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Phospholipase activities associated with the tonoplast from *Acer pseudoplatanus* cells: identification of a phospholipase A_1 activity

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Abstract

The study of phospholipase activities associated with the tonoplast of *Acer pseudoplatanus* was performed in vitro with *sn*-2-[^{14}C]acylphosphatidylcholine (PC) as a substrate. The hydrolysis of radiolabelled PC into [^{14}C]phosphatidic acid and [^{14}C]lyso-PC demonstrated the presence of phospholipase D and A_1 activities, respectively, associated with the tonoplast of *Acer pseudoplatanus*. The vacuolar sap did not show any significant phospholipase activity. In a second step, the properties of the phospholipase A_1 activity was studied using tonoplast endogenous PC labelled in vivo with [^{14}C]choline as a substrate. The phospholipase A_1 showed an optimal activity at pH about 6–6.5, did not necessarily require divalent cations, but was stimulated by Mg^{2+} and particularly by Ca^{2+}. This work presents the first evidence for the presence of phospholipases A_1 in plant cells.

Keywords: Tonoplast; Phospholipase A_1; Phospholipase D; (*Acer pseudoplatanus*)

1. Introduction

In higher plants, the lipolytic enzymes and their physiological functions are not well characterized [1]. Most reports are usually related to studies on physiological processes such as senescence or response to different stresses [2,3]. These studies have demonstrated that phospholipid catabolism in plants is achieved by the concerted action of membrane-bound enzymes including phospholipase D, phosphatidate phosphatase, lipolytic acyl hydrolases and lipoxygenases [2–4]. With the exception of phospholipase D, the literature on plant phospholipases is still very limited. Phospholipase D from various plant tissues has been well characterized [3]. Polyphosphoinositide phospholipase C activity has recently been identified in plant plasma membranes [5–7]. The detection of lysophospholipids in plant membranes suggests the presence of type A phospholipases [8–11] and a lysophospholipid generating phospholipase A activity was found to be activated by auxin in soybean cells [12]. A plant microsomal phospholipase A_2, with a high specificity towards PC with oxygenated *sn*-2-acyl groups, has nevertheless been unequivocally identified and characterized [13].

We previously reported that tonoplast from *Acer pseudoplatanus* cells contains small amounts of phosphatidic acid and lysophospholipids, which are produced together with free fatty acids, particularly after addition of Ca^{2+} [14]. These data suggest a possible involvement of phospholipase D and phospholipase A in the metabolism of vacuolar membrane lipids. In the present work, we have characterized phospholipase D and phospholipase A_1 activities associated with the tonoplast of *A. pseudoplatanus*.

2. Materials and methods

2.1. Chemicals

L-α-1-Palmitoyl-2-linoleyl[^{14}C]phosphatidylcholine (1.85 GBq/mmol) and [*methyl*-^{14}C]choline chloride (1.96 GBq/mmol) were purchased from Dupont NEN.

Abbreviations: DAG, diacylglycerol; FFA, free fatty acids; lyso-PC, lysophosphatidylcholine; lyso-PE, lysophosphatidylethanolamine; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; TLC, thin-layer chromatography.

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2.2. Plant material and tonoplast isolation

Cell suspensions of *Acer pseudoplatanus* L. were cultured in liquid medium as previously described [15]. The cells were harvested 8 days after the beginning of the culture, at the end of the exponential phase.

Vacuoles were obtained by ultracentrifugation of protoplasts on a discontinuous gradient of ficoll, as previously described [15]. Tonoplast was prepared from isolated vacuoles by an osmotic shock [14]. Purity of vacuole and tonoplast preparations was determined using marker enzymes of other cell compartments [14–16].

