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The anatomy of *native* CRAC channel(s)

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Abstract

The ubiquitous store-operated Ca^{2+} entry pathway mediated by plasma membrane Ca^{2+} release-activated Ca^{2+} (CRAC) channels regulates a wide variety of physiological functions. While it is clearly established that the ORAI1 protein is essential for *native* mammalian CRAC channels, the contribution of ORAI2 and ORAI3 have remained nebulous. The crystal structure of the sole Orai isoform in drosophila (*dOrai*) revealed a hexameric assembly, suggesting that mammalian CRAC channels are hexamers of ORAI. Nevertheless, the relative contribution of each isoform of the mammalian ORAI trio to the stoichiometry of native CRAC channels remains elusive. The recent generation of ORAI isoform single, double and triple knockout cell lines and tissue-specific knockout mice has shed light on how native ORAI isoform heteromerization fine tunes CRAC-mediated Ca^{2+} signaling.

Keywords

ORAI; STIM; Calcium signaling; Store-operated calcium entry; CRAC channels

Store-operated Ca^{2+} entry (SOCE)

Physiological calcium (Ca^{2+}) concentrations in the extracellular space (1–2 mM) and in the lumen of the endoplasmic reticulum (ER; 300 μM –1mM) are orders of magnitude higher than in the cytosol (50–100nM)[1]. Cytosolic Ca^{2+} concentrations are tightly regulated by means of channels, pumps, and transporters located on the surface of the plasma membrane (PM) and internal organelles. This tight control ensures efficient agonist mediated Ca^{2+} signaling while protecting from toxicity of elevated levels of cytosolic Ca^{2+} [1]. Stimulation of cells with agonists to G-protein coupled receptors or receptor tyrosine kinases that couple to

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CONFLICT OF INTEREST STATEMENT

The authors declare no competing interests.

isoforms of the phosphoinositide-specific phospholipase C (PLC) cause the release of luminal ER Ca^{2+} into the cytosol through ER-resident inositol 1,4,5-trisphosphate (IP_3) receptors. Eukaryotic cells have evolved a two-component store-operated Ca^{2+} entry (SOCE) pathway, which is mediated by the Ca^{2+} release activated Ca^{2+} (CRAC) channel, in response to depletion of ER Ca^{2+} stores[1–5]. The first component of SOCE/CRAC is the ER Ca^{2+} sensing proteins, stromal interacting molecules (STIM1 and STIM2) that change conformation upon ER Ca^{2+} depletion and translocate to ER-PM junctions [6,7]. Within these junctions, STIM proteins physically trap and gate ORAI proteins at the PM, the second component of SOCE representing the pore-forming subunits of CRAC channels[8–10]. SOCE has perhaps evolved first as means to replenish depleted ER Ca^{2+} but was coopted later during evolution for the purpose of cellular signaling that controls the induction of metabolic and transcriptional programs. CRAC channels are characterized by high Ca^{2+} selectivity with a reversal potential of $\sim +60$ mV, depotentiation of Na^+ currents when recorded in divalent free solutions, small unitary conductance, conduction of Sr^{2+} and Ba^{2+} ions, and two modes of Ca^{2+} -mediated negative feedback regulation in the form of fast (within milliseconds) and slow (within seconds to minutes) Ca^{2+} -dependent inactivation (CDI)[3,11]. Interestingly, a portion of STIM1 C-terminus is essential for ORAI1 fast CDI[12,13], and Ca^{2+} /calmodulin binding to STIM1 appears to disrupt STIM1/ORAI1 interactions to mediate slow CDI[14,15].

ORAI1 was discovered in genome-wide siRNA screens as an essential component of SOCE and CRAC channels[8–10]. ORAI2 and ORAI3 did not produce hits in those siRNA screens, but were identified by their significant sequence homology to ORAI1[8]. Each of the three mammalian ORAI isoforms can form functional CRAC channels when ectopically co-expressed with STIM1 in HEK293 cells[16,17]. However, while ORAI1 is clearly required for the function of *native* CRAC channels, the contribution of ORAI2 and ORAI3 has remained nebulous. Notwithstanding that mRNA of all three ORAI isoforms are ubiquitously co-expressed, it was reasonably proposed early after their discovery that ORAI2 and ORAI3 might encode CRAC channels in specialized cells from different tissues[17]. However, over a decade of studies suggested that this is largely not the case and the significance of ORAI2 and ORAI3 to native CRAC channel function have remained, until recently, elusive.

