ER-PM Contacts: Principals of Phosphoinositide and Calcium Signaling

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Abstract

The endoplasmic reticulum (ER) forms an extensive network of membrane contact sites with intra-cellular organelles and the plasma membrane (PM). Inter-organelle contacts have vital roles in membrane lipid and ion dynamics. In particular, ER-PM contacts are integral to numerous intra- and inter-cellular signaling pathways including phosphoinositide lipid and calcium signaling, mechano-transduction, metabolic regulation, and cell stress responses. Accordingly, ER-PM contacts serve important signaling functions in excitable cells including neurons, muscle, and endocrine cells. This review highlights recent advances in our understanding of the vital roles for ER-PM contacts in phosphoinositide and calcium signaling and how signaling pathways in turn regulate proteins that form and function at ER-PM contacts.

Introduction

Structures where the cortical endoplasmic reticulum (ER) closely apposes the plasma membrane (PM) are ubiquitously found in eukaryotic cells. These important intracellular structures are often referred to as ER-PM contacts or ER-PM junctions. In this review, I use the term ER-PM contacts to avoid possible confusion with intercellular junctions including tight junctions and adherens junctions. But why should we care about ER-PM contacts and what are their roles in cell signaling pathways? ER-PM contacts serve as important sites for membrane lipid and calcium (Ca^{2+}) dynamics that regulate numerous downstream signaling effectors including protein kinases, lipases, Rho GTPase family members, ion channels, and transcription factors in all eukaryotic cells.

Much of our current understanding of ER-PM contacts stems from seminal electron microscopy (EM) and physiological studies on excitable cells spanning from the 1950's to the early 2000's. In an EM study in published in 1957, Keith Porter and George Palade described ER-PM contacts as 'diads' and 'triads' in muscle cells [1], where they play important roles in calcium (Ca^{2+})-mediated excitation-contraction events [2,3]. An EM study by Jack Rosenbluth in 1962 subsequently reported 'subsurface cisterns' in neurons [4] that have recently undergone a resurgence in attention. A comparative EM study in 1974 found that ER-PM contacts in muscle cells and neurons share similar features [5]. Because 'diads and triads' and 'subsurface cisterns' had similar morphological features, it was suggested that ER-PM contacts might regulate specialized metabolic and signaling activities in excitable cells,

including ion transport and rapid membrane lipid dynamics. It turns out that they also share similar molecular compositions, as further revealed in current studies.

Independent pioneering biochemical and physiological studies on enzyme secretion in endocrine (and exocrine) cells suggested integral roles for ER-PM contacts in Ca^{2+} and phosphoinositide lipid signaling. In 1975, Robert Michell synthesized the findings from several groups including Mabel and Lowell Hokin as well as his own into an overarching hypothesis now widely known as the phosphoinositide cycle [6]. Along with important studies by Michael Berridge, Robin Irvine, and others, it is now generally understood that in response to physiological stimuli phospholipase C (PLC) hydrolyzes the phosphoinositide species phosphatidylinositol 4,5-bisphosphate, also known as $PI(4,5)P_2$, to generate the second messenger molecules diacylglycerol (DG) and soluble inositol 1,4,5-trisphosphate (IP_3) that activate downstream events, namely protein kinase C (PKC) signaling and IP_3 receptor-mediated Ca^{2+} release respectively [7]. However, the seminal studies by Hokin and Hokin, Michell, Berridge, Irvine, and their colleagues also indicated that DG species generated by PLC are rapidly recycled for $PI(4,5)P_2$ re-synthesis (Figure 1A). This important, but under-appreciated, aspect of the phosphoinositide cycle is absolutely critical to sustain repetitive rounds of the cycle in response to physiological stimuli. Accordingly, key lipid transfer events between the ER and PM must occur during the phosphoinositide cycle; this is because $PI(4,5)P_2$ is produced at the PM and phosphatidylinositol (PI) is synthesized in the ER (Figure 1A). Roles for ER-PM contacts in PLC signaling are not limited to membrane lipid dynamics alone. Subsequent landmark studies in the late 1980's to early 2000's by

Ole Petersen using pancreatic acinar cells, as well as work by James Putney, revealed a further involvement for ER-PM contacts in Ca^{2+} signaling events [8-10]. Following PLC- and IP_3 receptor-mediated ER Ca^{2+} release, a process termed store-operated Ca^{2+} entry (SOCE, also known as capacitive Ca^{2+} entry) generates additional cytoplasmic Ca^{2+} signals and refills ER Ca^{2+} stores (Figure 1A) [8-10]. Thus ER-PM contacts ultimately control $Ca^{2+}$and $PI(4,5)P_2$ pools required for cellular responses to various physiological stimuli.

Indeed, ER-PM contacts are now widely recognized as 'principal' sites for non-vesicular transport events in the phosphoinositide and Ca^{2+} signaling paradigm [11-13]. This is largely due to the identification of specific proteins that form and function at these important cellular structures. For example, groundbreaking studies in 2005 identified the ER-localized STIM1 protein as a regulator of the PM-localized Orai1 Ca^{2+} channel during SOCE (Figure 1B) [14-16]. In addition, the identification of lipid transfer proteins that function at ER-PM contacts has significantly expanded our understanding of the phosphoinositide cycle. Within the past few years alone, we have learned a great deal more about the regulated formation and function of distinct ER-PM contacts. In particular, recent studies have indicated that ER-PM contacts have a remarkable degree of plasticity. Namely, ER-PM contacts undergo dynamic and inducible remodeling events to generate inositide and Ca^{2+} signaling molecules as needed. Furthermore, ER-PM contacts can even be rapidly disassembled as a protective mechanism to prevent cytotoxic Ca^{2+} overload and catastrophic loss of $PI(4,5)P_2$ that would otherwise result in loss of cellular homeostasis and integrity.

