Metabolic evidence for PtdIns(4,5)P_2-directed phospholipase C in permeabilized plant protoplasts

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Comparison of the sequences of the genes encoding phospholipase C (PLC) which have been cloned to date in plants with their mammalian counterparts suggests that plant PLC is similar to PLCδ of mammalian cells. The physiological role and mechanism of activation of PLCδ is unclear. It has recently been shown that Ins(1,4,5)P_3 may not solely be the product of PtdIns(4,5)P_2-directed PLC activity. Enzyme activities capable of producing Ins(1,4,5)P_3 from endogenous inositol phosphates are present in *Dictyostelium* and also in rat liver. Significantly it has not been directly determined whether Ins(1,4,5)P_3 present in higher plants is the product of a PtdIns(4,5)P_2-directed PLC activity. Therefore we have developed an experimental strategy for the identification of D-Ins(1,4,5)P_3 in higher plants. By the use of a short-term non-equilibrium labelling strategy in permeabilized plant protoplasts, coupled to the use of a 'metabolic trap' to prevent degradation of [^{32}P]Ins(1,4,5)P_3, we were able to determine the distribution of ^{32}P in individual phosphate esters of Ins(1,4,5)P_3. The [^{32}]Ins(1,4,5)P_3 identified showed the same distribution of label in individual phosphate esters as that of [^{32}P]PtdIns(4,5)P_2 isolated from the same tissue. We thus provide *in vivo* evidence for the action of a PtdIns(4,5)P_2-directed PLC activity in plant cells which is responsible for the production of Ins(1,4,5)P_3 observed here. This observation does not, however, exclude the possibility that in other cells or under different conditions Ins(1,4,5)P_3 can be generated by alternative routes.

INTRODUCTION

In mammalian cells the binding of agonists to numerous cell surface receptors is known to activate PtdIns(4,5)P_2-directed phospholipase C (PLC) either through the action of heterotrimeric G-proteins (PLCβ) or by receptor protein tyrosine kinases (PLCγ) [1]. The release of Ins(1,4,5)P_3, the consequent mobilization of Ca^{2+}, and the release of diacylglycerol are central to PtdIns(4,5)P_2-mediated signalling regimes. In contrast, the mechanism of activation and the physiological function of PLCδ isoforms is not defined [1,2].

Although several plant cell types, most notably stomatal guard cells [3,4], have been shown to respond in a physiological manner to release of Ins(1,4,5)P_3 from a microinjected 'caged' compound, the lack of knowledge surrounding the identities of the many inositol phosphate species commonly found in plant cells, with some exceptions [5,6], potentially compromises the numerous claims of stimulated Ins(1,4,5)P_3 production (reviewed by Drøbak [7]). A recent report [8] has provided the first rigorous identification of D-Ins(1,4,5)P_3 in a higher plant cell and establishes an experimental system in which the metabolism of this important signalling molecule can be studied. [^{3}H]Ins(1,4,5)P_3 levels were enhanced by mastoporan, correlating with a decline in [^{3}H]PtdIns(4,5)P_2. This result corroborates an earlier observation [9] of the same phenomenon in the same experimental system in which Ins(1,4,5)P_3 levels were assayed by inhibition of Ins(1,4,5)P_3 binding. The manner in which mastoporan activates PLC in plants is unclear.

Various studies, e.g. [9,10], have utilized receptor-binding assays based around the use of crude animal Ins(1,4,5)P_3 receptor preparations to characterize 'Ins(1,4,5)P_3' levels in plant cells. With the exception of two studies [11,12], no attempt has been made to verify that the Ins(1,4,5)P_3-receptor-cross-reactive material is Ins(1,4,5)P_3 and not any of the numerous InsP_3s or inositol phosphates identified in animal cells that we might reasonably expect to be present in plant cells and which cumulatively could interfere with the assay of Ins(1,4,5)P_3 present in very small amounts. Various InsP_3s have been identified in *Spirodela polyrhiza* L. [5] and barley aleurone [6]. In a study of guard cells [11], the authors correlated a decline in radiolabelled PtdInsP_2 with increases in Ins(1,4,5)P_3 measured by receptor-binding assay. Significantly perhaps, PtdIns(4,5)P_2 is not the predominant PtdInsP_2 isomer in stomatal guard cells [13,14]. The former study [11] also reported the identification of Ins(1,3,4)P_3, although not the resolution of this compound from Ins(1,4,5)P_3. If plants contain the numerous InsP_3 isomers identified in animal cells [15,16], it is likely that the unresolved 'Ins(1,3,4)P_3/Ins(1,4,5)P_3' peak contains other InsP_3 isomers also, possibly those identified in other plants [5,6].

In consideration of the correlation between lipid labelling and Ins(1,4,5)P_3 levels measured by receptor binding, it is also worth taking note of the inherent difficulties in interpretation of short-term non-equilibrium labelling studies. The distinction between substrate cycling and the action of PLC has rarely been made. Both processes result in changes in the labelling of PtdInsP_2. In animal cells the levels of labelling in lipid may rise, fall or remain unchanged on receptor activation [17]. We refer the reader to [17] for a comprehensive description of the dynamics of inositol phospholipid turnover in stimulated and unstimulated cells. Also, in animal cells at least, not all PtdIns(4,5)P_2 turnover is directed to Ins(1,4,5)P_3. Apart from substrate cycling and PLC action, much is directed to PtdIns(3,4,5)P_3. PtdIns(4,5)P_2 also has other roles independent of PLC or phosphatidylinositol 3-kinase.

Significantly, none of the studies described above has afforded a direct experimental test that Ins(1,4,5)P_3 is the product of a

Abbreviations used: PLC, phospholipase C.
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PtdIns(4,5)P_2-directed PLC in plants. Thus the central tenet of the PtdIns(4,5)P_2/Ins(1,4,5)P_3-mediated signalling cascade has not been tested. This is not a trivial issue, as recent developments [18–20] suggest that Ins(1,4,5)P_3 may have metabolic origins other than in the activity of PtdIns(4,5)P_2-directed PLC, and so the possibility arises that Ins(1,4,5)P_3 may not be a signalling molecule in all scenarios. The PtdIns(4,5)P_2/Ins(1,4,5)P_3 paradigm has variants in animal cells, PLCβ, PLCδ, PLCγ, and may not be universal. The cDNA sequences of the plant PLC isoforms cloned to date [21–23] indicate that the plant PLC is of the δ type and there is little or no convincing evidence in higher plants that the production of stereochemically identified Ins(1,4,5)P_3 is activated directly in either a G-protein- or a receptor-tyrosine-kinase-dependent manner. Furthermore, in a mutant cell line (*plc*$^-$) of *Dictyostelium discoideum* in which PLC was disrupted and showed no measurable activity, the basal levels of Ins(1,4,5)P_3 were only slightly reduced [18]. A *Dictyostelium* gene for PLC was previously cloned by the same group and shown to be similar to the δ isoform of animal cells [19]. This raises the question as to what is the source of Ins(1,4,5)P_3 in *Dictyostelium* and indeed in plant cells. It was subsequently shown [20] that Ins(1,4,5)P_3 can be produced from endogenous inositol phosphates including Ins(1,3,4,5,6)P_5 by enzyme activities present in both *Dictyostelium* and rat liver. Ins(1,3,4,5,6)P_5 is a precursor in a pathway of InsP_6 synthesis in *Dictyostelium* which bears no direct relation to Ins(1,4,5)P_3 metabolism [24] and can therefore provide a precursor of Ins(1,4,5)P_3 independent of lipid metabolism. Similarly, Ins(1,3,4,5,6)P_5 is a precursor of InsP_6 in plant cells *in vivo* [5,25] and again is not related to inositol phospholipid metabolism.

