SYMPOSIUM REPORT

Orai, STIM1 and iPLA$_2\beta$: a view from a different perspective

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The mechanism of store-operated Ca^{2+} entry (SOCE) remains one of the intriguing mysteries in the field of Ca^{2+} signalling. Recent discoveries have resulted in the molecular identification of STIM1 as a Ca^{2+} sensor in endoplasmic reticulum, Orai1 (CRACM1) as a plasma membrane channel that is activated by the store-operated pathway, and iPLA$_2\beta$ as an essential component of signal transduction from the stores to the plasma membrane channels. Numerous studies have confirmed that molecular knock-down of any one of these three molecules impair SOCE in a wide variety of cell types, but their mutual relations are far from being understood. This report will focus on the functional roles of Orai1, STIM1 and iPLA$_2\beta$, and will address some specific questions about Orai1 and TRPC1, and their relation to SOC channels in excitable and non-excitable cells. Also, it will analyse the novel role of STIM1 as a trigger for CIF production, and the complex relationship between STIM1 and Orai1 expression, puncta formation and SOCE activation. It will highlight some of the most recent findings that may challenge simple conformational coupling models of SOCE, and will offer some new perspectives on the complex relationships between Orai1, STIM1 and iPLA$_2\beta$ in the SOCE pathway.

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Store-operated channels (SOCs) and store-operated Ca^{2+} entry (SOCE) are activated upon depletion of endoplasmic reticulum (ER) Ca^{2+} stores (Parekh & Putney, 2005), but the molecular mechanism of SOCE remains one of the intriguing mysteries in the field of Ca^{2+} signalling. Recent discoveries have resulted in the molecular identification of 1) STIM as a Ca^{2+} sensor in the endoplasmic reticulum (ER) that is capable of triggering a cascade of reactions leading to SOCE activation, 2) Orai (CRACM) as a plasma membrane (PM) channel that is activated by the store-operated pathway, and 3) iPLA$_2\beta$ as an essential component of signal transduction from the stores to plasma membrane channels. Molecular knock-down of any one of these three molecules (Orai, STIM1 and iPLA$_2\beta$) was shown to impair SOCE in a wide variety of cell types, but their relationship is far from being understood. Numerous review articles have been published within the last year (Lewis, 2007; Putney, 2007; Hewavitharana *et al.* 2007; Clapham, 2007) which nicely summarize the new knowledge about STIM1 and Orai1, and discuss currently popular models that suggest their conformational coupling as a mechanism of signal transduction and SOCE activation. This report will highlight some of the most recent findings that may challenge currently popular models of SOCE, and will offer some new perspectives on this complex mechanism.

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Orai1 and *TRPC1*: which gene encodes which channel?

One of the most important recent discoveries was the identification of Orai1 (Feske *et al.* 2006) or CRACM1 (Vig *et al.* 2006) as a pore-forming subunit of the CRAC channel (Feske *et al.* 2006; Vig *et al.* 2006; Yeromin *et al.* 2006; Luik *et al.* 2006; Lorin-Nebel *et al.* 2007). However, while most of the experts agree on the ability of the *Orai1* gene to encode a Ca^{2+}-selective SOC (CRAC) channel, and numerous studies demonstrated that molecular knock-down of Orai1 protein produces dramatic inhibition of I_{CRAC} and SOCE in all non-excitable

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cells tested so far (Roos *et al.* 2005; Liou *et al.* 2005; Zhang *et al.* 2005; Feske *et al.* 2006; Spassova *et al.* 2006; Peinelt *et al.* 2006; Soboloff *et al.* 2006*a*,*b*; Vig *et al.* 2006; Peel *et al.* 2006; Lorin-Nebel *et al.* 2007; Mignen *et al.* 2007), the role of Orai1 protein in a variety of excitable cells (in which SOCE is mediated by much less selective cation channels, cat-SOCs) remains an open question. Indeed, most investigators do not believe that the same *Orai1* gene could be responsible for the channels with such profoundly different selectivity to Ca^{2+} as CRAC and cat-SOC. Several recent models proposed cat-SOC to be formed by TRPC1 (Beech, 2005; Ambudkar, 2007), or a complex of Orai1 and TRPC1 subunits (Ambudkar, 2007; Ong *et al.* 2007; Yuan *et al.* 2007; Worley *et al.* 2007), or some other TRPC channels (Liao *et al.* 2007; Liao *et al.* 2008). So which gene encodes which channel, and how can the variety of SOC channels with different cation selectivity be created?

