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PHOSPHATIDYLINOSITOL PHOSPHOLIPASE C ACTIVITY IN POLLEN OF *LILIUM LONGIFLORUM*

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Key Word Index—*Lilium longiflorum*; Liliaceae; phosphatidylinositol; phospholipase C; pollen.

Abstract—Homogenates from pollen of *Lilium longiflorum* (cv. Arai) contain phosphatidylinositol phospholipase activity, which increased during germination. Analysis of radioactive products resulting from incubation of phosphatidylinositol, labelled in the *myo*-inositol, phosphate or glycerol moiety, showed that cleavage had only taken place between glycerol and phosphate. The activity is therefore of a phospholipase C-type (EC 3.1.4.10). It was stimulated by several divalent cations; Ca^{2+} was the most effective. Zn^{2+}, Cu^{2+} and EDTA are inhibitory. The activity was found in both the cytosol and particulate fractions.

INTRODUCTION

Phosphatidylinositol is one of the major membrane phospholipids in plant tissues. In rapidly growing cells such as pollen tubes, phospholipids are subject to intensive turnover [1, 2]. Besides its function as a membrane constituent in animal cells, phosphatidylinositol and its phosphorylated derivatives are key compounds in mediating a variety of physiological responses on specific stimuli, e.g. hormones and neurotransmitters [3, 4]. The latter process, which is known as the 'inositol lipid response' [5], involves an enhanced cleavage of these phosphoinositides, mobilization of Ca^{2+} and activation of protein kinases. The phosphoinositide cleavage is catalysed by phosphatidylinositol phospholipase C activity, which can also play a role in stimulus-independent turnover of this phospholipid.

There is only one report on phosphatidylinositol phospholipase C activity in higher plants. This enzymatic activity was observed in the cytosol fractions from celery, cauliflower and daffodils by Irvine *et al.* [6]. Both cytosol and particulate phosphatidylinositol phospholipase C activities are well documented in animal tissues [5]. They show affinity for unphosphorylated and phosphorylated forms of phosphatidylinositol [7, 8]. In a previous paper we reported the presence of phosphatidylinositol phospholipase activity in homogenates from *Lilium longiflorum* (L.) Thunb pollen [2]. In this communication we characterize the activity further.

RESULTS AND DISCUSSION

When phosphatidylinositol substrates, labelled in the *myo*-inositol, phosphate or glycerol moiety, were incubated with homogenates from *Lilium longiflorum* pollen, germinated previously for 4 hours, approximately 50% of the substrate was converted (Table 1) under the experimental conditions. Heat-treated homogenates (10 min, 100°) did not exhibit any phospholipase activity. Analysis of the labelled reaction products (Table 1) showed that they included inositol 1-phosphate and inositol 1,2-cyclic phosphate when the substrate was either phosphatidyl[^{3}H]inositol or [^{32}P]phosphatidylinositol, while [glycerol-^{3}H]phosphatidylinositol yielded tritiated 1,2-diacylglycerol. The products obtained were therefore consistent with cleavage between the glycerol and phosphate moieties of phosphatidylinositol, and thus with the presence of a phosphatidylinositol phospholipase C activity (EC 3.1.4.10). Michell [5] reported the same reaction products from phosphatidylinositol phospholipase C activity in animal tissues and Irvine *et al.* [6] from a similar enzymatic activity in celery. Free [^{3}H]inositol and [^{32}P]orthophosphate probably result from secondary cleavage of inositol phosphates by inositol phosphatase, reported to be present in the cytosol fraction from pollen of *Lilium longiflorum* [9]. We looked for, but did not observe, the formation of labelled lysophosphatidylinositol, glycerylphosphoryl inositol or phosphatidic acid. This suggests the absence of phosphatidylinositol phospholipase A_1, phospholipase A_2 and phospholipase D activities.

