Identification of a new polyphosphoinositide in plants, phosphatidylinositol 5-monophosphate (PtdIns5*P*), and its accumulation upon osmotic stress

Harold J. G. MEIJER*[1], Christopher P. BERRIE†, Cristiano IURISCI†, Nullin DIVECHA‡, Alan MUSGRAVE* and Teun MUNNIK*

*Swammerdam Institute for Life Sciences, Department of Plant Physiology, University of Amsterdam, Kruislaan 318, NL-1098 SM, Amsterdam, The Netherlands, †Department of Cell Biology and Oncology, Instituto di Ricerche Farmacologiche "Mario Negri", Consorzio Mario Negri Sud, 66030 Santa Maria Imbaro (Chieti), Italy, and ‡Division of Cellular Biochemistry, The Netherlands Cancer Institute, Plesmanlaan 121, NL-1066 CX, Amsterdam, The Netherlands

Polyphosphoinositides play an important role in membrane trafficking and cell signalling. In plants, two PtdIns*P* isomers have been described, PtdIns3*P* and PtdIns4*P*. Here we report the identification of a third, PtdIns5*P*. Evidence is based on the conversion of the endogenous PtdIns*P* pool into PtdIns(4,5)P_2 by a specific PtdIns5*P* 4-OH kinase, and on *in vivo* ^{32}P-labelling studies coupled to HPLC head-group analysis. In *Chlamydomonas*, 3–8 % of the PtdIns*P* pool was PtdIns5*P*, 10–15 % was PtdIns3*P* and the rest was PtdIns4*P*. In seedlings of *Vicia faba* and suspension-cultured tomato cells, the level of PtdIns5*P* was about 18 %, indicating that PtdIns5*P* is a general plant lipid that represents a significant proportion of the PtdIns*P* pool. Activating phospholipase C (PLC) signalling in *Chlamydomonas* cells with mastoparan increased the turnover of PtdIns(4,5)P_2 at the cost of PtdIns4*P*, but did not affect the level of PtdIns5*P*. This indicates that PtdIns(4,5)P_2 is synthesized from PtdIns4*P* rather than from PtdIns5*P* during PLC signalling. However, when cells were subjected to hyperosmotic stress, PtdIns5*P* levels rapidly increased, suggesting a role in osmotic-stress signalling. The potential pathways of PtdIns5*P* formation are discussed.

Key words: G-protein activation, lipid kinases, lipid metabolic pathways, lipids, PPIs, signal transduction, water relations.

INTRODUCTION

Polyphosphoinositides (PPIs) are only minor lipids in eukaryotic cells but play a major role in cell biology. They are precursors of second messengers [e.g. PtdIns(4,5)P_2 is hydrolysed by phospholipase C (PLC) to produce diacylglycerol and Ins(1,4,5)P_3] and also regulate various enzymic activities, K^+ channels and cytoskeletal proteins [1–5]. PPIs regulate cellular activity by interacting with proteins that contain PPI-binding domains. These include CalB, pleckstrin homology (PH) and FYVE domains, for which different PPI-binding specificities have been determined [6–8]. Through binding different PPIs, cytosolic target proteins can be recruited to specific membranes where they play roles in signal transduction, regulation of enzyme activity, cytoskeletal rearrangements or membrane trafficking [1,6,8].

Until now, two isoforms of PtdIns*P* have been recognized in plants, PtdIns3*P* and PtdIns4*P* [4,9,10]. PtdIns3*P* is thought to be the precursor of PtdIns(3,4)P_2 [11–13] or PtdIns(3,5)P_2 [14,15] whereas PtdIns4*P* is seen as the precursor of PtdIns(4,5)P_2. All three PtdInsP_2 isomers have been identified in plants [4,11–13,15,16]. A third PtdIns*P* isomer has been discovered in animal cells, PtdIns5*P* [17], the *in vitro* substrate for type-II PtdIns5*P* 4-OH kinase and present as a minor fraction of the PtdIns*P* pool in mouse fibroblasts. In platelets, PtdIns5*P* levels were found to transiently increase upon thrombin stimulation [18], indicating that it is directly or indirectly involved in signalling.

In this study, we have investigated the composition of the PtdIns*P* pool in plant cells and provide evidence for the existence of PtdIns5*P*. We also monitored changes in metabolism during rapid PtdIns(4,5)P_2 synthesis (G-protein activation) and rapid PtdIns(3,5)P_2 synthesis (hyperosmotic stress). PtdIns5*P* levels only changed during hyperosmotic stress, suggesting a role in osmotic-stress signalling.

MATERIALS AND METHODS

Materials

PtdIns and PtdIns*P* were purified from bovine brain. Reagents for lipid extraction and subsequent analysis, as well as Silica 60 TLC plates, were from Merck (Darmstadt, Germany). [^{32}P]P_i (carrier-free) and [γ-^{32}P]ATP (110 TBq/mmol) were from Amersham International. Synthetic mastoparan was bought from Calbiochem (La Jolla, CA, U.S.A.). Glass beads (200–400 mesh) were obtained from Sigma-Aldrich. Commercially available *myo*-[^{3}H]inositol-labelled HPLC standards were from New England Nuclear (Boston, MA, U.S.A.).

Plant material

Chlamydomonas moewusii (strain Utex 10; Culture Collection of Algae, University of Texas, Austin, TX, U.S.A.) was grown as described in [19]. Cell suspensions were produced by flooding 2–4-week-old plates overnight with 20 ml of HMCK (10 mM Hepes, pH 7.4/1 mM $MgCl_2$/1 mM $CaCl_2$/1 mM KCl), giving a cell concentration of $(1–2) \times 10^7$ cells·ml^{-1}. Suspension cultures of tomato (Msk8) were grown as described by Van der Luit et al. [20]. *Vicia sativa* (vetch) seedlings were grown in modified Fåhreus slides as described by Den Hartog et al. [21].

Phospholipid labelling, extraction and analysis

Chlamydomonas lipids were labelled, extracted and separated by TLC as described previously [22]. *V. sativa* seedlings were labelled

Abbreviations used: PLC, phospholipase C; PPI, polyphosphoinositide; PtdOH, phosphatidic acid; GroPIns, glycerophosphoinositol.

[1] To whom correspondence should be addressed (e-mail hmeijer@science.uva.nl).

© 2001 Biochemical Society

in 160 μl of medium (2.72 mM $CaCl_2$/1.95 mM $MgSO_4$/0.08 mM ferric citrate/10 mM Hepes, pH 6.5) containing 0.59 MBq of [^{32}P]P_i/seedling [21]. Tomato cells were labelled as described in [20]. Lipids were extracted by adding 3.75 vol. of chloroform/methanol/HCl (50:100:1, by vol.), and by using 1 vol. of 0.9 % NaCl and 3.75 vol. chloroform to induce phase separation. Extractions were processed further and lipids separated by TLC, as described previously [22]. Radiolabelled phospholipids were visualized by autoradiography (X-Omat AR film, Kodak) and quantified by PhosphoImaging (Storm from Molecular Dynamics and BAS 2000 from Fuji).