In our recently published article (Zarayskiy *et al.* 2007) we presented evidence that when Orai1 and TRPC1 proteins are endogenously present in the same cells, they form two totally different channels that can both respond to agonist and IP_3-dependent stimulation. Figure 1 summarizes these findings and shows that while Orai1 forms a store-operated channel that is activated exclusively upon depletion of Ca^{2+} stores (via an $iPLA_2\beta$-dependent pathway), TRPC1 forms an IP_3 receptor-operated channel (IP_3ROC) that is activated via its conformational coupling with IP_3 receptor. In RBL-2H3 cells endogenously expressing both Orai1 and TRPC1, we unmasked and characterized the whole-cell current through IP_3ROC channels, which was hidden behind some familiar

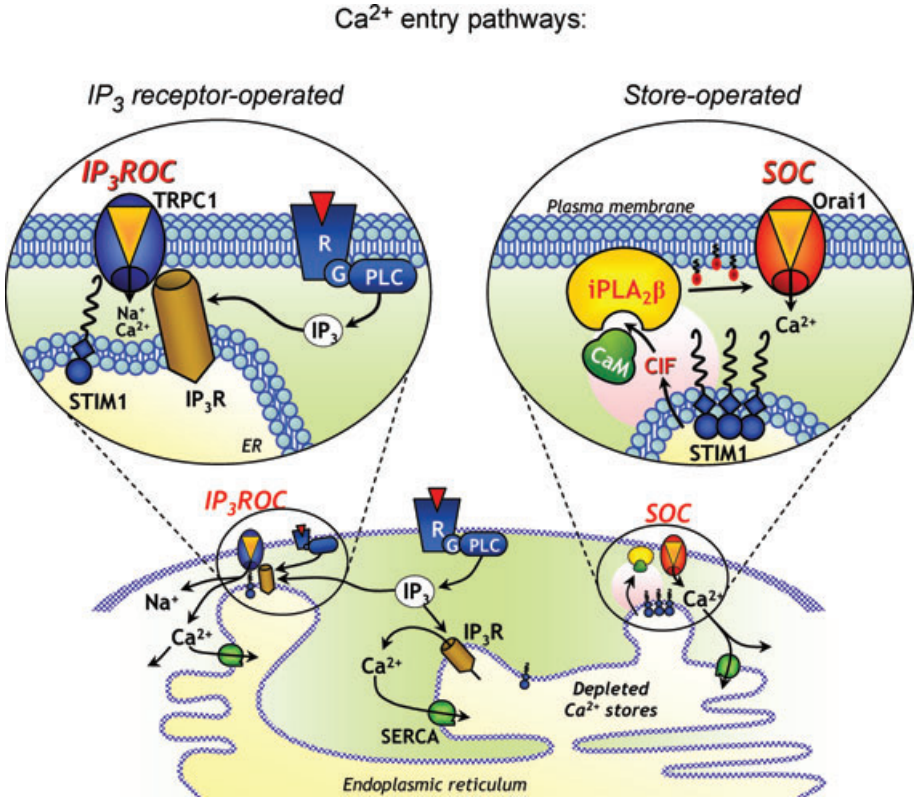

Figure 1. Two Ca^{2+} entry pathways can be mediated by two distinct channels, Orai1-dependent SOC and TRPC1-dependent IP_3ROC
Both pathways may be activated upon receptor (R) and G-protein (G)-mediated stimulation of phospholipase C (PLC) that leads to IP_3 production. The store-operated pathway (on the right) is activated upon depletion of Ca^{2+} stores, which leads to oligomerization and accumulation of STIM1 in ER membrane in close proximity to plasma membrane. STIM1 triggers production of calcium influx factor (CIF) that displaces inhibitory calmodulin (CaM) from a plasma membrane bound Ca^{2+}-independent phospholipase A_2 ($iPLA_2\beta$), which produces lysophospholipids and activates Orai1-dependent SOC channels that are responsible for the store-operated Ca^{2+} entry (SOCE). The IP_3 receptor-operated pathway (on the left) does not require store depletion, and is activated by IP_3-dependent conformational coupling of IP_3 receptor (IP_3R) and TRPC1-encoded plasma membrane channel (IP_3ROC). From Zarayskiy *et al.* 2007, ©Landes Bioscience.

fingerprints of I_{CRAC}, a current through the classical Ca^{2+}-selective SOC (CRAC) channels. We discriminated these currents by their molecular identity, selectivity and totally different requirements for store depletion, iPLA$_2\beta$ and conformational coupling to IP_3 receptor. Among many differences, we found that only *Orai1*-encoded CRAC channel, but not *TRPC1*-encoded IP_3ROC channel, requires the presence and functional activity of iPLA$_2\beta$, and while I_{CRAC} was absent, I_{IP3ROC} could be easily activated in the cells in which iPLA$_2\beta$ was knocked down. In contrast, TRPC1, but not CRAC, channels were fully dependent on activation of IP_3R. It is important to mention that despite clear molecular, biophysical and functional differences, both Orai1 and TRPC1 channels can be activated by agonists (and elevation of IP_3, but through totally different pathways), and both channels appear to be sensitive to 2-aminoethoxydiphenyl borate (2-APB) (which is widely used as SOC inhibitor). Because of these similarities, Ca^{2+} entry