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PHOSPHOINOSITIDES AND CALCIUM SIGNALING. A MARRIAGE ARRANGED IN ER-PM CONTACT SITES

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SUMMARY

Calcium (Ca^{2+}) ions are critically important in orchestrating countless regulatory processes in eukaryotic cells. Consequently, cells tightly control cytoplasmic Ca^{2+} concentrations using a complex array of Ca^{2+} -selective ion channels, transporters, and signaling effectors. Ca^{2+} transport through various cellular membranes is highly dependent on the intrinsic properties of specific membrane compartments and conversely, local Ca^{2+} changes have profound effects on the membrane lipid composition of such membrane sub-domains. In particular, inositol phospholipids are a minor class of phospholipids that play pivotal roles in the control of Ca^{2+} -dependent signaling pathways. In this review, we will highlight some of the recent advances in this field as well as their impact in defining future research directions.

Keywords

Phosphatidylinositol; phosphatidylinositol 4-kinase; calcium; membrane contact sites; Ca^{2+} channels

INTRODUCTION

Since the seminal observation of Sydney Ringer that Ca^{2+} ions are critical for the contractile apparatus of the heart [1], it has become well recognized that Ca^{2+} is also indispensable for the control of striated muscle contractility as well as for neuronal excitability and neurotransmission [2]. Research in the 70's and 80's subsequently showed that Ca^{2+} also serves as an important signaling molecule in non-excitable cells by communicating the activation status of specific classes of cell surface receptors in a manner similar to cyclic adenosine monophosphate (cAMP); the first established second messenger molecule. Many

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Conflict of interest statement

The Authors declare no conflict of interest

• The Authors would like to dedicate this review article to the memory of the late Sir Michael J. Berridge, who has been inspirational throughout the authors' studies on calcium signaling

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of these important discoveries coincided with those that revealed the importance of unique changes in inositol phospholipid turnover during cell activation in response to external signals [3]. These studies have laid the foundation of the discovery that phospholipase C (PLC)-mediated hydrolysis of phosphatidylinositol 4,5-bisphosphate [PI(4,5)P₂] liberates inositol 1,4,5-trisphosphate, a water-soluble second messenger molecule, and membrane-embedded diacylglycerol (DAG). InsP₃ production then directly stimulates the release of Ca²⁺ from intracellular Ca²⁺ stores leading to the increased cytosolic Ca²⁺ levels that trigger downstream cellular responses [4]. While these seminal studies firmly established the connection between phosphoinositide lipids and plasma membrane (PM)-initiated Ca²⁺ signals, they could not foresee the complexity of the interrelationship between these two signaling components that would be uncovered over the last 30 years.

Membrane contact sites are the hotspots for certain forms of Ca²⁺ entry

Discovery of the endoplasmic reticulum (ER)-localized STIM and PM-localized Orai proteins [reviewed in [5]] effectively opened up the modern era of investigations into the molecular details of the process of store-operated Ca²⁺ entry (SOCE) as initially postulated by Jim Putney [6]. SOCE is activated when the Ca²⁺ stores within the ER become depleted, which is sensed by the luminal EF hand domain of the ER-localized, single membrane-spanning, STIM1 protein. During its activation, STIM1 proteins cluster and undergo a molecular rearrangement (for details see [7]) that unmasks a small cytoplasmic segment of STIM1, called the SOAR or CAD domain, for interaction and activation of Orai1 Ca²⁺ channels present within the PM. Because of the distinct localization of these two protein components, their interaction must take place at sites where the ER and the PM make close appositions, which are commonly called ER-PM contact sites. In fact, discovery of the STIM1-Orai1 interaction drew significant attention to this specialized membrane interface. However, ER-PM contact sites not only serve as locations for SOCE, but conversely, Ca²⁺ entering through SOCE, or other Ca²⁺ entry routes, has profound impact on the structure of these contact sites and directly control lipid transport processes that determine the local lipid composition of these specialized membrane domains.

