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# *Dictyostelium* uses ether-linked inositol phospholipids for intracellular signalling

Jonathan Clark, Robert R. Kay, Anna Kielkowska, Izabella Niewczas, Louise Fets, David Oxley, Len R. Stephens and Phillip T. Hawkins

*Corresponding author: Phillip Hawkins, The Babraham Institute*

**Review timeline:**

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| Submission date: | 07 April 2014 |
| Editorial Decision: | 28 April 2014 |
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## Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

*Editor: Andrea Leibfried*

1st Editorial Decision 28 April 2014

Thank you for submitting your manuscript entitled 'Dictyostelium uses ether-linked inositol phospholipids for intracellular signalling'. I have now received the reports from all referees.

As you can see below, all referees appreciate your findings and referee #1 and #2 support publication, suggesting only minor amendments of your manuscript. Referee #3, however, raises a couple of serious concerns and does not think that the data provided support your conclusions sufficiently. This referee points out that more direct evidence for the existence of ether-linked inositol-containing glycerophospholipids is required and that several technical concerns need to be addressed. Upon further consultation, referee #2 agreed with referee #3 that a more direct evidence for the existence of this lipid species should be provided.
Given the clear comments provided, I would like to invite you to submit a revised version of the manuscript, addressing the concerns of the referees. Please contact me in case of further questions. I should also add that it is EMBO Journal policy to allow only a single round of revision and that it is therefore important to address all concerns raised at this stage.

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REFEREE COMMENTS

Referee #1:

 

This study by Clark et al. describes a new molecular makeup of phosphoinositides in Dictyostelium. The authors set out to use the unique mass spectrometric analysis developed in their laboratory to accurately measure absolute amount changes and fatty-acid side chain composition of all phosphoinositides in Dictyostelium. They found that the acyl-chain backbone of phosphoinositides did not conform to any of the known or expected fatty acid composition and suggested a unique hitherto unrecognized structure. With meticulous work the authors identified the phosphatidylinositol species as a plasmanylinositol where the sn-1 position contains an ether linkage formed with palmitol (16:0) whereas the sn-2 position is a an ester linked C18:1 acyl. They then found that this new backbone (C34:1e) is highly enriched in all inositides and also in phosphatidic acid (PA) whereas the other phospholipids (PC,PS,PE) and DAG contained this backbone only in smaller amounts. The authors then used several Dictyostelium strains stimulated by cAMP or genetically modified to demonstrate that the plasmanyl inositides showed all the expected changes that had previously been assumed to be those of the diacyl-derivatives as known from mammalian cell and tissues. The authors discuss the implications of these novel findings especially those that come from the difference between the proportions of the plasmanyl vs. diacyl PA and DAG as they serve as precursors for the plasmanyl-inositides and other phospholipids, respectively.

These are novel findings with great biological significance. The unique acyl side chain composition of the PI species vs. other phospholipids has been noted for a long time, yet its significance is still not understood. The authors are pioneering the mass spectrometric analysis of phosphoinositides, which gives extremely valuable information about the way these lipids are interconverted. The new discovery of the plasmanyl-inositides in Dictyostelium (and perhaps in other lower organisms) has a great potential to address several outstanding questions regarding the importance and significance of the unique side chains that keep these lipids in the membrane. These studies are professionally done, clearly presented and are interesting for a wide range of readers. There are only minor points that the authors may want to address.

1. The arachidonyl side chain in the sn-2 position has very important implications in mammalian systems as the PLA2-mediated liberation of arachidonic acid (AA) serves as a precursor in the cycloxygenase and lipoxigenase pathways. The lack of arachidonyl on the sn-2 position in plasmanyl-PI suggest that either these lipid mediators are not generated in Dictyostelium, or their precursor is liberated form other phospholipids. Is there any literature related to these questions? Even if not, it may be useful to mention these implications in the Discussion.

2. The authors state in the Discussion: "The presence of an ether linkage renders these molecules insensitive to alkaline deacylation" Should not alkaline hydrolysis still remove the acyl from the sn-2 position? If it would, may be the sentence can be rephrased.

3. The authors may want to cite more recent reviews in the Introduction (such as Balla's in Phys. Reviews) in addition to the excellent ones they cited.

Referee #2:

The work of Hawkins and co-workers is based on a very important new technique for the mass spectrometry analysis of highly charged lipids that usually does not require chemical processing of the sample other than methylation and hence preserves the complete lipid composition. It works well in our lab as well. The technique is highly suitable for detecting unusual lipid species and here the authors publish the first example of such a discovery. They identify a series of C16:0 ether-linked phosphatidylinositols with the typical phosphate modifications on the inositol (PI to PIP3) from Dictyostelium. Two tasks needed to be addressed: first, what is the precise structure of the etherlipids and second, are these lipids interconverted similar to the well described diacyl phosphoinositides.

