Rapid turnover of polyphosphoinositides in carnation flower petals

Munnik, T.; Musgrave, A.; de Vrije, T.

DOI
10.1007/BF00191611

Publication date
1994

Published in
Planta

Citation for published version (APA):

General rights
It is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), other than for strictly personal, individual use, unless the work is under an open content license (like Creative Commons).

Disclaimer/Complaints regulations
If you believe that digital publication of certain material infringes any of your rights or (privacy) interests, please let the Library know, stating your reasons. In case of a legitimate complaint, the Library will make the material inaccessible and/or remove it from the website. Please Ask the Library: https://uba.uva.nl/en/contact, or a letter to: Library of the University of Amsterdam, Secretariat, Singel 425, 1012 WP Amsterdam, The Netherlands. You will be contacted as soon as possible.
Rapid turnover of polyphosphoinositides in carnation flower petals

Teun Munnik*, Alan Musgrave1, Truus de Vrije1

1 Agrotechnological Research Institute (ATO-DLO) P.O. Box 17, 6700 AA, Wageningen, The Netherlands
2 Department of Molecular Cell Biology, University of Amsterdam, Kruislaan 318, 1098 SM, Amsterdam, The Netherlands

Received: 29 April 1993 / Accepted: 11 August 1993

Abstract. Carnation (Dianthus caryophyllus L. cv. White Sim) petal discs were radiolabelled with [32P]orthophosphate and the lipids were extracted and analysed by thin-layer chromatography and autoradiography. Phospholipids were identified by co-migration with standards using thin-layer chromatography with different solvent systems. Results showed that [32P]orthophosphate was rapidly incorporated into the minor lipids phosphatidic acid (PtdOH), phosphatidylinositol monophosphate (PtdInsP), and phosphatidylinositol bisphosphate (PtdInsP2), and relatively slowly into the structural lipids phosphatidylcholine, -ethanolamine, -glycerol and -inositol. Pulse-chase experiments revealed that the label was rapidly lost from PtdOH, PtdInsP and PtdInsP2 while the structural lipids remained radiolabelled. The amount of PtdInsP and PtdInsP2 was found to constitute 0.45% and 0.013%, respectively, of the total phospholipids, on a molar basis. Together these results show that the turnover of the chemically low-abundant polyphosphoinositides is relatively high compared with the major structural phospholipids. Phosphatidylinositol monophosphate was further characterized by showing that it incorporates myo-[3H]inositol and that its major fatty-acid constituents are palmitic acid and linoleic acid. Furthermore, we present evidence for the presence of both phosphatidylinositol 3-phosphate and phosphatidylinositol 4-phosphate isomers. The significance of these results is discussed with respect to plant phosphoinositide signal transduction.

Key words: Dianthus – Phosphatidylinositol 3-phosphate – Phospholipid turnover – Polyphosphoinositides – Signal transduction

Introduction

Calcium is recognized as being involved in numerous events during growth and development of plants and, more recently, evidence is increasing for its role as a second messenger in signal-response coupling (reviewed by Hepler and Wayne 1985; Kauss 1987; Poovaiah and Reddy 1987; Trewavas and Gilroy 1991).

The involvement of polyphosphoinositides in Ca2+ mobilization is well established in animal cells. These lipids are generated from phosphatidylinositol (PtdIns) by two consecutive phosphorylations resulting in the formation of phosphatidylinositol 4-phosphate (PtdIns(4)P) and phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P2). In response to an agonist, a phosphoinositide-specific phospholipase C is activated and hydrolyses PtdIns(4,5)P2 to yield the second messengers inositol 1,4,5-trisphosphate (Ins(1,4,5)P3) and diacylglycerol (DAG). Insitol-1,4,5-trisphosphate release calcium ions from intracellular stores while DAG, in concert with Ca2+, activates protein-kinase C. Together they form a ubiquitous transduction mechanism controlling a host of processes in the cell (Nishizuka 1988; Berridge and Irvine 1989; Berridge 1993).

Many of the basic components of the InsP3/Ca2+/ DAG second-messenger system outlined above have been shown to be present in plant systems, and a growing body of evidence suggests that responses to environmental factors (e.g. light, osmotic stress) and to several phytohormones could be mediated by these messengers (reviewed by Lehle 1990; Einspahr and Thompson 1990; Trewavas and Gilroy 1991).