PPI standards

^{32}P-Labelled PtdIns3*P* was synthesized using phosphoinositide 3-kinase activity that was immunoprecipitated from platelet-derived growth factor-stimulated Rat-1 cells by anti-phosphotyrosine antibodies as described in [15]. ^{32}P-Labelled PtdIns4*P* was prepared from PtdIns using recombinant Myc-tagged type-II PtdIns5*P* 4-OH kinase [23] that was immunoprecipitated from transgenic *Escherichia coli* cells. [^{32}P]PtdIns5*P* was synthesized using recombinant Myc-tagged type-I PtdIns4*P* 5-OH kinase which was immunoprecipitated from transiently transfected COS cells. All reactions were performed in 100 μl containing 50 mM Tris, pH 7.4, 10 mM $MgCl_2$, 80 mM KCl, 1 mM EGTA (PIP kinase buffer), 1 nmol of PtdIns and 1.85 MBq of [γ-^{32}P]ATP. After quenching the reactions, lipids were extracted and separated by TLC [24].

The glycerophosphoinositols [^{3}H]GroPIns, [^{3}H]GroPIns4*P* and [^{3}H]GroPIns(4,5)P_2 were produced by standard monomethylamine deacylation [25] of the corresponding ^{3}H-labelled PPIs. These lipids were generally obtained from equilibrium-loaded [^{3}H]Ins-labelling of Swiss 3T3 cells. Cells were maintained in Dulbecco's modified Eagle's medium plus 10 % fetal bovine serum, as described previously [26]. After adding cold methanol (−20 °C) and scraping the cells from the Petri dish, the lipids were obtained by drying the lower phase of a chloroform/methanol/water/HCl (100:100:48:1; by vol.) extract under nitrogen gas. Deacylation was carried out at 52 °C for 45 min in a monomethylamine mixture (40 % aqueous monomethylamine/water/*n*-butyl alcohol/methanol; 4.5:1:1.125:5.875, by vol.). After lyophilization, samples were resuspended in 500 μl of water, to which 700 μl of *n*-butyl alcohol/petroleum ether (40–60 °C fraction)/ethyl formate (20:4:1, by vol.) was added. The lower phase was re-extracted with 500 μl of the same mixture and lyophilized again. The resulting glycerophosphoinositols were purified by HPLC as described below.

The [^{32}P]GroPIns5*P* standard was produced by dephosphorylation of [^{32}P]GroPIns(3,5)P_2. The latter was isolated from ^{32}P-labelled *Chlamydomonas* cells that were osmotically stressed, and deacylated [15]. The resulting head group was then dephosphorylated selectively at the 3-position by washed human red blood cell ghosts in 25 mM Hepes, pH 7.0/2 mM EGTA/10 mM EDTA. EDTA was included in the incubation medium to alter the specificity of the 5-phosphatase to allow specific removal of the 3-phosphate, and hence the production of [^{32}P]GroPIns5*P* [27]. After incubation overnight, the reaction was halted by the addition of 700 μl of water and 100 μl of 19.25 % perchloric acid. After 30 min on ice, the protein was removed by centrifugation. The supernatant was neutralized to pH 7.5 by the addition of 200 μl of 50 % (w/v) $KHCO_3$ and kept on ice for 1 h. Precipitated potassium chlorate was removed by centrifugation and the supernatant was used for HPLC analysis.

Isolation of PPIs from *Chlamydomonas*

Lipids from non-labelled *Chlamydomonas* were dried and suspended in 500 μl of PPI-binding solution (chloroform/methanol/20 mM ammonium formate; 5:10:2, by vol.). After addition of 20 μl of neomycin-linked glass beads [28], samples were incubated at 4 °C for 15 min. The beads were then centrifuged (10000 ***g***), the supernatant removed and the beads washed three times with PPI-binding solution. Adsorbed lipids were eluted from the beads by adding carrier lipids [phosphatidylserine and phosphatidic acid (PtdOH), 5 nmol each] and extracted as described above. This procedure was developed in order to remove a kinase inhibitor from the lipid extracts. Using 25–200 μl of radioactive cell cultures, 98.2 ± 2.0 % of the PtdIns*P* was recovered. The only other lipid effectively purified was PtdInsP_2 (95.2 ± 0.6 %). Only small proportions of PtdOH (6.0 ± 1.3 %), phosphatidylglycerol (5.8 ± 0.4 %), PtdIns, phosphatidylcholine and phosphatidylethanolamine (< 0.5 %) were recovered by this procedure.

Kinase assays

In vivo-labelled PtdIns*P*s were purified by TLC and eluted from the silica gel. After resuspending them in 10 mM Tris buffer by sonication, double-strength PIP kinase buffer, which included enzymes and 200 μM ATP, was added. Phosphorylation was carried out in 100 μl at 30 °C for 15 h and the reaction was then stopped with 375 μl of cold (−20 °C) chloroform/methanol (1:2, v/v). Lipids were isolated and separated as described above.

When non-labelled lipids were used, they were isolated from neomycin beads as described above, suspended in 50 μl of diethyl ether and 50 μl of 10 mM Tris buffer (pH 7.4) and sonicated for 15 s. Diethyl ether was removed by vacuum centrifugation. Lipid phosphorylation was initiated by adding 50 μl of double-strength PIP kinase buffer containing 1 μl of the enzyme indicated and 74 kBq of [γ-^{32}P]ATP in the presence of 5 μM ATP. The lower amount of ATP resulted in increased substrate specificity. The mixture was incubated at 30 °C for 2 h with shaking. As a control for enzyme specificity, non-labelled PtdIns*P* was phosphorylated by type-II PtdIns5*P* 4-OH kinase or type-I PtdIns4*P* 5-OH kinase using [γ-^{32}P]ATP. The resulting type-II and type-I PtdIns(4,5)P_2s were dephosphorylated with a specific recombinant 5-phosphatase. All label lost from type-II PtdIns(4,5)P_2 was recovered in PtdIns4*P*, whereas all the ^{32}P-label from type-I PtdIns(4,5)P_2 was lost as P_i, with no radioactive counts remaining in PtdIns*P*.

HPLC analysis of ^{3}H/^{32}P-labelled PPIs

Deacylated lipids were routinely separated by anion-exchange HPLC at a flow rate of 1.0 ml·min^{-1} on a Partisil 10 SAX column (Jones Chromatography, Mid Glamorgan, U.K.) using a non-linear water (buffer A)/1.0 M ammonium phosphate, pH 3.35 (phosphoric acid; buffer B) gradient [26]. Because the peaks of GroPIns4*P* and GroPIns5*P* were separated by less than 20 s, a modified gradient was utilized: 0–45 min, 0.0–1.5 % buffer B; 45–46 min, 1.5–2.4 % buffer B; 46–80 min, 2.4–4.5 % buffer B; 80–81 min, 4.5–6.0 % buffer B; 81–141 min, 6.0–35.0 % buffer B; 141–142 min, 35–100 % buffer B; 142–147 min, 100 % buffer B; 147–150 min, 100–0 % buffer B; 150–180 min, 0 % buffer B wash. Individual peaks were identified following periodate treatment by HPLC analysis [27] and the use of ^{3}H-labelled standards, including inositol, GroPIns, Ins1*P*, Ins3*P*, Ins4*P*, GroPIns3*P*, GroPIns4*P*, Ins(1,4)P_2, Ins(1,5)P_2, GroPIns(3,4)P_2, GroPIns(4,5)P_2, Ins(1,3,4)P_3 and Ins(1,4,5)P_3.

