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Evidence for and Subcellular Localization of a Ca-Stimulated Phospholipase D from Maize Roots

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ABSTRACT

Autolytic lipid changes in corn (*Zea mays* L.) root crude homogenates and isolated membranes were examined by the use of high performance thin-layer chromatography. In the absence of added $CaCl_2$, losses in phosphatidylcholine and other phospholipids corresponds to increase in fatty acids without the accumulation of either phosphatidic acid or lyso-phosphatidylcholine. However, in the presence of 1 millimolar $CaCl_2$, phosphatidylcholine concentrations declined more rapidly with an immediate increase in phoshatidic acid, and slower rate of fatty acid accumulation. Autolytic phospholipid degradation yielded primarily free fatty acids in the absence of Ca and phosphatidic acid in the presence of 1 millimolar $CaCl_2$, suggesting the presence of an acyl hydrolase and phospholipase D activities. Differential centrifugation studies indicate that 50 to 80% of the crude homogenate's phospholipase D activity is membrane-bound. Density centrifugation experiments suggest that the membrane-bound phospholipase D activity is localized primarily on mitochondrial membranes.

Our interest in phospholipid degradation originates from a desire to minimize the loss of lipid constituents from membranes during isolation from plant tissues. Inactivation of membrane-bound CCR[1] from wheat aleurone and the vanadate-sensitive proton pump from maize roots during isolation was associated with degradation of membrane lipids (2, 28). The inactivation of CCR during membrane isolation was substantially reduced by the inclusion of choline and o-phosphorylcholine, putative inhibitors of plant PLase D (28). Such results suggested that PLase D activity was responsible for the inactivation of this enzyme. Scherer and Morre (23) reported that the amount of PA + phosphatidylinositol in soybean membranes increased during isolation, suggesting the existence of a PLase D that utilized endogenous phospholipids as substrates. The loss of proton transport by maize root membranes during incubation at 0 to 4°C was associated with an increase in membrane permeability to protons and the loss of membrane PC (2). Other polar lipids, besides PC, were also degraded; however, changes in PC accounted for a majority of the losses. Resupplying the proton pump with phospholipids by a reconstitution restored transport activity to initial levels (2). Such results indicated that net proton transport was inhibited because of increased leakage of proton resdulting from phospholipid degradation. Losses in membrane phospholipids from corn root microsomes did not correspond to an accumulation of either lysophospholipids or PA (2), suggesting that neither PLase A_2 nor D was responsible for the degradation. However, the exact nature of the lipolytic enzymes responsible for the phospholipid degradation in maize root homogenates has not been identified and is the focus of this report. Results from this study indicate the presence of at least two lipolytic enzyme activities, a Ca-stimulated PLase D and an acyl hydrolase.

[1] Abbreviations: CCR, antimycin A-insensitive NADH-dependent Cyt *c* reductase; PLase, phospholipase; PA, phosphatidic acid; PC, phosphatidylcholine; BTP, Bis-Tris-Propane; CCO, Cy *c* oxidase; G6PDH, glucose-6-phosphate dehydrogenase; PEPC, phospho*enol*pyruvate carboxylase; PHM, phosphohexosemutase.

MATERIALS AND METHODS

Preparation of Crude Homogenates and Membrane Fractions

Roots (40–50 g fresh weight) from 3 d old maize seedlings (*Zea mays* L. cv WF9 × Mo17) were grown on filter paper moistened with 0.1 mM $CaCl_2$ and harvested as described previously (19). Roots were homogenized by mortar and pestle for 3 min in the presence of 50 mM Mes titrated to pH 6.0 with BTP, 0.25 M sucrose, and 5 mM DTT using 3 mL of buffer per g of roots at 0 to 4°C. The brei was filtered through four layers of cheesecloth, and the resulting filtrate was used as the crude homogenate. Homogenates and membranes prepared as above contained less than 1 μM Ca^{2+} as determined by Ca-selective electrode (data not shown). Protein concentration was determined after precipitation by TCA in the presence of deoxycholate by a modification of the Lowry method (1).

