Evidence for substrate-cycling of 3-, 3,4-, 4-, and 4,5-phosphorylated phosphatidylinositols in plants

Charles A. BREARLEY* and David E. HANKE

Department of Plant Sciences, University of Cambridge, Downing Street, Cambridge CB2 3EA, U.K.

Short-term ^{32}P labelling and enzymic dissection of inositol phospholipids was used to study the turnover of 3-, 3,4-, 4-, and 4,5-phosphorylated phosphatidylinositols in the plant *Spirodela polyrrhiza* L. Analysis of label in the whole headgroup reveals that phosphatidylinositol 3- and 4-monophosphates (PtdIns3P and PtdIns4P) and phosphatidylinositol 3,4- and 4,5-bisphosphates [PtdIns(3,4) P_2 and PtdIns(4,5) P_2] all turn over with a half-life of approximately 2–5 h. Analysis of the labelling of individual phosphomonoesters and phosphodiester of these lipids indicates a rapid equilibration of label between the 4- and 5-monoester phosphates of PtdIns(4,5) P_2 within 5 h and largely independent of changes of labelling in the diester. We observed substantially slower equilibration of label (within ~ 27 h) between the mono-

ester and diester of PtdIns4P. These studies therefore indicate that PtdIns4P and PtdIns(4,5) P_2 participate in substrate-cycling reactions, evidence for which has been described experimentally only in erythrocytes, and give confirmation *in vivo* of the previous detection of inositol phospholipid phosphomonoesterase activity. Similar analyses of label in PtdIns3P and PtdIns(3,4) P_2 reveal the likely participation of these molecules in substrate cycles and hence for the first time the presence of PtdIns3P 3-phosphatase and PtdIns(3,4) P_2 4-phosphatase activities in plants. PtdIns3P and PtdIns(3,4) P_2 undergo turnover at rates similar to those of PtdIns4P and PtdIns(4,5) P_2 . Estimates are made of the relative sizes of the pools of phospholipid participating in the turnover process.

INTRODUCTION

It is well established that higher plants possess the phospholipid components of the phosphatidylinositol cycle (PI-cycle) and enzyme activities that have been shown to catalyse the interconversions of these components [1]. For the purpose of this study the PI-cycle is defined as those reactions by which PtdIns(4,5) P_2 is synthesized and by which the inositol moiety of the *myo*-inositol 1,4,5-trisphosphate [Ins(1,4,5) P_3] headgroup cleaved from PtdIns(4,5) P_2 by the action of PIC is itself released and returned to PtdIns(4,5) P_2 . Recently [2] we have provided direct evidence that the pathway of synthesis of PtdIns(4,5) P_2 proceeds in plants as it does in animal cells. Thus the synthetic components of the 'animal' PI-cycle have been convincingly established for the higher plant kingdom. There is, however, little direct evidence of the manner in which the 'plant' PI-cycle is controlled or, indeed, whether a cycle as such exists. Similarly, despite numerous claims for stimulated inositol phospholipid 'turnover' in a variety of experimental systems (reviewed in [1]), and leaving aside concerns regarding the rigour of identification of inositol phospholipids in plants [3], a convincing demonstration that the increase in 'turnover' involves biosynthesis and is not purely a degradative event has not been forthcoming.

Estimates of the relative proportions of different inositol phospholipids give values for the ratio PtdIns(4,5) P_2 :PtdIns4P that are much lower than those generally reported in animal cells. Such analyses have been interpreted [1] as implying that for

PtdIns(4,5) P_2 to perform a signalling role in plants the stimulated phosphoinositidase C (PIC)-mediated turnover of this molecule must be extremely rapid. Given that the binding of Ins(1,4,5) P_3 to a putative receptor in beet tonoplast membrane shows binding parameters similar to those for receptors from animal cells [4] and assuming that this turns out to be a general phenomenon, the latter interpretation seems reasonable.

With a recent and notable exception [5], a rigorous stereochemical identification of Ins(1,4,5) P_3 and hence a description of PIC action *in vivo* in higher plants has been lacking. Cho et al. were able to substantiate an earlier report of stimulated production of Ins(1,4,5) P_3 in mastoporan-treated carrot suspension culture [6] and provide support to a report [7] describing the effects of mastoporan in stimulating the production of a component cross-reactive for Ins(1,4,5) P_3 receptor in crude extracts of soybean suspension culture cells. That higher plants are capable of responding to a physiological stimulus by elevation of Ins(1,4,5) P_3 specifically remains to be demonstrated.

