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REGULATION OF CA^{2+} ENTRY BY INOSITOL LIPIDS IN MAMMALIAN CELLS BY MULTIPLE MECHANISMS

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Abstract

Increased phosphoinositide turnover was first identified as an early signal transduction event initiated by cell surface receptors that were linked to calcium signaling. Subsequently, the generation of inositol 1,4,5-trisphosphate by phosphoinositide-specific phospholipase C enzymes was defined as the major link between inositide turnover and the cytosolic Ca^{2+} rise in response to external stimulation. However, in the last decades, phosphoinositides have been emerging as major regulatory lipids involved in virtually every membrane-associated signaling process. Phosphoinositides regulate both the activity and the trafficking of almost all ion channels and transporters contributing to the maintenance of the ionic gradients that are essential for the proper functioning of all eukaryotic cells. Here we summarize the various means by which phosphoinositides affect ion channel functions with special emphasis on Ca^{2+} signaling and outline the principles that govern the highly compartmentalized roles of these regulatory lipids.

INTRODUCTION

The requirement of calcium ions for contractility of the heart was recognized by Sydney Ringer in 1883 marking the beginning of the development of our understanding of the role of Ca^{2+} in muscle contraction (see [1] for historical details). Calcium since became known as one of the most universal intracellular signaling molecules that regulates virtually every aspect of a cell's life and death. These not only include rapid processes such as contraction and secretion but also long-term responses such as regulation of metabolic enzymes and ultimately gene expression. To act as an effective intracellular signal cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) must be kept at a low (~100 nM) resting level, but also needs to rapidly rise to high levels (up to 10–100 μM) and quickly return to baseline. Therefore, the delicate control of cytoplasmic Ca^{2+} concentration has been a high priority during evolution. The source of Ca^{2+} for the $[Ca^{2+}]_i$ increase, in most cases, is the extracellular fluid but cells can also use Ca^{2+} stored in organelles, a mechanism highly evolved in skeletal muscle. Rapid release of Ca^{2+} from intracellular stores [mostly the endoplasmic reticulum (ER)] is a general mechanism to rapidly elevate cytosolic Ca^{2+}, but increased influx of Ca^{2+} is usually necessary to elicit a full biological response.

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The mechanism of Ca^{2+} signal generation in so-called non-excitable tissues has become a center of interest when a group of hormones and neurotransmitters acting on cell surface receptors was found to activate cells without production of cAMP, the then recently discovered "second messenger" (see [2]). These stimuli termed "calcium-mobilizing agonists" were often linked with cGMP production and increased turnover of phosphatidylinositol (PtdIns) and both Ca^{2+} release and influx responses [3]. For a period, it was believed that the source of the internal Ca^{2+} release was the mitochondria, an organelle known for its ability to take up and release significant amounts of Ca^{2+} [4]. Two major discoveries have finally provided with an explanation of how the Ca^{2+} signal was generated. First, it was recognized that PtdIns(4,5)P_2 breakdown by PLC in the plasma membrane (PM) is the first step in the increased turnover of PtdIns after hormonal stimulation [5,6], and second, it was demonstrated that one of the products of this reaction, Ins(1,4,5)P_3 was capable of releasing Ca^{2+} from non-mitochondrial internal Ca^{2+} stores [7]. With the finding of the Ins(1,4,5)P_3 receptors (InsP_3Rs) in the ER [8] and identifying them as Ca^{2+} release channels [9], the link between PtdIns turnover and Ca^{2+} release has been established.

Finding the mechanism responsible for the subsequent Ca^{2+} influx has proven to pose a greater challenge. In 1986, James Putney postulated that during stimulation of calcium mobilizing receptors, depletion of the ER Ca^{2+} stores was sufficient to activate Ca^{2+} influx without the need for any of the messengers formed by PLC action [10]. This mechanism has become known as store-operated Ca^{2+} entry (SOCE) and was believed not to depend on phosphoinositides other than indirectly through Ins(1,4,5)P_3, a regulator of Ca^{2+} release from the ER. The nature of the channel responsible for SOCE remained elusive and for several years TRPC channels had been the most favored candidates [11]. TRP channels are non-specific cation channels first identified in *Drosophila* eye as the proteins responsible for a characteristic light-induced change in the membrane potential (transient receptor potential) in electric recordings from the eye [12]. After cloning of several similar channels from mammalian sources [13], research on TRP have dominated the field of SOCE [11]. However, the ion selectivity and I/V profile of TRP channels in electrical recordings did not match those of I_{CRAC}, the electrophysiological correlate of SOCE previously identified in mast cells and T-cells [14,15], both of which display massive SOCE, questioning whether TRP channels were responsible for the Ca^{2+} influx in these cells. The other unsolved question was the means by which the decreased luminal ER Ca^{2+} ($[Ca^{2+}]_{ER}$) is communicated to the PM to activate Ca^{2+} entry. The most accepted model termed "conformational coupling" assumed some sort of molecular proximity between the ER and the PM, where ER-resident proteins could regulate PM ion channels by direct interaction [16], although the existence of a diffusible messenger has been also considered [17]. The final answers to these questions were found recently when the ER proteins, STIM1 and -2, were discovered as the ER Ca^{2+} sensors and the Orai1/CRACM proteins as essential component of SOCE and expression of these two proteins reconstituted both I_{CRAC} and SOCE (see[18–20]. However, it should be noted that SOCE may not be exclusively attributed to the Orai channels, as recent evidence suggests that STIM1 can also communicate to TRPC proteins [21,22] and that elimination of either Orai1 or TRPC channels can decrease the native SOC pathway in some cells [23].

