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SYMPOSIUM REVIEW

Ca^{2+} and lipid signals hold hands at endoplasmic reticulum–plasma membrane contact sites

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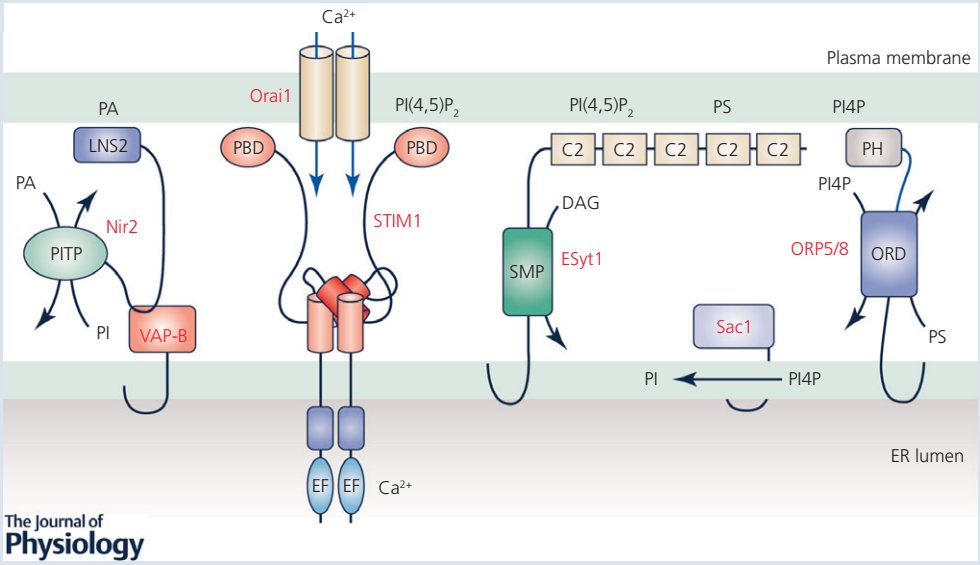

Abstract Discovery of the STIM1 and Orai proteins as the principal components of store-operated Ca^{2+} entry has drawn attention to contact sites between the endoplasmic reticulum (ER) and the plasma membrane (PM). Such contacts between adjacent membranes of different cellular organelles, primarily between the mitochondria and the ER, had already been known as the sites where Ca^{2+} released from the ER can be efficiently channelled to the mitochondria and also where phosphatidylserine synthesis and transfer takes place. Recent studies have identified contact sites between virtually every organelle and the ER and the functional importance of these small specialized membrane domains is increasingly recognized. Most recent developments have highlighted the role of phosphatidylinositol 4-phosphate gradients as critical determinants of the non-vesicular transport of various lipids from the ER to other organelles such as the Golgi or

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PM. As we learn more about membrane contact sites it becomes apparent that Ca^{2+} is not only transported at these sites but also controls both the dynamics and the lipid transfer efficiency of these processes. Conversely, lipids are critical for regulating the Ca^{2+} entry process. This review will summarize some of the most exciting recent developments in this rapidly expanding research field.