Apart from evidence of PIC involvement in plant inositol phospholipid turnover, we still have very little evidence of the rate or the manner of turnover of inositol phospholipids in 'unstimulated' cells or the relative contributions of different turnover processes in 'stimulated' cells. Whether plants show the form of substrate-cycling between PtdIns4P and PtdIns(4,5) P_2 demonstrated in erythrocytes [8,9], or between phosphatidylinositol (PtdIns) and PtdInsP, has not been addressed directly. Thus the distinction between PIC-mediated turnover resulting in

Abbreviations used: GroPIIns, glycerophosphoinositol; GroPIIns3P and 4P, glycerophosphoinositol 3- and 4-monophosphates; GroPIIns(3,4) P_2 and GroPIIns(4,5) P_2 , glycerophosphoinositol 3,4- and 4,5-bisphosphates; Ins(1,4,5) P_3 , *myo*-inositol 1,4,5-trisphosphate; PtdGro, phosphatidylglycerol; PtdIns 3-kinase, phosphatidylinositol 3-kinase activity; PIC, phosphoinositidase C; PI-cycle, phosphatidylinositol cycle; PtdIns, phosphatidylinositol; PtdIns3P and 4P, phosphatidylinositol 3- and 4-monophosphates; PtdIns(3,4) P_2 and PtdIns(4,5) P_2 , phosphatidylinositol 3,4- and 4,5-bisphosphates; PtdIns(3,4,5) P_3 , phosphatidylinositol 3,4,5-trisphosphate; SAX, strong anion-exchange.

Nomenclature: where enantiomers are defined the D-nomenclature is used.

* To whom correspondence should be addressed.

the loss of the whole inositol phosphate headgroup, i.e. mono- and di-ester phosphates, and phosphomonoesterase-mediated turnover removing a single monoester phosphate, has not been established in stimulated or unstimulated plant cells. Such a distinction has great bearing on the interpretation of studies employing [^{32}P]orthophosphate labelling strategies and particularly for short labelling periods.

Only recently have reports (e.g. [10]) directly addressed the rates of turnover of inositol phospholipids in unstimulated cells, though the conclusions reached, that inositol phospholipids turn over more rapidly than the so-called structural phospholipids, phosphatidylcholine and phosphatidylethanolamine, are implicit in results of earlier short-term ^{32}P labelling studies [2,11,12]. The evidence presented [10] suggests that the headgroups of PtdIns4P and PtdIns(4,5) P_2 have a half-life of the order of 5 h, although the route of turnover of these components was not determined. With the foregoing in mind we have set out to investigate the rate of turnover of individual phosphomonoesters and phosphodiester of inositol phospholipids in the aquatic plant *Spirodela polyrhiza*, to address the evidence for the routes of turnover, and to re-examine some of the metabolic evidence pertaining to the synthesis of inositol phospholipids in this plant. A full understanding of stimulated inositol phospholipid turnover in plants requires a description of the turnover process in the unstimulated state.

MATERIALS AND METHODS

Plant material

Spirodela polyrhiza plants were maintained in axenic culture as described [13].

Pulse-labelling

Plants were labelled with [^{32}P]P $_i$ essentially as described [2]. Briefly, cultures of plants were transferred to phosphate-free medium 2–3 d before labelling. This treatment increases phosphate uptake 3-fold [14]. Plants were subsequently washed in phosphate-free media and labelled for 60 min with [^{32}P]P $_i$ (0.35 mCi/ml). At the end of the labelling period NaH_2PO_4 was added to bring the concentration of phosphate to 10 mM, and the plants were incubated at 25 °C. At intervals sample plants were removed, washed briefly (1 min) with water, 10 mM NaH_2PO_4 , then with water again, blotted dry, weighed and frozen in liquid nitrogen.

Extraction

A perchloric acid extract was prepared, lipids were extracted from the cell debris and deacylated (all steps as described [2]).

Dissection of phospholipids and HPLC of the products

The water-soluble products of lipid deacylation were resolved by Partisphere strong anion-exchange (SAX) HPLC according to [15]. Peak fractions corresponding to glycerophosphoinositol 4-monophosphate (GroPIIns4P) and glycerophosphoinositol 4,5-bisphosphate [GroPIIns(4,5) P_2], detected by Čerenkov counting with a Canberra Packard A500 Series Radiomatic Flo-detector (Canberra Packard, Pangbourne, Bucks., U.K.), were desalted, mixed with ^3H -labelled standards obtained from *Spirodela* and dissected by enzymic or chemical means. GroPIIns(4,5) P_2 was treated with human erythrocyte ghosts under conditions designed to remove the 5-phosphate. GroPIIns4P, and on occasions GroPIIns(4,5) P_2 , was treated with alkaline phosphatase in an assay that included a trap of 0.25–1 mM glycerophosphoinositol

(GroPIIns) to limit attack on the diester of [^{32}P]-glycerophosphoinositol monophosphate (GroPIInsP) species. The products of dissection were neutralized and reapplied under the same elution conditions to a Partisphere SAX HPLC column (all steps as described [2]). The proportion of the ^{32}P label in individual phosphomonoesters and diesters was determined from the $^{32}\text{P}/^3\text{H}$ ratio of the GroPIIns and GroPIInsP species and of their unmetabolized parent compounds resolved on HPLC, or in some experiments (when only 2 reaction products, e.g. P $_i$ and GroPIIns or P $_i$ and GroPIInsP, were formed) from the ^{32}P content of these fractions.

