View metadata, citation and similar papers at core.ac.uk
brought to you by CORE
provided by Elsevier - Publisher Connector

FEBS Letters 579 (2005) 2868–2872 FEBS 29563

# Effects of lipid kinase expression and cellular stimuli on phosphatidylinositol 5-phosphate levels in mammalian cell lines

Hilary F. Roberts[a], Jonathan H. Clarke[a], Andrew J. Letcher[a], Robin F. Irvine[a], Katherine A. Hinchliffe[b,*]

[a] Department of Pharmacology, University of Cambridge, Tennis Court Road, Cambridge CB2 1PD, UK
[b] University of Manchester, Faculty of Life Sciences, G38 Stopford Building, Oxford Road, Manchester M13 9PT, UK

Received 25 February 2005; revised 11 April 2005; accepted 14 April 2005

Available online 27 April 2005

Edited by Francesc Posas

**Abstract Phosphatidylinositol 5-phosphate (PtdIns5P) is a relatively recently discovered inositol lipid whose metabolism and functions are not yet clearly understood. We have transfected cells with a number of enzymes that are potentially implicated in the synthesis or metabolism of PtdIns5P, or subjected cells to a variety of stimuli, and then measured cellular PtdIns5P levels by a specific mass assay. Stable or transient overexpression of Type IIα PtdInsP kinase, or transient overexpression of Type Iα or IIβ PtdInsP kinases caused no significant change in cellular PtdIns5P levels. Similarly, subjecting cells to oxidative stress or EGF stimulation had no significant effect on PtdIns5P, but stimulation of HeLa cells with a phosphoinositide-specific PLC-coupled agonist, histamine, caused a 40% decrease within 1 min. Our data question the degree to which inositide kinases regulate PtdIns5P levels in cells, and we discuss the possibility that a significant part of both the synthesis and removal of this lipid may be regulated by phosphatases and possibly phospholipases.**
**© 2005 Federation of European Biochemical Societies. Published by Elsevier B.V. All rights reserved.**

*Keywords:* Phosphatidylinositol; Phosphatidylinositol 5-phosphate; Type II phosphatidylinositol phosphate kinase; Mass assay

## 1. Introduction

The polyphosphoinositol lipids are now recognised to be a diverse family of compounds with a wide range of physiological functions [1,2]. The most recent of these lipids to be discovered, phosphatidylinositol 5-phosphate (PtdIns5P) [3], is the favoured substrate for Type II PtdInsP kinases (Type II PIPkins) [3], and it has been suggested to have several intracellular functions, including the stimulation of inositol lipid phosphatases such as myotubularin [4], PTEN [5], or an unidentified PtdIns(3,4,5)$P_3$ 5-phosphatase [6]. PtdIns5P has also been suggested to regulate actin polymerization [7], and the activity of the candidate tumour suppressor protein ING-2 [8].

PtdIns5P is present constitutively within cells, but its levels are subject to acute regulation: PtdIns5P levels increase in platelets stimulated by thrombin [9], in insulin-stimulated adipocytes [7] and, within the nucleus, at G1 in the cell cycle [10]. Furthermore, levels decrease in osmotically shocked adipocytes or fibroblasts [11]. At present, the mechanism underlying these dynamic changes is not understood, and indeed the physiological pathways by which PtdIns5P is synthesised and catabolised are themselves unclear. Two classes of inositol lipid kinase, the Type I and Type III PIPkins, can generate PtdIns5P from the inositol lipid PtdIns in vitro [12,13], but in neither case is it clear whether this constitutes a physiological mechanism for PtdIns5P production. Although overexpression of PIKfyve, the mammalian Type III PIPkin, increases cellular PtdIns5Plevels by about 80% in 3T3L1 adipocytes [11,12], it is not certain if this is by direct synthesis or by catabolism of PtdIns(3,5)$P_2$, the probable major product of PIKfyve. Significantly, phosphoinositide 3-phosphatases of the myotubularin family also increase PtdIns5Plevels when transfected into cells [4,13], suggesting that the observed effects of PIKfyve transfection on PtdIns5P may indeed be indirect. Similarly, the major physiological role of the Type I PIPkins is the synthesis of the multi-functional inositol lipid phosphatidylinositol (4,5)-bisphosphate (PtdIns(4,5)$P_2$). Whether or not they also regulate PtdIns5P levels in vivo remains to be determined.