Structural features that determine how ER-PM contact sites respond to Ca²⁺ changes

There has been an enormous interest in membrane contact sites (MCS) with several excellent recent reviews [8–10]. Here we only briefly summarize a few aspects of these special compartments that are relevant to Ca²⁺ signaling. Contacts between the ER and other organelles are defined as areas where the two membranes are in close apposition (10–30nm). Such sites are enriched in proteins that either act as tethers that provide the structural framework for these sites, or proteins whose functions are confined within these structures. The distinction between these groups is not absolute as most tethers also have important functional roles within contact sites and all proteins that localize to these areas also likely contribute to maintaining their structure. Early studies in Yeast identified a small group of proteins, called the tricalbins (Tcb1/2/3), which are important for maintaining ER-PM contacts [11]. Tcbs and their mammalian homologues, the extended synaptotagmins (E-Syt1/2/3) [12, 13], are anchored to the ER through an N-terminal hydrophobic hairpin while also interacting with the PM through numerous C2 domains that bind to acidic phospholipids within the inner leaflet of the PM, with preference to binding

phosphoinositides. There are noted differences in the architecture of specific E-Syts, mainly in terms of the number of C-terminal C2 domains they contain and the requirement of Ca^{2+} for their interactions with PM-localized phosphoinositides. Specifically, E-Syt2 and E-Syt3 interact with $\text{PI}(4,5)\text{P}_2$ within the PM at resting Ca^{2+} levels, whereas PM association of E-Syt1 is dynamically regulated by Ca^{2+} : high Ca^{2+} levels (in the $>10\text{ }\mu\text{M}$ range) strongly promote E-Syt1 binding to the PM [12]. Notably, at such high cytoplasmic Ca^{2+} levels, PM $\text{PI}(4,5)\text{P}_2$ levels are known to be substantially reduced and even the levels of another important anionic lipid species, phosphatidylserine (PS), are decreased within the PM due to PS externalization [14]. This raises several questions, regarding the identity of the acidic phospholipid that supports PM association of E-Syt1 under these conditions. However, while such very high Ca^{2+} levels are never reached in the entirety of the cytoplasm under physiological conditions, they can exist close to sites of Ca^{2+} entry, especially in confined spaces such as ER-PM contacts. Therefore, STIM1/Orai1-mediated Ca^{2+} entry provides a powerful means of controlling E-Syt functions through local Ca^{2+} changes. Importantly, cells lacking all three E-Syts still display normal SOCE [12].

E-Syts also contain a conserved synaptotagmin-like, mitochondrial and lipid-binding protein (SMP) domain that has been described in numerous proteins that are found in many MCS, not just those established between the ER and PM [15]. SMP domains are suitable for lipid transport as they can form a hydrophobic tunnel inside their β -barrel structure, which can extend up to $90\text{ }\text{\AA}$ in dimeric states [16]. Importantly, in the case of E-Syt1, one of the C2 domains exerts an intramolecular autoinhibition to the SMP domain, which can be relieved at high Ca^{2+} concentrations [17], and it was shown that E-Syts can transport diacylglycerol (DAG) under these high Ca^{2+} conditions [18].

Recent studies have identified another SMP domain-containing protein, called TMEM24, which is also found in ER-PM contacts and regulated by Ca^{2+} [19]. TMEM24, initially described in pancreatic β cells [20], is highly expressed in the brain, primarily within neurons, while its paralog C2CD2L is more widely expressed, but is particularly enriched in liver [21]. These proteins are anchored to the EM through an N-terminal transmembrane domain that is followed by an SMP domain and a medial C2 domain. They also contain a highly conserved C-terminal segment that is rich in basic residues and mediates the association of TMEM24 with the PM. Importantly, PM association of TMEM24 is regulated by PKC-mediated phosphorylation of C-terminal serine residues that disrupt PM localization, while dephosphorylation of this segment is carried out by the Ca^{2+} regulated protein phosphatase, calcineurin [19]. Evidently, TMEM24 was shown to play a role in the generation of oscillatory Ca^{2+} signals and insulin release in response to elevated glucose in pancreatic β cells [20]. It was proposed that the ability of the TMEM24 protein to transport phosphatidylinositol (PI) was responsible for its role in maintaining glucose-induced Ca^{2+} signals [19].

