FEBS Letters

FEBS PRESS science publishing by scientists

REVIEW ARTICLE

A central role for phosphatidic acid as a lipid mediator of regulated exocytosis in apicomplexa

Hayley E. Bullen[1] and Dominique Soldati-Favre[2]

1 Burnet Institute, Melbourne, Australia
2 Department of Microbiology and Molecular Medicine, CMU, University of Geneva, Switzerland

Correspondence
H. E. Bullen, Burnet Institute, 85 Commercial Road, Melbourne, Vic. 3004, Australia
Fax: +613 9282 2126
Tel: +613 9282 2226
E-mail: hayley.bullen@burnet.edu.au

(Received 20 June 2016, revised 10 July 2016, accepted 11 July 2016, available online 26 July 2016)

doi:10.1002/1873-3468.12296

Edited by Wilhelm Just

Lipids are commonly known for the structural roles they play, however, the specific contribution of different lipid classes to wide-ranging signalling pathways is progressively being unravelled. Signalling lipids and their associated effector proteins are emerging as significant contributors to a vast array of effector functions within cells, including essential processes such as membrane fusion and vesicle exocytosis. Many phospholipids have signalling capacity, however, this review will focus on phosphatidic acid (PA) and the enzymes implicated in its production from diacylglycerol (DAG) and phosphatidylcholine (PC): DGK and PLD respectively. PA is a negatively charged, cone-shaped lipid identified as a key mediator in specific membrane fusion and vesicle exocytosis events in a variety of mammalian cells, and has recently been implicated in specialised secretory organelle exocytosis in apicomplexan parasites. This review summarises the recent work implicating a role for PA regulation in exocytosis in various cell types. We will discuss how these signalling events are linked to pathogenesis in the phylum Apicomplexa.

Keywords: apicomplexa; exocytosis; lipid signalling; phosphatidic acid

Over the past 15 years, research has unravelled that different phospholipids are involved in numerous cellular functions. For instance phosphoinositides (PIs) contribute to a much broader range of biological processes than originally anticipated such as vesicular trafficking, endocytosis and exocytosis, modulation of lipid distribution and metabolism, and regulation of ion channels [1]. Structurally, PIs are amphiphilic molecules containing a glycerol backbone, two nonpolar fatty acid tails and phosphate groups substituted with inositol head groups. Differential phosphorylation of the inositol head-group generates different PIs carrying from one to three negative charges. Phospholipids can be further classified based on their occupation of space within membrane bilayers; cylinders (e.g. phosphatidylcholine, PC), cones (phosphatidic acid, PA) and inverted cones (lysophosphatidylcholine, LPC) [2]. While PIs play diverse roles within cells, this review will focus specifically on the key contribution of PA during regulated exocytosis.

Regulated exocytosis refers to the process of membrane fusion between specialised vesicles (including secretory granules) and the plasma membrane (PM) in response to extrinsic stimuli and elevated cytosolic calcium levels [3]. In mammalian cells, exocytosis is involved in a broad array of events from neuronal synapse signalling to insulin granule release [3]. In

Abbreviations
ANCAs, anti-neutrophil cytoplasmic antibodies; CDPK, calcium-dependant protein kinase; DGK, diacylglycerol kinase; GC, guanylate cyclase; LPAAT, lysophosphatidic acid acyl transferase; LPC, lysophosphatidylcholine; NSF, *N*-ethylmaleimide-sensitive factor; PA, phosphatidic acid; PDEs, phosphodiesterases; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PH, pleckstrin-homology; PI-PLC, phosphoinositide-phospholipase C; PIs, phosphoinositides; PKG, protein kinase G; PLD, phospholipase D; PM, plasma membrane; PX, phox-homology.

Apicomplexan parasites, such as *Toxoplasma gondii* and *Plasmodium* spp., the specialised organelles termed micronemes release their contents including adhesins, perforins and proteases in a regulated manner, critically contributing to invasion and egress from infected cells [4]. During regulated exocytosis, secretory vesicles dock to the plasma membrane via the use of specific machinery termed SNAREs (soluble *N*-ethylmaleimide-sensitive factor (NSF) attachment protein receptors) present on both the vesicles (v-SNAREs) and the target membrane (t-SNAREs). Interaction of these complexes pulls the membranes into close enough apposition to enable membrane fusion and subsequent vesicle exocytosis [3]. Multiple PIs are involved in this process, however, PA is currently emerging as another important signalling lipid during exocytosis across a wide range of cell types [3,5].

Phosphatidic acid can be generated through the activities of either phospholipase D (PLD), diacylglycerol kinase (DGK) or lysophosphatidic acid acyl transferase (LPAAT). While LPAAT has been shown to have some activity in PA generation for synaptic vesicle trafficking in ribbon synapses [6], LPAAT-derived PA is most commonly utilised for structural and metabolic processes and thus will not be discussed further here. PLD hydrolyses structural phospholipids such as PC and phosphatidylethanolamine (PE) to form PA and has been associated with plant stress responses [7] and mammalian exocytotic events [8–10]. Conversely, DGK phosphorylates phosphoinositide-phospholipase C (PI-PLC)-derived diacylglycerol (DAG) to form PA, which critically contributes to microneme exocytosis in apicomplexan parasites [11] and degranulation of neutrophils and mast cells [12–14] (Fig. 1). While the signalling cascades culminating in PA generation are important for PA-associated exocytosis, the biophysical properties of PA also underpin its ability to participate in this process. Specifically, PA is conical in shape within the membrane and thus packs poorly in planar bilayers, consequently generating areas of negative curvature that are preferred by target membranes involved in fusion/fission events [3,15]. The roles played by PA within this process are thus rather complex. This review covers the recent findings on the implications of PA in regulated exocytosis related to pathogenesis, the players in PA production and the characterisation of sensors detecting PA in the cells.

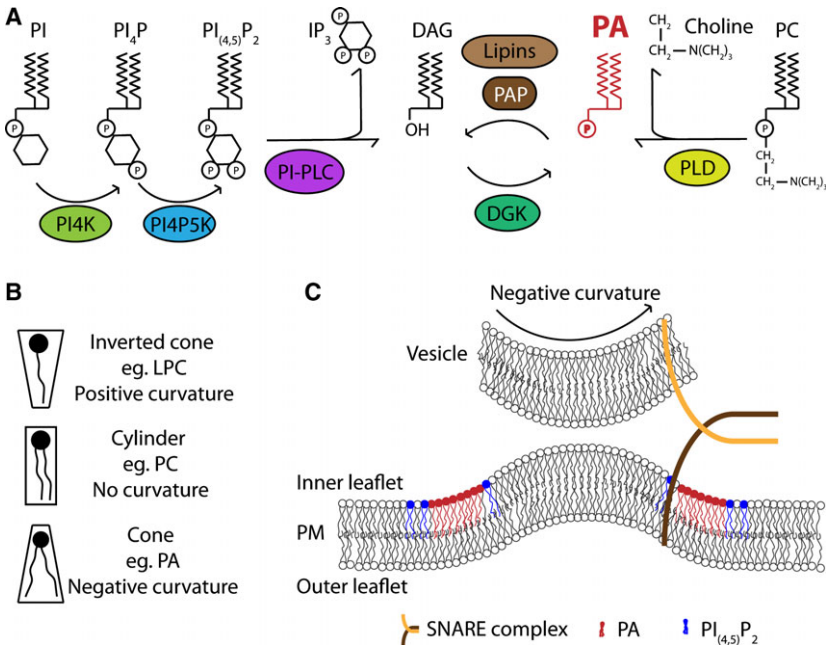


Fig. 1. Phosphatidic acid generation and its role in modulating negative membrane curvature. (A) The DGK and PLD pathways involved in the bulk of PA production from PI and PC for membrane signalling purposes. DAG, diacylglycerol; PA, phosphatidic acid; PC, phosphatidylcholine; PI-PLC, phosphoinositide-phopsholipase C; PAP, phosphatidic acid phosphatase; DGK, diacylglycerol kinase; PLD, phospholipase D. (B) Phospholipid conformation alters the shape of lipid bilayers, inducing either no curvature, positive curvature or negative curvature of the leaflet. LPC, lysophosphatidylcholine. (C) Schematic of basic vesicle fusion events involving SNARE complexes. PA (red) induces negative membrane curvature while lipid microdomains enriched in PA and PI(4,5)P2 are thought to aid in the clustering of docking/fusion machinery and may also regulate SNARE complex assembly. Adapted from [2,3].

