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Phosphatidylinositol 4,5-bisphosphate specific phospholipase C in *Pharbitis nil* membranes

P.L.R. BONNER*, ***, S. L. PRIOR*, A. M. HETHERINGTON**
and P.J. LUMSDEN*

*Department of Applied Biology, University of Central Lancashire, Preston PR1 2HE, U.K.**
*Institute of Environmental and Biological Sciences, Lancaster University, Lancaster LA1 4ZQ, U.K.***

Abstract

Phosphatidylinositol 4,5-bisphosphate specific phospholipase C has been detected in a membrane preparation from *Pharbitis nil* cotyledons. The enzyme has a pH optimum of 6.8 and activated by calcium ions, deoxycholate, phosphatidylinositol and phosphatidylethanolamine. The enzyme is inhibited to varying degrees by Tween 20, Triton X100, zinc, copper, cobalt and manganese ions and phosphatidylserine. G-protein activators do not affect the activity of *Pharbitis nil* phospholipase C. Analysis of the products of the reaction by HPLC shows inositol 1,4,5-trisphosphate from phospholipase C and inositol bisphosphate from inositol-1 and -5 phosphatase activity.

Introduction

The turnover of inositolphospholipids has proved to be central for the signal transduction of some hormones, neurotransmitters and light in animals (Majerus *et al.* 1986). Agonist binding to a receptor leads to increased activity of phosphatidylinositol 4,5-bisphosphate [PtdIns $(4,5)P_2$] phospholipase C resulting in elevated levels of inositol 1,4,5-trisphosphate [Ins$(1,4,5)P_3$] and diacylglycerol. The increased levels of Ins $(1,4,5)P_3$ affect a release of intracellular calcium which then activates a number of enzymes (Berridge 1987).

A number of reports indicate that a similar mechanism may operate in plant tissue.

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Abbreviations: PtdIns - phosphatidylinositol; PtdIns$(4,5)P_2$ - phosphatidylinositol (4,5) bisphosphate; Ins $(1,4,5)P_3$ - inositol 1,4,5-trisphosphate; PVPP - polyvinylpolypyrrolidone; DTT - dithiotreitol; CHAPS - 3-[(3-cholamidopropyl)-dimethylammoniol}-1-propanesulfonate.
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***To whom correspondence should be addressed.

The pulvini of *Samanes saman* show transient increases in Ins(1,4,5)P_3 levels in response to light (Morse *et al.* 1987) and Ins(1,4,5)P_3 has been shown to release calcium from the vacuolar membrane vesicles of oat root (Schumaker and Sze 1987) and beetroot (Alexandre *et al.* 1990). PtdIns(4,5)P_2 phospholipase C has been detected in membrane preparations from *Dunaliella salina* (Einspahr *et al.* 1989), *Avena sativa* (Tate *et al.* 1989) and celery stems (McMurray and Irvine 1988). However, only the *Samanea saman* system is clearly linked to a physiological response.

The induction of flowering in the short-day plant *Pharbitis nil* occurs in response to a dark period of sufficient duration. Dark time measurement appears to be controlled by a circadian rhythm (Vince-Prue 1983). The rhythm (dark time measurement) has been deduced from the changing flowering response to pulses of red light given during an otherwise inductive dark period. The phase of this rhythm, like other circadian rhythms, is also controlled by light (Lumsden and Furuya 1986). The possible involvement of inositol phosphate metabolism in the transduction of these light signals is currently being investigated. We report some characteristics of a PtdIns(4,5)P_2 phospholipase C present in the membrane fraction of *Pharbitis nil* cotyledons.

Material and methods

Material: Seeds of *Pharbitis nil* were soaked in concentrated sulphuric acid for 30 min and left in running tap water for 6 h. The seeds were germinated in damp vermiculite for 3 d in the dark and 3 d in continuous light (*Phillips* warm white fluorescent tubes, irradiance 110 μmol m^{-2} s^{-1}, temperature 25 ± 1 °C).

Enzyme extraction: The cotyledons were extracted in 50 mM Tris/HCl pH 7.2 containing; 5 mM 2-mercaptoethanol, 3 mM EDTA, 0.25 M sucrose and 5 % (m/v) PVPP. The extract was strained through muslin, centrifuged at 13 000 *g* for 20 min, then centrifuged at 80 000 *g* for 40 min. The resulting precipitate was resuspended in 5 mM Tris/MES pH 7.2 containing 0.25 M sucrose, 1 mM DTT, and 10% (v/v) ethanediol and stored at -70 °C in suitable aliquots.

