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REVIEW

The glycerophosphoinositols: cellular metabolism and biological functions

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Abstract The glycerophosphoinositols are cellular products of phospholipase A_2 and lysolipase activities on the membrane phosphoinositides. Their intracellular concentrations can vary upon oncogenic transformation, cell differentiation and hormonal stimulation. Specific glycerophosphodiester phosphodiesterases are involved in their catabolism, which, as with their formation, is under hormonal regulation. With their mechanisms of action including modulation of adenylyl cyclase, intracellular calcium levels, and Rho-GTPases, the glycerophosphoinositols have diverse effects in multiple cell types: induction of cell proliferation in thyroid cells; modulation of actin cytoskeleton organisation in fibroblasts; and reduction of the invasive potential of tumour cell lines. More recent investigations include their effects in inflammatory and immune responses. Indeed, the glycerophosphoinositols enhance cytokine-dependent chemotaxis in T-lymphocytes induced by SDF-1α-receptor activation, indicating roles for these compounds as modulators of T-cell signalling and T-cell responses.

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Abbreviations

AC	Adenylyl cyclase
APCs	Antigen-presenting cells
BBB	Blood–brain barrier
Ca-CaMKII	Ca^{2+}/calmodulin kinase II
CHO	Chinese hamster ovary
DESI	Desorption electrospray ionisation
ECM	Extracellular matrix
ERK1/2	Extracellullar regulated kinase 1 and 2
ESI-MS/MS	Electrospray ionisation tandem mass spectrometry
FAB	Fast atom bombardment
FXR	Farnesoid X receptor
GDE	Glycerophosphodiesterase
GDNF	Glial-derived neurotrophic factor
GEF	Guanine-nucleotide-exchange factor
GP-PDEs	Glycerophosphodiester phosphodiesterases
GRK	G protein-coupled receptor kinase
GroPIns	Glycerophosphoinositol
GroPIns3*P*	Glycerophosphoinositol 3-phosphate
GroPIns4*P*	Glycerophosphoinositol 4-phosphate
GroPIns45P_2	Glycerophosphoinositol 4,5-bisphosphate
GroPIns345P_3	Glycerophosphoinositol 3,4,5-trisphosphate
HPLC	High-performance liquid chromatography
IMLF	Immortalised mouse lung fibroblast
KO	Knock-out
LXR	Liver X receptor
LysoPtdIns	Lysophosphatidylinositol

MALDI	Matrix-assisted laser desorption/ ionisation
MAPKs	Mitogen-activated protein kinases
MFS	Major facilitator superfamily
NMR	Nuclear magnetic resonance
PDE	Phosphodiesterase
PDGF	Platelet-derived growth factor
PHS	Phosphate:H^+ symporter
PKA	Protein kinase A
PLA_2	Phospholipase A_2
PLB	Phospholipase B
PLC	Phospholipase C
PTCs,	Papillary thyroid carcinomas
PtdIns	Phosphatidylinositol
PtdIns4*P*	Phosphatidylinositol 4-phosphate
PtdIns45P_2	phosphatidylinositol 4,5-bisphosphate
PtdIns345P_3	Phosphatidylinositol 3,4,5-trisphosphate
RBL	Rat basophilic leukaemia
RGS	Regulator of G protein signalling
RNAi	RNA interference
SAPK	Stress-activated protein kinase
TLC	Thin layer chromatography
TSH	Thyroid-stimulating hormone
WT	Wild-type

Lipid-derived messengers

It is now widely accepted that the roles of lipids go well beyond membrane structure and energy metabolism, also encompassing numerous aspects of cell signalling [1]. The first concept of such "bioactive" roles for lipids arose from early observations that the cellular levels of lipids and their metabolites can vary concurrently with cell exposure to exogenous stimulation and environmental stress, and with the physiological functions of the cells themselves. Since then, the main pathways of lipid metabolism have been defined, with the identification of lipid messengers and their receptors or targets clarifying the key roles of the bioactive lipids in the modulation of cellular responses. However, these analyses have been accompanied by difficulties in deciphering these many cellular functions, which derive not only from technical problems of working with lipids and their metabolites, but also from the vast range of areas in cell biology where these are important. Furthermore, the complexity of the interconnections and cross-regulation of the different lipid signalling pathways is immense, whereby imbalances in these networks can contribute to the pathogenesis of many human diseases [2].

We consider here, in particular, the glycerophosphoinositols, which are relatively less well-known components of this lipid-signalling world. Their metabolism and function need to be considered in the framework of the well-known central roles in cellular signalling that have been assigned to the turnover of the membrane phosphoinositides, where among the other components involved, phosphatidylinositol 4,5-bisphosphate (PtdIns45P_2) is the substrate of two main receptor-regulated enzymes: phosphoinositide-specific phospholipase C (PLC) on the one hand (Fig. 1, box), with the production of diacylglycerol and inositol 1,4,5-trisphosphate [3]; and class I phosphoinositide

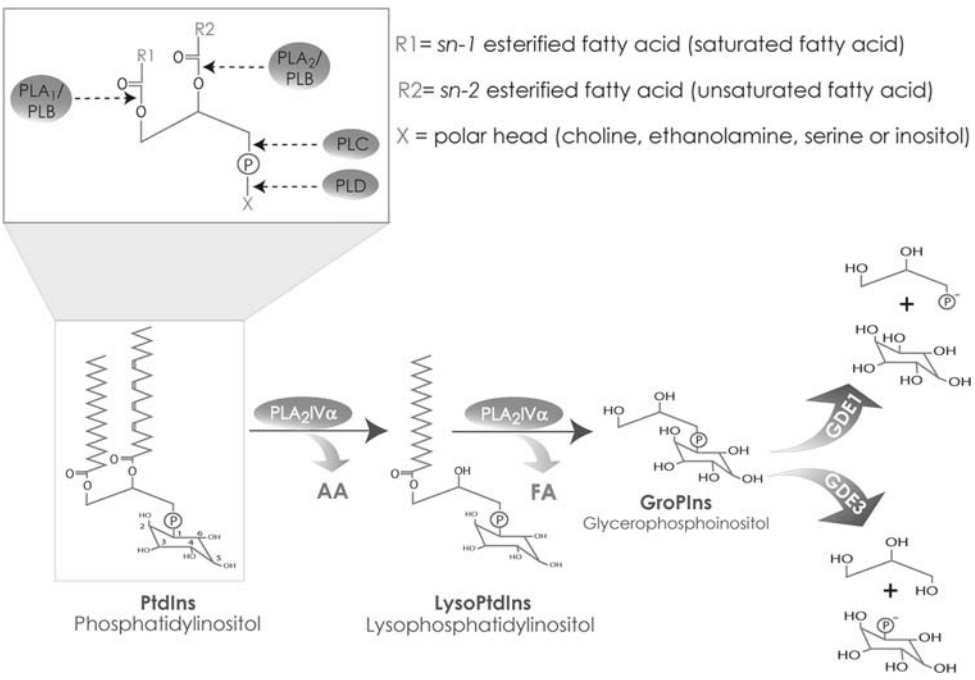

Fig. 1 Metabolism of GroPIns. Phospholipase cleavage sites (*box* phospholipase B, PLB, has both PLA_1 and PLA_2 activities) and schematic representation of the formation of LysoPtdIns and GroPIns from PtdIns via two sequential steps catalysed by $PLA_2IV\alpha$, and the subsequent catabolism of GroPIns as mediated by the GDEs (see text for details), *AA* arachidonic acid, *FA* generic fatty acid [25]

3-kinase on the other, with the production of phosphatidylinositol 3,4,5-trisphosphate (PtdIns345P_3) [4]. These main PtdIns45P_2 metabolites are the primary second messengers for activation of numerous enzyme systems devoted to the modulation of intracellular calcium signalling, further soluble inositol polyphosphate production [5], protein phosphorylation cascades [6] and further membrane polyphosphoinositide lipid production [7]; these last, in turn, regulate cellular functions or provide docking sites for lipid-binding proteins [8].

In considering other phosphoinositide metabolites, the glycerophosphoinositols fall among the active products of phospholipid metabolism that arise from activation of phospholipase A_2 (PLA_2), which also include the monoacylglycerols, the lysophospholipids, and the various products of arachidonic acid metabolism [9–11].

On a similar basis, another class of precursors of active lipid metabolites is constituted by the sphingolipids, characterised by a backbone containing the aliphatic amino-alcohol sphingosine [1]. Sphingomyelin is the substrate for sphingomyelinase, with the production of ceramide, which can be further converted into sphingosine by a hydrolysis step catalysed by ceramidase. Both ceramide and sphingosine are active per se in inducing cell-cycle arrest and apoptosis, and in promoting inflammation [12–14], and they are substrates of kinases that produce ceramide 1-phosphate and sphingosine 1-phosphate, respectively; these signalling molecules exert opposite effects, as mitogenic activities and pro-survival actions [15, 16].

All the signalling molecules considered above are now known to be involved at different levels in cellular functions ranging from inflammation and oncogenesis to metabolic syndromes [2]. These effects are mediated through actions that are both intracellular, even if the identification of their intracellular receptors has sometimes only been hypothesised and not experimentally proven, and extracellular, with the release of some of these signalling molecules activating plasma membrane receptors through autocrine and/or paracrine mechanisms. For instance, to cite some examples, the receptors of sphingosine 1-phosphate have been identified among the lysophospholipid receptor family of G protein-coupled receptors [17], which include the receptor for lysophosphatidic acid [18]. Other lipid metabolites, such as fatty-acid and sterol derivatives, can signal through nuclear hormone receptors, including the farnesoid X receptor, FXR [19], the liver X receptor, LXR [20], and the peroxisome-proliferator-activated receptor [21]. These signalling molecules can thus regulate gene transcription, thereby controlling lipid metabolism, storage, transport and elimination [22]. As for other extracellular stimuli, also for the lipid metabolites, the functional responses are strictly cell-type dependent as a consequence of the different signalling networks present in different target cell models [2].

