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Phosphoinositides regulate ion channels

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Abstract

Phosphoinositides serve as signature motifs for different cellular membranes and often are required for the function of membrane proteins. Here, we summarize clear evidence supporting the concept that many ion channels are regulated by membrane phosphoinositides. We describe tools used to test their dependence on phosphoinositides, especially phosphatidylinositol 4,5-bisphosphate, and consider mechanisms and biological meanings of phosphoinositide regulation of ion channels. This lipid regulation can underlie changes of channel activity and electrical excitability in response to receptors. Since different intracellular membranes have different lipid compositions, the activity of ion channels still in transit towards their final destination membrane may be suppressed until they reach an optimal lipid environment.

Keywords

Phosphatidylinositol 4,5-bisphosphate; Phospholipase C (PLC); G-protein coupled receptor (GPCR); Potassium channel; Calcium channel; Transient receptor potential channels (TRP channels)

1. Introduction

This article considers some key points in the regulation of ion channels by phospholipids. The prescient original concept, from the work of Hilgemann and Ball [1], was that ion channels in the plasma membrane interact with and need local membrane phosphoinositide phospholipids for proper function. We know by now that phosphoinositides do regulate a large number of ion channels. The major themes of our essay are as follows: Definitive evidence and a fairly complete story of ion channel regulation exist for a select few exemplar channels. Partial evidence and a suggestive picture exist for many more, and

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negative evidence or no positive evidence exists for the remainder. Some channels may not be sensitive to phosphoinositides at all. Some ion channels have an obligate requirement for one species of phosphoinositide, whereas others may accept a broader range of phosphoinositides or anionic lipids. Some channels do not function without their preferred lipid and others function differently with and without that lipid. Some channels interact with lipid partners so strongly (high affinity) that the binding site probably remains saturated during most physiological changes of the lipid concentration. Others bind more loosely (low affinity). For the low-affinity channels, variations of lipid levels may act as physiological signals that regulate the computational properties of neurons and the transport properties of secretory cells.

Other articles in this issue review several important properties of phosphoinositide phospholipids that we take here as given. Phosphoinositides derive by phosphorylation from the parent membrane lipid phosphatidylinositol (PI) by combinatorial phosphorylation of the 3, 4, and 5 positions of the inositol head group, so that seven phosphorylated forms exist [2, 3]. The polyphosphoinositides are the most negatively charged of all membrane lipids. They are found only in the cytoplasmic leaflet of the surface membrane and of organellar membranes. All except for PI itself are relatively minority lipids (at most a few mole percent in the membrane) and can serve signaling functions. The different species of phosphoinositides have markedly different abundance in membranes of different organelles of the cell so they act like zip codes or signature motifs of those membranes (Fig. 1; [4]). They are interconverted on a seconds time scale by lipid kinases and lipid phosphatases at the budding and fusing steps of membrane traffic. Finally, phosphoinositides are subject to regulation by membrane receptors. For example, the signature lipid of the plasma membrane, phosphatidylinositol 4,5-bisphosphate (PI(4,5)P₂), can be depleted by receptor activation of phospholipase C (PLC), and a small fraction of PI(4,5)P₂ can be converted to the potent signal PI(3,4,5)P₃ by receptor activation of lipid 3-kinases [5].

2. Two hypotheses

What is regulation by lipids good for? We discuss two hypotheses diagramed in Fig. 1 and Fig. 2. We think both are correct. Ion channels are large integral membrane proteins that become irreversibly imbedded in the lipid bilayer during their synthesis and assembly in the endoplasmic reticulum (ER). They remain membrane imbedded at all times as they traffic through the Golgi and usually continue on to other target membranes via transport vesicles. The vast majority of ion channels that have been studied so far are those targeted to the plasma membrane. During this traffic, integral membrane proteins experience the different signature lipids of each cellular compartment through which they pass. One major hypothesis, regulation in space, has been tested only in very limited ways: It proposes that the phospholipid composition of each compartment controls the activity of channels in transit so that until they arrive at the target membrane, channels may be turned off or altered in function in an adaptive manner [6]. This bold cell biological hypothesis might be broadly relevant to many other integral membrane proteins whether transporters, receptors, enzymes, or ion channels.

A second hypothesis is regulation in time by receptors. While integral membrane proteins like ion channels are residing in the plasma membrane, receptors coupled to PLC or to lipid 3-kinases may become activated and transiently change key membrane lipids sufficiently to modulate channel function. Thus, the electrical and ion-transport properties of the cell may be regulated dynamically by phosphoinositide signaling. As was already pointed out, such regulation in time would apply to channels that do not bind the lipids with very high affinity since the membrane concentration of lipids is likely changed by less than one order of magnitude during receptor activation of enzymes. At times, receptor-induced lipid changes may be graded and modest and their signals and effects on channels may be graded and modest.

3. Tools for study

To study such hypotheses requires tools. Ideally, we should measure the subcellular location and concentrations of the phosphoinositide species, and we should manipulate them up and down. At the same time, we need good assays of the functional changes of the regulated ion channels. Preferably, the measurements, manipulations, and functional assays could be used together in real time on living cells. Fortunately, the techniques and apparatus of patch clamp electrophysiology lend themselves nicely to approaching these ideals. Electrical recording from a cell on a microscope stage is intrinsically a real-time method with millisecond time resolution. Cell photometry or imaging with relevant multicolored fluorescent probes are easily done at the same time on the microscope, and bathing solutions with agonists and inhibitors can be changed by local perfusion, within seconds. Epifluorescence, confocal, and total internal reflection fluorescence (TIRF) microscopy are all possible.

Fluorescently labeled pleckstrin homology domains (PH domains) or other domains with binding specificity for a subset of phosphoinositides are widely used molecular tools for detecting various phosphoinositides [7, 8]. The first was PH-PLC δ 1-GFP, which binds selectively to the plasma membrane lipid PI(4,5)P₂, showing as a bright fluorescent ring around the edge of a cell in confocal microscopy [9, 10]. It also recognizes inositol 1,4,5-trisphosphate. The probe translocates away from the plasma membrane if PI(4,5)P₂ is depleted. Other probes recognize PI(4)P, PI(3,5)P₂, etc. Translocation can be followed from the changes of brightness of regions of interest in confocal imaging. Alternatively, translocation of the same fluorescent probes can be studied with Förster resonance energy transfer (FRET) if a suitable FRET-pair of colors is chosen to label the PH domain and a second protein of known location. There will be FRET when they are closely apposed at the same membrane and a loss of FRET when they move apart. A common FRET pair is PH-PLC δ 1-YFP and a specific membrane anchor tagged with CFP or even PH-PLC δ 1-CFP itself.

A second set of tools are methods to perturb the phosphoinositides of the cell, preferably fast, specific, and compatible with living cells. Several methods manipulate exogenous or endogenous enzymes. Many of these have been outlined by De Camilli and Idevall-Hagren [11] so a short list will suffice. Fig. 2 shows a small subset of phosphoinositide metabolism that includes the enzymes of interest here. The standard biosynthesis of PI(4,5)P₂ proceeds

from PI through PI(4)P to PI(4,5)P₂. PI is converted to PI(4)P by a lipid 4-kinase. PI(4)P is converted back to PI by a 4-phosphatase and into PI(4,5)P₂ by a PI(4)P 5-kinase. PI(4,5)P₂ is converted back to PI(4)P by a 5-phosphatase. PI(4,5)P₂ is also cleaved into diacylglycerol and IP₃ by receptor-activated PLC, mobilizing several second messengers and signaling pathways. The conventional approaches of cell biology would be overexpression of the enzyme or of a dominant-negative mutant by transfection, or knockdown by siRNA, or knockout by gene deletion. These methods can change phosphoinositide levels within hours or days.

