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A new approach to measuring phosphoinositides in cells by mass spectrometry

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A B S T R A C T

The phosphoinositide family of phospholipids, defined here as PtdIns, PtdIns3P, PtdIns4P, PtdIns5P, PtdIns(3,4)P_2, PtdIns(3,5)P_2, PtdIns(4,5)P_2 and PtdIns(3,4,5)P_3, play pivotal roles in organising the location and activity of many different proteins acting on biological membranes, including those involved in vesicle and protein trafficking through the endolysosomal system and receptor signal transduction at the plasma membrane. Accurate measurement of the cellular levels of these lipids, particularly the more highly phosphorylated species, is hampered by their high polarity and low cellular concentrations. Recently, much progress has been made in using mass spectrometry to measure many different lipid classes in parallel, an approach generally referred to as 'lipidomics'. Unfortunately, the acidic nature of highly phosphorylated phosphoinositides makes them difficult to measure using these methods, because they yield low levels of useful ions; this is particularly the case with PtdIns(3,4,5)P_3. We have solved some of these problems by methylating the phosphate groups of these lipids with TMS-diazomethane and describe a simple, integrated approach to measuring PtdIns, PtdInsP, PtdInsP_2 and PtdInsP_3 classes of lipids, in parallel with other phospholipid species, in cell and tissue extracts. This methodology is sensitive, accurate and

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robust, and also yields fatty-acyl compositions, suggesting it can be used to further our understanding of both the normal and pathophysiological roles of these important lipids.

Introduction

Inositol lipids, or phosphoinositides, comprise a distinct family of eight phospholipids, one or more of which are found in all eukaryotic cells; these are PtdIns, PtdIns3P, PtdIns4P, PtdIns5P, PtdIns(3,4)P_2, PtdIns(3,5)P_2, PtdIns(4,5)P_2 and PtdIns(3,4,5)P_3 (Fig. 1). They are interconverted by lipid kinases, phosphatases and phospholipases which acutely regulate their levels in response to different environmental cues. Several, perhaps all, of these lipids play major regulatory roles in cells by dictating the localisation and function of proteins which act on the membrane in which they reside (Di Paolo and De Camilli, 2006). In most examples studied to date, this involves the specific recognition of a phosphorylated inositol headgroup by a conserved protein domain, for example the binding of the PtdIns(4,5)P_2 or PtdIns(3,4,5)P_3 headgroups by distinct families of pleckstrin homology (PH) domains (Lemmon, 2008). Moreover, at least two of these lipids are involved in reactions which represent the rate limiting steps in the generation of 'second-messengers' in response to activation of cell surface