The examples given above represent only a fraction of the growing body of information on the metabolism and functions of the lipid-derived messengers: we thus refer the readers to recent reviews addressing these different classes of lipids and lipid-derived compounds [1, 2, 7]. Here, instead, as indicated above, our aim is to focus on the glycerophosphoinositols as a specific class of phosphoinositide metabolites. Besides their biological functions (detailed below), our interest in the glycerophosphoinositols also resides in their bridging two central signalling pathways: the phosphoinositide/inositol phosphate cascades and the PLA_2/arachidonic acid pathways. Thus, their metabolism has to be envisioned as an integral part of the maintenance of the phosphoinositide/inositol phosphate cellular pools, while their formation and activity parallel the initiation of the arachidonic acid signalling cascade.

The glycerophosphoinositols

As indicated above, the glycerophosphoinositols are bioactive water-soluble products of PLA_2 activity (Fig. 1) ([23–25] and references therein). The detection of the glycerophosphoinositols in cell extracts has basically paralleled that of the parent phosphoinositides and the range of inositol polyphosphates found in cells. Since the earliest investigations into these compounds, they were considered generally only as products of phosphoinositide metabolism, and very little information was available regarding their metabolism and function [25–29]. The glycerophosphoinositols gained attention as potential active metabolites in studies showing increases in their cellular levels associated with Ras transformation in epithelial cells; their presence and potential for related functions then became apparent in several cell lines [25, 30, 31]. In the following, we will summarise this information, with emphasis on the more recent discoveries around the functions of the glycerophosphoinositols in the regulation of cell proliferation and T-cell responses.

The metabolism of the glycerophosphoinositols

The glycerophosphoinositols can be considered ubiquitous phosphoinositide metabolites; their formation involves PLA_2 and lysolipase activities, which start from the membrane phosphoinositides and sequentially form the corresponding lysophosphoinositides (with a parallel release of arachidonic acid) and then the glycerophosphoinositols (Fig. 1). The enzymes now known to be involved in the formation of the glycerophosphoinositols

Table 1 Intracellular concentrations of GroPIns in selected cell lines

Cell line	GroPIns (μM ± SEM)	Cell volume (pl ± SE)	Cell type
FRT-Fibro	172 ± 25	1.62 ± 0.14	Fibroblast
FRT-Fibro-Ha	925 ± 155	1.92 ± 0.07	Fibroblast, H-Ras transformed
FRTL5	434 ± 48	0.84 ± 0.07	Thyroid
KiKi	272 ± 34	1.71 ± 0.11	Thyroid
PC-KiKi	133 ± 12	0.97 ± 0.02	Thyroid, K-Ras transformed
PC-Ha	44 ± 3	1.57 ± 0.08	Thyroid, H-Ras transformed
PC-Src	90 ± 7	2.22 ± 0.04	Thyroid, Src transformed
$11^{+/+}$	433 ± 40	4.39 ± 0.23	Mouse embryo fibroblasts esp8$^{+/+}$
$4^{-/-}$	317 ± 4	1.82 ± 0.04	Mouse embryo fibroblasts esp8$^{-/-}$
Swiss 3T3	328 ± 16	2.48 ± 0.14	Fibroblast
293	439 ± 12	1.11 ± 0.05	Kidney epithelial, adenovirus transformed
MDA	400 ± 30	1.53 ± 0.10	Prostate epithelial adenocarcinoma
OVCAR3	200 ± 22	2.20 ± 0.03	Ovarian epithelial adenocarcinoma
MCF7	185 ± 19	1.64 ± 0.10	Mammary epithelial adenocarcinoma
Hela	134 ± 10	1.39 ± 0.08	Cervical epithelial adenocarcinoma
L cells	89 ± 10	0.74 ± 0.19	Mouse fibrosarcoma
IMLF WT	85 ± 2	1.21 ± 0.02	Immortalised mouse lung fibroblast
IMLF KO	58 ± 1	1.21 ± 0.03	Immortalised mouse lung fibroblast PLA$_2$IVα$^{-/-}$
Jurkat T-cells	45 ± 1	0.86 ± 0.02	T-cell leukaemia
RAW 264.7	115 ± 9	0.77 ± 0.03	Macrophage
MC3T3	107 ± 5	1.33 ± 0.02	Osteoblast
$PCCl_3$	270 ± 20	0.97 ± 0.03	Thyroid
PC-PTC3	420 ± 20	1.11 ± 0.03	Thyroid, RET/PTC3 transformed

Intracellular concentrations of GroPIns in parent, transformed and carcinoma cell lines were analysed by liquid chromatography–tandem mass spectrometry under their normal growth conditions as described [30]

SEM Standard error of the mean, *SE* standard error

include PLA$_2$IVα (as detailed below and in [23, 31]) and phospholipase B (PLB; [32–34]).

Most of the investigations aimed at the definition of the enzymatic activities involved in the formation of the glycerophosphoinositols have been carried out recently, and have been made possible due to the improved analytical approaches that have also enabled the cellular handling of these compounds [30, 35, 36]. PLA$_2$IVα has been recently identified as the enzyme involved in the formation of glycerophosphoinositol (GroPIns) and GroPIns 4-phosphate (GroPIns4*P*), from their parent lipids phosphatidylinositol (PtdIns) and PtdIns 4-phosphate (PtdIns4*P*), respectively [23]. Interestingly, PLA$_2$IVα catalyses both the deacylation steps that are necessary for the formation and modulation of GroPIns, as indicated by in vitro studies performed using recombinant PLA$_2$IVα and purified lipids (Fig. 1). Thus, a single enzyme, PLA$_2$IVα, possesses both phospholipase and lysolipase activities, which hydrolyse PtdIns to lysophosphatidylinositol (LysoPtdIns), and LysoPtdIns to GroPIns, respectively [23] (Fig. 1). This dual enzymatic activity has also been reported in rabbit platelets and with human recombinant cytosolic PLA$_2$ for micelles containing lysophosphatidylcholine, l-oleoyl-lysophosphatidylcholine, and 1-palmitoyl-lysophosphatidylcholine [37–40].

While this applies to PLA$_2$IVα-expressing cells and to hormone-induced formation of the glycerophosphoinositols (see below), the contributions of other phosphoinositide-hydrolysing enzymes (e.g. other PLA$_2$ isoforms, lysolipases, PLB isoforms) are essential for the maintenance of the basal levels of the glycerophosphoinositols [25, 30]. This is also indicated by the intracellular levels of GroPIns detected in immortalised mouse lung fibroblasts from PLA$_2$IVα knock-out mice (IMLF KO), which are not different from those of control fibroblasts from wild-type animals (IMLF WT) [41] (Table 1). Instead, the receptor-stimulated production of GroPIns is completely blocked in the PLA$_2$IVα IMLF KO cells, but not in the controls (Table 2). Thus, it appears that PLA$_2$IVα mediates mainly the transient increases in the levels of the glycerophosphoinositols (Tables 1, 2).

PLA$_2$ activity and GroPIns-mediated functions

PLA$_2$IVα belongs to the PLA$_2$ superfamily, the 19 members of which differ in their substrate specificities, cellular localisation and calcium requirements for their enzymatic activities [42, 43]. Among these members, the group IV, calcium-sensitive, cytosolic PLA$_2$s are ubiquitously expressed and have activities specific for phospholipids

Table 2 Levels of GroPIns in cells stimulated with calcium ionophores

Cell line	GroPIns stimulation (% increase over basal)	Cell type
IMLF WT	180[a]	Immortalised mouse lung fibroblast
IMLF KO	90[a]	Immortalised mouse lung fibroblast $PLA_2IV\alpha^{-/-}$
RBL	70[b]	Rat basophilic leukaemia
THP-1	300[b]	Human acute myeloid leukaemia
Mono Mac 6	130[b]	Human monocyte
RAW 264.7	130[c]	Mouse leukaemia monocytic macrophage

The levels of GroPIns were evaluated by HPLC separation of water-soluble cellular extracts following [^{3}H]-inositol equilibrium labelling of cells, as described in [35]. Cells were stimulated with calcium ionophores at the indicated concentrations and for the indicated times, and the intracellular levels of GroPIns are expressed as percentage increases over basal (unstimulated) levels (unpublished data; see also [25] for further examples)

[a] 5 μM A23183 + 1 μM PMA (20 min, 37°C)

[b] 5 μM A23183 (15 min, 37°C)

[c] 5 μM Ionomycin (45 min, 37°C)