Production of PA for membrane signalling

Phospholipase D

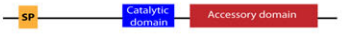
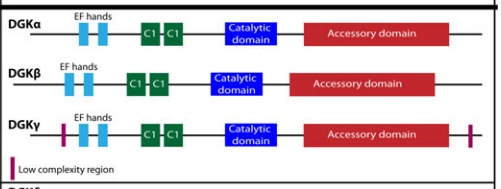
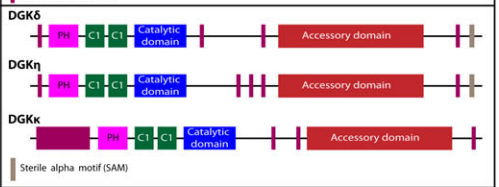
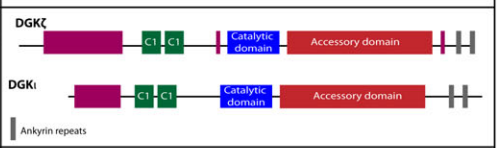
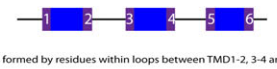
Phospholipase D was first discovered in plants [16] and in the years since, extensive research has better defined its activity in a variety of cell types. As mentioned previously, PA can be generated via multiple sources, however, historically it is PLD which has shown the strongest links to regulated exocytosis [3]. In cells in which PLDs are present, generally multiple isoforms can be found, each distinguishable by the different domain configurations they display. These differential configurations determine their activity, localisation and roles within various cellular processes [17]. PLDs most commonly contain N-terminal C2 domains, which serve to regulate PLD activity through calcium binding [17,18] and bias the PLD to utilise PC, PE and phosphatidylglycerol (PG) as substrates for PA production [19]. PLD isoforms that do not contain a C2 domain are regulated by pleckstrin-homology (PH) and phox-homology (PX) domains [17,18], which serve to determine PI-binding specificity. Within mammalian cells, deletion studies have suggested that the PX domain is critical for PLD activity while the PH domain is dispensable [20,21], however, the essentiality of these domains in plant cells is yet to be determined. In addition to these domains, all eukaryotic PLDs identified to date also contain two duplicated HKD motifs (*HxKxxxD/E*), which are known to form the active site of the PLD and thus are critical for PLD activity [17]. These domains act in concert to produce PA and thus elicit specific downstream signalling events.

Studies investigating the activity of PLD have commonly used the primary alcohol 1-butanol [22]. PLD is a transphosphatidylase that can use short-chain primary alcohols such as 1-butanol to generate phosphatidylalcohol products. These products are not normally found in biological membranes and are relatively inert and stable, thus stimulating their formation by addition of small amounts of primary alcohols is commonly used to measure PLD activity. Addition of higher levels of 1-butanol, however, diverts PLD activity away from PA production and therefore acts as a PLD inhibitor [23]. While this is a useful tool *in vitro,* the association of PLD activity with cancer metastasis has prompted the development of inhibitors acting specifically on PLD isoforms, which can be used for *in vivo* studies, and potentially be developed as novel therapeutics. Numerous naturally occurring compounds and proteins have been identified which inhibit PLD activity, including but not limited to Fodrin (the nonerythroid form of spectrin) [24], synaptojanin (a PI (4,5)P2 5-phosphatase) [25] and ceramide [8,26], however, there are issues surrounding the PLD specificity of these compounds and thus their therapeutic efficacy is limited. Small molecule inhibitor screens have, however, identified more specific compounds such as halopemide and its derivatives, most notable of which is FIPI (5-Fluoro-2-indolyl des-chlorohalopemide) [27]. Unlike halopemide which is a specific PLD2 inhibitor [27], FIPI shows activity against both PLD1 and PLD2 [27,28] and therefore may be a useful therapeutic tool. The bioavailability of these compounds is, however, limited thus there has been further work to identify novel compounds and halopemide derivatives more efficacious for therapeutic intervention [29–31].

Diacylglycerol kinase

While PLDs are well known to be associated with PA production for exocytosis, more recently, the DGKs are proving to play significant roles in both PA and DAG signalling underpinning exocytosis [32]. DGKs are widely evolutionarily conserved and their roles in PA production have been studied in bacteria [33], plants [34], multicellular organisms including drosophila [35], *Caenorhabditis elegans* [36] and apicomplexan parasites [11]. It is, however, the mammalian DGK enzymes that are the best characterised to date with 10 known isoforms [32]. These isoforms have been identified through differences in their regulatory and accessory domains and have established tissue and substrate specificity within mammalian cells as outlined in Table 1. The DGK catalytic domain does, however, remain evolutionarily conserved [32]. There is a wide repertoire of DGK accessory domains and based on the configuration of these domains, mammalian DGKs have been divided into five major types: I–V [32]. DGK accessory domains include: C1-type domains believed to be responsible for DAG binding (DGKε, θ), EF-hands involved in calcium binding (DGKα, β and γ), PDZ domains for nuclear localisation (DGK ζ and ι), PH domains for PI binding (DGK δ, η and κ), ankyrin repeats (DGK ζ and ι), and ras association (RA) domains (DGKθ) [32]. Plant DGKs have diverged into three clusters with cluster I resembling mammalian type III (DGKε), while the remaining plant DGKs in clusters two and three only contain kinase domains and are thus much smaller than other mammalian DGK isoforms [32]. Apicomplexan DGKs cannot be readily grouped with any of the mammalian DGKs, however, as with the plant DGKS, based on the presence of two N-terminal C1

Table 1. Classification, domain architecture and roles of DGKs and PAPs in *Homo sapiens* and the Apicomplexa. Adapted from [11,32,91–94].

		Protein	Gene ID	Characteristics	Domain structure
Apicomplexan	DGKs	DGK1	TGME49_202460 (*Toxoplasma gondii*) NCLIV_022470 (*Neospora caninum*) PF14_0681 (*Plasmodium falciparum*) PBANKA_133460 (*Plasmodium berghei*) PCHAS_133920 (*Plasmodium chabaudi*) PKH_123390 (*Plasmodium knowlesi*) PVX_116900 (*Plasmodium vivax*) XP_001611607.1 (*Babesia bovis*) cgd4_4340 (*Cryptosporidium parvum*)	Involved in PA production at the *T. gondii* plasma membrane for microneme secretion. Inducible knock-down indicates it is also required for additional lipid signalling and its down regulation results in parasite death.	
		DGK2	TGME49_259830 (*Toxoplasma gondii*) NCLIV_027060 (*Neospora canninum*)	Localises to the dense granules and parasitophorous vacuole. Unknown role.	
		DGK3	TGME49_239250 (*Toxoplasma gondii*) NCLIV_015910 (*Neospora canninum*) PFI1485c (*Plasmodium falciparum*) PBANKA_0831200 (*Plasmodium berghei*) PCHAS_0831500 (*Plasmodium chabaudi*) PKH_0729100 (*Plasmodium knowlesi*) PVX_099990 (*Plasmodium vivax*) cgd3_2630 (*Cryptosporidium parvum*)	Localises to the micronemes. Unknown role.	
	PAP	PAP1/Lipin	TGGT1_230690 (*Toxoplasma gondii*) NCLIV_031180 (*Neospora caninum*) PF3D7_0303200 (*Plasmodium falciparum*) PBANKA_040180 (*Plasmodium berghei*) PCHAS_040270 (*Plasmodium chabaudi*) PKH_083700 (*Plasmodium knowlesi*) PVX_119285 (*Plasmodium vivax*)	Soluble protein located in the cytosol of *T. gondii*. Undentified function but likely converts PA to DAG as a component of lipid metabolism.	
		PAP2	TGGT1_247360 (*Toxoplasma gondii*) NCLIV_063870 (*Neospora caninum*) SN3_01700035 (*Sarcocystis neurona*) HHA_247360 (*Hammondia hammondi*)	Six-pass transmembrane protein. Most likely to perform the function of converting PA to DAG at parasite membranes.	
Homo sapien	DGKs	Type I	α: P23743.3 β: Q9Y6T7.2 γ: P49619.3	Upon activation have been shown to rapidly translocate from the cytosol to the PM and are involved in vesicle exocytosis.	
		Type II	δ: Q16760.4 η: Q86XP1.1 κ: Q5KSL6.1	Contain a PH domain and a SAM domain that can bind to the ER and affect anterograde transport.	
		Type III	ε: P52429.1	Has a unique substrate specificity for arachidonate-DAG making it the most important DGK for PI re-synthesis.	
		Type IV	ζ: Q13574.3 ι: O75912.1	DGKζ interacts with sorting nexin 27 (SNX27) and is required for its correct localisation to endosomes.	
		Type V	θ: P52824.2	Potentially involved in neurotransmission however its exact role remains incompletely understood.	
	PAP	Type I	PAP1/Lipin: Q14693.2	Not membrane associated. Mg2+ dependent, involved in lipid metabolism. Translocates from the cytosol to microsomes during triacylglycerol synthesis.	
		Type II	PAP2a: AB000888 PAP2b: AB000889 PAP2c: AF056083	Contain 6 transmembrane domains and are Mg^{2+} independent. PAP2a/b are most active towards PA. PAP2c is most active towards LPA. PAP2 activity is believed to be involved in signal transduction.	