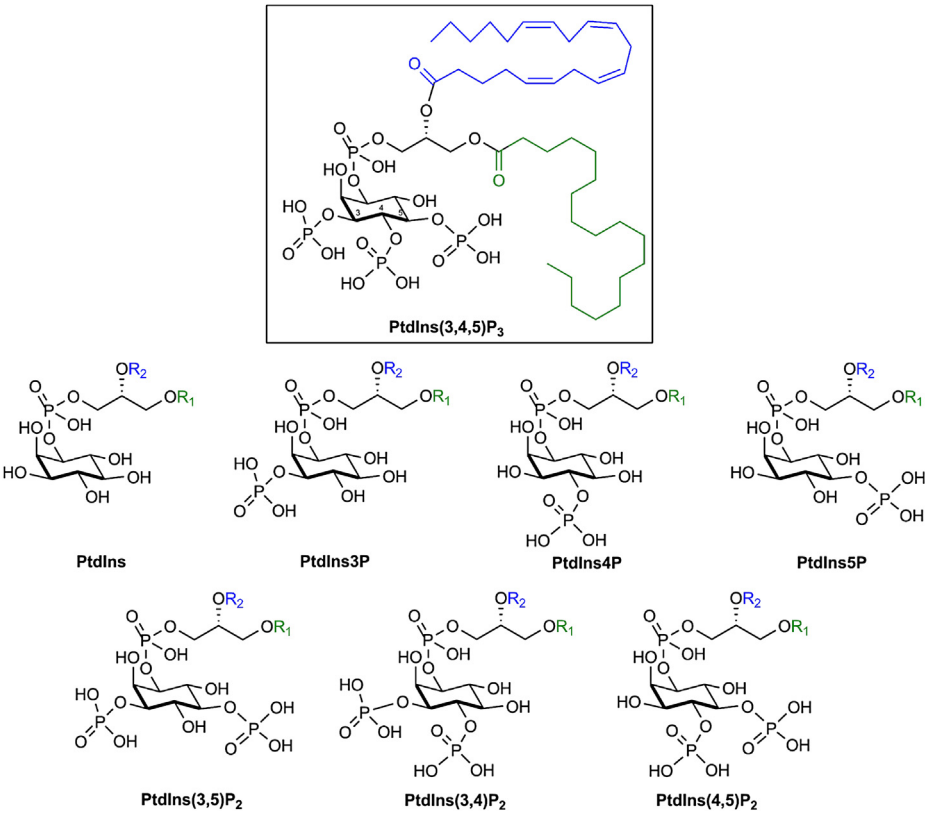


Fig. 1. The structures of phosphoinositides found in eukaryotic cells. The structure of PtdIns(3,4,5)P_3 is shown with 18:0 (stearoyl, green) and 20:4 (arachidonoyl, blue) acyl groups at the *sn*-1 and *sn*-2 positions, respectively, as an example of the major molecular species found in mammalian cells. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

receptors, namely the phospholipase C-catalysed cleavage of PtdIns(4,5)P_2 to form diacylglycerol (DAG) and Ins(1,4,5)P_3 (Kadamur and Ross, 2013) and Class I phosphoinositide 3-kinase (PI3K)-catalysed phosphorylation of PtdIns(4,5)P_2 to PtdIns(3,4,5)P_3 (Hawkins et al., 2006). DAG, Ins(1,4,5)P_3 and PtdIns(3,4,5)P_3 are now well established to play major roles in coordinating cellular responses to a huge range of different extracellular stimuli.

Despite major advances in our understanding of the cell biology and enzymology of this important family of phospholipids, there are still surprisingly few studies which focus on the measurement of the cellular levels of the lipids themselves. There are several reasons for this, but they mostly stem from problems associated with the relatively low cellular levels of the more highly phosphorylated phosphoinositides (e.g. PtdIns(4,5)P_2 and PtdIns(3,4,5)P_3 are usually present at less than 1% and 0.05% respectively, of total cellular phospholipids in mammalian cells) (Stephens et al., 1993). Thus, the most effective methods for measuring these lipids have usually used some form of enzyme-linked assay, or radiolabelling with ^{32}P-Pi or ^{3}H-inositol precursors followed by HPLC of deacylated headgroups (Guillou et al., 2007; Jones et al., 2013; Rusten and Stenmark, 2006; Sauer et al., 2009; Stephens et al., 1991). Each of these methods requires specialised reagents, or is very laborious, and has limited applicability across a range of sample formats (e.g. radiolabelling usually excludes the use of tissue biopsies). The recent development of systematic approaches to measuring lipids in cells by mass spectrometry, 'lipidomics', offers the promise of a new approach to this problem with high sensitivity, high throughput and large dynamic range (Ivanova et al., 2009, Postle et al., 2007, Wenk et al., 2003). However, despite some progress, this approach has been hampered by the low yield of ions generated in the mass spectrometer derived from the more highly phosphorylated phosphoinositides, particularly PtdInsP$_3$ and PtdInsP$_2$ (Milne et al., 2005; Pettitt et al., 2006; Vadnal and Parthasarathy, 1989).

