JGP Journal of General Physiology

COMMENTARY

Rushing to maintain plasma membrane phosphoinositide levels

Tamas Balla

In this issue of the *Journal of General Physiology*, Myeong and colleagues investigate the mechanisms by which mammalian cells maintain the levels of phosphatidylinositol (PI) 4,5-bisphosphate ($PI(4,5)P_2$) within the plasma membrane (PM) in response to strong PLC stimulation downstream of M1 muscarinic receptor (M1R) activation. These studies use an impressive array of molecular and pharmacological approaches, as well as comprehensive mathematical modeling, to conclude that the slow recovery of $PI(4,5)P_2$ levels in the PM during prolonged M1R stimulation is not due to receptor desensitization, but rather reflects the activation of the PI 4-kinase α (PI4KA) enzyme with an important contribution also coming from PI 4-phosphate (PI4P) derived from the Golgi complex. As detailed below, these studies address important questions regarding phosphoinositide distribution and homeostasis, which have deep roots in the cell signaling field.

$PI(4,5)P_2$ is a minor phospholipid that is located primarily within the inner leaflet of the PM and plays critical roles in the control of numerous cellular functions in eukaryotic cells. The importance of $PI(4,5)P_2$ was first recognized when it was discovered that it serves as the lipid precursor of important second messengers, inositol 1,4,5-trisphosphate ($InsP_3$) and diacylglycerol (DG), which are generated following agonist binding to cell surface receptors that are coupled to the activation of PLC enzymes (Berridge, 1984). Subsequently, $PI(4,5)P_2$ was also found to control many more essential cellular processes, including direct control of ion channel and transporter dynamics, actin cytoskeleton remodeling, and complex regulation of the vesicular trafficking machinery that governs both endo- and exocytic pathways (Balla, 2013). $PI(4,5)P_2$ is primarily generated from PI by sequential phosphorylation events that are catalyzed by PI 4-kinases (PI4Ks) and PI4P 5-kinases (PIP5Ks), with possible minor contributions from PI5P 4-kinases (also called type II PIP kinases) that use the minor lipid, PI 5-phosphate (PI5P), as their substrate. There are multiple isoforms and splice variants of all of these enzymes (Balla, 2013), which likely selectively contribute to unique localized $PI(4,5)P_2$ pools that could be dedicated to the regulation of specific signaling modalities.

Being such an important regulatory signal, cells must tightly control membrane $PI(4,5)P_2$ levels, especially against the actions of the numerous PLC and phosphoinositide 5-phosphatase enzymes that both contribute to $PI(4,5)P_2$ degradation. The importance of understanding how $PI(4,5)P_2$ homeostasis is balanced was already recognized a long time ago, when it was discovered that the products of PLC activation are rapidly recovered and used for the rapid resynthesis of $PI(4,5)P_2$ (recently reviewed in Chang and Liou, 2016). This metabolic cycle was dubbed the "PI-cycle"; however, the description of this metabolic turnover raises several important questions, especially considering that the synthesis of PI takes place in the ER, whereas $PI(4,5)P_2$, as well as the lipid products generated from its hydrolysis, are located in the PM. Consequently, for this cycle to occur, the DG formed as a result of PLC activation, or its rapidly formed metabolite, phosphatidic acid (PA), must reach the ER from the PM. At the same time, the PI that is synthesized within the ER, must reach membrane compartments where resident phosphoinositide kinases can generate the PI4P and $PI(4,5)P_2$ that finally end up in the PM (Fig. 1). For a long time it was assumed that PI4P and $PI(4,5)P_2$ synthesis both take place in the PM as the enzymatic activities yielding these lipids were found enriched in the PM. Additionally, these processes were extensively studied in membranes derived from red blood cells, which primarily contain PM and almost no or very little other membrane compartments. Only with the comprehensive cloning and subsequent mapping of the cellular localizations of the various PI4K and PIP5K enzymes was it possible to revisit these questions with better spatial definitions. Additional advances using imaging methods that reliably monitor the steady-state distributions of PI4P and $PI(4,5)P_2$ have since revealed that, in addition to the PM, PI4P is present in high amounts in the Golgi complex and endosomes. It is also important to note that at least three of the four mammalian PI4K enzymes (PI4KB, PI4K2A, and PI4K2B) are localized to the Golgi complex and some endosomal compartments (Minogue and Waugh, 2012; Boura and Nencka, 2015).

