Article

Dictyostelium uses ether-linked inositol phospholipids for intracellular signalling

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Abstract

Inositol phospholipids are critical regulators of membrane biology throughout eukaryotes. The general principle by which they perform these roles is conserved across species and involves binding of differentially phosphorylated inositol head groups to specific protein domains. This interaction serves to both recruit and regulate the activity of several different classes of protein which act on membrane surfaces. In mammalian cells, these phosphorylated inositol head groups are predominantly borne by a C38:4 diacylglycerol backbone. We show here that the inositol phospholipids of *Dictyostelium* are different, being highly enriched in an unusual C34:1e lipid backbone, 1-hexadecyl-2-(11Z-octadecenoyl)-*sn*-glycero-3-phospho-(1'-myo-inositol), in which the *sn*-1 position contains an ether-linked C16:0 chain; they are thus plasmanylinositols. These plasmanylinositols respond acutely to stimulation of cells with chemoattractants, and their levels are regulated by PIPKs, PI3Ks and PTEN. In mammals and now in *Dictyostelium*, the hydrocarbon chains of inositol phospholipids are a highly selected subset of those available to other phospholipids, suggesting that different molecular selectors are at play in these organisms but serve a common, evolutionarily conserved purpose.

Introduction

Inositol phospholipids are believed to be ubiquitous amongst the eukaryotes, where they play crucial roles in organising a wide variety of cellular functions, particularly vesicular trafficking and signal transduction (Balla, 2013; Di Paolo & De Camilli, 2006; Michell, 2008). Eight of these lipids are commonly described, the most abundant of which is phosphatidylinositol (PtdIns). PtdIns is distributed throughout the intracellular membrane systems of eukaryotes and usually represents approximately 10% of total cellular phospholipids. The other inositol phospholipids, where present, are much less abundant and carry one or more phosphate groups on their inositol ring, namely PtdIns3P, PtdIns4P, PtdIns5P, PtdIns(3,4)P2, PtdIns(3,5)P2, PtdIns(4,5)P2 and PtdIns(3,4,5)P3. These more highly phosphorylated inositol lipids are metabolically interconverted by a series of reactions catalysed by specific kinases and phosphatases, and characteristically exhibit a much more restricted cellular distribution relating to their function.

Phylogenetic surveys indicate that genes encoding type-III PI3K and PI4K inositol lipid kinases (making PtdIns(3)P and PtdIns(4)P, respectively) and PI(4)P5K (making PtdIns(4,5)P2) are likely to be present across the eukaryotes. In contrast, Class I PI3Ks, producing PtdIns(3,4,5)P3 at the plasma membrane, are absent in plants and fungi, but are found in the Amoebozoa, such as *Dictyostelium discoideum* (Brown & Auger, 2011; Engelman *et al*, 2006; Zhou *et al*, 1995).

In mammalian cells, PtdIns(3)P and PtdIns(3,5)P2 play important roles in endosomal/lysosomal trafficking and the induction of autophagy (Burman & Ktistakis, 2010; Raiborg *et al*, 2013). PtdIns(4)P and PtdIns(4,5)P2 play roles in plasma membrane identity, cytoskeletal organisation and secretion (Di Paolo & De Camilli, 2006; Saarikangas *et al*, 2010). PtdIns(4,5)P2 also plays a major role as the substrate for two major signal transduction pathways; PLC-catalysed conversion of PtdIns(4,5)P2 to the messenger molecules diacylglycerol and inositol 1,4,5-trisphosphate (Kadamur & Ross, 2013) and Class I PI3K-catalysed conversion of PtdIns(4,5)P2 to the messenger PtdIns(3,4,5)P3 (Hawkins *et al*, 2006).

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To a greater or lesser extent, all of the phosphorylated inositol lipids signal via the binding of their specific inositol phosphate head groups to several defined protein domains, the best studied of which are the binding of PtdIns(3,4,5)P3 to a subfamily of PH-domain containing proteins at the inner leaflet of the plasma membrane and the binding of PtdIns(3)P to FYVE or PX domains at the cytoplasmic face of endosomes and autophagosomes (Lemmon, 2008). These lipids are thus envisaged to act as regulatable membrane scaffolds dictating the localisation and function of proteins at the surface in which they reside.

Thus far, the composition of the hydrocarbon chains in inositol phospholipids has received very little attention. In part, this is because they are envisaged to merely anchor the inositol phospholipid in the bilayer, with minimal influence on the functional interaction of the head group with specific protein domains. However, it is also because most of the widely used techniques to measure these lipids in cell extracts do not yield the composition of the hydrocarbon chains and, indeed, often rely on anion-exchange chromatography of their deacylated derivatives (Guillou et al, 2007). Mass spectrometry-based approaches have the potential to define the full structures of lipids, but they have generally lacked sufficient sensitivity to accurately measure the more highly phosphorylated inositol lipids (Pettitt et al, 2006). We have recently solved some of these problems through chemical derivatisation of the phosphate groups on the inositol ring, allowing sensitive detection of PI, PIP, PIP2 and PIP3 species (the abbreviation PI(P)n is used here to refer to inositol phospholipids without implying the nature of the hydrocarbon linkage to the glycerol unit; PtdIns(P)n is used where the linkages are known to be esters, that is defining a 'phosphatidyl' unit) (Clark et al, 2011; Kielkowska et al, 2014). However, these methods do not distinguish between regioisomers of these lipids, that is, PI3P/PI4P/PI5P and PI(3,4)P2/PI(3,5)P2/PI(4,5)P2; PI(3,4,5,)P3 is the only known isomer of PIP3 found in cells. The mass spectrometric analysis of the fatty acyl/alkyl composition of highly phosphorylated inositol lipids is still in its infancy and has focused thus far on lipid extracts of mammalian origin, confirming that these lipids possess the characteristic diacylglycerol backbone of their precursor, PtdIns (Holub, 1986). The acyl groups that make up this backbone are highly selected: typically stearoyl (C18:0; 18 carbons: 0 double bonds) at the sn-1 position and arachidonoyl (C20:4) at the sn-2 position (C38:4 in total), particularly in primary tissue samples, with less abundant species containing palmitoyl (C16:0), oleoyl (C18:1) and linoloyl (C18:2) groups (Anderson et al, 2013; Lee et al, 2012; Milne et al, 2005; Rouzer et al, 2006). This raises the questions of whether the same C38:4 backbone is used in other organisms and whether there is the same strong selection for this (or another) backbone in the inositol lipids compared to the heterogeneity of backbone available in the other phospholipids.

The social amoeba *Dictyostelium discoideum* has genetic and cell biological features commending it as a model organism for investigating biological organisation across species (King & Insall, 2009; Muller-Taubenberger et al, 2013). *Dictyostelium* cells possess 7 recognisable PI3K genes, 5 of which have Ras-binding domains indicative of regulation through Ras (Hoeller & Kay, 2007; Zhou et al, 1995), a clear PTEN homologue (Iijima & Devreotes, 2002), and a number of PIP3-binding effector proteins, including a homologue of the protein kinase PKB/AKT (Meili et al, 2000; Zhang et al, 2010). Genetic studies show that PI3K signalling is required for efficient

macropinocytosis (Buczynski et al, 1997; Hoeller et al, 2013; Zhou et al, 1998) and for the relay of cyclic-AMP signals during aggregation (Insall et al, 1994; Loovers et al, 2006). Its role in chemotaxis to cyclic-AMP and folic acid is much more controversial (Kay et al, 2008). Although PIP3 is made in response to both chemoattractants (see later) and can be polarised towards the leading edge of chemotaxing cells (Parent et al, 1998), genetic elimination of PIP3 signalling does not prevent efficient chemotaxis to either chemical and indeed improves chemotaxis to folic acid (Hoeller et al, 2013; Hoeller & Kay, 2007; Takeda et al, 2007; Veltman et al, 2014). There is also evidence that PIP3 is involved in bleb-driven cell movement (Zatulovskiy et al, 2014), and as PIP3 is produced at phagosomes (Dormann et al, 2004), it is likely to be involved in phagocytosis. Despite this intense interest in phosphoinositide signalling, very few measurements have been published quantifying changes in the inositol lipids themselves in this organism.

We applied our recent mass spectrometry techniques to analyse inositol phospholipids from *Dictyostelium*. To our surprise, we discovered that the vast majority of the inositol phospholipids in this organism are ether lipids, with a novel plasmanyl-C34:1e structure (the 'e' is used here to indicate that one of the hydrocarbon chains is linked to the glycerol via an ether linkage). This C34:1e species is much more enriched in the inositol phospholipids than the other major phospholipid classes, suggesting it has been selected to convey specific properties to this pool. We also show through the use of appropriate mutants that these plasmanylinositols are used by the PI3K signalling pathway and define the kinetics of C34:1e PIP3 production in response to chemoattractants.

