Plant Physiol. (1989) 91, 1266–1269
0032-0889/89/91/1266/04/$01.00/0

Received for publication April 25, 1989
and in revised form August 25, 1989

Communication

Phosphoinositides in Barley Aleurone Layers and Gibberellic Acid-Induced Changes in Metabolism[1]

Pushpalatha P. N. Murthy*, Jill M. Renders, and Lisa M. Keranen

Department of Chemistry and Chemical Engineering, Michigan Technological University, Houghton, Michigan 49931

ABSTRACT

Phospholipids of barley (*Hordeum vulgare* L. cv Himalaya) aleurone layers were labeled with *myo*-[2-^{3}H]inositol or [^{32}Pi], extracted, and analyzed by physical (chromatography) and chemical (deacylation) techniques. Three phospholipids were found to incorporate both *myo*-[2-^{3}H]inositol and [^{32}Pi]—phosphatidylinositol, phosphatidylinositol-monophosphate, and phosphatidylinositol-bisphosphate. Stimulation of [^{3}H]inositol prelabeled aleurone layers with GA_3 showed enhanced incorporation of label into phosphatidylinositol within 30 seconds and subsequent rapid breakdown. Stimulation of phosphatidylinositol labeling observed in these studies is the earliest response of aleurone cells to gibberellic acid reported.

The plant hormone gibberellin is involved in the regulation of a number of diverse processes, including germination, shoot elongation, and fruit formation. Gibberellin-induced α-amylase synthesis and secretion during germination has been extensively studied and the involvement of Ca^{2+} in this process is well established (4, 12, 14). However, the molecular mechanism of hormone perception, message transduction, and biochemical regulation is unknown.

Membrane-bound phosphoinositides mediate signal transduction across cell membranes in a wide variety of animal cells (3, 6). In these systems, ligand-receptor interaction on the extracellular face of the membrane leads to activation of a membrane-bound phospholipase-C which cleaves $PtdInsP_2$[2] to diacylglycerol and Ins(1,4,5)P_3. Ins(1,4,5)P_3 releases calcium from endoplasmic reticulum, thereby increasing intracellular free calcium levels. At these elevated levels, calcium modifies enzyme activity by binding directly or as a Ca^{2+}-calmodulin complex. In addition, the released diglyceride activates protein kinase-C.

[1]Supported by a grant from the National Science Foundation (DCB-8609038).

[2]Abbreviations: $PtdInsP_2$, phosphatidylinositol bisphosphate; Ins(1, 4, 5)P_3, *myo*-inositol-1,4,5-trisphosphate; PtdInsP, phosphatidylinositol phosphate; PtdCh, phosphatidylcholine; LPtdCH, lysophosphatidylcholine; LPtdIns, lysophosphatidylinositol; PtdIns, phosphatidylinositol; GroPIns, glycerophosphoinositol; GroPInsP, glycerophosphoinositol phosphate; $GroPInsP_2$, glycerophosphoinositol bisphosphate.

The presence of polyphosphoinositides, PtdInsP and $PtdInsP_2$, has been shown in a number of plant cells, including carrot (5, 8, 22), *Samanea saman* (18), *Catharanthus roseus* (10), cultured parsley (20), and soybean cells (20). More recently, Irvine *et al.* (11) have reported on the presence of phosphatidylinositol-(4)-phosphate and phosphatidylinositol-(4,5)-bisphosphate in pea leaf discs. There is evidence to show that $PtdInsP_2$ is involved in signal transduction in *S. saman* (19) and *C. roseus* (9).

We are interested in the question of whether polyphosphoinositides are involved in gibberellin action. In this paper we describe the incorporation of [^{3}H]inositol and [^{32}Pi] into phospholipids of barley (*Hordeum vulgare* L. cv Himalaya) aleurone layers and the effect of GA_3 on prelabeled PtdIns.

