

CALCIUM ACTIVATION OF MAIZE ROOT PHOSPHOLIPASE D

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ABSTRACT: Calcium activation of plant lipases which occurs at relatively high concentrations is poorly understood. The effects of divalent cations on the activities of the soluble and membrane-bound phospholipase D were compared. Both the soluble and membrane-bound activities were stimulated by 1 mM Ca and to a lesser extent by 1 mM Ba, but not by 1 mM Mg, Mn, Zn, or Cd. The two enzyme forms differ in the concentration dependence of Ca activation. The membrane-bound activity exhibited some activity in the absence of Ca, whereas no activity was observed with the soluble enzyme without Ca. Maximal activation was observed at micromolar concentration with the membrane-bound enzyme compared to millimolar levels for the soluble enzyme. The effects of Ca on the structure of substrate vesicles for the soluble enzyme were determined by following changes in fluorescence of incorporated probes. Calcium had little effects on membrane structure at micromolar concentration, but alter the bilayer structure at millimolar concentrations. Thus, there was a strong association between the concentration dependence of the soluble phospholipase D activity and changes in membrane structure.

INTRODUCTION

Calcium is required for the growth and development of plants (1). In the past 10 years, evidence has been compiled that cytoplasmic levels of Ca regulate cellular functions (2). Usually the level of Ca in the cytoplasm of plant cells is maintained at rather low levels averaging 0.1 to 0.2 μM . In response to stimuli or changes in ontogeny, Ca levels may rise to higher levels but never exceed micromolar concentrations. This rise in cytoplasmic Ca concentration is believed to alter cellular

metabolism by activating specific enzymes (2). Many types of enzymes, including protein kinases, NAD kinase, and Ca-translocating ATPases, are activated by micromolar concentrations of Ca (2). The increase in cytoplasmic Ca is believed to lead to the production of other regulatory metabolites, especially those derived from phospholipids (3). Consistent with such a role, there are numerous phospholipid degrading enzymes that are activated by Ca (4,5,6). However, millimolar levels of Ca are necessary to stimulate many lipolytic hydrolases. Most of these enzymes that require such high concentrations of Ca for activation are soluble rather than membrane-bound. The reason why these soluble lipases require such high levels of Ca is poorly understood. It has been proposed previously that the Ca was necessary for charge compensation as zwitterionic PC is converted to the negatively charged PA (7), however, the validity of this hypothesis has been strongly criticized. Membrane-bound plant lipases tend to need lower concentrations of Ca (between 0.1 and 0.2 mM) for maximum activation (8,9). At present, it is difficult to make all encompassing comparisons between the soluble and membrane-bound lipases reported previously because the comparisons are made across species and different types of lipolytic enzymes. We recently discovered soluble and membrane-bound forms of phospholipase D (PLase D) in corn root homogenates (10). The activity of these two forms of PLase D from the same tissue was characterized to determine if the localization of the lipolytic enzyme to membranes alters its activation by Ca.

MATERIALS AND METHODS

Preparation of Soluble and Membrane-bound Phospholipase D: Three-day old maize seedlings (cv. WF9 x Mo17) were grown on filter paper moistened with 0.1 mM CaCl₂ and harvested as described previously (11). Roots were homogenized by mortar and pestle in the presence of 50 mM Mes titrated to pH 7.0 with Bis-Tris-Propane, 5 mM dithiothreitol, and 0.1 mM EGTA at 0° to 4°C using 3 mL of buffer per g of roots. After thoroughly homogenizing for 3 to 5 min, the brei was filtered through four layers of cheesecloth. The resulting filtrate was then centrifuged at 1,000g for 5 min at 4°C to remove starch grains, unbroken cellular fragments and other large particulates. To separate the soluble PLase D from the membrane-bound activity, the 1,000g supernatant was centrifuged at 90,000g for 60 min at 4°C. The supernatant was used as the source for the soluble PLase D activity. The membrane pellet was resuspended in homogenizing buffer and used as the source for the membrane-bound PLase D activity. Protein concentration was

determined after precipitation by trichloroacetic acid in the presence of deoxycholate by a modification of the Lowry method (12).

