • Intracellular Ca^{2+} • Thrombosis • Inflammation

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13.1 General Background on Ligand-Gated Ca^{2+}-Permeable Receptor Ion Channels

Surface-expressed, ligand-gated P2X receptors represent a unique mechanism whereby cytosolic Ca^{2+} can be elevated during cellular activation [1, 2]. The main difference between this class of ion channel and other Ca^{2+} mobilization pathways is the rapid coupling of ligand binding to pore opening; consequently membrane currents and calcium increases can be detected almost instantaneously following agonist application [3–5]. This compares with a latency of several hundreds of milliseconds for Ca^{2+} increases downstream of G-protein-coupled receptor (GPCR) activation [6, 7] due to the time required for the hydrolysis of phosphatidylinositol 4,5-bisphosphate and for released inositol 1,4,5 trisphosphate (IP_3) to diffuse to the IP_3 –dependent Ca^{2+}-permeable channels on intracellular stores. P2X receptors consist of a family of seven subunits which are widely expressed and can form a range of homotrimeric and heterotrimeric functional channels [2]. They are non-selective cation channels with significant Ca^{2+}-permeability (P_{Ca}/P_{Na} in the range 1.1–4 depending upon the receptor type and ionic conditions [2]). Although non-excitable cells have been reported to express several other types of ligand-gated cation channels (examples are glutamate-, serotonin- and acetylcholine-stimulated channels), their role in Ca^{2+} signalling is less clear. For example, platelets express a number of ionotropic glutamate receptor subunits of the AMPA and kainate family, however all evidence points towards functional channels that are selective for monovalent cations [8, 9]. For this reason, this chapter will consider only P2X receptors. Furthermore, discussion will concentrate on the P2X1 receptor, which is widely expressed in both excitable and non-excitable cells and has been extensively studied in terms of its role in Ca^{2+} signalling. Much of this information has been derived from experiments in the platelet (summarised in Figs. 13.1 and 13.2), where P2X1 is the only P2X receptor subtype expressed [10–12] and contributes to the responses to several agonists in vitro [13] and to thrombosis in vivo [14, 15]. We will also consider more recent evidence for a contribution by P2X1 receptors to physiological and pathophysiological responses via activation of T lymphocytes and neutrophils [16–19].

13.2 Structure, Activation and Regulation of P2X Cation Channels

13.2.1 Molecular Structure and Activation

The cloning of the first P2X receptor subunits over 20 years ago [20, 21] was a catalyst for understanding the role of extracellular ATP as a signalling molecule and the molecular diversity/properties of the receptors [22]. The seven mammalian P2X receptor subunits (P2X1-7) have intracellular amino and carboxyl termini, two

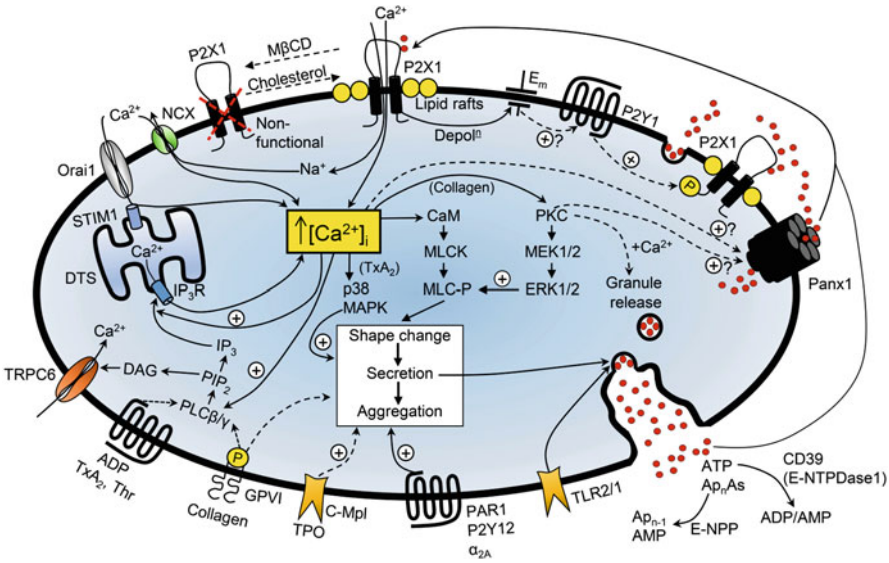

Fig. 13.1 Regulation of P2X1 receptors and interplay with other Ca^{2+}-mobilising pathways in the platelet: refer to text for full explanation. *Panx1* pannexin1, *TxA_2* thromboxane A_2, *MβCD* methyl-β-cyclodextrin, *PAR1* protease activated receptor 1, *thr* thrombin, *Depol^n* depolarization, *NCX* Na^+/Ca^{2+} exchanger, *C-Mpl* thrombopoietin receptor, *CaM* calmodulin, *TRPC6* canonical transient receptor potential channel 6, *TPO* thrombopoietin, *TLR1/2* Toll-like receptor1/2, *IP_3* inositol 1,4,5 trisphosphate, *PLC* phospholipase-C, *PIP_2* phosphatidylinositol 4,5-bisphosphate, *DAG* diacylglycerol, *PKC* protein kinase C, *Em* membrane potential, *Ap_nA* diadenosine polyphosphates, *α_{2A}* alpha$_{2A}$-adrenergic receptors, *GPVI* glycoprotein VI, *MLCK* myosin light chain kinase, *MLC-P* phosphorylated myosin light chain, *ERK2* extracellular receptor kinase 2, *p38 MAPK* p38 mitogen activated protein kinase, *MEK* MAPK/ERK kinase, *E-NTPDase1* ecto-nucleoside 5′-triphosphate diphosphohydrolase1, *E-NPP* ectonucleotide pyrophosphatase/phosphodiesterase (the enzyme products will be AMP + Ap_{n-1}, i.e. AMP + ATP for Ap_4A), *DTS* dense tubular system

transmembrane domains and a large extracellular ligand binding loop [23]. The subunits assemble to form a range of homo- and hetero-trimeric ATP-gated cation channels with properties dependent on the subunit composition [23]. The crystallization of the zebrafish P2X4 receptor in agonist-free and ATP-bound forms was a major advance, providing detailed structural insight into agonist binding and the gating of the receptor/channel [24, 25]. The crystal structures show the trimeric assembly of subunits with the ATP binding site(s) forming at the subunit interfaces and the second transmembrane domains lining the channel pore. ATP binding induces conformational change in the nucleotide binding pocket (≈40 Å from the pore-spanning transmembrane region) resulting in an iris-like expansion of the transmembrane helices and channel gating. A wide range of biochemical and electrophysiological studies have supported these conclusions and identified residues involved in the inter-subunit ATP binding site and formation of the channel pore [22, 26, 27].

Cysteine scanning mutagenesis has been used by several groups to investigate the pore-forming second transmembrane domain [28–30]. Silver and cadmium can

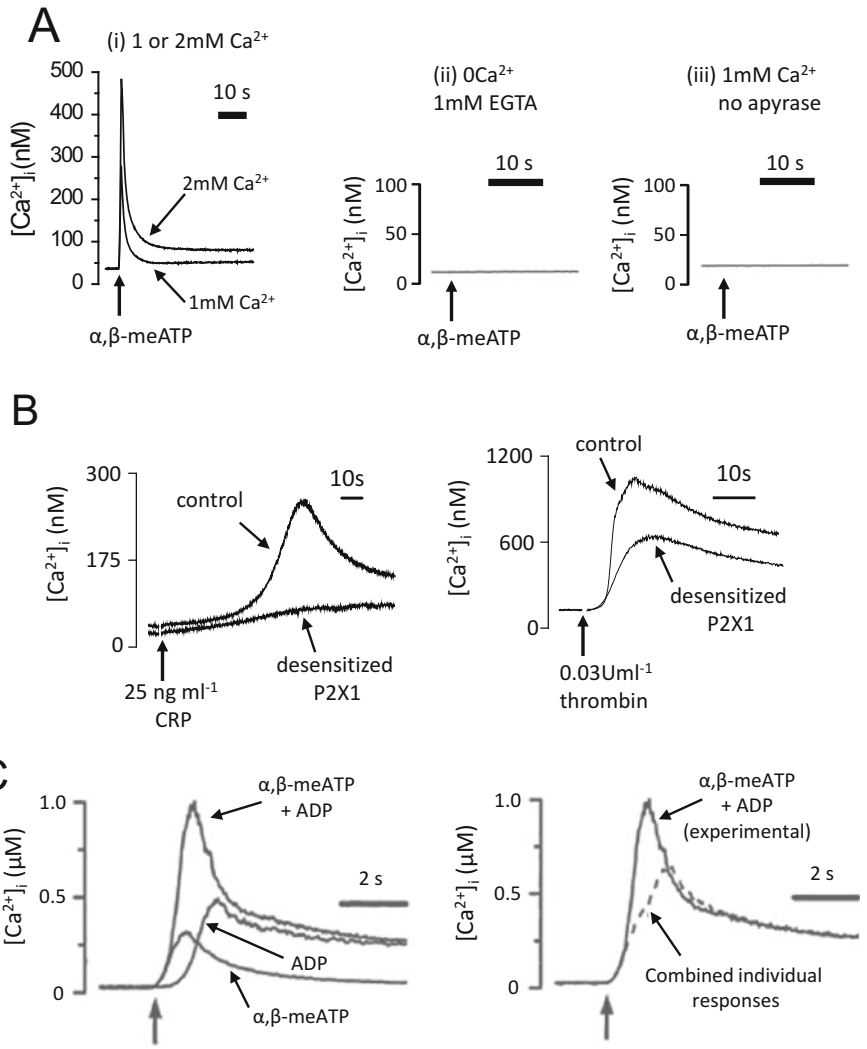

Fig. 13.2 P2X1 receptor-dependent intracellular Ca^{2+} responses in human platelets: average measurements from stirred suspensions of platelets loaded with the fluorescent indicator fura-2. **A** Selective activation of P2X1 receptors by a maximal concentration (10 μM) of the non-hydrolysable ATP analogue α,β-meATP leads to a rapid, transient increase in intracellular free Ca^{2+} in human platelets (i). An increase in extracellular Ca^{2+} results in a significant increase in peak response (i), whilst Ca^{2+} responses are abolished by removal of extracellular Ca^{2+} (ii) or omission of the ATP-degrading enzyme apyrase from the resuspension buffer due to receptor desensitization (iii). **B** Examples of the contribution of P2X1 receptors, activated by ATP released via exocytosis and/or Panx-1, to responses downstream of major platelet receptors; CRP (collagen receptor peptide) stimulates the TKR GPVI and thrombin co-stimulates the GPCRs PAR1 and PAR4; P2X1 function was prevented by desensitization with a low concentration of α,β-meATP prior to addition of extracellular Ca^{2+} (see [13]). **C** Synergy between P2X1 and P2Y receptors. The *left panel* shows responses to individual or combined addition of 10 μM α,β-meATP and 10 μM ADP. The *right panel* shows the experimental response to co-addition of these agonists (trace from the *left panel*) together with the response predicted by summation of the individual responses. α,β-meATP selectively activates P2X1 receptors whilst ADP generates Ca^{2+} responses primarily through P2Y1 receptors (Taken from [12, 13, 52, 148] with permission)

permeate the P2X receptor channel and the characterization of the rates of modification of cysteine mutants (in the absence or presence of ATP) by these metals highlights not only the residues that line the channel pore but also the location of the channel gate [30–32] and confirms findings from structural studies. P2X receptors show appreciable permeability to Ca^{2+} ([5]; see below). Mutagenesis studies on the P2X2 receptor have identified three polar pore-lining residues around the channel gate (Thr336, Thr339 and Ser340) that are important for high Ca^{2+} permeability [33], suggesting that these residues are forming the selectivity filter. Polar residues are found at two corresponding positions in the human P2X1 receptor (336-Ser335 and 339-Gly340, human P2X1 receptor), but at the position corresponding to 340 there is non-polar isoleucine (Iso341). However the contribution of these residues to Ca^{2+} permeability at the P2X1 receptor has not been determined and there does not appear to be a consensus of residues at this location within the P2X receptor family. For the rat P2X1 receptor, juxta-membrane acidic amino acids at the extracellular vestibule contribute to the high Ca^{2+} permeability of the receptor [34].

In myeloid cells such as platelets, megakaryocytes and neutrophils, the P2X1 receptor functions as a homomeric channel [12, 14, 19]. One of the characteristic features of the P2X1 receptor is its rapid desensitization; the channel closes with a time constant of ≈200–500 ms during the continued application of maximal concentrations of ATP. However, at lower concentrations of ATP the desensitization is slower, reflecting the cumulative activation of the receptors, and in this way P2X1 receptor activation can give rise to longer lasting, smaller rises in intracellular calcium [35]. Studies on chimeric P2X receptors and point mutants have shown that the molecular basis of the desensitizing phenotype is dominated by the intracellular domains and involves complex interactions with the transmembrane regions [36, 37] as well as structural changes around the ATP binding pocket [38].

13.2.2 Regulation of P2X1 Receptor Function by Cellular Signals and Agonist Removal

The activity of cell surface P2X1 receptors can be dynamically regulated by a range of mechanisms. Activation of Gαq-coupled receptors potentiates P2X1 receptor-mediated responses via a protein kinase C (PKC)-dependent mechanism [39, 40], likely via phosphorylation of a regulatory protein associated with the amino terminus [41]. Cholesterol depletion and disruption of lipid rafts inhibits P2X1 receptor-mediated responses [42, 43] and this is likely through an interaction with the pre-transmembrane 1 intracellular amino terminal region [44]. Purification of the P2X1 receptor from cell membranes has indicated interactions with the actin cytoskeleton and heat shock protein 90 [45, 46]. Disruption of the actin cytoskeleton inhibited P2X1 receptor mediated responses. Interestingly, stabilization of the actin cytoskeleton abolished the inhibitory effects of cholesterol depletion, suggesting that lipid rafts regulate the P2X1 receptor through stabilization of the cytoskeleton [45]. In platelets, approximately 85 % of receptors were not associated with lipid

rafts, and this proportion are predicted to be non-functional [43]. Therefore, it is interesting to speculate that high cholesterol levels may enhance P2X1 receptor responses and thus thrombosis.

Another factor that regulates P2X receptor responses is the rate of removal of the agonist. Diffusion will contribute to the movement of ATP away from the cell surface in all cells, and blood flow will exert profound effects on the duration of agonist exposure to P2X receptors on endothelial cells, platelets, erythrocytes and leukocytes. In addition, enzymatic degradation by ectonucleotidases plays a key regulatory influence, particularly E-NTPDase1 (CD39) [47–49]. Within in vitro experiments on human platelets, a ten-fold increase in the concentration of extracellular apyrase (a form of NTPDase from potato that displays similar properties to human CD39 [50]) reduced the activation of P2X1 receptors following collagen exposure [51]. This may result from "buffering" and/or active degradation of released ATP. On the other hand, a basal level of ATP-degrading activity is essential to prevent P2X1 desensitization in vitro [52] and in vivo [53] (see Fig. 13.2Aiii). Platelets, leukocytes, endothelial cells and blood-borne microparticles all express CD39 [54–58] and plasma also has ATP degrading activity [59–61]. Ectonucleotidase activity on the surface of platelets is insufficient alone in preventing P2X1 receptor desensitization in these cells, as receptor activity is rapidly lost following resuspension in apyrase-free saline [52]. The relative importance of plasma, microparticle or cellular CD39 in protecting platelet P2X1 desensitization in vivo remains to be ascertained. Although never directly tested, it is likely that loss of NTPDase1 activity in vivo results in complete desensitization of P2X1 in platelets and other blood cells since NTPDase1$^{-/-}$ mice suffer from chronically desensitized platelet P2Y1 [62] and *vas deferens* smooth muscle P2X1 receptors [53]. It is worth noting that P2X1 receptors are more readily lost in cellular preparations compared to other Ca^{2+}-mobilising pathways [52]. In addition, since it is not common practice in many laboratories to include apyrase in cellular preparations, studies will often be conducted under conditions where this cation channel has become completely desensitized (due to ATP release during cell preparation/culture), leading to an underestimation of its overall contribution. Of relevance and importance for future work, P2X1 receptor signals are at least partially recoverable in preparations of both platelets and continuous cell lines by exposure to a nucleotide-degrading environment [61, 63].

13.3 Sources of Ligand for Activation of P2X1 Receptors

13.3.1 The Importance of ATP as the Physiological Agonist of P2X1 Receptors

The principal physiological stimulus for gating P2X1 receptors is an increase in extracellular ATP. Although early studies in both platelets and heterologous expression systems suggested that ADP is also able to gate the opening of P2X1 receptors

[3, 6, 21, 64, 65], it is now recognised that this activity is entirely due to the 1–2 % contamination of commercial samples with ATP [66, 67]. It is clear that platelets possess one ATP-gated receptor (P2X1) and two ADP-gated receptors (P2Y1 and P2Y12) [68, 69]. Whilst ATP can activate both these GPCRs at high levels of expression in heterologous systems [70–72], it is an antagonist rather than an agonist of P2Y receptors in the platelet due to the lower receptor density levels [73–75]. Therefore, ATP stimulates platelets entirely through its ability to activate P2X1 receptors. Other possible physiological agonists of P2X1 include the diadenosine polyphosphates (Ap_nAs), and adenosine polyphosphoguanosines (Ap_nGs) [76–81], which are secreted from platelets. Ap_4A and Ap_5A are the most abundant of these compounds in platelet dense granules, albeit at levels substantially lower than ATP [82–84]. It is also important to note that Ap_nAs and Ap_nGs are only partial agonists at P2X1 receptors in rat mesenteric arteries and have a potency ten-fold lower than ATP [35, 79]. However, it is possible that diadenosine polyphosphates may outlast ATP in the circulation as they are degraded more slowly than ATP [85–87], and thus may act as longer-range or longer duration P2X1 agonists. Other possible physiological P2X1 agonists are uridine adenosine tetraphosphate (Up_4A) and adenosine tetraphosphate (Ap_4) which are released from endothelial cells and reach plasma concentrations sufficient to induce potent P2X1-dependent vasoconstriction [88, 89]. However, at present ATP is the only agonist with a clear physiological role in platelet P2X1 receptor activation.

There are three well-established routes whereby cells can be exposed to an increase in extracellular ATP and thereby gate the opening of P2X receptors. Firstly, there is simple diffusion from the cytoplasm as a result of general damage to the cell membrane following injury or necrosis. Secondly, several cells including platelets store ATP at high concentrations in specialised vesicles that are released via exocytosis. Thirdly, the pannexin family of anion-permeable channels represent a route whereby cellular signals can promote ATP efflux from healthy cells or during apoptosis. Exocytosis and pannexins represent routes for ATP release following cellular stimulation, for example by agonists of GPCRs or tyrosine kinase-linked receptors (TKRs). In addition, both of these ATP efflux pathways may also contribute to the background (i.e. constitutive) release of ATP that has been reported in many cells [90–92].

13.3.2 Release of Cytoplasmic ATP Following Cellular Damage

Cytoplasmic ATP concentrations are in the order of a millimolar or more in a healthy cell as a consequence of the ubiquitous use of this nucleotide as a cellular energy source. The plasma membrane is not normally permeable to ATP, and extracellular ATP levels are generally kept very low with the aid of ectonucleotidases [47]. It is therefore easy to understand how damage to the plasma membrane can

generate the micromolar extracellular concentrations of ATP that are required to stimulate P2X receptors (for example the EC_{50} for stimulation of P2X1 receptors is approximately 1 μM [2, 20, 21]). ATP levels in whole blood samples a few seconds after vascular injury have been reported to be in the range 0.2–2 μM depending upon the species, increasing to 20 μM ≈3–5 min after injury [93]. Damaged cells in the injured vessel walls were identified as the source of this ATP and it is likely that the ATP concentrations are much higher near the site of injury. Therefore, in theory this represents a potent signal to activate P2X receptors on platelets and other cells at the site of vascular injury, although the relative importance of this initial source of ATP within thrombus formation has not been clearly defined.

13.3.3 Exocytosis

Many non-excitable cells store ATP in secretory vesicles that can be released following regulated exocytosis [94]. For example, the dense granules of human platelets contain ≈0.5 M ATP, which is released via an exocytotic pathway involving SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptor) family proteins, assisted by accessory factors [95, 96]. It is clear that this is the major mechanism of ATP release in many cells [95, 97, 98], the exception being mature human erythrocytes which do not contain secretory vesicles [99, 100]. As in excitable cells, an increase in intracellular Ca^{2+} is a key stimulus for secretion in non-excitable cells, although other signalling events will contribute [95, 101–104]. Bulk phase measurements in platelet suspensions show that ATP can reach 1–5 μM following activation by a number of stimuli [105–109], however a surface-attached luciferase shows a considerably higher peak of 15–20 μM after simulation by thrombin [98, 110]. The EC_{50} for activation of P2X1 by ATP is 1 μM and the EC_{90} is ≈10 μM, therefore the extracellular level of this nucleotide following exocytosis can achieve near-maximal stimulation of the channel [78]. Autocrine activation of P2X receptors in patch clamp studies occurs as a series of transient inward currents in a quantal manner resembling the fundamental nature of exocytosis. This also suggests that vesicular ATP release and channel activation occurs at several different sites across the surface of an individual cell [111–113]. Similar multiple secretory events from single platelets have also been observed using electrochemical measurements of serotonin, which is stored and released from dense granules [114, 115]. In these real time voltammetric measurements, a high concentration of Ca^{2+} ionophore stimulated many more secretory events per platelet than the estimated total number of dense granules (≈6–8) [98, 116–118], This suggests that each dense granule will release its content via multiple exocytotic events rather than in an all or nothing manner. Theoretically, this could allow repeated activation of P2X receptors; however given the rapid desensitization and slow recovery (minutes) of the P2X1 receptor, it is unclear to what extent repeated release from each granule contributes to the substantial autocrine/paracrine activation of the ionotropic receptor observed in platelets [13].

P2X receptor activation itself can lead to granular secretion, as occurs after P2X7 receptor stimulation in mast cells [119–121]. Another possibility whereby exocytosis can use P2X receptors to amplify responses is via insertion of additional channels from internal membranes into the plasma membrane. This has been demonstrated in alveolar type II epithelial cells, where P2X4 receptors on the lamellar bodies lead to "Fusion-activated Ca^{2+} entry" when these organelles are inserted into the plasma membrane [122].

13.3.4 Release through Pannexin Channels

Pannexins are anion-permeable channels which also allow passage of molecules up to a molecular weight of approximately 1 kDa and serve as a route for non-lytic and non-vesicular regulated release of cytosolic ATP [123, 124]. Three isoforms exist, pannexin-1, -2 and -3, which are structurally related to the connexin family that form gap junctions between adjacent cells. Connexins can also form ion channels in the plasma membrane of a solo cell, when they are referred to as hemichannels or connexons. Consequently, the term hemichannel has been ascribed to pannexin channels, however this is a misnomer since pannexins do not form gap junctions due to glycosylation of the extracellular domain [125–127]. Pannexin-1 (Panx1) is ubiquitously expressed whereas Panx2 is found in the brain and central nervous system and Panx3 is restricted to the bone and skin [128]. Panx1 is expressed on several non-excitable cell types, including macrophages, platelets, astrocytes, airway epithelial cells and erythrocytes (reviewed in [100, 129]). Panx1 channel opening has been reported in response to oxygen-glucose deprivation, caspase cleavage, mechanical stimulation and elevation of $[Ca^{2+}]_i$ [123, 130–133]. However, we are really only at an early stage in understanding the cytosolic signals and environmental cues that regulate pannexins. It is clear that they represent an important route for release of ATP in several non-excitable cell types, particularly in erythrocytes that lack the capacity for regulated exocytosis [99, 100]. It is worth noting that although other ion channels have been proposed as pathways for cytosolic ATP efflux, including the cystic fibrosis transmembrane regulator, the maxi-anion channel and connexin hemichannels, clear evidence only exists for pannexins in this role [100].

Several studies have reported interactions between Panx1-dependent ATP release and activation of purinergic P2 receptors across a number of cell types [99, 134–140]. Recently, it has been shown that agonist-dependent stimulation of platelets causes efflux of the anionic dye calcein (similar molecular weight to ATP) through Panx1 channels. This ATP efflux pathway amplified platelet functional responses (e.g. aggregation) at low concentrations of agonist via activation of P2X1 receptors [129]. Another recent study has confirmed the presence of Panx1 in human and murine platelets and highlights that the channel serves as a particularly important route for collagen-evoked ATP release and subsequent aggregation through activation of P2X1 receptors [141]. Furthermore, this study identified a single nucleotide polymorphism 400C>A, which causes a switch from glutamine to

histidine within the N-terminal domain region and represents a gain in function mutation leading to increased ATP release and collagen-evoked responses. Panx1-dependent ATP release and activation of P2X receptors also plays key roles during immune responses [17, 137, 142, 143], including clearance of apoptotic cells [144–146].

13.4 Mechanisms whereby P2X1 Receptors can generate Cytosolic Ca^{2+} Signals

There are three main mechanisms whereby P2X1 receptor activation can generate or potentiate an increase in intracellular Ca^{2+}. The first is direct Ca^{2+} entry via opening of the P2X1 trimeric channel by extracellular ATP (or possibly other agonists such as diadenosine polyphosphates, see above). The second is via potentiation of phospholipase-C (PLC)-stimulated Ca^{2+} mobilization downstream of GPCRs or TKRs. Finally, since P2X1 receptors will generate substantial cellular entry of Na^+, this may lead to secondary Ca^{2+} entry via reverse Na^+/Ca^{2+} exchange. This final route is a focus of Chap. 4 of this volume and is therefore only briefly considered.

13.4.1 Direct Entry of Ca^{2+} via the Ligand-Gated Ion Channel

P2X1 receptors show a high permeability to Ca^{2+} (P_{Ca}/P_{Na} ≈3.9) [5, 147]. With a resting membrane potential of −50 to −70 mV in most non-excitable cells and a 10,000-fold concentration gradient for Ca^{2+} (1 mM outside and 100 nM inside), a large inward driving force exists for this cation. Selective stimulation of P2X1 receptors by ATP or the non-hydrolysable analogue α,β-methyleneATP can generate substantial increases in intracellular Ca^{2+}, typically in the range 100–500 nM following supramaximal agonist application to fura-2-loaded washed platelet suspensions [51, 52, 148, 149] (see Fig. 13.2Ai). Since P2X1 receptor responses show a steady decline from the outset within in vitro preparations [52], this may be an underestimation of maximal Ca^{2+} responses achievable in vivo through this ionotropic receptor. Increases in extracellular Ca^{2+} cause marked increases in P2X1-evoked intracellular Ca^{2+} responses [148], presumably via a combination of greater competition for the ion channel pore and enhanced driving force. In native tissues where P2X1 is the predominant isoform, it has been estimated that ≈6–10 % of current flow through this channel under physiological conditions is mediated by Ca^{2+} [150–152] (a similar estimate, ≈8 % has been reported for human P2X4 receptors in an expression system [153]). P2X1 receptors are permeable to multiple physiological cations, including Na^+, K^+, Ca^{2+}, Mg^{2+}, and under normal ionic conditions the reversal potential for current flow through this conductance is close to 0 mV [21, 147]. Its opening will therefore promote depolarization from the standard resting potential of ≈−50 to −70 mV in non-excitable cells. Thus, it can be considered that

Ca^{2+} influx, Na^{+} entry and membrane depolarization are the three "primary" signals resulting from P2X receptor activation.

When platelet preparations are generated in a manner that limits desentization of P2X1 receptors (e.g. by addition of sufficient apyrase, see Sect. 13.2.2), activation of this channel contributes to the Ca^{2+} response stimulated by multiple platelet agonists (see Fig. 13.2B) as a consequence of ATP released from granules or Panx1 channels (see Sects. 13.3.3 and 13.3.4). The percentage contribution by P2X1 depends upon the type and level of stimulation of the GPCR or TKR. For collagen-dependent GPVI and thrombin-evoked PAR receptors, P2X1 contributes more at low, compared to high, levels of agonist. For thromboxane A_2, similar enhancements due to P2X1 activation are observed at low and mid-range agonist concentrations and for ADP, P2X1 only contributes at high levels of P2Y receptor activation. This presumably reflects a combination of the amplitude of the PLC-dependent Ca^{2+} response, together with the extent to which secretion (and/or Panx1) is stimulated. Strikingly, at low levels of collagen, P2X1 can contribute up to 92 % of the peak response, which decreases to 45 % at high collagen levels [154] (see Fig. 13.2B *left panel*). This is consistent with the greater contribution of P2X1 to aggregation responses at low compared to high collagen concentrations [14, 155]. Ca^{2+} responses mediated via another tyrosine kinase-linked platelet receptor, Toll-like receptor 1/2, are also substantially amplified by ATP release and P2X1 activation. Greater amplification by P2X1 for TKRs compared to GPCRs likely reflects the efficiency with which the signalling cascade stimulates secretion (and Panx1 activation) compared to IP_3-dependent Ca^{2+} mobilization at low levels of agonist.

Two factors could explain the high level of efficiency with which P2X1 contributes to TKR or GPCR-evoked Ca^{2+} responses. Firstly, the sites of release of ATP could be closely located to P2X1 receptors. Evidence has been presented for co-localization of P2X1 receptors and Panx1 channels in platelets [129] and both proteins translocate (along with P2X4 receptors) to the immune synapse during interaction of T cells with antigen-presenting cells [137]. There is no direct evidence for localization of P2X1 and exocytotic sites of release, however this could potentially be a function of lipid rafts. These microdomains contain both P2X1 receptors and SNARE proteins and raft disruption leads to inhibition of P2X1 responses and exocytosis [43, 156]. Secondly, the speed of activation of P2X receptors means that ATP does not need to be in the proximity of the channel for very long to gate opening, and thus trigger significant Ca^{2+} entry, prior to being swept away from the cell surface as will occur within the circulation.

13.4.2 P2X-Dependent amplification of Phospholipase-C-stimulated Ca^{2+} Signals

Studies in platelets and a non-excitable cell line have shown that when P2X1 receptors and P2Y1 receptors are co-stimulated, the Ca^{2+} increase is super-additive, i.e. greater than the sum of the responses to each receptor alone [12, 157] (see

Fig. 13.2C). This effect is due to enhancement of the P2Y1 receptors by the P2X1-evoked Ca^{2+} influx without any evidence for a role for the Na^+ entry or membrane depolarization that also occur following activation of the ionotropic receptor (see above). This effect is not restricted to the P2Y1 receptor as it also occurs with co-activation of P2X1 and thrombin-stimulated PAR1 or acetylcholine-stimulated muscarinic M1 receptors [157]. The underlying cause of the synergy is Ca^{2+} influx and a general increase in free Ca^{2+} (rather than a P2X1-specific subplasma membrane cytosolic Ca^{2+} response) as ionomycin similarly enhanced P2Y1 receptors. It is likely that Ca^{2+} works to enhance the GPCR-evoked Ca^{2+} mobilization by an effect on PLC and IP_3 receptors, both of which are known to be stimulated by Ca^{2+} increases in the concentration range observed following P2X1 stimulation [158–161], although the relative importance of these two targets was not resolved. Interestingly, the potentiation of GPCR Ca^{2+} signals by P2X1 declines with time after addition of the ionotropic receptor agonist, lasting for over a minute following a maximal response. This could represent an important means whereby P2X1 receptors potentiate platelet function since it can be argued that ATP will often be in excess of ADP immediately following release from damaged endothelial cells and ADP will subsequently rise in concentration due to ectonucleotidase activity [47]. Although no evidence was obtained for a role of Na^+ influx or membrane depolarization in the synergy between P2X1 and P2Y1 receptors [157], Gq-protein-coupled receptors in some cell types are potentiated by membrane depolarizations [162–165] of the magnitude expected following opening of P2X receptors [166].

13.4.3 P2X1-Dependent Secondary Entry through Reverse Na^+/Ca^{2+} Exchange Activity

If 6–10 % of the P2X1-evoked inward current is carried by Ca^{2+} under physiological concentrations (see above), the remainder will be due to Na^+ entry. Indeed, P2X1 receptors are known to generate significant increases in intracellular Na^+ in platelets [167]. This may allow reverse Na^+/Ca^{2+} exchange (NCX) activity to generate Ca^{2+} entry, depending upon the membrane potential (as discussed in [168]). A NCX blocker has been used to suggest that reverse NCX activity occurs in platelets following P2X1 activation [169]. Readers are directed to another Chapter in this volume (Chap. 4) for more information on the role of reverse NCX activity as a pathway for Ca^{2+} entry.

13.5 Signalling through P2X1 Receptors

All P2X1-dependent functional responses require Ca^{2+} influx [14, 52, 148, 170, 171] and there is a strong correlation between the intracellular Ca^{2+} increase and shape change response to the P2X1 agonist α,β-meATP [52, 148]. The Ca^{2+} increase

activates calmodulin dependent myosin light chain kinase (MLCK) leading to myosin light chain phosphorylation (MLC-P) that is presumably responsible for the cytoskeletal events underlying P2X1-evoked shape change and granule localization [171]. P2X1 does not stimulate shape change through Rho-kinase in the platelet [172]. Selective P2X1 activation has also been shown to stimulate transient phosphorylation of ERK2, a member of the mitogen-activated protein kinase family [173]. Based upon experiments with extracellular EGTA, the broad spectrum PKC inhibitor GF109203-X and the calmodulin inhibitor W-7, P2X1-evoked ERK2 phosphorylation requires extracellular calcium and activation of both calmodulin and PKC [171, 173]. However, since P2X1-evoked ERK2 phosphorylation is slow compared to the shape change, the main role of this signalling event is envisaged to be enhancement of aggregation at low to intermediate levels of collagen [171, 173, 174]. This role for ERK2 in P2X1-induced potentiation of collagen responses is further supported by studies using transgenic mice over-expressing the human P2X1 receptor in the megakaryocytic cell lineage [174]. It is, however, unclear why concentrations of ADP that normally stimulate Ca^{2+} responses of larger amplitude and longer duration compared to those generated by P2X1 receptors [148, 171, 173] fail to activate ERK2 phosphorylation [173]. Recently, it has been shown that ERK2 does not contribute to the potentiation of P2Y1 receptor-evoked Ca^{2+} responses by P2X1 [157]. However, pharmacological inhibition of ERK1/2 prevented the potentiation of aggregation responses resulting from co-activation of these two receptors in human platelets [157]. This concurs with the fact that P2X1-evoked ERK2 phosphorylation is relatively slow compared to the shape change [173] and that inhibition of ERK1/2 with the MEK1/2 antagonist U0126 does not alter α,β-meATP-evoked shape change [171]. However, U0126 does not influence the amplification of thromboxane A_2 responses by P2X1, including aggregation and P-selectin surface expression; instead this requires activation of the p38 MAPK [175]. In T lymphocytes stimulated by hypertonic stress, P2X1, along with P2X4 and P2X7, also stimulate interleukin-2 production through activation of p38 MAPK [176].

13.6 The Relevance of Non-Excitable Cell P2X1 Receptors in Health and Disease

13.6.1 Thrombosis and Haemostasis

Selective activation of P2X1 receptors in human and murine platelets leads to shape change and granule centralization without significant aggregation [14, 15, 52, 148, 155, 170, 173]. However, release of ATP and thus secondary activation of P2X1 receptors contributes to aggregation induced by low levels of collagen [14, 155], PAR1 agonist [15] or thromboxane A_2 [175]. In addition, P2X1 receptors potentiate aggregation when coactivated alongside low level stimulation of receptors for thrombin [15], ADP [157], thrombopoietin and adrenaline [170]. P2X1 receptors also

amplify thrombus formation on a collagen-coated surface under flow, with the greatest contribution observed at levels of shear experienced within small arteries or arterioles and with little role at venous shear rates [14]. Genetic deletion or selective pharmacological block of P2X1 receptors in mice reduces thrombus formation in small arteries following laser injury [14, 15, 177] and also limits the fatal consequences of thromboembolism induced by co-injection of collagen and adrenaline [14, 177]. Furthermore, overexpression of human P2X1 receptors in a murine model enhances P2X1 receptor-dependent responses in vitro and in vivo [172, 174]. Neutrophils also accumulate at sites of vascular injury where they contribute to fibrin formation [178, 179] and indeed are one of the first cells to adhere to the inured vessel wall [180]. Interestingly, a recent study has reported that P2X1 receptors on both neutrophils and platelets are responsible for the reduced arterial thrombosis observed following vascular injury in $P2X1^{-/-}$ mice [19]. An increase in intracellular Ca^{2+} was observed in neutrophils activated at the sites of vascular injury prior to thrombus formation although whether this is entirely due to Ca^{2+} influx through P2X1 receptors was not studied, presumably because neutrophil adherence was virtually absent in $P2X1^{-/-}$ mice. Together, these studies suggest that P2X1 receptors may represent a useful target to prevent arterial thrombosis, particularly since bleeding times are little affected by loss of function of this receptor [14, 177].

An important property of P2X1 receptors that distinguishes them from other Ca^{2+} permeable ion channels in the platelet is their resistance to inhibition by cyclic nucleotides [4, 51, 113, 181]. Nitric oxide and prostacyclin are crucial signals whereby the endothelium can inhibit platelet function in the intact circulation by elevating cyclic GMP and cyclic AMP, respectively [182]. These cyclic nucleotides inhibit Ca^{2+} signals via actions on PLC and also IP_3 receptors, which will result in reduced activation of virtually all Ca^{2+} mobilization pathways (TRPC6, IP_3R, store-operated Orai1 channels) with the exception of ATP-gated P2X1 receptors [51, 182–184]. In addition, the platelet dense granule secretory responses downstream of GPVI collagen-receptors and the innate immune receptor TLR1/2 are also partially resistant to elevations of cytosolic cyclic nucleotides [51]. This may allow P2X1 receptors to act as a "primer" of platelet responses since they will be more active in the initial stages of vascular injury when residual effects of NO and PGI_2 are present.