This review highlights the latest developments on roles for ER-PM contacts in phosphoinositide and Ca^{2+} signaling.

ER-PM Contacts in Phosphoinositide Signaling

$PI(4,5)P_2$ plays a critical role in several cellular processes including exocytosis, endocytosis, phagocytosis, viral budding, cytoskeletal organization, cytokinesis, ion channel regulation, and cell signaling cascades [17]. Numerous effector proteins directly bind $PI(4,5)P_2$ through conserved domains, such as PH, ENTH, and C2 domains, or through clusters of basic charged residues on proteins. In addition, phosphorylation of $PI(4,5)P_2$ by class I PI 3-kinases (PI3Ks) produces $PI(3,4,5)P_3$ that regulates the PDK-AKT-mTor signaling nexus. Furthermore, activation of receptors coupled to PLC triggers $PI(4,5)P_2$ hydrolysis and the generation of DG, IP_3, and Ca^{2+} second messengers during the phosphoinositide cycle (Figure 1A) [6,7] that in turn induce PKC signaling, transcriptional responses, actin cytoskeletal dynamics, and regulated exocytosis. Because $PI(4,5)P_2$ has so many vital roles in cell signaling pathways, its metabolism must be precisely controlled to ensure that its signaling functions are not compromised.

Due to their importance in cell signaling, phosphoinositide lipid and Ca^{2+} dynamics taking place at ER-PM contacts have vital roles during immune cell responses, excitation-contraction coupling in muscle cells, neuronal activity, as well as the regulated exocytosis of zymogens and hormones. For example, glucose stimulation of β-cells results in $PI(4,5)P_2$ hydrolysis that is accompanied by increased Ca^{2+} influx and elevated cytoplasmic Ca^{2+} levels [18]. Elevated intracellular glucose

results in ATP production and inactivation of the ATP-sensitive potassium channel (K_{ATP}) leading to increased cytoplasmic K^+ and β-cell depolarization. This membrane potential activates a L-type voltage-gated calcium channel (LTCC/Cav) at the PM and Ca^{2+} influx into β-cells. Intracellular Ca^{2+} accumulation further promotes Ca^{2+}-stimulated PLC activity, $PI(4,5)P_2$ hydrolysis, and the generation of additional DG, IP_3, and Ca^{2+} second messengers (Figure 1A) [6,7] as well as PKC signaling that trigger the initial exocytosis of a readily-releasable pool of insulin vesicles. Gq- and Gs-coupled receptors further induce PLC and cAMP signaling events to efficiently trigger insulin secretion in β-cells. In addition to its role in second messenger generation, $PI(4,5)P_2$ directly binds several components of the exocytic machinery (*e.g.* syntaxin, Munc, CAPS, synaptotagmin) [19]. Consequently, $PI(4,5)P_2$ and Ca^{2+} oscillations must be precisely regulated during pulsatile insulin secretion in pancreatic β-cells.

Dynamic events at ER-PM contacts control flux through the phosphoinositide cycle. Two recent studies (within the current period of the review) have described distinct roles for the extended synaptotagmins (E-Syts) and TMEM24 proteins at ER-PM contacts in the control of pulsatile insulin secretion [20,21]. Upon Ca^{2+}-stimulated exocytosis, the Ca^{2+}-activated E-Syts are proposed to transfer DG from the PM to the ER via their SMP (synaptotagmin-like mitochondrial lipid-binding protein domain) domains (Figures 1A and 2A) [22]. Loss of E-Syt function results in prolonged DG accumulation in the PM, Ca^{2+} influx, and insulin secretion [21,22]. Thus Ca^{2+}-triggered exocytosis is coupled to Ca^{2+}-dependent E-Syt function that in turn attenuates exocytosis. This may be important for pulsatile insulin secretion, although

it is not yet entirely clear why insulin secretion is prolonged upon loss of E-Syt1. However, previous work suggested that E-Syt1 is necessary for $PI(4,5)P_2$ re-synthesis upon repetitive rounds of PLC signaling [23] and this may be a critical step for pulsatile waves of insulin secretion. As mentioned, upon glucose stimulation of β-cells, $PI(4,5)P_2$ hydrolysis is accompanied by increased Ca^{2+} influx and elevated cytoplasmic Ca^{2+} levels [18]. How might $PI(4,5)P_2$ oscillations (rounds of hydrolysis and re-synthesis) control Ca^{2+} oscillations and pulsatile insulin secretion? While IP_3 receptor-mediated Ca^{2+} dynamics are well known, key aspects of this regulatory system involve the K_{ATP} channel that sets the membrane potential in β-cells. At the resting basal state (low glucose), $PI(4,5)P_2$ facilitates K_{ATP} channel activity keeping intracellular K^+ low and thus LTCC/CaV activity low [24]. Glucose-stimulated ATP production and subsequent binding of ATP to the K_{ATP} channel decreases productive K_{ATP}-$PI(4,5)P_2$ interactions leading to β-cell depolarization and LTCC/CaV-mediated Ca^{2+} influx, Ca^{2+}-stimulated PLC activity and $PI(4,5)P_2$ hydrolysis for second messenger generation, and insulin secretion. $PI(4,5)P_2$ also promotes LTCC/CaV activity [24], but physiological PLC-mediated drops in $PI(4,5)P_2$ levels are modest due to dynamic activities taking place at ER-PM contacts that drive $PI(4,5)P_2$ re-synthesis during the phosphoinositide cycle (Figure 1) as well as additional regulatory failsafe mechanisms that prevent catastrophic collapses in $PI(4,5)P_2$ levels (see below). Importantly, $PI(4,5)P_2$ re-synthesis may reset the resting basal state through ATP consumption and by directly stimulating K_{ATP} channel activity, resulting in pulsatile insulin secretory events.

