

Accepted Manuscript

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PII: S0968-0004(19)30081-7
DOI: doi:[10.1016/j.tibs.2019.04.001](https://doi.org/10.1016/j.tibs.2019.04.001)
Reference: TIBS 1568

Published in: *Trends in Biochemical Sciences*

Cite this article as: Robinson CV, Rohacs T, Hansen SB, Tools for understanding nanoscale lipid regulation of ion channels, *Trends in Biochemical Sciences*, doi:[10.1016/j.tibs.2019.04.001](https://doi.org/10.1016/j.tibs.2019.04.001)

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Tools for understanding nanoscale lipid regulation of ion channels

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Keywords

signaling lipids, mass spectrometry, cryo-electron microscopy, super-resolution imaging, lipid rafts

Abstract

Anionic phospholipids are minor but prominent components of the plasma membrane and necessary for ion channel function. Their persistence in bulk membranes, in particular phosphatidylinositol 4,5-bisphosphate (PIP₂), initially suggested they act as channel cofactors. However, recent technologies have established an emerging system of nanoscale signaling to ion channels based on lipid compartmentalization (clustering), direct lipid binding, and local lipid dynamics that allow cells to harness lipid heterogeneity to gate ion channels. The new tools to study lipid binding are set to transform our view of the membrane and answer important questions surrounding ion channel delimited processes, such as mechanosensation.

Challenges and opportunities for lipid regulation of ion channels

Ion channels in lipid membranes can be regulated by extracellular ligands[1], post-translational modification[2], and the lipid that surround them[3–5]. Phosphatidylinositol 4,5 bisphosphate (**PIP₂**) (see Glossary), a component of cell membranes, was the first known [6] and remains the most prominent anionic **signaling lipid** to affect ion channels [3,7–9]. As such, PIP₂ has served as a model for studying this type of regulation. Initial studies (e.g. cardiac) suggested the concentration of PIP₂ remains relatively high and unresponsive in the bulk plasma membrane during physiological changes, giving rise to a theory that PIP₂ primarily functions as a cofactor for ion channels [3,4]. In contrast, PIP₂ was also recognized for its

potential role to dynamically regulate cell function [10], including localized signaling. For a detailed consideration of theories related to local PIP₂ signaling, the reader is referred to Hilgemann 2007 [4]. Thus, it was hypothesized that the putative signaling role of PIP₂ was dependent on its **lateral mobility** in the membrane, and its possible local sequestration and metabolism. However, the tools to confirm the role of PIP₂ at nanoscale resolution were lacking [4]. Tools for directly measuring membrane lipids bound to ion channels were also lacking leaving the following questions unanswered: which lipids bind to channels, what are their binding affinities, where exactly do lipids bind, how are the lipids organized relative to the channels, and how are they regulating the channel?

This review first highlights the new tools for detecting membrane lipid-ion channel interactions and heterogeneity of cell membranes at nanometer scales, and then discusses how these tools have been used to show how cells harness heterogeneity of membranes to gate channels. The review ends by discussing how evolving views on lipid regulation will likely transform lipids from mostly cofactors and structural support to central orchestrators of biological function. The data herein spans molecular to cellular mechanisms. Many of the principles outlined here likely apply to the lipid regulation of transporters and many other classes of membrane proteins.

Advanced technologies for detecting membrane lipid-ion channel interactions

The tools described here are advancements that have occurred over the last five years. Specifically, mass spectrometry, detergent-based lipid binding assays, cryo-electron microscopy (EM), and super-resolution imaging techniques are discussed. Classical tools for describing interactions of ion channels with lipids have been described previously. Combining these biochemical technologies may prove to be a powerful strategy to define lipid binding to ion channels.

Mass spectrometry

It has been challenging to identify the type of membrane lipid bound to an ion channel with no prior knowledge of what that lipid might be. This is because both ion channels and lipids are hydrophobic, hence they non-specifically aggregate, masking the specific interaction. Absent a direct detection mechanism, the most common method has been to test many candidate lipids either immobilized [3] or in a functionally reconstituted system [11,12] in order to find one that binds and has a functional effect on the ion channel.

Mass spectrometry was initially limited by expensive instrumentation and technical expertise but the introduction of commercial platforms have alleviated these problems to some extent [13]. Advances in mass spectrometry now enable the direct detection of a lipid binding to an ion channel [13,14]. Electrospray ionization is used to propel the lipid-ion channel complex into a gaseous state from solution in a micelle but without disrupting the specific lipid interaction (Figure 1A). Nonspecific lipids are stripped away by the input of energy while the bound lipid is directly detected inside the mass spectrometer. This technique has been used to characterize diverse families of ion channels, such as mechanosensitive channel (**MscL**), aquaporin Z, ammonia channel (**AmtB**) [14], and, most recently, the technique has defined PIP₂ interactions that influence downstream G-protein coupling [15]. Hence, the technique has broad utility. One of the great strengths of the approach is that the oligomeric state of the ion channel and detection of lipid binding can be deduced with the same experiment [16] and, in theory, the technique has the potential to identify a lipid species with no prior knowledge of the lipid. However, defining which lipids are regulating the function of a structure as opposed to only conferring stability typically requires electrophysiology experiments [17].

Soluble lipid binding assay

Knowing the binding affinity (K_d) of a lipid for an ion channel is perhaps the most important property for understanding its function in the membrane, as only the K_d can reveal the relevance of a particular lipid concentration in the membrane for that channel. Furthermore, the K_d s of two distinct lipids can reveal their ability to compete for binding with an ion channel. Lastly, the K_d can help to distinguish a ligand from a cofactor. A ligand will have an affinity similar to its cellular concentration that allows it to come on and off to perform its function [5].