We therefore set out to test whether Ins(1,4,5)P_3 is the product of PtdIns(4,5)P_2-directed PLC in plant cells. We devised a permeabilization strategy coupled to short-term non-equilibrium labelling which allows us to determine the order of addition of individual phosphate groups to the *myo*-inositol moiety of Ins(1,4,5)P_3. This has allowed us to distinguish between the different potential routes of Ins(1,4,5)P_3 production.

Previous studies of Ins(1,4,5)P_3 metabolism in plants have been restricted to cell-free extracts. It has been suggested [7] that the *in vitro* breakdown of Ins(1,4,5)P_3 by plant extracts represent a significant difference between plants and animals. Without the support of a rigorous stereochemical and enantiomeric description of the inositol phosphates of plant cells or the metabolic studies that have been documented for animal cells, it is unclear whether the *in vitro* breakdown of Ins(1,4,5)P_3 documented in plant cell extracts represents *in vivo* metabolism. By introduction of [^{3}H]Ins(1,4,5)P_3 into permeabilized plant protoplasts in which we have confirmed the presence of a PtdIns(4,5)P_2-directed PLC acting on newly synthesized lipid substrate, we have also examined the routes of metabolism of Ins(1,4,5)P_3 *in vivo*.

MATERIALS AND METHODS

Plant material

Seeds of *Commelina communis* L. were sown in 5.5 × 14 cm pots containing Levington potting compost. Plants were grown at 25 °C in a Fi-totron 600H chamber, with lighting provided by fluorescent tubes (100–130 μmol photons·m^{-2}·s^{-1} photosynthetically active radiation) in a 16 h light/8 h dark cycle. Plants were watered daily with reverse osmosis water and supplemented once a week with Hoagland's solution [26].

Protoplast preparation

Experiments were carried out using the youngest fully expanded leaves of 5–6-week-old plants of *C. communis* L. The mid-rib of each leaf was removed and the lower epidermis stripped from each lamina. The lamina was then floated abaxial surface down on to the holding medium [0.4 M mannitol, 0.1 mM $CaCl_2$, 0.5 mM ascorbic acid, 0.5 mM dithiothreitol (Sigma Chemical Co Ltd., Poole, Dorset, U.K.), 10 mM Mes buffer adjusted to pH 5.5 with KOH] contained in a 9 cm diameter Petri dish. Once the Petri dish was fully covered with stripped leaf lamina, the holding medium was replaced with the enzyme solution. This solution was prepared in the holding medium and included 2 % (w/v) cellulase 'Onozuka' RS (Yakult Pharmaceutical Company Ltd.), 2 % (w/v) Cellulysin (Calbiochem, Behring Diagnostic, La Jolla, CA, U.S.A.), 0.026 % (w/v) pectolyase Y23 (Sigma), 0.26 % (w/v) bovine albumin (Sigma), 10000 units of penicillin and streptomycin. The tissue was agitated with a Variomag model R100 shaker (Luckham, Sussex, U.K.) at a frequency of 0.5 Hz at 30 °C in the dark for 2 h.

After incubation, the Petri dish was 'tapped' gently on the bench. This ensured the release of the protoplasts. The Petri dish was then 'tapped' again in fresh holding medium. The cell suspension was collected and filtered through 100 μm and 50 μm metal test seives (Endecotts Ltd., London, U.K.). This procedure was repeated three or four times. The filtered cell suspension was centrifuged at 100 ***g*** max for 5 min at 15 °C. The pellet was washed twice and the protoplasts suspended in electroporation medium comprised of 450 mM mannitol, 20 mM KCl, 10 mM $MgCl_2$, 60 μM MgATP, 0.3 mM $CaCl_2$, 1 mM EGTA and 20 mM Hepes buffer, pH 7.6 (20 °C). In the [γ-^{32}P]ATP-labelling experiments described below Ins(1,4,5)P_3 was included at 1 mM concentration. In experiments designed to study the metabolism of Ins(1,4,5)P_3, unlabelled Ins(1,4,5)P_3 was included at 1, 10 and 100 μM concentrations.

Electroporation

Protoplasts were electroporated at a cell density of approx. 1×10^7 cells/ml in electroporation medium containing 100 μCi/ml [γ^{32}P]ATP (specific radioactivity > 3000 Ci/mmol), obtained from Amersham International (Amersham, Bucks., U.K.). Cell suspensions (0.2 ml/well) were held in a four-well multidish (Nunc, InterMed, Roskilde, Denmark) and electroporated with two 100 μF pulses of 5 ms duration, 500 V/cm field strength (15 s interval between pulses) from a Hoefer Pro Genetor II electroporation apparatus (Hoefer Scientific Instruments, Newcastle-Under-Lyme, Staffs, U.K.), fitted with a PG 220P-2.5 circular electrode. Cells were incubated on the bench at ambient temperature for 5 min.

Protoplast extraction

Reactions were stopped by the addition of 1.5 ml of chloroform/methanol (1:2 v/v). Lipids were extracted in the organic phase after additions, each with vigorous mixing, of 0.5 ml of 2.4 M HCl, 0.5 ml of 1 mM EDTA and 0.5 ml of chloroform. After brief centrifugation (1000 $\boldsymbol{g}_{max}$ for 3 min) to aid phase separation, the upper and lower aqueous phases were separated. The acidified aqueous phase was held on ice before addition typically of 11370 d.p.m. of D-*myo*-[1-^{3}H]Ins(1,4,5)P_3 (21 Ci/mmol), supplied by NEN/Dupont (Stevenage, Herts., U.K.), to act as a recovery marker, and 25 μg of $HClO_4$-washed charcoal to remove ATP [27]. Charcoal was precipitated by centrifugation for 5 min at 17500 $\boldsymbol{g}_{max}$ at 4 °C. The supernatant was adjusted to approx. pH 7 by the addition of triethylamine, diluted 5–10-fold and applied to a 0.4 ml column of Dowex AG1 (X8; formate form) resin. A putative InsP_3 fraction was separated from P_i by elution with ammonium formate and desalted by freeze drying. In some

experiments labelling reactions were quenched by the addition of 0.5 ml of ice-cold 3.5 % $HClO_4$. In these experiments lipids were extracted from the cell debris [28].

HPLC

Freeze-dried samples of the aqueous phase were rehydrated and applied to a Whatman Partisphere SAX HPLC column eluted isocratically with 520 mM NaH_2PO_4 (modified from Wreggett and Irvine [29]). Radioactivity was monitored by on-line Čerenkov counting in a Canberra Packard Radiomatic A-500 series flow detector (Canberra Packard, Pangbourne, Berks, U.K.) with an integration interval of 6 s. Peak fractions eluted with a retention time characteristic of InsP_3 were collected after the detector, desalted and applied to an Adsorbosphere SAX HPLC column eluted isocratically with 250 mM $NH_4H_2PO_4$/H_3PO_4 pH 3.35 (20 °C). Fractions (1.0 min) were collected after the detector, and radioactivity was estimated by dual-label scintillation counting. Purified InsP_3 fractions were desalted.