Although the link between SOCE and phosphoinositides has been firmly established (via Ins (1,4,5)P_3 production) several studies suggested that a variety of other ion channels and transporters can also be regulated by PLC-coupled receptors and ultimately by membrane phosphoinositides (see [24,25]). Therefore, the phosphoinositide-regulation of ion channel and membrane transport activities has emerged as a research topic parallel to the questions on SOCE and became an important new aspect of neuroscience and cell biology [26,27]. A third thread of research converging on this subject matter originated from the questions of how newly synthesized channels are delivered to the PM and whether the channels were active within the internal membranes en route to their final destination in the PM (see [28]). Even more

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importantly, the removal and insertion of ion channels using internalization and recycling machineries of the cells, was recognized as a way of rapidly regulating the number of channels available in the PM. These processes linked ion channels to the general questions of cell biology namely membrane assembly and movements within the cells. In this review we will try to highlight some examples of the numerous distinct ways of regulation of ion channels by phosphoinositides. Because of the extensive literature in each of these topics, this review will not provide detailed in depth discussion available in several comprehensive reviews, but will attempt to emphasize the important overlaps between these otherwise disparate research areas that all relate to some aspect of Ca^{2+} signaling.

PHOSHOINOSITIDE REGULATION OF CALCIUM SIGNALING

Regulation of the SOCE pathway by phosphoinositides

With the recent discovery of the STIM and Orai/CRACM proteins, the basic mechanism by which Ca^{2+} depletion of the ER leads to Ca^{2+} influx has been largely uncovered [18–20]. As detailed in several recent reviews [19,29,30], the ER-resident single transmembrane protein STIM1 undergoes oligomerization upon Ca^{2+} unbinding in its Ca^{2+} sensing luminal EF-hand domain. This oligomerization induces an interaction between the ER-bound STIM1 protein and the Orai/CRACM channels located in the PM, leading to the opening of the latter and stabilization of ER-PM contact sites [31,32]. STIM1 contains a polybasic sequence at its very C-terminus and its similarity to polybasic sequences found in other proteins that interact with negatively charged membrane phospholipids raised the possibility that STIM1-PM interactions are also facilitated by anionic phospholipids [31]. However, STIM1 constructs lacking the polybasic domain still can respond to Ca^{2+} depletion with oligomerization and activate Orai1-mediated Ca^{2+} influx indicating, that polybasic domains are not essential for SOCE [22,33, 34].

Nevertheless, the pivotal role of the luminal ER [Ca^{2+}] in the control of SOCE together with the central importance of Ins(1,4,5)P_3 in the regulation of Ca^{2+} release from the ER, tightly links SOCE to Ins(1,4,5)P_3 formation (Fig. 1A). Any intervention that reduces the level of Ins (1,4,5)P_3 – (such as inhibition of PLC, or depletion of its substrate, PtdIns(4,5)P_2), immediately reduces SOCE provided that the ER Ca^{2+} uptake mechanism is functional. This was demonstrated recently when rapid elimination of the PM PtdIns(4,5)P_2 led to a quick reversal of SOCE activation by a Ca^{2+}-mobilizing agonist [35]. Similarly, several reports have shown that inhibition of the phosphatidylinositol 4-kinase(s) (PI4Ks) that supply the PM with PtdIns4*P* [and hence PtdIns(4,5)P_2] leads to a depletion of PtdIns(4,5)P_2 and a diminishing Ins(1,4,5)P_3 generation in cells stimulated by Ca^{2+}-mobilizing agonists (e.g. [36,37] ultimately resulting in an inhibition of SOCE. These results, however, did not tell whether SOCE itself requires phosphoinositides. This question can only be studied if SOCE is activated without Ins (1,4,5)P_3, namely by inhibition of the SERCA Ca^{2+} pump [by thapsigargin or the reversible inhibitor 2,5-di-(tert-butyl)-1,4-hydroquinone, TBHQ]. A few studies have investigated this question. Broad et al [38] has reported that high concentration of the PI3K inhibitors wortmannin (Wm) and LY294002 inhibited SOCE and endogenous I_{CRAC} in rat basophilic leukemia cells. They showed that this inhibition was not caused by the lack of $InsP_3$ or DAG and it correlated better with changes in PtdIns4*P* than in PtdIns(4,5)P_2 levels [38]. These authors also showed that the PLC inhibitor, U73122 also inhibited the SOCE and I_{CRAC} but only if added before its activation and the drug failed to close SOCE once it got activated. Rosado et al. also analyzed the effects of LY294002 on SOCE in platelets and concluded that the inhibitory effect was not due to depolarization [39]. The interpretation of the experiments is complicated by the fact that Wm at concentrations used in these studies (but not LY294002) also inhibits MLCK, and MLCK inhibitors have been shown to inhibit SOCE [40]. This question was recently reexamined in our group using overexpressed STIM1 and Orai1 proteins.

