Phosphorylation of phosphatidylinositols in isolated plant membranes

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This report establishes the presence of phosphoinositide kinases in isolated plant membranes. Microsomal membrane fractions (10 000–50 000 × *g*) of shoots and roots of dark-grown wheat (*Triticum aestivum* L. cv. Drabant) phosphorylated phosphatidylinositol to form phosphatidylinositol monophosphate (PIP), and PIP to form phosphatidylinositol bisphosphate when supplied with [γ-^{32}P]ATP. Added exogenous substrate stimulated the reactions, as did Triton X-100 when exogenous substrate was present. Plasma membranes, isolated from the microsomal membranes by aqueous polymer two-phase partitioning, phosphorylated PI and PIP at much higher rates than did the microsomal membranes. This suggests that a major part of the phosphoinositide metabolism is localized in the plasma membranes.

Lipid kinase *Phosphatidylinositol phosphorylation* *Phosphoinositide* *Polyphosphoinositide* *Plant membrane* *Plasma membrane*

1. INTRODUCTION

PI is present in all plant membranes and generally comprises 1–10% of the acyl lipids [1]. PI is also present in animal membranes and is one of the lipids most rapidly turning over. For numerous animal tissues, PI has been reported to undergo stepwise phosphorylation to PI-4P and PI-4,5P_2 [2]. Interaction of external signals (hormones, neurotransmitters, light, etc.) with certain animal cell surface receptors induces hydrolysis of PI-4,5P_2 to form diacylglycerols and inositol trisphosphate. Diacylglycerols activate the Ca^{2+}- and phospholipid-sensitive protein kinase C, while inositol trisphosphate is released into the cytoplasm to function as a messenger for mobilizing intracellular Ca^{2+} [2,3].

Abbreviations: PI, phosphatidylinositols; PIP, phosphatidylinositol monophosphates; PI-4P, phosphatidylinositol 4-monophosphates; PIP_2, phosphatidylinositol bisphosphates; PI-4,5P_2, phosphatidylinositol 4,5-bisphosphates

In plants, several regulators of growth and development affect the intracellular content of Ca^{2+} [4], but no metabolic steps between signal perception and increased cytoplasmic Ca^{2+} content have yet been reported for plant tissues. Recently, Boss and Massel [5] reported the existence of phosphoinositides in plant cells by incubating a suspension culture of carrot cells overnight with [2-^{3}H]inositol which resulted in incorporation of inositol into several phosphoinositides, including PI, PIP, and PIP_2. However, no attempt has so far been made to localize phosphoinositide metabolism to any plant cell membrane. We here report that membranes isolated from shoots and roots of wheat, when supplied with [γ-^{32}P]ATP, are capable of phosphorylating both PI and PIP and that these reactions are enriched in plasma membrane fractions as compared to microsomes.

2. MATERIALS AND METHODS

PI-4P and PI-4,5P_2 were obtained from Sigma. [γ-^{32}P]ATP was synthesized according to Chang et

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al. [6]. All other chemicals used were of analytical grade.

The shoots and roots of 7-day-old dark-grown wheat seedlings (*Triticum aestivum* L. cv. Drabant) were harvested and the respective microsomal fractions (10000 × *g* for 15 min–50000 × *g* for 60 min) isolated [7]. Plasma membranes were isolated from the microsomal membranes by aqueous polymer two-phase partitioning as described [7]. Shoot and root membranes were always isolated from the same plants and assayed in parallel. Isolated membrane fractions were kept on ice 1–5 h prior to use.

The phosphorylation assay mixture contained in a final volume of 50 μl: 2.5 μmol Hepes, pH 7.8, 0.75 μmol $MgCl_2$, 50 nmol DTE, 75 nmol [γ-^{32}P]ATP (spec. act. 250–450 cpm/pmol), 25 μg membrane protein, and, when present, 20 nmol PI or 40 nmol PI-4P (sonicated solutions), and 0.125 mg Triton X-100 [8]. When Triton X-100 was used, it was added to the membranes 5 min prior to addition of phosphoinositides. The reactions were started by addition of ATP. The incubation time was 2 min for PI phosphorylation and 4 min for PI-4P and endogenous PI and PIP phosphorylation, all at 22°C. In time course experiments, samples were withdrawn from a scaled-up assay mixture, at indicated times.