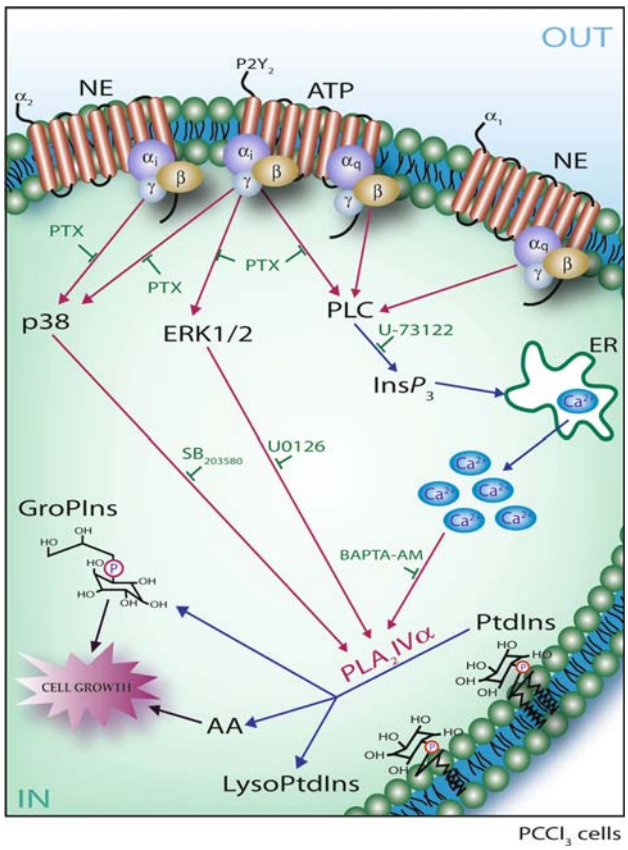

Fig. 2 Schematic representation of the signalling cascade in the formation of GroPIns in $PCCl_3$ cells. In $PCCl_3$ cells, activation of the adrenergic (*NE*; α_1, α_2) and purinergic (*ATP*; *P2Y*$_2$) receptors can lead to pertussin toxin (*PTX*)-sensitive activation of p38 SAPK (*p38*), ERK1/2 and PLC. These, in turn, can either directly phosphorylate and activate $PLA_2IV\alpha$, or in the case of $P2Y_2$ receptor activation, promote an increase in $[Ca^{2+}]_i$ that is relevant for $PLA_2IV\alpha$ translocation to intracellular membranes and for ERK1/2 activation. A $[Ca^{2+}]_i$ increase can also be induced by a PTX-insensitive pathways. $PLA_2IV\alpha$ activation results in hydrolysis of PtdIns and release of LysoPtdIns, and then GroPIns and arachidonic acid (AA). The inhibitors used in determining these cascades are indicated. *InsP*$_3$ inositol 1,4,5-trisphosphate, *PTX* pertussis toxin, *U-73122* PLC inhibitor, *U0126* ERK1/2 inhibitor, *BAPTA-AM* calcium chelator, *SB*$_{203580}$ p38 SAPK inhibitor, *ER* endoplasmic reticulum; *αi,q*, α subunits of the Gi,q G proteins; *β*, *γ*, *β* and *γ* subunits of these G proteins. Modified from Fig. 7 in [23]

with an arachidonic acid in the *sn-2* position. The group IV PLA_2s were recently divided into six subtypes: α, *β*, *γ*, *δ*, *ε* and *ζ* [44], with the α-subtype being the most common and having the peculiarity of also possessing lysolipase, transacylase and esterase activities [43].

The specific PLA_2 isoform involved in the synthesis of GroPIns was initially defined in $PCCl_3$ wild-type cells (an immortalised epithelial cell line derived from Fisher rat thyroid that retains markers of differentiation, [45]) and later confirmed as $PLA_2IV\alpha$ in oncogene-transformed $PCCl_3$ cells [23, 31] (Figs. 2 and 3). Accordingly, in $PCCl_3$ cells, upon mobilisation of intracellular calcium through purinergic or adrenergic receptor stimuli or the addition of a calcium ionophore, the levels of GroPIns increase in parallel with the release of arachidonic acid, indicating that receptor signalling that leads to the formation of GroPIns requires calcium, and a calcium-dependent PLA_2 activity. Interestingly, both the purinergic and the adrenergic systems regulate thyroid function, through cascades initiated by a rise in intracellular calcium [46–48].

$PLA_2IV\alpha$ activity is also regulated by phosphorylation [40]; Ser505 is the relevant residue modified by mitogen-activated protein kinases (MAPKs), an essential prerequisite for agonist-induced arachidonic acid release in Chinese hamster ovary (CHO) cells [49, 50], but not for thrombin activity in platelets [51]. In macrophages, this Ser505 phosphorylation has been shown to be relevant when transient increases in intracellular calcium take place [52], while in chondrocytes, the concomitant activation of the ERK1/2 and p38 SAPK signalling pathways is required for ATP-induced activation of cytosolic PLA_2 [53], with both of these kinases phosphorylating the same residue on the phospholipase sequence [40].

The full pathway of $PLA_2IV\alpha$ activation after hormonal stimulation in $PCCl_3$ cells also involves ERK1/2 and p38 SAPK phosphorylation of $PLA_2IV\alpha$. Thus, specific kinase inhibitors can prevent this agonist-induced phosphorylation of $PLA_2IV\alpha$, and hence the consequent formation of GroPIns and arachidonic acid release stimulated by receptor

activation. A schematic representation of this signalling cascade is given in Fig. 2 (see also [23]).

Different approaches have been used to confirm the coupling of these receptors to PLA_2IVα activation and the consequent formation of GroPIns. These have included the use of PLA_2 inhibitors, down-regulation of PLA_2IVα by RNA interference (RNAi) and over-expression of wild-type PLA_2IVα and of a dominant-negative deletion mutant ($PLA_2IV_{1–522}$ [54]); this last acts by competing with the endogenous PLA_2IVα for membrane binding [23]. These diverse approaches were combined, considering that the majority of the commercially available PLA_2 inhibitors lack specificity [55]. This is especially true for inhibitors of group IV PLA_2s, which are potentially of great interest as anti-inflammatory agents (due to the involvement of these PLA_2 metabolites in inflammation [56]). Indeed, several groups are working on the development of potent and selective inhibitors of group IV PLA_2α, which led to the recent identification of some leads based on the 1,2,4-tri-substituted pyrrolidine backbone [57]. Among these, pyrrophenone completely blocks ATP-induced and nor-epinephrine-induced production of GroPIns and LysoPtdIns in $PCCl_3$ cells, in line with the identification of PLA_2IVα as the relevant enzyme in this cascade.

From the functional point of view, hormone-induced formation of GroPIns has been related to the regulation of cell growth. In thyroid cells, the control of proliferation and of differentiation are strictly coupled [47, 58, 59]: a TSH-regulated cAMP signal promotes both cell growth and cell differentiation, while signals activated by different oncogenes can impair differentiation and stimulate TSH-independent proliferation [60, 61]. ATP and norepinephrine clearly stimulate $PCCl_3$ cell growth in a PLA_2IVα-dependent manner, an effect mimicked by the PLA_2-derived metabolites arachidonic acid and GroPIns [23]. Importantly, GroPIns is active when added to cells at concentrations that are similar to the intracellular levels (see also Table 1), implying that GroPIns is a good candidate for a role in the signalling pathways of purinergic- and adrenergic-receptor-induced thyroid cell growth. Thus, these studies define GroPIns as a potential cell mediator involved in the regulation of cell proliferation [23]. Data linking the PLA_2/GroPIns cascade to cell proliferation were also given in an earlier report that indicated that NIH 3T3 fibroblasts over-expressing PtdIns-transfer protein α (SPI8 cells) are characterised by a twofold to threefold increase in the cellular levels of LysoPtdIns and GroPIns, suggesting that in these cells a PtdIns-specific PLA_2 is constitutively active. This modulation was clearly associated with an increased growth rate and morphological changes of these SPI8 cells, although direct roles for either LysoPtdIns or GroPIns in these processes have not been detailed [62].

PLA_2 activity in RET-transformed cells

As mentioned above, oncogenic transformation of thyroid cells results in the loss of TSH control of cell proliferation, implying the involvement of alternative regulatory pathways, as could be the case for the PLA_2IVα-dependent cascade outlined above (see also [23]). In support of this, a PLA_2 activity associated with Ras-induced transformation has previously been reported in thyroid cells: this activity is specific for the phosphoinositides and can be detected by increases in the levels of the glycerophosphoinositols, which are paralleled by increases in LysoPtdIns and arachidonic acid [24, 25, 30, 63–65] (Table 1).

In the thyroid, chromosomal aberrations cause gene recombinations that can generate a constitutively active RET/PTC oncogene chimera that has been implicated in the development of papillary thyroid carcinomas (PTCs) [66, 67]. The RET proto-oncogene encodes a tyrosine kinase receptor for growth factors of the glial-derived neurotrophic factor (GDNF) family, and its signalling was believed to involve mainly the Grb/Ras/MAPK and phosphoinositide-3-kinase cascades [61], with no evidence for a role of PLA_2. Cells transformed by the RET/PTC oncogene (PC-PTC cells [60]) are, however, characterised by an increased PLA_2IVα activity and higher intracellular levels of GroPIns, as compared to normal $PCCl_3$ cells, suggesting that this cascade is part of the signalling activated by this oncogene [31] (Table 1).

This peculiarity of PC-PTC cells, whereby they show higher intracellular concentrations of GroPIns (420 μM, as compared to 270 μM in $PCCl_3$ cells; see [30] and Table 1), led to the identification of a novel signalling cascade that is initiated by the RET oncogene and involves activation of a PLA_2 and formation of GroPIns. The enzyme that operates downstream of active RET/PTC is PLA_2IVα, as indicated by pharmacological (the specific PLA_2IVα inhibitor pyrrophenone) and molecular (RNAi) approaches [31]. In addition, while knock-down of PLA_2IVα reduced the levels of GroPIns, as expected, it did not affect other inositol-containing aqueous compounds derived from PLC activity (i.e. the inositol polyphosphates). This PLA_2IVα activation is thus a direct consequence of RET kinase activity, which phosphorylates p38 SAPK, which, in turn, phosphorylates and regulates PLA_2IVα, as outlined in the scheme in Fig. 3 [31].