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We found that the movements of STIM1 were slightly impaired in cells acutely depleted in PtdIns(4,5)P_2 but this had no impact on SOCE. However, PtdIns4P depletion either by using PI3K inhibitors at concentrations that inhibit type-III PI4Ks, or by PLC activation by agonists had a significant inhibitory effect on SOCE. These inhibitory effects correlated with changes in PtdIns4*P* rather than PtdIns(4,5)P_2, and were not related to STIM1 movements. (Korzeniowski et al. submitted). These studies raise the possibility that Orai1 activation requires PtdIns4*P* generation. These findings are even more intriguing as the PI4K that is responsible for the generation of the PM pool of PtdIn4*P* is mostly ER localized in mammalian cells [41]. This makes the ER-PM contact sites stabilized by STIM1-Orai1 interaction a special cellular compartment potentially important for lipid transfer between the two membranes.

Regulation of Ca^{2+} transport by the plasma membrane phosphoinositides

The first reports on a direct role of membrane PtdIns(4,5)P_2 on a calcium transport system was the finding of a stimulatory effect of acidic phospholipids on the PM Ca^{2+} pump that was similar to those elicited by calmodulin or limited proteolysis [42]. Similarly, purified sarcoplasmic reticulum Ca^{2+} ATPase was shown to contain tightly associated phosphoinositides suggesting a possible regulatory interaction between the Ca^{2+} pump and these phospholipids [43]. However, fewer studies addressed whether the lipid regulation shown in isolated membranes or purified proteins does play a role in an intact cell system. Our studies using Wm at concentrations that inhibit type-III PI 4-kinases in combination with agonist-induced PLC activation showed that cells depleted in PtdIns(4,5)P_2 still can reduce their $[Ca^{2+}]_i$ very effectively with Ca^{2+} extrusion [44]. On the other hand, LY294002 was shown to inhibit Ca^{2+} extrusion in platelets at concentrations that also inhibit PI 4-kinases and perhaps other enzymes [45].

Although the effect of anionic phospholipids on the Na^+/Ca^{2+} exchange activity in reconstituted systems have been described earlier [46], Donald Hilgemann was the first to demonstrate that the effects of ATP on the Na^+/Ca^{2+} exchanger (NCX1) and on K_{ATP} potassium channels in giant excised patches of the heart were mediated by the synthesis of anionic phospholipids, mainly PtdIns(4,5)P_2 [47]. However, the changes in phosphoinositides in the heart are not particularly robust after hormonal influences and it is still puzzling whether Na^+/Ca^{2+} exchange activities are controlled by changing PtdIns(4,5)P_2 levels or the high PtdIns(4,5)P_2 of the PM is required as a permissive environment restricting the transport activity of the NCX1 protein to the PM [48]. It is noteworthy, though, that Ca^{2+}-calmodulin has a strong stimulatory effect on ATP-induced PtdIns(4,5)P_2 (but not PtdIns4P) synthesis in crude cardiac membranes [49] and osmotically-induced shrinkage increases both PtdIns4*P* and PtdIns(4,5)P_2 in several cell types [49]. However, whether PtdIns(4,5)P_2 levels change during contraction and relaxation in the heart is yet to be determined.

The reversal of the inactivation (termed run-down) of the Na^+/Ca^{2+} exchanger or the K_{ATP} potassium channel in excised macropatches by PtdIns(4,5)P_2 and ATP (via synthesis of PtdIns(4,5)P_2)[47] has established an experimental paradigm that was followed by a large number of studies revealing the PtdIns(4,5)P_2 requirement of several ion channels [24,25]. These included potassium channels of a great variety (see below) as well as calcium conductive channels, such as the voltage-gated P/Q [50] and N-type [25,51] Ca^{2+} channels as well as several members of the TRP family of non-selective cation channels [52]. Recently this list was extended to some of the ligand-gated channels such as the NMDA receptors [53] and the ATP-gated P2X4 [54]. In almost all of these studies the question was raised whether PtdIns(4,5)P_2 is merely a requirement for the proper functioning of the channels or it is actually regulated by PtdIns(4,5)P_2 changes that occur during activation of PLC-coupled receptor mechanisms. This question has been unequivocally answered in some cases, such as the M-current (mediated by the KCNQ potassium channels) [55,56] or the cold and menthol sensitive

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TRPM8 channels [57,58]. The activity of the KCNQ channels follows very tightly the changes in PM PtdIns(4,5)P_2 so much so that it can be used as a PtdIns(4,5)P_2 sensor [55]. However, there are other examples where the role of PtdIns(4,5)P_2 is more controversial, being reported as inhibitory as well as a stimulatory factor. For example, an apparently opposing effects of PtdIns(4,5)P_2 are thought to depend on the levels of the lipid in the case of P/Q channels. It was proposed that a high affinity lipid binding site site stabilizes the channel, whereas binding of PtdIns(4,5)P_2 to a putative lower affinity site would switch the channel into a “reluctant” mode [51]. In another example, TRPV1 channels were shown to be sensitized by PLC-coupled agonists apparently via reduction of PtdIns(4,5)P_2 [59] but the same lipid was found to be required for the recovery of TRPV1 channels from desensitization [60]. These seemingly contradictory findings were resolved when it was shown that PtdIns(4,5)P_2 depletion contributes to the desensitization of the TRPV1 channels at high capsaicin concentration, but at low capsaicin doses PtdIns(4,5)P_2 inhibited the same channels [61]. The inositol lipid regulation of TRPM8 and TRPV1 channels are discussed in detail in this issue by Tibor Rohacs.