MATERIALS AND METHODS

Labeling with Radioactive Precursors

Barley seeds (*Hordeum vulgare* L. cv Himalaya, 1979) were deembryonated, surface-sterilized (for 30 min in 1% [w/v] NaOCl, made by 5-fold dilution of commercial bleach), washed with sterile water, and allowed to imbibe on wet sterile sand for 4 d at room temperature. To label the *myo*-inositol-derived compounds with tritium, aleurone layers were removed from imbibed seeds as described by Chrispeels and Varner (7) and incubated in solution (2 mL per 5 layers) containing 20 mM succinate buffer (pH 5.0), 20 mM $CaCl_2$, and [2-^{3}H]inositol ([^{3}H]inositol from American Radiolabeled Chemicals, Inc. was purified just before use, by passing through a column containing 1 mL of Dowex-1, formate form, and eluting with water) at 25°C. To label Pi-derived compounds with ^{32}P, [^{32}Pi] (20 μCi per 5 layers; ^{32}Pi in 0.02 N HCl [New England Nuclear] was neutralized in 0.02 N NaOH just before use) was used instead of [^{3}H]inositol. The radioactive medium was removed, and the layers were washed free of radioactivity. To determine the effect of GA_3, aleurone layers prelabeled with [^{3}H]inositol were incubated in media containing succinate buffer (20 mM; pH 5.0) and $CaCl_2$ (20 mM) with or without 5 μM GA_3. In addition, GA_3-triggered α-amylase secretion was measured in each experiment to establish that the aleurone layers were physiologically responsive (7). Details of each experiment appear in figure legends.

Phospholipid Extraction and Separation

Phospholipids were extracted by modified acidic Bligh-Dyer method (1). Ice-cold methanol, 2.0 mL, was added to the aleurone layers and the solution frozen in liquid N_2. The thawed solution was sonicated and 1.7 mL chloroform:methanol (1:2, v/v), 1.1 mL chloroform, and 0.5 mL of 2.4 N HCl were added sequentially. The aleurone layers were then ground in a homogenizer with a little sand. The aqueous and organic layers were separated by centrifugation, the lower layer was withdrawn, and the upper aqueous layer was reextracted with 1.1 mL $CHCl_3$. The two organic extracts were combined and washed with 2.5 mL of 50% aqueous methanol and 0.3 mL of 2.4 N HCl. The organic phase was separated and dried under N_2 and the phospholipids mixed with standard phospholipids and redissolved in 100 μL chloroform:methanol (1:2, v/v). A 10 μL aliquot was removed for counting and the rest was divided into two parts. Each part was chromatographed on a potassium oxalate-coated silica-gel plate (13) and developed in an acidic solvent system, $CHCl_3$:CH_3OH:CH_3COCH_3:H_2O:CH_3CO_2H (80:26:30:14:24, v/v) or a basic solvent system, $CHCl_3$:CH_3OH:NH_4OH:H_2O (90:90:8:22, v/v). The standards were visualized with phosphomolybdate spray (15), with Dragendorf reagent (15) (to visualize PtdCh and LPtdCh), and by charring. ^{32}P-Labeled phospholipids from barley aleurone layers were revealed by autoradiography. In the case of [^{3}H]inositol labeled phospholipids, standards were visualized by phosphomolybdate spray, and regions corresponding to the standards were scraped and counted, or in some cases, the plate was scraped in 0.5 cm sections and counted by liquid scintillation.

Deacylation of Radiolabeled Phospholipids

Deacylation was carried out according to Berridge (2). [^{3}H] Inositol-labeled phospholipids from barley aleurone layers were mixed with a mixture of PtdIns, PtdInsP, and $PtdInsP_2$ (3 mg total) and treated with 1.5 mL of methylamine: methanol:water:*n*-butanol (5:4:3:1, v/v) at 53°C for 30 min. To the concentrated residue was added 2 mL of water, and the fatty acid amides were extracted twice with 2.4 mL of *n*-butanol:hexane:ethyl formate (20:4:1, v/v). The aqueous layer was concentrated, and the product glycerophosphoinositides were separated by high voltage paper electrophoresis on Whatman No. 1 sheets in 0.06 M sodium oxalate buffer (pH 1.5) at 4000 V for 15 min in a Gilson HVE apparatus (1). GroPIns, GroPInsP, and $GroPInsP_2$ were identified by comigration with standards.

Data Manipulation

Because it was not altogether clear which normalization data are best, data were normalized in three ways—normalization to cell protein, normalization to cpm in lipid-soluble extract, and normalization to cpm in all inositol containing compounds, lipids, and inositol phosphates. The percentage of stimulation compared to control varied from 350 to 400 depending on the normalization method.

