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# The Long and Arduous Road to CRAC

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## Abstract

Store-operated calcium (SOC) entry is the major route of calcium influx in non-excitable cells, especially immune cells. The best characterized store operated current, $I_{CRAC}$, is carried by calcium release activated calcium (CRAC) channels. The existence of the phenomenon of store-operated calcium influx was proposed almost two decades ago. However, in spite of rigorous research by many laboratories, the identity of the key molecules participating in the process has remained a mystery. In all these years, multiple different approaches have been adopted by countless researchers to identify the molecular players in this fundamental process. Along the way many crucial discoveries have been made, some of which have been summarized here. The last couple of years have seen significant breakthroughs in the field–identification of STIM1 as the store $Ca^{2+}$ sensor and CRACM1 (Orai1) as the pore forming subunit of the CRAC channel. The field is now actively engaged in deciphering the gating mechanism of CRAC channels. We summarize here the latest progress in this direction.

Intracellular free $Ca^{2+}$ is crucial for many cellular processes. Although endoplasmic reticulum (ER) serves to store the intracellular pool of $Ca^{2+}$, it is limited in its capacity to store and needs to be refilled when depleted. Depletion of ER $Ca^{2+}$ activates plasma membrane (PM) localized $Ca^{2+}$ influx channels known as store-operated $Ca^{2+}$ channels (SOCs). This fundamental act of store-operated calcium influx is believed to have many performers. Nevertheless, calcium release-activated calcium (CRAC) channels have been universally accepted as the protagonists, especially in the context of immune cells. The biggest challenge, as the act unfolds, has been the identity of the key performers, including the CRAC channels themselves. The 20 year old quest began with the hypothesis of store-operated or capacitative calcium entry by Jim Putney in 1986 [1] and subsequent demonstration of calcium release-activated calcium (CRAC) currents ($I_{CRAC}$) in mast cells by Hoth and Penner in 1992 [2]. Since then many laboratories around the world have been chasing the identity of key players in the act of store-operated calcium influx. Over the years, many genes have been claimed to code for the CRAC channel. Recently, however, two key players have been identified, STIM1, the store $Ca^{2+}$ sensor and CRACM1 (Orai1), the pore forming CRAC channel subunit. The path to identifying CRAC has been long but not fruitless. Along the way, several other genes with important immunological functions have been identified but the curtains to this act have not yet been drawn. Our laboratory joined the hunt almost 15 years ago and we summarize here multiple different approaches that were adopted by us over these years.

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## 1. The antibody strategy: identification of a new mast cell pathway

Immune cell signaling through antigen receptors, the high affinity receptor for immunoglobulin E (Fc ε RI) and the T cell receptor (TCR), results in the coordinated activation of tyrosine kinases that leads to calcium mobilization from intracellular stores. The resulting activation of CRAC channels localized in the plasma membrane (PM) is crucial for proliferation and cytokine secretion in T lymphocytes [3]. Mast cells require sustained $Ca^{2+}$ increase for degranulation of allergic mediators and cytokine secretion in response to Fc ε RI aggregation [4]. Thus, one of the very first approaches adopted by us to identify the CRAC modulators involved raising monoclonal antibodies against mast cell surface molecules and selecting clones that inhibited degranulation. This approach resulted in the identification of two PM-expressed molecules CD81 and CD63, that indeed regulate degranulation but they do so not by modulating calcium influx. CD81 and CD63 are important tetraspanin membrane molecules that form multi-molecular complexes with a broad array of molecules including ECM protein-binding beta integrins. Antibodies recognizing CD81 inhibited Fc epsilon RI-mediated mast cell degranulation without affecting tyrosine phosphorylation, calcium mobilization, or leukotriene synthesis [5]. Similar data were obtained with antibodies against CD63. These results revealed an unsuspected calcium-independent parallel pathway of antigen receptor regulation, which was later identified to be Fyn/Gab2/PI3K dependent and which is critical for both degranulation and adhesion. [6–8]. Both anti-CD63 and anti-CD81 antibodies target the Gab2 pathway to inhibit degranulation while leaving calcium mobilization intact. These findings introduced the possibility of using anti-CD63 and anti-CD81 antibodies as therapeutic agents in allergic disorders.

