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Homeostatic Regulation of the PI(4,5)P₂-Ca²⁺ Signaling System at ER-PM Junctions

Chi-Lun Chang and Jen Liou*

Department of Physiology, UT Southwestern Medical Center, Dallas, TX 75390, USA

Abstract

The phosphatidylinositol 4,5-bisphosphate (PI(4,5)P₂)-Ca²⁺ signaling system is important for cell activation in response to various extracellular stimuli. This signaling system is initiated by receptor-induced hydrolysis of PI(4,5)P₂ in the plasma membrane (PM) to generate the soluble second messenger inositol 1,4,5-trisphosphate (IP₃). IP₃ subsequently triggers the release of Ca²⁺ from the endoplasmic reticulum (ER) store to the cytosol to activate Ca²⁺-mediated responses, such as secretion and proliferation. The consumed PM PI(4,5)P₂ and ER Ca²⁺ must be quickly restored to sustain signaling responses, and to maintain the homeostasis of PI(4,5)P₂ and Ca²⁺. Since phosphatidylinositol (PI), the precursor lipid for PM PI(4,5)P₂, is synthesized in the ER membrane, and a Ca²⁺ influx across the PM is required to refill the ER Ca²⁺ store, efficient communications between the ER and the PM are critical for the homeostatic regulation of the PI(4,5)P₂-Ca²⁺ signaling system. This review describes the major findings that established the framework of the PI(4,5)P₂-Ca²⁺ signaling system, and recent discoveries on feedback control mechanisms at ER-PM junctions that sustain the PI(4,5)P₂-Ca²⁺ signaling system. Particular emphasis is placed on the characterization of ER-PM junctions where efficient communications between the ER and the PM occurs, and the activation mechanisms of proteins that dynamically localize to ER-PM junctions to provide the feedback control during PI(4,5)P₂-Ca²⁺ signaling, including the ER Ca²⁺ sensor STIM1, the extended synaptotagmin E-Syt1, and the PI transfer protein Nir2. This review is part of a Special Issue entitled The Cellular Lipid Landscape.

1. Introduction

Phosphatidylinositol 4,5-bisphosphate, or PI(4,5)P₂, is an inositol-containing phospholipid that is enriched at the inner leaflet of the plasma membrane (PM) [1]. PI(4,5)P₂ governs many cellular functions including membrane trafficking, ion channel and transporter activity, and cytoskeleton-PM interactions [2, 3]. Aberrant metabolism of PI(4,5)P₂ has been implied in diseases such as cancers, oculocerebrorenal syndrome of Lowe (OCRL), and infectious diseases [1, 4]. A remarkable function of PI(4,5)P₂ is its ability to mediate Ca²⁺ signaling following stimulation of cell surface receptors [5]. Receptor-induced signal is transduced from the PM to the endoplasmic reticulum (ER) by the soluble second messenger inositol

*Correspondence: Jen.Liou@UTSouthwestern.edu.

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1,4,5-trisphosphate (IP₃), generated via PI(4,5)P₂ hydrolysis by receptor-activated phospholipase C (PLC). IP₃ binds and opens a Ca²⁺ channel, namely the IP₃ receptor (IP₃R), in the ER membrane to release Ca²⁺ from the ER store into the cytosol. The increase in cytosolic Ca²⁺ levels activates an extensive array of Ca²⁺ effectors in the cell. This PI(4,5)P₂-Ca²⁺ signaling system is fundamental to many cellular processes in various cell types [6].

PI(4,5)P₂ at the PM and Ca²⁺ in the ER are consumed to generate IP₃ and cytosolic Ca²⁺ signals in the PI(4,5)P₂-Ca²⁺ signaling system. It is important to quickly restore PM PI(4,5)P₂ and ER Ca²⁺ levels in order to maintain the homeostasis and sustain Ca²⁺ signaling in stimulated cells. Following IP₃-induced depletion of the ER Ca²⁺ store, a mechanism is activated to open PM Ca²⁺ channels and bring in Ca²⁺ from the extracellular space to sustain cytosolic Ca²⁺ levels, and to refill the ER Ca²⁺ store [7]. This homeostatic mechanism is called store-operated Ca²⁺ entry (SOCE). Defective SOCE causes human diseases including severe combined immunodeficiency and muscular dysplasia [8]. The molecular basis of SOCE activation was identified in recent years. It involves the interaction of the ER Ca²⁺ sensor STIM1 and the PM Ca²⁺ channel Orai1 at ER-PM junctions where the ER forms close appositions with the PM. The observation of STIM1 translocation to ER-PM junctions to activate SOCE following ER Ca²⁺ depletion revived interests in understanding these membrane junctions and their roles beyond excitation-contraction (E-C) coupling in muscles [9, 10]. The functions and regulation of ER-PM junctions have since become areas of intense investigation [11-13].

The homeostasis of PM PI(4,5)P₂ in stimulated cells is maintained by a process called the PI cycle that promotes the resynthesis of PI(4,5)P₂ after agonist-induced breakdown [1]. After receptor-induced hydrolysis and the release of IP₃ to the cytosol, the remaining diacylglycerol (DAG) part of PI(4,5)P₂ in the PM is converted to phosphatidic acid (PA). PA is utilized to generate PI by enzymes resided in the ER for resynthesis of PI(4,5)P₂ in the PM. Although the first evidence of the PI cycle, known as the “phospholipid effect”, was reported in 1953 by Lowell and Mabel Hokin [14-16], the inter-organelle lipid exchange pathway of the PI cycle was not revealed until 2015, when the PI transfer protein (PITP) Nir2 was identified as the PI-PA exchanger [17-19]. Similar to STIM1, Nir2 translocates to ER-PM junctions to support PI(4,5)P₂-Ca²⁺ signaling following receptor stimulation [18, 20, 21]. Thus, ER-PM junctions are pivotal in the homeostatic regulation of PM PI(4,5)P₂ and ER Ca²⁺ in stimulated cells.

This review begins with a brief historical overview of selected milestones in the research of the PI(4,5)P₂-Ca²⁺ signaling system to provide context for more recent mechanistic studies. The detailed history of studies in the field can be found in three recent reviews [1, 6, 22]. Thereafter, the focus is on recent developments that contribute to our current understanding of the molecular and cellular mechanisms underlying SOCE and the PI cycle that maintain the homeostasis of Ca²⁺ and PI(4,5)P₂, respectively. A special emphasis on advances in the study of ER-PM junctions, the hubs for SOCE and the PI cycle, is also included.

2. The PI(4,5)P₂-Ca²⁺ Signaling System: an Historical Overview

2.1 PI, PI4P, and PI(4,5)P₂

PI is the most abundant inositol-containing lipid in eukaryotic cells [1]. Each PI molecule contains an *sn*-1,2-DAG backbone linked to the hydroxyl group at position 1 of a *myo*-inositol (*cis*-1,2,3,5-*trans*-4,6-cyclohexanehexol) via a phosphodiester bond. One marked feature of PI in animal cells is the enrichment of stearic acid (18:0) at the *sn*-1 position and arachidonic acid (20:4) at the *sn*-2 position of its DAG backbone [1, 22, 23]. It remains unclear how this enrichment takes place and whether it has a functional significance. PI is synthesized from CDP-DAG and *myo*-inositol by PI synthase in the ER and in ER-derived mobile compartments, and is delivered to the PM and other organelles via vesicular transport and PITPs [24-26]. After delivery, PI is subsequently phosphorylated at the hydroxyl groups at positions 3, 4, and/or 5 by various kinases at different membranes to generate seven structurally distinct phosphoinositides, providing membrane identities for selective recruitment of proteins to support organelle functions [1, 3, 27].