Results

Inositol phospholipids in *Dictyostelium* are C34:1e plasmanylinositols

We recently described a new HPLC-ESI mass spectrometry method to analyse phosphorylated inositol lipids in cellular lipid extracts (Kielkowska et al, 2014). This method uses methylation of acidic phosphate groups with TMS-diazomethane, reverse phase chromatography on a C4 column, fragmentation of the lipids at the phosphodiester phosphate and measuring the charged molecular species derived from the neutral loss of a specific methylated head group. We applied this methodology to measure inositol phospholipids in lipid extracts from *Dictyostelium discoideum* grown in axenic medium. Neutral loss scans corresponding to the loss of the inositide head groups (inositol, methylated inositol phosphate or methylated inositol bisphosphate) indicated that the most abundant inositide species had unexpected and unusual masses (m/z 837.6, 945.6 and 1053.6 respectively; Fig 1). The corresponding glycerol fragment had an m/z of 563.6, which suggested either it was derived from a diacylglycerol containing a fatty acyl group with an odd number of carbons or, that one chain was attached to the glycerol via an ether linkage. Both of these possibilities would appear to have the same mass at the resolution of the mass spectrometer used, although fatty acids with an odd carbon chain length are rarely found in eukaryotes.

To resolve this ambiguity, a sample of methylated PIP2 was isolated by HPLC and an accurate mass obtained on an Orbitrap mass spectrometer capable of working at a higher mass resolution.

Figure 1. The major molecular species of inositol phospholipids present in lipid extracts of *Dictyostelium discoideum* possess a C34:1e backbone.
Lipid extracts were prepared from *D. discoideum* grown in axenic medium, then methylated with TMS-diazomethane and analysed by HPLC-ESI mass spectrometry. Neutral loss scans are shown which describe the major species of PI, PIP and PIP2 present (the mass of the individual neutral fragments corresponding to the mass of methylated inositol phosphate 'head groups' are listed in parentheses and differ by multiples of 108, the mass of a methylated phosphate). The most abundant species detected in each case corresponded to the generation of a glycerol fragment with an m/z of 563.6, suggesting the presence of either one ether-linked hydrocarbon chain plus one acyl chain (C34:1e) or two acyl chains with an odd number of total carbon atoms (C33:1). Further high-resolution mass analysis (Supplementary Fig S1A) and fragmentation (Supplementary Fig S1B) confirmed the presence of C16:0 alkyl and C18:1 acyl chains. Similar results were obtained when lipid extracts were prepared from *D. discoideum* grown on bacteria (Supplementary Fig S1C) or in a fully defined medium containing no added fatty acids (Supplementary Fig S1D).

that first exchanges an acyl chain in the *sn*-1 position of the glycerol for an alcohol, in a reaction catalysed by alkyl-DHAP synthase (Nenci *et al*, 2012). We therefore attempted to confirm the structure and pathway for synthesis of a putative C34:1e lipid by feeding *Dictyostelium* cells a C16:0 alcohol (hexadecan-1-ol or palmitol) which contained two deuterium nuclei at the C1 position. Both deuteriums were efficiently incorporated into the C34:1e structure, indicating that the palmityl ether was present (Fig 2). The high proportion of deuterium incorporation (80% of PIP2 molecules had incorporated two deuteriums by 490 min; Supplementary Fig S2), and the lack of any significant incorporation of a single deuterium nucleus, ruled out significant metabolism at the C1 of D2-palmitol and hence more indirect routes of deuterium incorporation. Importantly, the lack of incorporation of a single deuterium also ruled out the presence of a vinyl ether linkage, a characteristic feature of a group of ether lipids called plasmalogens (Brites *et al*, 2004).

Dictyostelium C34:1e lipids were sensitive to hydrolysis by phospholipase A2 (Scott *et al*, 1990), indicating that the C18:1 acyl chain is in the *sn*-2 position (Fig 3A). Finally, the position of the C=C double bond in the C18:1 chain was identified by ozonolysis to be delta-11, identifying the acyl chain as the 11-ocatadecenoyl group (Fig 3B). It is most likely that the double bond is the *cis* isomer because this is the isomer which is most commonly found in biologically important fatty acids and because Z11-ocatadecenoic acid has recently been shown to be an abundant fatty acid in *Dictyostelium discoideum* (Blacklock *et al*, 2008). Thus, the structures of the most abundant species of inositol lipids in *Dictyostelium discoideum* are defined as 1-hexadecyl-2-(11Z-octadecenoyl)-*sn*-glycero-3-phospho-(1'-myo-inositol) and phosphates thereof.

We also analysed the major species of inositol phospholipids in *Dictyostelium discoideum* grown on bacteria or in a fully defined medium with no added fatty acids, SIH medium (Han *et al*, 2004) and, in each case, found a similarly high proportion of the C34:1e species (Supplementary Fig S1C and D), indicating that this structure is independent of the availability of particular fatty acyl chains in the medium. Further, we additionally examined three other species of social amoebae—*Dictyostelium purpureum*, *Dictyostelium mucoroides* and *Polysphondylium violaceum*—and in each case found that the major species of PIP2 is the same mass as that from *D. discoideum* (m/z 1053.6), suggesting that the use of plasmanylinositols is common amongst these amoebae (unpublished observations).

A value of m/z for MH$^+$ of 1053.5809 was obtained (Supplementary Fig S1A). The theoretical m/z for a C34:1e ether/acyl PIP2 is 1053.5804, whereas the theoretical m/z for the alternative C33:1 diacyl compound would be 1053.5440 (which was not seen). Further fragmentation studies of the PIP2 species revealed an ion with a m/z of 265.4 (Supplementary Fig S1B), suggestive of a C18:1 acyl cation. If this was indeed a C18:1 acyl cation, then the other chain would most likely be a C16:0 ether-linked chain, which was consistent with the other ions observed (see Supplementary Fig S1B). Previous work with other organisms has shown that ether-containing phospholipids are synthesised by a metabolic pathway

Figure 2. Determination of the alkyl chain structure in *Dictyostelium discoideum* inositol lipids.
Methylated lipid extracts were prepared from *D. discoideum* grown in axenic medium in the absence (left panel) or presence (right panel) of D2-hexadecan-1-ol for 120 min and then analysed by HPLC-ESI mass spectrometry. The mass data are shown with a centroid presentation to allow differences of one mass unit to be more easily discerned. Both the unlabelled and labelled signals show the typical pattern obtained in mass spectra of compounds predominantly made up of carbon, hydrogen and oxygen atoms, which is the result of the natural abundance of ^{13}C. D2-hexadecan-1-ol was synthesised with both deuterium nuclei in the C1 position, and the mass data indicate both deuteriums were efficiently incorporated into PIP2; that is, *m/z* peaks were shifted by precisely two mass units. There was no detectable increase in the *m/z* 1054.6 signal, indicating no significant incorporation of a single deuterium nucleus, and hence the absence of a vinyl ether linkage to the C16 (palmityl) chain (illustrated by the coloured structures). A more detailed description of the rate and extent of D2-hexadecan-1-ol incorporation is shown in Supplementary Fig S2.

The C34:1e plasmanyl motif is enriched in inositol lipids compared to other phospholipid classes

The striking selection for a particular combination of acyl tails in mammalian phosphoinositides raises the speculation that this selectivity could be functionally important; but equally with only this single example, it could be happenstance. We therefore asked whether a similar molecular selectivity exists in the *Dictyostelium* phosphoinositides. The relative abundances of molecular species of PC, PS, PE and PA in *Dictyostelium* were assessed by analogous neutral loss scans to those described above for the inositol phospholipids (Fig 4). It is immediately apparent that the C34:1e species is the most abundant form of PA, but is a smaller component of the other major phospholipid pools, which have the greater proportion comprised of a mixture of diacyl- and acyl/ether-species (relative comparison of these molecular species is given in Supplementary Fig S3).

The most abundant species of PI, PIP, PIP2 and PA were targeted for more careful comparison by multiple reaction monitoring (MRM), which indicated that the vast majority of each of these lipid pools was comprised of the C34:1e species under both axenic (Fig 5) and bacterially fed conditions (unpublished observations). The accepted pathway for *de novo* synthesis of PI in eukaryotes is via the formation of CDP-DG from PA and CTP, catalysed by CDP-DG synthase (Saito *et al*, 1997), followed by the formation of PI from CDP-DG and inositol, catalysed by PI synthase (Paulus & Kennedy, 1960). Thus, the enrichment of C34:1e species of PI/PIP/PIP2 is naturally explained by the relative abundance of C34:1e PA. However, the other major phospholipids are derived from diacylglycerol by base activation (the Kennedy Pathway; Vance & Vance, 2004). Analogous neutral loss scans of DG were not possible, so we quantified selected DG species based on the major species detected

for PI and PC, described above. The results indicated that the DG pool appears to be comprised mostly of diacyl-species, with only small quantities of C34:1e (Supplementary Fig S4). This suggests that the major pools of PA and DG are maintained with very different molecular compositions, consistent with their differential roles as the sources of PI and other phospholipids, respectively.

C34:1e plasmanylinositols are used in *Dictyostelium* signalling pathways

Previous work has characterised important roles for the PI3K signalling pathway in the response of *Dictyostelium* amoebae to chemoattractants. Thus far, however, measurements of the inositol phospholipids themselves in this organism have rarely been attempted, with most work using *in vivo* reporters instead. Although the levels of PIP3 were too low to obtain good neutral loss spectra (see above), specifically targeting the C34:1e species produced a very good signal/noise ratio and accurate measurement was readily achievable (see Supplementary Fig S5). We first measured changes in the levels of C34:1e-PI, -PIP, -PIP2 and -PIP3 in response to added cyclic-AMP using an adenylyl cyclase mutant to reduce background levels of cyclic-AMP and thus sharpen the response (*acaA$^-$*; Fig 6). Addition of cyclic-AMP produced a very fast and transient rise in PIP3, with a minimal associated drop in PIP2. The level of PIP3 peaked at approximately 5 s. A very similar pattern of changes was seen in the response of the *Ax2* strain to a different chemoattractant, folic acid (Fig 7).