RESULTS AND DISCUSSION

When aleurone layers were incubated with *myo*-[2-^{3}H] inositol, incorporation began slowly (2 h) and reached maximum level by 24 h. The [^{3}H]-inositol labeled phospholipids were mixed with standard phospholipids and separated on oxalate-impregnated silica-gel plates by developing in a basic solvent system, Folch (Fig. 1), or an acidic solvent system, Skipski (data not shown). Development in the basic solvent system achieved much better separation of the phosphoinositides than the acidic solvent system. [^{3}H]Inositol labeled three phospholipids. Two compounds comigrate with standard PtdIns (from soybean) and standard PtdInsP (from bovine brain) in both basic (Fig. 1, lane 1) and acidic (data not shown) solvent systems. Compound 4, not visible in the radioautograph, showed chromatographic mobility similar to, but not exactly the same as, standard $PtdInsP_2$ from bovine brain. Radioactivity trailed the standard $PtdInsP_2$ in both solvent systems. Compounds with similar chromatographic characteristics have been reported in suspension-cultured tomato cells (8). The difference in R_F between compound 4 and $PtdInsP_2$ from bovine brain could be due to differences in the fatty acid composition at the 1- and 2-carbons of the glycerol moiety (16). Since the chromatographic data were suggestive but inconclusive, further characterization of the [^{3}H]inositol-labeled phospholipids was necessary. To achieve this, [^{3}H] inositol-labeled phospholipids were deacylated and radioactivity in individual glycerophosphoinositolphosphates determined after separating the product mixture by high voltage paper electrophoresis. Three radioactive fractions corresponding to GroPIns, GroPInsP, and $GroPInsP_2$ contained radioactivity in the ratio 100:0.5:0.1 (221,000:982:200 cpm above background), similar to the ratio in the starting phosphoinositides, PtdIns, PtdInsP, and compound 4, 100:0.7:0.2 (143,000:1050:298 cpm above background), thereby provid-

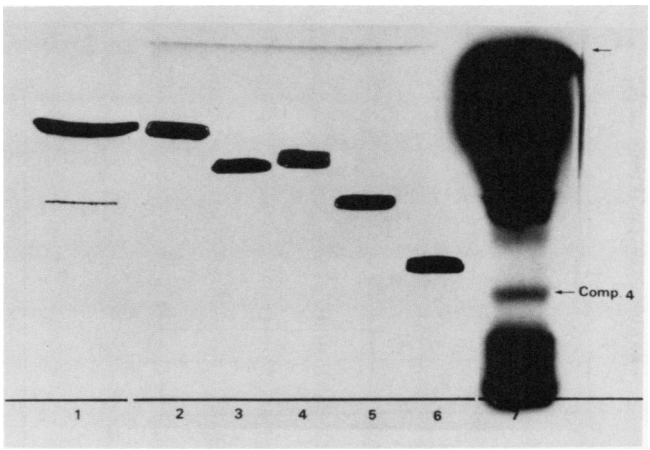

Figure 1. Separation of radiolabeled phospholipids extracted from barley aleurone cells, on oxalate-impregnated silica-gel plates developed in Folch solvent system. Lane 1 is an autoradiograph of [^{3}H] inositol labeled phospholipids; lanes 2 to 6 are standards PtdIns, LPtdIns, LPtdCh, PtdInsP, and $PtdInsP_2$, respectively; lane 7 is an autoradiograph of ^{32}Pi-labeled phospholipids overexposed to show compound 4 (with R_F lower than $PtdInsP_2$) that incorporates both [^{32}Pi] and [^{3}H]inositol.

ing strong evidence that compound 4 is $PtdInsP_2$. In keeping with observations in other plant tissues, total incorporation of *myo*-[2-^{3}H]inositol into phospholipids was very low. This could be due to the incorporation of inositol into nonlipid cell wall polysaccharides (17) like arabinose, xylose, and glucuronic acid (21) or due to the relative impermeability of cell wall to inositol or both.