When membranes in the pH 6.0 crude homogenate were subjected to density centrifugation, all of the membranes were recovered at about 40% (w/w) sucrose (data not shown). This apparent aggregation of membranes was prevented when roots were homogenized at pH 7.5 (data not shown). Therefore, when studying subcellular localization of the PLase D activity, roots were homogenized in 50 mM BTP (titrated to pH 7.5 with Mes), 0.25 M sucrose, and 5 mM DTT. After grinding by mortar and pestle and straining as above, the filtrate was centrifuged at 1,100*g* for 5 min and the resulting supernatant was used as the crude homogenate. Pellets from subsequent, faster centrifugation steps were difficult to resuspend if the 1,100*g* centrifugation step was omitted. The crude homogenate was subjected to the differential centrifugation scheme of Nagahashi and Hiraike (20). Mitochondrial enriched mem-

branes were recovered after centrifuging at 6,000*g* for 20 min. Microsomes and cytosolic proteins were separated from the 6,000*g* supernatant after centrifuging at 90,000*g* for 40 min. Both membrane pellets were resuspended in 2 to 3 mL of 15% (w/w) sucrose, 5 mM Hepes (pH 7.7), and 2 mM DTT and were subjected to further purification by discontinuous sucrose gradients essentially as described by Hodges and Leonard (6). Briefly, the sucrose gradient consisted of 6 mL steps of 20, 25, 30, 34, and 38% (w/w) sucrose and a final 5 mL step of 45% (w/w) sucrose. Each sucrose step contained 5 mM Hepes-BTP (pH 7.8) and 1 mM DTT. After centrifuging at 25,000 rpm in SW 28 rotor for 2.5 h, the gradient was fractionated with the interfaces being identified by an increase in absorbance at 280 nm.

Cyt *c* reductase and oxidase were assayed as described by Hodges and Leonard (6) except 25 μM antimycin A was added to reductase assay and Triton X-100 was substituted for digitinon in the oxidase assay. Vanadate-sensitive ATPase activity was determined essentially as described previously (2). Release of inorganic phosphate was assayed using 5 μL of membranes diluted to 100 μL with reaction mixture containing 17.5 mM Mes-BTP (pH 6.45), 50 mM KNO_3, 1.0 mM EGTA, 2.0 mM ATP, and 2.5 mM $MgSO_4$ with and without 0.2 mM vanadate by the formation of the molybdate-malachite green complex (26). Activity of the Triton X-100-stimulated IDPase was assayed by the procedure of Nagahashi and Nagahashi (21) following inorganic phosphate release by the formation of the molybdate-malachite green complex (26).

The degree of cytosolic contamination in membrane fractions was assessed by the recovery of the activities of G6PDH (25, 27), PEPC (7), and PHM (25, 27). All three enzyme activities were monitored by changes in absorbance at 340 nm using the protocols described by Kuby and Noltmann (8), Kringsted *et al.* (7), and Slein (24), respectively.

Assay following Loss of Endogenous Phospholipids

To follow the loss of endogenous lipids, 1.0 mL aliquots of crude homogenates, resuspended membrane pellets, or fractions from sucrose gradients were pipeted into 1 × 15 cm culture tubes with screw top caps and incubated for 0 to 4 h at 28°C with shaking. Assays conducted in the presence of 1 mM Ca^{2+} were prepared by the addition of a 1 M stock of $CaCl_2$. To adjust the pH of membrane fractions buffered at pH 7.5 or 7.7, 0.1 mL of 0.5 M Mes titrated to pH 6.0 with BTP was added per mL. The reaction was terminated by extracting the lipids with organic solvents as described by Moreau and Isett (16). The organic phase was stored at −20°C until the day of analysis. Changes in amounts of lipid constituents were followed by high performance TLC coupled with densitometry as described below. Increases in PA and fatty acids with concomitant losses in other phospholipids were taken as evidence for the activities of PLase D and acyl hydrolase, respectively (22). Changes in membrane lipids were followed rather than the metabolism of exogenously supplied lipolytic substrates, because there was no correlation between the rate of autolytic loss of phospholipids and the hydrolysis of lipolytic substrates in a recent survey of plant species and tissues (15).