Other more dynamic enzyme methods can be implemented in the middle of an experiment. These methods, which act in only 1-10 s, include recruitment of transfected enzymes to the membrane using rapamycin-induced dimerization or light-induced optogenetic dimerization. Most typically, the recruited enzyme is a lipid 5-phosphatase that converts PI(4,5)P₂ to PI(4)P after being recruited to the plasma membrane. The optogenetic dimerization is reversible, whereas the rapamycin dimerization is not. Although these enzyme-based methods for PI(4,5)P₂ depletion are effective and easy to use, it should be kept in mind that lipid kinases, lipid synthesis, and cellular regulatory feedbacks also usually continue. With this compensation, depletion cannot be 100%. Further, although a 5-phosphatase depletes PI(4,5)P₂, we should remember that it produces PI(4)P concomitantly.

Additional valuable fast perturbation methods are unique to electrophysiological approaches. These manipulations can be done while monitoring the function of an ion channel. They include (i) electrical activation of a unique voltage-sensing lipid phosphatase (VSP) by depolarizing voltage pulses using whole-cell clamp, (ii) rapid perfusion with enzyme inhibitors, although these drugs are usually not fully selective, and (iii) excising patches of membranes with a patch pipette and applying agents directly to the exposed cytoplasmic surface. After excision, the production of PI(4,5)P₂ in the patch may stop and PI(4,5)P₂ gradually becomes depleted over a minute or two. Channel function declines in parallel, a process called "channel rundown." Applying test phosphoinositide analogs can restore function. Dose-response curves as different concentrations of water-soluble phosphoinositide analogs (e.g. diC₈ analogs) are applied to the cytoplasmic face allow comparison of the efficacy of various analogs and of the relative lipid affinities of different channels. Applying antibodies against specific lipids also blocks lipids and channel function as does screening of the polyanionic lipids with polycations such as Mg²⁺ or poly-lysine. Again lipid analogs can restore function.

The methods we listed have contributed significantly to our growing understanding of lipid dependence of ion channels, but none is definitive and all have reservations. The strongest support of any lipid-regulation hypothesis requires self consistent evidence from several independent techniques. We cannot evaluate methods extensively here, but give a few biases. Since phosphoinositides play major roles in key cellular processes, we favor techniques that perturb the lipid within seconds so that channel effects can be monitored before membrane traffic and the cytoskeleton are disrupted and before compensatory metabolism, new gene expression, or other modulatory processes change the state of the cell. Activation of PLC through receptors provides a quick screen for potential phosphoinositide dependence, but in addition to depleting PI(4,5)P₂, it produces several

other powerful messengers. Thus positive results then need to be followed by confirmations like recruiting a 5-phosphatase to the plasma membrane with rapamycin or activating VSP with depolarizations, two fast methods. Since VSP produces PI(4)P from PI(4,5)P₂, with positive results one needs to consider possible actions of both phosphoinositides. With these fast methods, one needs to be sure that the necessary proteins are expressed and, for VSP, that the depolarizing voltage step reaches the membrane under study. One also should ensure with a lipid indicator that the appropriate lipid has been depleted. The three methods use enzymes in intact cells, which we regard as favorable because the baseline and the perturbed levels of endogenous lipid pools are likely to remain within or near a physiologically relevant range.

A different class of experiment is conveniently done with channel expression in oocytes. A patch of membrane is excised in the inside-out configuration and the PI(4,5)P₂ is manipulated. It may be allowed to run down in ATP-free solution or to be masked with poly-lysine or with specific antibodies. Exogenous diC₈ lipids are then perfused back on the patch to show recovery. In these procedures, we do not know that the lipid levels are in the physiological range, and the endogenous lipids may become replaced by lipids of much shorter chain length possibly in membrane domains where they are not normally found. The membrane concentration may become excessively high or low. This useful style of experiment may give strong suggestions of lipid dependence, but we feel it is best corroborated by other gentler methods as well.

We finish with a comment on inhibitors and on lipid indicators. The future will probably bring useful inhibitors of steps in lipid synthesis, but for now the drawbacks outweigh the advantages. The ideal would be inhibitors that block formation of specific pools of phosphoinositides. Right now we are uncertain which enzyme subtypes are where in the cell and what pools they serve. Most lack selective inhibitors. Wortmannin is the only agent in significant use. It blocks several kinases at different concentrations. Most relevant for this chapter is the blockage at high concentrations of type III PI 4-kinases, which seem to be required for reformation of PI(4,5)P₂ after depletion by PLC.

4. Structural basis of PI(4,5)P₂ activation of ion channels

How does the low-abundance plasma membrane lipid PI(4,5)P₂ regulate the activity of so many ion channels? All integral membrane proteins sit in intimate contact with a sea of lipids, so it is not surprising that many ion channels have evolved to interact with and recognize the distinctive high negative charge and headgroup geometries of polyphosphoinositides. Two recent papers show crystal structures of PI(4,5)P₂ in complex with members of the inwardly rectifying (Kir) potassium channel family: Kir2.2 [12] and G protein-coupled inwardly-rectifying potassium channel GIRK2 (Kir3.2; [13]). Both of these plasma membrane channels require PI(4,5)P₂ to be active. The lipid is incorporated right into the channel structure. The structures show specific ion channel-lipid interactions and conformational changes that suggest channel activation when the lipid binds.

Here we focus on the Kir2.2 channel for which phosphoinositide binding is sufficient to activate the wild-type channel in physiological experiments [13, 14]. The x-ray crystal

structure of Kir2.2 in complex with a short-chain analog of PI(4,5)P₂, reveals a tetrameric channel composed of a transmembrane domain that forms the potassium-selective pore, and a large cytoplasmic domain that participates in gating (Fig. 3A). One PI(4,5)P₂ molecule binds to each of the four channel subunits at the interface between the transmembrane and cytoplasmic domains and induces a translation of the cytoplasmic portion towards the membrane portion (upward in the figure). In effect, the cytoplasmic and transmembrane domains are pulled together by the lipid to favor an active conformation of the channel. The lipid head group sits roughly at the same interfacial level of the membrane as the other head groups of the bulk lipid bilayer with which it can exchange. The PI(4,5)P₂ binding site of each Kir2.2 subunit comprises amino acid residues from two distinct regions. First, the glycerol backbone and 1-phosphate of PI(4,5)P₂ interact with the inner and outer helices of the channel transmembrane domain (Fig. 3A,B), orienting the inositol head group towards the cytoplasmic domain of the channel. Second, the positioning of the negatively charged 4- and 5-phosphates of the inositol head group of PI(4,5)P₂ promotes an electrostatic interaction with positively charged lysine and arginine residues of the cytoplasmic domain and induces the formation of the interfacial "tether helix". These molecular events draw the two domains 6 Å closer to each other and induce a rotation of the inner helices into a conformation that more closely resembles an open channel. In the structure of GIRK2 (Kir3.2) with PI(4,5)P₂, there are only modest changes that do not open the channel. In contrast, in the structure of a constitutively-active GIRK channel (GIRK R201A) in complex with PI(4,5)P₂ there are striking changes in channel conformation, with rotation of the cytoplasmic domains and a rotation and splaying apart of the inner helices, promoting gate opening [13].