## 2. The *C. elegans* genome strategy: identification of three novel TRPM channels

Having failed to identify CRAC channels with the monoclonal antibody strategy, we designed a bio-informatic approach to screen the entire genome of *C. elegans* for proteins that were larger than 1000 amino acids. Since many ion channel subunits consist of six transmembrane (TM) helices, this feature was used as an additional criteria to filter the proteins. The resulting candidates were then compared with the EST data base generated from lymphocytes and the overlapping proteins were analyzed for their role in store operated calcium influx.

This approach led to the identification of three members of the transient receptor potential (TRP) superfamily, TRPM2 (previously designated TRPC7 or LTRPC2) [9], TRPM7 (designated as LTRPC7) [10] and TRPM4 [11] with crucial physiological functions.

The members of the transient receptor potential (TRP) superfamily have been in the forefront as likely candidates for store operated calcium entry for over a decade. The gene for TRP channel was first cloned from Drosophila photoreceptors [12], and the TRP proteins were demonstrated to form $Ca^{2+}$ permeable channels [13], that are activated downstream of G-protein-coupled receptor/PLC signaling pathway [14]. This finding was rapidly followed by cloning and identification of multiple mammalian homologs. The mammalian homologs have been divided into seven subfamilies (TRPC, TRPV, TRPM, TRPN, TRPA, TRPP and TRPML), many of which perform crucial functions in lymphocyte ion metabolism. Many TRP channels are non-selective $Ca^{2+}$ permeable channels. Since most $Ca^{2+}$ permeable channels show some sort of feedback regulation by $Ca^{2+}$ to prevent harmful increases in $[Ca^{2+}]_i$ levels, the activity of several TRP proteins, including TRPC3, TRPV5 (ECaC), TRPV6 (CaT1), TRPM2 (LTRPC2) and TRPM7 (LTRPC7), is affected either positively or negatively by changes in $[Ca^{2+}]_i$. It is therefore not surprising that some of the TRP channels have earlier been suspected to constitute the CRAC channel [15]. However, a closer scrutiny in many cases

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has revealed that these effects are only modulatory. We have briefly described the three TRP channels that we identified and characterized in search of the store-operated channels.

## TRPM2: The ADP-ribose gated non-selective channel

TRPM2 is a calcium-permeable channel activated in response to free ADP-ribose (ADPR), a product of NAD hydrolysis and a breakdown product of calcium-mobilizing second messenger cyclic ADP ribose (cADPR) [9]. In addition, TRPM2 has ADPR pyrophosphatase activity, however, this activity is not required for gating of its channel activity [9,16]. Whether nicotinamide adenine dinucleotide (NAD), a precursor of ADPR, has any effect on channel activation or not is debatable [9,16]. TRPM2 has also been shown to be activated by micromolar levels of $H_2O_2$ and agents that produce reactive oxygen/nitrogen species [17]. Thus a role for TRPM2 in cell death has been proposed. However, these effects are likely to be indirect since oxidative stress can result in the production of ADP-ribose intracellularly [18,19]. Recently a role for sirtuin, silent information regulator 2 (Sir2), metabolites has been proposed in TRPM2 gating. Sirtuins are protein deacetylases that regulate gene expression, apoptosis, metabolism, and aging [20]. Finally, the two second messengers, cyclic ADP-ribose (cADPR) and nicotinic acid adenine dinucleotide phosphate (NAADP), both of which have been critically implicated in the regulation of calcium signaling in T lymphocytes, strongly activate native TRPM2 channels in Jurkat T cells [21–23]. Therefore, it seems plausible that cADPR and NAADP and ADPR, represent physiological co-activators of TRPM2 channels that contribute to $Ca^{2+}$ influx in T lymphocytes and presumably other cell types that express this channel.

