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Identification of Ca^{2+}-stimulated polyphosphoinositide phospholipase C in isolated plant plasma membranes

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A polyphosphoinositide phospholipase C has been identified in highly purified plasma membranes from shoots and roots of wheat seedlings. The enzyme preferentially hydrolysed phosphatidylinositol 4-phosphate and phosphatidylinositol 4,5-bisphosphate and had a different phosphoinositide substrate profile from soluble phospholipase C. The enzyme activity was lower in plasma membranes isolated from light-grown shoots than from dark-grown ones, whereas no differences in activity between plasma membranes from light- and dark-grown roots were seen. Maximum activity of the membrane-bound enzyme was observed around pH 6. It was activated by micromolar concentrations of Ca^{2+}, but not by GTP or GTP analogues. The enzyme may participate in signal transduction over the plant plasma membrane.

Guanine nucleotide; Phosphatidylinositol 4-phosphate; Phosphatidylinositol 4,5-bisphosphate; Signal transduction; Plasma membrane; (Wheat)

1. INTRODUCTION

The various steps in the signal transduction mechanism leading to an increased concentration of cytosolic Ca^{2+} have been well characterised in animal cells [1,2]. The first steps in this mechanism generating PIP_2 from PI by two consecutive phosphorylation reactions were recently identified also in plant membranes [3]. Since inositol trisphosphate, presumably formed by cleavage of PIP_2, triggers the release of Ca^{2+} from plant microsomes [4] and vacuolar vesicles [5], the presence of phospholipase C in plant cells catalysing the hydrolysis of PIP_2 can be postulated. However, so far only phospholipase C hydrolysing PI has been identified in soluble [6,7] and particulate [7,8] fractions of plant tissues. We wish to report here the presence and some properties of a polyphosphoinositide-specific phospholipase C in highly purified plasma membranes from wheat seedlings.

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Abbreviations: GTPγS, guanosine 5'-(γ-thio)triphosphate; PI, phosphatidylinositol; PIP, phosphatidylinositol 4-phosphate; PIP_2, phosphatidylinositol 4,5-bisphosphate

2. EXPERIMENTAL

Shoots and roots of 7-day-old wheat seedlings (*Triticum aestivum* L. cv. Drabant) grown in dark or under white fluorescent light (8 h light period at 80 $W \cdot m^{-2}$ at plant level and 16 h dark period) were harvested and respective microsomal fractions isolated [9]. Highly purified plasma membranes were prepared by two-phase partitioning [9]. Cytosols were obtained by further centrifugation of the 30 000 × *g* microsomal supernatants at 100 000 × *g* for 90 min. Fractions were im-

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mediately frozen and stored in liquid nitrogen until used, or were used within 5 h (stored on ice). Protein was determined [10] using bovine serum albumin as standard. [^{3}H]PI was purified from baker's yeast [11] after incubation with *myo*-[^{3}H]inositol (Amersham). [^{3}H]PIP and [^{3}H]PIP_2 were from New England Nuclear. Non-labelled PI was from Lipid Products (South Nutfield, Surrey, England), while PIP and PIP_2 were purified from a phosphoinositide-rich brain extract (type I, Sigma) by chromatography on immobilised neomycin [11]. GTPγS was from Boehringer.

The standard incubation mixture contained 50 mM Tris-maleate, pH 6.0, 10 μM free Ca^{2+} (EGTA-$CaCl_2$ mixture as in [12], assuming that the stability constant is the same at pH 6.0 as at pH 7.4), 0.2 mM phosphoinositide (PI, PIP, PIP_2 in micellar solution) and 4 μg of membrane protein in a final volume of 50 μl. The reaction was started by addition of phosphoinositide, which was prepared by evaporating the lipid in solvent to dryness under a stream of nitrogen followed by sonication for 5–10 min in the incubation buffer. Incubations (in duplicate or triplicate) were for 5 min at 25°C. They were stopped by addition of 1 ml chloroform/methanol (2:1,v/v). After addition of 250 μl of 1 M HCl, vortexing and 30 s centrifugation in a Beckman microfuge, the radioactive reaction products were recovered in the upper phase to be measured by liquid scintillation counting.