© 2001 Biochemical Society

The latter gradient produced a 1.5 min peak-to-peak separation of GroPIns4*P* and GroPIns5*P* (see Figure 4B, below) and was exploited to separate the ^{32}P-labelled samples. Standard [^{3}H]GroPIns4*P* was included with every sample to correct for minor HPLC run-to-run variability and hence to allow more precise calculation of the relative levels of GroPIns4*P* and GroPIns5*P*. Furthermore, the radioactivity in these GroPInsP fractions (see also Figures 5 and 6 below) was determined by dual-label scintillation counting to a 0.5 % sample error (at the low isotope concentrations).

RESULTS

PtdIns5*P* is present in plant cells

To investigate whether *Chlamydomonas* cells contain PtdIns5*P*, non-stimulated cells were incubated for 2.5 h in [^{32}P]P_i and the radioactive lipids were extracted and separated by TLC. The [^{32}P]PtdIns*P* spot was then isolated and the lipids incubated with a type-II PtdIns5*P* 4-OH kinase (isolated from transgenic *E. coli* cells) in the presence of 200 μM non-radioactive ATP. If the PtdIns*P* spot contained [^{32}P]PtdIns5*P*, it would be converted to ^{32}P-labelled PtdIns(4,5)P_2. The reaction products were subsequently chromatographed together with PtdInsP_2 standards. As illustrated in Figure 1(A), the PtdIns5*P* 4-OH kinase phosphorylated [^{32}P]PtdIns*P* into two products: one co-migrated with PtdIns(4,5)P_2, the other with PtdIns(3,4)P_2. The production of PtdIns(4,5)P_2 indicates that PtdIns5*P* is present in this alga. The production of PtdIns(3,4)P_2 confirms the presence of PtdIns3*P* [29,30]. It also shows that the enzyme uses substrates other than PtdIns5*P* under certain conditions, substantiating earlier reports [17,18,31]. However, type-II PtdIns5*P* 4-OH kinase does not phosphorylate PtdIns4*P*, therefore the PtdIns(4,5)P_2 could not have arisen from the phosphorylation of PtdIns4*P* [17,32]. After 15 h of incubation, approx. 10 % of the original [^{32}P]PtdIns*P* was converted to [^{32}P]PtdIns(3,4)P_2, indicating that at least 10 % of the pool exists as PtdIns3*P*, which is in close agreement with results obtained previously [29,30]. Based on the same arguments, about 8 % of the *Chlamydomonas* [^{32}P]PtdIns*P* pool was PtdIns5*P*.

To determine whether PtdIns5*P* is also present in other plant cells, PtdIns*P* spots from radiolabelled vetch roots (*V. sativa*) and tomato suspension cultures were isolated and treated with PtdIns5*P* 4-OH kinase as described above. As for *Chlamydomonas*, two products were formed that co-migrated with PtdIns(4,5)P_2 and PtdIns(3,4)P_2 (Figure 1B). For vetch and tomato, about 8 % of the label was phosphorylated to PtdIns(3,4)P_2, which is again in agreement with previous estimates of higher-plant PtdIns3*P* levels [4,11,16,33,34]. Approx. 18 % was phosphorylated to [^{32}P]PtdIns(4,5)P_2, indicating that PtdIns5*P* represents a significant fraction of the [^{32}P]PtdIns*P* pool.

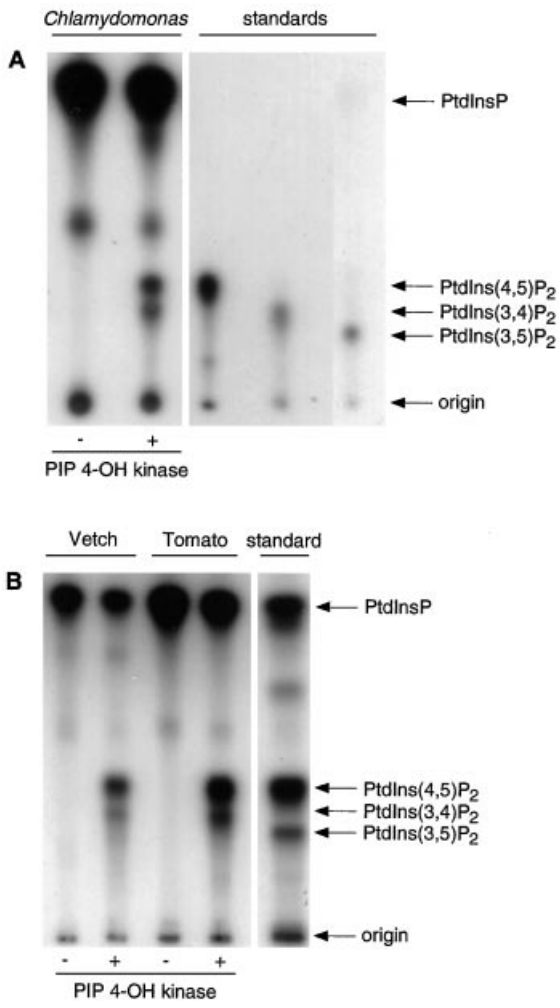


Figure 1 PtdIns5*P* is present in plant cells

Radioactive PtdIns*P* spots from ^{32}P-prelabelled plant cells were isolated from the TLC plate and incubated for 15 h with a type-II PtdIns5*P* 4-OH kinase at 30 °C in the presence of 200 μM ATP. Lipids were then extracted and separated by TLC and visualized by autoradiography. (**A**) *Chlamydomonas* together with standards of PtdIns(4,5)P_2, PtdIns(3,4)P_2 and PtdIns(3,5)P_2. (**B**) Vetch (*V. sativa*) and tomato lipids together with standard lipids extracted from osmotically stressed *Chlamydomonas* cells containing PtdIns*P*, PtdIns(4,5)P_2 and PtdIns(3,5)P_2 [15].

PtdIns4*P* but not PtdIns5*P* is decreased when PLC is activated

PLC signalling in *Chlamydomonas* can be activated strongly by using the wasp-venom peptide mastoparan [24,35]. Upon stimulation, most of the radiolabelled PtdIns(4,5)P_2 is metabolized within the first 30 s, accompanied by the formation of Ins(1,4,5)P_3 [24,35]. This is associated with a substantial decrease in radiolabelled PtdIns*P*, which is thought to reflect the conversion of PtdIns4*P* to PtdIns(4,5)P_2, to maintain PLC substrate levels [36]. HPLC analysis of the [^{32}P]PtdIns*P* head groups revealed that the decrease was not due to metabolism of PtdIns3*P*, and therefore was thought to reflect PtdIns4*P* metabolism [24]. However, we must now acknowledge that the decrease in PtdIns*P* could be due to enhanced metabolism of PtdIns5*P*, as postulated for animal systems [17,18].