The mechanism of direct inositol lipid regulation of these Ca^{2+} conductive channels remains elusive (Fig. 1B). Almost all of these channels contain clusters of basic residues in their membrane-adjacent regions facing the cytosol or within their C-terminal tails. In some TRP channels there are sequences identified as “half PH domains” that can form intermolecular PH domains potentially binding phosphoinositides [62], but lipid regulation was also mapped to the TRP domains of their cytoplasmic tails [57,63]. An interesting and common feature of the PtdIns(4,5)P_2 regulation of many potassium and TRP channels is that the lipid alters the interaction of the channels with other specific regulators. In several instances this regulator is calmodulin, which competes with PtdIns(4,5)P_2 such as in TRPC6, TRPV1, voltage-gated Ca^{2+} channels and KCNQ potassium channels [64]. Other examples include PtdIns(4,5)P_2 modulation of the interaction of Kir3 potassium channels with βγ-subunits [65], or the PtdIns(4,5)P_2-mediated decrease of the apparent ATP affinity of K_{ATP} channels [66]. The direct interaction of phosphoinositides with ion channels whether regulatory or permissive is a fascinating research area and adds to the complexity by which phosphoinositide changes can influence the ionic homeostasis of the cells.

Regulation of the removal or insertion of channels in the plasma membrane by phosphoinositides

Delivery of ion channels and transporters from a reserve pool found in subplasmalemmal vesicles could be a mechanism by which the number of channels can be rapidly increased in the PM. Conversely, channels can be rapidly moved from the membrane by endocytosis to decrease their number in the membrane (Fig. 1C). In addition, the constitutive trafficking of Ca^{2+} channel subunits to the site of their assembly is important to supply the PM with functional channels. Regulation of membrane trafficking and fusion events, therefore, can indirectly influence ion fluxes through the membrane. Since phosphoinositides play important roles in both exo- and endocytosis [67], they provide an additional means to channel regulation.

PI3K-dependent trafficking of voltage-gated Ca^{2+} channels to the PM has been recently reported in myoblasts and COS-7 cells [68]. This is mediated by PtdIns(3,4,5)P_3 induced Akt activation and phosphorylation on a Ser residue of the β2a subunit of the N-type Ca^{2+} channels [68]. An IGF1-induced PI3K-dependent increase of high voltage-activated Ca^{2+} channels in cerebellar neurons has also been described [69]. Another well-documented example of regulated channel insertion into the PM is the epithelial sodium channel (ENaC) (even though this is not directly related to Ca^{2+} signaling), a channel that also is regulated by PtdIns(4,5)P_2 once in the PM [70]. ENaC channels rapidly incorporate into the apical membrane by a Rho and PIP 5-kinase-dependent mechanism [71], whereas their removal from the membrane and degradation is inhibited by a PI3K-mediated mechanism [72].

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There are other documented examples of the phosphoinositide-regulation of trafficking of TRP channels [28]. A curious stimulatory effect of PLCγ1 expression on the activity of TRPC3 channels (which did not require the PLC activity of the protein) has been described [73]. This was attributed to the enhanced surface expression of the channels aided by an intermolecular PH domain formation between the 1/2 PH-domains of PLCγ1 and the other half within the intracellular tails of TRPC channels and the presumed inositol lipid binding of the hybrid PH domain [62]. It has been shown recently that growth factor stimulation enhances the insertion of TRPC5 channels from vesicular pools by a PI3K and Rac1 mediated mechanism that also involves PIP5KIα [74]. Similarly, EGF-stimulated insertion of TRPC4 channels into the PM was also reported. Channels of other TRP subfamilies, including TRPV2 and TRPV1 were also shown to translocate to the PM following NGF or IGF-1 stimulation [75,76]. One of the most prominent examples of channel regulation by insertion into and removal from the PM is found in *Drosophila* eye, where the TRP-like, or TRPL channel was shown to undergo a light-induced internalization into a storage compartment from which the channel gets reinserted to the photoreceptor membrane during recovery in the dark [77,78].

Several studies have shown the removal of ion channels from the PM as a way of reducing channel activity. The Kir1.1 K^+ channels (also known as ROMK) in the kidney are down regulated by clathrin-dependent endocytosis during dietary potassium restriction by a mechanism involving the endocytic adaptor protein, ARH and the “NPXY” internalization motif present in these channels [79]. Overexpression of PI 4-kinase and PIP 5-kinase significantly decreased rather than increased the current densities of NXC1, and a targeted expression of PI4KIIα in the heart of transgenic mice resulted in a large decrease of NXC1 current density without a change in expression levels and an increased rate of clathrin-mediated endocytosis [80]. These data strongly suggested that the effects of phosphoinositides on the endocytosis and recycling of these channels are dominant during long-term experiments.

These examples demonstrate that the acute regulation of channel activity in the PM by phosphoinositide changes is complemented with phosphoinositide control of the trafficking and distribution of these channels between the various membranes, adding a new level of complexity to the control of Ca^{2+} influx into the cell.