Mechanisms for the removal of PtdIns5P are also incompletely understood. It has previously been suggested that an important function of Type II PIPkins is to control levels of PtdIns5P by phosphorylating it to PtdIns(4,5)$P_2$ [1,3]. Nuclear levels of PtdIns5P can be regulated in this way [8] by overexpression of the predominantly nuclear [14] Type IIβ PIPkin, but it remains open as to the extent to which Type II PIPkins regulate PtdIns5P at normal expression levels. Recently, an alternative route of PtdIns5P catabolism has been suggested by the discovery of an apparently PtdIns5P-specific phosphatase [15], but again the significance of this protein in regulating PtdIns5P levels remains to be determined.

In the work presented here, we have sought to increase understanding of PtdIns5P by using the mass assay we developed previously [9] to measure PtdIns5P in cells subjected to a variety of stimuli, or transfected with a range of enzymes that have been implicated in PtdIns5P metabolism. Our data throw new light on some of the previous suggestions that have been

*Corresponding author. Fax: +44 161 2755600.
*E-mail address:* katherine.a.hinchliffe@manchester.ac.uk (K.A. Hinchliffe).

*Abbreviations:* PtdIns5P, phosphatidylinositol 5-phosphate; PtdIns-(4,5)$P_2$, phosphatidylinositol (4,5)-bisphosphate; PIPkin, PtdInsP kinase

0014-5793/$30.00 © 2005 Federation of European Biochemical Societies. Published by Elsevier B.V. All rights reserved.
doi:10.1016/j.febslet.2005.04.027

made about the metabolism and possible functions of this enigmatic lipid.

## 2. Materials and methods

### 2.1. Materials

PtdIns5P was from Echelon. Anti-p38 MAP kinase antibody was from Cell Signalling Technology, as was an antibody specific for the Thr180/Tyr182 phosphorylated form of p38 MAP kinase. The general anti-phosphotyrosine antibody was clone 4G 10 from Upstate. Constructs for GFP-tagged PIPkin IIα and IIβ were as in Ciruela et al. [14]. The construct for GFP-Type Iα PIPkin (using the mouse nomenclature) was as in [16], and a kinase-dead construct (see [17]) was a gift from Dr. M.L. Giudici. Pharbin, an inositide 5-phosphatase [18,19], was cloned out of the original pYX-Asc vector from an IMAGE clone (BC052717) into the FLAG tagged vector pCMVTag 2A (Stratagene) and into the GFP vector pEGFPC2 (Clontech).

### 2.2. Cell biology

HeLa and Cos-7 cells were cultured in Dulbecco's modified Eagle's Medium with 10% FCS in 5% $CO_2$. Cos-7 cells were transfected with FuGENE 6 transfection reagent (Boehringer); plasmid cDNAs were prepared using an endotoxin-free Maxi-prep kit (Qiagen). HeLa cells were transfected using calcium phosphate precipitation [16,17]. HeLa cells stably transfected with human Type IIα PIPkin were made using a commercial TET-On system (Clontech), though it was found that the line created had lost its tetracycline inducibility, so they were used directly as a stably transfected line (see Fig. 1).

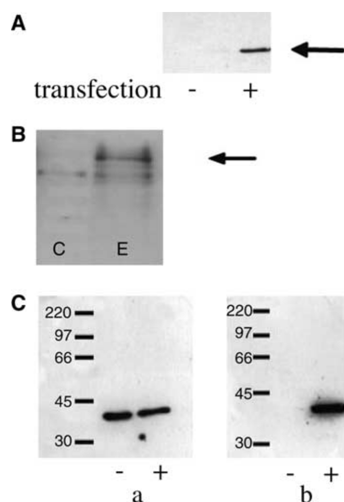


Fig. 1. (A) Stable transfection of HeLa cells. The parent HeLa cells (−), and a clone stably transfected with Type IIα PIPkin used in the experiments included in Table 1 (+), were Western blotted with a monoclonal antibody to Type II PIPkins [30,31]. Arrow indicates the position of Type IIα PIPkin. Type II PIPkin immunoreactivity can be demonstrated in anti-Type II PIPkin immunoprecipitates from parental HeLa cells (KAH, unpublished), but the levels of the endogenous protein are too low to produce a signal in the conditions used in this blot. (B) Stimulation of HeLa cells with EGF. Anti-phosphotyrosine Western blot of HeLa cells in the presence (E) and absence (C) of a 5-min stimulus with EGF. The position of the EGF receptor is shown by the arrow. Samples of these cells were analysed for PtdIns5P content (Table 2). (C) Treatment with $H_2O_2$. HeLa cells were incubated with (+) or without (−) 600 μM $H_2O_2$ for an hour, and the degree of phosphorylation of p38 MAP kinase determined by Western blotting with an anti-phospho-(Thr180/Tyr 182) p38 MAP kinase antibody (b). Total p38 MAP kinase content is shown in (a). Samples of these cells were analysed for PtdIns5P content (Table 2). Positions of molecular weight markers (in kDa) are indicated.

