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Phosphoinositides in Barley (*Hordeum vulgare* L.) Aleurone Tissue[1]

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[^{3}H]Inositol labeling of barley (*Hordeum vulgare* L. cv Himalaya) aleurone layers and analysis of phospholipids by deacylation revealed the presence of phosphatidylinositol (PtdIns), PtdIns3P, and PtdIns4P but not PtdInsP_2 species. In contrast to an earlier report (P.P.N. Murthy, G. Pliska-Matyshak, L.M. Keranen, P. Lam, H.H. Mueller, N. Bhuvarahamurthy [1992] Plant Physiol 98: 1498–1501) systematic chemical degradation of PtdIns revealed no evidence of a second isomer of PtdIns. Evidence of the widespread occurrence of 3-phosphorylated PtdIns within the plant kingdom is presented.

The discovery of 3-phosphorylated PtdIns in *Spirodela polyrhiza* (Brearley and Hanke, 1992, 1993), stomatal guard cells of *Commelina communis* (Parmar and Brearley, 1993), and *Chlamydomonas* (Irvine et al., 1992) gives new urgency to the study of phosphoinositides in plants and raises questions as to the function of these molecules within the plant kingdom. Their discovery also brings to the fore the repeated calls (Irvine, 1990a, 1990b; Brearley and Hanke, 1992; Drøbak, 1992) for utmost rigor in the identification of plant phosphoinositides. With some exceptions (Coté et al., 1989; Irvine et al., 1989, 1992; Brearley and Hanke, 1992; Quarmby et al., 1992; Brearley and Hanke, 1993; Parmar and Brearley, 1993), few studies have used chromatographic techniques capable of resolving the different isomers of phosphoinositides from each other, let alone from other inositol- and phosphate-containing lipids or the products of their dissection. This is not a trivial matter and cannot presently be excused on the grounds of very low levels of 3-phosphorylated lipids relative to 4-phosphorylated counterparts because reliable measurements of the chemical levels of these compounds have not been made, nor have conditions of equilibrium labeling been met. It is significant then that in stomatal guard cells (Parmar and Brearley, 1993) the predominantly labeled PtdInsP_2 is PtdIns(3,4)P_2 and not PtdIns(4,5)P_2 and that in fungi (Auger et al., 1989; Lakin-Thomas, 1993) PtdIns3P is at least as abundant as PtdIns4P. Consequently, the extent of the presence of 3-phosphorylated PtdIns within the plant kingdom is undefined, a question of some relevance to their function.

We regard the barley (*Hordeum vulgare* L.) aleurone layer as a prime candidate for the study of phosphoinositide involvement in signal transduction. The tissue has the advantage of being a single-cell type, readily available in reasonable quantities, showing well-defined physiological and biochemical responses to several plant-growth regulators. That the tissue is nonmeristematic allows us to investigate whether 3-phosphorylated PtdIns are restricted to meristematic tissues. Thus, given an early report (Murthy et al., 1989) of GA_3-stimulated turnover of PtdIns in barley aleurone and the reasons stated above, we consider that the biochemistry of phosphoinositide metabolism in this tissue deserves further study. In doing so we have also addressed a report (Murthy et al., 1992) describing evidence for the presence of two isomers of PtdIns in barley aleurone layers.

MATERIALS AND METHODS

Isolation and Labeling of Aleurone Tissue

Aleurone tissue was isolated by the method of Jacobsen et al. (1985) with modifications. Seeds of barley (*Hordeum vulgare* L. cv Himalaya, 1993 harvest) obtained from Cambridge University Botanic Gardens (Cambridge, UK) were de-embryonated and surface sterilized in bleach (2.5% available chlorine) for 40 min. The de-embryonated grains were washed five times in sterile water and allowed to imbibe for 3 d under aseptic conditions on filter paper in 9-cm Petri dishes containing 5 mL of 10 mM L-Arg, 20 mM $CaCl_2$, and 20 μM chloramphenicol.

Aleurone layers from 30 grains were manually isolated from the starchy endosperm under aseptic conditions. Isolated aleurone layers were incubated at 25°C in the dark for 40 h in 1.6 mL of a medium comprising 20 mM succinate buffer (pH 5.0 with NaOH, 25°C), 20 mM $CaCl_2$, and 20 μM chloramphenicol and containing 100 μCi of *myo*-[2-^{3}H]inositol (Amersham).

[1] This work was supported by grants from the Science and Engineering Research Council (UK) and Agriculture and Food Research Council (UK). Nomenclature: The positions of phosphate substituents in InsP are numbered with reference to the D-1 substitution site. The D/L-prefix is used where the InsP exist as racemic mixtures of the two enantiomers and the D- and/or L- prefix occurs when the mixtures of enantiomers are undefined.

