

# Phosphatidic acid – a simple phospholipid with multiple faces\*

Jolanta Zegarlińska, Magda Piaścik, Aleksander F. Sikorski and Aleksander Czogalla✉

Laboratory of Cytochemistry, Faculty of Biotechnology, University of Wrocław, Wrocław, Poland

**Phosphatidic acid (PA) is the simplest glycerophospholipid naturally occurring in living organisms, and even though its content among other cellular lipids is minor, it is drawing more and more attention due to its multiple biological functions. PA is a precursor for other phospholipids, acts as a lipid second messenger and, due to its structural properties, is also a modulator of membrane shape. Although much is known about interaction of PA with its effectors, the molecular mechanisms remain unresolved to a large degree. Throughout many of the well-characterized PA cellular sensors, no conserved binding domain can be recognized. Moreover, not much is known about the cellular dynamics of PA and how it is distributed among subcellular compartments. Remarkably, PA can play distinct roles within each of these compartments. For example, in the nucleus it behaves as a mitogen, influencing gene expression regulation, and in the Golgi membrane it plays a role in membrane trafficking. Here, we discuss how a biophysical experimental approach enabled PA behavior to be described in the context of a lipid bilayer and to what extent various physicochemical conditions may modulate the functional properties of this lipid. Understanding these aspects would help to unravel specific mechanisms of PA-driven membrane transformations and protein recruitment and thus would lead to a clearer picture of the biological role of PA.**

**Key words:** phosphatidic acid, protein-lipid interaction, signaling, membrane curvature, membrane model systems

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✉e-mail: [aleksander.czogalla@uwr.edu.pl](mailto:aleksander.czogalla@uwr.edu.pl)

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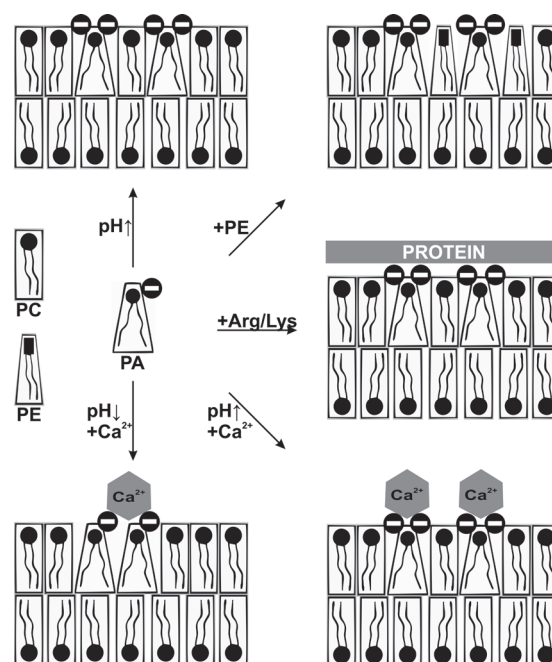
**Abbreviations:** BARS, brefeldin-A ADP-ribosylated substrate; CL, cardiolipin; COPI, coat protein I; DAG, diacylglycerol; DAGPP, diacylglycerolpyrophosphate; DGK, diacylglycerol kinase; DHAP AT, dihydroxyacetone phosphate acyltransferase; DMPA, dimyristoyl phosphatidic acid; DOPA, dioleoyl phosphatidic acid; DPPA, dipalmitoyl phosphatidic acid; ER, endoplasmic reticulum; G-3-P AT, glycerol-3-P acyltransferase; LPA, lysophosphatidic acid; LPAAT, lysophosphatidic acid acyltransferase; MitoPLD, mitochondrial PLD; PA, phosphatidic acid; PAP, phosphatidic acid phosphohydrolase; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PLD, phospholipase D; PM, plasma membrane; PS, phosphatidylserine

## INTRODUCTION

Phosphatidic acid (PA) is a minor component of biological membranes (about 1% of phospholipids) (Buckland & Wilton, 2000). Nevertheless, its presence is critical due to the multiple roles played by this lipid within the living cell. For example, PA is a central element in synthesis and turnover of glycerophospholipids and is

essential in numerous cellular functions, such as vesicular trafficking, signal transduction, cytoskeletal organization and cell proliferation (Yang & Frohman, 2012; Carman & Henry, 2013; Pleskot *et al.*, 2013). Most of these tasks are accomplished via binding of a broad range of effector proteins and thus through regulation of their functions. PA is also able to remodel membranes by changing their physicochemical properties when it is locally concentrated, which can also affect protein functionality (Yang *et al.*, 2008; Jang *et al.*, 2012).

