Lipid signaling to membrane proteins: From second messengers to membrane domains and adapter-free endocytosis

Donald W. Hilgemann,[1] Gucan Dai,[2] Anthony Collins,[3] Vincenzo Lariccia,[4] Simona Magi,[4] Christine Deisl,[1] and Michael Fine[1]

[1]Department of Physiology, University of Texas Southwestern Medical Center, Dallas, TX
[2]Department of Physiology and Biophysics, University of Washington, Seattle, WA
[3]Saba University School of Medicine, The Bottom, Saba, Dutch Caribbean
[4]Department of Biomedical Sciences and Public Health, School of Medicine, University "Politecnica delle Marche," Ancona, Italy

Lipids influence powerfully the function of ion channels and transporters in two well-documented ways. A few lipids act as bona fide second messengers by binding to specific sites that control channel and transporter gating. Other lipids act nonspecifically by modifying the physical environment of channels and transporters, in particular the protein–membrane interface. In this short review, we first consider lipid signaling from this traditional viewpoint, highlighting innumerable *Journal of General Physiology* publications that have contributed to our present understanding. We then switch to our own emerging view that much important lipid signaling occurs via the formation of membrane domains that influence the function of channels and transporters within them, promote selected protein–protein interactions, and control the turnover of surface membrane.

Introduction

Lipids and lipid signaling profoundly impact the function of ion channels and transporters, and the molecular mechanisms by which lipids act are now being rapidly elucidated. More than 50 JGP contributions illustrate specific regulation of ion channels by phosphoinositides, both transient regulation caused by phosphoinositide concentration changes and constitutive regulation related to channel localization in membrane compartments with differing phosphoinositide content. Less specifically, lipids modify the function of channels and transporters by modifying local membrane structure and protein–membrane interfaces. Membrane tension, fluidity, curvature, and asymmetry are subjects of more than 200 JGP publications. The lipids involved include diacylglycerols, ceramides, free fatty acids, fatty acid metabolites, lysolipids, and cholesterol. Arachidonic acid is addressed in 26 JGP publications, while cholesterol and anionic lipids are each addressed in more than 200 JGP articles. An emerging area of interest is the formation of proteolipid domains in cell membranes. Diverse studies indicate that membrane proteins and lipids can form ordered domains that modify membrane protein function and may catalyze unique membrane protein interactions, such as concerted channel gating. Domains initiate as nanoscale proteolipid aggregates and, upon coalescence, can achieve lifetimes of up to several seconds. Domains can vesiculate inwardly as adapter-free endocytosis or outwardly as ectosome shedding, and they may potentially serve as platforms for local lipid metabolism. Palmitoylation is a common protein modification that promotes participation in ordered domains. Amphipathic compounds, such as lysolipids and detergents, and phospholipids with small head groups, such as ceramide, can also catalyze domain coalescence. A parallel mechanism is that membrane domains can be formed via membrane cytoskeleton fences that restrict long-distance lipid diffusion without restricting local lipid diffusion. Clearly, the development of improved means to study membrane domains and to test emerging ideas about their formation will be a major challenge for membrane physiology in the 21st century.

Lipid signaling is complex

In 1969, a prescient JGP article explicitly raised the question of how mechanistically the lipid bilayer can influence membrane protein conformations (Wallach, 1969). The answers were not simple at that time, and they are not simple now. A few of the physical mechanisms of interest are tabulated in Fig. 1 A. Second messenger functions are exemplified by the regulation of K channels by phosphatidylinositol (4,5) bisphosphate (PIP_2) as it occurs in sympathetic neurons (Kruse et al., 2016). Numerous biophysical mechanisms by which the interface between membrane proteins and the bilayer can be modified are outlined in recent articles of the Olaf Andersen group (Lundbaek and Andersen, 1994; Andersen, 2013; Bruno et al., 2013; Rusinova et al., 2013, 2015), three of which were published in JGP.

Correspondence to Donald W. Hilgemann: Donald.Hilgemann@utsouthwestern.edu

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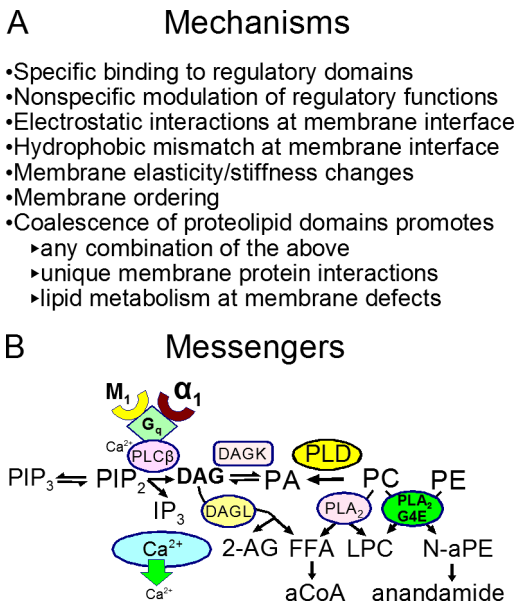

Figure 1. **Mechanisms involved in lipid signaling to ion channels and transporters. (A and B)** A host of biophysical mechanisms (A) and lipidic messengers (B) are involved in lipid regulation of membrane proteins. PLCs cleave one signaling lipid, PIP_2, and generate two signaling molecules, IP_3 and DAG. Phospholipase Ds (PLDs) and DAG kinases both generate the signaling molecule, PA. DAG lipases cleave one signaling lipid, DAG, and generate the cannabinoid 2-AG, whereas FFAs serve as precursors to many other signaling lipids. PLA_2 generates the signaling lipid LPC, as well as FFAs. The acyltransferase PLA2G4E generates LPC as it transfers an acyl chain from PC to PE to form N-aPE, the precursor of anandamide.