through TRPC1-dependent IP_3ROC can sometimes be confused with Orai1-dependent SOCE. Part of the confusion may arise from the ability of SERCA inhibitors to produce a significant Ca^{2+} rise in the cytosol, which, in the absence of efficient intracellular Ca^{2+} buffering, may lead to Ca^{2+}-induced activation of phospholipase C (PLC) and subthreshold IP_3 production, which may be enough for secondary activation of *TRPC1*-encoded IP_3ROC. Also, store depletion and iPLA$_2\beta$-dependent activation of SOCE can trigger activation of some other signalling cascades that can further activate different kinds of ion channels and physiological responses. As one of many examples, Ca^{2+} entry through CRAC was shown to activate cPLA2 (Chang & Parekh, 2004), which is a major source of arachidonic acid (AA). AA and its products can be further involved in numerous signalling cascades, and can also trigger activation of some other Ca^{2+}-conducting channels, such as ARC channel (Shuttleworth *et al.* 2004). New knowledge of the specific properties and possible coexistence of *Orai1*-encoded SOC and *TRPC1*-encoded IP_3ROC should help avoid further confusion about these channels, and open new exciting possibilities for their independent study.

Results from the new transgenic mice which lack either the *Orai1* (Vig *et al.* 2008) or the *TRPC1* (Dietrich *et al.* 2007*b*; Liu *et al.* 2007) gene brought new insights into the physiological roles of *Orai1* and *TRPC1*-encoded channels. *Orai1*$^{-/-}$ mice appeared to have severe abnormalities, with a dominant phenotype of impaired mast cell function, and strong potential for malfunctioning of other cells and organs that may be affected by Orai1 protein deficiency. In contrast, *TRPC1*$^{-/-}$ mice appeared to be rather healthy. The only major defect found so far was in its salivary gland function (Liu *et al.* 2007). Agonist induced activation of Ca^{2+} entry and poorly selective cation currents (which are thought to be mediated by cat-SOC in these cells) were found to be significantly compromised in *TRPC1*$^{-/-}$ animals. However, cat-SOC and SOCE appeared to be fully normal in vascular SMC, and *TRPC1*$^{-/-}$ mice did not show any noticeable flaws in their vascular function (Dietrich *et al.* 2007*a*). Thus, TRPC1 did not appear to be important for SOCE in vascular SMC, in which the role of cat-SOC and SOCE is well established by the work done in many different laboratories. Following all these studies, we were not surprise to find that the molecular knock-down of TRPC1 did not affect SOCE in both non-excitable RBL cells (Zarayskiy *et al.* 2007) and excitable vascular SMC (Yang & Bolotina, unpublished observations). However, a significant line of evidence suggests that the mechanism of SOCE activation can be identical in excitable and non-excitable cells types, which brings us back to the question on the molecular identity of the cat-SOC channels.