ORAI1 is an essential pore-forming subunit of *native* CRAC channels

In addition to the discovery of ORAI1 in unbiased genome-wide siRNA screens[8–10], several key findings cemented the position of ORAI1 as the *bona fide* pore-forming protein of CRAC channels and spawned a flurry of studies focused on ORAI1 in the past 15 years. These findings include: i) a point mutation in ORAI1 (R91W) that results in a severe combined immunodeficiency (SCID) syndrome due to lack of SOCE and CRAC currents in lymphocytes[8]. ii) Mutations within the putative pore region of ORAI1 generated either a dead CRAC channel or a channel with altered ion selectivity[18,19], and iii) the reconstitution of large CRAC currents by the ectopic co-expression of ORAI1 with STIM1[20–22]. The collective focus on ORAI1 resulted in the rapid identification of ORAI1 as a crucial component of SOCE in a growing number of primary cell types and identified mutations or altered expression of ORAI1 (and STIM1) as a contributor to several

diseases[23–25]. Except for few cases discussed below, only ORAI1 was consistently revealed as critical for *native* CRAC channel function in a number of cell lines and primary cells from different tissue origins, with no discernible roles for ORAI2 and ORAI3. The resolution of the crystal structure of the sole Orai isoform in invertebrates, drosophila Orai (dOrai), at 3.35 angstrom revealed that six dOrai subunits are concentrically organized in a three-fold symmetry to form a channel pore with six glutamates (E106 in human ORAI1) on the extracellular side forming the selectivity filter[26]. Subsequent studies utilizing concatenated oligomers of human ORAI1 suggested that the human CRAC channel is likely a hexamer[27,28]. Nevertheless, a key question remained unanswered for the past 15 years: If ORAI1 alone is required for *native* CRAC channels and ORAI2 and ORAI3 are not universally encoding CRAC channels in primary cell types from different mammalian tissues and organs, what then is the signaling function of ORAI2 and ORAI3?

Ectopically expressed ORAI2 and ORAI3 are structurally and functionally distinct from ORAI1

Mammalian ORAI1 has the closest homology to the founding invertebrate member, drosophila Orai (dOrai). Evolutionarily, ORAI2 arose in vertebrates while ORAI3, the last ORAI isoform, appeared in mammals where the *Orai3* gene is thought to have duplicated from *Orai1*[29]. While there is significant sequence homology between the pore region of all ORAI isoforms, their N- and C-termini are different, especially the C-terminus of ORAI3, which is significantly shorter compared to ORAI1 and ORAI2[30]. These structural differences likely contribute to the unique pharmacological profile of CRAC channels generated by ectopic co-expression of individual ORAI isoforms with STIM1. For example, while all three ectopically expressed ORAI isoforms are blocked by low concentrations of lanthanides (1–5 μM Gd^{3+})[31], the pharmacological compound 2-aminoethoxydiphenyl borate (2-APB) potentiates ORAI3 independently of store depletion while blocking ORAI1 and ORAI2[31–35]. A small molecule termed IA65 was discovered recently and shown to selectively potentiate ORAI1 with no significant effect on ORAI3[36] (Table 1). As mentioned above, side by side comparisons of ORAI1, ORAI2 and ORAI3 individually co-expressed with STIM1 in HEK293 cells showed that all three ORAI isoforms can support CRAC currents. However, ORAI1 supported the largest peak CRAC currents with ORAI2 and ORAI3 generating currents that were ~50% and ~20% the size of ORAI1, respectively[16,17,31]. These differences in peak currents between ORAI isoforms could be explained by differences in their fast CDI. ORAI3 showed the largest fast CDI, whereas ORAI1 showed the smallest with ORAI2 fast CDI falling in between[16,17]. Quite interestingly, Only ORAI1 is sensitive to slow CDI whereas ORAI2 and ORAI3 are insensitive[17] (Table 1).