Authentication of inositol phospholipid identity

The method of choice for identifying inositol phospholipids in plant tissues is SAX HPLC of the water-soluble products of phospholipid deacylation. This is primarily a consequence of the observation that plants possess lipids that co-migrate with authentic inositol phospholipids on TLC systems, incorporate inositol and P $_i$ but seem not to be susceptible to deacylation [11] and so may not be glycerophospholipids but rather sphingolipids. The identification of these lipids as sphingolipids is, however, entirely circumstantial and should itself be treated with caution. HPLC of water-soluble deacylation products also has the advantage that removal of the fatty acyl groups removes the uncertainty that surrounds the identification of plant inositol phospholipids by reference to their co-chromatography, often not absolute, with authentic standards from mammalian sources, which differ in fatty-acid composition. Thus the rigour of analysis possible by combination of [^3H]inositol labelling and SAX HPLC of deacylation products seems uncompromised. The situation is less clear when [^{32}P]P $_i$ -labelling is coupled to HPLC.

The results of early studies of phospholipid turnover in *Spirodela* [16,17], in which TLC was used to identify phospholipids, suggested that under conditions of short-term ^{32}P labelling the chloroplast lipid phosphatidylglycerol (PtdGro) turned over most rapidly of all the phospholipids and accounted for most of the label in phospholipids. Significantly, these studies were performed before the discovery of PtdIns4P and PtdIns(4,5) P_2 in plants. From our own studies [2] of inositol phospholipid metabolism in *Spirodela* we are led to the conclusion that the phospholipid identified formerly [16,17] as PtdGro is in fact PtdIns4P. Thus HPLC of deacylated lipid extracts obtained from short-term ^{32}P -labelled *Spirodela* reveals a single major product that coelutes precisely with [^3H]GroPIIns4P. However, the problem then arises as to where the product of deacylation of PtdGro runs on this HPLC system. It is not unreasonable to assume that glycerophosphoglycerol derived from PtdGro and GroPIIns derived from PtdIns will run in similar positions on a chromatographic system that relies on interaction between a positively charged support and negatively charged solutes for separation. Both molecules possess a phosphodiester as the functional group most likely to have the strongest influence on ion-exchange chromatography. Therefore before undertaking a detailed characterization of turnover of individual phosphate esters in inositol phospholipids of *Spirodela* we have sought to determine the position of elution of glycerophosphoglycerol on the SAX HPLC gradient that we routinely use to study deacylates of labelled phospholipids. [^{14}C]PtdGro was prepared from [^{14}C]glycerol 3-phosphate-labelled pea chloroplasts. Lipids were extracted and resolved on TLC (all steps as previously [18]). A band that eluted in a position corresponding to that of a standard of dipalmitoyl phosphatidylglycerol was detected by autoradiography, scraped from the TLC plate, eluted with solvent and deacylated. Subsequent chromatography on SAX HPLC

Figure 1 Partisphere SAX HPLC of deacylated PtdIns and PtdGro

[^{14}C]PtdGro was obtained from [^{14}C]Gro3P-labelled chloroplasts and deacylated. The water-soluble products were mixed with [^3H]GroPIs obtained from [^3H]inositol-labelled *Spirodela* and applied to a Partisphere SAX HPLC column. Fractions (0.2 min) were collected and radioactivity estimated by dual-label scintillation counting. Key to symbols: $\circ$, ^3H ; $\bullet$, ^{14}C .

revealed (Figure 1) that the product of deacylation of putative PtdGro coeluted precisely with an authentic standard of [^3H]GroPIs obtained from [^3H]inositol-labelled *Spirodela*. Further confirmation that the most heavily labelled lipid obtained from *Spirodela* under conditions of short-term ^{32}P -labelling is PtdIns4P and not PtdGro was obtained by TLC on oxalate-impregnated silica of intact ^{32}P -labelled lipids. The results (not shown) confirm that the major labelled lipid is PtdIns4P and further show that PtdGro, which is very well resolved from PtdIns and PtdIns4P in this system, constitutes only a very small fraction of the total label recovered in lipid and is a fraction of that recovered in PtdIns. Densitometry of autoradiographs of TLC-resolved lipids from extracts of tissue labelled with ^{32}P for 60 min reveals that PtdGro represents approximately half of the label in PtdGro and PtdIns combined. This result indicates that the GroPIs peak previously identified [2] in deacylates of ^{32}P -labelled lipids contains some GroPGro.

RESULTS

Analysis of the distribution of label in phospholipids revealed marked differences in the pattern of labelling between 'structural' lipids, such as phosphatidylcholine and phosphatidylethanolamine, and those that have roles in cell signalling assigned to them, i.e. inositol phospholipids. Thus in agreement with earlier reports [16,17] we observed a progressive increase in the labelling of phosphatidylcholine, phosphatidylethanolamine and PtdIns/PtdGro. In contrast, the level of labelling in PtdIns3P, PtdIns4P, PtdIns(3,4) P_2 and PtdIns(4,5) P_2 , the data for all of which are shown in Figure 2, increased approximately 2–3-fold in the first 2 h of chase before declining to ~25–40% of the peak value by 6 h of chase. Thus the half-life of labelling of the headgroups of both the 3- and 4-phosphorylated PtdInsP and PtdIns P_2 species was ~2 h.