Phosphatidic acid is the simplest membrane glycerophospholipid. However, despite its simple chemical structure, the nature of the lipid itself is much more complex. Its head group charge can be altered by various environmental components, including those that undergo spatial and temporal fluctuations within a cell (Fig. 1). Numerous PA-binding proteins of various functions and cellular localizations have been so far identified in yeast, plant and mammalian cells (Testerink & Munnik, 2005; (Stace & Ktistakis, 2006). Although the exact character



**Figure 1. Impact of different conditions on phosphatidic acid (PA) protonation state.**

In the center the molecule of PA carries one negative charge at physiological pH. Increasing pH or bilayer concentration of PE (see Fig. 3) causes deprotonation of PA (top). Proteins with basic amino acid residues (Arg, Lys) bind to PA through the hydrogen bond switch mechanism, increasing the negative charge of PA (right). Binding of calcium to PA is pH-dependent. When PA is carrying one negative charge, one molecule of calcium binds two molecules of PA. When PA carries two negative charges, it binds with calcium in a 1 to 1 ratio (bottom).



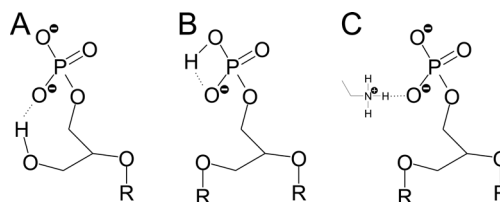
through the activity of DAG kinases (DGK), and acylation of lysoPA through the action of LPAAT. Also, for these enzymes different isoforms were characterized (ten for DGK and six for LPAAT in mammals cells) (Coleman & Lee, 2004; Shulga *et al.*, 2011; Gonzalez-Baro & Coleman, 2017). Signaling PA produced by these enzymes is involved in numerous cellular processes, e.g. membrane and vesicle trafficking, cell survival and proliferation or cytoskeletal organization.

As a result of the variety of substrates and metabolic pathways described above, different species of PA may vary in length and saturation level of their fatty acyl chains. For example, since PLDs process mostly the cellular pool of PC species, their activity preferably results in generation of PA containing one saturated (e.g. palmitoyl-) and one unsaturated (e.g. oleoyl-) acyl chain. These variations are important as they may be at least partly responsible for the differences in the PA-effector interactions, in analogy to sphingomyelin-interacting transmembrane proteins (Contreras *et al.*, 2012). Indeed, it has been shown in three different but well-characterized PA protein sensors (Spo20 and Opi1, both originating from *Saccharomyces cerevisiae*, and mammalian PDE4aA1) that each of them interacts with PA-enriched lipid bilayers exhibiting differences in acyl chain length and saturation level (Kassas *et al.*, 2017). Thus, the differences in molecular architecture between different species of PA may affect the biological activity of the lipid. Remarkably, a similar phenomenon was also described for diacylglycerols (Nadler *et al.*, 2013). These data clearly show that acyl chain composition strongly influences the lipid-protein recognition. Some hints regarding this issue come from the mTOR signaling pathways that are modulated by PA. It has been revealed that PA is required for the stabilization of two mTOR protein complexes: mTORC1 and mTORC2 (Toschi *et al.*, 2009; Foster, 2013). Active mTORC1 stimulates protein synthesis and cell growth by phosphorylation of multiple proteins, including ribosomal S6 kinase (S6K1). On the other hand, catalytically active mTORC2 phosphorylates a different set of substrates, including a well-characterized Akt kinase (Oh & Jacinto, 2011; Laplante & Sabatini, 2012). More detailed investigation of the issue showed that different PA species may not be equal in terms of mTOR activation. PA derived from the PLD pathway activates both mTOR protein complexes; however, PA produced in the glycerolipid synthesis pathway inhibits mTORC2 complex formation (Zhang *et al.*, 2012). Not only the PA derived from different enzymatic pathways, but also PA obtained by different types of PLD has different effect for activating/inhibiting the appropriate effector. Brefeldin-A ADP-ribosylated substrate (BARS) is required in one of the first steps in fission of transport vesicles formed by the COPI coatomer (coat protein I). It has been shown that induction of membrane curvature by BARS is dependent on PA (Yang *et al.*, 2008). However, only PA synthesized by PLD2, and not by PLD1, exhibited such activity. PA synthesized from DAG through DGK also did not have any influence on COPI vesicle formation. Thus, the presence of PA generated by PLD2, together with BARS, is essential for vesicle formation. It was shown that depletion of PLD2 inhibited the transformation of Golgi tubules into vesicles (Yang *et al.*, 2008). These differences may actually be related to the discrete intracellular localization of the enzymes (Fig. 2B), since PLD1 is mainly localized in intracellular membranes, such as endosomes, lysosomes, and Golgi, whereas PLD2 is found mostly in the plasma membrane (PM) (Liscovitch *et al.*, 2000; Freyberg *et al.*, 2001; Selvy