13.6.2 Inflammation and Sepsis

Extracellular ATP is an important signal during the early stages of infection and inflammation [185]. In contrast to platelets, most leukocytes express multiple P2X receptor subtypes, particularly P2X7, 4 and 1 [143, 186]. P2X7 receptors, formerly known as P2Z receptors, have well-established roles in immune cell responses [121, 187, 188]. More recently, P2X1 receptors have also been shown to be important in activation of T cells by antigen-presenting cells [137] and to stimulate neutrophil chemotaxis [18, 189]. It is unclear why Rho kinase is responsible for P2X1-evoked

neutrophil migration [189] but not involved in the shape change responses mediated by this receptor in the platelet, which instead depend upon calmodulin-dependent myosin light chain kinase (MLCK) [171]. Neutrophils are key mediators of the innate immune response and known to be recruited to infected tissues to fight bacterial infection, however when excessively activated they can lead to tissue damage, as occurs in sepsis [190, 191]. At sites of inflammation, neutrophils adhere to the activated endothelium prior to migration across the vascular wall [192]. In an animal model of sepsis, loss of P2X1 receptors reduced neutrophil migration from the circulation and protected against tissue damage following injection of LPS [18]. However, opposite results were obtained using similar LPS concentrations by another group [193], who observed enhanced neutrophil migration into the lungs and reduced animal survival in $P2X1^{-/-}$ compared to wild type mice. The reason for these contrasting results remains unclear. It is worth noting that whilst P2X1 receptors contribute to neutrophil signalling, they also promote thrombus formation (see above) which can either accentuate tissue damage or improve host protection by trapping and destroying invading organisms [194].

13.7 Conclusions

Extracellular nucleotides are important autocrine and intercellular signalling molecules, mediating their effects through surface receptors that fall into two categories, ligand gated P2X and G-protein-coupled P2Y receptors. Ca^{2+}-permeable P2X receptors play key roles in a variety of cell types, including non-excitable cells such as platelets and leukocytes, and thus represent therapeutic targets for treatment of thrombosis and modulation of immune responses. In the platelet it is well established that Ca^{2+} influx is the main signalling event that stimulates P2X1 receptor-dependent functional responses, alone or in synergy with other receptor signalling pathways, although Na^{+} influx will also occur. It is likely that the substantial contribution of ATP-gated P2X receptors to cellular responses results from their significant Ca^{2+} permeability, efficient stimulation by ATP release and rapid activation kinetics compared to other Ca^{2+} mobilization pathways.

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Part III
Role of Cellular Microdomains and Organelles in Calcium Entry

Chapter 14
The Calcium Entry-Calcium Refilling Coupling

Ziane Elaib, Francois Saller, and Regis Bobe

Abstract Calcium ions (Ca^{2+}) are versatile messengers that need to be tidily regulated in time and space in order to create a large number of signals. The coupling between Ca^{2+} entry and Ca^{2+} refilling is playing a central role in this Ca^{2+} homeostasis. Since the capacitative Ca^{2+} entry has been described, different mechanisms have been proposed in order to explain how the Ca^{2+} entry could be under control of intracellular store Ca^{2+} depletion. Today, in addition of STIM1 and Orai1, the two major elements of SOCe, increasing attention is put on the role of the transient receptor potential canonical (TRPC), that can form protein clusters with Orai1, and Sarco/endoplasmic reticulum Ca^{2+}ATPases (SERCAs), that refill the stores and are also located in the same environment than SOC clusters. Altogether, these proteins elaborate either Ca^{2+} microdomains in the vicinity of the membrane or larger Ca^{2+} increases overtaking the whole cell. The coupling between Ca^{2+} entry and Ca^{2+} refilling can possibly act much further away from the plasma membrane. Ca^{2+}, uptaken by SERCAs, have been described to move faster and further in the ER than in the cytosol and to create specific signal that depends on Ca^{2+} entry but at longer distance from it. The complexity of such created Ca^{2+} currents resides in the heteromeric nature of channels as well as the presence of different intracellular stores controlled by SERCA2b and SERCA3, respectively. A role for mitochondria has also been explored. To date, mitochondria are other crucial compartments that play an important role in Ca^{2+} homeostasis. Although mitochondria mostly interact with intracellular stores, coupling of Ca^{2+} entry and mitochondria cannot be completely rule out.

Keywords SERCA • STIM1 • Orai1 • TRPC channels

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14.1 Introduction

Calcium ions (Ca^{2+}) are universal second messengers that create a ubiquitous signal transduction pathway that is functional in every cellular type and species. Ca^{2+} signal regulates various processes including cell proliferation, response to the environment, death and apoptosis…

In a review published in 2006, R.J.P. Williams presented a paradigm in which the role of calcium as second messenger could be the result of evolution to adapt cells to life in an oxidized environment [1]. When cyanobacteria changed the face of the world and killed nearly all living species (mostly prokaryotic cells), surviving cells evolved to internally compartmentalized eukaryotic cells. These cells needed protection, ability to recognize their environment as well as to coordinate the internal activity of their new compartments; a new messenger was needed. As life had a long experience in dealing with calcium ions, widely common molecules that were rejected out cells for billions of years because of their ability to form insoluble salts (precipitates) with inorganic and organic anions carbon and phosphate, calcium ions became the messengers that not only coordinated the action of intracellular compartments but also triggered cell responses to the environment.

As Ca^{2+} signaling is very versatile, the question is to figure out how a single cation can code for a multitude of cellular responses, (i.e.) how distinct signals can be generated and how cell compartments can decipher specific messages.

The Ca^{2+} signal has, in order to be efficient and specific, to be tightly modulated in time and space. Multiple partners exist, forming cell specific calcium toolkits that are organized to control calcium flux and to translate Ca^{2+} signals into cellular activity. At rest, cytosolic Ca^{2+} is maintained around 50–200 nM. Upon activation its concentration will increase to create a global Ca^{2+} signal that can propagate over large distances (10–100 μm) in the range of μM and/or formation of microdomains with very high level of Ca^{2+} concentration (50–100 μM) in the vicinity of the Ca^{2+} channels. These latter events only spread over 20 nm and, Ca^{2+} concentration drops rapidly as ions are buffered and diluted in the cytosol [2, 3].

The increase in cytosolic calcium is due to its influx from the extracellular medium through the plasma membrane (PM) (extracellular Ca^{2+} concentration ranges between 1 and 2 mM in aqueous conditions) and its depletion from intracellular stores, mainly (but not only) the endoplasmic reticulum (ER). The storage capacity of the ER is limited and Ca^{2+} reuptake is performed by Ca^{2+} pumps termed Sarco/Endoplasmic Reticulum Ca^{2+}ATPases (SERCA) that control the Ca^{2+} concentration in the ER lumen. As SERCAs compete with other calcium transporters, such as Na/Ca exchangers or plasma membrane Ca^{2+}ATPases (PMCA) that extrude Ca^{2+} to the extracellular medium, a continuously loss of intracellular calcium storage would be observed if a subsequent calcium entry from extracellular medium was not organized to help cells to refill their intracellular stores in calcium. This Ca^{2+} entry is controlled by the ER lumen concentration and termed SOCe (Store operated calcium entry). If the SOC process was initially designed to maintain the concentration of stored Ca^{2+}, it now appears as a crucial actor that shapes the calcium signal within the cells.

14.2 Store Operated Calcium Entry (SOCe)

The classical way of non-excitable cell stimulation associates G protein coupled receptor and phospholipase activation. The newly formed inositol 1,4,5-trisphosphate (IP_3) will open its receptor channel (IP_3R) inserted in the endoplasmic reticulum (ER) membrane and will allow the mobilization of Ca^{2+} [4–6]. SOCe is a ubiquitous pathway in non-excitable cells as well as in some excitable cell types [7]. This event is associated to Ca^{2+} influx across the plasma membrane (PM). This paradigm of the existence of a store-dependent Ca^{2+} influx was proposed 30 years ago by J. W. Putney as Capacitive Calcium Entry (CCE) [8]. The use of Thapsigargin (Tg), a selective noncompetitive inhibitor of the SERCAs that is isolated from the plant *Thapsia garganica* and triggers a "passive" depletion of the ER stored Ca^{2+} without receptor activation, have permitted to establish that the store depletion by itself was responsible for a Ca^{2+} influx similar to those recorded as SOCe and, more important, independently of IP_3 [9, 10].

While the idea of having a Ca^{2+} entry from the extracellular medium under the control of intracellular organelles catches on, it was very difficult to have a clear insight into the underlying mechanisms until recently. Since, a number of studies have focused on the current specificities of this SOCe [11–14].

The first question was to understand how Ca^{2+} mobilization was able to activate the Ca^{2+} entry. At least three major hypothetic mechanisms have been advanced.

First, the existence of a soluble mediator from intracellular stores was proposed and termed "calcium influx factor" (CIF) [15]. This messenger was described to be released into the cytoplasm upon Ca^{2+} mobilization in Jurkat cells and is still unknown. Probably due to the lack of its molecular identity, very few studies are since focusing on CIF. Additionally, as CIF is "purified" or isolated after a long process, it is difficult to ascertain its specificity [16]. However, a recent publication showed that only CIF produced by agonists or Thapsigargin activation is able to induce a 2-APB (SOCe inhibitor) sensitive Ca^{2+} influx when injected in non-activated cells. Similar treatments, with components isolated in the same way than CIF but from non-activated cells, have no effect. Such data suggest in contrast, that the existence of a putative CIF is not ruled out [17].

The second proposed hypothesis was a conformational coupling between IP_3 receptor (IP_3R) and SOC channels in an ER-PM junction [18]. In this model, upon activation, IP_3R is able to activate Ca^{2+} channels and even to create a long channel that could transport Ca^{2+} directly from the extracellular medium into the ER lumen. While the idea of having the IP_3R working in a reverse mode was not established, it was later proposed that signaling clusters containing Transient receptor potential canonical channel type 1 (TRPC1) (see below for the roles of TRPC in SOCe) and IP_3R exist [19], both being able to bind directly through a CIRB domain positioned in the C-Terminal part of TRPC1 [20]. TRPC1 was described to associate with IP_3R in platelets upon store depletion, and to participate to extracellular Ca^{2+} influx into the cell [21, 22].

The last and most successful model was the existence of a direct interaction between ER resident proteins and Ca^{2+} channels.

14.2.1 Molecular Identity and Mechanism of Store Operated Channel

It took nearly 20 years to validate the whole concept of the SOC channel formation and the two key proteins involved in SOCe were almost concomitantly identified. First, the role of the Ca^{2+} sensor protein STIM1 (stromal interacting molecule 1) was highlighted in 2005 using a RNA interference-based screening in Drosophila and HeLa cells that showed that its knockdown strongly reduced SOCe [23, 24]. The second player, Orai1, was identified a year later also in Drosophila and human cells. Both RNAi screens in *Drosophila* and gene mapping on lymphocytes from a family with a severe combined immunodeficiency (SCID) revealed that Orai1 deficiency was associated to a defect in SOCe [25–27].

14.2.1.1 STIM1

STIM1 is a ubiquitously expressed protein which is widely expressed from Drosophila to mammalian cells. STIM1 is a single transmembrane protein of 77 kDa inserted predominantly in the ER [28] and distributed evenly along its membrane [29]. This protein is the crucial link between ER and Ca^{2+} channel in the PM. The luminal part of the protein contains two EF-hand (canonical and hidden EF-hand domains (cEF) and (hEF), respectively) domains and an adjacent sterile α motif (SAM) (Fig. 14.1a). Only the cEF domain can bind Ca^{2+} and confers to STIM1 its sensor function [23, 30, 31].

When Ca^{2+} stores are at rest, the estimated luminal Ca^{2+} concentration is about 500 μM [32]. At this level, Ca^{2+} binding to cEF allows the formation of a stable complex between EF and SAM domains. Upon ER depletion, Ca^{2+} dissociates from the STIM1 EF-hand domain, thus destabilizing the EF-hand-SAM complex (Fig. 14.1c). Both EF-hand and SAM domain will then expose their hydrophobic regions leading to the oligomerization of STIM1 [31, 33, 34]. It seems that the clustering of

Fig. 14.1 (continued) are composed of four transmembrane domains (TM1 (*blue*), TM2 (*purple*), TM3 *dark red*) and TM4 (*green*)). Two regions (CAD binding domains) are exposed into the cytosol and localized at both extremities of the proteins. On the right part of the figure using the same color code for the transmembrane domains of Orai is a top down schematic view of the hexameric Orai structure inserted into the plasma membrane with a Ca^{2+} (*red dot*) in the channel. (**c**) STIM1/Orai1 activation model. (*a*) Under resting conditions, the STIM1 Ca^{2+} binding to EF-hand domain masks SAM domains and keeps cytosolic domains folded and inactive. (*b*) Depletion of Ca^{2+} within the ER induces structural reorganization that uncovers SAM domains allowing oligomerization of STIM1. This also extends binding/activating domains towards the plasma membrane and (*c*) leads to clustering of Orai through interaction between SOAR and CAD domains

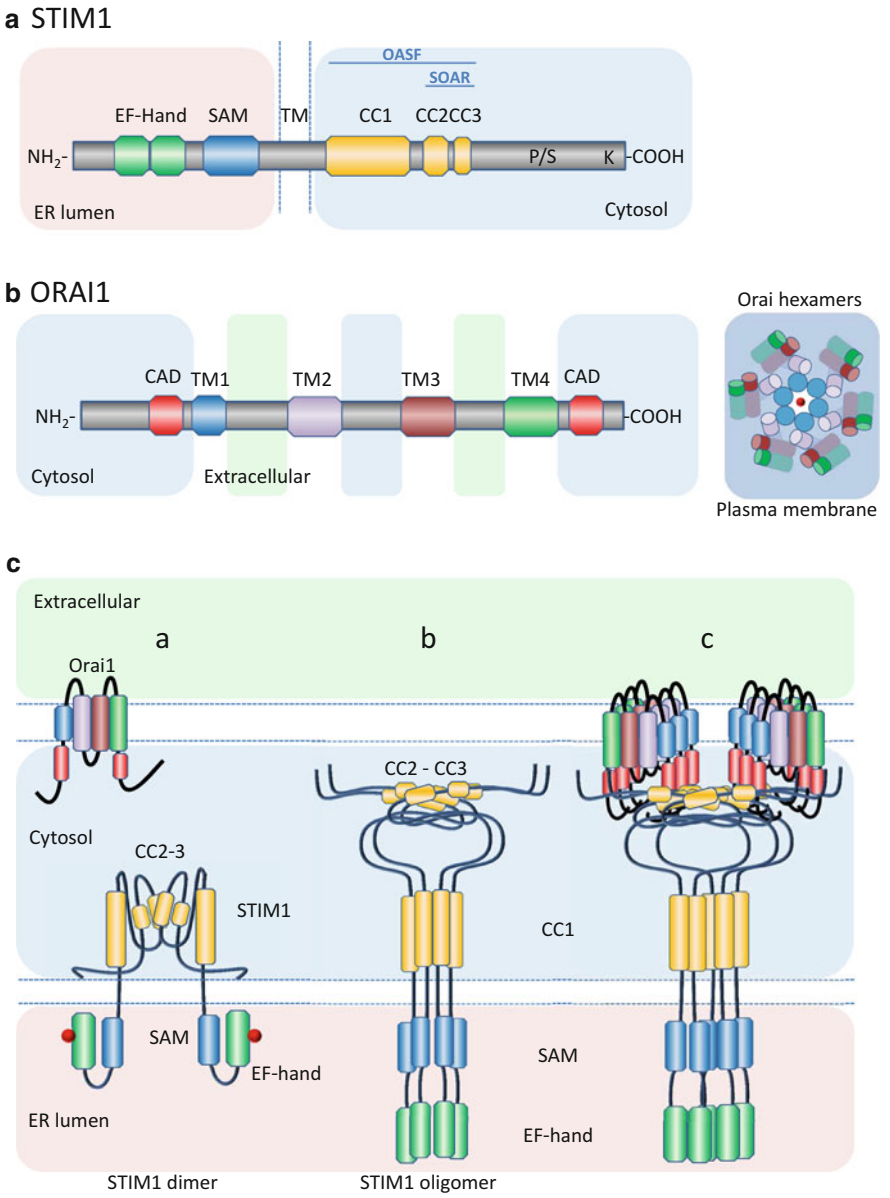

Fig. 14.1 Functional domains of STIM1 and Orai1 and model of coupling between STIM1 and Orai1. (**a**) Schematic representation of the main functional domains of STIM1. The N-terminal part of STIM1 (amino acids 1–214) is in the ER lumen and includes both EF-Hand domains (canonical and hidden EF-hand) and a sterile α motif (*SAM*) followed by transmembrane domain. In the cytosolic part of the protein are the coiled coil domains (CC1, CC2 and CC3) that form the region involved in the binding and activation of Orai (STIM-Orai activation region (SOAR, amino acids 345–444) or Orai activating small fragment (OASF, amino acids 233–450) and. The C-terminal part of STIM1 includes a serine/proline rich domain (P/S) and a lysine-rich domain (K). (**b**) Schematic representation of the main functional domains of Orai1. Orai monomers

EF-hand and SAM is critical, as similar oligomerization resulting in SOC activation was obtained by replacing EF-hand domain with FKBP-rapamycin binding domain under rapamicyn analogue stimulation [35].

To activate the Ca^{2+} channel, STIM1 clusters have to translocate to the plasma membrane to form punctate structures <25 nm from the plasma membrane (which corresponds to ER-PM junctions) [36]. It has been suggested that STIM1 could be preferentially localized within specific regions into the ER membrane, the so called "precortical subdomains" that will form cortical ER upon Ca^{2+} depletion [37]. A role for microtubule and F-actin polymerization seems to be involved, depending on the ER stores involved in the SOC (see below).

Of note, STIM2 shares similar structure with STIM1 and although its Ca^{2+} dissociation constant (Kd) is higher that of STIM1 (400 μM vs 200 μM respectively) [38], which could make STIM2 a better sensor for Ca^{2+} depletion [38], the unfolding of EF-hand – SAM domains for STIM2 appears to be much slower resulting in a lower SOC activation than STIM1 [39].

14.2.1.2 ORAI1

In mammalian cells, three genes code for three homologs: Orai1, Orai2 and Orai3. Orai monomers include four transmembrane domains (TM1–TM4) with a particular role of TM1 in the pore formation of the channel [40], the N- and C-terminal tails being cytoplasmic. Recent biochemical and fluorescence studies suggest that the SOC channel is composed of 6, and not 4 [41, 42] monomers that can form homo or heteromers of Orai (although Orai1 seems to be critical for the channel activity [43, 44]). The hexamer is organized in a concentric form with the most aqueous transmembrane α helice (TM1) in the central part of the pore (Fig. 14.1b) [42, 45, 46]. Orai proteins are inserted homogenously in the PM in resting cells but rapidly associate in clusters inside raft domains of the PM upon Ca^{2+} depletion and co-localize above the STIM1 clusters [47, 48]. The clustering by itself is not sufficient to open the channel and the direct interaction between STIM1 and Orai1 also leads to its activation.

14.2.2 Coupling Between STIM1 and Orai1

The coupling between STIM1 and Orai1 involves several domains of these proteins, all localized in the cytosol. Various regions named CAD, SOAR, OASF or Ccb9, all similarly located in the cytosolic part of STIM, have been identified in 2009 as being critical for the Orai activation (Fig. 14.1a) [48–51]. All of them cover the CC2 (aa 363–389) and CC3 (aa 399–423) regions of STIM1 that form a hairpin motif.

Two regions termed "CRAC activation domain" (CAD) have been proposed to bind STIM1 based on mutations in Orai and in silico studies; one is N-terminal (aa 73–85) and one is C-Terminal (aa 272–292) [52–54]. Whether this two CAD form

distinct binding regions or can form a unique binding site for STIM1 is still unknown and has to be determined. Interestingly, these regions seem to share structural homology with the STIM1 CC2 and CC3 domains, suggesting the possible formation of complex structure between the domains of both proteins [54].

The proposed mechanism suggests that, at rest, activation sites of STIM1 are masked by interaction with other flanking domains (possibly CC1 domain) of STIM1, thus preventing any interaction with Orai1. Upon Ca^{2+} depletion, lack of Ca^{2+} binding to EF-hand leads to homo association of CC1 (similarly to the SAM domains). This structural reorganization unmasks the CC2–CC3 domains and allows a physical extension of these domains in the cytoplasm [55] letting them to become available for Orai1 binding [52]. When binding to STIM1, Orai proteins will also change their structural conformation and either open their gates to Ca^{2+} or become highly specific for Ca^{2+} [56, 57] (Fig. 14.1c). Indeed, Orai channels display a selectivity for Ca^{2+} that is 1000 times higher than that for Na^{+} [58] when they are activated by STIM1. However, the mutant V102C/A, which appears to be constitutively activated, displays a poorly selective activity in absence of STIM1 and can regain its selectivity when co-expressed with STIM1 [59].

This first part of the chapter explained the relationship between ER and SOCe activation via STIM1 and ORAI1. Although STIM1 and Orai1 are crucial in coupling between ER and SOCe activation, other players participate in SOCe. First, a number of regulators of SOCe interact with either STIM1 or Orai1 (for review see [60, 61]). In addition other proteins can also form Ca^{2+} channels. A special attention was given to TRPC, that role as SOCe was strongly considered, until Orai1 was identified, a matter of debate.

14.2.3 TRPC

Transient receptor potential canonical (TRPC) are members of the superfamily of Transient receptor potential proteins. Since the discovery of its role as Ca^{2+} channel downstream of PLCγ2 activation, the TRPC proteins were proposed to be part of the SOC channel complex [62]. Probably because TRPC family is large and presents members with distinct functional capacities, whether TRPC function as SOCe has been highly debated [63, 64]. Among the TRPC family (TRPC1-7), TRPC1 is the most often associated to SOCe and was described to act as a STIM1-dependent Ca^{2+} channel in many studies [65–69]. Its expression in cell lines potentiates SOCe induced either by PLC activation or pharmacological (Tg) Ca^{2+} depletion [70].

It is however possible that TRPC form a macro-complex with Orai1 which therefore would explain their impact on SOCe without forming the channel by itself. Accordingly, direct association of Orai1 with TRPC3 and TRPC6 has been observed in HEK293 and COS1 cells [71, 72].

Most likely, as it has been described in blood platelets for TRPC1, TRPC play multiple role in the Ca^{2+} entry and their participation implies a higher level of complexity in the regulation of the Ca^{2+} entry controlled by store depletion [73].

Activation of TRPC1 downstream of Ca^{2+} depletion and their direct association with STIM1 has very recently been confirmed with a new single ion channel detection technique which further ascertains their possible role as SOCe [74].

Additionally to their action as SOCe, some members of the family, TRPC3, 4 6 and 7 have been described to be activated via diacylglycerol (DAG) upon PLC activation by G-protein coupled receptor stimulation. These activations are parallel to the Ca^{2+} mobilization but independent of Ca^{2+} depletion [67, 75, 76] and behave as ROC. Moreover, some authors suggest a direct link between IP_3R and TRPC, which lets suppose another way of store dependent Ca^{2+} entry regulation [19, 65, 77].

Thus, these three distinct pathways all activate TRPC channels, two being dependent on Ca^{2+} store depletion and one being parallel to the activation pathway of the Ca^{2+} depletion, might co-exist in the same cells (Fig. 14.2). Therefore, some TRPC members appear to be more inclined to participate to the SOC, like TRPC1, while some others seem to be more sensitive to DAG or IP_3R depending on their level of expression, their stoichiometry with STIM1 expression and the cellular models.

Finally, the whole system complexity increases again as TRPC can form heterodimer, thus sharing the properties of the different subunits that constitute the channels.

The paradigm of the crosstalk between Ca^{2+} entry and Ca^{2+} refilling is based on the observation that the fundamental reason for store-operated Ca^{2+} entry is that cells loose Ca^{2+} during signaling, Ca^{2+} mobilized from the ER to the cytoplasm is later transported outside the cell by plasma membrane Ca^{2+}ATPases (PMCA) or by the Na^{+}/Ca^{2+} exchanger (NCX). To ensure sustained signaling, the Ca^{2+} ions that have been extruded must return back to the store. Direct measurements of Ca^{2+} concentrations inside different organelles using nucleus, mitochondria or ER targeted aequorins have established that the impact of Ca^{2+} influx (by increasing extracellular Ca^{2+}) on Ca^{2+} uptake was 30 times larger in the ER than in other organelles [78]. Therefore, the third (or fourth taking into account TRPC) player in the coupling between Ca^{2+} entry – Ca^{2+} refilling has to be the Ca^{2+} pumps inserted in the ER membrane, the sarco/endoplasmic reticulum Ca^{2+}ATPase (SERCA).

14.3 SERCA

The SERCA family is composed of three genes giving rise to multiple isoforms though alternative splicing. SERCA2b isoform is ubiquitously expressed and plays a housekeeping function in the Ca^{2+} homeostasis. SERCA3 isoforms were firstly observed in non-muscular cells but some SERCA3 isoforms have now been described in smooth muscle as well [79–81].

The function of SERCA is to transport cytosolic Ca^{2+} into the ER, an action that will affect SOCe in two different ways depending on the point of view. From the ER point view, SERCA increase luminal ER Ca^{2+} concentration and allow STIM1 to return back to its inactivate status [82]. At the same time, Ca^{2+} uptake from the

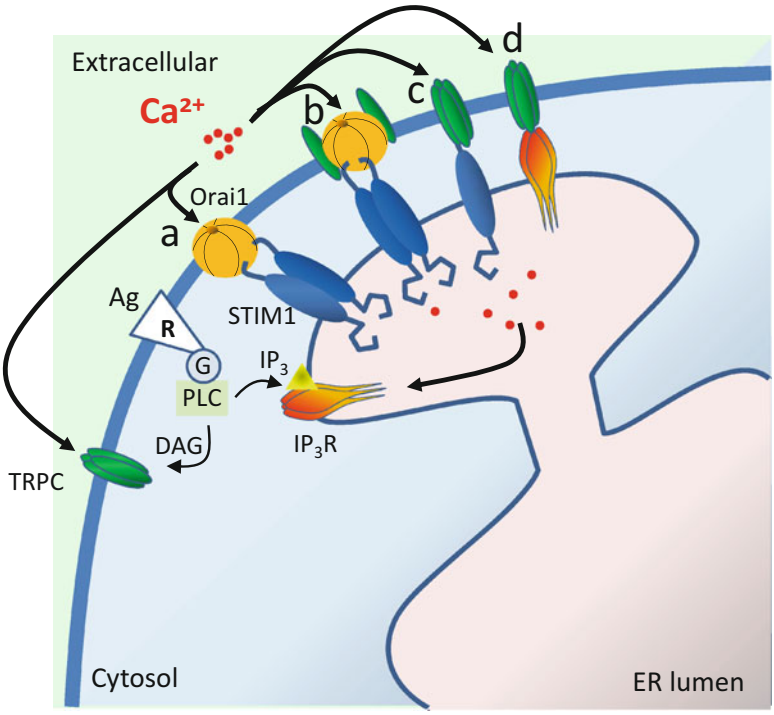

Fig. 14.2 Possible mechanism of store operated Ca^{2+} influx involving Orai1 and TRPc. Upon agonist stimulation of its receptor, PLC activation generates IP_3 and DAG. The latter is able to directly activate Ca^{2+} influx via TRPC channels. Produced IP_3 results in the depletion of Ca^{2+} from the ER via IP3R. (*a*) STIM1, the Ca^{2+} sensor inserted in the ER membrane oligomerizes and translocated to the ER-MP junction where it binds to and activates Orai1. (*b*) TRPC have been proposed to associate with Orai1 in a multiprotein complex or (*c*) to act as SOC channel when directly activated by STIM1. (*d*) Finally it has also been suggested that TRPC could be directly activated by IP_3R to directly refill the ER store. Abbr. 1,2-diacylglycerol (*DAG*); inositol trisphosphate (IP_3); inositol trisphophaste receptor (IP_3R); G protein-couple receptors (*R*); Ca^{2+} release-activate Ca^{2+} channel protein 1 (*Orai1*); phophoslipase (*PLC*); stromal interaction molecule 1 (*STIM1*); transient receptor potential cation channel (*TRPC*)

cytosol will avoid formation of high Ca^{2+} concentration microdomains at the mouth of channel and therefore will prevent channel inactivation [83, 84]. The combination of both actions explains why SERCA are so important in the shaping of Ca^{2+} signals. When over-expressed in cells, SERCA proteins are very efficient in reducing the need for Ca^{2+} entry. In agreement with this paradigm, it has been observed in smooth muscle cells that increased in SERCA2a expression leads in higher Ca^{2+} uptake and decrease in Ca^{2+} entry, showing a direct link between cell Ca^{2+} uptake capacity and SOCe activation [85].

Interestingly, recent reports show evidence that SERCA2 or SERCA3 associated with STIM1 upon Ca^{2+} depletion. This was inferred by confocal colocalization and co-immunoprecipitation [63, 86–88], although no FRET could have been obtained when SERCA-GFP and STIM1-CFP were co-expressed in HEK293T [89].

Additionally, while reduction in STIM1 expression using siRNA does not regulate SERCA activity in permeabilized cells, it decreased both SOCe and Ca^{2+} transport into ER in intact cells, which suggests that STIM1-SERCA association is more a spatial organization than a functional cluster [78, 90]. The current paradigm is that SERCA proteins form a crown around STIM1 when STIM1 is activated and associated with ORAI1 (Fig. 14.3b). Therefore, the Ca^{2+} pumps are localized just under the Ca^{2+} entry in order to refile the Ca^{2+} store from the high Ca^{2+} concentration microdomains formed at the mouth of the SOC.

In addition, Lemonnier et al. also demonstrated an association between SERCA and TRPC7, which appears to preserve the non capacitative Ca^{2+} influx (which is dependent on DAG) of the channel and suggests that the inhibition of TRPC7 by high cytosolic Ca^{2+} concentrations is suppressed by Ca^{2+} uptake into the stores similarly to what is described for SOC. Of note, the fact that the interaction between SERCA and TRPC7 is not disrupted in the presence of high concentrations of BAPTA indicates a more intimate relation between both proteins [83].

The fact that both SERCA2 and SERCA3 proteins have been involved in the regulation of SOCe and associated with STIM1, adds a new level of complexity in the organization of the crosstalk between Ca^{2+} influx and Ca^{2+} refilling. Since 1997, it has been proposed that both Ca^{2+} pump types could be part of distinct stores [91]. Based on electronical imaging and using inhibitors that could preferentially inhibit SERCA2 (Tg) or SERCA3 (tBHQ) associated to F-actin polymerization disruption by Cytochalisin-D or Latrunculin-A, the presence of at least two distinct SOCe has been observed in platelets (Fig. 14.4) [92, 93].

Acidic granules have been associated to SERCA3 [87, 94]. These granules are thought to be localized near the plasma membrane even in basal conditions. Therefore, formation of the ER-MP junction, necessary for activation of SOCe, does not need cytoskeleton reorganization. This was enlightened by the absence of effect (although some potentialization was actually observed) of F-actin polymerization inhibitors, on the SOCe induced by Ca^{2+} depletion using tBHQ that specifically inhibits SERCA3 [93]. These acidic granules have also been proposed to be sensitive to nicotinic acid adenine dinucleotide phosphate (NAADP), whose associated channel seems to be the two pores channels (TPC) [95, 96]. These granules are supposed to play a role of trigger of Ca^{2+} signaling starting before intracellular Ca^{2+} events that are sensitive IP_3R [97].

It is possible to imagine that these granules also act as SOCe trigger. Their submembrane localization can lead to a rapid association between STIM1 and Orai1 and the fast SERCA3 Ca^{2+} pumps can also participate preventing to reach high Ca^{2+} concentration in microdomains that could block the channel (Fig. 14.4a).

In contrast, SERCA2b-associated stores are strongly dependent on the actin polymerization and the similar experiments (pretreatments of cells with F-actin polymerization inhibitors) result in a decreased SOCe in response to Ca^{2+} depletion by Tg which preferentially inhibits SERCA2b [93]. This suggests a deeper insertion in the cytosol that could be associated to a slower process of activation in which STIM1 proteins has to relocalize to the ER-PM junctions, as observed in RBL cells (a tumor mast cell line) wherein STIM1 oligomers diffusion rate was evaluated to

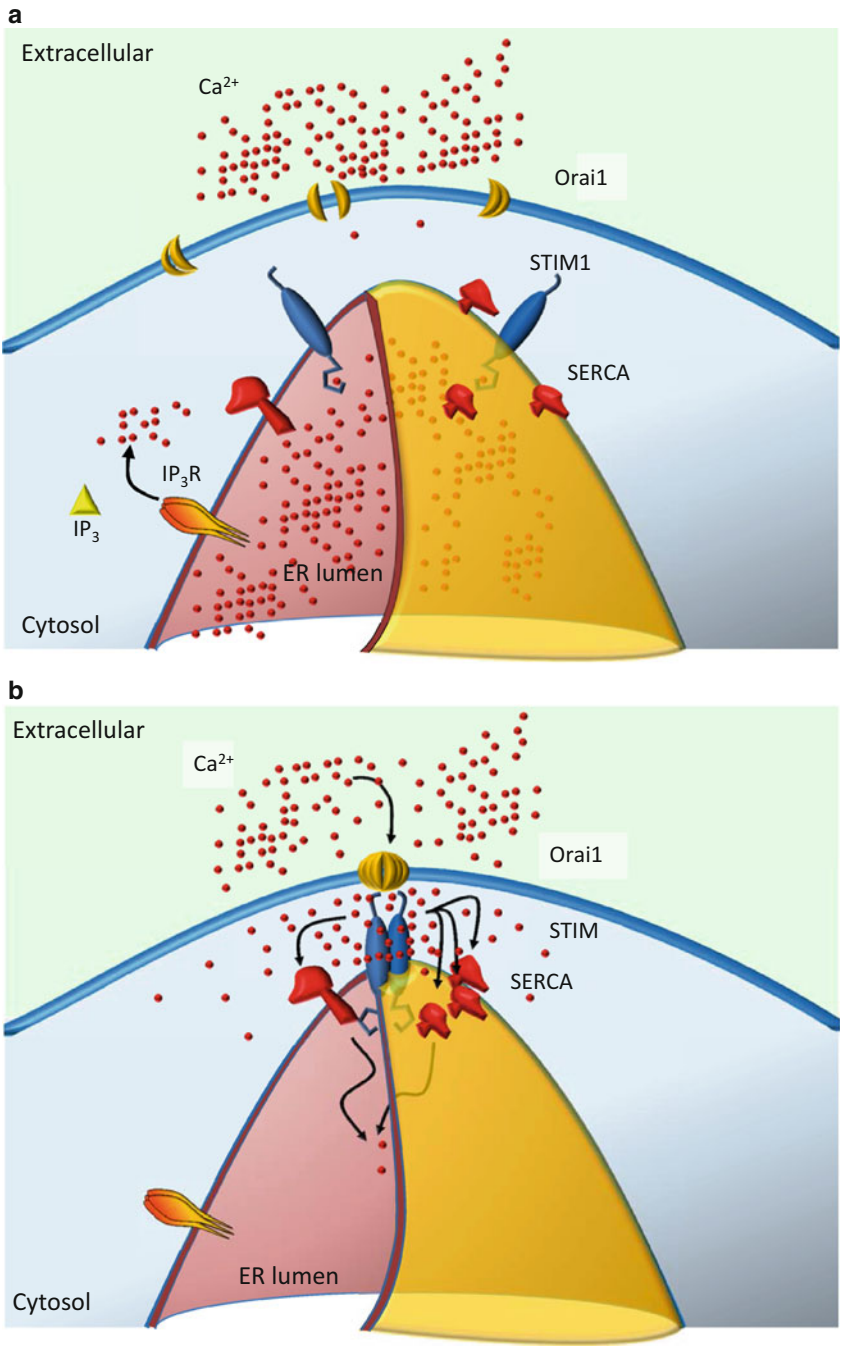

Fig. 14.3 Spatial organization of the SOC partners Orai1, STIM1 and SERCA. (**a**) Under resting conditions, high Ca^{2+} concentrations in the ER lumen keep SERCA and STIM1 homogenously at the surface of the ER membrane. Similarly, Orai is diffusely organized on the plasma membrane (*PM*). (**b**) Upon Ca^{2+} depletion though IP3R, STIM1 proteins oligomerise and translocate to form cluster under PM raft domains wherein Orai1 also form hexamers. SERCA pumps are also concentrated around STIM1 oligomers. Such organisation will avoid massive diffusion of Ca^{2+} ions in the cytosol and Ca^{2+} are re-uptaken by the pumps as soon as they reach the cytosol

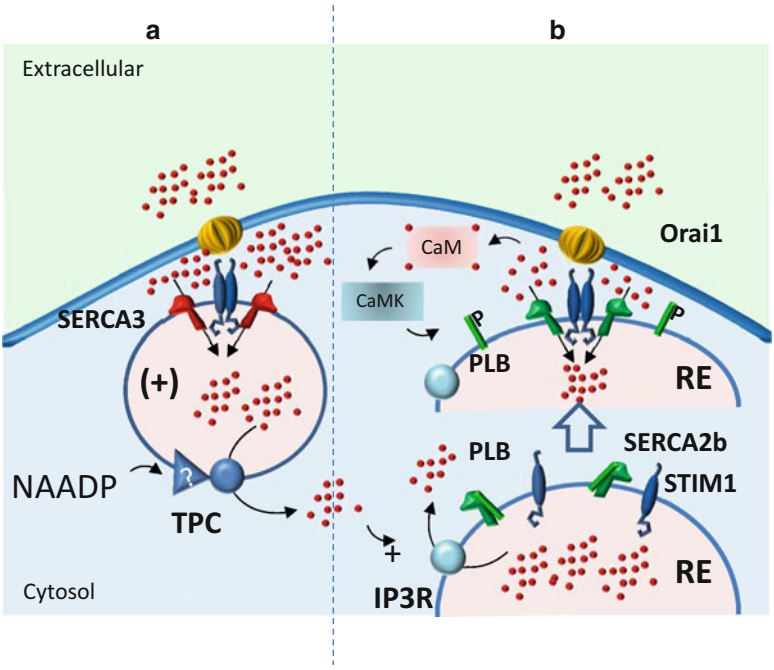

Fig. 14.4 Hypothetical representation of two SERCA3 and SERCA2b associated SOCe pathways. (**a**) Acidic granules (+) are enriched in SERCA3 and localized near the plasma membrane. Ca^{2+} depletion can produce a rapid activation of SOC as STIM1 is already close to the ER-PM junction. Presence of SERCA that are activated in high Ca^{2+} environment will also rapidly capture cytosol Ca^{2+} back into the granule avoiding its diffusion to the whole cell. Alternatively, depletion though TPC channels (NAADP receptor?) can act as trigger to potentialize IP_3R. (**b**) Ca^{2+} depletion through IP3R will induced STIM1 activation and translocation to the ER-PM junction. Orai1 activation will produce high Ca^{2+} microdomains that can activate Calmodulin (*Cam*)-dependent kinase (*CamK*) leading to the phosphorylation of phospholamban (*PLB*) and its dissociation from SERCA2b resulting in the activation of SERCA

be 0.05 $\mu m^2/s$. As the authors estimated that STIM1 will only form a cluster at a distance of 2 μm, STIM1 oligomers would take about 40s to achieve such a relocalization [82].