Turnover of mono- and di-ester phosphates of inositol phospholipids

Inositol phospholipids separated by SAX HPLC were desalted and dissected enzymically. GroPIs3P and GroPIs4P were

Figure 2 Pulse-chase analysis of [^{32}P]orthophosphate labelling of inositol phospholipids

Spirodela plants were pulse-labelled for 60 min in phosphate-free 1/2-strength Hutner's medium containing [^{32}P]orthophosphate, washed and incubated (chased) for periods up to 27 h in 1/2-strength Hutner's medium containing 10 mM phosphate. A perchloric acid extract was prepared and a lipid extract obtained. Phospholipids were deacylated, the water-soluble products resolved on Partisphere SAX HPLC and their radioactivity determined by Čerenkov counting of collected fractions. All steps were as described in Materials and Methods. Data for inositol phospholipids only are indicated. Key to symbols: $\circ$, PtdIns3P; $\square$, PtdIns4P; $\bullet$, PtdIns(3,4) P_2 ; $\blacksquare$, PtdIns(4,5) P_2 .

dissected with alkaline phosphatase (Figure 3). GroPIs(4,5) P_2 was dissected with alkaline phosphatase (Figure 4a) and separately with erythrocyte ghosts under conditions designed to remove the 5-phosphate (Figure 4b), whereas GroPIs(3,4) P_2 was dissected with erythrocyte ghosts under conditions designed to remove the 3-phosphate (dissection not shown). Changes in the distribution of ^{32}P label between the diester and monoester phosphates of PtdInsP and PtdIns P_2 species after 60 min pulse-labelling with [^{32}P]P $_i$ and subsequent chase of label with 10 mM P $_i$ are shown in Tables 1 and 2.

PtdIns4P and PtdIns(4,5) P_2

We have recently provided [2] direct evidence *in vivo* that the synthesis of PtdIns(4,5) P_2 proceeds in plants as it does in animal cells. Thus at short (30 min) periods of ^{32}P labelling, the diester phosphate of PtdIns4P contains only a very small proportion (~0.5%) of the label in the molecule and presumably much less than that in PtdIns(4,5) P_2 , whereas the 5-monoester contains most of the label in PtdIns(4,5) P_2 . The data presented (Tables 1 and 2) show a progressive increase in the labelling of the diester phosphate of both PtdIns4P and PtdIns(4,5) P_2 throughout the chase, such that at approximately 27 h of chase the diester and monoester phosphates of PtdIns4P have come almost to equilibrium, with the diester of PtdIns(4,5) P_2 containing ~6% of the label in that molecule. In one experiment the proportion of label in the 5-phosphate of PtdIns(4,5) P_2 fell to <50% of the total within 2 h of chase, whereas in a second experiment in which an estimation of the contribution of the diester to labelling of PtdIns(4,5) P_2 was made the value was ~54%. If we extrapolate back to very short periods of labelling when we would expect the label to be located exclusively (100%) in the 4- and 5-monoesters

Figure 3 Dephosphorylation of GroPIns3P and GroPIns4P with alkaline phosphatase

GroPIns3P (a) and GroPIns4P (b) obtained by deacylation of lipids extracted from [^{32}P] P_i -labelled *Spirodela* were resolved on Partisphere SAX HPLC and desalted. Aliquots were incubated with 1.25–2.5 units of alkaline phosphatase in an assay containing a trap of unlabelled GroPIns for 10 min at 25 °C. The reaction was stopped by boiling for 5 min and the supernatant was neutralized and applied to a Partisphere SAX HPLC column. Fractions were collected and their radioactivity measured by Čerenkov counting. All steps were as described in Materials and Methods.

of PtdIns4P and PtdIns(4,5) P_2 respectively, it becomes clear that the 4-monoester of PtdIns4P turns over with a half-life of approximately 27 h whereas the half-life of the 5-monoester of PtdIns(4,5) P_2 is much shorter (~2–6 h). It is therefore evident that the 4- and 5-phosphates of PtdIns(4,5) P_2 are closer to equilibrium with each other than they are with the diester, and are also closer to equilibrium with each other than are the mono- and di-ester phosphates of PtdIns4P with each other.

PtdIns3P and PtdIns(3,4) P_2

PtdIns3P and PtdIns(3,4) P_2 have only recently been identified in higher plants [2,10,19–21] and little is known of their metabolism. It is evident that PtdIns(3,4) P_2 synthesis in *Spirodela* proceeds by 4-phosphorylation of PtdIns3P [2]. Recently Munnik and co-workers [22] have shown that in *Chlamydomonas* PtdIns3P turns over as quickly as PtdIns4P. In the present study we have examined the nature of turnover of PtdIns3P and PtdIns(3,4) P_2 . In light of the much lower level of labelling of PtdIns(3,4) P_2 than PtdIns(4,5) P_2 we considered that an analysis of the labelling of the diester phosphate in PtdIns(3,4) P_2 was precluded. Although we cannot be absolutely certain that the diester does not contribute appreciably to the labelling of the whole molecule, we have rationalized our experimental strategy on the basis that in all animal systems studied so far under conditions of short-term ^{32}P labelling the diester phosphates of PtdIns(3,4) P_2 and PtdIns(3,4,5) P_3 are essentially unlabelled. Also, if the labelling of the diester in PtdIns(3,4) P_2 is comparable to

Figure 4 Dephosphorylation of GroPIns(4,5) P_2 with alkaline phosphatase and human erythrocyte ghosts