Erythrocyte ghost treatment of InsP_3

Desalted InsP_3 fractions from four electroporation treatments were pooled and freeze-dried. The pooled sample was incubated with human erythrocyte ghosts in an assay (0.4 ml volume) containing 12.5 mM Hepes, pH 7.2, 10 mM $MgCl_2$, 1 mM EGTA and human erythrocyte ghosts (2 mg/ml protein). The assay was designed [30] to remove 5-positioned phosphates from inositol phosphates. Erythrocyte ghosts were prepared as described [31]. At intervals, 0.1 ml aliquots of the reaction mixture were withdrawn and quenched by the addition of 5 μl of 70 % (w/v) $HClO_4$. Samples were held on ice for 15 min and membranes pelleted after the addition of 0.8 ml of ice-cold water. The supernatants were applied without further process to a Partisphere SAX HPLC column eluted isocratically with 520 mM NaH_2PO_4. Fractions were collected and radioactivity estimated by dual-label scintillation counting.

Identification and dissection of glycerophosphoinositol phosphates

The water-soluble products of lipid deacylation were resolved on Partisphere SAX HPLC, desalted and subsequently dissected by enzymic and chemical means [32].

Standards

[^{32}P]Ins(1,4)P_2 was prepared by acid hydrolysis [16] of [^{32}P]-GroPIns4*P* obtained from a lipid extract of the labelled cells. [^{32}P]Ins(1,5)P_2 and [^{32}P]Ins(4,5)P_2 were prepared by alkaline hydrolysis of PtdIns(4,[^{32}P]5)P_2 [33]. PtdIns(4,[^{32}P]5)P_2 was the product of a purified PtdIns4*P* 5-kinase and was the gift of N. Divecha of the Department of Biochemistry, IAPGR, Babraham Hall, Babraham, Cambridge, U.K. D/L-[^{14}C]Ins3*P*, [^{14}C]Ins2*P* and D/L-[^{14}C]Ins4*P* were prepared by acid treatment of L-*myo*-[U-^{14}C]Ins1*P* (D-[^{14}C]Ins3*P*). L-*myo*-[U-^{14}C]Ins1*P* (specific radioactivity, 52 mCi/mmol) was obtained from Amersham International. D-*myo*-[1-^{3}H]Ins(1,4,5)P_3 (specific radioactivity, 21 Ci/mmol) was obtained from NEN/Du Pont.

RESULTS

Metabolism of inositol phospholipids

An analysis of labelling in lipid deacylates revealed the profile indicated in Figure 1. All of the inositol phospholipids commonly identified in plant cells by these procedures were observed and, except for PtdIns(3,4)P_2, their identities confirmed by co-elution with authentic [^{3}H]inositol-labelled standards. Although there is little consensus on the relative levels, or levels of labelling at least, of 3-phosphorylated [PtdIns3*P*, PtdIns(3,4)P_2] and 'conventional' 4-phosphorylated inositides [PtdIns4*P* and PtdIns(4,5)P_2] in plant cells, where such distinctions have been made it is apparent that the predominance at the total tissue level of PtdIns4*P* over PtdIns3*P* and of PtdIns(4,5)P_2 over PtdIns(3,4)P_2 is characteristic of photosynthetic tissue [32,34,35] and holds for lower plants [36,37]. The same situation is maintained under short-term non-equilibrium labelling regimes [32,34,35,37] and is probably a consequence of similar rates of turnover of the two classes of lipid. It is perhaps significant that, in these short-term labelling experiments employing [γ-^{32}P]ATP, the pattern of labelling of the 3- and 'conventional' 4-phosphorylated lipids was similar to that reported for [^{32}P]P_i labelling of carnation petals [34], *Chlamydomonas eugametos* [37] and *Spirodela polyrhiza* [32,35]. The latter system is a much reduced plant in which we can be reasonably sure that we are studying the metabolism of inositol phospholipids in frond mesophyll cells predominantly.

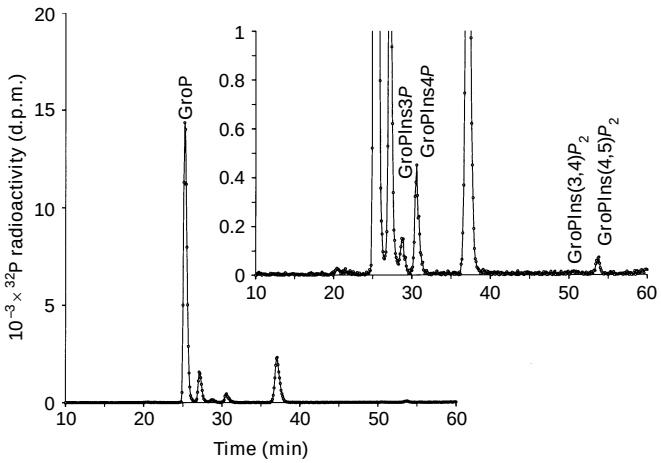

Figure 1 ^{32}P-labelled lipids in permeabilized mesophyll protoplasts

Mesophyll protoplasts were isolated from *C. communis* L. and labelled after transient permeabilization by electroporation in the presence of [γ-^{32}P]ATP. Lipids were extracted, deacylated and the water-soluble products resolved on a Partisphere SAX HPLC column. The ^{32}P content of the column eluate was determined by on-line Čerenkov counting (0.5 ml flow cell, 6 s integration interval). All steps were as described in the Materials and methods section. Abbreviations: GroP, glycerol phosphate; GroPIns3*P*, glycerophosphoinositol 3-monophosphate; GroPIns4*P*, glycerophosphoinositol 4-monophosphate; GroPIns(4,5)P_2, glycerophosphoinositol 4,5-bisphosphate. The approximate position of elution of GroPIns(3,4)P_2 (glycerophosphoinositol 3,4-bisphosphate) on such gradients is indicated.

In a notable departure from the foregoing, the [γ-^{32}P]ATP labelling of *C. communis* mesophyll protoplasts reported here revealed the presence of a phospholipid, evidenced by the generation on deacylation of what we assume to be a water-soluble headgroup, which was eluted shortly after glycerophosphoinositol 4-monophosphate (GroPIns4*P*) and accounted for a major part of the label incorporated into phospholipid. Although we have yet to identify this compound, the application of a number of dissection strategies, including mild periodate cleavage (deglyceration), acid and alkaline hydrolysis and, based on chromatographic mobility, lack of identity of the molecular fragments generated with any of the glycerophosphoinositol phosphates observed in Figure 1 (results not shown), suggests

Table 1 Distribution of label among individual phosphates of PtdIns4*P*, PtdIns(4,5)P_2 and Ins(1,4,5)P_3

[^{32}P]GroPIns4*P* and [^{32}P]GroPIns(4,5)P_2 obtained from deacylates of [γ-^{32}P]ATP-labelled lipids were purified on Partisphere SAX HPLC, desalted, mixed with [^{3}H]inositol-labelled standards and dissected with either erythrocyte ghosts under conditions designed to selectively remove the 5-phosphate or alkaline phosphatase. The products of dissection were separated by Partisphere SAX HPLC using the same gradient as that employed to separate different GroPIns*P* species and the radioactivity in individual phosphates of the different GroPIns*P*s was determined. [^{32}P]Ins(1,4,5)P_3◦, obtained from the same protoplast preparation that yielded the [^{32}P]GroPIns4*P* and [^{32}P]GroPIns(4,5)P_2 analysed, was purified by sequential HPLC on Partisphere SAX and Adsorbosphere SAX columns. The purified sample was desalted, mixed with authentic [^{3}H]Ins(1,4,5)P_3 and dissected with erythrocyte ghost 5′-phosphatase activity. The products of reaction were resolved on a Partisphere SAX HPLC column and the radioactivity in the different phosphates determined (all steps described in the Materials and methods section). Values in parentheses refer to duplicate electroporations. Given the very low labelling of the 1-phosphate in PtdIns4*P* we assume (see the text for rationale) that the labelling of the 1-phosphate is negligible in PtdIns(4,5)P_2. n.d., Not determined.