Additionally, it is important to keep in mind that SERCA3 and SERCA2 activities are differently regulated. SERCA2b has a much higher affinity for Ca^{2+} and this could allow those pumps to work at basal cytosolic Ca^{2+} level unless to be negatively controlled by phospholamban (PLB), a cAMP-dependent kinase whose phosphorylation by PKA and CAMKII kinase can disrupt its association with SERCA2b and can increase the SERCA affinity [98, 99]. This signaling cascade can provide a useful mechanism that specifically controls the SERCA2b Ca^{2+} uptake (Fig. 14.4b). Here, the Ca^{2+} uptake involves different signaling cascades including protein movements on membrane and in the cytosol, which could take a longer time to be func-

tional. It is therefore possible that the resulting SOCe is larger than the one depending on SERCA3-associated granule and overtakes the whole cytosol.

14.4 Coupling Response Between Mitochondria and SOCe

Mitochondria are multifunctional organelles that control a large number of cellular processes. Mitochondria are the cell energy factories that burn oxygen during oxidative phosphorylation to produce the cellular ATP used for biochemical reactions. They contain two membranes. In contact with the cytosol, is an outer membrane that is permeable, due to the abundant expression of voltage dependent anion selective channel (VDAC), acting as a general diffusion pore for small hydrophilic molecules such as ADP and ATP [100]. The larger Ca^{2+} uptake into the mitochondrial matrix occurs predominantly through the impermeable inner membrane via the ruthenium red-sensitive mitochondrial Ca^{2+} uniporter (MCU) complex.

Therefore, mitochondria are another crucial compartment that plays an important role in Ca^{2+} homeostasis.

Early models even proposed mitochondria as intermediate stores for Ca^{2+} originating from the extracellular medium before being transferred into the ER [101–103]. To do so, mitochondria should be located between the PM and ER. With the latter demonstration of the ER-PM junction and the direct interaction between STIM1 and Orai1 it is now hard to imagine such a role [104, 105].

Nevertheless, Mitochondria adjacent to ER and in the proximity of the SOCe channel, could take large amount of Ca^{2+} which dissipates high Ca^{2+} concentration in the ER-PM environment, facilitating STIM1 oligomerization and preventing Orai1 inactivation by Ca^{2+} [106, 107].

Ca^{2+} pumping by both SERCA and PMCA is an ATP-dependent process that is favored by the presence of activated mitochondria. By supplying ATP to the PMCA, mitochondria favor the transport of Ca^{2+} ions from the lumen of the ER to the extracellular medium when signaling is triggered, and thus favor SOCE activation. In contrast, by energizing SERCA, mitochondria improve store refilling and prevent SOCE activation (for review see [108]. Whether mitochondria enhance or reduce the level of ER depletion thus depends on the spatial organization of the organelles and on the relative contribution of mitochondria in buffering Ca^{2+} action (in the vicinity of Ca^{2+} depletion and Ca^{2+} entry channels) and in supplying ATP for SERCA and PMCA.

However, In more recent reports, it seems that Ca^{2+} uptake by mitochondria is essentially independent of SOC [78] and is much more sensitive to Ca^{2+} release by the ER than Ca^{2+} originating from the extracellular medium [109, 110]. Both organelles are finely tuned and interact one with each other. Although no direct link has ever been established between STIM1 and mitochondria, mitochondria and ER stores have been reported to be in close contact and to be able to exchange Ca^{2+} [111–113].

Additionally, recent findings also suggest that mitochondria can control the SOC independently of the Ca^{2+} buffering action through its action on STIM1 trafficking. Some authors have proposed that under depolarization, mitochondria might have a positive effect of STIM1 migration and subsequent SOC activation, a mechanism that appears to be dependent on mitofusin 2 (Mfn2) [114].

14.5 Ca^{2+} Tunnelling: Link Between SOCe and Store Depletion

Lastly, an important part of the relationship between ER refilling and SOC Ca^{2+} entry could rely on the Ca^{2+} tunnelling which has been described preferentially in highly polarized cells; i.e. pancreatic acinar cells. Such a mechanism allows the intracellular propagation of Ca^{2+} signals from a local entry at the basolateral membrane to distant targets several μm away. In these cells, Ca^{2+} across the cytosol would have been blocked by the greater buffering capacity of the cytosol, including mitochondria [115]. This supports the concept that entering Ca^{2+} is taken up into the ER by the SERCA, where it rapidly diffuses to the apical region and it is released through IP_3R. In the ER, Ca^{2+} ions can travel much easier (lower Ca^{2+} buffer activities and lower capacities of Ca^{2+} binding proteins of the ER lumen compared to the cytosol) [116, 117]. A similar mechanism is also proposed for induction of SOCe dependent Ca^{2+} signalling activation, such as NFAT, without induction of a global Ca^{2+} signal and faraway of the SOCe Ca^{2+} microdomains [118].

14.6 Conclusions

In a great number of cell types, Ca^{2+} signal is a message coded by change in Ca^{2+} concentration from steady increase to high oscillation frequencies with a period ranging from a few seconds to a few minutes. These Ca^{2+} oscillations are thought to control a wide variety of cellular processes, and are often organized into intracellular and intercellular Ca^{2+} waves.

SOCe plays a crucial part in those processes. Although the first goal of this Ca^{2+} influx into the cytosol via SOCe is to refill intracellular stores after their depletion, the impact of SOCe is major under physiological conditions. The existence of the SOC process is based on the crosstalk between distinct compartments. SERCA, the ER Ca^{2+} pump, STIM1 and Orai1 and TRPC1 can be localized at the ER-PM junction. This results in a very efficient refilling of intracellular stores.

The crosstalk between SOCe and intracellular stores allows a complex and finely tuned regulation of Ca^{2+} events either at the mouth of the channel, between distinct Ca^{2+} stores including mitochondria, or even far more distant from the SOC clusters into the cell via the diffusion of the message inside the ER. This is why this crosstalk appears much more than a simple process that refills intracellular stores but is recognized as a crucial member of the Ca^{2+} signalling.

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Chapter 15
Microdomains Associated to Lipid Rafts

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Abstract Store Operated Ca^{2+} Entry (SOCE), the main Ca^{2+} influx mechanism in non-excitable cells, is implicated in the immune response and has been reported to be affected in several pathologies including cancer. The basic molecular constituents of SOCE are Orai, the pore forming unit, and STIM, a multidomain protein with at least two principal functions: one is to sense the Ca^{2+} content inside the lumen of the endoplasmic reticulum(ER) and the second is to activate Orai channels upon depletion of the ER. The link between Ca^{2+} depletion inside the ER and Ca^{2+} influx from extracellular media is through a direct association of STIM and Orai, but for this to occur, both molecules have to interact and form clusters where ER and plasma membrane (PM) are intimately apposed. In recent years a great number of components have been identified as participants in SOCE regulation, including regions of plasma membrane enriched in cholesterol and sphingolipids, the so called lipid rafts, which recruit a complex platform of specialized microdomains, which cells use to regulate spatiotemporal Ca^{2+} signals.

Keywords Lipid rafts • SOCE • TRPC channels • STIM1 • Orai1 • ER/PM junction

Abbreviations

AC8	Adenylyl cyclase 8
APC	Adenomatous polyposis coli
ARC channels	Arachidonic acid gated channels
ASM	Airway smooth muscle
CaM	Calmodulin
cAMP	Cyclic adenosine monophosphate
$Ca_v1.2$	Voltage gated Ca^{2+} channel 1.2
Cav-1	Caveolin 1
CNS	Central nervous system

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CRAC	Cholesterol recognition/amino acid consensus
CRACR2A	Ca^{2+} release-activated Ca^{2+} channel regulator 2A
DRMs	Detergent-resistance membranes
EAE	Experimental autoimmune encephalomyelitis
ER	Endoplasmic reticulum
ERM	Ezrin/radixin/moesin domain
ER-PM junctions	Endoplasmic reticulum-plasma membrane junctions
FCS	Fluorescence correlation spectroscopy
FRET	Fluorescence resonance energy transfer
GPI	Glycosylphosphatidyl inositol
HEK293	Human embryonic kidney 293
IBD	Inflammatory bowel disease
Icrac	Ca^{2+} release-activated Ca^{2+} current
IP_3R	Inositol trisphosphate receptor
IS	Immunological synapse
MS	Multiple sclerosis
MβCD	Methyl-β-cyclodextrin
NFAT	Nuclear factor of activated T-cells
PDGF	Platelet-derived growth factor
PI_4P	Phosphatidylinositol 4-phosphate
PIP_2	Phosphatidylinositol 4,5-biphosphate
PIP_3	Phosphatidylinositol (3,4,5)-trisphosphate
$PIP_5KI\beta$	Phosphatidylinositol 4-phosphate-5-kinase I isoform β
$PIP_5KI\gamma$	Phosphatidylinositol 4-phosphate-5-kinase I isoform γ
PKB	Protein kinase B
PM	Plasma membrane
PMCA	Plasma membrane Ca^{2+}-ATPase
POST	Partner of STIM1
RYR	Ryanodine receptor
SCID	Severe combined immunodeficiency
SERCA	Sarco/endoplasmic reticulum Ca^{2+}-ATPase
SG	Salivary gland
SOCE	Store operated Ca^{2+} entry
SOCIC	Store operated Ca^{2+} influx complex
SPCA2	Secretory pathway Ca^{2+}-ATPase
TCR	T-cells receptors
TIRFM	Total internal reflexion fluorescence microscopy
TRPC	Transient receptor potential canonical
VGCC	Voltage gated Ca^{2+} channels

15.1 Introduction

The vision that we had not long ago about the plasma membrane (PM) was that proposed by the Singer and Nicolson model, a fluid mosaic where proteins and lipids convey their dynamics following Brownian motion [1]. This intrinsic fluidity excludes ordered phases. However, coincident with the development of new technologies, novel concepts have emerged and new attributes have appeared decorating a different view of the PM [2, 3]. Perhaps one of the greatest changes in our original idea about the PM is that of lateral heterogeneity, which presents the lipid bilayer as formed by heterogeneous patches or domains enriched in selective types of lipids and proteins [4].

Organization of the PM domains demands a great diversity of lipids that change structural and functional features of the membrane. Eukaryote cells possess three main classes of membrane lipids: glycerophospholipids, sphingolipids and sterols. Glycerophospholipids and sphingolipids can vary to produce a combinatorial diversity of more than 1,000 different structures, meanwhile sterols in mammalian cell membranes are formed only by cholesterol [4] (Fig. 15.1a).

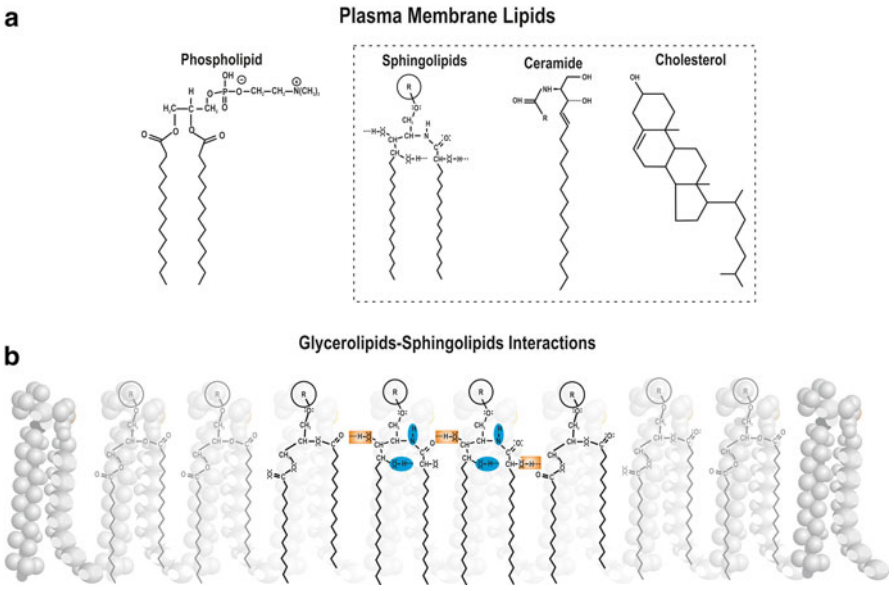

Fig. 15.1 Lipid components of rafts. (**a**) The drawing illustrates the four main lipids from the plasma membrane (PM). Outside the box are the phospholipids, the general components of membranes. Inside the box, the three major constituents of lipid rafts. Head polar groups are on *top*, the *R* group of sphingolipid and ceramide backbone results in a great diversity of lipids. (**b**) Inter and intra molecular interactions forming dynamic lipid rafts. In the center of the membrane two molecules of sphingolipids interact between them and with contiguous glycerolipids through hydrogen bonds (*rectangles*). In *ovals* intramolecular hydrogen bonds of sphingolipids constrain the lipid rafts

PM domains enriched in sphingolipids and cholesterol are components of the so called lipid rafts. Highly ordered and dynamic regions, restricted at the nanoscale size and with life spanning of only a few nanoseconds [5]. Lipid rafts plasticity results from thermodynamically favorable lipid-lipid, protein-protein and protein-lipid interactions [6, 7], and represent a recruitment center and a signaling platform for a large number of proteins. Lipid rafts have been shown to play important roles in diverse cellular functions, like trafficking, adhesion and Ca^{2+} signaling [8, 9]. This last, of great importance in the immune system, since T-cell activation requires a sustained rise of Ca^{2+} in order to activate the transcription factor NFAT, involved in antigen recognition [10].

The molecular mechanism in charge of increasing intracellular Ca^{2+} in T-cells is ubiquitous in non-excitable cells, the so-called Store Operated Ca^{2+} Entry (SOCE), a macromolecular protein complex for Ca^{2+} signaling associated to lipid rafts [9, 11–13]. The molecular assembled of SOCE begins immediately after the depletion of Ca^{2+} from the ER, followed by Ca^{2+} influx from the extracellular space [9]. The minimum proteins in charge of this mechanism are Orai and STIM. Orai by forming the pore channel and STIM by sensing Ca^{2+} which produce oligomerization and activation of Orai, when Ca^{2+} levels drop inside the ER [14]. Activation of Orai by STIM is an intricate and complex mechanism, which involves the physical movement of segments of the ER to bring them in close proximity with the PM, resulting in the formation of the so-called ER-PM junctions [15–17].

This chapter will describe some of the properties making lipid rafts excellent centers for the recruitment for SOCE components. We will review the recent methodologies that have identified SOCE as a macromolecular complex assembled in lipid rafts. From a molecular point of view, we will discuss the evidence supporting a complex multi-step mechanism of assembly and disassembly of SOCE components. Then, we will tackle on the dynamics of ER-PM junctions and their role in the establishment of Ca^{2+} microdomains. Finally, we will address the physiological and pathophysiological implications of alterations in the restricted organization of SOCE components in lipid rafts.

15.2 Platforms of Signaling, the Concept of Lipids Rafts

The first notions about the presence of heterogeneity domains at the PM came from the comparison of the lipid composition in membranes isolated from epithelial polarized cells, showing lateral and asymmetric distribution of sphingolipids and cholesterol from glycerophospholipids [18, 19]. Simons and van Meer hypothesized a lipid sorting process initiated at the Golgi complex, as responsible for the observed asymmetric distribution of sphingolipids in the PM. They proposed also a fundamental role of intermolecular interactions between hydrogen bonds of sphingolipids to maintain restricted domains [20] (Fig. 15.1b). Later, the advent of physicochemical experiments on model membranes came to recognize cholesterol as a key component promoting liquid ordered phases in artificial membranes [21, 22]. Finally, the concept of raft emerged for first time in 1992, with the studies of Brown and Rose, who developed a

new assay consisting in the treatment of cells with nonionic Triton X-100 detergent at 4 °C. Through this technique these authors showed that the association of sphingolipids and glycosylphosphatidylinositol (GPI) anchored proteins was found on the Triton X-100-insoluble membrane fractions [23]. Since that initial study, isolation of Triton X-100-insoluble membrane fractions would become the major biochemical procedure to examine proteins embedded in detergent-resistance membranes (DRMs), which are considered in many studies as synonymous of lipid rafts [24].

DRMs extraction is a widespread technique to identify proteins residing in lipid rafts. However, the biological significance of DRMs must be taken with caution. The evidence shows robust correlation regarding chemical composition of membrane domains and DRMs [25]. Liposomes and cholesterol enriched model membranes have shown to be detergent insoluble [26, 27]. Conversely, cells that have been depleted of cholesterol present an increment in membrane detergent solubility [28]. However, evidence supporting DRMs in the biology context is limited because is unknown if the lipid content resembles exactly those of lipid rafts in living cells. Other criticism to DRMs isolation are the need to use detergents and the fact that such domains cannot be isolated at 37 °C (physiological temperature at which such domains would reside in a biological environment) [29]. In addition to the unknown process that takes place during the detergent extraction, which causes a drastic perturbation of the PM and may alter lipid organization, limiting the conclusions obtained by using this technique [30].

In the last decade our compression of lipid rafts has been improved by the use of new powerful techniques, in particular by new microscopy technology, which takes lipid rafts from being an in vitro elusive domain to well-defined regions in the PM of living cells. Liposomes and artificial membranes were left aside and exchanged for complete cells to study biological membranes. By using a wide range of methodologies, covering from elegant colocalization assays [31] to atomic force microscopy [32] and a great diversity of spectroscopy techniques, covering FRET [33, 34], FCS [35–37], TIRFM [38], molecular tracking and super resolution microscopy [39, 40], all together condense general attributes of lipid rafts, describing them as discrete regions with short time lifespans (<1 s), covering small areas (10–200 nm) and being highly dynamic [40]. In the rest of the chapter we will refer to DRMs as lipid rafts, taking in consideration the limitations of using a biochemical procedure for its isolation.

15.3 Store Operated Ca^{2+} Entry Components, Assembling the Complex

A plethora of molecules participate and regulate SOCE. We have previously referred to these multiprotein interactions as the Store Operated Ca^{2+} Influx Complex (SOCIC) [9]. Several proteins from the cytoskeleton [41, 42], endoplasmic reticulum [9, 43, 44], plasma membrane [45] and cytoplasm [46] in conjunction with lipid rafts at the PM play a role orchestrating the correct assembly and regulation of SOCE (Figs. 15.2 and 15.3).

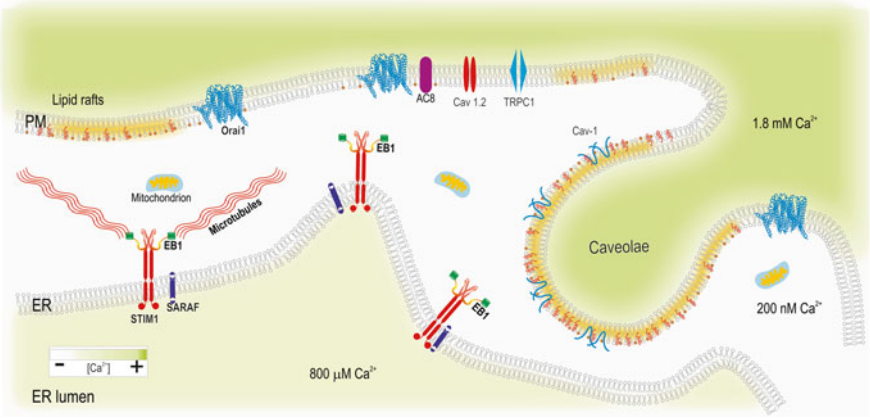

Fig. 15.2 SOCE at resting state. Model of SOCE components when the endoplasmic reticulum (*ER*) is replenish of Ca^{2+}. *Shadow patches* in PM depicts lipid rafts, Orai1, TRPC1 and adenylyl cyclase 8 (*AC8*) are inactive. $Ca_v1.2$, a voltage gated Ca^{2+} channel may be active, depending on the membrane potential. STIM1 moves continuously through the ER membrane by tracking microtubules via direct binding to EB1, who in turn tracks the tip of microtubules. Caveolae shows abundance expression of caveolin-1 and cholesterol and is presented as an invagination in the PM. Cholesterol molecules are shown in major abundance in the inner face of the PM. Solid polar heads of lipids in the PM represent PIP_2

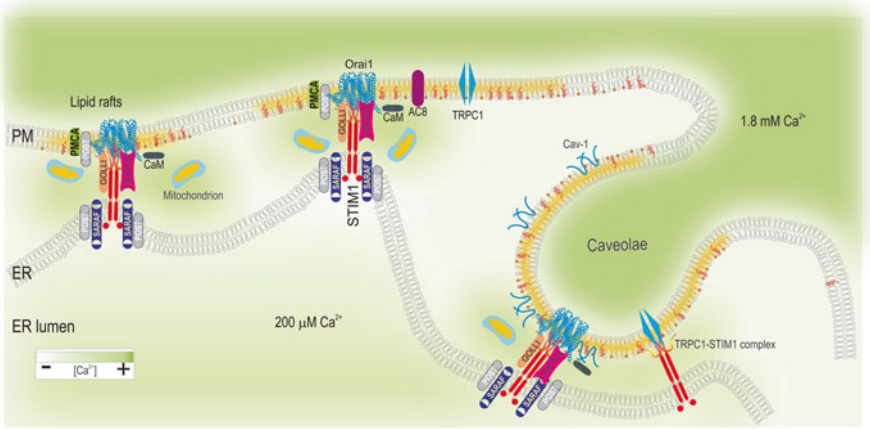

Fig. 15.3 SOCE in ER Ca^{2+} depletion conditions. SOCIC complex is illustrated associated to lipid rafts. STIM1 interacts with Orai1 to recruit a large number of regulators as Golli, CRACR2A, calmodulin (*CaM*), SARAF, POST and mitochondria. High density of proteins is supported in ER-PM junctions. In addition the SOCIC complex can be found in caveolae. Here, STIM1 may interact with and activate TRPC1. Cholesterol molecules are shown in major abundance in the inner face of the PM. Solid polar heads of lipids in the PM represent PIP_2. Focalized Ca^{2+} microdomains are developed beneath lipid rafts as signaling centers

STIM1 and Orai1 are the basic components of SOCE [47]. Together they generate a highly Ca^{2+} selective current with strong inward rectification, which resembles several of the biophysical properties of the so-called I_{crac} (Ca^{2+} release-activated Ca^{2+} current) [48]. However, depending of the cell type, the ionic current activated upon depletion of the ER does not recapitulate completely the I_{crac} initially described in mast cells, suggesting a heterogeneous molecular identity of the protein involved in ion conduction [49]. Indeed, several studies point to other possible players, like members from the transient receptor potential canonical (TRPC) channels, as putative ER-depletion activated channels [50]. Furthermore, there is experimental evidence showing direct interactions and gating of TRPC1 by STIM1 [51, 52]. Even more, a study shows that Orai1 may be required for the interaction between TRPC1 and STIM1 [53]. More recently, using a novel method combining single molecule imaging with single channel electrophysiological recordings we have shown the stoichiometry of STIM1-activated TRPC channels [54].

STIM1 appears to interact with a wide variety of proteins [55]. In resting state, when ER is filled of Ca^{2+}, STIM1 interacts with the microtubule tracking protein EB1, traveling continuously throughout the ER [41, 56]. Indeed, cytoskeleton plays an important role in SOCE regulation [57]. Short exposition to colchicine, to disrupt microtubules, enhances SOCE. Conversely, microtubules stabilization attenuates it [58]. At resting state, STIM1 interacts with a luminal ER oxidoreductase (ERp57), which associates to the amino terminal region of STIM1. Abolishing such interaction by using cells deficient in ERp57, results in an increment of Ca^{2+} entry when SOCE is activated [59]. The precise molecular role of ERp57 in SOCE modulation has not been elucidated to this date.

On the other hand, when ER depletion occurs, STIM1 undergoes a series of molecular rearrangements in order to activate Orai1. The active conformation of STIM1 is visualized as STIM1 clusters known as puncta [60]. These structures drive the recruitment of a great variety of molecules, including channels different from Orai and TRPC. STIM1 has been shown to associate and activate ARC channels [61, 62], leukotriene C_4-regulated Ca^{2+} channels [63] and mediates suppression of voltage-gated Ca^{2+} $Ca_v1.2$ activity [64].

The C-terminal of STIM1 is the target of intense modulation, aside from the autoinhibitory domain in its resting state [65, 66]. Golli-BG21, a myelin basic protein present in T-cells and oligodendrocytes, interacts with this STIM1 region, down regulating Ca^{2+} entry. Conversely, golli-deficient cells show increase Ca^{2+} entry mediated by SOCE [67]. Furthermore, other studies have shown a multitude of molecules regulating this cytosolic region of STIM1, including SARAF, an ER resident protein, which is found bound to STIM1 in resting conditions and having a role during depletion, where SARAF relocates to puncta to down regulate SOCE [44]. Conversely, the protein adenomatous polyposis coli (APC) facilitates translocation of STIM1 to ER-PM junctions, by binding to STIM1 upon depletion of the ER and inducing the dissociation of STIM1 from EB1 [42]. Later on, the translocation of STIM1 to ER-PM junctions is stabilized by septin4 and E-Syt1 [68].

Orai1 associates to a broad spectrum of proteins that modulate its function. CRACR2A associates to Orai1 and stabilizes STIM1-Orai1 puncta. On the other

hand, calmodulin modulates Ca^{2+}-dependent inactivation of Orai1, by binding in the same region of CRACR2A [46, 69] (Fig. 15.3).

Other novel proteins have been identified recently, for instance Junctate, an ER resident protein, plays a critical role in clustering Orai1 and STIM1 at ER-PM junctions [70]. Partner of STIM1 (POST), a ten transmembranal protein localized in the PM and ER, constitutively binds to Orai1 and interacts with STIM1 only after ER depletion, while promoting the formation of a complex integrated by SERCA, PMCA, Na^+/K^+-ATPase, importins and exportins [71]. Interestingly, while POST reduction does not alter SOCE, is reported that the activity of PMCA is notably reduced [71, 72]. The biological significance of decreasing Ca^{2+} clearance by PMCA results in a sustained NFAT activity in T-cells, a preliminary step in the immunological response [73].

In human airway smooth muscle (ASM), Caveolin-1 (Cav-1), a principal component of caveolae, has an important role organizing SOCE elements. Overexpressing Cav-1 results in an increment of Orai1 expression [74]. Cav-1 also targets TRPC1 to the PM and promotes its association to STIM1 [11, 75, 76]. In addition, during *Xenopus laevis* oocyte meiosis, Orai1 internalizes by a mechanism dependent on caveolin and dynamin [77].

Caveolae and the immunological synapse (IS) convey a remarkable molecular organization in the cell. Caveolae serve as a center for trafficking and signal transduction [8] and assembly of the IS represents a key step in T-cells activation and antigen-presenting cells recognition. Both structures trigger a well-modulated signaling cascade [78] which brings together several proteins in charge of processing all the signals. Another interesting example is adenylyl cyclase 8 (AC8) which produces cyclic adenosine monophosphate (cAMP) and whose activation works synergistically with specific Ca^{2+} signals arising from SOCE [79, 80]. AC8 interacts with N-terminal of Orai1, which facilitates its own dynamic microdomain formation involving the cortical cytoskeleton; such microdomain assembly occurs in cholesterol rich domains of the PM [81].

15.4 SOCE in Lipid Rafts

The first evidence pointing out to SOCE assembly in lipid rafts domains come from the studies of Murata et al. and Prakash et al. in endothelial cells and human ASM cells, respectively. Endothelial cells lacking Cav-1 showed a drastic misallocation of TRPC1 and TRPC4 and a diminished Ca^{2+} entry. Conversely, Cav-1 reconstituted cells showed a functional recovery of Ca^{2+} influx and adequate localization of TRPC1 and TRPC4. In addition, functional Cav-1 microdomains incorporate IP3R [82]. On the other hand, silencing Cav-1 in ASM resulted in the attenuation of SOCE [83]. The group of Rosado reported a reduction of thapsigargin-evoked Ca^{2+} entry and the dissociation of STIM1, Orai1 and TRPC1 in cells exposed to MβCD, an agent that reduces cholesterol from the plasma membrane [84, 85].

Noteworthy, caveolae and lipid rafts differ at different levels. First, caveolae are well defined structures that conform invaginations of the cell surface varying from 25 to 150 nm [86], whereas lipid rafts do not produce membrane invaginations. Second, the presence of caveolae is cell-specific, for example in endothelia and muscle cells caveolae are highly abundant, while in lymphocytes and neurons are absent [87]. Caveolae are also rich in caveolin-1, whose function is to stabilize these structures, but in addition are enriched in a variety of molecular components present in lipid rafts, and hence the biochemical similarity of both. Lipid rafts and caveolae are detergent-insoluble and enriched in cholesterol. Because of these similarities caveolae is considered a subtype of lipid raft [8].

There is abundant experimental evidence showing that TRPC1 interacts with Cav-1 [82, 83, 88–90]. However, the main regulator of TRPC1 inside rafts seems to be STIM1 [11, 13], whose interaction carry on important changes on TRPC1 channel properties [11]. Our group has shown in HEK293 cells a dual activity of TRPC1, which is determined by its interaction with STIM1 at PM microdomains. TRPC1 function as a store-operated channel when is associated to STIM1 and this interaction promotes TRPC1 insertion into lipid rafts domains. However, when TRPC1 is not associated to STIM1, the channel functions as an agonist-activated channel, outside of lipid rafts [11]. Pani et al. confirmed these results showing that interaction between TRPC1 and STIM1 is performed inside lipid rafts, where they function as platforms for gathering TRPC1 and STIM1 only after ER depletion [13]. In addition, activation of SOCE promotes TRPC1 dissociation from Cav-1 in a STIM1-dependent form [76]. Such interaction supposes a scaffold function of Cav-1 to partition proteins into lipid rafts domains.

On the other hand, partitioning of SOCE components in discrete domains at the PM, enriched in cholesterol, have been shown as important in the modulation of SOCE [11, 13, 84, 91]. The precise mechanism responsible for this observation remains largely unexplored. Nevertheless, one repetitive observation is that cells treated with MβCD (an agent that reduces cholesterol levels at the PM) results in the attenuation of SOCE in a wide variety of cells [12, 13, 91]. Most surprisingly, the cholesterol depletion effect on SOCE may be recovered by overexpression of Orai1 and STIM1 [91]. Noteworthy, STIM1 puncta formation, which is a prerequisite for SOCE activation, is inhibited in cells treated with MβCD [13].

Even though activation of SOCE occurs in lipid rafts, seems that lipid rafts are required only during SOCIC assembly, once the STIM1-Orai1-TRPC1 complex is formed; MβCD has no effect on SOCE or the complex itself [12]. STIM1 appears to have a determinant role orchestrating SOCE components into lipid rafts, the actual model of how STIM1 interacts with Orai1 suggests a diffusion trap, which postulate that a STIM1-PM association precedes the interaction with Orai1, probably by a mechanism facilitated by PIP_2 and PIP_3 [92], which are particular abundant in lipid rafts [93] (Fig. 15.3).

15.5 Targeting SOCE to Lipid Rafts

Assuming that lipid rafts are a highly ordered arrangement of membrane domains, independently of the protein content and that occur spontaneously, as with liquid-ordered phases in model membranes, this suggest the existence of discrete regions in charge of directing proteins to the rafts. Even though, all the evidence of raft-targeting motifs was obtained in liquid-ordered phases and DRMs [94, 95], well-defined raft-targeting motifs may recognize three types of lipidations, which could increase the probability of finding a protein associated to lipid rafts, especially on proteins without transmembrane domains [94, 96]. Acylation with myristic or palmitic acid, GPI-linkage and direct interaction with cholesterol are the three main mechanisms associated to lipid raft targeting [97, 98]. There are several reports showing a clear effect of myristate/palmitate modifications in directing integrins [99] and G proteins subunits [94] to lipid rafts. Regarding SOCE, an important myristoylation on the N terminus of golli-BG21 allows its PM association, resulting in the reduction of SOCE when golli-BG21 is overexpressed. Eliminating the myristoylation domain produces a recovery of Ca^{2+} entry in the same way than golli-deficient cells have an enhanced Ca^{2+} influx [100]. This important finding suggests that golli must be present in rafts in order to operate properly.

Meanwhile, cholesterol has been shown to have a carrier effect gathering proteins in cholesterol-rich domains. Many cholesterol-binding motifs have been identified in different proteins [101]. Perhaps one of the most prominent of such binding domains is the Cholesterol Recognition/Amino acid consensus (CRAC) motif, a consensus sequence first identified in the benzodiazepine receptor [102]. For example, Cav-1, on top of its palmitoylation residues [103], presents three well characterized CRAC domains, which may function reorganizing cholesterol from plasma membrane in a first step of raft assembly [104, 105]. However, the main function of caveolin is to recruit proteins to caveolae [106], and Orai1 possesses a caveolin consensus-binding site in its N terminus, which is important to internalize the channel in *X. laevis* oocytes [77]. Cav-1 and Orai1 interact forming a complex [74]. Similarly, salivary gland (SG) cells of a knockout mouse lacking Cav-1 showed a drastic reduction in SOCE. Interestingly, the mechanism for SOCE reduction appears to be the translocation of the TRPC1-STIM1 complex outside of the lipid rafts [90]. Furthermore, TRPC channels have a conserved Cav-1 binding domain within N and C termini, which could serves to direct this protein to lipid rafts by Cav-1 [88].

STIM1 possesses an ERM (ezrin/radixin/moesin) homology domain [107]. This region is located in the cytosolic part of STIM1 and covers the fragment in charge of activating Orai1 and TRPC1 [51]. ERM domains are coiled-coil structures that mediate interactions between cytoskeleton and PM proteins. A dynamic interplay between active and inactive forms is regulated by phosphorylation, which results in the exposition of binding sites for proteins of the cytoskeleton and PM [108]. However, the best-characterized motif to bind lipids in STIM1 is the polybasic region, a lysine rich motif proximal to its C-terminal region, which interacts with the PM via anionic phospholipids [109, 110] (Fig. 15.4). The polybasic region of

STIM1 interacts with PIP_2 and PIP_3 [111]. Deletion mutants of STIM1 lacking this polybasic region oligomerize normally forming puncta but are not translocated near the PM after ER depletion [112]. Using phosphatidyl kinases inhibitors, Korzeniowski et al. showed that depletion of PI4P, a precursor of PIP_2, causes a decrease in Orai1-mediated Ca^{2+} entry, but STIM1 puncta formation was not affected, which suggest that SOCE is also sensitive to PI4P in addition to PIP_2 depletion [113]. Something interesting is that overexpression of Orai1 results in recovery of STIM1 translocation to the PM, supporting the idea that STIM1 puncta involves interaction with the PM, but a stronger binding with Orai1 rescues STIM1 translocation when phosphoinositides are depleted [114].

In contrast, very little is known about how transmembrane proteins are targeted to lipid rafts [94]. Calloway et al. studied an arginine rich sequence on the N-terminal region of Orai1, suggesting that this polybasic region may be involved in directing the channel to distinct PIP2 pools [115] (Fig. 15.4).

Other example of a SOCE member with raft-targeting motifs is adenylyl cyclase 8, which presents a well-identified raft-targeting motif. Cooper's group, using an elegant approach with combinatorial chimeras from adenylyl cyclases containing raft and non-raft-targeted isoforms, showed that the membrane targeting sequence in adenylyl cyclases lies on cytoplasmic domains, illustrating an example of the major role of cytoplasmic and not transmembrane domains in directing a protein to lipid rafts [116] (Fig. 15.4).