To examine the genetic dependence of the response to cyclic-AMP, we used mutants lacking key enzymes in inositol phospholipid signalling pathways. C34:1e inositol lipids were measured in these mutants before and after a 5-s stimulation with cyclic-AMP (Fig 8; note that because these mutants were created in the *Ax2* strain,
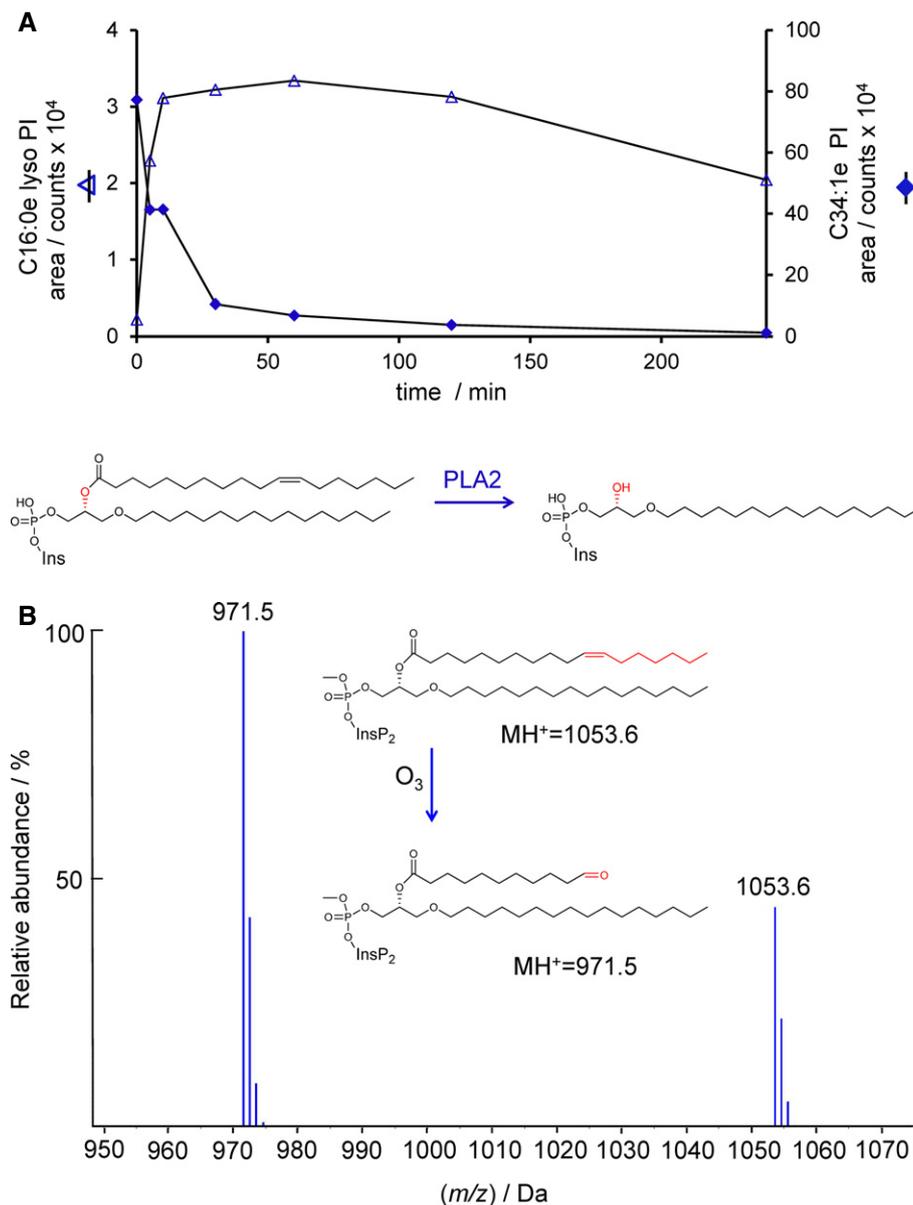
Figure 3.   Determination of the acyl chain structure in *Dictyostelium discoideum* inositol lipids.

A   The PI in *D. discoideum* is susceptible to hydrolysis by PLA2. Lipid extracts prepared from *D. discoideum* were mixed with phospholipase A2 (PLA2; from bee venom), and the levels of PI and lyso-PI measured over time by HPLC-ESI mass spectrometry. The quantitative conversion of PI to lyso-PI demonstrates the presence of an acyl chain in the *sn*-2 position.

B   The PIP2 in *D. discoideum* contains an 11-octadecenoyl acyl chain. Methylated lipid extracts prepared from *D. discoideum* were subjected to ozonolysis. The mass of the fragment generated indicates the presence of an 11-octadecenoyl chain.

endogenous production of cyclic-AMP likely led to a somewhat de-sensitised response compared to the *acaA⁻* strain described above). Loss of all of the five PI3Ks with a Ras-binding domain (Hoeller & Kay, 2007) prevented any PIP3 response to cyclic-AMP, though a basal level of PIP3 was still detectable; this indicates that these PI3Ks are the cyclic-AMP-sensitive enzymes responsible for PIP3 synthesis, but another minor pathway of PIP3 synthesis must also exist in this organism. Loss of the PIP3 phosphatase PTEN (Iijima & Devreotes, 2002) resulted in a hugely elevated basal and stimulated level of PIP3, indicating it is a major PIP3 phosphatase in this organism. Loss

of PI4P5K dramatically reduced the levels of PIP2 as previously reported (Fets *et al*, 2014), but remarkably, this diminished level could still support substantial cyclic-AMP-stimulated PIP3 synthesis, conflicting with previous measurements made using an ELISA.