Recently, a proximity based assay emerged for determining the K_d of PIP₂ bound to a fully assembled potassium channels [18] (Figure 1B). To measure the lipid K_d , the channel was tagged with a fluorescent protein, purified in detergent, and then competition of a fluorescent lipid probe with a lipid ligand was detected by fluorescence resonance energy transfer (FRET) and bioluminescent resonance energy transfer (BRET). The soluble environment led to detecting a K_d similar to traditional ligand binding constants which are convenient for thinking about lipids as ligands and comparing their interaction within protein sites. BRET is more sensitive than FRET (~100x) but has the drawback of needing a chemical substrate to generate the signal [18]. Previous binding of PIP₂ to soluble cytoplasmic fragments is unlikely to reflect the true affinity of the lipid [19].

CryoEM

By determining the structure of lipid-ion channel complexes, lipid binding sites in a membrane protein can be identified and the lipids function can be postulated. Lipids that function as ligands or cofactors likely bind to the transmembrane domain and cause a conformational change that opens the channel. Lipids that bind to disordered loops outside the membrane likely affect channel trafficking or localization. For example, X-ray crystallography produced one of the first convincing demonstrations of a lipid bound to an ion channel—the structure of PIP₂ bound to inward rectifying potassium channel subtype 2.2 (K_{ir}2.2). PIP₂ was seen bound to a specific site in the ion channel's transmembrane domain [20], causing a conformational change consistent with the lipid **gating** the channel [5,20,21].

Limitations of X-ray crystallography made it difficult to study lipid-ion channel interactions more completely. Recently, cryoEM has emerged as a powerful alternative to X-ray crystallography [22]. Primarily, cryoEM has the advantage that a structure can be determined in a lipid bilayer by reconstituting the channel into **nanodiscs** [23], a native membrane-like state.

The heat- and capsaicin-activated transient receptor potential (**TRP**) vanilloid 1 (TRPV1) was the first ion channel for which a high-resolution cryoEM structure was reported in a nanodisc [23]. TRP channels remain at the forefront of the cryoEM resolution revolution, and structures of representative members of all seven sub-families have been reported [24], four of which have been determined in complex with signaling lipids [22,25–27]. The first TRP ion channel structure in lipid nanodiscs reported that the capsaicin binding site of TRPV1 under resting conditions was occupied by a phosphoinositide, another component of cell membrane. It was thus proposed that this lipid acted as competitive antagonist in these conditions [22]. This paper showed the power of the nanodisc system for determining lipid protein interactions in membrane like conditions.

Additional examples which use the power of cryoEM to determine the structure of lipid-ion channel complexes are found in the Ca²⁺ selective channel TRPV5 [26], the lysosomal channel TRPML1 [27], and the γ -aminobutyric acid A (GABA_A) receptor [28]. TRPV5 is constitutively active in the presence of basal levels of PIP₂, and a recent study used cryoEM to determine the structure of PIP₂ bound to TRPV5 in lipid nanodiscs (Figure 1C). This study found that PIP₂ induced a conformational rearrangement of the channel that widens the conductance pathway, allowing Ca²⁺ ions to pass. This structure offered the first glimpse of TRP channels in PIP₂

dependent open and closed states [26]. TRPML1 is activated by PI(3,5)P₂, a phosphoinositide found in the lysosomal membrane, and it is inhibited by PIP₂ which is found in the plasma membrane [27]. Structures of this channel with either lipid showed that both lipids bind to the intracellular portions of the first two transmembrane segments, sites distinct from lipid binding in TRPV1, TRPV5 and TRPM8 [25,27]. Finally, using CryoEM, a PIP₂ binding site was discovered in the α 1 subunit of GABA_A receptor, which belongs to a separate super family of pentameric ligand gated ion channels that mediate fast inhibitory neurotransmission [28]. The site was speculated to contribute to localization of the channel to **lipid domains** within the plasma membrane, but the role of lipid regulation in GABA_A was not previously known and will require further study.

Overall, CryoEM has an enormous potential to provide direct information not only on how lipids interact with ion channels but also on the conformational changes they induce in the channel protein. From the large number of recently reported ion channel structures, relatively few have been solved with their regulatory lipid bound, but given the rapid progress in the field, we will likely see more of them soon.

Super-resolution imaging

Another question that has been difficult to answer is if signaling lipids are restricted in their lateral mobility in the membrane. Determining restricted lateral mobility is critical for establishing local gradients of lipids in cell membranes. Local gradients are also critical for establishing lipids as potential central regulators of ion channel function [4]. By typical confocal microscopy, PIP₂ appears relatively diffuse (i.e. there is no obvious gradient) [4,29]. But confocal microscopy diffraction is limited to ~200 nm resolution—higher resolution tells a different story [29,30].

Recently, super resolution imaging defined at least three types of lateral lipid heterogeneity (domains/clusters) in cell membranes. These include the classic lipid rafts (also called **GM1 domains**) [31], PIP₂/protein charged clusters [29,32], and phosphatidyl inositol triphosphate (PIP₃) clusters [33] (See Figure 2A). The existence of lipid heterogeneity establishes nanoscale locations within a membrane where a lipid-regulated ion channel can be activated or inhibited.

Cross-correlation analysis is a technique used to characterize colocalization of two molecules in the plasma membrane. This is particularly useful for direct stochastic optical reconstruction microscopy (dSTORM) and allows a quantitative measurement of the average

distance between two populations of molecules (Figure 1D) [34]. This analysis can be important since the resolution is sufficient to observe two localized molecules as separate signals. Cross-correlation can also be used to quantitatively measure the nanoscale movement or translocation of a protein or signaling lipid between lipid domains or clusters. For example, mechanical shear causes phospholipase D2 (**PLD2**) to shift from GM1 domains to PIP₂ clusters [32]. Due to the very small distances traveled (42 nM) this was only seen with super resolution imaging. Similar analysis should be amenable to putative nanoscale movement of channels between lipid domains.

