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Review

The uses and limitations of the analysis of cellular phosphoinositides by lipidomic and imaging methodologies☆

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ABSTRACT

The advent of mass spectrometric methods has facilitated the determination of multiple molecular species of cellular lipid classes including the polyphosphoinositides, though to date methods to analyse and quantify each of the individual three PtdInsP and three $PtdInsP_2$ species are lacking. The use of imaging methods has allowed intracellular localization of the phosphoinositide classes but this methodology does not determine the acyl structures. The range of molecular species suggests a greater complexity in polyphosphoinositide signaling than yet defined but elucidating this will require further method development to be achieved. This article is part of a Special Issue entitled Tools to study lipid functions.

1. Introduction

Lipids have a range of cellular functional roles — structural, metabolic and signaling. To facilitate this plethora of functions a number of distinct, though frequently interconnected and interdependent, metabolic and cellular lipid signaling pathways are present in cells. The control of these involves the regulated expression and in some cases the regulated activity of key enzymes, amongst these it is widely accepted that the phosphoinositide 3-kinases (PI3kinase), which catalyse the 3-phosphorylation of inositol phospholipids, are major signaling enzymes controlling a range of cellular functions, see [1] for review. The three classes of PI3kinases (Classes I, II and III), along with PtdIns 4 and 5 kinases, together with the action of a number of phosphatases bring about the generation of distinct inositol phospholipids phosphorylated on the 3-, 4- and 5-positions of the inositol ring (see Table 1). Each of these phosphoinositides interacts with protein targets, through specific, or strongly selective binding domains, thereby localizing the signaling proteins to specific membrane sites with the consequent activation of signaling cascades, in some cases with an additional effect upon catalytic activity [2]. A number of protein domains have been shown to bind the different phosphoinositides, though probably the best characterized are the PH (plexstrin homology) domains. Greater than 100 PH domain-containing proteins have been identified in mammalian cells and they are commonly considered to be $PtdIns3,4,5P_3$ specific, however, whilst many PH domains indeed bind $PtdIns3,4,5P_3$, others can bind $PtdIns3,4P_2$ or $PtdIns4,5P_2$ and not all are specific for one lipid. In contrast to the other bisphosphates, $PtdIns3,5P_2$ does not interact with PH domains, though it has been demonstrated to bind PROPPIN domains [3]. $PtdIns3,4P_2$ and PtdIns3P can both bind to PX domains [4] whilst PtdIns3P also binds to FYVE domains [5]. PtdIns4P regulates the activity of a number of cellular processes, though there is less clarity regarding the existence of a specific binding domain. Nevertheless, binding of this lipid to the PH domains in the lipid transfer proteins CERT, OSBP, ORP9L and FAPP2 has been demonstrated which promotes their transfer to the Golgi complex (see [6] for review). It has been further proposed that PtdIns5P can regulate cell function through binding to an additional binding partner — the PHD domain (PlantHomeoDomain) in nuclear proteins [5].

This fidelity associated with the phosphorylated headgroups has been considered to be the mechanistic basis of signaling specificity, however it is important to recognize that phospholipids are not characterized solely by their headgroups, rather there is a great diversity in the glycerol-based backbone, this can be differences between acyl, alkyl or alkenyl chains and additionally in their number of carbons and double bonds in the fatty acids. The diversity in these structures, and this applies not just to the phosphoinositides but also indeed to most lipids, has only become apparent through developments in analytical mass spectrometry-based lipidomics which has facilitated the determination of detailed structures. This review article considers this aspect of lipid signaling, highlighting the essential role mass spectrometry is playing in uncovering the fundamental complexity of lipid structures and addresses the potential this poorly considered area of physiological diversity has for biological specificity.

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Table 1
Molecular species of inositol phospholipids detected by mass spectrometry.
The species detected are listed with appropriate references, depending on the methodology some authors did not detect each species where more than one group have identified a particular species which gives confidence of its authenticity it is underlined.

Phosphoinositide	Species detected	Refs
Phosphatidylinositol	32:0, 32:1, 32:2 34:0, 34:1, 34:2, 34:3 36:0, 36:1, 36:2, 36:3, 36:4, 36:5 38:0, 38:1, 38:2, 38:3, 38:4, 38:5, 38:6 40:3, 40:4, 40:5, 40:6, 40:7	[11,12,38]
Phosphatidylinositol phosphate (PtdIns3P, PtdIns4P, PtdIns5P)	32:0, 32:1, 32:2 34:0, 34:1, 34:2, 34:3 36:0, 36:1, 36:2, 36:3, 36:4 38:0, 38:1, 38:2, 38:3, 38:4, 38:5, 38:6 40:3, 40:4, 40:5, 40:6	[11,12,32]
Phosphatidylinositol bisphosphate ($PtdIns(3,4)P_2$, $PtdIns(4,5)P_2$, $PtdIns(3,5)P_2$)	32:0, 32:1, 32:2 34:0, 34:1, 34:2, 34:3 36:0, 36:1, 36:2, 36:3, 36:4 38:0, 38:1, 38:2, 38:3, 38:4, 38:5, 38:6 40:3, 40:4, 40:5, 40:6	[8,11–13,32]
$PtdIns(3,4,5)P_3$	32:0 34:1, 34:2 36:1, 36:2, 36:3, 36:4 38:2, 38:3, 38:4, 38:5	[8,11,13,32]