Non-vesicular lipid transfer has a strong connection to Ca^{2+} signaling at ER-PM contacts

While SMP domain-containing proteins are able to function as regulators of lipid transport between membranes, many other proteins capable of mediating non-vesicular lipid transfer between organelles have been identified in MCS. These include oxysterol binding protein

(OSBP) and its related proteins (ORPs), members of the StAR domain-containing family of proteins (GRAMD/Lam/Ysp/Aster), ceramide transfer protein (CERT), and the Class II PI transfer proteins (PITPs; Nir2/3)[22]. Some of these proteins have been shown to transfer specific lipids between the ER and other organelles, including significant roles at ER-Golgi contacts, but the ones that function between the ER and the PM all have strong connections to Ca^{2+} signaling generated from the PM.

Class II PITPs—Nir2/3 are the mammalian orthologues of the fly RdgBa protein and belong to the Class II subfamily of PITPs [23]. They both possess an N-terminal PITP lipid-binding domain and are anchored to the ER via an FFAT (double phenylalanines in an acidic track) motif that strongly interacts with the ER-localized proteins, vesicle-associated membrane protein-associated (VAP)A and B. Nir2 was shown to transport phosphatidic acid (PA) from the PM to the ER during receptor-induced activation of PLC and phospholipase D (PLD). It is believed that Nir2, and probably Nir3, help to shuttle PA from the PM to the ER where it can be used as a precursor for the re-synthesis of PI during prolonged PLC activation [24]. Nir2, which is found throughout the ER in unstimulated cells, with enrichment in ER-Golgi contacts, rapidly translocates to ER-PM contacts during PLC activation due to the binding of its C-terminal LNS2 domain to PA [25]. This PM attachment is likely helped by local DAG, which is generated in the PM during receptor stimulation [24]. Without Nir2, PI synthesis and delivery to the PM is impaired, which eventually leads to depletion of PI4P and PI(4,5)P₂ within the PM [13, 25, 26]. The light-induced retinal degeneration observed in the RdgB fly has also been linked to the dual PA/PI transfer function of the protein [27], underscoring the importance and evolutionary conservation of this process.

Although not a genuine tether or lipid transfer protein, the recently identified Kv2.1 voltage gated potassium channel is also localized to ER-PM contacts, responds to changes in Ca^{2+} , and is linked to the lipid transfer functions mediated by Nir2/3. This channel is found in the PM and has a C-terminal tail that contains a phosphorylation-dependent FFAT-like motif that can interact with VAPA and VAPB [28, 29]. Kv2.1 was also shown to interact with Nir2/3 and facilitate their recruitment to ER-PM contact sites. Moreover, the same study also found that brain PI4P and PI(4,5)P₂ contents were significantly reduced in Kv2.1 knockout mice[30].

ORP proteins—Several members of the ORP family of lipid transfer proteins also work in ER-PM contact sites [31]. In particular, ORP5 and ORP8 are anchored to the ER via their C-terminal TM domains and interact with the PM via an N-terminus that includes a pleckstrin-homology (PH) domain [32]. These proteins utilize the PI4P gradient that is set Pre-between the PM and the ER by the PM-localized phosphatidylinositol 4-kinase (PI4K) isoform, PI4KA, and the ER-localized PI4P phosphatase Sac1 to deliver PS from the ER to the PM [32]. Since PM interaction of ORP5 and ORP8 is sensitive to both PI4P and PI(4,5)P₂ levels, the lipid transfer activity of these proteins depends on the PM content of these phosphoinositides. This creates a regulatory loop that controls the levels of PM PI(4,5)P₂ through adjusting the PM pool of PI4P available for PI(4,5)P₂ synthesis [33, 34]. Another member of the ORP family, ORP3, also works at ER-PM junctions but its activity is

uniquely regulated by PKC-mediated phosphorylation [35]. ORP3 is ER bound via interaction of its tandem FFAT motifs with ER-localized VAPA/B, but its PM association through binding to phosphoinositides only manifests after PKC activation [36]. Ca^{2+} has been shown to play an important role in the ORP3 activation process, most likely through PKC. Although the exact lipid that is transported by ORP3 still waits identification, active ORP3 is capable of depleting PI4P from the PM. Importantly, activated ORP3 inhibits SOCE and it may exert its inhibitory effect even when it cannot transport PI4P [36].