The reactions were stopped by addition of 600 μl ice-cold chloroform/methanol/water (1:2:0.6, by vol.) and phosphoinositides extracted as modified from Schacht [9]. After 0.5–1 h on ice, 150 μl chloroform containing 0.05% (w/v) 2,6-di(*tert*-butyl)-*p*-cresol and 8–12 μg product lipid (PI-4P, PI-4,5P_2) was added and mixed, followed by addition of 120 μl of 2.4 M HCl. After mixing and phase separation, the lower phase was collected and the upper phase reextracted twice with chloroform. The combined lower phases were washed twice with 1 M HCl/methanol (1:1, v/v), and the resulting lipid extract taken to dryness under nitrogen and dissolved in chloroform. After overnight storage at −18°C under nitrogen, samples were chromatographed on silica gel H plates (Merck 60H), impregnated with 1% (w/v) potassium oxalate and dried (120°C) for 2 h prior to use. The solvent system was chloroform/methanol/25% ammonia (Merck)/H_2O (45:45:3.5:10, by vol.). The plates were run for 2 h, and exposed to iodine vapor to localize the lipids. After evaporation of the iodine, silica gel areas that had co-chromatographed with PI-4P and PI-4,5P_2 were scraped off the plates and transferred to scintillation vials, supplied with 0.4% (w/v) Omnifluor (New England Nuclear) and 33% (v/v) Triton X-100 in toluene. The radioactivity was determined by liquid scintillation counting.

Proteins were determined according to Markwell et al. [10].

The data presented are representative of 2–5 independent experiments. All data in tables and figures correspond to 25 μg membrane protein. The reactions were, however, linear between 10 and 40 μg membrane protein (not shown).

3. RESULTS

Microsomal membranes isolated from wheat shoots and roots phosphorylated endogenous PI to form PIP (table 1). In both membrane fractions, phosphorylation of endogenous PIP occurred at approx. 1/10 the rate of phosphorylation of endogenous PI (table 1). Exogenously added substrate stimulated PI phosphorylation 1.5-times,

Table 1

Endogenous and exogenous phosphorylation of PI and PIP (PI-4P) in microsomal membranes isolated from shoots and roots of dark-grown wheat

Substrate	Triton X-100	Shoot microsomes	Root microsomes
PIP formation			
–	–	100	100
–	+	33	44
PI	–	165	155
PI	+	909	649
PIP_2 formation			
–	–	11	8
–	+	0.5	0.6
PI-4P	–	27	23
PI-4P	+	51	39

Activities are expressed in percent, with the phosphorylation rate of endogenous PI set as 100%. The rates were 0.027 and 0.042 nmol/mg membrane protein per min for shoot and root microsomes, respectively. For further details, see section 2

Table 2

Phosphorylation of PI and PI-4P in microsomes and plasma membranes isolated from shoots and roots of dark-grown wheat

Substrate	Shoot		Root	
	Microsomes	Plasma membranes	Microsomes	Plasma membranes
PI	0.27	1.83	0.22	0.91
PI-4P	0.021	0.074	0.018	0.056

PI (20 nmol) or PI-4P (40 nmol) and Triton X-100 were added to the incubation medium. Activities are expressed as nmol/mg membrane protein per min. See section 2 for further details

and PIP phosphorylation about 3-times in both shoot and root microsomal fractions (table 1). The inclusion of Triton in the assay inhibited phosphorylation of endogenous substrate in both shoot and root microsomal fractions, but stimulated phosphorylation of exogenously added substrates (table 1). This stimulation was highest in shoot microsomes, so that the initial rate of exogenous PI phosphorylation was similar in both shoot and root microsomal membrane fractions (tables 1 and 2, fig.1).

The initial rates of phosphorylation and also the relation between shoot and root membrane activities varied between separate series of experiments. Sometimes root microsomes showed higher initial phosphorylation rates (table 1), sometimes shoot microsomes did (table 2). However, for both shoot and root microsomal membrane fractions, the ratio between the rates of phosphorylation of endogenous and exogenous PI in the presence or absence of Triton X-100 were always similar between different series of experiments. The same was true also for PIP phosphorylation.