Regulation of Ca^{2+} influx by phosphoinositides indirectly by the changing membrane potential

The amount of Ca^{2+} entering a cell through various Ca^{2+} conductive channels is primarily determined by the open and closed state of the channel pores. Activation and inactivation of voltage gated Ca^{2+} channels are clearly regulated by the membrane potential, but even channels that are not gated by voltage are sensitive to the membrane potential as the amount of Ca^{2+} flowing through them depends on the electrochemical gradient of Ca^{2+}. This is especially true for the I_{CRAC} channels that show a pronounced inward rectification and hence this form of Ca^{2+} entry is very sensitive to depolarization [81]. It is because of this feature of Ca^{2+} entry why phosphoinositide-regulation of the various potassium channels is relevant to Ca^{2+} signaling. Potassium channels of a great variety show a wide range of functions. Inwardly rectifying K^+ (Kir) channels regulate the pattern of firing of action potentials, contribute to metabolic regulation (via control of insulin secretion) or determine potassium homeostasis (ROMK). A large family of two-pore K^+ channels serve as “background channels” determining the basal membrane potential in many neurons and neuroendocrine tissues [82], while members of the KCNQ family of K^+ channels underlie the M-current (KCNQ2, -3, -5) found in neurons as well as the I(Ks) current (KCNQ1) of cardiac tissues [83]. As outlined above, all these K^+ channels show a dependence and/or regulation by PM phosphoinositides that have been discussed in many recent reviews and will not be further detailed here [84]. However, it is important to keep in mind that phosphoinositide changes can have profound influence on both

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the basal membrane potential or on the shape of action potentials via their influence on these K^+ channels, which, in turn, affects Ca^{2+} signaling (Fig. 1B). Such an indirect effect should not be mistaken with the direct regulation of Ca^{2+} conductive channels by phosphoinositides.

ORGANIZATION OF LOCALIZED CHANGES AND MICRODOMAINS IN PHOSPHOINOSITIDE REGULATION

The extent to which phosphoinositide regulation is compartmentalized is being increasingly recognized. The PM has long been considered as the relevant site of polyphosphoinositide synthesis and PLC-mediated hydrolysis from a Ca^{2+} signaling standpoint. The only question regarding compartmentalization was how the ER-synthesized PtdIns reaches the PM to serve as precursor for polyphosphoinositide synthesis leading to the identification of the PI transfer proteins [85,86]. This view has suddenly changed when the yeast PI 3-kinase, Vps34p and its lipid product, PtdIns3P were identified as key regulators of vacuolar sorting in yeast [87] and endocytic trafficking in mammalian cells [88]. This was followed by the surprising localization of several PI 4-kinase and phosphoinositide phosphatase isoforms in the Golgi and endocytic compartments [89–91] challenging our understanding of how PtdIns4*P* is made and reaches the PM in mammalian cells [92]. Therefore, when thinking of compartmentalization, most of us refer to the unique phosphoinositides composition of various intracellular membranes. While this view is certainly valid and important in defining the inositide signature of internal membranes, it is becoming evident that further compartmentalization of phosphoinositides takes place even within the PM, Golgi or any membrane within the cell.

Some of these compartments can be physically unique, such as the apparent enrichment of phosphoinositides in detergent resistant membrane domains often referred to as “rafts” [93]. Pike and Casey [94] suggested that agonist-sensitive phosphoinositides are associated with caveolin-rich rafts in A431 cells. Indeed, the type II PI 4-kinase was found in and purified from detergent resistant (though non-caveolar) membrane fractions [95]. Similarly, several important signaling molecules related to phosphoinositide synthesis and hydrolysis are present in “rafts” [96,97] leading to the general notion that phosphoinositides are mostly “raft” associated. However, “raft” is a term that is not linked to any specific membrane (although most believe it is part of the PM) and thorough attempts using the recently available phosphoinositide visualization tools could not confirm that PtdIns(4,5)P_2 are enriched in “rafts” in the PM [98]. Moreover, the majority of the PI4KIIα enzyme, mentioned above, has been localized to the TGN and endosomal membrane network as opposed to the PM [89–91], although, undoubtedly this activity is present in the PM (the best example being the red blood cell membrane). Nevertheless, the possibility still remains that some phsophoinositide regulation is linked to “rafts” and in this context it is relevant that among the Ca^{2+} conductive channels, the TRPC1 channels are enriched in caveoli [99] and are responsible to the cyclodextrin sensitivity of SOCE in some cell types [100].

Regardless of whether inositides are segregated in physically definable compartments, strong evidence suggests that phosphoinositides are functionally compartmentalized. On the one hand, this is a result of the interaction of phosphoinositides, such as PtdIns(4,5)P_2 with multiple effector proteins (Fig. 2A). These protein-phosphoinositide interactions display a variety of affinities and dissociation rates. This implies that during activation of a PLC or phosphoinositide phosphatase enzyme, the decreasing free PtdIns(4,5)P_2 level will not be uniformly followed by a similar decreases in the amount of PtdIns(4,5)P_2 bound to various interacting molecules. Proteins that bind PtdIns(4,5)P_2 with high affinity and slow dissociation rate may not “sense” immediately the decreasing lipid levels while those from which the lipid rapidly dissociates will “read” the free lipid changes very closely. This affinity-driven functional compartmentalization was shown to play a role for example in the differential inositide sensitivities of two-pore K^+ channels [101]. Since the lateral diffusion of free