PI4P and PI(4,5)P₂ were the first two discovered phosphoinositides and were called diphosphoinositide and triphosphoinositide, respectively, in some early literatures [28-30]. PI4P is found in multiple organelles, including the Golgi apparatus, endosomes, and the PM [31]. The enrichment of PI4P in these organelles has recently been shown to provide a driving force for lipid export from the ER via lipid transfer proteins (LTPs) at organelle junctions [32-35]. PI(4,5)P₂ is predominantly found at the inner leaflet of the PM. PI(4,5)P₂ at the PM can be generated from PI via sequential phosphorylation at the 4- and 5- positions in the inositol head group by kinases localized at the PM [1, 36]. The roles of PI, PI4P, and PI(4,5)P₂ in receptor-induced signaling were revealed many years after the initial characterizations of these phospholipids.

2.2 The PI Cycle in Stimulated Cells

In 1953, Mabel and Lowell Hokin first discovered that enzyme secretion stimulated by cholinergic agonists in pancreas slices was accompanied by an increase in the incorporation of ³²P into phospholipids, which they called the “phospholipid effect” [14]. In subsequent studies, the Hokins found that ³²P was incorporated primarily into PI and PA [15, 37]. Moreover, they found that the rate of ³H-inositol incorporation into PI was significantly enhanced upon stimulation, but the rate of ¹⁴C incorporation into the DAG backbone was only slightly increased. These results indicated that the phosphoryl-inositol group of PI is renewed in response to extracellular stimuli, whereas the DAG backbone is recycled. Based on these results and other findings, the Hokins proposed a cycle of reactions leading to the increases in PI and PA turnover following stimulation [38]. As illustrated later by Robert Michell (Figure 1A), this PI cycle begins with the cleavage of the phosphoryl-inositol group of PI accompanied by the production of DAG. PA is generated from DAG by DAG kinases (DGKs); ³²P incorporation occurs at this step. PA is then converted into CDP-DAG, and PI is synthesized from CDP-DAG; it is at this step that ³H-inositol incorporation takes place [38, 39]. The causal relationship between the enhanced PI turnover and stimulated secretion of enzymes remained unclear at the time.

One intriguing phenomena later noted in the field was that most stimuli that enhanced PI turnover also elicited Ca^{2+} -mediated responses, even though the requirement of extracellular Ca^{2+} for the enhanced PI turnover was under debate [1, 39]. One common feature of these stimuli was that their effects on target cells were mediated through cell surface receptors. In 1973, Lapetina and Michell identified a PM-associated PLC that mediates PI breakdown and suggested that this enzyme might be controlled by cell surface receptors [40]. Based on these observations, together with other accumulative evidence, Michell proposed an advanced version of the PI cycle in his seminal review in 1975 (Figure 1B) [39]. In this model, Michell suggested that receptor activation-mediated PI breakdown at the PM is the key to trigger PI turnover. Michell also noted that mechanisms must exist to facilitate lipid interchange between the PM and the ER in order to feed PA generated at the PM into the PI synthesis machinery in the ER membrane as well as to provide the PM with PI. Based on the findings of soluble phospholipid exchange proteins [41], Michell hypothesized that cytosolic lipid exchange/transfer proteins mediate the PI cycle by quickly shuttling lipids between the PM and the ER. This model provided substantial conceptual advances and prompted further research to resolve the relationship between PI turnover and Ca^{2+} -mediated responses. The next question was whether PI turnover was simply in the upstream of Ca^{2+} -mediated responses, or these two were independent signaling events following receptor stimulation.

2.3 $\text{PI}(4,5)\text{P}_2$ Hydrolysis, IP_3 Production, and Ca^{2+} Release from the ER Store

In 1979, Fain and Berridge clarified the relationship between the enhanced PI turnover and Ca^{2+} -mediated responses by directly measuring trans-epithelial $^{45}\text{Ca}^{2+}$ flux induced by serotonin in the blowfly salivary gland [42]. They demonstrated that PI breakdown and trans-epithelial Ca^{2+} flux were kinetically coupled, whereas Ca^{2+} -mediated responses, such as fluid secretion, could be un-coupled from PI breakdown under certain conditions. In addition, by carefully removing Ca^{2+} from intracellular stores and extracellular space, they found that the presence of Ca^{2+} was not required for PI breakdown. These observations suggested that PI breakdown is involved in gating Ca^{2+} flux. A few months later, the same authors presented evidence that Ca^{2+} flux was downstream of PI resynthesis after its breakdown during prolonged receptor activation [43]. The next question became evident: what is the mechanism that links PI breakdown to Ca^{2+} flux?

To seek the link, the field started to re-evaluate previous observations of $\text{PI}(4,5)\text{P}_2$ breakdown after receptor activation. It has been shown in early studies that $\text{PI}(4,5)\text{P}_2$ has a high basal rate of ^{32}P incorporation, suggesting that it may have important functions [44]. In 1964, $\text{PI}(4,5)\text{P}_2$ -specific PLC generating DAG and IP_3 was isolated and characterized [45]. In the same year, $\text{PI}(4,5)\text{P}_2$ breakdown after receptor activation was reported by the Hokins' group [46]. They found that ^{32}P incorporation into $\text{PI}(4,5)\text{P}_2$ decreased under the same experimental conditions that resulted in enhanced PI and PA labeling. A later study demonstrated that inositol-bisphosphate, which can only be generated by PI4P or $\text{PI}(4,5)\text{P}_2$ breakdown, accumulated following cell stimulation [47]. The breakdown of $\text{PI}(4,5)\text{P}_2$ following receptor stimulation was examined in details in the late 1970s [48]. Nevertheless, most studies of receptor-stimulated effects on the turnover of inositol-containing lipids at that time were focused on PI, and $\text{PI}(4,5)\text{P}_2$ was generally ignored because it is of low abundance in most tissues, and was difficult to extract due to its unusual chemical properties

[49]. These factors also led to contradictory results of PI(4,5)P₂ metabolism from ³²P labeling experiments following receptor activation.

In 1983, several reports demonstrated a rapid accumulation of IP₃ after receptor-induced breakdown of PI(4,5)P₂ [50-52]. In the same year, the collaboration of Schulz, Irvine, and Berridge elegantly demonstrated that the addition of exogenous IP₃ to permeabilized rat pancreatic acinar cells was sufficient to induce Ca²⁺ release from a non-mitochondrial Ca²⁺ store [53]. A few years later in 1989, several groups identified the IP₃R in the ER membrane as the Ca²⁺ release channel activated following receptor stimulation [54-56]. Together, these findings uncovered the initial steps of the PI(4,5)P₂-Ca²⁺ signaling system, and linked receptor-induced PI(4,5)P₂ hydrolysis at the PM to the release of Ca²⁺ from the ER store by IP₃ production.

2.4 Store-Operated Ca²⁺ Entry (SOCE)

Cytosolic Ca²⁺ levels are tightly controlled at a low concentration (~100 nM, which is 5,000-20,000 times lower than Ca²⁺ concentrations in the ER store or in the extracellular space) in resting cells by the activities of sarco/endoplasmic reticulum Ca²⁺-ATPase (SERCA) and plasma membrane Ca²⁺-ATPase (PMCA) in the ER and the PM, respectively [57]. It was well recognized in the 1980s that receptor stimulation triggered a biphasic cytosolic Ca²⁺ response comprising of an initial transient increase caused by the release of an intracellular Ca²⁺ store, and a subsequent sustained response resulted from activation of Ca²⁺ entry across the PM [58]. Since the intracellular store provides finite sources of Ca²⁺, it is important to activate Ca²⁺ influx to sustain Ca²⁺ signaling and refill the Ca²⁺ store for cell homeostasis. Early studies indicated a mechanism for coordinated regulation of the initial intracellular Ca²⁺ release and the subsequent Ca²⁺ influx [59]. Based on the observation that the Ca²⁺ level of the intracellular store negatively operated Ca²⁺ influx across the PM in agonist-stimulated cells, the first model of this mechanism, later recognized as SOCE, was proposed by James Putney in 1986 (Figure 1C) [7]. Subsequent studies using thapsigargin (TG), a SERCA inhibitor that empties the ER Ca²⁺ store without activating the IP₃R [60], demonstrated that ER Ca²⁺ depletion is necessary and sufficient to activate SOCE [61]. These observations revealed the requirement of an ER-to-PM signaling pathway that links the reduction of ER Ca²⁺ levels to Ca²⁺ influx across the PM as depicted by James Putney in the revised model of SOCE (Figure 1D) [62]. Additional evidence in support of SOCE came from the detection of a Ca²⁺ current corresponding to Ca²⁺ influx activated in agonist-stimulated mast cells and T cells [63, 64]. This current, which is highly selective for Ca²⁺ over monovalent cations and has extremely low conductance, was named as Ca²⁺ release-activated Ca²⁺ current, abbreviated as I_{CRAC} [65]. Many models were proposed to explain this important ER-to-PM signaling pathway that activates SOCE or I_{CRAC} [66]. Nonetheless, the molecular mechanisms of SOCE had remained mysterious for nearly two decades since the first model was proposed.