Structure elucidation: the most reliable way for proving the structure would be to synthesize the lipid chemically and compare its properties. As this is not trivial, the authors relied on fragmentation results from mass spec analysis. I think the analysis is thorough and the outcome is at least likely to be correct. What surprises me is that the authors find a fragment that has lost the alkyl group. This should be close to impossible even inside the spectrometer. It would be much more likely to loose

 

one or several methyl groups from the lipid modification. This makes me a bit suspicious about the overall structure. In addition, I am a bit uneasy with the fact that deuterated palmitol is apparently easily incorporated into the etherlipids. I acknowledge that the authors explain the formation of 1-alkyl-DHAP and subsequent reduction into PA. Would feeding a deuterated palmitoic acid lead to the same species? Would feeding a shorter alcohol (myristol) lead to corresponding ether lipids?

It needs to be stressed that the authors were likely only able to identify the alkyl/acyl composition by mass spec, because of the extremely high abundance of one distinct species.

Biochemistry: the authors show convincingly that the ether-PIs are substrates for the typical kinases and phosphatases.

In the discussion, the author stress that evolution might (coincidentally) have used a slightly different pathway to produce a signaling network similar to the DAG-based lipids. This doesn't go along well with the fact, that only one particular fatty acid/ether composition is found. To me, this hints towards significant evolutionary pressure. What should be discussed is the possibility that this system is unable to produce molecules such as 2-AG and this might be an advantage in the interplay with other organisms, i.e. by not releasing an 'eat me' signal (pure speculation on my side).

In conclusion, I don't see mistakes on the author side in determining the structure of the new lipid and I have no better suggestion. Yet, I am only 98% convinced that the lipid has the proposed structure. Nevertheless, I recommend publication with minor revisions.

Minor points:

In the abstract, the various phosphoinositide species found should be named.

The authors should explain the fragment differences (e.g. 108) in the legend of Figure 1 to the non-specialist.

The linoic acid in the lipid should be called octadecenoyl... etc.

The speculation on the pool for diacyl PA on page 8 omits that these species might originate from the feeding bacteria.

Referee #3:

The manuscript by Clark et al. argues for the existence of ether-linked inositol-containing glycerophospholipids in Dictyostelium. To substantiate this finding the authors report several experiments where they monitor the abundance of the putative phosphorylated ether-linked inositol-containing glycerophospholipids. Overall, the manuscript presents a potentially interesting finding that can be relevant for a broad readership with interests in lipid biochemistry and membrane biology. However, the presented evidence for the existence of ether-linked inositol-containing glycerophospholipids is unfortunately only indirect and circumstantial. The applied analytical approach (low resolution mass spectrometry) cannot unequivocally resolve whether the detected inositol-containing glycerophospholipids comprise even numbered ether-linked alkyl residues, esterified odd numbered acyl residues, or a mixture of both? The authors should be required to provide direct evidence by i) high resolution mass analysis of PI lipids, and ii) NMR spectrometry of PI lipids. These approaches are used when documenting the structure of "novel" lipids (see for example: Penkov et al. 2010 (PMID: 21053225)). Based on the above-mentioned limitation the manuscript cannot be recommended for publication.

General comments that should be addressed:

- The authors use the incorporation of palmitol labeled with deuterium at position C-1 to indirectly show the existence of inositol-containing glycerophospholipids. In the material and methods the authors state that 10 min treatment result in "around 20% deuterated PIP2". In figure 2 the ratio between labeled and unlabelled PIP2 is approx 1:1. This indicates that the authors did not use 10

 

min labeling for the data shown in figure 2. Upon longer time treatment with palmitol one expects oxidation of palmitol to palmitic acid and release of deuterium. Released deuterium can exchange with H atoms next to carbonyl groups (e.g. at fatty acid moieties: R-CH2-CO-R => R-CD2-CO-R). Consequently, a mass shift of 2 Da can occur both by H+-D+ exchange as well as the biosynthetic incorporation as suggested by the authors. Hence, using treatment with deuterium-labelled palmitol is not adequate for claiming the existence of ether-linked inositol-containing glycerophospholipids.
A note related to figure 2: the distribution of the red -CD2- spectrum is showing a scrambled isotope pattern; this could potentially be due to H+-D+ exchange.
Another note related to figure 2: please show a spectrum of labeled and unlabeled PI 34:1e.

- The authors also use PLA2 treatment to indirectly show the existence of inositol-containing glycerophospholipids. In figure 3A the authors show a time course experiment of the conversion of "PI 34:1e" to "LPI 16:0e". Surprisingly, the level of "LPI 16:0e" starts decaying after 60 min of treatment. This could be explained by the authors monitoring the isobaric odd-chain LPI 15:0 or a mixture of LPI 15:0 and LPI 16:0e? Hence, using PLA2 treatment is not adequate for claiming the existence of ether-linked inositol-containing glycerophospholipids.