Another ORP that affects PM phosphoinositide levels is ORP2, which delivers cholesterol from the ER into the PM and extracts PI(4,5) P_2 from the PM [37]. Curiously, ORP2 lacks a PH domain for PM interaction but it does have an FFAT motif for binding to VAP within the ER. Although ORP2 was shown to alter PM cholesterol and PI(4,5) P_2 levels, its intracellular localization to lipid droplets and the Golgi suggests a more complex involvement in several aspects of lipid metabolism [38].

GRAMD/Aster proteins—Lastly, some members of the growing GRAMD/Aster family of sterol transport proteins are located in ER-PM contacts and transport cholesterol from the PM into the ER [39, 40]. Notably, GRAMD1/Aster proteins are the mammalian orthologues of the yeast Lam/Ltc proteins that also transport sterols between the ER and PM in contact sites in yeast [41, 42]. GRAMD1a-c [40], which were independently identified and named Aster-A, -B and -C [39], contain a C-terminal transmembrane domain localizing the protein to the ER and an N-terminal GRAM domain that is structurally related to PH domains, which ensures PM localization. In between these domains, GRAMD1 proteins possess a lipid transfer VAS_t domain that shows similarity to the START-related lipid binding domains. Aster A,B and C show different tissue distributions, and Aster B is critical for transport of cholesterol from the PM to the ER in steroidogenic tissues [39]. Intriguingly, the GRAM domain of Aster-B shows affinity to PS and PA, *in vitro*, but only interacts with the PM after the PM is loaded with cholesterol [39]. Under cholesterol rich conditions, Aster-B is found in ER-PM contacts but only partially co-localizes with contact sites marked by E-Syt2, E-Syt3, or Orp5 [39]. A separate study showed that GRAMD1a (corresponding to Aster-A) did not co-localize with E-Syt2 or E-Syt3 [40]. This study also identified two additional proteins, GRAMD2a and GRAMD2b, that possess the C-terminal ER-targeting transmembrane and GRAM domains but lack the VAS_t lipid transfer domain [40]. The same study found that GRAMD2 did localize to ER-PM contacts by binding to PI(4,5) P_2 , and perhaps to a lesser extent PI4P, and does mark the contact sites to which STIM1 translocates upon depletion of ER Ca^{2+} stores [40]. Importantly, during STIM1 activation, the STIM1 puncta that form only initially co-localize with GRAMD2, but the two proteins slowly laterally separate [40]. This is most likely due to spatial constraints that are imposed by the short GRAMD2 protein on the size of the ER-PM contacts, which is similar to what was observed earlier using engineered inducible short ER-PM linkers along with STIM1/Orai1 [43]. This tightening of the ER-PM contacts by GRAMD2 may also contribute to the lack of co-localization between the larger GRAMD1a and the shorter GRAMD2 proteins when expressed as a GFP-fusion constructs [40]. It is important to note that while STIM1 puncta are smaller in GRAMD2 depleted cells, the Ca^{2+} flux through SOCE is unaffected [40].

Membrane lipid composition controls various forms of Ca^{2+} entry

The reason so much attention is paid to ER-PM contacts and lipid transfer processes is because of their impact on SOCE and other forms of Ca^{2+} entry. However, it is important to note that not all ER-PM contact proteins affect Ca^{2+} signaling. For example, as mentioned earlier, the E-Syts and GRAMD2 proteins are dispensable for SOCE. While an extensive list of the Ca^{2+} regulatory processes are affected by various classes of lipids, as reviewed recently in [44], here we will focus on those lipid components of the PM that have been implicated in the regulation of SOCE, some of which also control other forms of Ca^{2+} entry.

Phosphoinositides—STIM1 contains a polybasic segment at its C-terminus that was found to be important for the optimal positioning of the protein within ER-PM contact sites for interactions with Orai1 channels upon ER Ca^{2+} store depletion [45]. This polybasic segment is dispensable when STIM1 and Orai1 proteins are overexpressed, which made it more difficult to characterize this regulation. Most studies refer to $\text{PI}(4,5)\text{P}_2$ as a PM interacting partner of the STIM1 polybasic segment, but investigations into this question indicate that PI4P plays an equal, if not more important role. For example, genetic [46] or pharmacological [47] inactivation of PI4KA , the enzyme responsible for generating PI4P in the PM, strongly inhibits SOCE or the Ca^{2+} current associated with SOCE (I_{CRAC}) even though, curiously, $\text{PI}(4,5)\text{P}_2$ levels were not found to be decreased within the PM. These data, however, do not rule out the possibility that $\text{PI}(4,5)\text{P}_2$ changes in specific membrane domains might still be the ultimate regulators of the STIM1 activation process. For example, one of the Ras association domain family proteins, RASFF4 , was shown to regulate SOCE via $\text{PI}(4,5)\text{P}_2$ generation [48]. Alternatively, the Ca^{2+} -dependent inactivation of SOCE, which is mediated by the STIM1-interacting molecule SARAF (SOCE-associated regulatory factor), was also shown to depend on the lateral segregation of the complex into $\text{PI}(4,5)\text{P}_2$ -rich PM domains [49].