GroPIns(4,5) P_2 obtained by deacylation of lipids extracted from [^{32}P] P_i -labelled *Spirodela* was resolved on Partisphere SAX HPLC and desalted. Aliquots were incubated with alkaline phosphatase (a) or with human erythrocyte ghosts (b). Aliquots were incubated for 20 min at 25 °C with 1.25–2.5 units of alkaline phosphatase in an assay containing a trap of unlabelled GroPIns. The reaction was stopped by boiling for 5 min and the supernatant was neutralized and applied to a Partisphere SAX HPLC column. Fractions were collected and their radioactivity was measured by Čerenkov counting. Other aliquots were incubated with human erythrocyte ghosts in the presence of 10 mM MgCl_2 and absence of EDTA for 75 min. The reaction was quenched with HClO_4 and the protein precipitated by centrifugation. The supernatant was neutralized and applied to a Partisphere SAX HPLC column. Fractions were collected and their radioactivity was measured by Čerenkov counting. All steps were as described in Materials and Methods.

that in PtdIns(4,5) P_2 , it would represent no more than 6% of the total label at 6 h of chase. The data presented in Tables 1 and 2 show the changes in the distribution of ^{32}P label between the diester and monoester phosphates of PtdIns3P and between the 3- and 4-monoester phosphates of PtdIns(3,4) P_2 after pulse-labelling and subsequent chase, making the assumption that label in the diester of PtdIns(3,4) P_2 is negligible. Thus by extrapolating to very short labelling times, when labelling will be exclusively restricted to the 4-monoester, it becomes clear that within 2 h of chase the contribution of the 4-phosphate has decreased to 44.8% as the 3-phosphate has become labelled. By the same arguments as used above, this result suggests that the equilibration of label between the 3- and 4-phosphates of PtdIns(3,4) P_2 is quicker than between the monoester and diester of PtdIns3P. That is, PtdIns3P and PtdIns(3,4) P_2 are closer to equilibrium with each other than are PtdIns and PtdIns3P.

Estimation of the pool sizes of inositol phospholipids participating in turnover

The close approach of the degree of labelling of the monoester

Table 1 ^{32}P labelling of diester phosphate of PtdIns3P and PtdIns4P

GroPInsP species obtained by deacylation of ^{32}P -labelled lipids were purified on Partisphere SAX HPLC, desalted, and dissected with alkaline phosphatase. The products of dissection were separated on Partisphere SAX HPLC and radioactivity was determined by Čerenkov counting. All steps were as described in Materials and Methods.

	Percentage of ^{32}P label in diester phosphate		
	PtdIns4P		PtdIns3P
	Expt. 1	Expt. 2	Expt. 2
Pulse (h)			
0*	0	0	0
1	1.7	2.1	1.8
Chase (h)			
1	4.9	4.2	7.6
2	7.5	8.1	8.2
4	21.2	15.2	10.5
7.5	28.0	24.7 ^{6h}	15.9 ^{6h}
27	46.1		

* Value obtained by extrapolation.

phosphates of PtdIns4P and PtdIns(4,5) P_2 on the one hand, and of PtdIns3P and PtdIns(3,4) P_2 on the other, implies that the two monoester phosphates of PtdIns(3,4) P_2 and PtdIns(4,5) P_2 have almost come to equilibrium with the monoester phosphates of PtdIns3P and PtdIns4P respectively. That is, the PtdIns3P pool is in equilibrium with PtdIns(3,4) P_2 and the PtdIns4P pool is in equilibrium with PtdIns(4,5) P_2 . Thus the total label in the respective pools, taking into account the number of monoester phosphates on each of the molecules, and assuming no contribution from the diester, gives the relative pool sizes. Moreover, by definition, these are essentially the pool sizes of inositol phospholipids participating in the turnover process.

Thus data from two experiments yield values for the ratio of pool sizes PtdIns4P:PtdIns(4,5) P_2 of $\sim 40:1$. Similar analysis

for the approximate equilibration of label between the diester and monoester phosphates of PtdIns4P at 27 h of chase yields a ratio of 11:1. Combination of the two estimates gives a PtdIns:PtdIns4P:PtdIns(4,5) P_2 ratio of 440:40:1, which is in very good agreement with the values 385:35:1 calculated by Munnik et al. [10]. The latter estimate was obtained by combining a ratio for PtdInsP:PtdIns P_2 [principally PtdIns4P:PtdIns(4,5) P_2] of 35:1 obtained from total ^{32}P labelling of the headgroup with an independent estimate of the ratio PtdIns:PtdInsP (again principally PtdIns:PtdIns4P) from [^3H]inositol labelling.

The very good agreement between the two estimates from quite different derivations is striking and is probably partly due to the minor contribution of the 3-isomers to total PtdInsP and PtdIns P_2 in the earlier data [10]. However, some caution is required in view of the observation that in stomatal guard cells at 18 h of ^{32}P labelling, the extent of labelling of PtdIns(3,4) P_2 is greater than that of PtdIns(4,5) P_2 , whereas PtdIns3P is much less strongly labelled than PtdIns4P [21]. Clearly, where analysis of labelling is restricted to the level of total labelling of PtdInsP and PtdIns P_2 and not of individual isomers or their individual phosphoesters, estimates of pool sizes will ultimately be dependent on the period of labelling purely as a consequence of the rates of turnover of individual isomers. The effect may be minor in mass terms but different rates of hydrolysis and resynthesis could vary widely for different positions, 3, 4 and 5, on the inositol ring.