*et al.*, 2011; Kolesnikov *et al.*, 2012). Moreover, intracellular transporters, such as Ups1 in yeast, which mediates PA transport between outer and inner mitochondrial membranes, contribute to localization of PA. Ups1 assembles with Mdm35, which stabilizes the lipid transporter (Connerth *et al.*, 2012). Transfer of PA between the plasma membrane and ER is facilitated by Nir2 (Kim *et al.*, 2016). In cells with depleted Nir2, activation of PLC leads to PA accumulation in plasma membrane. It is worth mentioning that PA transported to ER serves as a precursor for PI synthesis (through CDP-DAG pathway) and then PI is transferred from ER to PM by the Nir2 protein (Kim *et al.*, 2016) (Fig. 2B). Interplay between local production of PA within membranes of various composition, fatty acyl chain configuration of the lipid and its biological activity still remains an open question.

### HEAD GROUP CHARGE AND HYDROGEN BOND FORMATION BY PA

The extraordinary nature of PA stems from its ability to change its protonation state within the physiological pH range. The electric charge of an acid can be predicted from the  $pK_a$  values of its head group and local pH changes. Thus, its electrical charge can be estimated from the Henderson-Hasselbalch (H-H) formula (Kooijman & Burger, 2009). The phosphomonoester displays two  $pK_a$  values:  $pK_{a1}$  and  $pK_{a2}$ , equal to 3.2 and 7.9 respectively. Exhibiting these two  $pK_a$  values, PA also shows the unusual ability to form intramolecular hydrogen bonds. However, these features are only relevant in the context of bilayers composed of PC. Under physiological conditions, the ionization of PA is thought to be sensitive to the molecular organization of the lipid membrane, in particular to the presence of other lipids, such as PE or cholesterol (Kooijman *et al.*, 2005a; Kooijman & Burger, 2009), and thus formation of hydrogen bonds is affected (Fig. 3). The deprotonation effect of the PA monoester head group, observed in PC:PE mixed bilayers, is caused by intermolecular hydrogen bond formation. In contrast to PC, which possesses a quaternary amine, PE has a primary amine, which makes it a much better hydrogen bond donor (Kooijman *et al.*, 2005a). Moreover, the arrangement of the PE and PC head groups in the membrane differs, the PE head groups being more parallel to the membrane surface (Langner & Kubica, 1999). This intermolecular hydrogen bond between two membrane lipids will destabilize the intramolecular hydrogen bond within the phosphomonoester head group, decreasing the second dissociation constant (to  $6.89 \pm 0.05$ )