domains, apicomplexan DGKs are most similar to mammalian type III DGK (DGKε) [11].

There are currently two main chemical compounds available to investigate the temporal effects of DGK inhibition *in vitro* and these include R59022 [37] which has recently been shown to inhibit microneme secretion in *T. gondii*, and R59949 [38] which has been used to investigate the temporal role of DGK upregulation in cancer cells [39]. These compounds are selective for different isotypes and recent work has determined that R59022 most strongly targets DGK isotypes I/III and V, while R59949 targets preferentially isotypes I and II [40].

PA and regulated exocytosis

Regulated exocytosis occurs at specific sites within cells and formation of zones competent for exocytosis requires a plethora of signalling pathways culminating in the precisely localised and timed release of secretory granules. Here, we will focus specifically on the roles played by PA in this process and the effects it has on signalling events, recruitment of specific effector proteins and membrane curvature. Mechanistically, efficient vesicle fusion requires both v- and t-SNAREs, which when in a close enough proximity form a trans-SNARE complex to promote fusion of the two membranes [41]. Formation of this complex requires that the outer membrane leaflet maintains a negative curvature while the inner membrane leaflet maintains a positive curvature [3,42] (Fig. 1). Through X-ray diffraction studies, PA has been shown to produce negative membrane curvature [15] and thus is often present in the target plasma membrane [42] during regulated exocytosis. Furthermore, PA has also been shown to modulate intracellular calcium levels [14], which in turn regulates SNARE fusion by binding the calcium-sensor protein synaptotagmin [43]. These membrane and signalling effects of local and timely PA accumulation can be mechanistically and chemically grouped into three main categories to define the involvement of PA in regulated exocytosis: (a) PA can recruit proteins involved in vesicle fusion or priming events (such as SNAREs) to facilitate efficient vesicle exocytosis [44–46], (b) PA can stimulate the production of other PIs such as PI(4,5)P2, which is also involved in mediating exocytosis through both generating areas competent for SNARE complex formation and also for further signalling processes surrounding efficient exocytosis [3,47], (c) PA forms a cone shape in lipid bilayers and thus generates regions physically competent for fusion machinery functioning by promoting regions of negative curvature [15,48].

Given the complexity of the roles PA plays in exocytosis, the following section focuses predominantly on exocytosis in the context of PA affecting membrane curvature in a variety of different cell types.

PA in neurotransmission

Proper brain functioning necessitates effective communication between neurons, a process modulated by calcium flux and the subsequent fusion of synaptic vesicles with the plasma membrane at the synaptic cleft. The ensuing release of neurotransmitters into this cleft facilitates rapid neuronal communication. While this process is complex, a variety of studies using chromaffin cells or Aplysia neurons have implicated PLD1 as playing a significant role in producing the PA required for efficient and localised exocytosis of secretory granules or synaptic vesicles [8–10]. Specifically, studies have demonstrated that plasma membrane PA derived from PLD1 activity is a prerequisite for normal exocytotic functioning [9]. PA elicits these effects through generating a fusion competent site for SNARE complex formation, while also altering the biophysical properties of the target membrane to one maintaining negative curvature and thus promoting vesicle fusion [9,10]. Given the nature of synaptic vesicle exocytosis, recycling of the membranes via endocytosis is also a requirement of efficient and prolonged neuronal messaging. To this end, recent studies have linked DGKθ to a role in synaptic vesicle recycling through promoting endocytosis at these sites [49], underlining again the importance of PA in vesicle fusion/fission processes.

PA in immune functioning: neutrophil and mast cell exocytosis

The exocytotic release of azurophilic granules from neutrophils is widely studied, and their dis-regulated release is associated with increased tissue damage and inflammation during autoimmune disease [12]. Of specific focus has been the effect of anti-neutrophil cytoplasmic antibodies (ANCAs) on neutrophil exocytosis as they have been implicated in various pathologies [12]. Studies have revealed that both PA and calcium are required for ANCA-stimulated neutrophil exocytosis [12]. In this instance, PA likely induces negative membrane curvature to promote SNARE complex formation and subsequent vesicle fusion, while also serving to modulate intracellular calcium levels which promote numerous other steps in the signalling pathways leading to vesicle exocytosis [12,50]. It is known that DGK rather than PLD is the key enzyme responsible for PA production involved in this cascade within neutrophils [12,13]. Similarly, recent studies have revealed that DGKγ rather

than PLD regulates mast cell degranulation through the production of PA, however, in these studies PA has only been implicated in the modulation of calcium levels leading to exocytosis rather than having any specific effect on membrane curvature [14].

PA in acrosomal exocytosis

The acrosome is a secretory granule present in the head of sperm and its secretion is essential for physiological fertilisation. Unlike other forms of exocytosis such as synaptic vesicle fusion [49], there is no recycling of membranes and the acrosome is secreted only once [51]. Despite this difference, the mechanisms underpinning regulated acrosomal exocytosis are homologous to those involved in other forms of mammalian exocytosis. Acrosomal exocytosis has been intensively studied and thus the signalling pathway culminating in plasma membrane fusion has been relatively well defined. Specifically, upon sperm activation, there is an increase in cytoplasmic calcium, which in turn activates an ARF6 signalling cascade [52] that culminates in the assembly of SNARE complexes and subsequent membrane fusion and acrosome release [51]. Within this cascade, ARF6 promotes PLD activity and thus PA accumulation in the plasma membrane. The presence of PA is predicted to enhance membrane curvature of the inner leaflet of the plasma membrane, while also promoting formation of PI(4,5)P2 for SNARE complex association [52–54] (Fig. 1).

PA sensors

The list of proteins that bind PA is ever increasing and the roles played by these proteins are diverse [55]. Difficulties arise in predicting PA-binding proteins due to the fact that PA effectors generally do not display well-defined lipid-recognition domains, and instead utilise either positively charged and/or surface-exposed hydrophobic residues [55]. This is not always the case, however, and several mammalian and apicomplexan parasite proteins have been found to bind PA through PH domains ([11,56], D. Jacot, N. Tosetti, I. Pires, J. Stock, A. Graindorge, Y-F. Hung, R. Tewari, I. Kursula & D. Soldati-Favre, unpublished results). Given the number of PA-binding proteins currently identified, here we will focus on a few key examples in mammalian cells and yeast.