Lipids that can influence membrane–protein interfaces in all cells including diacylglycerols (DAGs), ceramides, free fatty acids (FFAs), and FFA metabolites, as well as cholesterol and PIP_2 (Rusinova et al., 2013). The complexity grows exponentially with the number of lipids considered, and it explodes with the realization that lipid-metabolizing enzymes simultaneously generate and deplete multiple bioactive lipids. Fig. 1 B highlights this principle for PLCs. As example, PLC-β becomes activated when M_1 muscarinic receptors or α_1 adrenergic receptors are activated and couple to G_q proteins (Falkenburger et al., 2013). The PLC-β activity controls not one signaling lipid, but a network of lipids, while it simultaneously generates inositol triphosphate (IP_3) and thereby Ca signals. DAG is generated, PIP_2 is depleted, and PIP_2 depletion potentially depletes phosphatidylinositol (3,4,5) trisphosphate (PIP_3; Howes et al., 2003). It is even suggested that PIP_3 phosphatase activity can in certain circumstances promote accumulation of PIP_2 (Li et al., 2014). Next, DAG is phosphorylated by DAG kinases to generate phosphatidic acid (PA; Gomez-Cambronero, 2014; Bullen and Soldati-Favre, 2016), or it is cleaved by DAG lipases to generate FFAs and arachidonylglycerol (2-AG). All three of the reaction products are universal signaling lipids (Okazaki and Saito, 2014), and FFAs are the precursors of additional highly active metabolites. The activated forms of FFAs, long-chain acyl coenzyme As (CoAs), directly regulate both transporters (Hamming et al., 2008) and channels (Shumilina et al., 2006), whereas prostaglandins and cannabinoids are agonists at their own specific receptors (Sang and Chen, 2006). 2-AG is also implicated to regulate A-type K channels by a direct mechanism and thereby control neuronal pacemaking (Gantz and Bean, 2017).

Expanding still further this complexity, the same lipid signaling molecule can often be generated by multiple biochemical pathways. Given that lipids typically traffic extensively during and subsequent to their synthesis (Blom et al., 2011), this multiplicity may or may not contribute to compartmentalization of lipid signaling. Whereas 2-AG, as diagrammed in Fig. 1 B, can be a product of the PLC pathway, cannabinoids are also generated in other branches of lipid metabolism (Rahman et al., 2016). Anandamide synthesis, for example, can be initiated by the transfer of an acyl chain from phosphatidylcholine (PC), possibly located in the outer monolayer, to the head group of a phosphatidylethanolamine (PE) in the inner monolayer, via an acyl transferase (PLA2G4E) that only recently has been identified (Ogura et al., 2016). Once again, multiple signaling molecules are generated. Lysophosphatidylcholines (LPCs), which are commonly generated by PLA_2, are also generated when the acyl chain of PC is removed and transferred to the head group of PE, thereby forming N-acyl-PE (N-aPE). The cannabinoid anandamide is formed subsequently by the cleavage of N-aPE. In this connection, it is an intriguing question whether this unusual, three-legged lipid intermediate, N-aPE, might itself have signaling functions in the cytoplasmic leaflet of the surface membrane.

A good signaling lipid can be hard to find

Invertebrate vision illustrates very well the complexities just outlined. The no receptor potential *Drosophila* visual mutant, *norpA* (Paj et al., 1976), characterized biochemically in a JGP paper in 1978 (Ostroy, 1978), provided the first very persuasive support, albeit indirect support, for the notion that specific membrane lipids can act as specific membrane-delimited second messengers to regulate ion channels. Biochemical work in the early 1980s revealed that in vision-impaired *norpA* mutants, DAG was not being phosphorylated to generate PA (Yoshioka et al., 1984). Soon, it was clarified that the disrupted enzyme was not a DAG kinase but rather a PLC (Yoshioka et al., 1985). Subsequently, the transient receptor potential *TRP* proteins that mediate invertebrate vision were identified (Montell and Rubin, 1989), and they were determined to be cation channels that could initiate light responses by depolarizing photoreceptor cells in the ommatidium (Montell

and Rubin, 1989). It remained then only to identify the second messenger generated by PLC activity.

Ironically, these seminal discoveries did not lead quickly to the identification of a second messenger that mediates invertebrate phototransduction. IP_3 appeared to be required for *Limulus* photo responses (Brown et al., 1984), and it was later verified to be critical in *Limulus* in another JGP contribution (Fein, 2003). However, IP_3 was eliminated as a possible second messenger in *Drosophila*, and interest turned to FFAs generated by DAG lipases subsequent to PIP_2 cleavage to DAG (Chyb et al., 1999). Later, results for DAG kinase mutants shifted interest to DAG itself (Hardie et al., 2002). However, the role of DAG in *Drosophila* phototransduction became less convincing over the next decade. Recently, it has been proposed that PIP_2 depletion within the membrane, together with acidification that can occur during PLC activity, underlies the photo response in *Drosophila* (Hardie and Juusola, 2015). Given this history, the field remains unsettled, and there are indeed good reasons for uncertainty. The functions of *TRP* and *TRP*L channels, although better understood in *Drosophila* as a result of contributions to JGP (Saari et al., 2017), are strongly dependent on the cell type in which they are expressed, thereby confounding analysis of their biochemical regulation in expression systems (Lev et al., 2012). Although regulatory proteins may play a role, it seems certain that the lipidic environment is important. Resolution of the remaining open questions will be challenging, and one possible outcome is an involvement of multiple lipid messengers.