On the day of lipid analysis, the organic solvent was removed from samples at 60 to 70°C under a stream of N_2 and the dried lipid film was dissolved in 100 μL of either 1:1 (v/v) chloroform:methanol or 65:30:2.5 (v/v/v) chloroform:methanol:water. High performance silica gel LHP-K plates (200 microns, 10 × 10 cm with celite preadsorbent layer from Whatman[2]) were cleaned and activated by submerging in methanol for 5 min and heating at 87°C for 15 min (RJ Maxwell *et al.* unpublished results). Clean, activated plates were stored desiccated up to 3 d prior to use. Typically, up to 11 standards and samples (0.5–2 μL) were applied per plate. Lipids were separated using a modification of the solvent system of Kupke and Zeugner (9). For the simultaneous separation of both neutral and polar lipids, one plate was developed sequentially with four sets of solvents. Initially, plates were developed twice in 65:30:2.5 (v/v/v) chloroform:methanol:water until the solvent front had migrated 3.5 cm from the origin. Next, plates were developed in 80:20:1.5 (v/v/v) hexane:ether:acetic acid followed by 80:20 (v/v) hexane:ether until the solvent front was within 0.5 cm of the top of the plate. The original development system of Kupke and Zeugner (9) did not utilize the fourth solvent elution. The three solvent developments poorly resolved the two galactolipids, digalactosediacylglyceride and monogalactosediacylglyceride, from diacyclglyceride. Complete resolution of the two galactolipids was achieved when plates were developed a fourth time in 80:20 (v/v) hexane:ether (data not shown). All solvents for extractions and chromatographic separations were HPLC grade obtained from either B&J or JT Baker Chemical Co. After each chromatographic step the plate was air-dried for 10 min at 18 to 22°C. After the fourth development, the plate was dipped in 10% (w/v) $CuSO_4 \cdot 5\ H_2O$ and 10% (v/v) concentrated H_3PO_4, and incubated at 120°C for 20 min to visualize the lipids.

The intensity of spots was determined by densitometry using a CAMAG TLC Scanner II operating with a tungsten light source at 440 nm in the reflectance mode under such conditions that the intensity of the entire spot was recorded. Densitometric quantitation of various phospholipids such as phosphatidylethanolamine, PC, and PA from egg yolk were similar (data not shown). Lipids with only saturated acyl chains were not visualized unless greater than 2 μmol of lipid was present per spot. Intensity of PC increased by about 20% with each double bond present per acyl chain (data not shown). Standards for quantitation were prepared to mimic the acyl composition of maize root phospholipid. Methyl esters of the fatty acids in phospholipids and total lipids from maize root homogenates were prepared and subjected to quantitative separation by GLC by the method of Maxwell and Marmer (11). Such analyses indicated that total lipids and a variety of classes of phospholipids under several conditions contained about 75% linoleic, 15% oleic, and 10% palmitic and 18:0 stearic acids (data not shown). Areas of spots from maize root lipids were converted to mass quantities using a weighted response factor of a 5:1 ratio of linoleic to oleic fatty acids. The sensitivity of densitometry for lipids with at least one double bond per acyl chain was about 1

[2] Reference to brand or firm does not constitute endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

nmol per lipid and the response range was broad and linear extending up to 200 nmol (data not shown). A detection limit of 1 nmol per lipid compares favorably with limits expected from assays following the conversion of radiolabeled lipids by methods similar to those reported by Witt *et al.* (29).

RESULTS

Autolytic Lipid Losses

The four successive development high performance TLC technique provided a method by which the degradation of phospholipids to end products could be followed quantitatively (Fig. 1), a procedure not possible with our previous single development TLC protocol coupled with total phosphorus analysis. It is readily apparent by comparing the lower two traces in Figure 1 that losses of phospholipids corresponded to increases in fatty acid production without the accumulation of lyso intermediates, PA, or diacylglyceride. Other phospholipids, such as phosphatidylethanolamine, phosphatidylinositol, and phosphatidylserine, declined abruptly as well as the trace levels of di- and triacyclglyceride initially present. PA levels were relatively constant over 15 min (Fig. 1); however, levels declined when the incubation time was increased (data not shown). Concentrations of sterol and sterol esters were constant. These results confirm our previous observation that under normal assay conditions PC losses did not correspond to increases in either lyso-PC or PA.

Further studies examined the loss of PC and the accumulation of fatty acids and PA. The fate of PC was followed because it is the predominant phospholipid in maize root homogenates and losses of PC paralleled that of total lipid phosphorus (2). The effect of Ca^{2+} on autolytic lipid losses in corn root homogenates was assessed initially because Ca^{2+} was found to greatly enhance the degradation of PC by acyl hydrolase in potato leaf extracts (17). During the first hour in the absence of Ca, there was a sharp decrease in PC content and an increase in fatty acid concentration (Fig. 2). The rate of both PC loss and fatty acid production slowed as the incubation period was increased. During the first hour, PA levels were relatively constant. Beyond 1 h, PA slowly declined.