Mammalian: Raf1 kinase

Raf-1 kinase is an essential serine threonine kinase within the MAPK cascade first shown to bind PA in canine kidney cells [57]. To participate in the MAPK cascade, recruitment to the correct subcellular location is necessary and studies have shown that this membrane recruitment is dependent on its association with PA [58] via a stretch of amino acids high in both positively charged and hydrophobic residues. While PA serves to direct Raf1 kinase to the correct location during the signalling cascade, it is not believed to be involved in the activation of the kinase and instead is thought to enable its correct localisation for activation by Ras and subsequent propagation of the MAPK cascade [59].

Sos

Also within the Ras signalling pathway is Sos; guanine nucleotide exchange factor Son of sevenless. Following recruitment to the plasma membrane, Sos promotes the conversion of Ras-GDP to its active form Ras-GTP as part of the Ras signalling cascade. The critical determinant in this membrane recruitment is believed to be the Sos PH domain, which has been found to have specificity for both PI(4,5)P2 [60,61] and PA [56]. Despite this dual specificity, it has been demonstrated that PA, not PI(4,5)P2 binding, is necessary for Sos recruitment to the plasma membrane for Ras activation [56].

PI4P5K

PI4P5K acts to phosphorylate PI4P to generate PI(4,5)P2 which is a substrate for PI-PLC and thus facilitates the generation of the key signalling intermediates DAG and IP3 (Fig. 2). These intermediates are used in a plethora of signalling events and thus their controlled production is essential. There are various types of PI4P5K and type I has been shown to be specifically regulated by PA [62]. Accordingly, the enzymatic activity of this enzyme increases by up to 20-fold in the presence of PA [63]. This is in contrast to the other PA binders outlined here as their activity has not been shown to increase as a direct result of PA binding. That said, the precise PA binding site of PI4P5K is yet to be identified and thus further investigation into the regulatory role of PA is required.

Yeast Spo20p

Spo20p is a member of the SNAP25 family of SNAREs, serving to mediate vesicle fusion at the prospore membrane during sporulation [64]. The PA-binding domain of Spo20p (amino acids 51–91) is responsible for ensuring the correct localisation of

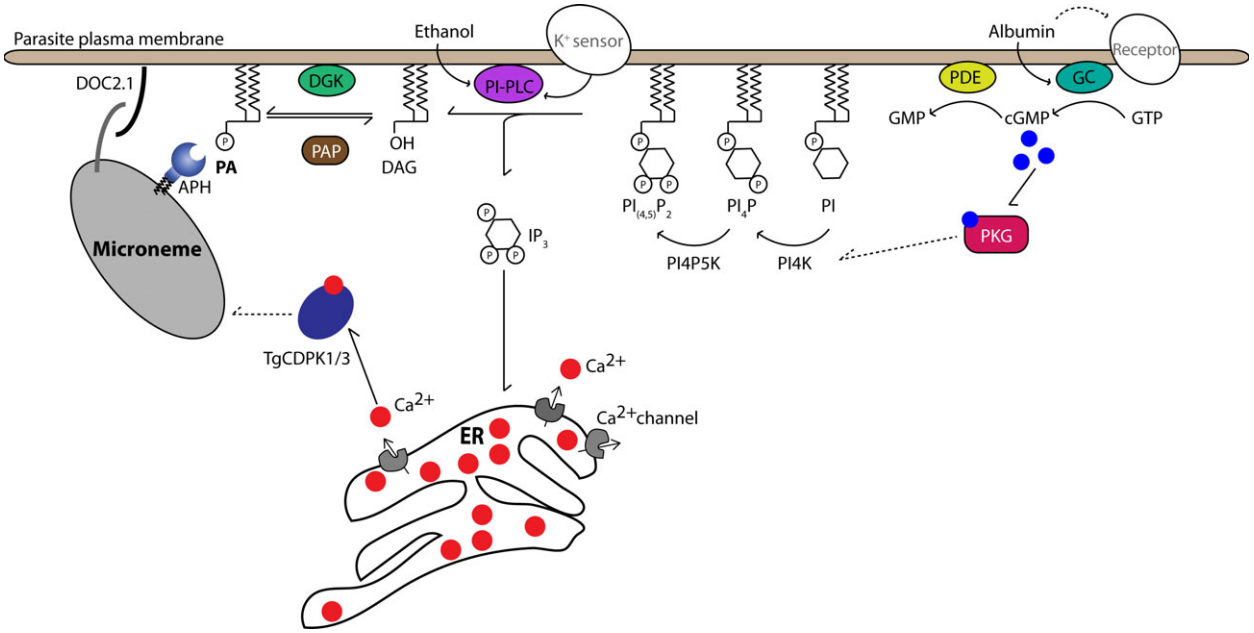


Fig. 2. The involvement of PA in microneme secretion events underpinning parasitism in Apicomplexa. Stimulation of GC, possibly via exposure to albumin, elicits the conversion of GTP to cGMP which is bound by PKG. Recent work has suggested that PKG promotes the activity of PI4K to generate PI4P from PI, which is subsequently further phosphorylated to form the PI-PLC substrate PI(4,5)P2. PI-PLC is likely the target of artificial ethanol stimulation as well as an as yet unidentified potassium (K+) sensor. PI-PLC splits PI(4,5)P2 into IP3 and DAG. IP3 stimulates calcium release, likely from the ER and the released calcium goes on to activate CDPKs. DAG generated by PI-PLC is converted to PA by DGK1 and ultimately bound by APH on the microneme surface, possibly signalling to the closely juxtaposed DOC2.1 SNARE complex to initiate membrane fusion. DGK, diacylglycerol kinase 1; PAP, phosphatidic acid phosphatase; PI-PLC, phosphoinositide-phospholipase C; PDE, phosphodiesterase; GC, guanylate cyclase; PKG, protein kinase G; TgCDPK1/3, *T. gondii* calcium-dependent protein kinase 1 and 3; APH, acylated pleckstrin homology domain containing protein; PA, phosphatidic acid; DAG, diacylglycerol. Adapted from [11].

Spo20p to the prospore membrane during sporulation [45] and has been used as the basis for a variety of PA biosensors including Spo20pGFP and PASS (PA biosensor with superior sensitivity). Spo20pGFP has been used to investigate membrane ruffling during phagocytosis [65], live cell imaging of PA dynamics in tobacco pollen tubes [66], and PA distribution and production during microneme secretion signalling in the Apicomplexa [11]. The drawback of this system is that the mutant form of Spo20p (Spo20pMut), which is reported to be unable to bind PA [45], continues to bind PA in PIP-strip assays in spite of its clear inability to bind PA *in vivo* [11].

PASS was derived from the PA-binding domain of yeast Spo20p (Spo20-PABD) fused to the nuclear export sequence of protein kinase A alpha for use in both regular fluorescence microscopy and fluorescence lifetime imaging microscopy–fluorescence resonance energy transfer (FILM/FRET) [67,68]. PASS is exclusively localised to the cytoplasm, unlike the original Spo20p, which was predominantly localised to the nucleus, and has been used in imaging PA in breast cancer cells [67,68].

In focus – apicomplexan PA signalling in exocytosis

The phylum apicomplexa groups a variety of pathogens of both human and agricultural importance, most notably of which being *Plasmodium falciparum* and *P. vivax,* the major etiologic agents of malaria in humans, and *T. gondii,* the causative agent of toxoplasmosis. Disease pathogenesis is associated with the asexual stage of these parasites' life cycles, thus a significant proportion of research to date has focused on these invasive stages. *T. gondii* has attracted attention, both considered as a model for some shared aspects of the biology of the Apicomplexa, as well as a significant human pathogen in its own right, causing severe and often fatal diseases in immunocompromised individuals, while also causing severe congenital diseases when acquired *in utero*.