Overall, the studies described in this chapter established the framework of the PI(4,5)P₂-Ca²⁺ signaling system: receptor stimulation induces the hydrolysis of PM PI(4,5)P₂ by PLC to generate IP₃ that triggers Ca²⁺ release from the ER for cell activation. In this system, PM PI(4,5)P₂ can be resynthesized after hydrolysis via the PI cycle, and ER Ca²⁺ is refilled after

depletion via SOCE to sustain receptor-induced signaling. Both the PI cycle and SOCE provide homeostatic regulation of the PI(4,5)P₂-Ca²⁺ signaling system and require fast communications between the ER and the PM in receptor-stimulated cells. In the next chapters, we will focus on recent discoveries of molecular mechanisms underlying SOCE and the PI cycle. These discoveries revealed the important role of ER-PM junctions, where the ER and the PM are within 10-20 nm from each other (Figure 2A), in the PI(4,5)P₂-Ca²⁺ signaling system.

3. Regulation of Ca²⁺ Homeostasis via SOCE at ER-PM Junctions

3.1 Activation of SOCE by STIM1 and Orai1

A major breakthrough in understanding the molecular and cellular mechanisms of SOCE was the identification of STIM1 and Orai1 [9, 67-70]. STIM1 and its homolog STIM2 were identified as the ER Ca²⁺ sensor of the ER-to-PM SOCE pathway using RNA interference screens in 2005 [9, 67]. In the subsequent year, Orai1 was identified as the PM Ca²⁺ channel that synergistically activates SOCE with STIM1 [68-75]. Both STIM1 and STIM2 are single transmembrane ER proteins with an N-terminal Ca²⁺-sensing EF hand-SAM (EF-SAM) domain in the ER lumen, and a C-terminal cytosolic region with three coiled-coil domains and a polybasic tail (Figure 2B) [76]. The minimal Orai1-activating domain of STIM1, roughly corresponding to the CC2-CC3 domain, was identified by several group [77-79]. In the resting state, inactive STIM1 binds Ca²⁺ via its luminal EF hand motif, and localizes throughout the ER membrane (Figure 2C) [9]. Following stimulation-induced ER Ca²⁺ depletion, STIM1 undergoes conformational changes and rapidly forms oligomers due to loss of Ca²⁺ binding [80, 81]. The exposed polybasic tail in STIM1 oligomers binds PM phosphoinositides, resulting in STIM1 redistribution to ER-PM junctions [80, 82-84]. The translocation of STIM1 to ER-PM junctions was sometimes referred as puncta formation based on the initial finding that cells overexpressing YFP-tagged STIM1 showed bright fluorescence signals in numerous puncta near the cell periphery following ER Ca²⁺ depletion [9]. The close appositions between the ER and the PM at ER-PM junctions allow STIM1 in the ER to trap and activate the PM-localized Orai1 to elicit SOCE [85]. Overall, STIM1 mediates a feedback mechanism that maintains ER Ca²⁺ homeostasis during PI(4,5)P₂-Ca²⁺ signaling by dynamically translocating to ER-PM junctions upon IP₃-induced drop of ER Ca²⁺ levels to activate SOCE, and bringing in Ca²⁺ from the extracellular space to the cytosol to refill the ER store (Figure 2D). Similar to STIM1, STIM2 activates Ca²⁺ influx via activation of Orai1 [86]. Interestingly, STIM2 translocates to ER-PM junctions and activates Ca²⁺ influx upon smaller decreases in ER Ca²⁺ comparing with STIM1, suggesting a role of STIM2 in maintaining basal Ca²⁺ homeostasis. Recent studies identified several proteins, including junctate, CRACR2A, α -SNAP, SARAF, Surf4, POST, septins, and STIMATE, that modulate SOCE mediated by STIM1 and Orai1 at ER-PM junctions [87-94]. Many of these proteins directly bind to STIM1 and/or Orai1, suggesting that the interaction of STIM1 and Orai1 may be regulated by complex mechanisms. Detailed description of studies on STIM1, Orai1, and SOCE can be found in a recent review [8].

3.2 ER-PM Junctions: the Hubs for SOCE and More

ER-PM junctions were first described by Porter and Palade in 1957 in muscle cells studied using electron microscopy (EM) [95]. Similar structures were later reported in neurons [96, 97]. In general, the distance between the ER and the PM at ER-PM junctions is in the range of 10 to 20 nm [10, 98-101]. The narrow distance allows proteins in one compartment to directly interact with proteins or lipids in the other; however, membrane fusion between these two compartments has not been reported at these junctions. The ER and the PM are often aligned in parallel over lengths of approximately 200 nm at ER-PM junctions in cells without overexpressing ER-PM tethering proteins [10, 99]. In addition, ER-PM junctions in many non-excitable cell types occupy less than 1% of the PM [99, 101]. The detection of these small and sparse membrane junctions using conventional microscopy had been a challenge that was recently overcome by developments of genetically-encoded fluorescence markers of ER-PM junctions [20, 92, 102, 103].

In the literature throughout the last half century, a variety of nomenclatures have been used to describe ER-PM junctions in different cell types and organisms. These terms include dyad and triad junctions (or dyads and triads), E-C units, and peripheral couplings in muscle cells [104]; subsurface cisterns in neurons (SSC's) [96]; PM-associated ER and cortical ER in yeast [105, 106]; subrhabdomic cisternae (SRC) in *Drosophila* photoreceptor cells [107]; and ER-PM contacts or ER-PM contact sites. We prefer the term junctions as this nomenclature emphasizes that these are the loci at which the ER and the PM are joined in both physical proximity and functional coupling [11].

The roles of dyads and triads in muscle contractile functions were widely accepted in the 1980s, but the functional relevance of ER-PM junctions in non-excitable cells had remained elusive despite their structural similarities to dyads and triads in muscles [98]. The discovery of the universal SOCE signaling pathway activated by the direct interaction of STIM1 in the ER and Orai1 in the PM at ER-PM junctions indicated that these minute subcellular structures are fundamental to inter-organelle communications in most, if not all, cells. Indeed, multiple lines of evidence recently demonstrated the importance of ER-PM junctions in the metabolism of several lipids [17-20, 33, 34, 108, 109]. This review will focus on the findings of ER-PM junctions relevant to the $PI(4,5)P_2$ - Ca^{2+} signaling system.

STIM1 overexpression caused a significant expansion of ER-PM junctions which greatly facilitated their detection in EM studies [10, 99, 100]. This observation was expected as STIM1 contains both the ER and the PM targeting motifs, and thus can mediate ER-PM tethering. Nevertheless, a requirement for STIM1 in the formation of ER-PM junctions has not been demonstrated in cells lacking STIM1. Intriguingly, STIM1 puncta formed repeatedly in almost identical positions in live cells upon multiple rounds of stimulation [110], suggesting that ER-PM junctions pre-exist, and that proteins other than STIM1 maintain ER-PM junctions in resting cells.