2.3. Phospholipase assays

Tonoplast vesicles (equivalent to 80 nmol PC) were incubated in 0.8 ml of a solution containing 5 nmol of *sn*-2-[^{14}C]linoleyl-PC, 10 mM EDTA or 2 mM Ca^{2+} or 2 mM Mg^{2+}, 0.1 M Mes-NaOH (pH 5.0) or 0.1 M Tris-HCl (pH 7.0) and 0.1% (v/v) Triton X-100. Assays were carried out for 30 min at 37°C. Three control reactions were performed (i) at $t = 0$ min, (ii) with boiled tonoplast and (iii) with 20 I.U. of bee venom phospholipase A_2 (pH 7.5, 1 mM Ca^{2+}). The reactions were stopped and the lipids extracted by addition of isopropanol/chloroform, as previously described [10].

Tonoplast phospholipase A_1 activity was characterized by measuring the release of radiolabelled lyso-PC from endogenous [*choline*-^{14}C]PC.

Labelling of tonoplast PC was carried out by addition of 0.37 MBq [*methyl*-^{14}C]choline-Cl to the cell culture. The addition of radiolabelled choline was performed 16–18 h before removing cells for tonoplast preparation.

The standard incubation mixture contained 100 mM Mes-NaOH (pH 6.0), 0.25 M mannitol, 2 mM Ca^{2+} and radiolabelled tonoplast vesicles (equivalent to 65 nmol PC, with a specific radioactivity of 205 000 dpm/μmol PC) in a final volume of 0.8 ml. Assays were carried out for 30 min at 37°C. The reactions were stopped and the lipids extracted by addition of isopropanol/chloroform [10].

2.4. Analytical procedures

The lipids extracted from the incubation mixtures were separated on silica gel TLC plates (Silica Gel 60, Merck) developed in two dimensions, with chloroform/methanol/28% aqueous ammonia (65:35:5, v/v) in the first dimension and chloroform/methanol/acetone/acetic acid/water (50:10:20:10:5, v/v) in the second dimension. The lipid areas were stained with I_2, scraped off plates, placed in scintillation vials and counted after the addition of 5 ml of scintillation fluid.

3. Results

Tonoplast was obtained by osmotic lysis of pure preparations of vacuoles isolated from protoplasts derived from *A. pseudoplatanus* cells. The extent of contamination was: 3% plasmalemma (UDP-glucose sterol glucosyltransferase and glucan synthetase II); 0% cytoplasm (glucose-6-*P* dehydrogenase); 2.5% Golgi (IDPase and glucan synthetase I); 3.1% endoplasmic reticulum (antimycin A-insensitive NADH–cytochrome-*c* reductase); 0% mitochondria (cytochrome-*c* oxidase); 2.8% peroxisomes (catalase); 2.3 and 3.4% vacuolar sap (α-mannosidase and acid phosphatase, respectively) [14–16].

At first, the phospholipase activities in tonoplast were studied by addition of *sn*-2-[^{14}C]linoleyl-PC to tonoplast vesicles in the presence of 0.1% Triton X-100 and by following the *sn*-2-[^{14}C]PC catabolism by analysis of radioactive products. Since phospholipases have been shown to be Ca^{2+} dependent, the effect of addition of EDTA, Ca^{2+} and another divalent ion (Mg^{2+}) on the catabolism of *sn*-2-[^{14}C]PC was investigated. The results presented in Table 1 indicate that 90 to 95% of radioactivity lost from PC was recovered in lyso-PC and phosphatidic acid (PA). The production of radioactive PA appeared to be Ca^{2+}-dependent, since no production of PA was detected in the presence of EDTA and Mg^{2+}. Moreover, PA production was stimulated at acid pH. The release of radioactive

Table 1
Phospholipase activities associated with the tonoplast of *A. pseudoplatanus*