When aleurone layers were incubated with [^{32}Pi], incorporation of label into phospholipids reached maximum levels after 18 to 20 h. ^{32}P-Labeled lipids from aleurone layers were mixed with standard phospholipids and separated as described above. Autoradiography showed that [^{32}Pi] labeled 10 to 12 compounds, two of which comigrated with PtdIns (from soybean) and PtdInsP (from bovine brain) in both acidic and basic solvent systems. As illustrated in Figure 1, lane 7, compound 4 again trailed standard $PtdInsP_2$. Relative incorporation of [^{32}Pi] into phosphoinositides amounts to 10 to 12% of total incorporation into phospholipids. Relative incorporation among the phosphoinositides PtdIns, PtdInsP, and $PtdInsP_2$ is in the ratio 100:10:1. The ratio of [^{32}Pi] incorporation among phospholipids is significantly different from that observed in animal tissues, where the quantitatively minor PtdInsP and $PtInsP_2$ incorporate more [^{32}P] than PtdIns (PtdIns:PtdInsP:$PtdInsP_2$ = 1:2:4) (1), suggesting that PtdIns-kinase and PtdInsP-kinase, which are very active in animal tissues (3), are not as active in barley aleurone layers.

To examine the effect of GA_3 on PtdIns metabolism, aleurone layers were prelabeled with [^{3}H]inositol for 25 h, washed free of isotope, and then treated with GA_3. Figure 2 illustrates the results. Addition of GA_3 increased incorporation of [^{3}H] inositol into PtdIns 375-fold within 15 s. By 60 s, radioactivity in PtdIns of stimulated cells had decreased to control levels and remained so for the next 20 min. This result suggests the GA_3 triggers a rapid stimulation of PtdIns biosynthesis followed by rapid breakdown. The breakdown of PtdIns may be due to the stimulation of phospholipase-A_2 (to yield LPtdIns) or phospholipase-C (to yield inositol-1-P and diacylglycerol) or PtdIns-kinase (to yield PtdInsP).

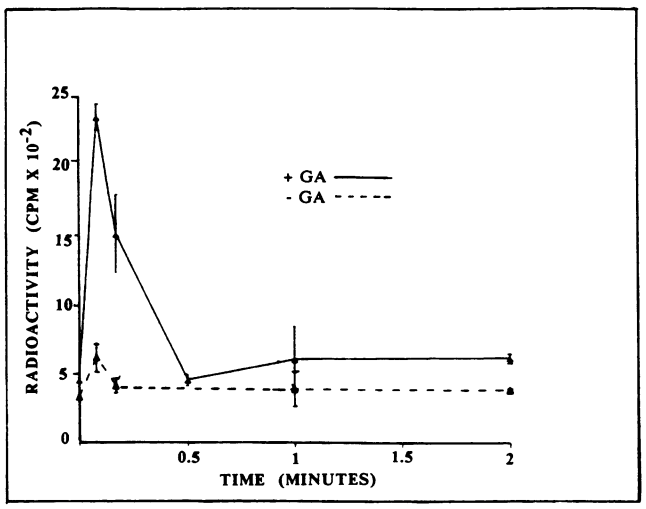

Figure 2. Changes in [2-^{3}H]inositol labeled PtdIns upon gibberellin addition. Aleurone layers were labeled with [^{3}H]inositol (50 μCi/5 layers) for 25 h, washed free of isotope, and incubated with 5 μM GA_3. Reactions were quenched with cold methanol at indicated times and phospholipids extracted, separated on silica-gel plates (basic solvent system), regions corresponding to PtdIns, scraped, and counted. Results are presented as mean of triplicates ± SEM.

In summary, two compounds labeled by both [^{32}Pi] and [^{3}H]inositol cochromatograph with PtdIns and PtdInsP. A third compound has chromatographic mobility similar to but not exactly the same as $PtdInsP_2$. Further characterization by deacylation provided strong evidence that the compound is $PtdInsP_2$. On stimulation of [^{3}H]inositol prelabeled aleurone layers with GA_3, we observe enhanced incorporation of radioactivity into PtdIns within 30 s followed by rapid loss. This stimulation of PtdIns labeling is the earliest response of aleurone cells to GA_3 reported so far. This observation suggests that PtdIns mediates GA_3 action in barley aleurone layers.