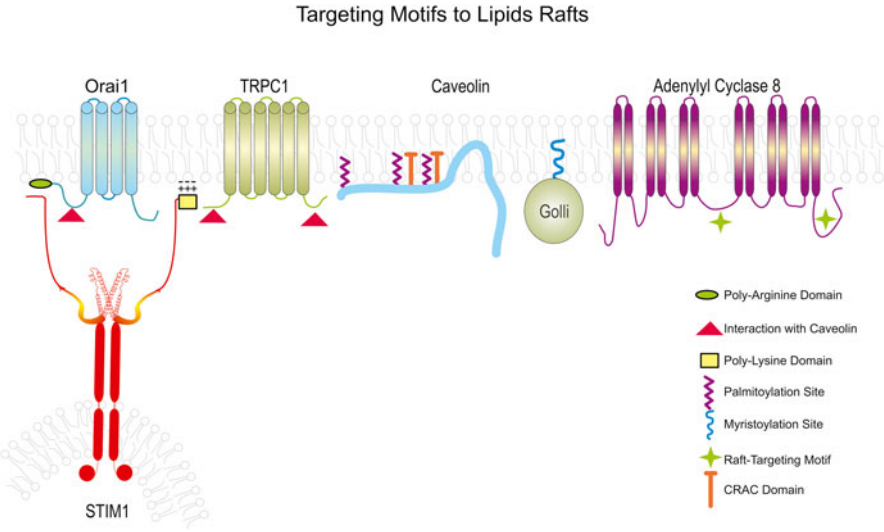

Fig. 15.4 Targeting motifs to lipid rafts. Domains reported to guide SOCIC components to lipid rafts. STIM1 possesses a poly-lysine domain that interacts with PIP_2 (*square*). Orai1 and TRPC1 present caveolin-1 binding domains (*triangle*) and in addition Orai1 has a poly-arginine domain suggested to interact with phospholipids (*oval*). Acylation of Cav-1 and Golli, palmitoylation and myristoylation are illustrated, respectively. Cav-1 also presents two CRAC domains and AC8 raft-targeting motifs

Nonetheless raft-targeting motifs assume the pre-existence of lipid rafts, a body of evidence indicates that rafts formation may be induced by proteins that will be part of the raft later on. Indeed, caveolin, flotillins and cytoskeleton regulate the assembling of caveolae and rafts [94, 117].

15.6 Methodologies Supporting SOCE in Rafts

The challenges of study lipid rafts are related to the very short life span and very small size of these PM domains. The definition of raft is intimately related to the methodology utilized to study them. For this reason we have highlighted the problems of using DRMs as synonymous of lipid rafts. While the lipid rafts is a general concept, DRMs are linked to the methodology employed to isolate such structures. For these reasons, it is important to discuss the diverse methodologies used to identify SOCE components in lipid rafts.

Using DRMs is a powerful and fast approach to identify proteins in detergent-resistant PM domains. However it is important to include a variety of controls in such studies. One important control frequently overlooked is the use of proteins that are not found in lipid rafts domains, as negative controls. That is the case of the transferrin receptor or actin, for example [13, 82].

DRMs isolation have permitted identify TRPC1, TRPC4 [82] and STIM1 [11, 13] on insoluble fractions. However, not all TRPC and STIM1 is present in DRM fractions, there are subpopulations recovered in soluble portions [13]. Unfortunately, the functional role of each subpopulation is unknown and understanding how they work in the context of its own microdomain represents a big methodological challenge.

Other biochemical assays yielding information about the presence of a protein of interest in lipid rafts have been co-immunoprecipitations, which involves the pull down of a bait protein to identify a second one (the prey) in conjunction with treatments that disrupts lipid rafts, like MβCD or filipin, agents that deplete cholesterol from the PM [91]. Rosado's group has reported that the interactions among Orai1, STIM1 and TRPC1 depend on the presence of normal levels of cholesterol at the PM [12, 84, 85]. At same time, the use of MβCD has permitted to investigate functional roles of SOCE in cholesterol depleted conditions, an interesting point is that cholesterol is required just for the activation and recruitment of SOCE components (the SOCIC assembly) but not for sustaining Ca^{2+} influx [12]. Others approaches used to examine the intricate relation of SOCE with specific components in lipid rafts are the use of commercially available lipid arrays, which allow the screening of proteins that may bind selected lipids, Zhou et al. used this approximation to confirm the interaction of STIM1 with PIP2 [111].

Today, major advances in understand lipid rafts are coming by spectroscopic approaches, in particular by microscopy techniques [39, 40]. These techniques provide a view of the unperturbed lipid environment on the cell, with high spatial and temporal resolution. Using a combined approach with electrophysiology and

FRET imaging, we have observed the dynamic interaction between STIM1-TRPC1. The association of both proteins precedes the ionic current trough TRPC1 channels. Removing cholesterol from the cell via incubation with MβCD abolished FRET signal and prevented the ionic current activation [11]. Calloway et al. showed with FRET an enhancement of STIM1-Orai1 interaction in mast cells overexpressing $PIP_5KI\beta$, which previously was reported to increase lipid rafts enriched in PIP_2 [118]. In contrast, overexpression of $PIP_5KI\gamma$, which also increases the production of PIP_2, did not enhance STIM1-Orai1 interaction. These results suggest that STIM1-Orai1 complex is established in membrane domains enriched in a subpopulation of PIP_2 and cholesterol, while a different subpopulation of PIP_2 contains less of the STIM1-Orai1 complex [115]. Recently, Maléth et al. confirmed this finding, showing that PIP_2 enriched domains support interactions between STIM1 and SARAF in membrane regions conformed by E-Syt1 and septin4 [68]. Lewis's group obtained similar results through single molecule tracking of STIM1-Orai1 complex in ER-PM junctions. Their results showed restriction of the STIM1-Orai1 complex to "corrals" that confined their diffusion. In addition, reduced events of free diffusible molecules were observed, suggesting a reversible binding of STIM1-Orai1 complex that escape to extrajunctional domains [92]. Such elegant techniques, including single molecule tracking, have provided a wealth of information about restricted movement of macromolecular complexes including those of STIM1-Orai1, STIM1-SARAF and STIM-TRPC1.

Recently, we have developed a combined single molecule-single channel determination technique, which allowed us to identify the stoichiometry of the STIM1-TRPC complex for several TRPC channels. Using this method we have determined also the stoichiometry for the TRPC-calmodulin complex. The power of this method is that allow us to study the stoichiometry of functional channels [54].

Other excellent tool to explore dynamic PM events of SOCE components is by TIRF microscopy, due to its high surface selectivity, which permits exciting fluorophores at distances not greater than 100 nm from the coverslip [38]. TIRF microscopy led to the identification of how clusters of STIM1 are inhibited when cholesterol was depleted from the PM [13], providing a possible explanation for SOCE reduction when lipid raft are disrupted. In a similar way, Sampieri et al. used TIRF to measure individual association events of STIM1 with PM microdomains in vivo [11]. By the use of the cholera toxin β subunit conjugated to a fluorophore, patches decorated with this conjugate represent areas with high concentration of the ganglioside GM1, an abundant constituent of lipid rafts [119]. The results show a clear preference of STIM1 to attach in areas decorated with the cholera toxin, suggesting an important role of lipid rafts in first steps of SOCIC assembly and SOCE activation.

Through TIRF microscopy Quintana et al. analyzed subplasmalemmal long-term Ca^{2+} signals in T-cells at IS or outside this domain. Taking advantage of T-cell function, IS was induced on coverslips coated with anti-CD3 antibodies. Interestingly, local Ca^{2+} at IS vicinity remained lower when compared to not-induced cells, suggesting a mechanism that prevents Ca^{2+}-dependent inactivation of Orai1 channels to sustain global Ca^{2+} signals and to activate the transcription factor NFAT [73].

With the advancement of methods like fluorescence correlation spectroscopy (FCS) and super-resolution microscopy more information regarding the elusive lipid rafts will be available in the near future, overcoming limitations of more traditional methods for the identification of lipid rafts elements, such as isolation of DRMs.

15.7 Plasma Membrane-Reticulum Endoplasmic Junctions

The endoplasmic reticulum (ER) is the main reservoir of Ca^{2+} in the cell [120]. In addition, proteins and lipid synthesis occurs here, positioning this organelle as a critical cellular structure in charge of Ca^{2+} signaling, protein traffic and lipid transfer [121]. To perform these functions, ER maintains highly modulated physical contact with cellular structures like mitochondria and the PM. In the case of the plasma membrane, these structures are known as ER-PM junctions [122].

ER-PM junctions are ubiquitous in eukaryote cells [121] and involves close apposition between two membranes by means of protein interactions, which provide the function of molecular bridges. These structures are highly dynamic, assembling and disassembling continuously. Some of the proteins functioning as molecular bridges are calsequestrin [123], RYR [124], junctions [123], mitsugumins [124], synaptotagmins [125], E-Syt1, Nir2 [126] and junctophilins. These last proteins are located in the ER, interacting with the PM most likely by binding to PIP_2 [121, 127].

The best characterized junctions are those for cardiac and skeletal striated muscles, where RYR and VGCC are key players in the junction formation [128]. Most interestingly, several SOCE components have been shown to play a role in these junctions. For instance, knockout mice lacking STIM1 present muscle fatigue and premature death due to myopathies, suggesting an important role for STIM1 in muscle ER-PM junction [129].

Translocation of STIM1 near to PM is a critical step prior to the activation of SOCE, thus Orai1 localization in ER-PM junctions is determined by interactions with STIM1 [130, 131]. Noteworthy, overexpression of STIM1 increases the number of ER-PM contacts, highlight the role of this protein as a molecular bridge [132]. PIP_2 and PIP_3 at the PM may have an important role in anchoring STIM1 molecules by its polybasic tail. However, the precise mechanism is poorly understood and appears to be very complex. Recently, the role of septins [133], ERp57, P100, golli, calmodulin and CRACR2A [134] in the translocation of STIM1 to ER-PM junctions has been evidenced. All these proteins perform a cooperative recruitment role to stabilize and regulate diverse SOCE components at ER-PM junctions.

SOCE in junctions is highly regulated by Ca^{2+}. The complex E-Syt1-septins have been shown to help in recruiting SARAF to ER-PM junctions and facilitate its interaction with STIM1, promoting slow Ca^{2+} dependent inactivation of Orai1 [68].

Calmodulin, CRACR2A and junctate possess Ca^{2+} binding domains important to inactivate or induce the disassembly of SOCE [46, 69, 70]. Junctate, for example, is

a partner of Orai1-STIM1 complex and possess a Ca^{2+}-binding EF-hand relevant to initiate the clustering of STIM1 independently of ER depletion [70]. Overexpression of junctate increases the number of ER-PM junctions, while IP3R and TRPC3 channels help to stabilize them [135]. Another element that facilitates STIM1 translocation is the protein APC, which interacts with EB1 at the tip of microtubules [136]. Reducing APC in the cell does not affect STIM1 oligomerization, but favors puncta assembly outside ER-PM junctions [42].

Electron microscopy studies on lymphocytes have shown the distance between PM and ER membranes during the ER-PM junction assembly, the distance determined was of ~17 nm [16]. It is at this junction where the SOCE macromolecular complex is recruited, but in addition, distribution of STIM1 puncta at ER-PM junctions can be highly polarized. Pancreatic acinar cells and lymphocytes are examples of this polarization in STIM protein. STIM1 is present in the basolateral membrane, while Orai1 distributes in all the plasma membrane [137], similarly to what takes place at the IS in lymphocytes, where STIM1 distributes in the vicinity of the IS while Orai1 is observed throughout the PM [138].

The proper ER structure is a critical for functional SOCE and morphological changes that undergoes after ER depletion are required for STIM1 redistribution [139]. Holowka et al. showed recently that acute addition of linoleic acid prevents STIM1 puncta formation and Orai1 interactions; these authors hypothesized that this effect is the result of perturbations in the ER membrane structure [140].

STIM1 distribution in ER-PM junctions brings important physiological consequences; the most relevant may be the control of Ca^{2+} increments through localized signals between ER and PM, which detonates particular physiological responses. This subject will be further explored in the next section.

15.8 Physiological and Pathophysiological Significance of SOCE Association to Microdomains

SOCE is a ubiquitous mechanism in non-excitable cells, providing Ca^{2+} to trigger a plethora of functions including proliferation, migration, differentiation, secretion and many others. In the immune system, the main role of SOCE is to trigger antigen recognition in T-cells, which is a central event in the immune response [10]. Microdomains play a role redirecting and restricting SOCE components to sites were T-cells receptors (TCR) contact with antigen-presenting B cells, in the so-called immunological synapse (IS) [138].

Through different methodologies, including biochemical approaches like isolation of DRMs [141, 142], or spectroscopy techniques like FRET [143], TIRF [144], fluorescence lifetime imaging [144, 145], evidence accumulates to provide a fantastic picture about the role of microdomains in forming the IS, where lipid rafts have a central role as platforms to recruit proteins that conform the machinery of the immune response [78].

SOCE maintenance in these microdomains promotes a sustained signal of Ca^{2+} to induce an effective NFAT activation [146], a key event to induce transcription of cytokine genes and other genes involved in the immune response [147]. NFAT activation results by a highly regulated Ca^{2+} signaling pattern, in which appropriate frequency and amplitude promotes activation of different transcription factors [148]. Dolmetsch et al. showed that activation of pro-inflammatory transcription factors, NF-κB and c-Jun N-terminal kinase depend on large and transient increments of Ca^{2+}, whereas NFAT activation occurs by low and sustained Ca^{2+} increments [149]. In this way, a unique messenger (Ca^{2+}) can promote different and selective responses [148]. Indeed, NFAT activation is highly selective to local Ca^{2+} increments resulting from the Orai1 microdomain distribution, meanwhile TRPC1 or TRPC3-mediated Ca^{2+} entry cannot activate NFAT, but instead induce the activation of NF-κB [150, 151].

Lioudyno et al. showed for first time the polarization of STIM1 and Orai1 during IS formation of human T-cells [138]. Polarization of SOCE components is accompanied by focalized increments of Ca^{2+}. In an elegant study, Quintana et al. showed in great detail the intricate mechanism that modulates Ca^{2+} microdomains in T-cells following IS formation. The authors observed accumulation of Orai1, STIM1, PMCA as well mitochondria in the vicinity of the IS. STIM1-Orai1 are assembled at IS and mitochondria plays a fundamental role on the clearance of Ca^{2+} to prevent Ca^{2+} dependent inactivation of Orai1 channels in the microdomain. Mitochondria inhibit PMCA, avoiding extrusion of Ca^{2+}, providing a continuous Ca^{2+} signal at the domain [73].

Disruption of Ca^{2+} entry mediated by Orai1 in T-Cells results in severe combined immunodeficiency (SCID). The SCID syndrome is characterized by propensity for fungal and viral infections and a deficiency in NFAT activation [45], as a consequence of mutations in Orai1 and STIM1 genes [45, 152].

Also interesting, knockout mice lacking STIM1 and Orai1 genes display T-cell-mediated autoimmunity problems, resulting in autoimmune inflammatory bowel disease (IBD) [153]. In the same way, SOCE is required to induce autoimmune CNS inflammation, mice lacking STIM1 and STIM2 present resistance to acquire experimental autoimmune encephalomyelitis (EAE), a model for multiple sclerosis (MS) [154, 155].

Other SOCE players have been shown to play a role in the modulation of immune cells. Golli-deficient T-cells increase Ca^{2+} entry mediated by SOCE followed by TCR stimulation, which produce in vitro hyperproliferation. On other hand, in vivo studies have shown tolerance to produce EAE. Authors hypothesize that Golli-deficient T-cells present excessive Ca^{2+} entry and subsequent alterations in NFAT signaling [100].

SOCE recently has been implicated in carcinogenesis processes [156], Fedida-Metula et al. showed an increment in proliferation promoted by SOCE in B16-BL6 cells, a melanoma model. SOCE activates calmodulin (CaM) and SRC kinase, which in turn inactivates PP2A phosphatase, a negative regulator of protein kinase B (PKB) [157]. An interesting observation is that SOCE promotes proliferation

via CaM and SRC kinase in a cholesterol dependent manner, resulting in a high sensitivity of PKB in cells depleted from cholesterol. The same study showed in vivo and in vitro retardation of tumor growth in cells exposed to MβCD [157]. SOCE also plays a role in the growth of mammary tumor cells, were SPCA2 gates Orai1 independently from STIM1 and ER Ca^{2+} content [158].

Lipid rafts and SOCE have been implicated in the proliferation of smooth muscle in the respiratory system where STIM1 and Orai1 modulate proliferation but also contraction [74, 159, 160]. Interesting data show that migration induced by PDGF is upregulated by the STIM1-Orai1 complex, having important consequences in remodeling airway epithelium in asthma [161]. SOCE activation in air smooth muscle cells is regulated by STIM1 but not STIM2 [160]. In addition, Cav-1 has been shown as an important component in SOCE. During inflammation processes Ca^{2+} entry-mediated by Orai1 contributes in a Cav-1 dependent-manner [74] and disruption of caveola domains with propofol, a lipid agent used for bronchodilatation, downregulates Ca^{2+} entry [159].

Cav-1 plays a role in intestinal epithelial cells as well, where TRPC1 functions as a store-operated channel, stimulating cell migration and producing rapid mucosal restitution after injury [89].

According to all the evidence obtained with cell lines, SOCIC components present an exquisite regulation by partitioning in lipid rafts, suggesting important consequences in the context of their native cell types. Notwithstanding SOCE partners and its regulation by rafts remains poorly explored, which could contribute in the future to better understand T-cell immunoreactivity, asthma, cancer and other pathophysiological conditions.

15.9 Conclusion

Since the original model proposed by Putney in 1986 [162], our understanding about the store-operated Ca^{2+} influx has evolved significantly to a sophisticated mechanism of intercommunication between the main Ca^{2+} reservoir (ER) and the PM. The use of novel methodologies helped us in the identification of many players in this complex signaling process. Among these players are a handful of proteins and lipids, including lipid-delimited domains such as lipid rafts.

An orchestrated symphony of intracellular signaling events transforms the initial trigger (depletion of the ER) into a profound rearrangement of the intracellular milieu, including the generation of de novo structures (ER-PM junctions) and the recruitment of a macromolecular complex into specialized microdomains (what we have named SOCIC). This sophisticated mechanism ensures the spatio-temporal modulation of intracellular Ca^{2+} concentrations, which in turn determines the type of cellular response.

Here we have reviewed the role of lipid rafts as centers involved in the modulation of Ca^{2+} signaling, we have analyzed the current evidence indicating that many

of the proteins responsible for SOCE are found in lipid rafts. Finally, we discussed some of the physiological repercussions of SOCE association to rafts domains and how alterations in these microdomains may lead to pathological disorders.

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Chapter 16
Role of Scaffolding Proteins in the Regulation of TRPC-Dependent Calcium Entry

Bruno Constantin

Abstract Plasma membrane ion channels, and in particular TRPC channels need a specific membrane environment and association with scaffolding, signaling, and cytoskeleton proteins in order to play their important functional role. The molecular composition of TRPC channels is an important factor in determining channel activation mechanisms. TRPC proteins are incorporated in macromolecular complexes including several key Ca^{2+} signaling proteins as well as proteins involved in vesicle trafficking, cytoskeletal interactions, and scaffolding. Evidence has been provided for association of TRPC with calmodulin (CaM), IP_3R, PMCA, $G_{q/11}$, RhoA, and a variety of scaffolding proteins. The interaction between TRPC channels with adaptor proteins, determines their mode of regulation as well as their cellular localization and function. Adaptor proteins do not display any enzymatic activity but act as scaffold for the building of signaling complexes. The scaffolding proteins are involved in the assembling of these Ca^{2+} signaling complexes, the correct subcellular localization of protein partners, and the regulation of the TRPC channelosome. In particular, these proteins, via their multiple protein–protein interaction motifs, can interact with various ion channels involved in the transmembrane potential, and membrane excitability. Scaffolding proteins are key components for the functional organization of TRPC channelosomes that serves as a platform regulating slow Ca^{2+} entry, spatially and temporally controlled $[Ca^{2+}]_i$ signals and Ca^{2+} -dependent cellular functions.

Keywords TRPC channels • Caveolin • Homer • NHERF • Syntrophin

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16.1 Introduction

The ability of a cell to perceive and correctly respond to its microenvironment is depending on very complex signaling systems. This includes various membrane and transmembrane proteins, which are at the interface between the extracellular and intracellular medium. An efficient cellular response is asking for a precise localization and coupling of various signaling components, such as transmembrane receptors, ion channels and transporters, signal transduction proteins, and the secondary effectors. This coordinated and highly controlled signaling system is depending on the assembling at the plasma membrane of macromolecular complexes including adaptor proteins or scaffolding proteins [1–3]. This class of proteins is responsible for the correct clustering and anchoring of signaling proteins at specific sub-cellular areas and membrane domains. Scaffolding proteins contains several binding sites, which confer the ability of interacting with multiple signaling proteins, membrane lipids and cytoskeleton proteins. Adaptor proteins do not display any enzymatic activity but act as scaffold for the building of signaling complexes. The scaffolding proteins are thus involved in the assembling of these complexes, the correct subcellular localization of protein partners, and the regulation of signaling proteins. In particular, these proteins, via their multiple protein–protein interaction motifs, can interact with various ion channels involved in the transmembrane potential, membrane excitability.

Adaptor proteins, through their interaction with calcium channels can be implicated in regulation of intracellular calcium signaling. Plasma membrane calcium channels support the entry of Ca^{2+} along its concentration gradient across the plasma membrane into the cell. Entry of Ca^{2+} is driven by the presence of a large electrochemical gradient across the plasma membrane. Various calcium channels, such as voltage-gated Ca^{2+} channels (Ca_v family), can be involved in this Ca^{2+} influx [4]. The expression of this family of proteins is a characteristic of "excitable cells," and these channels do require depolarization of the plasma membrane (PM) for activation. However, some of these, such as members of the low voltage-activated (low threshold of activation) Ca_v3 subfamily, are expressed in "non-excitable cell". Despite Ca_v expression, Ca^{2+} entry in non-excitable cells mostly occurs through non-voltage-gated channels [5]. These include ligand-gated channels (P2X purinergic ionotropic receptor families, for instance); Secondary messenger-operated channels (SMOC) linked to GPCR (G-protein-Coupled Receptor) activation and the production of secondary messengers; Store-Operated Channels (SOC); and Stretch-Activated Channels (SAC). The opening of these calcium channels is contributing to calcium entry in response to cell stimulation, and cells use a transient increase in cytosolic free calcium concentration ($[Ca^{2+}]_i$) for intracellular signaling. A tight control of intracellular $[Ca^{2+}]$ is essential for the survival and normal function of cells, which maintain resting calcium activity at a low level (around 100 nM) in order to keep a large dynamic range for the calcium signal. Membrane ionic channels and transporters, cytosolic calcium buffers and calcium buffering organelles regulate calcium influx, storage and extrusion to maintain $[Ca^{2+}]_i$ below the

activation thresholds and extraphysiological values. This precise control is essential for differential modulation, in an individual cell, of various signaling pathways and intracellular Ca^{2+}-regulated proteins involved in specific cellular processes. These include regulation of metabolism, proliferation, death, gene transcription, cell migration, exocytosis, and contraction [6].

16.2 Scaffolding Proteins and TRPC Channels

16.2.1 TRP Channels

The founding member of the TRP superfamily of cation channels is the *Drosophila* TRP channel. This superfamily consists of a large number of cation channels [7–9]. All TRPs contain six putative transmembrane domains, which are thought to assemble as homo- or hetero-tetramers to form cation selective channels. It is admitted that TRP channels display intracellular NH2 and COOH termini, as well as six transmembrane domains and an ion-selectivity P loop involved in the building of the pore. TRP channels form tetrameric assemblies around a central selectivity filter and gate, consistent with the structure of voltage-dependent K^+ channels [7–9] (Fig. 16.1).

On the basis of sequence homology, the TRP family can be divided in seven main subfamilies: the

TRPC ('Canonical') family; the TRPV ('Vanilloid') family; the TRPM ('Melastatin') family; the TRPP ('Polycystin') family; the TRPML ('Mucolipin') family; the TRPA ('Ankyrin') family; and the TRPN ('NOMPC') family. All functionally characterized TRP channels are cation channels and are permeable to Ca^{2+} with the exceptions of TRPM4 and TRPM5, which are only permeable to monovalent cations.

TRP channels participate in changes in $[Ca^{2+}]_i$ either by supporting Ca^{2+} entry through the plasma membrane, or via changes in membrane polarization, modulating the driving force for Ca^{2+} entry.

TRP channels are activated by a wide range of stimuli that include the binding of intra- and extracellular messengers, changes in temperature, chemical agents, mechanical stimuli, and osmotic stress [10, 11].

16.2.2 Scaffolding Proteins and TRP in Drosophila Photoreceptor

The first evidence for a TRP-containing macromolecular complex was demonstrated in *drosophila* photoreceptor, where the Inactive No After Potential D (INAD) provides the molecular scaffold [12, 13]. Interestingly, the *InaD* mutant

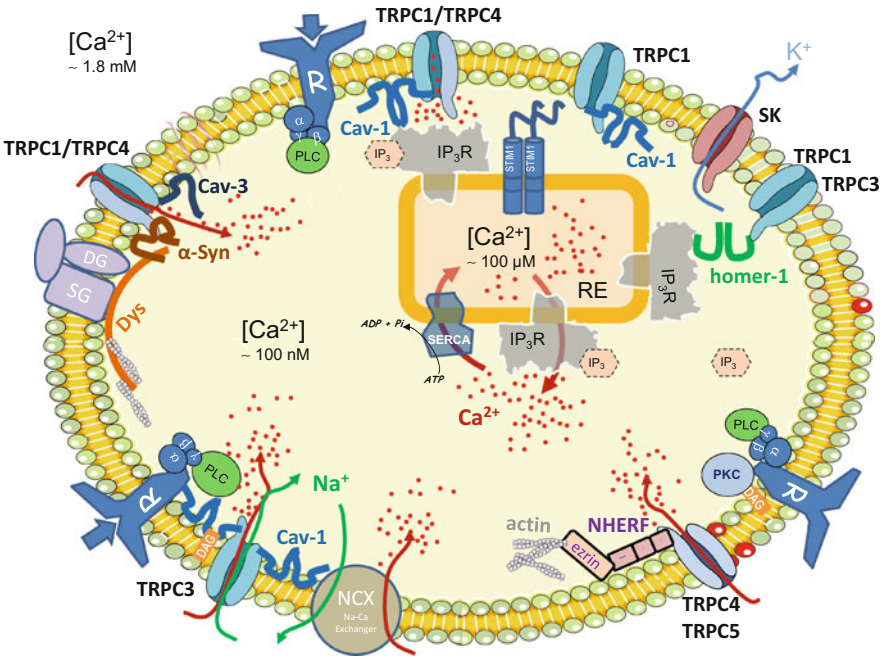

Fig. 16.1 Overview of the interplay between TRPC channels and scaffolding proteins. TRPC channels require scaffolding proteins for plasma membrane targeting, and incorporation in macromolecular signalplex controlling the opening of the channels and/or regulating their activity. It is proposed that Caveolin-1 regulates TRPC1 membrane targeting/retention as well as its association with STIM1. This is proposed to control SOCE activation through TRPC1 channels. Caveolin-1 is also involved in building a complex between TRPC1/TRPC4 heterotetramers and IP_3R, which is necessary for the proper activation of ROCE. The association of TRPC1 or TRPC3 channels with IP3R was also shown to be dependent on homer-1 and to maintain the channels in a close state. It was also suggested that caveolin-1 may associate TRPC3 channels and the Na^+/Ca^{2+} exchanger NCX in a same complex. TRPC4 and TRPC5 channels were also shown to associate with the PDZ domain of NHERF, the scaffolding protein regulating the membrane expression of the channels and the anchorage to ERM and actin cytoskeleton. Similarly, TRPC1/TRPC4 channels associates to the PDZ domain of α1-syntrophin in muscle, which anchors the channels to the dystrophin cytoskeleton and regulates the calcium entry

(*i*nactivation *n*o *a*fterpotential) is a drosophila phototransduction mutant that exhibits altered responses to light, suggesting a crucial role of the scaffolding protein in the signal transduction. INAD contains five PDZ domains and has the capacity of binding multiple proteins for constituting a signaling complex. Named "signalplex" by Craig Montell, this macromolecular complex is consisting in INAD binding with TRP [14], Phospholipase C (PLC) [15, 16], rhodopsin [16], protein kinase C (PKC) [16], calmodulin [17], and the NINAC (neither inactivation nor-afterpotential C) myosin III [18], and an eye-specific homologue of TRP, TRPL (TRP-like). Drosophila photoreceptors respond to light with an increase in membrane conductance, leading to a light-induced current (LIC) and membrane depolarization. Cells

from double mutants are almost totally unresponsive to light, indicating that TRP and TRPL account for most, of the LIC. Other proteins interact with this signalplex such as rhodopsin, calmodulin, FKBP59 and the nonconventional myosin III (NINAC), which is involved in efficiently terminating the photoresponse. The interaction with the scaffolding protein INAD is necessary for the proper localization of signaling proteins in the light-sensitive structures, the rhabdomeres. In *inaD* mutant flies, the localization of three INAD partners, TRP, PKC, and PLC, is severely altered [17, 19]. INAD plays an essential role in retention of these proteins to the rhabdomeres [20]. In addition, elimination of INAD or the INAD-binding sites in TRP, PKC, or PLC results in instability of these INAD-binding proteins [19, 20]. The interaction of the TRP/TRPL signalplex with the cortical actin cytoskeleton is mediated by the non-conventional myosin NINAC. This interaction also controls the gating of the channels [18] and regulates the translocation of TRPL [21]. The signalplex is thought to maintain the proper stoichiometry of signaling proteins in the rhabdomeres and to provide efficient transmission and termination of the signal.

The interaction between PDZ-containing protein, INAD and TRPC channels (see below) was also examined by heterologous expression of TRPC proteins in Sf9 insect cells [22]. The TRPC1, TRPC4, and TRPC5 channels co-immunoprecipitated with INAD, whereas TRPC3, TRPC6, and TRPC7 did not. This interaction could be explained by the homology of the PDZ domains (27.5 % similarity) of INAD and the Na+/H+-exchanger regulatory factor 1 (NHERF1), which is known to interact with TRL motif of TRPC4 and TRPC5 (see below). Thus INAD could form a binding domain comparable to NHERF1, allowing the binding of the TRL motif of TRPC4 and TRPC5.

16.2.3 TRPC Channels

Of the TRP families, the *Drosophila* TRP channel exhibits the greatest homology to the TRPCs, the first member of which, TRPC1, was cloned in 1995 [23]. A total of seven TRPC proteins have been described in mammals (TRPC1-TRPC7). In contrast to mice and rats, humans express only six TRPCs, because TRPC2 is a pseudogene [23]. TRPC channels are constituted of six predicted transmembrane domains (TM1-TM6), including a putative region between TM5 and TM6 involved in the constitution of the pore and assemble both as homo-tetramers or hetero-tetramers complexes. The cytosolic N-terminus is composed of three to four ankyrin repeats, a coiled-coil region involved in homo- or hetero-tetramerization of channels, and a putative caveolin binding domain. The cytoplasmic C-terminus region contains the TRP signature (EWKFAR), a highly conserved proline-riched motif (L<u>P</u>X<u>P</u>FXXX<u>P</u>S<u>P</u>K), the Calmodulin/IP_3 Receptor Binding (CIRB) region, and a coiled-coil region. All mammalian TRPCs build non-selective Ca^{2+}-permeable cation channels that demonstrate variable Ca^{2+}/Na^{+} permeability ratio, and support relatively non-selective cation currents [11, 24]. Several TRPC channels were

earlier described as SOC and SMOC [25, 26]. Upon activation of transmembrane receptors by extracellular ligand, stimulation of PLC and production of inositol 1,4,5-trisphosphate (IP3) and Diacylglycerol (DAG) activates or potentiates TRPC-dependent current and calcium entry, referred as receptor-operated Ca^{2+} entry (ROCE). Following IP3-stimulated calcium release through the opening of IP3R, some TRPC can be activated upon depletion of intracellular Ca^{2+} stores from the endoplasmic or sarcoplasmic reticulum (ER and SR) and contribute to Store-Operated Calcium Entry (SOCE). The use of antibodies against TRPC1 [27, 30], TRPC3 [27, 28], TRPC5 [29, 30], TRPC6 [30] and TRPC7 [30] inhibited SOCE and ROCE after stimulation of cells with an extracellular agonist. The Knockdown by siRNA of several native TRPC channels also reduced SOCE [26] including knockdown of TRPC4 in adrenal cells and corneal epithelial cells [31, 32], or of TRPC1 and TRPC6 in granulocytes [33]. In particular, the knockdown of TRPC1 was consistently reported to significantly reduce store depletion-dependent Ca^{2+} entry in a lot of different cell types, such as salivary gland [34], B-lymphocytes [35], skeletal muscle fibers [36], endothelial cells [37] embryonic neural stem cells [38], keratinocytes [39] and intestinal epithelial cells [40]. Endogenous heteromeric TRPC channels have also been suggested to contribute to SOCE or ROCE by using siRNA knock-down or antibodies, such as endogenous TRPC1/TRPC3 in human parotid gland ductal cells [41] and rat H19-7 hippocampal cell lines [42]; TRPC1/TRPC5 in vascular smooth muscles [43, 44]; TRPC1/TRPC6 in intestinal smooth muscles [45]; TRPC1/TRPC4 in endothelial cells [46], and in skeletal muscle cells [47]; and TRPC1/TRPC3/TRPC7 in HEK293 cells [48].

Gene deletion in mice of TRPC1 [49, 50], TRPC3 [51] and TRPC4 [52, 53] also highlighted the contribution of TRPC proteins in non-voltage activated Ca^{2+} entries such as SOCE and ROCE. Cells isolated from knock-out mice displayed alteration of cell function that could be related to the reduction of SOCE and of Ca^{2+}-dependent regulation. For instance, studies using salivary gland and pancreatic acinar cells from $TRPC1^{-/-}$ mice reported a strong reduction in SOCE and an alteration of fluid secretion and Ca^{2+}-dependent K^+ channel activation [49]. Acinar cells isolated from the submandibular glands of TRPC1 knockout mice showed significant inhibition of the outward Cl^- currents, suggesting TRPC1 channels are essential for the activation of calcium-activated chloride channel (CaCC) [50]. $Trpc1^{-/-}$ mast cells derived from the bone marrow (BMMCs) responded to antigen with enhanced calcium signaling but with little defect in degranulation or associated signaling. In contrast, antigen-mediated production of TNF-α, and other cytokines, was enhanced in the $Trpc1^{-/-}$ BMMCs, as were calcium-dependent events required for these responses [54]. These data suggest that TRPC1 promotes recovery from the anaphylactic response by repressing antigen-mediated TNF-α release from MCs. Smooth muscle cells from $TRPC4^{-/-}$ mice display loss of agonist-induced Ca^{2+} entry, resulting in impaired vasorelaxation, suggesting that TRPC4 is contributing to the regulation of blood vessel tone [52]. The defect in Ca^{2+} influx in TRPC4(–/–) endothelial cells was associated with a lack of thrombin-induced actin-stress fiber formation and a reduced endothelial cell retraction response [53]. TRPC4-dependent Ca2+

entry in mouse lung vascular endothelial cells was shown to be crucial for increased microvascular permeability [53]. In the gastric smooth muscle cells of TRPC4 knockout mice, the observations suggest that TRPC4 is an essential component of the NSCC activated by muscarinic stimulation in the murine stomach [55]. A constitutive background cation entry was also shown to be dependent on TRPC1/TRPC4 heteromers and TRPC1/C4-gene inactivation protects against development of maladaptive cardiac remodelling without altering cardiac or extracardiac functions [56].

Although TRPC channels has been shown to contribute to ROCE and SOCE, the core component of calcium selective Store-operated channels have been demonstrated to be the ER Ca^{2+} sensor protein STIM1 (STromal Interaction Molecule 1), and the calcium channel Orai1 [57, 58]. STIM1 is a type 1, single transmembrane-span protein that transmits the information of the ER Ca^{2+} load to the Ca^{2+} channels at the plasma membrane [59, 60]. A minimal sequence of STIM1(344–442) termed STIM1 Orai-activating region (SOAR) [61] is sufficient to fully activate the Orai channels and has a coiled-coil domain that interacts with the C-terminal coiled-coil domain of the Orais [61–64]. Although it is now clear that Orai1 is required for all forms of SOCE [57, 58], it was shown that TRPC1, TRPC4 and TRPC5, but not TRPC3, TRPC6 and TRPC7, interact with STIM1 and that STIM1 is required for the activity of TRPC1 [65, 66]. The ability of TRPC heteromers to respond to ER-Ca^{2+} store depletion has been suggested to depend on the presence of STIM1-activated TRPC, such as TRPC1 [67]. Other studies have shown that TRPC1 activation by STIM1, following store depletion, also requires functional Orai1 [68–71]. Moreover it was suggested that local Ca^{2+} entry mediated by Orai1 controls the recruitment of TRPC1 into the plasma membrane where TRPC1 channel is activated by STIM1 [72]. In different cell types, native Orai and TRPC1 channels were shown to contribute to SOCE after activation of STIM1 by store depletion [71, 73]. For instance, in developing myotubes SOCE were demonstrated to be dependent on STIM1, Orai1 and TRPC1 [74]. It was also shown that Ca^{2+} entry mediated by TRPC1 and TRPC4 participate in SOCE, and specifically allow the formation of normal-sized myotubes [75], but also is involved in alteration of calcium entry in dystrophic cells deficient in the dystrophin/syntrophin scaffold [47].