PLase D Assays: Membrane-bound PLase D activity was determined by following the autolytic conversion of endogenous phospholipids to phosphatidic acid (PA). Changes in membrane lipids were followed in the present study rather than the metabolism of exogenously lipolytic substrates, because there was no correlation between the rate of autolytic loss of phospholipids and the hydrolysis of exogenously supplied substrates in a recent survey of plant species and tissues (13). Aliquots containing 1.0 mL of membranes were adjusted to 1.1 mM CaCl₂ by the addition of 0.1 M stock solution and incubated for 5 to 180 min at 28°C. Variations in assay conditions are noted in the text and associated figures. Triplicate assays typically were performed. Reactions were terminated by extracting the lipids with organic solvents as described by Moreau and Isett (14). Activity was determined by the net increase in PA as measured by high performance thin-layer chromatography coupled to densitometry as described previously (10).

Exogenous phospholipids had to be supplied to the soluble fraction to detect PLase D activity. Typically, 1.0 mL of soluble fraction was diluted with 3.0 mL of homogenizing buffer containing 0.53 mM dioleoylphosphatidylcholine (di-18:1 PC) and 1.45 mM CaCl₂. After incubating at 28°C for up to 180 min, 1.0 mL aliquots of the assay media were extracted with organic solvents to terminate the reactions (14). Activity was followed by the increase in PA as measured by high performance thin layer chromatography coupled to densitometry as described previously (10). Phospholipids used as substrates were added as exhaustively sonicated suspensions. Substrates were prepared by dissolving the phospholipid in chloroform and removing the solvent under a stream of N₂ at 60° to 70°C. After storing under vacuum for 2 to 24 h, homogenizing buffer was added to the dried film to yield approximately 20 mg of lipid/ mL. The lipid suspension was sealed under N₂ and then sonicated in a bath type apparatus for three, five min intervals.

PLase D activity also was followed by the production of choline. The aqueous phase from the lipid extraction was clarified by centrifugation at 10,000g for 10 min at 4°C. The supernatant was then dried at 110°C under a stream of N₂. The residue was resuspended in 1 ml of water. The choline content was determined spectrophotometrically after reaction with choline oxidase as described previously (15). The recovery of choline added to samples averaged approximately 90% (data not shown).

Evaluation of Membrane Structure: The effect of Ca on the structure of phospholipid vesicles was estimated from changes in the polarization of fluorescence of diphenylhexatriene and a diphenylhexatriene analog of phosphatidylcholine, DPH-PC (16). Vesicles suspended in homogenizing buffer were equilibrated with 2 μ M diphenylhexatriene for 20 min at 20°C to 22°C. The DPH-PC analog was mixed with phospholipids in chloroform and vesicles were prepared by sonication as described above. Membranes incorporated with either diphenylhexatriene or DPH-PC were illuminated with vertically polarized light at 370 nm. Fluorescence intensities at the parallel and the perpendicular directions were measured at 424 nm. Polarization was calculated by the ratio of parallel intensity minus perpendicular intensity divided by the sum of the types of intensities.

RESULTS AND DISCUSSION

Time Course of Autolytic Changes in Membrane Lipids: The changes in lipids of isolated membranes and the soluble fraction supplied with di-18:1 PC as a function of time were assessed in the presence of 1 mM CaCl₂ to determine if experimental conditions were adequate to assay the rates of PLase D activity. First, changes in lipids in the isolated membranes were followed. Levels of membrane PC declined at a fairly constant rate of about 80 nmol/h/mg protein for at least 2 h (Fig 1). Between 2.5 and 3 h, the rate of PC loss tended to decrease. During the first h, losses in PC corresponded to increases in PA and choline. The loss in PC was approximately the same as the production of choline, indicative of degradation by PLase D. In the first hour, PA production (120 nmol/mg protein) was slightly greater than PC loss and choline production. The disparity between PC loss and increases in PA probably resulted from other phospholipids being utilized for PA production since the levels of other phospholipids such as phosphatidylinositol, phosphatidylethanolamine and phosphatidylserine also declined with incubation (data not shown).

