Phosphatidylinositol 4,5-bisphosphate phosphodiesterase in higher plants

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A phospholipase C which hydrolyses phosphatidylinositol 4,5-bisphosphate to release inositol trisphosphate was detected in a sedimentable fraction from celery and from some other higher plants. The particulate enzyme also hydrolyses phosphatidylinositol, whereas the soluble phosphatidylinositol phosphodiesterase described previously [Irvine, Letcher & Dawson (1980) *Biochem. J.* **192**, 279–283] acts only on phosphatidylinositol, and we were unable to detect activity of this soluble activity on phosphatidylinositol 4,5-bisphosphate. Activity of the particulate enzyme is markedly enhanced in the presence of deoxycholate, but not of other detergents; the particulate enzyme can also be solubilized by extraction with deoxycholate.

INTRODUCTION

An early response in the activation of animal cells by many agonists acting through interaction with cell-surface receptors is the hydrolysis of membrane phosphoinositides by a phospholipase C. The stimulated phosphodiesterase activity acts specifically on inositol phospholipids and, at least initially, preferentially on the polyphosphoinositides (for review see Downes & Michell, 1985). Such receptor-mediated cleavage of phosphoinositides gives rise to diacylglycerol and inositol phosphates, of which the predominant product will be inositol trisphosphate (InsP_3) from breakdown of phosphatidylinositol 4,5-bisphosphate (PtdInsP_2). The diacylglycerol acts as a second messenger by activating protein phosphorylation (Nishizuka, 1984; Nidel & Blackshear, 1986), and InsP_3 has been shown to initiate a signal cascade by releasing Ca^{2+} from intracellular stores (Berridge & Irvine, 1984; Berridge, 1986).

Recent evidence has suggested that the same intracellular signalling mechanisms may be used by higher plants (e.g. Drøbak & Ferguson, 1985; Schumaker & Sze, 1987) and, in particular, evidence that PtdInsP_2 exists in plants has been presented (Heim & Wagner, 1987). Previously, a Ca^{2+} -dependent phosphatidylinositol phosphodiesterase has been reported to occur in celery tissues (Irvine *et al.*, 1980), but whether or not this soluble enzyme could hydrolyse PtdInsP_2 was not determined. The present report examines this question, and also describes a particulate polyphosphoinositide phosphodiesterase in plant tissues that is Ca^{2+} -dependent and activated specifically by deoxycholate.

MATERIALS AND METHODS

Phospholipase assays

Phosphatidyl[^3H]inositol (Amersham) and phosphatidyl[^3H]inositol 4,5-bisphosphate (New England Nuclear) were dissolved in chloroform and washed exhaustively (six to eight times) with equal volumes of

methanol/water (1:1 v/v) and methanol/water/HCl (25:25:1, by vol.) respectively to eliminate water-soluble contaminants. Chloroform solutions of these radio-labelled substrates, together with appropriate amounts of unlabelled carrier substrates, were added to reaction tubes, the solvent was removed by evaporation under nitrogen, and the lipids were suspended in appropriate buffer solutions with mild sonication (2–3 min in a sonic bath). The standard reaction mixture for phosphodiesterase assays contained 50 nmol of [^3H]PtdIns (20 000–30 000 d.p.m. total) or 5 nmol of [^3H]PtdIns-(4,5) P_2 (15 000–20 000 d.p.m. total), 50 mM-Tris/maleate, pH 7.2, 1 mM- CaCl_2 , 0.15% sodium deoxycholate, and enzyme in a final volume of 0.5 ml. Variations of Ca^{2+} concentration in the range 1 nM–1 mM were obtained with EGTA buffers (Raaflaub, 1956; Burgess *et al.*, 1983). When other lipids were added, they were dissolved in chloroform, added with the substrate, and the solvent was evaporated before addition of the buffer solutions. All assays were incubated at 25 °C for 15 min under conditions where the reaction was linear with respect to amount of added enzyme.