We have recently solved this problem using chemical derivatisation of the acidic phosphate groups on these lipids (Anderson et al., 2013, Clark et al., 2011). This method allows sensitive detection of all phosphoinositide classes in parallel to other phospholipid families, but it does not yet allow resolution of different regio-isomers (i.e. resolution of the 3-, 4-, 5- species of PtdInsP or (3,4)-, (3,5)- and (4,5)-species of PtdInsP$_2$). This means, for mammalian cells at least, that PtdInsP and PtdInsP$_2$ estimates will be dominated by the most abundant isomers of these classes, PtdIns4P and PtdIns(4,5)P_2, respectively. However, since PtdIns(3,4,5)P_3 is the only PtdInsP$_3$ isomer found in eukaryotic cells, this lipid is measured without ambiguity. Moreover, phospholipid classes are known to represent families of molecular species which differ in their fatty-acyl composition (Shindou et al., 2009, van Meer et al., 2008). Thus, precise measurement of the masses of these species using mass spectrometry allows for the first time a systematic analysis of the fatty-acyl composition of PtdInsP$_2$ and PtdInsP$_3$.

We present here an overview of this methodology, explaining the rationale behind some of the steps involved and our recent experience of applying it across a range of different cell and tissue types.

An integrated approach to measuring polyphosphoinositides

An overview of the methodology we have developed is shown in Fig. 2. Technical details of individual steps can be found in (Anderson, Kielkowska, 2013; Clark, Anderson, 2011).

Extraction of lipids

We have found the most simple and effective method for quantitative extraction of all phosphoinositides and most bulk phospholipids from cell and tissue samples uses an acidified, two phase solvent system, first described by Folch (Folch et al., 1957). The use of acidification to suppress the ionisation of polyphosphoinositide phosphate groups, together with the relatively polar organic phase generated by this method, allows more effective partition of the highly polar phosphorylated phosphoinositides. Moreover, the acidified, methanol-containing organic phase was found to be an ideal environment for the subsequent derivatisation reaction using TMS-diazomethane (see below).

Solid tissue (typically 0.1–0.5 mg wet weight, initially frozen in liquid nitrogen) or cell suspensions (typically 10^5–10^6 cells) are initially extracted in a solvent mixture to yield a single phase containing chloroform, methanol and acidified aqueous solution in the proportions 1.25:2.5:1.0 (v/v). Any internal lipid standards (ISDs, see below) are added to this single phase at this point. We have often found bath

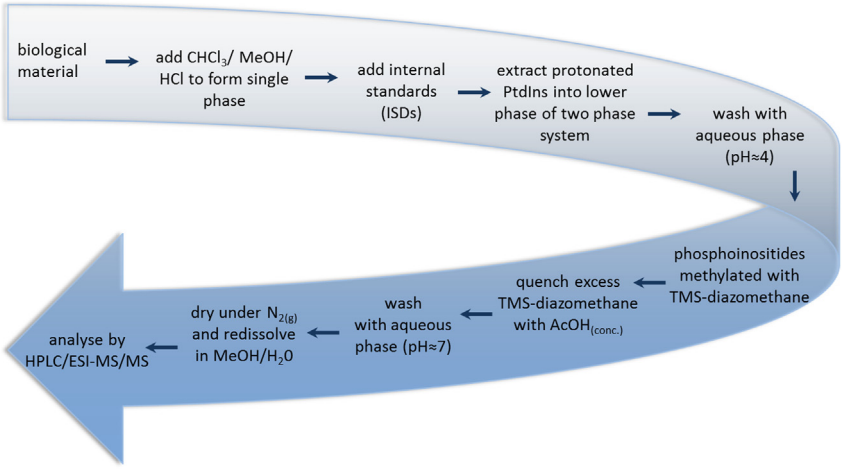


Fig. 2. A flow diagram illustrating the overall procedure for extracting, derivatising and measuring phosphoinositides by mass spectrometry.

or even probe sonication is required to break up tightly packed pellets in this solvent mixture, which is essential for effective lipid extraction. In cases where the solvent mixture will dissolve plastic containers (e.g. tissue culture dishes) we have found cell reactions can be terminated by the addition of ice-cold 1 M HCl; the cells can then be scraped, transferred to polypropylene tubes and cell pellets collected by centrifugation.