At this writing, only the two Kir channels have been crystallized in complex with PI(4,5)P₂. These informative structures revealed a binding interaction that may be generalizable. Given that all Kir channels have some sensitivity to PI(4,5)P₂ [14, 15], and the residues forming the PI(4,5)P₂ binding pocket are highly conserved within the Kir superfamily [12], we can expect such a mechanism for other Kir's. For example, docking simulations with Kir6.2 predicted a PI(4,5)P₂ binding site in good concordance with that of Kir2.2 [16-18]. For other kinds of channels, mutations of specific C-terminal basic residues do change apparent PI(4,5)P₂ sensitivity, but the definitive picture awaits further structures. From the incomplete evidence available, we do not anticipate that there will be a recognizable structural element that is common to all PI(4,5)P₂-sensitive ion channels. For example, they will not have the fold that defines a classic PH domain. Presumably, each class of channels has its own fold that brings together several basic residues from well-separated regions of the linear sequence to coordinate phosphoinositide phosphates.

In the next four sections we describe highlights of what is known about the phosphoinositide dependence of several classes of ion channels.

5. Regulation of potassium channels by phosphoinositides

Potassium-selective ion channels, typically formed from four homologous subunits as in Fig. 3, are the largest ion channel superfamily with over 80 members. Functionally they maintain the resting membrane potential of cells acting as dampers on excitation as well as

repolarizing the membrane during an action potential. We consider evidence for regulation of voltage-gated potassium (K_V) channels, inwardly-rectifying potassium (Kir) channels, and calcium-activated potassium (K_{Ca}) channels by plasma membrane $PI(4,5)P_2$. There are reports that other phosphoinositides can activate some of these channels to an extent, but $PI(4,5)P_2$ seems to be the principal phosphoinositide regulating potassium channel activity in the plasma membrane [19-21].

5.1. Voltage-gated potassium channels

We begin to summarize the regulation of several potassium channel superfamilies by phosphoinositides. For K_V channels, the clearest examples of regulation by $PI(4,5)P_2$ are the K_V7 channel family. These are slowly activating channels some of which are already open at rest. In particular it is accepted for the common neuronal KCNQ2/KCNQ3 ($K_V7.2/K_V7.3$) heterotetrameric potassium channels, that $PI(4,5)P_2$ is essential for their voltage-gated activity, and that depletion of $PI(4,5)P_2$ renders them inactive so the neuron becomes transiently more excitable until the lipid is resynthesized [22-24]. For many years it was unclear how agonists including acetylcholine could turn off the KCNQ2/3 current of sympathetic neurons (Fig. 4A,B). The answer was that receptor activation couples to PLC, which depletes the plasma membrane $PI(4,5)P_2$. The channels are relatively selective for this one phosphoinositide although they bind $PI(4,5)P_2$ with low enough affinity to be only ~70% activated at the normal resting $PI(4,5)P_2$ level [25]. Many lines of evidence supporting the requirement for $PI(4,5)P_2$ are summarized in Table S1: Activation of PLC through G_q -coupled receptors with subsequent hydrolyses of $PI(4,5)P_2$ causes rapid closure of the channels (Fig. 4A,B). Addition of soluble analogs of $PI(4,5)P_2$ to the cytoplasmic face of excised patches from cells expressing KCNQ2 and KCNQ3 increases the channel activity, and application of $PI(4,5)P_2$ -screening substances like poly-L-lysine leads to rapid loss of channel activity [21, 23, 26, 27]. Dephosphorylation of $PI(4,5)P_2$ to $PI(4)P$ by VSP (Fig. 4C,D) or by a chemically- or photo-recruitable lipid-5-phosphatase (Fig. 4E,F) causes loss of KCNQ2/3 channel activity [28-32]. Lastly, after $PI(4,5)P_2$ depletion, restoration of KCNQ2/3 channel activity follows the time course of $PI(4,5)P_2$ resynthesis at the plasma membrane [30, 33-35]. In the experiments with VSP, depolarizing pulses that are only a few hundred milliseconds long suffice to deplete the $PI(4,5)P_2$ and to turn off the channel. Thus the low-affinity channel-lipid complex must be short lived and rapidly turning over. In the structure of Fig. 3, we should imagine that the phosphoinositides are popping in and out of the channel protein, exchanging many times each second with the neighboring membrane phosphoinositides.

Many of these methods have been applied to other K_V channels (Table S1). All members of the K_V7 channel family require $PI(4,5)P_2$. KCNQ1 ($K_V7.1$) channels combine with the β -subunit KCNE1 to form the molecular correlate of the I_{Ks} current in the human heart. Depletion of $PI(4,5)P_2$ by voltage-sensing or rapamycin-recruitable lipid-5-phosphatases strongly inhibits KCNQ1/KCNE1 channel complexes [32], and excising patches from cells expressing KCNQ1/KCNE1 causes current rundown, which is prevented by application of $PI(4,5)P_2$ to the cytoplasmic face of the patch [23]. The presence of KCNE1 enhances the $PI(4,5)P_2$ sensitivity of KCNQ1 by a factor of ~100 [36]. An intriguing mechanistic concept is that $PI(4,5)P_2$ aids the conformational coupling between the channel voltage-sensing

domain and the gate in KCNQ1 [37]. In the absence of PI(4,5)P₂, voltage changes can move the voltage sensor, but the channel does not open, and mutations can make the pore open, but the movements of the voltage sensor are not affected. KCNQ4 (K_V7.4) channels are sensitive to activation of type 1 muscarinic acetylcholine receptors (M₁R), which activate PLC via G_q [38]. As for KCNQ1/KCNE1 and KCNQ2/3 channel complexes, depletion of PI(4,5)P₂ leads to closure of KCNQ4 channels and almost complete loss of channel activity. Again, in excised patches KCNQ4 channel activity can be restored by application of diC₈-PI(4,5)P₂ [23, 26]. The remaining channel of this family, KCNQ5 (K_V7.5), is similarly sensitive to PI(4,5)P₂ [23].

Other than for K_V7 channels, no experiments on other members of the K_V family have suggested that PI(4,5)P₂ is essential for their function, although it may have some effects. One member of the K_V1 channel family has clear phosphoinositide sensitivity. The gating properties of K_V1.2 channels are changed by lowering PI(4,5)P₂. In *Xenopus laevis* oocytes, depletion of PI(4,5)P₂ yields a decrease of current amplitude, a left-shift in the voltage dependence of the activation curve, and a marked slowing of channel deactivation [39]. Application of PI(4,5)P₂ to an excised patch restores current amplitude and reaccelerates channel deactivation. Comparable results are found in whole-cell recordings from tsA-201 cells following enzymatic depletion of PI(4,5)P₂ [40].

For the rest of the K_V family, the evidence for any kind of PI(4,5)P₂ effects is either negative or controversial (Table S1). We now mention several controversial examples. For each of them, our own experiments with verified enzymatic depletion of PI(4,5)P₂ using VSP or using a recruitable lipid-5-phosphatase showed no effect in intact tsA-201 cells [32]. On the other hand, experiments with membrane patches excised from *Xenopus* oocytes indicated that application of full-length PI(4,5)P₂ changed the channel gating. Thus, applying PI(4,5)P₂ to the cytoplasmic face of excised patches from oocytes expressing K_V1.4 or K_V3.4 channels or K_V1.1/K_Vβ1.1 or K_V1.5/K_Vβ1.3 channel complexes reduces channel inactivation. Subsequent application of poly-L-lysine to screen polyphosphoinositides reverses the effect and restores fast inactivation [41, 42]. Therefore, it still remains to be clarified whether these channels can be regulated by physiological changes in PI(4,5)P₂ levels and why these experiments give contrasting results.