Polyphosphoinositides have been identified in several plants, in various tissues (Sandelius and Sommarin 1990),
and have been demonstrated to be identical to those found in animals, namely PtdIns(4)P and PtdIns(4,5)P₂ (Côté et al. 1989; Irvine et al. 1989). In addition, several enzymes involved in their metabolism, such as PtdIns kinase, PtdInsP kinase, DAG kinase, phospholipase C, and PtdInsP monoesterase have been reported (Sandelius and Sommarin 1986; Heim and Wagner 1987; Pfaffmann et al. 1987; McMurray and Irvine 1988; Sommarin and Sandelius 1988; Einspahr et al. 1989; Tate et al. 1989; Sandelius and Sommarin 1990; Kamada and Muto 1991; Lundberg and Sommarin 1992; Wissing et al. 1989). While the basic elements of phosphoinositide metabolism have been shown to be present, relatively little is known about its turnover. Preferential incorporation of [³²P]Pi into PtdInsP in tomato suspension-cultures (Drobot et al. 1988), and into PtdInsP and PtdInsPs in brinjal leaf discs (Wagh et al. 1988), have indicated that their turnover might be higher than that of structural lipids.

In animals, the discovery of D3-phosphorylated phosphoinositides and their subsequent synthesis upon stimulation, have indicated the presence of another signalling pathway, even though its mode of action is not yet known (see Downes and Carter 1991; Irvine 1992; Panayotou and Waterfield 1992). Phosphatidylinositol 3-phosphate, the most abundant member of these lipids, but still only comprising 2-5% of the PtdInsP pool (Downes and Carter 1991), has also been found in yeast (Auger et al. 1989), Chlamydomonas (Irvine et al. 1992), and the higher plant Spirodela (Brearley and Hanke 1992).

We are exploring the InsP₃/Ca²⁺/DAG second-messenger system in carnation flower petals. Our first step was to identify the lipid precursors PtdInsP and PtdInsPs and to investigate whether their turnover characteristics are typical of signal precursors. For animals and the green algae Chlamydomonas and Dunaliella, turnover studies have shown that polyphosphoinositides are synthesized and broken down much faster than structural lipids (Müller et al. 1986; Palmer et al. 1986; Einspahr et al. 1988; Brederoo et al. 1991). Here, evidence for the rapid turnover of polyphosphoinositides in a higher-plant system is presented for the first time. This is demonstrated as the results from ³²P-incorporation and pulse-chase experiments. The lipid PtdInsP was further characterized by showing that it incorporates myo-[³H]inositol, by determining its fatty-acid composition and by demonstrating that it is present as two isomers, namely PtdIns(4)P and PtdIns(3)P. The presence of the latter is the second indication that another polyphosphoinositide signalling system is present in higher plants.

Materials and methods

Materials. Myo-[²H]inositol and [³²P]orthophosphate (³²Pi; carrier free) were obtained from Amersham (Buckinghamshire, UK). Organic solvents, TLC silica 60 plates and kieselgel 60 (63-200 μm) were from Merck (Darmstadt, Germany). Phospholipid standards (except lysoPtdInsP), phospholipase A₁ (Crotalus durissus terrificus Venom) and the rest of the reagents were from Sigma (St. Louis, Mo., USA). Lyso-PtdInsP was synthesized from PtdIns(4)P (bovine brain) according to the following modification of the method of Kates (1988). A dry film of PtdInsP (0.2 mg) was dispersed in 200 μl of 100 mM Tris-HCl (pH 8.9) in a bath sonicator (5 min). To this suspension 0.2 mg phospholipase A₁ in 100 μl of 60 mM CaCl₂ in the same Tris buffer was added. The reaction mixture was incubated at 30°C for 4 h after which phospholipase activity was blocked by 33 mM EDTA. Lipids were then extracted according to Bligh and Dyer (1959) in the presence of 0.5% HCl (v/v).

Plant material. Carnation flowers (Dianthus caryophyllus L. cv. White Sim) were obtained from commercial growers. They were cut in the developmental stage in which they are normally supplied to flower auctions and then transported dry to the laboratory. Stems were re-cut to 20 cm and placed in deionized water for 16 to 20 h under controlled environmental conditions (Woltering et al. 1993). Just before use, discs (0.8 cm diameter, approx. 10 mg FW) were cut from the basal region of the petals. From each flower only the petals of the outer whorl were used.