The role of microneme secretion

Micronemes are specialised secretory organelles located at the parasite apex, which discharge from the tip of

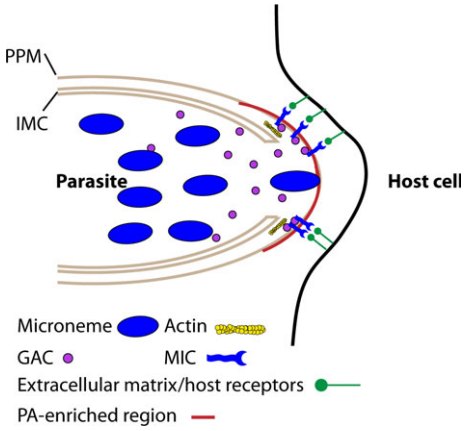


Fig. 3. Phosphatidic acid-binding proteins facilitate apical microneme fusion and glideosome engagement. Schematic representation of the apical pole of an extracellular *T. gondii* parasite. Secreted proteins are released from the micronemes at the apical pole of the parasite and subsequently interact between the parasite inner membrane complex (IMC) and parasite plasma membrane (PPM) with the gliding machinery. PA-enriched regions shown in red.

the parasite where the inner membrane complex stops and the plasma membrane is accessible for fusion (Figs 2 and 3). The contents of the micronemes participate in gliding motility, invasion and egress from infected cells and the protein constituents include a plethora of proteins acting as (a) perforin-like molecules containing MACPF (membrane attack complex pore forming) domains which are involved in eliciting efficient host cell egress [69–73], (b) adhesins and (c) proteases. The adhesins assemble as complexes of transmembrane and soluble proteins (MICs) that interact with the host extracellular matrix, or receptors on host cells [74]. These MICs are subjected to a complex array of proteolytic activities upon their exocytosis [75].

Signalling mechanisms underpinning apicomplexan PA production

The signalling cascade leading to microneme secretion is complex and involves both intrinsic and extrinsic signalling pathways [4]. Microneme exocytosis has been linked to changes in extracellular potassium levels [76,77] and changing cyclic nucleotide levels which are thought to indirectly increase intracellular calcium concentrations through phosphoinositide-phospholipase C (PI-PLC) activation. PI-PLC uses PI(4,5)P2 as a substrate for the generation of the second messengers IP3 and DAG [78] (Fig. 2). DAG is further converted into PA through the activity of DGKs [11] while IP3 goes on to stimulate the release of calcium, likely from the endoplasmic reticulum via unidentified receptors [79]. The complete repertoire of functions this calcium is involved in is yet to be fully elucidated, however, it is known to activate members of the calcium-dependant protein kinase (CDPK) family, which play important yet incompletely defined roles in microneme exocytosis [80–82]. Subsequent to CDPK activation, microneme secretion depends on a fusion event involving SNARE-like protein DOC2.1 [83,84], however, the precise mechanism and effectors underpinning this fusion event are yet to be determined (Fig. 2).

Phosphoinositide-phospholipase C can be seen as the central nexus for microneme secretion with critical processes occurring both upstream and downstream of its activation (Fig. 2). Upstream of PI-PLC activation is a diverse network of signalling molecules and effector proteins including cyclic nucleotides, protein kinases and phosphodiesterases (Fig. 2). Recent work has suggested that albumin may activate guanylate cyclase (GC) [85], which in turn converts GTP to cGMP. This then activates protein kinase G (PKG) leading to an increase in PI4P production, likely as a result of the upregulation of PI4K [86] (Fig. 2). This PI4P is then converted to PI(4,5)P2, thus forming a substrate for PI-PLC and the subsequent downstream production of DAG and PA. As expected, inhibiting PKG activity by use of specific inhibitors Compound 1 (a trisubstituted pyrrole) and Compound 2 (an imidazopyridine-based inhibitor), blocks microneme secretion [86,87]. Adding another layer of complexity to this pathway is the presence of phosphodiesterases (PDEs) whose roles are to convert cGMP and/or cAMP to GMP and AMP respectively (Fig. 2). Studies have shown that inhibiting PDE with either zaprinast or BIPPO promotes microneme secretion [88], likely as a result of the ensuing build up of cGMP. Overall, data available to date suggest that the interplay between all arms of this pathway is critical for ensuring effective microneme secretion and thus the survival and propagation of Apicomplexan parasites.

Specific contribution of PA signalling to parasitism by the apicomplexa

Downstream of PI-PLC signalling is the production of DAG and subsequently PA through the specific activity of DGK1 at the parasite periphery [11]. Conditional deletion of the *TgDGK1* gene resulted not only in the impairment of PA production and defects in microneme secretion [11] but also led to a severe loss in plasma membrane integrity due either to an accumulation of DAG or a depletion in PA, highlighting

the importance of lipid regulation in this unicellular organism. Alongside TgDGK1 there are two additional DGKs in *T. gondii* (DGK2 and DGK3), which show distinct localisations within the parasite [11]. TgDGK2 localises to the dense granules and accumulates in the parasitophorous vacuole, a specialised compartment encapsulating the parasite and protecting it from the host cellular defence mechanisms. Intriguingly, TgDGK3 localises to the micronemes [11]. The roles played by DGK2 and DGK3 are yet to be defined, however, their localisation would predict that they too are contributing to signalling and pathogenesis. Interestingly, only DGK1 and DGK3 are conserved across the Apicomplexan phylum, with DGK2 being present only in Coccidia, suggesting that DGK2 maintains a coccidian-specific function.

Phosphatidic acid can be converted back to DAG through the actions of phosphatidic acid phosphatases (PAP) and lipins, of which there are several within the Apicomplexa [11] (Table 1). PAPs can be targeted by the commercially available drug propranolol [89] and this compound has been shown to promote overstimulation of parasite microneme secretion [11]. This overstimulation is likely due to the build up of PA that occurs when the PAP can no longer function, but the remainder of the signalling pathway promoting DAG formation is still active (Fig. 2). A candidate PAP putatively involved in microneme secretion was identified in *T. gondii*, however, its gene is absent from other members of the Apicomplexa and it was found to be nonessential for *in vitro* growth of *T. gondii* [11] (Table 1). These observations are not surprising given that in other apicomplexan parasites such as *Plasmodium* spp., microneme secretion is an all-or-nothing event and thus there would be no need for such an 'off switch'. In light of the severe phenotype observed upon conditional depletion of DGK1, further investigation into the roles played by the apicomplexan lipins and PAPs are likely to shed further light on the complexity of signalling events modulated by DAG/PA regulation.

PA sensing in the apicomplexa: APH and GAC

Recent work completed within *T. gondii* has identified the first PA-sensing protein termed acylated-pleckstrin-homology domain (TgAPH) protein [11]. TgAPH is anchored on the surface of the micronemes in an acylation-dependant manner [11] and the conditional knockout of the gene revealed its essentiality for parasite survival and the key role of TgAPH in microneme secretion [11]. Presumably TgAPH senses changing PA levels at the parasite apex during signalling leading to microneme secretion by binding to PA at the plasma membrane and possibly by signalling to the closely juxtaposed DOC2.1 SNARE complex to initiate membrane fusion [11] (Fig. 2). While the downstream effects of APH binding PA are clear (microneme secretion), the precise mechanisms through which this is achieved are still poorly understood.

As part of the machinery leading to invasion and egress, the secreted transmembrane MICs interact in their cytoplasmic tail with the parasite actomyosin system in order to generate motility [90]. Importantly, a recently identified protein termed glideosome associated connector (GAC, TGME49_312630) is essential for motility and invasion and contains a PH domain that also binds to PA. Consequently PA acts as a lipid mediator that coordinates microneme secretion with the engagement of the released adhesins into the actomyosin system to promote gliding motility in the Apicomplexa.

Conclusions and perspectives

Phosphatidic acid is involved in diverse signalling events in a variety of different cell types and organisms, and the precise mechanisms and events involved in these signalling pathways are only recently beginning to be unravelled. Deeper investigation into the roles played by PA is likely to reveal a wider repertoire of both essential physiological process and diseases linked to PA dis/regulation and signalling. The development of a broader range of more specific and timely PA sensors as biological tools may help better unravel the PA-based signalling pathways in a range of cell types and lead to novel methods of intervention for a variety of diseases. Furthermore, blocking DGK-regulated PA generation in apicomplexan parasites may be key to treating parasitic infections caused by the Apicomplexa. In conclusion, PA signalling is an exciting and growing field of research with a great deal of overlap in a broad array of different cell types and organisms. As such, efforts made to understand PA signalling in one cell type are likely to inform research in other cell types, potentially leading to breakthroughs in the field of exocytosis.

