Osmotic stress-induced phospholipid signalling in plants

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Chapter 5

Identification of a new polyphosphoinositide in plants, PI5P, and its accumulation upon osmotic stress

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Polyphosphoinositides play an important role in membrane trafficking and cell signalling. In plants, two PIP isomers have been described, PI3P and PI4P. Here, we report the identification of a third, PI5P. Evidence is based on the conversion of the endogenous PIP pool into $PI(4,5)P_2$ by a specific PI5P 4-OH kinase, and on *in vivo* ^{32}P-labelling studies coupled to HPLC head-group analysis. In *Chlamydomonas*, 3-8% of the PIP pool was PI5P; 10-15% was PI3P and the rest was PI4P. In seedlings of *Vicia faba* and suspension cultured tomato cells, the level of PI5P was about 18%, indicating that PI5P is a general plant lipid that represents a significant proportion of the PIP pool. Activating PLC signalling in *Chlamydomonas* cells with mastoparan, increased the turnover of $PI(4,5)P_2$ at the cost of PI4P, but did not affect the level of PI5P. This indicates that $PI(4,5)P_2$ is synthesized from PI4P rather than from PI5P during PLC signalling. When cells were subjected to hyperosmotic stress however, PI5P levels rapidly increased, suggesting a role in osmotic stress signalling. The potential pathways of PI5P formation are discussed.

Introduction

Polyphosphoinositides (PPI) are only minor lipids in eukaryotic cells but play a major role in cell biology. They are precursors of second messengers [e.g. $PI(4,5)P_2$ is hydrolysed by PLC to produce diacylglycerol and inositol 1,4,5-trisphosphate] but also regulate various enzymatic activities, K^+ channels and cytoskeletal proteins (Corvera *et al.*, 1999; Hinchliffe *et al.*, 1998a; Leevers *et al.*, 1999; Munnik *et al.*, 1998a; Toker, 1998). 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 (Gillooly *et al.*, 2001; Katan and Allen, 1999; Lemmon and Ferguson, 2000). Through binding different PPIs, cytosolic target proteins can be recruited to specific membranes where they play a role in signal transduction, regulate enzyme activity, cytoskeleton rearrangements or membrane trafficking (Corvera *et al.*, 1999; Gillooly *et al.*, 2001; Lemmon and Ferguson, 2000).

Until now, two isoforms of PIP have been recognized in plants: PI3P and PI4P (Munnik *et al.*, 1998a; Munnik and Meijer, 2001; Stevenson *et al.*, 2000). PI3P is thought to be the precursor of $PI(3,4)P_2$ (Brearley and Hanke, 1993; Parmar and Brearley, 1995; Parmar and Brearley, 1993) or $PI(3,5)P_2$ (Dove *et al.*, 1997; Meijer *et al.*, 1999) whereas PI4P is seen as the precursor of $PI(4,5)P_2$. All three PIP_2 isomers have been identified in plants (Brearley and Hanke, 1992; 1993; Meijer *et al.*, 1999; Munnik *et al.*, 1998a; Parmar and Brearley, 1995; Parmar and Brearley, 1993). A third PIP isomer has been discovered in animal cells, PI5P (Rameh *et al.*, 1997), the *in vitro* substrate for type-II PIPkins (PI5P 4-kinase) and present as a minor fraction of the PIP pool in mouse fibroblasts. In platelets, PI5P levels were found to transiently increase upon thrombin stimulation (Morris *et al.*, 2000), indicating that it is directly or indirectly involved in signalling.

In this study, we have investigated the composition of the PIP pool in plant cells and provide evidence for the existence of PI5P. We also monitored changes in metabolism during rapid $PI(4,5)P_2$ synthesis (G-protein activation) and rapid $PI(3,5)P_2$ synthesis (hyperosmotic stress). PI5P levels only changed during hyperosmotic stress, suggesting a role in osmo-stress signalling.

Methods

Materials — PI and PIP 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 before (Munnik *et al.*, 1995). Cell suspensions were produced by flooding 2- to 4-week-old plates overnight with 20 ml 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) x 10^7 cells ml^{-1}. Suspension cultures of tomato (Msk8) were grown as described in Van der Luit *et al.* (2000). *Vicia sativa* seedlings were grown in modified Fåhreus slides as described in Den Hartog *et al.* (2001).

Phospholipid Labelling, Extraction and Analysis — *Chlamydomonas* lipids were labelled, extracted and separated by TLC as described earlier (Munnik *et al.*, 1996). *V. sativa* seedlings were labelled in 160 µl 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 [^{32}P]P_i/per seedling (Den Hartog *et al.*, 2001). Tomato cells were labelled as described (Van der Luit *et al.*, 2000). Lipids were extracted by adding 3.75 volumes 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 earlier (Munnik *et al.*, 1996). Radiolabelled phospholipids were visualized by autoradiography (X-Omat AR film, Kodak) and quantified by phosphoimaging (Storm, Molecular Dynamics and BAS 2000, Fuji).