The majority of the experts rule out the possibility that *Orai1* by itself may encode the channels with such profound differences in selectivity as Ca^{2+}-selective CRAC and poorly cation selective cat-SOC channels. However, our most recent studies of SMC (as a model for cat-SOC) and RBL cells (as a model for CRAC) brought us to a rather unexpected discovery, which may help resolve the molecular nature of cat-SOC channels. We obtained new evidence that the *Orai1* gene may in fact encode both CRAC and cat-SOC channels and molecular knock-down of Orai1 protein equally impairs I_{CRAC} in RBL and I_{catSOC} in vascular SMC. Most importantly, we discovered that RNA editing may be a molecular mechanism that can produce a post-transcriptional modification of Orai1 selectivity, and adjust it to the specific needs of excitable and non-excitable cells (Zarayskiy *et al.*, 2008). Thus, mounting evidence make us believe that *Orai1* may encode not only Ca^{2+}-selective CRAC in non-excitable cells, but also cat-SOC channels in a variety of excitable cells. At the same time, *TRPC1* can encode an important, but totally different, agonist-activated channel that is regulated via conformational coupling with the IP_3 receptor.

STIM1 and its new role as a trigger for CIF production

STIM1 is a protein predominantly located in the ER membrane; it has one transmembrane domain and an EF hand motif in its N terminus that may allow STIM1 to bind Ca^{2+} in the ER lumen and to function as a low affinity Ca^{2+} sensor in the stores (for a most recent review see Lewis, 2007). Upon Ca^{2+} depletion, STIM1 has been shown to 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, followed by SOCE activation (Liou *et al.* 2005; Mercer *et al.* 2006; Wu *et al.* 2006; Baba *et al.* 2006; Luik *et al.* 2006; Stathopulos *et al.* 2006; Liou *et al.* 2007; Ross *et al.* 2007; Li *et al.* 2007; Muik *et al.* 2008). The role of STIM1

in SOCE was originally discovered in *Drosophila* S2 (Roos *et al.* 2005) and in HeLa cells (Liou *et al.* 2005), and was confirmed by numerous other studies which showed that the molecular knock-down of STIM1 results in the disappearance of SOCE in virtually all cell types tested so far. Although it is now clear that STIM1 is an essential component of the SOCE pathway, the exact mechanism of its involvement in SOCE is far from being understood.

While most of research has focused on STIM1 accumulation in puncta and its interactions with Orai1 (which will be discussed below), we decided to look at the early ER-delimited events and examine the role of STIM1 from the perspective of a diffusible messenger model. Calcium influx factor (CIF) is known to be produced in the cells upon depletion of Ca^{2+} stores, and its production in the ER stores upon the drop of intraluminal Ca^{2+} concentration was further confirmed in our recent studies (Csutora *et al.* 2006, 2008). Although the molecular identity of CIF is still unknown, its presence and biological activity were detected by numerous groups in a wide variety of cell types ranging from yeast to human (for review see Bolotina & Csutora, 2005). The physiological target of CIF (iPLA$_2\beta$, which will be described below), and the mechanism of CIF-induced activation of SOCE (illustrated in Fig. 1) were identified (Smani *et al.* 2004), but very little is known about the molecular mechanism of CIF production in the stores.