### 2.3. Lipid extraction

Cells grown on 10 mm dishes had their medium removed, 1 ml ice cold 5% PCA was added to the dishes and the cells were scraped with a rubber policeman. The PCA extraction was removed to an Eppendorf tube on ice and the dish washed with a further 1 ml 5% PCA, which was pooled with the first extraction. Samples were spun at $5000 \times g$ for 10 min at 4 °C and the supernatant removed from the pellet. Lipids were then extracted from the pellet using acidified chloroform/methanol as in Morris et al. [9]. Before loading onto neomycin beads (see below), an aliquot was taken for total phospholipid measurement [20] so that PtdIns5P mass could be standardised relative to cellular phospholipid.

### 2.4. PtdIns5P mass assay

This also followed the procedure of Morris et al. [9], but with some minor alterations in loading and eluting the neomycin beads to improve reproducibility. 50 μl of a 50% suspension of neomycin beads [21,22] was pipetted into a glass microtube, and washed successively in 500 μl, of 5:10:2 (by volume) $CHCl_3$:$CH_3OH$:$H_2O$, 5:10:2 (by volume) $CHCl_3$:$CH_3OH$:ammonium formate (0.5 M final concentration of formate), then 5:10:2 (by volume) $CHCl_3$:$CH_3OH$:$H_2O$ and finally resuspended with 50 μl 5:10:2 (by volume) $CHCl_3$:MeOH:ammonium formate (final concentration 50 mM formate [22]). The lipids extracted from the tissue (above) were dried down, resuspended in a volume of 500 μl of 5:10:2 (by volume) $CHCl_3$:$CH_3OH$:ammonium formate (final concentration 50 mM formate) and added to the beads. The samples were then incubated at room temperature for 20 min, spun at $4000 \times g$ for 1 min and the supernatant was discarded. The beads were washed twice with 500 μl 50 mM formate buffer with the last residue taken off with a Hamilton syringe. The lipids were eluted twice with 2 M triethylbicarbonate (TEAB) [9] to which phosphatidylserine had been added to a final concentration of 14.25 μM; this acts as an inert carrier and also acts as a carrier in the PIPkin assay (below). The beads were first incubated with 250 μl 2 M TEAB + phosphatidylserine for an hour, spun for 2 min at $4000 \times g$ and the supernatant removed to a plastic microfuge tube using a Hamilton syringe. The beads were further washed with 100 μl 2 M TEAB + phosphatidylserine (also for 1 h), and the supernatant was combined with the first. The tubes were placed in a vacuum centrifuge overnight at 60 °C.

Lipid phosphorylation with recombinant Type IIα PIPkin was as in [9]. Phosphorylation was initiated by addition of 2 μCi [$\gamma$-$^{32}$P] ATP and 5 μM ATP to each tube, and extraction and separation of the PtdIns(4,5)$P_2$ product by thin layer chromatography was exactly as described previously [9]. PtdIns5P mass levels were determined with reference to a standard curve constructed in each individual experiment, using known amounts of synthetic PtdIns5P, and expressed relative to the amount of total cellular phospholipid present in each sample. There was some minor variability in mass levels between experiments, so the results are expressed as percentages of the control levels determined in each individual experiment.

### 2.5. Verification of assay specificity

In selected samples, the reliability of the assay was tested as previously described [9], to ensure that the PtdIns5P measurements were accurate. Briefly, the Ins(1,4,5)$P_3$ headgroup of the PtdIns(4,5)$P_2$ obtained in the radiolabelling step was chemically cleaved from the lipid and then dephosphorylated using recombinant Type I inositol 5-phosphatase. Separation of the products by hplc confirmed that more than 95% of the original lipid was radiolabelled at the D4 position (that is, 95% of the radiolabel released from Ins(1,4,5)$P_3$ by the 5-phosphatase was in Ins(1,4)$P_2$, and the rest was in inorganic phosphate), confirming that the assay is a reliable measurement of PtdIns5P levels. However, in one experimental protocol this procedure demonstrated that this was not so (see results), and the significance of this finding is discussed below.