In order to determine whether PtdIns4*P* and/or PtdIns5*P* contributed to the decrease in PtdIns*P*, conditions were selected in which both type-II (PtdIns5*P* 4-OH kinase) and type-I (PtdIns4*P* 5-OH kinase, isolated from transiently transfected COS cells) kinases specifically phosphorylated their substrates in a time- and concentration-dependent manner. An essential aspect of the procedure was that the extracted PPIs were first purified on neomycin beads, which removed an unknown kinase inhibitor from the *Chlamydomonas* lipid extracts (see the Materials and methods section). Phosphorylation was carried out for 2 h in the presence of 5 μM ATP and 74 kBq of [γ-^{32}P]ATP (rather than the 200 μM ATP previously used) using phosphatidylserine and PtdOH as carrier lipids to increase the kinase specificity. Under these conditions, type-I and type-II PtdIns*P* kinases phosphorylated PtdIns4*P* and PtdIns5*P* respectively, and the amount of [^{32}P]PtdIns(4,5)P_2 formed was linear with time

© 2001 Biochemical Society

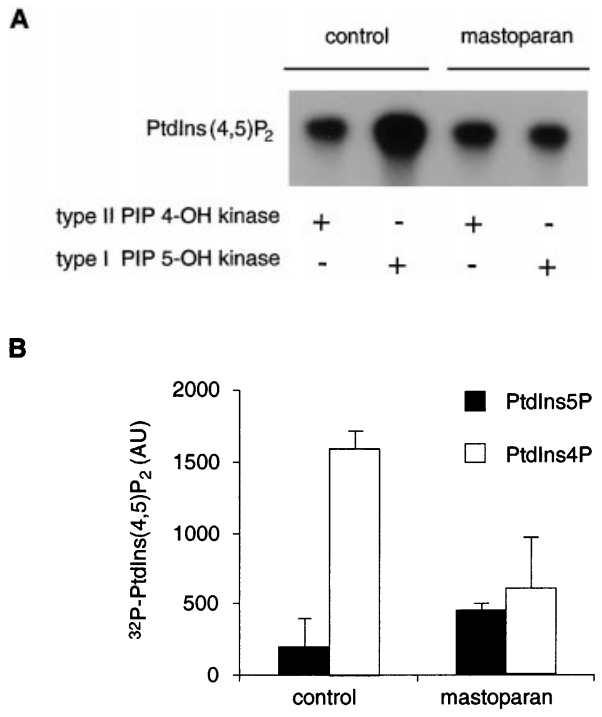


Figure 2 Stimulation of *Chlamydomonas* cells with mastoparan reduces PtdIns4*P* but not PtdIns5*P* levels

Non-labelled cells were treated with 2 μM mastoparan or buffer (control) for 30 s. Lipids were extracted and PPIs isolated using neomycin beads. PtdIns*P* species were then phosphorylated using either type-I or type-II kinase in the presence of [γ-^{32}P]ATP and 5 μM unlabelled ATP for 2 h at 30 °C, conditions under which only PtdIns(4,5)P_2 is formed. (**A**) Autoradiograph of the [^{32}P]PtdIns(4,5)P_2 formed. (**B**) Quantification of [^{32}P]PtdIns(4,5)P_2 generated by type-I PtdIns4*P* 5-OH kinase from PtdIns4*P* or by type-II PtdIns5*P* 4-OH kinase from PtdIns5*P*, from control ($n = 2$) or mastoparan-stimulated ($n = 3$) cells. Results (means $\pm$ S.E.M.) are presented as arbitrary units (AU).

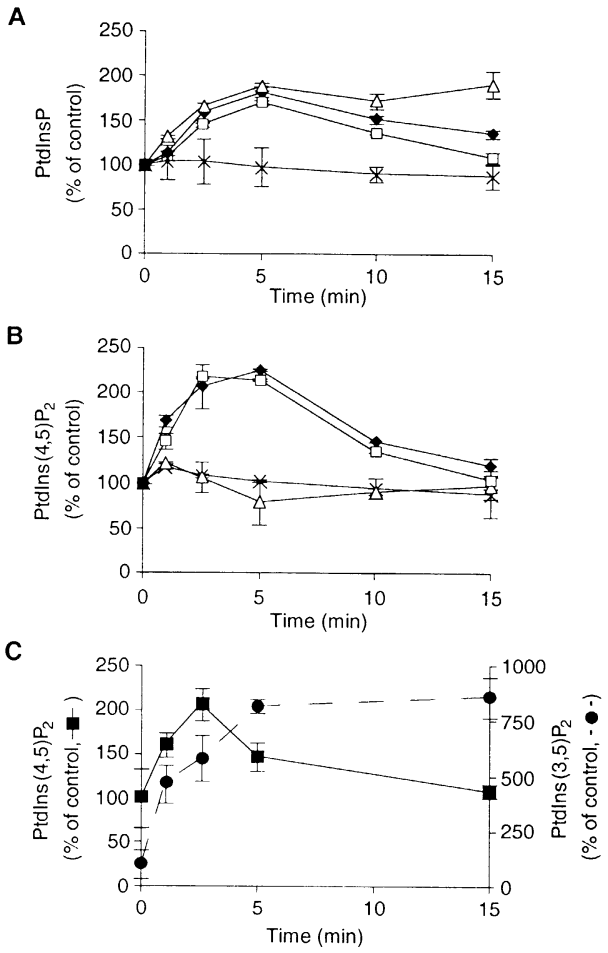


Figure 3 Effect of osmotic stress on PtdIns*P* and PtdInsP_2 synthesis

Chlamydomonas cells were prelabelled with [^{32}P]P_i for 1 h and treated with HMCK (control, $\times$), NaCl (150 mM, ◆), KCl (150 mM, □) or mannitol (270 mM, △). Lipids were extracted, separated by TLC and the radioactivity quantified by PhosphoImaging. The results for the total PtdIns*P* pool (**A**) and PtdIns(4,5)P_2 (**B**) are expressed as fold-increase with respect to non-treated cells at $t = 0$ min. (**C**) Changes in PtdIns(4,5)P_2 (■) and PtdIns(3,5)P_2 (●) in response to 300 mM NaCl as determined by HPLC. Results represent the means $\pm$ S.E.M. from two independent experiments.

and with respect to PtdIns*P* concentration. PtdIns3*P* was not phosphorylated under those conditions (results not shown). Subsequently, we used the assay to quantify the relative amounts of PtdIns5*P* and PtdIns4*P* in extracts of *Chlamydomonas* cells.

The PtdIns*P*s were extracted from non-labelled *Chlamydomonas* cells that had been treated with or without 2 μM mastoparan for 30 s [24], and purified using neomycin beads. Thereafter, they were incubated with either type-I or type-II kinase in the presence of [γ-^{32}P]ATP. After 2 h, reactions were stopped and the [^{32}P]PtdIns(4,5)P_2 formed visualized by autoradiography and quantified by PhosphoImaging. As illustrated in Figure 2, the level of PtdIns4*P*, shown as [^{32}P]PtdIns(4,5)P_2 formation by type-I kinase, was lowered significantly by mastoparan treatment. However, no significant change in the level of PtdIns5*P* was observed; [^{32}P]PtdIns(4,5)P_2 was produced by type-II kinase. This indicates that the hydrolysis of PtdIns(4,5)P_2 upon PLC activation is replenished by the phosphorylation of PtdIns4*P* by PtdIns4*P* 5-OH kinase. In contrast, the metabolism of PtdIns5*P* was unaffected and apparently does not contribute to PtdIns(4,5)P_2 synthesis under these conditions.

Multiple PPI isomers formed upon osmotic stress

Another stress condition that is known to affect the turnover of PPIs in plants is osmotic stress [9,15,33,37,38]. When ^{32}P-labelled *Chlamydomonas* cells were treated with osmotically equivalent concentrations of NaCl (150 mM), KCl (150 mM) or mannitol (270 mM), the [^{32}P]PtdIns*P* levels increased, reaching a maximum at 5 min, after which they returned to control values in the presence of the salts (Figure 3A). The salts also stimulated a rapid and transient increase in [^{32}P]PtdIns(4,5)P_2, whereas mannitol did not have this effect (Figure 3B). The formation of [^{32}P]PtdIns(3,5)P_2 followed a similar pattern, reaching a maximum after 5 min (results not shown) [15], which was stimulated by all osmotically active compounds. However, as shown in Figure 3(C), which represents additional data from HPLC analyses, 300 mM NaCl treatment transiently increased the level of [^{32}P]PtdIns(4,5)P_2, whereas the level of [^{32}P]PtdIns(3,5)P_2 was kept high for the duration of the experiment.