- The authors state that they culture Dictyostellium in HL5 axenic medium. According to the manufacturer, ForMedium, this medium is made using "Peptone and Yeast extract". Yeast extracts are commonly made using P. pastoris, which is able to synthesize odd-chain fatty acids. Hence, it is likely that the authors have been using a medium containing odd-chain fatty acids which could be salvaged by Dictyostellium and used for making odd-chain fatty acid-containing PI lipids that are isobaric with the proposed ether-linked PI lipids. On a related note, the authors should rule out the possibility that Dictyostellium can synthesize and incorporate branched-chain fatty acids (C. elegans is known to be able to do this).

- The authors show MS spectra of derivatizied PI lipids in Figure 1. It would be more informative for the reader if the authors would show MS/MS spectra of the putative PI lipids. The MS/MS data would provide more structural information than the MS data. Moreover, and pertaining to all figures with MS data, the authors should specify the centroid m/z values in all spectra. This will help readers acknowledge their claims.

- The authors state: "...for more careful quantification...". Careful quantification should include use of appropriate internal standards for quantification of PI, PIP, PIP2, PIP3 and other lipids. Moreover, one would expect for a paper to be published in the EMBO journal that the authors show data with error bars based on biological replicates and not only technical replicates. In the legend for figure 6, 7 and 8 the authors should write: "(mean +/- sd, n=3 technical replicates)".

- The authors state: "Our data indicates that the predominant alcohol used for this reaction in Dictyostelium is palmitol". How can the authors conclude this when they have not tested and compared incorporation of other substrates? Revise statement.

- In figure 4 the authors show neutral loss scans for detection of PA, PS, PC and PE. This mass spectral analysis is not explained in the material and methods. Moreover, the values for neutral scans are not specified anywhere. Please revise. Importantly, the authors should show a precursor ion scan or a neutral loss scan to show the profile of PI lipids. Again, please specify centroid m/z values for detected precursors. Finally, the scan for PC lipids is of extremely poor quality due to detector saturation. The scan should be replaced with better quality data and the corresponding panel in Expanded Fig 3 be remade.

- Figure 6 and 7: The quantification of PIPs based on peak area indicates that PIP2 is approx. 4-fold more abundant than PI and PIP1. This is confusing and should be commented on.

 

- The authors should establish if the wording "plasmanylinositols" is in accordance with IUPAC nomenclature. The authors should consistently use either "Inositol phospholipids" or "Inositol phospholipids". A more contemporary wording could be "inositol-containing glycerophospholipids"? (Fahy et al. 2005 (PMID: 15722563).

- The authors use redundant nomenclature for annotation of "Inositol phospholipids": The authors should consistently use either the "PIP system" or the "PtdIns system". Using both systems is confusing. The "PIP system" aligns with the system used for PA, PC, PE and DAG.

- The authors state: "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". Please insert the references: (Clark et al, 2011; Kielkowska et al, 2014) directly after this sentence.

- the authors state: "... although fatty acids with an odd carbon chain length are rarely found in nature". This statement is not correct. The mass of bacteria in our biosphere is larger than that of mammals. Revise statement.

- the abbreviation KK2 is used before its definition. Please correct.

1st Revision - authors' response 26 June 2014

Referee #1:
*1. The arachidonyl side chain in the sn-2 position has very important implications in mammalian systems as the PLA2-mediated liberation of arachidonic acid (AA) serves as a precursor in the cycloxygenase and lipoxigenase pathways. The lack of arachidonyl on the sn-2 position in plasmanyl-PI suggest that either these lipid mediators are not generated in Dictyostelium, or their precursor is liberated form other phospholipids. Is there any literature related to these questions? Even if not, it may be useful to mention these implications in the Discussion.*

This is an interesting point. We are not aware of any significant literature on the role of eicosanoids in Dictyostelium signalling and have added a relevant comment to the Discussion (P9, L8-9).

*2. The authors state in the Discussion: "The presence of an ether linkage renders these molecules insensitive to alkaline deacylation" Should not alkaline hydrolysis still remove the acyl from the sn-2 position? If it would, may be the sentence can be rephrased.*

We have reworded the discussion (P7, last two lines paragraph 2) to make it clearer that alkaline hydrolysis would not remove an ether linked chain (ie not produce the glycerophosphoesters required by previous analytical methods).

*3. The authors may want to cite more recent reviews in the Introduction (such as Balla's in Phys. Reviews) in addition to the excellent ones they cited.*

The Balla review is an excellent choice and we have inserted it (P2, L3), thank you.