This single phase is then split by the addition of further chloroform, methanol and 0.1 M HCl to yield final proportions of chloroform, methanol and acidified aqueous solution of 8:4:3 (v/v). After thorough mixing and brief centrifugation, this yields a two phase system in which the lipids of interest are quantitatively extracted into the organic phase. After washing it with acidified aqueous phase, the organic phase is carefully removed for derivatisation.

We have found this method is effective at quantitatively extracting phosphoinositides from a wide range of plant, animal tissues and cells. However, in some cases, where the total lipid content of the tissue is dominated by neutral lipids (like triglyceride in adipose tissue, or surfactant in bronchiolar lavages), the sensitivity of PtdIns(3,4,5)P_3 measurement can be substantially improved by first extracting and discarding the bulk neutral lipids using a non-acidified Folch phase partition, and then re-extracting the aqueous phase/protein interphase in the presence of additional acid. However, this approach removes most of the less polar lipids, including PtdIns, and thus many of the lipids with which measurements of PtdIns(3,4,5)P_3 and PtdIns(4,5)P_2 may wish to be compared.

Derivatisation

The phosphate groups of phosphoinositides are reacted with TMS-diazomethane for 10 min at room temperature, directly in the organic phase generated by the Folch extraction (see Fig. 3) (Clark, Anderson, 2011). If the conditions of the reaction are adhered to, there is minimal methylation of the free hydroxyls on the inositol ring and no modification of the fatty acyl side chains. Excess TMS-diazomethane is destroyed by the addition of acetic acid, and the derivatised lipids are purified through two further rounds of a neutral Folch phase partition (acidification is not required because the phosphates are now methylated, and should be avoided to prevent phosphate migration/hydrolysis during drying). The final organic phase is then dried gently, before re-suspension in a solvent mixture containing chloroform and methanol 8:2 (v/v).

Whilst the reaction with TMS-diazomethane was invented to radically improve the ionisation and stability of ions generated from PtdInsP, PtdInsP$_2$ and PtdInsP$_3$ in the mass spectrometer, we have

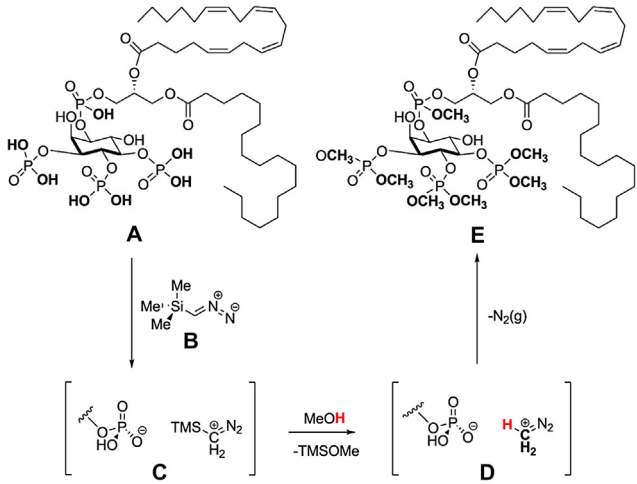


Fig. 3. The reaction of PtdIns(3,4,5)P_3 with TMS-diazomethane. TMS-diazomethane **(B)** reacts with hydroxyl groups of PtdInsP_3**(A)** to form unstable species **(C)**. MeOH in this reaction acts as a reagent to remove TMS in the form of a methyl ether, as well as a source of protons to facilitate formation of the reactive intermediate presented in structure **(D)**. The methylation reaction is fast, giving rise to the desired methylated PtdInsP_3**(E)** and releasing nitrogen gas as a by-product. Methylation of only one hydroxyl group is shown in the figure for simplicity.

found it also reacts in a predictable manner with other phospholipid species, allowing their detection in parallel (see below).