Discussion

Most work on phosphoinositide signalling in *Dictyostelium* hitherto has relied on *in vivo* reporters, such as the PIP3-binding PH-domain
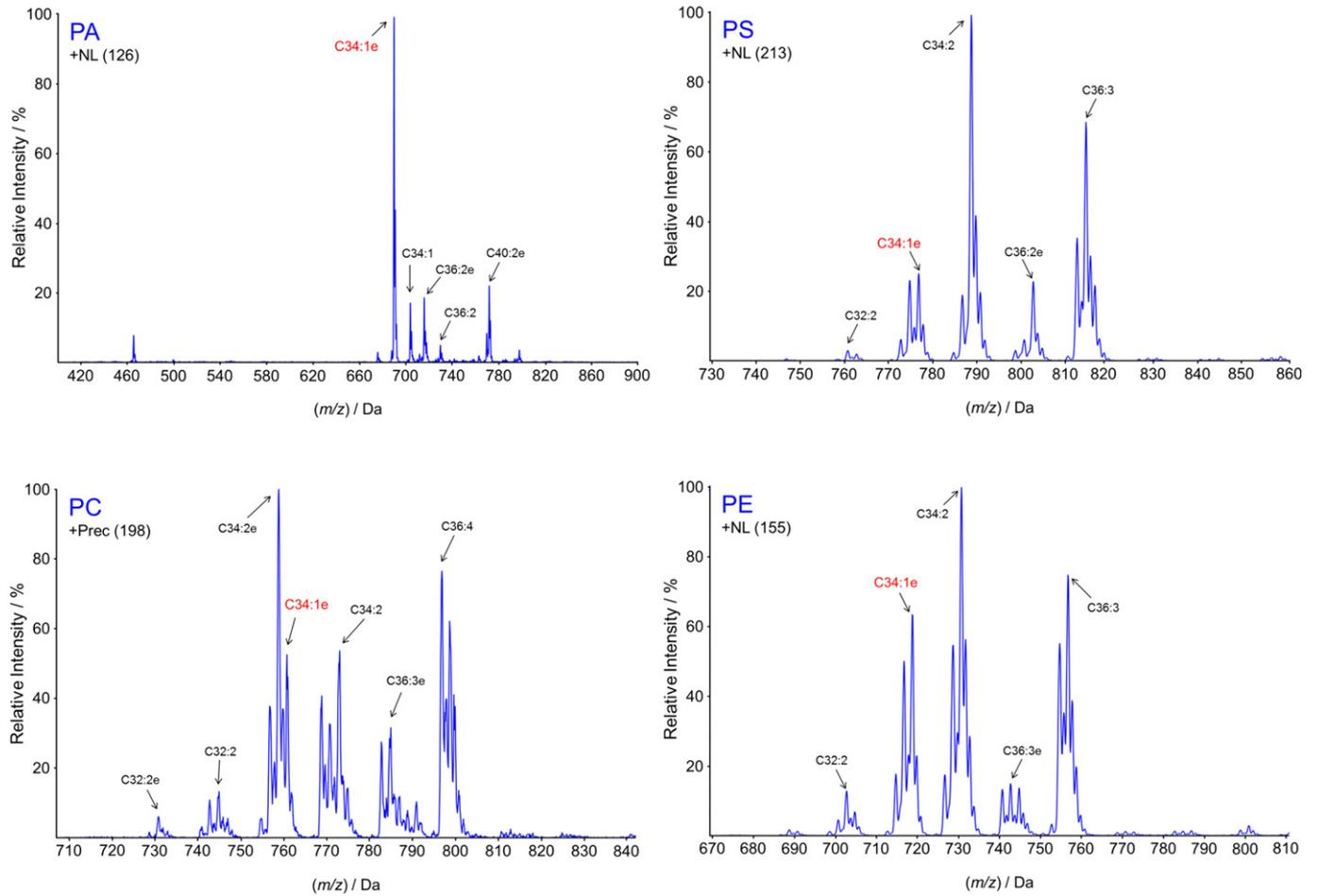
Figure 4. The molecular species of the major phospholipid classes in *Dictyostelium discoideum* are highly heterogeneous.
Neutral loss (PA, PS, PE) or precursor ion (PC) scans are shown describing the major molecular species of abundant phospholipids present in methylated lipid extracts of *D. discoideum* grown under axenic conditions (see Materials and Methods for the MRM transitions monitored). The C34:1e species highlighted in red is analogous to the major species of inositol lipids found in *D. discoideum*. Relative quantification of some of these species is given in Supplementary Fig S3.
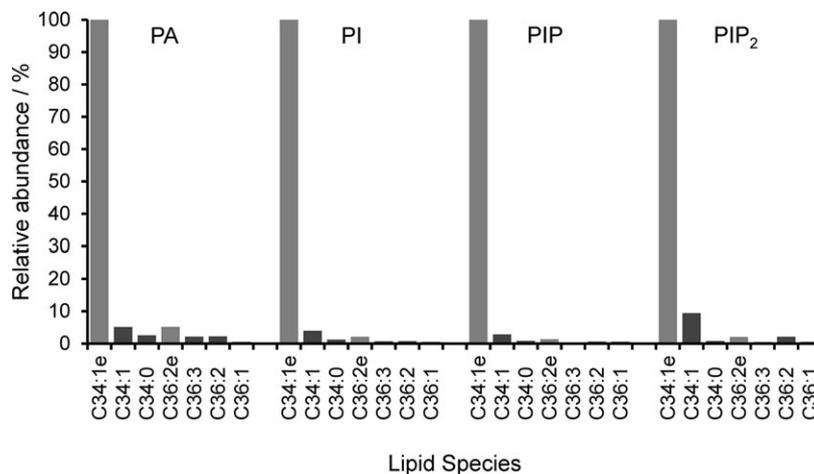
Figure 5. Measurement of the relative abundance of the major molecular species of inositol phospholipids and PA in *Dictyostelium discoideum*.
Methylated lipid extracts prepared from *D. discoideum* were grown under axenic conditions and analysed by HPLC-ESI mass spectrometry. MRM traces were integrated to provide relative abundances of the major species of PA, PI, PIP and PIP2 present.
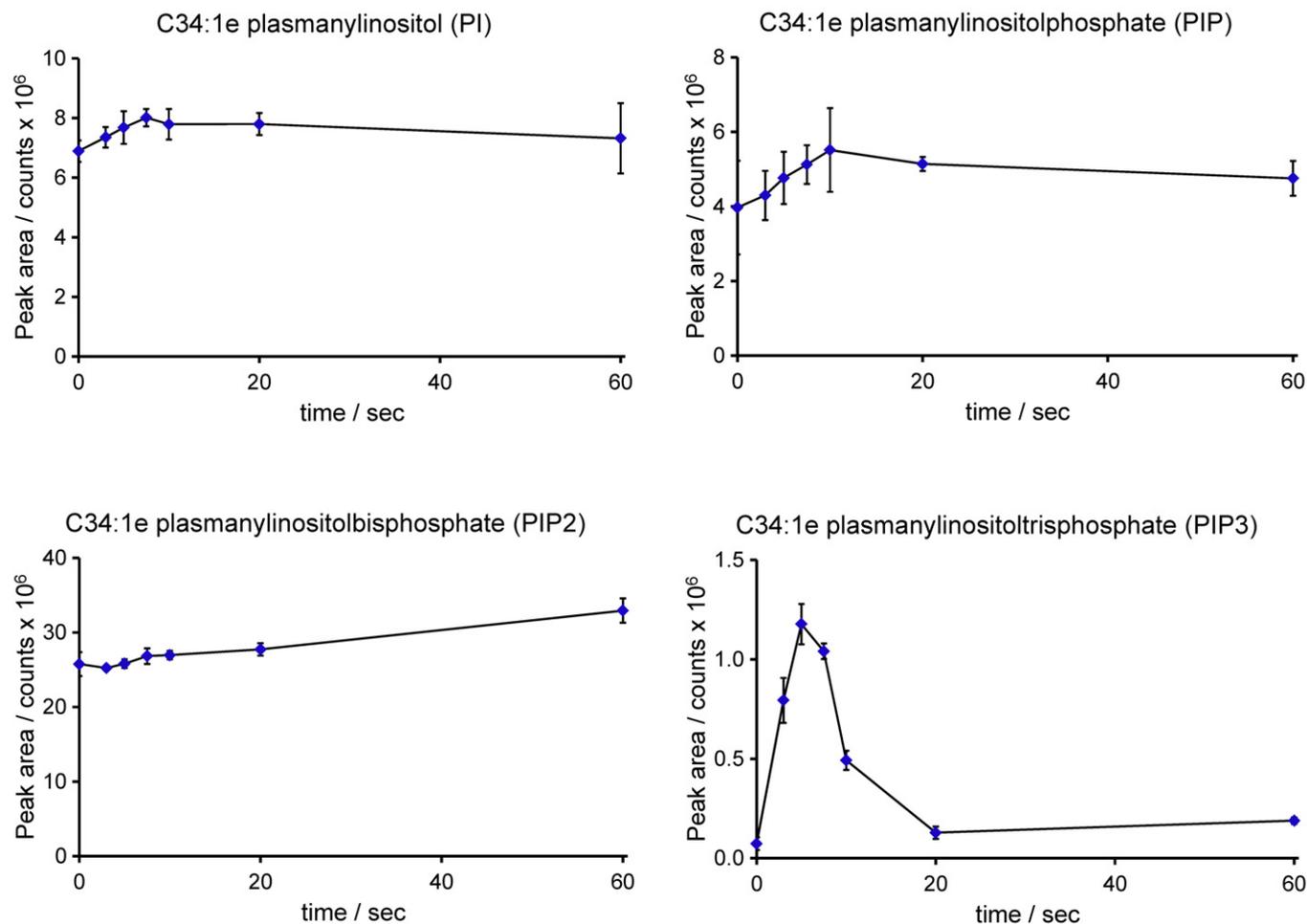
Figure 6. Changes in inositol phospholipids in response to cAMP in *Dictyostelium discoideum* (*acaA⁻*).

The *acaA⁻* strain of *D. discoideum* was starved and rendered competent to respond to cAMP. Individual samples of cells were then stimulated with 10 μM cAMP for the times shown. Methylated lipid extracts were prepared and analysed by HPLC-ESI mass spectrometry. Integrated MRM values (mean ± SD, *n* = 3 individual cell incubations) for the C34:1e species of PI, PIP, PIP2 and PIP3 are shown; for example, traces from which the integrations were performed are shown in Supplementary Fig S5. This experiment has been repeated three times with qualitatively very similar results. These data are uncorrected for differences in extraction and ionisation of different lipid classes, and so, no significance can be placed on differences between the signal intensity of PI, PIP, PIP2 or PIP3.

of CRAC fused to GFP (Parent *et al*, 1998), leaving the nature of the acyl/alkyl-glycerol backbone unknown. We have used a combination of mass spectrometry and further analytical techniques to define the major molecular species of inositol phospholipids: over 95% of the PI, PIP and PIP2 pools contain the C34:1e structure, with an ether-linked C16:0 chain (palmityl) in the *sn*-1 position and an ester-linked C18:1 chain (11-ocatadecenoyl) in the *sn*-2 position. The correct prefix for phospholipids with a saturated alkyl chain in the *sn*-1 position is plasmanyl, to distinguish them from plasmenyl-lipids (which contain a vinyl ether linkage in the *sn*-1 position, often referred to as plasmalogens) and phosphatidyl-lipids (which contain ester linkages in both *sn*-1 and 2 positions). The correct abbreviation for phosphatidylinositols is PtdIns(P)n, but often the more shorthand PI(P)n is used in the literature; we suggest the PI(P)n nomenclature is retained to include both phosphatidyl- and plasmanyl-inositols.