The initial product of phospholipid degradation during this first h appeared to PA. This is supported by the fact that very little production of free fatty acids was observed. In addition, total lipid phosphorus did not change, averaging 440 nmol/mg protein (data not shown). No production of either lyso-phospholipids or

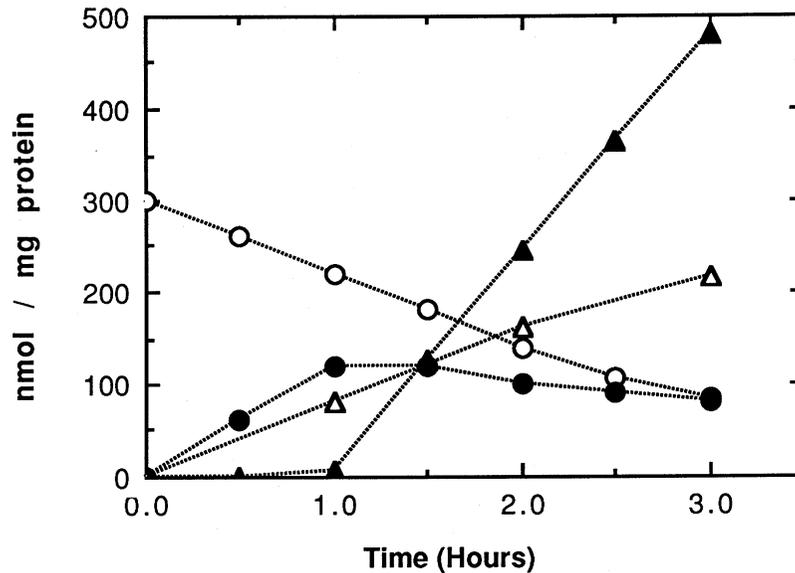


Figure 1. Time Course of Autolytic Changes in Lipids of Isolated Membranes. Aliquots of isolated membranes were incubated at 25°C for 0 to 3 h. The reactions were stopped by extracting the lipids with organic solvents and the samples were analyzed for choline, PC, PA and free fatty acids as described in Materials and Methods. Changes in the levels of fatty acids (▲) and PA (●), and the absolute levels of PC (○) and choline (△) are plotted as a function of time.

diacylglyceride was observed (data not shown). The lack of these two intermediates was reported previously with crude homogenates from maize roots (10). All of these results indicate that PA was the overwhelming product of phospholipid degradation during the first h and the rate of PA production was relatively constant. Therefore, the production of PA during this time appeared to be a reliable estimate of PLase D activity.

However, beyond 1 h, levels of PA declined as the concentrations of free fatty acids increased progressively. The concentration of choline continued to increase indicating that additional PC was being converted to PA by PLase D, despite the

drop in PA. These results indicate that after 1 h that PC was still being converted to PA and choline, but the PA was being rapidly broken down to free fatty acids. Between 1 and 3 h, the loss in total lipid phosphorus averaged about 110 nmol/mg protein/h. Free fatty acid production occurred at a rate of about 230 nmol/mg protein. Therefore, the theoretical stoichiometry of 2 free fatty acids produced per phospholipid degraded was realized. Therefore, changes in PA levels after 1 h of incubation were determined by both the rate of production from other phospholipids and the rate of degradation to free fatty acids.

The time course of lipid changes in the soluble fraction supplied with di-18:1 PC was significantly different from of the membrane fraction (Fig. 2). The di-18:1 PC was degraded to PA and choline at a rate that was substantially slower than that observed with the membrane fraction. The production of choline and PA balanced closely with the loss of di-18:1 PC. However, there was no detected production of free fatty acids, even when the incubation time was increased to 8 h (data not shown). These results indicate that the soluble fraction contained little if any lipolytic enzymes that were capable of degrading PA.

The results from Figures 1 and 2 indicate that both the soluble and membrane-bound PLase D activities could be estimated by the production of PA. In the case of membrane-bound PLase D, the assay time had to be less than 1 h because beyond 1 h significant amounts of the PA produced by PLase D were further degraded to free fatty acids.