	% of total radioactivity						
	control ($t = 0$)	boiled tonoplast	tonoplast + exogenous phospholipase A_2	tonoplast + 10 mM EDTA, pH 5	tonoplast + 2 mM Ca^{2+}, pH 5	tonoplast + 2 mM Mg^{2+}, pH 5	tonoplast + 2 mM Ca^{2+}, pH 7
Lyso-PC	0.6 ± 0.4	1.8 ± 0.6	0.9 ± 0.5	28.4 ± 2.2	27.0 ± 2.6	28.1 ± 2.7	28.7 ± 2.4
PC	98.9 ± 1.4	97.3 ± 1.8	0.8 ± 0.7	67.7 ± 3.2	59.3 ± 2.8	67.3 ± 3.1	61.4 ± 3.0
PA	0.1 ± 0.1	0.2 ± 0.1	0.1 ± 0.1	0.8 ± 0.4	11.1 ± 1.1	0.3 ± 0.3	6.8 ± 0.9
FFA	0.4 ± 0.2	0.5 ± 0.3	98.2 ± 2.5	2.6 ± 0.9	2.1 ± 0.7	3.6 ± 0.8	2.7 ± 1.1
DAG	-	0.2 ± 0.2	-	0.5 ± 0.4	0.5 ± 0.3	0.7 ± 0.4	0.4 ± 0.3

Tonoplast fraction (equivalent to 80 nmol PC) was incubated with 9.25 kBq of *sn*-2-[^{14}C]acyl-PC (5 nmol) in the presence of 0.1% (w/v) Triton X-100 for 30 min at 37°C. Lipids were separated by TLC and the radioactivity was measured in a liquid scintillation counter as described in Material and methods. The values shown are the average of three experiments, each performed in triplicate.

lyso-PC was, however, the same irrespective of the pH and the addition of EDTA or divalent ions (Table 1). A further incubation of 30 min in the presence of 2 mM Ca^{2+} showed an increase in both PA and lyso-PC formation (19.1% and 34% respectively at pH 5; 11.3% and 35.5% respectively at pH 7), while a further incubation of 30 min at pH 5 in the presence of 10 mM EDTA or 2 mM Mg^{2+} revealed only an increase in lyso-PC formation (34.5 to 37%). Almost no radioactivity was recovered in free fatty acids (FFA) in the different assays, but almost all the radioactivity was detected in FFA in the control after addition of bee venom phospholipase A_2 (Table 1). The experiments presented in Table 1 were also performed in the absence of Triton X-100 (data not shown). Under this condition and in the presence of 2 mM Ca^{2+} low phospholipase A_1 and phospholipase D activities were detected. These activities represented 22.5% and 44% of the phospholipase A_1 and phospholipase D activities, respectively, measured in the presence of Triton X-100 0.1%. The assays performed in the absence of detergent and in the presence of 10 mM EDTA did not show any significant PA or lyso-PC formation.

These results unequivocally demonstrate the association of phospholipase A_1 and phospholipase D activities with the tonoplast of *A. pseudoplatanus*.

Since phospholipases are present in membranous and soluble forms [3], the presence of vacuolar soluble phospholipase activities was also investigated. Incubation of liposomes, formed with *sn*-2-[^{14}C]PC and tonoplast lipids, with concentrated vacuolar sap did not reveal any significant accumulation of radioactive products (data not shown). This result suggests the absence of soluble phospholipase activity in the vacuolar sap.

In order to characterize the phospholipase A_1 activity, the properties of this enzyme were studied by following hydrolysis of endogenous tonoplast [*choline*-^{14}C]PC under various conditions. Radiolabelling of tonoplast PC molecules was previously performed by addition of [*methyl*-^{14}C]choline to the cell culture 16 to 18 h before harvesting cells (cf. Materials and methods). Phospholipase A_1 activity was expressed in amount of [*choline*-^{14}C]lyso-PC produced per assay (dpm/200 μg protein/30 min) and not in amount of [*choline*-^{14}C]PC hydrolysed, to avoid errors caused by simultaneous hydrolysis of [^{14}C]PC into PA by phospholipase D.

Phospholipase A_1 activity was measured over a pH range from 4 to 8.5. Fig. 1 shows that optimum phospholipase A_1 activity was observed at pH 6–6.5 with PC as substrate.