HPLC analyses were also used to determine which isomers contributed to the observed increase in [^{32}P]PtdIns*P*. A typical HPLC profile of a total extract of cells stimulated with 150 mM NaCl is shown in Figure 4(A). Individual peaks were identified using ^{3}H- or ^{32}P-labelled standards. The original HPLC gradient was able to separate GroPIns3*P* from GroPIns4*P* and GroPIns5*P*

© 2001 Biochemical Society

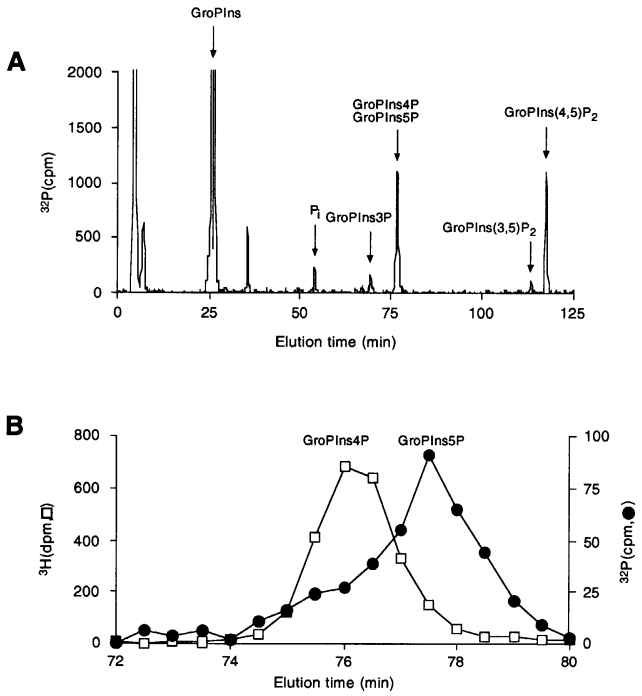


Figure 4 Anion-exchange HPLC of deacylated phospholipids

(**A**) Cells were radiolabelled for 1 h, treated for 2.5 min with 150 mM NaCl and the lipids extracted and deacylated. The water-soluble products were separated by HPLC. (**B**) [^{3}H]GroPIns4*P* and [^{32}P]GroPIns5*P* standards were separated by HPLC and collected as 30 s fractions. Radioactivity was determined by scintillation counting.

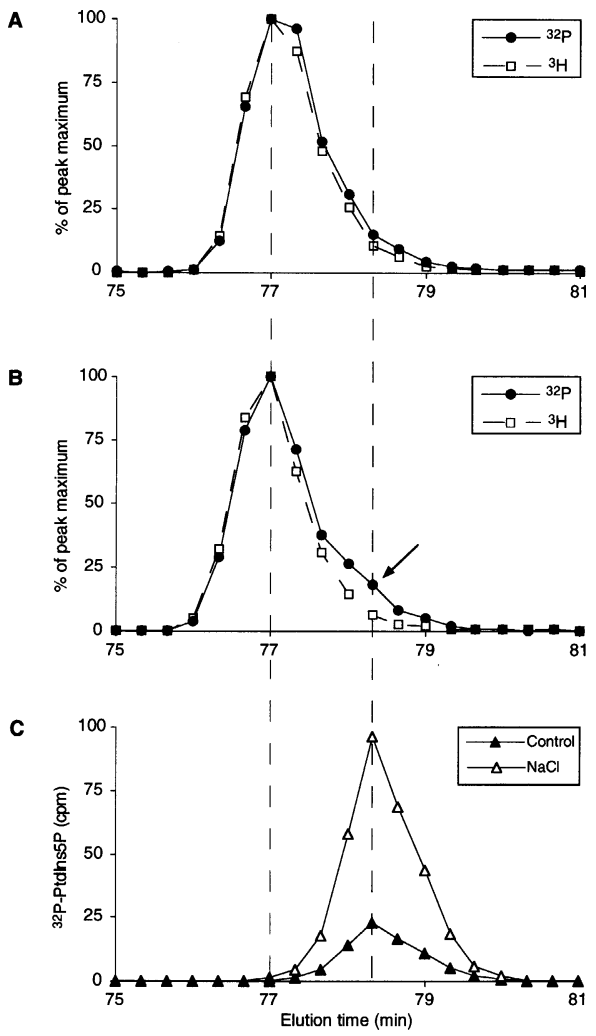


Figure 5 PtdIns5*P* is formed upon hyperosmotic stress

^{32}P-prelabelled *Chlamydomonas* cells were treated with or without 300 mM NaCl for 15 min. Lipids were isolated, deacylated and separated by HPLC after mixing with a deacylated [^{3}H]PtdIns4*P* standard. Fractions between 75 and 81 min were collected every 20 s, and their radioactivity measured. The results are shown for ^{32}P (●) and ^{3}H (□) for (**A**) control cells and (**B**) cells stimulated with 300 mM NaCl for 15 min. Data are expressed as the percentages of the peak maxima for either ^{32}P or ^{3}H after scintillation counting and subtraction of the (β-counter) background counts. The position of the [^{32}P]GroPIns5*P* shoulder is indicated by an arrow. (**C**) Based on the co-migration of deacylated ^{3}H- and ^{32}P-labelled PtdIns4*P*, the relative amounts of [^{32}P]PtdIns5*P* were determined. Data from control (▲) and NaCl-stimulated (△) cells are presented. The R_f values for the maximum levels of GroPIns4*P* and GroPIns5*P* are indicated by two vertical lines.

(Figure 4A), but the latter two were separated by only 20 s. The HPLC gradient was therefore modified such that [^{3}H]GroPIns4*P* and [^{32}P]GroPIns5*P* were separated by 1.5 min (see the Materials and methods section), peak-to-peak (Figure 4B).

Initial HPLC separations of deacylated ^{32}P-labelled *Chlamydomonas* lipids indicated that the amount of [^{32}P]PtdIns4*P* was dominantly high, masking the presence of [^{32}P]PtdIns5*P* that appeared as a shoulder on the PtdIns4*P* peak. Therefore, to distinguish between peak and shoulder, [^{3}H]GroPIns4*P* was included in all further runs, which had the effect of delineating the [^{32}P]PtdIns4*P* peak.

Cells were stimulated with 150 or 300 mM NaCl in time-course experiments, their lipids extracted and deacylated and a [^{3}H]GroPIns4*P* standard added prior to HPLC analysis. All HPLC runs were first monitored on-line without scintillant to define the ^{32}P profiles. Fractions containing the GroPIns4*P*/GroPIns5*P* peak were then analysed by scintillation counting to determinate the individual amounts of ^{3}H and ^{32}P. This procedure is illustrated by presenting the raw ^{32}P/^{3}H data from non-stimulated (Figure 5A) and NaCl-stimulated (Figure 5B) samples. When the ^{3}H and ^{32}P profiles were compared, the ^{32}P shoulder in the stimulated sample was clear (Figure 5B, arrow). The counts present in the [^{32}P]GroPIns4*P* samples were calculated by using the [^{3}H]GroPIns4*P* profile. By subtracting those counts from the total ^{32}P counts, a single peak emerged that eluted at the exact position of [^{32}P]GroPIns5*P* (Figure 4B). The [^{32}P]PtdIns5*P* peaks from both Figures 5(A) and 5(B) are presented in Figure 5(C), showing that NaCl treatment clearly increased the level of radiolabelled PtdIns5*P*.