Acknowledgements

HB was supported by the Indo–Swiss Joint Research Project (ISJRP-138850). DSF is HHMI senior international research scholar. We are grateful to Dr Nicolas Vitale for critical reading of the manuscript.

References

1 Balla T (2013) Phosphoinositides: tiny lipids with giant impact on cell regulation. *Physiol Rev* **93**, 1019–1137.

2 Simons K and Sampaio JL (2011) Membrane organization and lipid rafts. *Cold Spring Harb Perspect Biol* **3**, a004697.
3 Ammar MR, Kassas N, Chasserot-Golaz S, Bader MF and Vitale N (2013) Lipids in regulated exocytosis: What are they doing? *Front Endocrinol (Lausanne)* **4**, 125.
4 Lourido S and Moreno SN (2015) The calcium signaling toolkit of the Apicomplexan parasites *Toxoplasma gondii* and *Plasmodium* spp. *Cell Calcium* **57**, 186–193.
5 Tu-Sekine B, Goldschmidt H and Raben DM (2015) Diacylglycerol, phosphatidic acid, and their metabolic enzymes in synaptic vesicle recycling. *Adv Biol Regul* **57**, 147–152.
6 Schwarz K, Natarajan S, Kassas N, Vitale N and Schmitz F (2011) The synaptic ribbon is a site of phosphatidic acid generation in ribbon synapses. *J Neurosci* **31**, 15996–16011.
7 Arisz SA, van Wijk R, Roels W, Zhu JK, Haring MA and Munnik T (2013) Rapid phosphatidic acid accumulation in response to low temperature stress in Arabidopsis is generated through diacylglycerol kinase. *Front Plant Sci* **4**, 1.
8 Vitale N, Caumont AS, Chasserot-Golaz S, Du G, Wu S, Sciorra VA, Morris AJ, Frohman MA and Bader MF (2001) Phospholipase D1: a key factor for the exocytotic machinery in neuroendocrine cells. *EMBO J* **20**, 2424–2434.
9 Zeniou-Meyer M, Zabari N, Ashery U, Chasserot-Golaz S, Haeberle AM, Demais V, Bailly Y, Gottfried I, Nakanishi H, Neiman AM *et al.* (2007) Phospholipase D1 production of phosphatidic acid at the plasma membrane promotes exocytosis of large dense-core granules at a late stage. *J Biol Chem* **282**, 21746–21757.
10 Humeau Y, Vitale N, Chasserot-Golaz S, Dupont JL, Du G, Frohman MA, Bader MF and Poulain B (2001) A role for phospholipase D1 in neurotransmitter release. *Proc Natl Acad Sci U S A* **98**, 15300–15305.
11 Bullen HE, Jia Y, Yamaryo-Botte Y, Bisio H, Zhang O, Jemelin NK, Marq JB, Carruthers V, Botte CY and Soldati-Favre D (2016) Phosphatidic Acid-Mediated Signaling Regulates Microneme Secretion in Toxoplasma. *Cell Host Microbe* **19**, 349–360.
12 Holden NJ, Savage CO, Young SP, Wakelam MJ, Harper L and Williams JM (2011) A dual role for diacylglycerol kinase generated phosphatidic acid in autoantibody-induced neutrophil exocytosis. *Mol Med* **17**, 1242–1252.
13 Williams JM, Pettitt TR, Powell W, Grove J, Savage CO and Wakelam MJ (2007) Antineutrophil cytoplasm antibody-stimulated neutrophil adhesion depends on diacylglycerol kinase-catalyzed phosphatidic acid formation. *J Am Soc Nephrol* **18**, 1112–1120.
14 Sakuma M, Shirai Y, Ueyama T and Saito N (2014) Diacylglycerol kinase gamma regulates antigen-induced mast cell degranulation by mediating Ca(2+) influxes. *Biochem Biophys Res Commun* **445**, 340–345.
15 Kooijman EE, Chupin V, Fuller NL, Kozlov MM, de Kruijff B, Burger KN and Rand PR (2005) Spontaneous curvature of phosphatidic acid and lysophosphatidic acid. *Biochemistry* **44**, 2097–2102.
16 Hanahan DJ and Chaikoff IL (1947) A new phospholipide-splitting enzyme specific for the ester linkage between the nitrogenous base and the phosphoric acid grouping. *J Biol Chem* **169**, 699–705.
17 Hong Y, Zhao J, Guo L, Kim SC, Deng X, Wang G, Zhang G, Li M and Wang X (2016) Plant phospholipases D and C and their diverse functions in stress responses. *Prog Lipid Res* **62**, 55–74.
18 Elias M, Potocky M, Cvrckova F and Zarsky V (2002) Molecular diversity of phospholipase D in angiosperms. *BMC Genom* **3**, 2.
19 Qin C and Wang X (2002) The Arabidopsis phospholipase D family. Characterization of a calcium-independent and phosphatidylcholine-selective PLD zeta 1 with distinct regulatory domains. *Plant Physiol* **128**, 1057–1068.
20 Sung TC, Altshuller YM, Morris AJ and Frohman MA (1999) Molecular analysis of mammalian phospholipase D2. *J Biol Chem* **274**, 494–502.
21 Sung TC, Zhang Y, Morris AJ and Frohman MA (1999) Structural analysis of human phospholipase D1. *J Biol Chem* **274**, 3659–3666.
22 Hu T and Exton JH (2005) 1-Butanol interferes with phospholipase D1 and protein kinase Calpha association and inhibits phospholipase D1 basal activity. *Biochem Biophys Res Commun* **327**, 1047–1051.
23 Su W, Chen Q and Frohman MA (2009) Targeting phospholipase D with small-molecule inhibitors as a potential therapeutic approach for cancer metastasis. *Future Oncol* **5**, 1477–1486.
24 Lukowski S, Mira JP, Zachowski A and Geny B (1998) Fodrin inhibits phospholipases A2, C, and D by decreasing polyphosphoinositide cell content. *Biochem Biophys Res Commun* **248**, 278–284.
25 Chung JK, Sekiya F, Kang HS, Lee C, Han JS, Kim SR, Bae YS, Morris AJ and Rhee SG (1997) Synaptojanin inhibition of phospholipase D activity by hydrolysis of phosphatidylinositol 4,5-bisphosphate. *J Biol Chem* **272**, 15980–15985.
26 Venable ME, Bielawska A and Obeid LM (1996) Ceramide inhibits phospholipase D in a cell-free system. *J Biol Chem* **271**, 24800–24805.
27 Monovich L, Mugrage B, Quadros E, Toscano K, Tommasi R, LaVoie S, Liu E, Du Z, LaSala D, Boyar W *et al.* (2007) Optimization of halopemide for