Divalent Cation Activation: The effects of a variety of divalent cations at 1 mM concentration on the soluble and membrane-bound PLase D activities were compared to the absence of added cations. The soluble activity had no activity in the absence of metals whereas the membrane-bound PLase D had significant activity. In the presence of 1 mM Ca, the membrane-bound and soluble PLase D activity had rates of 124 ± 13 and 29 ± 3 nmol PA/mg protein/h, respectively, as compared to 36 ± 4 and 0 nmol PA/min/mg protein in the absence of metals. Membrane-bound PLase D activity in the absence of Ca could not be reduced further by the addition of high amounts of EGTA in the homogenizing buffer. When the EGTA in the homogenizing buffer was increased to either 1 or 10 mM, the PLase D varied 124 and 125 nmol PA/h/mg protein when assayed in the presence of 1 mM CaCl_2 as compared to 125 ± 11 nmol PA/h/mg protein when membranes were isolated in the presence of 0.1 mM EGTA and then assayed in the presence of 1 mM CaCl_2 . The

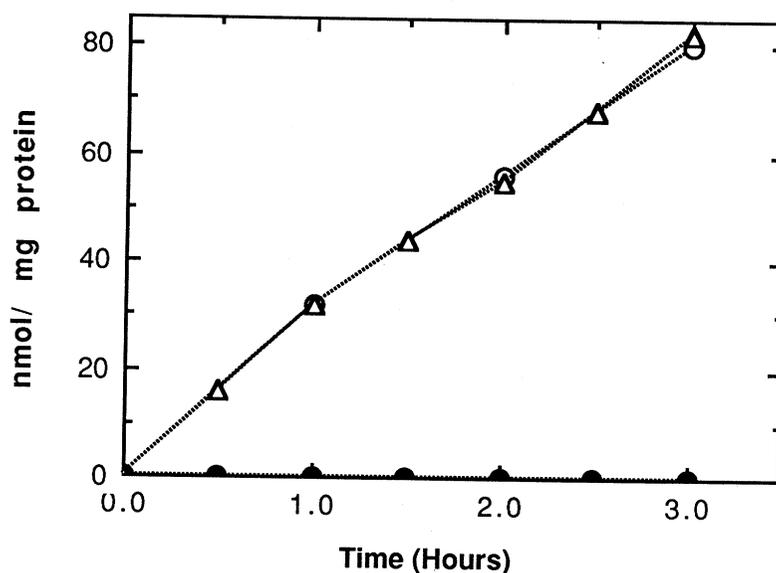


Figure 2. Time Course of Changes in Lipids of Soluble Fraction Supplied with Dioleoylphosphatidylcholine. Soluble fractions were incubated with di-18:1 PC in the presence of 1 mM CaCl₂ as described in the Materials and Methods. Incubation was terminated by extracting the lipids with organic solvents and the samples were analyzed for choline, PA and fatty acids as described in Materials and Methods. Levels of PA (Δ), choline (○) and fatty acids (●) are plotted as a function of time.

lack of inhibition by high EGTA in the homogenizing buffer suggests that the PLase D activity in the absence of metals was not due to contamination by Ca.

The selectivity of the PLase D activity for Ca was assessed by comparing the activity in the presence of 1 mM chloride salts of Ba, Mg, Cd, Zn, or Mn to that found with 1 mM CaCl₂. In this experiment, PLase D activity was 116 ± 9 and 33 ± 3 nmol PA/mg protein/h for membrane-bound and soluble sources, respectively, in the presence of 1 mM CaCl₂, and 24 ± 3 and 0 nmol PA/mg protein/h, respectively, in the absence of added cations. Of the other divalent cations tested,