Phosphatidylinositol phospholipase C activity is also observed in homogenates from ungerminated pollen of *Lilium longiflorum* (Fig. 1). It increases to a maximum by 2–4 hours of germination, at which time pollen tubes have begun to emerge from the grains and turnover of membrane phospholipids has increased [2]. The protein content of the pollen does not change significantly during the 8 hours germination period. In all other experiments phosphatidylinositol phospholipase C activity was examined in homogenates from 4 hours-germinated pollen. The time course of phosphatidylinositol phospholipase C activity was linear up until 15 min. The activity increased with increasing concentrations of homogenate added until at least 3.0 mg protein/ml were present in the incubation mixture.

Addition of divalent cations was not required for phosphatidylinositol phospholipase C activity (Table 2); however, addition of 1 mM EDTA completely inhibits the reaction. This indicates the presence of essential divalent

Table 1. Distribution of radioactivity in chloroform- and methanol–water-soluble products resulting from 15 min enzymolysis at 25° of phosphatidylinositol, labelled in the *myo*-inositol, phosphate or glycerol moiety, by a pollen tube homogenate containing 0.9 mg protein

Position of label in phosphatidylinositol	Distribution of label after incubation	
	Chloroform phase	Methanol–water phase
myo-[2-^{3}H]Inositol	55% Phosphatidylinositol	12% Inositol 8% Inositol phosphate 25% Inositol cyclic phosphate
[^{32}P]Phosphate	56% Phosphatidylinositol	13% Phosphate 6% Inositol phosphate 25% Inositol cyclic phosphate
[2-^{3}H]Glycerol	48% Phosphatidylinositol 51% 1,2-Diacylglycerol	1%

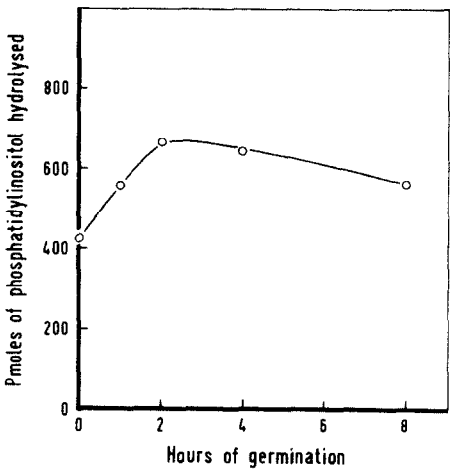

Fig. 1. Changes in phosphatidylinositol phospholipase C activity during germination of *Lilium longiflorum* pollen. At the times indicated, pollen was washed, homogenates prepared and phospholipase C activity determined as described in the Experimental section.

Table 2. Effect of divalent cations (1 mM) on the phosphatidylinositol phospholipase C activity in pollen tube homogenate

Divalent cation	Phosphatidylinositol phospholipase C activity (%)
None	73
Ca^{2+}	100
Co^{2+}	80
Mg^{2+}	86
Mn^{2+}	85
Zn^{2+}	17
Cu^{2+}	7

Results are expressed as percentage of the activity in the presence of 1 mM Ca^{2+}.

metal ions in the homogenate, a finding also observed with enzyme preparations from animal tissues [10, 11] and from celery [6]. In contrast to the phosphatidylinositol phospholipase C activity in this latter tissue, the *Lilium* homogenate retains its activity after exhaustive dialysis against Ca^{2+}-free 0.1 M potassium acetate buffer (pH 6.5). In addition dialysis restores enzymatic activity in homogenates, which have been inactivated by the addition of 1 mM EDTA. These results suggest a strong interaction between the divalent cation and the enzyme.