Polyphosphoinositide Standards — ^{32}P-Labelled PI3P was synthesized using phosphoinositide 3 kinase activity that was immunoprecipitated from PDGF stimulated Rat-1 cells by anti-phosphotyrosine antibodies as described (Meijer *et al.*, 1999). ^{32}P-Labelled PI4P was prepared from PI using recombinant Myc-tagged type-II PI5P 4-OH kinase (Divecha *et al.*, 1995) that was immunoprecipitated from transgenic *Escherichia coli* cells. [^{32}P]PI5P was synthesized using recombinant myc-tagged type-I PI4P 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 nmole of PI and 1.85 MBq of [γ-^{32}P]ATP. After quenching the reactions, lipids were extracted and separated by TLC (Munnik *et al.*, 1998b).

The glycerophosphoinositols [^{3}H]GroPIns, [^{3}H]GroPIns4P and [^{3}H]GroPIns(4,5)P_2 were produced by standard mono-methylamine deacylation (Clarke and Dawson, 1981) 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 Eagles medium plus 10% fetal bovine serum, as described previously (38). After adding cold (-20°C) methanol and scraping 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 mono-methylamine mixture (40% aqueous mono-methylamine/water/*n*-butyl-alcohol/methanol; 4.5:1:1.125:5.875; v/v). After lyophilization, samples were resuspended in 500 µl water, to which 700 µl *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 then lyophilized again. The resulting glycerophosphoinositols were purified by HPLC as described below. The [^{32}P]GroPIns5P 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 (Meijer *et al.*, 1999). 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 to allow specific removal of the 3-phosphate, and hence the production of [^{32}P]GroPIns5P (Stephens *et al.*, 1991). After overnight incubation, the reaction was halted by the addition of 700 µl H_2O and 100 µl 19.25% perchloric acid. After 30 min on ice, the protein was removed by

centrifugation. The supernatant was neutralised to pH 7.5 by addition of 200 µl 50% (w/v) $KHCO_3$ and kept on ice for 1 h. The precipitate was removed. and the supernatant used for HPLC analysis.

Isolation of PPIs from Chlamydomonas — Lipids from non-labelled *Chlamydomonas* were dried and suspended in 500 µl PPI-binding solution (chloroform/methanol/20 mM ammonium formate, 5:10:2 by vol.). After addition of 20 µl neomycin-linked glass beads (Schacht, 1978), samples were incubated at 4 °C for 15 min. Beads were then centrifuged (10000g), 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 [PS] and phosphatidic acid (PA), 5 nmole each] and extracted as described above. This procedure was developed because it removed a kinase inhibitor from the lipid extracts. Using 25 to 200 µl radioactive cell cultures, 98.2 ± 2.0% of the PIP was retrieved. The only other lipid effectively purified was PIP_2 (95.2 ± 0.6%). Only small proportions of PA (6.0 ± 1.3%), PG (5.8 ± 0.4%), PI, PC and PE (< 0.5%) were recovered by this procedure.

Kinase Assays — *In vivo*-labelled PIPs 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, including enzymes and 200 µM ATP, was added. Phosphorylation was carried out in 100 µl at 30°C in a 15 h incubation and then stopped with 375 µl of cold (-20°C) chloroform/methanol, (1:2 by vol.). 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 diethyl ether and 50 µl 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 [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 control for enzyme-specificity, non-labelled PIP was phosphorylated by type-II PI5P 4-OH kinase or type-I PI4P 5-OH kinase using [γ-^{32}P]ATP. The resulting type-II- and type-I $PI(4,5)P_2$s were dephosphorylated with a specific recombinant 5-phosphatase. All label lost from type-II-$PI(4,5)P_2$ was recovered in PI4P whereas all the ^{32}P from type-I-$PI(4,5)P_2$ was lost as P_i, with no counts remaining in PIP.

HPLC Analysis of ^{3}H/^{32}P-Labelled PPI — 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, UK) using a non-linear water (buffer A)/1.0 M ammonium phosphate, pH 3.35 (phosphoric acid; Buffer B) gradient (Berrie *et al.*, 1999). Because the peaks of GroPIns4P and GroPIns5P were only separated by less than 20 s, a modified gradient was utilised: 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 (Stephens *et al.*, 1991) and the use of ^{3}H-labelled standards, including Ins, GroPIns, Ins1P, Ins3P, Ins4P, GroPIns3P, GroPIns4P, $Ins(1,4)P_2$, $Ins(1,5)P_2$, $GroPIns(3,4)P_2$, $GroPIns(4,5)P_2$, $Ins(1,3,4)P_3$ and $Ins(1,4,5)P_3$. The latter gradient produced a 1.5 min peak-to-peak separation of GroPIns4P and GroPIns5P (see Fig. 4B) and was exploited to separate the ^{32}P-labelled samples. Standard [^{3}H]GroPIns4P was included with every sample to correct for minor HPLC run-to-run variability and hence allow for more precise calculation of the relative levels of GroPIns4P and GroPIns5P. The radioactivity in these GroPInsP fractions (see also Fig. 5 and Fig. 6) was determined by dual-label scintillation counting to a 0.5% sample error (low isotope concentrations).