**Figure 3. Schematic representation of hydrogen bond formation.**

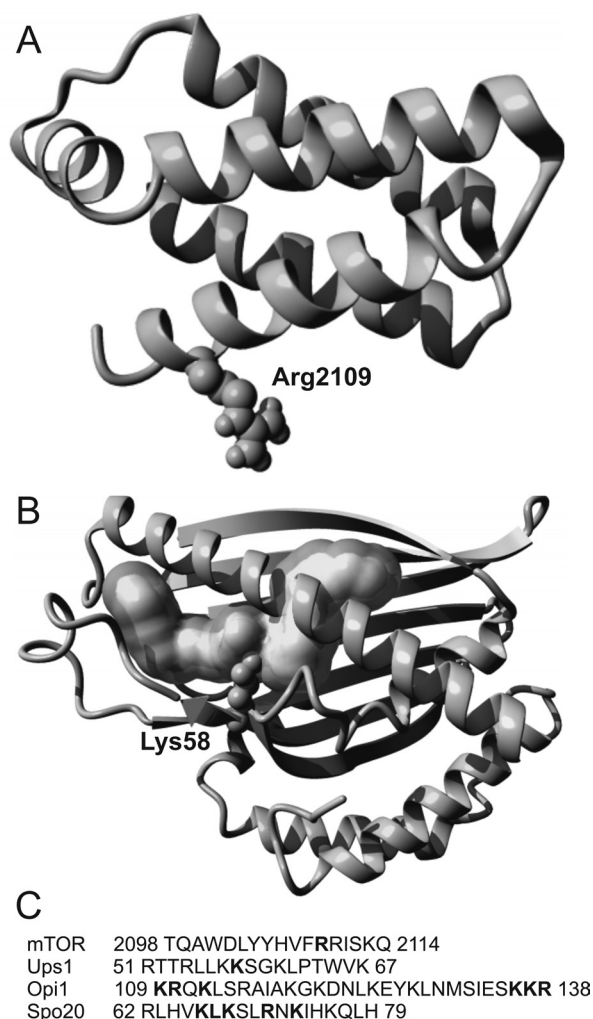
LPA (A) and PA (B) form intramolecular hydrogen bonds between deprotonated and protonated hydroxyl of their phosphomonoester head group. Moreover, LPA can create an intramolecular hydrogen bond between hydroxyl of the deprotonated phosphomonoester group and hydroxyl at the 2'-sn position of its glycerol backbone. The intermolecular hydrogen bond between PA and an amine group (C), primary amine group of PE or lysine/arginine side chains, is a hydrogen bond donor. Hydrogen bonds are represented by dotted lines.



(Kooijman *et al.*, 2005a). This issue is of high physiological importance due to the fact that in different cellular compartments (i.e. membranes of various composition) PA may interact with its effectors in different ways. For example, in the endoplasmic reticulum (ER), PA is surrounded by PC at a level over 50%, while in the inner leaflet of the plasma membrane phosphatidylserine (PS) and PE are present at much higher concentrations (Kooijman & Burger, 2009). This increased deprotonation of PA by PE was found to increase association of the Yas3p protein (an Opi1 family transcription repressor) with PA (Kobayashi *et al.*, 2013). Similar PE-assisted modulation of PA was observed while studying Spo20 (Horchani *et al.*, 2014), a protein from the SNARE family that mediates spore formation in yeasts.

Changes in composition of the water phase that surrounds the lipid bilayer are also highly influential in regard to the PA head group. PA has been described as a cellular pH-biosensor, the protonation state of which may fluctuate in response to slight changes in pH (Fig. 1). Thus, the binding of its effectors is pH-dependent. This was proposed for the transcription repressor Opi1, one of the PA-binding proteins in yeasts (Young *et al.*, 2010). The protein is sequestered at the ER membranes, where it interacts with PA. When PA is depleted or when the intracellular pH decreases, Opi1 migrates to the nucleus, where it regulates transcription of various genes, especially INO1, which is responsible for inositol synthesis (Carman & Henry, 2013). Under glucose starvation, cytoplasmic acidification occurs (pH decreases to around 6), which stimulates Opi1 release from the ER due to increased protonation of PA. Thus, the lipid mediates translation of intracellular pH fluctuations into changes in localization of the transcription repressor (Young *et al.*, 2010). Alterations of the PA molecule itself also had an influence on the binding characteristics. Methyl-PA, which has a methyl group substituted on the phosphate, has only one  $pK_a$  value. The binding specificity of Opi1 to the modified PA remains unaffected, but the binding is not that strong and is pH independent within the tested pH range (Young *et al.*, 2010). Another example of all of these phenomena is lipin-1, which binds with high specificity to PA in a pH- and membrane composition-dependent manner (Eaton *et al.*, 2013). Lipin-1 is a phosphatidic acid phosphatase, which is involved in the *de novo* synthesis of phospholipids and triacylglycerides. However, to fulfill its catalytic function, it has to translocate from the cytosol to the membrane, where it specifically binds to PA. Lipin-1 preferentially binds to di-anionic PA, meaning that such interactions occur preferably in the presence of hydrogen-bond donor molecules (PE or amphiphilic amines) and are dependent on pH (Eaton *et al.*, 2013).