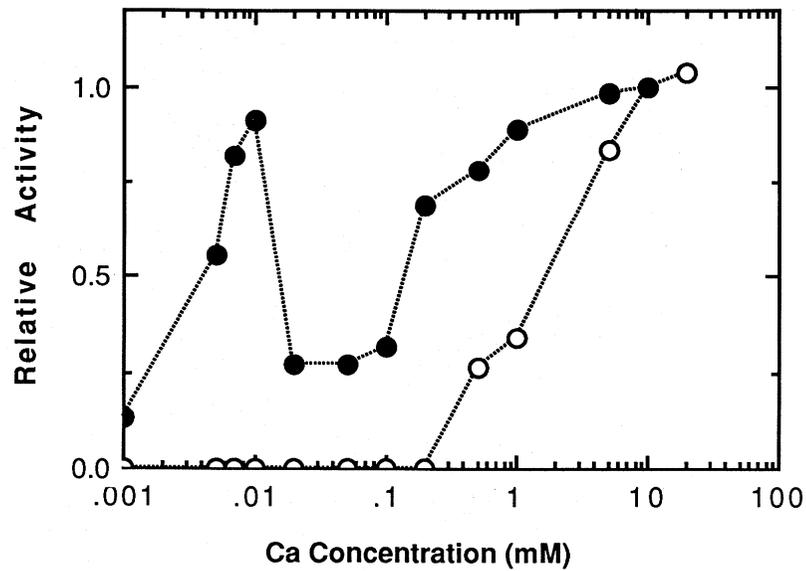


Figure 3. Effect of Ca Concentration on the Phospholipase D Activities. The production of PA after an hour of incubation was determined as described in Materials and Methods. Activities are plotted relative to that observed at 10 mM CaCl_2 , 122 ± 11 and 34 ± 4 nmol PA/h/mg protein for the membrane-bound (●) and soluble (○) PLase D, respectively .

only Ba was stimulatory. In the presence of 1 mM Ba, rates were about one-third that in the presence of Ca, 46 ± 6 and 13 ± 3 nmol PA/mg protein/h for the membrane-bound and soluble activities, respectively. No PA was formed in the presence of either 1 mM Cd or 1 mM Zn, when with the membrane-bound enzyme. Therefore, these two metals appeared to be inhibitory to the membrane-bound PLase D.

The membrane-bound PLase D showed a complex stimulation by Ca (Fig. 3) Activity increased about four-fold as the Ca concentration was increased from 1 to 10 μM . Activation of this PLase D activity by micromolar Ca concentrations could have *in vivo* significance (2). Increasing the Ca slightly above 10 μM inhibited activity. PLase D activity remained at this minimum level until the Ca concentration

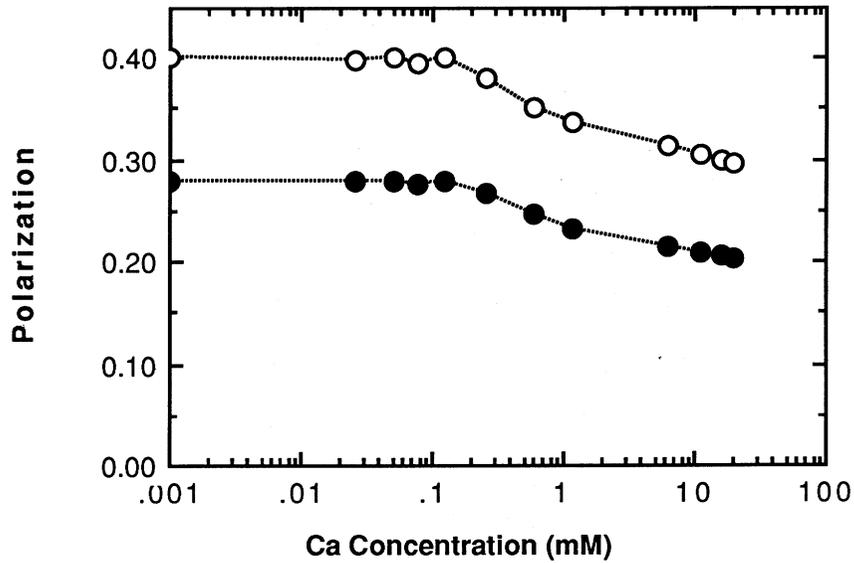


Figure 4. Effects of Ca Concentration on the Polarization of Lipophilic Probes Incorporated into Membranes. Fluorescence of diphenylhexatriene (○) and diphenylhexatriene-phosphatidylcholine (●) incorporated into di-18:1 PC vesicles. was determined at Ca concentrations from 1 μ M to 20 mM as described in Materials and Methods.

was increased above 0.1 mM. With increases in Ca beyond 0.2 mM, activity increased approaching saturation at concentrations above 10 mM.

The soluble PLase D activity had quite a different dependence of Ca (Fig. 3). There was no detectable activity at concentrations below 0.1 mM. PLase D activity increased rapidly with increasing Ca concentrations above 0.1 mM, saturating between 10 and 20 mM Ca. Therefore, the results reported here support the generality that membrane-bound lipases require less Ca for maximal activity than soluble forms of the enzyme.

Millimolar concentrations of Ca are known to catalyze the fusion of membrane vesicles (17). This acceleration is believed to occur because the Ca induces changes

in the structure of the lipids in the membranes vesicles. Could a similar phenomenon be involved in the activation of phospholipases?