Reaction products formed were analysed by ion-exchange chromatography on Dowex AG 1-X8 columns [13]. ^{3}H-labelled phosphoinositides remaining after incubation were also checked by thin-layer chromatography on silica gel H (Merck) impregnated with potassium oxalate [14,15].

3. RESULTS

Phospholipase C activity in plasma membranes and the soluble fraction obtained from wheat shoots and roots was tested using either added PIP_2, PIP or PI as substrate (table 1). Enzyme activity was found in both subcellular fractions, but the rate of hydrolysis of PIP_2 and PIP by plasma membranes was 5–20-times higher than that of PI, and the cytosolic activity was comparatively low using either of the substrates. PIP was the best substrate for the plasma membrane-bound enzyme regardless of membrane source (shoots or roots grown in either dark or light); the specific activity towards PIP_2 was approx. 70% of that towards PIP in each of these membranes. The rate of hydrolysis of both substrates was 1.5–2.5-times faster in shoot than in root membranes. It was also ~ 50% higher in shoot plasma membranes from dark-grown seedlings than from light-grown ones, whereas no hydrolytic differences were found in dark- and light grown root membranes. PI tended to be the best substrate for soluble phospholipase C under our experimental conditions, particularly after growing the seedlings in the dark.

The reaction products obtained upon incubation of PIP and PIP_2 with plasma membranes from

Table 1

Phospholipase C activity in plasma membranes and cytosol obtained from shoots and roots of dark- or light-grown wheat seedlings

Source	Phospholipase C activity (nmol/mg protein per min)					
	Plasma membranes			Cytosol		
	PIP_2	PIP	PI	PIP_2	PIP	PI
Shoots						
Dark	147 ± 14 (6)	194 ± 14 (3)	10.4 ± 0.7 (3)	9.7 ± 4.3 (2)	11.0 ± 4.5 (2)	27.8 ± 5.8 (3)
Light	100 ± 6 (3)	135 ± 6 (2)	9.9 (1)	6.6 (1)	8.6 ± 0.6 (2)	5.5 ± 0.4 (2)
Roots						
Dark	57.6 ± 7.8 (5)	79.5 ± 17.6 (3)	6.9 ± 1.7 (2)	3.0 ± 0.6 (2)	4.2 ± 2.2 (2)	15.5 ± 1.7 (3)
Light	57.0 ± 7.0 (4)	85.3 ± 22.8 (3)	10.9 (1)	5.6 ± 1.4 (2)	9.3 ± 0.9 (3)	14.2 ± 3.4 (3)

Each preparation was analysed in triplicate as described in section 2. Results are given ± SD, or deviation from the mean when two preparations were analysed. The number of preparations analysed are indicated in parentheses

dark- and light-grown shoots were analysed to verify that the reaction studied was due to phospholipase C activity. Of the water-soluble products, analysed by chromatography on Dowex AG 1-X8 [13], more than 95% eluted with inositol bisphosphate after incubation with PIP, and 85% and 15% with inositol tris- and bisphosphate, respectively, after incubation with PIP_2. The proportion of ^3H-labelled PIP_2, PIP and PI recovered in the organic phase after incubation (91:6:3, by label) was the same as in the PIP_2 added, while only [^3H]PIP was recovered when PIP was the substrate. These results show that the hydrolysis of PIP and PIP_2 catalysed by the plasma membrane fraction proceeds through a direct attack of phospholipase C on the polyphosphoinositides.