Multiplicity in lipid signaling

The interconnected nature of lipid signaling and the potential for involvement of multiple signaling lipids, just highlighted, raise similar problems across the board. One prevalent example is that PIP_2-sensitive transporters and channels are more often than not also affected by PA or other anionic lipids. Human inwardly rectifying K channels, for example, have one relatively specific site for PIP_2 and another nonspecific site where additional anionic lipids can bind (Cheng et al., 2011). Therefore, regulation of DAG kinases and PA hydrolases, as well as PLCs, becomes physiologically important. PIP_2 and PA both have substantial effects on many K_v channels, and the voltage sensitivity of some K_v channels is more affected by PA than PIP_2 (Hite et al., 2014). Our own interest in lipid signaling was stimulated by finding that multiple negatively charged lipids can profoundly activate cardiac Na/Ca exchange (NCX1) in isolated cardiac membranes (Hilgemann and Collins, 1992). At first, it appeared that phosphatidylserine and PA were the key modulators of Na/Ca exchangers (Hilgemann and Collins, 1992), but in giant membrane patches, PIP_2 unambiguously became the most active anionic lipid in the presence of ATP (Hilgemann and Ball, 1996). As expected, PIP_2 cleavage in response to activation of M_1 muscarinic receptors expressed in cell lines inhibited NCX1 currents (Yaradanakul et al., 2007).

Disappointing for us, others found that PLC activation in cell cultures did not inhibit NCX1 activity, monitored as ion flux (Chernysh et al., 2008). Furthermore, hormones that activate G_q signaling and PLCs were found in some circumstances to activate, rather than inhibit, NCX1 activity (Ballard and Schaffer, 1996; Stengl et al., 1998; Yaradanakul et al., 2007). In the case of α_1-adrenergic receptor activation by phenylephrine, an involvement of PKCs was indicated (Ballard and Schaffer, 1996), although many α-agonist effects in myocytes do not seem to involve PKCs (Endou et al., 1991). Certainly, PA is generated during the α_1 response, both via the PLC/DAG kinase pathway and via phospholipase Ds (Singer et al., 1996). Accordingly, PA rather than PIP_2 may be the most active lipid messenger at Na/Ca exchangers in cardiac myocytes, and the long-term activation of NCX1 by Ca elevations (Lu et al., 2016) may well reflect generation of PA by Ca-dependent DAG kinases (Liu et al., 2016; Boroda et al., 2017). In summary, and typical for PLC activation in any cell, the response of cardiac myocytes to PLC-β activation involves multiple lipids. PA may act primarily on Na/Ca exchangers, DAG on *TRP*C3, and *TRP*C6 channels (Onohara et al., 2006), and PIP_2 on delayed rectifier K channels (Bian and McDonald, 2007), but it remains possible that multiple lipids regulate each of these mechanisms.

Beyond the complexity that multiple lipids may regulate the same targets, individual signaling lipids can have different functions in different cell types. Table 1 summarizes work from more than 40 JGP contributions concerning the functions of PIP_2 in regulating ion channels. These articles explore a wide range of issues, from interactions of PIP_2 with polyamines in the regulation of K_{ATP} channels, to the specificity of PIP_2 as a regulator of different K channel types (e.g., Kir verus K_v), as well as very different channel types (ENaC versus HCN channels), and the potential of PIP_2 to regulate skeletal muscle excitation–contraction coupling. Space does not permit a detailed review of this work, but many details are available in review articles compiled previously (Logothetis and Nilius, 2007; Robertson, 2007). Especially in neurons, the second messenger function of PIP_2 to K_v channels has been firmly established (Hughes et al., 2007; Hernandez et al., 2008b; Hille et al., 2015; Dai et al., 2016). In other tissues, notably the heart, it remains more convincing that PIP_2 usually functions as a surface membrane marker that constitutively activates ion channels when they are localized to the cell surface (Hilgemann et al., 2001).

As usual in biology, the rules are not absolute. It is described for cardiac atrial myocytes that PIP_2-sensitive GIRK channels can desensitize as muscarinic receptor activation promotes PIP_2 cleavage (Jan and Jan,

Table 1. **Ion channel regulation by PIP_2: >40 JGP contributions**

Ion channel	JGP contributions
K_{ATP} channels	Koster et al., 1999; Ribalet et al., 2000; Shyng et al., 2000; Cukras et al., 2002; Pratt et al., 2011
GIRK channels	Petit-Jacques et al., 1999; Lacin et al., 2017
Multiple inward rectifier (Kir) channels	Lu et al., 2002; Xie et al., 2005; Lee et al., 2016
KCNQ channels	Suh et al., 2004; Hernandez et al., 2008a,b; Telezhkin et al., 2012; versus other K_v channels: Kruse et al., 2012
TRPV channels	Lee et al., 2005; Doerner et al., 2011; Ufret-Vincenty et al., 2015
Hyperpolarization-activated HCN ("I_F") channels	Pian et al., 2006
Epithelial (ENaC) Na channels	Pochynyuk et al., 2007
Large conductance Ca-activated (BK) K channels	Vaithianathan et al., 2008; Tian et al., 2015
CNG vertebrate vision channels	Dai et al., 2013
TRPC6/7 channels	Itsuki et al., 2014
ELK K channels	Li et al., 2015
TRPM3 channels	Badheka et al., 2015; Tóth et al., 2015
Skeletal muscle Ca release mechanism	Berthier et al., 2015
Ca_v channels	Park et al., 2017

CNG, cyclic nucleotide-gated; Ca_v, voltage-gated Ca; ELK, EAG-like.