The evidence presented here suggests that the active pools of PtdIns3P and PtdIns(3,4) P_2 are further from equilibrium than are those of PtdIns4P and PtdIns(4,5) P_2 . Thus an estimate of the relative pool sizes of these components will be rather less accurate than that for PtdIns4P and PtdIns(4,5) P_2 . Nevertheless, a description of such an estimate is of value as it is the only estimate so far for plant cells. The ratio PtdIns3P:PtdIns(3,4) P_2 (32:1) overestimates the relative amount of PtdIns(3,4) P_2 but does set the limits. The ratio for PtdIns:PtdIns3P (17:1) at 6 h of labelling will similarly be much more inaccurate. This is particularly evident when comparison is made with estimates from long-term [^3H]inositol labelling of *Spirodela* (data from [2]) which reveal

Table 2 ^{32}P labelling of individual phosphates of PtdIns(3,4) P_2 and PtdIns(4,5) P_2

GroPIns(4,5) P_2 species obtained by deacylation of ^{32}P -labelled lipids were purified on Partisphere SAX HPLC, desalted, and dissected with alkaline phosphatase [PtdIns(4,5) P_2], and erythrocyte ghosts under conditions designed to remove the 5-phosphate [PtdIns(4,5) P_2] or the 3-phosphate [PtdIns(3,4) P_2]. The products of dissection were separated on Partisphere SAX HPLC and their radioactivity was determined by Čerenkov counting. All steps were as described in Materials and Methods.

	Percentage of ^{32}P label in individual phosphates						
	PtdIns(4,5) P_2					PtdIns(3,4) P_2	
	Expt. 1		Expt. 2			Expt. 2	
	1 + 4	5	1	4	5	1 + 4	3
Pulse (h)							
0*	0	100	0	0	100	100	0
1	29.5	70.5	0.5	31.7	67.8	80.5	19.5
Chase (h)							
1	48.4	51.6	1.3	40.8	57.9	67.8	32.2
2	48.9	51.1	2.7	39.2	58.1	57.2	42.8
4	53.6	47.4	6.8	36.1	57.1	49.7	50.3
7.5	58.6	41.4	6.0 ^{6h}	38.6 ^{6h}	55.4 ^{6h}	44.8 ^{6h}	55.2 ^{6h}
27	56.7	43.3					

* Value obtained by extrapolation.

ratios PtdIns:PtdIns4P (7:1), PtdIns4P:PtdIns(4,5) P_2 (19:1), PtdIns:PtdIns3P (122:1) and PtdIns3P:PtdIns(3,4) P_2 (20:1). That these figures differ to greater or smaller extents from the present data may be an indication that turnover of the inositol moiety of the headgroup occurs by a different route from that of the phosphate moieties. Moreover, the evidence from animal systems is that the 3-phosphorylated lipids are not substrates for PIC.

DISCUSSION

Analysing the turnover of label in the whole phosphoinositide headgroup hides the turnover of individual phospho mono- and di-esters. The present data (Table 1) indicate that the monoester and diester phosphates of PtdIns4P come to equilibrium in ~ 27 h, whereas for PtdIns(4,5) P_2 , in which turnover of the 4- and 5-phosphate is largely independent of the diester, this occurs in ~ 4 h. It is therefore unlikely that the major part of PtdIns4P and PtdIns(4,5) P_2 turnover can be attributed to phosphodiesterase activity. We conclude that the equilibration of label in the 4- and 5-phosphates of PtdIns(4,5) P_2 , and between the di- and mono-ester phosphates of PtdIns4P, is a consequence of phosphomonoesterase/kinase cycles, the direct experimental test of which has been described only in work with erythrocytes [8,9,23]. These data therefore represent one of the very few demonstrations in any kingdom and the first demonstration in the plant kingdom that the inositol phospholipids PtdIns4P and PtdIns(4,5) P_2 , and PtdIns and PtdIns4P, take part in substrate-cycling reactions. It should be pointed out that 'substrate-cycling' is a metabolic definition borne out of analysis *in vivo* and not an extrapolation of descriptions of kinase and phosphatase activities *in vitro* in cell extracts. From the point of cell-signalling and particularly considering ^{32}P -labelling studies, the crucial experimental distinction between substrate-cycling via kinase/monoesterase reactions and PIC-mediated turnover of PtdIns(4,5) P_2 is the demonstration that the turnover of the monoester phosphates is independent of the diester phosphate, or more simply the coming to equilibrium of the labelling of the 4- and 5-monoester phosphates.

Although the existence of substrate-cycling is often equated with the state of readiness of a cell to respond quickly to a stimulus or a change in metabolic demand, the extent to which substrate-cycling alone can meet demand for inositol phospholipid synthesis, and hence Ins(1,4,5) P_3 generation on cell stimulation, has only very recently been addressed for animal cells. Thus detailed analysis reveals that substrate-cycling can account for a large proportion of the synthesis of PtdIns4P but only a small proportion of the synthesis of PtdIns(4,5) P_2 , necessitated on cell stimulation [23]. It has also been shown that in neutrophils the demand for PtdIns(4,5) P_2 synthesis is met by activation of PtdIns4P 5-kinase [23]. It is likely that this is a universal phenomenon in the animal kingdom, as is apparent in the results of Harden and co-workers [24]. Linear inositol phosphate production was sustained for up to 30 min in β -adrenergic receptor agonist-stimulated turkey erythrocyte ghosts maintained in the presence of an ATP-regenerating system.

