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Physiological roles of STIM1 and Orai1 homologs and CRAC channels in the genetic model organism *Caenorhabditis elegans*

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Summary

The nematode *Caenorhabditis elegans* provides numerous experimental advantages for developing an integrative molecular understanding of physiological processes and has proven to be a valuable model for characterizing Ca^{2+} signaling mechanisms. This review will focus on the role of Ca^{2+} release activated Ca^{2+} (CRAC) channel activity in function of the worm gonad and intestine. Inositol 1,4,5-trisphosphate (IP_3)-dependent oscillatory Ca^{2+} signaling regulates contractile activity of the gonad and rhythmic posterior body wall muscle contraction (pBoc) required for ovulation and defecation, respectively. The *C. elegans* genome contains a single homolog of both STIM1 and Orai1, proteins required for CRAC channel function in mammalian and *Drosophila* cells. *C. elegans* STIM-1 and ORAI-1 are coexpressed in the worm gonad and intestine and give rise to robust CRAC channel activity when coexpressed in HEK293 cells. STIM-1 or ORAI-1 knockdown causes complete sterility demonstrating that the genes are essential components of gonad Ca^{2+} signaling. Knockdown of either protein dramatically inhibits intestinal cell CRAC channel activity, but surprisingly has no effect on pBoc, intestinal Ca^{2+} oscillations or intestinal ER Ca^{2+} store homeostasis. CRAC channels thus do not play obligate roles in all IP_3 -dependent signaling processes in *C. elegans*. Instead, we suggest that CRAC channels carry out highly specialized and cell specific signaling roles and that they may function as a failsafe mechanism to prevent Ca^{2+} store depletion under pathophysiological and stress conditions.

1. Introduction

Non-mammalian model organisms such as *E. coli*, *Saccharomyces*, *Caenorhabditis elegans*, *Drosophila*, zebrafish and the plant *Arabidopsis* provide numerous experimental advantages for defining the molecular bases of complex physiological processes. *C. elegans* provides a particularly striking example of the experimental utility of non-mammalian model organisms [1;2]. Worms have a short life cycle, produce large numbers of offspring by sexual reproduction and can be cultured easily and inexpensively in the laboratory. Sexual reproduction occurs by self-fertilization in hermaphrodites or by mating with males. The reproductive and laboratory culture characteristics of *C. elegans* make it an exceptionally powerful model system for forward genetic analysis.

In addition to forward genetic tractability, *C. elegans* also has a fully sequenced and well-annotated genome. Genomic sequence and virtually all other biological data on this organism

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are assembled in readily accessible public databases (e.g., WormBase; <http://www.wormbase.org>). Numerous reagents including mutant worm strains and cosmid and YAC clones spanning the genome are freely available through public resources. Creation of transgenic worms is relatively easy, inexpensive and rapid. *C. elegans* gene expression can be specifically and potently targeted for knockdown using RNA interference (RNAi). Finally, *C. elegans* is a highly differentiated animal but is comprised of <1000 somatic cells. This relatively simple anatomy greatly facilitates the study of complex physiological processes.

2. IP₃-dependent Ca²⁺ signaling processes in *C. elegans*

C. elegans has proven to be a valuable model for characterizing Ca²⁺ signaling mechanisms that control diverse physiological processes (reviewed in [3]). For the purpose of this review, we will focus on IP₃-dependent Ca²⁺ signaling in the worm gonad and intestine, and on the role of Ca²⁺ release activated Ca²⁺ (CRAC) channel activity in these signaling pathways.

2.1 Gonad function

The gonad of adult hermaphrodite worms consists of two identical U-shaped arms connected via spermatheca to a common uterus [4;5](Figure 1A). Gonad arms are surrounded by thin, smooth muscle-like myoepithelial sheath cells. The distal portion of each arm contains germline nuclei that differentiate into either sperm or oocytes.

During the fourth larval stage, germline nuclei develop into sperm that are stored in the spermatheca (Figure 1A). In adult worms, germline nuclei differentiate into oocytes. Newly formed oocytes undergo oogenesis, which is a period of intense biosynthetic activity and rapid and massive growth. Oocytes accumulate in the proximal gonad arm in a single-file row of graded developmental stages. These oocytes remain in diakinesis of prophase I of the meiotic cell cycle until they reach the most proximal position in the gonad arm. During the late stage of oogenesis, an oocyte located immediately adjacent to the spermatheca re-enters the meiotic cell cycle, a process termed meiotic maturation (Figure 1A). Within 5-6 min after maturation is initiated, the oocyte is ovulated into the spermatheca where it is fertilized.

Prior to ovulation, sheath cells contract weakly at a basal rate of 7-8 contractions/min. Basal sheath contractions are triggered by release of major sperm protein (MSP) from sperm stored in the spermatheca. MSP also triggers meiotic maturation in the most proximally located oocyte [6]. The EGF-like protein LIN-3 is released from the maturing oocyte and binds to its receptor LET-23 located on sheath and spermatheca cells. This in turn activates PLC γ and the resulting IP₃ and Ca²⁺ signals induce ovulation by increasing the rate and force of sheath cell contractions and by triggering opening of the distal spermatheca [7-12] (Figure 1B).

2.2 Defecation rhythm

Defecation in *C. elegans* is a rhythmic process that occurs every 45-50 sec with little variation while the animal is feeding [13;14]. Defecation is mediated by sequential contraction of the posterior body wall muscles, anterior body wall muscles and enteric muscles.

In an effort to identify genes that regulate rhythmic biological processes, Iwasaki et al. [15] mutagenized worms and screened for animals with abnormal defecation cycles. Twelve *dec* (**d**efecation **c**ycle **d**efective) genes were identified that when mutated cause the cycle to slow down or speed up. *dec-4* encodes the single *C. elegans* IP₃ receptor [16]. This gene is also termed *itr-1* (**i**nositol **t**risphosphate **r**eceptor). Loss-of-function mutations in *itr-1* slow or eliminate the defecation cycle [16-18] whereas overexpression of the gene increases defecation rate [16].