Ether lipids have been widely described in other organisms, for example in humans plasmalogens make up approximately 20% of total phospholipid mass, with plasmenyl ethanolamine and plasmenyl choline being particularly abundant in mammalian brain and heart, respectively (Brites *et al*, 2004). Myeloid cells such as macrophages and neutrophils are also known to contain high levels of plasmanyl choline, which is the synthetic precursor of platelet activating factor (which has a 1-O-ether linkage at *sn*-1 and an acetyl-chain at *sn*-2) (Montrucchio *et al*, 2000). However, ether-linked inositol phospholipids have only been reported previously in trace quantities (Ivanova *et al*, 2010) or, as components of glyco-phosphatidylinositol (GPI) anchors (Kanzawa *et al*, 2009), and this is the first example where they represent the major species of inositol phospholipids present in any organism or tissue. The standard methods of inositol lipid analysis rely on base induced deacylation followed by anion-exchange chromatography of glycerophospho-esters (Guillou *et al*, 2007). This approach is not appropriate for *Dictyostelium* phosphoinositide analysis because the ether linkage is resistant to these conditions, and this may explain past failures to measure significant quantities of these lipids.
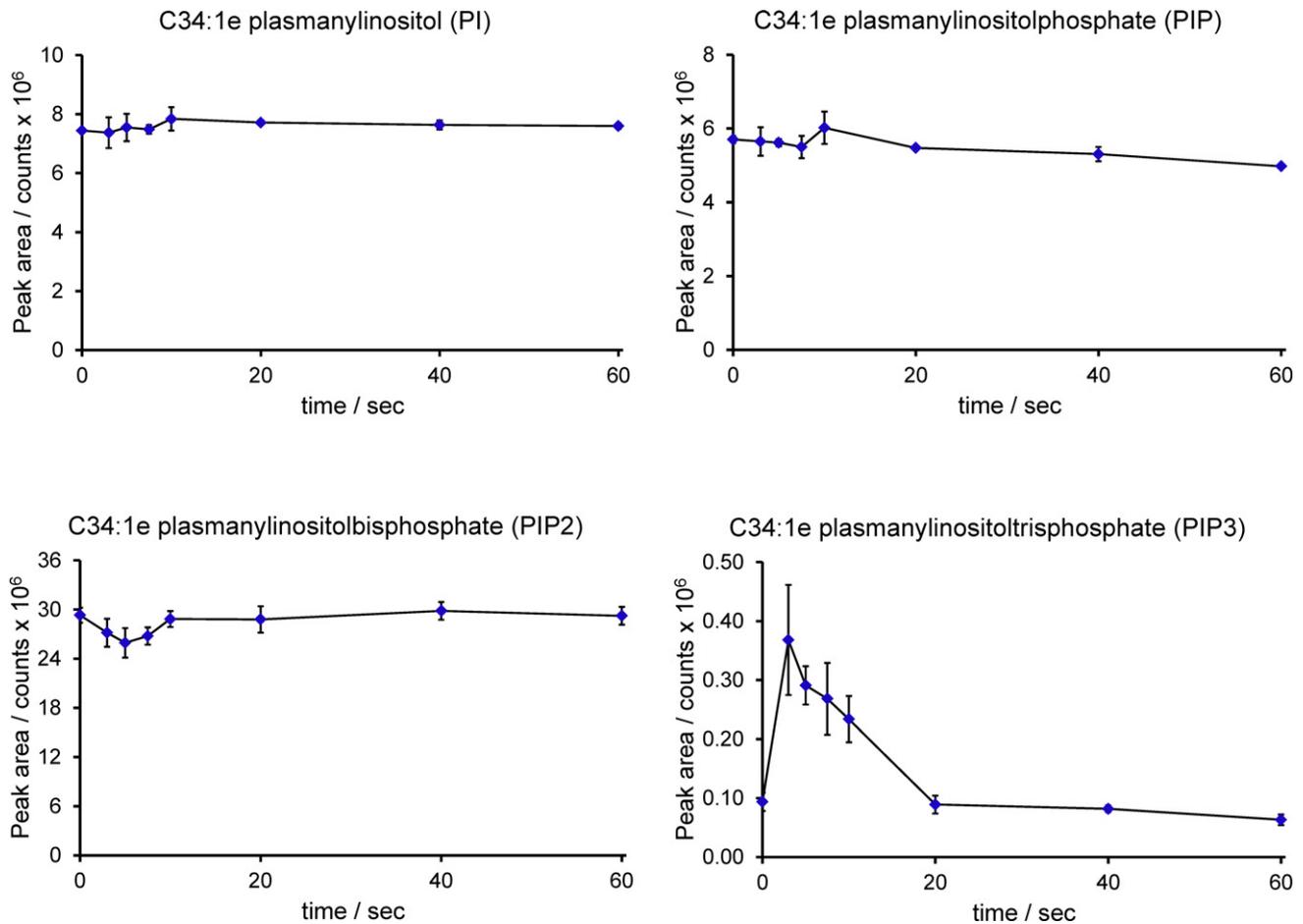
Figure 7.   Changes in inositol phospholipids in response to folic acid in *Dictyostelium discoideum* (*Ax2*).
The *Ax2* strain of *D. discoideum* was grown on bacteria and stimulated with 100 μM folic acid for the times shown. Methylated lipid extracts were prepared and analysed by HPLC-ESI mass spectrometry. Integrated MRM values (mean ± SD, n = 3 individual cell incubations) for the C34:1e species of PI, PIP, PIP2 and PIP3 are shown. This experiment has been repeated three times with qualitatively very similar results. These data are uncorrected for differences in extraction and ionisation of different lipid classes, and so, no significance can be placed on differences between the signal intensity of PI, PIP, PIP2 or PIP3.

A large body of literature implicates PI3K signalling pathways in the regulation of macropinocytosis and cell movement in *Dictyostelium*. Our results indicate that the previously identified 'Class 1' PI3Ks in this organism are responsible for chemoattractant stimulated formation of PIP3 and that the phosphatase PTEN is a major regulator of PIP3 levels. Further, our results define the extremely rapid time course for the formation of PIP3 in response to chemoattractants and imply that C34:1e PIP3 is responsible for PH-domain-mediated translocation of downstream effectors such as CRAC or PhdA (Funamoto *et al*, 2001; Parent *et al*, 1998). The relatively small and transient drop in the level of PIP2 in response to chemoattractants suggests pathways for resynthesis of this lipid are well matched to PLC and PI3K induced pathways of consumption, which is consistent with many analogous contexts in mammalian cells, although not easily reconciled with recent theories suggesting large drops in PIP2 enhance PI3K signalling via loss of membrane-bound PTEN (Kortholt *et al*, 2007). The ability to accurately measure the levels of inositol phospholipids in *Dictyostelium* should now allow future studies to gain a much better understanding of these signalling pathways in this organism.

The composition of the inositol phospholipids in *Dictyostelium* is strikingly biased, with the C34:1e configuration comprising about 95% of the total, whereas diacyl or other alkyl/acyl configurations are preferred in the other phospholipid classes. In principle, the molecular selector that causes this bias could act at various levels in the biosynthesis or turnover of these lipids, but our data suggest it acts prior to the synthesis of PA. Phosphoinositides arise from PA, which in most organisms is synthesised *de novo* and can be dephosphorylated to produce DGs. These DGs are then used for the synthesis of the other phospholipid classes (Kent, 1995). However, the PA and DG pools in *Dictyostelium* comprise very different fatty alkyl/acyl compositions. PA is mostly the C34:1e species that is highly represented in the PI pool, but the DGs contain higher proportions of the other species which predominate in other phospholipids. Ether-linked PA is synthesised via DHAP in a pathway that originates in peroxisomes (Wanders & Waterham, 2006). The key step is the formation of 1-alkyl-DHAP by alkyl-DHAP synthase from acyl-DHAP and a long chain alcohol (a reaction in which the original acyl chain is essentially swapped for the alcohol) (Nenci *et al*, 2012). Our data suggest that the predominant alcohol used for this
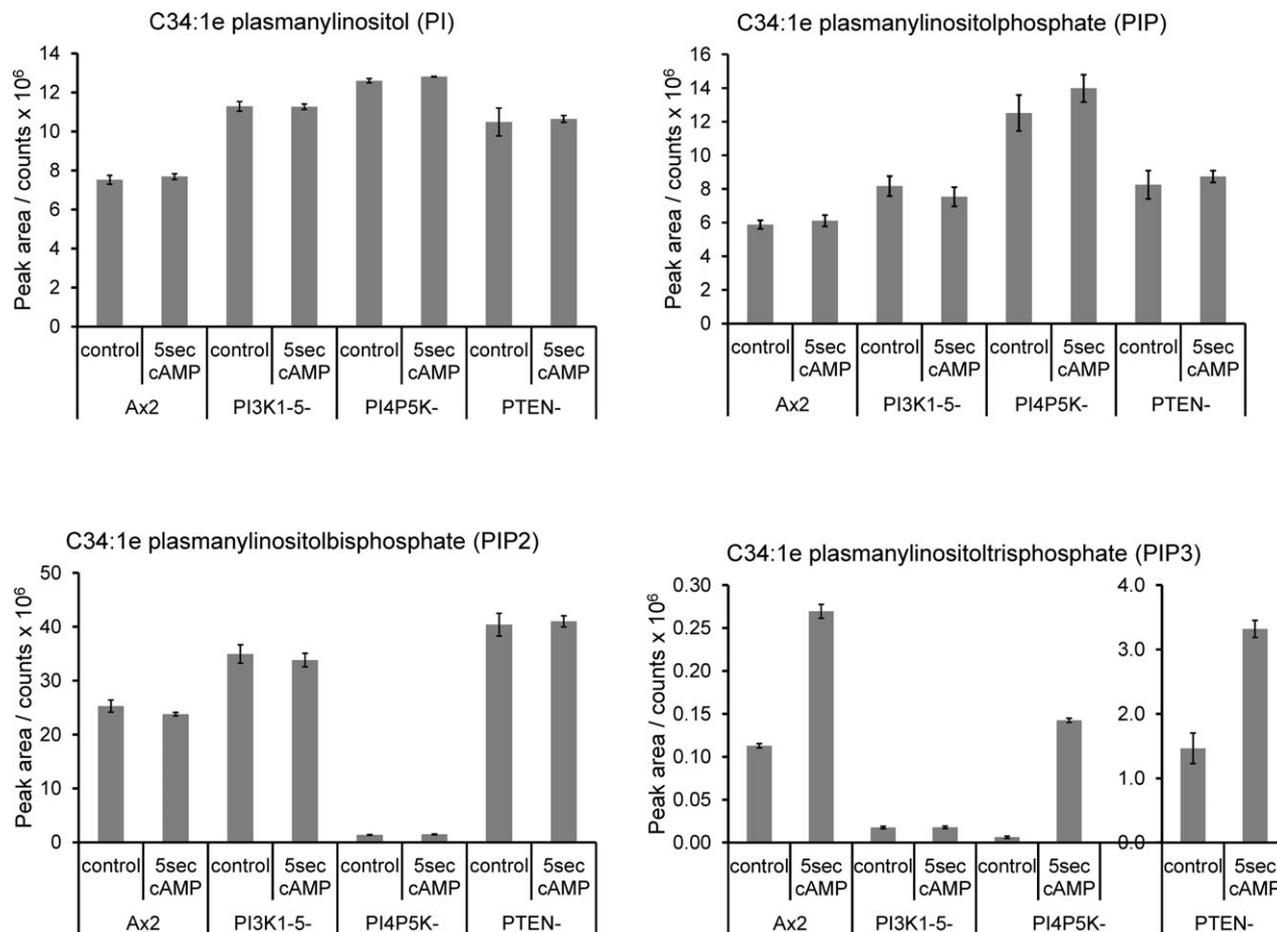
Figure 8. Changes in inositol phospholipids in response to cAMP in mutants of *Dictyostelium discoideum*.
The parental *Ax2* strain of *D. discoideum*, or the indicated mutant strains derived from it (PI3K1-5-, *pikA⁻*, *pikB⁻*, *pikC⁻*, *pikF⁻*, *pikG⁻*; PI4P5K-, *pikI⁻*; PTEN-, *ptenA⁻*) were grown on bacteria and then rendered competent to respond to cAMP. Individual samples of cells were then stimulated with 10 μM cAMP or vehicle for 5 s. Methylated lipid extracts were prepared and analysed by HPLC-ESI mass spectrometry. Integrated MRM values (mean ± SD, $n = 3$ individual cell incubations) for the C34:1e species of PI, PIP, PIP2 and PIP3 are shown. This experiment has been repeated three times with qualitatively very similar results. These data are uncorrected for differences in extraction and ionisation of different lipid classes and so no significance can be placed in differences between the signal intensity of PI, PIP, PIP2 or PIP3.