Results

PI5P is Present in Plant Cells

To investigate whether *Chlamydomonas* cells contain PI5P, 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]PIP spot was then isolated and the lipids incubated with a 4-OH kinase (type-II PIP kinase, isolated from transgenic *E. coli* cells) in the presence of 200 μM non-radioactive ATP. If the PIP spot contained [^{32}P]PI5P, it would be converted to ^{32}P-labelled PI(4,5)P_2. The reaction products were subsequently chromatographed together with PIP_2 standards. As illustrated in Figure 1A, the PIP 4-OH kinase phosphorylated [^{32}P]PIP into two products: one co-migrated with PI(4,5)P_2, the other with PI(3,4)P_2. The production of PI(4,5)P_2 indicates that PI5P is present in this alga. The production of PI(3,4)P_2 confirms the presence of PI3P (Irvine *et al.*, 1992; Munnik *et al.*, 1994a). It also shows that the enzyme uses substrates other than PI5P under certain conditions, substantiating earlier reports (Morris *et al.*, 2000; Rameh *et al.*, 1997; Zhang *et al.*, 1997). However, type-II PIP 4-OH kinase does not phosphorylate PI4P, therefore the PI(4,5)P_2 could not have arisen from the phosphorylation of PI4P (Kunz *et al.*, 2000; Rameh *et al.*, 1997). After 15 h of incubation, approximately 10% of the original [^{32}P]PIP was converted to [^{32}P]PI(3,4)P_2, indicating that at least 10% of the pool exists as PI3P, which is in close agreement with results obtained previously (Irvine *et al.*, 1992; Munnik *et al.*, 1994a). Based on the same arguments, about 8% of the *Chlamydomonas* [^{32}P]PIP pool was PI5P.

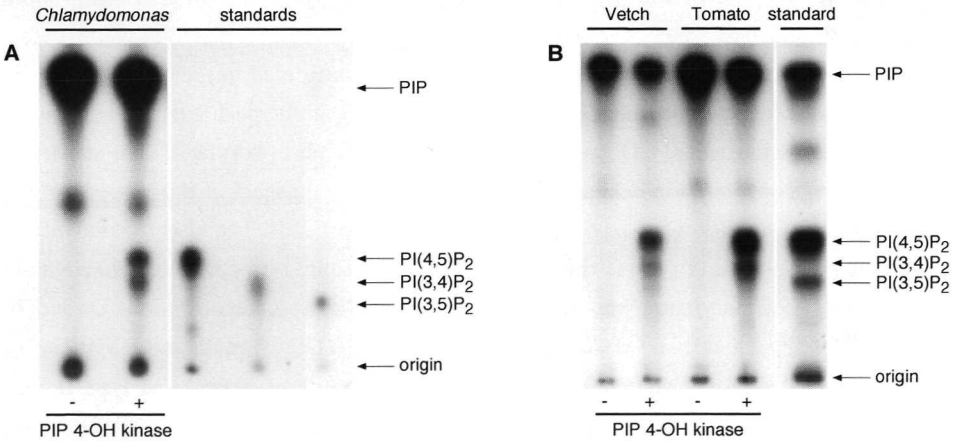


Fig. 1. PI5P is present in plant cells. Radioactive PIP spots from $^{32}P_i$-prelabelled plant cells were isolated from the TLC and incubated for 15 h with a type-II PIP 4-OH kinase at 30°C in the presence of 200 μM ATP. Lipids were then extracted and separated by TLC and visualized by autoradiography. A, *Chlamydomonas* together with standards of PI(4,5)P_2, PI(3,4)P_2 and PI(3,5)P_2. B, Vetch (*Vicia sativa*) and tomato lipids together with standard lipids extracted from osmotically stressed *Chlamydomonas* cells containing PIP, PI(4,5)P_2 and PI(3,5)P_2 (Meijer *et al.*, 1999).

To determine whether PI5P is also present in other plant cells, PIP spots from radiolabelled vetch roots (*Vicia sativa*) and tomato suspension cultures were isolated and treated with PIP 4-OH kinase as described above. As for *Chlamydomonas*, two products were formed that co-migrated with $PI(4,5)P_2$ and $PI(3,4)P_2$ (Figure 1B). For vetch and tomato, about 8% of the label was phosphorylated to $PI(3,4)P_2$, which is again in agreement with previous estimates of higher plant PI3P levels (Brearley and Hanke, 1992; 1993; Munnik *et al.*, 1998a; Munnik *et al.*, 1994b; Pical *et al.*, 1999). Approximately 18% was phosphorylated to $[^{32}P]PI(4,5)P_2$, indicating that PI5P represents a significant fraction of the $[^{32}P]PIP$ pool.