In the absence of Ca^{2+}, during the first hour PC declined by 13.5 nmol/mg protein were consumed during the first

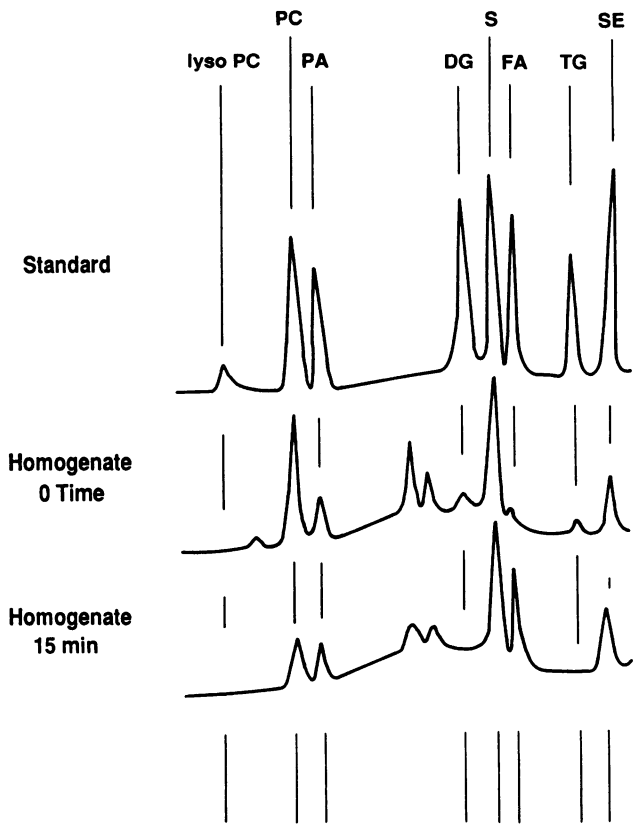

Figure 1. Densitometric tracings of high performance TLC plates demonstrating separation of neutral and polar lipids on one plate. The upper most trace (labeled standard) demonstrates the resolution of a mixture of phospholipids, galactolipids, fatty acids (FA), triacylglyceride (TG), diacylglyceride (DG), sterols (S), and sterol esters (SE). The lower two traces demonstrate the separation of lipids from corn root homogenates before and after 15 min incubation at 28°C.

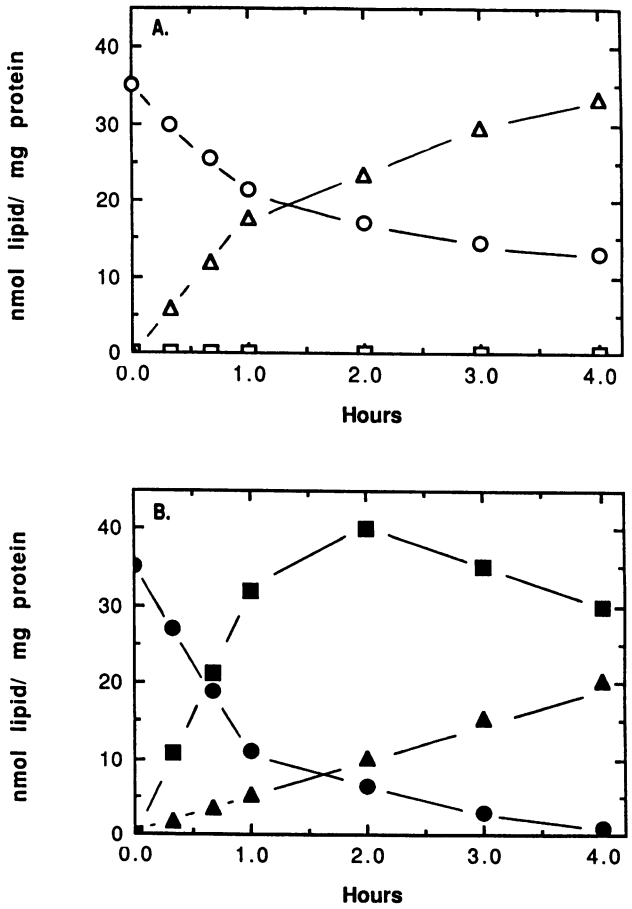

Figure 2. Changes in PA, PC, and fatty acid concentrations of corn root homogenate incubated 0 to 4 h at 28°C in the absence (upper panel) and presence of 1 mM $CaCl_2$ (lower panel). Levels of PC in the homogenate (○) as well as the change in fatty acid and PA concentrations are represented by (△) and (□), respectively. Data are the average of three replications. Standard errors of the mean did not exceed the size of the data symbol.