Dal Santo et al. [16] microinjected fura-2 into intestinal epithelial cells and demonstrated that an intracellular Ca^{2+} spike occurs just prior to the initiation of posterior body wall muscle contraction (pBoc). Calcium oscillations are slowed or absent in animals with loss-of-function mutations in *itr-1*. Rhythmic, IP_3 -dependent intestinal Ca^{2+} oscillations are also observed in transgenic worms expressing cameleon protein in the intestine and in isolated intestines loaded with Ca^{2+} sensitive fluorescent probes [17;18]. Dal Santo et al. [16] have proposed that IP_3 -dependent Ca^{2+} oscillations in intestinal cells control the secretion of a signal that regulates contraction of the posterior body wall muscles that initiate the defecation cycle (Figure 2).

3. Identification of a CRAC-like current in *C. elegans* intestinal cells

Our interest in the *C. elegans* intestine began with the recognition that it could potentially provide a powerful experimental system in which to develop an integrated genetic and molecular understanding of an oscillatory Ca^{2+} signaling pathway. Patch clamp electrophysiology is an important tool for defining the role of ion channels in generating Ca^{2+} oscillations. However, electrophysiological characterization of somatic cells in *C. elegans* is difficult due to the small size of the animal and the presence of a tough, pressurized cuticle that limits access. To circumvent this problem, we developed methods for primary culture of nematode embryo cells [19;20]. When placed in culture, embryo cells undergo terminal differentiation within 24 h. Individual cell types in culture are identified by isolating embryo cells from transgenic worms expressing cell specific GFP reporters.

Cultured intestinal cells express two Ca^{2+} currents [21]. One of these currents, ORCa (Outwardly Rectifying Calcium), displays strong outward rectification, inhibition by intracellular Mg^{2+} , and insensitivity to intracellular Ca^{2+} store depletion. I_{ORCa} resembles the Mg^{2+} -inhibited current (MIC), Mg^{2+} -nucleotide regulated metal ion current (MagNUM), and TRPM7 currents studied in mammalian cells (e.g., [22-25]).

C. elegans intestinal cells also express a store-operated Ca^{2+} channel (SOCC) current [21]. SOCC shares many characteristics with vertebrate and *Drosophila* CRAC channels (reviewed in [26]). These characteristics include activation by passive and active depletion of endoplasmic reticulum (ER) Ca^{2+} stores, strong inward rectification, high selectivity for Ca^{2+} over monovalent cations, and lack of voltage dependent gating (Figure 3).

4. STIM1 and Orai1 homologs in *C. elegans*

Two recent discoveries have dramatically advanced our understanding of the molecular basis of CRAC channel function and store-operated Ca^{2+} entry (SOCE). RNA interference screening in *Drosophila* S2 cells first identified stromal interaction molecule 1 (STIM1) as an essential component of CRAC channel activation [27]. Studies from several laboratories have established that *Drosophila* and human STIM1 homologs function as ER Ca^{2+} sensors [28-31]. In response to Ca^{2+} store depletion, STIM1 undergoes redistribution from a diffuse ER localization to a punctate localization [28;30;32-34] that corresponds to sites of ER-plasma membrane contact [32]. This redistribution in turn activates CRAC channels and SOCE [28-32;35]. The sites of punctate STIM1 localization also appear to be sites of localized Ca^{2+} influx and CRAC channel activity [35].

In an elegant study, Feske et al. [36] used linkage analysis and a *Drosophila* S2 cell genome-wide RNAi screen to identify Orai1 as an essential component of CRAC channel activity and as the gene mutated in patients with a rare form of severe combined immunodeficiency syndrome (SCID) (see also [37;38]). Immune cells of these patients lack functional CRAC channels and SOCE. Work from several laboratories has demonstrated that Orai1 homologs are essential components of the CRAC channel and likely function as pore subunits [39-41]. Coexpression of STIM1 and Orai1 homologs dramatically increases SOCE and CRAC channel

activity [39-44]. During SOCE/CRAC channel activation, Orai1 redistributes from a diffuse localization pattern in the plasma membrane and colocalizes with STIM1 puncta [34;35]. Coimmunoprecipitation studies suggest that STIM1 and Orai1 homologs bind to each other directly or through intermediary proteins [39;40]. Together, these observations have led to the hypothesis that redistribution and subsequent coassociation of STIM1 and Orai1 homologs in response to ER Ca^{2+} depletion activates CRAC channels and SOCE.

The *C. elegans* genome contains a single homolog of both STIM1 and Orai1 (Figures 4-5). *C. elegans stim-1* encodes a 530 amino acid protein that is most similar to human STIM1 versus STIM2. Human STIM1 and *Drosophila Stim* possess several conserved domains including an N-terminal signal peptide, an EF-hand Ca^{2+} binding motif, a SAM domain, a single predicted transmembrane domain, and a large C-terminal region predicted to encode a coiled-coil domain [45]. These motifs are conserved in *C. elegans* STIM-1 (Figure 4).

C. elegans orai-1 encodes a 293 amino acid protein that shares 34-38% amino acid identity with *Drosophila* and human Orai1 homologs. Sequence alignment of worm, fly and human Orai homologs reveals that the four predicted transmembrane (TM) domains show strong conservation of primary structure in all five proteins. In addition, the predicted intracellular loop between TM2 and TM3 is highly conserved. Glutamate residues located in TM1 and TM3 have recently been shown to play key roles in controlling CRAC channel ion selectivity [39; 40;46] and are fully conserved in worm, fly and human Orai homologs. Mutation of an arginine residue at the beginning of TM1 in human Orai1 is responsible for the loss of CRAC channel activity in lymphocytes of a subset of SCID patients [36]. This residue is conserved in worm and human Orai proteins and exhibits a conserved substitution with lysine in fly Orai (Figure 5).

Mutagenesis studies on *Drosophila* Orai [39] and human Orai1 [40] suggest that 2-3 acidic amino acid residues in the extracellular loop between TM1 and TM2 may function to attract polyvalent cations towards the channel pore and control channel selectivity. Interestingly, only one of these residues is conserved in *C. elegans* ORAI-1 and human Orai2 suggesting that these channels may exhibit selectivity properties distinct from other Orai homologs.