Interestingly, for two of these channels, K_V1.1/K_Vβ1.1 and K_V3.4, inactivation can be removed by activating PLC. This would deplete PI(4,5)P₂ but also generate diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP₃) and activate protein kinase C (PKC) (Fig. 2). Since direct pharmacological activation of PKC in these cells leads to the same change in channel inactivation, it is plausible that the observed changes in K_V1.1/K_Vβ1.1 and K_V3.4 channel behavior in this experiment are caused by phosphorylation of these channels by PKC rather than by depletion of PI(4,5)P₂ [32].

The last controversial channel of this group is K_V11.1 (hERG). Dialysis of PI(4,5)P₂ into cells via a patch-pipette leads to increased K_V11.1-mediated current amplitude, a left-shift in the activation curve, accelerated channel activation, and slowed channel inactivation [43, 44]. In apparent contrast, depletion of PI(4,5)P₂ in tsA-201 cells by either a voltage-sensing or a recruitable lipid-5-phosphatase does not influence K_V11.1 current amplitude or kinetics

[32]. Again it is unclear at present to what extent alterations in PI(4,5)P₂ levels might regulate K_V11.1 under physiological conditions.

In short, the evidence that KCNQ (K_V7) channels require PI(4,5)P₂ for normal activity is strong. In addition, K_V1.2 channel gating can be modified by physiological changes of PI(4,5)P₂. However, for the other tested members of the K_V channel family, PI(4,5)P₂ is apparently not essential, and whether it has modulatory effects under physiological conditions is controversial in several examples. For reasons not understood, the results seem to depend on choices of expression systems, recording configurations, and the tests tried.

5.2. Inwardly-rectifying potassium channels

Inwardly-rectifying potassium channels conduct best at negative potentials and are most important in regulating the resting potential. Unlike the K_V channel family, every member of the Kir channel family requires PI(4,5)P₂ [1, 14, 19, 20, 45-51]. There are many lines of evidence (Table S1), and as we have seen, even crystal structures of PI(4,5)P₂ complexes with Kir channels: Measurements of Kir currents in the inside-out patch-clamp configuration show rundown of the currents as soon as membrane patches are excised, and application of diC₈-PI(4,5)P₂ restores current amplitudes rapidly and completely [14]. Addition of an anti-PI(4,5)P₂ antibody to the cytoplasmic face of excised patches accelerates current rundown, as does the addition of Mg²⁺ that can screen PI(4,5)P₂ rendering it less available [49]. In addition, stimulation of the G_q-coupled M₁R receptor or of the EGF receptor reduces the activity of several, although not all, Kir channels [49, 52]. Both receptors activate PLC and initiate hydrolysis and depletion of PI(4,5)P₂.

The physiological modulation of Kir channels occurs through at least three different receptor actions, and the various channel subtypes show quite a range of susceptibilities to these signals. Two inhibitory signals are mediated by receptors coupled to G_q, depletion of PI(4,5)P₂ and channel phosphorylation via the DAG-PKC pathway. A third signal, excitatory this time, is mediated by βγ subunits of G proteins liberated by receptor activation of G_{i/o}. The Gβγ dimer acts directly on GIRK channels (the Kir3 subfamily) and not on the other Kirs.

We focus on the two inhibitory signals. They bear a kind of inverse relation to each other. The different Kir channels bind PI(4,5)P₂ with significantly different affinities [49]. Kir channels with high PI(4,5)P₂ affinity (Kir1, Kir2.1, Kir4) show only a small inhibition from PKC or from M₁R activation, whereas channels with low PI(4,5)P₂ affinity (Kir2.3, Kir3) are more strongly inhibited by PKC and M₁R activation. The Kir channel with the highest measured PI(4,5)P₂ affinity, Kir2.1, is insensitive to both PKC and M₁R activation. Site-directed mutagenesis bears out this generalization. While wild-type Kir2.3 channels, which have a relatively low PI(4,5)P₂ affinity, are inhibited by about 50% by PKC, increasing their PI(4,5)P₂ affinity by introducing a point mutation renders them insensitive to PKC. On the other hand, lowering the PI(4,5)P₂ affinity of the normally PKC-insensitive Kir2.1 channel by a point mutation introduces PKC-sensitivity to this mutant channel [49]. Thus, by varying the expression pattern of different Kir channels, a cell could adjust the relative strength of physiological stimuli necessary to cause a decrease in Kir channel conductance (an increase of electrical excitability) by either the PKC or the PI(4,5)P₂-depletion pathways.

Kir3 (GIRK) channels show a special kind of regulation as they not only depend on PI(4,5)P₂, but also require the binding of Gβγ subunits to be active. PI(4,5)P₂ and Gβγ act synergistically to activate these channels [45]. Excising membrane patches from *Xenopus* oocytes expressing GIRK channels leads to rundown of the current due to hydrolysis of PI(4,5)P₂, and subsequent application of Gβγ does not restore channel activity under these conditions. Application of PI(4,5)P₂ without Gβγ can restore channel activity over several minutes, but applying PI(4,5)P₂ together with Gβγ restores GIRK channel activity in only seconds, illustrating an interdependence of Gβγ and PI(4,5)P₂ actions.

5.3. Calcium-activated potassium channels

Activated when intracellular calcium rises, calcium-activated potassium channels in neurons gradually slow the rate of firing during trains of action potentials (spike frequency adaptation) and keep cells quiet and hyperpolarized after bursts of firing. There are three families: the small (SK), intermediate (IK) and big conductance (BK) K_{Ca} channels. SK and BK channels are reported to be modulated by phosphoinositides. Thus, the activity of SK channels from apical membranes of rat cortical-collecting duct decreases upon excision of membrane patches and is restored by addition of PI(4,5)P₂ to the cytoplasmic face [53]. Exposing the patches to inhibitors of phosphoinositide kinases decreases SK channel activity, and application of PI(4,5)P₂ restores it. The BK channels do not run down in excised patches, but nevertheless, channel activity could be increased by application of PI(4,5)P₂ to the cytoplasmic face of membrane patches excised from cerebral artery myocytes [54]. In expression systems, potentiation by PI(4,5)P₂ was evident when coexpressing BK β1 subunits (those present in the native vascular myocytes) but not β4 subunits (present in skeletal muscle). Mutagenesis of residues in the Ca²⁺-coordinating RCK1 domain of BK channels suggests a regulatory role of Ca²⁺ in the PI(4,5)P₂ sensitivity of BK channels [55]. With low cytoplasmic Ca²⁺ levels, the RCK1 domain limits channel activation by decreasing the PI(4,5)P₂ affinity of BK channels, whereas upon binding of Ca²⁺ to the RCK1 domain, this inhibitory influence is removed and PI(4,5)P₂ contributes to BK channel activation.

6. Regulation of voltage-gated Ca²⁺ channels by phosphoinositides

Voltage-gated calcium (Ca_V) channels mediate calcium influxes and regulatory calcium transients upon membrane depolarization, transients that initiate all the effector actions of electrically excitable cells [56]. Modulation of Ca_V channels by G protein-coupled receptors (GPCRs) tunes many physiological responses [57, 58]. We focus here on inhibition of Ca_V channel by membrane receptors coupled to G_q where depletion of plasma membrane PI(4,5)P₂ is one of the underlying signals.