hour (Fig. 2). During the same time period, fatty acids increased by about 17.4 nmol/mg protein. The loss of 13.5 nmol PC/mg protein should have yielded more fatty acids (*i.e.* theoretically, 27 nmol/mg protein *versus* the observed 17.4 nmol/mg protein). Such a discrepancy may indicate that intermediate products, such as diacyclglyceride and lyso-PC, were accumulated. However, increases in these compounds were not sufficient to be detected by either densitometry or phosphorus analysis. For example, lyso-PC could be detected by phosphorus analysis when a large proportion of the organic solvent extract was subjected to TLC. At 0 time, lyso-PC content was less than 5% that of PC, averaging 1.6 ± 0.7 nmol/mg protein. After 60 min of incubation, lyso-PC concentration was 2.1 ± 0.6 nmol/mg protein. This increase was not great enough to be significantly different from the 0 time measurement. Alternatively, the discrepancy between observed fatty acid production and the theoretical value may have resulted from differences in the degree of unsaturation of the initial PC and the produced fatty acids. If a particular species of PC was more susceptible to degradation than other species, the calibration curves used for the quantitation of densitometric data may have been incorrect. The end result would be an apparent change in the stoichiometry of PC conversion to fatty acids.

In the presence of Ca^{2+}, however, different results were obtained (Fig. 2). The initial rate of PC loss proceeded much faster than in the absence of Ca, 24 nmol PC loss/mg protein during the first hour, while the production of fatty acids was slower, 5.1 nmol/mg protein. The enhanced loss of PC corresponded to an increase in PA levels with an increase of 32 nmol/mg protein during the first hour. Decreases in PA and PC after 1 h of incubation were associated with increases in fatty acids.

In addition to the aforementioned high performance TLC four-solvent development method, the production of PA was confirmed by altering the relative mobility of the polar lipids by developing samples in three solvent systems: (a) 65:30:2.5 (v/v/v) chloroform:methanol:water; (b) 65:30:5 (v/v/v) chloroform:methanol:ammonium hydroxide; and (c) 85:15:10:3.3 (v/v/v/v) chloroform:methanol:acetic acid:water. In each case, the product of PC degradation in the presence of 1 mM $CaCl_2$ had a mobility corresponding to PA (data not shown). Quantitation of PA production by densitometry yielded similar values to those obtained by total phosphorus analysis (data not shown). For an experiment similar to that in Figure 2, 31 ± 2 nmol PA/mg protein were formed as determined by high performance TLC and densitometry as compared to 29 ± 6 nmol PA/mg protein as determined by TLC coupled with phosphorus analysis. Stimulation of PA production by $CaCl_2$ appeared to be due to the addition of Ca^{2+} since EGTA prevented activation (data not shown).

Localization of PLase D Activity

The above characterization indicated crude homogenates from maize roots contained a Ca-activated PLase D activity capable of converting endogenous phospholipids to PA and subcellular localization was chosen as the first step in the characterization of this activity. Preliminary experiments indicated that subcellular fractionation could not be accurately conducted at pH 6.0, pH of the PLase D assay, due to apparent membrane fusion or aggregation. Therefore, subcellular fractionation was conducted after homogenizing roots at pH 7.5. The amount of PLase D activity recovered in the 6000*g* pellet *versus* supernatant after homogenizing in the pH 6.0 buffer system was compared to the distribution using the pH 7.5 buffer to assess the pH dependency of the recovery of membrane bound PLase D activity. Eighty percent of the total PLase D activity was recovered in the 6000*g* pellet when roots were homogenized in pH 6.0 buffer. In comparison, only 59% of the total activity was recovered in the 6000*g* pellet when homogenized at pH 7.5. The specific activity per g of tissue was comparable, 51 and 54 nmol PA/h/g fresh weight at pH 6.0 and 7.5, respectively. Specific activities on a protein basis were comparable, 22.4 and 24.3 nmol PA/h/mg protein, respectively. Total activities on a protein basis was similar because the protein content of the homogenates were comparable (data not shown). These results suggested that there was a slight pH dependence of the recovery of the PLase D activity in the 6000*g* pellet. Similar results were found when roots were homogenized in either the pH 6.0 or 7.5 buffer, titrated to the other pH value, subjected to the 6000*g* centrifugation and assayed for PLase D activity (data not shown).