What then are the key roles for ER-PM contacts during the phosphoinositide cycle and pulsatile insulin secretion? $PI(4,5)P_2$ re-synthesis may be facilitated by the proposed role for E-Syt1 in DG recycling, as PLC-generated DG is sequentially converted to phosphatidic acid (PA), then CDP-DG, and then to PI during the phosphoinositide cycle (Figure 1A). A recent study has suggested that a DG kinase isoform involved in PA formation during the phosphoinositide cycle is stimulated by membrane curvature [25]. Intriguingly, recent cryo-electron tomography studies on ER-PM contacts in yeast revealed that the tricalbin proteins (E-Syt orthologs) generate cortical ER tubules and even peaks of extreme curvature on the ER membrane facing the PM [26•,27•]. Thus, E-Syt-mediated membrane sculpting may promote conversion of DG to PA and PI synthesis in the ER. However, impaired DG recycling cannot fully explain why Ca^{2+} oscillations and pulsatile insulin secretion are prolonged upon loss of E-Syt1. First, DG and DG-stimulated PKC activity are proposed to attenuate LTCC/CaV-mediated Ca^{2+} influx. Second, conversion of DG to PA also occurs at the PM and the Nir2 lipid transfer protein is proposed to efficiently deliver PA from the PM to the ER during the phosphoinositide cycle (Figures 1A and 1B; see below for further details on Nir2 and related proteins) [28]. Instead, E-Syt1 may execute some additional unknown function that facilitates $PI(4,5)P_2$ synthesis. Intriguingly, biochemical and structural studies demonstrate that the E-Syt SMP domains bind and transfer various phospholipids [22]. It has been suggested that the peaks of extreme curvature on the ER formed by the tricalbin proteins (E-Syt orthologs) may facilitate lipid transfer from the ER to the PM [26•,27•]. Possibly, E-Syt1 may transfer some as yet unidentified phospholipids from the ER to the PM that

promote PI(4,5)P_2 synthesis and in this manner resets the basal state for pulsatile insulin secretion.

Following its synthesis in the ER, PI must be delivered to the PM to continue the phosphoinositide cycle. In pancreatic β-cells and neurons, the SMP domain protein TMEM24 is proposed to transfer PI from the ER to the PM for PI(4,5)P_2 synthesis (Figures 1A and 2A) [20,29•]. Loss of TMEM24 impairs glucose-stimulated pulsatile insulin secretion and PI(4,5)P_2 re-synthesis in model β-cells [20]. However, the phosphoinositide cycle is not entirely disrupted upon loss of TMEM24 [20] or E-Syt1 [23]. This indicates that additional lipid transfer proteins function at ER-PM contacts during the phosphoinositide cycle. The Nir2 PI transfer protein (and its ortholog RdgB in *Drosophila melanogaster*) is also proposed to transport PI from the ER to the PM for phosphatidylinositol 4-phosphate, or PI4P, synthesis and thus PI(4,5)P_2 synthesis (Figures 1A and 1B) [23,28,30]. Following PLC activation, Nir2 targets to ER-PM contacts by binding PA and the ER-localized protein VAP (VAMP-associated protein; Figure 1B) [23,28]. VAP is a tail-anchored ER membrane protein that binds a FFAT (two phenylalanines in an acidic tract) motif in Nir2. At ER-PM contacts, Nir2 may transfer PA from the PM to the ER and in turn transfer PI from the ER to the PM during the phosphoinositide cycle (Figures 1A and 1B). E-Syt1 facilitates Nir2 recruitment and function at ER-PM contacts [23], suggesting that these two proteins function in concert. However, Ca^{2+} signals that trigger E-Syt1 activity instead attenuate TMEM24 function via Ca^{2+}- and PKC-dependent inhibitory phosphorylation events on TMEM24 (Figure 2A) [20,29•]. Thus, Ca^{2+} oscillations may control ER-PM contact site remodeling for the temporal and spatial coordination of

distinct E-Syt-mediated and TMEM24-mediated lipid transfer events during distinct steps of the phosphoinositide cycle.

Additional proteins are implicated in membrane lipid dynamics at ER-PM contacts. Seminal studies have demonstrated roles for members of the oxysterol-binding protein related protein (ORP) family in the transfer of newly synthesized cholesterol and phosphatidylserine (PS) from the ER in exchange for PI4P at the PM [31-35]. In mammalian cells, ORP5 and ORP8 are integral ER membrane proteins involved in PI4P/PS exchange at ER-PM contacts, while ER-localized VAP recruits additional ORPs (*e.g.* ORP3 and ORP6) to ER-PM contacts [36] (Figure 1B). In agreement with several pioneering ORP studies, quantitative lipidomic analyses of yeast cells have confirmed that ER-PM contacts and ORP family members (termed Osh proteins) are key regulators of PM lipid composition and organization [37•]. This study further revealed that Osh protein-mediated lipid exchange is critical for the synthesis of $PI(4,5)P_2$. PS and sterols, both enriched at the PM via ORP/Osh proteins and ER-PM contacts, synergistically activate the PI4P 5-kinase (PIP5K) via its specificity loop that may serve as a co-incidence detector for PI4P/PS/sterol lipid nanodomains. Thus, ER-PM contacts and ORP/Osh proteins create a PM lipid environment that supports PIP5K activity and $PI(4,5)P_2$ synthesis [37•]. Accordingly, ORP-mediated PI4P exchange reactions regulate the levels of multiple lipids at the PM. First, they exchange PI4P for other lipids including PS and sterols that facilitate $PI(4,5)P_2$ synthesis. In the process, ORP-mediated lipid exchange also keeps PI4P levels in check at the PM [31,35,37•,38]. Indeed, a more recent study found that depletion of ORP6 resulted in increased localization of a PI4P reporter at the PM in

neuronal cells [36]. As PI4P is the precursor for $PI(4,5)P_2$ synthesis, transient attenuation of ORP family members may elevate PI4P pools for rapid bursts of $PI(4,5)P_2$ synthesis as needed. In support of this notion, levels of PI4P simultaneously rise with drops in $PI(4,5)P_2$ and loss of the PI4P/PS exchange proteins ORP5 and ORP8 from ER-PM contacts promotes the flux of PI4P to $PI(4,5)P_2$ [39•]. Although speculative, ER-PM contacts may be remodeled from sites of ORP-mediated PI4P exchange to sites that exclusively promote PI4P and $PI(4,5)P_2$ synthesis via the E-Syt1, Nir2, and/or TMEM24 proteins during the phosphoinositide cycle. Future experiments are needed to examine the spatial and temporal regulation of ORP isoforms in response to stimuli that trigger PLC signaling and the phosphoinositide cycle,