2. Mass spectrometric analysis of phosphoinositides

We have previously reviewed the limited methodologies available for successfully determining the concentrations and structures of cellular phosphoinositides with a focus upon the use of mass spectrometry (MS) [7], however it is pertinent to consider briefly the underlying issues here. The major factors impeding easy analysis of phosphoinositides are directly related to those properties that make them such effective signaling molecules, primarily this is due to the charge associated with the phosphate groups located at specific positions on the inositol head group of the phospholipid. The high charge associated with the multiply phosphorylated phosphoinositides, which facilitates the lipid protein domain binding, also compromises organic extraction of lipids from membranes because of the strong interaction with such proteins, necessitating the use of acidic solvents. These acidic solvents, however, can bring about phosphate hydrolysis, or phosphate migration on the inositol ring if their use is not carefully controlled. A further issue associated with the presence of the charged phosphates is that they bind avidly to both glass and steel thereby seriously compromising recoveries during both extraction and separation, this therefore points to the need to use silanised surfaces wherever possible in order to minimize such losses.

The difficulties associated with the phosphoinositide phosphate groups prompted us to develop a method whereby the phosphate groups were methylated in order to protect them and thereby negate these charge effects [8]. Following separation by reverse phase chromatography, the structure of the derivatised phosphoinositide is determined by making use of neutral loss mass spectrometry. Whist this methodology allows for the determination of $PtdIns(3,4,5)P_3$ with a particular emphasis on correctly defining the acyl chain structure, it can only determine the total PtdInsP and $PtdInsP_2$ rather than the individual enantiomers. Consequently there remains a major need for further method development. Chromatographic modifications are suggesting that it should be possible to separate and thereby individually determine $PtdIns3,4P_2$ and $PtdIns4,5P_2$ concentrations, though it remains unclear if $PtdIns3,5P_2$ can also be separated and quantified. Recently, Lee et al. have developed a supercritical fluid chromatography methodology hyphenated to MS/MS in which methylated lipids are analysed [9]. This procedure undoubtedly improved the separation of a range of phospho- and sphingolipids allowing for simultaneous profiling, however it is not clear if this procedure can be adapted to phosphoinositide separation and analysis though this is theoretically potentially achievable. Nevertheless, the blocking of the phosphate groups reduces the differences associated with the different bisphosphates and suggests that novel strategies are required perhaps a change in the blocking group would be beneficial.

The distinct biological importance of PtdIns3P, PtdIns4P and PtdIns5P emphasizes the need to develop a methodology that will allow their characterization and quantification, it is only once this has been achieved that we will be able to fully understand and define the complexity of phosphoinositide signaling. Unfortunately, this has yet to be achieved for the derivatised lipids and indeed there are no reports of any successful chromatographic separation of each of the intact phosphatidylinositol phosphates. Historically, many groups separated the deacylated forms of phosphoinositides by ion-exchange HPLC, particularly making use of extracts prepared from radiolabelled cell samples which permits quantification of agonist-stimulated changes. This methodology was developed to the point where it was successful in separating the individual mono- and bisphosphorylated derivatives and thus could theoretically provide the basis for separation methodologies for the intact phospholipids, nevertheless, to date, no such successful method has been reported. Recently, Jones et al. [10] have reported the extensive analysis of zebrafish phosphoinositides using this methodology, these authors also made use of thin layer chromatographic separation of intact inositol phospholipids as well as utilizing neomycin-affinity chromatography to isolate the individual phosphoinositide classes and were therefore able to semi-quantify the phosphoinositide family of lipids, however this methodology was unable to provide structural discrimination.

Milne et al. [11] and Wenk et al. [12] have both reported analysis and measurement of phosphoinositides, however their methodologies involved direct infusion into the mass spectrometer and thus they were unable to discriminate the distinct PtdInsP and $PtdInsP_2$ enantiomers and their methods additionally suffered from the inevitable ion suppression effects associated with direct infusion. Pettitt et al. reported a limited separation of intact PtdIns5P from PtdIns3P and PtdIns4P using a silica column, unfortunately this limited separation was hampered by variations between batches of silica columns and further compromised by the acyl chain length and saturation having small, but significant effects upon stationary phase retention [13]. The methodology reported in this paper included the use of a detailed MS^3 analysis that permitted distinction between PtdIns3P and PtdIns4P thereby providing discrimination from PtdIns5P, however this complicated and inevitably poorly reproducible methodology has not gained widespread acceptance. Therefore, at present and until a satisfactory methodology is developed which will undoubtedly involve a novel separation