Phosphoinositides also regulate other forms of Ca^{2+} entry. It is increasingly evident that many membrane-spanning proteins strongly interact with phosphoinositides. This not only includes ion channels and transporters (see [50] for a list), but also G protein-coupled receptors and phospholipid flippases [51, 52]. Phosphoinositides indirectly regulate voltage-gated Ca^{2+} channels (see [50] for original citations), but their indirect activation can be the result of potassium channel closure that depolarizes the PM, a process that is also regulated by phosphoinositides [53]. An important and poorly understood phenomenon related to this regulation is that activation of SOCE results in the inhibition of voltage-gated Ca^{2+} channels. Apparently, this inhibition is mediated through interactions between the $\text{Ca(V)}1.2$ channels with the SOAR/CAD domain of STIM1 that also activates Orai1 [54]. This interesting interplay between SOCE and voltage-gated Ca^{2+} entry [55], could have important functional implications, especially in neuronal [56] or neuroendocrine tissues, and certainly warrants further exploration.

In addition to SOCE and voltage-gated Ca^{2+} channels, several members of the transient receptor potential (TRP) channels contribute to Ca^{2+} entry and are also regulated by phosphoinositides. Prominent among these are the cold and hot-sensing TRPM8 and TRPV1 channels, respectively [57]. The control of TRPV1 has been especially complicated as both

positive and negative regulatory influences have been associated with phosphoinositides [58]. A detailed discussion of the large body of literature associated with TRP channel regulation by phosphoinositides is beyond the scope of this review and can be found elsewhere [59].

Diacylglycerol and Phosphatidic Acid—The best-known ion channels regulated by DAG are selected members of the TRP channel family. Typically, they respond to PLC-activation, as established for their prototypical fly orthologue, TRP [60]. DAG has been shown to activate mammalian TRPC3/6/7 channels [61, 62] but their DAG sensitivity is also influenced by PI(4,5)P₂ [63]. Other TRP channels, such as TRP4 and TRP5 only respond to DAG after dissociation from Na⁺/H⁺ exchanger regulatory factors (NHERF) 1 and 2 [64]. Other DAG regulated channels include the two-pore domain potassium channels (K2P) such as TASK1 and TASK3 that are inhibited by DAG [65]. Activity of some of the K2P channels is also dependent on phosphoinositides [66] and through their control of membrane potential they can indirectly control Ca²⁺ signaling. Ion channels controlled by PA include the voltage-gated potassium (Kv) channels [67] and the K2P channels TREK1 and TREK2, whose activity is regulated by PLD2-mediated local PA production [68]. Remarkably, based on direct analysis of reconstituted TREK1 channels, PI(4,5)P₂ can compete with PA for binding to the channel and hence the balance of these two lipids determine channel activity [69].