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Abbreviations: GroPIns, glycerophosphoinositol; GroPIns3P, glycerophosphoinositol 3-phosphate; GroPIns4P, glycerophosphoinositol 4-phosphate; GroPIns(3,4)P_2, glycerophosphoinositol 3,4-bisphosphate; GroPIns(4,5)P_2, glycerophosphoinositol 4,5-bisphosphate; GroPIns(3,4,5)P_3, glycerophosphoinositol 3,4,5-trisphosphate; InsP, inositol phosphate(s); PtdIns, phosphatidylinositol(s); SAX, strong anion exchange.

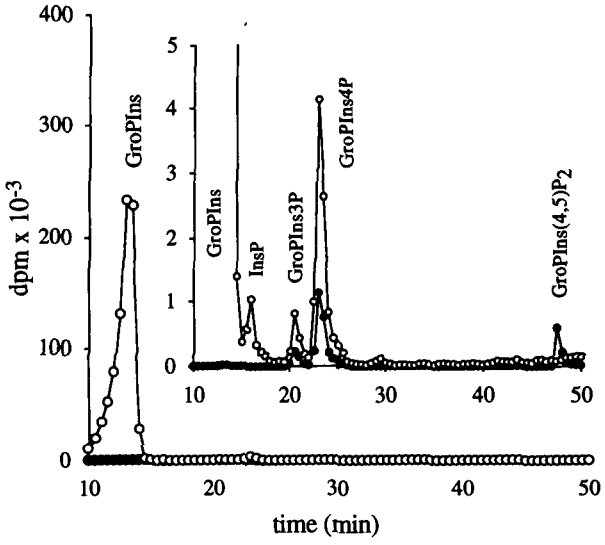

Figure 1. Anion-exchange separation of deacylation products from [^{3}H]inositol-labeled barley aleurone layers. Lipid extracts were deacylated, and the water-soluble products were mixed with [^{32}P]-GroPIns3P, [^{32}P]GroPIns4, and [^{32}P]GroPIns(4,5)P_2, obtained from [^{32}P]Pi-labeled *S. polyrhiza*, and applied to a Partisphere SAX HPLC column. Fractions, 0.5 min, were collected, and radioactivity was measured by dual-label scintillation counting. O, ^{3}H; ●, ^{32}P. The inset shows the same separation on a different scale.

Tissue Extraction

After labeling, aleurone layers were washed with water, blotted dry, weighed, and frozen in liquid N_2. At this stage an aliquot (50 μL) of phytate hydrolysate (40 μg of phosphorus, prepared according to the method of Wregget and Irvine, 1987) was added to minimize losses of InsP due to nonspecific binding during extraction. The tissue was ground in a liquid N_2-cooled mortar and pestle and extracted with 0.7 mL of 3.5% (w/v) $HClO_4$. The debris was pelleted by centrifugation at 13,000g_{max} for 5 min in a refrigerated microfuge.

Lipids were extracted from the cell debris, deacylated, and deglycerated by the methods described previously (Brearley and Hanke, 1993).

RESULTS

Deacylation of phospholipid extracts from [^{3}H]inositol-labeled aleurone tissue gave the profile detailed in Figure 1 on SAX HPLC. The inclusion of authentic standards of ^{32}P-labeled GroPIns3P, GroPIns4P, and GroPIns(4,5)P_2 obtained from *S. polyrhiza* allowed us to confirm an earlier report (Murthy et al., 1989) of the presence of PtdIns4P in barley aleurone cells. We have also revealed the likely presence of PtdIns3P, but at the level of labeling achieved we were unable to confirm the presence of PtdIns(4,5)P_2. The figure also reveals the presence of a trace of InsP, representing less than 0.1% of the radioactivity in the GroPIns peak and generated presumably by a side reaction during deacylation of the parent phospholipid. This peak was identified as InsP by nature of the fact that it eluted soon after the AMP internal standard (not shown in Fig. 1), whereas GroPIns elutes substantially before AMP on Partisphere SAX columns.

Conclusive identification of PtdIns was obtained by mild periodate treatment of an aliquot of the putative GroPIns peak identified in Figure 1. When mixed with authentic standards of D/L-[^{14}C]inositol-3-phosphate and inositol-2-phosphate and resolved on the isocratic HPLC system of Wregget and Irvine (1989), the product of deglyceration co-eluted precisely with D/L-inositol-3-phosphate and was eluted before inositol-2-phosphate (Fig. 2). The identification of only a single peak of D- and/or L-inositol-3-phosphate in this study is in contrast with the report of Murthy et al. (1992). Analysis of deacylated lipid extracts from barley aleurone protoplasts and wheat aleurone tissue also revealed only a single peak of labeled material with the chromatographic properties of GroPIns (our unpublished results).