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Abstract figure legend Molecules identified in endoplasmic reticulum (ER)–plasma membrane (PM) contacts sites. STIM1 is localized in the ER with a Ca^{2+}-sensing luminal EF hand and sterile alpha motif (SAM) domains. An intramolecular interaction keeps its Orai1 activation (SOAR) domain (red) inactive. The polybasic domain of STIM1 (PBD) interacts with acidic membrane phosphoinositides, such as phosphatidylinositol 4,5-bisphosphate ($PI(4,5)P_2$). The Orai1 channels in the PM are activated by the STIM1 SOAR domain only when the latter is released from the intramolecular clamp when the ER luminal Ca^{2+} concentration decreases. Extended synaptotagmin 1 (ESyt1) is anchored to the ER by an N-terminal membrane insertion hook and binds to the PM via C2 domains. $PI(4,5)P_2$ is one of the acidic lipids that interacts with at least one of the C2 domains. ESyt2 and -3 have similar architecture except they have fewer C2 domains; they also bind $PI(4,5)P_2$ (not shown). The SMP domain of ESyts can transfer diacylglycerol (DAG) when the cytoplasmic Ca^{2+} concentration reaches supra-micromolar levels. The phosphatidylinositol transfer protein (PITP), Nir2, is anchored to the ER through an interaction with the ER-localized VAP (-A and -B) proteins via the Nir2 FFAT sequence. It is recruited to the PM through interaction with the lipids, phosphatidic acid (PA) and DAG both produced from phospholipase C-mediated hydrolysis of $PI(4,5)P_2$. The PITP domain of Nir2 is able to transport phosphatidylinositol (PI) from the ER to the PM and phosphatidic acid (PA) in the reverse direction. The ORP5/8 proteins are anchored to the ER by their C-terminal transmembrane domains, whereas their N-terminal PH domains with the aid of adjacent polybasic sequences bind to PM phosphatidylinositol 4-phosphate (PI4P) (and possibly $PI(4,5)P_2$). The lipid transfer domain (ORD) of these proteins is capable of binding either PI4P or phosphatidylserine (PS) and exchanges these lipids between the two membranes using the PI4P gradient between the PM and the ER. This gradient is maintained by the action of the enzyme phosphatidylinositol 4-kinase α in the PM (not shown) and the Sac1 phosphatase in the ER.

STIM and Orai proteins constitute store-operated Ca^{2+} entry in endoplasmic reticulum–plasma membrane contact sites

It was over 10 years ago when the molecular basis of store-operated Ca^{2+} entry (SOCE) was finally identified. SOCE has long been recognized as a ubiquitous Ca^{2+} entry route that is activated upon stimulation of mammalian cells through Ca^{2+}-mobilizing cell surface receptors, and which is ultimately controlled by the Ca^{2+} filling state of the endoplasmic reticulum (ER) (Putney, 1986). Based on pioneering studies from the Meyer, Stauderman, Rao, Lewis, Penner and Kinet laboratories, stromal interaction molecule 1 (STIM1) was identified as the ER luminal Ca^{2+} sensor (Liou *et al.* 2005; Roos *et al.* 2005) and Orai1 as the plasma membrane (PM) Ca^{2+} channel (Feske *et al.* 2006; Prakriya *et al.* 2006; Vig *et al.* 2006). These proteins work together to constitute SOCE. Because of the localization of the two proteins in the ER and PM, respectively, they can only work at sites where the two membranes are in close proximity, i.e. in ER–PM contact areas (Lewis, 2007). This simple fact has drawn significant attention to this special compartment, which turned out to hold a great amount of exciting new biology to be discovered. Great progress has since been made and it is now well established that ER luminal Ca^{2+} decrease causes STIM1 molecules to change their conformation such that they undergo massive clustering in the ER and interact and activate the Orai1 channels in the PM. Several important reviews have summarized these advances (Hogan *et al.* 2010; Soboloff *et al.* 2012; Hogan & Rao, 2015; Derler *et al.* 2016*a*; Putney *et al.* 2017; Zhou *et al.* 2017). More recent studies have revealed structural features of the STIM1 and Orai1 proteins that are critical for the activation process. These include the identification of the part of the STIM1 molecule that interacts with the Orai1 channel (called SOAR, Yuan *et al.* 2009, or CAD, Park *et al.* 2009) and the characterization of an intramolecular interaction between the SOAR domain and the first membrane-adjacent coiled-coil domain of the cytoplasmic part of STIM1 that keeps the SOAR domain from activating Orai1 in the resting state (Muik *et al.* 2011; Zhou *et al.* 2013; Fahrner *et al.* 2014; Ma *et al.* 2015). Similar structural and mutational studies reveal the key interactive motifs that form the basis of interaction between SOAR and Orai1 and the mechanism of Orai1 channel activation (Derler *et al.* 2013; Gudlur *et al.* 2014; Zhou *et al.* 2016; Nwokonko *et al.* 2017; Palty *et al.* 2017).