In addition to interacting with the STIM1 protein and Orai channels, TRPC proteins are incorporated in macromolecular complexes including several key Ca^{2+} signaling proteins as well as proteins involved in vesicle trafficking, cytoskeletal interactions, and scaffolding. Evidence has been provided for association of TRPC with calmodulin (CaM), IP_3R, PMCA, $G_{q/11}$, RhoA, and a variety of scaffolding proteins [76, 77]. The interaction between TRPC channels with accessory proteins, determines their mode of regulation as well as their cellular localization and function. Scaffolding proteins are key components for the functional organization of TRPC channelosomes that serves as a platform regulating Ca^{2+} entry, and spatially and temporally controlled intracellular Ca^{2+} signals and Ca^{2+} -dependent cellular functions.

16.2.4 Caveolin and TRPC Channels

TRPC1 and TRPC3 have been found in glycosphingolipid- and cholesterol-enriched membrane microdomains referred to as caveolae. Caveolae play a key role in organizing receptor signaling complexes containing receptors and their accessory regulatory protein. Caveolae are also enriched with various proteins that participate in Ca^{2+} regulation and with Caveolin, a transmembrane scaffolding protein that nucleates signaling complexes [78]. Caveolin-1 has been described to interact with TRPC1, TRPC4 and TRPC3 channels (see below).

16.2.4.1 Caveolin-1 and TRPC1

TRPC1 and caveolin-1 were colocalized in the plasma membrane region of human submandibular gland and Madin-Darby canine kidney cells [79]. This work has shown Full-length caveolin-1 bound to both the N and C termini of TRPC1. Amino acids 271–349, which includes a caveolin-1 binding motif (amino acids 322–349), was identified as the caveolin-1 binding domain in the TRPC1 N terminus. They suggested a role for caveolin-1 interaction in membrane translocation of TRPC1. All TRPC members conserve a similar motif at the N-terminal part close to the first transmembrane domain TM1. Deletion of this region (amino acids 271–349 or 322–349) prevents the targeting of TRPC1 to the plasma membrane [79]. Importantly, TRPC1Δ271-349 exerted a dominant negative effect on endogenous SOCE and was associated with wild-type TRPC1. In addition, expression of a truncated caveolin-1 (Cav1Δ51-169), lacking its protein scaffolding and membrane anchoring domains (but not expression of full-length caveolin-1), disrupted plasma membrane targeting of TRPC1 [79]. Cav1Δ51-169 also suppressed thapsigargin- and carbachol-stimulated Ca^{2+} entry. These data demonstrate that plasma membrane localization of TRPC1 depends on an interaction between its N terminus and Cav1. Conversely, another study proposed a role of the TRPC1 C terminus domain in the regulation of calcium entry by the scaffolding protein [80]. Caveolin-1 associates with TRPC1 cation channels in human pulmonary artery endothelial cells (HPAECs). The role of the caveolin-1 scaffolding domain (CSD) in regulating thrombin-induced Ca^{2+} entry was demonstrated by using the cell-permeant antennapedia (AP)-conjugated CSD peptide, which competes for protein binding partners with caveolin-1. Immunoprecipitation studies demonstrated an interaction between endogenous TRPC1 and ectopically expressed hemagglutinin-tagged CSD. Moreover, Streptavidin-bead pull-down assay indicated strong binding of biotin-labeled AP-CSD peptide to TRPC1. A CSD binding consensus sequence was identified in the TRPC1 C terminus, and biotin-labeled AP-TRPC1 C terminus peptide interacts with caveolin-1. Finally, AP-TRPC1 peptide containing the CSD binding sequence markedly reduced the thrombin-induced Ca^{2+} entry. These results demonstrated a crucial role of caveolin-1 scaffolding domain interaction with TRPC1 in regulating thrombin-induced Ca^{2+} entry.

Caveolin-1 interaction is critical for TRPC1 targeting/retention and the scaffolding protein has been shown to play a dual role in regulation of Ca^{2+} entry. Overexpression of Cav-1ΔCSD construct, which decreases TRPC1– caveolin-1 interaction, actually showed an increase in SOCE [81]. In contrast, the TRPC1 COOH-terminal residues 781–789 truncated (TRPC1-CΔ781-789) mutant expression abolished SOCE in human dermal microvascular endothelial cell line (HMEC). TRPC1-CΔ781-789 binding to Cav-1 was markedly suppressed, but effectively interacted with IP_3R3. This work describes that CSD interacts with TRPC1 but also with IP_3R3. The negative regulation of SOCE could be explained on the basis of CSD interaction with IP_3R3.

Using mice deficient in caveolin-1, Murata et al. [82] demonstrated that the scaffolding protein governs in endothelial cells the localization and the interactions of TRPC1 and TRPC4. Cav-1 is associated with a dynamic protein complex consisting of TRPC4, TRPC1, and IP_3Rs, and the loss of Cav-1 impairs the localization of TRPC4 and agonist-simulated complex formation. Moreover, results with mice deficient in caveolin-1, showed that caveolin-1 is essential for ACh-mediated calcium entry and the co-precipitation of TRPCs with IP_3Rs. Both were rescued in caveolin-1 knock-out reconstituted with endothelium-specific caveolin-1. These data suggest that caveolin-1 is responsible for the assembly of an activated calcium-influx protein complex consisting of TRPCs and IP_3Rs in endothelial cells. More generally, caveolae are thought to organize a calcium signaling complex containing TRPC1 anchored to caveolin-1 where it associates with signaling proteins including IP_3R, calmodulin (CaM), plasma membrane calcium pump (PMCA), and $G_{\alpha q/11}$ [77, 83].

Caveolin-1 association with TRPC1 is thus important for membrane localization of the channel at microdomain but also for its activation by signaling pathways. It appears also to play a role in the interaction of TRPC1 with STIM1 and in participation of TRPC1 in SOCE. It was reported that caveolin-1 retains TRPC1 within the plasma membrane regions where STIM1 puncta are localized following store depletion [84]. This enables the interaction of TRPC1 with STIM1 that is required for the activation of TRPC1-SOCE. Silencing caveolin-1 in human submandibular gland (HSG) cells decreased plasma membrane retention of TRPC1 and TRPC1-STIM1 clustering. This also reduced TRPC1-dependent SOCE, without altering the formation of STIM1 puncta. Importantly, the work is describing a balance where activation of TRPC1-dependent SOCE was associated with an increase in TRPC1-STIM1 association and a decrease in TRPC1-caveolin-1 clustering. Overexpression of caveolin-1 decreased TRPC1-STIM1 clustering and SOCE, while silencing STIM1 or expression of STIM1 mutant prevented dissociation of TRPC1-caveolin-1 and activation of TRPC1-dependent SOCE [84]. It is thus proposed that the scaffolding protein associates with inactive TRPC1 at the plasma membrane and dissociates from the channel upon activation of TRPC1 by STIM1. Caveolin-1 seems to be essential for the selective organization of TRPC1-STIM1 channel assembly, but not for Orai1-STIM1 channels: Plasma membrane localization of TRPC1, its association with lipid raft microdomains and interaction with STIM1, are also disrupted in SG acinar cells from caveolin-1 –/– mice [85]. Forced expression of caveolin-1 in

SG acinar cells of caveolin-1 −/− mice restored agonist-stimulated TRPC1-STIM1 association, and SOCE. In contrast, Orai1–STIM1 interaction is not affected in SG acinar cells from caveolin-1 −/− mice, whereas agonist-induced and store-dependent calcium entries are significantly reduced [85].

In vascular smooth muscle cells (VSMC), Oxidized low-density lipoprotein (oxLDL) enhanced the cell surface expression of TRPC1, as shown by biotinylation of cell surface proteins, and induced TRPC1 translocation into caveolar compartment [86], which was dependent on cytoskeleton. Caveolin-1 silencing induced concomitant decrease of TRPC1 expression and reduced oxLDL-induced apoptosis of VSMC. The reported results show that caveolin-1-dependent translocation of TRPC1 channels are involved in apoptotic processes that play a major role in atherosclerosis. TRPC1 and caveolin-1 association has been also shown to participate in the formation of cell polarity [87]. Human bone osteosarcoma U2OS cells exhibit distinct morphological polarity during directional migration. The scaffolding protein caveolin-1, and the calcium channels TRPC1, and Orai1 were found to be concentrated at the rear end of polarized cells. The inhibition of store-operated Ca^{2+} entry, or Knockdown of TRPC1, but not knockdown of Orai1, reduced cell polarization. Furthermore, disruption of lipid rafts or overexpression of caveolin-1 contributed to the down-regulation of cell polarity.

16.2.4.2 Caveolin-1 and TRPC3

Similarly to TRPC1, TRPC3 is assembled in macromolecular complex containing $G_{\alpha q/11}$, PLCβ, IP_3R, SERCA, Ezrin and caveolin-1 [88]. TRPC3 is proposed to be included in a caveolar Ca^{2+} signaling complex linked to the cortical actin cytoskeleton. Interestingly, the TRPC3- IP_3R signaling complex has been shown to be internalized by conditions which stabilize the cortical actin cytoskeleton.

Coupling of TRPC3 channels with IP_3R-1 has been suggested to activate a cation current in arterial smooth muscle cells that induces vasoconstriction. Interestingly, Caveolin-1 knockdown using shRNA attenuates IP_3-induced vasoconstriction [89]. Moreover, a synthetic peptide corresponding to CSD sequence (amino acids 82–101) also attenuates vasoconstriction as well as IP_3-induced activation of cation current. Caveolin-1 has been shown to co-immunoprecipitate with TRPC3, and IP_3R-1 from cerebral artery lysate. TRPC3 channels co-localize with IP_3R-1 and Caveolin-1 in arterial smooth muscle cells and this localization is disrupted by the CSD peptide. These data indicate that Caveolin-1 co-localizes SR IP_3R1 and plasma membrane TRPC3 channels in close spatial proximity thereby enabling IP_3-induced coupling of these proteins, leading to calcium and sodium entry and in turn vasoconstriction of arterial smooth muscle cells. The same kind of macromolecular complex associating Caveolin-1 IP_3R-1 and TRPC3 channels has been found in mesenteric artery lysate [89].

In plasma membrane lysates from human prostate cancer cells, caveolin co-immunoprecipitated with p-PLCγ1 and TRPC3 [90]. Interestingly this macromolecular complex also contained the cell surface GRP78 that regulates apoptosis, and

TFII-I, a transcription factor, which regulates cellular proliferation and apoptosis. In addition to its transcriptional functions, cytosolic TFII-I regulates agonist-induced Ca^{2+} entry and cell surface localization of TFII-I is dependent on cell surface-associated GRP78, binding α_2 -macroglobulin. Down regulation of TFII-I expression activates agonist-induced Ca^{2+} entry and is associated with decreased cell surface expression of GRP78 and the induction of pro-apoptotic signaling.

Upon Angiotensine II-stimulation, a TRPC3-dependent NCX1-mediated Ca^{2+} influx was described into cardiomyocytes and was associated with an increased cell surface presentation of the two ion transport proteins [91]. Receptor/PLC-dependent membrane recruitment of cardiac TRPC3/NCX1 complexes was suggested to activate TRPC3 cation conductances, TRPC3-mediated local rises in $[Na^+]_i$ and reverse mode Na^+/Ca^{2+} exchangers-mediated Ca^{2+} entry. Interestingly, co-immunoprecipitation experiments revealed an interaction of NCX1 with TRPC3. GST pulldown-experiments revealed only a weak association of NCX with an N-terminal fragment of TRPC3, while a C-terminal domain efficiently retained NCX1. It is tempting to speculate that TRPC3 and NCX1 are co-transported as a preformed protein complex via vesicular trafficking and the interaction with caveolin. The TRPC3-NCX1 signaling complexes described here, are likely to play a significant role in cardiac physiology or/and pathophysiology.

16.2.4.3 Caveolin-3 and TRPC1

Gervasio and collaborators [92] reported the association of the scaffolding protein caveolin-3 with TRPC1, by showing co-localization at the sarcolemma and co-immunoprecipitation of endogenous proteins. Moreover, this work suggested by FRET assay and expression of TRPC1-CFP and caveolin-3-YFP, that the latter is necessary for localization of TRPC1 at the plasma membrane of myoblasts. This suggested the scaffolding protein caveolin-3 is necessary for localization of TRPC1 at muscle sarcolemma. These observations are in accordance with the idea that TRPC1 is part of a Dystrophin-Associated protein complex at the sarcolemma of skeletal muscle cells [93], which includes caveolin-3 but also syntrophin (see below).

16.2.5 Syntrophin and TRPC Channels

TRPC1 was found at the sarcolemma of adult mouse fibres [36, 47, 92, 94] and of developing mouse myotubes [47, 95]. This suggested that the cationic channels could be anchored to the subsarcolemmal cytoskeleton including spectrin and/or dystrophin. Indeed, TRPC1 isolated from adult mouse skeletal muscle and from developing myotubes was found to co-immunoprecipitate with dystrophin [95]. Dystrophin is providing in striated muscle the scaffold for multiple dystrophin-associated proteins (DAPs) and cytosolic adaptors, such as syntrophin. Through

syntrophins, the DAP-complex is thought to anchor various signaling molecules such as enzymes and channels near their functional site at the membrane. Syntrophins are multigene family of intracellular membrane-associated adaptor proteins. The syntrophin family consists of five homologous isoforms, α1-syntrophin, β1-syntrophin, β2-syntrophin, γ1-syntrophin and γ2-syntrophin [96, 97]. The α1-syntrophin is the predominant syntrophin isoform in skeletal and cardiac muscles [98]. These scaffold proteins are characterized by the presence of a N-terminal PH-1 domain (Plekstrin Homology) split in two halves (PH_N and PH_C) by insertion of a PDZ domain (Post Synaptic Density protein-95, Drosophila discs large protein, and the Zona occludens protein 1), a second Plekstrin Homology domain (PH-2) and a C-terminal domain unique to syntrophins (SU). The SU domain and PH-2 domain interact with the carboxy terminus of dystrophin [96, 97]. This adaptor protein binds transmembrane channels through a PDZ domain such as voltage-gated sodium channels [99–101] or the potassium channel Kir4.1 [102]. We demonstrated that native TRPC1 and TRPC4 could be co-immunoprecipitated with endogenous and recombinant Alpha1-syntrophin [47, 95]. Moreover, GST-pull down assays showed that the PDZ domain of the Alpha1-syntrophin could be involved in this association, and native TRPC1 and TRPC4 proteins could be precipitated with the PDZ-GST domain as bait. This suggested that TRPC1 and TRPC4 could bind to Alpha1-syntrophin and be a constituent of a costameric macromolecular complex anchored to the dystrophin-based cytoskeleton. As shown by Si RNA knock-down of Alpha1-syntrophin, TRPC-mediated Ca^{2+} entry evoked by depletion of SR Ca^{2+} stores were greatly increased in wild type skeletal myotubes [47, 95]. The TRPC-dependent Ca^{2+} entry was similar to the one observed in dystrophin-deficient myotubes. This suggests that the scaffolding protein is a necessary component of the dystrophin-associated protein complex for moderating the calcium entry through TRPC1 and TRPC4 channels. Moreover, forced expression of recombinant Alpha1-syntrophin, but not of Alpha1-syntrophin lacking the N-terminal part containing the PDZ domain, restored normal TRPC-dependent calcium entry in dystrophic myotubes. TRPC-mediated Ca^{2+} entry were increased by a PLC/PKC-dependent pathway in dystrophic muscle cells and was restored at normal levels by Alpha1-syntrophin expression both in mouse and human skeletal muscle cells [74, 103]. In addition, silencing α1-syntrophin also increased cation influx in a PLC/PKC-dependent pathway. We also showed that α1-syntrophin and PLCβ are part of a same protein complex [74] reinforcing the idea of their interaction in a calcium signaling complex. This alteration of TRPC-mediated Ca^{2+} entry may be achieved by the loss of a α1-syntrophin-mediated inhibitory mechanism. As α1-syntrophin is present in the same complex than PLCβ, it could act directly on PLC pathway as a regulatory molecule or indirectly through the modulation of trimeric G proteins, which can also interact with α1-syntrophin [96, 97]. These observations were providing the idea that TRPC association with the scaffolding protein and DAPs in skeletal muscle could constitute a signaling complex at costameres, which regulates the TRPC-mediated Ca^{2+} entry, and spatially and temporally controlled intracellular Ca^{2+} signals [93]. The lack of Alpha1-syntrophin at the plasma membrane was

shown to dysregulate TRPC-mediated Ca^{2+} entry, which may contribute to Ca^{2+} -dependent cellular necrosis.

16.2.6 Homer and TRPC Channels

TRPC1 also interacts with the highly conserved class II EVH1 [Ena (Enabled)/VASP (vasodilator-stimulated phosphoprotein) homology 1] domain [104] of the scaffolding protein Homer. A proline-rich motif (LPXPFXXXPSPK), downstream of the TRP domain (EWKFAR) is conserved in all members of the TRPC subfamily and TRPC channels exhibit two Homer-binding sites named type 1 (PPXXF or PXXF) [105] interacting with EVH1. Homer is able to dimerize through a Coiled-Coil motif, and the EVH domain of each monomer binds to a consensus PPXXF motif [106]. The coiled-coils support the assembling of Homer monomers into elongated tetramers [107]. The tetrameric Homer complex is thus thought to form a lattice with other scaffolds that bind Ca^{2+} signaling proteins in cellular microdomains [108–110]. The interaction of Homer-1 with TRPC channels and regulation of channel activity by Homer1 have been explored extensively.

TRPC1 is associated with Homer in brain protein extracts [105], and localizes in a complex containing IP_3 receptors and group 1 metabotropic glutamate receptors (mGluR1). Yuan and collaborators found a second binding site of Homer in the N-terminus, LPSSP, which does not include a phenylalanine, unlike the previous Homer binding sites (PPXXF). Homer expression was found to be crucial for mediating a TRPC1-IP_3R complex necessary for responses to G-protein-coupled receptor activation [105]. On the contrary expression of TRPC1 with mutation of the proline-rich motif disrupted Homer binding and resulted interestingly in a constitutive activity of channels with reduced agonist regulation. Homer was thus proposed to permit the assembly of an agonist responsive TRPC1-IP_3R complex. To further demonstrate the role of Homer in native cells, Ca^{2+} influx were measured in acini from Homer-$1^{-/-}$ mice [105]. Deletion of Homer1 resulted in the increase of spontaneous Ca^{2+} in pancreatic acinar cells. This study also demonstrated that the filling state of the ER dynamically controls the association of TRPC1 with Homer and of TRPC1 with IP_3R. The activation of TRPC1-dependent SOCE by depletion of the intracellular Ca^{2+} stores was associated with the dissociation of TRPC1–Homer complex, while store refilling led to reassembly of the TRPC1–Homer–IP_3R complex.

TRPC3 was also found in complex with IP3R and Homer1b, which is suggested to mediate both gating by IP3R and TRPC3 trafficking [111]. The authors proposed a model in which the assembly of the TRPC3-Homer-1b/c-IP_3Rs complexes by Homer1b/c mediates both the translocation of TRPC3-containing vesicles to the plasma membrane and gating of TRPC3 by IP_3Rs. In resting cells, complexes containing TRPC3, Homer-1b/c and IP_3R were found in part at the PM and in part in intracellular vesicles. The binding of IP_3 to the IP_3Rs dissociated the interaction between IP_3Rs and Homer1 but not between Homer-1 and TRPC3 to form

IP_3R-TRPC3-Homer-1b/c. The store-dependent translocation of the TRPC3 to the PM was dependent on IP_3R [111].

These studies suggest that Homer1 is essential to maintain the TRPC channels in the closed state. At the basal state, TRPC channels are present in a complex with IP_3Rs dependent on the long Homer-1b/c, which could keep the channel inactive. The N-terminal domain of the IP_3R is in the conformation that exposes its Homer-1-binding ligand. This allows the binding of IP_3R to Homer-1, which also binds to the Homer-1-binding site of the TRPC channels. It is proposed that activation of TRPC channels following cell stimulation is dependent on the dissociations of the complexes within Homer.

Interestingly, the homer-binding motif of the TRPC channels' C-terminal domains is separated by only four residues from the conserved DD/E residues interacting with the two terminal lysines K684 and K685 of the STIM1. A function of this K-domain in the ER protein STIM1 is gating of TRPC channels [65, 67, 112], although the K-domain is not required for activation of Orai1 by STIM1 [113]. These findings lead to propose a model for store-dependent activation of TRPC channels by STIM1 [114]. When the ER is filled with Ca^{2+}, which binds to the EF hand of STIM1, sequestration of STIM1 in the ER and formation of the TRPC-Homer-IP_3R channel complex keep the TRPC channels in the closed state. Upon cell stimulation, binding of IP_3 to the IP_3Rs dissociates the IP_3R-Homer-1-TRPC channel complexes, triggers the calcium release through IP_3Rs, which in turn dissociates Ca^{2+} from STIM1 EF hand and results in the clustering of STIM1 with the TRPC channels. Finally, the interaction of STIM1(K684,K685) with the DD/E residues of the TRPC channels is proposed to stabilize the TRPC channels open state.

The involvement of Homer 1 in maintaining TRPC channels in the closed state, could explain the consequences observed in skeletal muscle cells from mice lacking Homer-1 [95]. Homer-1 knockout myotubes displayed increased basal current density and spontaneous cation influx that was blocked by silencing of TRPC1. This spontaneous TRPC1-dependent cation entry was blocked by reexpression of Homer-1b, but not Homer-1a, suggesting Homer-1b is specifically necessary for preventing a constitutive activation of TRPC1 channels. Interestingly, In HEK293, dissociating the Homer-1b/c-IP_3R complex resulted in TRPC3 translocation to the plasma membrane, where it was spontaneously active [106].

In human platelets, a study suggested a role of Homer proteins in association of TRPC1 with the type II IP3 receptor (IP3RII), as well as in the binding of with Orai1 [115]. However, Treatment of human platelets with thapsigargin or thrombin enhanced the association of Homer-1 with STIM1 and Orai1 in a Ca^{2+}-dependent manner, while it resulted in a Ca^{2+}-independent association of Homer-1 with TRPC1 and IP3RII. The interference with Homer PPXXF motif by using a synthetic PPKKFR peptide reduced both STIM1-Orai1 and TRPC1- IP3RII associations and inhibited thrombin-evoked Ca^{2+} entry and thapsigargin-induced store-operated Ca^{2+} entry.

16.2.7 NHERF and TRPC4, C5 Channels

Several studies have identified different components of the signaling machinery involved in TRPC4 and TRPC5 mediated Ca^{2+} entry. These include the Na+/H+-exchanger regulatory factor 1 (NHERF) a scaffolding protein with PDZ domain. NHERF was first isolated as a cofactor required for protein kinase A-mediated inhibition of type 3 Na1/H1 exchanger localized in the renal brush-border membrane [116]. It was later determined to bind members of the ezrin/radixin/moesin (ERM) proteins [117–119]. The ERM proteins are known to link cell surface receptors to actin cytoskeleton and in many cases such link is mediated through NHERF. NHERF1, or ezrin/radixin/moesin(ERM)-Binding Protein 50 (EBP50), is a relatively small PDZ domain-containing protein characterized by two PDZ domains and a carboxyl terminal ezrin-binding domain. NHERF2 is quite similar to NHERF1 as it shares 44 % sequence homology with NHERF1 and contains two PDZ domains and a carboxyl terminal ezrin-binding domain [120, 121]. The two PDZ domains of NHERF bind to target proteins while its C terminus binds to the N terminus of ERMs [117, 122]. It has been shown that the first PDZ domain of NHERF interacts with a number of G-protein-coupled receptors and ion transporting proteins [121].

Murine TRPC4 and TRPC5 were shown to bind to the first PDZ domain of NHERF [123]. The same PDZ domain binds to the C termini of PLC-β1 and PLC-β2. The Association of PLC-β1, TRPC4, and NHERF was demonstrated in an HEK293 cell line stably expressing TRPC4 and in adult mouse brain by co-immunoprecipitation experiments. This was an indication that NHERF is bringing together the signaling molecules involved in the TRPC-mediated calcium entry pathway in mammalian cells.

TRPC4 and TRPC5, have a TRL COOH-terminal amino acid sequence, which represents a class 1 PDZ-binding domain. The binding of two partners to the same PDZ domain suggests that NHERF can form a homodimer via PDZ2 and the PDZ1 domains, bringing TRPC4 and TRPC5 in vicinity of the PLCβ1 and PLCβ2 [123, 124]. The role of the TRL motif was examined on the subcellular distribution of TRPC4 in HEK293 cell line [125], as well as the consequences of the interaction between EBP50 and the membrane-cytoskeletal adaptors of ERM family for the cell surface expression of TRPC4. Confocal immunofluorescence microscopy analysis showed that the mutant lacking the TRL motif accumulated into cell outgrowths with a punctate distribution pattern whereas the wild-type channel was distributed on the cell surface [125]. In Cells co-expressing TRPC4 and an EBP50 mutant lacking the ERM-binding site, TRPC4 was not present in the plasma membrane but co-localized with the truncated EBP50 in a perinuclear compartment. Deletion of the PDZ-interacting domain also decreased the expression of TRPC4 in the plasma membrane, as assessed by cell surface biotinylation experiments. These data demonstrated that the PDZ-interacting domain of TRPC4 controls its localization and surface expression in transfected HEK293 cells.

Immunohistochemical studies demonstrated expression of TRPC4 and NHERF-2 proteins in both the endothelial cells and pericytes [126] of microdissected

descending vasa recta (DVR). A complex containing both TRPC4 and NHERF-2 was also found in microdissected in renal medullary descending vasa recta. TRPC4 coimmunoprecipitated with NHERF-2 from renal medullary lysates, and NHERF-2 coimmunoprecipitated with TRPC4. The function of TRPC4 and the significance of its binding to NHERF-2 are unknown at present. Studies in TRPC4$^{-/-}$ mice showed a marked attenuation of agonist-induced increases in intracellular Ca^{2+} in endothelial cells and impaired agonist-induced vasorelaxation in preconstricted aortic rings from TRPC4-null mice [52]. These findings suggest that TRPC4 and NHERF-2 may play a role in the control of Ca^{2+} entry and in the regulation of medullary blood flow.

The functional effects of EBP50 on TRPC5 activity have been investigated with rat TRPC5 (rTRPC5), expressed in HEK293 cell [127]. Both rTRPC5 and the VTTRL deletion mutant were localized to the plasma membrane, and deletion of the VTTRL motif had no detectable effect on the biophysical properties of the channel when studied with patch-clamp technique. Co-expression of EBP50 with rTRPC5 led to a significant delay in the time-to-peak of the histamine-evoked, transient large inward current. Conversely, EBP50 did not modify the activation kinetics of the VTTRL-deletion mutant. It suggests that the VTTRL motif is not necessary for activation of TRPC5, but may mediate the modulatory effect of EBP50 on TRPC5 activation kinetics.

16.3 Concluding Remarks

Plasma membrane ion channels, and in particular TRPC channels need a specific membrane environment and association with scaffolding, signaling, and cytoskeleton proteins in order to play their important functional role. The molecular composition of TRPC channels is an important factor in determining channel activation mechanisms. Homomeric and heteromeric TRPC channels composed of TRPC3, TRPC6, and TRPC7 subunits are often termed classical ROCs, as they are activated by DAG through a PKC-independent mechanism that does not involve intracellular Ca^{2+} stores. TRPC channels containing TRPC1 subunits are gated by DAG via a PKC-dependent action, and also by STIM1 following stimulations that deplete intracellular Ca^{2+} stores. A recent work [44] showed that agents that deplete intracellular Ca^{2+} stores activated native heteromeric TRPC1/C5 channels in freshly isolated, contractile mesenteric artery vascular smooth muscle cells (VSMCs). It was shown that TRPC1 subunits are important for conferring activation by PKC, PIP_2 and PIP_3 on TRPC1/C5 channels. This work suggested that PKC is necessary for activation of native TRPC1/C5 channels by agents that deplete Ca^{2+} stores in VSMCs. In addition, PIP_2 and PIP_3 also activate native TRPC1/C5 channels and PKC activity is also important for this activation. The excitatory effects of PKC, PIP_2, and PIP_3 on TRPC1/C5 channel activities were absent in TRPC1$^{-/-}$ VSMCs. Moreover, TRPC1$^{-/-}$ VSMCs express homomeric TRPC5 channels, which are inhibited by PKC, PIP_2, and PIP_3. This illustrates that many cells and tissues

coexpress several members of the seven TRPCs, which can result in various channels with significantly different current properties, stimulation mechanisms and functional interactions. Moreover, Previous reports showed that the movement of TRPC1 to the plasma membrane is dependent on co-expression with TRPC4 or TRPC5 [128]. It suggests that TRPC1 is not able to reach the plasma membrane alone and at least without association with TRPC4 or TRPC5, or without association of a scaffolding protein such as caveolin-3 as observed by FRET assay and coexpression of TRPC1-CFP and caveolin-3-YFP in myoblasts [93]. Caveolin-1 interaction is also critical for TRPC1 [79] and for TRPC4 targeting/retention [82].

TRPC homotetramers and heterotetramers associate with cytoskeleton constituents, such as non-muscle actin and microtubules [76] and with various scaffolding proteins in order to be addressed to specific membrane domains, and to be incorporated in macromolecular complexes playing a role in cell signaling. In these "signalplex", the interaction of scaffolding and adaptor proteins plays a relevant role in the association of TRPC with other signaling molecules, which regulate the channel properties and the cation entry. Moreover, scaffolding proteins may be part of the mechanism triggering TRPC channel opening. A lack of the adaptor protein due to genetic defect may lead to TRPC channels over-activation or constitutive activation as observed with α-syntrophin and homer-1. Since the cation and calcium entry through TRPC channels is involved in the modulation of membrane excitability and of submembrane microdomains, this lack of regulation can have major consequences in calcium homeostasis and cell survival. Given the variety of cellular responses linked to the activation of these channels, many physiological or physiopathological functions have been shown to depend on TRPCs [129, 130]. A recent example is a pivotal role of TRPC channels, in particular TRPC1, in the striated muscle tissue and in the development of calcium mishandling observed in dystrophin-deficient skeletal and cardiac muscle cells [93]. Interestingly, enhanced TRPC1-mediated Ca^{2+} influx were dependent of PKC, supported by TRPC1/TRPC4 heteromers and regulated by the scaffolding protein α1-syntrophin. When incorporated in various signalplex, TRPC tetramers, can play different roles depending of the specific associations with different scaffolding proteins, signaling molecules, and with other channels, such as Orai or calcium-dependent K^+ channels. The presence of multiple TRPC signalplexs in the same cells can serves to mediate selective cellular functions. For example, it was shown in human salivary gland cell line HSG cells that TRPC1-mediated Ca^{2+} influx stimulates Ca^{2+}-activated K^+ channels and NFκB, while Orai1-mediated calcium entry activates NFAT but not TRPC-mediated Ca^{2+} influx [72]. The differential association of TRPC channels was also shown to lead to Antagonistic regulation of actin dynamics and cell motility [131]. It was shown that TRPC5 was in a molecular complex with Rac1, whereas TRPC6 was in a molecular complex with RhoA. TRPC5-mediated Ca^{2+} influx induced Rac1 activation, thereby promoting cell migration, whereas TRPC6-mediated Ca^{2+} influx increased RhoA activity, thereby inhibiting cell migration. Depending on the signaling repertory of a given cell and on the complement TRPC channels co-expressed, the various stimuli activating TRPC may lead to complex changes in membrane-potential, various mode of calcium entry, slow sustained increase in intracellular

calcium concentration and numerous Ca^{2+}-induced responses. Scaffolding proteins also play a pivotal role by incorporating TRPC channels in signalplex and specific membrane domain, leading to calcium microdomains and localized calcium signals with different cell function. For example, Ca^{2+} influx through TRPC1 channels has been shown to promote in *Xenopus* spinal neuron growth cones filopodial Ca^{2+} transients, and to activate calpain, which cleaves talin at the tips of filopodia [132]. Mislocalization of the TRPC channels can thus alter their function and consequently downstream localized Ca^{2+} signaling events that are dependent on these channels.

In conclusion, TRPC channels require scaffolding proteins for plasma membrane targeting, and incorporation in macromolecular signalplex controlling the opening of the channels and/or regulating their activity. An important role of Caveolins has been reported by several studies for TRPC membrane targeting/retention. This family of scaffolding protein was also involved in the association of TRPC channels with the calcium release channel IP3R, which seems to control the opening of the channels and the resulting calcium signals evoked by cell stimulation. The coupling of an agonist responsive TRPC-IP3R complex was also shown to be dependent on Homer scaffolding proteins. These studies suggest that Homer1 is essential to maintain the TRPC channels in the closed state. Clearly, Scaffolding proteins also help to build signaling complex comparable to the TRP/INAD/ and bring TRPC channels in the vicinity of signaling proteins that may regulate their activity. Scaffolding proteins such as NHERF were suggested to associate TRPC4 and TRPC5 with PLC, and we described also a putative TRPC/syntrophin/PLC complex in muscle.

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Chapter 17
Modulation of Calcium Entry by Mitochondria

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Abstract The role of mitochondria in intracellular Ca^{2+} signaling relies mainly in its capacity to take up Ca^{2+} from the cytosol and thus modulate the cytosolic [Ca^{2+}]. Because of the low Ca^{2+}-affinity of the mitochondrial Ca^{2+}-uptake system, this organelle appears specially adapted to take up Ca^{2+} from local high-Ca^{2+} microdomains and not from the bulk cytosol. Mitochondria would then act as local Ca^{2+} buffers in cellular regions where high-Ca^{2+} microdomains form, that is, mainly close to the cytosolic mouth of Ca^{2+} channels, both in the plasma membrane and in the endoplasmic reticulum (ER). One of the first targets proposed already in the 1990s to be regulated in this way by mitochondria were the store-operated Ca^{2+} channels (SOCE). Mitochondria, by taking up Ca^{2+} from the region around the cytosolic mouth of the SOCE channels, would prevent its slow Ca^{2+}-dependent inactivation, thus keeping them active for longer. Since then, evidence for this mechanism has accumulated mainly in immunitary cells, where mitochondria actually move towards the immune synapse during T cell activation. However, in many other cell types the available data indicate that the close apposition between plasma and ER membranes occurring during SOCE activation precludes mitochondria from getting close to the Ca^{2+}-entry sites. Alternative pathways for mitochondrial modulation of SOCE, both Ca^{2+}-dependent and Ca^{2+}-independent, have also been proposed, but further work will be required to elucidate the actual mechanisms at work. Hopefully, the recent knowledge of the molecular nature of the mitochondrial Ca^{2+} uniporter will allow soon more precise studies on this matter.

Keywords SOCE • Store-operated Ca^{2+} entry • Mitochondria • Endoplasmic reticulum • Orai • STIM • MCU • Ca^{2+} uniporter • Ca^{2+} microdomain • CRAC channels

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Abbreviations

$[Ca^{2+}]_M$	Mitochondrial matrix free $[Ca^{2+}]$
CICR	Ca^{2+}-induced Ca^{2+} release
CRAC channels	Ca^{2+}-release activated Ca^{2+} channels
ER	Endoplasmic reticulum
GDAP1	Ganglioside-induced differentiation-associated protein 1
$InsP_3$	Inositol 1,4,5-trisphosphate
IS	Immunological synapse or Immune synapse
JPH1	Junctophilin 1
MCU	Mitochondrial calcium uniporter
NFAT	Nuclear factor of activated T-cells
SERCA	Sarcoplasmic/endoplasmic-reticulum Ca^{2+}-ATPase
SOCE	Store-operated Ca^{2+} entry
STIM	Stromal interaction molecule
TRP	Transient receptor potential

17.1 Mitochondrial Ca^{2+} Transport

Ca^{2+} is an intracellular second messenger able to fulfill a variety of different functions in all cell types with no exception, both excitable and non-excitable cells. Let's just mention muscle contraction, neurotransmitter secretion, fertilization, proliferation, development, learning, memory or cell death [1]. Most of these functions are triggered by changes in cytosolic Ca^{2+}, but several intracellular organelles also play a very important role in cellular Ca^{2+} homeostasis, either because Ca^{2+} plays specific roles inside the organelle or because Ca^{2+}-fluxes in and out the organelle are able to regulate cytosolic $[Ca^{2+}]$. The first organelle that was known to have an important role in Ca^{2+} homeostasis was the endoplasmic reticulum (ER), that was shown to release Ca^{2+} in the presence of inositol 1,4,5-trisphosphate (IP_3) [2]. This organelle behaves as a Ca^{2+} store and in steady-state conditions has a very high $[Ca^{2+}]$ in its lumen, around 1–2 mM, as we have recently reported using a new double-mutated aequorin with very low Ca^{2+} affinity [3]. Different kinds of cell stimulation can induce a rapid release of the stored Ca^{2+} to the cytosol through several Ca^{2+} channels present in its membrane (IP_3 and ryanodine receptors, mainly).