6.1. Role of membrane PI(4,5)P₂ in the activation of Ca_V channels

Early studies showed that Ca_V2.2 channel (N-type) currents are partially suppressed by G_q-coupled M₁ muscarinic receptors in sympathetic neurons [59-62]. The signaling pathway was slow (~10 s for inhibition and ~100 s for recovery) and voltage independent. The underlying mechanisms were not clear for many years. Eventually, evidence accumulated that PI(4,5)P₂ is required for Ca_V channels to open in response to membrane potential

changes and therefore PI(4,5)P₂ depletion might underlie the muscarinic inhibition (Table S2). The first evidence, with membrane patches excised from frog oocytes, was that rundown of Ca_v2.1 channel activity was slowed by application of PI(4,5)P₂ or Mg-ATP and accelerated by depleting or sequestering PI(4,5)P₂ [63]. Subsequently, in sympathetic neurons the G_q-coupled muscarinic inhibition of Ca_v2.2 was shown to involve PI(4,5)P₂ hydrolysis by PLC, and the recovery of current paralleled PI(4,5)P₂ resynthesis [64, 65]. Additional direct evidence for a functional role of membrane PI(4,5)P₂ in the activation and modulation of Ca_v channels came from rapid activation of lipid phosphatases [66, 67]. Using both VSP and the rapamycin dimerization system with a 5-phosphatase, it was shown that PI(4,5)P₂ depletion alone sufficed to diminish the currents of several high-voltage-activated Ca_v channels but not those of low-voltage activated channels. Further, the recovery from this inhibition needed intracellular ATP-dependent PIP₂ resynthesis. As with receptor activation, the reduction of current was 30-50% and not 100%. And as for modulation of KCNQ currents by VSP, the partial suppression of Ca_v currents required a depolarizing pulse of only 100 ms, implying a rapidly exchanging, low-affinity PI(4,5)P₂ interaction. It should be noted that in a slightly different hypothesis, the classical slow receptor-mediated suppression of Ca_v current in sympathetic neurons has also been ascribed to inhibitory actions of arachidonic acid released from PI(4,5)P₂ by phospholipase A₂ [68].

6.2. Role of auxiliary β subunits in PI(4,5)P₂ regulation of Ca_v channels

As we already saw for some K_v channels, the PI(4,5)P₂ regulation of Ca_v channels changes with the coexpression of different Ca_v β subunits [67]. Ca_v β 2a subunits bind to the membrane through two palmitoyl side chains in the N-terminus, and the Ca_v channels with this subunit show slow inactivation of current and little PI(4,5)P₂ sensitivity [69-71]. Ca_v β 3 subunits are not lipidated and do not bind to the membrane until coupled with an α 1 channel subunit. Channels coexpressed with β 3 subunits inactivate more rapidly and show higher PI(4,5)P₂ sensitivity. It was proposed that the two palmitoyl chains of the β 2a subunit compete with the interaction of the two fatty acyl chains of PI(4,5)P₂ at the channel, reducing the requirement for PI(4,5)P₂. When the possibility of lipidation was removed by mutation of the β 2a subunit, the channel became PI(4,5)P₂-sensitive. In the same vein, when β 3 subunits are targeted to the membrane by adding a Lyn₁₁ lipidation signal, the chimeric subunit acts like the membrane tethered β 2a subunit and diminishes the PI(4,5)P₂ sensitivity, suggesting that the subcellular localization of the β subunit governs the phosphoinositide sensitivity of channels [67]. Such experiments raise the interesting concept that PI(4,5)P₂ at times may act as a competitor for binding of other lipids to targets and that lipidated proteins may sometimes assume the role of PI(4,5)P₂ [72-74]. Even though the Ca_v β subunit is a key determinant of regulation by PI(4,5)P₂, the binding sites for PI(4,5)P₂ on Ca_v channels seem still to be on the pore-forming α 1 subunit. The PI(4,5)P₂ sensitivity of Ca_v channels is not lost in channels whose N-terminus has been modified to prevent the inhibitory binding of G protein $\beta\gamma$ subunits [66].

6.3. PI(4,5)P₂ binds to Ca_v channels as a bidentate ligand

In analogy with the crystal structure of Kir2.2 (Fig. 3), one can speculate that a PI(4,5)P₂ molecule acts as a bidentate ligand crosslinking two parts of the Ca_v α 1 subunit and favoring active protein conformations [66, 72] (Fig. 5). The hydrophobic recognition site

can be disturbed by the palmitoyl groups of $\text{Ca}_V \beta 2a$ [66] or by direct incorporation of fatty acids, such as arachidonic acid or palmitic acid into the interaction sites [72, 73]. The hydrophilic interaction can fail when membrane $\text{PI}(4,5)\text{P}_2$ is depleted or screened [64, 66, 75].

7. Other plasma membrane channels regulated by phosphoinositides

7.1 TRP channels

Transient receptor potential (TRP) channels form a diverse superfamily. Most of them are non-selective cation channels usually with some Ca^{2+} permeability. They often mediate sensory or homeostatic modalities like stretch, temperature change, pH, vision (in arthropods), or osmotic sense, so their regulation has physiological impact. They are tetrameric complexes with an architecture similar to that of K_V channels, but their opening probability is only mildly increased by depolarization. So far, 20 out of 28 channels in the TRP superfamily have been said to be sensitive to $\text{PI}(4,5)\text{P}_2$ (summarized in Table S3), and for most of them, $\text{PI}(4,5)\text{P}_2$ promotes opening [76].

A well-studied example of $\text{PI}(4,5)\text{P}_2$ dependence is the cold-activated TRPM8 channel. It is expressed primarily in trigeminal and dorsal root ganglion neurons. TRPM8 is activated by low temperatures, voltage, and the "cooling" agents menthol and icilin [77, 78]. It is inhibited by ethanol and desensitized by calcium. TRPM8 channels are not responsive to those stimuli when $\text{PI}(4,5)\text{P}_2$ is absent. For instance, PLC-mediated hydrolysis of $\text{PI}(4,5)\text{P}_2$ reduces TRPM8 currents activated by temperature, menthol, or voltage [79-82]. It also reduces inhibition by ethanol [83]. Since by hydrolyzing $\text{PI}(4,5)\text{P}_2$ PLC also makes IP_3 and DAG, it could be thought that TRPM8 channels are responding to these products of lipid hydrolysis. However, when $\text{PI}(4,5)\text{P}_2$ levels are reduced without IP_3 or DAG production using the recruitable lipid 5-phosphatases, menthol-induced TRPM8 currents fall concomitantly [81, 84]. TRPM8 channels are also inhibited when $\text{PI}(4,5)\text{P}_2$ is scavenged by antibodies or screened with poly-L-lysine [79-81, 83]. Mutagenesis of positively charged amino acid residues in the conserved C-terminal TRP domain reduces the apparent affinity for $\text{PI}(4,5)\text{P}_2$. These residues required for $\text{PI}(4,5)\text{P}_2$ binding are located in a different part of the protein from that required for the activation by temperature, voltage, or cooling agents [85].

Based on such functional and structural experiments, the current model suggests that temperature, voltage, and $\text{PI}(4,5)\text{P}_2$ interact allosterically with each other. When $\text{PI}(4,5)\text{P}_2$ is present, less membrane depolarization or less cooling are needed to activate TRPM8 channels. Molecular simulations docking $\text{PI}(4,5)\text{P}_2$ on the TRPM8 channel suggest that the aliphatic chains of the lipid bind to a hydrophobic pocket in the transmembrane domain of the channel, modifying the position of the cytoplasmic domain [85]. Removal of $\text{PI}(4,5)\text{P}_2$ would allow the cytoplasmic domain to move back and render TRPM8 channels less sensitive to any stimulus. Thus, as in Kir channels, $\text{PI}(4,5)\text{P}_2$ is thought to regulate gating properties of TRPM8 channels by modifying the position of the cytoplasmic domain relative to the transmembrane domain.