Initial investigations in the subcellular localization of PLase D activity followed the distribution of various enzyme activities among steps from the differential centrifugation scheme of Nagahashi and Hiraike (20). The distribution of CCO and CCR was assessed to follow the separation of mitochondrial and other organelle membranes from the smooth membrane vesicles such as endoplasmic reticulum. The mitochondrial marker enzyme, CCO, was found primarily in the 6000*g* pellet whereas most of the endoplasmic reticulum marker enzyme, CCR, was found in the 90,000*g* pellet (Fig. 3). The degree of cytoplasmic contamination of the two membrane fractions was determined by the distribution of PHM, PEPC, and G6PDH. Over 75% of the three cytosolic markers (7, 25, 27) were recovered in the 90,000*g* supernatant. The specific activities for these three markers were significantly lower in the membrane pellets as compared to the crude homogenate. The 6,000*g* pellet contained most of the CCO activity, 78% of the homogenate's level, and the highest enrichment in specific activity, 2.6-fold greater than the crude homogenate. Similarly, the 6,000*g* pellet contained a majority of the PLase D activity, 55% and had the greatest enrichment in specific activity, 1.8 times that of the crude homogenate. The microsomal pellet contained significant quantities of both CCO and PLase D activities, about 25% of the activity in the crude homogenate. The sum of the PLase D activity in the two membrane fractions only comprised 80% of the crude activity.

To pursue further the localization of membrane-bound PLase D, the 6,000*g* and 90,000*g* pellets were fractionated by discontinuous sucrose density centrifugation. The endoplasmic reticulum, golgi, plasma membrane, and mitochondria as determined by the distribution of CCR, Triton-stimulated IDPase, vanadate-sensitive ATPase and CCO, respectively (18), were fairly well resolved with a distribution similar to that reported previously for corn root membranes (10). The distribution of PLase D activity closely followed that of the mitochondrial marker, CCO (Fig. 4).

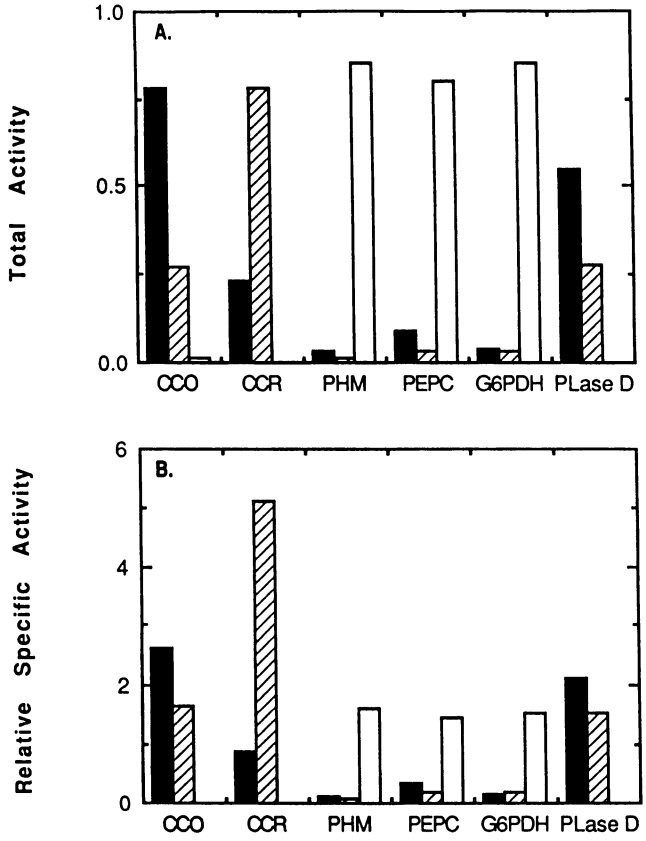

Figure 3. Distribution of marker enzymes and PLase D activity among fractions from differential centrifugation. Corn root homogenates (prepared with the pH 7.5 buffer) were subjected to successive centrifugation steps to yield a 6,000*g* pellet (solid black bar), 90,000*g* pellet (cross hatched bar), and 90,000*g* supernatant (open bar). Both pellets were resuspended in the pH 6.0 homogenizing buffer. The crude homogenate as well as the three fractions were assayed for CCO, CCR, PHM, PEPC, G6PDH, and PLase D. PLase D activity was determined by the formation of PA in the presence of 1 mM $CaCl_2$. The pH of the crude homogenate and supernatant was adjusted to pH 6.0 by the addition of Mes immediately prior to the assay. Panels A and B depict data for specific and total activity, respectively, relative to the crude homogenate. The specific activity in the crude homogenate for CCO, CCR, PHM, PEPC, and G6PDH in A/min/mg protein was 2.8, 2.0, 0.2, 0.1, and 0.7, respectively, and for PLase D 33 nmol PA/h/mg protein. The total activity in the crude homogenate for CCO, CCR, PHM, PEPC, and G6PDH in A/min was 240, 167, 17.8, 8.4 and 55.8, and for PLase D, 2880 nmol PA/h.