phospholipase D2 inhibition. *Bioorg Med Chem Lett* **17**, 2310–2311.
28 Su W, Yeku O, Olepu S, Genna A, Park JS, Ren H, Du G, Gelb MH, Morris AJ and Frohman MA (2009) 5-Fluoro-2-indolyl des-chlorohalopemide (FIPI), a phospholipase D pharmacological inhibitor that alters cell spreading and inhibits chemotaxis. *Mol Pharmacol* **75**, 437–446.
29 Ganesan R, Mahankali M, Alter G and Gomez-Cambronero J (2015) Two sites of action for PLD2 inhibitors: the enzyme catalytic center and an allosteric, phosphoinositide biding pocket. *Biochim Biophys Acta* **1851**, 261–272.
30 Lavieri R, Scott SA, Lewis JA, Selvy PE, Armstrong MD, Alex Brown H and Lindsley CW (2009) Design and synthesis of isoform-selective phospholipase D (PLD) inhibitors. Part II. Identification of the 1,3,8-triazaspiro[4,5]decan-4-one privileged structure that engenders PLD2 selectivity. *Bioorg Med Chem Lett* **19**, 2240–2243.
31 Lewis JA, Scott SA, Lavieri R, Buck JR, Selvy PE, Stoops SL, Armstrong MD, Brown HA and Lindsley CW (2009) Design and synthesis of isoform-selective phospholipase D (PLD) inhibitors. Part I: Impact of alternative halogenated privileged structures for PLD1 specificity. *Bioorg Med Chem Lett* **19**, 1916–1920.
32 Xie S, Naslavsky N and Caplan S (2015) Diacylglycerol kinases in membrane trafficking. *Cell Logist* **5**, e1078431.
33 Badola P and Sanders CR 2nd (1997) Escherichia coli diacylglycerol kinase is an evolutionarily optimized membrane enzyme and catalyzes direct phosphoryl transfer. *J Biol Chem* **272**, 24176–24182.
34 Arisz SA, Testerink C and Munnik T (2009) Plant PA signaling via diacylglycerol kinase. *Biochim Biophys Acta* **1791**, 869–875.
35 Liu Z and Huang X (2013) Lipid metabolism in Drosophila: development and disease. *Acta Biochim Biophys Sin (Shanghai)* **45**, 44–50.
36 Jose AM and Koelle MR (2005) Domains, amino acid residues, and new isoforms of *Caenorhabditis elegans* diacylglycerol kinase 1 (DGK-1) important for terminating diacylglycerol signaling in vivo. *J Biol Chem* **280**, 2730–2736.
37 de Chaffoy de Courcelles DC, Roevens P & Van Belle H (1985) R 59 022, a diacylglycerol kinase inhibitor. Its effect on diacylglycerol and thrombin-induced C kinase activation in the intact platelet. *J Biol Chem*. **260**, 15762–15770.
38 de Chaffoy de Courcelles D, Roevens P, Van Belle H, Kennis L, Somers Y & De Clerck F (1989) The role of endogenously formed diacylglycerol in the propagation and termination of platelet activation. A biochemical and functional analysis using the novel diacylglycerol kinase inhibitor, R 59 949. *J Biol Chem* **264**, 3274–3285.
39 Torres-Ayuso P, Daza-Martin M, Martin-Perez J, Avila-Flores A and Merida I (2014) Diacylglycerol kinase alpha promotes 3D cancer cell growth and limits drug sensitivity through functional interaction with Src. *Oncotarget* **5**, 9710–9726.
40 Sato M, Liu K, Sasaki S, Kunii N, Sakai H, Mizuno H, Saga H and Sakane F (2013) Evaluations of the selectivities of the diacylglycerol kinase inhibitors R59022 and R59949 among diacylglycerol kinase isozymes using a new non-radioactive assay method. *Pharmacology* **92**, 99–107.
41 Ryu JK, Jahn R and Yoon TY (2016) Review: Progresses in understanding N-ethylmaleimide sensitive factor (NSF) mediated disassembly of SNARE complexes. *Biopolymers* **105**, 518–531.
42 Vicogne J, Vollenweider D, Smith JR, Huang P, Frohman MA and Pessin JE (2006) Asymmetric phospholipid distribution drives in vitro reconstituted SNARE-dependent membrane fusion. *Proc Natl Acad Sci U S A* **103**, 14761–14766.
43 Fernandez-Chacon R, Konigstorfer A, Gerber SH, Garcia J, Matos MF, Stevens CF, Brose N, Rizo J, Rosenmund C and Sudhof TC (2001) Synaptotagmin I functions as a calcium regulator of release probability. *Nature* **410**, 41–49.
44 Manifava M, Thuring JW, Lim ZY, Packman L, Holmes AB and Ktistakis NT (2001) Differential binding of traffic-related proteins to phosphatidic acid- or phosphatidylinositol (4,5)- bisphosphate-coupled affinity reagents. *J Biol Chem* **276**, 8987–8994.
45 Nakanishi H, de los Santos P & Neiman AM (2004) Positive and negative regulation of a SNARE protein by control of intracellular localization. *Mol Biol Cell*. **15**, 1802–1815.
46 Rizzo M and Romero G (2002) Pharmacological importance of phospholipase D and phosphatidic acid in the regulation of the mitogen-activated protein kinase cascade. *Pharmacol Ther* **94**, 35–50.
47 Honda A, Nogami M, Yokozeki T, Yamazaki M, Nakamura H, Watanabe H, Kawamoto K, Nakayama K, Morris AJ, Frohman MA *et al*. (1999) Phosphatidylinositol 4-phosphate 5-kinase alpha is a downstream effector of the small G protein ARF6 in membrane ruffle formation. *Cell* **99**, 521–532.
48 Kooijman EE, Chupin V, de Kruijff B and Burger KN (2003) Modulation of membrane curvature by phosphatidic acid and lysophosphatidic acid. *Traffic* **4**, 162–174.
49 Goldschmidt HL, Tu-Sekine B, Volk L, Anggono V, Huganir RL and Raben DM (2016) DGKtheta catalytic activity is required for efficient recycling of presynaptic vesicles at excitatory synapses. *Cell Rep* **14**, 200–207.

50 Siddiqui RA and English D (1996) Phosphatidic acid binding to human neutrophils: effects on tyrosine kinase-regulated intracellular Ca2+ mobilization. *Cell Signal* **8**, 349–354.
51 Mayorga LS, Tomes CN and Belmonte SA (2007) Acrosomal exocytosis, a special type of regulated secretion. *IUBMB Life* **59**, 286–292.
52 Pelletan LE, Suhaiman L, Vaquer CC, Bustos MA, De Blas GA, Vitale N, Mayorga LS and Belmonte SA (2015) ADP ribosylation factor 6 (ARF6) promotes acrosomal exocytosis by modulating lipid turnover and Rab3A activation. *J Biol Chem* **290**, 9823–9841.
53 Begle A, Tryoen-Toth P, de Barry J, Bader MF and Vitale N (2009) ARF6 regulates the synthesis of fusogenic lipids for calcium-regulated exocytosis in neuroendocrine cells. *J Biol Chem* **284**, 4836–4845.
54 Lopez CI, Pelletan LE, Suhaiman L, De Blas GA, Vitale N, Mayorga LS and Belmonte SA (2012) Diacylglycerol stimulates acrosomal exocytosis by feeding into a PKC- and PLD1-dependent positive loop that continuously supplies phosphatidylinositol 4,5-bisphosphate. *Biochim Biophys Acta* **1821**, 1186–1199.
55 Stace CL and Ktistakis NT (2006) Phosphatidic acid- and phosphatidylserine-binding proteins. *Biochim Biophys Acta* **1761**, 913–926.
56 Zhao C, Du G, Skowronek K, Frohman MA and Bar-Sagi D (2007) Phospholipase D2-generated phosphatidic acid couples EGFR stimulation to Ras activation by Sos. *Nat Cell Biol* **9**, 706–712.
57 Ghosh S, Strum JC, Sciorra VA, Daniel L and Bell RM (1996) Raf-1 kinase possesses distinct binding domains for phosphatidylserine and phosphatidic acid. Phosphatidic acid regulates the translocation of Raf-1 in 12-O-tetradecanoylphorbol-13-acetate-stimulated Madin-Darby canine kidney cells. *J Biol Chem* **271**, 8472–8480.
58 Rizzo MA, Shome K, Watkins SC and Romero G (2000) The recruitment of Raf-1 to membranes is mediated by direct interaction with phosphatidic acid and is independent of association with Ras. *J Biol Chem* **275**, 23911–23918.
59 Krishna S and Zhong XP (2013) Regulation of lipid signaling by diacylglycerol kinases during T cell development and function. *Front Immunol* **4**, 178.
60 Zheng J, Chen RH, Corblan-Garcia S, Cahill SM, Bar-Sagi D and Cowburn D (1997) The solution structure of the pleckstrin homology domain of human SOS1. A possible structural role for the sequential association of diffuse B cell lymphoma and pleckstrin homology domains. *J Biol Chem* **272**, 30340–30344.
61 Kubiseski TJ, Chook YM, Parris WE, Rozakis-Adcock M and Pawson T (1997) High affinity binding of the pleckstrin homology domain of mSos1 to phosphatidylinositol (4,5)-bisphosphate. *J Biol Chem* **272**, 1799–1804.
62 Jenkins GH, Fisette PL and Anderson RA (1994) Type I phosphatidylinositol 4-phosphate 5-kinase isoforms are specifically stimulated by phosphatidic acid. *J Biol Chem* **269**, 11547–11554.
63 Moritz A, De Graan PN, Gispen WH and Wirtz KW (1992) Phosphatidic acid is a specific activator of phosphatidylinositol-4-phosphate kinase. *J Biol Chem* **267**, 7207–7210.
64 Neiman AM (1998) Prospore membrane formation defines a developmentally regulated branch of the secretory pathway in yeast. *J Cell Biol* **140**, 29–37.
65 Bohdanowicz M, Schlam D, Hermansson M, Rizzuti D, Fairn GD, Ueyama T, Somerharju P, Du G and Grinstein S (2013) Phosphatidic acid is required for the constitutive ruffling and macropinocytosis of phagocytes. *Mol Biol Cell* **24** (1700–12), S1–S7.
66 Potocky M, Pleskot R, Pejchar P, Vitale N, Kost B and Zarsky V (2014) Live-cell imaging of phosphatidic acid dynamics in pollen tubes visualized by Spo20p-derived biosensor. *New Phytol* **203**, 483–494.
67 Lu M, Tay LW, He J and Du G (2016) Monitoring phosphatidic acid signaling in breast cancer cells using genetically encoded biosensors. *Methods Mol Biol* **1406**, 225–237.
68 Zhang F, Wang Z, Lu M, Yonekubo Y, Liang X, Zhang Y, Wu P, Zhou Y, Grinstein S, Hancock JF *et al*. (2014) Temporal production of the signaling lipid phosphatidic acid by phospholipase D2 determines the output of extracellular signal-regulated kinase signaling in cancer cells. *Mol Cell Biol* **34**, 84–95.
69 Kafsack BF, Pena JD, Coppens I, Ravindran S, Boothroyd JC and Carruthers VB (2009) Rapid membrane disruption by a perforin-like protein facilitates parasite exit from host cells. *Science* **323**, 530–533.
70 Garg S, Agarwal S, Kumar S, Yazdani SS, Chitnis CE and Singh S (2013) Calcium-dependent permeabilization of erythrocytes by a perforin-like protein during egress of malaria parasites. *Nat Commun* **4**, 1736.
71 Deligianni E, Morgan RN, Bertuccini L, Wirth CC, Silmon de Monerri NC, Spanos L, Blackman MJ, Louis C, Pradel G and Siden-Kiamos I (2013) A perforin-like protein mediates disruption of the erythrocyte membrane during egress of *Plasmodium berghei* male gametocytes. *Cell Microbiol* **15**, 1438–1455.
72 Wirth CC, Glushakova S, Scheuermayer M, Repnik U, Garg S, Schaack D, Kachman MM, Weissbach T, Zimmerberg J, Dandekar T *et al*. (2014) Perforin-like protein PPLP2 permeabilizes the red blood cell membrane during egress of *Plasmodium falciparum* gametocytes. *Cell Microbiol* **16**, 709–733.
73 Roiko MS, Svezhova N and Carruthers VB (2014) Acidification activates *Toxoplasma gondii* motility and