The formation of [^{14}C]lyso-PC was remarkably activated by addition of Ca^{2+} and Mg^{2+} (Fig. 2). Addition of EDTA did not totally inhibit the phospholipase A_1 activity. The phospholipase A_1 activity in the presence of Ca^{2+} was 3- to 4-fold higher than that measured in the presence of EDTA. Moreover, addition of an excess of Ca^{2+} after 20 min incubation of tonoplast vesicles in the presence of

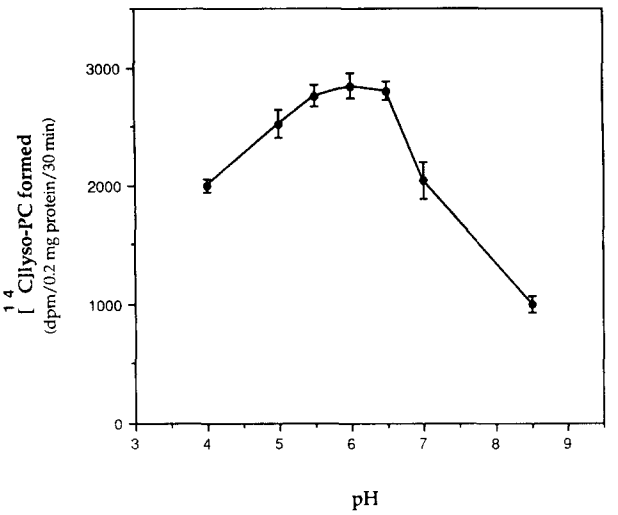

Fig. 1. Phospholipase A_1 activity as a function of pH. [^{14}C]Lyso-PC production was measured by incubating tonoplast vesicles (equivalent to 65 nmol PC) with 2 mM Ca^{2+} in 50 mM sodium citrate (pH 4.0, pH 5.0 and pH 5.5), 50 mM Mes-KOH (pH 5.5, pH 6.0 and pH 6.5) and 50 mM Tris-HCl (pH 7.0 and pH 8.5). Reactions were carried out at 37°C for 30 min. Data presented are the means of three independent determinations ± S.E.

EDTA stimulated the phospholipase A_1 activity (data not shown). These results show that phospholipase A_1 activity did not necessarily require divalent cations but was, however, stimulated by Ca^{2+} and Mg^{2+}. Phospholipase A_1 activity in the presence of Mg^{2+} represented 85% of that in the presence of Ca^{2+} (Fig. 2).

Since Ca^{2+} and Mg^{2+} did not stimulate the acyl hydrolysis of exogenous substrate (*sn*-2-[^{14}C]PC) in the presence of Triton X-100 (Table 1), the phospholipase A_1

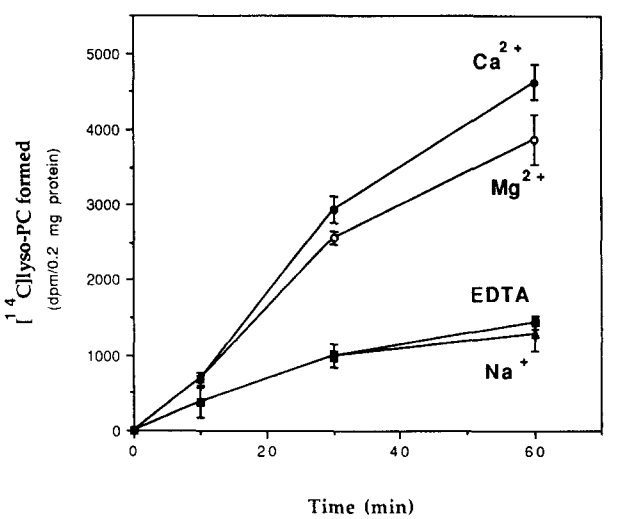

Fig. 2. Effect of ions on phospholipase A_1 activity. [^{14}C]Lyso-PC production was measured by incubating tonoplast vesicles (equivalent to 65 nmol PC) with 10 mM EDTA (■), 2 mM Mg^{2+} (○), 2 mM Ca^{2+} (●) or 2 mM Na^+ (▲) in 100 mM Mes-KOH, pH 6. Reactions were carried out at 37°C for 10, 30, 60 min. Data presented are the means of three independent determinations ± S.E.