In many animal cells showing agonist-responsive inositol phospholipid turnover, the levels of PtdIns4P and PtdIns(4,5) P_2 are similar. By contrast, in plant cells the levels of PtdIns(4,5) P_2 are much lower relative to PtdIns4P [1], which has been taken to indicate other functions for PtdIns4P turnover over and above demand for PtdIns(4,5) P_2 synthesis [25]. For GTP[S]-stimulated turkey erythrocyte membranes in which the metabolism of labelled Ins(1,4,5) P_3 , generated from PtdIns(4,5) P_2 , was inhibited by an excess of unlabelled Ins(1,4,5) P_3 , an analysis of the

contribution of PIC-mediated turnover of PtdIns4P to inositol phosphate production [24] revealed that PIC-mediated turnover of PtdIns4P could account for $\sim 12\%$ of stimulated inositol phosphate production. There are, however, no such data for plant cells, and limited evidence linked to rigorous stereochemical analysis of endogenous inositol phosphates for the action, let alone activation, of PIC *in vivo* in plants. The study by Cho et al. [5] has provided the first rigorous identification of Ins(1,4,5) P_3 in a higher-plant cell and shown that the cells are responsive to mastoporan. That higher plants respond to physiological stimuli by elevation of Ins(1,4,5) P_3 is indicated by a number of studies [6,7] (see also review in [1]), which have employed Ins(1,4,5) P_3 receptor-binding assays to measure Ins(1,4,5) P_3 levels. Without any indication of the stereochemical identity of inositol phosphates in plant cells or an indication of the K_d for binding of putative inositol trisphosphates to Ins(1,4,5) P_3 receptor preparations, it is difficult to assess the extent to which these measurements are compromised by interference from other inositol phosphates and ATP.

In summary, and in light of the limited evidence available, it is not possible to assess the contribution of substrate-cycling observed in the present study to demand for PtdIns(4,5) P_2 synthesis and Ins(1,4,5) P_3 production on plant cell stimulation. Nevertheless the increase in the labelling of the diester phosphate of PtdIns(4,5) P_2 indicates some unidirectional flux in the pathway PtdIns–PtdIns4P–PtdIns(4,5) P_2 . Whether this represents a basal level of PIC-mediated turnover through the PI-cycle is difficult to assess because of our complete lack of understanding of the contribution to phospholipid catabolism of various phospholipases in plants.

The cellular function of the extensive turnover of PtdIns4P evidenced by analysis of short-term labelling of the whole molecule [10], and of the substrate-cycling of this molecule described here, remains to be revealed. This turnover may itself indicate the readiness of the plant cell to respond to as yet unidentified stimuli.

In the absence of any indication from the animal kingdom that PtdIns3P, PtdIns(3,4) P_2 or PtdIns(3,4,5) P_3 are substrates for PIC, and by arguments similar to those presented above for PtdIns4P and PtdIns(4,5) P_2 turnover, we are drawn to the conclusion that the rapid turnover of PtdIns3P, PtdIns(3,4) P_2 and of their individual phosphates, albeit slightly slower than that of PtdIns4P and PtdIns(4,5) P_2 , is evidence for substrate-cycling between PtdIns3P and PtdIns(3,4) P_2 and between PtdIns and PtdIns3P. Clearly such a proposition invokes the presence of PtdIns(3,4) P_2 4-phosphatase and PtdIns3P 3-phosphatase activities previously unconsidered in plants, but with precedents, though currently only from analysis *in vitro*, in the animal kingdom. Inositol phospholipid phosphatase activities that are capable of removing the 3-phosphate from PtdIns3P, PtdIns(3,4,5) P_3 , and possibly PtdIns(3,4) P_2 have been detected in neutrophils and rat brain [26,27]. An activity capable of removing the 4-phosphate from PtdIns(3,4) P_2 has also been detected in neutrophils [26]. These data are reviewed in [28]. The relationship between the PtdIns(3,4) P_2 4-phosphatase and the PtdIns4P 4-phosphatase activities detected in animal cells is currently undefined [28], as is that of the activities detected here *in vivo* in plants.