Another organelle with an important role in cell Ca^{2+} homeostasis is mitochondria, the organelle responsible for aerobic energy production in the cell. The ability of mitochondria to take up large amounts of Ca^{2+} was known since the 1960s [4]. In fact, until the end of the 1970s, mitochondria were thought to be the main cellular Ca^{2+} store. Several findings changed then the view on this point. First, it was shown that Ca^{2+} uptake by mitochondria was carried out by a system (the uniporter) which had a very low Ca^{2+}-affinity (K_M above 10 μM) [5, 6]. In the same dates, the first measurements of cytosolic $[Ca^{2+}]$ in intact cells obtained with loaded dyes showed

that cytosolic [Ca^{2+}] under resting conditions was very low, around 100 nM, and that even after cell stimulation, it did not usually rise above 1–2 μM, that is, still tenfold below the K_M of the uniporter. Moreover, the finding that the endoplasmic reticulum had Ca^{2+} channels activated by IP_3 [2] suggested that the main responsible for intracellular Ca^{2+} release was the endoplasmic reticulum. As a consequence, mitochondria were thought not to have a significant role in Ca^{2+} homeostasis, at least under physiological conditions (although perhaps under pathological conditions). Then, in the beginning of the 1990s, the general view on this point changed again when experiments performed in the laboratory of T. Pozzan and R. Rizzuto using recombinant aequorin targeted to the mitochondria showed that mitochondrial [Ca^{2+}] ($[Ca^{2+}]_M$) was able to undergo rapid changes during cell activation [7]. That was the origin of a series of works by several research groups, demonstrating that mitochondria are very active players indeed in the control of the global cell Ca^{2+} homeostasis.

The rationale developed in those years to explain the apparent contradiction among the low Ca^{2+}-affinity of the uniporter and the fast mitochondrial Ca^{2+} uptake observed in intact cells was based in the concept of high-Ca^{2+} microdomains. So, it is right that mitochondria would be unable to take up much Ca^{2+} when exposed to the mean [Ca^{2+}] of the cytosol, around 1 μM. However, during cell stimulation, some mitochondria may be placed very close to the Ca^{2+} channels responsible for either Ca^{2+} release from the ER or Ca^{2+} entry from the extracellular medium. These particular mitochondria would be exposed to a much higher [Ca^{2+}] (a hot-spot or high-Ca^{2+} microdomain), enough to activate the uniporter and trigger fast mitochondrial Ca^{2+} uptake. A corollary of this hypothesis is that Ca^{2+} uptake by the mitochondrial network is heterogeneous, because only mitochondria close enough to those channels will take up significant amounts of Ca^{2+}, while the rest will remain with low [Ca^{2+}]. And, of course, the precise intracellular localization of mitochondria with respect to the Ca^{2+} channels becomes critical to determine the rate and amount of Ca^{2+} uptake. This point will be particularly important with regards to the interaction between mitochondria and capacitative Ca^{2+} entry.

Evidence for the multiple roles played by mitochondria in cell Ca^{2+} homeostasis has been continuously growing in the last years. First, it was already known that Ca^{2+} activates several Krebs cycle enzymes: pyruvate dehydrogenase, α-ketoglutarate dehydrogenase and isocitrate dehydrogenase [8], and it has been conclusively shown that an increase in $[Ca^{2+}]_M$ immediately leads to an increase in ATP production [9, 10]. This mechanism constitutes a basic homeostatic response that associates cell activation to energy production, in order to cover the energy requirements of the response to the stimulus. In addition, it has been recently shown that mitochondrial metabolism is also modulated by extramitochondrial [Ca^{2+}], thanks to the activity of several Ca^{2+}-dependent mitochondrial carriers, including both the aspartate/glutamate carriers from the malate/aspartate shuttle and the ATP-Mg/Pi carriers [11–13]. These mitochondrial carriers are in the inner mitochondrial membrane but sense cytosolic Ca^{2+} through EF-hand motifs placed in the intermembrane space. Another Ca^{2+}-dependent mitochondrial dehydrogenase is the FAD-glycerol phosphate dehydrogenase, a key component of the glycerol-3-phosphate shuttle, which

is also placed in the inner mitochondrial membrane and senses cytosolic Ca^{2+} in the intermembrane space [14]. This dehydrogenase has been recently shown to be the target of metformin, one of the most effective therapeutics for treating type 2 diabetes [15].

On the other hand, the fast mitochondrial Ca^{2+}-accumulation that occurs during cell stimulation constitutes in fact a mechanism of transient Ca^{2+} buffering (see [16] for a review). Because of the low-Ca^{2+}-affinity of their Ca^{2+} uptake mechanism, mitochondria are very well adapted to take up Ca^{2+} from local high-Ca^{2+} microdomains, such as those formed after the activation of plasma membrane or ER Ca^{2+} channels. These high-Ca^{2+} cytosolic microdomains are responsible for a variety of Ca^{2+}-dependent phenomena, such as neurotransmitter secretion or cardiac cell contraction. Mitochondria can therefore modulate these important cellular functions by acting as local Ca^{2+} sinks. For example, we have shown that mitochondria can modulate local cytosolic Ca^{2+} concentrations and Ca^{2+}-dependent catecholamine secretion in chromaffin cells [17, 18]. In these cells, a large fraction of the Ca^{2+} entering the cell during stimulation appears to be transiently buffered by mitochondria [18–20]. In fact, during chromaffin cell stimulation, mitochondria can undergo reversible and repetitive near millimolar $[Ca^{2+}]_M$ transients [17], as a consequence of the generation of local high Ca^{2+} microdomains of up to 20–40 μM, compared with a mean cytosolic $[Ca^{2+}]$ of 2 μM. The ability of mitochondria to buffer local high-Ca^{2+} microdomains has also been used to explain the modulation by mitochondria of the activity of several Ca^{2+} channels showing Ca^{2+}-dependent inactivation. The role of mitochondria in these cases would be to control the size of the local $[Ca^{2+}]$ microdomain close to the cytosolic mouth of the channel. Capacitative Ca^{2+} entry channels were the first ones shown to be modulated in this way by mitochondria [21]. It was proposed that mitochondrial Ca^{2+} uptake in the vicinity of the channels in the plasma membrane reduced the local $[Ca^{2+}]$ and avoided their slow Ca^{2+}-dependent inactivation, thus keeping the channels active for longer. The same idea was then used to explain the modulation by mitochondria of plasma membrane voltage-dependent Ca^{2+} channels [22, 23] and ER IP_3 receptor channels [24, 25] (see [26] for a review).

Largely in parallel with the history of the capacitative calcium entry, most of the mitochondrial Ca^{2+} transport systems have resisted for many years all the attempts to characterize them molecularly, in spite of the efforts of many research groups. However, the last 5 years have been very fruitful at this respect and we have now a much clearer picture of the molecules involved. First the mitochondrial Na^+/Ca^{2+} exchanger [27] and then the mitochondrial Ca^{2+} uniporter [28, 29] and all its different regulatory components have been described in these years (for comprehensive reviews, see [30, 31]). Among the unexpected findings obtained, let us mention two main points. First, the large complexity of the mitochondrial Ca^{2+} uniporter, which has not only a pore subunit (the MCU protein, that most probably forms tetramers in the membrane), but also a paralog with dominant negative function (MCUb), and at least five additional regulatory subunits: MICU1, MICU2, MICU3, EMRE and MICUR1, all of them with different and in some cases opposite functions, which are still not completely clarified. The second surprise came about 1 year ago, when the

group of T. Finkel developed a transgenic mouse with a knockout of the pore subunit (MCU), and it was not only viable, but in fact showed a very mild phenotype [32]. It is true that these mice are indeed only viable if they are maintained on a mixed genetic background, and that even in this case there appears to be still a significant amount of embryonic lethality [33]. But the survivors, which had no detectable protein expression of MCU and showed a complete abrogation of any fast mitochondrial calcium uptake in isolated mitochondria, were nevertheless nearly normal.

17.2 Capacitative Ca^{2+} Entry

Capacitative Ca^{2+} entry was first described nearly 30 years ago [34, 35] as a mechanism able to activate Ca^{2+} entry from the extracellular medium after the depletion of Ca^{2+} of the intracellular stores, namely, the endoplasmic reticulum. The pathway was later renamed store-operated Ca^{2+} entry (SOCE, [36]) and also Ca^{2+}-release activated Ca^{2+} channels (CRAC channels or I_{CRAC}, [37]) when referred to the Ca^{2+} current measured in the plasma membrane of immunitary cells after depletion of the intracellular Ca^{2+} stores. A large series of works has showed that SOCE is not only the predominant Ca^{2+}-entry pathway in non-excitable cells, but it is also present and very important in excitable cells, where it coexists with many different types of voltage-dependent and receptor-operated Ca^{2+} channels in the plasma membrane.

In spite of the long history of the SOCE channels, the molecular mechanism responsible for Ca^{2+} entry and the mechanism of signal transduction from the emptying of the Ca^{2+} stores to the activation of Ca^{2+} entry has been only elucidated in the last 10 years. The discovery in 2005 and 2006 of the main constituents of the CRAC channels, the family of STIM proteins as sensors of the ER [Ca^{2+}] and the family of Orai proteins as Ca^{2+} channels in the plasma membrane, finally provided the molecular tools required to explore the importance of the SOCE mechanism (for reviews, see [38–43]).

Co-expression of STIM1 and Orai1 generates Ca^{2+} currents identical to those of native I_{CRAC}, store-operated, with very high Ca^{2+} selectivity, inward rectifying and with extremely small single-channel conductance. However, although I_{CRAC} is certainly the best characterized Ca^{2+} current responsible for SOCE, it may not be the only one. Store-operated Ca^{2+} currents with different properties (particularly with lower Ca^{2+} selectivity or non-selective) have also been reported and named I_{SOC} to differentiate them from I_{CRAC} [44]. TRP channels, and particularly the TRPC subfamily, could play an important role in the I_{SOC} currents. Most of the members of the TRPC subfamily have been reported to generate store-operated Ca^{2+} currents and some authors sustain that these currents might be carried through homo or heteromeric combinations of different channel subunits involving TRPCs and/or Orai. Interaction of these plasma membrane homo/heteromeric channels with STIM1 in the ER would confer them store-operated properties. STIM1 has in fact been reported to interact physically both with Orai1 and TRPC channels [45–47].

17.3 Effect of Mitochondrial Ca^{2+} Buffering on SOCE Activation

The classical mechanism proposed for regulation by mitochondria of the activity of SOCE channels relies in the ability of mitochondria to reduce the size of the local Ca^{2+} microdomain around the inner mouth of the channel and thus avoid their Ca^{2+}-dependent inactivation. CRAC channels have two types of Ca^{2+}-dependent inactivation. Fast inactivation occurs with time constants of 10–100 ms and is due to Ca^{2+} binding at a site within less that 10 nm of the pore. The molecular determinants of this fast Ca^{2+}-dependent inactivation have been found both in Orai1 and STIM1, and a role of calmodulin has also been suggested [39, 48]. Because of the extremely small spatio-temporal scale of this phenomenon, mitochondria are not considered to be able to modulate the Ca^{2+} microdomain responsible for this fast inactivation. However, there is also a slow Ca^{2+}-dependent inactivation of CRAC channels that develops with a time course of tens of seconds and involves a larger high-Ca^{2+} spatial domain. It is this slow inactivation mechanism the one that could be modulated by mitochondria. The mechanism and molecular determinant of this slow Ca^{2+}-dependent inactivation is unknown, but it seems to be placed at around 100 nm distance from the CRAC channel pore [44]. In the case of TRPC1 channels, which also have Ca^{2+}-dependent inactivation, there is evidence that it may be mediated by calmodulin [49].

The first evidences for the modulation of SOCE by mitochondria through this mechanism were obtained in 1997 [21]. Both mitochondrial depolarization and inhibition of the electron transport chain enhanced the slow inactivation of the CRAC channel current in Jurkat T lymphocytes. Therefore, energized mitochondria were necessary to prevent the slow inactivation of the channels and facilitate a sustained Ca^{2+} entry through CRAC channels. T cell activation after exposure to antigen or other stimuli requires this prolonged Ca^{2+} entry in order to activate the Ca^{2+}-dependent protein phosphatase calcineurin. Then, dephosphorylation of the transcription factor NFAT by calcineurin unmasks the nuclear localization sequence of NFAT, which then translocates to the nucleus to activate gene transcription of T-cell activation genes [50]. In addition, mitochondria could also have a direct role in the activation of the CRAC current. Removal of cytosolic Ca^{2+} by mitochondria was proposed to compete with store refilling by SERCA pumps, increasing IP_3-induced ER depletion and leading to faster CRAC activation [51]. Thus, mitochondrial Ca^{2+} uptake would be essential both to activate and to sustain CRAC activity [52]. On the other hand, inhibition of mitochondrial Ca^{2+} efflux also diminished SOCE, suggesting that both uptake and release of Ca^{2+} from mitochondria were important to control SOCE [53].

In any case, some uncertainties remain on the role of mitochondria to control SOCE and the possible mechanism. The main mechanism proposed for mitochondrial modulation of SOCE involves local Ca^{2+} buffering to prevent the slow Ca^{2+}-inactivation of the channels. However, local Ca^{2+} microdomains in the SOCE Ca^{2+}-entry sites in T lymphocytes have been reported to be also essential to activate

ryanodine receptors via Ca^{2+}-induced Ca^{2+} release (CICR), thus inducing further Ca^{2+}-release to avoid store refilling and keep SOCE active [54]. Coupling of ryanodine receptors and CRAC channels through this Ca^{2+} microdomain would therefore be critical to keep SOCE active in T cells. A similar mechanism has been described for the selective activation of adenylate cyclase by SOCE in defined microdomains. Ca^{2+}-sensitive adenylate cyclase would interact with Orai1 in order to be activated by the local Ca^{2+} microdomain created by channel activation [55]. In addition, local Ca^{2+} microdomains around Orai1 channels are also essential for translocation of NFAT to the nucleus, probably because NFAT dephosphorylation depends on the local recruitment of calcineurin in the microdomain region [56–58]. Thus, the local $[Ca^{2+}]$ near CRAC channels has to rise above a certain threshold in order to trigger NFAT migration. It is therefore difficult to envision the role of mitochondria controlling the architecture of this local Ca^{2+} microdomain, which would be at the same time responsible for SOCE inactivation and essential to trigger CICR and calcineurin activation.

Other mechanisms for Ca^{2+}-dependent mitochondrial regulation of SOCE have also been proposed, among them local ATP production, which could chelate Ca^{2+} close to mitochondria, competition for Ca^{2+} uptake with the plasma membrane Ca^{2+} ATPase, which could contribute to sustain intracellular $[Ca^{2+}]$ increase and direct modulation of ER Ca^{2+}-release through the Ca^{2+}-dependence of IP_3 receptors [59–61]. In addition, Ca^{2+}-independent mechanisms for mitochondrial regulation of SOCE may also be present. In RBL-1 cells and mouse embryonic fibroblasts, store depletion did not induce any change in the pattern of mitochondrial distribution, and most mitochondria were far from the plasma membrane, between 500 nm and 1 μm. However, mitochondrial depolarization impaired the trafficking of STIM1 oligomers to ER-plasma membrane junctions and correspondingly inhibited SOCE [62]. This effect depended on the mitochondrion-shaping protein mitofusin-2. In the absence of mitofusin-2, STIM1 traffic and SOCE were no longer affected by mitochondrial depolarization. Therefore, mitochondria may act on SOCE by mechanisms unrelated to Ca^{2+} fluxes.

17.4 Mitochondrial Subcellular Localization and SOCE

In order for mitochondria to be able to control the local $[Ca^{2+}]$ around the SOCE channels in the plasma membrane, they should be placed very close to them. As mentioned above, the low Ca^{2+}-affinity of the mitochondrial Ca^{2+} uniporter puts a lower limit to the cytosolic $[Ca^{2+}]$ around mitochondria required to trigger fast Ca^{2+} uptake by the organelle. Thus, although mitochondria have been shown to take up Ca^{2+} at submicromolar $[Ca^{2+}]$ [63, 64], high rates of Ca^{2+} uptake are only obtained when extramitochondrial $[Ca^{2+}]$ rises above 2–3 μM [17, 64]. Given that most cellular stimuli induce under physiological conditions mean cytosolic $[Ca^{2+}]$ increases below 1 μM, mitochondria should be quite close to the Ca^{2+}-entry sites in order to activate fast Ca^{2+} uptake through the MCU. In chromaffin cells, for example, Ca^{2+}

entry through voltage-dependent Ca^{2+} channels generates local Ca^{2+} microdomains of 20–40 μM that trigger fast mitochondrial Ca^{2+} uptake in a mitochondrial population. However, more than 50 % of the mitochondria in each cell, those placed far away from the channels, hardly take up any Ca^{2+} [17, 18].

In immune cells, considerable evidence suggests that mitochondria actually move towards the SOCE sites during cell activation. In the Jurkat T cell line, stimulation of Ca^{2+} entry facilitated translocation along microtubules of the complete mitochondrial network towards the plasma membrane, in a Ca^{2+} and kinesin-dependent manner [65]. In T cells, the interface between an antigen-presenting cell or target cell and the lymphocyte is known as the immunological synapse or immune synapse (IS). The junction between T cells and antigen-presenting cells is characterized by close membrane appositions, separated from one another by 'synaptic' regions. Inside the T cell, membrane microdomains containing signaling proteins reorganize and several organelles redistribute toward the IS, including mitochondria and endoplasmic reticulum [66]. Thus, T cell stimulation causes a redistribution of the mitochondrial network towards the plasma membrane and the immunological synapse (IS), and some mitochondria approach to distances of 200 nm of the SOCE channels. In addition, mitochondria close to the IS took up more Ca^{2+} than those farther away, and redistribution of mitochondria to the IS was necessary to maintain Ca^{2+} influx across the plasma membrane and Ca^{2+}-dependent T-cell activation [67–69]. In fact, once formed the IS, mitochondria move towards them in a Ca^{2+}-dependent manner, and maintain SOCE active specifically at the IS, even in the absence of Orai1 accumulation there. Mitochondrial localization would therefore determine where in the plasma membrane should Ca^{2+} entry be active [70] (for reviews see [60, 71]).

However, there are still some findings awaiting explanation. Redistribution of mitochondria to the IS was impaired by knockdown of the mitochondrial fission factor dynamin-related protein 1 (Drp1). Moreover, Drp1 knockdown induced mitochondrial depolarization. However, Drp1 knockdown increased T-cell receptor signal strength and produced a larger and more durable intracellular $[Ca^{2+}]$ increase, in spite of the absence of mitochondria close to the IS [72]. On the other hand, silencing of the outer mitochondrial membrane protein GDAP1, mutations of which produce Charcot-Marie-Tooth neuropathy, induced abnormal distribution of the mitochondrial network in SH-SY5Y human neuroblastoma cells and decreased Ca^{2+} influx through SOCE [73]. Interestingly, junctophilin 1 (JPH1) overexpression was able to restore the SOCE activity in GDAP1-silenced cells [74]. JPH1 is a protein placed in the ER within the mitochondria-associated membrane (MAM) complexes (as well as GDAP1 from the mitochondrial side), but relocates during SOCE activation to co-localize with STIM1 in ER-plasma membrane junctions. Surprisingly, although mitochondrial blockers produced severe inactivation of SOCE in control cells, SOCE activity in JPH1-overexpressing cells was no longer inhibited by mitochondrial blockers, indicating that the restoration of SOCE activity by JPH1 in GDAP1 deficient cells was not related to the recovery of mitochondrial distribution or function [74].

In other non-excitable cell types, the evidence for the regulation of SOCE by mitochondria is weaker. In HeLa cells, expression of the mitochondrial fission promoter protein hFis1 induced a rapid and complete fragmentation of mitochondria, which redistributed away from the plasma membrane and clustered around the nucleus, even though they maintained a normal transmembrane potential and pH, and took up normally the Ca^{2+} released from intracellular stores upon agonist stimulation. Despite the dramatic morphological alteration, SOCE was only marginally reduced. However, disruption of mitochondrial potential with uncouplers or oligomycin/rotenone still reduced SOCE by 35 % [75]. Therefore, either close contact of mitochondria and Ca^{2+} influx channels is not required for SOCE modulation in these cells or mitochondrial inhibitors have additional effects directly on the channels. Also in HeLa cells, activation of SOCE has been shown not to produce significant local [Ca^{2+}] microdomains. While release of Ca^{2+} from the ER generated $[Ca^{2+}]_M$ levels five to tenfold higher in neighboring mitochondria than in the bulk cytosol, Ca^{2+} entry through SOCE generated similar [Ca^{2+}] levels in plasma membrane mitochondria and in deep mitochondria [76, 77]. These results suggest that mitochondria are excluded in these cells from the regions where SOCE occurs. In fact, in COS-7 cells, mitochondria were located either in the gaps between STIM1 puncta or in remote, STIM1-free regions, and the increase in $[Ca^{2+}]_M$ after Ca^{2+} addition was independent of the mitochondrion–STIM1 distance [78]. Similarly, in the cell lines RBL-2H3 and H9c2 cells, all mitochondria were shown to have contacts with the ER, but contacts between mitochondria and the plasma membrane were less frequent due to the presence of interleaving ER stacks [79, 80]. In fact, these findings provide structural basis for the observation that in some conditions Ca^{2+} reaches mitochondria only after crossing the ER [81].

These observations indicate that in these cells mitochondria are only exposed to Ca^{2+} diffusing laterally from the SOCE Ca^{2+}-entry sites, suggesting that the close apposition among ER and plasma membrane in the sites of STIM-Orai interaction represents indeed a physical barrier for access of mitochondria. These STIM-Orai interacting sites have also been shown to co-localize with SERCA pumps in non-excitable cells [82, 83]. For example, in HEK293 cells, SOCE activation generates subplasmalemmal high Ca^{2+} microdomains, but most of the entering Ca^{2+} is taken by the ER and not by mitochondria. This may also be due to the small size of the microdomains generated by SOCE, as SERCA pumps have a much higher Ca^{2+} affinity than mitochondrial uniporters [83].

In summary, mitochondrial localization with respect to SOCE appears to change considerably among different cell types [48]. In cells such as HeLa, RBL or HEK293, mitochondria are far away from the SOCE Ca^{2+} entry sites, while in T cells or Jurkat T lymphocytes cell activation triggers a fast movement of mitochondria toward the IS. Regarding excitable cells, there is also a significant variability. In vascular smooth muscle A10 cells, mitochondrial Ca^{2+} uptake was triggered by SOCE activation, but not by activation of voltage-dependent Ca^{2+} channels [84]. In other excitable cells, instead, mitochondrial Ca^{2+} uptake was much larger after voltage-dependent Ca^{2+} channels activation [17, 76].

17.5 Effect of MCU Knockout on SOCE Activation

The recent discovery of the main constituents of the mitochondrial Ca^{2+} uniporter provides a much better tool to assay the effects of mitochondrial Ca^{2+} uptake on Ca^{2+} entry through SOCE. Knockout of the pore component of the uniporter, the MCU, has been recently used to study the effect on SOCE. In RBL-1 cells, knockout of MCU considerably reduced Ca^{2+} entry after store depletion with thapsigargin [85]. MCU silencing by siRNA in MDA-MB-231 breast cancer cells also reduced serum- or thapsigargin-induced SOCE [86]. Similarly, in HeLa cells, MCU knockout reduced the rate of Ca^{2+} entry through SOCE and favored the development of slow inactivation [87].

We have also studied this point by using specific shRNA to silence the MCU protein in HeLa cell clones. However, our data do not show any inhibition of SOCE in the MCU-silenced clones with respect to scrambled clones. Figure 17.1 shows studies carried out using aequorin targeted to mitochondria or endoplasmic reticulum. The histamine-induced $[Ca^{2+}]_M$ peak was nearly abolished in the MCU-silenced cells (Fig. 17.1a), and addition of Ca^{2+} to permeabilized cells produced no increase in $[Ca^{2+}]_M$ in the MCU-silenced cells (Fig. 17.1b), showing that MCU activity was almost fully abolished in the silenced clone. In Ca^{2+}-depleted cells, refilling with Ca^{2+} of the ER was in fact faster in the silenced cells (Fig. 17.1c, d), suggesting that Ca^{2+}-entry through SOCE channels was not reduced in the MCU-silenced cells. Regarding Ca^{2+}-release through IP_3 receptors induced by the agonist histamine, it was somewhat slower in the MCU –silenced cells (Fig. 17.1e). This observation may be due to the increased feed-back inhibition by Ca^{2+} of IP_3 receptors in the absence of mitochondrial Ca^{2+} uptake.

Imaging studies of cytosolic $[Ca^{2+}]$ (Fig. 17.2) showed that the histamine induced cytosolic $[Ca^{2+}]_c$ increase was significantly larger in the MCU-silenced cells (Fig. 17.2a), probably because of the lack of mitochondrial Ca^{2+} uptake. When SOCE was explored directly by looking at the cytosolic $[Ca^{2+}]$ increase induced by Ca^{2+} addition to Ca^{2+}-depleted cells, Ca^{2+} entry through SOCE was found to be significantly increased in the MCU-silenced cells (Fig. 17.2b). Consistently, the rate of Mn^{2+} entry in Ca^{2+}-depleted cells (Fig. 17.2c) was also significantly increased in the MCU-silenced cells. Therefore, our data indicate that the absence of mitochondrial Ca^{2+} uptake did not inhibit SOCE in HeLa cells. In fact, a significant stimulation was observed. The reasons for the discrepancy with previously reported data [87] are unknown.

17.6 Conclusions

Intracellular Ca^{2+} signaling is mainly controlled by ER and mitochondria, two organelles with opposite roles in terms of Ca^{2+} homeostasis. The ER is the main intracellular Ca^{2+}-store, releases Ca^{2+} to the cytosol during cell activation and then

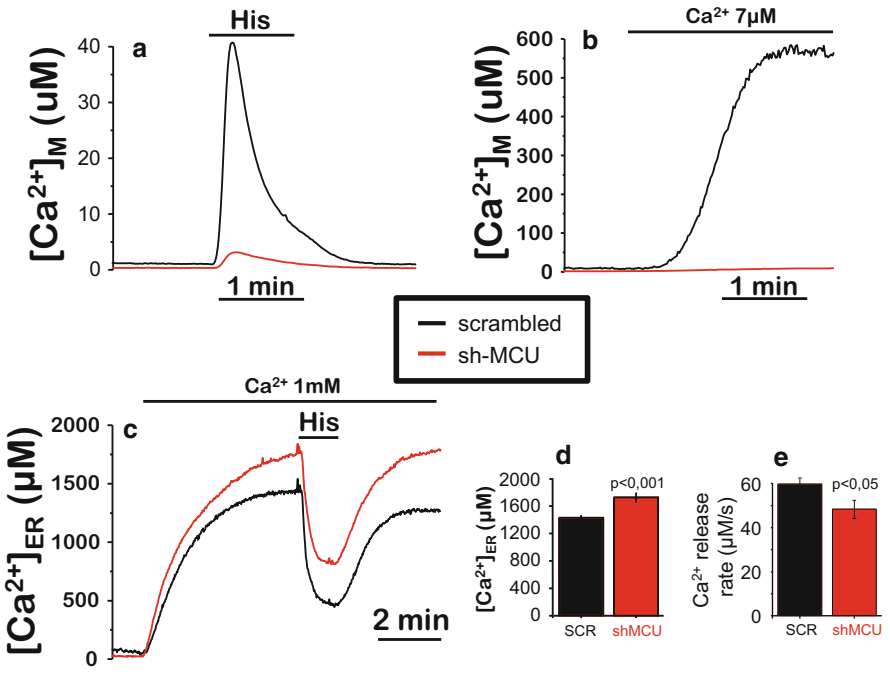

Fig. 17.1 Effect of MCU knockout on mitochondrial and ER [Ca^{2+}] dynamics. HeLa cell clones expressing either shRNA for the MCU protein or scrambled shRNA were transfected with either mitochondrially targeted mutated (**a**) or double mutated (**b**) aequorin or ER-targeted double-mutated aequorin (**c**). (**a**) Effect of stimulation with 100 µM histamine on $[Ca^{2+}]_M$. (**b**) Effect of perfusion of a 7 µM [Ca^{2+}] buffer in permeabilized cells (see [88] for other details). (**c**) Refilling of the ER with Ca^{2+} and Ca^{2+} release induced by 100 µM histamine (see Ref. [3] for other details). (**d**) Increase in the steady-state ER [Ca^{2+}] in MCU-silenced cells. (**e**) Decrease in the Ca^{2+} release rate in MCU-silenced cells

helps to end the Ca^{2+} transient by taking up Ca^{2+} back after the stimulation. In contrast, mitochondria have little [Ca^{2+}] under resting conditions, but have a large capacity to take up Ca^{2+} during cell stimulation and then release it again to the cytosol at the end of the Ca^{2+} transient. In addition, because of the low-Ca^{2+} affinity of the mitochondrial Ca^{2+} uptake mechanism, this organelle is specially adapted to buffer Ca^{2+} from high-Ca^{2+} microdomains such as those formed around the cytosolic mouth of open Ca^{2+} channels. The classical mechanism proposed for mitochondrial regulation of SOCE activity relies in the effect of mitochondrial Ca^{2+} uptake controlling the local Ca^{2+} microdomain around the SOCE channels, in order to prevent its Ca^{2+}-dependent inactivation. To be effective, this mechanism requires very close contacts between mitochondria and SOCE channels. However, mitochondrial subcellular localization with respect to SOCE channels appears to be highly variable among different cell types. While in immunitary cells mitochondria move to the IS during cell activation, in other cells there is little evidence for that. Figure 17.3 shows diagrams summarizing these mechanisms. In addition, activation of SOCE

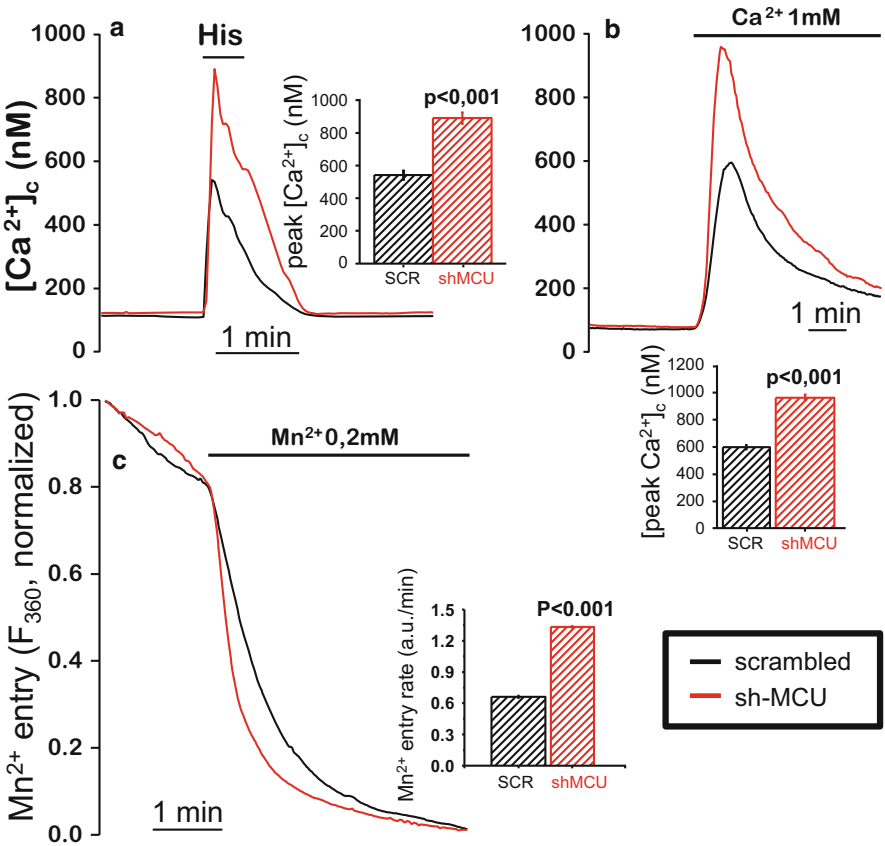

Fig. 17.2 Effect of MCU knockout on cytosolic [Ca^{2+}] dynamics and Mn^{2+} entry. The shMCU and scrambled HeLa cell clones were loaded with fura-2 and prepared for single-cell imaging experiments as described previously [25]. (**a**) Cells were stimulated with 100 μM histamine. Data are mean±s.e. from five experiments in each condition (221 cells scrambled, 191 cells shMCU). (**b**) Cells were depleted of Ca^{2+} with 10 μM benzohydroquinone and then SOCE was induced with 1 mM extracellular [Ca^{2+}]. Data are mean±s.e. from nine experiments in each condition (700 cells scrambled, 602 cells shMCU). **c** Cells were depleted of Ca^{2+} in Ca^{2+}-free medium and then Mn^{2+} entry was monitored at 360 nm fluorescence excitation. Data are mean±s.e. from five experiments in each condition (611 cells scrambled, 768 cells shMCU)

requires a close interaction among Orai (or TRPs) channels in the plasma membrane and STIM sensors in the ER. The steric hindrance due to the close apposition of plasma and ER membranes in the SOCE sites constitutes a barrier that limits the approach of mitochondria. Even in immune cells, this makes the distance of mitochondria to the SOCE sites to be at least 200 nm. In other cells the distance is even larger. On the other hand, the local Ca^{2+} microdomain created in the SOCE sites in immune cells seems to be also essential for T cell activation and NFAT dephosphorylation. Therefore, many uncertainties still remain on the role of mitochondria to control SOCE and the possible mechanism. Alternative pathways for mitochondrial modulation of SOCE, both Ca^{2+}-dependent and Ca^{2+}-independent, have also been proposed, but clear evidence for a specific mechanism is still lacking. The

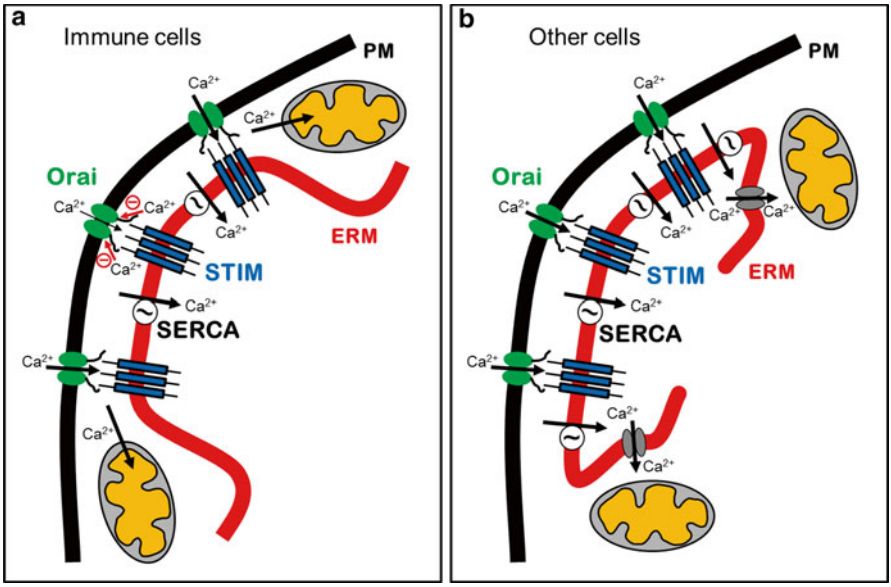

Fig. 17.3 A model of the role of mitochondria in the regulation of SOCE. (**a**) In immune cells, mitochondria move to the plasma membrane during cell activation and considerable evidence indicates that, after SOCE activation, mitochondrial Ca^{2+} uptake reduces the size of the local [Ca^{2+}] microdomain around nearby Orai-STIM complexes and protects Orai1 channels from Ca^{2+}-dependent inactivation. (**b**) In many other cell types, the available evidence suggests that mitochondria remain too far from the Orai-STIM complexes to be able to modulate directly the local Ca^{2+} microdomain. Mitochondria could anyway take up some of the incoming Ca^{2+} after passing through the ER, but the possible modulation of Ca^{2+} entry by mitochondria remains unclear. Alternative mechanisms of modulation based on mitochondrial ATP production, ER depletion induced by mitochondrial Ca^{2+} uptake, or others, have also been proposed. *PM* plasma membrane, *ERM* ER membrane, *SERCA* ER Ca^{2+} ATPase

recent advances in the knowledge of the molecular nature of the mitochondrial Ca^{2+} uptake mechanism open new ways to approach this problem directly, avoiding the use of mitochondrial inhibitors or protonophors and all their large side-effects.

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Chapter 18
Modulation of Calcium Entry by the Endo-lysosomal System

G. Cristina Brailoiu and Eugen Brailoiu

Abstract Endo-lysosomes are acidic organelles that besides the role in macromolecules degradation, act as intracellular Ca^{2+} stores. Nicotinic acid adenine dinucleotide phosphate (NAADP), the most potent Ca^{2+}-mobilizing second messenger, produced in response to agonist stimulation, activates Ca^{2+}-releasing channels on endo-lysosomes and modulates a variety of cellular functions. NAADP-evoked signals are amplified by Ca^{2+} release from endoplasmic reticulum, via the recruitment of inositol 1,4,5-trisphosphate and/or ryanodine receptors through a Ca^{2+}-induced Ca^{2+}- release (CICR) mechanism. The endo-lysosomal Ca^{2+} channels activated by NAADP were recently identified as the two-pore channels (TPCs). In addition to TPCs, endo-lysosomes express another distinct family of Ca^{2+}- permeable channels, namely the transient receptor potential mucolipin (TRPML) channels, functionally distinct from TPCs. TPCs belong to the voltage-gated channels, resembling voltage-gated Na^{+} and Ca^{2+} channels. TPCs have important roles in vesicular fusion and trafficking, in triggering a global Ca^{2+} signal and in modulation of the membrane excitability. Depletion of acidic Ca^{2+} stores has been shown to activate store-operated Ca^{2+} entry in human platelets and mouse pancreatic β-cells. In human platelets, Ca^{2+} influx in response to acidic stores depletion is facilitated by the tubulin-cytoskeleton and occurs through non-selective cation channels and transient receptor potential canonical (TRPC) channels. Emerging evidence indicates that activation of intracellular receptors, situated on endo-lysosomes, elicits canonical and non-canonical signaling mechanisms that involve CICR and activation of non-selective cation channels in plasma membrane. The ability of endo-lysosomal Ca^{2+} stores to modulate the Ca^{2+} release from other organelles and the Ca^{2+} entry increases the diversity and complexity of cellular signaling mechanisms.