While ORP family members have been intensely studied, their function in cell signaling remains paradoxical and even controversial. It has been argued that the primary function of the yeast Osh proteins may not be inter-organelle lipid transfer or even the control of sterol, PS, PI4P, or $PI(4,5)P_2$ levels. Instead, the Osh proteins are suggested to sequester lipids and integrate differential lipid-binding states (*e.g.* apo- or PI4P- or sterol-bound forms) to downstream signaling effectors including the Tor1 complex (TORC1) and Greatwall (Rim15) protein kinase for cell growth control [40,41]. Yet numerous studies in yeast and mammalian cells firmly establish that ORP family members control the composition of sterols, PS, PI4P, and $PI(4,5)P_2$ in cellular membranes and these findings should not be overlooked [31,32,35-39•,42]. Accordingly, the Osh proteins may modulate Tor1 and Rim15 kinase activity indirectly through the control of sterol, PS, PI4P, and/or $PI(4,5)P_2$ membrane lipid composition.

For example, cholesterol and sterol lipids have been suggested to regulate TORC1 activity at lysosomes/vacuoles [43,44]. Nonetheless, alternative models for ORP/Osh protein function cannot be refuted based on the data currently available. While evidence that the yeast Osh proteins directly interact with cell signaling effector proteins is lacking, there is evidence that mammalian ORPs do. This includes cholesterol-regulated OSBP scaffolding functions in ERK signaling [45] and more recent studies suggesting that ORP2 and ORP5 physically interact with and promote mTORC1 activity [46,47]. Moreover, ORP4L is proposed to bind $PI(4,5)P_2$, $G\alpha q$, and $PLC\beta 3$ to promote $PLC\beta 3$ activity necessary for leukemia stem cell growth and survival [48]. ORP4L phosphorylation enhances cholesterol binding, possibly at the expense of $PI(4,5)P_2$ binding [49], providing a potential regulatory mechanism for ORP4L activity. In summary, ORP family members promote $PI(4,5)P_2$ signaling [37•,49]; they also restrict $PI(4,5)P_2$ signaling [39•,42]; they have been shown to be lipid *transfer* proteins [31-35]; they have also been described as lipid *transmission* proteins [40,41]; they are necessary for cancer cell proliferation, survival, and migration [46,48,49]; they have also been described as tumor cell suppressors [40]. Thus while roles for ORP family members in membrane lipid transfer and regulation are well established, no singular descriptor seems to encompass their important cellular functions.

Additional lipid transfer proteins may also control PIP5K activity at ER-PM contacts. For example, StARD/GRAM family members (also known as Aster proteins) are proposed to extract ‘free’ accessible cholesterol from the cytoplasmic leaflet of the PM and transfer cholesterol back to the ER [50,51]. Aster/StARD/GRAM protein-

mediated delivery of cholesterol from the PM to the ER is used for bile synthesis in hepatocytes, steroid synthesis in adrenal cells, and the synthesis of cholesterol esters for storage in lipid droplets. Because these proteins extract cholesterol from the PM, they may also influence the organization and biophysical properties of the PM and thus dynamic events and activities taking place at the PM. It is not known whether modulation of PM cholesterol by mammalian Aster/StARD/GRAM domain proteins controls PIP5K activity. However, the yeast ortholog Ysp2 (Lam2/Ltc4) is inhibited by Ypk1 (AGC kinase ortholog) phosphorylation under stress conditions shown to promote $PI(4,5)P_2$ synthesis [52]. Thus, the transient attenuation of both ORP and Aster/StARD/GRAM family members may promote bursts of $PI(4,5)P_2$ synthesis at the PM via increases in PI4P and cholesterol content.

ER-PM Contacts in Store-Operated Calcium Entry

ER-PM contacts are key sites for Ca^{2+} dynamics controlled by STIM1-Orai1 coupling as well as CaV-ryanodine receptor (RyR) interactions (Figure 1). Recent studies (within the current period of review) have provided important new information regarding how ER-PM contacts are assembled to generate Ca^{2+} signals, how they are attenuated to maintain baseline Ca^{2+} levels, and even how they are disassembled to prevent cytotoxic Ca^{2+} overload that could lead to loss of cellular integrity.

Following IP_3 receptor-mediated ER Ca^{2+} release, the ER Ca^{2+} sensor STIM1 oligomerizes and translocates to ER-PM contacts where it activates store-operated Ca^{2+} entry (SOCE) via the Orai1 Ca^{2+} channel in the PM (Figure 1B) [14-16]. In addition, diads and triads in muscle cells are dynamically remodeled during exercise