Other signals downstream of PI(4,5)P₂ hydrolysis also regulate some TRP channels, increasing their activity. This raises the possibility that when PI(4,5)P₂ is being hydrolyzed, channel activity can both be stimulated by the products of the hydrolysis and at the same time reduced by PI(4,5)P₂ depletion. This potentially complex situation is well exemplified with TRPC channels. TRPC6/7 channels are activated by DAG, a product of PI(4,5)P₂ hydrolysis [86, 87]. Nevertheless, PI(4,5)P₂ is essential for TRPC6/7 function [88-91]. Therefore, weak or brief activation of PLC stimulates TRPC6/7 currents, and stronger or longer activation of PLC will inactivate them [91]. Evidently it is not necessary to hydrolyze a lot of PI(4,5)P₂ before DAG can exert its stimulatory effects on the channel, a situation analogous to the very small cleavage of PI(4,5)P₂ needed to get full diacylglycerol action on protein kinase C and full IP₃ action on calcium release [34, 35].

A few TRP channels may have negative regulation by PI(4,5)P₂. An interesting case is TRPC4. There the TRPC4α splice variant is negatively regulated when diC₈-PI(4,5)P₂ is dialyzed into the cell from the patch pipette [92], whereas the TRPC4β splice variant is not. The effect is attributed to changes in interactions with cytoskeletal elements. In addition, there is a case in which there is not yet agreement on the effect of PI(4,5)P₂ despite a lot of work by several groups. TRPV1 channels are sensitive to PI(4,5)P₂, and are described as activated or inhibited by PI(4,5)P₂--or both--[76]. Here, we summarize some evidence for these not fully reconciled and opposite actions.

Several authors suggest that, like other TRP channels, TRPV1 channels are activated by PI(4,5)P₂. For instance, capsaicin-induced TRPV1 currents in a membrane patch can be supported by application to the cytoplasmic face of diC₈ analogs of PI(4,5)P₂, PI(3,4,5)P₃, or PI(4)P, in order of decreasing potency, whereas currents are reduced by screening PI(4,5)P₂ with poly-L-lysine [93-96]. Rapid dephosphorylation of PI(4,5)P₂ with VSP or with translocatable 5-phosphatases decreases capsaicin stimulation of TRPV1 currents [95, 97-99]. These phosphatases reduce PI(4,5)P₂ levels and simultaneously produce PI(4)P, which is less effective than PI(4,5)P₂ at supporting TRPV1 currents. Similarly, PI(4,5)P₂ becomes depleted during minute-long applications of capsaicin because Ca²⁺ entering through the open channels activates PLC. In parallel, TRPV1 or TRPV2 channels close, a process called desensitization [94, 97, 100]. Blocking phosphorylation of PI at the 4 position or hydrolyzing PI(4,5)P₂ suppresses recovery from desensitization [101]. The loss of PI(4,5)P₂ is cited as the explanation of this agonist-induced Ca²⁺-dependent desensitization of channel responses.

Together, the preceding evidence is fully consistent with a PI(4,5)P₂ requirement for channel activity. On the other hand, certain experiments suggest inhibitory effects of PI(4,5)P₂. For instance, reducing PI(4,5)P₂ levels by adding an anti-PI(4,5)P₂ antibody increases TRPV1 currents, and reducing the pool of phosphoinositides in general by adding a phosphatidylinositol phospholipase (PI-PLC) increases TRPV1 currents [102, 103].

Possible PI(4,5)P₂ binding sites have been sought in the long C-terminus of the TRPV1 channel. Mutagenesis or deletions in regions claimed to be relevant either increase or decrease channel activity [85, 103, 104]. From this evidence, PI(4,5)P₂ is variously said to be an inhibitor or stimulator of channel activity. Alternatively, it has been suggested that the

effect of PI(4,5)P₂ on TRPV1 channels is mediated not by a domain located on the protein but by a different protein (pirt) that binds both PI(4,5)P₂ and TRPV1 channels [105].

One barrier to understanding regulation of TRPV1 in living cells is that the channels are simultaneously sensitive to a large number of different modulatory signaling pathways: phosphorylation, calcium, lipids, other interacting proteins, and voltage. Indeed the same problem applies to most types of channels. TRPV1 channels also seem to be sensitive to several acidic lipids so depletion of just one lipid may not give clear answers. One ideal would be to study the channels in the absence of extraneous signals and proteins. For TRPV1, this has been done by reconstituting purified channel protein in lipid vesicles containing defined lipids [106, 107]. Such channels are inactive in lipid bilayers containing only neutral lipids, but can be made active by including PI(4,5)P₂ or other negatively charged lipids. This shows that the purified channel and simple lipids alone suffice to reconstitute function and lipid regulation. When functionally reconstituted with negatively charged lipids, the channel activity decreases secondarily upon addition of high concentrations of PI(4,5)P₂. Thus PI(4,5)P₂ or some other negatively charged lipids are essential for activity, but high concentrations of PI(4,5)P₂ decrease activity. Senning et al. [96] recognized that reconstitution studies might be confounded by the obligatory symmetry of artificial liposome bilayers. They found with membrane patches excised from cells that the addition of PI(4,5)P₂ to the cytoplasmic face enhanced channel activity, whereas addition of high concentrations to the extracellular face inhibited it. This observation might resolve some of the apparent contradictions saying that there is an activating site in the inside face of the channel and an inhibiting site on the outside face. Since PI(4,5)P₂ and the other polyphosphoinositides are present only in the cytoplasmic leaflet in normal cells, the activating site would be the relevant one in a cellular environment.

7.2 ENaC channels

Epithelial sodium channels (ENaCs) mediate sodium transport in epithelia. Regulation of their activity controls the rate of sodium reabsorption in the kidney, lungs, and colon. They require PI(4,5)P₂. Transport through ENaC channels is suppressed by activation of G-protein coupled P2Y purinergic receptors. The suppression does not occur if PLC blockers are present. Activation of P2Y receptors simultaneously depletes PI(4,5)P₂ in the plasma membrane, activates PKC, and raises intracellular calcium, but neither the calcium signal nor PKC are necessary for the suppression of sodium transport [108]. PI(4,5)P₂ must be added to excised patches to prevent rundown of channel activity. Channel rundown is accelerated by screening PI(4,5)P₂ with poly-L-lysine, by adding an anti-PI(4,5)P₂ antibody, or by activating PLC. PI(4,5)P₂ and PI(3,4,5)P₃ promote ENaC activity [109, 110] but at different binding sites. Both bind to β and γ subunits of the channels; however, PI(4,5)P₂ binds to the N-terminal whereas PI(3,4,5)P₃ binds to the C-terminal [108, 109, 111-113].

7.3 P2X channels

The P2X receptor channels are cation-permeable channels that are opened by ATP and other purinergic ligands binding to the extracellular domain. They are strongly expressed in neurons but also in smooth muscle, platelets, and immune cells. The seven members (P2X1-P2X7) can form homomers or heteromers. All functional homomers are PI(4,5)P₂ sensitive

[114, 115]. The principal tests have been to decrease PI(4,5)P₂ with inhibitors of phosphatidylinositol 4-kinase and to apply exogenous PI(4,5)P₂ to excised patches (Table S3). In excised patches, P2X activity runs down, and application of PI(4,5)P₂ reduces rundown, whereas screening of PI(4,5)P₂ with polycations accelerates it. In *in vitro* binding assays, PI(4,5)P₂, as well as other phosphoinositides, bind the proximal C-terminal [114-116]. A physiological example of PI(4,5)P₂ actions is a decrease in P2X1-mediated smooth muscle contraction induced by reduction of PI(4,5)P₂ levels [117]. As with other PI(4,5)P₂ sensitive channels, P2X channel action is thought to be decreased by a variety of G-protein coupled and tyrosine kinase receptors that activate PLC and deplete PI(4,5)P₂. Given the wide distribution of P2X receptor channels in the nervous system, their sensitivity to receptor-mediated PI(4,5)P₂ hydrolysis could have relevant consequences in synaptic transmission and neural function.