Labelling with [³²P]Pi and myo-[³H]inositol. Petal discs were labelled for different periods of time in 25 mM 2-(N-morpholino)ethanesulfonic acid-KOH (Mes-KOH) buffer pH 6.0 with [³²P]Pi (carrier free) or myo-[²H]inositol. Per disc, 3.7 MBq [³²P]Pi or 185 kBq myo-[³H]inositol in 50 μl buffer was used. Discs were vacuum-infiltrated, resulting in the very rapid uptake of label into the petal tissue without physiological damage. Labelling started after releasing the vacuum, which was applied for 5 min. At the times indicated, labelling was stopped and the lipids extracted.

Pulse-chase experiments. For pulse-chase experiments, discs were labelled with [³²P]Pi for 3 h as described above. Petal discs were then gently blotted on tissue paper to remove as much label as possible and subsequently incubated in an excess (25 ml) of 25 mM Mes-KOH buffer (pH 6.0), containing 10 mM K₂PO₄. At subsequent times, labelling was stopped and the lipids extracted.

Lipid extraction. Labelling was stopped by adding each petal disc to 600 μl CHCl₃/CH₃OH/HCl (100:100:1, by vol.) and the lipids were directly extracted while rapidly freezing the tissue in liquid nitrogen and thawing. Subsequently discs were spun down in a micro centrifuge (10 000 g) for 2 min and the extracts collected. Discs were re-extracted with 450 μl CHCl₃/CH₃OH/HCl (100:100:1, by vol.) as described above. Lipid fractions were pooled and a two-phase system was induced by the addition of 500 μl CHCl₃ and 375 μl 0.9% NaCl. After mixing vigorously, the two-phase system was centrifuged for 2 min in a micro centrifuge and the upper-phase discarded. The organic lower phase was washed three times with 375 μl CH₃OH/H₂O/HCl (50:50:1, by vol.) and subsequently labelled was stopped and the lipids extracted.

Lipid analyses. Lipids were dissolved in CHCl₃/CH₃OH (3:1, v/v) and separated on potassium-oxide-impregnated silica 60 TLC plates (0.25 x 20 x 20 cm). The plates were first heat-activated at 115°C for at least 20 min, cooled to room temperature and, after sample application, equilibrated for 1 h above the TLC solvent. Chromatograms were developed with the alkaline solvent CHCl₃/CH₃OH/25%NH₄OH/H₂O (90:70:5:5:15, by vol.). Alternatively, an acidic TLC solvent was used: CHCl₃/CH₃COCH₃/CH₃OH/CH₃COOH/H₂O (40:15:14:3:7.5, by vol.). For two-dimensional separations, plates were first developed in the alkaline solvent, dried carefully, and subsequently chromatographed in the acidic solvent.

For the analysis of PtdIns(3)P- and PtdIns(4)P-isomers, PtdInsP spots were scraped from alkaline-developed chromatograms and eluted by extracting the lipids twice with CHCl₃/CH₃OH/HCl (100:100:1, by vol.). The extracts were combined, dried under a stream of nitrogen and stored at -20°C until further use.

Usually this was within one week.

Lipid analyses. Lipids were dissolved in CHCl₃/CH₃OH (3:1, v/v) and separated on potassium-oxide-impregnated silica 60 TLC plates (0.25 x 20 x 20 cm). The plates were first heat-activated at 115°C for at least 20 min, cooled to room temperature and, after sample application, equilibrated for 1 h above the TLC solvent. Chromatograms were developed with the alkaline solvent CHCl₃/CH₃OH/25%NH₄OH/H₂O (90:70:5:5:15, by vol.). Alternatively, an acidic TLC solvent was used: CHCl₃/CH₃COCH₃/CH₃OH/CH₃COOH/H₂O (40:15:14:3:7.5, by vol.). For two-dimensional separations, plates were first developed in the alkaline solvent, dried carefully, and subsequently chromatographed in the acidic solvent.

For the analysis of PtdIns(3)P- and PtdIns(4)P-isomers, PtdInsP spots were scraped from alkaline-developed chromatograms and eluted by extracting the lipids twice with CHCl₃/CH₃OH/HCl (100:100:1, by vol.). The extracts were combined, dried under a stream of nitrogen and separated on a borate TLC system as described by Walsh et al. (1991). Alternatively, PtdInsP-isomers were analysed by anion-exchange HPLC as described by Payrastre et al. (1992). Briefly, alkaline-TLC-separated PtdInsP was deacylated and the resulting glycerophosphoinositolphosphates (GROInsPs) sepa-
rated on a Partisphere SAX column (100 mm long, 4.6 mm i.d.; Whatman International, Maidstone, UK). Radioactivity eluting from the column was monitored and quantified by a Bertold LB506C detector (München, Germany), using the Cherenkov effect for $^{32}$P. $^{32}$P-labelled PtdIns(3)P and PtdIns(4)P isolated from platelets were used as a standard.