2000; Kobrinsky et al., 2000), consistent with a second messenger function. However, this appears to be species-dependent and may not occur in intact cardiac tissue. In guinea pig atria, for example, muscarinic receptor activation shortens action potentials rapidly and monotonically, and the underlying GIRK channels clearly remain active for many minutes with no sign of desensitization (Gertjegerdes et al., 1979). This is indicative of strong G_i signaling to activate GIRKs with only weak G_q signaling that might deplete PIP_2. As a second example, HERG (Ikr) K channels in ventricular myocytes can be inactivated by PIP_2 depletion and potently reactivated by PIP_2 (Bian and McDonald, 2007). Nevertheless, their inhibition by G_q-coupled α_1-adrenergic receptors (Bian and McDonald, 2007; Urrutia et al., 2016) appears to depend more on PKC activity (Urrutia et al., 2016) than on PIP_2 depletion. In this context, many studies show that the affinities of PIP_2 binding sites can be regulated by phosphorylation and by additional channel modulators (Du et al., 2004; Rapedius et al., 2005; Li et al., 2011; Zhang et al., 2014; Chen et al., 2015; Salzer et al., 2017). Accordingly, PIP_2 can be switched from being a constitutive, high-affinity channel activator to being a regulatory second messenger with lower channel affinity whose influence changes with changes of surface membrane PIP_2 levels.

That phosphoinositides indeed regulate ion channels in a membrane compartment–dependent manner is now supported by studies of ion channels in internal membrane compartments. D3 phosphoinositides, such as $PI(3,5)P_2$, activate TRPML channels that are localized to lysosomes (Zhang et al., 2012; Schmiege et al., 2017), where D3 inositides are prevalent, whereas $PI(4,5)P_2$ is inhibitory (Zhang et al., 2012). Similarly, the activity of Na-selective two-pore channels (TPCs), localized to endolysosomal membranes, requires D3 inositides (Lagostena et al., 2017; Nguyen et al., 2017).

Local PIP_2 signaling

After more than 20 years of electrophysiological PIP_2 studies, it remains an open question whether PIP_2 signaling can occur in a local manner. Superresolution microscopy has generated conflicting results about PIP_2 domains with antibody probes showing PIP_2 clustering (Wang and Richards, 2012), whereas less aggressive, lower-affinity PH domains reveal even distributions of PIP_2 with interspersed areas of PIP_2 sparsity (Ji et al., 2015). Findings that tend to support local PIP_2 signaling in cardiac myocytes, and therefore the presence of long-lived membrane domains, include the following. Although global PIP_2 changes very little with α_1-adrenergic stimulation (Nasuhoglu et al., 2002), multiple studies suggest that PIP_2-sensitive ion channels can experience PIP_2 depletion during α_1-receptor activation. This is the case for volume-activated anion channels that in myocytes require PIP_2 for their activation in response to cell swelling (Ichishima et al., 2010). Activation of α_1 receptors can block their activation by swelling, and multiple methods to deplete and enhance PIP_2 modulate this blockade as expected if α_1-adrenergic receptor activation locally depletes PIP_2.

More directly, provocative experiments using fluorescent phospholipids in cardiac myocytes suggested (a) that PIP_2 diffusion is strongly and selectively restricted by the membrane cytoskeleton of the sarcolemma (Cho et al., 2005a), (b) that this restriction can be readily disrupted by latrunculin-induced disruption of membrane cytoskeleton (Cho et al., 2005a), (c) that PIP_2 restriction by the cytoskeleton enables local signaling from specific receptors to specific PIP_2-sensitive inward rectifying K channels (Cho et al., 2005b), and (d) that GIRK channels are specifically localized to caveolae in which PIP_2 depletion is very pronounced during activation of colocalized endothelin receptors (Cui et al., 2010). That PIP_2 in caveolae can be regulated in a localized manner is supported further by an independent biochemical study showing that activation of α_1-adren-