The location of lipid regulation

Lipid based localization

Super resolution imaging has made critical advancements for answering the questions of how lipid heterogeneity emerges and how the cell harnesses heterogeneity to gate a channel. Classically, GM1 domains are thick, high in saturated lipids, and thought to utilize hydrophobicity to partition in the membrane [31]. In contrast, PIP₂ is polyunsaturated [5] and has been shown to cluster with charged proteins away from GM1 domains [29] (Figure 2A). Presumably the acyl chains of PIP₃ also facilitate the partitioning of PIP₃ away from PIP₂ [33] and GM1 domains [29,32]. Despite the advances of super resolution imaging, showing the absolute size, exact composition, and putative structures of the heterogeneity in membranes is still challenging [35]. But most agree that the membrane is not perfectly homogeneous in its distribution of lipids and proteins [36] and this alone is sufficient to postulate a mechanism for gating channels.

To harness heterogeneity for gating, a channel or a lipase that regulates the channels is targeted to lipid nanodomains [32,37]. The targeting sequesters the channel or lipase away from its ligand or substrate. Cellular mechanisms then allow the channel or lipase to translocate to its ligand or substrate over very short distances as a form of rapid regulation (Figure 2B). Best estimates from dSTORM imaging indicate translocation occurs in the 650 μ sec time frame faster than what is typically detectable by electrophysiology experiments [32].

Palmitoylation can regulate the targeting of proteins in and out of nanodomains [38,39]. For example, PLD2 contains two palmitoylation sites and a PIP₂ binding motif. dSTORM has shown PLD2 associates with GM1 domains, presumably using these acylations, and this inactivates it by sequestering the enzyme away from its substrate [32]. But PLD2 also binds PIP₂. PIP₂ is polyunsaturated hence, PIP₂ binding to PLD2 opposes the palmitoylation/GM1

localization and exposes the enzyme to PC resulting in the production of PA. The generated PA then activates TWIK-related K⁺ channel type 1 (K2P2.1, **TREK-1**) (Figure 2B-D, Box 1) [18,40]. In short, the cell harnesses lipid heterogeneity to gate the channel. Disruption of the lipid domains by either anesthetics or mechanical force circumvents the endogenous regulatory system and robustly activates PLD2 [32,37]. Furthermore, any channel could theoretically be palmitoylated directly and move in and out of a GM1 domain without an enzyme intermediary.

Numerous channels have been shown to be palmitoylated [2]. Initially palmitoylation was thought to simply increase hydrophobicity of a protein to localize the protein to the membrane [41]. This is clearly not the case with ion channels [42], as they are typically permanent integral membrane proteins usually with several transmembrane spanning segments. The role of palmitoylation localizes the channels to GM1 domains altering their membrane sorting [42] which then likely changes their exposure to lipid ligands [5].

The balance between GM1 and PIP₂ signaling can be regulated by lipid composition. When arachidonic acid is added, cells rapidly take it up causing a shift in the equilibrium between GM1 domains and the disordered region, e.g. PIP₂ cluster [43]. Adding cholesterol has the opposite effect, the membrane becomes thicker and favors GM1 domain formation and signaling. In theory, translocation of a channel to a GM1 domain would also expose the channel to thicker membranes or different anionic signaling lipids that could gate the channel[43]. In other words, the lipids can signal without any change in lipid concentration, simply by the channel translocation in the membrane.

Biological function of lipid signals

Lipid competition

The potentially broad applicability of the advanced tools and emerging nanoscale signaling raises the question, should lipids be more broadly viewed as central signals regulating biological function? In a biological membrane, a channel is exposed to numerous signaling lipids. Interestingly, among the PA regulated channels, almost all of them also bind PIP₂ and the binding typically has an antagonizing effect. In other words, the lipid competition appears to oppose both binding and function. For example, PIP₂ inhibits TREK-1 while PA activates and PA saturation inhibits Kir2 channels while PIP₂ activates (Box 1) [18,20]. In K_v channels, PIP₂ completely reversed the effect of PA shifting the V_{mid} back -40 mV toward resting membrane potentials[44].

Figure 3 shows a proposed model for lipid competition. Initially, PIP₂ is in high concentration and either activates or inhibits multiple classes of channels to achieve a coordinated resting membrane potential and to set cell excitability (Figure 3A). For channels regulated by both PA and PIP₂ (Figure 3B), a local PA competes out PIP₂ and can reverse the activity of the channel. In some sense, the PIP₂ could be thought of as a tonic competitor to PA signaling, i.e. a type of cofactor as previously speculated [3,4].

Lipid competition is also important for alcohol intoxication. PLD produces an unnatural lipid phosphatidylethanol (PEtOH) that accumulates in the membrane of cells and competes with PIP₂ and PA binding to potassium channels (Figure 3C) [45]. Blocking the production of the unnatural lipid blocked an intoxicating phenotype in flies [45], suggesting a single class of toxic lipid can affect the behavior of a whole animal.

Lipids as central regulators

In light of the emerging signaling roles of PIP₂ and PA, it is worth revisiting channels as downstream effectors in biological function. Of the 24 channels known to contribute to cardiac function, all 24 belong to families of channels regulated by PIP₂ or PA and 17 of the 24 have identified PIP₂ or PA regulation (Table 1). Their K_d's for PIP₂ and PA are not known; but experimentally, PIP₂ dictates muscle (skeletal) cell excitability [46,47]. Similarly, at least 24 channels are known to contribute to pain sensation in animals (Table 1). Of those 24, 19 are known to be regulated by PIP₂ or PA.

Only 5 of the channels are known to participate in both cardiac and pain processes suggesting subtype selective ligands could target pain over cardiac disease. Positive and negative allosteric modulators of lipid binding to ion channels could provide the therapeutic specificity.