Referee #2:
*Structure elucidation: the most reliable way for proving the structure would be to synthesize the lipid chemically and compare its properties. As this is not trivial, the authors relied on fragmentation results from mass spec analysis. I think the analysis is thorough and the outcome is at least likely to be correct.*

 

This is a good point. We have been able to chemically synthesise a C34:1e PA analogue and show that it has an identical retention time and fragmentation pattern to endogenous C34:1e PA isolated from Dictyostelium. At the time of synthesis we did not know where the double bond was and unfortunately made the delta 9 version. For clarity we have not included this work in the manuscript. We have been unable to chemically synthesise C34:1e inositol lipids so far, although we have made a couple of attempts. This is because the molecules have proved very labile to the final deprotection step, although a number of different methods have been tried. The synthesis needs to be re-started with a different protecting group strategy and is likely that this would take a significant number of months and is not guaranteed to be successful even then.

*What surprises me is that the authors find a fragment that has lost the alkyl group. This should be close to impossible even inside the spectrometer. It would be much more likely to loose one or several methyl groups from the lipid modification. This makes me a bit suspicious about the overall structure.*

Our fragmentation analysis of C34:1e PIP2 is shown in Fig E1B. It is assumed that this referee is talking about the ion with m/z of 225.3 ie the alkyl fragment itself (we do not identify a fragment which has lost only the alkyl group). This fragment shows a shift of 2 amu when the Dictyostelium has been fed deuterated palmitol, this would indicate that it is a fragment from the ether bond breakage. This spectrum was acquired in the ion trap mode, a series of reactions are possible in this mode and it is suggested that the ion is a decomposition product from the m/z 281.2 fragment, which also shows a 2 amu shift when the deuterated alcohol is used.

*In addition, I am a bit uneasy with the fact that deuterated palmitol is apparently easily incorporated into the etherlipids. I acknowledge that the authors explain the formation of 1-alkyl-DHAP and subsequent reduction into PA. Would feeding a deuterated palmitoic acid lead to the same species? Would feeding a shorter alcohol (myristol) lead to corresponding ether lipids?*

This issue is related to a similar point raised by referee 3 below. Deuterated palmitol was used because two deuteriums could be incorporated in the C1 position, which allowed us to confirm the presence of an alkyl-linked C16 chain. It is likely that a reductase would reduce a suitably labelled palmitic acid to palmitol, which we would expect to then be incorporated into the lipids that we identify here, however, the level of structural information obtained from the experiment would be less (because the deuterium could not be at C1 in palmitic acid because C1 is the carboxyl carbon). We did in fact initially feed a series of alcohols to Dictyostelium and measured their relative incorporation into inositol lipids. C4, C6 and C8 alcohols were not significantly incorporated. C10 and C12 alcohols caused dramatic reductions in growth rates and death (probably due to their known mitochondrial poisoning effects eg PMID:10869381). C14 alcohol (myristol) was incorporated to a very low extent (8% of PIP2 at 24 hrs; see Figure 1). Thus, D2-C16 alcohol (palmitol) was incorporated to a far greater extent than any other alcohol tested (80% of endogenous PIP2 incorporated D2-palmitol within 490 min; new Fig E2), indicating the presence of very efficient pathways for the biosynthesis of C34:1e lipids using palmitol. When taken together, the high proportion of D2-palmitol incorporation, the high resolution molecular mass, and the PLA2-derived identification of a C18:1 chain, point to only one possible conclusion, that the C34:1e pool of inositol lipids possess a relatively homogenous C16e/C18:1 backbone.

 

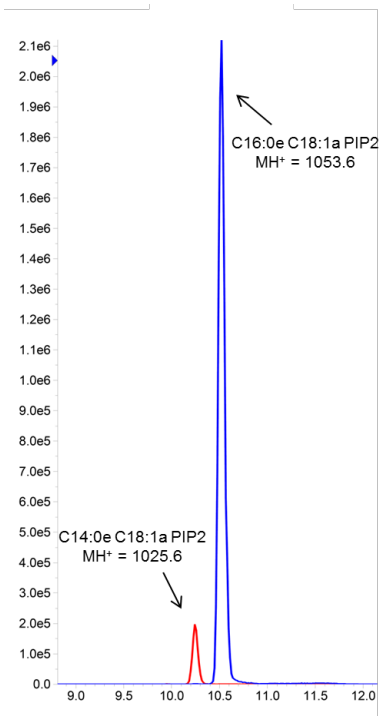


**Figure 1 The level of c14 alcohol incorporation into PIP2 of *D. discoideum* cells (Ax2 strain) after 24hr treatment with 0.4 mM 1-tetradecanol**

*In the discussion, the author stress that evolution might (coincidentally) have used a slightly different pathway to produce a signaling network similar to the DAG-based lipids. This doesn't go along well with the fact, that only one particular fatty acid/ether composition is found. To me, this hints towards significant evolutionary pressure. What should be discussed is the possibility that this system is unable to produce molecules such as 2-AG and this might be an advantage in the interplay with other organisms, i.e. by not releasing an 'eat me' signal (pure speculation on my side).*

This is an interesting idea but we're not aware of any evidence that the lipid backbone of phospholipids constitute an 'eat me' signal.