The changes in the structure of di-18:1 PC vesicles as a function of Ca concentration was evaluated by changes in the microviscosity of the lipid bilayer as reported by the polarization of the fluorescence of diphenylhexatriene (16). As the Ca concentration was increased up to 0.12 mM, there was little change in the polarization, indicating that there was very little changes in membrane structure at micromolar concentrations of Ca (Fig. 4). However, increasing Ca above 0.25 mM resulted in decreased polarization. The polarization of diphenylhexatriene decreased sharply between 0.25 and 11 mM Ca, tending to approach a minimum above 11 mM. These effects of Ca on the lipid environment of di-18:1 PC vesicles paralleled Ca activation of soluble PLase D activity. Such results argue in favor of the hypothesis that Ca activation involved an interaction between the metal and the lipid bilayer. However, definite proof awaits further experimentation. Perhaps, one approach to answer the mechanism of Ca activation of lipolytic enzymes is to identify substrates that do not form aggregates ion water and to use these substrates to study metal activation.

REFERENCES:

1. Mengel, K. and Kirby, E.A. 1978. Principles of Plant Nutrition. Der BundAG, Bern, Switzerland.
2. Marme, D. 1985. The role of calcium in the cellular regulation of plant metabolism. *Physiologie Vegetale* 23:945-953.
3. Zbell, B. and Walter-Back, C. 1988. Signal transduction of auxin on isolated plant cell membranes: indications for a rapid poly-phosphoinositide response stimulated by indoleacetic acid. *Journal Plant Physiology* 133: 353-360.
4. Lee, M. H. 1989. Phospholipase D of rice bran, I. Purification and characterization. *Plant Science* 59:25-33

5. Witt, G., Yelenosky, G. and Mayer, R.T. 1987. Purification of phospholipase D from citrus callus tissue. *Archives Biochemistry Biophysics* 259: 164-170.
6. Galliard, T. 1980. Degradation of acyl lipids: hydrolytic and oxidative enzymes, pp. 85-116. IN: Stumpf, P.K. (ed.) *The Biochemistry of Plants: A Comprehensive Treatise*, vol. 4, Lipids: Structure and Function. Academic Press, New York, NY.
7. Hill, M.J. and Beevers, H. 1987. Ca-stimulated neutral lipase activity in castor bean lipid bodies. *Plant Physiology* 84:272-276.
8. Paliyath, G. and Thompson, J.E. 1987. Calcium- and calmodulin-regulated breakdown of phospholipids by microsomal membranes from bean cotyledons. *Plant Physiology* 83:63-68.
9. Melin, P., Sommarin, M., Sandelius, A.S. and Jergil, B. 1987. Identification of Ca-stimulated polyphosphoinositide phospholipase C in isolated plasma membranes. *FEBS Letters* 223:87-91.
10. Brauer, D., Nungesser, E., Maxwell, R.J., Schubert, C. and Tu, S-I 1990. Evidence for and subcellular localization of a Ca-stimulated phospholipase D from maize roots. *Plant Physiology* 92:672-678.
11. Nagahashi, G. and Baker, A. F. 1984. B-glucosidase activity in corn roots homogenates: problems in subcellular fraction. *Plant Physiology* 76:861-864.
12. Bensadoun, A. and Weinstein, D. 1976. Assay of proteins in the presence of interfering materials. *Analytical Biochemistry* 70:241-250.
13. Moreau, R.A. 1987. Autolysis of phospholipids in homogenates of various plant tissues. *Phytochemistry* 26:1899-1902.
14. Moreau, R.A. and Isett, T.F. 1985. Autolysis of membrane lipids in potato leaf homogenates: effects of calmodulin and calmodulin antagonists. *Plant Science* 40:95-98.
15. Hise, M.K. and Mansbach, C.M. 1983. Determination of intracellular choline levels by an enzymatic assay. *Analytical Biochemistry* 135:78-82.

16. Shinitzky, M. and Inbar, M. 1976. Microviscosity parameters and protein mobility in biological membranes. *Biochimia Biophysica Acta* 433: 133-149.
17. Chernomordik, L.V., Melikyan, G.B. and Chizmadzhev, Y.A. 1987. Biomembrane fusion: A new concept derived from model studies using two interacting planar lipid bilayers. *Biochimia Biophyscia Acta* 906:309-352.