Pharmacological and genetic studies in yeast and mammalian cells have shown that the majority of the PM pool of PI4P is

Section on Molecular Signal Transduction, Eunice Kennedy Shriver National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, MD.

Correspondence to Tamas Balla: ballat@mail.nih.gov.

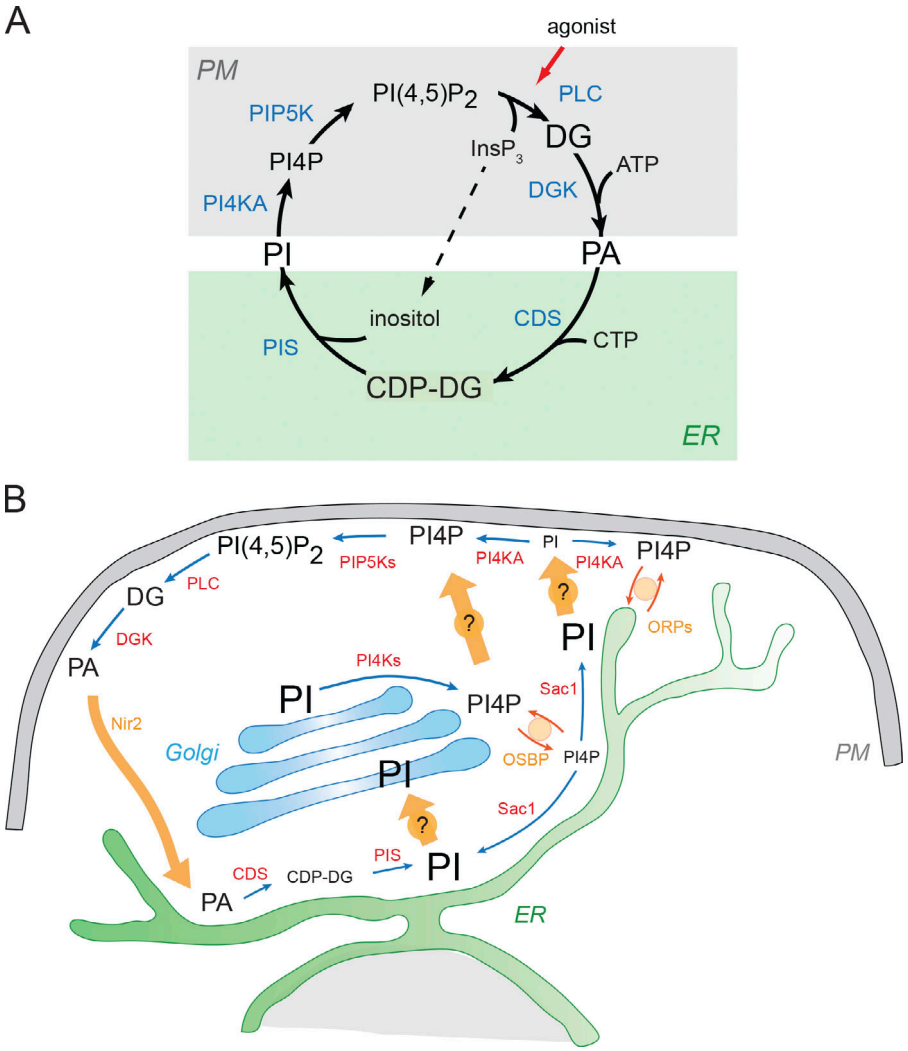

Figure 1. **Phosphoinositide synthesis and distribution in eukaryotic cells. (A)** The biochemical reactions contributing to the PI cycle. Agonists of receptors that couple to PLC activation lead to the hydrolysis of $PI(4,5)P_2$ in the PM (represented by the gray rectangle). Both products of the hydrolytic reaction that consumes $PI(4,5)P_2$, DG and $InsP_3$, are used as metabolic intermediates for the rapid resynthesis of PI. PI synthesis takes place within membranes of the ER (represented by the green rectangle) via a two-step enzymatic reaction that is catalyzed by the sequential activity of cytidine-diphosphate-DG (CDP-DG) synthase (CDS) and PI synthase (PIS; also called CDP-DG;*myo*-inositol 3-phospho-transferase [CDIPT]) using PA and *myo*-inositol as substrates, respectively. PI has to be transported from the ER to reach the PM, or other organelles, where it can be phosphorylated to generate PI4P by PI4K enzymes. PI4P can be further phosphorylated by PI4P-specific 5-kinases (type I PIP5Ks) to produce $PI(4,5)P_2$, primarily in the PM. At the same time, upon hydrolysis of resident $PI(4,5)P_2$, the DG formed in the PM is rapidly converted to PA by diacylglycerol kinases (DGKs) and it has been shown that the lipid transfer protein, Nir2, is involved in the transport of PA from the PM to the ER, while also transferring PI from the ER to the PM. It is possible, although not proven, that DG may also be directly transported from the PM and into the ER where is could also be converted to PA (not shown). **(B)** Cellular topology of the PI cycle reactions and lipid transport mechanisms. Enzymatic reactions are shown by blue arrows, whereas lipid transfer routes are shown with orange arrows. Note that the nonvesicular lipid counter-transport processes between the PM and ER and between the Golgi and the ER are driven by the PI4P gradient that is established by the resident PI4K and PI4P-phosphatase activities. Similar PI4P-driven lipid transport processes exist between the endosomes and the ER (not shown for simplicity). Oxysterol binding protein (OSBP) and its related proteins, the ORPs, have been found to carry out these lipid transport processes the