## 3. Results and discussion

### 3.1. PtdIns5P levels

The amount of PtdIns5P in unstimulated HeLa cells and Cos-7 cells was 302.14 ± 34.5 (S.E.M.; $n = 20$) and 223.3 ± 23.9

(S.E.M.; $n = 10$) pmol/μmol cellular phospholipid, respectively. If we assume that approximately 10% of cellular phospholipid content is inositol lipids, then overall the amount of PtdIns5P can be estimated as 0.2–0.3% of the inositol lipids in these cells, in good agreement with previous studies [3,11].

### 3.2. Effect of PIPkin transfection on PtdIns5P levels

As discussed above, it is possible that Type II PIPkins regulate PtdIns5P levels by phosphorylating PtdIns5P to PtdIns(4,5)$P_2$. To investigate this possibility, HeLa cells were stably transfected with type IIα PIPkin (Fig. 1A) and the impact on PtdIns5P levels determined. Somewhat surprisingly, despite the expression of considerable type IIα PIPkin immunoreactivity (Fig. 1A), there was no detectable difference in PtdIns5P levels between these cells and the parent cell line (Table 1). This might be due to adaptation of the cells to the chronic high expression levels of the enzyme (that is, an increased removal of PtdIns5P could be compensated for by increased synthesis). As a control for this, we also acutely transfected Cos-7 cells (to a 50–60% efficiency) with GFP-tagged Type IIα PIPkin, an approach that should decrease (though it cannot eliminate) the likelihood of a compensatory upregulation of synthesis. However, acute transfection also had no detectable effect on PtdIns5P levels (Table 1).

We also tested Type IIβ PIPkin in similar experiments. As discussed in Section 1, this is primarily a nuclear isoform [14], though as it was originally cloned from its association with a cell surface receptor [23], it is also likely to have a significant cytoplasmic component. Overexpression of Type IIβ PIPkin in Cos7 cells was similarly without detectable effect on cellular PtdIns5P levels (Table 1). This assay measures the PtdIns5P content of the whole cell, and does not discriminate between pools within the nucleus or elsewhere, but this result suggests that the inability of Type IIα PIPkin to affect total PtdIns5P levels (above) was not simply due to its inability to obtain access to a large nuclear pool of the lipid.

Although this lack of effect of Type II PIPkin transfection could be explained by the PIPkins not having access to the bulk of the cellular PtdIns5P due to compartmentalization, it is still likely that they can access any PtdIns5P pools available to the endogenous enzymes. Thus the failure of their marked overexpression to make an impact on the levels of PtdIns5P strongly suggests that these enzymes do not contribute to the regulation of resting PtdIns5P levels in the cell lines examined. This observation in turn calls into question the idea that a principal function of Type II PIPkins is to regulate cellular PtdIns5P levels.

As Type I PIPkins have also been reported to synthesise PtdIns5P from PtdIns in vitro [24], we also investigated whether transfection with one of these enzymes might increase PtdIns5P in intact cells. Note that for these experiments we used as a control cells transfected with a kinase-dead mutant with less than 1% of wild-type activity, to circumvent artefacts caused by high levels of Type I PIPkin transfection (see e.g. [17]). However, transfection of Type Iα PIPkin into Cos-7 cells produced no detectable difference in PtdIns5P levels (Table 1). This finding suggests that the Type I PIPkins are not responsible for regulating cellular PtdIns5P levels.

Table 1
Effect of transfection on mass levels of PtdIns5P in HeLa and Cos-7 cells

| Construct | %Control (±S.E.M.) | Significance (from control) |
|---|---|---|
| Type IIα PIPkin (stable) | 85.6 ± 13.24 ($n = 24$; 4 expts) | N.S. |
| Type IIα PIPkin (acute) | 102.8 ± 8.9 ($n = 8$; 2 expts) | N.S. |
| Type IIβ PIPkin | 85.6 ± 8.8 ($n = 12$; 3 expts) | N.S. |
| Type Iα PIPkin | 122.7 ± 29 ($n = 8$; 2 expts) | N.S. |

Data show the PtdIns5P levels for HeLa cells stably transfected with Type IIα PIPkin (see Section 2 and Fig. 1A), or for Cos-7 cells were transfected for 24 h with the constructs indicated. In all acute transfections the efficiency was confirmed by immunocytochemistry to be 50–60% (not shown). Results are expressed as percentage controls (these are either GFP-transfected cells, or for the Type Iα PIPkin, cells transfected with a kinase-dead Type Iα PIPkin construct).