Moreover, the PA head group charge can be also influenced by proteins. The number of identified PA effectors is large, but no distinguishable binding domain or structural motif responsible for interactions with this lipid has been described so far. Nevertheless, it may be concluded that PA-interacting proteins have some common features (Fig. 4). All of the best studied PA binding partners, e.g. Opi1 (Loewen *et al.*, 2004), Spo20 (Tritarelli *et al.*, 2004), and Raf-1 kinase (Ghosh *et al.*, 2003), have clusters of basic, positively charged amino acids in their PA-binding sites (Ghosh & Bell, 1997; Testerink & Munnik, 2005; Kooijman & Burger, 2009) (Fig. 4C). Thus, one would think that the interactions between PA and arginine/lysine residues would be solely electrostatic in nature, but also hydrogen bonding occurs, which altogether assures the specificity towards PA. This phenom-



**Figure 4. Features of selected proteins that interact with PA.**

(A) Ribbon diagram of FRB domain of the mTOR protein (PDB ID: 1AUE), Arg2109 directly involved in PA recognition is shown as a ball model. (B) Ribbon diagram of Ups1-Mdm35 complex, with Lys58 of Ups1 critical in PA binding shown as a ball model, and bound PA molecule shown as a surface model. (C) Examples of most broadly studied phosphatidic acid binding proteins with PA binding regions as sequence stretches, residues directly involved in PA recognition are in bold (Fang *et al.*, 2001; Loewen *et al.*, 2004; Nakanishi *et al.*, 2004; Watanabe *et al.*, 2015).

enon has been called the hydrogen bond switch model and was proposed for the first time by Kooijman (Kooijman *et al.*, 2007). Arginine and lysine residues possess amine groups in their side chain that are strong hydrogen bond donors and are prone to interact with membranes containing singly charged PA. At first, a protein probes the membrane until it finds PA and interacts with it by means of electrostatic attraction. Upon shortening the distance between the protein and the lipid, a hydrogen bond is formed and a proton dissociates from the phosphomonoester group of PA. This event switches the head group charge from  $-1$  to  $-2$  (Fig. 3C) and makes the electrostatic attraction stronger (Kooijman *et al.*, 2007). Substitution of lysines and/or arginines with alanines usually blocks protein binding to the PA-enriched membrane, proving the importance of these residues for specific binding to this lipid. Opi1 can be given as an example of such occurrence. Amino acids responsible for the Opi1-PA interaction are Lys<sub>136</sub>, Lys<sub>137</sub>, Arg<sub>138</sub> (Fig. 4C). Substitution of these residues with alanine

caused loss of pH sensitivity in the range of 6.4–7.6 (Young *et al.*, 2010). This means that only direct interactions between PA and its effector are pH dependent and confirms that presence of Arg and/or Lys residues is essential for such interactions. However, it is not necessary for the protein to have both types of residues within the binding domain. On the other hand, these two amino acid residues are not equal in terms of influencing the head group of PA, since arginine seems to be a weaker hydrogen bond donor. This difference is a consequence of the differences in the chemical structure of the side chains of both residues, and a bond formed with Arg is longer (by approx. 0.1 Å) than a bond formed by Lys (Kooijman *et al.*, 2007). Detailed analysis of the PA binding proteins shows that the presence of Arg and Lys is essential but not sufficient for stable interaction with the lipid. Development of a synthetic, tetravalent PA-binding motif, described as PAB-TP (phosphatidic acid-binding tetravalent peptide), showed that tryptophan, as well as histidine residues are required for stabilization of such interactions. This synthetic peptide contains an amino acid stretch with the sequence MARWHRHHH and is able to bind to phosphatidic acid present in lipid bilayers at a concentration as low as 1% (Ogawa *et al.*, 2015). All of these amino acid residues seem to play an important role in natural PA receptors – for example, the FRB domain of mTOR, which binds with high specificity to PA (Veverka *et al.*, 2008).