ORAI2 and ORAI3 heteromerize with ORAI1 to regulate CRAC channel function

The first subtle hint that ORAI1 alone might not encompass CRAC channels came earlier from ectopic co-expression of ORAI1 with STIM1 in HEK293 cells. The large CRAC currents generated in these experiments recapitulated most, but not all, of the biophysical

properties of *native* CRAC currents[21]. In ion substitution experiments, the Sr^{2+} and Ba^{2+} currents mediated by overexpressed STIM1 and ORAI1 were smaller than those typically observed for native CRAC channels[21], suggesting that some *native* regulatory factor or protein was missing from these overexpression experiments. A thorough study by Gwack et al. demonstrated using co-immunoprecipitations of tagged ectopically expressed proteins, that all ORAI isoforms physically interact[37]. FRET studies on ectopically expressed proteins also indicated that all three ORAI isoforms interact, and this interaction is not enhanced by ER store depletion[31]. While these reports indicated that overexpressed ORAI can heteromerize to form CRAC channels, the earliest evidence of a contribution of an ORAI isoform, other than ORAI1, to *native* CRAC was obtained from studies on ORAI1 knockout mice. The original report on ORAI1 knockout mice showed that mast cells from these mice have defective degranulation with a significant reduction of ~50% (but not a complete abrogation) of SOCE[38], suggesting a contribution of native ORAI2 and/or ORAI3 to the remaining SOCE. Surprisingly, and in stark contrast to human T-cells from SCID patients[8] or human cell lines[39], naïve T-cells from ORAI1 knockout mice showed very high mRNA expression of ORAI2 and unaltered proliferation, suggesting that, at least in the absence of ORAI1, ORAI2 can partially mediate *native* SOCE in mouse T-cells[38].

The first evidence that *native* ORAI2 and ORAI1 associate to form CRAC channels was provided by Vaeth et al in T-cells[40] and subsequently by Tsvilovskyy et al in mast cells[41]. Vaeth et al showed that SOCE in CD4^{+} T cells from T-cell specific ORAI1 knockout mice was partially reduced. Unexpectedly however, they discovered a significant increase in SOCE and CRAC currents in CD4^{+} T cells from global ORAI2 knockout mice, whereas the combination of ORAI2 and ORAI1 knockout in mouse T-cells abolished SOCE[40]. Tsvilovskyy et al reported increased SOCE in mast cells from ORAI2 knockout mice and this correlated with increased mast cell degranulation, core body temperature, and anaphylaxis[41]. These independent findings suggest that native ORAI1 and ORAI2 form heteromeric CRAC channels, where ORAI2 serves as a negative modulator of CRAC channel function[40,41]. Examination of dental enamel cells from ORAI2 knockout mice and dental enamel cell lines subjected to shRNA-mediated ORAI3 knockdown showed enhanced SOCE in the absence of ORAI2 or with knockdown of ORAI3[42], suggesting that ORAI2 and ORAI3 are negative regulators of SOCE. Indeed, a more recent side by side evaluation of the contribution of *native* ORAI isoforms to SOCE in HEK293 cells using CRISPR/Cas9-mediated single, double and triple *Orai* gene knockout revealed that both *native* ORAI2 and ORAI3 act as negative regulators of SOCE showing additive effects, with ORAI2 imparting larger negative regulation than ORAI3[31]. Together, these results support the notion that both ORAI2 and ORAI3 interact with ORAI1 to form native CRAC channels and serve as negative regulators that fine-tune CRAC channel activity.

However, studies in neurons paint a different picture for the role of ORAI2 in SOCE. Neurons from ORAI2 knockout mice have reduced SOCE and these mice are protected from stroke, presumably from reduced Ca^{2+} -mediated neuronal death[43]. This positive participation of ORAI2 in neuronal SOCE is likely due to the high level of expression of ORAI2 in neurons, where only ORAI2 mRNA was detected while mRNAs for ORAI1 and ORAI3 were not[44]. Consistent with these results, neurons from STIM2 knockout mice have reduced SOCE while those from STIM1 knockout and ORAI1 knockout mice have

unaltered SOCE [45], suggesting that neurons might have coopted the STIM2/ORAI2 pair for SOCE. Just like the case of ORAI2 in neurons, the first evidence that ORAI3 contributes to *native* SOCE was obtained from a subset of estrogen receptor positive breast cancer cell lines, which show high levels of expression of ORAI3[46,47].