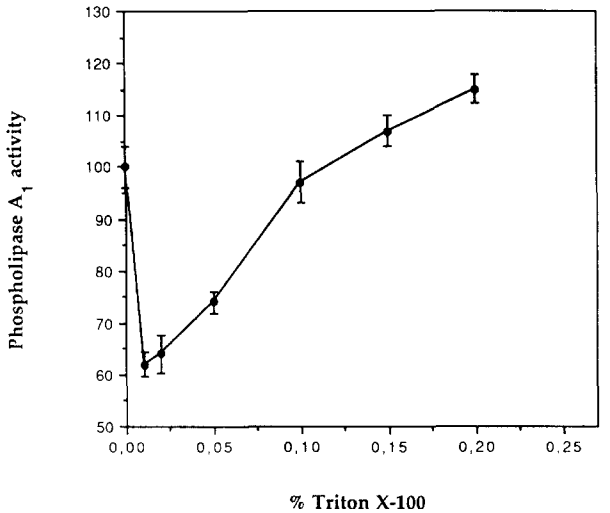

Fig. 3. Effect of Triton X-100 on phospholipase A_1 activity. [^{14}C]Lyso-PC production was measured by incubating tonoplast vesicles (equivalent to 65 nmol PC) with increasing Triton X-100 concentrations in 100 mM Mes-KOH, pH 6. Reactions were carried out at 37°C for 30 min. The 100% activity corresponded to phospholipase A_1 activity without Triton X-100. Data presented are the means of two independent determinations ±S.E.

activity was measured by following the hydrolysis of endogenous substrate [*choline*-^{14}C]PC in the presence of Triton X-100 (Fig. 3). Triton X-100 concentrations lower than 0.1% (v/v) inhibited phospholipase A_1 activity with a maximum inhibition (40%) for a Triton X-100 concentration range from 0.01 to 0.02%. 0.1% Triton X-100 had no effect, whereas Triton X-100 concentrations exceeding 0.1% stimulated phospholipase A_1 activity. As expected, the release of [*choline*-^{14}C]lyso-PC in presence of 0.1% Triton X-100 was the same irrespective of the addition of

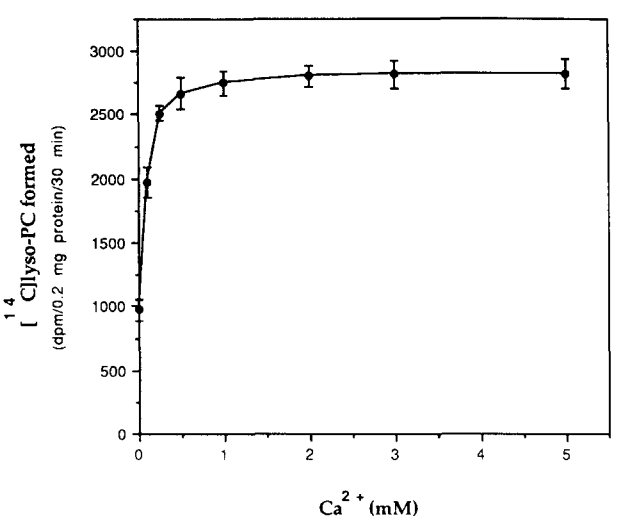

Fig. 4. Phospholipase A_1 activity as a function of Ca^{2+} concentration. [^{14}C]Lyso-PC production was measured by incubating tonoplast vesicles (equivalent to 65 nmol PC) with increasing Ca^{2+} concentrations in 100 mM Mes-KOH, pH 6. Reactions were carried out at 37°C for 30 min. Data presented are the means of three independent determinations ±S.E.

EDTA, Ca^{2+} or Mg^{2+} (not shown). Thus, this result is in good agreement with those reported in Table 1: 0.1% Triton X-100 seems to affect interactions between enzyme and substrate. These results obtained with PC were confirmed by following the formation of lyso-PE in the presence of Triton X-100 (not shown).