LITERATURE CITED

1. **Agranoff BW, Murthy P, Seguin EB** (1983) Thrombin-induced phosphodiesteratic cleavage of phosphatidylinositol bisphosphate in human platelets. J Biol Chem **258:** 2076–2078
2. **Berridge MJ** (1983) Rapid accumulation of inositol trisphosphate reveals that antagonists hydrolyse phosphoinositides instead of phosphatidylinositol. Biochem J **212:** 849–858
3. **Berridge MJ** (1984) Inositol trisphosphate and diacylglycerol as secondary messengers. Biochem J **220:** 345–360
4. **Bewley JD, Black M** (1983) Physiology and Biochemistry of Seeds, Vol I. Springer-Verlag, New York
5. **Boss WF, Massel MO** (1985) Polyphosphoinositides are present in plant tissue culture cells. Biochem Biophys Res Commun **132:** 1018–1023
6. **Chandra Sekar M, Hokin LE** (1986) The role of phosphoinositides in signal transduction. J Membr Biol **89:** 193–210
7. **Chrispeels MJ, Varner JE** (1967) Gibberellic acid-enhanced synthesis and release of α-amylase and ribonuclease by isolated barley aleurone layers. Plant Physiol **42:** 398–406
8. **Drøbak BK, Ferguson IB, Dawson AP, Irvine RF** (1988) Inositol-containing lipids in suspension-cultured plant cells. Plant Physiol **87:** 217–222
9. **Ettlinger C, Lehle L** (1989) Auxin induces rapid changes in phosphatidylinositol metabolites. Nature **331:** 176–178
10. **Heim S, Wagner KG** (1986) Evidence of phosphorylated phosphatidylinositols in the growth cycle of suspension cultured plant cells. Biochem Biophys Res Commun **134:** 1175–1181
11. **Irvine RF, Letcher AJ, Lander DJ, Drøbak BK, Dawson AP, Musgrave A** (1989) Phosphatidylinositol(4,5)bisphosphate and phosphatidylinositol (4)phosphate in plant tissues. Plant Physiol **89:** 888–892
12. **Jacobsen JV, Chandler PM** (1987) Gibberellin and abscisic acid in germinating cereals. *In* PJ Davies, ed, Plant Hormones and Their Role in Plant Growth and Development. Marinus Nijhoff Publishers, Dordrecht, pp 164–193
13. **Jolles J, Wirtz KWA, Schotman P, Gispen WH** (1979) Pituitary hormones influence polyphosphoinositide metabolism in rat brain. FEBS Lett **105:** 110–114
14. **Jones RL, Deikman J, Melroy D** (1986) Role of Ca^{2+} in the regulation of α-amylase synthesis and secretion of barley aleurone. *In* AJ Trewavas, ed, Molecular and Cellular Aspects of Calcium in Plant Growth and Development. Plenum Press, New York, pp 49–56
15. **Kates M** (1972) Techniques of Lipidology, North-Holland/American Elsevier, Amsterdam, p 437
16. **Keough K, MacDonald G, Thompson W** (1972) A possible relation between phosphoinositides and the diglyceride pool in rat brain. Biochim Biophys Acta **270:** 337–347
17. **Maiti IB, Loewus FA** (1978) *myo*-Inositol metabolism in germinating wheat. Planta **142:** 55–60

18. **Morse MJ, Crain RC, Satter RL** (1987) Phosphatidylinositol cycle metabolites in *Samanea saman* pulvini. Plant Physiol **83:** 640–644
19. **Morse MJ, Crain RC, Satter RL** (1987) Light-stimulated inositol phospholipid turnover in *Samanea saman*. Proc Natl Acad Sci USA **84:** 7075–7078
20. **Strasser H, Hoffmann C, Griseback H, Matern V** (1986) Are polyphosphoinositides involved in signal transduction of elicitor-induced phytoalexin synthesis in cultured plant cells? Z Naturforsch **41C:** 717–724
21. **Verma DC, Dougall DK** (1979) Biosynthesis of *myo*-inositol and its role as a precursor of cell-wall polysaccharides in suspension cultures of wild carrot cells. Planta **146:** 55–62
22. **Wheeler JJ, Boss WF** (1987) Polyphosphoinositides are present in plasma membranes isolated from fusogenic carrot cells. Plant Physiol **85:** 389–392