In yeast, ER-PM junctions are maintained by multiple families of proteins, including tricalbins, Scs2/Scs22, and Ist2 [111]. The orthologs of tricalbins, namely extended-synaptotagmins (E-Syts), were shown to mediate ER-PM tethering in mammalian cells [112]. E-Syts were named after synaptotagmin because of the similarity in domain

architecture [113]. E-Syts contain an N-terminal hydrophobic stretch that is anchored in the ER membrane (Figure 3A). These proteins also contain a cytosolic SMP (synaptotagmin-like mitochondrial lipid binding protein) domain followed by three to five C2 domains. The SMP domain of E-Syts belongs to a conserved superfamily of lipid-binding domains [114]. The SMP domain of E-Syt2 has been shown to bind phospholipids, implying a role of E-Syts in shuttling lipids at ER-PM junctions [115]. The very C-terminal C2 domain of E-Syt2 and E-Syt3 mediate PI(4,5)P₂-dependent PM targeting and enable ER-PM tethering (Figure 3B) [112, 113]. When fluorescent protein-tagged E-Syt2 and E-Syt3 were overexpressed in cells, they were enriched at ER-PM junctions and caused an expansion of ER-PM junctions [112]. Consistently, knockdown of all three E-Syts resulted in a significant reduction in the number of ER-PM junctions. Nonetheless, mice lacking both E-Syt2 and E-Syt3 were viable and developed normally [116], suggesting that components in addition to E-Syts provide ER-PM tethering in resting cells to support functions occurring at ER-PM junctions. Intriguingly, E-Syt2 also facilitated activated FGF receptor endocytosis to support FGF signaling [117, 118]. In combination with the finding that ER-PM junctions may serve as membrane protein trafficking hubs [119], these observations suggest a functional role of ER-PM junctions in receptor recycling.

In contrast to the enrichment of E-Syt2 and E-Syt3 at ER-PM junctions, VAP-A (vesicle-associated membrane protein-associated protein A) and VAP-B, the mammalian orthologs of Scs2/Scs22, are distributed throughout the ER. VAP proteins can bind the FFAT (an acronym for two phenylalanines (FF) in an Acidic Tract) motif present in several regulators of phospholipids [120], and thus can support the targeting of proteins, such as Nir2 and OSBP, to ER-PM junctions and ER-Golgi junctions, respectively [20, 35].

Junctate, another ER membrane protein, was found to contribute to the formation of ER-PM junctions [121]. Overexpression of junctate resulted in a significant increase in the number and size of ER-PM junctions, whereas knockdown of junctate had the opposite effect. Overexpressed junctate displayed a diffuse localization throughout the ER, suggesting that junctate interacts with other components, such as the IP₃R or STIM1-Orai1 complexes, to provide ER-PM tethering [91, 121, 122]. Additional molecular components contributing to the formation of ER-PM junctions likely exist, and are remained to be identified.

3.3 Dynamic Regulation of ER-PM Junctions during Ca²⁺ Signaling

Since ER-PM junctions are indispensable platforms for the communications between the ER and the PM, the regulation of the number, size, and/or shape of ER-PM junctions is expected to have significant functional impacts. Several studies demonstrated that the number of ER-PM junctions is dynamically regulated during Ca²⁺ signaling. It was first reported in the EM study by Gardiner and Grey in 1983, which showed that the abundance of ER-PM junctions correlates with Ca²⁺ signaling events during *Xenopus* oocyte maturation [98]. In addition, an increase in the number of ER-PM junctions in Jurkat T cells and in HeLa cells following ER Ca²⁺ depletion by TG was detected by EM [10, 99]. Using a genetically-encoded marker, MAPPER, that selectively labels ER-PM, the increase in the number of ER-PM junctions can be monitored during Ca²⁺ signaling in live cells [20]. Notably, the size and shape of ER-PM junctions labeled by MAPPER remained similar during Ca²⁺ signaling as measured

using stimulated emission depletion (STED) super-resolution microscopy (supplemental info in [20]). Interestingly, the number of ER-PM junctions in pancreatic acinar cells remained unchanged following TG treatment [100]. These results suggested that the abundance of ER-PM junctions may be differentially regulated depending on physiological needs of the cell.

The regulation of the gap distance between the ER and the PM at ER-PM junctions is also expected to have a functional significance [11]. Extending the gap distance at ER-PM junctions by ~6 nm using an engineered Sec22b protein led to impaired neurite growth [123]. By contrast, artificial constraint of the gap at ER-PM junctions restricted the accessibility of STIM1-Orai1 protein complexes to ER-PM junctions [102]. These observations imply that changes in the physical space at ER-PM junctions may lead to differential accommodation of proteins and ions enabling distinct cellular functions.

Recent studies demonstrated that E-Syt1 translocation to ER-PM junctions triggered a decrease in the gap distance at ER-PM junctions (Figure 3B) [20, 124]. Unlike E-Syt2 and E-Syt3, fluorescence protein-tagged E-Syt1 displayed a diffuse ER localization in resting cells. E-Syt1 translocates to ER-PM junctions following an increase in cytosolic Ca^{2+} to the lower micromolar range [20, 112, 125]. The translocation of E-Syt1 requires its cytosolic C2C domain for Ca^{2+} binding and its C2E domain for PM $\text{PI}(4,5)\text{P}_2$ binding [20, 112]. The E-Syt1-triggered decrease in the gap distance at ER-PM junctions was first suggested in live HeLa cells using TIRF (total internal reflection fluorescence) microscopy by applying MAPPER and MAPPER-s, a short form of MAPPER that restricts the gap distance of ER-PM junctions to 10 nm or less [20]. A recent cryo-electron tomography study further confirmed that the gap distance was reduced from 21.8 nm to 14.8 nm following TG treatment in COS-7 cells overexpressing E-Syt1 [124]. This study also demonstrated that overexpression of different ER-PM tethering proteins resulted in differences in the gap distance and in architecture at ER-PM junctions, suggesting the existence of structurally, and likely functionally, distinct ER-PM junctions. E-Syt1 may mediate the decrease in the gap distance through the interaction with other E-Syts at ER-PM junctions since they form hetero-oligomers [112, 118]. In support of this hypothesis, a model of E-Syt2 mediating a decrease in gap at ER-PM junctions was proposed in a recent structural study [126]. Further investigation is required to understand the mechanism underlying the decrease in the gap distance following E-Syt1 translocation to ER-PM junctions.

A decrease in the gap distance at ER-PM junctions mediated by E-Syt1 was shown to be important for $\text{PI}(4,5)\text{P}_2$ - Ca^{2+} signaling [20]. Knockdown of E-Syt1 resulted in defective $\text{PI}(4,5)\text{P}_2$ replenishment following receptor-induced hydrolysis. This defect was rescued by overexpression of wild-type E-Syt1 but not that of E-Syt1-D724A, a mutant incapable of translocating to ER-PM junctions to regulate the gap distance. Consistently, expression of MAPPER-s enhanced the replenishment of $\text{PI}(4,5)\text{P}_2$. Moreover, receptor-induced Ca^{2+} signaling was profoundly reduced when E-Syt1 knockdown cells were challenged with periodic stimulation. Given that knockdown of all three E-Syts failed to disrupt the onset of SOCE [112], the reduced Ca^{2+} signaling in E-Syt1 knockdown cells was likely due to a decrease in IP_3 -mediated Ca^{2+} release resulting from a defective replenishment of PM $\text{PI}(4,5)\text{P}_2$ and the subsequent IP_3 generation. Overall, these observations suggest that the

decrease in the gap distance at ER-PM junctions by E-Syt1 translocation facilitates PI(4,5)P₂ replenishment during PI(4,5)P₂-Ca²⁺ signaling.