PI4P But Not PI5P is Diminished When PLC is Activated

PLC signalling in *Chlamydomonas* can be strongly activated by using the wasp-venom peptide mastoparan (Munnik *et al.*, 1998b; Van Himbergen *et al.*, 1999). Upon stimulation, most of the radiolabelled $PI(4,5)P_2$ is metabolised within the first 30 s, accompanied by the formation of inositol 1,4,5-trisphosphate (Munnik *et al.*, 1998b; Van Himbergen *et al.*, 1999). This is associated with a substantial decrease in radiolabelled PIP, which is thought to reflect the conversion of PI4P to $PI(4,5)P_2$, to maintain PLC-substrate levels (Munnik, 2001). HPLC analysis of the $[^{32}P]PIP$-head groups revealed that the decrease was not due to metabolism of PI3P, and therefore was thought to reflect PI4P metabolism (Munnik *et al.*, 1998b). However, we must now acknowledge that the decrease in PIP could be due to enhanced metabolism of PI5P, as postulated for animal systems (Morris *et al.*, 2000; Rameh *et al.*, 1997).

In order to determine whether PI4P and/or PI5P contributed to the decrease in PIP, conditions were selected in which both type-II (PIP 4-OH) and type-I (PIP 5-OH, 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, because it removed an unknown kinase inhibitor from the *Chlamydomonas* lipid extracts (see *Experimental procedures*). Phosphorylation was carried out for 2 h in the presence of 5 μM ATP and 74 kBq $[\gamma\text{-}^{32}P]ATP$ (rather than the previously used 200 μM ATP) using PS and PA as carrier lipids to increase the kinase specificity. Under these conditions, type-I and type-II PIP-kinases phosphorylated PI4P and PI5P respectively, and the amount of $[^{32}P]PI(4,5)P_2$ formed was linear with time and with respect to PIP concentration. PI3P was not phosphorylated under those conditions (data not shown). Subsequently, we used the assay to quantitate the relative amounts of PI5P and PI4P in extracts of *Chlamydomonas* cells.

The PIPs were extracted from non-labelled *Chlamydomonas* cells that had been treated with or without 2 μM mastoparan for 30 s (Munnik *et al.*, 1998b), and purified

using neomycin beads. Thereafter, they were either incubated with type-I or type-II kinase in the presence of [γ–^{32}P]ATP. After 2 h, reactions were stopped and the [^{32}P]PI(4,5)P_2 formed visualized by autoradiography and quantitated by phosphoimaging. As illustrated in Figure 2, the level of PI4P (witnessed as [^{32}P]PI(4,5)P_2 formation by type-I kinase) was significantly lowered by mastoparan treatment. However, no significant change in the level of PI5P was observed ([^{32}P]PI(4,5)P_2 produced by type-II kinase). This indicates that the hydrolysis of PI(4,5)P_2 upon PLC activation is replenished by the phosphorylation of PI4P by PI4P 5-OH kinase. In contrast, the metabolism of PI5P was unaffected and apparently does not contribute to PI(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 (Meijer *et al.*, 2001; Meijer *et al.*, 1999; Munnik and Meijer, 2001; Munnik *et al.*, 2000; Pical *et al.*, 1999). 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]PIP levels increased, reaching a maximum at 5 min, after which they returned to control values in the case of the salts (Figure 3A). The salts also stimulated a rapid and transient increase in [^{32}P]PI(4,5)P_2, whereas mannitol did not have this effect (Figure 3B). The formation of [^{32}P]PI(3,5)P_2 followed a similar pattern, reaching a maximum after 5 min [not shown, (Meijer *et al.*, 1999) which was stimulated by all osmotica. However, as shown in Figure 3C, which represents additional data from HPLC analyses, 300 mM NaCl treatment transiently increased the level of [^{32}P]PI(4,5)P_2, whereas the level of [^{32}P]PI(3,5)P_2 was maintained high for the duration of the experiment.