7.4 CNG channels

Now we come to two families of channels whose activity is depressed by PI(4,5)P₂. The first is the cyclic nucleotide gated CNG family, channels that are opened by intracellular ATP or GTP. CNG channels of photoreceptors (cones and rods) and olfactory neurons require more cyclic nucleotide to be activated when levels of PI(4,5)P₂ or PI(3,4,5)P₃ increase [118-122]. The sensitivity to PI(4,5)P₂ varies with the specific channel subtype, being low for olfactory channels, as well as with the splice variant expressed. PI(3,4,5)P₃ binds the N-terminal of olfactory CNG channels [120], PI(4,5)P₂ and PI(3,4,5)P₃ bind both N- and C-termini of cone CNG channels [121]. Interestingly, the N-terminal PI(3,4,5)P₃-binding region also interacts with calmodulin [120]. There may be an occlusive functional interaction between the PI(3,4,5)P₃ and calmodulin regulatory mechanisms since PI(3,4,5)P₃ fails to decrease the activity of the channels while calmodulin is bound. Analogous cross-talk between phosphoinositides and a second regulatory mechanism, is observed for other ion channels such as GIRK channels.

7.5 TMEM16A channels

The recently discovered TMEM16-ANO1 channels are calcium-activated chloride channels that are widely expressed including in smooth muscle, neurons, and epithelial cells [123-125]. They too are apparently inhibited by PI(4,5)P₂. Calcium-activated chloride currents recorded in cell lines expressing TMEM16A channels increase when PI(4,5)P₂ levels are reduced, either by screening or by activation of PLC, and they are decreased when exogenous PI(4,5)P₂ is added. A direct interaction between TMEM16A channels and PI(4,5)P₂ is suggested since they co-immunoprecipitate [126]. Negative dependence of TMEM16A on PI(4,5)P₂ could explain the depolarization in smooth muscle cells that is induced by PLC-activating factors. Since TMEM16A channels are widely expressed, their regulation by PI(4,5)P₂ can have several, yet unexplored, physiological consequences.

8. Regulation of intracellular ion channels by phosphoinositides

Thus far, we have focused on ion channels in the plasma membrane and their dependence on PI(4,5)P₂. Now we turn to channels and phosphoinositides in other membranes of the cell. Consider Fig. 1. Ion channels, pumps and transporters, like other membrane proteins, are

synthesized, folded, and assembled within the endoplasmic reticulum (ER) before being transported to the Golgi where they undergo further post-translational modifications. On reaching the trans-Golgi network, membrane proteins are sorted based on specific sequences and earmarked for their final destination, be it other intracellular compartments or the cell surface. Sequential transport along the secretory pathway is mediated by vesicular intermediates that bud from one compartment and fuse with the next, creating almost continuous membrane flow from the ER to the plasma membrane.

As described earlier, ion channels need the coincidence of (i) an opening stimulus and (ii) the minor phosphoinositide PI(4,5)P₂ for full function (Tables S1, S2, S3). In simplified terms, each organelle can be thought of having its own 'signature' phosphoinositide (Fig. 1), although, in reality, organelle membranes probably have a more complex mixture of precursor, signature, and successor phosphoinositides [4, 98, 127-131]. Nevertheless, to discuss the spatial modulation hypothesis [6], we retain the concept that each organelle membrane has a simple phosphoinositide menu as described in Fig. 1. The phosphoinositides are kept in dynamic steady state through the actions of lipid kinases and lipid phosphatases [2]. Although there is evidence for PI(4,5)P₂ synthesis on other internal membranes, such as the Golgi [132], its free concentration there is thought to be much less than at the cell surface. Then ion channels and transporters that require PI(4,5)P₂ would probably be kept at low activity during trafficking through the secretory pathway. The location-specific control would prevent PI(4,5)P₂-requiring transporters and channels from spuriously disturbing the ion content, pH gradients, or electrical potentials across organelle membranes, as they traffic towards the plasma membrane. Conversely, some channels and transporters important for intracellular organelles may be swept forward by membrane flow to the plasma membrane. There, the high PI(4,5)P₂ concentration or lack of other appropriate activating phosphoinositides might turn off function. Having specific phosphoinositides to regulate membrane proteins ensures that organelle-specific functions and trafficking can occur on the same platform without deleterious side effects.

How can one study phosphoinositide requirements of intracellular channels? Traditionally, to determine whether a plasma membrane ion channel or transporter is sensitive to phosphoinositides, whole-cell or inside-out patch-clamp recordings have been made from cells overexpressing the membrane protein of interest while one or more of the 7 species of polyphosphoinositides is manipulated. Until recently small size and restricted accessibility have prohibited such patch-clamp analysis of intracellular organelles. One approach is to make the organelles large enough to patch clamp, and another changes their sorting signals to allow them to traffic to the cell surface. For example, the small molecule vacuolin-1 converts endosomes and lysosomes into large vacuoles by fusion, while leaving other cell processes, such as the actin cytoskeleton, receptor mediated endocytosis, and membrane traffic along the secretory pathway, intact [133]. In practice, the heterogeneous endolysosomal organelles can be enlarged from ~ 0.5 µm to ~ 5 µm diameter, facilitating patch clamp analysis [134-139].

To date, the electrical activity and phosphoinositide dependence of two classes of endolysosomal cation channels have been described well using vacuolin-enlarged organelles: TRPML of the mucolipin subfamily of TRPs [134, 136, 140], and the two pore-

segment channel 1 TPC1 [138, 139] (Fig. 1). TRPML1-3 and TPC1 and TPC2, are strongly inwardly rectifying channels of intracellular organelles, permeable to Ca^{2+} and Fe^{2+} or to Na^+ , respectively. Both kinds of channels have been implicated in endosomal and lysosomal trafficking. They are specifically activated in enlarged vesicles by the phosphoinositide of late endosomes and lysosomes, $\text{PI}(3,5)\text{P}_2$ (Fig. 1) [135, 136, 139]. For TRPML1, a small steady-state pool of channels appears at the plasma membrane that can be activated by "run-up" after patch excision or by adding $\text{PI}(3,4)\text{P}_2$, and that can be inhibited by $\text{PI}(4,5)\text{P}_2$, $\text{PI}(3,4)\text{P}_2$, and $\text{PI}(3,4,5)\text{P}_3$ [136]. The TRPML1 channel can be mutated to enhance surface expression to make these phenomena more evident. The finding that several phosphoinositides can inhibit the channel and only one can activate it suggests potent suppression of the activity of channels while in transit through "foreign" phosphoinositide/organelle environments until they reach a specific home location.