Both STIM1 and E-Syt1 are ER membrane proteins that translocate to ER-PM junctions by binding to PI(4,5)P₂ in the PM in stimulated cells. STIM1 translocation is induced by ER Ca²⁺ depletion, whereas E-Syt1 translocation can be independently induced by an increase in cytosolic Ca²⁺. It seems paradoxical that STIM1 and E-Syt1 translocate to ER-PM junctions in stimulated cells via PM PI(4,5)P₂ binding while PM PI(4,5)P₂ is being hydrolyzed. It is possible that the pool of PM PI(4,5)P₂ utilized for PLC-mediated hydrolysis is different from that for the recruitment of STIM1 and E-Syt1 to ER-PM junctions. A recent study suggested that ER-PM tethering by E-Syt1 may form PI(4,5)P₂-enriched micro-domains for STIM1 targeting [127]. Nevertheless, direct evidence for the enrichment of PI(4,5)P₂ at ER-PM junctions has not been demonstrated. Another explanation is that there is enough PI(4,5)P₂ at the PM for IP₃ generation and for the targeting of STIM1 and E-Syt1 to ER-PM junctions in stimulated cells.

4. Regulation of PI(4,5)P₂ Homeostasis via the PI Cycle

4.1 A Non-vesicular Mechanism for PI(4,5)P₂ Replenishment

It was observed more than 30 years ago that the level of PI(4,5)P₂ quickly recovered after the initial breakdown even in the presence of continuous receptor stimulation [50]. This observation indicated that a feedback mechanism is promptly activated to replenish PM PI(4,5)P₂ levels. The resulting near steady PM PI(4,5)P₂ levels may support the recruitment of STIM1 and E-Syt1 to ER-PM junctions as well as other PI(4,5)P₂-dependent functions in stimulated cells. The PM only contains a limited pool of PI for PI(4,5)P₂ re-synthesis [128]. Thus, cells must rely on PI synthesized in the ER to sustain PI(4,5)P₂ levels in the PM during PI(4,5)P₂-Ca²⁺ signaling. This hypothesis is supported by the findings that constitutive or inducible removal of PI from the ER by a bacterial PI-PLC resulted in defective replenishment of PI(4,5)P₂ in the PM [17, 24]. To maintain PM PI(4,5)P₂ levels, PI in the ER must be transported to the PM within a few minutes or less after receptor stimulation. Since diacylglycerol lipids are highly hydrophobic, the rate of spontaneous lipid transfer across an aqueous medium is extremely slow [129]. Blocking vesicular trafficking or depletion of PI(4,5)P₂ precursor lipids in the Golgi apparatus had minimal effects on PI(4,5)P₂ replenishment during PI(4,5)P₂-Ca²⁺ signaling [17, 130, 131]. These findings suggest that a non-vesicular mechanism is involved in the rapid delivery of PI in the ER to the PM for PI(4,5)P₂ replenishment.

Early on, Michell had hypothesized that cytosolic LTPs participate in the lipid shuttling process between the ER and the PM in the PI cycle [39]. The ability of LTPs to mediate lipid transport between membranes was demonstrated in numerous *in vitro* studies using radiolabeled or fluorescent lipids [132]. This inter-membrane lipid transport is likely caused by LTPs binding and facilitating lipid desorption from membranes [133]. It was proposed that lipid transfer between membranes via LTPs *in vivo* would be most efficient when LTPs function at membrane contact sites where LTPs can simultaneously bind two membrane compartments (Figure 4A) [26, 132, 134, 135]. It is likely that efficient shuttling of PI and PA between the ER and the PM in the PI cycle is mediated by LTPs docking at ER-PM

junctions. Consistent with this hypothesis, the decrease in the gap distance via E-Syt1 translocation or MAPPER-s expression facilitated the docking of Nir2, a PITP, at ER-PM junctions during PI(4,5)P₂-Ca²⁺ signaling [20].

4.2 PI Transfer Proteins

There are five PITPs in the human genome which are grouped into two classes based on sequence homology [26]. Class I PITPs consisting of PITP α and PITP β were first purified in the 1970s [136]. These two Class I PITPs only contain the conserved PITP domain that catalyzes the transfer of PI and phosphatidylcholine (PC) between membranes. Thus, Class I PITPs are PI-PC exchangers. The crystal structures of PITP α and PITP β revealed a hydrophobic cavity in the PITP domain that can be occupied by either PI or PC [137, 138]. This cavity is closed by a lid-like α -helix, and the phospholipid is completely buried in this cavity with the polar head group located deep in the cavity. Four residues, threonine 59, lysine 61, glutamate 86, and asparagine 90, in this cavity make hydrogen bonds with the inositol group of PI. These residues are conserved in many, if not all, PITPs in animal cells [137]. Mutation of any one of these four residues resulted in defective PI binding and transfer [139, 140]. Another conserved residue, cysteine 95, was shown to be important for PC binding and transfer [140, 141]. PITP α was identified as a soluble component essential for reconstitution of PLC signaling in permeabilized cells [142-144]. Subsequent studies demonstrated that both PITP α and PITP β support PI(4,5)P₂-related functions [26]. Mice without PITP α died shortly after birth and exhibited spinocerebellar degeneration, intestinal and hepatic fat accumulation, and hypoglycemia; while ablation of PITP β expression in mice resulted in embryonic lethality [145-147]. These results indicated the functional significance of PITP α and PITP β in physiology and development. Nonetheless, the molecular basis of how PITP α and PITP β support PLC signaling and PI(4,5)P₂-related functions remain unclear.

Class II PITPs are consisted of three proteins: PITPNC1 (also known as RdgB β) that only contains the PITP domain; Nir2 (also known as PITPNM1 or RdgB α I) that contains several C-terminal domains following the N-terminal PITP domain (Figure 4B); and Nir3 (also known as PITPNM2 or RdgB α II) that shares a similar domain architecture with Nir2. Early genetic studies revealed the importance of RdgB α , the *Drosophila* ortholog of Nir2 and Nir3, in photo-transduction [26]. In *Drosophila* photoreceptor cells, PI(4,5)P₂ is hydrolyzed by light-activated PLC during photo-transduction. Flies lacking RdgB α exhibited defective photo-transduction and retinal degeneration [148]. Moreover, electrophysiological studies using Kir channels as a PI(4,5)P₂ indicator demonstrated defective PI(4,5)P₂ replenishment following light exposure in the RdgB α mutant photoreceptor cells [149]. This result was recently confirmed by pseudopupil imaging using PLC δ -PH domain to detect PI(4,5)P₂ levels in *Drosophila* photoreceptors [19]. The PITP domain of RdgB α , but not PITP α , was sufficient to rescue the defects in light response and retinal degeneration in RdgB α mutant flies [19, 150]. These findings suggested that the PITP domain of RdgB α contains activities essential for maintaining PM PI(4,5)P₂ levels during photo-transduction, and that the activities cannot be provided by a PI-PC exchanger.

4.3 PI-PA Exchange Mediated by Nir2 at ER-PM Junctions

Although early genetic data indicated the role of RdgB α in PI(4,5)P₂ replenishment following light-induced hydrolysis, the mechanistic basis of how the PI cycle is mediated by PITPs remained unclear until recent studies of a human ortholog of RdgB α , namely Nir2. Nir2 is expressed in most tissues, and is named as the PYK2 N-terminal domain-interacting receptor (Nir) because it was identified as a PYK2-binding protein [151]. A role of Nir2 has been implicated in many cellular functions, including cytokinesis and secretion [152, 153]. Recent studies showed that Nir2 regulates PI(4,5)P₂-Ca²⁺ signaling [17-21]. Knockdown of Nir2 significantly reduced PI(4,5)P₂ replenishment following receptor-induced hydrolysis, whereas Nir2 overexpression had an enhancing effect. Moreover, Ca²⁺ signaling and other PI(4,5)P₂-dependent functions were abolished in cells depleted of Nir2 [17, 18, 21]. The PITP activity of Nir2 is required for PI(4,5)P₂ replenishment as mutations in the conserved inositol-interacting residues or deletion of the PITP domain led to defective PI(4,5)P₂ replenishment [20]. The PI transfer ability of the PITP domains of Nir2 and RdgB α has been shown *in vitro* [19, 150, 154]. Nonetheless, it is very difficult to demonstrate PI transfer among different membrane compartments *in vivo*, because PI specific biosensor is not currently available, and it is believed that PI is quickly converted to PI4P or PI(4,5)P₂ by kinases once it reaches the PM. Despite the technical difficulties, a recent study demonstrated that Nir2-enhanced PI(4,5)P₂ replenishment is dependent on PI transfer from the ER to the PM by employing an inducible approach to acutely remove PI in the ER membrane while monitoring PM PI(4,5)P₂ replenishment following receptor stimulation in live cells [17]. This finding strongly suggests that Nir2 mediates PI transfer from the ER to the PM to support PI(4,5)P₂ replenishment *in vivo*.