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Abbreviations

$[Ca^{2+}]_i$	Cytosolic Ca^{2+} concentration, Ca_V, voltage-gated Ca^{2+} channels
cADPR	Cyclic ADP ribose
CCE	Capacitative Ca^{2+} entry
CICR	Ca^{2+}-induced Ca^{2+} release
CRAC	Ca^{2+}-release-activated Ca^{2+}
DTS	Dense tubular system
ER	Endoplasmic reticulum
IP_3	Inositol 1,4,5-trisphosphate
IP_3R	Inositol 1,4,5-trisphosphate receptor
LPI	L-α-lysophosphatidylinositol
MDCK	Madin-Darby canine kidney
NAADP	Nicotinic acid adenine dinucleotide
Na_V	Voltage-gated sodium channels
PARs	Protease-activated receptors
PI(3,5)P2	Phosphatidylinositol 3,5-bisphosphate
PLC	Phospholipase C
RyR	Ryanodine receptor
SOCE	Store-operated calcium entry
STIM1	Stromal interaction molecule-1
TBHQ	2,5-di-(tert-butyl)-1,4-hydroquinone
TPCs	Two-pore channels
TRPC	Transient receptor potential canonical
TRPML	Transient receptor potential mucolipin.

18.1 Endo-lysosomal System as Ca^{2+} Store

Lysosomes are intracellular organelles discovered in the mid-1950s by Christian de Duve [1, 2], commonly associated with the intracellular digestion and macromolecules degradation [3]. Lysosomes were identified as vacuolar structures with an acidic pH, containing hydrolytic enzymes, surrounded by a membrane [1, 2]. Four digestive processes, namely receptor-mediated endocytosis, pinocytosis, phagocytosis and autophagy are mediated by the lysosomes [4]. The lysosomal system is a heterogeneous system, comprising of: primary/nascent lysosomes, early autophagic vacuoles, intermediate/late endosomes, pinocytic/phagocytic vacuoles and

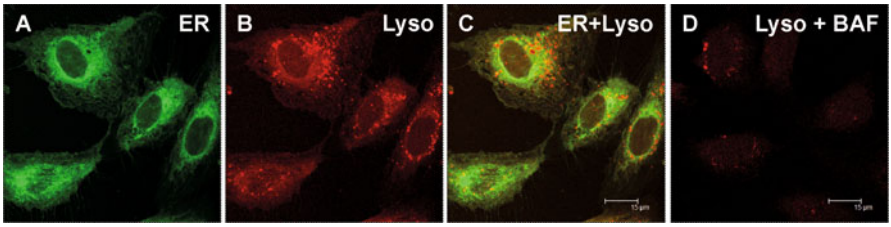

Fig. 18.1 Localization of the acidic compartments and the endoplasmic reticulum in human aortic endothelial cells (*HAEC*). Confocal images of endoplasmic reticulum labeled with ER-Tracker Green (**A**) and of endo-lysosomes labeled with LysoTracker Red (**B**) in HAEC. (**C**), a merged image of **a** and **b**. (**D**), pretreatment of HAEC cells with bafilomycin A1 (*BAF*), a V-type H^+ ATPase inhibitor, abolished the LysoTracker Red fluorescence

multivesicular bodies. Lysosomes play critical roles in proteolysis, an important regulatory mechanism, through an ubiquitin-proteasome system [5].

In the last few years, increasing evidence has established endo-lysosomes as a Ca^{2+} store. The endoplasmic reticulum (ER) is the best characterized intracellular Ca^{2+} store, endowed with a well-defined system of Ca^{2+} channels and pumps [6, 7]. In addition, mitochondria, Golgi apparatus, nucleus, caveolae, and acidic stores contain variable amount of Ca^{2+} and contribute to Ca^{2+} homeostasis [8, 9]. Acidic Ca^{2+} stores, in addition to the endo-lysosomal system, include acidocalcisomes, vacuoles, lysosome-related organelles, secretory vesicles and the Golgi complex [10].

The use of LysoTracker Red, a weak base that accumulates in acidic organelles [11] and of lysosomotropic agents that interfere with acidic compartments, such as bafilomycin A1 (Fig. 18.1), glycyl-L-phenylalanine-β-napthylamide (GPN), and monensin, facilitated the understanding of lysosomes as Ca^{2+} stores. Bafilomycin A1, a V-type ATPase inhibitor, prevents the acidification of lysosomes [12, 13]; the endo-lysosomal H^+ gradient is the driving force for most transporters [14]. GPN, a basic amine, is hydrolyzed by the lysosomal enzyme cathepsin C; accumulation of GPN in acidic organelles leads to osmotic swelling and, loss of lysosomal membrane barrier [15–17]. Monensin acts by collapsing the pH gradients across acidic organelles by forming a Na^+/H^+ exchanger [18]. The Ca^{2+} uptake by the endo- lysosomes occurs via a mechanism incompletely characterized; it requires the H^+ gradient generated by the V-type H^+ ATPase pump together with Na^+/H^+, Ca^{2+}/H^+ and perhaps Na^+/Ca^{2+} exchangers whose identity is still controversial [14, 19–21].

Lysosomal Ca^{2+} stores were identified in several cell models from various species from invertebrates to mammalians: snail neurons [22], human neutrophils [23], Drosophila melanogaster S2 cell lines [24], trypanosomatids parasites [25], mouse macrophages [26], Madin-Darby canine kidney (MDCK) cell lines [16, 27], bovine corneal endothelial cells [28], pancreatic acinar and beta cells [29], rat neurons [30, 31], human aortic endothelial cells [32], and human platelets [33].

On the other hand, defective Ca^{2+} handing by lysosomes disrupts endo-lysosomal membrane trafficking and has been involved in the pathogenesis of lysosomal stor-

age disorders such as mucolipidosis type IV, Niemann-Pick disease, Chediak–Higashi syndrome [21, 34–37], in acute pancreatitis [38, 39] and Alzheimer's disease [40].

18.2 NAADP Mobilizes Ca^{2+} from Endo-lysosomes

Nicotinic acid adenine dinucleotide phosphate (NAADP) is the most potent Ca^{2+} mobilizing second messenger [41–44]. NAADP is synthesized by the same enzyme, ADP-ribosyl cyclase and its homolog CD38, as cyclic ADP-ribose (cADPR) [45]. NAADP was first reported to produce Ca^{2+} mobilization from Ca^{2+} stores insensitive to inositol trisphosphate (IP_3) and cADPR in sea urchin eggs [46–49]. Early studies provide pharmacological and biochemical evidence that this Ca^{2+} store is the reserve granule, the functional equivalent of a lysosome in the sea urchin eggs [50].

Other studies reported that NAADP also released Ca^{2+} from acidic stores in pancreatic acinar cells [51], brain microsomes [52], pancreatic beta cells [29, 53], arterial smooth muscle cells [54], platelets [55] or astrocytes [56]. Our previous work indicates that NAADP releases Ca^{2+} from acidic lysosomal-like Ca^{2+} stores, distinct from endoplasmic reticulum, in the frog neuromuscular junction [57], intact mammalian neurons [31] or human aortic endothelial cells (HAEC) [32]. An example of NAADP-induced increased in cytosolic Ca^{2+} concentration $[Ca^{2+}]_i$, produced by intracellular microinjection of NAADP in preganglionic vagal neurons of nucleus ambiguus, is shown in Fig. 18.2.

NAADP-dependent Ca^{2+} release is characterized pharmacologically by its insensitivity to specific inhibitors of IP_3 receptors, ryanodine receptors or cADPR-mediated Ca^{2+} release [47, 49, 58, 59]. The study of NAADP-mediated Ca^{2+} release was facilitated by the synthesis of the cell-permeant form of NAADP, NAADP-acetoxymethylester (NAADP-AM) [60] and by the identification of Ned-19, a chemical probe that inhibits NAADP signaling (Fig. 18.2) while fluorescently labeling the NAADP receptors [61].

The second messenger role of NAADP was first proven in sea urchin eggs [62]; additional evidence supports NAADP as a second messenger in several other cells/tissues. In the nervous system, NAADP is a second messenger [63, 64] that modulates neurosecretion [65], neurite outgrowth [30], neuronal differentiation [66] and excitability [31]. NAADP has been involved in several other cellular functions such as T-cell activation [67], insulin secretion [68, 69], smooth muscle contraction [70, 71], fertilization [62, 72], cardiac contractility [73], platelets aggregation [55, 74], skeletal muscle differentiation [75], and angiogenesis [76].

In pancreatic secretory cells, vascular smooth muscle cells, endothelial cells, neurons, or myometrium, an increase in NAADP levels or NAADP-induced calcium response occurred during stimulation with agonists such as: cholecystokinin [29], glucagon-like peptide-1 (GLP-1) [77], insulin [78, 79], endothelin-1 [54, 80, 81], histamine [82, 83], glutamate [84], acetylcholine [32] and oxytocin [85]. More

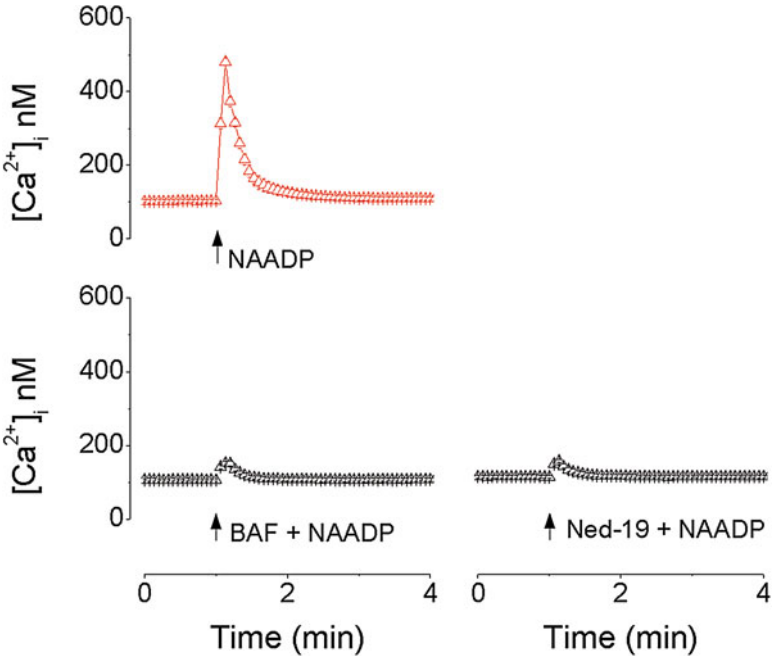

Fig. 18.2 NAADP mobilizes Ca^{2+} from acidic Ca^{2+} stores in vagal preganglionic neurons of nucleus ambiguus. Intracellular microinjection of NAADP (300 nM estimated intracellular concentration) (*red trace*) elicits a fast and transient increase in cytosolic Ca^{2+} concentration, $[Ca^{2+}]_i$. The NAADP-induced increase in $[Ca^{2+}]_i$ was abolished by bafilomycin A1 (BAF, 1 μM, 1 h), a V-type H^+ ATPase inhibitor, and by Ned-19 (1 μM, 15 min), an antagonist of NAADP signaling

recently, NAADP has been reported to be a second messenger in tracheal smooth muscle [86].

Intriguingly, in some cellular models, NAADP appears to have different roles depending on the agonist generating this second messenger. For example, in pancreatic β-cells, NAADP produced by activation of the cells with insulin modulates insulin synthesis and β-cells proliferation, while GLP-1-induced NAADP production potentiates insulin secretion and glucose-mediated Ca^{2+} signaling [77–79].

18.3 Functional Coupling of Acidic Ca^{2+} Stores with Other Intracellular Ca^{2+} Stores

One remarkable feature of the intracellular Ca^{2+} stores is that despite their structural segregation, they are functionally interacting [9, 87, 88]. Interactions between different Ca^{2+} stores and channels shape the Ca^{2+} signal and contribute to the diversity of cellular responses [89].

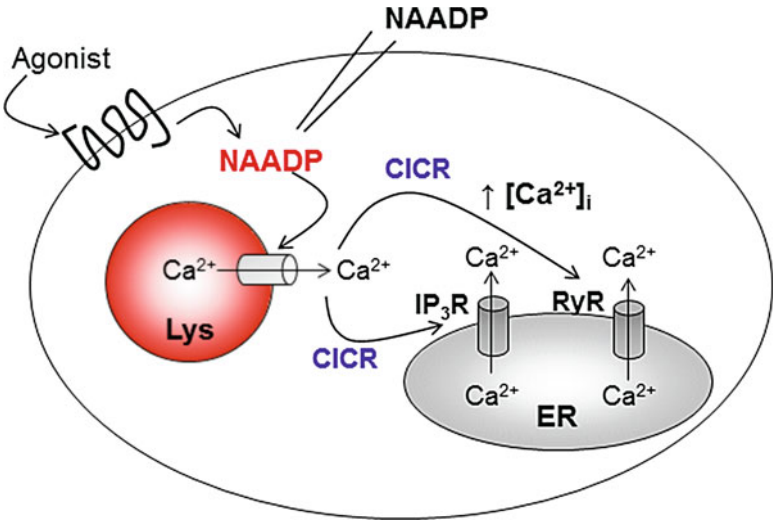

Fig. 18.3 NAADP-induced Ca^{2+} release activates Ca^{2+} induced Ca^{2+} release (*CICR*). NAADP produced in response to agonist stimulation, administered by intracellular microinjection, photolysis of caged NAADP, or administered as a cell permeant derivative (NAADP-AM), activates Ca^{2+} release from endo-lysosomes (*Lys*). This trigger Ca^{2+} is amplified by Ca^{2+} release from endoplasmic reticulum (*ER*) via inositol 1,4,5-trisphosphate receptors (*IP_3R*) or via ryanodine receptors (*RyR*) through a CICR mechanism

An early identified characteristic of NAADP-induced lysosomal Ca^{2+} release was its association with the recruitment of inositol trisphosphate and/or ryanodine receptors via Ca^{2+}-induced Ca^{2+} release (CICR) [30, 51, 54, 70, 87, 90–92]. Through this mechanism, a small, local release of Ca^{2+} from lysosomes is amplified via release of Ca^{2+} from ER leading to a global increase in Ca^{2+}. NAADP is thus thought to act as trigger during agonist-evoked Ca^{2+} signaling; the Ca^{2+} signal is then amplified by Ca^{2+}-sensitive Ca^{2+} release channels on the ER [90, 93, 94].

Early studies demonstrate that the lysosomal Ca^{2+} pool is functionally coupled with the IP_3-sensitive Ca^{2+} pool in MDCK cells [27]. At the frog neuromuscular junction, NAADP increases neurosecretion by releasing Ca^{2+} from an internal store via a two-pool model [65], supporting the functional coupling between the NAADP-sensitive store and ER stores [57]. In some cellular models that express both IP_3R and RyR, such as vascular smooth muscle [54] or SKBR3 breast cancer cells [95], Ca^{2+} release from acidic stores couples selectively to RyR. Conversely, in pancreatic acinar cells [51], starfish oocytes [92] and sea urchin eggs [90], NAADP appears to recruit both IP_3R and RyR; a diagram summarizing these mechanisms is illustrated in Fig. 18.3.

Recently, total internal reflection fluorescence (TIRF) microscopy revealed that lysosomes were closely associated with the ER, allowing them also to accumulate Ca^{2+} released by IP_3 receptors [17]. Furthermore, Ca^{2+} released from the ER may activate the NAADP pathway by stimulating Ca^{2+}-dependent NAADP synthesis and

by activating NAADP-regulated channels, supporting a bidirectional Ca^{2+} signaling between the ER and acidic organelles [96].

Intriguingly, the three main Ca^{2+}-mobilizing second messengers, IP_3, cADPR and NAADP were ascribed different functions in various systems, such as invertebrate Ascidian oocytes [97], mammalian pancreatic cells [51] or mouse smooth muscle [71]. A messenger-specific role of NAADP was identified in neuronal differentiation [66] neurite outgrowth [30] and endothelial function [32].

The differential recruitment of intracellular Ca^{2+}-mobilizing messengers and their target Ca^{2+} stores contribute to the specificity and versatility of Ca^{2+} signaling [98]. The functional coupling ("chatter") [99] between NAADP, IP_3, and cADPR has been involved in several Ca^{2+}-dependent events, including fertilization [62, 97], glucose sensing [29, 69, 77] or smooth muscle contraction [71].

18.4 Ca^{2+} Release Channels in the Endo-lysosomal System

Despite the early studies indicating that NAADP releases Ca^{2+} from endo-lysosomes, the molecular identity of endo-lysosomal Ca^{2+} release channels targeted by NAADP remained elusive for several years [100, 101]. Candidates NAADP receptors included RyRs [102, 103] and endo-lysosomal transient receptor potential mucolipin 1(TRPML1) channels [104]. In 2009, members of the two-pore channel (TPC) family, namely TPC1 [95] and TPC2 [105] or TPCN2 [106] were identified to mediate NAADP-evoked Ca^{2+} release from acidic organelles and thus, considered the long-sought after target channels for NAADP [14, 101, 107].

The role of TPCs as a target for NAADP is supported by several findings: overexpression of TPC1 or TPC2 increased the Ca^{2+} response to NAADP [56, 95, 107], and increased [^{32}P]NAADP binding [105]; while knocking down the TPCs expression abolished the response to NAADP [95, 105, 108]. Moreover, the pharmacology of TPCs [95, 105] was in agreement to that reported in earlier studies for the "NAADP receptors" [96, 101, 104, 109–111].

In addition, activation of NAADP-induced currents was identified in plasma membrane patches obtained from cells expressing plasma membrane-targeted TPC2 [112] and in bilayers reconstituted with TPC1 [113] or TPC2 [114–116]. Furthermore, the response to NAADP was abolished in cells from TPC2 knockout mice [71, 105].

Genomic analysis indicated that the animal kingdom express up to three family members (TPC1–3) [95, 105]. The three TPCN genes (*TPCN1*, *TPCN2* and *TPCN3*) are expressed in most vertebrate species [107, 117]. All three TPC isoforms were cloned from the sea urchin, while TPC3 is a pseudogene in humans and other primates [107].

TPC2 is expressed primarily in lysosomes, while TPC1 and TPC3 appear also to be expressed in endosomes [95, 105, 118, 119]. The restricted subcellular distribution of TPC1 compared to TPC2 leads to NAADP-induced localized Ca^{2+} transients in TPC1-expressing cells and global Ca^{2+} increases in cells overexpressing TPC2 [105].

In addition to TPCs, another distinct family of Ca^{2+} permeable channels, namely the transient receptor potential mucolipin (TRPML) channels are expressed in endo-lysosomes [120]. A segregated distribution has been reported: TRPML3, similarly to TPC1, are present mainly in endosomes, while TRPML1, similarly to TPC2 are expressed in lysosomes [14, 101, 120, 121]. Despite the fact that TPCs and TRPMLs are expressed in the same complex, they are functionally independent [120]. TRPMLs act as phosphatidylinositol 3,5-bisphosphate [PI(3,5)P2]-activated non-selective cation channels [34, 122], while TPCs were proposed as NAADP-gated channels [95, 105, 106]. Several aspects such as the functional relationships between TPCs and TRPMLs remain to be explored [14].

The role of TPCs as NAADP-regulated Ca^{2+} channels has been recently challenged [123, 124] leading to some controversies [101]. One study [124] suggested that TPCs are Na^+-selective channels regulated by PI(3,5)P2 and by ATP and not by NAADP. Another study [123] found that TPCs couple the cell's metabolic state to endo-lysosomal function.

However, an elegant study [19] has recently contributed to the reconciliation of the findings, by demonstrating that TPC2 is activated by NAADP and essential for NAADP-mediated Ca^{2+} release. The study confirmed the activation of TPC2 by PI(3,5)P2 and its permeability to Na^+, while identifying Mg^{2+}, MAPKs, JNK and P38 as novel regulators of TPC2 [19]. Changes in cytosolic Mg^{2+} associated with receptor activation and with changes in cytosolic ATP are sensed by TPC2 and affect the lysosomal membrane potential [19]. On the other hand, mTORC1, MAPKs, JNK and P38 kinases inhibit TPC2 [19].

While, several studies demonstrate indubitably that NAADP activates TPCs [19, 95, 105–107, 113–116], the actual site of NAADP binding remained unclear. Photoaffinity labeling studies indicate that binding of NAADP occurs at an accessory component within a larger TPC complex [125].

18.5 TPCs Regulation and Roles

TPCs are a family of intracellular ion channels, residing in the membranes of acidic organelles [10], initially cloned from rat kidney [126]. The TPC family is a member of voltage-gated superfamily of cation channels, resembling ancient voltage-gated Na^+ (Na_V) and Ca^{2+} channels (Ca_V) [127, 128]. Unlike Ca_Vs and Na_Vs channels that are composed of four homologous domains, TPCs consist of two-linked six transmembrane domains [128]. TPCs assemble as homo- and heterodimers through differential interactions between transmembrane regions [128–130].

Biophysical properties such as single-channel gating and conductance properties of purified human TPC2 have been recently reported [114]. The luminal Ca^{2+} concentration and the luminal pH control the sensitivity of TPCs to NAADP: low NAADP levels and low pH activate TPC2, while high NAADP levels and neutral pH inhibit the channel [114] Interestingly, Ned-19, an inhibitor of NAADP signaling [61], in the absence of NAADP and at high concentration (1 μM), closes TPC2

while at lower concentration (100 nM) potentiates the effect of NAADP (10 nM) by binding at a site distinct from NAADP [114].

To explore the evolution of TPCs, a TPC1 model was recently constructed using the crystal structure of a bacterial Na_V that only has one, instead of four, of the repeated domains [128]. Molecular docking analysis indicated that Ca_Vs or Na_Vs antagonists bind the pore region of TPC1 through common sites [128]. In addition, in functional, calcium imaging studies, the Ca_Vs or Na_Vs antagonists blocked the Ca^{2+} signals elicited by NAADP in TPC1-expressing cells [128].

TPCs play an important role in vesicular fusion and trafficking in endo-lysosomes, by promoting the local Ca^{2+} release [91, 119, 131, 132]. Emerging evidence support the role of TPC2 in endo-lysosomal trafficking [133, 134]. Fibroblasts and hepatocytes from TPC2 KO mice present a profound impairment of LDL- cholesterol and of EGF-receptor trafficking [133]. Additional, morphological and functional changes were identified in lysosomal fibroblasts from patients with Parkinson disease with a common mutation in LRRK2; the changes could be reversed by inhibition of TPC2 indicating that TPC2 acts downstream of pathogenic LRRK2 to regulate endo-lysosomal trafficking [134].

TPCs also trigger a global increase in cytosolic Ca^{2+} concentration and contribute to the amplification of a local Ca^{2+} signal by recruiting CICR at the lysosomal-ER junction [91, 131]. To dissociate the role of trigger of NAADP-TPCs from that of amplifier played by the ER, we examined the ability of NAADP to evoke Ca^{2+} signals in cells expressing plasma membrane-targeted TPC2 [112]. Redirecting TPCs to the plasma membrane produced a dissociation of NAADP-induced Ca^{2+} release from the ER-dependent Ca^{2+} release [112], indicating the requirement for TPCs targeting to the endo-lysosomal system. Another role described for TPCs is to regulate the cellular excitability via Ca^{2+} release from endo-lysosomal stores from the close proximity of the plasma membrane and the consequent activation of Ca^{2+}-activated channels at the plasma membrane [10, 78, 131, 132].

TPCs interact with many proteins with critical roles in Ca^{2+} homeostasis, trafficking, and membrane organization leading to important isoform-specific roles in organelle proliferation and cellular pigmentation [135]. Both TPC1 and TPC2 interact with several Rab isoforms, however, pigmentation phenotype required TPC2, but not TPC1 activity [135]. This is in agreement with the results of a genetic screen that identified coding variants of human TPC2 that are associated with hair color [136]. Similar to some other channels, TPCs are regulated by N-glycosylation [91]. Glycosylation serves to inhibit TPC function, as mutation of the N-glycosylation sites in TPC1 enhances NAADP-evoked Ca^{2+} release [137].

NAADP via TPC2-mediated Ca^{2+} release has been involved in angiogenesis in response to vascular endothelial growth factor (VEGF) [76]. In skeletal muscle C2C12 cells, that express both TPC1 and TPC2, NAADP promotes skeletal muscle differentiation; the effect was inhibited by NAADP antagonist, Ned-19 [61] or by the down-regulation of TPC1 and TPC2 [75]. TPCs have been proposed to contribute to autophagy signaling and protein homeostasis in skeletal muscle via mTOR pathway [123, 138].

In the megakaryoblastic cell line MEG01, expressing TPC1 and TPC2, NAADP releases Ca^{2+} from acidic lysosomal-like stores that promotes subsequent activation of ryanodine-sensitive Ca^{2+} release and amplification of Ca^{2+} signals via a CICR mechanism [108]. NAADP-induced Ca^{2+} release was impaired by TPC1 and TPC2 expression silencing, while TPC1 and TPC2 overexpression produced opposite results [108].

TPC2 has a critical role in coupling of the muscarinic receptors with Ca^{2+} release from acidic stores in mouse detrusor muscle [71]. NAADP-evoked contractions are abolished in detrusor muscle from TPC2 KO mice indicating that TPC2 is required for NAADP-mediated contractility. TPC2 contributes, in addition to IP_3R and RyR, to the response to the agonist carbachol [71]. Moreover, tissues from TPC2 KO mice have a reduced sensitivity in the IP_3-induced contraction, indicating a cross-talk between acidic stores and ER Ca^{2+} stores [71].

The endo-lysosomes are dynamic organelles and the TPCs are highly mobile proteins [139], facilitating the interactions with ER Ca^{2+} channels [140]. Emerging evidence indicates intimate functional and physical coupling between acidic stores and ER Ca^{2+} stores [140, 141]; membrane contact sites were identified between the ER and late endosomes [142], multivesicular bodies [143] or lysosomes [141]. The membrane contact sites between acidic stores and the ER are similar to those between the ER and other organelles, supporting local Ca^{2+} microdomain signaling [140, 141]. These findings support previous reports that NAADP signaling occurred at discrete "trigger" zones [54].

A recent computational model of the microdomains between lysosomes and ER suggests that the Ca^{2+} oscillations in the microdomains may produce global Ca^{2+} signals even in the absence of high microdomains Ca^{2+} concentrations [144, 145]. Moreover, the IP_3R-dependent global Ca^{2+} signals can be regulated by the distribution and density of TPCs [144, 145].

18.6 Ca^{2+} Release from Endo-lysosomes and Ca^{2+} Entry

In non-excitable cells, depletion of intracellular Ca^{2+} stores by IP_3 leads to store-operated calcium entry (SOCE) or capacitative calcium entry (CCE) through Ca^{2+} release-activated Ca^{2+} (CRAC) channels [146, 147]. There is limited information whether or not release of Ca^{2+} from endo-lysosomes leads to Ca^{2+} influx. Earlier studies indicate that depletion of lysosomal Ca^{2+} stores with GPN induced Ca^{2+} influx via CCE in bovine corneal endothelium [28]. On the other hand, depletion of acidic stores by fatty acids in amoeba [148] induced CCE.

A number of non-selective Ca^{2+}-permeable channels with different biophysical properties has been considered to carry the SOCE current, I_{CRAC} [147, 149–151]. The protein Orai1 (also named CRACM1 for CRAC modulator 1) has been proposed to form the pore of the channel mediating I_{CRAC} [152–154]. The activation of SOCE involves the participation of the stromal interaction molecule-1 (STIM1), an endoplasmic reticulum (ER) Ca^{2+} sensor [155, 156]. STIM-1 is present in the mem-

branes of the ER [157] and acidic Ca^{2+} stores [158]. When the intraluminal Ca^{2+} concentration decreases, STIM1 redistributes to ER-plasma membrane sites where the Orai1 subunit of the CRAC channel accumulates in the plasma membrane and CRAC channels open, activating SOCE [159–161].

The transient receptor potential (TRP) channels, particularly members of the TRP canonical (TRPC) subfamily have been involved in Ca^{2+} entry during SOCE [162]. In addition to interacting with Orai1, emerging evidence indicates that STIM1 also interact with TRPC channels [163]. Unlike STIM1, STIM2, a closely related protein, is considered a feedback regulator that inhibits SOCE [164].

Human platelets are endowed with two distinct Ca^{2+} stores: the dense tubular system (DTS) and acidic organelles (Fig. 18.4). Two mechanisms of SOCE, regulated by each Ca^{2+} compartment, were identified [33, 55, 165–167]. Human platelets express SERCA2b in the DTS; it has high sensitivity to thapsigargin, while it is insensitive to 2,5-di-(tert-butyl)-1,4-hydroquinone (TBHQ) [168]. Conversely, the platelet acidic Ca^{2+} stores express different isoforms of SERCA3 Ca^{2+} pump, sensitive to TBHQ and with low affinity for thapsigargin [74, 165, 169, 170]. In addition, the acidic stores express the vacuolar H^+-ATPase [165, 170].

In platelets, both mechanisms of SOCE are modulated by the actin cytoskeleton and microtubules [171]. Tubulin-cytoskeleton plays a dual role in SOCE: it facili-

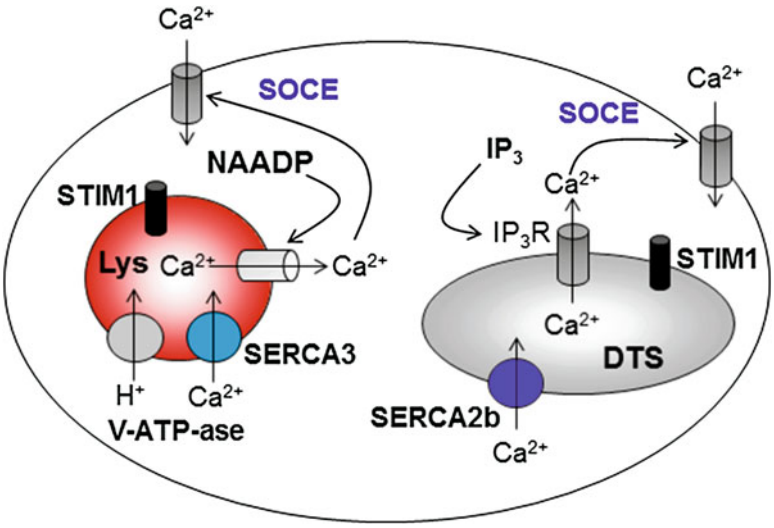

Fig. 18.4 Acidic stores-induced SOCE pathways in human platelets. In human platelets, SOCE may be induced by two mechanisms: by depletion of the acidic Ca^{2+} stores (*Lys*) or by depletion of the dense tubular system (*DTS*) [33]. NAADP releases Ca^{2+} from acidic stores; depletion of acidic organelles stimulates Ca^{2+} entry via plasma membrane Ca^{2+}-permeable channels via a SOCE mechanism. Depletion of DTS via IP_3 acting on IP_3R activates SOCE. STIM1 located on the membrane of acidic organelles or DTS, interacts with Orai 1 channels or with TRPC channels on plasma membrane, promoting the Ca^{2+} influx. SOCE is modulated by the actin cytoskeleton and microtubules. Acidic stores express SERCA3 and vacuolar H^+-ATPase, and DTS express SERCA2b pump [33, 167]

tates SOCE mediated by depletion of the acidic stores, while acting as a barrier that prevents constitutive SOCE regulated by DTS [172].

SOCE may be modulated by the interaction between the IP_3R type II situated in the membrane of DTS and the TRPC channels in the plasma membrane [173, 174]. Moreover, selective depletion of the intracellular Ca^{2+} stores in human platelets, induces the formation of heteromeric complexes between hTRPC1 and hTRPC6 and the interaction of both hTRPC1 and hTRPC6 with the IP_3R type II, SERCA2b and SERCA3 [171].

NAADP has been shown to release Ca^{2+} in human platelets from TBHQ-sensitive stores [55, 74], and NAADP-binding sites was demonstrated using a radioreceptor-binding assay [175]. Proposed mechanisms involved in SOCE activated by the depletion of acidic Ca^{2+} stores include the translocation of STIM1 and STIM2 and their association with Orai1 as well association of STIM1 with TRPC1 and TRPC6 [158, 176]. SOCE controlled by the acidic stores has been shown to be regulated by phosphatidylinositol 4,5-bisphosphate (PIP_2) [33].

In mouse pancreatic β-cells, NAADP, in addition to releasing Ca^{2+} via TPCs, evoked oscillatory non-selective cation currents; the effect was abolished by Ned-19 or in cells from TPC2 knockout mice [61, 105]. The underlying mechanism coupling the NAADP-induced Ca^{2+} release from acidic stores with the membrane depolarization was considered the activation of TRPM4/5 channels at the plasma membrane [78].

In human cells MEG01 and HEK293 cells, TPC2 may associate with STIM1 and Orai1 to modulate SOCE [170]. Silencing the expression of endogenous TPC2, but not of TPC1, attenuated both the rate of Ca^{2+} entry and the extent of SOCE stimulated by thapsigargin, without affecting the ability of cells to accumulate Ca^{2+} into the intracellular stores [177].

18.7 Functional Intracellular Receptors in Endo-lysosomes

Accumulating evidence indicates that in addition to effects mediated by the plasma membrane receptors, several agonists activate intracrine signaling, by acting on receptors located on intracellular membranes [178]. Work from our lab [179–184] and others [178, 185–187] support the idea that intracellular G protein-coupled receptors (GPCR) are functionally active [188]. Intracrine signaling has been better characterized for the renin-angiotensin system [178, 186, 187, 189, 190].

Intracrine signaling plays important roles in the physiology and pharmacology of GPCR ligands and expands the diversity and complexity of signaling mechanisms [178, 186]. Several lines of evidence, such as the presence of heterotrimeric G proteins in endoplasmic reticulum, Golgi, endo-lysosomes and nucleus, indicate that classical receptor signaling occurs at intracellular membranes [191–195]. The enzymes involved in NAADP and cADPR synthesis are also present at the membrane of acidic organelles [196, 197] and the release of second messengers from organelles contributes to Ca^{2+} signaling [198].

While receptor endocytosis is generally seen as distinct from cell signaling [199], relatively recent, endosomes-localized GPCRs were demonstrated to be present in the active form, supporting the view that canonical and non-canonical GPCR signaling occurs from endosomes, in addition to the plasma membrane [200–203]. For example, internalized beta-adrenoreceptors, thyroid stimulating hormone (TSH) or parathyroid hormone (PTH) receptors can initiate Gs-mediated signal transduction and consequent increase in cAMP from endosomes, in addition to the signal initiated from the plasma membrane [200, 202, 203]. Moreover, internalized protease-activated receptors (PARs), GPCRs that are activated by proteolysis, signal from endosomes through the recruitment of β-arrestins [201, 204]. Receptor signaling from endosomes is not surprising, since the endo-lysosomal membranes are organized into specialized domains where molecules can assemble into specific signaling complexes [199, 205].

Furthermore, peptides such angiotensin II [182, 184] or endothelin-1 [183], can be readily transferred from the cytosol inside the endo-lysosomal system via microautophagy [206]. In excitable cells, like neurons [184] or myometrium [182], as well as in U2OS cells transfected with AT_1 receptor [184], we identified that angiotensin II activates AT_1 receptors located on endo-lysosomes and activates phospholipase C (PLC) at the lysosomal membrane. The subsequent production of IP_3 activates IP_3R followed by the Ca^{2+} release from the ER. In neurons, the local Ca^{2+} release activates TRPC and produces membrane depolarization [184], while in myometrial cells, the Ca^{2+} response is further augmented by a CICR mechanism via ryanodine receptors activation [182].

Similarly, in endothelial cells that express endothelin-1 receptors type B (ET_B), or in cells transfected with ET_B, endothelin-1 (ET-1) activates ET_B receptors on endo-lysosomes, followed by activation of PLC, release of IP_3 and activation of IP_3R from the ER. The subsequent IP_3-induced increase in cytosolic Ca^{2+} activates endothelial nitric oxide synthase to produce nitric oxide [183].

The emerging paradigm of functional intracellular GPCRs is particularly significant in the case of lipid messengers that are generated intracellularly "on-demand" [207–209]. We examined the distribution and function of CB_1 and CB_2 cannabinoid receptors, and of the atypical cannabinoid receptors GPR55, GPCRs activated by lipids [180, 181, 210]. We monitored the cytosolic Ca^{2+} concentration during intracellular microinjection and extracellular application of the receptor agonists and identified distinct signaling mechanisms initiated by the intracellular versus plasma membrane receptors [180, 181, 210].