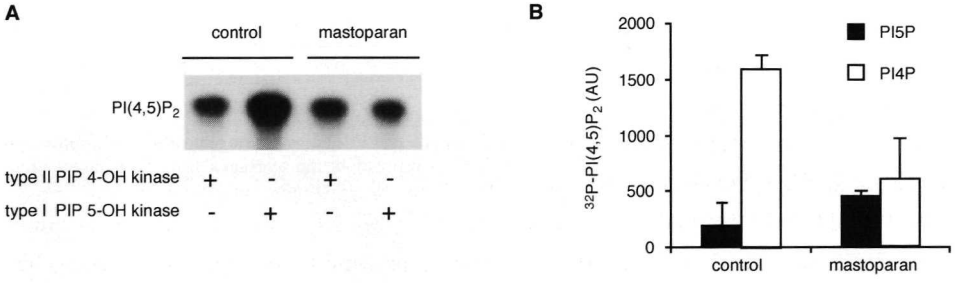


Fig. 2. Stimulation of *Chlamydomonas* cells with mastoparan reduces PI4P but not PI5P levels. Non-labelled cells were treated with 2 μM mastoparan or buffer (control) for 30 s. Lipids were extracted and PPIs isolated using neomycin beads. PIP species were then phosphorylated using either type-I or type-II kinase, in the presence of [^{32}P]-γATP and 5 μM cold ATP for 2 h at 30°C, conditions under which only PI(4,5)P_2 is formed. A, Autoradiograph of the [^{32}P]PI(4,5)P_2 formed. B, Quantification of [^{32}P]PI(4,5)P_2 generated by PI4P 5-OH kinase type-I from PI4P or PI5P 4-OH kinase type-II from PI5P, from control (n=2) or mastoparan stimulated cells (n=3). Results are presented as arbitrary units (AU) ± SE.

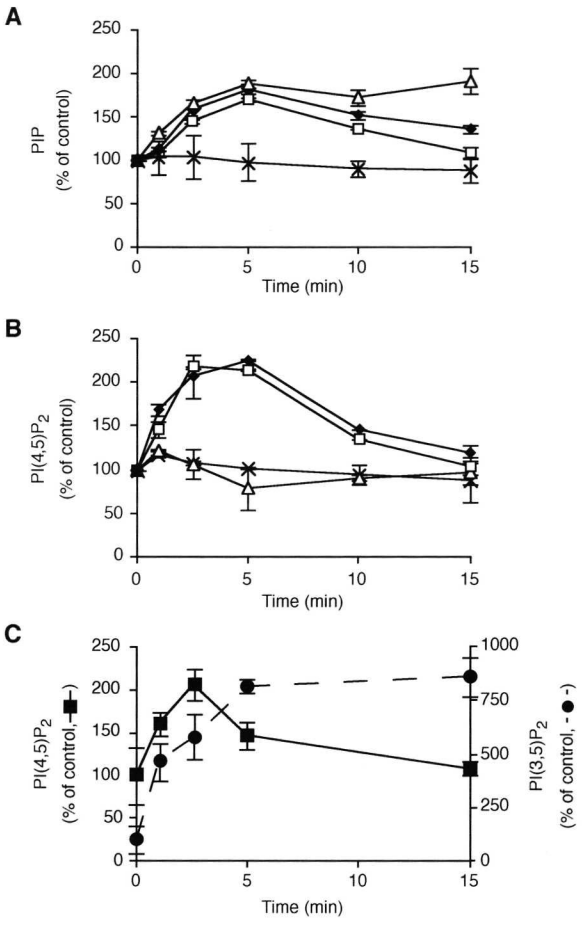


Fig. 3. Effect of osmotic stress on PIP and PIP_2 synthesis. *Chlamydomonas* cells were prelabelled with $^{32}P_i$ for 1 h and treated with HMCK (control, cross), NaCl (150 mM, closed diamond), KCl (150 mM, open square) or mannitol (270 mM, open triangle). Lipids were extracted, separated by TLC and the radioactivity quantified by phosphoimaging. The results for the total PIP pool (A) and $PI(4,5)P_2$ (B) are expressed as fold-increase with respect to non-treated cells at T=0. *C,* Changes in $PI(4,5)P_2$ (square) and $PI(3,5)P_2$ (circle) in response to 300 mM NaCl as determined by HPLC. Data represents the averages from two independent experiments (± SE).

HPLC analyses were also used to determine which isomers contributed to the observed increase in [^{32}P]PIP. A typical HPLC profile of a total extract of cells stimulated with 150 mM NaCl is shown in Figure 4A. Individual peaks were identified using ^{3}H- or ^{32}P-labelled standards. The original HPLC gradient was able to separate GroPIns3P from GroPIns4P and GroPIns5P (Figure 4A), but the latter two were separated by only 20 s. The HPLC gradient was therefore modified (see *Experimental Procedures*) such that [^{3}H]GroPIns4P and [^{32}P]GroPIns5P were separated by 1.5 min, peak-to-peak (Figure 4B).