	^{32}P content of individual phosphates (%)		
	1	4	5
PtdIns4*P*	0.8 (1.5)	99.2 (98.5)	
PtdIns(4,5)P_2	n.d.	32.5 (31.5)$^{(1+4)*}$	67.5 (68.5)
Ins(1,4,5)P_3◦	n.,d.	33.7$^{(1+4)*}$	66.3
Ins(1,4,5)P_3	0†	n.d.	n.d.

* Labelling of 1- and 4-phosphates combined.

† Labelling of the 1-phosphate of Ins(1,4,5)P_3 was determined by alkaline phosphatase treatment (see Figure 4 and text for details).

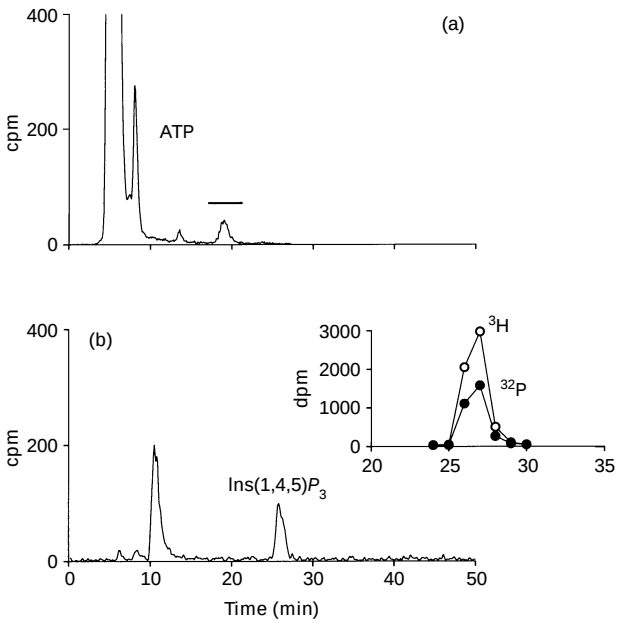

Figure 2 HPLC purification of [^{32}P]Ins(1,4,5)P_3 from permeabilized mesophyll protoplasts

Mesophyll protoplasts were isolated from *C. communis* L. and labelled after transient permeabilization by electroporation in the presence of [γ-^{32}P]ATP and 1 mM unlabelled Ins(1,4,5)P_3. Lipids were extracted. The acidified aqueous phase was treated with charcoal to remove nucleotides, neutralized and spiked with [^{3}H]Ins(1,4,5)P_3. The neutralized extract was applied to an ion-exchange column [Dowex AG1 (X8; formate form)] and an '$InsP_3$' fraction was obtained. The '$InsP_3$' fraction was desalted by freeze-drying, applied to a Partisphere SAX HPLC column and eluted (**a**). Peak fractions denoted by the bar in the chromatogram in (**a**) were desalted and applied to an Adsorbosphere SAX HPLC column (**b**). The ^{32}P content of the column eluates in (**a**) and (**b**) was determined by on-line Čerenkov counting (0.5 ml flow cell, 6 s integration interval). Aliquots of the fractions collected after the detector in (**b**) were counted for ^{3}H and ^{32}P content by dual-label scintillation counting (inset). All steps were as described in the Materials and methods section.

that this compound is not a derivative of the inositol phospholipids identified thus far in plants.

Labelling of individual phosphate esters in inositol phospholipids

Analyses of the relative labelling of phosphate monoesters and diesters in PtdIns4*P* revealed that label was located almost exclusively in the monoester (Table 1). The rationale for such a non-equilibrium experimental approach has been described [38] and has been applied to plants [14,32,35] and permeabilized cells [39]. The near-exclusive labelling of the monoester is entirely consistent with the biosynthesis of PtdIns4*P* by phosphorylation of phosphatidylinositol [32,35], and is probably a consequence of the extremely low level of labelling of phosphatidylinositol (Figure 1). Similarly, the low levels of labelling in a peak with the chromatographic properties of GroPIns(3,4)P_2 (Figure 1) precluded analysis of labelling in this peak. Dissection of GroPIns(4,5)P_2 with the inositol 5-phosphate phosphatase activity of human erythrocyte ghosts confirmed the synthesis of PtdIns(4,5)P_2 by 5-phosphorylation of PtdIns4*P*; 67.5% and 68.5% of total label was located in the 5-position in duplicate electroporations in a single experiment (Table 1) and 88.2% in an independent experiment (results not shown). Although we did not analyse the labelling of the diester, we can be reasonably sure that it did not contribute to the labelling of PtdIns(4,5)P_2 on the grounds that labelling of the diester of PtdIns4*P* (the expected precursor) was less than 1.5% of the total in that molecule.

Labelling and extraction strategy for identification of Ins(1,4,5)P_3

Before any analysis of InsP_3 species can be attempted in ^{32}P-labelling studies, especially in plants, it is necessary to remove contaminating labelled nucleotides, particularly ATP, which is present in vast excess in mass and labelling terms over inositol phosphates. It is reasonable to suppose that plants contain a number of InsP_3s, and the limited analysis of InsP_3s performed thus far in plants [5,6] confirms this. The InsP_3s detected in animal cells are not all readily resolved on Partisphere SAX HPLC columns. Partisphere WAX columns are also commonly used to separate InsP_3 species, and detailed analysis of isomers in avian erythrocytes has shown that, of six *myo*-[^{3}H]inositol-labelled isomers detected in avian erythrocytes [16], only three peaks of InsP_3 could be resolved. Other isomers have also been detected by the metal-dye detection technique of Mayr and coworkers [40]. With these considerations in mind, we used sequential HPLC on Partisphere SAX and Adsorbosphere SAX, which provides in our hands a more manageable alternative to Partisphere WAX, after acidified charcoal treatment [27] to remove nucleotides. Because of the large number of experimental manipulations between extraction and final resolution of individual InsP_3 fractions, we included at the point of extraction a spike of [^{3}H]Ins(1,4,5)P_3 to act as an internal recovery marker. Thus by simple analysis of the ^{3}H and ^{32}P counts of the purified product we obtain a reliable estimate of recovery.

Identification of D-Ins(1,4,5)P_3

Initial Partisphere SAX HPLC of Dowex AG1 X8-purified '$InsP_3$' fraction (Figure 2a) revealed a minor peak of ^{32}P label which was eluted approx. 6 min after a trace of [^{32}P]ATP carried through the extraction procedure and which itself was identified

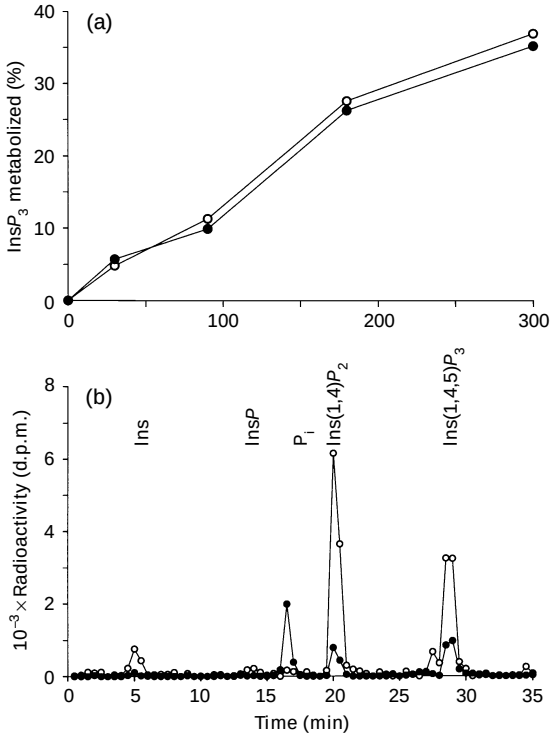

Figure 3 Erythrocyte ghost 5-phosphatase treatment of [^{32}P]Ins(1,4,5)P_3 obtained from permeabilized mesophyll protoplasts of *C. communis* L.