Lipid composition within special membrane domains controls STIM1/Orai1 function

While these structural studies are critically important to better understand the details of the molecular events leading to activation of STIM1/Orai1, it is equally intriguing how the lipid composition of the membrane determines the function of the STIM1/Orai1 complex. Soon after the discovery of STIM1 it was recognized that its cytoplasmic C-terminus contains a polybasic stretch that helps the protein to maintain PM contact even in the resting state (Liou *et al.* 2007; Park *et al.* 2009). While this part of STIM1 is dispensable for activation when both STIM1 and Orai1 are overexpressed, it certainly increases the efficiency of the activation process during physiological conditions. It has been debated what the STIM1 polybasic domain binds to in the PM, but anionic phospholipids have been the prime candidates. The consensus that appears to have been reached identifies phosphatidylinositol 4,5-bisphosphate ($PI(4,5)P_2$) as the lipid that binds the polybasic domain of STIM1 (Walsh *et al.* 2010; Maleth *et al.* 2014; Cao *et al.* 2015). It should be noted, though, that our studies addressing this question could not substantiate $PI(4,5)P_2$ as the major STIM1 PM anchor molecule, possibly because of the use of overexpressed proteins (Varnai *et al.* 2007; Korzeniowski *et al.* 2009). Yet, another inositol lipid, PI4P was suspected to control Orai1 channel activity, based on the sensitivity of SOCE and I_{CRAC} (the current carried by Orai1) to PI-kinase inhibitors that affected PI4P but not $PI(4,5)P_2$ levels (Rosado & Sage, 2000; Broad *et al.* 2001; Korzeniowski *et al.* 2009). While many ion channels and transporters require phosphoinositides (mainly $PI(4,5)P_2$ for proper function (see a list in Balla, 2013), this aspect of Orai channel regulation has not been fully explored. It will be important to see if the PI4P-mediated regulation of Orai1 channels can be better understood with the use of newly available more specific phosphatidylinositol 4-kinase (PI4K) inhibitors (Bojjireddy *et al.* 2014). In light of the new developments in the biology of contact sites and the fact that PI4P gradients shape the lipid composition of membranes, there are a number of possibilities regarding how changes in PM PI4P levels may indirectly affect the function of the Orai1 channels (see below).

Additional data also indicated that membrane microdomains and their special lipid composition play critical roles in the control of SOCE. Cholesterol depletion was reported to increase STIM1/Orai1-mediated Ca^{2+} influx or I_{CRAC} currents, an effect that was attributed to cholesterol interaction with the N-terminal segment of Orai1 (Derler *et al.* 2016*b*) and also with the STIM1 SOAR domain affecting its association with Orai1 (Pacheco *et al.* 2016). Even earlier studies had reported on the influence of cholesterol status on SOCE, showing its effect on changes in Ca^{2+}-dependent inactivation (Dionisio *et al.* 2011) and the control of STIM1 clustering and interaction with transient receptor potential channel (TrpC1) (Pani *et al.* 2008). Furthermore, additional studies suggested that cholesterol status also affects the $PI(4,5)P_2$-mediated lateral segregation of STIM1–Orai complexes (Calloway *et al.* 2011). This notion was extended in studies where the Ca^{2+}-dependent inactivation of Orai1 channels by the accessory protein SARAF (SOCE-associated regulatory factor) (Palty *et al.* 2012) was found to require partitioning of the STIM1/Orai1 complex to $PI(4,5)P_2$-enriched PM compartments (Maleth *et al.* 2014).