Interestingly, this RET-dependent PLA_2IVα pathway is of patho-physiological relevance, in that it is active in human papillary carcinoma cells and in thyroid tumour tissues (which have high levels of phosphorylated/active PLA_2IVα), indicating a relevant role for PLA_2IVα in promoting the mitogenic activity of the RET/PTC oncogene [31]. In addition, the RET/PTC–PLA_2IVα coupling is specific [31], since the PLA_2IVα isoform is not active in

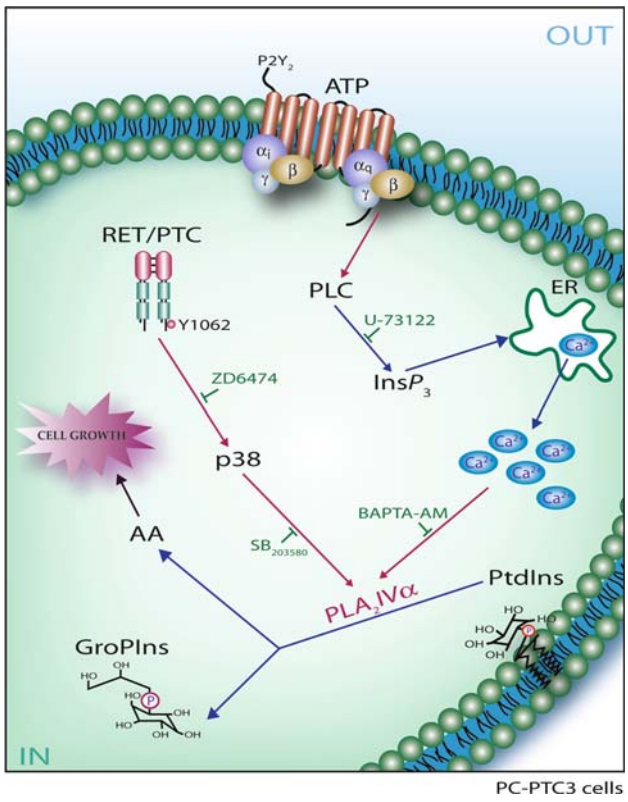

Fig. 3 Schematic representation of the signalling cascade leading to cell growth in RET/PTC-transformed cells. In PC-PTC3 cells, the pathway initiated by purinergic (*ATP*; *P2Y*$_2$) agonists is similar to that described in PCCl$_3$ cells (see Fig. 2). In addition, RET/PTC induces PLA$_2$IVα activation through p38 SAPK (*p38*)-dependent phosphorylation of PLA$_2$IVα, resulting in the release of arachidonic acid (AA) and GroPIns. *InsP*$_3$, inositol 1,4,5-trisphosphate, *ZD6474* RET inhibitor; see legend to Fig. 2 for other labels [31]

thyroid cells transformed by a different oncogene (i.e. BRAF V600E), which is frequently seen in papillary thyroid carcinomas [60].

In summary, the investigation of the metabolism of the glycerophosphoinositols in transformed thyroid cells has led to the identification of PLA$_2$IVα as a novel component of the signalling cascade initiated by the RET/PTC oncogene, and of its metabolite GroPIns as a marker of RET/PTC transformation.

Phospholipase B

As mentioned above, PLB enzymes are potentially involved in the formation of the glycerophosphoinositols, in that they can remove both the *sn-1* and *sn-2* fatty acids from the glycerol moiety of these phospholipids, and thus have phospholipase and lysolipase activities. Several PLBs have been identified in various microorganisms [32] and fungi [68], and in the brush-border membrane of mature enterocytes from guinea pig [69], rat [70] and rabbit [71].

The first characterisation of a human homologue of PLB was in epidermis [72]. Immunolabelling and in situ hybridisation analyses have provided evidence for PLB expression in the whole epidermis, with an accumulation in the basal layers. This location of PLB suggests a function in the hydrolysis of lipids from the blood circulation for the production of the free fatty acids that are required for epidermis barrier function.

More recently, a PLB precursor was isolated from the secretory organelles of human neutrophils that shows deacylation activity towards phospholipids, including phosphatidylcholine, PtdIns, phosphatidylethanolamine and their respective lysophospholipids [73]. Deacylation activity has also been detected in in vitro assays using a number of substrates, such as didecanoyl-phosphatidylcholine, dipalmitoyl-phosphatidylcholine, PtdIns, phosphatidylethanolamine and lysophosphatidylcholine, indicating that this PLB can act on a wide range of different membrane phospholipids. This enzyme is Ca^{2+} independent and is active across a broad pH range, with an optimum of 7.4. Finally, the presence of this PLB precursor in neutrophils may indicate a role in the defence against invading microorganisms and in the generation of lipid mediators of inflammation, as is the case for PLA$_2$/lysolipase activities.

Cellular levels

The cellular levels of the glycerophosphoinositols, and in particular those of GroPIns and GroPIns4*P*, have been monitored in a wide range of parental and transformed cells (Ras-transformed and tumour-derived cells, in general), as well as in differentiating cells [25, 30, 74]. The concentrations of GroPIns vary across these cells, ranging from the low micromolar to almost millimolar (from 44 to 925 μM; [30], see also Table 1). For GroPIns4*P* and GroPIns 4,5-bisphosphate (GroPIns45P_2), their relative levels are some 10-fold and 100-fold lower, respectively, than those of GroPIns, which are well within the levels of the other inositol-containing molecules (i.e. the inositol polyphosphates) [30, 35]).

Modulation of the levels of GroPIns has been seen in different tissues and cell lines under hormone stimulation, and cell development and differentiation (for review, see [25, 74–78]). More recently, an increase in the basal levels of GroPIns was reported for rat basophilic leukaemia cells (RBL cells) and two human monocytic (THP-1, Mono Mac 6) and a mouse macrophagic cell lines (RAW 264.7), upon calcium ionophore addition (Table 2). In this last system, the intracellular concentrations of GroPIns were measured under normal growth conditions by electrospray ionisation tandem mass spectrometry (ESI-MS/MS, identification and quantification of GroPIns; [36]) (Table 1),

and they were shown to be modulated by several potentially active compounds, including lipopolysaccharides from *Escherichia coli*, cytokines, and other pro-inflammatory agents that are present at inflammatory foci, thus suggesting a role for GroPIns in inflammatory responses. This observation opened a line of study that has uncovered a role of GroPIns4*P* in T-cell receptor signalling and T-cell responses (see below and [79]).

Catabolism of the glycerophosphoinositols

The glycerophosphodiester phosphodiesterases (GP-PDEs) are to date the best studied of the enzymes that are involved in the catabolism of the glycerophosphoinositols [80]. These GP-PDEs were initially characterised in bacteria, with functional roles in the production of metabolic carbon and phosphate sources from the glycerophospholipids [81, 82], and for adherence to and degradation of mammalian host-cell membranes [83].

In *Saccharomyces cerevisiae*, a predicted cytosolic protein encoded by the YPL110c gene has also been characterised as a glycerophosphodiesterase (GDE) that can hydrolyse glycerophosphocholine but not GroPIns [84], and the presence of other GDEs cannot yet be excluded [85].

To date, seven different mammalian GDEs have been cloned [80], all of which are characterised by a catalytic region of a sequence of 56 amino acids [86]; however, so far, the GDE substrate specificities and physiological functions have been defined only for GDE1 and GDE3. GDE1 was identified as an interactor of regulator of G protein signalling (RGS)16 in two-hybrid screening [86], and it showed preference for the glycerophosphoinositols over glycerophosphocholine as substrate, with GroPIns converted into inositol and glycerol phosphate [86] (Fig. 1). This is in contrast to the bacterial GP-PDEs, which show broad substrate specificities with respect to the alcohol moiety of the glycerophosphodiesters [81, 82]. This GDE1-mediated catabolism of GroPIns should also be hormone regulated, as GDE1 activity is under the control of G protein-coupled receptors [86].

GDE3 has been characterised as a marker of osteoblast differentiation and was isolated using a differential display method [87]. It is also a specific GroPIns inositolphosphodiesterase (EC 3.1.4.43) [88], although it shows different specificities, and it attacks the phosphodiester bond by hydrolysing GroPIns to glycerol and inositol 1-phosphate, showing a phospholipase C-like activity; instead, the GDE1 enzymatic activity resembles more a phospholipase D-like attack of the phosphodiester bond (Fig. 1). GDE1 and GDE3 can thus both mediate the catabolism of GroPIns, leading to different products, which might reflect specific functions of GroPIns and its catabolic products in given tissues and cell systems.

With regard to other functions of these GDEs, interesting data have been obtained in osteoblasts, where the activity of GDE3 can indeed be related to the physiology of these cells [88]. Thus, GDE3 expression in MC3T3-E1 osteoblasts can induce actin cytoskeleton disorganisation, resulting in a clear disassembly of stress fibres. Intriguingly, GroPIns4*P* is a well-characterised modulator of the actin cytoskeleton in fibroblasts, where exogenous addition of GroPIns4*P* induces ruffle formation and the appearance of stress fibres [89, 90]. Moreover, although a previous report simply showed GDE3 as an early marker of osteoblast differentiation [87], more recently we have shown that GDE3 has a role in inducing the osteogenic process [88]. The GDE3 expression pattern during MC3T3-E1 development showed that its mRNA levels peaked at days 5–7 of culturing cells in osteogenic medium, which corresponds to the extracellular matrix (ECM) development stage of the three osteoblast differentiation stages [87]. In addition, stable expression of GDE3 in osteoblasts is sufficient to induce osteoblast differentiation, which is characterised by a decrease in cell growth rate and a dramatic stimulation of alkaline phosphatase activity and calcium deposition [88]. Here, several aspects of skeletal development are mechanistically linked, including lineage specification and growth and differentiation of mesenchymal cells. In particular, temporal growth arrest is considered to have a critical role in triggering osteoblastic differentiation [87]. The hydrolysis of GroPIns has been shown to be one of the causes of this decreased growth rate of differentiated osteoblasts, since as seen for $PCCl_3$ rat thyroid cells [23], GroPIns stimulates cell growth; thus induced expression of GDE3 in differentiated osteoblasts will increase the metabolism of GroPIns, with a consequent slowing down of osteoblast proliferation [88].