[^{32}P]InsP_3 fractions which contained a 'spike' of [^{3}H]Ins(1,4,5)P_3, added at the point of cell extraction, were obtained by sequential HPLC on Partisphere and Adsorbosphere SAX columns. A pooled sample from four separate electroporations was desalted and presented as substrate to the inositol phosphate 5-phosphatase activity of human erythrocyte ghosts. Aliquots of the assay mixture were removed at intervals and processed for HPLC. Inositol phosphate and Pi products were separated from unchanged InsP_3 substrate on a Partisphere SAX column. Fractions were collected and the ^{3}H and ^{32}P content of the products and reactants were determined by dual-label scintillation counting. The extent of reaction, percentage conversion of InsP_3 substrate to products, is presented for both ^{3}H (○) and ^{32}P (●) substrates in (**a**). A separation of the products of a separate assay, in which similar proportions of ^{3}H and ^{32}P substrate were converted into product, is shown in (**b**).

by spiking the sample with unlabelled ATP before HPLC. Even after considerable prepurification, the amount of putative [^{32}P]InsP_3 resolved by SAX HPLC was a vanishingly small proportion of total ^{32}P applied to the column. Preliminary experiments showed that the putative [^{32}P]InsP_3 was co-eluted with [^{3}H]Ins(1,4,5)P_3 added before HPLC.

The putative [^{32}P]InsP_3 peak when desalted and applied to an Adsorbosphere SAX column (Figure 2b) resolved into two components, one which was eluted substantially before ATP and one of which was eluted with a retention time characteristic of InsP_3s. Dual-label scintillation counting of peak fractions (Figure 2b inset) revealed the precise co-elution of ^{3}H and ^{32}P label in constant isotopic ratio over the peak fractions. The ratio of ^{32}P label between the two peaks resolved on Adsorbosphere SAX (Figure 2b) varied considerably between samples.

Subsequent analysis of the putative ^{3}H- and ^{32}P-containing InsP_3 fraction was undertaken by presenting a sample pooled from four separate electroporations, each containing approx. 2000–3000 d.p.m. of ^{32}P label, at the time of analysis, and approx. 3000–4000 d.p.m. of [^{3}H]Ins(1,4,5)P_3, to human erythrocyte ghosts under conditions of high $MgCl_2$ (10 mM) designed to facilitate removal of the 5-phosphate of Ins(1,4,5)P_3. The progress of the reaction is indicated in Figure 3(a). The results are presented as percentage conversion of InsP_3 to product, defined as loss of label from InsP_3 for both ^{3}H and ^{32}P label. Products corresponding to Ins$P$, P_i and InsP_2 were detected, although the poor resolution of the latter two in this experiment did not allow us to determine the relative labelling of these components. Nevertheless, the identical kinetics of conversion into products of the putative [^{32}P]InsP_3 with the co-purified [^{3}H]Ins(1,4,5)P_3 standard provides beyond reasonable doubt a rigorous identification of D-Ins(1,4,5)P_3. In a second experiment three independent preparations of [^{32}P]InsP_3 were converted into products to the same extent as the co-purified [^{3}H]Ins(1,4,5)P_3 internal standard. The results of one such analysis in which 62.7 % and 65.2 % of [^{3}H]Ins(1,4,5)P_3 and [^{32}P]InsP_3 respectively were converted into product are shown in Figure 3(b). The data reveal the precise co-elution of the [^{3}H]Ins(1,4)P_2 product with the [^{32}P]InsP_2 product on an HPLC gradient which was shown (Figure 5) to resolve all three potential InsP_2 products of dephosphorylation of Ins(1,4,5)P_3.

Analysis of labelling in individual phosphates of Ins(1,4,5)P_3

The dissection of Ins(1,4,5)P_3 with what we can confirm experimentally to be erythrocyte ghost inositol phosphate 5-phosphatase activity afforded an analysis of the distribution of label among the different phosphates. Thus in the experiment summarized in Table 1, in which we recovered 67.5 % and 68.5 % of the label in duplicate determinations in the 5-phosphate of PtdIns(4,5)P_2, we recovered 66.3 % of the label in the 5-phosphate of the pooled Ins(1,4,5)P_3 fractions. The similarity in the values is striking and establishes that the 5-phosphate of both PtdIns(4,5)P_2 and Ins(1,4,5)P_3 is added after the 1- and 4-phosphates.

In considering the metabolic origins of Ins(1,4,5)P_3 in plants it is worth considering, in the light of the work described in the Introduction, that Ins(1,4,5)P_3 may be the product of metabolism of higher inositol phosphates. The absence, to date, of InsP_4s with the 1,4,5 motif {only Ins(3,4,5,6)P_4, D- and/or L-Ins(1,2,3,4)P_4 and D- and/or L-Ins(1,2,5,6)P_4 have been identified *in vivo* with any rigour [5,6,27]} limits discussion of the potential precursors of Ins(1,4,5)P_3 to InsP_5s and InsP_6. There is metabolic evidence that InsP_6 is synthesized in plants by sequential phosphorylation of Ins(3,4,5,6)P_4 at the 1- and 2-positions [25]. Various groups have reported the identification of Ins(1,3,4,5,6)P_5 2-kinase activity and other activities in plants [41,42]. We have detected Ins(3,4,5,6)P_5 1-kinase activity in mesophyll protoplasts (C. A. Brearley, P. N. Parmar and D. E. Hanke, unpublished work). The consequence of all this is that, if Ins(1,3,4,5,6)P_5 or InsP_6 is the precursor of Ins(1,4,5)P_3, then under the short-term non-equilibrium labelling conditions of our experiment the 1-phosphate should be more strongly labelled than both the 4- and 5-phosphates. Clearly it is not (see Table 1), so we can discount such a possibility. Consistent with this interpretation is the fact that we did not observe products of [γ-^{32}P]ATP labelling with chromatographic mobilities of InsP_5s or InsP_6 (results not shown). Material with the approximate chromatographic mobility of InsP_4 was detected. This material was eluted after Ins(1,3,4,5)P_4 on Partisphere SAX HPLC, and is therefore not Ins(1,4,5,6)P_4, nor was it a substrate for erythrocyte ghosts under conditions in which the polyphosphate 3- and 5-phosphatase activities were functional. We have detected impurities in commercially available [γ-^{32}P]ATP with chromatographic properties similar to InsP_4. Other workers studying InsP_4 kinases have purified [γ-^{32}P]ATP before use [43].