## TRPM7: The intracellular $Mg^{2+}$ sensor

The second channel that we identified using the bio-informatic screen of *C. elegans* genome is TRPM7, a $Ca^{2+}$and $Mg^{2+}$ permeant divalent cation channel. TRPM7 gating appears to be regulated by changes in the intracellular levels of $Mg^{2+}$ or MgATP, quite similar to native MagNuM or MIC channels [10,24,25]. TRPM7's C-terminal region possesses homology to a unique family of protein kinase domains designated as α-kinase family [26–28]. Therefore, in addition to TRPM2, TRPM7 also represents one of the few examples of channels coupled with intrinsic enzyme activity. As in the case of TRPM2's ADPR pyrophosphatase, the kinase activity of TRMP7 does not appear to influence the channel properties or gating mechanism [10,24,25]. On the contrary, the phosphotransferase activity of the TRPM7 α-kinase is modulated by free $Mg^{2+}$. In fact, it is likely that the proximity of the $Mg^{2+}$-responsive protein kinase to the channel pore or the site of $Mg^{2+}$ fluxes across the plasma membrane serves to regulate its kinase activity. Targeted deletion of TRPM7 in DT-40 B cells is lethal, therefore, these channels play a fundamental and non-redundant role in cellular physiology [10].

## TRPM4: The calcium activated non-selective (CAN) channel

The third channel we identified, TRPM4, belongs to the calcium activated non-selective (CAN) channel family. These channels are directly activated by elevations in cytoplasmic $Ca^{2+}$. They support large inward currents carried primarily by $Na^+$ that result in $Ca^{2+}$ dependent depolarization. Thus, their main function seems to mediate membrane depolarizations to support important cellular responses [29–31]. Remarkably, these channels do not undergo voltage or $Ca^{2+}$ dependent inactivation and therefore are capable of maintaining membrane depolarization. In non-excitable cells, this depolarization decreases the driving force for $Ca^{2+}$ influx through store-operated $Ca^{2+}$ channels [11]. This, in turn, can affect the shape and intensity of $Ca^{2+}$ signals initiated by receptor stimulation. Molecular suppression of TRPM4 converts oscillatory changes of $[Ca^{2+}]_i$ into long-lasting sustained elevations in $Ca^{2+}$ and leads to augmented IL-2 production. Therefore, it is conceivable that physiologically, TRPM4 exerts an important modulatory role on calcium dependent T lymphocyte effector functions such as NFAT translocation and IL-2 secretion [32]. In addition, recently, Vennekens et al. showed

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that TRPM4−/− bone marrow-derived mast cells had increased $Ca^{2+}$ influx compared to wildtype cells in response to FcepsilonRI stimulation [33]. As a result TRPM4−/− mast cells showed enhanced degranulation and cytokine secretion. These results further establish a critical physiological role for TRPM4 channels as regulators of $Ca^{2+}$ influx.