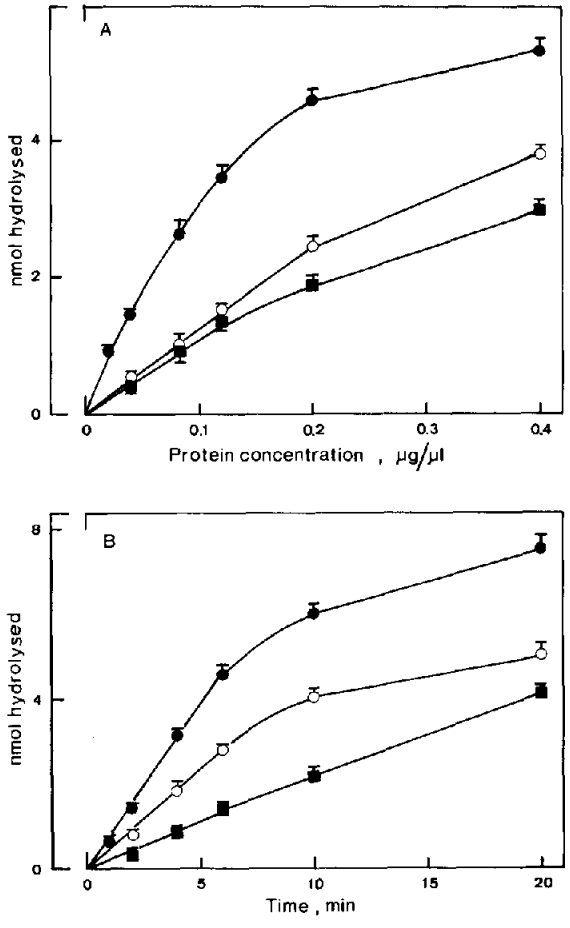

Fig. 1. Phospholipase C activity as a function of (A) protein concentration and (B) incubation time. Plasma membranes isolated from dark-grown wheat seedlings were used as enzyme source. See section 2 for further details. (○) Shoot membranes with PIP as substrate; (●) shoot and (■) root membranes with PIP_2 as substrate. Each point represents the mean of duplicate determinations with the deviation from the mean indicated.

As plant membrane phospholipase C had not been characterised before, the enzyme activity in plasma membranes purified primarily from shoots of dark-grown wheat seedlings was studied in some detail. The hydrolysis of PIP was proportional to the amount of shoot membrane protein up to 10 µg per incubation, whereas the hydrolysis of PIP_2 was largely proportional up to 4 and 6 µg in shoot and root membranes, respectively (fig. 1A). The reactions were linear with time for at least 6 min (fig. 1B).

Optimum phospholipase C activity was observed at pH 5.5–6 with PIP_2 as substrate (fig. 2), whereas the optimum range was much broader for PIP (pH 5.5–7.5). Fig. 3 shows that phospholipase C became substrate saturated at 0.2 mM PIP_2. When PIP was the substrate, the reaction increased linearly with substrate concentration up to 0.2 mM. The rate continued to increase also above this concentration, albeit more slowly.

The activity of phospholipase C of shoot membranes was dependent on Ca^{2+} using both PIP and PIP_2 as substrate (fig. 4). Similar results were obtained with root plasma membranes. Very little ac-

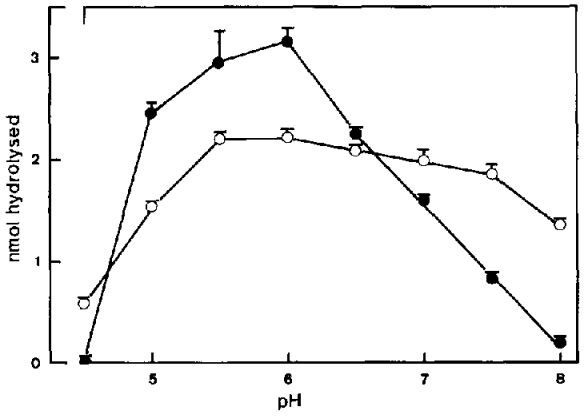

Fig. 2. Phospholipase C activity as a function of pH. Incubations in triplicate were performed as described in section 2 with 50 mM (final concentration) Tris-maleate buffer and 5 µg (PIP) or 4 µg (PIP_2) of plasma membranes purified from dark-grown wheat shoots. (○) PIP as substrate; (●) PIP_2 as substrate.

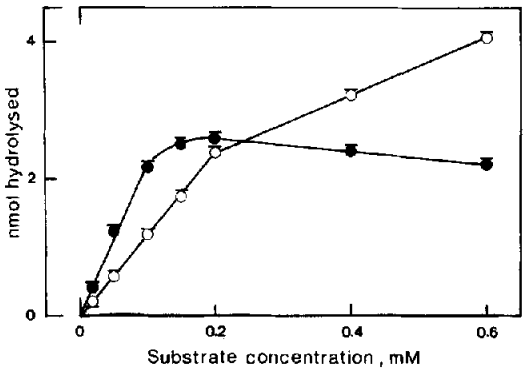

Fig. 3. Phospholipase C activity as a function of substrate concentration. Incubation in triplicate were performed as described in section 2 with (○) PIP or (●) PIP_2 as substrate. Plasma membranes from dark-grown wheat shoots were the source of enzyme.