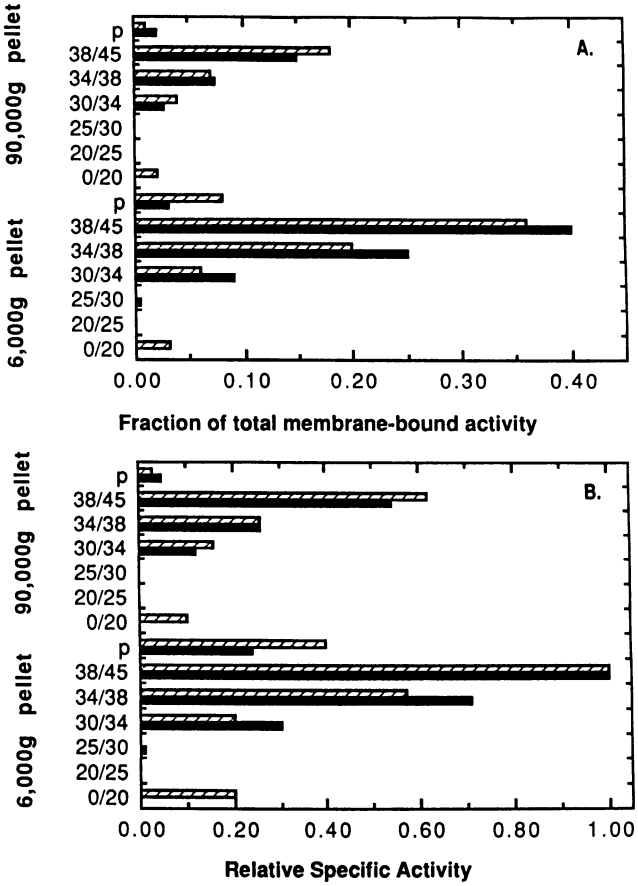

Figure 4. Distribution of CCO and PLase D activities among the fractions from density centrifugation of the 6,000*g* and 90,000*g* pellets. The two pellets prepared from differential centrifugation were applied to a discontinuous sucrose gradient that comprised six steps. After centrifuging the interface were recovered and assayed for either CCO (solid bars) or PLase D (cross hatched bars). Activity of PLase D was determined by the formation of PA in the presence of 1 mM $CaCl_2$. Fractions were adjusted to pH 6.0 immediately prior to the assay by the addition of 0.1 mL of 0.5 M Mes (titrated to pH 6.0 with BTP) per 1 mL of membrane fraction. Total activity (upper panel) of the two membrane pellets prior to discontinuous sucrose density centrifugation was 235 A/min and 2200 nmol PA/ for CCO and PLase D, respectively. The lower panel depicts the specific activity relative to the 38/45% (w/w) sucrose interface of the 6,000*g* pellet, which was 10.1 A/mg protein/min and 142 nmol PA/h/mg protein for CCO and PLase D, respectively.

DISCUSSION

Nature of Enzymes Involved in Autolytic Phospholipid Losses in Maize root Homogenates

In the absence of Ca, PA levels remained fairly constant during the first hour of incubation at 28°C, implying that the activity of the PLase D was not very rapid relative to the other lipolytic enzymes (Fig. 2). However, concentrations of other phospholipids declined as levels of fatty acids increased. Such results suggest that phospholipids were being converted to fatty acids and water soluble products. The enzyme which is most likely responsible for this conversion would be an acyl hydrolase or PLase B since no transient rise in either lysophospholipids or diacyglyceride were observed. The possibility that phospholipids were being converted to fatty acids by enzymes other than the acyl hydrolase cannot be discounted by the results in this study. However, each successive reaction in the pathway from phospholipids to free fatty acids would have to be more efficient than the preceding one since no intermediates were observed.

In the presence of 1 mM $CaCl_2$, the rate of loss of all phospholipids except PA was greater than in the absence of Ca. For example, the rate of PC loss increased from 13.5 in the absence to 24 nmol PC/h/mg protein in the presence of