efficiency of which requires the ER-localized Sac1 phosphatase that dephosphorylates PI4P within the ER to keep the bulk levels of this lipid low. Recent studies have highlighted the prominent PI and PI4P content of the Golgi, where PI4P is synthesized by four different PI4K enzymes, while there is a relatively low amount of PI in the PM, possibly as a result of rapid conversion to PI4P by PI4KA. The pathways by which bulk PI reaches the other organelles is yet to be identified, although there is evidence to suggest that the Nir2 protein could deliver PI in exchange for PA in ER–PM contacts. However, the mechanism by which PI4P present at the Golgi complex might reach the PM, as suggested by the present study by Myeong et al. (2020), also awaits identification. It is also possible that these transfer processes may involve both vesicular and nonvesicular lipid transfer components.

synthesized by the PI4KA enzyme (Audhya and Emr, 2002; Nakatsu et al., 2012; Bojjireddy et al., 2014), which functions in a tetrameric complex that is composed of TTC7 (A or B), Fam126A, and the palmitoylated EFR3 (A or B) protein that anchors the complex to the PM in mammalian cells (Lees et al., 2017). Curiously, inhibition or genetic inactivation of PI4KA does not lead to a decrease in the $PI(4,5)P_2$ levels in the PM, even though the PM pool of the PI4P precursor decreases to almost undetectable levels (Hammond et al., 2012; Nakatsu et al., 2012; Bojjireddy et al., 2014). Only after PLC activation and rapid depletion of $PI(4,5)P_2$ in the PM does PI4KA inhibition prevent restoration of the PM pool of $PI(4,5)P_2$ (Hammond et al., 2012; Bojjireddy et al., 2014). This apparent dissociation of the $PI(4,5)P_2$ content from PI4P levels revealed a further complexity for understanding PI4P biology. As it turned out, the majority of the PI4P within the cell is hydrolyzed by the ER-localized phosphatase Sac1, including regulatory control of the Golgi and PM pools of PI4P (Foti et al., 2001). The formation of PI4P in the Golgi complex, PM, or endosomes together with its dephosphorylation in the ER set up a PI4P gradient between the membranes of these organelles and the ER. This gradient is used to transport other important membrane lipids, including cholesterol and phosphatidylserine (PS), via nonvesicular lipid transport by proteins that are capable of facilitating the counter-transport of PI4P and specific lipid cargoes between the PI4P-rich membrane and the ER (recently reviewed in Antonny et al., 2018; Lipp et al., 2020).