ER–PM contacts control lipid dynamics between these organelles

The question of what molecules keep the ER in close contact with the PM has been first addressed in yeast. It was found that the synaptotagmin-like proteins, tricarbins (Tcb1/2/3), and three other proteins, one called Ist2 and two ER-resident proteins, Scs2 and Scs22, were responsible for maintaining the ER–PM connection. Elimination of all six proteins disrupted association of ER with the PM and caused major dysregulation of phosphoinositide signalling and induced the unfolded protein response in the ER (Manford *et al.* 2012). The mammalian homologues of the Tcb proteins, the extended synaptotagmins (ESyt1/2/3), were then subsequently found to be important for ER–PM tethering in mammalian cells (Chang *et al.* 2013; Giordano *et al.* 2013). An important feature of the ESyts and Tcbs is that they possess an SMP (synaptotagmin-like mitochondrial-lipid-binding protein) domain, which is found in many proteins that localize to membrane contact sites (Toulmay & Prinz, 2012). The structure of the SMP domain of ESyt2 revealed a β-barrel module similar to those found in the tubular-lipid-binding (TULIP) superfamily. In its dimeric state, the SMP domain of ESyt2 forms an approximately 90 Å-long cavity encompassing a hydrophobic channel that could serve as a tunnel to pass hydrophobic molecules, including lipids, through the aqueous phase that separates the two membranes in contact sites (Schauder *et al.* 2014; Jeong *et al.* 2017). Indeed, cells that lack all three ESyts showed a defect in removing diacylglycerol (DAG) from the PM during massive Ca^{2+}-induced phospholipase C (PLC) activation suggesting that they can participate in DAG transfer between the PM and other membranes (Saheki *et al.* 2016). This latter study also showed that lipid transfer by SMP domains *in vitro* requires Ca^{2+} concentrations in the hundred micromolar range (Saheki *et al.* 2016). It is noteworthy, though, that ESyt depleted or knockout cells appear to have normal SOCE mediated by STIM1/Orai1 (Giordano *et al.* 2013) and the fact that ESyt triple knockout mice show no obvious phenotype (Sclip *et al.* 2016) suggests that functionality of the ER–PM contacts can be maintained by compensatory mechanisms.

Another SMP domain-containing protein, TMEM24, has been shown to play an important role in pancreatic β cells for glucose-induced Ca^{2+} signals and insulin secretion (Pottekat *et al.* 2013), and it was suggested that it serves as a phosphatidylinositol (PI) transfer protein to supply the PM with PI for maintaining the PM $PI(4,5)P_2$ pool necessary for exocytosis of insulin-containing vesicles (Lees *et al.* 2017). ESyts also contain C-terminal C2 domains that make contacts with PM $PI(4,5)P_2$ and perhaps with other anionic phospholipids depending on the Ca^{2+} concentration (Chang *et al.* 2013; Giordano *et al.* 2013) (see below).

Other lipid transfer proteins are also linked to ER–PM contact sites. The Nir2 protein, which belongs to the family of PI transfer proteins (PITPs), was shown to translocate to ER–PM contact sites during activation of PLC-coupled receptors to support PI transfer from the ER to the PM during PLC-mediated consumption of PM $PI(4,5)P_2$ (Chang *et al.* 2013; Kim *et al.* 2013). Subsequent studies showed that the Nir2 protein and its fly orthologue, RdgBα, can also transfer phosphatidic acid (PA) from the PM to the ER at these contact sites when PLC is activated (Kim *et al.* 2015; Yadav *et al.* 2015). Recruitment of the Nir2 proteins to the contact sites is provided by their interaction with the ER-localized VAP-A and VAP-B proteins via the Nir2 FFAT (double phenylalanine in an acidic tract) domain (Peretti *et al.* 2008) on the one hand, and with the PA that is generated by PLC activation in the PM on the other (Chang *et al.* 2013; Kim *et al.* 2013, 2015). Although Nir2 may also function in other contexts at different contact sites, such as the ER and Golgi (Litvak *et al.* 2005), its dynamic recruitment to ER–PM contacts, together with its PI and PA transport functions, supports the reutilization of PA and maintenance of $PI(4,5)P_2$ during PLC action.