The effect of Ca^{2+} concentration on phospholipase A_1 activity was investigated (Fig. 4). Maximum stimulation after 30 min incubation was observed with a concentration of Ca^{2+} close to 1 mM.

The effects of pH, Ca^{2+} and Mg^{2+} on lyso-PE formation were also tested (data not shown). The results obtained confirmed those previously reported for lyso-PC formation. However, phospholipase A_1 may hydrolyse PC rather than PE: 40% of PC and 22% of PE were degraded into lyso-PC and lyso-PE respectively under standard conditions.

4. Discussion

The present work shows the degradation, by tonoplast, of *sn*-2-[^{14}C]acyl-PC as an exogenous substrate into radioactive PA and lyso-PC. These results clearly demonstrate the presence of phospholipase D and A_1 activities associated with the tonoplast of *A. pseudoplatanus*. Since deacylation of fatty acids occurs specifically at the *sn*-1 position of phospholipids, this enzyme can not be referred to a lipolytic acyl hydrolase which would not exhibit any positional specificity for acyl chains or substrate specificity for phospholipids, lysophospholipids and di- or monoacylglycerols [2–4]. Almost no phospholipase activity was detected in the vacuolar sap, in spite of the fact that the major lipolytic enzymes show both membranous and soluble forms and that soluble phospholipase D is usually compartmentalized in vacuoles [1,17].

The phospholipase D activity associated with the tonoplast of *A. pseudoplatanus* seems to be more active in the acidic pH range and specifically requires millimolar levels of Ca^{2+} (Table 1). These results are in good agreement with several previous reports about this enzyme [3]. In contrast to phospholipase D, which is well characterized from various plant tissues [1,3], in our knowledge, this is the first report of the presence of a phospholipase of the A_1 type in plant cells. Phospholipase A_1 efficiently hydrolyses PC, PE and PS [14], but PC is the privileged substrate. Phospholipases have been shown generally to be Ca^{2+} dependent [2]. The phospholipase A_1 activity in tonoplast of *A. pseudoplatanus* is not Ca^{2+}-dependent but the enzyme is greatly stimulated by Ca^{2+}. Highest stimulation was obtained with 2 mM Ca^{2+} but 100 μM Ca^{2+} gave a 55% stimulation. Nevertheless, this Ca^{2+} effect is not specific since Mg^{2+} induced 85% of the activity measured in the presence of Ca^{2+}, indicating that divalent cations may improve the enzyme/substrate interactions. Because Ca^{2+} or Mg^{2+} did not stimulate phospholipase

A_1 activity (exogenous substrate) in presence of 0.1% Triton X-100 as reported in Table 1, we looked for the effect of 0.1% Triton X-100 on Ca^{2+} or Mg^{2+} stimulation of the phospholipase A_1 activity (endogenous substrate). Our results indicate that the presence of 0.1% Triton X-100 suppresses stimulation of phospholipase A_1 activity by divalent cations, affecting probably the interactions between the substrate-enzyme complex and divalent cations. These results indicate that substrate presentation can also affect phospholipase A_1 activity.

It is of interest to establish the physiological significance of the tonoplast phospholipase A_1 activity. Phospholipid turnover is now a well characterized phenomenon in animal tissues and has also been suggested to occur in plant cells [2]. Possible roles for plant phospholipases A may be (i) protection of membranes from oxidative damage [13], (ii) involvement in auxin mediated responses [12], (iii) release of precursors for hormone synthesis, or (iv) production of secondary messengers involved, for example, in senescence or host-pathogen interactions [2,3,18,19]. Moreover, release of free fatty acids and lysophospholipids by phospholipase A_1 would locally and transiently modify the structural organization and the surface properties of the tonoplast. Such modifications could be involved in the regulation of tonoplast protein activities [20,21] or in membrane fusion processes. Further investigations about acyl chain-specificity of tonoplast phospholipase A_1, its location within the membrane and its mode of regulation will be necessary to clarify its physiological role.

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