Furthermore, potential interactions between *native* ORAI1 and ORAI3 were inferred from the high sensitivity to H₂O₂-mediated inhibition of SOCE in naïve CD4⁺ T cells and monocytes compared to differentiated cells. The enhanced resistance to H₂O₂ correlated with an increase in ORAI3 expression[48], as the redox-resistant ORAI3 lacks the equivalent of cysteine-195 that mediates H₂O₂ inactivation of ORAI1[49] (Table 1). Increased ORAI3 expression is believed to alter the composition of CRAC channels generating more redox-resistant channels and it seems that the presence of one ORAI3 subunit in a concatenated tetramer was sufficient to protect CRAC channels from oxidative inactivation[50]. Therefore, it appears that under certain conditions *native* ORAI2 and ORAI3 can function independently to mediate SOCE and that ORAI3 can serve as a positive modulator of SOCE in redox-rich environments where ORAI3 protects against redox-mediated SOCE inhibition.

***Native* CRAC channels: dynamic heteromers of all ORAIs tailored to diverse Ca²⁺ signaling events**

As it turned out, the negative regulation of CRAC channels by heteromerization of ORAI2 and/or ORAI3 with ORAI1 reveals only a portion of the full picture. In fact, most studies to date (discussed above) used protocols that cause maximal store depletion to generate supramaximal Ca²⁺ signals that typically manifest as a sustained cytosolic Ca²⁺ plateau[51]. Physiological Ca²⁺ signaling events *in vivo* are seldom of this large magnitude, except perhaps during cell death and primary cells *in vivo* tend to respond to very low concentrations of agonists compared to cultured cells[52]. Thus, physiological Ca²⁺ signals encompass a large repertoire of oscillatory Ca²⁺ events or spikes with diverse amplitudes and frequencies that increase with increasing concentrations of the stimulus and only manifest as plateaus with maximal agonist stimulation. With this in mind, CRISPR/Cas9-mediated knockout combinations of ORAI isoforms showed that *native* ORAI3 and ORAI2 can support regenerative Ca²⁺ oscillations but only ORAI1 supported Ca²⁺ plateaus. This behavior may be due to the strong basal interactions of ORAI2 and ORAI3 with STIM1 and the heightened fast Ca²⁺-dependent inactivation (CDI) of these two isoforms [31,53]. The preferential basal interaction of STIM1 with ORAI2 and ORAI3 over ORAI1 could potentially allow CRAC channels consisting of ORAI2 and/or ORAI3 to be rapidly activated and inactivated to suit the small and pulsatile oscillatory Ca²⁺ signaling in response to low and moderate physiological agonist stimulation. Of note, ORAI1/ORAI3 heteromeric assemblies involving basal interactions of ORAI3 with STIM1 were proposed to mediate a Ca²⁺ entry route activated independently of store depletion at low physiological concentrations of agonist and involving, through unknown mechanisms, arachidonic acid or its metabolite leukotriene C₄[54–57]. Whether this store-independent pathway is simply a variant of SOCE that is activated by low concentrations of agonists that might not achieve measurable ER depletion is an interesting question that requires further investigations.

These results imply that *native* CRAC channels either have a dynamic stoichiometry or present with a multitude of fixed stoichiometries, presumably depending on the relative expression levels of each of the three ORAI isoforms within a specific cell type. Heteromeric CRAC channels with higher proportions of ORAI2 and ORAI3 are expected to be gated by STIM1 at low agonist concentrations than those consisting predominantly of ORAI1. These heteromeric CRAC channels would generate a tempered SOCE activity and NFAT activation that is tailored to the strength of agonist stimulation. In fact, deletion of native ORAI2 and ORAI3 in HEK293 cells changed the cell oscillatory Ca^{2+} signal in response to low and moderate agonist concentrations into mostly Ca^{2+} plateaus and generated maximal NFAT4 and NFAT1 isoform nuclear translocation, essentially restricting the graded diversity of agonist-activated Ca^{2+} signals and the differential induction of NFAT isoforms normally activated by distinct magnitudes of Ca^{2+} signals[31]. The graded diversity of Ca^{2+} signals would be analogous to the graded intensity of light emitted by a dimmable electrical bulb. With ORAI1 alone the system is restricted to an On/Off switch while addition of ORAI2 and ORAI3 endows the system with the fine control of a dimmer switch.