Another lipid transfer mechanism at ER–PM contacts has been recently described that links non-vesicular phosphatidylserine (PS) transport to the PM with utilization of PI4P gradients that exist between the PM and ER. It has been known for a while that the PI4P pool made by the yeast Stt4 PI4K is kept under control by the Sac1 phosphatase located in the ER (Foti *et al.* 2001). More recent yeast studies showed that the ER-localized Sac1 can access PI4P produced in the PM with the aid of some yeast oxysterol binding protein homologues, such as Osh3 (Stefan *et al.* 2011). Curiously, in earlier studies, Sac1-deleted yeast strains showed a large accumulation of PI4P that did not translate to increased $PI(4,5)P_2$ levels (Guo *et al.* 1999; Hughes *et al.* 2000; Foti *et al.* 2001). These findings suggested that the PI4P accumulated in a compartment where the PIP 5-kinase was unable to convert it to $PI(4,5)P_2$, possibly in the ER. This apparent conundrum can be explained by the recent discovery that PI4P gradients can be utilized to support non-vesicular transport of cholesterol from the ER to the Golgi both in yeast and mammalian cells (de Saint-Jean *et al.* 2011; Mesmin *et al.* 2013) and, in a similar fashion, to control transport of PS from the ER to the PM (Maeda *et al.* 2013; Chung *et al.* 2015; Moser von Filseck *et al.* 2015). In the mammalian cell, the latter transport is mediated by the oxysterol binding protein related proteins (ORP5) and ORP8. Both of these molecules are anchored to the ER by their C-terminal hydrophobic domains and make contact with the PM via their PH domains that bind PI4P (Chung *et al.* 2015). Their lipid transfer domain is able to bind either PI4P or PS but not both at the same time. The PI4P gradients between the ER and the PM are maintained by the action of the PI4KA enzyme in the PM (Balla *et al.* 2007; Nakatsu *et al.* 2012; Bojjireddy *et al.* 2014) and the Sac1 phosphatase in the ER to which PI4P is delivered by the ORP proteins. This arrangement raises the question of how PM PI4P flux is divided between conversion to $PI(4,5)P_2$ and consumption for PI4P/PS exchange. Curiously, $PI(4,5)P_2$ levels can be maintained even when PI4P levels fall in the PM, as long as PLC is not activated, suggesting a functional dissociation between these two lipid pools in spite of their known substrate–product relationship (Hammond *et al.* 2012; Nakatsu *et al.* 2012; Bojjireddy *et al.* 2014). Another important conclusion of these findings is that PI4P metabolism has a great impact on PS levels and, conversely, PS overproduction has a major effect on PI4P levels both in the PM and other PI4P-rich compartments, such as the Golgi (Sohn *et al.* 2016).

Lastly, a new family of proteins called Lams (Elbaz-Alon *et al.* 2015; Gatta *et al.* 2015) or Ltcs (Murley *et al.* 2015) were identified in yeast as sterol transport proteins that function between contact sites of various organelles, including the ER and PM. Mammalian homologues of these proteins have been identified (Gatta *et al.* 2015; Murley *et al.* 2015) and it will be interesting to identify which if any of them works in ER–PM contacts and has an impact on lipid composition and Ca^{2+} regulation.

Ca^{2+} has important roles in the control of ER–PM contacts

It is hard not to note the close interrelationship between Ca^{2+} signals generated at the PM in ER–PM contact sites and the tethering molecules that maintain the structure and the lipid composition on either side of the contact region. First, the interaction of ESyt1 with the PM is regulated by Ca^{2+} (Chang *et al.* 2013; Giordano *et al.* 2013), while ESyt2 and ESyt3 use one of their C2 domains to interact with PM $PI(4,5)P_2$. It is not entirely clear what lipid is the main binding partner of ESyt1 and it may depend on the Ca^{2+} concentration. ESyt1 localization during Gq-mediated PLC activation suggests that moderate cytoplasmic Ca^{2+} elevations enhance binding to $PI(4,5)P_2$, which then becomes a limiting factor when high PLC activity induces $PI(4,5)P_2$ depletion. However, further

Ca^{2+} increases (probably reaching over 10 μM) again increase ESyt1 PM association even when $PI(4,5)P_2$ is no longer available (Saheki *et al.* 2016). This dual PM recruitment depending on the Ca^{2+} may reflect the participation of more than one of the five C2 domains found in this molecule and more than one lipid in the PM (Idevall-Hagren *et al.* 2015).