To determine where *stim-1* and *orai-1* are expressed in *C. elegans*, we generated transgenic worms expressing full-length STIM-1 and ORAI-1 proteins fused to either GFP or DsRed. Expression of the transgenes was driven by 1.9 kb and 4 kb of *stim-1* and *orai-1* promoter sequence, respectively. STIM-1 and ORAI-1 reporters are coexpressed in the intestine, gonad sheath cells and spermatheca. ORAI-1 appears to be localized primarily to the plasma membrane and/or a submembrane region while STIM-1 is expressed largely in an intracellular compartment [47].

5. *C. elegans* STIM-1 and ORAI-1 reconstitute CRAC channel activity

Coexpression of STIM1 and Orai1 homologs dramatically increases SOCE and CRAC channel activity in cultured mammalian and *Drosophila* cells [39-44]. Similarly, coexpression of *C. elegans* STIM-1 and ORAI-1 in HEK293 cells induces large SOCC currents (Figure 6A) [47]. These currents show strong inward rectification, reverse at membrane voltages $>+80$ mV and are not activated by membrane voltage.

The *C. elegans* intestinal cell SOCC current shares many similarities with mammalian I_{CRAC} . However, the current also exhibits some distinct differences. Unlike *Drosophila* and mammalian I_{CRAC} , *C. elegans* I_{SOCC} is not activated by low concentrations of 2-APB, has a relatively high Cs^+ permeability and shows much slower rundown or “depotentialization” when exposed to divalent cation-free (DVF) extracellular solution [21;26]. To determine whether

heterologous expression of STIM-1 and ORAI-1 recapitulates the properties of the endogenous intestinal I_{SOCC} , we characterized 2-APB sensitivity, Cs^+ permeability and depotentiation.

As was observed in cultured intestinal cells [21], low concentrations of 2-APB had no effect on the STIM-1/ORAI-1 induced I_{SOCC} . The effect of DVF bath on whole cell current was complex. Two cells with SOCC current densities of 6-7 pA/pF exhibited the typical response to DVF bath; upon removal of extracellular divalent cations, current amplitude increased rapidly and then underwent slow depotentiation. In one cell, bath Na^+ was successfully replaced with Cs^+ . The calculated Cs^+ permeability relative to Na^+ (i.e., P_{Cs}/P_{Na}) in this cell was 0.7 [47]. Both the rate of depotentiation and relative Cs^+ permeability are remarkably similar to what we have observed previously for the native SOCC expressed in cultured worm intestinal cells [21].

The two cells that exhibited the typical response to DVF bath had relatively low SOCC currents. We refer to these cells as “low current” cells. All other cells in which DVF experiments were performed successfully had current densities of 30-60 pA/pF and we refer to these as “high current” cells. The response to DVF bath in high current cells contrasted sharply with that of low current cells. Upon removal of bath divalent cations, whole cell current in high current cells was rapidly and nearly completely inhibited. Current inhibition was followed by slow current reactivation. The calculated P_{Cs}/P_{Na} for high current cells was 0.3 [47].

Several observations on high current cells suggested the possibility that the anomalous response to DVF bath might be due to high rates of Ca^{2+} influx and intracellular Ca^{2+} dependent channel regulatory mechanisms. Regulation of CRAC channels by intracellular Ca^{2+} concentration has been well described (e.g., [48;49]). To test the possible effects of high rates of Ca^{2+} influx on the response to bath divalent cation removal, cells were bathed in an extracellular solution containing 0.25 mM Ca^{2+} before being exposed to DVF medium. We refer to these cells as “low Ca^{2+} ” cells. In 10 out of 10 low Ca^{2+} cells, exposure to DVF bath caused an immediate increase in whole cell current. This current typically continued to activate slowly and then stabilized. Rapid rundown or “depotentialization” of the current was never observed. The mean P_{Cs}/P_{Na} in low Ca^{2+} cells was 0.2 [47].

To summarize, the ORAI-1/STIM-1 induced Ca^{2+} current has biophysical properties similar to those of the native SOCC current. The ORAI-1/STIM-1 induced SOCC is not activated by 5 μ M 2-APB and has a P_{Cs}/P_{Na} 2-7-fold higher than that reported for mammalian and *Drosophila* CRAC channels. In addition, whole cell currents that showed a normal response to DVF bath underwent depotentiation at a rate remarkably similar to that of the native channel. We conclude from these results and studies on *Drosophila* and human Orai homologs [39-41], that *C. elegans* expresses a bona fide CRAC channel encoded by *orai-1* and regulated by STIM-1.

The anomalous response of high current cells to DVF bath suggests that high rates of Ca^{2+} influx alter CRAC channel function and regulation. The underlying mechanism by which this occurs is unclear at present. However, further study of this phenomenon may shed light on the mechanisms by which both extracellular and intracellular Ca^{2+} regulate CRAC channel activity. Our findings also raise a cautionary note. We are unaware of studies showing that native CRAC channels undergo rapid inhibition in response to DVF extracellular solutions. Thus, it is likely that heterologous overexpression alters channel structure/function relationships and/or regulation. Conclusions drawn from heterologous expression studies on CRAC channel function, and in particular regulation, should be tempered by these concerns.

6. CRAC channels play essential roles in gonad function and fertility

Knockdown of either STIM-1 or ORAI-1 expression in worms using RNAi causes complete sterility. Video microscopy of anesthetized worms demonstrated that the rates of sheath cell contraction before and during ovulation are decreased by *stim-1* or *orai-1* RNAi [47;50]. As described earlier, contraction of sheath cells during ovulation is required to pull the spermatheca over the maturing oocyte.

Reduction in the rate and force of sheath cell contraction is expected to reduce fertility rather than result in complete sterility. The main reason for sterility in *stim-1(RNAi)* and *orai-1(RNAi)* worms is failure of the distal spermatheca to open thereby preventing oocytes from entering the spermatheca for fertilization.

To further examine the role of STIM-1 in gonad function, we mutated aspartate residues at positions 55 and 57 to alanine (i.e., D55A and D57A). These residues are located in the predicted Ca²⁺ binding EF-hand domain. Mutation of the analogous amino acids in *Drosophila* and human STIM1 homologs constitutively activates SOCE [28;30] and I_{CRAC} [29].