## 3. The drosophila genome strategy: identification of CRACM1

Since the above described second approach could not identify the CRAC channel, we adopted a third approach that involved performing a completely unbiased whole-genome RNAi screen in drosophila S2 cells. We performed a dye-based assay to measure the real time changes in $[Ca^{2+}]_i$ using an automated fluorometric imaging plate reader (FLIPR), in response to a commonly used sarcoplasmic and endoplasmic reticulum calcium adenosine triphosphatase (ATPase) (SERCA) inhibitor, thapsigargin. Analysis of real-time changes in calcium fluxes allowed us to easily cull false positive genes that affected ER release or baseline intracellular calcium levels [34]. 27 genes identified from the dye-based primary high-throughput screen were then re-analyzed in a secondary patch-clamp screen. Patch-clamp analysis identified a single gene *olf186-F* to be crucial for $I_{CRAC}$. Around the same time Feske et al. conducted the whole-genome RNAi screen. However, this group designed an assay to study the disruption of nuclear translocation of the nuclear factor of activated T cells (NFAT) in response to thapsigargin mediated elevated $[Ca^{2+}]_i$, using S2 cells stably transfected with NFAT-GFP [35]. Feske et al. combined the results of the high-throughput RNAi screen in Drosophila S2 cells with a modified linkage analysis to identify the mutated gene in two human SCID patients. They also identified *olf186-F* using the RNAi screen in S2 cells and found the human homolog of this gene to be mutated and located on chromosome 12 in SCID patients [35]. A third report closely followed the above described two reports and confirmed *olf186-F* to be crucial for SOC entry [36]. The human homologue of *olf186-F*, FLJ14466 was named as CRACM1 (for CRAC modulator) by us [34] and Orai1 by Feske at al. [35]. CRACM1 is a relatively small TM protein of 32.7 KDa with four predicted transmembrane domains. Over-expression of differentially tagged CRACM1 in HEK cells revealed that both the N- and C-terminal tails are present in the cytosol [34]. Insertion of a tag between the TM3-TM4 loop of CRACM1 confirmed the predicted extracellular location of this loop [37]. Soon after the discovery of CRACM1, a strong functional synergy between STIM1 (the ER $Ca^{2+}$ sensor described below) and CRACM1 was identified by us [38] and others [36,39,40]. So much so that when co-expressed in different cell lines, STIM1 and CRACM1 reconstituted 50–100 fold larger $I_{CRAC}$ currents that were biophysically similar to endogenous $I_{CRAC}$ [38]. These observations pointed towards the possibility that CRACM1 forms the channel itself. Since many ion channels multimerize to form a functional ion pore, we first tested the propensity of CRACM1 to form homopolymers. Co-immunoprecipitation of differentially tagged versions of CRACM1 showed that CRACM1 can form homopolymers with itself [41]. Although multimers of CRACM1 do not form a conventional ring of charged residues, we hypothesized that this may in fact form the basis of the somewhat unconventional pore selectivity of the CRAC channel. Several groups adopted the classical approach to mutate the conserved transmembrane glutamate residues of CRACM1, in positions 106 and 190, to study the changes in the selectivity and permeation of the CRAC channel [37,41,42]. The E106Q mutation of CRACM1 not only prevented the reconstitution of large $I_{CRAC}$ currents but also completely suppressed small endogenous CRAC currents. A charge conserving mutation of this glutamate to an aspartate residue E106D (or alanine E106A) resulted in relatively smaller currents and altered selectivity for $Ca^{2+}$ [41]. The E190Q mutation, on the other hand, resulted in very subtle changes in channel selectivity [41]. These results provided the unequivocal evidence that CRACM1 multimers form the pore-subunit of the CRAC channel with E106 residue conferring the high $Ca^{2+}$ selectivity. In addition to the two glutamates, there is a string of three aspartate residues in the first extracellular (TM1-TM2) loop. A double mutation in the first two aspartate residues D110/112A increased sodium permeation under calcium free conditions suggesting that these

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residues may contribute to the selectivity profile of CRAC channels, presumably by facilitating $Ca^{2+}$ binding to this site thereby contributing to the higher selectivity of $Ca^{2+}$ ions against monovalent cations [41,42]. Thus the 20 year long mystery shrouding the store-operated calcium influx finally seems to have been solved by adopting an unbiased approach.

## 4. STIM1: The store $Ca^{2+}$ sensor

An important regulatory step in the CRAC signaling pathway is at the level of communication of store depletion to CRAC channels. A couple of years ago, STIM1, an ER resident, single spanning transmembrane protein with a MW of 77 KDa was identified as the crucial calcium sensor coupling the process of store depletion with calcium influx through the CRAC channels [43,44]. STIM1 possesses an N-terminal EF-hand domain and a SAM domain facing either the lumen of the endoplasmic reticulum or outside when present in the plasma membrane. In addition, a coiled-coil domain and several others are present in the C-term cytosolic tail [45]. Upon store depletion, STIM1 has been shown to relocate to punctae like structures beneath the plasma membrane [43] or in the plasma membrane [46]. Mutating EF hand results in constitutive activation of $I_{CRAC}$ and translocation of STIM1 to punctae or plasma membrane [46,47]. Whether STIM1 actually inserts into the PM following store depletion has remained controversial. For instance, Zhang et al. showed that the amount of STIM1 pulled down with biotinylated plasma membrane proteins increases upon store depletion [46]. These studies, however, did not rule out the possibility of an indirect pull down of ER STIM1 that is bound to a membrane resident biotinylated protein. Luik et al, on the other hand, showed that STIM1 redistributes into punctae that are located close to PM using TIRF [47]. This study and several others [43,48] failed to detect the N-terminal tagged STIM1 exposed extracellularly and therefore proposed a role for junctional/punctae aggregated-STIM1 in CRAC channel activation. Recently, Hauser et al. tagged STIM1 with CFP at either the N-term end after the signal peptide (SP) or at the C-terminal tail end and showed that while both the tagged versions of STIM1 translocated upon store depletion, the N-term CFP tagged version did not insert into the plasma membrane whereas C-term tagged STIM1 did, as seen by cell-surface labeling of unpermeabilized cells over-expressing the two different versions [49]. Thus protein tags might affect STIM1 trafficking and prevent its insertion into the plasma membrane, depending on their size and location. Taken together, the above studies suggest that plasma membrane insertion of STIM1 following store depletion is unlikely to be crucial for CRAC channel activation since irrespective of its localization, STIM1 is capable of activating CRAC currents. Interestingly, a fraction of STIM1 is constitutively expressed in the plasma membrane and an earlier independent study assigned a crucial role to PM localized STIM1 in CRAC channel activation by using a monoclonal antibody (mAb) against the N-terminal STIM1 sequence [50]. STIM1 monoclonal antibody suppressed CRAC currents substantially when applied to the outside of intact cells. The same antibody also inhibited the constitutively active CRAC currents in cells expressing the STIM1 EF hand mutant. Contrary to this study, the cytosolic fragment of STIM1, STIM1-CT, was shown to be sufficient to constitutively activate $I_{CRAC}$ in the absence of endogenous PM expressed STIM1 [51,52]. The role for constitutively expressed STIM1 in the plasma membrane therefore remains to be resolved. Although STIM1-CT was sufficient to constitutively activate CRAC currents [51], the amplification in calcium influx normally seen with the co-expression of WT-STIM1 and CRACM1 was not observed with STIM1-CT and CRACM1 co-expression (our unpublished data). Further studies will likely resolve the role of PM expressed STIM1 in the activation of CRAC currents under physiological conditions.