Although the addition of exogenous divalent cations is not essential for enzymatic cleavage of phosphatidylinositol, it does stimulate the reaction. Ca^{2+} is the most effectively stimulatory ion species (Table 2), while Mg^{2+}, Mn^{2+} and Co^{2+} have less effect. The activity increases with the addition of Ca^{2+} up to 1 mM and levels off at higher concentrations. Addition of Zn^{2+} and Cu^{2+} inhibit the activity (Table 2). Similar effects of divalent cations are observed with the phosphatidylinositol phospholipase C activities from celery [6] and from several animal tissues [12–15]. Phosphatidylinositol phospholipase C activity from *Lilium longiflorum* is stable at −20° for several weeks as is the celery enzyme [6]. When the pollen tube homogenate is centrifuged at 100000 *g* for 1 hour, 65% of the phospholipase C activity at pH 6.5 is recovered in the cytosol fraction, which contained 80% of the protein. The remaining 35% of the phospholipase activity is recovered in the particulate fraction containing the membrane components of the cell. Repeated washing of the particulate fraction did not affect its activity.

Since this investigation was directed towards the characterization of phosphatidylinositol phospholipase C activity, we can only speculate on its physiological role. A function in membrane turnover, as was also suggested by Irvine *et al.* [6] for this enzyme activity in celery, can be readily accepted. Other types of phospholipase activity have not been detected for phosphatidylinositol in these cells in which a high turnover of all phospholipids has been observed [2]. A relation with a possible 'inositol lipid response' cannot be excluded, although this phenomenon has not yet been described in a higher plant. The present observation of phosphatidylinositol phospholipase C activity and the occurrence of polyphosphoinositides in pollen of *Lilium longiflorum* [2] indicate the presence of the necessary components for such a response.

EXPERIMENTAL

Chemicals used were of reagent grade. Specifically-labelled forms of phosphatidylinositol, used as substrates for phospholipase assays, were prepared by incubating 25 mg of *Lilium longiflorum* (cv Arai) pollen for 8 hr at 25° with 40 μCi *myo*-[2-^{3}H]inositol, [^{32}P]orthophosphate or [2-^{3}H]glycerol, according to Helsper *et al.* [2]. All radiochemicals were obtained from Amersham International. After extraction of lipids according to Helsper *et al.* [2] labelled phosphatidylinositol was purified by TLC on activated Kieselgel 60 in $CHCl_3$–MeOH–HOAc (60:25:8). Areas corresponding to phosphatidylinositol were scraped off, extracted with 4.0 ml $CHCl_3$–MeOH (1:1) and stored at −20°.

Homogenates from *Lilium longiflorum* pollen, which had been germinated for 4 hours, if not mentioned otherwise, were obtained as described by Helsper *et al.* [2], diluted to 3 mg protein/ml and dialysed for 24 hr against 0.1 M KOAc buffer (pH 6.5). Dialysed preparations were stored at −20°.

For phospholipase assays 50 μl of the $CHCl_3$–MeOH solns, containing *ca* 1 nmol of radioactive phosphatidylinositol, were evaporated to dryness at 25° under an N_2 stream in conical centrifuge tubes. Then 0.3 ml pollen tube homogenate (0.9 mg protein) was added together with 0.6 ml of 0.1 M KOAc, pH 6.5, containing also $CaCl_2$. Standard incubations were carried out with phosphatidyl[^{3}H]inositol as substrate for 15 min at 25° in 0.9 ml total vol. containing 1 mM $CaCl_2$. The reaction was stopped by heating for 10 min at 100°. Lipoidal and water-soluble compounds were separated as described by Helsper *et al.* [2]. Phospholipase C activities were calculated from the amount of tritium released into the aq. phase.

Radioactivity in diacylglycerol, synthesized from [glycerol-^{3}H]phosphatidylinositol, was determined after TLC separation on activated Kieselgel 60 in $CHCl_3$–MeOH–HOAc (60:25:8) or in petrol–Et_2O–HCOOH (60:40:1). Radioactivity in inositol 1-phosphate, inositol 1,2-cyclic phosphate, orthophosphate and inositol were determined after separation by anion exchange chromatography on Dowex-1 (formate form) according to Berridge *et al.* [3]. Further identification of inositol phosphates was achieved by paper electrophoresis and PC [16]. Liquid scintillation analysis was carried out with 5.0 ml Tritosol [17] in a Philips PW 4540 liquid scintillation analyser. The results presented in this paper are the mean of at least two experiments.

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