by the recruitment of STIM1-Orai1 assemblies to activate SOCE and prevent fatigue [53]. We are still learning more about the formation and regulation of STIM1-Orai1 assemblies at ER-PM contacts. For example, a recent study has shown that the EB1 protein traps STIM1 at microtubule plus ends and delays STIM1 translocation to ER-PM contacts [54]. This may provide a mechanism to prevent inappropriate SOCE and toxic Ca^{2+} overload. In opposite fashion, the ER-localized GRAMD2a protein promotes STIM1 translocation to ER-PM contacts [55]. GRAMD2a binds $PI(4,5)P_2$ and other anionic lipids at the PM via its GRAM domain and is proposed to preform ER-PM contacts that become occupied by STIM1 during SOCE. GRAMD2a is not required for SOCE however [55], and it may be worthwhile to examine additional potential roles for GRAMD2a at ER-PM contacts. Several studies have further examined how additional proteins are organized around STIM1-Orai1 assemblies. For example, two studies have described potential roles for cortical septin and actin assemblies in SOCE and ER-PM contact formation, respectively. Septin 4 is proposed to promote STIM1-Orai1 contacts, possibly by restricting Orai1 diffusion in the PM [56]. Likewise, F-actin appears to stabilize Nir2-containing ER-PM contacts [57]. Of note, SOCE recruits E-Syt1 to ER-PM contacts [58] and Nir2 co-localizes with E-Syt1 at ER-PM contacts [23], and thus F-actin may spatially coordinate E-Syt1 and Nir2 activities at sites of SOCE. Indeed, another recent study confirms that the Ca^{2+}-activated E-Syt1 protein forms close ER-PM contacts (spanning approx. 20 nm between the ER and PM) in the surrounding proximity of STIM1-Orai1 contacts (also spanning approx. 20 nm between the ER and PM) [59]. Intriguingly, the cortical ER is further extended around the E-Syt1 ER-PM contacts suggesting that additional ER

proteins may surround the STIM-Orai1 and E-Syt1 zones [59]. Possibly, VAP-Nir2 complexes surround or neighbor the STIM-Orai1 and E-Syt1 ER-PM contacts consistent with previous work [23]. As phosphoinositide lipids regulate STIM1 and Orai1 [11] (Figure 1B), the localized recruitment of Nir2 and E-Syt1 may modulate SOCE during the phosphoinositide cycle. Another recent study has found that the ER-localized ANO8 protein is recruited to STIM1-Orai1 contacts in a $PI(4,5)P_2$-dependent fashion [60•]. ANO8 in turn recruits ER-localized SERCA2 Ca^{2+} pumps to the surrounding cortical ER to refill ER Ca^{2+} stores (see Figure 1A). This may also facilitate a process known as Ca^{2+}-mediated SOCE inactivation to attenuate Ca^{2+} influx and prevent cytotoxic Ca^{2+} overload [60•].

ER-PM Contacts in Neuronal Soma and Dendrites

While ER-PM contacts, or 'subsurface cisterns', were first reported in neurons nearly 60 years ago [4], we are still learning important details about the proteins that form and function at these sites. Extensive ER-PM contacts (micrometer scale in length) are readily observed in resting neurons at axon initial segments, proximal dendrites, and the soma where they cover approximately 10-15% of the PM [5,61]. Interestingly, non-conductive Kv2.1 and Kv2.2 K^+ rectifier channels are clustered in ER-associated PM domains [62,63]. It is now apparent that clustering of Kv2 channels at ER-PM contacts is mediated through interactions with the ER-localized protein VAP (VAMP-associated protein; Figure 1B) [64••-66••]. VAP is a tail-anchored ER membrane protein that binds a non-conventional FFAT (two phenylalanines in an acidic tract) motif in the carboxy-terminal cytoplasmic tail of Kv2 channels [64••,66••].

Phosphorylation of serine residues in the non-conventional FFAT motif promotes Kv2-VAP contacts (Figure 2B) [64••,66••]. Kv2 channel activity (or inactivity) is not regulated at VAP-mediated ER-PM contacts *per se* [67], suggesting that Kv2-VAP contacts may have roles independent of K^+ rectifier channel activity. Accordingly, additional ion channels co-localize with Kv2 clusters including the PM L-type Ca^{2+} channel (LTCC) CaV2.1 [63], the ryanodine receptor (RyR) ER Ca^{2+} release channel [68], and the ER-localized junctophilin protein that associates with LTCC-RyR contacts in muscle cells (Figure 1B) [2,69]. Thus, ER-PM contacts in neuronal soma and muscle cells share common molecular compositions and functions in Ca^{2+} regulation, just as predicted by a morphological study decades ago [5]. Indeed, a recent study has found that VAP-mediated Kv2 clustering augments LTCC opening and RyR-mediated Ca^{2+} transients in resting neurons (Figure 2B) [70•]. Interestingly, the PI transfer proteins TMEM24 and Nir2 also associate with Kv2-VAP contacts in neurons [29•,71•], but they may not occupy ER-PM contacts simultaneously, as they are differentially regulated during the phosphoinositide cycle [21,29•]. Importantly, levels of neuronal PI, PI4P, and $PI(4,5)P_2$ species were reduced in brains from Kv2.1 knockout mice [71•], suggesting that Kv2-VAP contacts may be involved in the phosphoinositide cycle. A previous study also found that STIM1 and Orai1 localize to Kv2 clusters suggesting that Kv2-VAP contacts may also be sites for SOCE (Figure 2B) [63]. Intriguingly, Kv2 clusters are also hotspots for the regulated exocytosis and endocytosis of channels and receptors [72]. Possibly, phosphoinositide and Ca^{2+} signaling events taking place at Kv2-VAP contacts may regulate cell-surface targeting of Kv2, CaV2, Orai1, as well as NMDA (N-methyl-D-aspartate) and AMPA (α-amino-

3-hydroxyl-5-methyl-4-isoxazolepropionic acid) receptors that may impact long term potentiation and neuronal excitability.