Transgenic worms expressing STIM-1(D55A;D57A) are sterile. We did not detect developing embryos or unfertilized oocytes in the uteri of STIM-1(D55A;D57A) worms indicating that the fertility defect is due at least in part to defects in ovulation. Constitutive activation of CRAC channels by the STIM-1 EF-hand mutant likely disrupts cellular Ca²⁺ homeostasis and signaling events, which in turn disrupts the contractile activity of gonad sheath and spermatheca cells. Figure 1B is a schematic diagram showing the IP₃ signaling pathways that regulate sheath cell contraction and opening of the distal spermatheca. The precise role of CRAC channels in this pathway is uncertain, but they could function to refill depleted stores and/or modulate cytoplasmic Ca²⁺ levels.

7. CRAC channels are not required for oscillatory Ca²⁺ signaling or ER Ca²⁺ homeostasis in the worm intestine

As noted above, IP₃-dependent oscillatory Ca²⁺ signaling in intestinal epithelial cells triggers posterior body wall muscle contraction (pBoc) required for defecation. Surprisingly, we found that RNAi knockdown of STIM-1 or ORAI-1 has no effect on pBoc rhythm or the characteristics of intestinal Ca²⁺ oscillations [47;50]. These results could be explained if RNAi was ineffective in silencing *stim-1* and *orai-1* expression. However, a number of experimental observations argue strongly against this possibility. First, STIM-1 or ORAI-1 RNAi inhibits mean CRAC channel current in cultured intestinal cells >90% (Figure 6B). Fluorescence levels in worms expressing STIM-1 or ORAI-1 GFP reporters are reduced >90% or suppressed completely by *stim-1* and *orai-1* RNAi, respectively. Finally, transgenic worms expressing STIM-1(D55A;D57A) exhibit prolonged and arrhythmic pBoc cycles. This defect is fully suppressed by *stim-1* or *orai-1* RNAi [47;50]. The ability of *orai-1* RNAi to suppress the effects of the STIM-1 EF hand mutant on pBoc rhythmicity demonstrates that the two genes function together in common pathway.

Endoplasmic reticulum Ca²⁺ homeostasis is not only important for intracellular Ca²⁺ signaling, but also for proper protein synthesis and folding. Disruption of ER Ca²⁺ homeostasis triggers the unfolded protein response (UPR), an intracellular signaling and transcriptional/translational program activated by the accumulation of unfolded proteins in the ER lumen (reviewed by [51;52]). In mammalian cells, inhibition of the sarcoplasmic/ER Ca²⁺ ATPase (SERCA) with thapsigargin depletes ER Ca²⁺ stores and triggers the UPR (e.g., [53;54]). We

reasoned that if CRAC channels are essential for refilling ER Ca^{2+} stores during oscillatory Ca^{2+} signaling, then inhibition of channel activity should induce the UPR in the worm intestine.

To monitor the intestinal UPR, we utilized a transgenic worm strain expressing an *hsp-4* transcriptional GFP reporter. *hsp-4* encodes a *C. elegans* homolog of the ER chaperone protein GRP78/BiP and is expressed in the worm intestine and induced by ER stress [55]. Exposure of worms for 6 h to 10 $\mu\text{g}/\text{ml}$ of tunicamycin, which induces ER stress, causes a nearly 7-fold increase in intestinal *hsp-4::GFP* expression [50].

To determine whether store Ca^{2+} depletion activates the UPR in *C. elegans*, we knocked down SERCA expression by feeding worms *sca-1* double stranded RNA-producing bacteria for 30 h. *sca-1* encodes the *C. elegans* SERCA homolog [56]. *sca-1* RNAi causes a nearly 3-fold increase in *hsp-4::GFP* expression. In contrast, knockdown of STIM-1 expression by RNAi has no effect on *hsp-4::GFP* expression [50].

Interestingly, knockdown of *sca-1* and *stim-1* together causes a 30-45% increase *hsp-4::GFP* expression compared to *sca-1* alone. Taken together, these results indicate that *C. elegans* CRAC channels are not obligate components of all IP_3 -dependent Ca^{2+} signaling pathways nor are they essential for ER Ca^{2+} homeostasis under normal physiological conditions. However, the effects of combined *sca-1* and *stim-1* RNAi on the UPR suggest that CRAC channels may contribute to the regulation of store Ca^{2+} levels during experimental manipulations that induce extreme store Ca^{2+} depletion.

8. Are CRAC channels essential components of all IP_3 -dependent Ca^{2+} signaling pathways?

Despite their presence in organisms as diverse as roundworms, fruit flies and humans, and their widespread expression in functionally diverse mammalian cell types [57-59], the physiological roles of CRAC channels are largely unknown. It is widely stated in the literature that CRAC channels and SOCE are essential for generation of IP_3 -dependent Ca^{2+} signals and for maintenance of ER Ca^{2+} levels during Ca^{2+} signaling events [57-59], but in most cell types, direct evidence supporting this notion is lacking. Interestingly, in SCID patients with a loss-of-function mutation in Orai1 [36], the only non-immunological defects observed are nonprogressive muscle hypotonia and mild psychomotor and mental retardation [60]. This finding is unexpected if CRAC channels play a ubiquitous and essential role in Ca^{2+} signaling. It is possible that human Orai2 and Orai3 proteins function as SOC channels and compensate for loss-of-function mutations in Orai1. However, *C. elegans* has only a single Orai1 homolog. Our findings that CRAC channels are not required for oscillatory Ca^{2+} signaling and ER Ca^{2+} homeostasis in the worm intestine raise interesting and important questions about store depletion and store-operated Ca^{2+} channels.