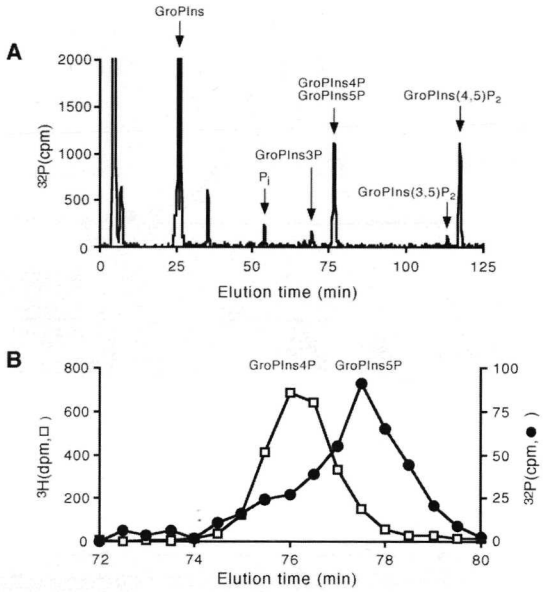


Fig. 4. Anion-exchange HPLC of deacylated phospholipids. A, Cells were radiolabelled for 1 h, treated for 2.5 min with 150 mM NaCl and the lipids extracted and deacylated. The water-soluble products were separated by HPLC. B, [^{3}H]GroPIns4P and [^{32}P]GroPIns5P standards were separated by HPLC and collected as 30 s-fractions. Radioactivity was determined by scintillation counting.

Initial HPLC separations of deacylated ^{32}P-labelled *Chlamydomonas* lipids indicated that the amount of [^{32}P]PI4P was dominantly high, masking the presence of [^{32}P]PI5P that appeared as a shoulder on the PI4P peak. Therefore, to distinguish between peak and shoulder, [^{3}H]GroPIns4P was included in all further runs to delineate the [^{32}P]PI4P peak.

Cells were stimulated with 150 or 300 mM NaCl in time-course experiments, their lipids extracted and deacylated and a [^{3}H]GroPIns4P standard was then added prior to HPLC analysis. All HPLC runs were first monitored on-line without scintillant to define the ^{32}P-profiles. Fractions containing the GroPIns4P/GroPIns5P 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 samples (Figure 5B). When the ^{3}H- and ^{32}P-profiles are compared, the ^{32}P-shoulder in the stimulated sample is clear (Figure 5B; indicated by arrow). The counts present in [^{32}P]GroPIns4P were calculated by using the [^{3}H]GroPIns4P profile. By subtracting those counts from the total ^{32}P counts, a single peak emerged that eluted at the exact position of [^{32}P]GroPIns5P (Figure 4B). The [^{32}P]PI5P peaks from both Figure 5A and B are presented in Figure 5C, showing that NaCl treatment clearly increased the level of radiolabelled PI5P.

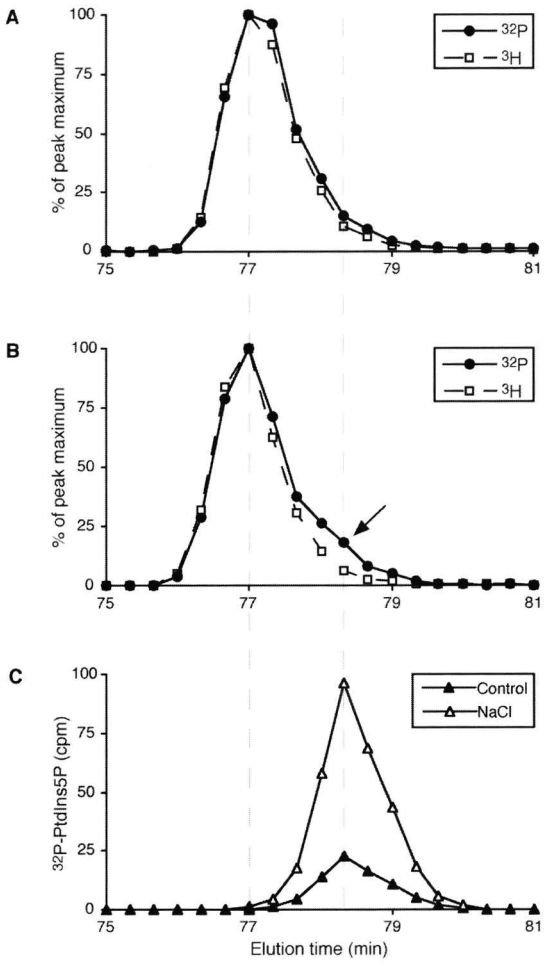


Fig. 5. PI5P is formed upon hyperosmotic stress. ^{32}P-prelabelled *Chlamydomonas* cells were treated with or without 300 mM NaCl for 15 min. Lipids were isolated, deacylated and separated by HPLC after mixing with a deacylated [^{3}H]PI4P standard. Fractions between 75 and 81 min were collected every 20 s, and their radioactivity measured. The results are shown for ^{32}P (solid line, circles) and ^{3}H (dotted line, squares) 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 (beta-counter) background counts. The position of the [^{32}P]GroPIns5P shoulder is indicated by an arrow. C, Based on the co-migration of deacylated ^{3}H- and ^{32}P-labelled PI4P, the relative amounts of [^{32}P]PI5P were determined. Data from control (closed triangle) and NaCl-stimulated cells (open triangle) are presented. The Rfs for the maximum levels of GroPIns4P and GroPIns5P are indicated by two vertical lines.