Mass spectrometry

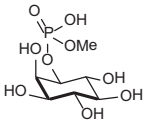
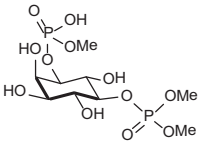
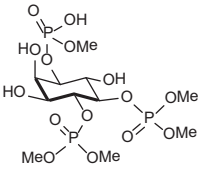
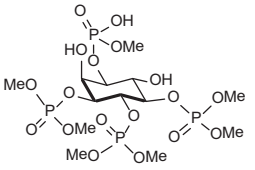
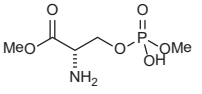
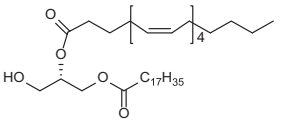
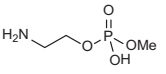
Samples of derivatised lipid extract are then partially purified and concentrated by HPLC through an in-line C4 column before infusion into a triple quadrapole mass spectrometer in positive ion mode.

We have found the most useful fragmentation for phosphoinositides, yielding the highest quantity of unambiguous ions, is cleavage at the diester phosphate bond, generating the loss of a neutral headgroup and the detection of a positively charged DAG^+. We have found this type of fragmentation can also be used for a range of other phospholipids, allowing useful parallel measurements (see Table 1). We routinely measure 20–30 individual lipid species from a single extract, typically 1–5 molecular species from 5 to 10 different lipid classes.

The general work plan for a particular cell or tissue type is to first run neural loss scans (NLSs) to identify the range of DAG species that are associated with a particular headgroup; some typical NLSs for $PtdInsP_2$ derived from a mammalian tissue and a cultured cell line are shown in Fig. 4. This then allows the identification of the particular molecular species to target for accurate quantification. Thus far, we have found all phosphoinositide species derived from mammalian tissue are relatively molecularly homogenous for the 38:4 fatty-acyl species (Fig. 4; 18:0 in the sn-1 position and 20:4 in the sn-2 position) (Anderson, Kielkowska, 2013; Lee et al., 2012), as first noticed for PtdIns (Holub, 1986). However, cells grown in culture, even primary cells grown for short periods of time, rapidly synthesise a more diverse range of phosphoinositide molecular species; (Fig. 4), possibly because the serum used is relatively deprived of 20:4 (arachidonate) or its metabolic precursor, 18:2 (linoleic acid) (Clark, Anderson, 2011; Rouzer et al., 2006). There is still very little work describing molecular species of phosphoinositides across different cells, tissues and organisms and therefore this is usually an important step in the analysis.

Once the major molecular species of phosphoinositides present have been established, the next step is to accurately quantify them by integrating Multiple Reaction Monitoring (MRM) chromatograms (see Fig. 5). The integrated ion currents ('peak areas') are directly related to quantity, but each molecular species and, in particular, each lipid class is likely to have different efficiencies of ionisation and fragmentation and thus they cannot generally be compared with each other without some form of cross-standardisation. This is usually achieved by the inclusion of ISDs that are sufficiently chemically similar to the endogenous

Table 1
This table shows a list of phospholipid classes that we have successfully measured in lipid extracts derivatised with TMS diazomethane.

Lipid type	Neutral fragment lost upon fragmentation in the collision cell	
	Structure	Mass [Da]
PtdIns		274
PtdInsP		382
$PtdInsP_2$		490
$PtdInsP_3$		598
Phosphatidic acid		126
Phosphatidylserine		213
Phosphatidylcholine		626
Phosphatidylethanolamine		155