In general, PIP₂ appears to increase the activity of most channels and thus the permeability of the membrane (see Table 1). PIP₂ also alters the excitability of the membrane. For cardiac channels, PIP₂ tends to coordinate a decrease in excitability primarily by opening K⁺ channels. K⁺ channels work opposite Ca²⁺ and Na⁺ channels to decrease excitability of the cell. PIP₂ can simultaneously activate a hyperpolarizing potassium channel (e.g. K_{ir}s) and decrease depolarizing Na⁺ or Ca²⁺ channel (e.g. **Ca_v** channels) and depolarizing Cl⁻ channels (e.g. **TMEM16A**) [48]. The competition of PA with PIP₂ is likely to have the opposite effect, a decrease in permeability and ion channel opening.

Direct versus indirect lipid regulation

Many channels appear to be both activated and inhibited by PIP₂ manipulation in cells, giving rise to a debate of PIP₂'s direct effect (e.g. TREK-1 [18], TRPV1 [49,50], K_v [51], **HCN** [52], Ca_v [53], and TRP [54]). A likely contributor to the uncertainty is the fact that PIP₂ has at least three independent roles in the membrane that include the following: localizing proteins to disordered regions [32], directly binding to ion channels [5], and generating **second messenger signals** [3]. Distinguishing direct binding of lipid versus an indirect role (e.g. through localization of the channel or an enzyme) can be challenging, especially when manipulations take place in the membrane.

TREK-1 is perhaps the best understood example of dual regulation [18,55]. When saturated with PIP₂, TREK-1 is antagonized [18] (tonic inhibition). But PIP₂ also localizes PLD2 near TREK-1 which indirectly activates TREK-1. Hence, PIP₂ has cellular mechanisms that both activate and inhibit TREK-1. Whether these same localized and indirect mechanism contribute to the lack of apparent lipid specificity in other channels [7] is not known.

However, many ion channels have disordered loops and could bind a PA producing enzyme or PIP₂ depleting enzyme. Phospholipase C (**PLC**) co-purifies with TRPV1 [56], glutamate receptors [57], the nAChR [58], and cation channels [59].

CONCLUDING REMARKS

Functionally reconstituting ion channels in lipid vesicles and testing ion transport of the purified channels is still the best technique to observe the putative action of a lipid with a channel. However, a reconstitution system is not without problems. Foremost, determining what lipids and proteins are needed for accurate reconstitution to recapitulate biological function is difficult. Also, there is variability in how the channel tolerates purification. Lastly, even in a purified systems, signaling molecules such as PIP₂ can both activate and inhibit depending on the lipid composition or concentration [18]. Future advancements will need to address these concerns.

However, the advanced techniques described have significantly progressed the understanding of how cells harness lipid heterogeneity to gate ion channels. This system has the advantage that the concentration need not change across the entire membrane to dynamically activate or inhibit a channel [60]. The effect of heterogeneity will need to be worked out for each channel individually by identifying all the pertinent lipids bound to each channel, characterizing their direct and indirect interactions, and establishing their nanoscale

function in the cells (see Outstanding Questions). The core tools are now available and set to change the role of lipids in ion channel regulation and biological function.

Acknowledgements

We apologize to colleagues whose work was not cited due to space limitations. Work in the authors' laboratories is supported by a Director's New Innovator Award to S.B.H. (1DP2NS087943-01) from the National Institutes of Health. We thank Nick Petersen from the laboratory of S.B.H. for helpful comments.

GLOSSARY

AmtB: is an ammonium transport protein from *Escherichia coli*.

Ca_v: are voltage activated calcium channels that typically initiate action potentials by allowing Ca²⁺ ions to pass into the cytosol of a neuron.

GM1(monosialotetrahexosylganglioside) domains: is a lipid domain comprised of GM1 lipids and cholesterol. GM1 lipids are the prototypic ganglioside (glycolipid).

KcsA: is a potassium channel from *Streptomyces lividans*.

K_v: are voltage gated potassium channels that typically hyperpolarize a neuron (terminate an action potential) by releasing potassium from inside the cell.

Lateral mobility: is the ability of membrane resident proteins and lipids to laterally diffuse in the two-dimensional plane of the plasma membrane.

Lipid domains: are regions within a membrane where lipids are restricted in their lateral mobility due to lipid-lipid partitioning or clustering of lipids with proteins. For example long chain saturated lipids and cholesterol (GM1 domains) partition away from polyunsaturated lipids (e.g. PIP₂) The restricted movement leads to lipid heterogeneity which is harnessed by the cell to regulate ion channel gating.

MscL: is the large conductance of mechanosensation channel found in bacteria. MscL is activated by membrane stretch and helps bacteria avoid lysis due to osmotic swell.

Nanodiscs: are isolated lipid bilayers supported by a ring of scaffolding proteins. The lipid bilayer in the nanodiscs provides the most native-like environment for determining the structure, of a lipid bound to an ion channel.

Palmitoylation: is a post translational modification to a protein that places a saturated lipid (typically 14-16 carbons). Palmitoylation can localize an otherwise soluble protein to GM1 domains in the plasma membrane.

Phosphatidylinositol biphosphate (PIP₂): is a glycol lipid (contains an inositol sugar) with two phosphorylation sites —typically located at the 4' and 5' positions of the inositol ring. When not in the 4,5 positions, the phosphates are indicated in parenthesis e.g., PI(3,5)P₂. PIP₃ contains 3 phosphates located in the 3,4, and 5' positions. The placement of phosphates in the ring is critical for many aspects of anionic lipid signaling to ion channels. See [61] for further reading.

Phospholipase D (PLD) is a membrane associated enzyme that hydrolyzes phosphatidylcholine (PC) into phosphatidic acid (PA) [62], an anionic signaling lipid similar to PIP₂, and choline. PLD can also generate phosphatidylglycerol (PG) when glycerol is present [63]. The PLD isoform 2 (PLD2) activates TREK-1 channels.

Phospholipase C (PLC): is a membrane associated enzyme that hydrolyzes phospholipids between the glycerol and phosphate. In the case of PIP₂, PLC generates diacylglycerol and inositol triphosphate.