The reaction was terminated by sequential addition of methanol (0.5 ml), chloroform (1.0 ml), and, in the case of the experiments using [^3H]PtdIns-(4,5) P_2 , HCl (0.02 ml), followed immediately by vigorous stirring with a vortex mixer. The phases were separated by centrifugation (2000 g for 10 min), and the upper phases were removed and added to a second set of tubes containing 1.0 ml of the lower phase from an equilibrated mixture of chloroform/methanol/water (2:1:1, by vol.). The tubes were mixed as before, centrifuged (2000 g for 10 min), and 0.4 ml of the washed upper phase was added to a scintillation minivial. A portion of the original lower phase from the first set of tubes (usually 0.1 ml) was also added to a minivial. The chloroform was evaporated from both sets of vials under a stream of nitrogen, and 4 ml of scintillation fluid (Emulsifier Scintillator 299 TM; Packard) was added. The ratio d.p.m. in upper phase/d.p.m. in lower phase was used to calculate the mass of product formed after correction for d.p.m. in the upper

Abbreviations used: InsP_3 , inositol trisphosphate; PtdInsP_2 , phosphatidylinositol 4,5-bisphosphate.

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phase of samples incubated without added enzyme. Identifications of inositol phosphate products in the enzyme hydrolysates were carried out by anion-exchange chromatography on QMA Sep-paks as described by Wreggett & Irvine (1987).

Plant extracts

Celery (*Apium graveolens*) was cooled to 5 °C and the lower white portion of stalk, freed of fibrous cords, was chopped finely, then ground with washed sand with a chilled mortar and pestle. A similar procedure was adopted for homogenization of other plant materials. The use of mechanical blenders was avoided to prevent excess frothing and heating, which led to less active preparations in trial runs. An equal volume of water or buffered solution containing glycerol (see below) was added, and the tissue was thoroughly reground, then filtered through several layers of gauze. Debris was removed by centrifugation at 600 *g* for 10 min and the low-speed supernatant was retained. Membrane particles were then sedimented by centrifugation at 100 000 *g* for 60 min and finally resuspended in a measured volume of the homogenization medium. The particulate suspension and the high-speed supernatant were stored in small batches at -20 °C. Solubilization of the particulate enzyme could be achieved by addition of 0.2 vol. of 5 % sodium deoxycholate in the presence of 10 % (v/v) glycerol (final concn.) to the particle suspension, sonication for 1 min in the sonic waterbath, followed by standing on ice for 20–30 min. The suspension was centrifuged at 100 000 *g* for 60 min, and the pellet was discarded. The supernatant, which contained 50–80 % of the original activity, was stored at -20 °C. Protein was determined by a modified Lowry method (Peterson, 1977).

The phosphodiesterase activities were first measured in aqueous extracts prepared as described by Irvine *et al.* (1980). However, experience indicated that in particulate fractions the particles aggregated with time and that membrane-bound enzyme activities were unstable to freezing and thawing or storage at -20 °C. Addition of glycerol stabilized the particulate enzyme to long-term storage at low temperature. Consequently, subsequent preparations used 10 % glycerol/1 mM-Tris/maleate (pH 7.2)/0.1 mM-EDTA as the homogenizing and suspension medium. This medium was also used for studies with detergent solubilization of the phosphodiesterase, and for other plant extracts.

RESULTS

Soluble phosphodiesterase

The original work of Irvine *et al.* (1980) established that dialysed soluble extracts of celery and some other plants could hydrolyse PtdIns in the presence of Ca²⁺ in the millimolar range and at neutral pH, with the release of phosphoinositol and 1:2-cyclic phosphoinositol in the ratios expected for cleavage by a phospholipase C-like phosphodiesterase. Subsequent studies by Irvine *et al.* (1984) and Wilson *et al.* (1984), with extracts of rat brain or purified sheep seminal-vesicle phosphoinositide phosphodiesterase respectively, indicated that the mammalian phosphodiesterase would act on both PtdIns and polyphosphoinositides. In both these studies there was, however, a preference for PtdInsP₂ at neutral pH in the presence of Ca²⁺ in the micromolar range and with