Kv2-VAP contacts can be rapidly disassembled as a protective mechanism to attenuate Ca^{2+} influx and prevent cytotoxic Ca^{2+} overload. It is well documented that upon neuronal activity, for example upon glutamate-induced NMDA receptor Ca^{2+} influx, Kv2 clusters are dispersed and Kv2 K^+ rectifier channel activity (K^+ exit) is induced in a Ca^{2+}- and calcineurin-dependent manner to reset the voltgage gradient (Figure 2B) [67]. Disruption of clustered non-conductive Kv2 channels also occurs upon hypoxic or ischemic injury to suppress neuronal excitability and prevent cytotoxic Ca^{2+} overload as a neuroprotective mechanism against these insults [73]. Likewise, two recent studies found that ER-PM contacts are disassembled upon neuronal activation [29•,74•]. This may attenuate LTCC-RyR and SOCE Ca^{2+} transients as a protective mechanism to prevent Ca^{2+} overload upon neuronal stimulation (Figure 2B). Consistent with this idea, Kv2 channels have been shown to modulate Ca^{2+} transients in dendrites [62]. Thus, Kv2-VAP contacts may be formed and dispersed as needed to ensure Ca^{2+} and phosphoinositide lipid homeostasis. As Kv2 channels are also expressed in muscle and pancreatic β-cells [67], it will be important to examine roles for Kv2-VAP contacts in excitation-contraction coupling and pulsatile insulin secretion.

Summary

Seminal EM studies in the 1950s to 1970s provided the first glimpse of ER-PM contacts revealing some similar morphological features in neurons and muscle cells. It is exciting to now learn that they also share common molecular compositions and functions in membrane lipid and ion transport. Several groundbreaking studies have further revealed that ER-PM contacts display a remarkable degree of heterogeneity and are dynamically remodeled to generate and phosphoinositide and Ca^{2+} signals as needed. It will be important to further examine the spatiotemporal relationships between the numerous factors that function at these important intracellular structures. Furthermore, just as the pioneering EM studies on neurons and muscle cells, future morphological investigations on ER-PM contacts in additional cells and tissues may lead the way to new breakthroughs in our current understanding of cell signaling and physiology.

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Competing interests

The author declares no conflicts of interest.

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- • of special interest
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Figure legends

Figure 1. ER-PM cross talk serves vital roles in phosphoinositide metabolism and Ca^{2+} dynamics. **(A)** Examples of membrane lipid and Ca^{2+} transport taking place at ER-PM contacts are shown. Phosphatidylinositol (PI) is sequentially converted to PI4P and PI(4,5)P_2 by PI kinase activities at the PM. In response to physiological stimuli, phospholipase C (PLC) hydrolyzes PI(4,5)P_2 to generate the second messenger molecules diacylglycerol (DG) and inositol trisphosphate (IP_3). PLC-generated DG is converted back to PI, PI4P, and PI(4,5)P_2 in a process known as the "phosphoinositide cycle". This allows repetitive rounds of membrane lipid and Ca^{2+} signaling in response to the activation of cell-surface receptors that are coupled to PLC signaling. Because PLC generates DG at the PM and PI is synthesized in the ER, lipid transfer between the ER and PM is integral to the phosphoinositide cycle. The extended-synaptotagmins (E-Syt) proteins are proposed to transfer DG from the PM to the ER. In addition, DG may be converted to phosphatidic acid (PA) by DG kinase (DGK) at the PM. Nir2 (as well as Nir3 and RdgB proteins) then transfer PA from the PM to the ER. Multiple proteins are proposed to function as PI transfer proteins (PITP) including Nir2 and TMEM24. In addition to lipid transfer, ER-PM contacts are also important sites for Ca^{2+} transport and signaling. Generation of soluble IP_3 by PLC activates the ER-localized IP_3 receptor (IP_3R) resulting in ER Ca^{2+} release. Together, Ca^{2+} and DG activate downstream effectors including protein kinase C, transcription factors, and exocytic machinery (not shown). IP_3 receptor (IP_3R)-mediated ER Ca^{2+} release triggers Ca^{2+} influx across the PM by process known as store-operated Ca^{2+} entry (SOCE). SOCE generates additional cytosolic

Ca^{2+} signals to trigger downstream events including exocytosis and transcriptional responses (not shown). SOCE is also crucial for refilling capacitive ER Ca^{2+} stores through the sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA). In addition to SOCE, ER-PM contacts are important sites for coupling PM L-type voltage-gated Ca^{2+} channels (LTCC) to ER ryanodine receptors (RyR) during excitation-contraction coupling in muscle cells. Recent studies now reveal that these systems may also be regulated in neuronal soma and dendrites by an unconventional role for K^+ rectifier Kv2 channels.

(B) Examples of proteins that assemble and function at ER-PM contacts are provided. Nir2 is proposed to function as a PI and PA transfer protein at ER-PM contacts. ER-localized VAP (VAMP-associated protein) and PA recruit Nir2 to ER-PM contacts. Nir2 is proposed to transfer PA (generated by DG kinase at the PM) from the PM to the ER and subsequently transfer PI from the ER to the PM during the phosphoinositide cycle. VAP also recruits certain ORP family members, such as ORP6, to ER-PM contacts. ORPs are proposed to extract newly synthesized lipids, such as cholesterol (Sterol), from the ER for transfer to target membranes including the PM. Upon arrival at the target membrane (the PM in this case), exchange for PI4P is thought to occur. PI4P bound to ORP can then be presented directly to PI4P phosphatases, such as ER-localized Sac1, or dumped into the ER (or other membrane compartments) for subsequent hydrolysis by PI4P phosphatases. Both PI4P clearance mechanisms, direct phosphatase presentation (*in trans* or in transit) and ER delivery, promote lipid transfer and PI4P regulation at ER-PM contacts. In this fashion, ORP family members act as important regulators of PM lipid