Besides the PITP domain, Nir2 possesses targeting motifs for the ER and the PM (Figure 4B). The FFAT motif of Nir2 interacts with the ER membrane proteins VAPs [155]. A C-terminal region containing the DAG-binding DGBL domain and the PA-binding LNS2 domain is important for PM targeting [17, 18, 21]. Overexpressed Nir2 was mainly cytosolic with moderate ER association in resting cells, indicating that Nir2 adopts a partially closed conformation obscuring the ER- and the PM-targeting motifs [17, 18]. The detection of Nir2 in membrane fractions of HeLa cells and *Drosophila* head extract likely reflects the exposure of the ER- and/or the PM-targeting motifs during biochemical preparations *in vitro* [19, 156]. During PI(4,5)P₂-Ca²⁺ signaling, Nir2 rapidly translocated to ER-PM junctions [17, 18, 20]. This observation is consistent with EM studies showing that RdgB α localized to SRC, a specialized ER domain juxtaposed to the PM, in *Drosophila* photoreceptor cells [107, 157]. Nir2 translocation to ER-PM junctions was abolished in the Nir2-FM-D1128A mutant containing point mutations disrupting its VAP- and PA-binding motifs [17]. In addition, inhibition of PA production following PI(4,5)P₂ hydrolysis by pharmacological inhibitors of PLC or DGK significantly reduced Nir2 translocation to ER-PM junctions. Consistently, addition of exogenous PA triggered Nir2 translocation in the absence of receptor stimulation. These observations indicate that Nir2 detects receptor-induced PI(4,5)P₂ hydrolysis by binding to PA in the PM via its C-terminal LNS2 domain. A recent report showed that addition of DiC8-DAG also induced binding of Nir2 to the PM, suggesting that Nir2 interacts with the PM via combined DAG and PA binding [18]. The accumulation of Nir2 at ER-PM junctions was further facilitated by the decrease in the gap

distance at ER-PM junctions by E-Syt1 translocation [20]. These findings suggest that Nir2 translocation to ER-PM junctions is tightly gated during PI(4,5)P₂-Ca²⁺ signaling by DAG and PA production via PI(4,5)P₂ hydrolysis and E-Syt1 translocation. Intriguingly, Nir2 has been shown to localize in the Golgi apparatus and to maintain the Golgi DAG pool [152, 156]. It would be of importance to understand how Nir2 functions in the Golgi apparatus.

The enhanced PI(4,5)P₂ replenishment mediated by Nir2 appears to be dependent on its localization at ER-PM junctions. Unlike wild-type Nir2, overexpression of the Nir2-FM-D1128A mutant failed to enhance PI(4,5)P₂ replenishment [17]. Interestingly, overexpression of the Nir2 PITP domain alone, which distributed in the cytosol, was able to promote PI(4,5)P₂ replenishment in receptor-stimulated cells albeit with much slower kinetics comparing with those overexpressing the full-length Nir2 [17]. These observations are consistent with genetic studies in *Drosophila* showing that the PITP domain of RdgB α is sufficient to rescue retinal defects [19, 150], and support the notion that docking at ER-PM junctions enhances the efficiency of lipid transfer by Nir2. Given that the Nir2-FM-D1128A mutant contains the PITP domain but is unable to enhance PI(4,5)P₂ replenishment, the functional PITP domain appeared to be structurally hindered in resting cells. The binding of Nir2 to PA in the PM and/or VAPs in the ER following receptor stimulation likely opens up the structure and exposes the functional PITP domain to mediate lipid transport at ER-PM junctions. Based on these results, it was proposed that Nir2 is a feedback regulator for PI(4,5)P₂ homeostasis during PI(4,5)P₂-Ca²⁺ signaling by sensing PA production following PI(4,5)P₂ hydrolysis and translocating to ER-PM junctions to support PI(4,5)P₂ replenishment by mediating ER-to-PM PI transfer (Figure 4C) [17]. Ca²⁺-induced E-Syt1 translocation to ER-PM junctions further couples Ca²⁺ signaling to PI(4,5)P₂ homeostasis by ensuring efficient docking of Nir2 at ER-PM junctions [20].

Moreover, recent studies demonstrated that Nir2 facilitates the clearance of PA from the PM during PI(4,5)P₂-Ca²⁺ signaling, and the PITP domain of Nir2 mediates PA transfer *in vitro* [18, 19]. Thus, Nir2 is a PI-PA exchanger that shuttles lipids across the membranes at ER-PM junctions to complete the PI cycle. PITPNC1 can also transfer PA, indicating that PA transfer is a conserved function in Class II PITPs [154]. PM-to-ER PA transfer seems to be important for the PI cycle since inhibition of PA production at PM resulted in a reduced rate of PI resynthesis [26]. The fact that reconstitution of PITP α , a PI-PC exchanger, failed to rescue the defects caused by RdgB α mutation in *Drosophila* also suggests the importance of the PM-to-ER PA transfer in PI(4,5)P₂ replenishment [19, 150]. Interestingly, PA generated by phospholipase D2 (PLD2) from PC failed to support CDP-DAG production in the PI cycle despite that it triggered Nir2 translocation to ER-PM junctions (unpublished data mentioned in [18]). This observation is consistent with a previous report showing that only PA derived from the action of PLC, but not that of PLD, was converted into PI in permeabilized neutrophils [158]. These findings suggest that Nir2 preferentially transfers a subgroup of PA molecules generated by PLC-induced PI(4,5)P₂ hydrolysis. It is of importance to investigate whether Nir2 contributes to the enrichments of stearic acid (18:0) at the *sn*-1 position and arachidonic acid (20:4) at the *sn*-2 position in the DAG backbone of PI. A revised PI cycle showing that Nir2 supports PI(4,5)P₂-Ca²⁺ signaling by facilitating PI-PA exchange at ER-PM junctions is depicted in summary of recent discoveries made by multiple groups [17-21] (Figure 5).

Nir2 translocation to ER-PM junctions may be regulated by a self-inactivation mechanism involving binding to PA in the PM by the C-terminal LNS2 domain, and transferring PA in the PM to the ER by the N-terminal PITP domain. Thus, when PA levels at the PM drop below the critical concentration for LNS2 binding to the PM, Nir2 reverts to the cytosol and PI(4,5)P₂ replenishment is subsequently inactivated. This elaborated mechanism is exemplified by recent work on OSBP, a multi-domain LTP that binds PI4P at the Golgi membrane via the N-terminal PH domain and transfers PI4P from the Golgi apparatus to the ER via its C-terminal lipid transfer domain to mediate sterol-PI4P exchange at ER-Golgi contact sites [35].