### 3.3. Role of phosphatases in PtdIns5P production

These generally negative results with kinases led us to consider the contribution of inositol lipid phosphatases to regulating PtdIns5P levels. It is already known that the lipid phosphatase myotubularin and its homologues can produce PtdIns5P via the hydrolysis of PtdIns(3,5)$P_2$ [13]. Furthermore, the bacterial lipid phosphatase IpgD generates large amounts of PtdIns5P when introduced into vertebrate cells, by hydrolysing PtdIns(4,5)$P_2$ [25]. Whilst no mammalian orthologue of that activity has been identified, a number of PtdIns(4,5)$P_2$ 5-phosphatases are found in the human genome. The possibility that a PtdIns(4,5)$P_2$ 5-phosphatase might also generate PtdIns5P physiologically is one that has not been considered, even though Rameh et al. [3] showed that one member of the 5-phosphatase family, SHIP-1, does have significant (about 5%) 4-phosphatase action on PtdIns(4,5)$P_2$ in vitro.

To investigate this possibility, we transfected Cos7 cells with another inositol lipid 5-phosphatase, Pharbin ([18], also cloned by Kong et al. [19]). Pharbin appeared significantly to increase PtdIns5Plevels as measured by the mass assay. However, following the assay we analysed the PtdIns(4,5)$P_2$ that was generated in vitro by removing its headgroup and dephosphorylating it with Type I inositol polyphosphate phosphatase (see Section 2). This analysis revealed that there was considerable incorporation of radiolabel into the D5 position (not shown), which means that PtdIns4P in the extract from the Pharbin-transfected cells was making a significant contribution as a substrate. This assay relies crucially on the high preference of the Type II PIPkins for PtdIns5P over PtdIns4P, such that even though there is much more PtdIns4P in cells than PtdIns5P, the assay is still specific for the latter [9]. But it is important to note that the enzyme will also phosphorylate PtdIns4P in vitro if provided with very high levels of this lipid [3,9]. We conclude that, in this set of experiments, the assay was compromised because it was detecting a huge increase in cellular PtdIns4P engendered by the 5-phosphatase action of Pharbin on cellular PtdIns(4,5)$P_2$. This finding highlights directly the limitations of the PtdIns5P assay when PtdIns4P levels are very high, and it emphasises the need to check the assay's veracity by analysing the distribution of radioactivity in the PtdIns(4,5)$P_2$ between the 4 and 5 phosphates whenever PtdIns5Plevels appear to increase.

Overall, the kinase expression data do not support the idea that PtdIns5P levels are regulated by inositol lipid kinases.

Instead, they favour the emerging suggestion that the predominant route of PtdIns5P production in cells is via dephosphorylation of a more highly phosphorylated inositol lipid (PtdIns(3,5)$P_2$, PtdIns(4,5)$P_2$ or both – see above and Section 1). A further piece of evidence that supports this conclusion lies in the observation that PtdInsP substrates prepared from mammalian brains that have been removed, chilled and processed within approximately 1 h post-mortem contain lower levels of PtdIns5P than those from brains processed less rapidly (R.F.I. and A.J.L. unpublished observations). PtdIns5P generated post-mortem is most likely to result from phosphatase rather than from kinase activity, and this suggests that such phosphatases are able to make significant contributions to PtdIns5P content. These observations, together with the recent discovery of a specific PtdIns5P phosphatase [15], suggest that phosphatases may be more important than kinases in the regulation of PtdIns5P levels in vivo.

### 3.4. Effects of cellular stimuli on PtdIns5P levels

As mentioned above, a number of stimuli are reported to cause fluctuations in cellular PtdIns5P levels. To identify further treatments that also modify PtdIns5P levels, HeLa cells were subjected to a number of diverse stimuli, and the effects (if any) on PtdIns5P levels measured. The results are presented in Table 2. Stimulation with epidermal growth factor (EGF) at a concentration that stimulated tyrosine phosphorylation of the EGF receptor (Fig. 1B) had no significant effect on PtdIns5P levels (Table 2). Similarly, oxidative stress (600 μM hydrogen peroxide, applied for 1 h, conditions which promoted robust phosphorylation of p38 MAP kinase: Fig. 1C) was without effect on PtdIns5P content (Table 2).