Lipids separated by TLC were detected using iodine vapour and-or phosphate stain (Kates 1988). Their identity was established by co-chromatography with standards. Radioactive lipid spots were detected by autoradiography (X-Omat S; Kodak, Rochester, N.Y., USA; O/N exposure), then scraped off and quantitated by liquid scintillation counting. Alternatively, $^{32}$P-incorporation was measured by scanning the autoradiogram with an Ultrosan XL (LKB Products, Bromma, Sweden) and expressed as arbitrary units (AU) per mm.

Fatty-acid analyses of PtdInsP. Phospholipids were isolated by Bligh and Dyer (1959) extraction in the presence of 0.5% HCl (v/v) from 50 g of outer petals, of which the basal green part was discarded, and purified by silicic-acid column chromatography. After first eluting neutral lipids and other contaminants in 100% CHCl$_3$ (10 column vol.) and secondly eluting the glycolipids in 100% CH$_3$COCH$_3$ (30 column vol.), phospholipids were eluted with 6 column volumes of CHCl$_3$/CH$_3$OH/25%NH$_4$OH/H$_2$O (90:70:5.5:15, by vol.). Fractions enriched in PtdInsP were combined and the constituent lipids separated by TLC using the same alkaline solvent. The PtdInsP band was scraped from the chromatogram and partly used for fatty-acid methylation. Some of the silica-lipid was re-extracted and run on TLC. Only one spot, which co-migrated with a PtdInsP standard, was detected after charring with sulphuric acid. Analysis of the fatty-acid methyl esters by gas chromatography (GC) and GC-mass spectrometry (GC-MS) was performed essentially as described by Huijberts et al. (1992).

Yeast lipids. Saccharomyces cerevisiae (CBS1782; CBS, Baarn, The Netherlands) was grown overnight at 25°C in vigorously aerated medium (2.5 g per 1 yeast extract, 5.0 g per 1 tryptone (Oxoid, Uni-path, Basingstoke, UK) and 0.5% glucose). The cell suspension (4 ml) was washed twice with 10 mM Mes-KOH (pH 5.0) and resuspended in the same buffer with $^{32}$P$_{2}$Pi (7.4 MBq per 500 µl suspension). Labelling was stopped by adding perchloric acid to a final concentration of 5%. The lipids were extracted from the cell pellet (5 min, 10 000 g) as described above.

Results

Separation and identification of phospholipids. Figure 1A, B shows the patterns of $^{32}$P-labelled phospholipids from carnation petals after separation by one-dimensional TLC using an alkaline or acidic solvent. Identification of these lipids was established by mixing the $^{32}$P$_{2}$Pi-labelled lipid extract with commercial lipid standards prior to TLC. In the presence of an excess of standard, the appropriate radioactive spot was distorted (Fig. 1A, B, lanes E, F) while the behaviour of other spots was unaffected. In this way all the major spots were identified. Most of the $^{32}$P was incorporated into the structural lipids phosphatidylcholine (PtdCho), phosphatidylethanolamine

Fig. 1A–C. Separation and identification of phospholipids from carnation flower petals. Petal discs were radiolabeled with $^{32}$P$_{2}$Pi for 3 h and the lipids were extracted, separated by different TLC systems and detected by autoradiography. Phospholipids were identified by adding an excess lipid standard to the radioactive petal extract using three different TLC systems. A Alkaline TLC; B acidic TLC. Additions: lane D, -; lane E, excess PtdIns(4,5)P$_2$; lane F, excess PtdIns(4)P. C Two-dimensional TLC: I, alkaline TLC solvent; II, acidic TLC solvent. Identity of spots: 1, PtdGro; 2, PtdEttn; 3, PtdOH; 4, PtdCho; 5, PtdIns; 6, PtdInsP; 7, PtdInsP$_2$; 8, lyso-Ptd-Gro; 9, lyso-PtdEttn; 10, lyso-PtdCho; 11, lyso-PtdIns; 12, lyso-Ptd-OH; 13, lyso-PtdInsP; 14, origin; other spots unknown
the minor spots remains unknown but they could be between PtdInsP and PtdInsP₂. The identity of some of the alkaline TLC system, while lyso-PtdInsP migrated were identified. They all migrated faster than PtdInsP in dimension and the acidic solvent in the second (Fig. 1C). The lysoforms of the structural lipids and lyso-PtdOH were established by two-dimensional chromatograms and the identification of structural lipids PtdCho (26%), PtdEtn (19%), PtdGro (17%) and PtdIns (16%). These percentages are not the same as the relative masses of the phospholipids for they are the lipid precursors of InsP₃ and DAG, and on PtdOH because it might be the product of DAG-kinase activity and as such can give a measure of the rate of formation of these signal molecules. Carnation petal discs were incubated with [³²P]Pi and vacuum-infiltrated for 5 min. Thereafter, lipids were extracted at regular intervals and separated on TLC. The results are presented in Fig. 2. Incorporation of [³²P] into the lipid extract increased during the first 24 h (Fig. 2A) and continued up to at least 53 h (data not shown), reflecting [³²P]-incorporation into the structural lipids (Fig. 2B). The rates of incorporation into PtdIns and PtdGro, and into PtdCho and PtdEtn were similar, indicative of their shared biosynthetic pathways. In contrast, incorporation into the minor lipids PtdInsP and PtdInsP₂ usually reached a maximum within 6 h, and then declined. Phosphatidic acid showed a labelling pattern similar to that of the structural lipids, though at a lower level (Fig. 2C).