Looking at the early events in ER that follow store depletion and precede puncta formation, we discovered (Csutora *et al.* 2008) that CIF production is tightly coupled with STIM1 expression and requires functional integrity of glycosylation sites in its intraluminal SAM domain. Molecular knock-down or overexpression of STIM1 resulted in a corresponding impairment or amplification of CIF production. We demonstrated that CIF production is one of the earliest STIM1-dependent events in the ER lumen, which precedes STIM1 accumulation in puncta and activation of SOCE. We also found that the inherent deficiency in SOCE in the NG115 cell line can be the result of deficiency in STIM1 protein and CIF production. Expression of wild-type STIM1 in these cells was sufficient to fully rescue their ability to produce CIF and SOCE. We have established NG115 cells as a very useful model for studying the behaviour of exogenously expressed STIM1 (and its mutants) without involvement of endogenous STIM1 background. Using his model we discovered that STIM1 with mutated glycosylation sites in its SAM domain does not trigger CIF production, and fails to activate SOCE (Csutora *et al.* 2008), but remains fully capable of sensing store depletion and accumulating in puncta (Gwozdz *et al.* unpublished observations).

Taken together, these data demonstrate a novel role for STIM1 as a trigger for CIF production in the ER, which seems to be essential for SOCE activation. When the free Ca^{2+} concentration in the ER lumen drops and Ca^{2+} is lost from its EF hand, the change in STIM1 conformation may allow glycosylation sites in its SAM domain to interact with (and trigger activation of) CIF-generating machinery in ER. Subsequent accumulation of STIM1 into puncta in close proximity to the plasma membrane may allow effective and fast delivery of CIF to its plasma membrane target, iPLA$_2\beta$. CIF-induced activation of iPLA$_2\beta$ can further transduce the signal to Orai1, leading to Ca^{2+} entry. Importantly, without CIF production mere translocation of STIM1 to the vicinity of plasma membrane may not be sufficient for SOCE activation. Thus, STIM1-dependent activation of CIF production may go hand-in-hand with STIM1's ability to sense intraluminal Ca^{2+} and to accumulate in the vicinity of the plasma membrane, providing important molecular, functional and structural prerequisites for SOCE activation.

STIM1 and Orai1: what is their relationship, and what does it mean for SOCE activation?

The ability of STIM1 and Orai1 to accumulate and colocalize in punctate structures along the plasma membrane (for recent review see Lewis, 2007), and the reported coimmunoprecipitation of overexpressed STIM1 and Orai1, fuelled the return of the direct conformational coupling models of SOCE. Originally, conformational coupling models suggested a direct coupling of SOC channel with the IP_3 receptor (IP_3R) (Irvine, 1990; Petersen & Berridge, 1996). This idea got strong support from the reported coimmunoprecipitation and functional coupling of some TRPC channels with IP_3R (Kiselyov *et al.* 1998, 1999; Boulay *et al.* 1999; Rosado & Sage, 2000, 2001), but these models were abandoned after demonstration that triple IP_3R knockout DT40 chicken B-cells (in which all three known types of IP_3R were deleted) have a totally normal SOCE (Prakriya & Lewis, 2001; Bakowski *et al.* 2001; Ma *et al.* 2001). Recently, the conformational coupling idea returned, but instead of the IP_3R and TRPC, new models propose direct conformational coupling and signal transduction from STIM1 to Orai1 to be a mechanism for SOCE activation (Hisatsune & Mikoshiba, 2005; Huang *et al.* 2006; Lopez *et al.* 2006; Soboloff *et al.* 2006*b*; Spassova *et al.* 2006; Yeromin *et al.* 2006). This idea was strongly supported by several important findings. First, coimmunoprecipitation of overexpressed STIM1 and Orai1 was shown in S2 cells (Yeromin *et al.* 2006) and HEK293 cells (Vig *et al.* 2006). Second, overexpression of both proteins resulted in remarkable amplification (50–100 fold) of I_{CRAC} (so-called monster CRAC) (Peinelt *et al.* 2006; Soboloff *et al.* 2006*b*). Third, Förster resonance energy transfer (FRET) between STIM1 and Orai1 labelled with fluorescent proteins was reported (Muik *et al.* 2008), indicating their very close spatial