Interestingly, new substrates of GDE1 have also been discovered recently among the glycerophospho-*N*-acyl ethanolamines, suggesting physiological involvement of GDE1 in the biosynthesis of anandamide (*N*-arachidonoyl ethanolamine), an endogenous ligand for the brain cannabinoid receptor [91]. In contrast to GDE1, the other membrane-associated GDEs, including GDE3, are not active on the glycerophospho-*N*-acyl ethanolamines [91]. However, based on our analysis of different potential physiological substrates of GDE3, it cannot be excluded at this stage that molecules other than GroPIns might be more physiologically relevant for GDE3 activity, and might also include the phosphorylated glycerophosphoinositols (e.g. GroPIns4*P*).

Based on hydropathy analysis, all but one of the GDEs contain multiple transmembrane regions [80] and appear to be membrane bound [91]; the exception here is GDE5. As

GDE5 is therefore cytosolic, it would at present represent the only good candidate for the regulation of the intracellular levels of GroPIns; however, our recent data indicate that GDE5 cannot hydrolyse GroPIns or GroPIns4*P* (unpublished observations).

The topology model of GDE1 predicts the facing of its N- and C-termini into the cytoplasm, and its catalytic domain into the extracellular space or the lumen of the endoplasmic reticulum [86]. The catalytic domain of GDE3 is also exposed in this way, and thus active towards the extracellular space, as also indicated by the requirement of calcium in the millimolar range for GDE3 activity and by the absence of hydrolysis of the intracellular pool of GroPIns when GDE3 is over-expressed [88]. Since the metabolism of the glycerophosphoinositols has been shown to occur intracellularly [23, 25, 31], it implies that GroPIns would have to reach the outer leaflet of the plasma membrane to interact with the catalytic domain of these GDEs. Thus, the release of the glycerophosphoinositols into the extracellular medium can initiate their catabolism or promote effects on their paracrine targets [79].

The transporter of the glycerophosphoinositols

An intriguing aspect of the biology of the glycerophosphoinositols is that, even when added exogenously, these hydrophilic compounds exert their functions intracellularly. In addition, they have been shown to be released from cells, and to be processed by specific extracellular phosphodiesterases (see above and [85, 92]). This catabolic pathway might be activated under specific conditions (e.g. high-intracellular Ca^{2+}, PLA_2 activation), where high intracellular levels of GroPIns are reached and the gradient formed across the plasma membrane favours the release of this GroPIns [23, 25, 63]. The released GroPIns could also be part of paracrine or autocrine signalling, where the GroPIns released can act on the cell of origin or on nearby cells.

Clearly, the hydrophilic nature of the glycerophosphoinositols is not compatible with their rapid partitioning across the membrane (and thus with their diffusion through the membrane), and therefore a transporter-assisted permeation was investigated as the potential mechanism that promotes their crossing of the plasma membrane. The evidence related to the mechanism of the membrane transport of GroPIns first came from studies in yeast. In this cellular system, GroPIns is secreted as a product of phosphoinositide catabolism; moreover, under conditions of scarce inositol supply or in phosphate-starved yeast cells, GroPIns can be taken up again from the extracellular medium and be incorporated into the phosphoinositides [92–96]. This transport of GroPIns involves a permease of 57.3 kDa (characterised by the SDRIGR(K/R) sugar-transport motif around amino acid 329 and by 12 potential membrane-spanning domains) that is encoded by the 1,556-bp GIT1 gene [95, 96]. This transport is specific, in that it is not significantly affected by glycerol or inositol, and only inhibited in part by glycerol 3-phosphate, glycerophosphocholine and glycerophosphoethanolamine [95]. This Git1p-mediated release of GroPIns is followed by its hydrolysis, providing an extracellular reservoir of crucial nutrients (inositol and phosphate) when PtdIns or GroPIns, but not free inositol, are available [85].

Git1p [95, 96] is a member of the phosphate:H^+ symporter (PHS) subfamily of the major facilitator superfamily (MFS) of transporters, which includes members from yeast, fungi and plants, but not from bacteria, animals and other eukaryotes [97]. Identification of the human Git1p orthologue was based initially on a search by homology, which would result in very low scores between human and yeast proteins [97], and by a phylogenetic study between the different families of the MFS tree. The results indicated that the human protein that has the highest homology with Git1p (through the PSI-BLAST search) is Glut2, which itself belongs to the sugar-porter family of proteins that is highly related to the PHS family of Git1p.

The hGlut2 protein [98] contains 12 membrane-spanning (TMS) helices that are characterised by a QLS motif in helix 7 and an STSIF motif in loop 7 (which participate in substrate recognition), a five-residue motif (RXGRR) located between TMS2 and TMS3, and a similar motif between TMS8 and TMS9 (which is involved in interactions with the negative charges of the membrane–lipid head groups) [97, 99, 100]. With the exception of the QFS motif, these motifs are also present in the yeast Git1p structure [101].

Biochemical investigation into the function of Glut2 (with specific inhibitors, and by RNAi and competition studies) have indicated that Glut2 can indeed mediate the specific uptake of GroPIns into cells, as seen in cells that endogenously express the transporter (HEK293 cells) and in HeLa cells over-expressing human Glut2 [101]. Indeed, Glut2 shows a tenfold higher apparent affinity for GroPIns ($K_m = 80$ μM) than for glucosamine ($K_m = 0.8$ mM), a sugar that is involved in the synthesis of the proteoglycans that has previously been shown to be transported by Glut2 [102, 103]. This transporter affinity is in line with the intracellular concentrations of GroPIns, and the lack of competition at physiological concentrations of glucose or glucosamine indicate that GroPIns should indeed be an endogenous ligand for Glut2 [101].

Of note, knowing that Glut2 is predominantly expressed in liver, kidney and small intestine [98] raises the point that it should not be the only transporter of GroPIns in mammals. Thus, while Glut2-mediated transport provides a

proof-of-principle that the glycerophosphoinositols can permeate the cell membrane by a transporter-driven mechanism, similar to that reported in yeast for Git1, other yet-to-be-identified proteins must facilitate the transport of GroPIns in tissues that do not express Glut2 [101].

The biological functions of the glycerophosphoinositols

The different glycerophosphoinositols have been shown to have a broad range of functions and targets. As indicated above, in epithelial cells, these compounds are specifically produced by the PLA_2IVα isoform, and endogenously produced GroPIns controls TSH-independent cell proliferation [23]. Cellular growth and iodide uptake of thyroid cells can also be regulated by GroPIns4*P*, through inhibition of adenylyl cyclase (AC) [104]. In addition, GroPIns4*P* is involved in the mechanisms controlling T-cell signalling, again through the involvement of AC, and here also a guanine-nucleotide-exchange factor (GEF) of Rac1, Vav [79]. In fibroblasts, exogenous addition of GroPIns4*P* induces actin ruffling via activation of another GEF of Rac1, Tiam1 [90], and this time in a cAMP-independent manner. GroPIns and GroPIns4*P* also reduce the ability of metastatic cells to degrade the ECM in in vitro assays [105].

Other targets reported for these compounds include PLC, as GroPIns4*P* has been described as a selective and competitive inhibitor of guinea pig uterus-derived PtdIns-specific PLCβ [106], and consequently it has also been used as an inhibitor to determine PLC involvement in caffeine-induced contraction of endothelium-intact arterial strips [107]. Other studies have indicated that the glycerophosphoinositols are substrates of PtdIns-specific PLCδ1, with the phosphate groups on the inositol ring important for substrate binding (the 4′-phosphate) and for catalysis (both the 4′- and 5′-phosphates) [108]. Consequently, PLCδ1 shows a lower activity towards GroPIns [108], which has also been confirmed in a further study that identified the specific amino acids involved in the enzymatic cleavage of the phosphoinositide phosphodiester bond by PLC [109].

Other potential physiological effects of the glycerophosphoinositols include the inhibition of the cardiac sarcolemmal Na^+/Ca^{2+} exchanger in vesicle preparations by both GroPIns4*P* and GroPIns45P_2 [110]. The same authors also showed that α1-adrenergic agonists stimulate PLA_2 and lysolipase activities in rat right ventricle slices, forming GroPIns4*P*, which could then inhibit the Na^+/Ca^{2+} exchanger, thus contributing to the positive inotropic effects of these agonists [111]. Again, these effects of the glycerophosphoinositols are also believed to be due to competition for the same binding sites that recognise the phosphoinositides, particularly as the latter are also known to stimulate the same sarcolemmal Na^+/Ca^{2+} exchanger [110]. More recently, GroPIns has been evaluated with the aim of developing novel strategies for blood–brain barrier (BBB) repair. GroPIns showed a dramatic similarity with respect to the steroidal hormones and synthetic analogues such as dexamethasone when used to counteract BBB failure [112]. Thus, at least in an in vitro model of the BBB based on co-culture of endothelial cells and glia, GroPIns can improve BBB function by promoting both BBB formation and repair [112].