reaction in *Dictyostelium* is palmitol. Alkyl-DHAP is then thought to leave the peroxisome, after which it is reduced to 1-alkyl-glycerol 3-phosphate by an acyl/alkyl-DHAP reductase and then acylated at the sn-2 position by a fatty acyl-CoA acyl transferase located in the ER (Wanders & Waterham, 2006). Given the enrichment of delta11-ocatadecenoyl in *Dictyostelium* PA, this would predict the presence of an acyl transferase with specificity for 1-palmityl-glycerol-3-phosphate and delta11-ocatadecenoyl-CoA. In this regard, an active and unusual fatty acyl-CoA elongase has recently been described in *Dictyostelium*, which converts delta9-palmitoyl-CoA to delta11-o-catadecenoyl-CoA, providing the likely source of substrate for this reaction (Blacklock *et al*, 2008). In contrast, diacyl PA is thought to be synthesised by successive acylation of glycerol 3-phosphate directly in the ER (Shindou *et al*, 2009). It seems unlikely that there are novel and active pathways in *Dictyostelium* which re-model C34:1e-PA into diacyl DGs, and therefore, it seems most probable that there is a pool of diacyl PA which is synthesised *de novo* but does not accumulate to significant levels, perhaps because it is rapidly channelled into diacylglycerols. However, further work

will be needed to establish the synthetic route of C34:1e PA in *Dictyostelium* and delineate how the diverse pools of PA and DGs are created.

A key issue raised by our results is why evolution has driven the creation of such an unusual structure for inositol phospholipids in *Dictyostelium* and why the inositol phospholipids are relatively molecularly homogeneous and distinct from the much more hetero-geneous species of the other major classes of phospholipid. Inositol phospholipids are crucial signalling molecules designed to interact with extrinsic membrane proteins via their phosphorylated inositol lipid head groups. During macropinosome formation and in other situations, PIP3 forms a dense signalling patch on the membrane, and it is possible that the 1-O-ether linkage conveys molecular prop-erties that are particularly suited to this signalling role. The lack of a carbonyl in the sn-1 position may allow the lipid to be more tightly integrated into the membrane, alter its packing with other lipids or subtly alter the orientation of the head group. Previous studies with plasmalogens suggest ether-linked lipids are more resistant to oxidants, but this property is thought to be due primarily to the

vinyl ether bond (Brites *et al*, 2004; Zoeller *et al*, 1999), which is absent in plasmanyl lipids. The lack of an ester linkage at *sn*-1 would also make these lipids insensitive to hydrolysis by a PLA1 activity. It seems unlikely however that any such physical or chemical properties of ether-linked lipids would be more pertinent to the roles of inositol phospholipids in *Dictyostelium* than in other organisms.

There are still very few careful studies of the molecular composition of inositol phospholipids across species. Most of the work has been done in mammalian cells, where phosphatidylinositols are highly enriched in C38:4 species (C18:0, C20:4) compared to most other lipid classes (Holub, 1984; MacDonald *et al*, 1975). The mechanisms creating this enrichment are unclear, but are probably derived from the substrate specificity of key enzymes in the *de novo* synthesis pathway for PtdIns and remodelling via specific acylCoA-PtdIns transferases (Anderson *et al*, 2013; D'Souza & Epand, 2013; Lee *et al*, 2012). The specific properties conveyed by this enrichment in C38:4 species are also unclear. It has been suggested that these lipids may act as a source of PLA2-liberated C20:4 for lipoxygenases and cycloxygenases (Gijon *et al*, 2008), or it represents a mechanism to segregate PA and DG derived from inositol lipids from PA and DG involved in the metabolism of triacylglcerol or other phospholipids. Some evidence in favour of this notion is the specificity of some PKC isoforms for C38:4 DG derived from PLC-catalysed hydrolysis of PtdInsP2 (Madani *et al*, 2001). Of relevance here, neither eicosanoid biosynthesis, nor DG-regulated PKCs, have been convincingly described in *Dictyostelium*.

Dictyostelium, though having distinct phosphoinositides from mammals, also displays lipid tails chemically distinct from other phospholipids, which suggests that the evolutionary selective processes that gave rise to this distinction may have a common functional significance. Thus, it is plausible that the enrichment of C34:1e species of inositol phospholipids in *Dictyostelium* represents a molecular signature for these lipids that allows common intermediates to be distinguished by their lipid origin; thus, enabling intermediates such as PA or DG to be used as specific signals and/or selectively reused for re-synthesis of inositol phospholipids. Whatever the answers, these are clearly important questions to resolve since they impact on our understanding of how inositol phospholipid signalling pathways are organised and how they may interact with other metabolic pathways regulating lipid synthesis and nutrition.

Materials and Methods

Dictyostelium methods

Ax2 (Kay laboratory strain of *Dictyostellium discoideum*) was used as wild-type, with the following mutant strains derived from it: HM1366 (*acaA⁻*; adenylyl cyclase null, isolated by Oliver Hoeller); HM1200 (*pikA⁻*, *pikB⁻*, *pikC⁻*, *pikF⁻*, *pikG⁻*; PI3K quintuple null) (Hoeller & Kay, 2007); HM1289 (*ptenA⁻*; PTEN null, isolated by Oliver Hoeller) and HM1513 (*pikI⁻*; PIP5K null) (Fets *et al*, 2014). *Dictyostelium* cells were grown at 22°C either in shaken suspension in HL5 axenic medium + glucose (ForMedium) at 180 rpm, or on SM plates in association with *Klebsiella aerogenes* bacteria (Kay, 1987). Axenic growth was used as standard, but growth on bacteria was used for folate stimulation, the phosphoinositide signalling mutants and for *Dictyostelium purpureum*, *Dictyostelium mucoroides* and *Polysphondylium violaceum*. In some experiments, *Dictyostellium discoideum* was also grown in fully defined SIH medium (Han *et al*, 2004).

Cells were labelled with D2-palmitol by first preparing an emulsion of 25 mM D2-palmitol (from 1 M stock in chloroform) in 25 mg/ml BSA by sonicating in a bath-sonicator at 100 watts for 40 s. This emulsion was diluted to 0.4 mM D2-palmitol, 0.4 mg/ml BSA into either HL5 growth medium or KK2 (16.5 mM KH_2PO_4, 3.8 mM K_2HPO_4, 2 mM $MgSO_4$, 0.1 mM $CaCl_2$, pH 6.2); labelling was rapid with around 20% deuterated PIP2 seen after 10 min.

For lipid measurements, cells were washed free of nutrients by repeated centrifugation (200 g × 2 min) and resuspension in KK2, then starved at 10^7/ml in KK2 with shaking at 180 rpm. Folate stimulation was performed after 30 min of starvation, whereas cyclic-AMP stimulation was performed after 5.5 h, during which time the cells were pulsed with 100 nM cyclic-AMP every 6 min after the first hour, to bring them to a responsive state, then pelleted, washed once and resuspended to the original concentration in KK2. Samples were prepared in 2-ml Safelock tubes by adding 750 μl acidified chloroform–methanol (chloroform/methanol/1M HCl, 1:2:0.097) to 170 μl of cell suspension and stored on dry ice for further

Table 1. The parent masses, neutral loss masses and glycerol fragment ions for the different mass spectrometry experiments used are shown in the table.