These recent developments have posed further challenges for understanding the connection between PI4P production and the

regulation of $PI(4,5)P_2$ turnover within the PM. Is the PM indeed the sole source of PI4P for $PI(4,5)P_2$ production? This question is especially relevant in light of recent studies that showed that the PM has very little PI when compared with other organelles, especially the Golgi complex (Pemberton et al., 2020; Zewe et al., 2020). It is against this background that the studies by Myeong et al. (2020) provide important new insights into the question of how the $PI(4,5)P_2$ levels are maintained in the PM.

Myeong et al. (2020) have set out to investigate the mechanism(s) by which cells slowly recover $PI(4,5)P_2$ within the PM from its greatly reduced level following prolonged stimulation of M1Rs. The obvious first thought is that the receptor slowly desensitizes during prolonged agonist exposure. However, unlike some other G protein-coupled receptors, M1Rs do not show massive desensitization after agonist stimulation. Moreover, analysis of the products of $PI(4,5)P_2$ hydrolysis did not suggest their diminished production, as would be expected if the receptors were to desensitize. Here, the authors also employed a detailed modeling approach that builds upon their previous mathematical description of the enzymatic reactions comprising the PI-cycle (Falkenburger et al., 2010; Falkenburger et al., 2013). In silico simulations of the diminishing PLC activity supported the conclusion that receptor desensitization does not explain the experimental findings related to changes in the levels of $PI(4,5)P_2$ or its hydrolytic products. Next, using pharmacological inhibitors of PI4KA and PI4KB, the authors confirmed that it is the PI4KA enzyme that is required for PI4P and $PI(4,5)P_2$ regeneration during M1R stimulation. Using their mathematical simulations of either an increased PIP5K or PI4K activities during M1R stimulation, they found that only the latter was able to faithfully mimic the experimental findings. Collectively, these data suggested that M1R activation likely leads to substantial increases in PI4KA activity.

The question then arose where in the cell does the increased PI4P generation take place? Myeong et al. (2020) measured PI4P levels both in the PM and at the Golgi complex using established biosensors and found that PM levels of PI4P were maintained at the expense of reductions to the Golgi PI4P content when cells were stimulated, and even when PI4KA was inhibited. This suggested that Golgi-derived PI4P contributed to the maintenance of PM PI4P. To directly test this possibility further, the authors used a rapamycin-inducible heterodimerization system that uses the enzymatic activity of an engineered PI4P 4-phosphatase, pseudojanin, which was originally described by Hammond et al. (2012), to acutely deplete PI4P levels specifically at the Golgi complex. These experiments showed that depletion of Golgi PI4P had a profound effect on both PI4P and $PI(4,5)P_2$ regeneration within the PM during M1R stimulation. These data were also consistent with previous reports that Golgi PI4P contributes to $PI(4,5)P_2$ maintenance during agonist stimulation (Szentpetery et al., 2010; Dickson et al., 2014). This conclusion was further supported using a refinement of the mathematical model that now included compartmental separation of the Golgi and PM pools of PI4P.