To follow the formation of all PtdIns*P* isomers, time-course experiments were performed with 150 and 300 mM NaCl. As shown in Figure 6(A), 150 mM NaCl induced a transient 2.5-fold increase in PtdIns5*P* and a more sustained 4.5-fold increase when 300 mM NaCl was used. Similar results were obtained when the type-II PtdIns5*P* 4-OH kinase assay was used instead of HPLC (results not shown). Treatment affected all the PtdIns*P* isomers. For example, a 6-fold increase in [^{32}P]PtdIns3*P* was found after treating cells for 5 min with 300 mM NaCl (Figure 6B). [^{32}P]PtdIns4*P* also increased in concentration, although the effect was only 1.6-fold (Figure 6C).

Analysing the composition of the PtdIns*P* pool in non-stimulated cells via HPLC again emphasized that PtdIns4*P* is the dominant isomer in *Chlamydomonas*. Using this technique

© 2001 Biochemical Society

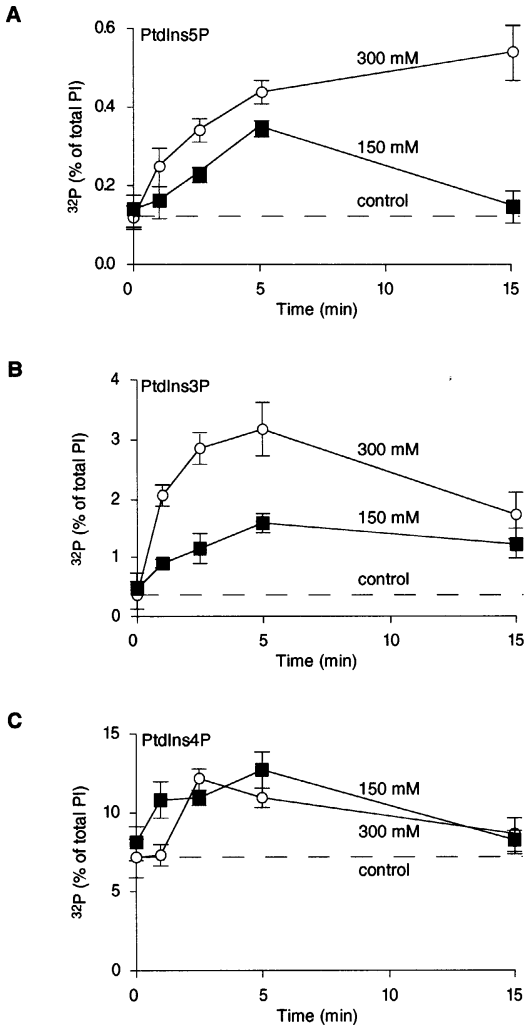


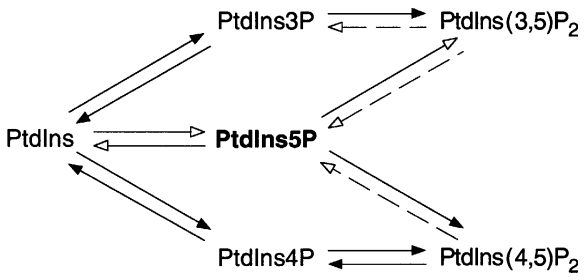
Figure 6 Time-dependent synthesis of PtdIns*P* isomers during hyperosmotic stress

Cells were labelled for 1 h and treated with 150 mM (■) or 300 mM (○) NaCl for the times indicated. The lipids were then extracted, deacylated and separated by HPLC. The radioactivity in (**A**) PtdIns5*P*, (**B**) PtdIns3*P* and (**C**) PtdIns4*P* was determined as described in the text. The data represent the means ± S.E.M. from four individual samples from two independent duplicate experiments and are expressed as percentages of the total radioactivity in the phosphoinositides. Control levels during experiments did not change and are represented by dashed lines.

PtdIns5*P* was estimated to be only about 3% of the PtdIns*P* pool.

DISCUSSION

We have shown here that PtdIns5*P* is present in plants. This isomer was previously unrecognized because it co-migrates with PtdIns4*P* in all TLC systems and the two can only be distinguished enzymically or via extensive HPLC analysis ([17,39–43] and this study). In *Chlamydomonas*, PtdIns5*P* is estimated to represent between 3 and 8% of the total PtdIns*P* pool, whereas for vetch and tomato the percentage is higher, approx. 18%. In mammalian fibroblasts, PtdIns5*P* was estimated to be 2% of the PtdIns4*P* pool [17]. More recently, Morris et al. [18] showed by enzymic analysis that PtdIns5*P* is present in resting platelets, although no indication was given of its level.



Scheme 1 Putative pathways on the metabolism of PtdIns5*P*

Solid arrows indicate metabolic routes that have been established for plants or mammals, while others that have been demonstrated *in vitro* are indicated by open-headed arrows. Those without any confirmation *in vitro* or *in vivo* are indicated by dashed lines.

These data indicate that PtdIns5*P* is a minor but distinct fraction of the PtdIns*P* pool in eukaryotic cells.

How PtdIns5*P* is synthesized is not known. A diagram representing the possible routes is given in Scheme 1. The simplest route involves PtdIns being phosphorylated by PtdIns 5-OH kinase. Although PtdIns*P* kinases are capable of producing this lipid from PtdIns *in vitro*, there is no evidence that this happens *in vivo* [41,42,44]. A second possibility is that PtdIns(4,5)P_2 is 4-dephosphorylated. However, no such phosphatase has so far been described [2,45]. During thrombin treatment of platelets, both the PtdIns5*P* levels and PtdIns5*P* 4-OH kinase activity increased, suggesting that PtdIns5*P* was involved in the formation of PtdIns(4,5)P_2 rather than its degradation [18,46]. In our experiments, when mastoparan activated the breakdown of PtdIns(4,5)P_2, it had no significant effect on PtdIns5*P* levels. Similarly, all non-salt osmotically active compounds induced an increase in radiolabelled PtdIns*P* and PtdIns(3,5)P_2 levels without affecting PtdIns(4,5)P_2. Therefore it seems unlikely that PtdIns5*P* originates from PtdIns(4,5)P_2. However, PtdIns5*P* could be formed by dephosphorylation of PtdIns(3,5)P_2. This novel PtdInsP_2 isomer is synthesized by phosphorylation of PtdIns3*P*, but its further metabolic fate is unknown [2,14,15,39]. Whereas there is much that we do not know, we do know that PtdIns5*P* can be phosphorylated to produce PtdIns(4,5)P_2 or PtdIns(3,5)P_2 [2,17,47], but further investigation is required to establish the actual pathways involved in PtdIns5*P* metabolism.

To investigate the function of PtdIns5*P*, two conditions known to influence PtdIns*P* levels were studied. First, PLC was activated by treating *Chlamydomonas* cells with mastoparan, because it dramatically decreases PtdIns(4,5)P_2 and PtdIns*P* levels within the first 30 s to produce Ins(1,4,5)P_3 [24,35]. In contrast to PtdIns4*P*, which was reduced by two-thirds, the amount of PtdIns5*P* was not affected. Since the hydrolysis of PtdIns(4,5)P_2 by PLC is followed immediately by resynthesis [24], this result clearly indicates that PtdIns4*P*, but not PtdIns5*P*, is used as a precursor. Thus PtdIns5*P* does not seem to have a role in PLC signalling, at least not when activated by mastoparan.