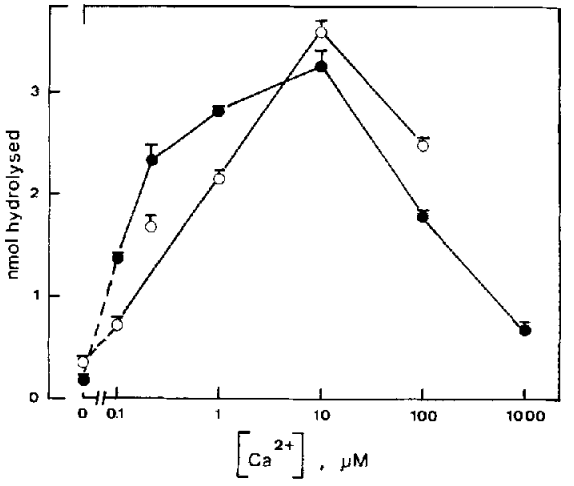

Fig. 4. Phospholipase C activity as a function of Ca^{2+} concentration. The concentration of free Ca^{2+} was varied by different EGTA-$CaCl_2$ mixtures [12]. Incubations in triplicate were performed as described in section 2 with (○) PIP or (●) PIP_2 as substrate and plasma membranes from dark-grown wheat shoots as enzyme source.

tivity was found in the absence of Ca^{2+}, but already at 0.3 μM of the divalent cation the rate had increased to 50% (PIP) or 70% (PIP_2) of respective maximum activities, which were reached at 10 μM $CaCl_2$ for both substrates. Higher concentrations of Ca^{2+} were inhibitory. 1 mM Mg^{2+} could only partly replace Ca^{2+} as divalent cation (15% of the activity obtained with 10 μM $CaCl_2$), and the enzyme was inactive with Mn^{2+} or Co^{2+}.

Phospholipase C activity in shoot plasma membranes was also tested in the presence of up to 0.1 mM GTPγS using PIP or PIP_2 as substrate. However, this non-hydrolysable GTP analogue, as GTP itself, failed to activate the enzyme.

4. DISCUSSION

Our work has identified phospholipase C activity capable of hydrolysing polyphosphoinositides in shoot and root plasma membranes of wheat seedlings at rates comparable to those obtained with plasma membranes of animal tissue [16]. The enzyme was dependent on micromolar concentrations of Ca^{2+} for activity as is also the case in several animal tissues [2,16–18]. It efficiently catalysed the hydrolysis of both PIP and PIP_2, while PI was an inefficient substrate, thereby displaying a different phosphoinositide substrate profile than the comparatively inactive soluble phospholipase C from the same tissues. This indicates that membrane-bound and soluble phospholipase C are different enzymes, as also seems to be the case in animal tissues [16].

The plasma membrane-bound enzyme may be a transducing link in cases when external signals affect metabolic events in plant cells through the release of calcium from intracellular stores, presumably mediated by inositol trisphosphate. The mechanism whereby such external signals regulate the enzyme is still unclear. Unlike phospholipase C in animal membranes [16–18], guanine nucleotides failed to stimulate the plant enzyme. However, guanine nucleotides have been shown to increase the formation of inositol phosphate in isolated sycamore membranes prelabelled with [^{3}H]inositol [8]. Since our membranes were treated extensively before incubation, they may have lost any GTP-binding regulatory proteins, which, furthermore, appear to be rather loosely attached to membranes [19].

Interestingly, the plant enzyme efficiently catalysed the hydrolysis of both PIP and PIP_2. It is not known whether the generation of inositol bisphosphate from PIP is of any physiological significance in vivo, or whether it reflects the incubation conditions in vitro. However, phospholipase C of isolated rat liver plasma membranes is specific for PIP_2 [16]. Therefore, an intracellular messenger function for inositol bisphosphate in plants cannot be ruled out entire-

ly, although this substance is inefficient in releasing Ca^{2+} from vacuolar membranes [5].

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