physiological concentrations of K⁺ and Mg²⁺, provided that the substrate was presented as a non-bilayer mixture with other phospholipids. These conditions were accordingly applied to the soluble plant enzyme in an attempt to detect phosphodiesterase activity against the polyphosphoinositides. Although we confirmed that the soluble celery extract (high-speed supernatant fraction) was active in hydrolysing PtdIns, negligible hydrolysis of PtdInsP₂ was observed in dialysed or undialysed extracts under a wide variety of conditions. These included: variations of pH from 5.5 to 8.5; variations in Ca²⁺ over the range 1 nM–10 mM; additions of 80 mM-K⁺ or 1 mM-Mg²⁺; the presence of lipid mixtures simulating a natural membrane bilayer; additions such as excess diacyl glycerols or phosphatidylethanolamine to induce perturbation of the bilayer state of the substrate; the presence of a range of non-ionic, anionic, cationic or amphoteric detergents. In summary, the soluble celery enzyme was, in our hands, different from the rat brain enzyme in that under none of these conditions were we able to persuade it reproducibly to hydrolyse PtdInsP₂; we cannot rule out the possibilities either that it has a very weak activity, or that some special conditions, which we did not try, would enable it to hydrolyse PtdInsP₂.

Particulate activity

When PtdInsP₂ was presented in a bilayer mixture to the membrane particulate fraction of celery at neutral pH, this fraction cleaved PtdInsP₂ in a Ca²⁺-dependent reaction, as did a soluble rat brain fraction included as a control (Table 1). The phosphodiesterase activity against PtdInsP₂ and PtdIns in celery particles was markedly enhanced by addition of sodium deoxycholate above 0.1 % in the reaction medium (Table 1, Fig. 1). Hydrolysis of PtdIns by soluble extracts of celery was also stimulated by deoxycholate, but little hydrolysis of PtdInsP₂ could be detected. Of a wide range of detergents including Triton X-100, Triton X-114, Tween 20, octyl glucoside, digitonin, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulphonic acid ('Chaps'), Zwittergent or Miranol H2M, none was able to mimic the activation by deoxycholate in stimulating the particulate PtdInsP₂ phosphodiesterase activity, and most were inhibitory in the presence of optimal concentrations of deoxycholate. Sodium cholate was unable to replace deoxycholate, but sodium glycodeoxycholate also activated the PtdInsP₂ phosphodiesterase (results not shown). The activity was not affected by addition of 80 mM-KCl and was inhibited by 1 mM-MgCl₂, hence these cations were omitted from subsequent assays.

It is possible that in the particulate fraction the PtdInsP₂ is being hydrolysed first by phosphomonoesterase(s) to PtdIns, which is then in turn being hydrolysed by the phosphodiesterase. Two pieces of evidence argue against this. Firstly, if a 10–20-fold excess of PtdIns was added to the assays containing [³H]PtdInsP₂, there was no decrease of the radioactivity in the water-soluble products (in fact a slight stimulation occurred; see Fig. 3 and below). Secondly, more direct evidence was obtained by examining the aqueous radioactive products from hydrolysis of PtdInsP₂, and the major product was InsP₃ (Table 2). The formation of some inositol, its monophosphate and bisphosphate suggests that inositol phosphate phosphatase(s) (and possibly also inositol-lipid phosphomonoesterases) are present in celery tissue. It is clear from the data in Table 2

Table 1. Hydrolysis of [^3H]PtdIns and [^3H]PtdIns P_2

[^3H]PtdIns (50 nmol) or [^3H]PtdIns P_2 (5 nmol) was incubated as described in the text with celery supernatant or particles derived from 50 mg of fresh celery or supernatant from 1 mg of fresh rat brain. Data are calculated as nmol of product formed in 15 min at 25 °C and are means of duplicates, with similar results obtained in a separate experiment (n.d. = not detectable).

Expt. no.	Additions	Celery				Rat brain	
		Supernatant		Particles		Supernatant	
		PtdIns	PtdIns P_2	PtdIns	PtdIns P_2	PtdIns	PtdIns P_2
1	10 mM- CaCl_2	0.88	n.d.	0.18	0.05	n.d.	0.19
	1 mM- CaCl_2	1.00	n.d.	1.61	0.12	7.05	0.40
2	1 mM- CaCl_2	0.63	n.d.	1.85	0.11	5.70	0.84
	1 mM- CaCl_2 + 0.1 % sodium deoxycholate	12.65	0.02	8.45	0.69	8.08	1.03

that the major mechanism of hydrolysis of PtdIns P_2 under these conditions is a phospholipase C.