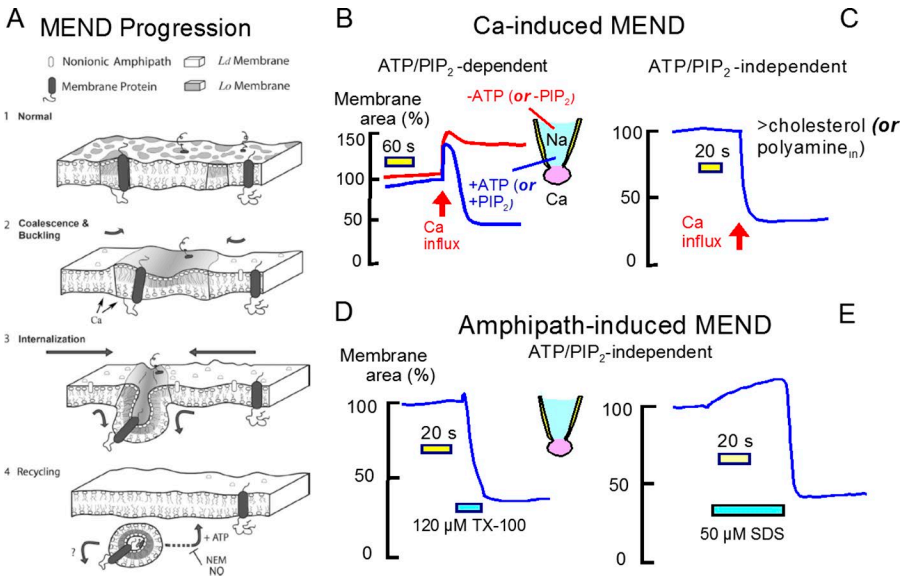

Figure 2. **Massive endocytosis or MEND. (A)** Formation of ordered membrane domains that can internalize by MEND (Fine et al., 2011). (1) The outer monolayer consists of nanoscale liquid ordered (*Lo*) and liquid disordered (*Ld*) domains with lifetimes of microseconds up to a few seconds. Affinity differences for *Lo* versus *Ld* domains are less than one log unit. (2) Palmitoylation of membrane proteins promotes the coalescence of larger *Lo* domains, as do amphipathic compounds that displace cholesterol from *Ld* to *Lo* domains. Line tension between domains may now promote the plasmalemma to buckle. (3) Accumulation of proteins with large cytoplasmic domains in *Lo* domains promotes membrane budding. (4) Membrane scission remains a key biophysical problem. "Line tension" between *Lo* and *Ld* domains will minimize the perimeters of *Lo* domains and promote domain budding, but it cannot account energetically for excision of vesicles <400 nm in diameter. One speculation is that inverted V-shaped lipids accumulate in "neck" regions with high curvature and catalyze the final "pinch" to excise vesicles. MEND generates vesicles that follow normal trafficking pathways to endosomes, and the contents of endocytized vesicles can recycle back to the plasmalemma via ATP-dependent processes that are inhibited by n-ethylmaleimide (NEM) and oxidative stress. **(B)** Delayed MEND triggered by Ca influx for 5 s (blue trace) is blocked in the absence of ATP (red trace) but can be restored by perfusion of PIP_2 into cells (Lariccia et al., 2011). **(C)** MEND occurs very rapidly in the presence of high-cytoplasmic Ca when the cytoplasm is enriched with polyamines (e.g., spermidine) or the membrane is enriched with cholesterol. There is no requirement for ATP. **(D)** Application of TX-100 at a sublytic concentration induces MEND within a few seconds without significant conductance changes and with no requirement for ATP. **(E)** Application of ionic detergents, such as SDS, at low concentrations appears to block constitutive endocytosis. MEND occurs when SDS is washed off, indicating that ordered domains with long lifetimes were formed in the presence of SDS. (B–E) Tracings of records published in the two JGP articles referenced in this legend.

ergic receptors depletes PIP_2 in caveolae of myocytes but not in the bulk of the cardiac sarcolemma (Morris et al., 2006). Given the potential importance of these findings for an understanding of PIP_2 signaling in myocytes, it remains paramount that these conclusions are addressed by further studies, preferably with additional methodological approaches. That membrane cytoskeleton and/or other membrane-associated proteins can strongly impede the diffusion of lipids in membranes is unambiguously established for phagosomes (Golebiewska et al., 2011; Ostrowski et al., 2016) and for lipid diffusion within the cytoplasmic leaflet of epithelial cells, where tight junctions form a diffusion barrier between apical and basolateral cell surfaces (van Meer and Simons, 1986).

Membrane domains as regulators of membrane protein interactions and function

It has been advocated for decades that the formation of proteolipid domains within biological membranes promotes selected protein–protein interactions and membrane trafficking events (van Meer and Simons, 1988). How, in detail, domains form and regulate these functions remains controversial. Our group was forced to think in terms of membrane domains after finding that cells can internalize large fractions of their surface membrane without involvement of conventional endocytic proteins (Yaradanakul et al., 2007). Fig. 2 A describes the progression of massive endocytosis (MEND) in cartoon form. During these responses, membrane regions internalize that are more ordered, and therefore bind amphipathic compounds poorly. More than 20 amphipaths were analyzed, including detergents, hydrophobic ions, and fluorescent membrane probes (Hilgemann and Fine, 2011). Those membrane regions that are more disordered, and bind amphipaths more avidly, remain at the cell surface. The capacitance records typically do not reveal discrete events, indicating that the vesicles formed are not unusually large. Triggers of MEND include large Ca transients, amphipathic compounds, membrane protein palmitoylation, sphingomyelinase activities, and metabolic stress.

The JGP review process enabled us to publish a series of articles describing very large datasets concerning these endocytic processes (Fine et al., 2011; Hilgemann and Fine, 2011; Lariccia et al., 2011). At least three different Ca sensors could eventually be distinguished (Hilgemann et al., 2013), and MEND could be shown to occur in several different flavors, some of which are illustrated in Fig. 2 (B–E) using capacitance recording to monitor surface membrane area in BHK cells. Ca influx for 5 s via constitutively expressed Na/Ca exchangers

routinely causes initially an exocytic response that expands the surface membrane by >30%. After cytoplasmic Ca recedes, this response is followed by loss of 50% of the cell surface over 2 min (blue trace in Fig. 2 A), and this response was verified optically to be endocytosis rather than ectosomal membrane shedding (Lariccia et al., 2011). In the absence of cytoplasmic ATP (red trace in Fig. 2 A), MEND is blocked but can be rapidly restored after a Ca transient by perfusing PIP_2 into cells (Lariccia et al., 2011). In other circumstances, illustrated in Fig. 2 C, MEND occurs with no dependence on ATP or PIP_2, and the rapid progression of MEND requires the continued presence of a high cytoplasmic Ca concentration. This type of Ca-activated MEND is promoted by polyamines, such as spermidine, and by enhancing the membrane cholesterol content.