The diversity of these effects has prompted further investigations into the mechanisms of action of these compounds, as summarised below:

Modulation of adenylyl cyclase activity

GroPIns4*P* reduces cAMP levels in a number of cell systems, including fibroblasts, and thyroid epithelial, RBL and RAW 264.7 cells, and Jurkat T-cells [75, 79, 104, 113]. With FRTL5 thyroid cell membranes, GroPIns4*P* causes a $\sim$50% inhibition of the AC activity stimulated by the GTP-binding protein Gs activator aluminium fluoride. This inhibition is also seen in intact cells, where as a consequence, GroPIns4*P* inhibits cAMP-dependent cellular functions, such as iodide uptake and thymidine incorporation. In this cell system, the inhibition is evident only when the Gs protein is activated (either directly by cholera toxin or aluminium fluoride, or through receptor coupling). In parallel, GroPIns4*P* can also inhibit cholera toxin-induced ADP-ribosylation of Gs, suggesting that GroPIns4*P* acts directly on Gs [75, 104], although no direct binding of GroPIns4*P* to Gs or other G proteins has been reported [25].

Thus, GroPIns4*P* represents both an endogenous modulator and a potential pharmacological tool for the regulation of cAMP levels in cells. This becomes relevant for those hormonal cascades that, by producing GroPIns4*P*, could directly regulate cAMP-dependent signalling cascades. In Swiss 3T3 fibroblasts and RBL cells, the direct stimulation of AC by forskolin is also inhibited by GroPIns4*P* [113]. A possible explanation of this different behaviour is that, since the interaction of forskolin with some AC isoforms requires an activated Gs protein [114], the action of GroPIns4*P* could in principle be due to the Gs activity [113]. Instead, in NIH 3T3 fibroblasts, the GroPIns4*P* effects on AC appear to be mediated by activation of Gαi, since GroPIns4*P* can increase GTPγS binding to plasma membranes and inhibit AC in a pertussin-toxin-dependent manner, indicating the involvement of Gαi ([90] and unpublished observations). Thus, in specific cell systems, GroPIns4*P* might activate Gi, or intervene in the signalling of a membrane receptor coupled to Gi (Fig. 4).

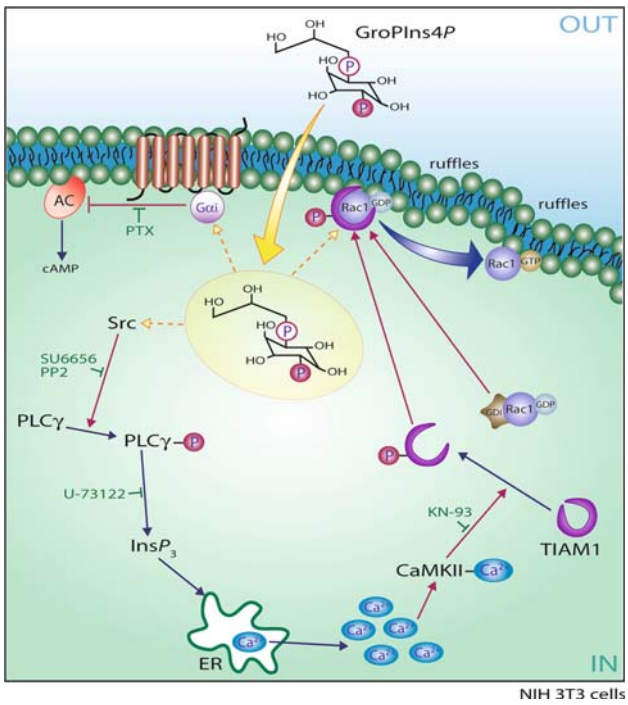

Fig. 4 Schematic representation of the GroPIns4*P* pathway modulating the actin cytoskeleton in NIH 3T3 cells. GroPIns4*P* induces the activation of the Src kinase (*Src*), which phosphorylates and activates PLCγ, leading to InsP_3 production and consequently $[Ca^{2+}]_i$ increase. This results in activation of Ca-CaMKII, which phosphorylates Tiam1, facilitating its translocation to the plasma membrane and enhancing its exchange activity toward Rac1 and the formation of membrane ruffles. Alternative GroPIns4*P* pathways could involve a direct stabilisation of the Tiam1 and Rac1 interaction, or modulation of adenylyl cyclase (AC) in a pertussis toxin (*PTX*)-sensitive manner. The inhibitors used in determining these cascades are indicated. *InsP_3* inositol 1,4,5-trisphosphate, *GDI* generic Rac1 GDP dissociation inhibitor, *PP2* and *SU6656* Src inhibitors, *KN93* CaMKII inhibitor; see legend to Fig. 2 for other labels. Modified from Fig. 9 in [90]

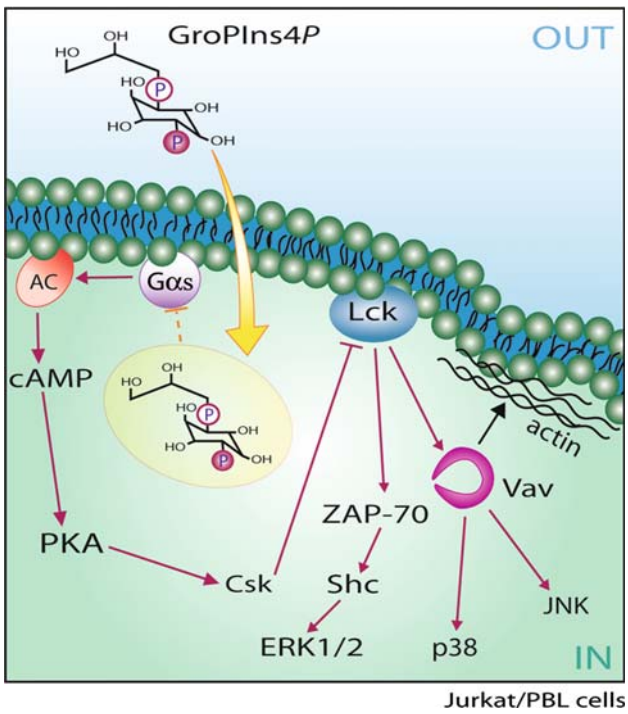

Fig. 5 Schematic representation of the GroPIns4*P* signalling cascade regulating chemotaxis in T-lymphocytes. Exogenously added GroPIns4*P* acts at the G protein level, inhibiting Gs (αs)-dependent cAMP production and hence PKA activity. This consequently removes the PKA block of the Lck regulator Csk, resulting in the activation of two partially independent pathways by Lck: ZAP-70/Shc/ERK1/2 and Vav/p38/JNK. These two pathways that arise partially through Vav activation and the reorganisation of the actin cytoskeleton are involved in chemotaxis of T-cells in responses to proinflammatory stimuli. Modified from Fig. 7 in [79]

Inhibition of cAMP production by GroPIns4*P* has also been reported in Jurkat T-cells [79]. T-cell treatment with GroPIns4*P* (and to a lesser extent, with GroPIns) resulted in a synergistically enhanced SDF-1α-dependent chemotaxis, without affecting the low levels of spontaneous cell migration. As in other cells, these activities of GroPIns4*P* are dependent on its ability to antagonise the production of cAMP (both basal and forskolin-stimulated), resulting in attenuation of protein kinase A (PKA) activity (as determined by assessing the phosphorylation state of intracellular PKA substrates using an antibody specific for the phosphorylated PKA consensus, R-X-X-pT-X-X/R-R-X-pS-X-X) [79]. These data were obtained both in the presence and the absence of the generic phosphodiesterase inhibitor, IBMX, and they suggest that the inhibitory effects of GroPIns4*P* on cAMP production are not exerted at the level of the phosphodiesterase, but more likely through a Gs protein-mediated action (Fig. 5).

However, the recent identification and characterisation of new PDE isoforms [115] that are specific for cAMP hydrolysis indicate that IBMX is not as general a PDE inhibitor as was originally thought. Further, the possibilities that the mechanism of action of GroPIns4*P* is based on the increased catabolism of cAMP and that a PDE could be the GroPIns4*P* target cannot be completely excluded. In particular, some of these PDEs (PDE8, PDE10) are highly expressed in lymphocytes (where can be up-regulated with cell activation) and in thyroid cells [115, 116], both systems in which GroPIns4*P* has been shown to be active on AC. Alternatively, depending on the cell system, different G proteins might be the targets of GroPIns4*P* activity, or the effects of GroPIns4*P* might be exerted on cellular factor(s) regulating these G proteins (such as RGS or GRK [117–119]).

Modulation of Rho GTPase function: the GEF, Tiam1

GroPIns4*P* modulates the activity of the small GTPases of the Rho family, thus affecting the organisation of the actin

cytoskeleton and leading to the formation of membrane ruffles and stress fibres [89, 90]. In fibroblasts, the mechanism of action of GroPIns4*P* added exogenously involves the activation of a phosphorylation cascade that acts through the tyrosine kinase Src, PLCγ and Ca^{2+}/calmodulin kinase II (Ca-CaMKII). This results in Tiam1 translocation to the plasma membrane, and thence to the formation of membrane ruffles via Rac1 activation (Fig. 4; [89, 90]). In parallel, the fraction of GTP-bound Rac1 is increased, as was revealed by affinity precipitation of the active Rac1 GTPase, with the different glycerophosphoinositols showing different kinetics and potencies of their effects: GroPIns4*P* induces a pronounced and sustained activation of Rac1, whereas the activation by GroPIns and GroPIns45P_2 is only transient and weak. This differential modulation may explain the ability of GroPIns4*P,* and not GroPIns and GroPIns45P_2, to promote rearrangements of the actin cytoskeleton [89].