γ-aminobutyric acid A receptor (GABA_A): is a chloride conducting inhibitory channel activated by the neurotransmitter γ-aminobutyric acid.

Gating: is the process of a channel switching from a conducting to a non-conducting state in response to a ligand or voltage. In the conducting state, ion channels allow the passage of ions through the plasma membrane. Gating in response to voltage is 'voltage-gated'; to extracellular neurotransmitter-ligand is 'ligand-gated'; and to lipid is 'lipid-gated'.

Signaling lipids: are typically low abundant anionic lipids (i.e. negative charged lipids) that signal to cells. Signaling lipids include membrane-resident lipids (e.g. PIP₂ and PA) that bind to and gate ion channels. The hydrolysis of PIP₂ can further signal through the generation of second messenger signals.

Second messenger signals: are intracellular signals released by the activation of an extracellular stimulus. For example, classical activation of G-protein coupled receptor (GPCR) subtype G_q activates PLC which releases intracellular inositol triphosphate from PIP₂ [3].

Transmembrane member 16A (TMEM16A): is a calcium-activated chloride channel (CaCC).

Transient receptor potential (TRP): are a large family of cation channels that mediate a variety of sensations including pain, temperature, taste, pressure, and vision.

TWIK-related AA-stimulated K⁺ channel (TRAAK): is a homolog of TREK-1 [40]. The similar response and affinity to PA suggests native TRAAK is likely regulated by a PA producing enzyme and responds to local high concentrations of PA [18].

TWIK-related K⁺ channel subtype 1 (TREK-1): is an inhibitory mechanosensitive K₂P channel that is activated by phosphatidic acid and directly binds PLD.

Two pore domain potassium channels (K₂P): are dimeric potassium leak channels that form a pseudo-tetrameric pore.

V_{mid} voltage activation: is the applied voltage to a membrane that half maximally activates a voltage-gated channel. PA shifts the V_{mid} of K_v1.2/2.1 chimera by +40 mV (towards positive potentials). Hence, the channel requires much stronger depolarizing current to open when PA is present [44].

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Box 1. Lipid localization mediated by protein-protein interactions.

TREK-1, an anesthetic-sensitive inhibitory mechanosensor, was initially classified as a PIP₂ activated channel [64]. Recently, phosphatidic acid (PA), a signaling lipid that facilitates activation of the nicotinic acetylcholine receptor (nAChR) [30-32], was shown to activate TREK-1 [18,40] and at least 10 other ion channels including mechanosensitive channels piezo1-2 [65], the nAChR [66], MscL [67,68], and KcsA [69,70]. TREK-1 and its homolog, TRAAK in artificial systems [18,40], are activated by the localization of PLD2 near to the channel's disordered C-terminus [40]. The role of PLD2 and the finding that PIP₂ directly antagonizes the effect of PA and phosphatidylglycerol (**PG**) on TREK-1 in a reconstituted system [18] argues that TREK-1 is a PA or PG-gated channel.

Presumably, the activation depends on both the K_d and local concentration of PA. The K_d of PA for TREK-1 is 9 μ M [18], and it can be calculated, by the formula $1/\pi r^2 h N_A$, that its local concentration near TREK-1 is ~3.5 mM in the presence of PLD2. PA could diffuse away if lateral mobility is unrestricted, but experimentally this does not appear to be the case [40].

Figure 1A shows a schematic of channel activation by local production of PA. Since TREK-1 is mostly inactive absent PLD2 in the membrane [40] we can estimate the effective 'non-PLD2' exposure to PA to be less than 500 nM—a concentration sufficiently low not to activate the channel. This mechanism is opposite the one previously considered for local PIP₂ depletion [4]. In that mechanism, PIP₂ remains high in the membrane and local PIP₂ hydrolysis produces a localized graduation that depletes the lipid [4]. The local high concentration of PA and the potential to modulate channels with low affinity for PA suggest an emerging class of putative PA regulated channels (Figure 1B).

If we consider the PIP₂ scenario with an ion channel (Figure 1C), PIP₂ remains high and the channel binds PIP₂ with a K_d ~10-fold below the concentration in the membrane such that the channel is active in the resting state. Local depletion then inactivates the channel. The model is supported by binding constants for PIP₂-gated inward rectifier potassium channels (**K_{irs}**). K_{irs} bind with 100-250 nM affinity [18] and the estimated concentration of PIP₂ in membranes is 1-10 μ M [4], thus PIP₂ is bound in the resting state and localization of PLC to a channel would inactivate the channel.

Figure 1. Lipid activation of ion channels through localized synthesis. (A) Co-localization of TREK-1 with phosphatidic acid (PA) very convincingly established a lipid-protein based

localization mechanism as a means of its activation [40]. Indirectly it also establishes the fact that local lipid gradients in a cell must exist sufficient to activate a channel. The putative concentration, local to the channel (grey cylinders), is indicated by an inset circle. PA, shown with black stick and red circle, is in concentrations low relative to a channel's K_d . Localization of phospholipase D (PLD) and local production of PA results in a local high concentration of PA (>1 mM) and channel activation (gating). Local PA concentration is estimated from a single molecule of PA produced within 100 Å of the channel. Additional putative enzymes that could generate local PA include diacylglycerol kinase (DAGK) and lysophosphatidic acid acyltransferase (LPAAT). (B) Emerging class of PA regulated channels. At least 11 channels are known to respond directly to PA. Both nAChR and TRAAK require relatively high concentrations of PA suggesting local high concentrations may contribute to their activation [18,66]. $K_v1.2/2.1$ is a channel chimera [44]. TREK is activated by a mixture of anionic lipids [71]. (C) PIP_2 tonic concentration is high in the bulk membrane (~ 1 -10 μ M, red shading) [4] relative to a channel's K_d (~ 200 nM), shown as black sticks with two red spheres. A hypothetical ion channel is shown with phospholipase C (PLC) bound to a disorder loop (black line). Local hydrolysis depletes PIP_2 and decreases PIP_2 binding resulting in a closed channel. Additional putative enzymes that could locally deplete PIP_2 include phospholipase A_2 (PLA_2) and phosphoinositide 3 kinase (PI3K). Bulk PIP_2 and PA concentrations are estimated from K_{ir} and K_2P lipid binding constants [18].