Enzyme assay: [^{3}H] PtdIns(4,5)P_2 was dissolved in chloroform and dried under nitrogen onto the surface of a glass vessel and resuspended in buffer by mild sonication. The reaction mixture (100 μl) contained 50 mM Tris/MES pH 6.8, 5 nmol [^{3}H] PtdIns(4,5)P_2, 0.05 % (m/v) sodium deoxycholate and 1 mM $CaCl_2$. The reaction was started by the addition of 0.5 mg of enzyme and was linear up to 1 h at 30 °C. Products of the reaction were extracted in chloroform/methanol/HCl (200:100:2) (Jackowski and Rock 1989) and the aqueous fraction measured by liquid scintillation counting. Protein was estimated using bicinchoninic acid in the presence of 0.1 % (v/v) Triton X100 to solubilise membrane proteins and bovine serum albumin as a standard (Smith *et al.* 1985).

Analysis of aqueous products by HPLC: Aqueous products of the phospholipase C reaction were dried under nitrogen and dissolved in water. The products were then injected onto a *Partisil-10 SAX HPLC* column (Whatman, U.K.) and eluted with a gradient of amonium formate pH 3.7 (with phosphoric acid) (Morse *et al.* 1987). The flow rate was 1 ml min^{-1} and the radioactivity in the fractions was measured by liquid scintillation counting. Authentic inositol phosphate standards were obtained from Amersham, U.K. and the inositol (4,5) bishosphate isomer was generated by incubating [^{3}H] Ins(1,4,5)P_2 with alkaline phosphatase at pH 6.8 and separating the products by HPLC.

All reagents were of the highest grade available from Sigma.

Results and discussion

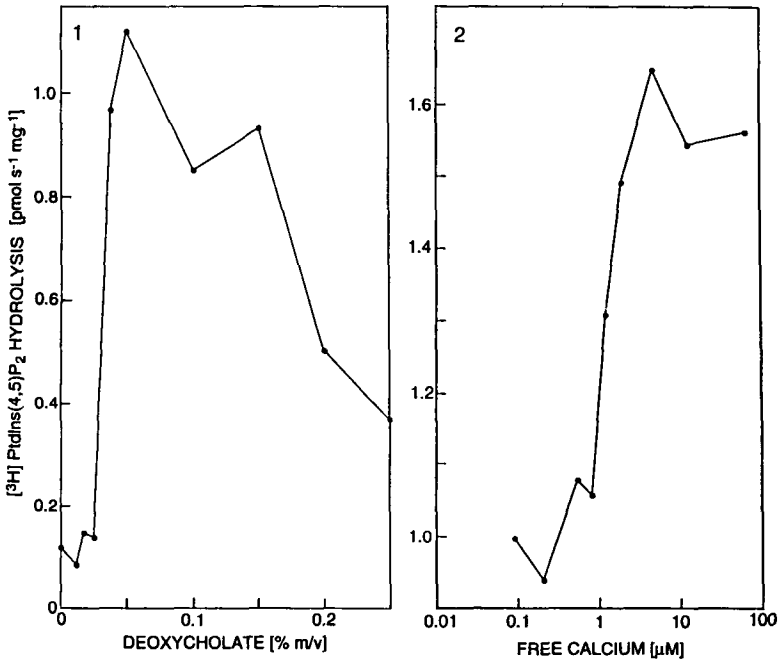

Fig. 1. The effect of deoxycholate on [^{3}H] PtdIns(4,5)P_2 in the presence of 1 mM Ca^{2+}. These values are mean of two results from one preparation; essentially similar results have been obtained with three different preparations.

Fig. 2. The effect of Ca^{2+} on [^{3}H] PtdIns(4,5)P_2 hydrolysis in the presence of 0.05 % (m/v) deoxycholate, with Ca^{2+} concentrations varied by using Ca^{2+}/EGTA buffers. Free Ca^{2+} was determined using *Eqcal* computer programme (Biosoft, Ltd., U.K.). The specific activity of the enzyme preparation in the presence of 1 mM EGTA was 0.83 pmol s^{-1} mg^{-1}. These values are mean of two results from one preparation; essentially similar results have been obtained from three different preparations.

The PtdIns(4,5)P_2 phospholipase C from *Pharbitis nil* had a pH optimum of 6.8. and showed increased activity in the presence of increasing concentrations of the anionic detergent sodium deoxycholate (Fig. 1). The maximum stimulation of activity was achieved at 0.05 % (m/v) deoxycholate. The enzyme from celery stem was maximally stimulated by 0.15 % (m/v) deoxycholate (Mc Murray and Irvine 1988). The effect of other detergents at 0.05 % (m/v) was various: N-lauryl sarcosine and CHAPS activated the enzyme, cetylpyridinium chloride. Triton X100, Tween 20 and dodecyl-β-D-maltoside inhibited PtdIns(4.5)P_2 phospolipase C activity. Activation by deoxycholate is a characteristic of PtdIns(4.5)P_2 phospholipase C enzymes isolated from animal tissue (Crooke and Bennet 1989).