Calcium, of course, will have an impact on phosphoinositides, especially during receptor activation of PLC, and through those changes can affect both ESyt PM interactions (see above), they can also affect the ORP5 and ORP6-mediated PI4P/PS exchange process. Since these ORPs maintain PM contact through PI4P interaction, their PM engagement and hence lipid transfer function is limited by the available PI4P in the PM as PI4P levels in the PM usually follow the same kinetics as $PI(4,5)P_2$ during strong PLC activation. Turning off ORPs this way will affect PS transport and perhaps that of other lipids that may impact the STIM1/Orai1 function. Another recent study identified RASFF4, one of the Ras association domain family proteins, as regulators of SOCE via controlling $PI(4,5)P_2$ production thus maintaining ESyt2 and ESyt3 PM contacts (Chen *et al.* 2017). Higher cytoplasmic Ca^{2+} increases that are evoked by Ca^{2+} ionophores, or during PM damage and repair can activate PLC without receptor stimulation, but also initiate more profound changes in the distribution of other lipids, such as DAG (see above) and PS. PS externalization occurs via Ca^{2+} activation of scramblases such as the TMEM16F (Suzuki *et al.* 2010; Bevers & Williamson, 2016; Brunner *et al.* 2016; Gyobu *et al.* 2017). Incidentally, TMEM16F is a member of the anoctamin family of 10 proteins (hence also called ANO6). Several members of this family are chloride channels (Kunzelmann *et al.* 2016) and, notably, they are the mammalian orthologues of the yeast Ist2 that was identified as one of six yeast proteins important for maintaining ER–PM contacts (see above). Anoctamins are Ca^{2+}-regulated proteins that have been linked to a variety of diseases and it is hotly debated whether their Cl^- channel or scramblase activities are more important. It is noteworthy, though, that yeast Ist2 is primarily ER localized contacting the PM with its C-terminal polybasic domains, whereas the cellular distribution of anoctamins and their role(s) in stabilizing ER–PM contacts is still poorly understood (Kunzelmann *et al.* 2016).

Morphological data indicate that ER–PM contact sites are heterogeneous: some of the cortical ER juxtaposed to the PM has a very narrow lumen excluding even ER luminally targeted proteins, such as green fluorescent protein (GFP) (Orci *et al.* 2009; Giordano *et al.* 2013; Fernandez-Busnadiego *et al.* 2015; Wu *et al.* 2017). Overexpression of STIM1 causes enlargement of the ER–PM contact areas (even without expression of Orai1) and can generate stacks of ER beneath the PM (Orci *et al.* 2009). It is not clear if the morphological differences reflect functionally distinct contact areas and what signalling modalities are associated with them.

Concluding remarks

The physiology and pathology of ER–PM contacts has become one of the most exciting research areas since the discovery of STIM1/Orai1 proteins. More and more proteins are discovered that work at these contact sites and it is a recurring question whether they are simply recruited to these sites to fulfil their function or they contribute to the structural stability of this compartment. ER–PM contacts are critically important for non-vesicular lipid transfer and also for SOCE, but the possible presence of anoctamine Cl^- channels and even potassium channels (Fox *et al.* 2015; Fu *et al.* 2017) in these sites suggest that other less explored functions may add to their complexities. Even from this limited overview one theme has to emerge: that is the close interrelationship between Ca^{2+} and lipid signals that influence one another in profound ways. While most attention is focused on the lipid composition of the PM at these contact zones, relatively little is known about the lipid composition of the ER side of the contacts and how those lipids affect ER-related processes, including Ca^{2+} release and uptake pathways. Lastly, these new developments have made it clear that the imaging tools we have been using to determine lipid distribution are too crude to assess the local enrichment of these lipids around specific structures or molecular clusters. Another important point to realize is that *in vitro* lipid transfer assays based on transport between lipid vesicles may not be sufficient to understand how these processes are organized in the intact cell. We have to find ways to monitor lipid transfer in the intact cell where the fine structure is undisturbed. In summary, ER–PM contacts represent an exciting research field that not only shapes our current concepts regarding lipid distribution and metabolism, but also poses methodological challenges that we have to meet to fully understand the impact of this membrane organization on specific cellular functions.

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Additional information

Competing interests

None declared.

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