Figure 1 New tools for detecting lipid ion channel interactions. (A) Schematic representation of the processes occurring in the mass spectrometry of membrane protein lipid interactions. (i) Ionization of the protein complex takes place while in the protective detergent micelle. (ii) Activation within the source, at high energy, is required to disrupt the micelle. (iii) Isolation of discrete m/z values is achieved in the quadrupole and is followed by activation in the collision cell (iv). (v) Following time-of-flight analysis the protein and direct protein lipid interaction is measured. (B) Detergent solubilized phosphatidylinositol 4,5-bisphosphate (PIP₂) binding assay for potassium channels. The purified channel and lipids are solubilized in detergent and the binding of a fluorescent-PIP₂ probe is detected by fluorescence resonance energy transfer (FRET) assay from a genetically encoded fluorescent protein (FP). (C) Cryo-electron microscopy (cryoEM), PIP₂ resolved in the structure of TRPV5 channel (PDB 6DMU). A channel is purified and reconstituted into nanodiscs, i.e. a protein lipid particle, and imaged with electron microscopy. An atomic resolution structure with the lipid bound is then determined by single particle reconstruction. (D) Super resolution imaging (direct stochastic optical reconstruction microscopy (dSTORM)) of native TREK-1 and PIP₂ in C2C12 myoblast cell. Cross-correlation of TREK-1 and PIP₂ shows colocalization of the channel with PIP₂ at specified distances in cellular membrane. The atomic scale resolution of an ion channel structure ((i.), TREK-1 PDB 4XKD) is depicted relative to the nanoscale resolution of the membrane imaged by dSTORM and the cellular resolution (iii.) imaged by diffraction limited confocal microscopy of the plasma membrane (PM). See also Figure 2A.

Figure 2 Lipid partitioning and clustering gives rise to lipid heterogeneity. (A) Lipids partition into at least three domains or regions of the cell membrane. The regulation of a channel depends on its partitioning with agonizing or antagonizing anionic signaling lipids (e.g. PIP₂ and PIP₃). Ceramide-1-phosphated (C1P) is an anionic ceramide comprised of a phosphate head group (red dot) similar to phosphatidic acid and expected to localize to GM1 domains. The endoplasmic reticulum (ER) is shown for reference and scale. (B) PLD2 is activated by substrate presentation. GM1 domains sequester PLD2 away from its substrate phosphatidylcholine (PC, orange circles). Increases in PIP₂ attract PLD2 away from the GM1 domains localizing the enzyme near PC allowing for hydrolysis. (C) Cross correlation analysis of PLD association with GM1 domains before and after disruption with methyl-beta-cyclodextrin. After disruption, PLD fails to correlate with GM1 at any distance (radius in nm, error bars shown as standard error of the mean (n=7-8)). (D) PIP₂ activation of PLD2 can then indirectly activate TREK-1. The enzyme localizes to the disordered C-terminus (grey strand) of TREK-1 through a PLD2 binding site (green segment). PLD2 produces anionic lipid phosphatidic acid (PA) (green lipid) which binds to lipid binding site (blue cylinder) which opens the channel. This indirect mechanism contrasts with PIP₂ directly activating a channel shown in (A).




Figure 3 Examples of ion channel regulation by lipid competition. In most excitable cells, K^+ channels are inhibitory and Ca^{2+} and Na^+ channels are excitatory. (A) Lipids can regulate this excitability by increasing the opening of K^+ channels (i, open) and decreasing the gating or voltage sensitivity of Ca^{2+} and Na^+ channels (ii, closed). In general, PIP_2 (red shading) increases permeability (flow of ions) of the plasma membrane, but numerous channels are exceptions. (B) In some instances, endogenous PA (green shading) can compete with PIP_2 to reverse its effects (coordinated dysregulation). (C) In the presence of ethanol, PLD2 produces an unnatural lipid phosphatidylethanol (PEtOH, blue shading) which can competitively displace and reverse the effect of the endogenous lipid ligand (adapted from Chung et al. [45] under a creative commons license (CC BY-NC-ND 4.0)).

Super Family	Cardiac ^a	Permeability ^c	Excitability ^d	Ref ^e	Pain ^b	Permeability ^c	Excitability ^d	Ref ^e
K2P	TASK-1 ⁱ	PIP2 ↑	↓	[64]	TRESK ⁱ	Anionic ↑	↓	[71]
	TWIK-1/2 ⁱ			?	TREK-1	PA ^h ↑ (PIP2 ^h ↑↓?)	↓↑	[18,64]
	TRAAK ⁱ	PA ^h ↑ (PIP2 ↑?)	↓	[18,64]	TRAAK	PA ^h ↑ (PIP2 ↑?)	↓	[18,64]
Nav ⁱ	Nav1.5			?	Nav1.3/1.7/ 1.8/1.9			?
Cav ⁱ	Cav1.2	PIP2 ↑	↓^	[53]	Cav2.2	PIP2 ↑	↓ ^g	[53]
	Cav3.1/3.2			?				
Kir ⁱ	Kir2.1/2.2	PIP2 ↑ (PA ↓)	↓	[72,73]	Kir2.1	PIP2 ↑ (PA ↓)	↓	[72,73]
	Kir6.2	PIP2 ↑	↓	[73]	Kir6.2	PIP2 ↑	↓	[73]
	Kir3.1/3.4	PIP2 ↑ ^f	↓	[73]	Kir3.2	PIP2 ↑	↓	[73]
Kv ⁱ	Kv1.4/1.5/1.7	PIP2 ↑ ^f	↓	[51]	Kv1.1/1.2	PA ^h ↓ ^g (PIP2 ^h ↑↓)	↑	[44]
					Kv2.1/2.2	PA ^h ↓ ^g	↓	[44]
	Kv 3.1/3.4	PIP2 ↑ ^f	↓	[51]	Kv3.4	PIP2 ↑ ^f	↓	[51]
	Kv 4.2/4.3	PIP2 neg		[74]	Kv4.3	PIP2 neg		[74]
	Kv7.1	PIP2 ↑	↓	[75]	Kv7.2-3,5	PIP2 ↑	↓	[74,76]
	hERG ⁱ	PIP2 ↑	↓	[77]				
HCN ⁱ	HCN2/4	PIP2 ↑↓	↓ ^g	[52]				
P2X ⁱ					P2X2/P2X3	PIP2 ↑	↑	[78]
TRP ⁱ					TRPV1	PIP2 ^h ↓↑?	↓↑	[49,50]
					TRPM3	PIP2 ^h ↑	↑	[79,80]
					TRPM8	PIP2 ^h ↑	↑	[81]