*Minor points:*
*In the abstract, the various phosphoinositide species found should be named.*
The formal nomenclature of C34:1e PIs has been added to the abstract (L8).

*The authors should explain the fragment differences (e.g. 108) in the legend of Figure 1 to the non-specialist.*
The '108' refers to the mass of a methylated phosphate group ie the unit of difference between differentially phosphorylated PIs; we have added a clearer explanation to the legend of Fig1.

*The linoic acid in the lipid should be called octadecenoyl... etc.*
We only use linoloyl in the introduction (P3, L12), where we believe the use of common names is more appropriate; we have added the abbreviation C18:2 for consistency with our references to other acyl groups mentioned in the same sentence.

*The speculation on the pool for diacyl PA on page 8 omits that these species might originate from the feeding bacteria.*
We have now grown Dictyostelium on a defined medium with no added fatty acids or alcohols (SIH) and observed very similar fatty acyl diversity in PC/PE/PS pools and uniformity in the C34:1e-PA pool as we observed in Dictyostelium grown on axenic medium or bacteria (see the response to referee 3 below).

Referee #3:
*The manuscript by Clark et al. argues for the existence of ether-linked inositol-containing glycerophospholipids in Dictyostelium. To substantiate this finding the authors report several*

 

*experiments where they monitor the abundance of the putative phosphorylated ether-linked inositol-containing glycerophospholipids. Overall, the manuscript presents a potentially interesting finding that can be relevant for a broad readership with interests in lipid biochemistry and membrane biology. However, the presented evidence for the existence of ether-linked inositol-containing glycerophospholipids is unfortunately only indirect and circumstantial. The applied analytical approach (low resolution mass spectrometry) cannot unequivocally resolve whether the detected inositol-containing glycerophospholipids comprise even numbered ether-linked alkyl residues, esterified odd numbered acyl residues, or a mixture of both? The authors should be required to provide direct evidence by i) high resolution mass analysis of PI lipids, and ii) NMR spectrometry of PI lipids. These approaches are used when documenting the structure of "novel" lipids (see for example: Penkov et al. 2010 (PMID: 21053225). Based on the above-mentioned limitation the manuscript cannot be recommended for publication.*

While it is true that the resolution of the QTRAP 4000 mass spectrometer we used could not resolve the difference between an ether linked alkyl lipid (C34:1e; m/z for $MH^+$ 1053.5804) and an odd numbered acyl chain (C33:1; m/z for $MH^+$ 1053.5440), we did not claim this, but presented additional evidence supporting the ether-linked structure.
We have now purified methylated PIP2 from Dictyostelium lipid extracts and obtained an accurate mass on a high resolution mass spectrometer of m/z for $MH^+$ of 1053.5809 (new Fig E1A and text P4, second paragraph). This new data confirms our original structural designation. We consider that this new data, together with the indirect evidence provided by the analysis of D2-palmitol-labelled samples, definitively identifies the C34:1e structure. In our view, the purification of Dictyostelium PIs in sufficient quantity and purity to allow analysis by NMR would be arduous, technically challenging and that the additional data generated would not add sufficiently to warrant the investment of resources into this exercise.

*General comments that should be addressed:*
*- The authors use the incorporation of palmitol labeled with deuterium at position C-1 to indirectly show the existence of inositol-containing glycerophospholipids. In the material and methods the authors state that 10 min treatment result in "around 20% deuterated PIP2". In figure 2 the ratio between labeled and unlabelled PIP2 is approx 1:1. This indicates that the authors did not use 10 min labeling for the data shown in figure 2.*

Both referees 2 and 3 had difficulty in interpreting the data and arguments presented in Fig 2, for which we apologise. We have now redrawn this figure, re-written the legend and added a time course of D2-palmitol incorporation (new Fig E2). The key finding is that a very high proportion of Dictyostelium C34:1e PIP2 incorporated both deuteriums from the D2-palmitol; there was no measurable incorporation of a single deuterium at any time point. We can see no plausible explanation for this result other than that a D2-C16 chain was incorporated via an alkyl linkage. Any indirect mechanism of incorporation would result in a much lower proportion of both deuteriums being incorporated into the pool of this lipid, or a single deuterium being incorporated into the C34:1e structure (see below).