Do ER Ca^{2+} stores become sufficiently depleted to activate CRAC channels during physiologically relevant Ca^{2+} signaling events? With the exception of immune cells (reviewed in [61]), CRAC channel and SOCE activation in most cell types has been observed only under conditions of extreme store depletion experimentally induced by SERCA inhibition, supraphysiological IP_3 receptor activation, exposure to high concentrations of ionomycin and/or increases in cytoplasmic Ca^{2+} buffering (e.g., [62-64]). Direct measurements of store Ca^{2+} levels during physiologically relevant Ca^{2+} signaling events are lacking. In the one detailed study conducted to date, little or no change in store Ca^{2+} levels was detected during acetylcholine-induced Ca^{2+} oscillations in pancreatic acinar cells. Only during stimulation with supraphysiological acetylcholine concentrations was store depletion observed [65].

The relationship between store emptying and CRAC channel activation has been examined indirectly in a number of cell types. Many studies suggest that store depletion is not tightly coupled to SOCE whereas other work suggests a more direct coupling (reviewed in [59]). The apparent absence of direct coupling has been taken as indirect evidence that CRAC channels are regulated by specialized ER stores or ER microdomains (e.g., [59;66-68]). If specialized ER Ca^{2+} stores exist, then STIM1 homologs may exhibit a heterogeneous distribution in the ER membrane. It will thus be important to carefully define the intracellular localization of native STIM1 proteins and determine whether they co-localize with other components of the ER Ca^{2+} signaling machinery. In addition, while technically demanding, it will be important to measure store Ca^{2+} levels under conditions that mimic physiologically relevant Ca^{2+} signaling events.

If CRAC channels are not essential components of all Ca^{2+} signaling pathways, why are they so widely observed and why have the channel's functional/structural properties been conserved from worms to humans? One possibility is that they may provide cells with a failsafe mechanism for protecting store Ca^{2+} levels during pathophysiological insults, exposure to cellular stressors and during high rates of protein synthesis. Bacterial toxins [69;70], viral proteins [71;72] ischemia [73] and oxidants [74;75] induce store Ca^{2+} loss and depletion. Two recent studies have shown that SOCE is activated by ER Ca^{2+} loss through the translocon [76;77]. Failure to maintain store Ca^{2+} levels under pathophysiological and stress conditions and during increases in translational activity will disrupt ER protein synthesis, folding and processing, which can cause cell injury and ultimately lead to cell death [51;52]. Consistent with this idea, we observed that *stim-1* RNAi does not trigger a UPR in the intestine indicating that CRAC channels are not required for ER Ca^{2+} homeostasis under normal physiological conditions. However, the intestinal UPR induced by RNAi knockdown of the SERCA encoding gene *sca-1* is increased further when STIM-1 expression is knocked down at the same time [50]. Thus, under conditions of extreme store depletion induced by an unphysiological event (i.e., reduced SERCA expression and activity), *C. elegans* CRAC channels appear to play a role in regulation of store Ca^{2+} levels.

CRAC channels clearly play critical signaling roles in some cell types. An important role for CRAC channels in immune cell signaling is well established (reviewed by [61]) and they are essential for gonad function and fertility in *C. elegans* [47;50]. The functional properties of CRAC channels are likely specialized for certain signaling mechanisms. CRAC channels have a very high Ca^{2+} selectivity and thus will have little effect on membrane potential when they are active and mediating Ca^{2+} influx. While CRAC channels are not gated directly by voltage, membrane potential will alter Ca^{2+} flux by changing the electrical driving force for Ca^{2+} . In addition, CRAC channels have been reported to undergo slow voltage dependent changes in macroscopic conductance (reviewed in [59;61]). Thus, variable patterns of CRAC channel mediated Ca^{2+} influx can be induced by the activity of other plasma membrane ion channels that affect membrane potential. This in turn increases the complexity and information content of Ca^{2+} signals as well as modulates the rate of store refilling.

Calcium influx through CRAC channels appears to occur at discrete membrane locations where STIM1 proteins accumulate in response to store depletion [35]. Such compartmentalization of Ca^{2+} entry likely provides a mechanism for specifically regulating downstream cellular and ER signaling molecules that colocalize with STIM1 proteins and CRAC channels. Regulation of CRAC channel activity by store Ca^{2+} depletion also provides a way to coordinate compartmentalized Ca^{2+} influx with Ca^{2+} efflux through IP_3 receptors and signaling pathways that control levels of IP_3 and associated lipid second messengers.

9. Conclusions and future perspective

The identification of Orai1 and STIM1 proteins has revolutionized our understanding of SOCE. However, numerous important questions remain to be answered. In particular, our studies in *C. elegans* raise several issues. As noted above, little evidence exists linking CRAC channels to the regulation of physiologically relevant cellular processes. It is therefore essential to define the signaling pathways in which CRAC channels normally function. Direct measurement of the dynamics of ER Ca²⁺ levels during physiologically relevant Ca²⁺ signaling events is also needed. CRAC channels are essential for the normal functioning of mammalian immune cells and *C. elegans* gonad cells. Do Ca²⁺ stores in these cells become depleted enough to activate CRAC channels because they have a limited volume and/or because rates of ER Ca²⁺ uptake are slow relative to total store capacity and rates of passive Ca²⁺ leak and efflux through activated IP₃ receptors? It is likely that the functional properties of the ER Ca²⁺ stores are specifically tailored to the signaling requirements of the cell and the role of CRAC channels in those signaling pathways.

Recent studies suggest that STIM1 regulates other types of channels including TRPC1 [78; 79] and the ARC channel [80]. Interestingly, when we generated transgenic worms expressing the STIM-1 EF-hand mutant under the control of the promoter for *let-858*, a gene that functions in most *C. elegans* cell types [81], the animals showed numerous serious defects and survived very poorly (Yan, Lamitina and Strange, unpublished observations). This suggests that CRAC channels function in more cell types than revealed by our GFP reporter studies [47;50]. Alternatively, STIM-1 and/or the EF-hand mutant may have functions in addition to regulating the activity of CRAC channels. The forward and reverse genetic tractability of *C. elegans* may be particularly useful in elucidating the physiological roles and regulation of STIM-1, CRAC channels and SOCE.