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phosphoinositides is fast, it is not possible to generate a sharp lipid gradient in any particular membrane without association of the lipids with proteins with slower lateral mobility. The dissociation rate of the lipid from the binding protein (the effector) together with the lateral mobility of the latter will determine how far the lipid can diffuse on the “back” of an effector molecule. This has been elegantly analyzed recently using pleckstrin homology (PH) domains with various affinities to PtdIns(4,5)P_2 [102]. This mechanism, however, alone is probably not sufficient to generate functional inositide gradients. What is also needed is the highly localized production (or elimination) of the lipids in the close vicinity of the effector molecule. This model assumes that the inositol lipid kinases (and/or phosphatase) are in close association with the effector molecule (for this review, an ion channel) that is to be regulated by the lipid. This arrangement has several advantages: the localized lipid change can control the channel without any “spilling” effect on neighboring proteins, and the lipid binding specificity of the channel is less critical for the regulation. The specificity is provided by the kinase and phosphatase that is tightly associated with the signaling complex. Although this signaling organization represents the highest degree of specificity and is most likely widely used in biology, it is the most difficult to demonstrate experimentally. Such highly localized lipid changes dedicated to specific effector molecules will probably evade detection by the widely used GFP-PH domain lipid binding tools unless the probe originates from the very effector molecule itself. There are already few examples of the use of ion channels as reporters of phosphoinositide changes [55]. This organization explains why inositide kinases or phosphatases with apparently identical localization and biochemical activity can assume non-redundant functions. Recent examples are the distinct roles of two PIP 5-kinase isoforms, both localized in phagocytic cups, in actin polymerization and phagocytosis [103], or the non-redundant role of PI 4-kinase IIIβ and PI4KIIα, both localized to the Golgi, in supporting the transfer function of the ceramide transfer protein [104]. Although this level of specificity has not been clearly demonstrated for Ca^{2+} conductive channels there are some indications that such specific control mechanisms also exists in ion channel regulation. Interaction of ion channels with enzymes that control phosphoinositides has been reported. One of the TRP channels, TRPM7 (TRP-PLIK) was identified as an interactor with the C-terminal tail of PLCβ1 [105]. Subsequently it was shown that TRPM7 associates with PLCβ1 and is regulated by PLC-activating agonists [106], although the direction of regulation remains controversial [107]. In another example, proteomic analysis of proteins associated with the purinergic P2X7 channel has identified PI4KIIIα as part of the channel-associated complex [108]. The recently described voltage-regulated inositide 5-phosphatase isolated from ciona intestinalis (ci-VSP) also suggests that rapid localized changes in phosphoinositide levels regulated by voltage could be directly linked to ion channel function [109].

Unfortunately, it is extremely difficult to analyze the existence and the importance of direct interaction of inositide kinases or phosphatases with ion channels. RNAi-mediated depletion, or expression of dominant negative versions of these enzymes, has grave consequences on the vesicular trafficking of the cells likely altering the distribution of these channels. Interruption of the association between the two proteins or acute inhibition of the enzymes might be a better approach to analyze the significance of these protein-protein interactions leading to localized delivery of phosphoinositides to ion channels.

CONCLUDING REMARKS

Postulating and revealing the connection between phosphoinositides and calcium signaling [2,110] have been one of the greatest milestones in the last 50 years in signal transduction research. Yet, none of us who witnessed these developments could have foreseen the complexity, depth and variety of inositide-regulation of cellular Ca^{2+} homeostasis. This short summary highlighted three aspects of Ca^{2+} channel regulation, namely the classical link between PLC-mediated Ins(1,4,5)P_3 production, Ca^{2+} release and the store-operated Ca^{2+}

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entry, the direct effect of PM phosphoinositides on channel gating, and the regulation of channel trafficking by phosphoinositides. This, however, is an artificial separation, because these regulatory means are closely interrelated and work in concert all the time. Appreciating this complexity is crucial for the right interpretation of our experimental data. Often the only way to change phosphoinositides is to express or knock down the enzymes that either form them (the kinases) or eliminate them (the phosphatases). However, these manipulations require a long time period before the effects can be analyzed. During this time the assembly, trafficking or elimination of the channels may have a more important and lasting effects on apparent channel activity than the actual change in the phosphoinositides in the membrane. This is one of the many reasons why development of specific inositide kinase inhibitors is so desirable and also why we and others have been working on methods by which inositides can be changed within the intact cell acutely in a matter of seconds [56,111,112]. These new tools should facilitate our understanding on the importance of each of the inositide-dependent regulatory processes for each of the channels in a real cellular setting. Such studies are still needed because the fundamental question of what makes these lipids so versatile and universally adaptable signaling cues remains to be answered.