*Upon longer time treatment with palmitol one expects oxidation of palmitol to palmitic acid and release of deuterium. Released deuterium can exchange with H atoms next to carbonyl groups (e.g. at fatty acid moieties: R-CH2-CO-R => R-CD2-CO-R). Consequently, a mass shift of 2 Da can occur both by H+-D+ exchange as well as the biosynthetic incorporation as suggested by the authors. Hence, using treatment with deuterium-labelled palmitol is not adequate for claiming the existence of ether-linked inositol-containing glycerophospholipids.*

The oxidation of palmitol to palmitic acid presumably occurs through first the action of alcohol dehydrogenase to give an aldehyde, and then aldehyde dehydrogenase to give the acid, in the overall reaction
Alcohol + $2NAD^+$+H2O -> Acid + 2NADH
In the case of the deuterated alcohol, the reaction would produce 2NADD (ie ‘deuterated NADH’), which would then presumably be diluted within a large pool of cellular NADH. In principle, the NADD could be utilised to reduce the acid or aldehyde back down to give either the correctly labelled D2- palmitol again, or if NADH was utilised, a palmitol with only one deuterium; either way, a much smaller proportion of D2-labelled C34:1e lipid, or the presence of D1-labelled lipid, would be seen.

 

The exchange reaction that the reviewer alludes to is a reaction that can occur between a methylene adjacent to a carbonyl, catalysed by acid or base, which occurs in a protic solvent such as water. If any of the deuterium were to end up in the bulk water, rather than combined to $NAD^+$, it would not be at a sufficiently high concentration for this type of exchange to be seen.

*A note related to figure 2: the distribution of the red -CD2- spectrum is showing a scrambled isotope pattern; this could potentially be due to H+-D+ exchange.*

The red-CD2- spectrum in Fig 2 did not show a 'scrambled' isotope pattern; it described a mixture of a non-deuterium-labelled series of +1 mass unit peaks derived from the natural abundance of the C-13 isotope, combined with the same series of peaks shifted by two mass units, due to the incorporation of two deuterium nuclei. We have now re-drawn this figure with a centroid presentation of masses to make this clearer and added further explanation to the legend and text (P4, second paragraph). We apologise for the confusion this figure has caused.

The key point, is that the +1 mass unit peak seen in the unlabelled sample (due to the natural abundance of $^{13}C$) did not increase in relative intensity when Dictyostelium were labelled with D2-palmitol (see Fig 2 and new Fig E2). Therefore, there was no measurable incorporation of a single deuterium nucleus, rather, a high proportion (approx. 50% at the time point shown in Fig 2) of the endogenous series of peaks were shifted by the addition of +2 mass units.

*Another note related to figure 2: please show a spectrum of labeled and unlabeled PI 34:1e.*

It should now be clear that we have shown labelled and unlabelled data.

*- The authors also use PLA2 treatment to indirectly show the existence of inositol-containing glycerophospholipids. In figure 3A the authors show a time course experiment of the conversion of "PI 34:1e" to "LPI 16:0e". Surprisingly, the level of "LPI 16:0e" starts decaying after 60 min of treatment. This could be explained by the authors monitoring the isobaric odd-chain LPI 15:0 or a mixture of LPI 15:0 and LPI 16:0e? Hence, using PLA2 treatment is not adequate for claiming the existence of ether-linked inositol-containing glycerophospholipids.*

The PLA2 experiment was performed solely to show the position of the acyl group on the glycerol, it was not to show the existence of ether-linked inositol containing glycerophospholipids. We do not attach any significance to the small loss of LPI C16:0e from the system after prolonged incubations (the experiment had essentially gone as far as it was going to after about 40-50min with the majority of the PI C34:1e being cleaved) and suggest it's most likely a physical effect of liposomes coming out of solution (settling/ sticking to the glass vial), so that the effective concentration being sampled has dropped.

*- The authors state that they culture Dictyostellium in HL5 axenic medium. According to the manufacturer, ForMedium, this medium is made using "Peptone and Yeast extract". Yeast extracts are commonly made using P. pastoris, which is able to synthesize odd-chain fatty acids. Hence, it is likely that the authors have been using a medium containing odd-chain fatty acids which could be salvaged by Dictyostellium and used for making odd-chain fatty acid-containing PI lipids that are isobaric with the proposed ether-linked PI lipids. On a related note, the authors should rule out the possibility that Dictyostellium can synthesize and incorporate branched-chain fatty acids (C. elegans is known to be able to do this).*

We believe that our evidence definitively describes the C34:1e structure of inositol lipids ie these lipids do not contain odd-numbered fatty acyl/alkyl chains or branched chain fatty acids (the new accurate mass data rules out these alternatives). Nonetheless, the extent to which the alkyl/acyl compositions of Dictyostelium phospholipids are influenced by their diet is an interesting one; we therefore grew Dictyostelium in a defined medium with no added fatty acids (SIH) and compared the lipid species present with those derived from Dictyostelium grown in HL5 or bacteria. The pattern of molecular species found was remarkably similar in each case (new Figs E1C and E1D), suggesting Dictyostelium did not 'salvage' odd-chain fatty acids for making PI lipids.