The hydrogen bond switch model also explains PA recognition via proteins over other negatively charged lipids, e.g. phosphatidylserine. This lipid also carries a negative charge and its concentration in biological membranes (particularly in the inner leaflet of the plasma membrane) is relatively high, yet PA binding partners either do not recognize PS or recognize it very weakly. The main reason for this is that, theoretically, PA protonation states vary between three values (0, –1, –2), whereas PS has only two (0 and –1). PA recognition has been summarized in the hydrogen bond switch model, where the “switch” represents the ability of PA to be present in various protonation states (Kooijman *et al.*, 2007). Modification of the PA head group by esterification effectively inhibits binding of effectors, as it makes the switching mechanism no longer possible (Ghosh & Bell, 1997). The electrostatic/hydrogen bond switch model also explains the protein recognition of PA over lysophosphatidic acid (LPA). The latter has a phosphomonoester head group, similarly to PA; however, instead of two acyl chains there is only one and a hydroxyl group at the glycerol backbone. This difference in the chemical architecture of the LPA molecule makes the hydrogen bond switch irrelevant, as the hydrogen bond formed between the hydroxyl group and phosphate head group disables hydrogen bonding with neighboring molecules (Fig. 3) (Pascher & Sundell, 1985; Kooijman *et al.*, 2005a).

## MEMBRANE CURVATURE INDUCED BY PA

PA is generally considered as a lipid that induces negative curvature in lamellar lipid structures, which facilitates membrane fusion and fission in various organelles, e.g. in mitochondria (Choi *et al.*, 2006) and formation of secretory vesicles during cellular trafficking (Huang *et al.*, 2005; Yang *et al.*, 2008). Local enrichment in PA within the membrane may be responsible for the negative curvature formation but also facilitates recruitment of a particular set of proteins to the membrane (Yang & Frohman, 2012).

In general, the shape of lipid molecules is determined by the packing parameter, and it determines the ability of lipids to form distinct lipid aggregates in an aqueous environment (Cullis *et al.*, 1986; Mouritsen, 2011). The packing parameter ( $P$ ) is equal to  $v/(al)$ , where  $v$  is the molecular volume,  $a$  is the polar head group cross sectional area, and  $l$  is the length of the molecule. For different lipids, the packing parameter may be equal to, higher or lower than one (Escribá *et al.*, 1997; Mouritsen, 2011; Jouhet, 2013), while for phosphatidic acid the value of this parameter is always higher than one. It means that under physiological conditions it has the shape of a cone, which allows loose packing of PA head groups in the membrane and hence a stronger propensity to create packing defects within the bilayer. This enables relatively easy penetration of the hydrophobic core of the bilayer by PA-recognizing proteins. Moreover, cone-shaped lipids, such as PE and PA ( $P > 1$ ), tend to induce curvature of a negative character, and create structures such as the inverted micelles or inverted hexagonal phase ( $H_{II}$ ) (Cullis & de Kruijff, 1986; Chernomordik & Kozlov, 2003; Di Paolo & De Camilli, 2006; Zimmerberg & Kozlov, 2006).

Negative curvature induced by PA can be regulated by pH, temperature and salt concentrations (Kooijman *et al.*, 2005b). Ion concentration and pH influence the actual size of the PA head group. Because the PA head group is negatively charged, its cross sectional area is apparently bigger due to the electrostatic repulsion (e.g. at higher PA concentration or when other negatively charged phospholipids are present in the membrane environment). This effect can be modulated by monovalent and divalent cations or by variations in pH which change ionization of the PA head groups (Träuble & Eibl, 1974; Cullis & de Kruijff, 1986; Pomorski *et al.*, 2014). While studying the effects of salt concentration, it was found that in pure water PA behaves as if it had a cylindrical shape. Studies at pH 7.0 and physiological salt concentration (150 mM of NaCl) proved that the PA-enriched monolayer has a negative spontaneous curvature under these conditions. Remarkably, further increase in salt concentration did not enhance this effect. Such results were obtained for di-oleoyl PA/di-oleoyl PE (DOPA/DOPE) mixed lipid monolayers, with DOPA concentration ranging from 0 to 35 mol%. It has been suggested that this effect correlates with decreased repulsion between head groups, as electrolytes reduce it, resulting in smaller effective size of the lipid head groups (Kooijman *et al.*, 2005b). In another set of experiments, a supported lipid bilayer made of DOPC/DOPA/NBD-PC was examined in buffers containing 250 mM KCl (50 mM MES, 0.1 mM EDTA, pH 5) (Cambrea & Hovis, 2007). When the ionic strength of the buffer above the supported bilayer (distally to the support) was lowered (50 mM KCl), the bilayer tended to bend away from the glass slide, and this effect was observed only for a narrow range of PA concentrations (11–20 mol%). When PA was exchanged for PG, which is also a negatively charged lipid, the bilayer did not bend away from the support. This shows that besides specific ionic conditions, a particular concentration of PA in the bilayer is also essential for curvature generation (Cambrea & Hovis, 2007).