In light of these new kinetic data, two major questions remain to be addressed. The first concerns the cellular localization of PI4KA. Initial reports of the endogenous PI4KA localization suggested that this enzyme was present in the ER (Wong et al., 1997), but the resolution of the images reported in this early study did not allow for a precise localization of the protein to be determined. Later studies using expressed and epitope-tagged versions of the protein showed ER localization and a very minor presence in the PM (Balla and Balla, 2006). This contrasted the importance of the protein for the maintenance of the PM PI4P levels. Only after the discovery of the accessory proteins (EFR3, TTC7, and Fam126A) that form a tetrameric complex with PI4KA has it been shown that at least an important fraction of the enzyme is PM localized (Baird et al., 2008; Nakatsu et al., 2012). Myeong et al. (2020) now also show that overexpressed PI4KA, together with the necessary EFR3, TTC7, and Fam126A proteins, show a signal overlapping with the Golgi compartment. The second question concerns the mechanism by which PI4KA activity is enhanced by M1R stimulation. In a previous report, it was shown that PI4KA activity was stimulated by PKC, as revealed by the use of a low-dosage agonist exposure or by direct pharmacological activation of PKC (Tóth et al., 2016). Myeong et al. (2020) have also found that PI4P levels in the PM showed an increase at a low level of M1R stimulation or with PKC activation. However, in their study, these increases were not prevented by pretreatment with PI4KA inhibitors. Therefore, the mechanism by which PI4KA activity is regulated remains to be determined.

Taken together, these studies raise several important questions for the field. First and foremost, by what mechanism does Golgi-generated PI4P reach the PM? Although several members of the oxysterol binding protein family have been shown to transport PI4P, their elimination actually increased, rather than decreased the PI4P and $PI(4,5)P_2$ levels in the PM (Ghai et al., 2017; Sohn et al., 2018). This is consistent with their proposed functions related to the consumption, rather than building of PI4P gradients between the PM and the ER. Identification of such Golgi-to-PM PI4P delivery pathways, whether mediated by relatively nonspecific vesicular membrane transport or more specific nonvesicular lipid transfer pathways, will be an important step moving forward. Second, the question of how PI4KA is anchored to the Golgi complex needs further studies. Is the enzyme found with the same accessory proteins in the Golgi, or there are alternative binding partners? Related to this question is finding the molecular mechanism by which the activity of the PI4KA enzyme is regulated; either directly or within the known macromolecular assembly with TTC7, Fam126, and EFR3. Does the PI4KA enzyme itself or its associated proteins undergo phospho-regulation? Is the activity of the enzyme controlled by substrate (PI) delivery either at the Golgi or at the PM? These are exciting questions to be explored by future studies.

The concept of the PI cycle was introduced nearly 45 yr ago (Michell, 1975), but simple and important questions remain to be answered. As we gather more information about its various constituent elements and their subcellular distribution, one cannot help but be amazed by the complexity of this process. Every time we feel a new piece has been successfully added to the puzzle, more questions always appear to surface. This study by Myeong et al. (2020) is not only an important contribution to this field, but also is an inspiration for the continuation of our search for answers.

Acknowledgments

Jeanne M. Nerbonne served as editor.

The author declares no competing financial interests.

References

Antonny, B., J. Bigay, and B. Mesmin. 2018. The Oxysterol-Binding Protein Cycle: Burning Off PI(4)P to Transport Cholesterol. *Annu. Rev. Biochem.* 87:809–837. https://doi.org/10.1146/annurev-biochem-061516-044924

Audhya, A., and S.D. Emr. 2002. Stt4 PI 4-kinase localizes to the plasma membrane and functions in the Pkc1-mediated MAP kinase cascade. *Dev. Cell.* 2:593–605. https://doi.org/10.1016/S1534-5807(02)00168-5

Baird, D., C. Stefan, A. Audhya, S. Weys, and S.D. Emr. 2008. Assembly of the PtdIns 4-kinase Stt4 complex at the plasma membrane requires Ypp1 and Efr3. *J. Cell Biol.* 183:1061–1074. https://doi.org/10.1083/jcb.200804003