In order to determine the labelling of the 1-phosphate of

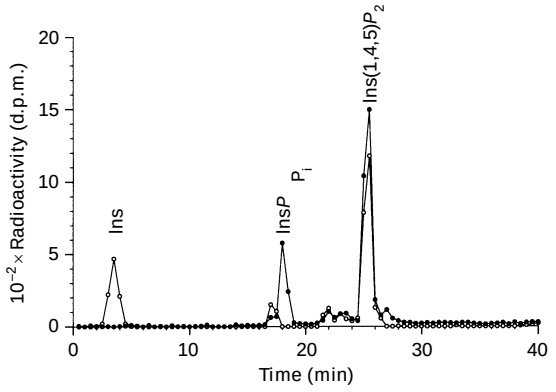

Figure 4 HPLC analysis of the products of alkaline phosphatase treatment of Ins(1,4,5)P_3 obtained from permeabilized mesophyll protoplasts

A [^{32}P]Ins(1,4,5)P_3 fraction which contained a 'spike' of [^{3}H]Ins(1,4,5)P_3, added at the point of cell extraction, was obtained by sequential HPLC on Partisphere and Adsorbosphere SAX columns. The sample was desalted and presented as substrate to alkaline phosphatase in an assay (0.52 ml volume) containing 10 mM Hepes/KOH, pH 7.5, 35 μM Ins(4,5)P_2 (obtained from Sigma) and 50 units of calf intestinal alkaline phosphatase (obtained from Boehringer, Lewes, Sussex, U.K.). An aliquot of the substrate was removed before the addition of enzyme for determination of the ^{32}P/^{3}H ratio. Incubation was at 25 °C for 1 min. The reaction was stopped by the addition of 5 μl of $HClO_4$ and the products were processed for HPLC. Products were resolved on a Partisphere SAX HPLC column eluted with a gradient of NaH_2PO_4 derived from buffer reservoirs A and B containing water and 2.5 M NaH_2PO_4 respectively. Time (min), percentage B: 0, 0; 5, 0; 65, 100. Fractions (0.5 min) were collected and the ^{3}H and ^{32}P contents of the products and reactants were determined by dual-label scintillation counting. ○, ^{3}H; ●, ^{32}P.

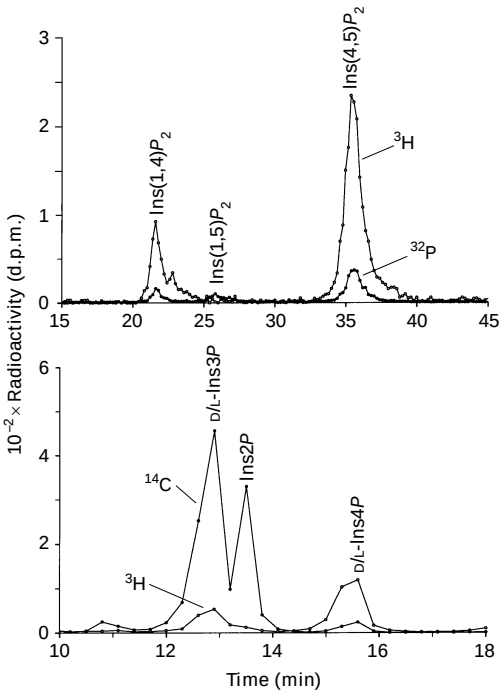

Figure 5 HPLC analysis of the products of metabolism of Ins(1,4,5)P_3 in permeabilized mesophyll protoplasts

(**a**) Mesophyll protoplasts were permeabilized in electroporation medium containing *myo*-[1-^{3}H]Ins(1,4,5)P_3 and 1 μM unlabelled Ins(1,4,5)P_3. Protoplasts were labelled for 5 min and extracted with $HClO_4$. The $HClO_4$ extract was neutralized and an aliquot (one-fifth of the neutralized sample) was mixed with authentic standards of [^{32}P]Ins(1,4)P_2, [^{32}P]Ins(1,5)P_2 and [^{32}P]Ins(4,5)P_2. The sample was applied to a Partisphere SAX HPLC column and eluted. The ^{3}H and ^{32}P contents of the column eluate were monitored by on-line scintillation counting (2 ml flow cell, 12 s integration interval). All steps were as described in the Materials and methods section. Upper trace, ^{3}H; lower trace, ^{32}P. Only that part of the chromatogram in which InsP_2s were eluted is shown. (**b**) An aliquot of the products of a separate electroporation experiment similar to (**a**) was mixed with authentic standards of D/L-[^{14}C]Ins3*P*, [^{14}C]Ins2*P* and D/L-[^{14}C]Ins4*P* applied to a Partisphere SAX column and eluted. Fractions were collected and counted for ^{3}H and ^{14}C by dual-label scintillation counting. Upper trace, ^{3}H; lower trace, ^{14}C. Only that part of the chromatogram in which inositol (Ins) and Ins*P*s were eluted is shown. We found no evidence for phosphorylation of Ins(1,4,5)P_3 in these experiments.

Ins(1,4,5)P_3 and hence whether it is added before or after the 4- and/or 5-phosphates, we examined the ^{32}P/^{3}H ratio of the products of alkaline phosphatase treatment of Partisphere SAX- and subsequent Adsorbosphere SAX-purified [^{32}P]Ins(1,4,5)P_3. Again, the labelled protoplasts from which [^{32}P]Ins(1,4,5)P_3 was extracted were spiked at the point of extraction with [^{3}H]Ins(1,4,5)P_3. The product of attack by alkaline phosphatase on Ins(1,4,5)P_3 is Ins(4,5)P_2. The results of this experiment are shown in Figure 4. An aliquot of the ^{3}H/^{32}P substrate was removed before addition of alkaline phosphatase for determination of the ^{32}P/^{3}H ratio of the starting material. The products of the assay were resolved by Partisphere SAX HPLC and reveal a single major InsP_2 product, Ins(4,5)P_2, and trace amounts of what we assume to be Ins(1,4)P_2 and Ins(1,5)P_2. The ^{32}P/^{3}H ratio of Ins(1,4,5)P_3 substrate was identical with that of Ins(4,5)P_2 product (1.27 compared with 1.28), indicating that the 1-phosphate makes no contribution to the ^{32}P labelling of Ins(1,4,5)P_3. The 1-phosphate is therefore added before both the 4- and 5-phosphates. Thus considering the other data in Table 1 (i.e. those obtained by dissection of a separate preparation of Ins(1,4,5)P_3 with erythrocyte ghost 5-phosphatase activity), we can say that the 1-phosphate is less strongly labelled than the 4-phosphate and the 4-phosphate less so than the 5-phosphate. We conclude that under these experimental conditions, which favour labelling of those inositol phospholipids and inositol phosphates that turn over rapidly, Ins(1,4,5)P_3 is the product *in vivo* of a PtdIns(1,4,5)P_2-directed PLC.

Our data are also inconsistent with production of Ins(1,4,5)P_3 from Ins(1,3,4,5,6)P_5 as demonstrated *in vitro* in *Dictyostelium* extracts [20]. Ins(1,3,4,5,6)P_5 is produced by 5-phosphorylation of Ins(1,3,4,6)P_4 *in vitro* in *Dictyostelium* extracts and Ins(1,3,4,6)P_4 by 1-phosphorylation of Ins(3,4,6)P_3 [24]. Thus, under our labelling conditions, and even assuming that mesophyll protoplasts can also synthesize Ins(1,3,4,5,6)P_5 in the same manner *in vivo*, there is no evidence yet that plants possess Ins(1,3,4,6)P_4 or a Ins(1,3,4,6)P_4 5-kinase, in which case the 1-phosphate would be more strongly labelled than the 4-phosphate. This is not the case (Table 1).