Lipid chain	Neutral loss mass	213.03	155.03	198.09	126	274	382.04	490.05	598
	Glycerol MRM fragment	PS	PE	PC	PA	PI	PIP	PIP2	PIP3
C34:1 ae	563.503	776.53	718.53	761.59	689.5	837.503	945.543	1053.6	1161.5
C34:1 aa	577.535	790.57	732.57	775.63	703.54	851.535	959.575	1067.6	1175.54
C36:3 ae	579.53	792.56	734.56	777.62	705.53	853.53	961.57	1069.6	1177.53
C36:2 ae	589.52	802.55	744.55	787.61	715.52	863.52	971.56	1079.6	1187.52
C36:1 ae	591.55	804.58	746.58	789.64	717.55	865.55	973.59	1081.6	1189.55
C36:3 aa	601.519	814.55	756.55	799.61	727.52	875.519	983.559	1091.6	1199.52
C36:2 aa	603.535	816.57	758.57	801.63	729.54	877.535	985.575	1093.6	1201.54
C36:1 aa	605.55	818.58	760.58	803.64	731.55	879.55	987.59	1095.6	1203.55
		Parent mass for each species							

processing. In stimulation experiments, 160 µl cells were added to 10 µl of 170 µM cyclic-AMP or 1.7 mM folic acid (to give 10 µM and 100 µM respectively), then the response was terminated as above. All samples were prepared in triplicate. Protein was determined after solubilisation of pellets in 10 mM NaOH using the Bradford dye-binding assay, with BSA as a standard.

Lipid extraction and methylation

Incubations of *Dictyostelium discoideum* (170 µl) terminated as above, and forming a single phase was extracted using an acidified Folch phase partition, as described previously (Folch *et al*, 1957; Kielkowska *et al*, 2014). The final lower phase from the lipid extraction (approx. 1 ml) was mixed with 50 µl TMS-diazomethane (hazardous) for 10 min at RT; excess TMS-diazomethane was then quenched with 6 µl acetic acid and lipids re-extracted through a neutral pH Folch phase partition, as described previously (Clark *et al*, 2011; Kielkowska *et al*, 2014). The final lower phases were dried *in vacuo* or under a stream of N_2 and then lipids re-dissolved by bath sonication in 100 µl of methanol:H_2O (80:20 v/v).

HPLC-ESI mass spectrometry

Methylated lipid extracts (typically 40 µl) were separated by HPLC on an in-line C4 column before infusion into a triple quadrapole mass spectrometer in positive ion mode, as described previously (Clark *et al*, 2011; Kielkowska *et al*, 2014).

Mass spectrometer parameters used were the same as described in Clark *et al* (2011) unless specified otherwise. Fragmentations were carried out with a range of collision energies using the same settings for the other machine parameters, as described previously (Clark *et al*, 2011; Kielkowska *et al*, 2014). MRM transitions monitored and neutral loss masses are shown in the Table 1. Note that the PC data were acquired as parents of the 198.09.

Synthesis of d$_2$-palmitol

Methyl palmitate (2.5 g, 9.24 mmol) was dissolved in 20 ml dry tetrahydrofuran and cooled in an ice bath. 1 M lithium aluminium deuteride (20 ml, 20 mmol) was added in tetrahydrofuran under an inert atmosphere. The mixture was stirred for 30 min, then removed from the ice bath, allowed to warm up and stirred further at room temperature for 1 h. The mixture was then poured into 100 ml ice and 50 ml 2 M hydrochloric acid added, extracted into ethyl acetate, dried over magnesium sulphate and concentrated under vacuum. Yield was 2.1 g, 93%. GC-MS data showed the compound to be correct, 226.27 (M-18)$^+$. Fragmentation data were as expected by comparison with the literature. Purity by GC and TLC was greater than 90%.

PLA2 hydrolysis

Preparation of the liposomes: lipids from 4×10^7 cells grown under axenic conditions were extracted (acid Folch), dried under nitrogen and then dried further under vacuum for 2 h. 1.8 ml of HEPES buffer (20 mM, pH 7.2) and 200 µl of 1 M CaCl$_2$ were added, and then, the solution was vortexed and bath sonnicated for a total time of 15 min. The sample was then subjected to nine cycles of freezing in $CO_{2(s)}$/acetone and thawing, which yielded a cloudy solution of liposomes; the liposomes were stored at −20°C until use.

A stock solution of phospholipase A2 was prepared (Sigma-Aldrich; 1 µg/µl in H_2O). 10 µl of PLA2 solution was mixed with 50 µl of liposomes and samples incubated at room temperature for 5, 10, 30, 60, 120 or 240 min (a sample without PLA2 was prepared as a control). Enzyme activity was quenched with 170 µl 2 M HCl, and then, each sample was vortexed and prepared for HPLC-ESI mass spectrometry, as described above.

Ozonolysis

Ozone was generated at the anode of an electrolytic cell made from two platinum electrodes separated by a glass walled tube in 0.4 M sulphuric acid. The ozone was carried into the samples using a flow of nitrogen across the top of the glass tube containing the anode. A standard laboratory power pack was used set at 30 V, and a current of between 500 and 700 mA was measured.

Samples were extracted and methylated as usual and suspended in 200 µl methanol. This was then cooled to −78°C, and the ozone/nitrogen mix bubbled into the samples for 45 min. 2 µl dimethyl sulphide was then added and the sample allowed to warm up to room temperature, when 40 µl water was added and the sample loaded onto the mass spectrometer.

Author contributions

JC, RRK, PTH and LRS designed the study, performed experiments and wrote the paper. AK designed and performed experiments and contributed to writing the paper. IN synthesised critical reagents. LF provided a valuable mutant. DO performed high-resolution mass spectroscopy.

Conflict of interest

The authors declare that they have no conflict of interest.

References

Anderson KE, Kielkowska A, Durrant TN, Juvin V, Clark J, Stephens LR, Hawkins PT (2013) Lysophosphatidylinositol-acyltransferase-1 (LPIAT1) is required to maintain physiological levels of PtdIns and PtdInsP(2) in the mouse. *PLoS One* 8: e58425

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

Blacklock BJ, Kelley D, Patel S (2008) A fatty acid elongase ELO with novel activity from *Dictyostelium discoideum*. *Biochem Biophysl Res Comm* 374: 226–230

Brites P, Waterham HR, Wanders RJ (2004) Functions and biosynthesis of plasmalogens in health and disease. *Biochim Biophys Acta* 1636: 219−231

Brown JR, Auger KR (2011) Phylogenomics of phosphoinositide lipid kinases: perspectives on the evolution of second messenger signaling and drug discovery. *BMC Evol Biol* 11: 4

Buczynski G, Grove B, Nomura A, Kleve M, Bush J, Firtel RA, Cardelli J (1997) Inactivation of two *Dictyostelium discoideum* genes, DdPIK1 and DdPIK2, encoding proteins related to mammalian phosphatidylinositide 3-kinases, results in defects in endocytosis, lysosome to postlysosome transport, and actin cytoskeleton organization. *J Cell Biol* 136: 1271−1286

Burman C, Ktistakis NT (2010) Regulation of autophagy by phosphatidylinositol 3-phosphate. *FEBS Lett* 584: 1302−1312

Clark J, Anderson KE, Juvin V, Smith TS, Karpe F, Wakelam MJ, Stephens LR, Hawkins PT (2011) Quantification of PtdInsP3 molecular species in cells and tissues by mass spectrometry. *Nat Meth* 8: 267−272

Di Paolo G, De Camilli P (2006) Phosphoinositides in cell regulation and membrane dynamics. *Nature* 443: 651−657

Dormann D, Weijer G, Dowler S, Weijer CJ (2004) In vivo analysis of 3-phosphoinositide dynamics during *Dictyostelium* phagocytosis and chemotaxis. *J Cell Sci* 117: 6497−6509

D'Souza K, Epand RM (2013) Enrichment of phosphatidylinositols with specific acyl chains. *Biochim Biophys Acta* 1838: 1501−1508

Engelman JA, Luo J, Cantley LC (2006) The evolution of phosphatidylinositol 3-kinases as regulators of growth and metabolism. *Nat Rev Genet* 7: 606−619

Fets L, Nichols JM, Kay RR (2014) A PIP5 kinase essential for efficient chemotactic signaling. *Curr Biol* 24: 415−421

Folch J, Lees M, Sloane Stanley GH (1957) A simple method for the isolation and purification of total lipides from animal tissues. *J Biol Chem* 226: 497−509

Funamoto S, Milan K, Meili R, Firtel RA (2001) Role of phosphatidylinositol 3′ kinase and a downstream pleckstrin homology domain-containing protein in controlling chemotaxis in *Dictyostelium*. *J Cell Biol* 153: 795−810

Gijon MA, Riekhof WR, Zarini S, Murphy RC, Voelker DR (2008) Lysophospholipid acyltransferases and arachidonate recycling in human neutrophils. *J Biol Chem* 283: 30235−30245

Guillou H, Stephens LR, Hawkins PT (2007) Quantitative measurement of phosphatidylinositol 3,4,5-trisphosphate. *Meth Enzymol* 434: 117−130

Han SI, Friehs K, Flaschel E (2004) Improvement of a synthetic medium for *Dictyostelium discoideum*. *Process Biochem* 39: 925−930