In addition to the white stalk portion of celery plants, significant but lower activity of PtdIns P_2 phosphodiesterase assayed with deoxycholate present could be obtained from the green portions. Activity was found in stalks of other plants (daffodil, leek, onion), leafy portions (lettuce, chicory, brassicas), in the flowery umbels (cauliflower, calabresi) or in vegetable roots (turnip, parsnip, carrot) (Table 3). End-products of the

Table 2. Products of [^3H]PtdIns and [^3H]PtdIns P_2 hydrolysis

Products from the hydrolytic reactions described in Table 1 are expressed as percentages of total radioactivity (d.p.m.) in the hydrolysate. Results are means of duplicates, and similar results were obtained in a separate experiment.

	Rat brain supernatant		Celery particles
	PtdIns	PtdIns P_2	PtdIns P_2
Inositol	56.9	3.0	9.3
Inositol monophosphate	42.6	9.0	1.4
Inositol bisphosphate	0.4	25.3	11.7
Ins P_3	0.0	62.7	77.6

Table 3. [^3H]PtdIns P_2 hydrolysis in plant particle preparations

Assay conditions were as in Table 1. Data are the results of duplicate determinations, one experiment for each tissue examined.

		Activity (nmol/h per mg of membrane protein)
Stems	Celery	50.6
	Onion	34.9
	Leek	18.7
	Daffodil	12.7
	Asparagus	5.3
Leaves	White cabbage	20.9
	Lettuce	2.8
	Spinach	1.3
Flowers	Cauliflower	30.7
	Calabresi	14.0
Roots	Turnip	19.7
	Carrot	9.4

Fig. 1. Effect of deoxycholate on celery PtdIns P_2 hydrolysis

The hydrolysis of [^3H]PtdIns P_2 was determined as described in Table 1 and in the text, with particles derived from 50 mg of fresh celery and with various concentrations of sodium deoxycholate in the presence of Ca^{2+} (1 mM). Similar results were obtained in a separate experiment.

hydrolyses were not determined in these experiments, and consequently phospholipase D or other hydrolytic reactions cannot be excluded in these other plant species. Nevertheless, we suggest that the membrane-bound PtdInsP_2 phosphodiesterase activity (when assayed in the presence of deoxycholate) is probably widespread in the plant kingdom.

Properties of the deoxycholate-stimulated PtdInsP_2 phosphodiesterase

As shown previously for the soluble enzyme, the phosphodiesterase activity of the particulate celery enzyme was observed in the neutral pH range with $[\text{H}]\text{PtdIns}$ or $[\text{H}]\text{PtdInsP}_2$ as substrates (results not shown), although with deoxycholate present little significance can be attached to this, because at this pH (approx. 7) the ionization of the deoxycholic acid is maximal, and it is the deoxycholate anion which is the active species (Irvine & Dawson, 1978); activity in the absence of deoxycholate, although detectable, was in our hands too weak for quantitative experiments. In order to determine the effects of varying the Ca^{2+} concentration, a series of Ca^{2+} -EGTA buffers at pH 7.2 were prepared (Burgess *et al.*, 1983) to yield the free Ca^{2+} concentrations shown in Fig. 2. The hydrolysis of both PtdIns and PtdInsP_2 was markedly activated at concentrations of Ca^{2+} in the intracellular range of $0.1\text{--}1\ \mu\text{M}$, though again the presence of deoxycholate makes the significance of this difficult to assess; full stimulations by Ca^{2+} of the particulate enzyme were produced in the millimolar range at neutral pH. In summary, we can say that the particulate PtdInsP_2 phosphodiesterase activity is Ca^{2+} -dependent, but cannot draw any definite conclusions about whether it is Ca^{2+} -controlled.

Effects of other lipids on the hydrolysis of PtdInsP_2

Fig. 2. Effect of Ca^{2+} concentration on celery PtdInsP_2 hydrolysis

The hydrolysis of $[\text{H}]\text{PtdInsP}_2$ was determined as described in the text in the presence of 0.15% sodium deoxycholate, with Ca^{2+} concentrations varied by using Ca -EGTA buffers (Raaf laub, 1956; Burgess *et al.*, 1983), with particles derived from 50 mg of fresh celery. Similar results were obtained in a separate experiment.