An essential role for the Src/Ca^{2+}/CaMKII pathway in GroPIns4*P*-induced ruffle formation in NIH 3T3 cells is clearly indicated by the lack of effect of GroPIns4*P* in SYF cells (embryonic fibroblasts that do not express the three ubiquitously expressed Src isoforms of Src, Fyn and Yes [120]). This ruffle induction by GroPIns4*P* can be rescued in these SYF cells upon over-expression of Src, directly demonstrating the link between GroPIns4*P* and the Src cascade [90]. At variance with this finding, however, is that stimulation of growth factor receptors (e.g. the PDGF receptor, which is known to regulate actin polymerisation) can induce ruffle formation in SYF cells both before and after Src over-expression, indicating that growth factor signalling in these cells diverges from that of GroPIns4*P*. Thus, while the tyrosine kinase Src is essential for GroPIns4*P*-induced membrane ruffling in both NIH 3T3 and SYF fibroblasts, the growth factors appear to use alternative pathways, bypassing the Src-dependent phosphorylation cascade. However, GroPIns4*P* does not exert these actions by directly binding to Src or by directly stimulating Src kinase activity, since GroPIns4*P* cannot activate this kinase per se, or even in combination with cell lysates (used as a supply of potential cofactors) when used in in vitro kinase assays with purified Src [90].

Since Src kinases are regulated by phosphorylation/dephosphorylation events [121], a regulatory mechanism might involve the action of GroPIns4*P* on one of the protein–tyrosine phosphatases that activate Src kinases by removing the inhibitory phosphate (on tyrosine 527), such as PTPα, Shp1 and Shp2 [122]. Evidence of this mechanism has not been reported. Alternatively, GroPIns4*P* could favour an interaction between Src and one of its cofactors.

An intriguing observation is that, besides initiating this Src-dependent phosphorylation cascade, GroPIns4*P* also directly regulates the interaction between Tiam1 and Rac1. In in vitro pull-down experiments using purified Rac1-GST and lysates from HEK293 cells over-expressing Tiam1-HA, GroPIns4*P* increased the Tiam1–Rac1 interaction by 2.8-fold [90]. Therefore, with Rac1 and Tiam1, GroPIns4*P* induces both their plasma membrane co-localisation and their direct interaction, which in turn leads to the formation of membrane ruffles. Since GroPIns4*P* does not directly activate Rac1 [89], Tiam1 represents the main target of both the GroPIns4*P*-induced regulatory cascade and the GroPIns4*P*-facilitated interactions between Tiam1 and Rac1, to form a multi-protein complex. Whether these two mechanisms coexist or whether they have independent roles under different physiological conditions in mutant cells remain to be determined. A possibility is that GroPIns4*P* interacts with a scaffolding protein, which could then bring Tiam1 and Rac1 together, either through conformational changes or by increasing their mutual affinity following incremental formation of the resulting protein complex (Fig. 4).

Modulation of the immune response: the GEF, Vav

Recent studies on the glycerophosphoinositols have focussed on inflammatory and immune responses, as some of the cells involved in these functions have potent PLA_2 activities that generate the glycerophosphoinositols. Of note, Jurkat T-cells, the human leukaemic T-cell line widely used as model system for the T-cell receptor signalling pathway [123], are characterised by low endogenous levels of GroPIns (Table 1) and however, it is very likely that under physiological conditions T-cells will be exposed to high GroPIns4*P* levels released by other immune cells as they respond to external/inflammatory stimuli. Several haematopoietic cell lines have potent PLA_2 activities and can produce large amounts of the glycerophosphoinositols in response to proinflammatory stimuli, including macrophages and dendritic cells (antigen-presenting cells, APCs, that engage in close partnerships with T-cells) [25]. Thus, under physiological/pathological conditions, the glycerophosphoinositols, and in particular GroPIns4*P*, can be released by APCs and will thus be able to modulate T-cell activity.

Interestingly, GroPIns4*P* enhances the Jurkat T-cell and peripheral blood lymphocyte chemotaxis that is dependent on the homeostatic chemokine SDF-1α [79], while GroPIns shows minor enhancement and GroPIns45P_2 consistently reduces T-cell chemotaxis slightly. Actin reorganisation is a critical element for T-cell chemotaxis [124], and this process requires the activation of Rho family GTPases that is mediated by the GEF Vav [125, 126]. Jurkat T-cell treatment with GroPIns4*P* resulted in a robust phosphorylation of Vav and an enhancement of actin polymerisation.

Although numerous mechanisms may account for this Vav activation, the agonistic activity of GroPIns4*P* on the Src-family kinase Lck, together with the failure of GroPIns4*P* to trigger Vav phosphorylation in Lck-deficient cells (JCaM1 cells) and the reconstitution of GroPIns4*P* synergism on SDF-1α-dependent chemotaxis in JCaM1 cells with Lck expression, are all fully consistent with a mechanism of Vav activation by GroPIns4*P* mediated through Lck (Fig. 5). GroPIns4*P* has also been shown to induce Lck-dependent activation of ZAP-70, Shc and ERK1/2, with delayed kinetics (Fig. 5).

These activities of GroPIns4*P* are dependent on its ability to inhibit cAMP production and PKA activation. In addition, GroPIns4*P* has a potent modulatory effect on lymphocyte proliferation induced by T-cell-receptor activation (unpublished observations). These data clearly indicate a role for the glycerophosphoinositols as modulators of T-cell signalling, and establish a mechanistic basis for the effects of GroPIns4*P* on F-actin dynamics in the immune response.

Modulation of extracellular matrix degradation

The glycerophosphoinositols also have effects that are potentially relevant to tumour biology. These consist of the ability of GroPIns and GroPIns4*P* to inhibit migration through the ECM of human mammary carcinoma (MDA-MB-231) cells and human melanoma (A375MM) cells [105]. Both GroPIns and GroPIns4*P* do not significantly alter the ability of these cells to follow a chemotactic gradient, but instead they inhibit the ability of these cells to degrade the ECM (as shown in in vitro assays of gelatin degradation). Thus, treatment with GroPIns and GroPIns4*P* reduces both the total area of degradation and the mean area per individual degradation event [105].

The actions of the glycerophosphoinositols on the invasivity of tumour cells cannot be clearly linked to their regulation of the actin cytoskeleton, or to other cellular functions known to be implicated in tumour invasion (adhesion, both between cells and with the ECM, proteolytic degradation of and penetration into the ECM, and mobility towards the target tissue) ([105] and unpublished observations). In addition, the reduced efficiency of degradation is also unlikely to be exerted at the level of the matrix metalloproteases, the family of endopeptidases that degrade all the components of the ECM [127]. Indeed, when the role of the glycerophosphoinositols on the activation of latent matrix metalloproteases was evaluated using zymographic analysis with gelatin and a fluorimetric analysis with DQ-gelatin, treatments with the different glycerophosphoinositols did not modulate the enzymatic activities of these matrix metalloproteases. Further investigation of ECM degradation concerning its localisation at the site of formation of the invadopodia, the highly specialised cellular structures that are involved in cell invasion and can be identified by markers such as actin and cortactin [128], indicated that the effects of the glycerophosphoinositols here might be exerted at the level of invadopodia formation. Indeed, GroPIns and GroPIns4*P* reduce the number of both degradation events and invadopodia structures, while they do not have significant effects on invadopodia organisation (as assessed by immunofluorescence of specific markers [105]).

However, a full mechanistic definition of this GroPIns- and GroPIns4*P*-induced impairment of tumour cells to degrade the ECM remains in need of further investigation. At this stage, however, it is reasonable to expect that this action of the glycerophosphoinositols can be exploited for the identification and development of novel anticancer drugs. Indeed, the different glycerophosphoinositols are being assessed in a number of models of tumour-cell invasion, to fully characterise their potential for prevention of tumour spreading. In parallel, the synthesis and evaluation of newly synthesised stable derivatives of the glycerophosphoinositols is underway, which represents a complementary approach that is necessary for the full pharmacological exploitation of these compounds.

Methods of detection

The identification of the most extensively studied glycerophosphoinositols, GroPIns and GroPIns4*P*, has required the development of some specific protocols for their separation and analysis. The different protocols that have been designed with the glycerophosphoinositols more specifically in mind are now provided in our recent methodological review on these aspects (see [35]).

In considering the hormone-induced formation of GroPIns4*P*, particular attention needs to be paid to the rapid dephosphorylation processes occurring under physiological conditions [63]. A membrane-associated phosphatase activity that is specific for GroPIns4*P* and is calcium- and pH-dependent (100–1,000 nM $[Ca^{2+}]_i$, pH 7.6) has been described [63]. Thus, the detection of this compound in some cell systems is possible only if general phosphatase inhibitors, such as vanadate, are routinely used during cell modulation followed by detection of the phosphorylated glycerophosphoinositols [23, 25, 35]. Other cellular enzymatic activities controlling GroPIns4*P* levels include its calcium-insensitive phosphorylation to GroPIns45P_2 and a cytosol-associated PLC-like activity acting on GroPIns45P_2 [63]. Finally, reacylation of GroPIns to PtdIns (but not of GroPIns4*P* to PtdIns4*P*) has been detected in cells and in membrane fractions (see [25], for details [129]).