After 24 h most of the [³²P] was incorporated into the structural lipids PtdCho (26%), PtdEtn (19%), PtdGro (17%) and PtdIns (16%). These percentages are not the same as the relative masses of the phospholipids for they are the product of the very rapidly labelled polyphosphoinositides. The latter could not be readily detected by colorimetric means because of their small quantities, but after 24 h labelling, PtdInsP accounted for 10% of the total incorporated [³²P]. Similarly, PtdOH also incorporated a disproportionately large amount of label (12%).

The preferential incorporation of [³²P] into the minor lipids was even more apparent when labelling within the first hour was studied (Figs. 3, 4A). Clearly PtdInsP and PtdOH incorporated more label than the major structural lipids, and while PtdInsP₂ only incorporated as much as PtdEtn, this was far in excess of its relative mass. Of the structural lipids PtdIns was the first to be labelled, much as PtdEtn, this was far in excess of its relative mass. The preferential incorporation of [³²P] into the minor lipids was even more apparent when labelling within the first hour was studied (Figs. 3, 4A). Clearly PtdInsP and PtdOH incorporated more label than the major structural lipids, and while PtdInsP₂ only incorporated as much as PtdEtn, this was far in excess of its relative mass. Of the structural lipids PtdIns was the first to be labelled, while it is the least abundant (Brown et al. 1991).

If the rapid [³²P]-incorporation into PtdInsP, PtdInsP₂ and PtdOH was caused by their rapid turnover, it should be possible to chase the label out by incubating the petal discs with excess non-labelled phosphate after a relatively short [³²P]Pi pulse. Accordingly, discs were labelled for 3 h with [³²P]Pi and then chased for 20 h with 10 mM PO₄³⁻. The results are presented in Figs. 4B and 5. Within 6 h, most of the label was lost from the minor lipids PtdOH (80%), PtdInsP (87%) and PtdInsP₂ (70%) while hardly any label was lost from each of the major structural lipids.
Fig. 3. Time course of $^{32}$P-incorporation into phospholipids of carnation flower petals. Petal discs were incubated with $[^{32}P]Pi$ by vacuum-infiltration. After 5 min the vacuum was released and labelling time was started. At the times indicated, lipids were extracted, separated by TLC, and the radioactivity per spot determined by densitometry. Inset: $^{32}$P-incorporation into PtdInsP$_2$. These data are representative of those from three independent experiments.

Myo-$[^{3}H]$inositol labelling. Carnation petal discs were incubated with myo-$[^{3}H]$inositol and their lipids extracted at regular intervals. A $^{32}$P-labelled petal extract was...
mixed with the extract in order to identify the positions of the individual phospholipids. After TLC and autoradiography, the silica was scraped off the plate in 5-mm sections and each was measured for its $^3$H-content by liquid scintillation counting. Figure 6A, B shows the autoradiogram of the $^{32}$P-labelled marker phospholipids (Fig. 6B) together with the distribution of $^3$H throughout the chromatogram (Fig. 6A). The extract in this figure was from petal discs labelled for 23 h. Most of the label was incorporated into PtdIns and PtdInsP with a minor fraction in lyso-PtdIns and lyso-PtdInsP. It was not possible to detect $^3$H in the PtdInsP$_2$ spot illustrated here, but in some samples the $^3$H-counts were just above background. Three components incorporated $^3$H but did not contain $^{32}$P. One of them migrated near PtdInsP$_2$ and lyso-PtdInsP and might represent the polar phytolipid reported previously (reviewed by Hetherington and Drobak 1992).