Metabolism of Ins(1,4,5)P_3 in electroporated plant cells

In order to characterize the *in vivo* routes of metabolism of Ins(1,4,5)P_3, mesophyll protoplasts were electroporated in the presence of [^{3}H]Ins(1,4,5)P_3. Electroporated protoplasts were quenched with $HClO_4$. The extracts were neutralized, mixed with ^{32}P-labelled standards and applied to a Partisphere SAX HPLC column (Figure 5a). Two peaks of InsP_2 product were detected on an HPLC gradient which resolved all three potential products of Ins(1,4,5)P_3 dephosphorylation. The major dephosphorylation product was Ins(4,5)P_2, and Ins(1,4)P_2 was a minor component. In the absence of any indication of phosphorylation of Ins(1,4,5)P_3 under these experimental conditions, we can be reasonably sure that the InsP_2s detected were the direct products of dephosphorylation of Ins(1,4,5)P_3. In which case the resolution of all three potential products by Partisphere SAX HPLC and

the co-elution of the products of [^{3}H]Ins(1,4,5)P_3 dephosphorylation with authentic standards of [^{32}P]Ins(1,4)P_2 and [^{32}P]Ins(4,5)P_2, but not Ins(1,5)P_2, identifies the InsP_2 products unambiguously. Analysis of the inositol monophosphate products of Ins(1,4,5)P_3 metabolism was performed on a Partisphere SAX HPLC column eluted isocratically with 40 mM NaH_2PO_4. The results (Figure 5b) show that the principal product was co-eluted precisely with an authentic standard of [^{14}C]Ins4P, prepared by acid treatment [16] of L-[^{14}C]Ins1P, and after both D/L-[^{14}C]Ins1P and [^{14}C]Ins2P. Without the inclusion of a standard of Ins5P we cannot be sure that Ins4P was resolved from Ins5P. A smaller amount of [^{3}H]Ins$P$ which was co-eluted with D/L-[^{14}C]Ins1P and hence which, considering that the starting material must be [^{3}H]Ins1P, was also detected. The presence of a sizeable peak, relative to the Ins$P$ products, of [^{3}H]inositol (Figure 5a) means that we are unable to say whether metabolism via Ins1P or Ins4P/Ins5P is the greater. Nevertheless, the predominance of Ins(4,5)P_2 and the products of further breakdown combined (Figures 5a and 5b) indicates that dephosphorylation via Ins(4,5)P_2 is the principal route of inactivation of the 'second messenger' Ins(1,4,5)P_3 in permeabilized protoplasts.

Insensitivity of Ins(1,4,5)P_3 dephosphorylation to LiCl

The inclusion in the electroporation medium of LiCl at concentrations up to 50 mM had little effect on either the 5- or 1-specific dephosphorylation of Ins(1,4,5)P_3. The ratio of the two products Ins(4,5)P_2/Ins(1,4)P_2, in one experiment, in the absence and presence of 0.1, 1, 10 and 50 mM LiCl, was 4.7, 4.8, 4.6 and 4.5 respectively. The lack of a marked effect of Li^+ was observed on all occasions. In many mammalian cells, e.g. in rat and mouse cerebral cortex, *in vivo* inhibition by millimolar concentrations of LiCl of the inositol phosphatase(s) responsible for recycling of inositol from Ins(1,4,5)P_3 occurs most markedly at the level of inositol monophosphate phosphatase [44]. Reductions in the agonist-stimulated accumulation of Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 as a consequence of reduced supply of inositol for inositol phospholipid synthesis is a corollary. The initial metabolism of Ins(1,4,5)P_3 in animal cells has been attributed to a 5-specific phosphatase and to a 3-kinase [45]. In contrast with lower plants [37], there is no evidence yet for Ins(1,4,5)P_3 3-kinase activity in higher plants; the identification of Ins(1,3,4)P_3 in stomatal guard cells [11] is questionable, and the lack of phosphorylation of Ins(1,4,5)P_3 in these experiments does not allow for such a possibility. The demonstration [46] that in LiCl-challenged muscarinic cholinoceptor-stimulated rat cerebral cortex slices much metabolism occurs via Ins(4,5)P_2 therefore offers an explanation of the general insensitivity to LiCl of the *in vitro* breakdown of Ins(1,4,5)P_3 in higher plants. Thus, in plants much *in vitro* breakdown of Ins(1,4,5)P_3 apparently proceeds via Ins(4,5)P_2, and most studies [47–49] indicate that such breakdown, and also that which proceeds via Ins(1,4)P_2, is Li^+-insensitive. The apparent Li^+-insensitivity of Ins(4,5)P_2 production from Ins(1,4,5)P_3 suggests that the plant Ins(1,4,5)P_3 1-phosphatase observed here shows similar properties to the activity in rat cerebral cortex [46]. A caveat not favoured by the authors [46], must be introduced here that the production of Ins(4,5,)P_2 in rat cerebral cortex may originate from Ins(1,3,4,5)P_4 and not Ins(1,4,5)P_3, in which case we may not be studying comparable enzyme activities. Alternatively, the uncompetitive nature of inhibition by Li^+ of Ins(1,4)P_2/Ins(1,3,4)P_3 1-phosphatase [45] and inositol monophosphate phosphatase [50] has the consequence that inhibition is only manifested at high substrate concentrations [44,45]. Consequently, if the Ins(1,4,5)P_3 1-phosphatase and 5-phosphatase activities detected here were uncompetitively inhibited by Li^+ then the inhibition might not be manifested at the substrate concentrations employed here. However, the insensitivity to Li^+ of the Ins(1,4,5)P_3 1-phosphatase activity assayed at micromolar substrate concentration in homogenates of *Nicotiana tabacum* [47] and pea roots [48] argues against this possibility.

In order to test the possibility that the levels of substrate were below that at which uncompetitive inhibition is manifested, we performed additional experiments at supraphysiological levels of Ins(1,4,5)P_3. Thus, when protoplasts were permeabilized in the presence of 10 μM and even 100 μM Ins(1,4,5)P_3 at 10 mM LiCl, there was no difference in the pattern of inositol phosphates produced between the respective controls and LiCl treatments (results not shown). We conclude that the Ins(1,4,5)P_3 1- and 5-phosphatase activities detected here are insensitive to Li^+.

That plant enzymes responsible for the dephosphorylation of lipid-derived inositol phosphates might be Li^+-sensitive was recently suggested. A gene with homology to the inositol monophosphate phosphatase of bovine brain [51] was cloned in tomato [52]. The authors were careful to point out that, from sequence comparison, it was not possible to identify the cloned gene as that encoding an inositol monophosphate phosphatase specifically. The gene product was expressed in *Escherichia coli* and, in an assay that measured dephosphorylation of D-[^{14}C]Ins3P (L-[^{14}C]Ins1P), dephosphorylation was found to be Li^+-sensitive. The activity was only tested against one inositol phosphate substrate and one which, from an Ins(1,4,5)P_3 signalling perspective, in higher plants at least considering the lack of Ins(1,4,5)P_3 3-kinase, is not physiologically relevant. That is to say Ins3P is the enantiomer of the physiologically relevant substrate Ins1P. It has also been shown that there are multiple inositol monophosphatase activities in *Dictyostelium discoideum* which differ in their substrate specificities and sensitivities to inhibition by Li^+ [53]. Ins3P is the product of D-*myo*-inositol 3-phosphate synthase and so it is possible that the enzyme encoded in tomato by the cloned gene family is responsible for dephosphorylation of Ins3P and the *de novo* synthesis of inositol rather than the recycling of inositol from signalling lipid turnover. Thus the physiological significance of the gene cloned in tomato remains to be established. The high levels of inositol in plant cells may obviate the requirement for rapid and direct recycling of inositol. Also, it remains to be established directly that a 'phosphatidylinositol cycle' exists in plants.