In the following experiments, phosphoinositide phosphorylation was assayed in presence of exogenously added substrate and Triton X-100. Time course experiments (fig.1) showed that phosphorylation of PI to form PIP was linear with time for 6–8 min and 2–3 min in shoot and root microsomal membrane fractions, respectively. In root microsomes, the reaction reached a plateau after 10–12 min, while the shoot microsomes continued to phosphorylate PI for over 30 min (fig.1). With only PI added as substrate, PIP_2 was also formed. However, in both shoot and root microsomal fractions, the rate of PIP_2 formation was higher when exogenous PI-4P was added (fig.1). In both shoot and root microsomes, PIP_2

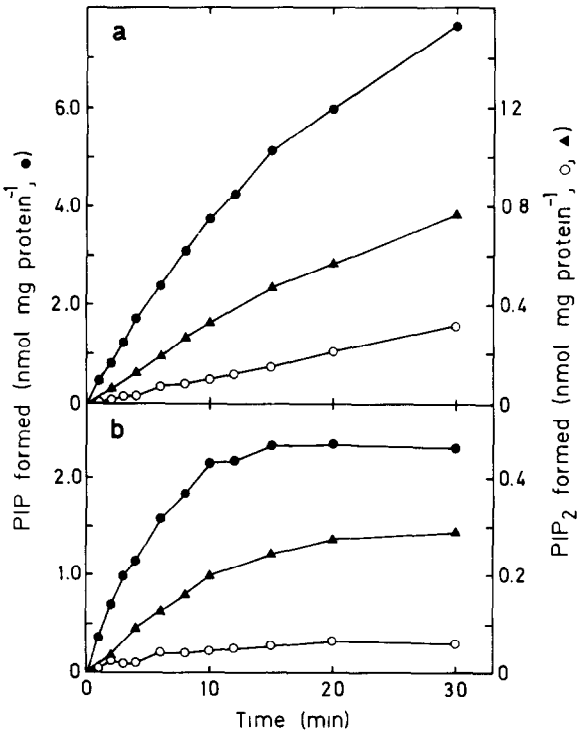

Fig.1. Time course of phosphoinositide phosphorylation in microsomal membranes isolated from dark-grown wheat. (a) Phosphorylation in presence of Triton X-100 of added PI (20 nmol) to form PIP (●) and PIP_2 (○), and of added PI-4P (40 nmol) to form PIP_2 (▲) in microsomal membranes of wheat shoots. (b) As in (a), except for root microsomal membranes. For further details, see section 2.

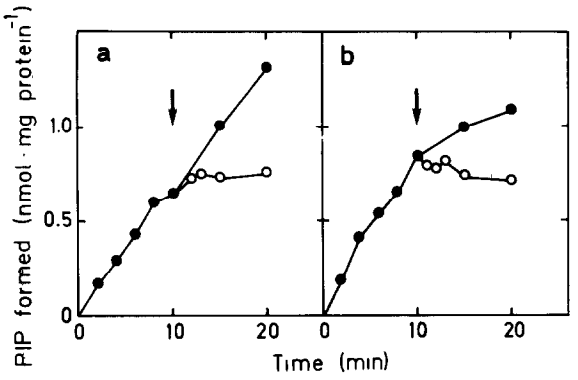

Fig.2. ATP dependence of PI phosphorylation in microsomal membranes isolated from dark-grown wheat. (a) Phosphorylation of PI (20 nmol added) in presence of Triton X-100 in microsomal membranes of wheat shoots (●). (○) PI phosphorylation after addition (arrow) of hexokinase (5 units) and glucose (0.5 μmol). (b) As in (a), except for root microsomal membranes. For further details, see section 2.

formation showed time dependencies similar to respective PIP formation.

Addition of hexokinase and glucose to deplete ATP, showed that PIP formation was in both shoot and root microsomes dependent on ATP, and that the PIP formed prior to ATP depletion was not significantly degraded (fig.2).

To study membrane localization of phosphoinositide metabolism in more detail, plasma membrane fractions were isolated from the microsomal fractions by aqueous polymer two-phase partitioning. The rate of PI phosphorylation on a protein basis was several-fold higher in the isolated plasma membrane fractions compared to the parent microsomal membrane fractions (table 2). Shoot plasma membranes were twice as active in PI phosphorylation as the root plasma membranes. PIP_2 formation was enriched in both shoot and root plasma membrane fractions as compared to respective microsomal membrane fraction.