Not only monovalent ions influence the ability of PA to generate curvature. Several studies have shown that the negative curvature generated by PA is more pronounced at low pH and in the presence of divalent cations (Verkleij *et al.*, 1982; Farren *et al.*, 1983; Kooijman *et al.*, 2003). Early investigation of DOPA aggregation at

different pH values showed that the lipid at pH higher than or equal to 6.0 prefers lamellar organization (Farren *et al.*, 1983). Much attention was also given to investigate the influence of divalent ions on structural and thermotropic properties of PA (van Dijck *et al.*, 1978; Farren *et al.*, 1983; Kouaouci *et al.*, 1985). The requirement of calcium ions for the formation of non-lamellar structures by this lipid may be related to the fact that these ions usually play an important role in endo- and exocytosis (Yao *et al.*, 2009; Yamashita *et al.*, 2010). Introducing  $\text{Ca}^{2+}$  or  $\text{Mg}^{2+}$  ( $\text{Me}^{2+}/\text{DOPA}$  molar ratio = 0.5) at pH up to 6.0 caused formation of structures in the  $\text{H}_{\text{II}}$  phase (Farren *et al.*, 1983). Increasing the level of divalent cations ( $\text{Me}^{2+}/\text{DOPA}$  molar ratio = 1.0) induced disorganization of these structures, and formation of an intermediate between the lamellar and  $\text{H}_{\text{II}}$  phases (Farren *et al.*, 1983). At high pH (~11) calcium binds PA in the molar ratio of 1:1, whereas at physiological and acidic pH calcium binds to PA in the molar ratio of 1:2 (Fig. 1) (Boughriet *et al.*, 1988; Takahashi *et al.*, 1995; Kooijman *et al.*, 2003). This effect of  $\text{Ca}^{2+}$  binding seems to be strongly reliant on the PA head group charge, which arises from its  $\text{pK}_{\text{a2}}$ . Other studies using differential scanning calorimetry and X-ray diffraction to analyze aggregates composed of two saturated PA species, di-myristoyl PA (DMPA) and di-palmitoyl PA (DPPA), showed that when calcium ions were present (at physiological pH), no gel-to-liquid crystalline phase transition was observed within temperature ranging from 10°C to 80°C (van Dijck *et al.*, 1978; Liao & Prestegard, 1981). On the other hand, each of the two factors (divalent ions and pH fluctuations) separately did not affect the curvature of membranes containing PA, but taking together high divalent cation concentrations and low pH drives PA to such a behavior. Similar results were obtained using  $^{31}\text{P}$ -NMR (Kooijman *et al.*, 2003). When single-lipid systems composed of DOPA were studied at neutral pH, no negative curvature was observed, even in the presence of  $\text{Ca}^{2+}$  ( $\text{Me}^{2+}/\text{DOPA}$  molar ratio = 1.0). Remarkably, doubling the concentration of  $\text{Ca}^{2+}$  or  $\text{Mg}^{2+}$  or changing the pH to 5.0 (in the absence of divalent cations) gave the same results. In conclusion, in a single lipid bilayer system, DOPA forms a lamellar phase, which is to a large extent independent of pH or divalent ion concentration. However, when alternative conditions were examined (pH 5 and  $\text{Ca}^{2+}/\text{DOPA}$  molar ratio = 0.2 and higher), it caused the local phase transition to the  $\text{H}_{\text{II}}$  phase. These observations may be related to cellular physiology, as each subcellular compartment is unique in terms of pH value and ion concentrations. Examination of the DOPA bilayer behavior under conditions resembling the Golgi lumen (0.3 mM  $\text{Ca}^{2+}$ , pH range from 6.0 to 7.2) showed that below pH 6.8 the bilayer presented a mixed lamellar/  $\text{H}_{\text{II}}$  character, with a 5% and 60%  $\text{H}_{\text{II}}$  bilayer at pH 6.4 and 6.0, respectively. Thus, the conditions in the Golgi lumen favor the  $\text{H}_{\text{II}}$  phase of DOPA (Kooijman *et al.*, 2003). Moreover, the effect of calcium and pH was assessed in mixed-lipid systems (PE with 10% PA) (Kooijman *et al.*, 2003). The results confirmed that in the presence of divalent cations and in a slightly acidic environment PA behaves like a cone-shape lipid. Hence, one can assume that the bilayer-to- $\text{H}_{\text{II}}$  phase transitions (for PA-rich membranes) would be more likely in an intra-Golgi environment, rather than under cytosolic conditions.