Given the level of labeling achieved in our study and a conservative estimate of the lower limit of detection for a peak of approximately 800 dpm above background, we can conclude that, if another resolvable isomer of GroPIns were present, it must represent less than 0.1% of the label in the conventional isomer derived from 1,2-diacylglycero-3-(D-*myo*-inositol-1-phosphate).

These methods do not allow us to distinguish between alternative structures of the parent lipid, namely 1,2-diacylglycero-3-(D-*myo*-inositol-1-phosphate) or the entirely theo-

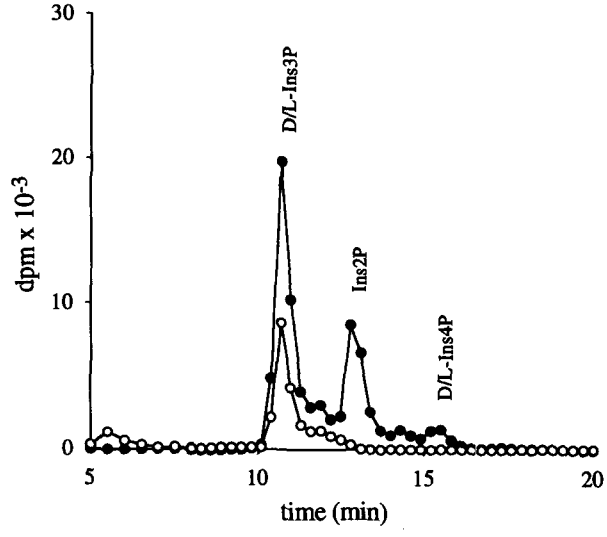

Figure 2. Anion-exchange separation of products of mild periodate treatment of putative [^{3}H]GroPIns. A putative [^{3}H]GroPIns obtained by deacylation of a lipid extract from [^{3}H]inositol-labeled aleurone tissue was treated with periodate under conditions designed to remove glycerol moieties. The products were desalted, mixed with D/L-[^{14}C]inositol-3-phosphate (D/L-Ins3P), [^{14}C]inositol-2-phosphate (Ins2P), and D/L-[^{14}C]inositol-4-phosphate (D/L-Ins4P), applied to a Partisphere SAX HPLC column, and eluted with 40 mM-NaH_2PO_4. Fractions, 0.5 to 8 min and 0.3 min thereafter, were collected, and radioactivity was measured by dual-label scintillation counting. O, ^{3}H; ●, ^{14}C. An increase in the phosphate concentration of the eluant to 240 mM, sufficient to elute inositol bisphosphates, failed to resolve any other peaks of inositol-labeled material. This analysis has been performed twice on independent tissues.

retical 1,2-diacylglycero-3-(D-*myo*-inositol-3-phosphate). Both of these structures would give monophosphates that form the respective members of an enantiomeric pair, namely D-inositol-1-phosphate and D-inositol-3-phosphate, on deacylation and subsequent deglyceration. These enantiomers are not resolved by SAX HPLC. The HPLC trace also shows a minor peak of what we assume to be D/L-[^{14}C]inositol-4-phosphate obtained by limited trans-migration of phosphate on acid treatment of the parent [^{14}C]inositol-3-phosphate.

DISCUSSION

It is becoming clear through the application of rigorous techniques (Brearley and Hanke, 1992, 1993; Parmar and Brearley, 1993) that, with the exception of PtdIns(3,4,5)P_3 identified in stimulated animal cells, higher plants possess the entire complement of phosphoinositides commonly observed in animal cells. It is intriguing that a recent report (Murthy et al., 1992) identifies a novel isomer of PtdIns, containing either a phosphodiester linkage to the 5 position of *myo*-inositol or alternatively an inositol isomer other than *myo*-inositol in barley aleurone tissue. It seems to us that the experimental data do not necessarily substantiate the conclusion.

One-dimensional TLC was used to purify PtdIns. Subsequent deacylation and HPLC yielded two peaks of putative GroPIns, which were treated with periodate under conditions designed to remove glycerol moieties. The earlier eluting of the two GroPIns peaks yielded a single peak of labeled material that co-eluted precisely with authentic [^{3}H]inositol-1-phosphate. Few would argue, on the grounds of the chromatographic data presented, that the first of the two putative GroPIns peaks is anything other than GroPIns derived from 1,2-diacylglycero-3-(D-*myo*-inositol-1-phosphate).