The involvement of membrane domains became increasingly likely with indications that conventional endocytic proteins, such as clathrin, dynamins, and actin, were not involved in MEND. As anticipated, reagents that modify mechanical properties of membranes (Lundbaek and Andersen, 1994; Lundbæk et al., 2004) and promote complex membranes to form domains (Staneva et al., 2005) powerfully induced MEND with no requirement for ATP and without significant conductance changes. Fig. 2 D illustrates the induction of MEND by Triton X-100 (TX-100, 120 µM). Like TX-100, ionic detergents such as SDS can also displace cholesterol laterally (Caritá et al., 2017), and SDS induces MEND at concentrations 10-fold lower than its critical micelle concentration. These MEND responses, illustrated in Fig. 2 E, take place with the caveat that detergent must be removed for MEND to occur. An explanation of this pattern must take into account that SDS does not cross membranes and has no effect from the cytoplasmic side in pipette perfusion experiments. Our suggestion is that, although SDS promotes the formation of domains that can internalize, the ionized head groups of SDS molecules also prevent the final fusion of the outer monolayer with itself that must occur as vesicles pinch off. SDS can be washed off cells much faster than the membrane can reorganize laterally back to its "ground" state. Accordingly, domains induced by SDS must have lifetimes equivalent to the few seconds over which MEND takes place in these experiments when SDS is washed off.

The induction of MEND by sphingomyelinases (Zha et al., 1998; Lariccia et al., 2011) is also implicated to involve the formation of submicroscopic membrane phase separations. Equivalent experiments using giant artificial vesicles demonstrate that ceramides, generated during sphingomyelin cleavage by sphingomyelinases, associate into domains and catalyze membrane vesiculation in the direction expected from their inverted V shape (Holopainen et al., 2000). A correlate to this result, described in a provocative JGP article (Combs et al., 2013), is that the loss of head group mass by sphingomyelinase activity causes large hyperpolarizing shifts in the activation of K_v channels by decreasing the energy required for K channel voltage sensors to move outwardly.

From the different MEND types, delayed ATP/PIP_2-dependent MEND has been studied most extensively up to now. As illustrated in Fig. 3, mitochondria appear to initiate delayed MEND through a pathway that uses CoA as a second messenger (Hilgemann et al., 2013; Lin et al., 2013). In brief, CoA is synthesized on the outer surface of mitochondria and is then actively concentrated at least 50-fold into the mitochondrial matrix (Idell-Wenger et al., 1978; Tahiliani and Neely, 1987) via voltage-dependent transporters (Tahiliani, 1989; Tahiliani et al., 1992), perhaps using a nucleotide exchange mechanism (Fiermonte et al., 2009). The opening of large-diameter permeability transition pores (PTPs) in response to Ca or metabolic stress in mitochondria releases CoA back into the cytoplasm, where it initiates a wave of acyl-CoA (aCoA) synthesis via aCoA synthetase activity (Idell-Wenger et al., 1978; Sepp et al., 2014). Possibly, aCoA supports domain coalescence via direct amphipathic effects on the cytoplasmic monolayer of the surface membrane. In addition, however, the aCoA wave supports palmitoylation of membrane proteins. This clearly requires additional Ca-mediated "permissive" signals at the surface membrane, whereby both PKC activation and transient generation of reactive oxygen species can be effective (Hilgemann et al., 2013; Lin et al., 2013). Functionally, palmitoylation and PIP_2 seem to support domain coalescence in a similar fashion. A possible explanation is that one acyl chain of PIP_2, bound to at the edge of a membrane protein, can bend laterally and engage in hydrophobic interactions with neighboring proteins, including the acyl chains of palmitoylated proteins that protrude similarly.

That Ca can effectively promote endocytosis, as well as exocytosis, was described insightfully in a 1979 JGP article analyzing pinocytosis that occurs in *Amoeba proteus* (Prusch and Hannafin, 1979). Other types of Ca-dependent endocytosis that might be related to delayed MEND are "bulk endocytosis" (Cheung et al., 2010) and "excess endocytosis" (Engisch and Nowycky, 1998), which occurs in secretory cells. Although recent work on bulk endocytosis suggests an involvement of actin and tropomyosin in some secretory cells (Gormal et al., 2017), Ca-dependent endocytosis that occurs in rat calyx of Held terminals appears to be rather similar to Ca-activated MEND (Yue et al., 2017). Ca-activated endocytosis in astrocytes (Jiang and Chen, 2009) has very similar characteristics to the fast forms of Ca-activated MEND (Lariccia et al., 2011).

As portrayed in Fig. 4, the manipulations that lead to MEND presumably drive small aggregates or clusters of proteins and lipids to coalesce into larger domains

that eventually become large enough to form vesicles (Lingwood and Simons, 2010; Schmid, 2017). During this progression, increased ordering of the membrane may enable collective conformational changes of lipids that in turn can mediate functional interactions between membrane proteins without direct protein–protein contacts (Sarasij et al., 2007; García-Sáez and Schwille, 2010). Of course, this requires that the proteins involved significantly perturb the bilayer when they undergo conformational changes. Recent reconstitution studies using complex lipid mixtures reveal that conformational changes of Na/K pumps indeed significantly affect the bilayer (Bhatia et al., 2016). As expected if those modifications are significant and can affect neighboring proteins, Na/K pump activity in cardiac myocytes appears to impact Na/Ca exchangers by mechanisms that do not involve Na concentration changes (Lu and Hilgemann, 2017). In this light, it will be of great interest to determine how and if membrane ordering and domain formation may facilitate the concerted gating of clusters of voltage-gated ion channels (Choi, 2014; Moreno et al., 2016) as well as channel cross talk that can occur between distinct channel types (Vivas et al., 2017).