proximity. Fourth, overexpression of the C-terminus of STIM1 was reported to be sufficient for SOCE activation (Huang *et al.* 2006). All these data strongly supported the idea of direct conformational coupling of STIM1 to Orai1 as a rather straightforward mechanism of signal transduction and Orai1 activation. However these studies neither ruled out nor considered the possibility that intermediate steps and molecular components may be required for their co-localization, mutual interaction and signal transduction from ER to PM.

In our most recent (unpublished) work we looked at the relationship between STIM1 and Orai1 from this new perspective, and tested several important predictions of the conformation coupling models. In contrast to what would be expected, if direct interaction of STIM1 with Orai1 underlies puncta formation and I_{CRAC} activation, we found that: (1) STIM1 can form puncta independently of Orai1 expression and there is no strict correlation between STIM1 and Orai1 accumulation in puncta, (2) STIM1 expression may be neither required nor sufficient for I_{CRAC} and SOCE activation, and (3) without CIF production mere translocation of STIM1 to the vicinity of plasma membrane is not sufficient for SOCE activation (Csutora *et al.* 2008). Strikingly, we found that normal I_{CRAC} can be activated even in the cells in which STIM1 was knocked down. In this case, cell deficiency in STIM1 could be easily compensated by a direct activation of iPLA$_2\beta$ by cell dialysis with CIF (which is a simple short-cut in the SOCE pathway, which we described in our earlier publications (Smani *et al.* 2004; Csutora *et al.* 2006, 2008). These data extend our findings on the role of STIM1 as a trigger for CIF production (Csutora *et al.* 2008), and strongly suggest that STIM1 may be upstream, and Orai1 downstream, from CIF and iPLA$_2\beta$, which may be required as functional transducers of the signal from STIM1 in the ER to Orai1 in the plasma membrane (Fig. 1). The need for additional intermediate(s) between STIM1 and Orai1 was strongly supported by recent findings from Dr Balla's group (Varnai *et al.* 2007), which demonstrated that STIM1 and Orai1 colocalization does not occur in narrow (4–6 nm) junctions, but happens only in the areas where a larger 12–14 nm gap exists between the ER and the plasma membrane. To explain these observations, the need for an additional linker between STIM1 and Orai1 was postulated by the authors.

Thus, although direct coupling of ER-resident STIM1 to PM-resident Orai1 may be rightfully 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. It is important to emphasize that the apparent requirement for molecular intermediate(s) between STIM1 and Orai1 does not contradict any of the studies that demonstrated their close spatial and functional proximity, including reported FRET between STIM1 and Orai1, as colocalization of fluorescently tagged proteins within ~10 nm space between ER membrane and plasma membrane may allow energy transfer (Liou *et al.* 2007; Muik *et al.* 2008) even without their direct physical interaction.

The idea that additional molecules and functional steps may be involved in signal transduction from STIM1 in the ER to Orai1 in the PM, and new experimental evidence (Csutora *et al.* 2008; Gwozdz *et al.* unpublished observations) which places STIM1 upstream and Orai1 downstream from CIF and iPLA$_2\beta$ (Fig. 1) further highlight the important role of iPLA$_2\beta$ in the SOCE mechanism. So, what is so special about this element of the SOCE pathway?