Second, *Chlamydomonas* cells were subjected to hyperosmotic stress, based on reports that such treatment affects phospholipid levels in plant cells [15,33,37,38,48–50], reviewed in Munnik and Meijer [9]. We found that the radiolabelled PtdIns*P* pool increased, and HPLC analysis established that PtdIns5*P* contributed to that increase. The rapid increase in PtdIns5*P* suggests that it plays a role in signalling, as a signal itself, a signal

© 2001 Biochemical Society

precursor or as an attenuation product of PtdIns(3,5)P_2 metabolism. In analogy with proteins binding specifically to PtdIns3*P* via their FYVE domains [1,3], PtdIns5*P* might also function as a ligand for certain FYVE domains and may thereby be involved in the membrane targeting of proteins.

HPLC analyses showed that levels of PtdIns3*P* and PtdIns4*P*, as well as PtdInsP_2 levels, increased in response to osmotic stress. The increase in PtdIns(3,5)P_2 formation (Figure 3C) was maintained for 10 min and contrasted with the transient increase in the PtdIns(4,5)P_2, which was restricted to the first 2 min of treatment. This difference in kinetics, in itself, suggests that these PtdInsP_2 isomers have different functions. Furthermore, whereas PtdIns(3,5)P_2 synthesis was stimulated by all osmolytes tested, PtdIns(4,5)P_2 only accumulated when cells were stimulated with salts, but not when they were treated with mannitol. This indicates that PtdIns(4,5)P_2 accumulates as a result of ionic rather than osmotic stress and therefore that *Chlamydomonas* cells are capable of discriminating between such stresses [9]. The combined increase in both PtdInsP_2 isomers has previously been reported for yeast cells treated with 0.9 M NaCl [14]. This resulted in a more than 10-fold increase in PtdIns(3,5)P_2 and a 1.7-fold increase in PtdIns(4,5)P_2. In contrast, when *Arabidopsis thaliana* was subjected to hyperosmotic stress, a dramatic increase in PtdIns(4,5)P_2 was found, independent of the osmotic agent used [33]. These authors did not report changes in PtdIns(3,5)P_2 or PtdIns(3,4)P_2. During our own HPLC analyses of *Chlamydomonas*, no PtdIns(3,4)P_2 was detected. This implies that the PtdIns(3,4)P_2 previously thought to be in *Chlamydomonas* [29,30] is PtdIns(3,5)P_2. PtdIns(3,4)P_2 has also been reported in other plants [11,13,16]; however, the recent identification of PtdIns(3,5)P_2, together with the knowledge that these two isomers behave similarly under many separation conditions, makes it necessary to reassess their identities.

This work was supported financially by a Funke fellowship, the Netherlands Organisation for Scientific Research (NWO), the Royal Netherlands Academy of Arts and Science (KNAW), the Italian Association for Cancer Research (AIRC), Telethon Italia and the Netherlands Cancer Institute (NKI). We thank our colleagues in the collaborating laboratories and in particular Dr Daniela Corda for their scientific discussions. We are grateful to John van Himbergen, Martine den Hartog and Clive D'Santos for providing tomato and *Vicia* samples and the neomycin beads, respectively.

REFERENCES

1 Corvera, S., D'Arrigo, A. and Stenmark, H. (1999) Phosphoinositides in membrane traffic. Curr. Opin. Cell Biol. **11**, 460–465
2 Hinchliffe, K. A., Ciruela, A. and Irvine, R. F. (1998) PIPkins1, their substrates and their products: new functions for old enzymes. Biochim. Biophys. Acta **1436**, 87–104
3 Leevers, S. J., Vanhaesebroeck, B. and Waterfield, M. D. (1999) Signalling through phosphoinositide 3-kinases: the lipids take centre stage. Curr. Opin. Cell Biol. **11**, 219–225
4 Munnik, T., Irvine, R. F. and Musgrave, A. (1998a) Phospholipid signalling in plants. Biochim. Biophys. Acta **1389**, 222–272
5 Toker, A. (1998) The synthesis and cellular roles of phosphatidylinositol 4,5-bisphosphate. Curr. Opin. Cell Biol. **10**, 254–261
6 Gillooly, D. J., Simonsen, A. and Stenmark, H. (2001) Cellular functions of phosphatidylinositol 3-phosphate and FYVE domain proteins. Biochem. J. **355**, 249–258
7 Katan, M. and Allen, V. L. (1999) Modular PH and C2 domains in membrane attachment and other functions. FEBS Lett. **452**, 36–40
8 Lemmon, M. A. and Ferguson, K. M. (2000) Signal-dependent membrane targeting by pleckstrin homology (PH) domains. Biochem. J. **350**, 1–18
9 Munnik, T. and Meijer, H. J. G. (2001) Osmotic stress activates distinct lipid and MAPK signalling pathways in plants. FEBS Lett. **498**, 172–178
10 Stevenson, J. M., Perera, I. Y., Heilmann, I., Persson, S. and Boss, W. F. (2000) Inositol signaling and plant growth. Trends Plant Sci. **5**, 252–258
11 Brearley, C. A. and Hanke, D. E. (1993) Pathway of synthesis of 3,4- and 4,5-phosphorylated phosphatidylinositols in the duckweed *Spirodela polyrhiza* L. Biochem. J. **290**, 145–150
12 Parmar, P. N. and Brearley, C. N. (1993) Identification of 3- and 4-phosphorylated phosphoinositides and inositol phosphates in stomatal guard cells. Plant J. **4**, 255–263
13 Parmar, P. N. and Brearley, C. A. (1995) Metabolism of 3- and 4-phosphorylated phosphatidylinositols in stomatal guard cells of *Commelina communis* L. Plant J. **8**, 425–433
14 Dove, S. K., Cooke, F. T., Douglas, M. R., Sayers, L. G., Parker, P. J. and Michell, R. H. (1997) Osmotic stress activates phosphatidylinositol-3,5-bisphosphate synthesis. Nature (London) **390**, 187–192
15 Meijer, H. J. G., Divecha, N., van den Ende, H., Musgrave, A. and Munnik, T. (1999) Hyperosmotic stress induces rapid synthesis of phosphatidyl-D-inositol 3,5-bisphosphate in plant cells. Planta **208**, 294–298
16 Brearley, C. A. and Hanke, D. E. (1992) 3- and 4-phosphorylated phosphatidylinositols in the aquatic plant *Spirodela polyrhiza* L. Biochem. J. **283**, 255–260
17 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 (London) **390**, 192–196
18 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
19 Munnik, T., Arisz, S. A., de Vrije, T. and Musgrave, A. (1995) G protein activation stimulates phospholipase D signalling in plants. Plant Cell **7**, 1997–2010
20 Van der Luit, A. H., Piatti, T., van Doorn, A., Musgrave, A., Felix, G., Boller, T. and Munnik, T. (2000) Elicitation of suspension-cultured tomato cells triggers the formation of phosphatidic acid and diacylglycerol pyrophosphate. Plant Physiol. **123**, 1507–1516
21 Den Hartog, M., Musgrave, A. and Munnik, T. (2001) Nod factor-induced phosphatidic acid and diacylglycerol pyrophosphate formation: a role for phospholipase C and D in root hair deformation. Plant J. **25**, 55–66
22 Munnik, T., de Vrije, T., Irvine, R. F. and Musgrave, A. (1996) Identification of diacylglycerol pyrophosphate as a novel metabolic product of phosphatidic acid during G-protein activation in plants. J. Biol. Chem. **271**, 15708–15715
23 Divecha, N., Truong, O., Hsuan, J. J., Hinchliffe, K. A. and Irvine, R. F. (1995) The cloning and sequence of the C isoform of PtdIns4*P* 5-kinase. Biochem. J. **309**, 715–719
24 Munnik, T., van Himbergen, J. A. J., ter Riet, B., Braun, F., Irvine, R. F., van den Ende, H. and Musgrave, A. (1998b) Detailed analysis of the turnover of polyphosphoinositidides and phosphatidic acid upon activation of phospholipases C and D in *Chlamydomonas* cells treated with non-permeabilizing concentrations of mastoparan. Planta **207**, 133–145
25 Clarke, N. G. and Dawson, R. M. (1981) Alkaline O leads to N-transacylation. A new method for the quantitative deacylation of phospholipids. Biochem. J. **195**, 301–306
26 Berrie, C. P., Iurisci, C. and Corda, D. (1999) Membrane transport and *in vitro* metabolism of the Ras cascade messenger, glycerophosphoinositol 4-phosphate. Eur. J. Biochem. **266**, 413–419
27 Stephens, L. R., Hughes, K. T. and Irvine, R. F. (1991) Pathway of phosphatidylinositol(3,4,5)-trisphosphate synthesis in activated neutrophils. Nature (London) **351**, 33–39
28 Schacht, J. (1978) Purification of polyphosphoinositides by chromatography on immobilized neomycin. J. Lipid Res. **19**, 1063–1067
29 Irvine, R. F., Letcher, A. J., Stephens, L. R. and Musgrave, A. (1992) Inositol polyphosphate metabolism and inositol lipids in a green alga, *Chlamydomonas eugametos*. Biochem. J. **281**, 261–266
30 Munnik, T., Irvine, R. F. and Musgrave, A. (1994) Rapid turnover of phosphatidylinositol 3-phosphate in the green alga *Chlamydomonas eugametos*: signs of a phosphatidylinositide 3-kinase signalling pathway in lower plants? Biochem. J. **298**, 269–273
31 Zhang, X., Loijens, J. C., Boronenkov, I. V., Parker, G. J., Norris, F. A., Chen, J., Thum, O., Prestwich, G. D., Majerus, P. W. and Anderson, R. A. (1997) Phosphatidylinositol-4-phosphate 5-kinase isozymes catalyze the synthesis of 3-phosphate-containing phosphatidylinositol signaling molecules. J. Biol. Chem. **272**, 17756–17761
32 Kunz, J., Wilson, M. P., Kisseleva, M., Hurley, J. H., Majerus, P. W. and Anderson, R. A. (2000) The activation loop of phosphatidylinositol phosphate kinases determines signaling specificity. Mol. Cell **5**, 1–11
33 Pical, C., Westergren, T., Dove, S. K., Larsson, C. and Sommarin, M. (1999) Salinity and hyperosmotic stress induce rapid increases in phosphatidylinositol 4,5-bisphosphate, diacylglycerol pyrophosphate, and phosphatidylcholine in *Arabidopsis thaliana* cells. J. Biol. Chem. **274**, 38232–38240
34 Munnik, T., Musgrave, A. and de Vrije, T. (1994) Rapid turnover of polyphosphoinositides in carnation flower petals. Planta **193**, 89–98
35 Van Himbergen, J. A. J., ter Riet, B., Meijer, H. J. G., van den Ende, H., Musgrave, A. and Munnik, T. (1999) Mastoparan analogues activate phospholipase C- and phospholipase D activity in *Chlamydomonas*: a comparative study. J. Exp. Bot. **50**, 1735–1742