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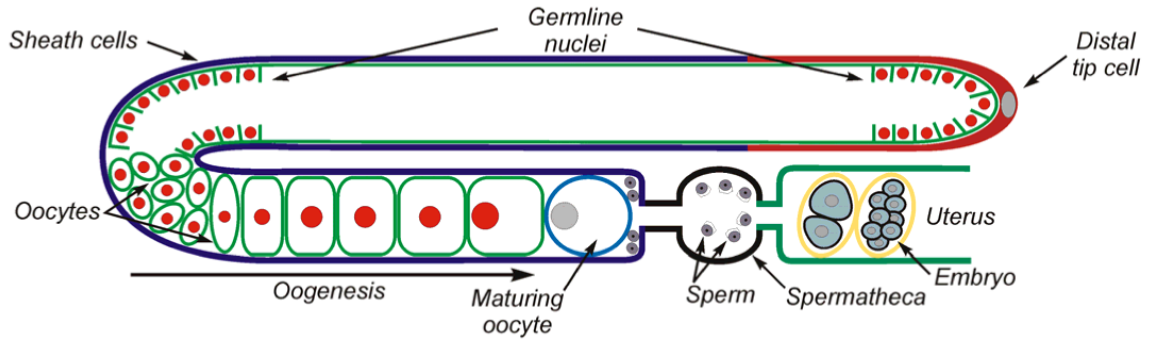
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(A)



(B)

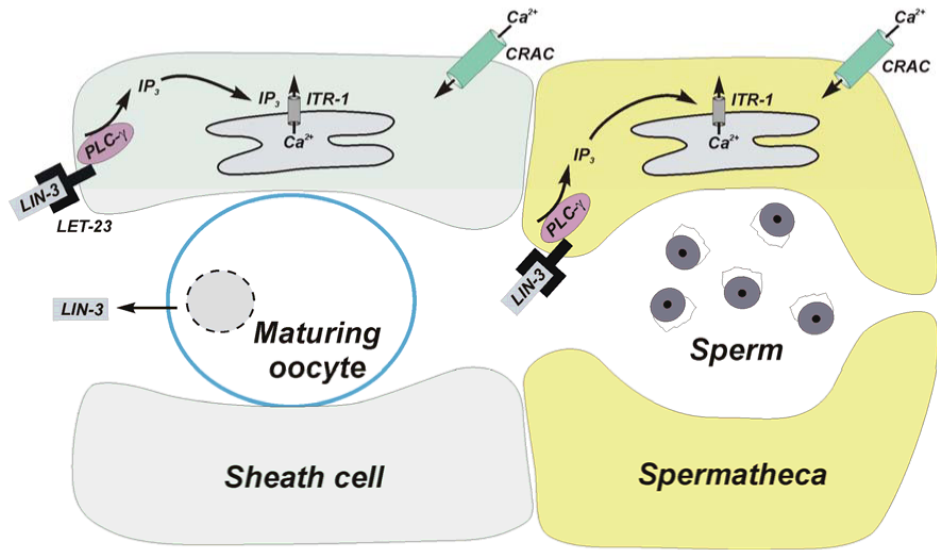


Figure 1. 1,4,5-trisphosphate-dependent Ca^{2+} signaling regulates gonad function and fertility in *C. elegans*. (A) Schematic diagram of the gonad in an adult hermaphrodite worm. (B) Model illustrating IP_3 and Ca^{2+} signaling events that regulate ovulation in *C. elegans*. Oocyte undergoing meiotic maturation releases LIN-3, an EGF-like molecule that binds to the LET-23 receptor expressed in sheath and spermatheca cells. LIN-3/LET-23 binding activates PLC- γ producing IP_3 that in turn activates the IP_3 receptor ITR-1. Release of ER Ca^{2+} and plasma membrane Ca^{2+} influx through CRAC channels encoded by *orai-1* and regulated by *stim-1* trigger sheath cell ovulatory contractions and opening of the gonad-spermatheca valve. Contracting sheath cells pull the spermatheca over the maturing oocyte.