The incorporation of $^3$H into the lipid extract, PtdIns and PtdInsP was linear for 4 h but then the incorporation into the named lipids leveled off (Fig. 6C). Within the first hours, more than 99% of the label was recovered in PtdIns and PtdInsP (Fig. 6A), only later was $^3$H incorporated into the unidentified compounds. This indicates that the latter have a lower turnover, or that metabolic products of $[^3H]$inositol were first formed and then incorporated into these lipids.

**Characterization of PtdInsP.** The fatty-acid composition of polyphosphoinositides in animals is characterized by the presence of arachidonic acid at the C2 position. Also in the green alga *Chlamydomonas*, a long-chain polyunsaturated fatty acid (C22:4) seems characteristic of these lipids (Brederoo et al. 1991). In carnation flowers the major fatty acid of PtdInsP were palmitic (16:0) and linoleic (18:2) acid (95%) which are also the common fatty acids of the structural phospholipids (Brown et al. 1991). This is consistent with a previous report of the composition of polyphosphoinositides in carrot cells (Van Breemen et al. 1990). No fatty acids of C20 or longer were found in the lipid extract (data not shown).

In animal systems the idea of the existence of a novel polyphosphoinositide signalling pathway involving phosphorylation of inositol at the D3-position is gaining interest. In plants there is also evidence for its existence, judging from reports of a PtdIns(3)P isomer (Brearley and Hanke 1992; Irvine et al. 1992). Consequently, we tested whether PtdIns(3)P was part of the PtdInsP pool in carnation petals. As a means of identification, two TLC systems were used; an alkaline system in which the PtdIns(3)P isomer migrates just below PtdInsP$_4$ (Whitman et al. 1988; Stephens et al. 1991), and a recently described borate system, in which the migration order is reversed (Walsh et al. 1991). As markers for the two PtdIns$_P$ iso-
mers, we used a 32P-labelled lipid extract from the yeast *Saccharomyces cerevisiae* which was reported to contain PtdIns(3)P and PtdIns(4)P in a 1:1 ratio (Auger et al. 1989). Accordingly, a 32P-labelled petal extract was separated by alkaline TLC (Fig. 7A), the lower part of the PtdlnsP spot was scraped off and, after elution, the lipids were subjected to borate TLC (Fig. 7B). Two spots were found, the upper representing putative PtdIns(3)P and the lower PtdIns(4)P (Fig. 7B). The Rf values for the PtdlnsP isomers were slightly lower than reported by Walsh et al. (1991), 0.45 and 0.38 for PtdIns(3)P and PtdIns(4)P, respectively. However, we always found that a lyso-PtdCho standard migrated between the PtdlnsP isomers, as reported by Walsh et al. (1991). Phosphatidylinositol 3-phosphate was also labelled by [3H]inositol, though at much lower levels than the PtdIns(4)P isomer. The 3H : 32P-ratios were 1:10 and 1:4 for PtdIns(3)P and PtdIns(4)P, respectively, after 23 h of labelling. These results suggest that the parent lipids differ from each other in the pattern of synthesis or metabolic turnover, as has been found for human neutrophils (Stephens et al. 1991).

To support the putative identification of PtdIns(3)P, the upper and lower halves of the 32P-labelled PtdInsP spot were again scraped from an alkaline-TLC plate, but now the lipids were deacylated and the resulting GroPInsPs analysed by an anion-exchange HPLC system that is capable of resolving both isomers (Payrastre et al. 1992). The results are presented in Fig. 8. In both upper and lower samples, the major radioactive component was identified as GroPIns(4)P but in the lower sample, a minor peak eluted exactly at the position of a GroPIns(3)P standard. On average 2.2 ± 0.32% (n = 3) of the total GroPtdlnsP pool was present as GroPtdlnsP3 after 23 h of 32P-labelling. The fact that this product was only found in the lower half of the alkaline-TLC-separated PtdInsP spot substantiates the claims in the literature (Whitman et al. 1988; Stephens et al. 1991; Walsh et al. 1991) and our experience reported above (Fig. 7A, B) that Ptdlns(3)P can be identified by its TLC characteristics. The two minor peaks in both HPLC chromatograms (Fig. 8A, B) eluting at approx. 12 and 20 min are degradation products that probably arose during isolation and deacylation of the PtdInsP spots. They have been tentatively identified as glycerophosphoinositol and Pi.