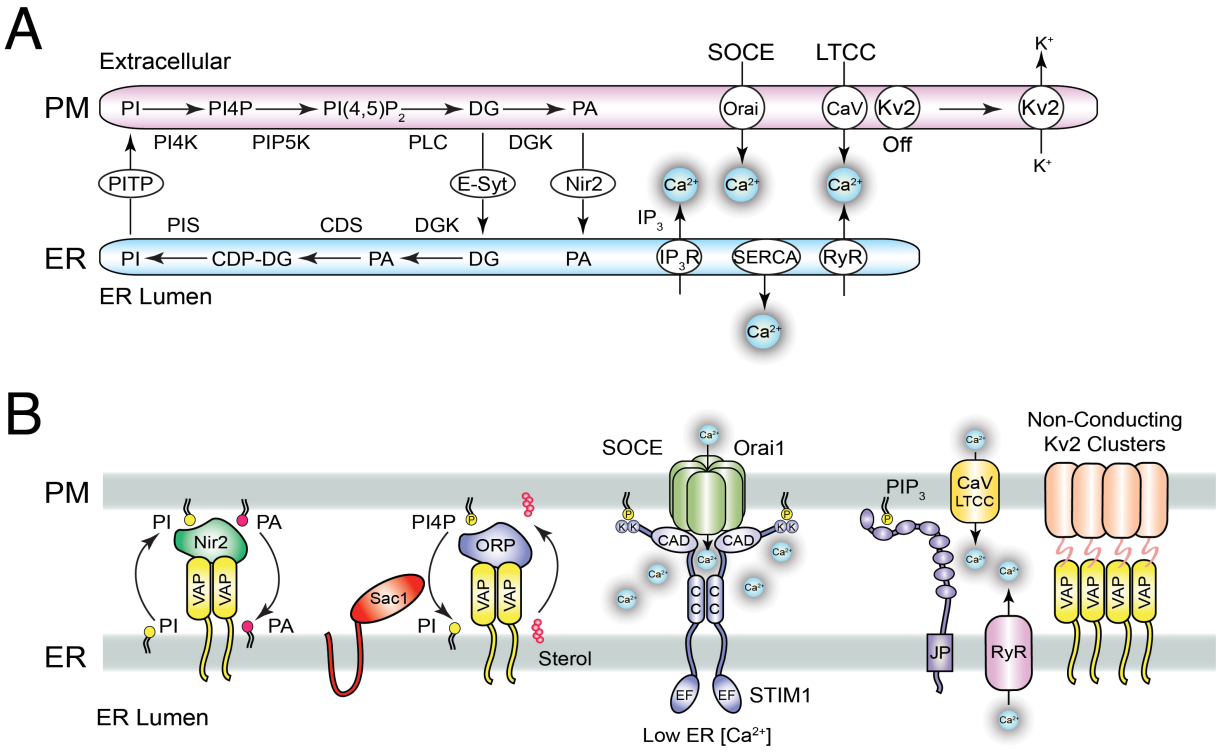
composition. ER-PM contacts are also important sites for Ca^{2+} transport and signaling. Following IP_3R-mediated ER Ca^{2+} release, luminal ER Ca^{2+} stores are replenished by STIM protein function in the store-operated Ca^{2+} entry (SOCE) pathway. The ER-localized STIM protein senses ER Ca^{2+} levels via a Ca^{2+}-binding EF hand domain. Upon depletion of ER Ca^{2+}, STIM proteins oligomerize via their cytoplasmic coiled-coil domains (CC) and translocate to ER-PM contact sites. A polybasic lysine-rich region (K) within the cytoplasmic tail of STIM binds to phosphoinositide lipids in the PM. STIM oligomers subsequently interact with and activate PM-localized Orai1 Ca^{2+} channels (also known as Ca^{2+} release-activated channels, CRAC) at ER-PM junctions. The CRAC-activation domain (CAD) within the STIM proteins directly binds and activates Orai1 channels, resulting in Ca^{2+} influx necessary for cytosolic Ca^{2+} signals and for refilling ER Ca^{2+} stores through SERCA transporters. In addition to SOCE, L-type voltage-gated Ca^{2+} channels (LTCC) in the PM engage with ryanodine receptors (RyR) in the ER. Upon activation, RyR releases ER Ca^{2+} stores to amplify cytoplasmic Ca^{2+} signals necessary for cytoskeletal-mediated excitation-contractions in muscle cells. The junctophilin (JP) protein also localizes to LTCC-RyR contacts in muscle cells. Junctophilin is an integral ER membrane protein with several cytoplasmic MORN repeats proposed to bind the phosphoinositide species $PI(3,4,5)P_3$, or PIP_3, in the PM. In a surprising and important twist, recent studies reveal that ER-localized VAP also interacts with PM Kv2 clusters to form ER-PM contacts in neurons that reportedly engage with Nir2, STIM1-Orai1, and LTCC-RyR couplings and thus reveal conserved modes for ER-PM cross talk. **(A and B)** Additional abbreviations are: PI4K, phosphatidylinositol 4-

kinase; PIP5K, PI4P 5-kinase; CDS, CDP-diacylglycerol synthase; CDP-DG, CDP-diacylglycerol; PIS, phosphatidylinositol synthase. The schematic cartoons do not reflect temporal or spatial dimensions.

Figure 2. ER-PM contacts and Ca^{2+} signals participate in mutual cross talk. It has been generally accepted that ER-PM contacts control Ca^{2+} dynamics. Recent studies have revealed new ways in which Ca^{2+} signals can in turn control ER-PM contacts. This beneficial cross talk may ensure the proper timing of the phosphoinositide cycle and prevent cytotoxic Ca^{2+} overload. **(A)** Cytoplasmic Ca^{2+} oscillations ensure the timing of lipid transport and metabolism during phosphoinositide cycle. The TMEM24 protein serves as a phosphatidylinositol transfer protein (PITP) in excitable cells. TMEM24 PITP activity is precisely controlled during the phosphoinositide cycle. TMEM24 activity is kept low by PLC- and ER Ca^{2+} release-induced PKC activity. This may ensure that PI transfer to the PM does not occur when PLC and PKC activity is high. In contrast, these initial signaling events (PLC-generated DG and Ca^{2+}) rapidly trigger Ca^{2+} influx by SOCE and recruit the E-Syt1 protein to ER-PM contacts where it executes then DG transfer to the ER. These opposing activities may ensure the proper timing of lipid transfer and metabolism during the phosphoinositide cycle. **(B)** Kv2 channel-VAP form ER-PM contacts in resting neurons. Phosphorylated Kv2 channels form PM clusters and interact with ER-localized VAP. Kv2-VAP contacts appear to facilitate phosphoinositide metabolism, SOCE, and LTCC-RyR coupling in resting neurons. Importantly, upon neuronal stimulation or ischemic insult, Kv2 cluster-VAP contacts are disassembled in a Ca^{2+}- and calcineurin (CaN)-dependent