In endothelial cells, we provided functional and morphological evidence that CB_2 receptors are localized, in addition to the plasma membrane, at the endo-lysosomes, and their activation mobilizes NAADP-sensitive acidic-like Ca^{2+} stores and IP_3-sensitive Ca^{2+} stores [181]. Our results support the functionality of intracellular CB_2 receptors and their ability to couple to Gq proteins and to elicit Ca^{2+} signaling [181]. In CB_1-transfected cells, anandamide, acting on CB_1 receptors situated on acid-filled Ca^{2+} stores, activates IP_3-dependent and NAADP-dependent Ca^{2+} pathways [180]. Similarly, activation of intracellular GPR55 by L-α-lysophosphatidylinositol (LPI) elicited NAADP-dependent Ca^{2+} mobilization from

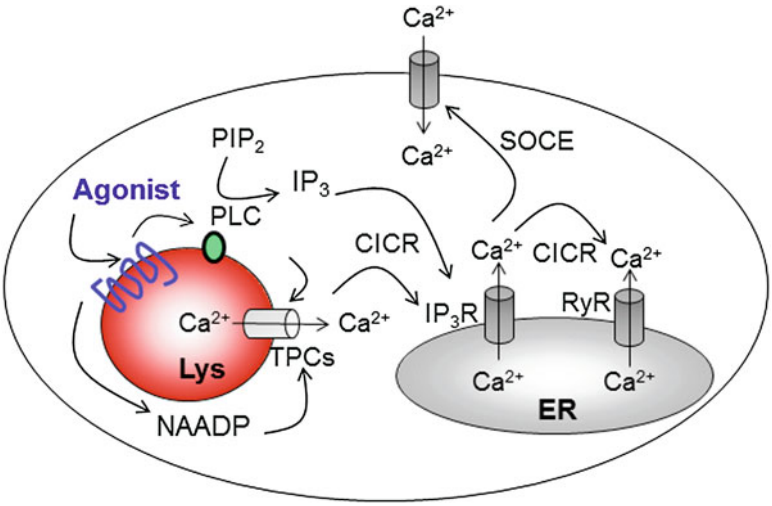

Fig. 18.5 Proposed mode of action of agonists via activation of intracellular receptors. Agonists such as angiotensin II [182, 184], endothelin-1 [183], anandamide [180], 2-arachidonoyl glycerol (2-AG) [181], and L-α-lysophosphatidylinositol (*LPI*) [210], act on receptors (AT_1, ET_B, CB_1, CB_2, and GPR55, respectively) situated on acid-filled Ca^{2+} stores (endo-lysosomes, *Lys*). Receptor activation may lead to the activation of lysosomal phospholipase C (*PLC*), and generation of IP_3 from phosphatidylinositol 4,5-bisphosphate (*PIP_2*), or to NAADP production. IP_3 releases Ca^{2+} via IP_3 receptors (*IP_3R*) situated on endoplasmic reticulum (*ER*). NAADP releases Ca^{2+} from endo-lysosomes via TPCs. IP_3- or NAADP-induced increase in $[Ca^{2+}]_i$ may activate ryanodine receptors (*RyR*) and further potentiates Ca^{2+} release from the ER via a Ca^{2+}-induced Ca^{2+} release (*CICR*) mechanism. Depletion of Ca^{2+} from organelles may also activate the store-operated Ca^{2+} entry (*SOCE*) mechanism and promote the Ca^{2+} influx via plasma membrane Ca^{2+} channels

acidic-like Ca^{2+} stores, mechanisms distinct from those elicited by the activation of plasma membrane GPR55 [210]. A diagram summarizing mechanisms elicited by activation of endo-lysosomal GPCRs is presented in Fig. 18.5.

18.8 Conclusions

Emerging evidence supports endo-lysosomes as intracellular Ca^{2+} stores targeted by NAADP, the most potent Ca^{2+}-mobilizing second messenger. NAADP, produced in response to several agonists, elicits Ca^{2+} release via TPCs, and modulates a variety of cellular functions such as fertilization, neurosecretion, contraction and differentiation. Ca^{2+} release from endo-lysosomes is amplified by Ca^{2+} release from the ER; depletion of acidic Ca^{2+} stores, similar to the ER, elicits store-operated Ca^{2+} entry. The physiological significance of the Ca^{2+} signaling originating from endo-lysosomes and the pathophysiological consequences of defective Ca^{2+} handling at this level are just beginning to be understood.

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Chapter 19
Remodeling of Calcium Entry Pathways in Cancer

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Abstract Ca^{2+} entry pathways play important roles in control of many cellular functions, including long-term proliferation, migration and cell death. In recent years, it is becoming increasingly clear that, in some types of tumors, remodeling of Ca^{2+} entry pathways could contribute to cancer hallmarks such as excessive proliferation, cell migration and invasion as well as resistance to cell death or survival. In this chapter we briefly review findings related to remodeling of Ca^{2+} entry pathways in cancer with emphasis on the mechanisms that contribute to increased store-operated Ca^{2+} entry (SOCE) and store-operated currents (SOCs) in colorectal cancer cells. Finally, since SOCE appears critically involved in colon tumorogenesis, the inhibition of SOCE by aspirin and other NSAIDs and its possible contribution to colon cancer chemoprevention is reviewed.

Keywords Store-operated Ca^{2+} entry • Cancer • Colorectal cancer • Aspirin • NSAID

19.1 Intracellular Ca^{2+} Homeostasis and Ca^{2+} Entry Pathways

Intracellular Ca^{2+} is a very versatile second messenger involved in the control of many different physiological and cellular processes in the short and the long-term. Unlike other messengers, Ca^{2+} is not created or destroyed by cells, but transported down electrochemical gradients through specific channels, or transported back against gradients at the expense of the energy stored as ATP (Ca^{2+} pumps) or coupled with transport of other ions (Ca^{2+} exchangers). Ca^{2+} channels, pumps and exchangers are located in the plasma membrane and endomembranes of the endoplasmic reticulum (ER), mitochondria and other cell organelles. Unlike other ions,

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the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$) is so little (100 nM) that even minimal changes in channel activity may induce large increases in $[Ca^{2+}]_{cyt}$. Thus, this cation is unique in the sense that has been selected by nature to carry signals inside cells and organelles. Changes in $[Ca^{2+}]_{cyt}$ may be restricted in time and space resulting in elementary events or Ca^{2+} microdomains that can regulate specifically located cellular functions such as exocytosis in plasma membrane, cell respiration and ATP synthesis in mitochondria or gene transcription in the nucleus. Alternatively, elementary events may give rise to regenerative waves leading to sustained, global changes associated to long-term events like cell growth, differentiation or death. Thus, the study of the intracellular Ca^{2+} homeostasis is a matter of ion transport across boundaries that requires the use of sophisticated methodologies for recording Ca^{2+}-driven currents (patch-clamp) or measuring the tiny concentrations of Ca^{2+} in the cytosol or the very variable Ca^{2+} concentrations in organelles and/or subcellular environments using live cell imaging and targeted calcium probes with different affinities for Ca^{2+}.

Ca^{2+} pumps and transporters contribute significantly to the maintenance of resting $[Ca^{2+}]_{cyt}$ and to the recovery of basal $[Ca^{2+}]_{cyt}$ after stimulation. However, most increases in $[Ca^{2+}]_{cyt}$, are rather due to activation of Ca^{2+} entry pathways at the plasma membrane and Ca^{2+} release channels at the ER. In the RE, IP_3 receptors and ryanodine receptors are ligand-gated Ca^{2+} channels that mediate Ca^{2+} release from stores. Ca^{2+} release channels induce transient increases in $[Ca^{2+}]_{cyt}$ but their activity may secondarily activate Ca^{2+} channels in plasma membrane that are gated by the filling state of Ca^{2+} stores. In mitochondria, the main Ca^{2+} channel is the so-called mitochondrial Ca^{2+} uniporter (MCU), a Ca^{2+}-activated Ca^{2+} channel recently characterized at the molecular level [1, 2]. Activation of this channel in physiological conditions leads to mitochondrial Ca^{2+} uptake and removal of Ca^{2+} from cytosol. This is due to the fact that mitochondria inner membrane shows a strong mitochondrial potential, negative inside the mitochondrial matrix, thus favoring Ca^{2+} influx into mitochondria provided that cytosolic Ca^{2+} is large enough to activate the MCU [3, 4]. At the plasma membrane there are many different types of Ca^{2+} channels, including receptor-operated and voltage-operated Ca^{2+} channels that are widely expressed in excitable cells (ROCCs and VOCCs) together with voltage-independent channels that are particularly relevant in non-excitable cells. The most important Ca^{2+} entry pathway in non-excitable cells is the store-operated Ca^{2+} entry (SOCE), a Ca^{2+} entry pathway ubiquitous and responsible for the entry of Ca^{2+} after agonist-induced activation of phospholipase C and emptying of intracellular Ca^{2+} stores.

SOCE is activated physiologically after the emptying of the intracellular Ca^{2+} stores induced by physiological agonists producing IP_3 or, pharmacologically, after inhibition of the sarcoplasmic and endoplasmic reticulum Ca^{2+} ATPase (SERCA) pump with thapsigargin or similar SERCA antagonists. SOCE usually remains active until Ca^{2+} stores become filled again. Interestingly, SOCE is also regulated by mitochondria. Mitochondrial control of SOCE is due to the ability of these organelles to take up Ca^{2+}, thus preventing refilling of Ca^{2+} stores and preventing also the Ca^{2+}-dependent inactivation of IP_3 receptors and the own Ca^{2+} release activated channels responsible for SOCE. This mechanism ensures the efficient emptying of

Ca^{2+} stores and SOCE activation and is believed to be critical to maintain Ca^{2+} entry in those signaling pathways in which a sustained activation is required. This is the case, for instance, of the activation of the nuclear factor of activated T (NFAT) cells during the immunological synapsis. In this case, a sustained entry of Ca^{2+} through SOCE is needed to promote IL2 gene expression and the clonal expansion of the activated T cell [5].

For years, the molecular basis of SOCE had remained elusive. However, the molecular players involved in SOCE began to be crack after the discovery of the TRP superfamily of ion channels. Some TRP channels were held during quite some time responsible for SOCE. However, more recently, the protein families STIM and ORAI were described to be the cornerstone of SOCE becoming now fully accepted as responsible for Icrac and SOCE in multiple cell types. At the molecular level, SOCE begins with the emptying of intracellular Ca^{2+} stores. Emptying, in this case, means that Ca^{2+} concentration inside the ER decreases from around 700 μM before stimulation to about 200 μM after agonist-induced Ca^{2+} release, as revealed by ER targeted probes with very low affinity for Ca^{2+} [6]. This "emptying" is detected by a sensor named Stromal Interaction Molecule 1 (STIM1) which, upon dissociation of Ca^{2+} ions from Ca^{2+} binding sites, undergoes oligomerization and its interaction with ORAI1, a Ca^{2+} channel located in specific places of the plasma membrane. This interaction opens Ca^{2+} specific CRAC channels, thus enabling the entry of Ca^{2+} into the cytosol. The whole mechanism is reversed when Ca^{2+} stores become filled again [7–9]. There is another Ca^{2+} sensor at the ER called STIM2, which has a lower affinity for Ca^{2+} and is believed to be activated only after moderate depletion of Ca^{2+} stores [10]. However, its role in SOCE remains controversial. In addition, two other ORAI family members (ORAI2 and ORAI3) may also be involved in SOCE but their role is also poorly known [11]. Finally, other types of channels, including some of the TRP superfamily, particularly TRPC channels may contribute to SOCE as well by forming channel complexes with STIM1 and ORAI1 or simply forming alternative store-operated channels less selective for Ca^{2+} [12, 13].

Other Ca^{2+} entry pathways also widely expressed are now collectively termed as store-independent Ca^{2+} entry (SICE) pathways. They are less known and most of them are characterized only at the functional level but not at the molecular level. They include channels gated by araquidonic acid, diacylglycerol (DAG) and stretch activated channels. Most of these channels may well be mediated by TRP channels and/or channel complexes made thereof. Other chapters in this issue describe in more detail characteristics and molecular basis related to store-independent Ca^{2+} entry pathways.

19.2 Ca^{2+} Entry Remodeling in Cancer

A series of recent reports suggest that changes in intracellular Ca^{2+} homeostasis (remodeling) may be critically involved in various forms of cancer. This is not surprising if we consider that the so many cellular processes exacerbated in the

Table 19.1 Changes in expression levels of voltage-operated Ca^{2+} channels [VOCCs] in tumor cells relative to normal cells or tissue samples

Channel isoform	Type of tumor	Research model	Change	Reference
$Ca_v1.1$	Colorectal	Cell lines and tissue samples from patients	↑ mRNA	[20]
$Ca_v1.2$	Colon	Cell lines and tissue samples from patients	↑ mRNA	[21]
$Ca_v3.1$	Colorectal	Cell lines and tissue samples from patients	↓ mRNA	[22]
	Gastric	Cell lines and tissue samples from patients	↓ mRNA	[22]
$Ca_v3.2$	Prostate	Tissue samples from patients	↑ Protein	[23]

transformed cell such as excessive cell proliferation, migration and invasion capabilities as well as apoptosis resistance and cell survival are regulated by intracellular Ca^{2+} [14–16]. A Ca^{2+} entry pathway that has been implicated in cancer is SOCE [17, 18] which could offer new therapeutic possibilities against cancer as suggested earlier [19]. However, some other Ca^{2+} entry pathways and channels have been also related to tumorogenesis. For example, it has been reported that voltage-gated Ca^{2+} channels (VOCCs) are overexpressed in various types of tumors (Table 19.1).

Thus, epithelial tumors express L-type [21] and T type VOCCs and their inhibition may prevent cell proliferation [24]. It is not clear the role of VOCCs in epithelial cells or even if they may work as channels. For example, plasma depolarization with medium containing a high concentration of K^+, a typical experimental maneuver intended to activate voltage-gated Ca^{2+} entry in Ca^{2+} imaging experiments in excitable cells [25], has no effect in epithelial cells, either normal or tumoral. Accordingly, increased expression of VOCCs in cancer cells may not be related to intracellular Ca^{2+} homeostasis. Expression of other Ca^{2+} channels of the TRP superfamily, particularly canonical TRP channels not so selective for Ca^{2+}, has been reported as well to be altered in different tumors (Table 19.2). Other channels of the same superfamily have been also reported to be either overexpressed or downregulated in different tumors, particularly TRPV4, TRPV6 and TRPM8 in carcinomas and other tumors [34, 37, 38]. The role of these channels in cancer remains unclear but recent evidence suggests a role for some of these channels in cancer hallmarks. For instance, it has been shown that the calcium selective channel TRPV6 is able to translocate to the plasma membrane via Orai1-mediated mechanism controlling cancer cell survival [39]. This expanding issue is being covered by excellent recent reviews [40]. See Table 19.3 for further details on changes in the expression of these channels in different tumors.

More recently, it has proposed an essential role for SOCE and/or its molecular players in tumorigenesis. For example, STIM1 and Orai1 may be critical for migra-

Table 19.2 Changes in expression levels of TRPC channels in tumor cells relative to normal cells or tissue samples

Channel isoform	Type of tumor	Research model	Change	Reference
TRPC1	Breast	Tissue samples from patients	↑ mRNA and protein	[26]
TRPC3	Ovary	Tissue samples from patients	↑ Proteína	[27]
	Breast	Tissue samples from patients	↑ mRNA	[28]
TRPC4	Kidney	Cell lines	↓ mRNA	[29]
TRPC6	Esophagus	Tissue samples from patients	↑ mRNA and protein	[30, 31]
	Gastric	Tissue samples from patients	↑ mRNA and protein	[32]
	Glioma	Tissue samples from patients	↑ mRNA and protein	[33]
	Liver	Tissue samples from patients	↑ mRNA and protein	[34]
	Breast	Tissue samples from patients	↑ mRNA and protein	[26, 28]
		Cell lines and Tissue samples from patients	↑ mRNA and protein	[35]
	Prostate	Cell lines	↑ mRNA	[36]

tion of breast cancer cells and metastasis [27]. STIM1 plays an important role in cell growth and migration in cervical cancer [54]. Indeed STIM1 is overexpressed in 70 % of cervical cancers, which has been associated with an increased risk of metastasis. As a matter of fact, the suppression of STIM1 inhibits human glioblastoma cell proliferation and induces G0/G1 phase arrest [55]. Moreoever, STIM1 and Orai1 mediate CRAC channel activity and are essential for human glioblastoma invasion [56]. Orai1 could be also overexpressed in breast cancer [57]. The entry of Ca^{2+} mediated by Orai1 regulates also proliferation and survival in glioblastoma cells and hepatoma [34].

The role of other molecular players involved in SOCE in tumorogenesis is unknown except for the case of Orai3 that has been proposed to be an estrogen receptor α-regulated Ca^{2+} channel that promotes tumorigenesis [58]. Likewise, Orai3 has been also reported to constitute a native SOCE regulating non-small cell lung adenocarcinoma cell proliferation [59]. Therefore, multiple recent evidence suggests an unexpected role for intracellular Ca^{2+} remodeling, particularly SOCE in cancer [15, 16]. See Table 19.4 for further details on changes in expression of molecular players involved in SOCE in different forms of cancer.

Table 19.3 Changes in expression of TRPV4, TRPV6 and TRPM8 in tumor cells relative to normal cells or tissue samples

Channel isoform	Type of tumor	Research model	Change	Reference
TRPV4	Skin	Tissue samples from patients	↓ mRNA and protein	[41]
	Bladder	Tissue simple and mouse cell line	↓ mRNA and protein	[42]
TRPV6	Breast	Tissue samples from patients	↑ mRNA and protein	[26, 37, 43]
	Prostate	Tissue samples from patients	↑ mRNA	[44, 45]
		Tissue samples from patients	↑ Proteína	[37]
	Thyroid	Muestras de tejido de pacientes	↑ Proteína	[37]
	Colon	Tissue samples from patients	↑ Proteína	[37]
	Ovary	Tissue samples from patients	↑ Proteína	[37]
	Lung	Tissue samples from patients	↓ mRNA and protein	[46]
TRPM8	Pancreas	Cell lines and tissue samples from patients	↑ mRNA and protein	[47]
	Prostate	Tissue samples from patients	↑ mRNA and protein	[48–51]
	Androgen-independent prostate tumor	Tissue samples from patients	↓ mRNA	[51]
	Breast	Tissue samples from patients	↑ mRNA and protein	[26, 48]
		Cell lines and tissue samples from patients	↑ mRNA and protein	[52]
		Tissue samples from patients	↑ mRNA and protein	[48]
		Tissue samples from patients	↑ mRNA	[48]
		Cell lines and tissue samples from patients	↑ Protein	[53]
	Colorectal	Tissue samples from patients	↑ mRNA	[48]
	Lung	Tissue samples from patients	↑ mRNA	[48]

Table 19.4 Changes in expression of ORAI and STIM family members in tumor cells relative to normal cells or tissues

Channel isoform	Type of tumor	Research model	Change	Reference
ORAI1	Breast	Cell lines	↑ mRNA	[57]
	Glioblastoma	Cell lines and tissue samples from patients	↑ mRNA	[56]
	Glioma	Tissue samples from patients	↑ Protein	[60]
	Melanoma	Cell lines	↑ Protein	[61]
	Kidney	Tissue samples from patients	↑ Protein	[62]
ORAI3	Breast	Cell lines and tissue samples from patients	↑ mRNA	[63]
	Prostate	Tissue samples from patients	↓ mRNA	[64]
		Tissue samples from patients	↑ mRNA	[65]
STIM1	Glioblastoma	Tissue samples from patients	↑ mRNA	[66]
	Cervix	Tissue samples from patients	↑ protein	[54]
	Breast	Cell lines	↑ mRNA	[57]
	Lung	Tissue samples from patients	↑ protein	[55]
	Liver	Cell lines and Tissue samples from patients	↑ mRNA	[27]
	Melanoma	Cell lines	↑ protein	[61]
	Colon	Tissue samples from patients	↑ mRNA and protein	[21]
STIM2	Glioblastoma	Tissue samples from patients	↑ mRNA	[67]
	Colon	Tissue samples from patients	↑ mRNA	[68]

19.3 Ca^{2+} Entry Pathways in Normal and Colon Cancer Cells

We have recently reported a deep remodeling of SOCE in colorectal cancer [69]. For investigating Ca^{2+} remodeling in colon cancer we have compared Ca^{2+} entry pathways in a series of normal human colonic mucosa cell lines and human colon adenocarcinoma cells. We found that SOCE is significantly larger in colorectal cancer cells than in normal cells [69]. Both normal and tumor cells differed also in their rate of cell proliferation with tumor cells showing always larger rates of cell proliferation. Interestingly, there was a clear correlation between SOCE and the rate of cell proliferation suggesting that the larger rate of cell proliferation of tumor cells follows changes in SOCE [69]. Consistently, SOCE inhibition with antagonists prevents not only tumor cell proliferation but also cell invasion as tested by Matrigel invasion assays. Therefore, enhancement of SOCE in colon cancer cells may contribute not only to increased cell proliferation characteristic of tumor cells but also to cell invasion, both being critical hallmarks of cancer [69].

Differences between normal and tumor cells regarding Ca^{2+} entry and Ca^{2+} release induced by physiological agonists were also studied in detail. ATP and

Carbachol, two physiological agonists that activate G protein-coupled receptors and phospholipase C, increase $[Ca^{2+}]_{cyt}$ in both normal and tumor cells. However, the rises in $[Ca^{2+}]_{cyt}$ are much larger in tumor cells compared with normal colonic epithelium cells. Analysis in Ca^{2+} free medium revealed that the two agonists released more Ca^{2+} in tumor cells than in normal cells. Results on agonist-induced Ca^{2+} entry were surprising: In fact, both agonists induced Ca^{2+} entry only in tumor cells but not in normal cells, despite that both released Ca^{2+} from intracellular stores. To avoid contribution of possible differences in expression of GPCRs in normal and tumor cells, experiments were carried out using caged IP_3. Again, the IP_3-induced increase of $[Ca^{2+}]_{cyt}$ was larger in tumor cells than normal cells [69]. However, these results could be due to either differences in the level of expression and/or activity of IP_3 receptors and/or to differences in the extent of Ca^{2+} store content. Experiments using ionomycin or cyclopiazonic acid in Ca^{2+} free medium revealed that Ca^{2+} stores were paradoxically larger in normal cells than in tumor cells. In other words, Ca^{2+} stores in tumor cells are partially empty. These data together with the data derived form Ca^{2+} release experiments, always much larger in tumor cells, suggest that in tumor cells Ca^{2+} stores are nearly depleted and any minimal stimulation is sufficient to reach the threshold for SOCE activation. However, in normal cells, where Ca^{2+} stores are full and physiological stimulation releases only a limited amount of Ca^{2+}, SOCE threshold is beyond reach leading to no Ca^{2+} entry. These data may explain why agonists do activate Ca^{2+} entry only in tumor cells but not in normal mucosa cells [69]. Of course, we have to take into account that these data derive from the analysis of a few, non isogenic cell lines serving as models of normal and tumor colon cells. Results must be confirmed in additional normal and tumor samples derived from the same specimen.

19.4 Store-Operated Currents in Normal and Colon Cancer Cells

To identify functional and pharmacologically SOCs in normal and tumor cells we have used planar patch-clamp in the voltage clamp configuration [69]. The depletion of Ca^{2+} stores with thapsigargin in normal colonocytes induces a small, voltage-independent, inward rectifying current that is highly selective for Ca^{2+} and sensitive to La^{3+} and to low concentrations of 2-APB [69]. Therefore, this current is very similar, if not identical, to the Icrac current originally described as responsible for SOCE in mast and T cells. In tumor cells, however, SOCs are very different. Emptying of Ca^{2+} stores induces two types of currents: First, a current similar to Icrac recorded in normal cells with the only exception that current density is larger in tumor cells. Second, we observe a different Isoc, absent in normal cells, with a large outward component, not selective for Ca^{2+} and sensitive to high concentrations of 2-APB [69]. The data suggest that SOCE in normal cells is mediated by Orai1 and STIM1, the molecular players previously reported to be involved in Icrac in

mast and T cells. However, tumor cells would have two streams, one should be Icrac but with larger current density probably mediated by changes in expression of Orai1 and Stim1. The other current could be mediated by a TRP channel. Accordingly, data suggest that store-operated currents may be mediated by different channels in normal and tumor cells.

19.5 Molecular Basis of Remodeling of Ca^{2+} Entry Pathways in Colorectal Cancer

Molecular candidates involved in SOCE and SOCs in normal and colon cancer cells have been investigated using conventional and quantitative RT-PCR. All members of the STIM and Orai families (Orai1, 2, 3 and STIM1 and 2) are expressed in normal and tumor cells. Other TRPC channels including TRPC1 and TRPC4 channels are expressed as well in both normal and tumor cells. However, other genes that have been related to SOCE are expressed only in normal cells but not in tumor cells including TRPV6 and TRPM8 suggesting a loss of function during tumorogenesis. Finally, some other related channels are missing in both cell types. Quantitative RT-PCR and Western blotting show significant increases in the expression of many of the above genes. Interestingly, the expression pattern of the different molecular players involved in SOCE and SOCs is roughly similar in normal and tumor cells except that most genes are rather increased in tumor cells relative to normal cells. At the protein level, Orai1, Orai2, and Orai3 proteins are increased significantly in tumor cells. Similar results are obtained with TRPC1 and STIM1. Surprisingly, although STIM2 gene expression is increased in tumor cells, STIM2 protein is nearly lost in tumor cells [69].

Using siRNA against each of these specific molecular players, we have established that SOCE and SOCs in normal cells are mediated by the interactions between ORAI1, STIM1 and STIM2. However, in tumor cells, SOCs and SOCE are more complex and include not only STIM1 and Orai1 but also TRPC1. The increased expression of both may contribute to explain the increase in SOCE and agonist-induced Ca^{2+} observed in tumor cells relative to normal cells (Fig. 19.1).

Therefore, SOCs in colon carcinoma cells are made of different molecular players than in colon normal mucosa cells. Differences involve likely changes in channel complexes including the appearance of TRPC1 and the disappearance of STIM2 from those complexes. This view is supported by silencing experiments. TRPC1 silencing has no influence on Icrac in normal cells but it decreases not only the outward component associated to the non-selective current present only in tumor cells but also the inward component. Moreover, ORAI1 silencing decreases Icrac in normal cells and both components inward and outward in tumor cells. Thus, these data suggest strongly that ORAI1 and TRPC1 form a channel complex in tumor cells but not in normal cells. The data invite speculation as to what is the role played by TRPC1 in SOCE. Silencing experiments reveal that TRPC1 knockdown does not

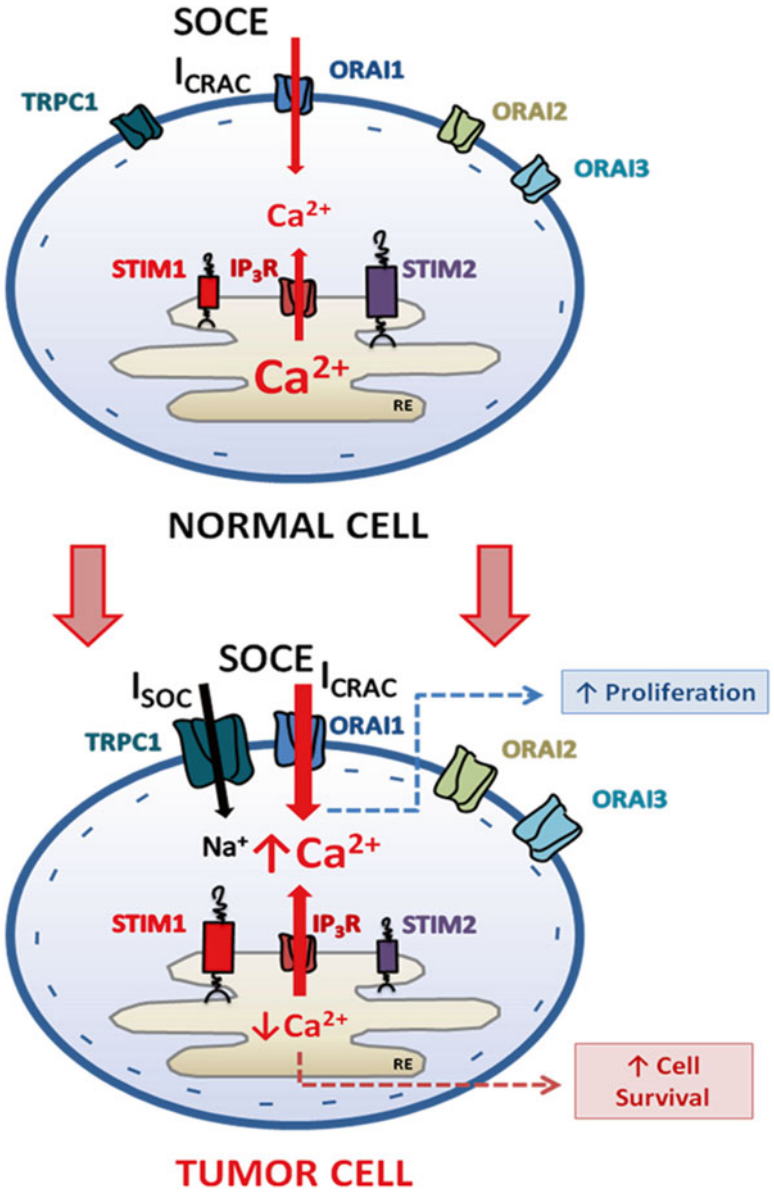

Fig. 19.1 Ca^{2+} entry remodeling in colorectal cancer. Normal human colon cells (*above*) show a small SOCE mediated Orai1 and STIM1 and a large Ca^{2+} store content associated with the large expression of STIM2. The cells also express TRPC1, TRPC4, TRPC7, TRPV6 and TRPM8. Cells of human colon adenocarcinoma (*below*) present large and modified SOCE mediated by increases in expression of Orai1, TRPC1 and STIM1, and partial depletion tank contents mediated decreased expression of STIM2. These cells lack TRPC7, TRPV6 and TRPM8. Remodeling may contribute to increased proliferative capacity, invasion and survival of tumor cells [69]

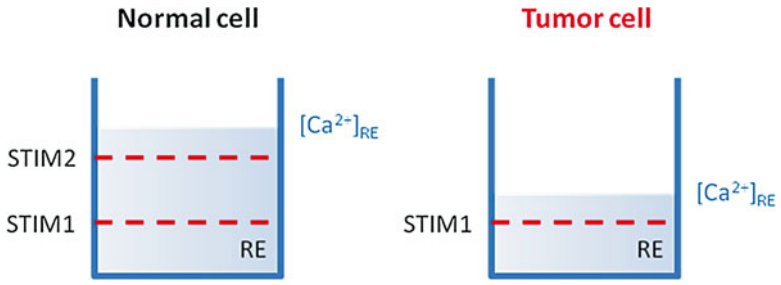

Fig. 19.2 Loss of Stim2 in tumor cells decreases Ca^{2+} store content in tumor cells. Normal cells express both Stim1 and Stim2, ER Ca^{2+} sensors with different affinities for Ca^{2+}. Loss of Stim2 in colon cancer cells leaves Stim1 as the only sensor available that refills Ca^{2+} stores poorly leading to partially depleted stores in tumor cells

decrease SOCE induced by thapsigargin in tumor cells. Therefore, TRPC1 might play roles different from supporting Ca^{2+} entry in tumor cells. Since this channel permeates mostly Na^{+}, a possible role could be to depolarize plasma membrane and limit Ca^{2+} entry. Alternatively, TRPC1 may be involved in changes in cell volume related to cell cycle.

What is the biological significance of STIM2 loss in tumor cells? STIM2 is a Ca^{2+} sensor inside the ER with low affinity for Ca^{2+}. This means that STIM2 should sense Ca^{2+} concentrations inside the ER when Ca^{2+} stores are filled at around >500 μM. A little decrease below this value should activate STIM2 and likely SOCE to keep the store filled. Consistently with this view, STIM2 knockout in normal cells decreases SOCE. In tumor cells, the loss of STIM2, leaves STIM1 in charge of sensing and refilling the stores. However, STIM1 is a different sensor with lower affinity for Ca^{2+} than STIM2 (Kd values around 300 μM). In this scenario, in which STIM1 would became activated only if stores are substantially depleted, the loss of STIM2 should lead to a partial depletion of Ca^{2+} stores (Fig. 19.2).

The above possibility was tested experimentally using siRNA against STIM2 in normal cells [69]. The decrease in STIM2 expression leads to the partial depletion of Ca^{2+} stores suggesting that STIM2 loss in tumor cells may contribute to Ca^{2+} remodeling by modifying Ca^{2+} store content. This partial depletion may have two relevant functional consequences. First, it may move Ca^{2+} store content close to the threshold for SOCE activation. Second, as Ca^{2+} store content has been related to the intrinsic pathway for cell death, loss of STIM2 in tumor cells may favor resistance to cell death and cell survival. Consistently, STIM2 silencing in normal cells decreases Ca^{2+} store content and increases resistance to apoptosis induced by H_2O_2 [69]. Therefore, the reciprocal shift in STIM1/STIM2 observed in colon cancer cells may be critical in colorectal tumorogenesis. Consistently with this view, it has been recently reported that STIM1 overexpression promotes colorectal cancer progression, cell motility and COX-2 expression [36].

In summary, colon cancer cells display enhanced and modified store and agonist-induced Ca^{2+} entry together with enhanced agonist-induced Ca^{2+} release and decreased Ca^{2+} store content. These differences are likely mediated by reciprocal

changes in the expression of ORAI1, STIM1, TRPC1 and STIM2. These changes contribute to increased cell proliferation, invasion and survival. Therefore, although the above results must be confirmed in samples from colorectal cancer patients, the data strongly suggest a critical role for changes in SOCE and SOCs in colon cancer tumorigenesis. In fact, with the logical limitations on the role of SOCE in the immune response against tumors, SOCE antagonists could be considered for colon cancer. Previous results are consistent with this view. For example, it has been extensively documented in in vitro assays, animal testing and even clinical trials with high risk patients that aspirin may efficiently prevent colon cancer. Interestingly, our previous results clearly showed that the main aspirin metabolite salicylate inhibits SOCE off site and colon cancer cell growth in a mitochondria-dependent manner suggesting that aspirin may prevent colon cancer acting on SOCE [70].

19.6 Aspirin Prevents Cancer

A large series of epidemiological evidences suggest that aspirin and other non-steroidal anti-inflammatory drugs (NSAIDs) prevent colorectal cancer and other forms of cancer including breast cancer [71]. A recent meta-analysis showed that NSAIDs decreased the frequency of adenomas, colorectal cancer and deaths related to colorectal cancer in 57 out of the 59 studies carried out between 1988 and 2006 [72]. Yet, the lack of clinical trials and the risk of secondary effects associated to chronic NSAID use prevented recommendation of aspirin. Recently, a few clinical trials have been completed in high risk patients including Lynch syndrome patients, a type familial colorectal cancer with 100 % chances of developing colorectal cancer. Aspirin protected largely (63 %) against cancer in these high risk patients which has resulted in recommendation of aspirin for high risk patients of colorectal cancer [73]. Ongoing clinical trials suggest that combinations of aspirin or other NSAIDs with additional chemopreventive compounds may be highly efficient in preventing polyp formation and cancer death in patients that had undergone surgery for tumor removal. These are high risk patients with a 50 % chance of recurrence and death. Similar trials are underway in other forms of familial cancer including breast cancer associated to driving mutations in BRCA1 and 2.

Basic studies carried out in cell lines and animal models of cancer indicate that aspirin and other NSAIDs inhibit tumor cell proliferation and growth, cell migration and invasion and tumor growth in animal models of cancer. Interest on the action mechanism is growing since the realization that a large part of the effects are largely independent of the anti-inflammatory activity of these drugs. This view is based in that antitumor activity remains in tumor cells lacking expression of COX, the classic target of anti-inflammatory compounds. Moreover, structural analogues like R-flurbiprofen that lack ant-inflammatory activity are also efficient in preventing tumor cell growth. Therefore, even though COX-mediated synthesis or prostanoids may contribute to inflammation and colon tumorogenesis and NSAIDs may act partially by preventing inflammation, other targets of aspirin and other NSAIDs

are likely involved in the antitumor actions of these drugs. We and others have shown previously that the aspirin metabolite and other NSAIDs may inhibit SOCs and SOCE in colon cancer cells likely providing a candidate mechanism for cancer chemoprevention by these compounds and a novel target for cancer chemoprevention.

19.7 Aspirin and Other NSAIDs Inhibit SOCE

NSAIDs inhibit SOCE and colon cancer cell proliferation [74]. However, no mechanism of action was provided. A few years ago, our group proposed that the main metabolite of aspirin, salicylate, could prevent tumor cell growth by inhibiting SOCE off site in a mitochondria-dependent manner [70, 75]. Salicylate is a mild mitochondrial uncoupler. This is due to the fact that the negative charge of the carboxylic residue is delocalized throughout the aromatic ring of salicylate. Thus, salicylic acid is neutral and capable of entering the cell and the mitochondrial matrix down a chemical gradient without restriction. However, once inside mitochondria, the matrix pH favors salicylic acid dissociation leading to salicylate formation that can exit the matrix favored by the negative mitochondrial potential. The net result is the release of protons inside mitochondria and mitochondrial uncoupling, partially depolarizing mitochondria and limiting the electromotive force for mitochondrial Ca^{2+} uptake [70]. Inasmuch as SOCE is strongly regulated by mitochondria, salicylate effect promotes the Ca^{2+} -dependent inactivation of Icrac and inhibition of SOCE. Since a sustained SOCE pathway is required for cell proliferation, the result is that salicylate inhibits cell proliferation via inactivation of a cancer-relevant Ca^{2+} channel. This mechanism may contribute to explain the antiproliferative effects of aspirin on T lymphocytes and vascular smooth muscle cells [70, 76, 77]. Interestingly, it has been recently reported that STIM1 overexpression promotes colorectal cancer progression, cell motility and COX-2 expression [36]. Accordingly, the effects of NSAIDs on SOCE may inhibit also colorectal cancer progression by preventing SOCE-mediated expression of COX-2.

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