## 5. Mechanism of activation of CRAC channels-The next milestone!

Over the years, many general models have been proposed to explain the activation of store operated calcium influx. Although these models predate the discovery of the two crucial

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molecular players, STIM1 and CRACM1, revisiting some of them in light of the new findings may help in decoding the next big puzzle–the molecular mechanism of activation of CRAC channels. The first model proposed the existence of a diffusible messenger that would link store depletion to CRAC channel activation [53]. Even though the issue of STIM1 and CRACM1 communication currently remains unresolved, a direct interaction between the two molecules would make the diffusible messenger unnecessary. On the other hand, communication over short distances or molecular interaction between ER localized STIM1 and PM localized CRACM1 across two intracellular subcompartments, as proposed by Luik et al. [47] fits better with the secretion-like coupling hypothesis proposed by Gill and colleagues in 1999 [54,55]. According to this hypothesis, store depletion initiates trafficking of peripheral ER to the PM and when the ER and PM are optimally juxtaposed, coupling between IP3 receptors in the ER (presumably via STIM1) and SOC channels in the PM occurs. A third model proposed that upon store depletion SOCs are inserted into PM via an exocytotic mechanism. This hypothesis was based on the observations that exocytosis blocking drugs inhibited $I_{CRAC}$ [56,57]. The latest studies have shown that the pore forming subunit of CRAC channel, CRACM1, is constitutively present in the PM [34,37]. Although these findings rule out the requirement of exocytosis for transporting the CRAC channel in response to store depletion, movement of STIM1 to plasma membrane or junctional ER may require the exocytotic machinery. In fact, a careful analysis of the genes inhibiting calcium influx but not $I_{CRAC}$ (from the recent high-throughput screens) reveals, amongst others, candidates commonly known to be involved in vesicular transport such as SNAP and SNARE (syntaxin5) proteins [34,36].

Like in the case of many voltage gated channels [58], phosphorylation seems to play a modulatory role in store operated $Ca^{2+}$ entry [59,60]. Phorbol ester mediated activation of PKC inhibits store operated $Ca^{2+}$ entry in the Jurkat cells, human neutrophils, peripheral T-lymphocytes, and rat basophilic leukemia cells [61–63]. In contrast to PKC, activation of PKA has shown opposing regulatory effects on CRAC channel function [64]. Serine/threonine phosphatase inhibitors have been reported to inhibit activation of the CRAC pathway [65,66]. CRACM1 has several putative serine/threonine phosphorylation sites and there is evidence of serine phosphorylation on CRACM1 (our unpublished data). Therefore, it remains to be seen whether phosphorylation plays a role in the activation of CRAC channels.

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