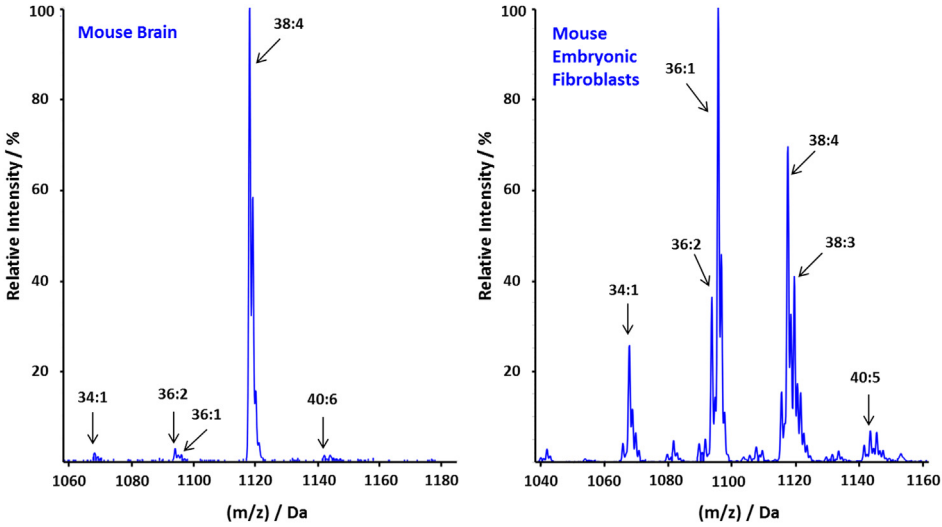


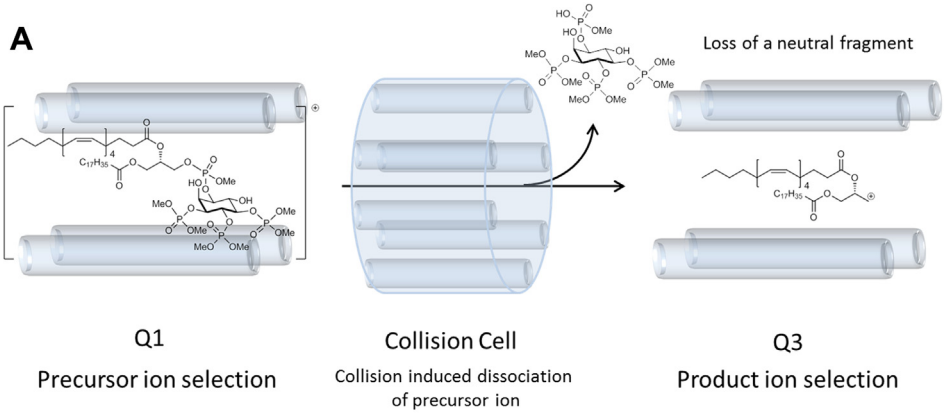
Fig. 4. Examples are presented for neutral loss scans (NLSs) measuring the DAG^+ species generated by the loss of the derivatised headgroup of $PtdInsP_2$ (490.04 amu). The major peaks are annotated with the total fatty-acyl composition of the individual DAG species (e.g. 38:4 is equivalent to 18:0/20:4, sn-1/sn-2); the trace has not been 'de-isotoped' and thus each molecular species is represented by a series of +1 masses related to the natural abundance of ^{13}C. Note that mouse brain contains predominantly the 38:4 species, but mouse embryonic fibroblasts (MEFs) have a wider diversity of molecular species, probably related to their culture conditions.

molecules that they can correct for differential recoveries through all steps of the procedure (if they are added at the point of initial solvent extraction, this will also include recoveries through the extraction and derivatistion steps, as well as during HPLC and mass spectrometry). Unfortunately, for most phosphoinositide species, the relevant ISDs are not commercially available and require a great deal of expertise to synthesise chemically. The most obvious form of ISD is the precise equivalent to the endogenous species except for the incorporation of heavy nuclei, which allow 'mass distinction'. However, in case of deuterated-C38:4 ISDs care should be taken as the molecules are unstable when stored in solution for extended periods of time. The presence of double bonds in the structure makes these ISDs prone to oxidation which becomes a problem, especially when quantitative measurements are to be performed. As an alternative, we have synthesised biologically irrelevant C16:0/C17:0 versions of these lipids (C17:0 is rarely found in nature). We have found C33:0-PtdIns, -$PtdInsP_2$ and -$PtdInsP_3$ ISDs are stable, create homogenous solutions in chloroform/methanol mixtures and correct well for the recoveries of their endogenous counterparts. Using ISDs, we have found that recoveries of endogenous lipids tail off dramatically if too much tissue/cells are used (Fig. 6) and thus it is important to define a working range of sample size; typically we aim for 0.5 million cells and dilute solid tissue samples to give equivalently concentrated lipid extracts.