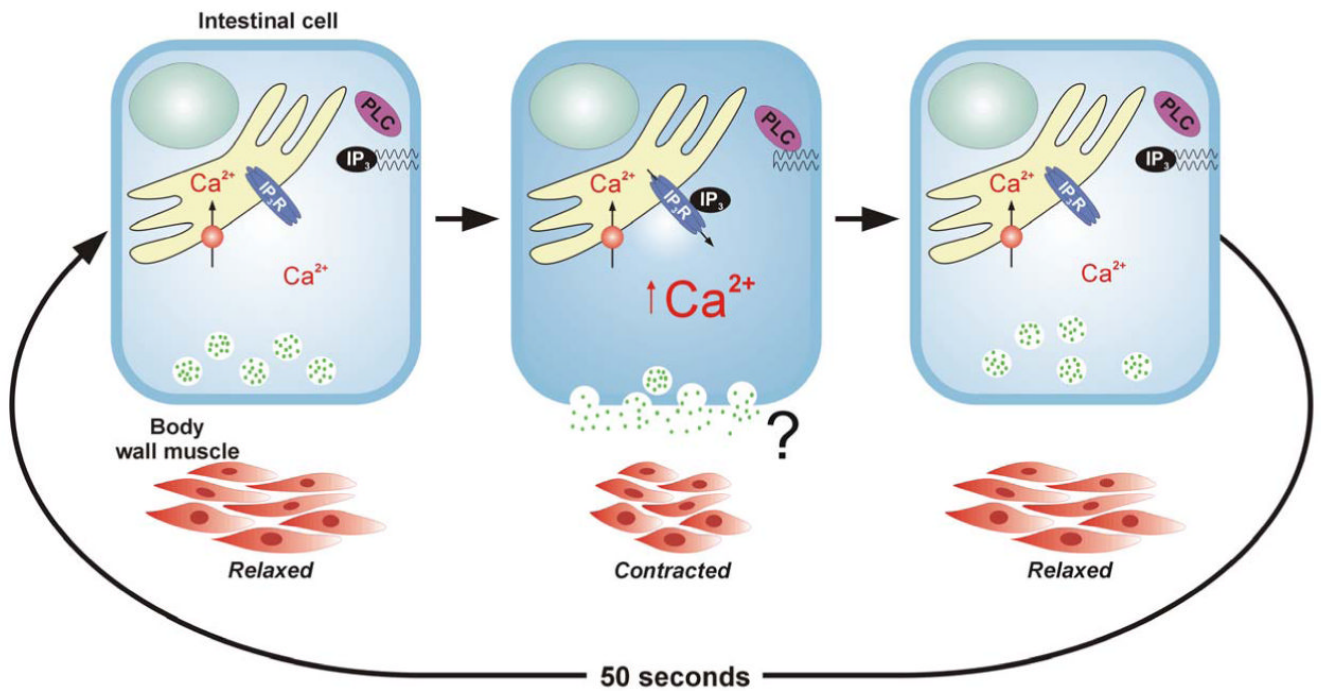


Figure 2. Model illustrating role of intestinal cell IP₃ and intracellular Ca²⁺ in regulating defecation cycle. Cyclical elevation of cytoplasmic Ca²⁺ levels driven in part by IP₃-dependent release of Ca²⁺ from intracellular stores triggers exocytic secretion from intestinal cells of an unidentified messenger that induces posterior body wall muscle contraction (pBoc).

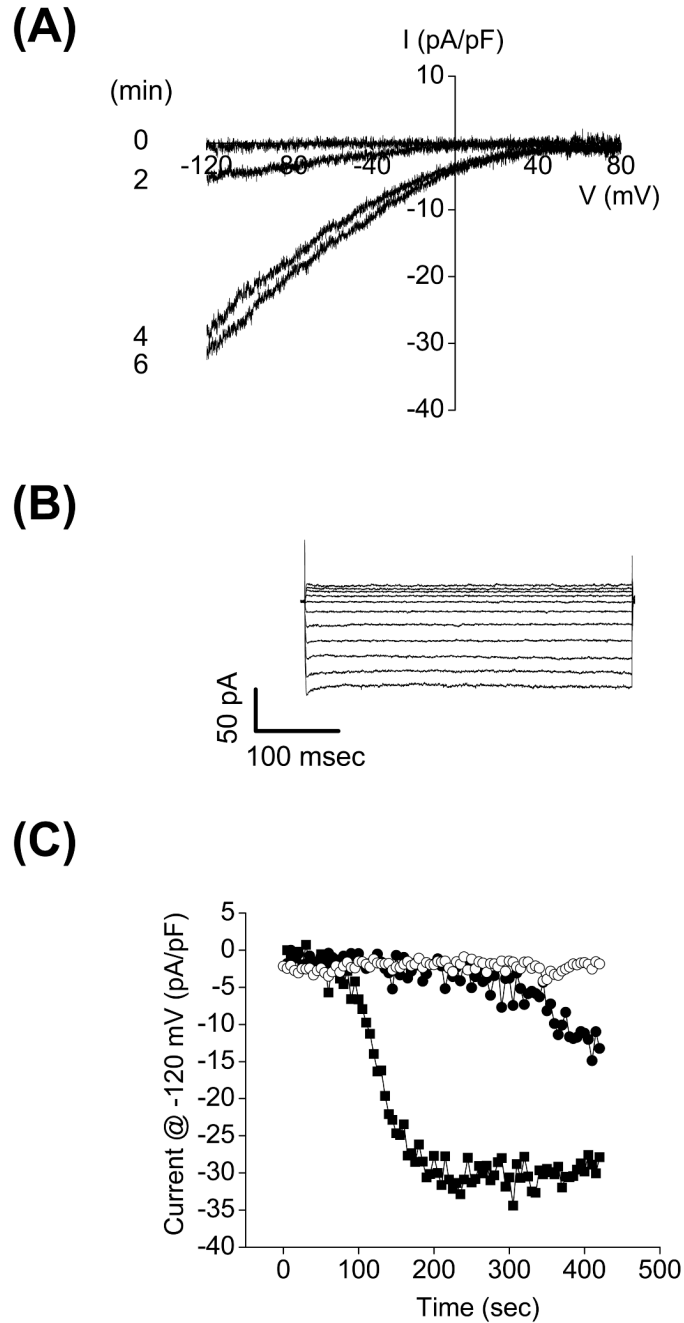


Figure 3.

Activation of CRAC-like current in cultured *C. elegans* intestinal cells. (A) An inwardly rectifying CRAC-like current activates when Ca^{2+} stores are depleted by inclusion of $10 \mu\text{M}$ IP_3 in the patch pipette solution. Currents were elicited by ramping membrane voltage from -120 mV to $+80 \text{ mV}$ at 200 mV/sec every 5 sec . (B) Inwardly rectifying whole-cell currents elicited by stepping membrane voltage from -120 mV to $+80 \text{ mV}$ from a holding potential of 0 mV . Steps were 400 msec in duration. Currents were measured after store depletion induced activation was complete. (C) Changes in whole cell current observed in the absence of store depletion (open circles), during passive store depletion (solid circles), and during active store depletion induced by IP_3 (solid squares).