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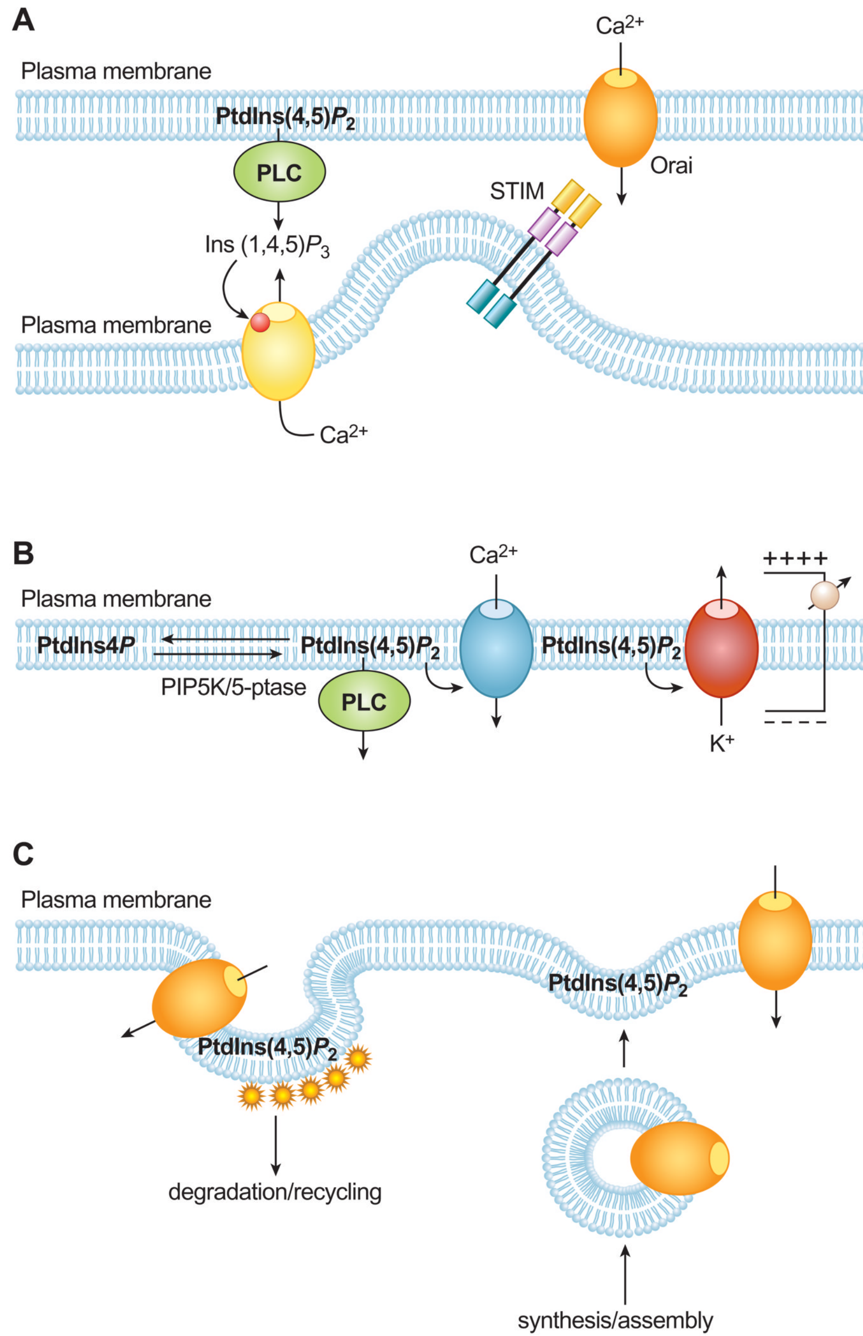

Figure 1.

Different forms of regulation of ion channels relevant to Ca^{2+} signaling. (A) In the canonical phosphoinositide signaling pathway, phospholipase C (PLC) activation from cell surface receptors leads to the generation of Ins(1,4,5)P_3, which, in turn, releases Ca^{2+} from the endoplasmic reticulum (ER) Ca^{2+} stores via Ins(1,4,5)P_3 receptor-channels. The decreased ER luminal Ca^{2+} is sensed by the ER resident STIM proteins that respond with oligomerization and interaction with PM (PM) Orai/CRACM channels at ER-PM junctional sites activating Ca^{2+} influx. This form of Ca^{2+} entry is indirectly linked to Ins(1,4,5)P_3 production, but direct regulation of the Orai channels and possibly STIM interaction with the PM by phosphoinositides has also been proposed. (B) Direct regulation of ion channel (and

transporter) activities by PM phosphoinositides. Here the lipid directly interacts with some molecular component of the channel usually by electrostatic interaction involving positively charged regions of the channel facing the cytoplasm. Phosphoinositide levels can change as a result of PLC activation, or due to a shift in the balance of 5-phosphatase and PIP 5-kinase ctivities. Channels with the highest affinity to inositides may not respond to decreasing inositide levels if they tightly hold on to their bound lipid, therefore, the same lipid change can evoke a variety of activity changes depending on the lipid affinities of the channels. Potassium channels are also known to be sensitive to phosphoinositide changes and because they have significant impact on the membrane potential, they can indirectly affect Ca^{2+} entry by changing the electrochemical driving force of Ca^{2+}. This is especially important for voltage-gated Ca^{2+} channels or for the inwardly rectifying I_{CRAC} channels. (C) Ion channels are also moved around within the cells as a result of membrane trafficking. The synthesis and assembly of channels usually occures in the ER and the channels are glycosylated in the Golgi from where they are heading their final destination, the PM. It is now believed that most channels are not active within the internal membranes, although there are examples suggesting otherwise. PtdIns(4,5)P_2 is present in the PM and channels and transporters that require this lipid for activity gain functionality only upon insertion in the PM. In the opposite process, active channels can be removed from the PM by various interanlization routes, some clathrin-dependent, some –independent. Internalized channles are moved into recycling compartments from where they can recycle back to the membrane or head to degradation. All of these trafficking events are regulated by phosphoinositides.