4.4 Functions of other Nir Proteins

Nir3, a homolog of Nir2, is expressed in most human tissues [151], although it may be mainly expressed in the retina and the dentate gyrus [159]. Similar to Nir2, Nir3 translocates to ER-PM junctions and mediates PI(4,5)P₂ replenishment following receptor stimulation [17]. Intriguingly, analysis of domain-swapped chimeras of Nir2 and Nir3 demonstrated that Nir3 harbors weaker PITP activity toward PI(4,5)P₂ replenishment, but is more sensitive to PA production than Nir2. These properties allow Nir3 to sense small amounts of PA production, and translocate to ER-PM junctions to maintain basal PM PI(4,5)P₂ levels. By contrast, Nir2 mediates robust PI(4,5)P₂ replenishment, but only following receptor stimulation. Thus, Nir2 and Nir3 work in tandem to support PI(4,5)P₂ homeostasis in response to different physiological conditions. These observations may explain why reconstitution of Nir2, but not Nir3, fully rescued the defects in RdgB α mutant flies [159, 160], since photoreceptors are subjected to intense light stimulation and require ample amounts of PI that would be provided by the stronger PITP Nir2 to support the signaling machinery. In addition, the domain-swapping experiment also indicated that the PITP domain and the ER-PM junction targeting motifs work primarily as modules. It will be important to identify the residues responsible for the substantial differences in activities of Nir2 and Nir3.

Besides Nir2 and Nir3, another Nir protein called Nir1 (PITPNM3) has been identified. Nir1 contains the ER- and the PM-targeting motifs but lacks the PITP domain (Figure 4B) [151, 161]. Thus, Nir1 may serve as an ER-PM tethering protein at ER-PM junctions. The functional significance of Nir1 has been demonstrated in a human disease called autosomal dominant cone dystrophy [162]. Patients with a mutation in the gene encoding Nir1 have defective color vision, low visual acuity, and abnormal cone responses. In addition, Nir1 has been shown to bind to CCL18 to promote the phosphorylation of Akt, and its expression is associated with lymph node and distant metastasis in patients with invasive ductal carcinoma [163]. In RdgB α mutant flies, reconstitution of Nir1 slowed the retina degeneration phenotype, but failed to restore photo-transduction [161]. It is of interest to further examine the subcellular localization and the functions of Nir1.

Previous studies have shown that Nir proteins bind to Ca²⁺ but not Mg²⁺ via their acidic regions [151]. Notably, the VAP-binding FFAT motif resides in this acidic region. This raises interesting questions of whether activation of Nir2 and Nir3 requires Ca²⁺ binding to the acidic regions, and whether Ca²⁺ binding affects the ER targeting via the VAP-FFAT

interaction. Answers to these questions may further expand our understanding of the crosstalk between Ca^{2+} and $\text{PI}(4,5)\text{P}_2$ at ER-PM junctions. In addition, Nir proteins were identified as PYK2-binding partners, suggesting a correlation of ER-PM junctions and receptor signaling. Moreover, the importance of Nir2 at the organismal level remains unclear because of contradictory results derived from Nir2-deficient mice [164, 165]. Further studies are needed to resolve this controversy. Finally, mice lacking Nir3 are fertile and appear healthy [164]. Generating mice with deficiencies in both Nir2 and Nir3 is likely to reveal the importance of these functional redundant PITPs during development.

5. Concluding Remarks

The $\text{PI}(4,5)\text{P}_2$ - Ca^{2+} signaling system is fundamental for cells to respond to extracellular stimuli. Inspired by the initial observation of the “phospholipid effect” in 1953, tremendous research efforts eventually established the framework of the $\text{PI}(4,5)\text{P}_2$ - Ca^{2+} signaling system in 1983. Since then, signaling events and cellular functions activated by the $\text{PI}(4,5)\text{P}_2$ - Ca^{2+} signaling system have been studied extensively. Nonetheless, the molecular mechanisms underlying the feedback control that resets this signaling system for further activation and maintain $\text{PI}(4,5)\text{P}_2$ / Ca^{2+} homeostasis have remained unresolved until recently. In the past decade, the discovery of STIM1 that mediates SOCE by its dynamic translocation to ER-PM junctions has revolutionized our understanding of the feedback regulation of Ca^{2+} during $\text{PI}(4,5)\text{P}_2$ - Ca^{2+} signaling. Moreover, the identification of E-Syt1 and Nir2 that dynamically translocate to ER-PM junctions has revealed the feedback mechanisms maintaining PM $\text{PI}(4,5)\text{P}_2$ homeostasis during $\text{PI}(4,5)\text{P}_2$ - Ca^{2+} signaling. The feedback control of the $\text{PI}(4,5)\text{P}_2$ - Ca^{2+} signaling system mediated by STIM1, E-Syt1, and Nir2 at ER-PM junctions starts quickly following receptor stimulation and persists during $\text{PI}(4,5)\text{P}_2$ - Ca^{2+} signaling. Remarkably, STIM1, E-Syt1, and Nir2 translocate to ER-PM junctions during $\text{PI}(4,5)\text{P}_2$ - Ca^{2+} signaling to mediate their functions in response to signals generated in different subcellular compartments: ER Ca^{2+} depletion for STIM1, cytosolic Ca^{2+} increase for E-Syt1, and PA production at the PM for Nir2. These three proteins all have homologs that target to ER-PM junctions by mechanisms conserved within their protein families. Notably, STIM2 and Nir3 have been shown to work in tandem with their homologs STIM1 and Nir2, respectively, at ER-PM junctions to achieve distinct levels of feedback under different physiological states. Further understanding of these three families of proteins as well as ER-PM junctions, the hubs for the homeostatic regulation of $\text{PI}(4,5)\text{P}_2$ - Ca^{2+} signaling, will shed light on our knowledge of other membrane junctions/contact sites and the spatiotemporal regulation of cell signaling.

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Highlights

- Key discoveries established the PI(4,5)P₂-Ca²⁺ signaling system are summarized
- The PI cycle and SOCE mediate homeostatic regulation of the PI(4,5)P₂-Ca²⁺ signaling system.
- SOCE is mediated by STIM1-Orai1 interaction at ER-PM junctions.
- The PI cycle requires Nir2-mediated inter-organelle PI-PA exchange at ER-PM junctions
- ER-PM junctions are hubs for homeostatic regulation of the PI(4,5)P₂-Ca²⁺ signaling system

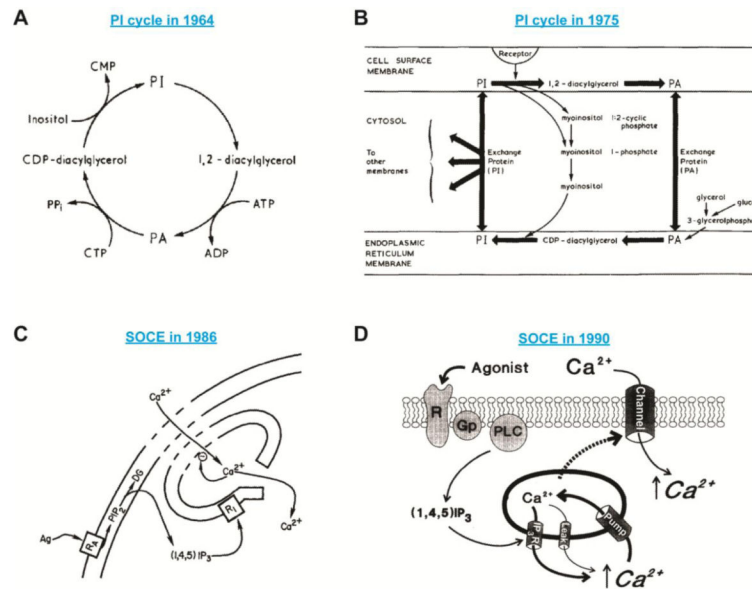


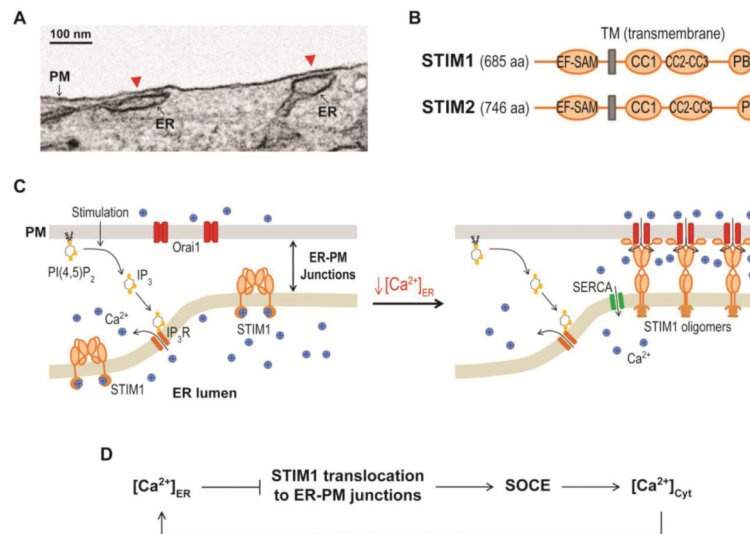
Figure 1.
Models of the PI cycle and SOCE.