To follow the formation of all PIP isomers, time-course experiments with 150 and 300 mM NaCl were performed. As shown in Fig 6A, 150 mM NaCl induced a transient 2.5-fold increase in PI5P and a more sustained 4.5-fold increase when 300 mM NaCl was used. Similar results were obtained when the type-II PI5P 4-kinase assay was used instead

of HPLC (data not shown). Treatment affected all the PIP isomers. For example, a 6-fold increase in [^{32}P]PI3P was found after treating cells for 5 min with 300 mM NaCl (Figure 6B). [^{32}P]PI4P also increased in concentration, although the effect was only 1.6-fold (Figure 6C). Analyzing the composition of the PIP pool in non-stimulated cells via HPLC again emphasized that PI4P is the dominant isomer in *Chlamydomonas*. Using this technique PI5P was estimated to be only about 3% of the PIP pool.

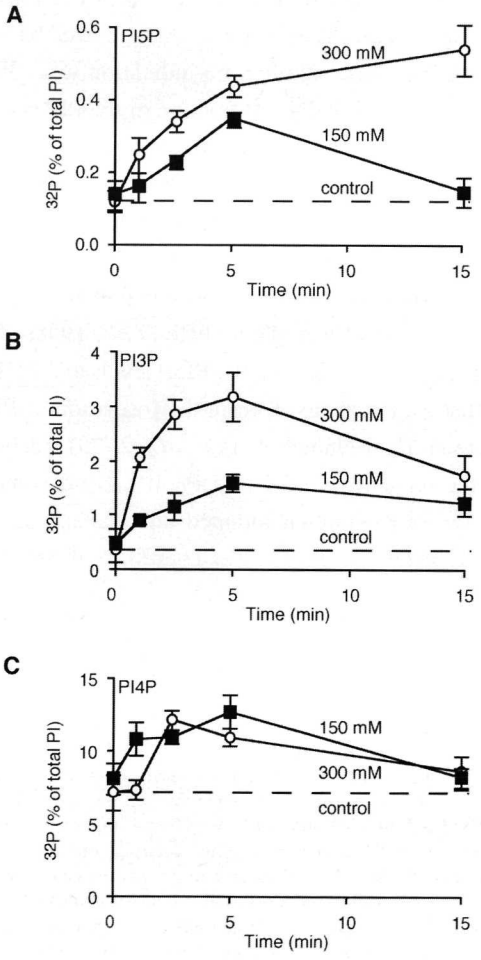


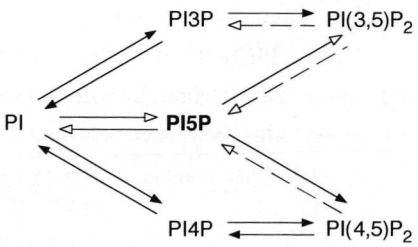
Fig. 6. Time-dependent synthesis of PIP isomers during hyperosmotic stress. Cells were labelled for 1 h and treated with 150 mM (closed squares) or 300 mM (open circles) NaCl for the times indicated. The lipids were then extracted, deacylated and separated by HPLC. The radioactivity in A, PI5P, B, PI3P, and C, PI4P was determined as described (see text). The data represent the means of four individual samples from two independent duplicate experiments (± SE) 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.

Discussion

We have shown here that PI5P is present in plants. This isomer was previously unrecognized because it co-migrates with PI4P in all TLC systems and the two can only be distinguished enzymically or via extensive HPLC analysis (Hughes *et al.*, 2000; Itoh *et al.*, 1998; McEwen *et al.*, 1999; Rameh *et al.*, 1997; Sbrissa *et al.*, 1999; Whiteford *et al.*, 1997), this report]. In *Chlamydomonas*, PI5P is estimated to represent between 3% and 8% of the total PIP pool, whereas for vetch and tomato the percentage is higher, 18%. In mammalian fibroblasts, PI5P was estimated to be 2% of the PI4P pool (Rameh *et al.*, 1997). More recently, Morris *et al.* (Morris *et al.*, 2000) showed by enzymatic analysis that PI5P is present in resting platelets, although no indication was given of its level. These data indicate that PI5P is a minor, but distinct fraction of the PIP pool in eukaryotic cells.