However, stimulation with histamine produced a significant decrease in PtdIns5P levels (Table 2). In HeLa cells, histamine activates PtdIns(4,5)$P_2$ breakdown via the activation of phospholipase C [26]. We confirmed, using Fura-2, that our HeLa cells gave a robust $Ca^{2+}$ signal in response to histamine within 30 s (not shown). To determine whether the observed effect (Table 2) was due to activation of protein kinase C (PKC) downstream of PLC activation, we tested the effects of the phorbol ester TPA on PtdIns5P levels, and of the PKC inhibitor chelerythrine on the histamine-induced change. TPA produced a small (though non-significant) reduction in PtdIns5P levels (Table 2), but the inability of the PKC inhibitor to prevent the effect of histamine treatment on PtdIns5P levels (Table 2) suggests that PKC activation is not required for the observed decrease. Furthermore, thapsigargin was also without effect (Table 2), suggesting that the elevation of cytosolic free $Ca^{2+}$ concentration provoked by histamine receptor activation is also not involved in regulating PtdIns5P levels.

The effects of histamine on HeLa cells are apparently all mediated via $H_1$ receptor-stimulated PI-PLC activation [27], so the simplest (though not the only) remaining explanation of these results is the possibility that they are due to direct hydrolysis of PtdIns5P by activated PI-PLC. The structure of PI-PLC suggests that PtdIns5P could act as a substrate [28], and indeed PLC $\delta_1$ has been shown to catalyse the breakdown of PtdIns5P in vitro [29]. The possible physiological consequences of such a signalling pathway are unclear, however, if PLC-mediated PtdIns5P hydrolysis does underlie the observed effect of histamine on PtdIns5P levels in HeLa cells, the extent of the effect suggests that at least 40% of the total cellular PtdIns5P is found in a plasma membrane pool accessible to PLC.

Whatever the explanation, the finding that a stimulus that activates PLC leads to a rapid decrease in PtdIns5P levels in HeLa cells is somewhat surprising, given our earlier demonstration that thrombin stimulation of platelets, which causes a marked activation of platelet PLC, leads to an increase in PtdIns5P levels [9]. We cannot presently account for this difference, though it is interesting to note that the increase seen with thrombin was significantly delayed relative to the known time-course of PLC activation [9]. It is possible that, in platelets, stimulation also causes a marked increase in PtdIns5P production that may mask the effects of early PtdIns5P hydrolysis, but this suggestion remains speculative.

Table 2
Effect of pharmacologial agents on mass levels of PtdIns5P in COS-7 cells

| Treatment | %Control (±S.E.M.) | Significance (from control) |
|---|---|---|
| EGF | 90.3 ± 25.2 ($n$ = 7; 2 expts) | N.S. |
| $H_2O_2$ | 88.3 ± 27.4 ($n$ = 8; 2 expts) | N.S. |
| Histamine | 62.5 ± 1.7 ($n$ = 11; 3 expts) | $P < 0.05$ |
| Thapsigargin | 93.7 ± 10.8 ($n$ = 8; 2 expts) | N.S. |
| Phorbol ester | 71.7 ± 10.8 ($n$ = 8; 2 expts) | N.S. |
| Histamine + chelerythrine | 48.4 ± 6.9 ($n$ = 4; 1 expt) | $P < 0.05$ |

HeLa cells were stimulated as described and PtdIns5P levels measured. All data are expressed as % control cells in the same individual experiment. Treatments used were: EGF, 5 min; $H_2O_2$, 600 μM for 60 min; histamine, 100 μM for 1 min; thapsigargin, 1 μM 1 min; TPA, 1 mg/ml for 1 min; histamine + chelerythine, 30 min pre-treatment with 10 μM chelerythrine, then 100 μM histamine for 1 min.

## 4. Conclusions

In conclusion, we have demonstrated that overexpressing Type I or Type II PIPkins does not significantly affect PtdIns5P levels in Cos-7 or HeLa cells, supporting the idea that lipid phosphatase activity is perhaps more likely to be of major importance in regulating levels of this lipid mediator. Furthermore, we have shown that stimulation of HeLa cells with histamine provokes a marked decrease in PtdIns5P levels, though whether this reduction results from phosphatase or from phospholipase activation remains to be established.