Balla, T. 2013. Phosphoinositides: tiny lipids with giant impact on cell regulation. *Physiol. Rev.* 93:1019–1137. https://doi.org/10.1152/physrev.00028.2012

Balla, A., and T. Balla. 2006. Phosphatidylinositol 4-kinases: old enzymes with emerging functions. *Trends Cell Biol.* 16:351–361. https://doi.org/10.1016/j.tcb.2006.05.003

Berridge, M.J. 1984. Inositol trisphosphate and diacylglycerol as second messengers. *Biochem. J.* 220:345–360. https://doi.org/10.1042/bj2200345

Bojjireddy, N., J. Botyanszki, G. Hammond, D. Creech, R. Peterson, D.C. Kemp, M. Snead, R. Brown, A. Morrison, S. Wilson, et al. 2014. Pharmacological and genetic targeting of pPI4KA reveals its important role in maintaining plasma membrane PtdIns4p and PtdIns(4,5)p2 levels. *J. Biol. Chem.* 289:6120–6132. https://doi.org/10.1074/jbc.M113.531426

Boura, E., and R. Nencka. 2015. Phosphatidylinositol 4-kinases: Function, structure, and inhibition. *Exp. Cell Res.* 337:136–145. https://doi.org/10.1016/j.yexcr.2015.03.028

Chang, C.L., and J. Liou. 2016. Homeostatic regulation of the PI(4,5)P2-Ca^{2+} signaling system at ER-PM junctions. *Biochim. Biophys. Acta.* 1861(8 Pt B, 8 Pt B):862–873. https://doi.org/10.1016/j.bbalip.2016.02.015

Dickson, E.J., J.B. Jensen, and B. Hille. 2014. Golgi and plasma membrane pools of PI(4)P contribute to plasma membrane PI(4,5)P2 and maintenance of KCNQ2/3 ion channel current. *Proc. Natl. Acad. Sci. USA.* 111:E2281–E2290. https://doi.org/10.1073/pnas.1407133111

Falkenburger, B.H., J.B. Jensen, and B. Hille. 2010. Kinetics of PIP2 metabolism and KCNQ2/3 channel regulation studied with a voltage-sensitive phosphatase in living cells. *J. Gen. Physiol.* 135:99–114. https://doi.org/10.1085/jgp.200910345

Falkenburger, B.H., E.J. Dickson, and B. Hille. 2013. Quantitative properties and receptor reserve of the DAG and PKC branch of G_q-coupled receptor signaling. *J. Gen. Physiol.* 141:537–555. https://doi.org/10.1085/jgp.201210887

Foti, M., A. Audhya, and S.D. Emr. 2001. Sac1 lipid phosphatase and Stt4 phosphatidylinositol 4-kinase regulate a pool of phosphatidylinositol 4-phosphate that functions in the control of the actin cytoskeleton and vacuole morphology. *Mol. Biol. Cell.* 12:2396–2411. https://doi.org/10.1091/mbc.12.8.2396

Ghai, R., X. Du, H. Wang, J. Dong, C. Ferguson, A.J. Brown, R.G. Parton, J.W. Wu, and H. Yang. 2017. ORP5 and ORP8 bind phosphatidylinositol-4, 5-biphosphate ($PtdIns(4,5)P_2$) and regulate its level at the plasma membrane. *Nat. Commun.* 8:757. https://doi.org/10.1038/s41467-017-00861-5

Hammond, G.R., M.J. Fischer, K.E. Anderson, J. Holdich, A. Koteci, T. Balla, and R.F. Irvine. 2012. PI4P and PI(4,5)P2 are essential but independent lipid determinants of membrane identity. *Science.* 337:727–730. https://doi.org/10.1126/science.1222483

Lees, J.A., Y. Zhang, M.S. Oh, C.M. Schauder, X. Yu, J.M. Baskin, K. Dobbs, L.D. Notarangelo, P. De Camilli, T. Walz, and K.M. Reinisch. 2017. Architecture of the human PI4KIIIα lipid kinase complex. *Proc. Natl. Acad. Sci. USA.* 114:13720–13725. https://doi.org/10.1073/pnas.1718471115