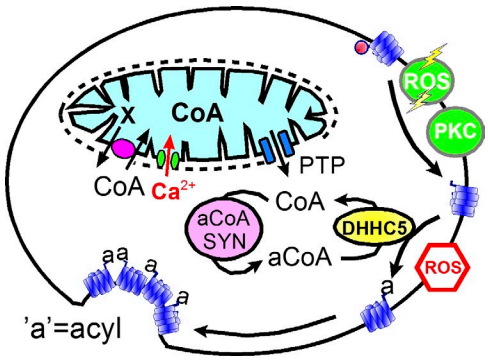

Figure 3. **Proposed molecular basis of delayed palmitoylation-dependent MEND.** Adapted from Hilgemann et al. (2013). CoA is synthesized on the outer surface of mitochondria and accumulated to high concentrations in the mitochondrial matrix via a voltage-dependent nucleotide (X)/CoA exchange mechanism (Fiermonte et al., 2009). CoA is released to the cytoplasm by transient activation of PTPs when mitochondrial accumulate Ca and/or generate oxidative stress. A wave of long-chain aCoA synthesis promotes palmitoylation of surface proteins via the activity of aCoA transferases (DHHCs), especially via the surface membrane DHHC5. PKCs and transient generation of reactive oxygen species (ROS) appear to play permissive roles for the palmitoylation of DHHC5 substrates, which subsequently accumulate in *Lo* domains that vesiculate inwardly during MEND.

Membrane domains mushroom in life-or-death circumstances

Yeast can survive without clathrin (Lemmon and Jones, 1987), mammalian cells can survive without dynamins (Park et al., 2013), and one speculative explanation is that primitive endocytic processes related to MEND enable this survival. At least, it seems reasonable to assume that over the course of evolution, cells developed complex lipidic membranes before developing the intricate protein machinery that underlies classical membrane trafficking. Accordingly, MEND-related endocytic processes might have been critical for survival of evolving cells, and MEND might have served as a template upon which more selective endocytic processes and signaling mechanisms developed. Speculatively, at least, this would explain why life-or-death metabolic stress appears to activate MEND. Beside cardiac cells in an ischemic zone of the myocardium, neoplastic cells within solid tumors must cope with extreme metabolic stress to survive (Noman et al., 2015), as do degenerating neurons with limited mitochondrial function (Pluta et al., 2013). In solid tumors, MEND-related endocytosis might provide an endocytic flux of nutrients in parallel with classical pinocytosis (Recouvreux and Commisso, 2017), whereas in degenerating neurons, MEND-related endocytosis might propagate cell demise by internalizing deranged proteins (e.g., prions

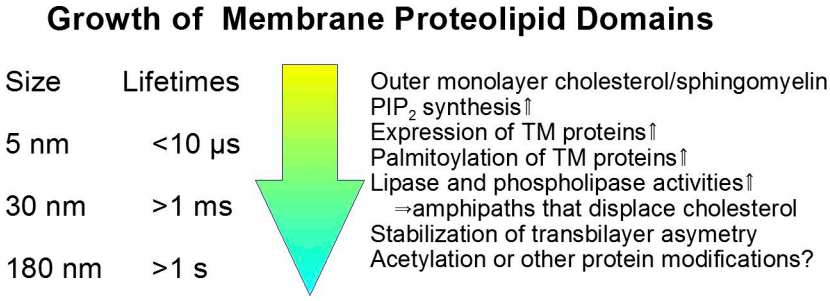

Figure 4. **Potential hierarchy of membrane domain entities from clusters to vesicles.** Adapted from Lingwood and Simons (2010). *Lo* membrane domains begin as aggregates or clusters of lipids around one to a few membrane proteins (Schmid, 2017). Lifetimes of such clusters will in general be in the microsecond range, although lifetimes of phosphoinositides bound to membrane proteins can be extraordinarily long, namely tens of seconds (Huang et al., 1998; McKenna and Ostap, 2009). Clusters can then coalesce to domains that grow large enough to form vesicles and that potentially achieve lifetimes long enough to allow local phosphoinositide metabolism. Assuming that membrane reorganization, which leads to MEND, indeed reflects growth of membrane domains, coalescence will depend on cholesterol/sphingomyelin content of the outer monolayer, PIP_2 synthesis, the expression of transmembrane proteins that can be palmitoylated, PKC activities that appear to promote palmitoylation, the maintenance of transbilayer phospholipid asymmetry that supports outer monolayer ordering, and G-protein signaling that controls lipase activities generating MEND-catalyzing amphipathic compounds.

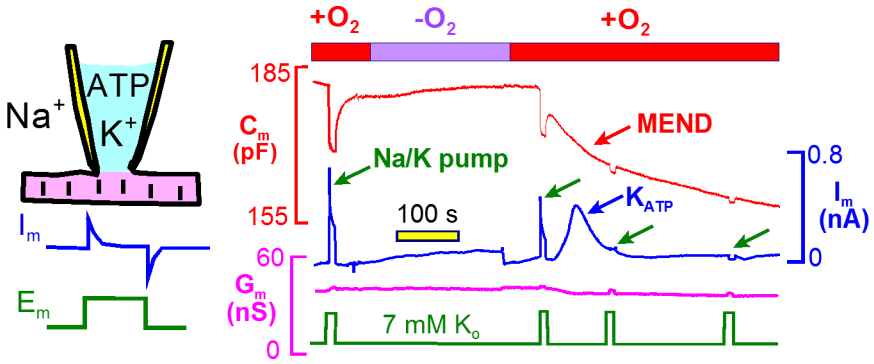

Figure 5. **MEND is a key event in ischemia/reperfusion injury.** MEND occurs in cardiac myocytes upon reintroduction of oxygen after an anoxic episode, in parallel with a transient activation of K_{ATP} channels and the complete loss of Na/K pump activity. Capacitance (C_m) and conductance (G_m) were monitored online via square wave voltage perturbation (20 mV/3 ms; Wang and Hilgemann, 2008). The extracellular solution contained 130 mM Na, and 7 mM Na was substituted for 7 mM K to activate Na/K pump currents as indicated. The cytoplasmic solution contained 100 mM K, 25 mM Na, and 6 mM MgATP. Results are consistent with the hypothesis that during reoxygenation, mitochondria generate a cytoplasmic wave of aCoA that activates K_{ATP} channels and that promotes MEND, possibly both via direct amphipathic effects on the sarcolemma and via membrane protein palmitoylations. Both MEND and Na/K pump inhibition may also be supported by PLA_2 activities that generate additional amphipaths, such as LPC. Conventional endocytic mechanisms might also become involved, as is suggested to occur during ischemia (Yang et al., 2016).

or amyloid proteins) secreted by neighboring cells (Stopschinski and Diamond, 2017).