Table 1 Lipid coordinated biological function.

^aChannels involved in cardiac function [82].

^bChannels involved in pain [83,84]

^cThe lipid effect the flow of ions across the membrane (permeability); up arrow (increase), or down arrow (decrease).

^dThe lipid effect on the ability of a neuron to depolarize (excitability); up arrow (increase), or down arrow (decrease).

^eReference (Ref).

^fAffects excitability through inactivation.

^gAffects excitability through voltage or cGMP dependence.

^hLipid effect determined with purified reconstituted channel.

ⁱAbbreviations for ion channel names are as follows: voltage activated calcium channel (Cav), hyperpolarization-activated cyclic nucleotide-gated channel (HCN), human Ether-à-go-go-Related Gene (hERG), inward rectifying potassium channel (Kir), voltage activated potassium channel (Kv), voltage activated sodium channel (Nav), ATP-gated P2X receptor (P2X), TWIK-related acid-sensitive K⁺ channel (TASK), TWIK-related spinal cord potassium channel (TRESK), transient receptor potential (TRP), two-pore weak inward rectifier channel (TWIK). Additional letters and numbers after the abbreviations, indicate the channel's subtype.

OUTSTANDING QUESTION (2000 characters, including spaces, required)

- Which channels directly bind PIP_2 and which ones are indirectly modulated through PIP_2 activation of regulatory proteins?
- Which channels if any translocate between lipid domains as a response to changes in physiological conditions?
- What are the relevant affinities for PIP_2 and related anionic signaling lipids binding to ion channels? What lipids types bind to ion channels and do they compete with each other in a complex lipid environment?
- How many ion channels are regulated by lipid signaling through the dynamic localization of lipid modifying enzymes?
- Does a central lipid signaling-network coordinate biological function through localized lipid signals? If yes, how many of the channels participate and in what biological systems?

Highlights

- Mass spectrometry, cryoEM, and super resolution microscopy are advanced tools set to evolve the role of phosphatidylinositol 4,5 biphosphate (PIP₂) and other anionic lipids in the regulation of ion channel function.
- Cells harness lipid heterogeneity to gate a channel.
- Phosphatidic acid (PA) regulated channels are an emerging class of lipid regulated channels.
- Nanoscale lipid gradients open up the possibility for membrane resident anionic lipids to centrally coordinate biological processes by locally regulating ion channel function.

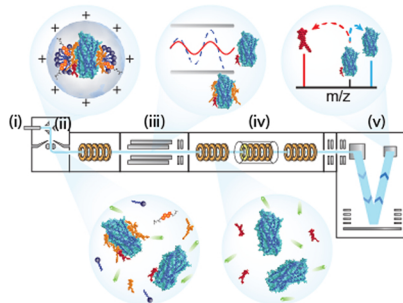
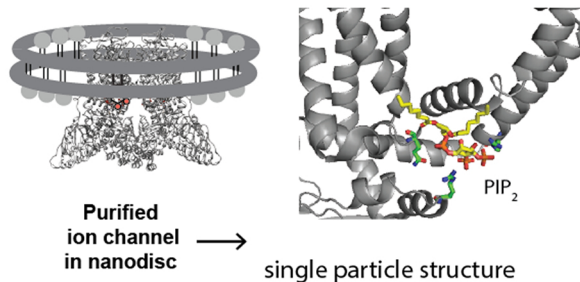
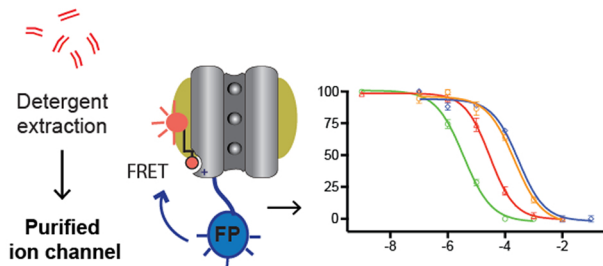
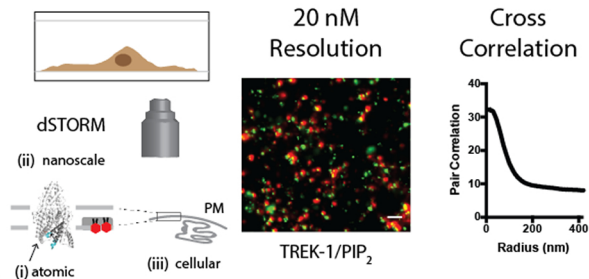
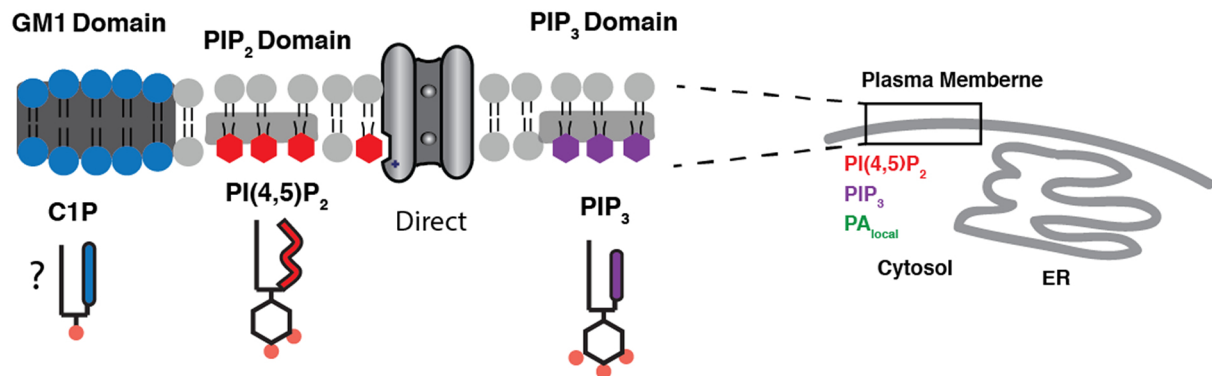
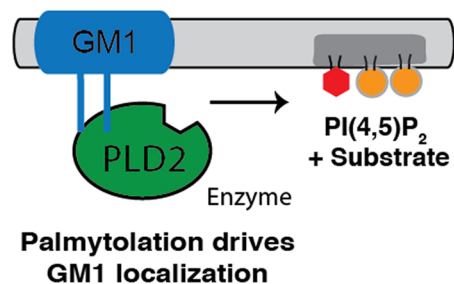
(A)**Mass Spectrometry****(C)****Cryo-Electron Microscopy****(B)****Soluble Lipid Binding Assay****(D)****Super Resolution Imaging**