4. DISCUSSION

Both root and shoot microsomal membrane fractions incorporated $^{32}P_i$ from [γ-^{32}P]ATP into several endogenous lipid substrates, forming mainly phosphatidic acid, PIP, and PIP_2. The rate of phosphoinositide phosphorylation increased when exogenous substrate was supplied, and Triton X-100 further increased this phosphorylation (table 1). However, Triton X-100 inhibited phosphorylation of both PI and PIP when only endogenous substrate was present (table 1), indicating that the low content of these lipids in the membranes could not sustain enzyme activity when membrane structure had been impaired. The effects of added substrate and Triton X-100 are consistent with those reported for phosphoinositide kinases in isolated rat liver membranes [8]. The initial rates of PI phosphorylation in the presence of Triton X-100 were similar between root and shoot microsomes (tables 1 and 2). However, shoot microsomes retained a high activity of PI phosphorylation much longer than did root microsomes (fig.1). The same difference also occurred between shoot and root plasma membrane fractions (not shown). One explanation for this phenomenon could be that other ATP-consuming reactions occurred to a greater extent in root membranes than in shoot membranes under the present assay conditions.

Phosphorylation of PI leading to formation of both PIP and PIP_2 indicated that these two phosphorylation steps occurred in the same membrane, as in vitro lipid transfer between membranes does not occur to any greater rate spontaneously. PIP formed by phosphorylation of PI was not significantly degraded (fig.2), which indicated that phosphatase activities that cause degradation of this lipid in animal tissues, were not active in the isolated plant membranes under the present assay conditions.

PI and PIP phosphorylation activities were higher in plasma membrane fractions than in corresponding microsomal membrane fractions (table 2). For shoot membranes, 3–4% of the microsomal membrane protein was recovered in the plasma membrane fraction, together with 20–40% of the total PI phosphorylation activity of the microsomes, the data for root membranes and for PIP phosphorylation were similar. Since probably not all plasma membrane vesicles present in the microsomal fraction were recovered, it is not clear whether phosphoinositide phosphorylation is localized only in plasma membranes. For animal tissues, no general agreement exists as to localization of the phosphoinositide kinases, although a plasma membrane localization is often assumed

[2]. PI and PIP phosphorylation probably do not occur to the same extent in membranes of different cell types or in cells of different ages and it is important to point out that for membrane isolations, we used the whole leaves, including coleoptiles, and the whole roots.

This is to our knowledge the first report showing that phosphorylation of PI and PIP occurs in isolated plant membranes. As phosphoinositide metabolism plays a crucial role in several signal perception mechanisms in animal tissues [2,3], the role(s) of polyphosphoinositides, if any, as signal transducers in plants merit attention.

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REFERENCES

[1] Harwood, J.L. (1980) in: Lipids: Structure and Function (Stumpf, P.K. ed.) The Biochemistry of Plants, vol.4, pp.1–55, Academic Press, New York.
[2] Hokin, L.E. (1985) Annu. Rev. Biochem. 56, 205–235.
[3] Berridge, M.J. and Irvine, R.F. (1984) Nature 312, 315–321.
[4] Hepler, P.K. and Wayne, R.O. (1985) Annu. Rev. Plant Physiol. 36, 397–439.
[5] Boss, W.F. and Massel, M.O. (1985) Biochem. Biophys. Res. Commun. 132, 1018–1023.
[6] Chang, K.J., Marcus, N.A. and Cuatrecasas, P. (1974) J. Biol. Chem. 249, 6854–6865.
[7] Sommarin, M., Lundborg, T. and Kylin, A. (1985) Physiol. Plant. 65, 27–32.
[8] Lundberg, G.A., Jergil, B. and Sundler, R. (1985) Biochim. Biophys. Acta 846, 379–387.
[9] Schacht, J. (1978) J. Lipid Res. 19, 1063–1067.
[10] Markwell, M.A.K., Hass, S.M., Bieber, L.L. and Tolbert, N.E. (1978) Anal. Biochem. 87, 206–210.