*- The authors show MS spectra of derivatizied PI lipids in Figure 1. It would be more informative for the reader if the authors would show MS/MS spectra of the putative PI lipids. The MS/MS data would provide more structural information than the MS data. Moreover, and pertaining to all figures with MS data, the authors should specify the centroid m/z values in all spectra. This will help readers acknowledge their claims.*

 

We have re-labelled Fig 1 and re-written the legend to make it even clearer that they represent neutral loss scans. Examples of MS (new Fig E1A) and MS/MS (Fig E1B) spectra are shown to support our arguments, we have also checked through all the relevant figures to ensure the key centroid m/z values are clearly labelled.

*- The authors state: "...for more careful quantification...". Careful quantification should include use of appropriate internal standards for quantification of PI, PIP, PIP2, PIP3 and other lipids. Moreover, one would expect for a paper to be published in the EMBO journal that the authors show data with error bars based on biological replicates and not only technical replicates. In the legend for figure 6, 7 and 8 the authors should write: "(mean +/- sd, n=3 technical replicates)".*

We used the term 'for more careful quantification' with reference to MRM versus neutral loss scans. We agree that for absolute quantification of amounts internal standards are required (which, as explained above, we have not yet been able to synthesise). We have replaced 'quantification' with 'comparison' to avoid confusion (P5, paragraph 4).
The data in figs 6,7,8 were derived from individual incubations of Dictyostelium, which most scientists would describe as biological replicates, rather than technical replicates (where multiple measurements are performed on the same sample); it really comes down to your definition of 'sample'. We then repeated the whole experiment with at least three different original cultures of Dictyostelium on different days to check we obtained the same qualitative results (it is difficult to combine the data from these separate experiments because the absolute amounts of material are slightly different). We have rewritten the relevant legends to make this clearer.

*- The authors state: "Our data indicates that the predominant alcohol used for this reaction in Dictyostelium is palmitol". How can the authors conclude this when they have not tested and compared incorporation of other substrates? Revise statement.*

We have changed 'indicates' to 'suggests' (P8, L10). The C34:1e structure predominates in the PA and PI lipid pools, this does suggest that palmitol is the predominant alcohol used by Dictyostelium for PA-ether synthesis; if it wasn't you would expect to see a higher proportion of other species. Our experience of using other shorter chain alcohols is that they are poorly incorporated, myristol (the C14 alcohol) is only incorporated into PIP2 at a level of 8% after 24hrs. These experiments were complicated, as described above, by the known mitochondrial toxicity of lower alcohols and so has been left out of this manuscript for clarity.

*- In figure 4 the authors show neutral loss scans for detection of PA, PS, PC and PE. This mass spectral analysis is not explained in the material and methods. Moreover, the values for neutral scans are not specified anywhere.*

The values we used are listed in the mass spectrometry section of the methods; a reference to this table has been added to the legend to Fig 4.

*Please revise. Importantly, the authors should show a precursor ion scan or a neutral loss scan to show the profile of PI lipids. Again, please specify centroid m/z values for detected precursors.*

We show neutral loss scans, with relevant centroid m/z values labelled, in Fig 1.
We have also re-presented the mass spec data in Figs 2 and 3B as 'centroid presentations'.

*Finally, the scan for PC lipids is of extremely poor quality due to detector saturation. The scan should be replaced with better quality data and the corresponding panel in Expanded Fig 3 be remade.*

We repeated the PC scans with different amounts of sample and a better quality scan is now included in Fig 4.

*- Figure 6 and 7: The quantification of PIPs based on peak area indicates that PIP2 is approx. 4-fold more abundant than PI and PIP1. This is confusing and should be commented on.*

 

We didn't comment on this precisely because we don't have internal standards to perform absolute quantification of these lipids (see above). We have now added appropriate comments to the legends of Figs 6,7 and 8.

*- The authors should establish if the wording "plasmanylinositols" is in accordance with IUPAC nomenclature. The authors should consistently use either "Inositol phospholipids" or "Inositol phospholipids". A more contemporary wording could be "inositol-containing glycerophospholipids"? (Fahy et al. 2005 (PMID: 15722563).*

We did indeed check the IUPAC guidelines; the nomenclature of these new lipids is obviously something we are keen to get right.
We believe 'inositol-containing glycerophospholipids' is too clumsy and readers will assume 'inositol phospholipids' refers to glycerophospholipids.