egress by enhancing protein secretion and cytolytic activity. *PLoS Pathog* **10**, e1004488.
74 Soldati D, Dubremetz JF and Lebrun M (2001) Microneme proteins: structural and functional requirements to promote adhesion and invasion by the apicomplexan parasite *Toxoplasma gondii*. *Int J Parasitol* **31**, 1293–1302.
75 Dowse T and Soldati D (2004) Host cell invasion by the apicomplexans: the significance of microneme protein proteolysis. *Curr Opin Microbiol* **7**, 388–396.
76 Moudy R, Manning TJ and Beckers CJ (2001) The loss of cytoplasmic potassium upon host cell breakdown triggers egress of *Toxoplasma gondii*. *J Biol Chem* **276**, 41492–41501.
77 Singh S, Alam MM, Pal-Bhowmick I, Brzostowski JA and Chitnis CE (2010) Distinct external signals trigger sequential release of apical organelles during erythrocyte invasion by malaria parasites. *PLoS Pathog* **6**, e1000746.
78 Fang J, Marchesini N and Moreno SN (2006) A *Toxoplasma gondii* phosphoinositide phospholipase C (TgPI-PLC) with high affinity for phosphatidylinositol. *Biochem J* **394**, 417–425.
79 Budu A and Garcia CR (2012) Generation of second messengers in Plasmodium. *Microbes Infect* **14**, 787–795.
80 Garrison E, Treeck M, Ehret E, Butz H, Garbuz T, Oswald BP, Settles M, Boothroyd J and Arrizabalaga G (2012) A forward genetic screen reveals that calcium-dependent protein kinase 3 regulates egress in Toxoplasma. *PLoS Pathog* **8**, e1003049.
81 Lourido S, Tang K and Sibley LD (2012) Distinct signalling pathways control Toxoplasma egress and host-cell invasion. *EMBO J* **31**, 4524–4534.
82 McCoy JM, Whitehead L, van Dooren GG and Tonkin CJ (2012) TgCDPK3 regulates calcium-dependent egress of *Toxoplasma gondii* from host cells. *PLoS Pathog* **8**, e1003066.
83 Farrell A, Thirugnanam S, Lorestani A, Dvorin JD, Eidell KP, Ferguson DJ, Anderson-White BR, Duraisingh MT, Marth GT and Gubbels MJ (2012) A DOC2 protein identified by mutational profiling is essential for apicomplexan parasite exocytosis. *Science* **335**, 218–221.
84 Jean S, Zapata-Jenks MA, Farley JM, Tracy E and Mayer DC (2014) Plasmodium falciparum double C2 domain protein, PfDOC2, binds to calcium when associated with membranes. *Exp Parasitol* **144**, 91–95.
85 Brown KM, Lourido S and Sibley LD (2016) Serum albumin stimulates protein kinase G-dependent microneme secretion in *Toxoplasma gondii*. *J Biol Chem* **291**, 9554–9565.
86 Brochet M, Collins MO, Smith TK, Thompson E, Sebastian S, Volkmann K, Schwach F, Chappell L, Gomes AR, Berriman M *et al*. (2014) Phosphoinositide metabolism links cGMP-dependent protein kinase G to essential Ca(2)(+) signals at key decision points in the life cycle of malaria parasites. *PLoS Biol* **12**, e1001806.
87 Wiersma HI, Galuska SE, Tomley FM, Sibley LD, Liberator PA and Donald RG (2004) A role for coccidian cGMP-dependent protein kinase in motility and invasion. *Int J Parasitol* **34**, 369–380.
88 Howard BL, Harvey KL, Stewart RJ, Azevedo MF, Crabb BS, Jennings IG, Sanders PR, Manallack DT, Thompson PE, Tonkin CJ *et al*. (2015) Identification of potent phosphodiesterase inhibitors that demonstrate cyclic nucleotide-dependent functions in apicomplexan parasites. *ACS Chem Biol* **10**, 1145–1154.
89 Baron CL and Malhotra V (2002) Role of diacylglycerol in PKD recruitment to the TGN and protein transport to the plasma membrane. *Science* **295**, 325–328.
90 Heintzelman MB (2015) Gliding motility in apicomplexan parasites. *Semin Cell Dev Biol* **46**, 135–142.
91 Hooks SB, Ragan SP and Lynch KR (1998) Identification of a novel human phosphatidic acid phosphatase type 2 isoform. *FEBS Lett* **427**, 188–192.
92 Kai M, Wada I, Imai S, Sakane F and Kanoh H (1997) Cloning and characterization of two human isozymes of Mg2+-independent phosphatidic acid phosphatase. *J Biol Chem* **272**, 24572–24578.
93 Kanoh H, Kai M and Wada I (1997) Phosphatidic acid phosphatase from mammalian tissues: discovery of channel-like proteins with unexpected functions. *Biochim Biophys Acta* **1348**, 56–62.
94 Long JS, Pyne NJ and Pyne S (2008) Lipid phosphate phosphatases form homo- and hetero-oligomers: catalytic competency, subcellular distribution and function. *Biochem J* **411**, 371–377.