Ca^{2+}-independent phospholipase A$_2\beta$ (iPLA$_2\beta$): what makes it fit into SOCE pathway?

iPLA$_2$ (or PLA$_2$ group VI) (Balsinde & Dennis, 1997; Winstead *et al.* 2000) is a family of intracellular phospholipases that do not require Ca^{2+} for their activation and function. At least five isoforms of iPLA$_2$ have already been identified (Balsinde & Balboa, 2005), which utilize catalytic serine to perform the hydrolysis of the middle (*sn*-2) ester bond of substrate phospholipids, producing free fatty acids and lysophospholipids. One particular isoform, iPLA$_2\beta$ (group VIA), demonstrates the most complex structure and function. It has multiple splice variants (Turk & Ramanadham, 2004), can undergo caspase cleavage and post-translational modifications (Song *et al.* 2004), and has several unique features which puzzled researchers for many years. One of them is its Ca^{2+}-independence (the ability to function in the presence of strong Ca^{2+} chelators), and at the same time its ability to bind the Ca^{2+}–CaM complex. The signature feature of iPLA$_2\beta$ is that it contains a CaM-binding domain in the C-terminus, which, along with the IQ motif, forms a pocket, enabling CaM to bind and inhibit iPLA$_2\beta$ activity (Wolf & Gross, 1996*b*; Jenkins *et al.* 2001). Removal of CaM from iPLA$_2\beta$ results in its activation. An analysis of the known iPLA$_2\beta$ sequences (human, rat, mouse, hamster and *Drosophila*) revealed that CaM binding domains represent one of the most highly conserved regions in the entire iPLA$_2\beta$ protein. Based on the molecular and biochemical studies (Wolf & Gross, 1996*a*; Mancuso *et al.* 2000; Jenkins *et al.* 2001) it is believed that in the absence of CaM the active site of iPLA$_2\beta$ interacts with the CaM-binding domain leading to a catalytically competent enzyme. Binding of CaM disrupts this interaction, resulting in the loss of iPLA$_2\beta$ activity.

A few years ago, we discovered that iPLA$_2\beta$ is an important molecular determinant of SOCE (Smani *et al.* 2003, 2004), and can be a physiological target for CIF, which we found to displace inhibitory CaM from iPLA$_2\beta$.

This results in activation of iPLA$_2\beta$, which triggers a PM-delimited cascade of reactions that lead to activation of SOC and Ca^{2+} entry (Fig. 1). For all the details of this pathway we will refer the readers to our earlier review (Bolotina & Csutora, 2005), and the papers that described the role of iPLA$_2\beta$ in SMC, platelets, Jurkat T lymphocytes, RBL-2H3, neuroblastoma/glioma, and some other cell types (Smani *et al.* 2003, 2004; Csutora *et al.* 2006; Zarayskiy *et al.* 2007). The role of iPLA$_2\beta$ in SOCE was further confirmed by many other investigators in a growing number of cell types, including astrocytes (Singaravelu *et al.* 2006), keratinocytes (Ross *et al.* 2007), skeletal muscle (Boittin *et al.* 2006), fibroblasts (Martinez & Moreno, 2005), prostate cancer cells (Vanden Abeele *et al.* 2004), and others. In all these studies molecular knock-down and/or functional inhibition of iPLA$_2\beta$ caused full impairment of SOCE. It is important to emphasize that genetic screening of *Drosophila melanogaster* performed and published by Vig *et al.* (2006) picked up not only STIM1 and Orai1, but also an orthologue of iPLA$_2\beta$ encoded by the CG6718 gene, which has a very high level of homology (up to 85% in main structural domains) to the human iPLA$_2\beta$. Along with STIM1 and Orai1, knock-down of iPLA$_2\beta$ (CG6718) showed a dramatic impact on SOCE activation that was remarkably identical to that of STIM1 (see supplemental data in Vig *et al.* 2006).

Conclusion

Recent developments in the SOCE field have resulted in major breakthrough discoveries that have opened new exciting areas for future study. Orai1, STIM1 and iPLA$_2\beta$ proved to be the essential components of the SOCE mechanism, and further studies are required to elucidate how these three molecules can work together to create fast, effective and specific transduction of the signal from the depleted stores to the plasma membrane channels. There is no surprise that the SOCE mechanism emerges as a rather complex phenomenon that may require concerted action and close interaction of all currently known and still to be discovered molecules. It may involve components of different models that historically have been thought to be mutually exclusive, but in the end may come together in one multidimensional SOCE mechanism.

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