### Discussion

We have presented evidence for the lipids PtdInsP and PtdlnsP2 being in carnation flower petals. The 32P-labelled inositides co-migrated exactly with bovine PtdIns(4)P and PtdIns(4,5)P2 standards (Fig. 1) and with the well-characterized polyphosphoinositides from *Saccharomyces cerevisiae* (Auger et al. 1989) and *Chlamydomonas eugametos* (Irvine et al. 1989, 1992; Bréderoo et al. 1991) in both alkaline and acidic TLC systems, and in combination as two-dimensional TLC (data not shown). Myo-[3H]inositol was incorporated into PtdInsP but the level was very low, limiting our ability to detect its incorporation into PtdlnsP2. Much of the problem lay in the poor uptake together with metabolism of that which was taken up, and judging from our own observations (data not shown) and previous reports, this is typical of plant cells (Côté et al. 1987; Drobak et al. 1988; Côté et al. 1989; Rincón et al. 1989; Rincón and Boss 1990). The

---

**Fig. 8A, B.** HPLC head-group analysis of TLC-purified PtdInsP. Lipid extracts from 24-h [32P]-labelled carnation petal discs were separated by alkaline TLC and autoradiographed. The regions corresponding to the upper and lower halves of the PtdlnsP spot were excised, the lipid deacylated, and the products resolved by Partisphere SAX anion-exchange HPLC. Glycerophosphoinositol phosphates were identified by comparison with the elution times of deacylated 32P-labelled PtdIns(3)P and PtdIns(4)P from mammalian cells. Samples were co-injected with AMP, ADP and ATP to monitor (A260) the elution characteristics. A Upper half of the alkaline-TLC-separated PtdlnsP spot, and B the lower half.

**Fig. 9A, B.** Schematic representation of phosphoinositide turnover and generation of phosphatidic acid. A Phosphoinositide turnover. B Several routes to generate PtdOH: 1) phosphorylation of DAG by DAG kinase; 2) PtdOH as metabolic intermediate in phospholipid biosynthesis resulting from acylation of glycerol-3-phosphate to lyso-PtdOH and again to PtdOH by acetyl CoA transferase; and 3) hydrolysis of structural phospholipid by phospholipase D.
32P-labelling kinetics of PtdInsP and PtdInsP2 during incorporation and pulse-chase experiments were obviously different from those of the structural lipids and were characteristic of lipids expressing a high turnover rate. Identical findings in animal and lower plant cells suggest that this phenomenon reflects their function as signal precursors (Müller et al. 1986; Majerus et al. 1986; Palmer et al. 1986; Einspahr et al. 1988; Brederoo et al. 1991).

Analysis of PtdInsP in carnation petals showed the presence of both PtdIns(3)P and PtdIns(4)P. Since PtdIns(3)P has also been found in Chlamydomonas (Irvine et al. 1992) and in the aquatic monocotyledon Spirodella polyrhiza (Brearley and Hanke 1992) its presence in carnation suggests that this signalling system, which is still being elucidated for animal cells (Downes and Carter 1991; Irvine 1992; Panayotou and Waterfield 1992), is also common in plants. The low level of PtdInsP2 in carnation petals prevented us from analyzing this lipid for the presence of 3-isomers, but since the most prevalent isomer PtdIns(3,4)P2 constitutes less than 1% of the PtdInsP2 pool in animal and lower-plant cells (Downes and Carter 1991; Irvine et al. 1992), we presume that the PtdInsP2 found in carnation is predominantly PtdIns(4,5)P2, as has been demonstrated earlier for other plants (Côté et al. 1989; Irvine et al. 1989).