*Acknowledgements:* We thank Dr. M.L. Giudici for the kinase-dead Type Iα PIPkin clone, and Dr. P.T. Hawkins and other members of the Irvine lab for helpful suggestions. H.F.R. was supported by a Studentship from the Department of Pharmacology, University of Cambridge, J.H.C. and A.J.L. by the Wellcome Trust, R.F.I. by the Royal Society, and K.A.H. by the M.R.C.

## References

[1] Hinchliffe, K.A., Ciruela, A. and Irvine, R.F. (1998) PIPkins, their substrates and their products: new functions for old enzymes. Biochim. Biophys. Acta 1436, 87–104.

[2] Payrastre, B., Missy, K., Giuriato, S., Bodin, S., Plantavid, M. and Gratacap, M. (2001) Phosphoinositides: key players in cell signalling, in time and space. Cell. Signal. 13, 377–387.

[3] Rameh, L.E., Tolias, K.F., Duckworth, B.C. and Cantley, L.C. (1997) A new pathway for synthesis of phosphatidylinositol-4,5-bisphosphate. Nature 390, 192–196.

[4] Schaletzky, J., Dove, S.K., Short, B., Lorenzo, O., Clague, M.J. and Barr, F.A. (2003) Phosphatidylinositol-5-phosphate activation and conserved substrate specificity of the myotubularin phosphatidylinositol 3-phosphatases. Curr. Biol. 13, 504–509.

[5] Campbell, R.B., Liu, F. and Ross, A.H. (2003) Allosteric activation of PTEN phosphatase by phosphatidylinositol 4,5-bisphosphate. J. Biol. Chem. 278, 33617–33620.

[6] Carricaburu, V., Lamia, K.A., Lo, E., Favereaux, L., Payrastre, B., Cantley, L.C. and Rameh, L.E. (2003) The phosphatidylinositol (PI)-5-phosphate 4-kinase type II enzyme controls insulin signaling by regulating PI-3,4,5-trisphosphate degradation. Proc. Natl. Acad. Sci. USA 100, 9867–9872.

[7] Sbrissa, D., Ikonomov, O.C., Strakova, J. and Shisheva, A. (2004) Role for a novel signaling intermediate, phosphatidylinositol 5-phosphate in insulin-regulated F-actin stress fiber breakdown and GLUT4 translocation. Endocrinology 145, 4853–4865.

[8] Gozani, O., et al. (2003) The PHD finger of the chromatin-associated protein ING2 functions as a nuclear phosphoinositide receptor. Cell 114, 99–111.

[9] Morris, J.B., Hinchliffe, K.A., Ciruela, A., Letcher, A.J. and Irvine, R.F. (2000) Thrombin stimulation of platelets causes an increase in phosphatidylinositol 5-phosphate revealed by mass assay. FEBS Lett. 475, 57–60.

[10] Clarke, J.H., Letcher, A.J., D'Santos, C.S., Halstead, J.R., Irvine, R.F., Divecha, N., 2001. Inositol lipids are regulated during cell cycle progression in the nuclei of murine erythroleukaemia cells. Biochem. J. 357, 905–910.

[11] Sbrissa, D., Ikonomov, O.C., Deeb, R. and Shisheva, A. (2002) Phosphatidylinositol 5-phosphate biosynthesis is linked to PIKfyve and is involved in osmotic response pathway in mammalian cells. J. Biol. Chem. 277, 47276–47284.

[12] Sbrissa, D., Ikonomov, O.C. and Shisheva, A. (1999) PIKfyve, a mammalian ortholog of yeast Fab1p lipid kinase, synthesizes 5-phosphoinositides. Effect of insulin. J. Biol. Chem. 274, 21589–21597.

[13] Tronchere, H., Laporte, J., Pendaries, C., Chaussade, C., Liaubet, L., Pirola, L., Mandel, J.L. and Payrastre, B. (2004) Production of phosphatidylinositol 5-phosphate by the phosphoinositide 3-phosphatase myotubularin in mammalian cells. J. Biol. Chem. 279, 7304–7312.

[14] Ciruela, A., Hinchliffe, K.A., Divecha, N. and Irvine, R.F. (2000) Nuclear targeting of the β isoform of Type II phosphatidylinositol phosphate kinase (phosphatidylinositol 5-phosphate 4-kinase) by its α-helix 7. Biochem. J. 364, 587–591.