Hawkins PT, Anderson KE, Davidson K, Stephens LR (2006) Signalling through Class I PI3Ks in mammalian cells. *Biochem Soc Trans* 34: 647−662

Hoeller O, Kay RR (2007) Chemotaxis in the absence of PIP3 gradients. *Curr Biol* 17: 813−817

Hoeller O, Bolourani P, Clark J, Stephens LR, Hawkins PT, Weiner OD, Weeks G, Kay RR (2013) Two distinct functions for PI3-kinases in macropinocytosis. *J Cell Sci* 126: 4296−4307

Holub BJ (1984) 1982 Borden Award lecture. Nutritional, biochemical, and clinical aspects of inositol and phosphatidylinositol metabolism. *Can J Physiol Pharmacol* 62: 1−8

Holub BJ (1986) Metabolism and function of myo-inositol and inositol phospholipids. *Ann Rev Nutr* 6: 563−597

Iijima M, Devreotes P (2002) Tumor suppressor PTEN mediates sensing of chemoattractant gradients. *Cell* 109: 599−610

Insall R, Kuspa A, Lilly PJ, Shaulsky G, Levin LR, Loomis WF, Devreotes P (1994) CRAC, a cytosolic protein containing a pleckstrin homology domain, is required for receptor and G protein-mediated activation of adenylyl cyclase in *Dictyostelium*. *J Cell Biol* 126: 1537−1545

Ivanova PT, Milne SB, Brown HA (2010) Identification of atypical ether-linked glycerophospholipid species in macrophages by mass spectrometry. *J Lipid Res* 51: 1581−1590

Kadamur G, Ross EM (2013) Mammalian phospholipase C. *Ann Rev Physiol* 75: 127−154

Kanzawa N, Maeda Y, Ogiso H, Murakami Y, Taguchi R, Kinoshita T (2009) Peroxisome dependency of alkyl-containing GPI-anchor biosynthesis in the endoplasmic reticulum. *Proc Natl Acad Sci USA* 106: 17711−17716

Kay RR (1987) Cell differentiation in monolayers and the investigation of slime mold morphogens. *Meth Cell Biol* 28: 433−448

Kay RR, Langridge P, Traynor D, Hoeller O (2008) Changing directions in the study of chemotaxis. *Nat Rev Mol Cell Biol* 9: 455−463

Kent C (1995) Eukaryotic phospholipid biosynthesis. *Annu Rev Biochem* 64: 315−343

Kielkowska A, Niewczas I, Anderson KE, Durrant TN, Clark J, Stephens LR, Hawkins PT (2014) A new approach to measuring phosphoinositides in cells by mass spectrometry. *Adv Biol Reg* 54: 131−141

King JS, Insall RH (2009) Chemotaxis: finding the way forward with *Dictyostelium*. *Trend Cell Biol* 19: 523−530

Kortholt A, King JS, Keizer-Gunnink I, Harwood AJ, Van Haastert PJ (2007) Phospholipase C regulation of phosphatidylinositol 3,4,5-trisphosphate-mediated chemotaxis. *Mol Biol Cell* 18: 4772−4779

Lee HC, Inoue T, Sasaki J, Kubo T, Matsuda S, Nakasaki Y, Hattori M, Tanaka F, Udagawa O, Kono N, Itoh T, Ogiso H, Taguchi R, Arita M, Sasaki T, Arai H (2012) LPIAT1 regulates arachidonic acid content in phosphatidylinositol and is required for cortical lamination in mice. *Mol Biol Cell* 23: 4689−4700

Lemmon MA (2008) Membrane recognition by phospholipid-binding domains. *Nat Rev Mol Cell Biol* 9: 99−111

Loovers HM, Postma M, Keizer-Gunnink I, Huang YE, Devreotes PN, van Haastert PJ (2006) Distinct roles of PI(3,4,5)P3 during chemoattractant signaling in *Dictyostelium*: a quantitative *in vivo* analysis by inhibition of PI3-kinase. *Mol Biol Cel* 17: 1503−1513

MacDonald G, Baker RR, Thompson W (1975) Selective synthesis of molecular classes of phosphatidic acid, diacylglycerol and phosphatidylinositol in rat brain. *J Neurochem* 24: 655−661

Madani S, Hichami A, Legrand A, Belleville J, Khan NA (2001) Implication of acyl chain of diacylglycerols in activation of different isoforms of protein kinase C. *FASEB J* 15: 2595−2601

Meili R, Ellsworth C, Firtel RA (2000) A novel Akt/PKB-related kinase is essential for morphogenesis in *Dictyostelium*. *Curr Biol: CB* 10: 708−717

Michell RH (2008) Inositol derivatives: evolution and functions. *Nat Rev Mol Cell Biol* 9: 151−161

Milne SB, Ivanova PT, DeCamp D, Hsueh RC, Brown HA (2005) A targeted mass spectrometric analysis of phosphatidylinositol phosphate species. *J Lipid Res* 46: 1796−1802

Montrucchio G, Alloatti G, Camussi G (2000) Role of platelet-activating factor in cardiovascular pathophysiology. *Physiol Rev* 80: 1669−1699

Muller-Taubenberger A, Kortholt A, Eichinger L (2013) Simple system−substantial share: the use of Dictyostelium in cell biology and molecular medicine. *Eur J Cell Biol* 92: 45−53

Nenci S, Piano V, Rosati S, Aliverti A, Pandini V, Fraaije MW, Heck AJ, Edmondson DE, Mattevi A (2012) Precursor of ether phospholipids is

synthesized by a flavoenzyme through covalent catalysis. *Proc Natl Acad Sci USA* 109: 18791−18796

Parent CA, Blacklock BJ, Froelich WM, Murphy DB, Devreotes PN (1998) G Protein signaling events are activated at the leading edge of chemotactic cells. *Cell* 95: 81−91

Paulus H, Kennedy EP (1960) The enzymatic synthesis of inositol monophosphatide. *J Biol Chem* 235: 1303−1311

Pettitt TR, Dove SK, Lubben A, Calaminus SD, Wakelam MJ (2006) Analysis of intact phosphoinositides in biological samples. *J Lipid Res* 47: 1588−1596

Raiborg C, Schink KO, Stenmark H (2013) Class III phosphatidylinositol 3-kinase and its catalytic product PtdIns3P in regulation of endocytic membrane traffic. *FEBS J* 280: 2730−2742

Rouzer CA, Ivanova PT, Byrne MO, Milne SB, Marnett LJ, Brown HA (2006) Lipid profiling reveals arachidonate deficiency in RAW264.7 cells: structural and functional implications. *Biochem* 45: 14795−14808

Saarikangas J, Zhao H, Lappalainen P (2010) Regulation of the actin cytoskeleton-plasma membrane interplay by phosphoinositides. *Physiol Rev* 90: 259−289

Saito S, Goto K, Tonosaki A, Kondo H (1997) Gene cloning and characterization of CDP-diacylglycerol synthase from rat brain. *J Biol Chem* 272: 9503−9509

Scott DL, Otwinowski Z, Gelb MH, Sigler PB (1990) Crystal structure of bee-venom phospholipase A2 in a complex with a transition-state analogue. *Science* 250: 1563−1566

Shindou H, Hishikawa D, Harayama T, Yuki K, Shimizu T (2009) Recent progress on acyl CoA: lysophospholipid acyltransferase research. *J Lipid Res* 50(Suppl): S46−S51

Takeda K, Sasaki AT, Ha H, Seung HA, Firtel RA (2007) Role of phosphatidylinositol 3-kinases in chemotaxis in *Dictyostelium*. *J Biol Chem* 282: 11874−11884

Vance JE, Vance DE (2004) Phospholipid biosynthesis in mammalian cells. *Biochem Cell Biol* 82: 113−128

Veltman DM, Lemieux MG, Knecht DA, Insall RH (2014) PIP3-dependent macropinocytosis is incompatible with chemotaxis. *J Cell Biol* 204: 497−505

Wanders RJ, Waterham HR (2006) Biochemistry of mammalian peroxisomes revisited. *Ann Rev Biochem* 75: 295−332

Zatulovskiy E, Tyson R, Bretschneider T, Kay RR (2014) Bleb-driven chemotaxis of *Dictyostelium* cells. *J Cell Biol* 204: 1027−1044

Zhang P, Wang Y, Sesaki H, Iijima M (2010) Proteomic identification of phosphatidylinositol (3,4,5) triphosphate-binding proteins in *Dictyostelium discoideum*. *Proc Natl Acad Sci USA* 107: 11829−11834

Zhou KM, Takegawa K, Emr SD, Firtel RA (1995) A phosphatidylinositol (PI) kinase gene family in *Dictyostelium discoideum*: biological roles of putative mammalian p110 and yeast Vps34p PI 3-kinase homologs during growth and development. *Mol Cell Biol* 15: 5645−5656

Zhou K, Pandol S, Bokoch G, Traynor KA (1998) Disruption of *Dictyostelium* PI3K genes reduces [32P]phosphatidylinositol 3,4 bisphosphate and [32P] phosphatidylinositol trisphosphate levels, alters F-actin distribution and impairs pinocytosis. *J Cell Sci* 111: 283−294

Zoeller RA, Lake AC, Nagan N, Gaposchkin DP, Legner MA, Lieberthal W (1999) Plasmalogens as endogenous antioxidants: somatic cell mutants reveal the importance of the vinyl ether. *Biochem J* 338: 769−776