DISCUSSION

We have described in the foregoing, a cell permeabilization strategy that when coupled to exhaustive analytical techniques, allows the detection of labelled D-Ins(1,4,5)P_3 in plant cells. This, more than the description of lipid metabolism evident in the labelling of inositol phospholipids and their individual phosphate esters, validates permeabilized plant cells as a system for the study of plant signal transduction.

We have previously shown for guard cells [13] and *Spirodela polyrhiza* (mesophyll cells predominantly [35]) that PtdIns4P and PtdIns(4,5)P_2 are components of a substrate cycle. Thus, in intact cells, the metabolic flux PtdIns → PtdIns4P → PtdIns(4,5)P_2, measured by increases in the labelling of the diester of PtdIns(4,5)P_2, was a fraction of that in the PtdIns4P–PtdIns(4,5)P_2 couple [35]. With few identifications of Ins(1,4,5)P_3 to date in higher plants, direct comparisons of the levels of PtdIns(4,5)P_2 and Ins(1,4,5)P_3 are largely impossible. Moreover, measurements of Ins(1,4,5)P_3 production by receptor-binding assay are not reconcilable with the radiolabelling methods used

to identify PtdInsP_2, as the units of measurement are different. Thus a causal relationship between lipid metabolism and Ins(1,4,5)P_3 production has not been established by these studies.

Returning to the current data, at the time of erythrocyte ghost treatment, approx. 2 weeks after extraction and analysis of lipids indicated in Figure 1, after considerable prepurification and associated losses of Ins(1,4,5)P_3, we presented approx. 13000 d.p.m. of [^{3}H]Ins(1,4,5)P_3 and 10000 d.p.m. of [^{32}P]Ins(1,4,5)P_3 to a preparation of erythrocyte ghosts. For the four independent preparations of Ins(1,4,5)P_3 pooled for analysis, this represents a recovery of [^{3}H]Ins(1,4,5)P_3 of approx. 25%. Taking into account the half-life of ^{32}P, the percentage recovery of Ins(1,4,5)P_3 and a total of ^{32}P label recovered in PtdIns(4,5)P_2, it is evident that labelled Ins(1,4,5)P_3 is in considerable (approx. 25-fold) excess over PtdIns(4,5)P_2. It is likely, although it has not been tested here, that the elevation of [^{32}P]Ins(1,4,5)P_3 to levels in considerable excess of [^{32}P]PtdIns(4,5)P_2 is a consequence of the 'trapping' of label in a large pool of unlabelled Ins(1,4,5)P_3. The corollary of this is that the apparently low level of Ins(1,4,5)P_3 in plants cells [7], not withstanding the dearth of stereochemical descriptions of inositol phosphates in plant tissues, is a consequence of rapid metabolism of Ins(1,4,5)P_3 by inositol phosphate phosphatases.

The *in vivo* production and metabolism of Ins(1,4,5)P_3 by 1- and 5-specific dephosphorylation demonstrated here confirms the expectations of the strictly *in vitro* approach to Ins(1,4,5)P_3 breakdown displayed in earlier studies and may yet afford an opportunity to compare PtdIns(4,5)P_2 turnover and Ins(1,4,5)P_3 production under stimulatory and non-stimulatory regimes. Considering the observations of Van Haastert and co-workers [18–20] of other potential routes to Ins(1,4,5)P_3 in *Dictyostelium* and rat liver and our lack of knowledge of how PLCδ is activated in animals or plants, we still lack direct *in vivo* evidence linking a physiologically relevant signal to the stimulated production specifically from PtdIns(4,5)P_2 of enantiomerically characterized D-Ins(1,4,5)P_3 in higher plants.

Although a recent report [11] raises the possibility that Ins(1,4,5)P_3 production in stomatal guard cells is activated by physiological signals, e.g. abscisic acid, the circumstantial observation that increases in Ins(1,4,5)P_3, measured with a radioreceptor-binding assay, correlated with reduction in levels of ^{32}P labelling in PtdInsP_2, is compromised by the less than secure identification of PtdInsP_2 as PtdIns(4,5)P_2. It has been shown under short- and long-term [^{32}P]P_i labelling conditions that the predominant PtdInsP_2 isomer in *Commelina* guard cells is not PtdIns(4,5)P_2 [13,14]. Thus, despite the observation that the products of mild alkaline hydrolysis of total [^{32}P]lipid were co-eluted with the products of co-hydrolyzed authentic [^{3}H]PtdIns(4,5)P_2, the HPLC separation of the products (Figure 2 of [11]) was of such low resolution that it is not likely that the experiment could distinguish between the products of hydrolysis of different PtdInsP_2 isomers. It is also surprising that hydrolysis generated greatly different ratios of Ins(1,4,5)P_3/GroPIns(4,5)P_2 product for ^{32}P lipid and co-hydrolysed [^{3}H]PtdIns(4,5)P_2. The proportions should be the same if the two nuclides label the same compound.

The report also claimed to distinguish abscisic acid-induced changes in the levels of [^{32}P]P_i-labelled Ins(1,3,4)P_3, Ins(1,4,5)P_3 and Ins(1,4)P_2 in guard cells (Table 1 of [11]). Again, the inability of the authors to resolve Ins(1,3,4)P_3 from Ins(1,4,5)P_3 (Figure 6a of [11]) and the identification of these compounds solely by co-elution with ^{3}H standards on low-resolution HPLC leaves some doubt as to the identity of the labelled compounds. Surprisingly, Ins(1,4)P_2 was identified by comparison with previous (1987) chromatograms. We would expect [^{32}P]ATP to be prominent in the chromatograms and in vast excess of inositol phosphates, as has been shown for guard cells of *Commelina* [13].

In experiments quite independent of the [^{32}P]P_i labelling described above, the authors report that unlabelled guard-cell-derived Ins(1,4,5)P_3, purified by binding to a crude Ins(1,4,5)P_3 receptor preparation, was converted by erythrocyte ghosts into product with similar kinetics to endogenous Ins(1,4,5)P_3 present in the ghost preparation. Without knowing what contribution the guard-cell-derived Ins(1,4,5)P_3 made to the endogenous Ins(1,4,5)P_3 in the ghost preparation (this information was not provided), it is difficult to assess whether the guard-cell-derived Ins(1,4,5)P_3 made any contribution at all to the total Ins(1,4,5)P_3 breakdown. If it made no contribution, then the kinetics of breakdown would have been the same. Significantly, measurements of Ins(1,4,5)P_3 content of guard cells treated with abscisic acid were performed on whole cell extracts without purification of the extract by receptor binding or alternative means. Thus it is not clear whether other cellular components, whether labelled or not, interfered with the assay.

Returning to the present data, the establishment of a technique for the identification of Ins(1,4,5)P_3 affords an alternative strategy to the use of InsP_3-receptor-binding assays for measurement of Ins(1,4,5)P_3 and so avoids the potential problem of the validity of such assays for measurement of a single InsP_3 against a background of numerous other inositol phosphates, some of which, e.g. Ins(2,4,5)P_3 which is one of the major InsP_3 isomers by mass in avian erythrocytes [40], are potent mobilizers of Ca^{2+} [54]. Given how little we know about plant inositol phosphate metabolism, and what we do know suggests that Ins(1,4,5)P_3 is only a tiny part of this, the establishment of alternative techniques for the investigation of Ins(1,4,5)P_3 metabolism that are not compromised by questions of inositol phosphate identity is a useful development.

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