Figure 1

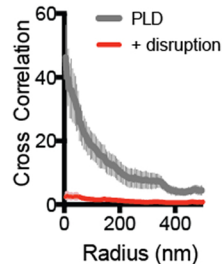
(A)



(B)



(C)



(D)

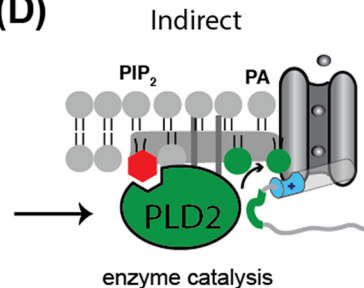
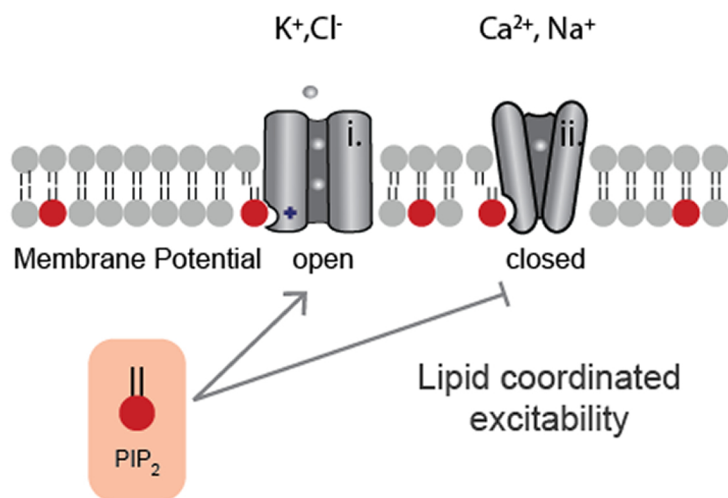
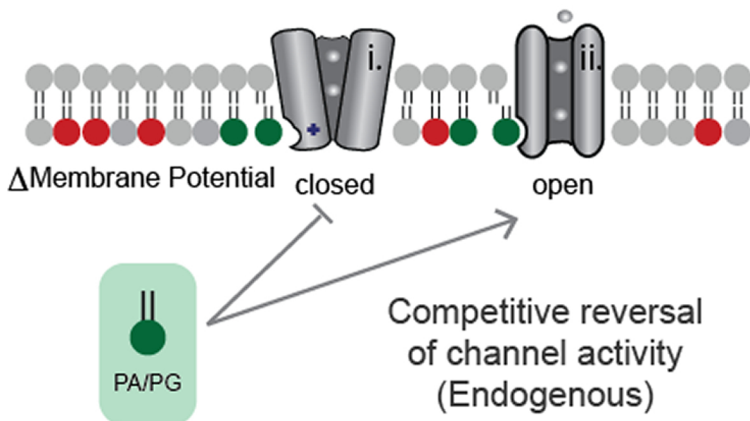


Figure 2

(A)



(B)



(C)

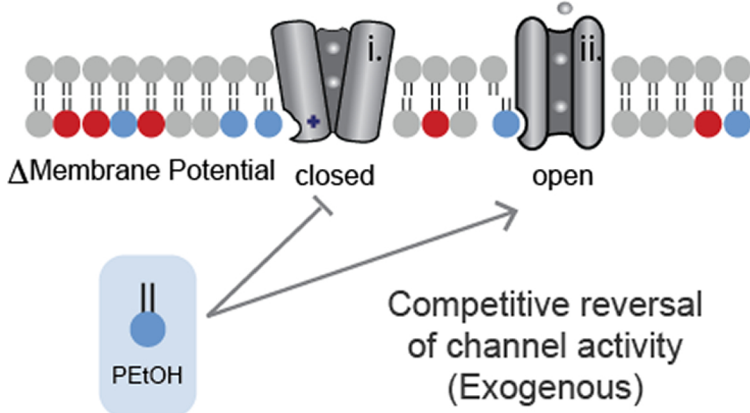


Figure 3