Lipp, N.F., S. Ikhlef, J. Milanini, and G. Drin. 2020. Lipid Exchangers: Cellular Functions and Mechanistic Links With Phosphoinositide Metabolism. *Front. Cell Dev. Biol.* 8:663. https://doi.org/10.3389/fcell.2020.00663

Michell, R.H. 1975. Inositol phospholipids and cell surface receptor function. *Biochim. Biophys. Acta.* 415:81–47. https://doi.org/10.1016/0304-4157(75)90017-9

Minogue, S., and M.G. Waugh. 2012. The phosphatidylinositol 4-kinases: don't call it a comeback. *Subcell. Biochem.* 58:1–24. https://doi.org/10.1007/978-94-007-3012-0_1

Myeong, J., L. de la Cruz, S.-R. Jung, J.-H. Yeon, B.-C. Suh, D.-S. Koh, and B. Hille. 2020. Phosphatidylinositol 4,5-bisphosphate is regenerated by speeding of the PI 4-kinase pathway during long PLC activation. *J. Gen. Physiol.* 152. https://doi.org/10.1085/jgp.20012627

Nakatsu, F., J.M. Baskin, J. Chung, L.B. Tanner, G. Shui, S.Y. Lee, M. Pirruccello, M. Hao, N.T. Ingolia, M.R. Wenk, and P. De Camilli. 2012. PtdIns4P synthesis by PI4KIIIα at the plasma membrane and its impact on plasma membrane identity. *J. Cell Biol.* 199:1003–1016. https://doi.org/10.1083/jcb.201206095

Pemberton, J.G., Y.J. Kim, J. Humpolickova, A. Eisenreichova, N. Sengupta, D.J. Toth, E. Boura, and T. Balla. 2020. Defining the subcellular distribution and metabolic channeling of phosphatidylinositol. *J. Cell Biol.* 219:e201906130. https://doi.org/10.1083/jcb.201906130

Sohn, M., M. Korzeniowski, J.P. Zewe, R.C. Wills, G.R.V. Hammond, J. Humpolickova, L. Vrzal, D. Chalupska, V. Veverka, G.D. Fairn, et al. 2018. $PI(4,5)P_2$ controls plasma membrane PI4P and PS levels via ORP5/8 recruitment to ER-PM contact sites. *J. Cell Biol.* 217:1797–1813. https://doi.org/10.1083/jcb.201710095

Szentpetery, Z., P. Várnai, and T. Balla. 2010. Acute manipulation of Golgi phosphoinositides to assess their importance in cellular trafficking and signaling. *Proc. Natl. Acad. Sci. USA.* 107:8225–8230. https://doi.org/10.1073/pnas.1000157107

Tóth, J.T., G. Gulyás, D.J. Tóth, A. Balla, G.R. Hammond, L. Hunyady, T. Balla, and P. Várnai. 2016. BRET-monitoring of the dynamic changes of inositol lipid pools in living cells reveals a PKC-dependent PtdIns4P increase upon EGF and M3 receptor activation. *Biochim. Biophys. Acta.* 1861:177–187. https://doi.org/10.1016/j.bbalip.2015.12.005

Wong, K., Meyers ddR, and L.C. Cantley. 1997. Subcellular locations of phosphatidylinositol 4-kinase isoforms. *J. Biol. Chem.* 272:13236–13241. https://doi.org/10.1074/jbc.272.20.13236

Zewe, J.P., A.M. Miller, S. Sangappa, R.C. Wills, B.D. Goulden, and G.R.V. Hammond. 2020. Probing the subcellular distribution of phosphatidylinositol reveals a surprising lack at the plasma membrane. *J. Cell Biol.* 219:e201906127. https://doi.org/10.1083/jcb.201906127