As documented in Fig. 5, the metabolic settings that support MEND in cardiac myocytes are often also coincident with those that promote K_{ATP} channel openings, which underlie ST-segment elevation in the EKG (Long et al., 2010; Stoller et al., 2010). Fig. 5 presents a routine recording of these events in an isolated, patch-clamped murine cardiac myocyte ($n > 30$). Using standard physiological solutions with 6 mM MgATP included in the pipette solution, myocytes are superfused with thoroughly O_2-depleted solutions for a period of 5 min. During the anoxic period, membrane conductance and capacitance (i.e., sarcolemma area) are entirely stable. Upon reintroducing oxygen, however, membrane capacitance begins to fall within 40 s, and a large outward K_{ATP} potassium current develops transiently. During the same time period over which K_{ATP} current rises and falls, and over which membrane area decreases by ∼18%, Na/K pump activity decreases to negligible values and remains suppressed for long periods of time.

These results appear fundamental, starting with the fact that K_{ATP} current is activated during reoxygenation in these protocols, not during anoxia. To explain in detail this sequence of events, it must eventually be determined whether ATP becomes depleted as K_{ATP} channels activate and whether conventional endocytic proteins become involved. At this time, however, the events appear consistent with the pathway outlined in Fig. 3. When mitochondria are stressed, they initiate a cytoplasmic aCoA wave that promotes the direct activation of K_{ATP} channels (Shumilina et al., 2006) and the progression of MEND. Na/K pump activity may become suppressed in part via endocytosis of pumps, in part via inhibitory effects of palmitoylating Na/K pump subunits, and in part as a response to additional lipidic messengers that affect the function of ion channels and transporters. Bioactive lipids generated in ischemia include acylcarnitines (Yamada et al., 1994), FFAs, and lysolipids (DaTorre et al., 1991). LPC, in particular, has powerful inhibitory effects at both K_{ATP} channels (Eddlestone, 1995) and Na/K pumps (Oishi et al., 1990).

Other lipidic events that may occur include the following. PIP_2 may become depleted as a result of mitochondrial Ca release via PTPs with subsequent PLC activation. PIP_2 may become depleted if PTP openings promote reverse ATP synthase activity that results in ATP depletion. Strong support for the involvement of a MEND-like process in these events comes from seminal studies published in the 1970s, as well as recent work: sarcolemmal membrane particles, which likely reflect Na/K pumps, were described to aggregate during reperfusion injury in 1977 (Ashraf and Halverson, 1977), the density of Na/K pumps in the cardiac sarcolemma was described to decrease substantially during reperfusion injury in 1976 (Beller et al., 1976), and this decrease was recently shown to be uninfluenced by disruption of a classical dileucine clathrin endocytosis motif present in the α subunits of Na/K pumps (Pierre et al., 2011).

The mountains beyond the mountains

In conclusion, lipid signaling regulates ion transporters and channels much more extensively and powerfully than was previously envisioned. PIP_2 is a chameleon that can act as a second messenger or as a constitutive activator, and it can be switched between these roles by classical cell signaling mechanisms. Although PIP_2 has received an extraordinary amount of scientific attention, DAGs almost certainly have equally profound roles at ion channels and transporters. We predict that new signaling roles for most of the lipids highlighted in Fig. 1 B will be revealed in the coming few years. The idea that membrane domains bring proteins together and thereby promote functionally important protein–protein interactions is not a new one (van Meer and Simons, 1988). However, the idea of a raft evokes images

of isolated signaling complexes in a sea of membrane, not the endocytosis of large fractions of the surface membrane. Clearly, we are only beginning to understand how membrane domains organize and regulate important membrane processes. A key challenge will be to elucidate how the "unconventional" mechanisms considered here are related to classical signaling and trafficking mechanisms. JGP is, in our experience, a superb forum within which relevant new studies can be reviewed, improved, and disseminated to advance this fast-growing area of membrane physiology.

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The interest of our group in the regulation of ion transporters and channels extends over three decades, and it has been our privilege to benefit from the peer review system of JGP over this entire time. From our early work on transmembrane Ca movements in the heart (Hilgemann, 1986) via our studies of different forms of Na-coupled transport (Hilgemann et al., 1992; Lu and Hilgemann, 1999; Kang et al., 2003; Fuster et al., 2008), to work on unconventional membrane turnover (Lariccia et al., 2011), and back to research on Na homeostasis in the heart (Lu and Hilgemann, 2017), the JGP peer review system has been a bedrock of critical help. What makes the difference is the selfless commitment by those involved to foster and improve new scientific work, independent of fads and fashion. In this light, we express our sincere gratitude to all involved and dedicate this article to two of JGP's leaders over decades, Olaf S. Andersen and David C. Gadsby. As editors for work from our group, and innumerable other groups, Olaf and David provided consistently substantive and uncompromising criticism, professional mentoring from the big picture down to line-by-line error corrections, and unrelenting encouragement for us to improve. Their legacies inspire us to continue to try in a difficult time for basic science. Their examples should inspire us all to engage similarly.

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