Studies on this small sample of intracellular channels support the hypothesis of regulation in space. There seems to be a clear dichotomy between channels that function at the plasma membrane and channels that function on intracellular organelles. Extending this concept to $\text{K}_{\text{v}}7.2/7.3$ channels (KCNQ2/3) leads us to theorize that they will have higher activity at the plasma membrane than at other places in the cell. This assumes that locations proximal and distal to the plasma membrane do not have sufficient $\text{PI}(4,5)\text{P}_2$ to active the channel. Perhaps in the future there will be examples of channels that are active across multiple membrane compartments. They would be expected to (i) be activated by multiple phosphoinositides, or (ii) be localized to distinct specific domains with phosphoinositides different from the signature phosphoinositide of an organelle.

9. Concluding challenges

Regulation of ion channels by phosphoinositides is a widespread phenomenon with interesting consequences for cell signaling and during membrane traffic. Certain phenomena are now very clear, but the studies raise important general cell biological questions, points that need to be clarified to understand the extent of the regulation. We speak of signature phosphoinositides in specific membranes, but how different are their levels from those of the other lipids in the same membrane? Is it just two-fold or as much as 100-fold? Further for each organelle and the plasma membrane, do we need to consider a locally sorted mosaic of membrane microdomains that concentrate specific lipids and proteins? Then discussions of traffic would be much more complex, and each membrane has to be imagined as made up of multiple rapidly exchanging "compartments" with different properties. We speak of depletion of certain lipids by receptor-activated lipases. How much are $\text{PI}(4,5)\text{P}_2$ and other phosphoinositide levels reduced when endogenous receptors are activated under physiological conditions? Many channels have been shown to be phosphoinositide sensitive using the extreme experimental conditions we have listed. How lipid-sensitive does a channel have to be for actual regulation during physiological membrane traffic or by receptor activation? Is localization of the channel with respect to $\text{PI}(4,5)\text{P}_2$ domains a decisive factor for channel regulation? In many cases we have described, other cellular signals and auxiliary proteins compete with or potentiate lipid signals, either directly or through allosteric interactions. Is this crosstalk the origin of functional differences between

different cell types or in different disease states? Will many other classes of membrane proteins be found to be lipid regulated in the same ways as ion channels clearly are?

Much remains to be done in the future.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Phosphoinositides regulate functional characteristics of many ion channels.
- For study, need defined perturbations of phosphoinositides levels in living cells.
- Phosphoinositides may silence ion channel during trafficking.

Fig. 1.

Organelle phosphoinositide signature helps regulate ion channel function. Schematic representation of the predominant subcellular localization of phosphoinositide species in each organelle. PI(4,5)P₂ and PI(3,4,5)P₃ are concentrated at the plasma membrane (PM). PI(3,4)P₂ is found mostly in early endocytic pathways distal to the plasma membrane. PI(4)P is principally concentrated in the Golgi complex, but can also be found at the plasma membrane and secretory pathways (SGs). PI(3)P is located in early endosomes (EE) and PI(3,5)P₂ on late endosomal (LE), multi-vesicular body (MVB), and lysosomal compartments. PI is found in the endoplasmic reticulum (ER). Grey arrows represent the continuous flow of membrane between organelles. Note that heterotetrameric KCNQ2/3 ion channels are closed (no arrow) during trafficking and retrieval from the plasma membrane, but are open (with arrow) at the plasma membrane due to the presence of appropriate activating phosphoinositide (PI(4,5)P₂) and membrane voltage.

Fig. 2.

Metabolic synthesis and cleavage of PI(4,5)P₂. A) Production of PI(4,5)P₂ from PI in two steps of phosphorylation. Lipid kinases transfer phosphates from ATP to the inositol 4- and 5-hydroxyls, and lipid phosphatases remove the phosphates in continuous steady-state cycles. B) G_q-couple receptors activate PLCβ, which cleaves PI(4,5)P₂ into soluble inositol 1,4,5-trisphosphate (IP₃) and membrane-bound diacylglycerol. IP₃ releases calcium from intracellular stores and diacylglycerol recruits and activates PKC.

Fig. 3.

Structural basis of PI(4,5)P₂ activation of Kir2.2. A) X-Ray crystal structures of the Kir2.2 homotetrameric channel in the absence (left, blue α -carbon chain, Protein Data Base coordinates, PDB: 3JYC) and presence of PI(4,5)P₂ (right, gray, PDB: 3SPI) [12]. The Kir2.2 tetramer is viewed from the membrane side with the extracellular face up. The approximate position of the plasma membrane (PM) lipid bilayer is represented as grey bars. Purple spheres represent potassium ions on the axis of the pore. Each channel subunit is in complex with a single PI(4,5)P₂ molecule represented as spheres and colored according to atom type: carbon, grey; phosphorous, orange; and oxygen, red. Binding of PI(4,5)P₂ induces a translation of the cytoplasmic domains towards the transmembrane domains and overall 6 Å compression of the channel (compare red arrows) and an opening of the inner helix 'activation' gate. B) A single Kir2.2 subunit in complex with a single PI(4,5)P₂ molecule. The vertical line is the pore axis with four K⁺ ions visible. Inset: detailed view of the PI(4,5)P₂ binding site. Helices (shown as ribbon) are colored grey. All side chains are shown as sticks. Di-C₈-PI(4,5)P₂ is shown as sticks and colored according to atom type: carbon, grey; phosphorous, orange; oxygen, red; and nitrogen blue. N-O hydrogen bonds are dashed lines. Upon PI(4,5)P₂ binding, a flexible linker contracts to form a compact helical structure, the cytoplasmic domain translates and becomes tethered to the transmembrane domain, this causes the inner gate to open. Note the electrostatic map of the same subunit behind the Kir2.2 structure (blue is positive; red negative; white neutral). The PI(4,5)P₂ binding site comprises numerous basic residues (blue) that interact electrostatically with the negatively charged phosphates of PI(4,5)P₂.

Fig. 4.

Depletion of plasma membrane PI(4,5)P₂ by different approaches closes KCNQ2/3 channels. A) Current traces of KCNQ2/3 channels activated by a depolarizing pulse from -60 mV to -20 mV in the whole-cell configuration before (black) and after (red) activation of muscarinic acetylcholine receptor type I (M₁R) by 10 μM oxotremorine methiodide (Oxo-M) in tsA-201 cells. Test pulse protocol is shown above the current traces. B) KCNQ2/3-mediated tail current amplitude against time in the same experiment as A. Orange bar indicates Oxo-M application. C-F) Same type of experiment as in A and B, but PI(4,5)P₂ was depleted either by a voltage-pulse to +100 mV to activate a voltage-sensitive lipid-5-phosphatase (VSP, panels C and D), or by recruitment of a lipid-5-phosphatase (pseudojanin) to an anchor protein at the plasma membrane (LDR) by addition of 5 μM rapamycin (panels E and F).

Fig. 5.

Bidentate model of $\text{Ca}_v2.2$ channel regulation by $\text{PI}(4,5)\text{P}_2$ with different coexpressed Ca_v β subunits. A) Interactions between $\text{Ca}_v2.2$ and $\beta 2a$ subunit. The two palmitoyl fatty acyl chains of Ca_v $\beta 2a$ compete with the those of $\text{PI}(4,5)\text{P}_2$ at the channel, reducing the need for $\text{PI}(4,5)\text{P}_2$. See text. B). Interactions between $\text{Ca}_v2.2$, $\text{PI}(4,5)\text{P}_2$, and $\beta 3$ subunit. Non lipidated Ca_v $\beta 3$ subunits do not compete with $\text{PI}(4,5)\text{P}_2$ binding. The electrostatic interaction of $\text{PI}(4,5)\text{P}_2$ with the channel is disturbed after dephosphorylation by a lipid $\text{PI}(4,5)\text{P}_2$ 5-phosphatase. See text.