© 2001 Biochemical Society

36 Munnik, T. (2001) Phosphatidic acid: an emerging plant lipid second messenger. Trends Plant Sci. **6**, 227–233
37 Meijer, H. J. G., Arisz, S. A., van Himbergen, J. A. J., Musgrave, A. and Munnik, T. (2001) Hyperosmotic stress rapidly generates lyso-phosphatidic acid in *Chlamydomonas*. Plant J. **25**, 541–548
38 Munnik, T., Meijer, H. J. G., ter Riet, B., Hirt, H., Frank, W., Bartels, D. and Musgrave, A. (2000) Hyperosmotic stress stimulates phospholipase D activity and elevates the levels of phosphatidic acid and diacylglycerol pyrophosphate. Plant J. **22**, 147–154
39 Whiteford, C. C., Brearley, C. A. and Ulug, E. T. (1997) Phosphatidylinositol 3,5-bisphosphate defines a novel PI 3-kinase pathway in resting mouse fibroblasts. Biochem. J. **323**, 597–601
40 Itoh, T., Ijuin, T. and Takenawa, T. (1998) A novel phosphatidylinositol-5-phosphate 4-kinase (phosphatidylinositol-phosphate kinase IIγ) is phosphorylated in the endoplasmic reticulum in response to mitogenic signals. J. Biol. Chem. **273**, 20292–20299
41 McEwen, R. K., Dove, S. K., Cooke, F. T., Painter, G. F., Holmes, A. B., Shisheva, A., Ohya, Y., Parker, P. J. and Michell, R. H. (1999) Complementation analysis in PtdInsP kinase-deficient yeast mutants demonstrates that *Schizosaccharomyces pombe* and murine Fab1p homologues are phosphatidylinositol 3-phosphate 5-kinases. J. Biol. Chem. **274**, 33905–33912
42 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
43 Hughes, W. E., Woscholski, R., Cooke, F. T., Patrick, R. S., Dove, S. K., McDonald, N. Q. and Parker, P. J. (2000) SAC1 encodes a regulated lipid phosphoinositide phosphatase, defects in which can be suppressed by the homologous Inp52p and Inp53p phosphatases. J. Biol. Chem. **275**, 801–808
44 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
45 Majerus, P. W., Kisseleva, M. V. and Norris, F. A. (1999) The role of phosphatases in inositol signaling reactions. J. Biol. Chem. **274**, 10669–10672
46 Hinchliffe, K. A., Irvine, R. F. and Divecha, N. (1998) Regulation of PtdIns*4P* 5-kinase C by thrombin-stimulated changes in its phosphorylation state in human platelets. Biochem. J. **329**, 115–119
47 Fruman, D. A., Meyers, R. E. and Cantley, L. C. (1998) Phosphoinositide kinases. Annu. Rev. Biochem. **67**, 481–507
48 Cho, M. H., Shears, S. B. and Boss, W. F. (1993) Changes in phosphatidylinositol metabolism in response to hyperosmotic stress in *Daucus carota* L. cells grown in suspension culture. Plant Physiol. **103**, 637–647
49 Drøbak, B. K. and Watkins, P. A. (2000) Inositol (1,4,5)trisphosphate production in plant cells: an early response to salinity and hyperosmotic stress. FEBS Lett. **481**, 240–244
50 Einspahr, K. J., Peeler, T. C. and Thompson, Jr, G. A. (1988) Rapid changes in polyphosphoinositide metabolism associated with the response of *Dunaliella salina* to hypoosmotic shock. J. Biol. Chem. **263**, 5775–5779

Received 4 June 2001/10 August 2001; accepted 28 September 2001

© 2001 Biochemical Society