This methodology can be used to measure phosphoinositides and other lipid species in tissue and cell extracts with high levels of sensitivity and reproducibility, and a significantly greater throughput compared to most existing methods (40–60 samples can be routinely processed in a day). Importantly, this method represents a major advance in our ability to detect the very low levels of $PtdIns(3,4,5)P_3$ that are generated by cell surface receptor stimulation of Class I PI3Ks (see Fig. 7). This opens up the possibility of directly assessing Class I PI3K activity in tissue biopsies derived from pathophysiological contexts in which PI3Ks are deregulated, including human cancers and immune cell dysfunctions.

Future prospects

One of the major limitations of the current methodology is the inability to resolve regioisomers of polyphosphoinositides. Potential solutions to this problem could include improved HPLC separations



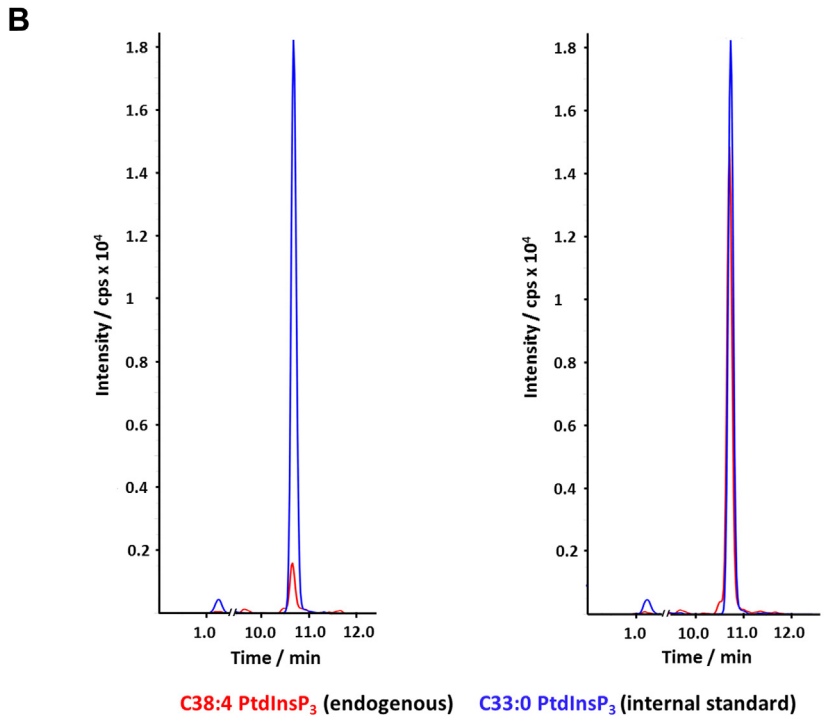


Fig. 5. A. A cartoon describing the fragmentation used to measure C38:4-PtdInsP$_3$(Q1/Q3 1225.573/627.535), illustrating the principle of the MRM transitions used. B. Examples of MRM traces used for measuring PtdInsP$_3$ in extracts derived from control (left panel) and EGF-stimulated (1 min, 2 ng/ml; right panel) MEFs, showing the excellent signal to noise ratios that can be achieved. Ion current is plotted against HPLC elution time and integrated areas under the peaks are used to determine quantity of material. The trace measuring endogenous C38:4-PtdInsP$_3$(red) is overlaid with the trace measuring the C33:0-PtdInsP$_3$ internal standard (blue); the ratio between these two areas (response ratio) gives the relative quantities of endogenous material corrected for any losses through the entire procedure.