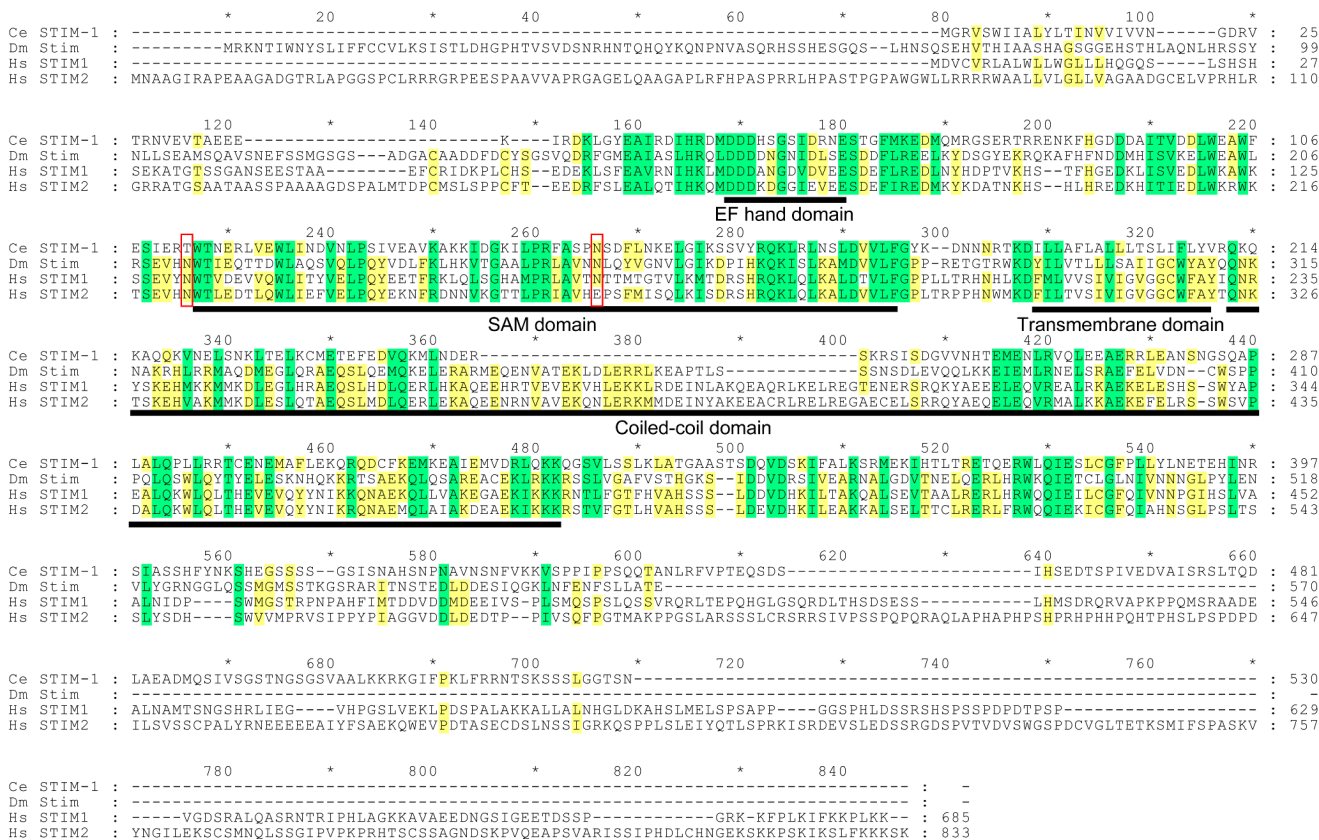


Figure 4. Amino acid sequence alignment of *C. elegans* (Ce) STIM-1 with *Drosophila* (Dm) Stim and human (Hs) STIM1 and STIM2 homologs. Yellow and green shading indicates sequence identity and conserved amino acid substitutions, respectively. Conserved domains are underlined in black. Red boxes show location of N-linked glycosylation sites in human STIM1. Percent similarity and identity of EF-hand, SAM, transmembrane and coiled-coil domains are 58.3% and 50.0%, 50.7% and 37.7%, 27.8% and 5.6%, and 57.4% and 21.3%, respectively. Alignment was performed using Vector NTI software (InforMax, Bethesda, MD). Protein domains were identified by SMART (http://smart.embl-heidelberg.de/smart/change_mode.pl).

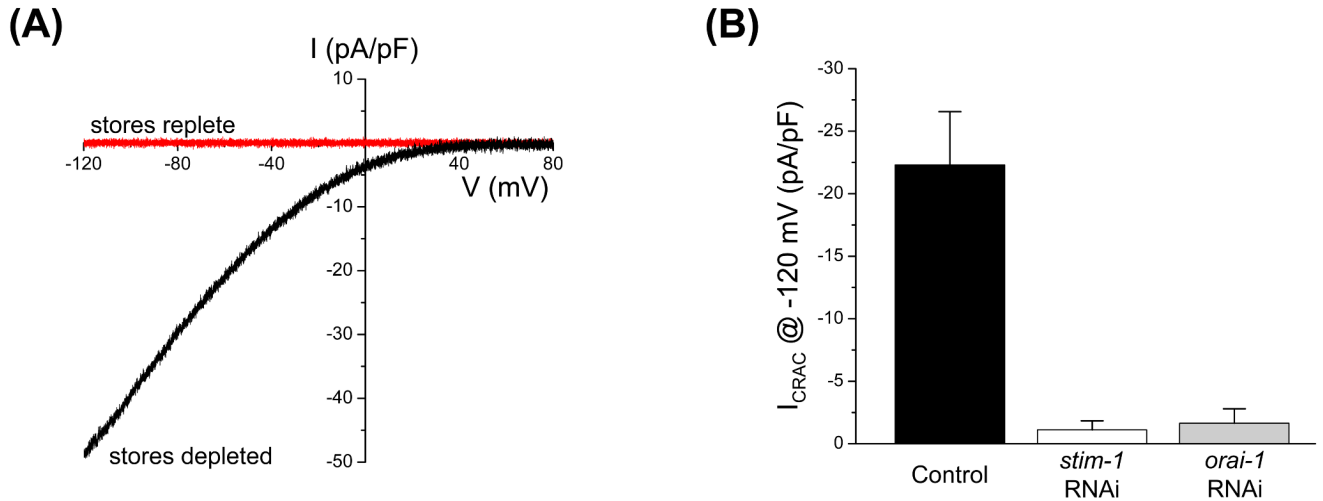


Figure 6.

C. elegans CRAC channel activity is mediated by STIM-1 and ORAI-1. (A) Current-to-voltage relationships of currents observed in a HEK293 with replete stores coexpressing ORAI-1 and STIM-1 and in an ORAI-1/STIM-1 coexpressing HEK293 cell in which stores were depleted. Current-to-voltage relationships were plotted for currents measured 5 min after obtaining whole cell access. Store depletion was induced by dialyzing cells with a pipette solution containing 10 mM BAPTA, 10 μ M IP₃ and ~18 nM free-Ca²⁺. Depletion of stores was prevented using a pipette solution containing 10 mM BAPTA, 2 mM ATP and ~140 nM free-Ca²⁺. (B) Effect of *stim-1* or *orai-1* RNAi on endogenous CRAC channel activity measured in cultured *C. elegans* intestinal cells. Values are means \pm S.E. (n=7-21)