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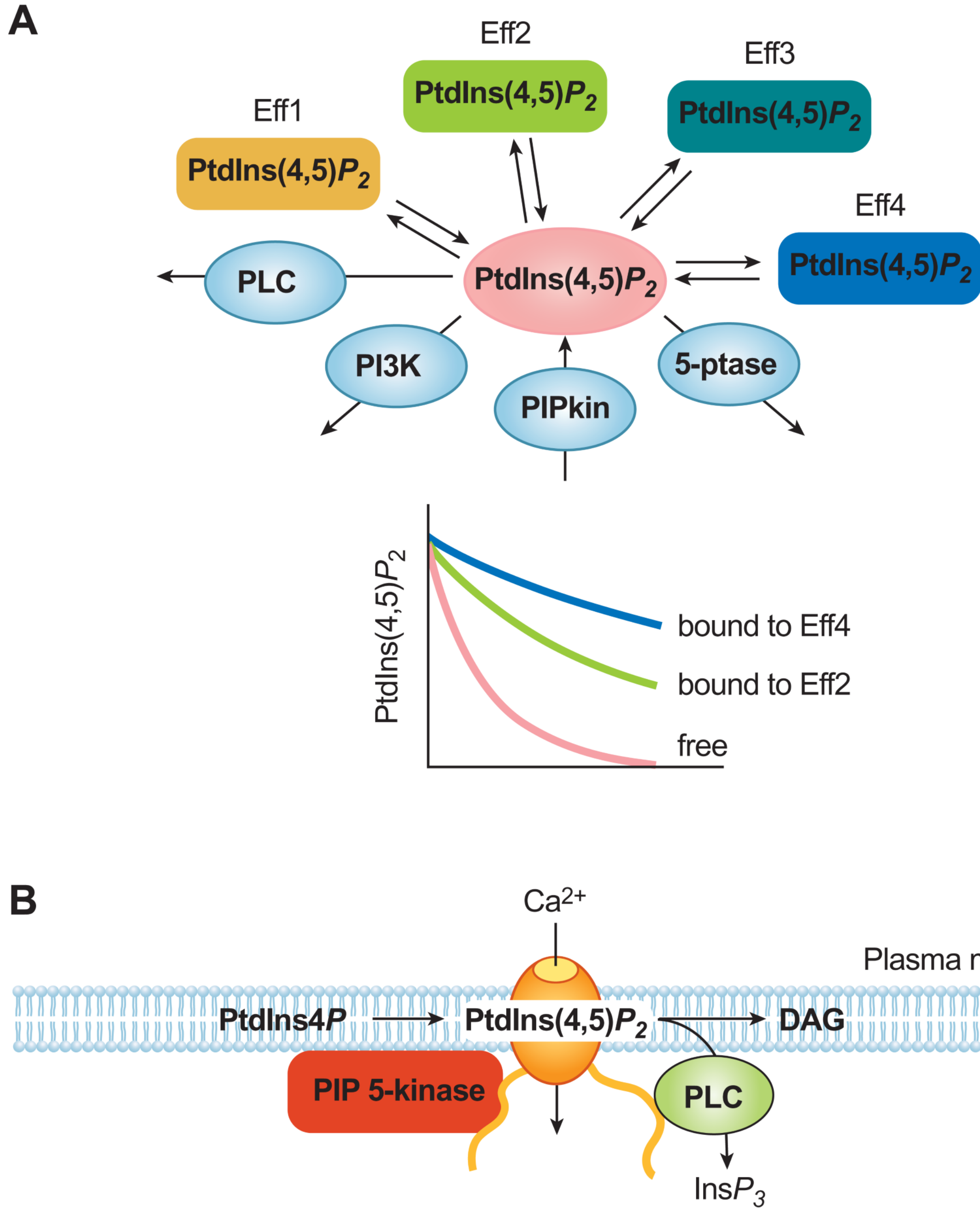

Figure 2.

Principles governing the compartmentalized regulation of ion channels (or other effectors) by phosphoinositides. (A) PtdIns(4,5)P_2 in the plasma membrane exists in free form but is also bound to several proteins regulated by this lipid (effectors, Eff1-4). These could be channels, transporters, actin binding proteins, clathrin adaptors, enzymes and several other proteins. The enzymes that produce and convert these lipids [including PIP 5-kinases (PIPkin), phosphoinositide 5-phosphatases (5-ptase), Class I PI 3-kinases (PI3K) and phospholipase C (PLC) enzymes] act on the unbound fraction (in pink) which is in a dynamic equilibrium with the protein bound fractions (Eff1-4). Changes in the level of free PtdIns(4,5)P_2 are reflected differently in the pools bound to the various effectors depending on their rates of dissociation.

This represents a functional compartmentalization that does not necessarily mean a phisical segregation but may result in different metabolic turnover rates. (B) Theoretical example of the regulation of an effector (ion channel) by a phosphoinositide dedicated to the protein. Here the associated kinase or phosphatase ensures that the phosphoinositide is not diffusing away from the effector and does not contribute to the larger “shared” pool of the inositol lipid in question.

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