Fig. 3. Effects of added lipids on celery PtdInsP_2 hydrolysis

The hydrolysis of $[\text{H}]\text{PtdInsP}_2$ was determined as described in the text in the presence of 0.15% sodium deoxycholate and 1 mM - Ca^{2+} with particles derived from 50 mg of fresh celery. Abbreviations: BL, bilayer lipid mixture (Irvine *et al.*, 1984) (100 nmol); PI, phosphatidylinositol (50 nmol); PS, phosphatidylserine (50 nmol); PE, phosphatidylethanolamine (50 nmol); PC, phosphatidylcholine (50 nmol); DG, 1,2-diolein (50 nmol); SM, sphingomyelin (50 nmol). Results are means of duplicates, and are pooled from a series of experiments.

were generally stimulatory (Fig. 3). The presence of a mixture of phospholipids with cholesterol in proportions to simulate composition of a natural bilayer (Irvine *et al.*, 1984) stimulated the activity over 2-fold, as did the addition of phosphatidylinositol, phosphatidylserine sphingomyelin or phosphatidylethanolamine. Phosphatidylcholine was inhibitory in the presence of other lipids. Addition of diacylglycerol, which also reportedly activates phospholipase actions by perturbing the usual bilayer structures of membranes (Hofmann & Majerus, 1982; Dawson *et al.*, 1983, 1984), produced a smaller stimulation of the PtdInsP_2 phosphodiesterase, but neither stearylamine nor oleic acid had any detectable effect on the activity.

In summary, minor effects of a number of lipids were observed, but it should be emphasized that all these stimulatory and inhibitory effects occurred in the presence of 0.15% sodium deoxycholate. In its absence none of these stimulators was effective (or at least not effective enough to produce detectably more activity). We think, therefore, that the deoxycholate is serving not only to activate the enzyme by presenting the substrate in the correct conformation, but also by solubilizing the enzyme from the membrane so that it has access to the substrate. Were it possible to assay the enzyme without deoxycholate present, then the effect of some of these amphiphiles might be greater [as, for example, the soluble rat brain PtdInsP_2 phosphodiesterase studied by Irvine *et al.* (1984)].

G-protein stimulators

A number of mammalian cells show evidence for the involvement of guanine-nucleotide-binding proteins in the coupling of membrane receptors to hydrolysis of the phosphoinositides and mobilization of Ca^{2+} (for review see Taylor & Merritt, 1987). However, no effect of GTP, GTP analogues or AlF_3 could be detected on the celery particulate PtdInsP_2 phosphodiesterase activity.

DISCUSSION

A Ca^{2+} -dependent phosphodiesterase has been demonstrated in particulate fractions from celery and other higher plants which hydrolyses both PtdIns and PtdInsP_2 , and is markedly activated by deoxycholate. In our hands the hydrolysis of PtdIns by the enzyme was always greater than that of PtdInsP_2 , though obviously we cannot be sure that this would be so *in vivo*. Contamination of the particulate fraction with the soluble enzyme (which, we show here, undoubtedly favours PtdIns) could obscure a greater preference of a true particulate activity for PtdInsP_2 . Thus the true substrate specificity of the particulate activity *in vivo* is unknown, but it may be that it favours polyphosphoinositides over PtdIns , as does the mammalian membrane-bound equivalent when it is activated by receptors (for references see Taylor & Merritt, 1987).

Preliminary attempts to purify the enzyme(s) indicated that 50–80% of the membrane-bound activity can be extracted in soluble form with deoxycholate (see the Materials and methods section), though subsequent removal of deoxycholate by dialysis resulted in inactivation, and so problems described above in studying the enzyme's properties remain. We did make one attempt to purify the enzyme by anion-exchange chromatography in the presence of deoxycholate, but, as shown for the mammalian enzyme by Low & Weglicki (1983), hydrolytic activities against PtdIns and PtdInsP_2 were not separable.

Finally, the low activity of the soluble celery PtdIns phosphodiesterase (Irvine *et al.*, 1980) against PtdInsP_2 leads to the question of what the usual substrate and function of this soluble enzyme are, and at present we have no answer to this. We suggest that the membrane-bound activity studied here may be a receptor-controlled enzyme analogous to that found in many mammalian systems (Taylor & Merritt, 1987), and its presence strengthens the likelihood that the polyphosphoinositide signalling system is used by higher plants (see, e.g.,

Drøbak & Ferguson, 1985; Schumaker & Sze, 1987; Heim & Wagner, 1987).

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