How PI5P is synthesized is not known. A diagram representing the possible routes is given in Scheme 1. The simplest route involves PI being phosphorylated by PI 5-OH kinase. Although PIP-kinases are capable of producing this lipid from PI *in vitro*, there is no evidence that this happens *in vivo* (McEwen *et al.*, 1999; Sbrissa *et al.*, 1999; Tolias *et al.*, 1998). A second possibility is that $PI(4,5)P_2$ is 4-dephosphorylated. However, no such phosphatase has so far been described (Hinchliffe *et al.*, 1998a; Majerus *et al.*, 1999). During thrombin treatment of platelets, both the PI5P levels and PI5P 4-OH kinase activity increased, suggesting that PI5P was involved in the formation of $PI(4,5)P_2$ rather than its degradation (Hinchliffe *et al.*, 1998b; Morris *et al.*, 2000). In our experiments, when mastoparan activated the breakdown of $PI(4,5)P_2$, it had no significant effect on PI5P levels. Similarly, all non-salt osmotica induced an increase in radiolabelled PIP and $PI(3,5)P_2$ levels without affecting $PI(4,5)P_2$. Therefore it seems unlikely that PI5P originates from $PI(4,5)P_2$. However, PI5P could be formed by dephosphorylation of $PI(3,5)P_2$. This novel PIP_2 isomer is synthesized by phosphorylation of PI3P, but its further metabolic fate is unknown (Dove *et al.*, 1997; Hinchliffe *et al.*, 1998a; Meijer *et al.*, 1999; Whiteford *et al.*, 1997). Whereas there is much that we do not know, we do know that PI5P can be phosphorylated to produce $PI(4,5)P_2$ or $PI(3,5)P_2$ (Fruman *et al.*, 1998; Hinchliffe *et al.*, 1998a; Rameh *et al.*, 1997), but further investigation is required to establish the actual pathways involved in PI5P metabolism.

To investigate the function of PI5P, two conditions known to influence PIP levels were studied. First, PLC was activated by treating *Chlamydomonas* cells with mastoparan, because it dramatically decreased $PI(4,5)P_2$ and PIP levels within the first 30 s to produce $Ins(1,4,5)P_3$ (Munnik *et al.*, 1998b; Van Himbergen *et al.*, 1999). In contrast to PI4P, which was reduced by two thirds, the amount of PI5P was not affected. Since the hydrolysis of $PI(4,5)P_2$ by PLC is immediately followed by resynthesis (Munnik *et al.*, 1998b), this result clearly indicates that PI4P but not PI5P, is used as precursor. Thus PI5P does not seem to have a role in PLC signalling, at least not when activated by mastoparan.



Scheme 1. Putative pathways on the metabolism of PI5P. 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 arrows. Those without any conformation *in vitro* or *in vivo* are indicated by dashed arrows.

Second, *Chlamydomonas* cells were subjected to hyperosmotic stress, based on reports that such treatment affects phospholipid levels in plant cells (Cho *et al.*, 1993; Drøbak and Watkins, 2000; Einspahr *et al.*, 1988; Meijer *et al.*, 2001; Meijer *et al.*, 1999; Munnik *et al.*, 2000; Pical *et al.*, 1999), reviewed in Munnik and Meijer (Munnik and Meijer, 2001). We found that the radiolabelled PIP pool increased, and HPLC analysis established that PI5P contributed to that increase. The rapid increase in PI5P suggests that it plays a role in signalling, either as a signal itself, a signal precursor or as an attenuation product of $PI(3,5)P_2$ metabolism. In analogy with proteins binding specifically to PI3P via their FYVE domains (Corvera *et al.*, 1999; Leevers *et al.*, 1999), PI5P might also function as a ligand for certain FYVE-domains and thereby be involved in the membrane targeting of proteins.

HPLC analyses showed that PI3P and PI4P, as well as PIP_2 levels increased in response to osmotic stress. The increase in $PI(3,5)P_2$ formation (Figure 3C) was maintained for 10 min and contrasted with the transient increase in the $PI(4,5)P_2$, that was restricted to the first 2 min of treatment. This difference in kinetics in itself suggests that these PIP_2 isomers have different functions. Furthermore, whereas $PI(3,5)P_2$ synthesis was stimulated by all osmolytes tested, $PI(4,5)P_2$ only accumulated when cells were stimulated with salts, but not when they were treated with mannitol. This indicates that $PI(4,5)P_2$ accumulates as a result of ionic rather than osmotic stress and therefore that *Chlamydomonas* cells are capable of discriminating between such stresses (Munnik and Meijer, 2001). The combined increase in both PIP_2 isomers has previously been reported for yeast cells treated with 0.9 M NaCl (Dove *et al.*, 1997). This resulted in a more than ten-fold increase in $PI(3,5)P_2$ and a 1.7-fold increase in $PI(4,5)P_2$. In contrast, when *A. thaliana* was subjected to hyperosmotic stress, a dramatic increase in $PI(4,5)P_2$ was found independent of the osmotic agent used (Pical *et al.*, 1999). They did not report changes in $PI(3,5)P_2$ or $PI(3,4)P_2$. During our own HPLC analyses of *Chlamydomonas*, no $PI(3,4)P_2$ was detected. This

implies that the $PI(3,4)P_2$ previously thought to be in *Chlamydomonas* (Irvine *et al.*, 1992; Munnik *et al.*, 1994a) is $PI(3,5)P_2$. $PI(3,4)P_2$ has also been reported in other plants (Brearley and Hanke, 1992; 1993; Parmar and Brearley, 1995), however, the recent identification of $PI(3,5)P_2$, together with the knowledge that these two isomers behave similarly under many separation conditions, makes it necessary to reassess their identities.

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