The PtdIns(4.5)P_2 phospholipase C from *Pharbitis nil* showed activation in the presence of calcium ions (Fig. 2); the activity doubled between 1 and 10 μM calcium. Zinc, copper, manganese and cobalt ions (1 mM) were inhibitory to varying degrees. Activation by low calcium concentrations (μM) is characteristic of membrane bound PtdIns(4.5)P_2 phospholipase C enzymes from animal and plant tissue, while cytosolic phosphatidylinositol (PtdIns) specific phospholipase C enzymes are activated by higher (*ca.* 1 mM) calcium concentrations (Einspahr *et al.* 1989, Tate *et al.* 1989, Crooke and Bennett 1989). PtdIns(4,5)P_2 phospholipase C from *Pharbitis nil* was not inhibited by mM calcium concentrations.

Table 1. [^{3}H] PtdIns(4,5)P_2 hydrolysis in the presence of unlabelled phospholipides.

	[^{3}H] PtdIns(4,5)P_2 hydrolysis [pmol s^{-1} mg^{-1}]	[% of control]
Control - 5 mM[^{3}H] PtdIns(4,5)P_2	0.31	100
+ 50 nmol phosphatidylcholine	0.31	100
+ 50 nmol phosphatidylglycerol	0.31	100
+ 50 nmol phosphatidylethanolamine	2.10	663
+ 50 nmol phosphatidylinositol	1.10	347
+ 50 nmol phosphatidylserine	0.21	68

In the presence of 50 nmol phosphatidylcholine, phosphatidylserine or phosphatidylglycerol the PtdIns(4.5)P_2 phospholipase C from *Pharbitis nil* showed no activation (Table 1). In the presence of 50 nmol phosphatidylethanolamine and phosphatidylinositol the enzyme was activated 8- and 4-fold respectively. It is difficult to demonstrate that lipids have a direct regulatory effect on phospholipase C activity because they also affect the physical properties of the phospholipid substrate (Crooke and Bennet 1989). However, the results from Table 1 contrast with the PtdIns(4,5)P_2 phospholipase C from celery stem (McMurray and Irvine 1988) which showed a 2- to 3-fold activation in the presence of all the phospholipids. The PtdIns(4,5)P_2 phospholipase C from *Pharbitis nil* showed no activation or inhibition in the presence of 5 mM NaF, 5 mM NaF + 10 mM $AlCl_3$, CTP or GTP-γ-S (results not shown).

$Ins(1,4,5)P_3$ is never the major product, presumably due to the action of the inositol-1 and -5 phosphatases (Fig. 3). This resembles the pathway for inositol phosphate metabolism in the slime mould *Dictyostelium discoideum* (Van Lookeren Campagne *et al* 1988), *Pisum sativum* (Drobak *et al.* 1991) and *Nicotiana tabacum* (Joseph *et al.* 1989). The inositol-1 phosphatase present in *Pharbitis nil* membranes can be inhibited by 10 mM molybdate but at this concentration the $PtdIns(4,5)P_2$ phospholipase C is also inhibited. ATP at 20 mM does not reduce inositol phosphatase activity. The inositol-5 phosphatase present in *Pharbitis nil* membranes can be inhibited by 2,3-bisphosphoglycerate (results not shown), an inhibitor of the 5 phosphatase in animal tissue (Berridge 1987) and plant tissue (Joseph *et al.* 1989).

We have presented evidence for a $PtdIns(4,5)P_2$ phospholipase C in a membrane preparation from *Pharbitis nil* cotyledons. In addition there is evidence that the necesary phosphatases are present to remove the second messenger $Ins(1,4,5)P_3$ produced by $PtdIns(4,5)P_2$ phospholipase C activity.

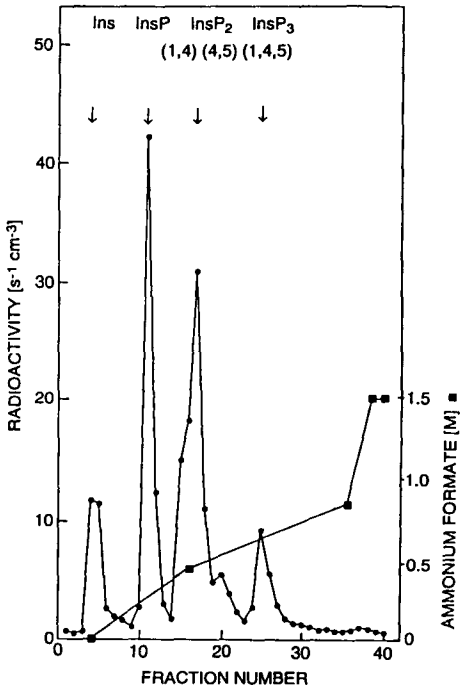

Fig. 3. Indentification of the products of [^{3}H] $PtdIns(4,5)P_2$ hydrolysis by HPLC (flow rate 0.016 $cm^3\ s^{-1}$; 1 cm^3 fractions). The labelled products of the reaction were identified based on the elution times of authentic standards. The specific activity of the enzyme preparation used was 13.3 pmol $mg^{-1}\ s^{-1}$.

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