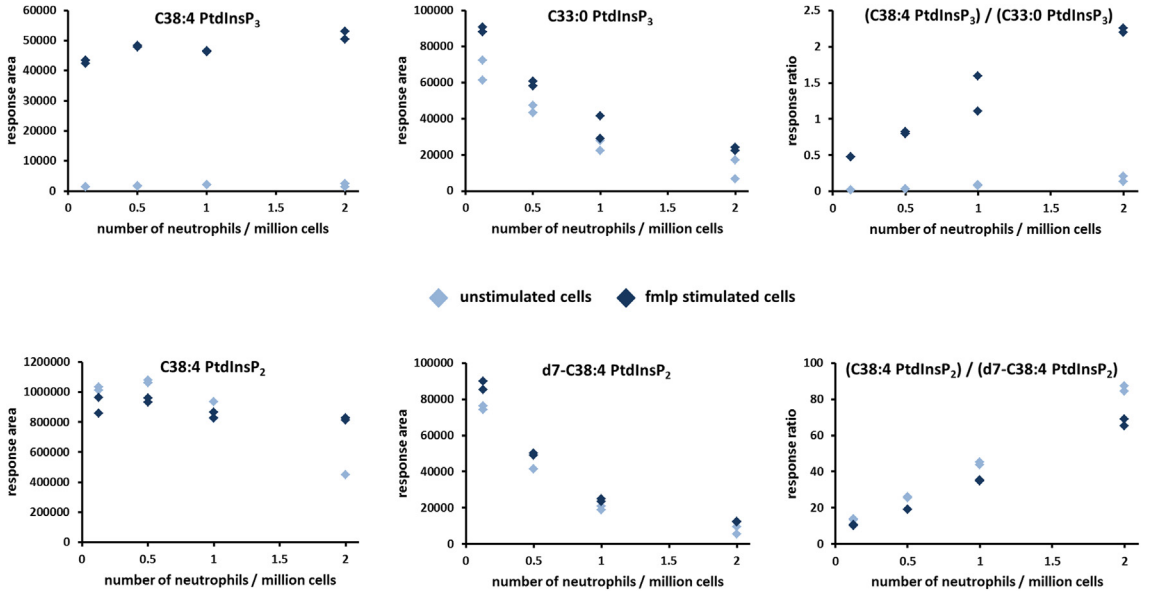


Fig. 6. An example showing how the recovery of endogenous $PtdInsP_2$ and $PtdInsP_3$ is related to the recovery of ISDs (d7-C38:4 PtdIns(4,5)P_2 and C33:0 PtdIns(3,4,5)P_3) from lipid extracts prepared using increasing numbers of mouse neutrophils. There is clearly no advantage in using cell numbers beyond 1×10^5.

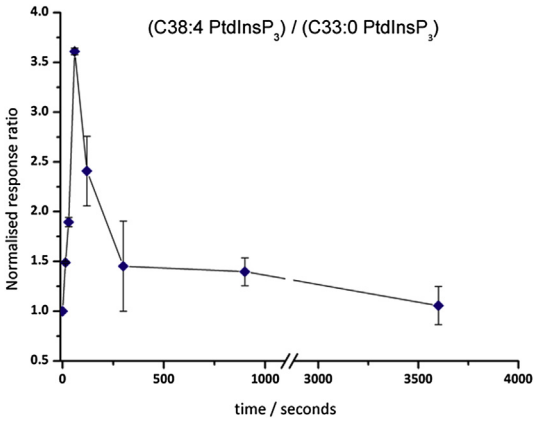


Fig. 7. An example of measuring changes in the levels of C38:4-PtdInsP$_3$ during EGF stimulation of MEFs (2 ng/ml EGF added at time 0).

which resolve the isomers prior to infusion into the mass spectrometer, though this will be difficult using the intact lipid species and classical 'reverse phase' systems (since they will often be molecularly diverse because of fatty-acyl heterogeneity and because the basis of the separation is largely hydrophobic). However, it may also be possible to devise new chemical approaches to the derivatisation of the headgroup or to use a different fragmentation strategy to distinguish between the different phosphorylated positions on the inositol headgroup. Another more tractable but nonetheless essential advance is the chemical synthesis of a more comprehensive set of standards (ISDs) that can be used for the robust and accurate quantification of a wider variety of phosphoinositides and other phospholipids.

Conflict of interest statement

Phillip Hawkins, Len Stephens and Jonathan Clark have paid consultancy agreements with Glaxo SmithKline UK.

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