Phosphatidylserine—Enrichment of PS in the inner leaflet of the PM is also important for the maintenance and regulation of Ca²⁺-dependent signaling pathways. PS asymmetry is maintained by phospholipid flippases that use ATP hydrolysis to flip PS from the outer to the inner leaflet of the PM, and which, incidentally, are also regulated by PI(4,5)P₂ [52, 70, 71]. In contrast, PS externalization is carried out by PS scramblases, such as TMEM16F, the activity of which is controlled by cytoplasmic Ca²⁺ increases [72–75]. Interestingly, although TMEM16F is a member of the anoctamin family of proteins (corresponding to ANO6), not all anoctamins function as lipid scramblases. In fact, it has been shown that several anoctamin proteins actually function directly as chloride channels [76]. Moreover, the diverse family of anoctamins have recently come into focus as they are the mammalian orthologues of the yeast Ist2 protein. Ist2 is one of six yeast proteins, along with the tricalbins and the VAP orthologue Scs2, discussed above, that are identified as essential for maintaining ER–PM contacts [11]. The topological conservation between Ist2 and anoctamins is not complete, however, as Ist2 is primarily ER localized and contacts the PM with a C-terminal polybasic domain, whereas the location of anoctamins and their role(s) in stabilizing ER–PM contacts are still poorly understood [76]. A recent study identified ANO8 as important for stabilizing ER–PM contacts and organizing several proteins that control SOCE, including STIM1/Orai1 [77]. The exact mechanism by which PS contributes to the control of Ca²⁺ channels has not been explored in depth. However, being the major contributor to the negative electrostatic charge of the inner leaflet of the PM [78], it is possible that PS could stabilize the transmembrane helices of integral proteins via lateral interactions with the positive residues that often define their lipid phase boundaries.

Cholesterol—The proposed existence of lipid microdomains within the PM that are defined as being rich in cholesterol and sphingolipids has been fueled by the notion that the PM has a high relative cholesterol content [79]. Many signaling processes have been proposed to be active in such microdomains that are also variably considered to be rich in phosphoinositides [80], although this latter view has not received general acceptance [81]. Regardless of which side of this debate one stands, there is a vast amount of literature showing regulation of the activity of various Ca^{2+} channels, including TRPC channels and SOCE, by the cholesterol content of the PM. However, the effects of cholesterol depletion on SOCE activity are variable and also appear to show differences based on the tissues studied and experimental conditions. For example, reducing cholesterol content in the PM was shown to induce Orai1 channel internalization and lateral mobility that was dependent on caveolin 1 expression [82]. Another study showed the requirement of cholesterol for establishing STIM1/Orai1 interactions within the PM [83]. In contrast, other studies showed that cholesterol depletion increased SOCE via regulating interactions between the STIM1 SOAR domain with Orai1 [84] or through direct cholesterol regulation of Orai1 itself [85]. In another mode of regulation, TRPC1 channel association with STIM1 during activation of SOCE was shown to occur in specialized “lipid raft” domains [86].

Several studies suggested that cholesterol content exerts its effects by modifying lateral $\text{PI}(4,5)\text{P}_2$ distribution or interactions with STIM1/Orai1. One study suggested that the activity of STIM1/Orai1 complexes is positively affected by $\text{PI}(4,5)\text{P}_2$ in ordered lipid domains, while the opposite was true in disordered membrane domains [87]. A very recent study has also shown that the activity of $\text{PI}4\text{P}$ -specific 5-kinase that generates $\text{PI}(4,5)\text{P}_2$ is controlled by the PS and cholesterol content of the PM and that this regulation could very well be limited to ER-PM contact sites where the local lipid composition can be significantly different from the bulk of the PM [88]. It is apparent from these studies that some effects of cholesterol content and distribution is actually indirect, translated to phosphoinositide changes and their interaction with the Ca^{2+} influx machinery.

Concluding Remarks

Early studies seeking to establish the connection between phosphoinositide changes and Ca^{2+} signaling revolved around the question of whether PI turnover was the cause or the consequence of Ca^{2+} signals in hormone stimulated cells. It only took 50 years to realize that the arguments on both sides were partially correct as the two events are inseparable. The last 10 years have expanded our knowledge and identified new molecules and regulatory mechanisms that add to the complexity of this question. In particular, the importance of MCS and localized lipid domains for the integrated control of both inositol lipid metabolism and the activity of Ca^{2+} entry channels are only beginning to emerge. These recent developments have been aided by the establishment of experimental approaches and molecular tools that allow for the detection and controlled local manipulation of membrane lipid composition and Ca^{2+} signals. These new insights have since provoked new questions and completely changed our views regarding the spatial and temporal properties of signal propagation and containment within the PM. The emergent regulatory paradigms have also highlighted the fact that the tools that were introduced 10 years ago and used to remarkable effects are no longer good enough to probe and understand these complex processes at a

deeper level. Many new, and some not so new, questions need answers. How membrane lipids can be “extracted” and loaded into lipid transfer proteins or SMP-based lipid tunnels, or how they can then be “inserted” into already existing membranes are critical questions to answer for a deeper understanding of membrane dynamics and energetics. What molecules and processes determine the extent to which local lipid signals can propagate and dissipate? Exciting new data are already emerging on lipid dynamics and the complex interactions between membrane lipids and lipid-binding or lipid-modifying proteins using supported lipid bilayers [89].