*- The authors use redundant nomenclature for annotation of "Inositol phospholipids": The authors should consistently use either the "PIP system" or the "PtdIns system". Using both systems is confusing. The "PIP system" aligns with the system used for PA, PC, PE and DAG.*

We have made a significant effort to avoid mixing up the "PIP system" with the "PtdIns system". We explain in the Introduction (P3, first paragraph) and the Discussion (P7, first paragraph) that the prefix 'PtdIns' is an abbreviation for phosphatidylinositol lipids ie lipids which contain two ester-linkages. Historically, scientists in this field have used PtdIns (correctly) and PI (inaccurate, but common) interchangeably to describe these lipids. We propose that PI can be retained as a 'catch all' abbreviation to cover both acyl and ether linked inositol phospholipids, where the lipid backbone composition is irrelevant to the point being made (eg PI3K converts PIP2 to PIP3). Where the lipid backbone is relevant, the structures should be described in more detail and the terms phosphatidyl or plasmanyl should be used.

*- The authors state: "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". Please insert the references: (Clark et al, 2011; Kielkowska et al, 2014) directly after this sentence.*

We have now moved these references to the preceding sentence.

*- the authors state: "... although fatty acids with an odd carbon chain length are rarely found in nature". This statement is not correct. The mass of bacteria in our biosphere is larger than that of mammals. Revise statement.*

We have replaced 'nature' with 'Eukaryotes'.

*- the abbreviation KK2 is used before its definition. Please correct.*

This has been corrected.

2nd Editorial Decision 09 July 2014

I have now received comments from two of the original referees of your manuscript who are both satisfied with the amount of revisions. I attach their comments below. Please deposit your mass-spec data in a public database and include the accession numbers into your manuscript. Please also address the few remaining concerns of referee #3 to allow official acceptance of your paper for publication here.

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Referee #2:

The authors addressed all points raised by this reviewer. The paper is now ready to be published in EMBO J. Congratulations.

Referee #3:

The authors have addressed most of my criticisms satisfactorily. However, the authors should revise the manuscript to address the following issues:

- The authors use the word 'novel' to describe the lipid. How can a molecule that has existed for millions of years be 'novel'? Please use a more appropriate wording.

- the authors should know that it is convention to write m/z in italic. Please correct this mistake throughout the manuscript.

- the authors use multiple styles of shorthand notation for glycerolphospholipids, e.g. "34:1 a/e is equivalent to C34:1e", PtdIns and PI). This is confusing and unnecessary. Please simplify by using only one style of shorthand notation throughout the manuscript.

2nd Revision - authors' response 29 July 2014

Thank you for decision letter, we very much appreciate the time and effort you and the referees have invested in improving this manuscript.

To address the remaining issues:

*1. Please deposit your mass-spec data in a public database and include the accession numbers into your manuscript.*

The public mass spec databases are really designed for large ‘proteomics’ studies or to provide ‘examples’ of mass spectra to aid people in the identification of particular molecules. In our case, the primary mass spec data used to identify novel inositol lipids in Dictyostelium are actually presented in the main Figures and in supplementary material, so deposition in a database would essentially be duplication. If there is a specific database you have in mind however, we would of course be happy to do this

*2. Please also address the few remaining concerns of referee #3 to allow official acceptance of your paper for publication here.*
*- The authors use the word 'novel' to describe the lipid. How can a molecule that has existed for millions of years be 'novel'? Please use a more appropriate wording.*

Obviously ‘novel’ to the human race is not novel enough. We’ve replaced ‘novel’ in the abstract with ‘unusual’ but we’ll accept your editorial guidance here.

*- the authors should know that it is convention to write m/z in italic. Please correct this mistake throughout the manuscript.*

We’ve placed ‘m/z’ in italic where we can; in some traces that have been taken directly from the mass spectrometer (eg expanded figures 1a, b) they have remained un-italicised to show that the traces are un-manipulated.

*- the authors use multiple styles of shorthand notation for glycerolphospholipids, e.g. "34:1 a/e is equivalent to C34:1e", PtdIns and PI). This is confusing and unnecessary. Please simplify by using only one style of shorthand notation throughout the manuscript.*

We have converted all examples of ‘34:1a/e’ to ‘C34:1e’ and made the appropriate changes to the text and figure legends.

 

We have re-checked through the manuscript and believe we have used 'PtdIns' and 'PI' nomenclature correctly; they do not, and should not, be used to represent the same thing, PtdIns lipids are a subset of PI lipids in which both hydrocarbon chains are known to be linked to the glycerol via ester linkages. This is clearly explained in the Introduction and Discussion sections, as outlined in our 'point by point response' to referees.

 