In addition, a complete investigation into the metabolic pathways involved in basal and hormone-modulated levels of the glycerophosphoinositols requires the full analysis of the interconversions of the inositol-containing molecules that can originate from the parent phosphoinositides. Thus, techniques to evaluate the lipid and aqueous components of phosphoinositides breakdown include the separation of the phosphoinositides, of LysoPtdIns (produced via $PLA_{1/2}$ activities on PtdIns), of the cyclic inositol phosphates (produced via PtdIns/LysoPtdIns-specific PLC activities), and of the glycerophosphoinositols and the inositol phosphates [35]. Conventional chromatographic techniques (TLC, HPLC) were the mainstays of the first investigations into the metabolism of the glycerophosphoinositols, with their more recent complementation with mass spectrometry approaches that have allowed the definition of the molecular masses of these compounds, particularly when, for example, accurate definitions of their cellular levels are required ([30, 36]; and see also above).

Among the mass spectrometry techniques available for the glycerophospholipids, the best performance has been obtained using ESI-MS/MS, which provides higher sensitivity and lower interference due to matrix-derived noise with fast atom bombardment (FAB) and matrix-assisted laser desorption/ionisation (MALDI) mass spectrometry [130]. In addition, the ESI source is more versatile, as it is compatible with nearly all tandem mass spectrometers. The tandem mass spectrometric approach has allowed the complete identification of the glycerophospholipid structures, including the polar head groups, the fatty acid substituents, and the location of the radyl groups on the glycerol backbone. When knowledge of the mechanisms of fragmentation of various phospholipid classes becomes available, this technique will be useful for analysis not only of complex lipid structures [131] but also of the composition of biological specimens [130, 132]. A variant of this ESI-MS/MS method is desorption electrospray ionisation (DESI), which is an ambient ionisation technique that allows the direct analysis of biological samples, such as from tissues, without the homogenisation, extraction and chromatographic steps required for ESI-MS/MS [133]. Even if the sensitivity of this method is lower when compared to ESI-MS/MS, most of the phospholipids can be positively identified by this mass spectrometry method, which has also been coupled to imaging mass spectrometry that can show the distribution of particular lipids over sample areas to a spatial resolution of 250 μm. This imaging technique has been applied to distinguish between cancerous and non-cancerous tissues, and to identify types and stages of cancers, by monitoring alterations in the specific phospholipid composition [134]. NMR spectroscopy also represents another key tool for understanding the metabolic profiling of lipids, and lipid metabolites in intact tissues and in tissue extracts. Recently, this technique has been applied to the analysis of the relative metabolite peak areas of the glycerophosphoinositols in aqueous extracts and in intact tissues of normal prostate and prostate tumour tissues from C57BL/6J mice [135]. In mice with malignancy, significant decreases in the levels of GroPIns and glycerophosphocholine were reported.

The use of different techniques in determining the cellular levels of the glycerophosphoinositols has also provided novel information related to the inositol pool homeostasis. Thus, a consideration that has derived from directly comparing both standard [^{3}H]-inositol-loading and mass determinations in different cell lines is that there is a preferred pathway of inositol incorporation into and retention in the phosphoinositide pool under limiting inositol conditions [30]. For example, under conditions of increased metabolic activity, such as receptor stimulation or cell transformation, the polyphosphorylated phosphoinositide levels (i.e. PtdIns4*P*, PtdIns45P_2) can be maintained at the expense of PtdIns and the turnover of the glycerophosphoinositols and inositol [30]. This became evident by directly comparing cells with low ($\sim$250 μM) and high ($\sim$100 mM) cytosolic inositol levels, whereby they are both able to actively maintain their intracellular concentrations of the various inositol-containing compounds [30]. The main indication of this study was that the different inositol pools that characterise cells of different origins also need to be taken into consideration. This is due to isotope dilution effects under [^{3}H]-inositol equilibrium radiolabelling, whereby the intracellular concentrations of inositol can indeed contribute significantly towards the total inositol pool under cell culture conditions. Thus, comparisons across different tissues and cells should also be verified by mass measurements. These latter can be based on lipid-derived inositol 1,4,5-trisphosphate binding assays or on mass spectrometry techniques (see, for example, [30, 36]).

One consequence of these isotope dilution effects under [^{3}H]-inositol equilibrium radiolabelling is that the relationships between Ras-transformation and the intracellular concentrations of GroPIns that were initially reported were shown to hold for some, but not all, of the cell lines examined (e.g. when parental and Ras-transformed cells were compared; [24, 25, 74, 75, 136]). Instead, under conditions of cell transformation using temperature shifts, such as with temperature-sensitive clones, the levels of the glycerophosphoinositols can be reliably evaluated by equilibrium radiolabelling of the inositol pool [24]). This also holds for the evaluation of the different glycerophosphoinositols after hormone stimulation [23, 25, 113], in cells induced to differentiate, as for hepatocytes and neuronal cells [74], and during differentiation of various haematopoietic cells lines [76–78].

Open questions

From the information reported in the literature and summarised above, it emerges that besides being essential components of phosphoinositide metabolism, the glycerophosphoinositols participate in the regulation of cell functions such as proliferation and actin cytoskeleton organisation and dependent processes. The observed increase in the intracellular levels of GroPIns4*P* following inflammatory stimuli in macrophages suggests that the glycerophosphoinositols also mediate a paracrine mechanism relevant in the immune response: under physiological (or pathological) conditions, GroPIns4*P* might be released by cells, such as activated macrophages, and act on T-cells, enhancing their response to chemokines [79]. Thus, the production of the glycerophosphoinositols during an infection would be followed by their release into the extracellular space and by the increased recruitment of T-cells at the site of the infection; this would then lead to improved bacterial clearance. Similarly, the glycerophosphoinositols might contribute to anti-tumour immunity in cancers that are characterised by high levels of these compounds, whereby they are released in the extracellular space, and, by a mechanism similar to that described above, they enhance the recruitment of T-cells promoted by chemotactic signals released by cancer cells [79].

These proposed mechanisms might indeed represent the main physiological function of the glycerophosphoinositols, in that they involve cell types that are characterised by high and regulated levels of these compounds; however, this conclusion awaits a direct demonstration of this paracrine effect, which could be derived, for example, in cell co-cultures that can mimic this process. Alternatively, the identification of the direct molecular targets of the glycerophosphoinositols would also predict/be diagnostic of the cell types controlled by the glycerophosphoinositols.

As mentioned above, some of the effects of the glycerophosphoinositols have been related to an action at the level of the heterotrimeric G proteins (Gαs or Gαi) and of the GEF of the Rho GTPases. It is clear, however, that these proteins are not the direct targets of the glycerophosphoinositols. A number of candidates identified by a proteomic approach are at present being validated, which could lead to the full definition of the multiple mechanisms of action of these compounds.

Among the possibilities, the role of a membrane receptor specific for the glycerophosphoinositols, or indeed for only one of the phosphorylated forms, remains to be fully explored. Thus, while direct specific bindings of GroPIns and GroPIns4*P* to intact cells, membranes and membrane fractions have not been resolved [63, 90, 101], it cannot be excluded that in specific cell systems the glycerophosphoinositols can directly initiate receptor-mediated signalling, notwithstanding the observation that both GroPIns and GroPIns4*P* can permeate the cell membrane [63, 101]. Interestingly, the uptake of GroPIns4*P* has been followed both in intact and in permeabilised cells, with amounts of GroPIns4*P* that remain associated to intact cells double that of permeabilised cells (unpublished observations). This indicates that GroPIns4*P* can indeed accumulate in cells, while also binding to an immobile intracellular fraction. This is consistent with the absence of specific membrane binding in the same cell system, and with the observation that about 50% of the GroPIns4*P* that permeates into intact cells remains soluble (which is lost in permeabilised cells). Thus, we hypothesise that GroPIns4*P* can bind to a protein complex that it is not lost upon cell permeabilisation, as would be the case for the GroPIns4*P*–Tiam–Rac1 complex schematised in Figure 4.

To complicate this picture further, other glycerophosphoinositols that derive from other different phosphoinositides (such as GroPIns3*P* and GroPIns345P_3) can also modulate signalling enzymes, including AC, with different specificities and potencies (unpublished observations), indicating the crucial roles that the number and/or position of the phosphate groups on the inositol ring have in the interactions of these compounds with their specific targets. While these data were obtained in in vitro assays, it is conceivable that the full range of phosphorylated glycerophosphoinositols are present and active in different cell systems and/or compartments, possibly with the same degree of multiple targets and functions that have been reported so far for the phosphorylated inositols and the phosphoinositides. However, investigations into these polyphosphorylated glycerophosphoinositols are not readily feasible by conventional approaches [35]. This is largely due to their very low abundance (a few μM at most) and potentially relatively slow (or indeed fast) turnover, and hence problems with their being detectable using standard [^{3}H]-inositol equilibrium labelling and HPLC analysis. Similarly, difficulties for mass spectrometry analysis arise from this low abundance combined with the lower sensitivities of detection under the presently available negative-ion ESI-MS/MS technique (some 10- to 20-fold less sensitive than for GroPIns) [30, 35].

Efforts to obtain synthetic analogues and stable derivatives of the glycerophosphoinositols, are in progress in several laboratories. This will obviously help with the full elucidation of the physiological functions of the different glycerophosphoinositols and help in their exploitation as lead compounds, as has been proposed for their use as inhibitors of tumour invasion [25, 75, 105] and as bioactive molecules to enhance T-cell immunity in immunodeficiencies and in cancers [79].

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