(A) An illustration of the first PI cycle model proposed by Mabel and Lowell Hokin in 1964. © Michell and BBA, 1975. Originally published in *Biochim Biophys Acta*, 415 (1975) 81-147.

(B) An advanced version of the PI cycle model proposed by Robert Michell in 1975. The ideas that the PI cycle is an inter-organelle pathway and that cytosolic exchange proteins mediate PI/PA shuttling between the ER and the PM were incorporated into this model. © Michell and BBA, 1975. Originally published in *Biochim Biophys Acta*, 415 (1975) 81-147.

(C) The first model of SOCE proposed by James Putney in 1986. Ag, agonist; R_A , receptor; PIP_2 , $PI(4,5)P_2$; $(1,4,5)IP_3$, IP_3 ; R_I , IP_3R . © Putney and *Cell Calcium*, 1986. Originally published in *Cell Calcium*, 7 (1986) 1-12.

(D) A revised model of SOCE proposed by James Putney in 1990. The dashed arrow indicates an unknown mechanism that links ER Ca^{2+} depletion to the opening of a Ca^{2+} channel at the PM. Ca^{2+} first enters the cytosol and is then pumped into the ER lumen to refill the ER Ca^{2+} store. R, receptor; Gp, G-protein. © Putney and *Cell Calcium*, 1990. Originally published in *Cell Calcium*, 11 (1990) 611-624.

**Figure 2.**

Activation of SOCE by STIM1 and Orai1 at ER-PM junctions.

(A) An electron microscopy image of ER-PM junctions (red arrow heads) in a MAPPER-expressing HeLa cell.

(B) Diagrams of the domain structure of human STIM1 and STIM2. EF-SAM, EF hand and sterile alpha motif; TM, transmembrane; CC1, CC2, and CC3, coiled coil domain 1,2, and 3; PB, polybasic.

(C) STIM1 translocation to ER-PM junctions induced by a decrease in ER Ca^{2+} levels during $\text{PI}(4,5)\text{P}_2$ - Ca^{2+} signaling resulting in SOCE by Orai1 activation.

(D) Schematic representation of feedback regulation of ER Ca^{2+} levels mediated by dynamic translocation of STIM1 to ER-PM junctions.

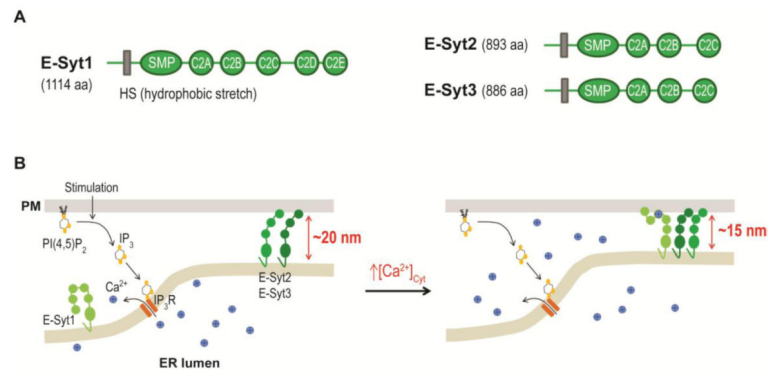


Figure 3.

Regulation of ER-PM junctions by E-Syts.

(A) Diagrams of human E-Syt1, E-Syt2, and E-Syt3 domain structures. HS, hydrophobic stretch; SMP, synaptotagmin-like-mitochondrial-lipid binding protein domain.

(B) E-Syt2 and E-Syt3 provide ER-PM tethering in resting cells. The increase in cytosolic Ca^{2+} levels during $\text{PI}(4,5)\text{P}_2$ - Ca^{2+} signaling triggers E-Syt1 translocation to ER-PM junctions, resulting in a reduction in the gap distance.

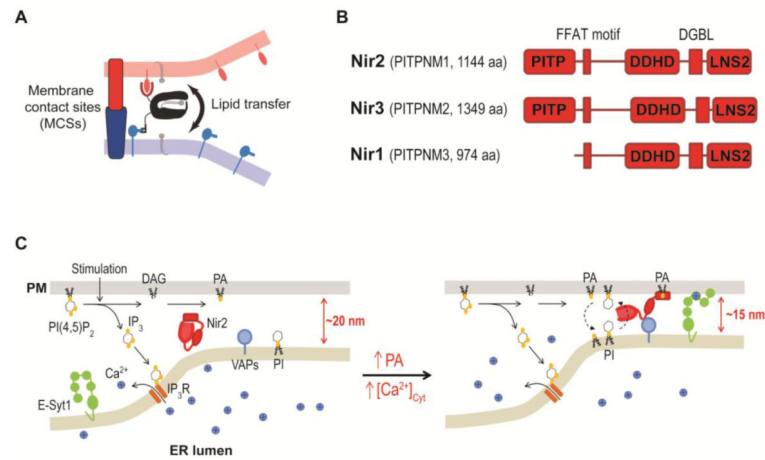


Figure 4. PI(4,5)P₂ homeostasis mediated by Nir2 at ER-PM junctions during PI(4,5)P₂-Ca²⁺ signaling.

(A) Efficient lipid transfer at membrane contact sites illustrated by Tim Levine in 2004. © Levine and TICB, 2004. Originally published in Trends in Cell Biology, 14 (2004) 483-490.

(B) Domain structures of human Nir2, Nir3, and Nir1.

(C) Nir2 is a feedback regulator for PI(4,5)P₂ homeostasis. Nir2 senses PA produced by PI(4,5)P₂ hydrolysis and mediates PI/PA transfer at ER-PM junctions to support PI(4,5)P₂ replenishment during PI(4,5)P₂-Ca²⁺ signaling. The decrease in the gap distance via E-Syt1 translocation facilitates the accumulation of Nir2 at ER-PM junctions.

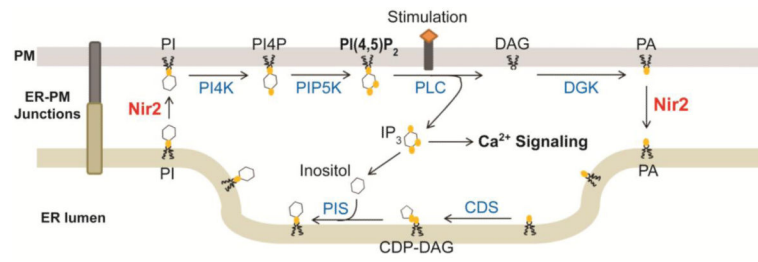


Figure 5. updated model of the PI cycle with Nir2 mediating PI-PA exchange at ER-PM junctions. PI4K, PI-4 kinase; PIP5K, PI4P-5 kinase; CDS, CDP-DAG synthase; PIS, PI synthase.