fashion. This results in reduced ER-PM contacts and increased K^+ rectifier activity (K^+ exit) to reset the voltgage gradient as a protective mechanism to attenuate Ca^{2+} influx and prevent cytotoxic Ca^{2+} overload.

Figure 1.


A
Extracellular
PM
PI
PI4P
PI(4,5)P2
DG
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LTCC
Orai
CaV
Kv2
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PI4K
PIP5K
PLC
DGK
PITP
E-Syt
Nir2
Ca2+
IP3
PIS
CDS
DGK
ER
CDP-DG
IP3R
SERCA
RyR
ER Lumen
B
PM
ER
PI
PA
Nir2
VAP
Sac1
PI4P
ORP
Sterol
SOCE
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CAD
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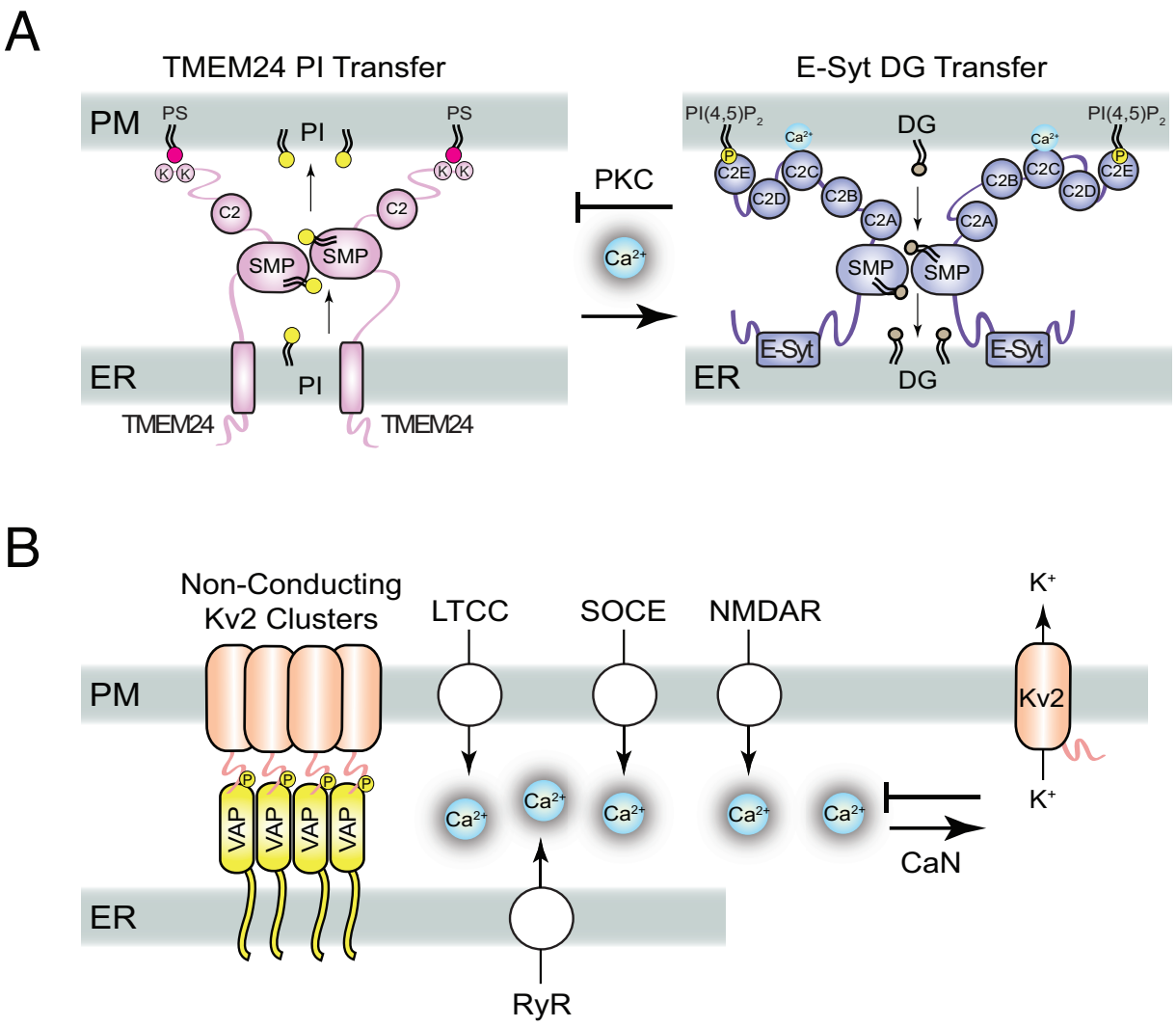
ER Lumen

Figure 2.


A
TMEM24 PI Transfer
E-Syt DG Transfer
PM
ER
PS
PI
C2
SMP
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PKC
Ca2+
PI(4,5)P2
DG
C2E
C2C
C2D
C2B
C2A
E-Syt
B
Non-Conducting
Kv2 Clusters
LTCC
SOCE
NMDAR
VAP
RyR
Kv2
K+
CaN