Conclusions

Currently, there is no structural or proteomic evidence shedding light on the stoichiometry or stoichiometries of CRAC channels in native tissues or data ruling out the coexistence of certain stoichiometries, including homomers of each ORAI isoform. Nevertheless, knockout models and data on Ca^{2+} oscillations mediated by native SOCE in HEK293 cells cannot be reconciled with the exclusive presence of ORAI isoform homomers[31]. Rather, these data support the idea that ORAI isoforms heteromerize to form *native* CRAC channels (Figure 1). This heteromerization ensures the graded diversity of CRAC-mediated Ca^{2+} signaling events in response to the full range of agonist strengths and the specific activation of a given transcription factor isoform (e.g. NFAT) at its specific range of cytosolic Ca^{2+} concentration. The presence of ORAI2 and ORAI3 within the *native* CRAC channel ensures the differential activation of NFAT4 (requiring a low Ca^{2+} signal) and NFAT1 (requiring a high Ca^{2+} signal) isoforms at distinct agonist concentrations. With CRAC channels containing ORAI1 alone, the Ca^{2+} signal is already maximal at lower concentrations of agonist leading to mostly concomitant NFAT4 and NFAT1 nuclear translocation[31]. Obviously, it is difficult to currently predict if there is only one *native* CRAC channel species with a specific fixed ORAI isoform stoichiometry or a dynamic one, or that many ORAI combinations generate a wide variety of *native* CRAC channels. The latter situation is more likely and the diversity of *native* CRAC channels in any given cell type would likely depend on the relative expression of each ORAI isoform in these cells. At this stage, the presence of functional homomeric CRAC channels composed of one *native* ORAI isoform cannot be ruled out (Figure 1). Because native ORAI2 and ORAI3 either alone or together are not capable of supporting NFAT isoform nuclear translocation in HEK293 cells[31], CRAC channels composed exclusively of ORAI2 and/or ORAI3, if they widely exist, likely signal to different downstream pathways and transcription factors. Similarly, whether *native* CRAC channels are predominantly heteromers of two or three ORAI isoforms or both remain unclear. The choreography of *native* CRAC channels becomes even more complex when we consider the

dynamic and privileged interactions of STIM1 and STIM2 with individual ORAIs during CRAC channel activation at the full range of physiological agonist stimulation[58,59]. Furthermore, relative contributions of STIM1 and STIM2 are likely dependent on the levels of expression of their splice variants and their potential regulation by posttranslational modifications in a given cell type. For instance, in monocytes, neurons, and colorectal cancer cells, knockdown or knockout of STIM2 has a profound effect on SOCE, causing a reduction by >50%, whereas STIM2 knockout in many cell types including HEK293 cells reduces SOCE by only ~10–15%[39,45,48,60]. Currently, important tools such as reliable antibodies capable of specifically detecting endogenous levels of ORAI2 and ORAI3 are missing. Further, the crystal structures of mammalian ORAI1, ORAI2 and ORAI3 have yet to be resolved. These structures and those of STIM/ORAI interactions would provide critical insights into the molecular composition of the native CRAC channel in various primary cell types, and these insights would be of crucial importance to specific targeting of this important Ca^{2+} entry route for the purpose of disease therapy.

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Figure 1. Native CRAC channels.

Native CRAC channels are likely heterohexamers of all three ORAI isoforms but their precise stoichiometry or stoichiometries remain unclear. The incorporation of ORAI2 and/or ORAI3 within a hexamer negatively regulates ORAI1 and fine tunes CRAC-mediated Ca^{2+} signaling. Whether native CRAC channels can also exist as homohexamers of each ORAI isoform remains an open question.

Table 1.

Functional and pharmacological characteristics of ORAI1, ORAI2 and ORAI3.

	ORAI1	ORAI2	ORAI3
Basal STIM1 Interaction	+	+++	+++
Activated STIM1 interaction	+++	+++	++
Fast CDI	+	++	+++
Slow CDI	Yes	No	No
Supports Ca ²⁺ Oscill ations	Yes	Yes	Yes
NEAT1/4 activation	Yes	No	No
Redox Sensitive	Yes	Yes	No
1A65	Potentiated	ND	No effect
2-APB	Blocked	Blocked	Potentiated
1–5 μM Gd ³⁺	Blocked	Blocked	Blocked

ND, not determined;

+,_{low};

++,_{medium};

+++,_{high}.