[15] Pagliarini, D.J., Worby, C.A. and Dixon, J.E. (2004) A PTEN-like phosphatase with a novel substrate specificity. J. Biol. Chem. 279, 38590–38596.

[16] Hinchliffe, K.A., Giudici, M.L., Letcher, A.J. and Irvine, R.F. (2002) Type IIalpha phosphatidylinositol phosphate kinase associates with the plasma membrane via interaction with type I isoforms. Biochem. J. 363, 563–570.

[17] Giudici, M.L., Emson, P.C. and Irvine, R.F. (2004) A novel neuronal-specific splice variant of Type I phosphatidylinositol 4-phosphate 5-kinase isoform gamma. Biochem. J. 379, 489–496.

[18] Asano, T., Mochizuki, Y., Matsumoto, K., Takenawa, T. and Endo, T. (1999) Pharbin, a novel inositol polyphosphate 5-phosphatase, induces dendritic appearances in fibroblasts. Biochem. Biophys. Res. Commun. 261, 188–195.

[19] Kong, A.M., et al. (2000) Cloning and characterization of a 72-kDa inositol-polyphosphate 5-phosphatase localized to the Golgi network. J. Biol. Chem. 275, 24052–24064.

[20] Rouser, G., Fleischer, S. and Yamamoto, A. (1970) Two dimensional then layer chromatographic separation of polar lipids and determination of phospholipids by phosphorus analysis of spots. Lipids 5, 494–496.

[21] Schacht, J. (1978) Purification of polyphosphoinositides by chromatography on immobilized neomycin. J. Lipid Res. 19, 1063–1067.

[22] Palmer, F.B. (1981) Chromatography of acidic phospholipids on immobilized neomycin. J. Lipid Res. 22, 1296–1300.

[23] Castellino, A.M., Parker, G.J., Boronenkov, I.V., Anderson, R.A. and Chao, M.V. (1997) A novel interaction between the juxtamembrane region of the p55 tumor necrosis factor receptor and phosphatidylinositol-4-phosphate 5-kinase. J. Biol. Chem. 272, 5861–5870.

[24] Tolias, K.F., Rameh, L.E., Ishihara, H., Shibasaki, Y., Chen, J., Prestwich, G.D., Cantley, L.C. and Carpenter, C.L. (1998) Type I phosphatidylinositol-4-phosphate 5-kinases synthesize the novel lipids phosphatidylinositol 3,5-bisphosphate and phosphatidylinositol 5-phosphate. J. Biol. Chem. 273, 18040–18046.

[25] Niebuhr, K., et al. (2002) Conversion of PtdIns(4,5)P(2) into PtdIns(5)P by the S.flexneri effector IpgD reorganizes host cell morphology. EMBO J. 21, 5069–5078.

[26] Bootman, M.D., Berridge, M.J. and Taylor, C.W. (1992) All-or-nothing $Ca^{2+}$ mobilization from the intracellular stores of single histamine-treated HeLa cells. J. Physiol. 450, 163–178.

[27] Tilly, B.C., Tertoolen, L.G., Remorie, R., Ladoux, A., Verlaan, I., de Laat, S.W. and Moolenaar, W.H. (1990) Histamine as a growth factor and chemoattractant for human carcinoma and melanoma cells: action through $Ca^{2+}$-mobilizing $H_1$ receptors. J. Cell Biol. 110, 1211–1215.

[28] Essen, L.O., Perisic, O., Cheung, R., Katan, M. and Williams, R.L. (1996) Crystal structure of a mammalian phosphoinositide-specific phospholipase C delta. Nature 380, 595–602.

[29] Liu, Y., Bruzik, K.S., Ananthanarayanan, B. and Cho, W. (2003) New aspects of formation of 1,2-cyclic phosphates by phospholipase C-delta1. Bioorg. Med. Chem. 11, 2471–2475.

[30] Brooksbank, C.E., Hutchings, A., Butcher, G.W., Irvine, R.F. and Divecha, N. (1993) Monoclonal antibodies to phosphatidylinositol 4-phosphate 5-kinase: distribution and intracellular localization of the C isoform. Biochem. J. 291, 77–82.

[31] Hinchliffe, K.A., Irvine, R.F. and Divecha, N. (1996) Aggregation-dependent, integrin-mediated increases in cytoskeletally associated PtdInsP2 (4,5) levels in human platelets are controlled by translocation of PtdIns 4-P 5-kinase C to the cytoskeleton. EMBO J. 15, 6516–6524.