To determine the amounts of PtdInsP and PtdInsP2, quantitative radioactivity data are needed after all the phospholipids have reached isotopic equilibrium. In a multicellular tissue segment, this is difficult to achieve because gradients of the labelled precursor are formed and large turnover differences between polyphosphoinositides and structural phospholipids exist (this paper; Côté and Crain 1992). We therefore made estimates based on longer labelling times (23–53 h) in which the phosphoinositol ratios had become stable. Calculated from 32P-incorporation data, and corrected for the amount of phosphate per molecule, a molar PtdInsP: PtdInsP2 ratio of 35:1 (n = 6) was found. In other experiments using myo-[3H]inositol a constant PtdIns: PtdInsP molar ratio of 11:1 (n = 6) was detected. Combining the data, the molar ratio between PtdIns: PtdInsP: PtdInsP2 was approx. 385:35:1. Since PtdIns accounts for 5% of the total phospholipids (Brown et al. 1991), the amounts of PtdInsP and PtdInsP2, on a molar basis, are 0.45% and 0.013%, respectively, of the total phospholipids, or 8.3% and 0.23%, respectively, of the phosphoinositides. Others have reported ratios of PtdIns: PtdInsP: PtdInsP2 ranging from 300:17:1 to 10:1:1 (Hetherington and Dröbak 1992). So while the PtdIns: PtdInsP ratio is typical of plants in general, the PtdInsP2 level seems very low, but nonetheless consistent with reports on carrot, tomato, pea and Catharanthus roseus cells (Boss and Massel 1985; Heim and Wagner 1986; Dröbak et al. 1988; Irvine et al. 1989; Rincón et al. 1989). In this context, it should be noted that some authors may have overestimated their PtdInsP2 quantities due to co-migration of lyso-PtdInsP (Hetherington and Dröbak 1992) or to non-equilibrium labelling data as mentioned above (Côté and Crain 1992).

The difference in pool sizes of PtdInsP and PtdInsP2 (35:1) is in contrast to their similar turnover rate and shows that most of the PtdInsP is not immediately phosphorylated to PtdInsP2 (Fig. 9A). This is not because they exist in independent locations, for the available information suggests that both lipids and the enzymes regulating their levels are concentrated in the plasma membrane (Melin et al. 1987; Pfaffmann et al. 1987; Wheeler and Boss 1987; Einspahr et al. 1988, 1989; Sommarin and Sandelius 1988; Tate et al. 1989; Sandelius and Sommarin 1990; Brederoo et al. 1991; Kamada and Muto 1991; Melin et al. 1992; Lundberg and Sommarin 1992; Pical et al. 1992; Wissing and Wagner 1992). Consequently, most of the PtdInsP must be degraded by phosphomonoesterase and/or phosphodiesterase activity faster than it is further phosphorylated to PtdInsP2.

In animal systems, the hydrolysis of PtdInsP by phospholipase C is reflected in a rapidly 32P-labelled PtdOH pool due to subsequent phosphorylation of the resulting DAG by DAG kinase (Berridge 1992). Similar claims have been made for the green algae Chlamydomonas and Dunaliella (Einspahr et al. 1988; Brederoo et al. 1991; Ha and Thompson 1991; Musgrave et al. 1992) and the same process is presumed to occur in higher plants (Heim and Wagner 1990). In carnation, the rapid incorporation and loss of 32P-label from PtdOH resembles the labelling kinetics of PtdInsP and PtdInsP2, suggesting that the two are again causally related. However, we emphasise that this is not necessarily the case. For example, the amount of PtdInsP2 present seems too small to account for all the DAG that is phosphorylated to PtdOH. Phospholipase C hydrolysis of PtdInsP could provide another source of DAG but this reaction has not been demonstrated in vivo in plants (only in vitro, see Sandelius and Sommarin 1990) and is thought not to occur in vivo in animals (Irvine 1992). Moreover, PtdOH is an intermediate in the biosynthesis of structural phospholipids (Fig. 9B). As such, the small PtdOH pool must be turning over rapidly to be metabolised to the large pools of structural lipids, even though any one of the latter only accumulates label relatively slowly. Theoretically, phospholipase D can also generate labelled PtdOH from labelled structural lipids, but the rapid labelling of PtdOH in the absence of labelled structural lipids argues strongly against this possibility. Nonetheless, this discussion illustrates that more data are needed to establish the source of PtdOH. Subcellular localisation of the rapidly labelled PtdOH pool could clarify the problem because phospholipid biosynthesis occurs at the endoplasmatic reticulum and plastid (Ohlrogge et al. 1991) while DAG kinase is predominantly localized in the plasma membrane (Lundberg and Sommarin 1992; Wissing and Wagner 1992).

We thank Theo de Rijk and Gern Huijbers (ATO-DLO) for the GC-MS analysis of fatty acids, Eddy Smid (ATO-DLO) for yeast cultures, Hans Klerk (University of Amsterdam) for his discussions concerning isotopic labelling, and Jeanine Brederoo (University of Amsterdam) and Ernst Woltering (ATO-DLO) for critical readings of this manuscript. We are indebted to Bernard Paystrastre (INSERM Unité 326, Toulouse, France) for analyzing the PtdInsP isomers by HPLC.
References


Catharanthus roseus