These are all facets of plant inositol phospholipid metabolism whose functions cannot yet be rationalized with the functions of these lipids in plants. The recent detection [29] of a PtdIns 3-kinase in carrot cells and its association with, among other cellular components, the cytoskeleton, begin to offer some suggestion of the sort of processes with which PtdIns 3-kinase and PtdIns3P might be associated. It remains to be established

whether these turn out to be of the mitogenic, trophic or cell-cycle control nature indicated in animal cells or of the type of vacuolar protein-sorting role evidenced in yeast. The recent cloning of PtdIns 3-kinase isoforms from soybean [30] and *Arabidopsis* [31], their similarity to the VPS34 PtdIns 3-kinase of yeast [32], and the demonstration that a chimaeric gene in which the coding sequence of the carboxy-terminal end of VPS34 is replaced by the plant sequence rescues the yeast mutant [31], are strongly suggestive of a membrane trafficking role for this enzyme and 3-phosphorylated lipids in plants. The presence of PtdIns(3,4) P_2 in plant cells [2,21] but not yeast and of the enzymes of its synthesis and turnover suggest additional roles for this lipid and ultimately PtdIns3P, even if only as a precursor of PtdIns(3,4) P_2 . The possibility arises that PtdIns3P 4-kinase and PtdIns(3,4) P_2 4-phosphatase allow attenuation of the levels of PtdIns3P. Nevertheless we should also be alert to the possibility that plants may possess more than one PtdIns 3-kinase.

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REFERENCES

- Drabak, B. K. (1992) *Biochem. J.* **288**, 697–712
- Brearley, C. A. and Hanke, D. E. (1993) *Biochem. J.* **290**, 145–150
- Irvine, R. F. (1990) in *Inositol Metabolism in Plants* (Morre, D. J., Boss, W. F. and Loewus, F. A., eds.), p. xiii, Wiley-Liss, New York
- Brosnan, J. M. and Sanders, D. (1993) *Plant Cell* **5**, 931–940
- Cho, M. H., Tan, Z., Erneux, C., Shears, S. B. and Boss, W. F. (1995) *Plant Physiol.* **107**, 845–856
- Drabak, B. K. and Watkins, P. A. C. (1994) *Biochim. Biophys. Res. Commun.* **205**, 739–745
- Legendre, L., Yueh, Y. G., Crain, R., Haddock, N., Heinsteins, P. F. and Low, P. S. (1993) *J. Biol. Chem.* **268**, 24559–24563
- King, C. E., Hawkins, P. T., Stephens, L. R. and Michell, R. H. (1989) *Biochem. J.* **259**, 893–896
- Muller, E., Hegewald, H., Jaroszewicz, K., Cumme, G. A., Hoppe, H. and Frunder, H. (1986) *Biochem. J.* **235**, 775–783
- Munnik, T., Musgrave, A. and de Vrije, T. (1994) *Planta* **193**, 89–98
- Drabak, B. K., Ferguson, I. B., Dawson, A. P. and Irvine, R. F. (1988) *Plant Physiol.* **87**, 217–222
- Wagh, S. S., Menon, K. K. G. and Natarajan, V. (1988) *Biochim. Biophys. Acta* **962**, 178–185
- Smart, C. C. and Trewavas, A. J. (1983) *Plant Cell Environ.* **6**, 507–514
- McPharlin, I. R. and Bielecki, R. L. (1987) *Aust. J. Plant Physiol.* **14**, 561–572
- Stephens, L. R., Hawkins, P. T. and Downes, C. P. (1989) *Biochem. J.* **259**, 267–276
- Bielecki, R. L. (1968) *Plant Physiol.* **43**, 1297–1308
- Bielecki, R. L. (1972) *Plant Physiol.* **49**, 740–745
- Mudd, J. B., Andrews, J. E. and Sparace, S. A. (1987) in *Methods in Enzymology*, Vol. 148 (Packer, L. and Douce, R., eds.), pp. 348–355, Academic Press, London
- Brearley, C. A. and Hanke, D. E. (1992) *Biochem. J.* **283**, 255–260
- Brearley, C. A. and Hanke, D. E. (1994) *Plant Physiol.* **104**, 1381–1384
- Parmar, P. N. and Brearley, C. A. (1993) *Plant Journal* **4**, 255–263
- Munnik, T., Irvine, R. F. and Musgrave, A. (1994) *Biochem. J.* **298**, 269–273
- Stephens, L. R., Jackson, T. R. and Hawkins, P. T. (1993) *Biochem. J.* **296**, 481–488
- Harden, T. K., Hawkins, P. T., Stephens, L. R., Boyer, J. L. and Downes, C. P. (1988) *Biochem. J.* **252**, 583–593
- Drabak, B. K. (1991) in *Essays in Biochemistry*, Vol. 26 (Tipton, K. F., ed.), pp. 27–37, Portland Press, London
- Stephens, L. R., Hughes, K. T. and Irvine, R. F. (1991) *Nature (London)* **351**, 33–39
- Lips, D. L. and Majerus, P. W. (1989) *J. Biol. Chem.* **264**, 19911–19915
- Stephens, L. R., Jackson, T. R. and Hawkins, P. T. (1993) *Biochim. Biophys. Acta* **1179**, 27–75
- Dove, S. K., Lloyd, C. W. and Drabak, B. K. (1994) *Biochem. J.* **303**, 347–350
- Hong, Z. and Verma, D.-P. S. (1994) *Proc. Natl. Acad. Sci. U.S.A.* **91**, 9617–9621
- Welters, P., Takegawa, K., Emr, S. D. and Chrispeels, M. J. (1994) *Proc. Natl. Acad. Sci. U.S.A.* **91**, 11398–11402
- Schu, P. V., Takegawa, K., Fry, M. J., Stack, J. H., Waterfield, M. D. and Emr, S. D. (1993) *Science* **260**, 88–91