Miscibility of lipids can vary in respect to several aspects (Carruthers & Melchior, 1983; Vaz, 1994), which may result in the increase of local concentration of PA, and thus may induce negative curvature and at-

tract PA-binding proteins. The mixing behavior of PA/PC mixtures has been investigated using multiple experimental methods, such as Langmuir monolayers, differential scanning calorimetry (DSC) and molecular dynamics (MD) simulations (Garidel *et al.*, 1997a; Garidel *et al.*, 1997b; Estrela-Lopis *et al.*, 2004; Vila-Vicosa *et al.*, 2014; Kwolek *et al.*, 2015; Santos *et al.*, 2015). Miscibility of PA/PC species of the same (e.g. DPPA/DPPC or DMPA/DMPC) or different chain lengths (e.g. DPPA/DMPC) depends on pH, as the latter affects ionization of PA. Moreover, miscibility depends on the PA concentration. At neutral pH, PA carries one negative charge per molecule; therefore head group repulsion between PA molecules and hydrogen bonds between PA and PC favors mixing of these lipids. When pH drops to acidic (4.0), hydrogen bonds between PA molecules become more preferred, which results in clustering (Garidel *et al.*, 1997a; Vila-Vicosa *et al.*, 2014; Santos *et al.*, 2015). Non-ideal mixing behavior was also observed with increasing PA content in the mixture (Estrela-Lopis *et al.*, 2004; Kwolek *et al.*, 2015). For example, phase separation for DPPA-rich domains and DPPC-rich domains could be observed in mixed monolayers containing 12 mol% of PA (Estrela-Lopis *et al.*, 2004). Furthermore, results from MD simulations are in correlation with the “umbrella model” (Huang & Feigenson, 1999), according to which PA molecules bearing one negative charge are partially shielded by large head groups of PC. In these simulations PA molecules were located about 0.2–0.4 nm deeper in the bilayer than PC. In the case of di-anionic PA, ionic bridges were formed with sodium ions, leading to clustering of PA (Broemstrup & Reuter, 2010; Kwolek *et al.*, 2015). All of these observations indicate that lipid packing and miscibility are sensitive to pH. Presence of PE in the membrane at slightly acidic pH triggers the PA-induced negative curvature. On the other hand, inclusion of diacylglycerol (DAG), which is also a lipid that prefers formation of  $\text{H}_{\text{II}}$  phases, decreases protein binding to PA (Putta *et al.*, 2016). Moreover, lipids which support positive membrane curvature seem to significantly lower the protein binding to PA. A gradual increase (5–15 mol%) of lysophosphatidylcholine (LPC) in the membrane lowered Opi1 binding to PA, whereas LPC at higher concentrations (25 mol%) completely inhibited this binding. A similar effect of decreased PA binding was also observed with two other proteins, which are defined as PA-binding partners: ECA1 and the PH2 domain of PDK1 protein, which suggests that this effect is more common (Putta *et al.*, 2016).

## CONCLUDING REMARKS

The unique physicochemical properties of PA, which are manifested by modulatory effects on the lipid bilayer and specific recruitment of proteins, allow it to play multiple specific roles in cellular physiology. For example, PA is essential for membrane sculpting processes and numerous signaling pathways. Discrete fluctuations in the environment of PA molecules can affect their protonation state, which drastically changes their interactions with protein partners. Although much is known about the nature of PA, its full characteristics are still unknown. Major questions concern the exact mechanisms of binding with its protein effectors and whether there are some common features of PA binding domains that distinguish them from other lipid-binding domains. Another intriguing question is how divalent ions affect protein binding to PA and whether this mechanism is



common for all of these ions. Namely, are these effects related to signaling processes that involve PA and where  $\text{Ca}^{2+}$  ions usually play a pivotal role, contrary to e.g.  $\text{Mg}^{2+}$  ions? These and many other issues still await exploration, making PA one of the most interesting membrane lipids.

### Conflict of interest statement

The Authors declare no conflict of interest.

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