A recurring theme throughout this review is the central role of PI(4,5)P₂ in the control of almost all structural and signaling elements within the PM or found at ER-PM contact sites. Whether it is a direct regulation of ion channels, transport of other lipids, or the maintenance of membrane contacts, PI(4,5)P₂ consistently plays a central role. It is then paramount to understand what factors determine how much PI(4,5)P₂ is to be made from PI4P. This is particularly important given that the PM pool of PI4P is not only used as a precursor of PI(4,5)P₂ synthesis, but is also as an essential currency that supports non-vesicular transport of structural lipids at ER-PM contact sites. While answers to these questions can be partially obtained using generic cells or *in vitro* biophysical methods, how these processes are adapted to the needs of specialized cells in a tissue-specific context and how their defects contribute to the development of disease states are exciting future directions that will keep us all busy for a long time to come.

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Figure 1. Interrelationship between Ca^{2+} influx and non-vesicular lipid transport in ER-PM contact sites

(A) Store operated Ca^{2+} entry is triggered by the concerted communication between the ER-localized STIM1 and PM-localized Orai1 proteins in ER-PM contact sites. The polybasic domains (PBD) of STIM1 is kept at the PM by interaction with phosphoinositides PI4P and PI(4,5)P₂. Several proteins involved in non-vesicular lipid transport are also located in ER-PM contact sites. Many of these proteins are regulated by Ca^{2+} either directly or via phospholipase C (PLC)-mediated changes in phosphoinositides. Extended synaptotagmins (E-Syt1/2/3) maintain contacts with the PM through their C2 domains and are anchored to the ER by an N-terminal membrane anchoring hook. They also contain SMP domains that can form lipid tunnels and can transport diacylglycerol (DAG). The oxysterol binding protein related ORP5 and ORP8 are anchored to the ER via a C-terminal transmembrane domain and interact with the PM phosphoinositides through their N-terminal sequences containing a pleckstrin homology (PH) domain. These proteins consume the PI4P gradient between the two membranes to transport phosphatidylserine (PS) from the ER to the PM. GRAMD1/Aster proteins are fixed in the ER by their N-terminal membrane spanning domain and interact with the membrane by a GRAM domain. This interaction is facilitated by PM cholesterol. The VAS domains of these proteins transfer cholesterol from the PM to the ER. The PI transfer proteins, Nir2 and Nir3 interact with the ER through interaction with the ER-localized VAPA/B proteins and bind to the PM when DAG and PA are produced during receptor activation. Their N-terminal lipid transfer domains transfer PA from the PM to the ER and most likely also delivers PI in the opposite direction. Important lipid transfer proteins, such as TMEM24 or ORP3, both regulated by Ca^{2+} and protein kinase C (PKC) are omitted for clarity. See text for discussion of these proteins.

(B) Schematic diagram depicting the connection between Ca^{2+} signals and the various proteins that work in ER-PM contact sites. Local or global cytoplasmic Ca^{2+} increases directly activate enzymes such as PLC or PKC isoforms or the PS scramblase TMEM16F or E-Syt1. As a result, several lipids are changing in the PM, possibly confined to contact sites if the Ca^{2+} signal is limited. Those lipid changes, in turn, affect the lipid transport proteins, primarily by controlling their PM association, leading to secondary changes in lipids in these membrane compartments. Such lipid changes then affect Ca^{2+} channels including SOCE. Color codes: Rectangles filled red are enzymes controlled by Ca^{2+} . Rounded rectangles filled light yellow are lipids. Phosphoinositides are labeled with red. Rounded rectangles

filled light green, are non-vesicular lipid transfer proteins. GRAMD2 does not have a lipid transfer domain, therefore, it is lighter colored in a rectangle. Rounded rectangles filled light blue are multi transmembrane proteins with flippase or scramblase function. Dark blue ovals are channels capable of Ca^{2+} transport.