The second eluting of the two GroPIns peaks yielded on periodate treatment a single peak of radiolabeled material that eluted after an authentic standard of [^{3}H]inositol-4-phosphate. It is significant then that it has been reported (Shears, 1989; Menniniti et al., 1990) that on Partisil SAX columns inositol-5-phosphate elutes before inositol-4-phosphate. This observation therefore precludes the possibility that that parent lipid contains the partial structure inositol-5-phosphate.

The alternative conclusion that the parent lipid may contain an isomer of inositol other than *myo*-inositol is equally not supported by the data presented. The failure to include relevant standards and the absence of confirmatory structural information, afforded by co-elution with these standards on treatment designed to deacylate and deglycerate, throws some doubt on the identification of the parent lipid as a PtdIns. TLC alone is not sufficient for the rigorous identification of plant inositol phospholipids (Irvine, 1990a, 1990b; Brearley and Hanke, 1992; Drøbak, 1992). Compounds that are clearly not PtdIns, which label from both [^{3}H]inositol and ^{32}Pi, have been described in tobacco leaves (Kaul and Lester, 1975, see refs. therein to the work of Carter) and in fungi (Lester et al., 1974). Others that share the chromatographic properties of inositol phospholipids have been observed in plants (Drøbak et al., 1988) and in animals, e.g. in the work of Larner's group, which is described below.

In view of the forgoing arguments we believe that the data presented do not in themselves warrant the specific claim that there is a second isomer of PtdIns in barley aleurone. The possibility, however, cannot be discounted that *chiro*-inositol-containing PtdIns may yet be found in plants.

A precedent is set for the existence of *chiro*- (but not *neo*- or *scyllo*-), inositol-containing glycans and possibly lipids in various animal tissues by the work of Larner and co-workers (Pak and Larner, 1992; Pak et al., 1992a, 1992b; Asplin et al., 1993). Nevertheless, the extent of the structural analysis of such molecules does not yet match that of conventional PtdIns or that of some of the *myo*-inositol-containing PtdIns glycans of protozoa and higher eukaryotes (see review by McConville and Ferguson, 1993). Specifically, the nature of the chemical linkage between the component parts of putative *chiro*-inositol-containing PtdIns has not been established. We suggest, therefore, that any reference to *chiro*-inositol-containing PtdIns must be speculative.

Bearing in mind all of these limitations and in contrast to the previous report (Murthy et al., 1992), our experiments revealed only a single isomer of GroPIns. On mild periodate treatment this compound yielded a single peak of inositol monophosphate, which co-eluted with authentic D/L-[^{14}C]-inositol-3-phosphate and before inositol-2-phosphate and D/L-inositol-4-phosphate. This result is consistent with the existence of only one isomer of PtdIns.

We are in agreement with Murthy et al. (1989) that the the level of labeling in PtdIns4P is only a small fraction of that achieved in PtdIns. We were unable at the level of labeling achieved to conclusively identify any PtdInsP_2 species in aleurone tissue. Our failure to detect, and the failure of Murthy et al. (1989) to label, PtdInsP_2 appreciably, would seem to preclude the study of PtdInsP_2 in barley aleurone tissue. We have by the use of HPLC and the inclusion of authentic standards revealed the likely presence of PtdIns3P. This observation is important because only by the adoption of rigorous approaches to phosphoinositide analysis does it become apparent (see below) that 3-phosphorylated lipids are as ubiquitous as their 4-phosphorylated counterparts.

The likely presence of PtdIns3P in barley aleurone and the confirmed presence of 3-phosphorylated PtdIns in stomatal guard cells clearly discount the possibility that 3-phosphorylated PtdIns are restricted to meristematic tissues in higher plants. This raises the possibility of the comparative study of their function in two nondividing tissues that are both responsive to the plant-growth regulator ABA but that have very different functions. That the two tissues are nonmeristematic affords the advantage that any evidence of phospholipid or InsP turnover can be divorced from any changes in metabolism associated with growth, cell division, and large-scale developmental changes. Clearly, 3-phosphorylated PtdIns show a wide phylogenetic range between higher and lower kingdoms. Although rigorous analysis is currently restricted to monocots, we also have evidence (not published) of deacylation products of inositol-containing lipids from soybean and mung bean with the chromatographic properties of authentic GroPIns3P. Moreover, within monocots 3-phosphorylated PtdIns are present in two highly specialized and terminally differentiated tissues.

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