1 mM $CaCl_2$ (Fig. 2). In addition, Ca altered the products that accumulated during phospholipid metabolism. In the presence of Ca, levels of PA rose sharply, whereas the rate of fatty acid accumulation was slow throughout the incubation period. This rapid rise in PA in the presence of Ca and a lack of a change in PA levels in the absence of Ca could indicate the presence of a Ca-stimulated PLase D activity. Alternatively, Ca could be inhibiting the conversion of PA to fatty acids. However, such an explanation does not account for the increased loss of PC and other phospholipids in the presence of Ca. Therefore, the most straightforward explanation of such results is that most of the phospholipids in the presence of 1 mM $CaCl_2$ were sequentially converted first to PA by a PLase D and then at slower rate to fatty acids possibly by an acyl hydrolase or PLase B activity. Alternatively, PA could be sequentially degraded first by PA phosphatase to diacylglyceride and then to fatty acids by either a lipase or acyl hydrolase. Plant tissues are known to contain PA phosphatase (4). However, there is no evidence for the existence of PA phosphatase in maize root homogenates at present. Following the degradation of PA by high performance TLC, no accumulation of diacylglyceride was observed (data not shown). Additionally, there was no PA-stimulated inorganic phosphate production in crude homogenates. Therefore, two possibilities exist. First, PA phosphatase is not functional in maize root homogenates and PA is degraded directly to fatty acids. Alternatively, PA is sequentially converted to fatty acids via diacylglyceride intermediates in such a way that the second step is so vastly more efficient that no diacylglyceride accumulates.

The results in Figure 2 are the first reported indications of the presence of a Ca-stimulated PLase D activity from maize roots. Previous results on autolytic lipid degradation from our laboratory were conducted in the absence of Ca^{2+} and did not indicate the presence of PLase D (2). The Ca-stimulation of the PLase D may indicate that this lipid degradative enzyme is highly regulated and involved in the responses of root cells to the environment. The results of this study demonstrated clearly that the immediate products of phospholipid degradation differed widely in the absence and presence of 1 mM $CaCl_2$. One possible role of this PLase D activity is to supply PA for the synthesis of phosphtidylinositol and its associated inositol phosphate derivatives according to the scheme of Michelle *et al.* (13). Inositol polyphsphates, in particular inositol triphosphates, are thought to act as second messengers to regulate cellular metabolism, (13, 30). Therefore, Ca activation of PLase D activity may allow for increased production of phosphatidylinositol in response to an increased demand for inositol polyphosphatases. In addition, Ca-stimulated PLase D activity could supply diacylglyceride, which can act as a second messenger in cells having protein kinase C activity (13).

Previous reports also have identified Ca as a possible regulatory ion in phospholipid metabolism. Paliyath and Thompson (22) concluded that the conversion of PC to PA was rather unaffected by Ca with homogenates from soybean cotyledons. Instead, Ca appeared to affect the rate of conversion of PA to fatty acids (22). In contrast to the results with soybeans, Ca appeared to affect the rate of PA formation in corn root homogenates. Such discrepancies may indicate that the PLase D from different tissues serve different *in vivo* functions. Many plant lipolytic enzymes including acyl hydrolase or PLase B (14, 16), PLase C (12), PLase D (29), and neutral lipase (5) are Ca-stimulated. The significance of Ca-activation of lipolytic enzymes remains uncertain (5).

Localization of PLase D

Plant PLase D are known to exist in both soluble and membrane-bound forms (3). Purification and characterization of soluble PLase D from a variety of dicot species has been reported (29 and references cited therein). Less is known about membrane-bound plant PLase D activity. Since our interest in phospholipid metabolism stems from a desire to minimize membrane losses during isolation, the subcellular localization of the Ca-stimulated PLase D activity was characterized. Significant levels of PLase D activity were found in the two membrane fractions generated by differential centrifugation with the 6,000*g* pellet containing almost twice as much activity as the 90,000*g* pellet (Fig. 3). However, the activity in both membrane fractions comprised only 80% of the activity in the crude homogenate. One possible reason for the discrepancy between the sum of the two membrane fractions and the crude homogenate is a partial inactivation of the PLase D activity. The levels of PLase D activity in the crude homogenate and isolated membrane fractions were stable at 0 to 4°C over 24 h incubation (data not shown), suggesting inactivation was not occurring. Alternatively, the incomplete recovery could indicate that significant levels of PLase D activity were present in the 90,000*g* supernatant which could not be assayed because of the absence of endogenous lipids. When the supernatant was supplied with exogenously added phospholipid, such as dioleoyl-PC, Ca-stimulated PA production was observed (data not shown). Therefore, the 90,000*g* supernatant did contain detectable levels of PLase D activity, which may account for the activity not recovered in the two membrane pellets.

The higher recovery of PLase D activity in the 6,000*g* pellet as compared to the 90,000*g* pellet suggests that this activity is localized on a membrane of relative high density such as one from an organelle, in contrast to a smooth membrane vesicles (Fig. 3). Such a conclusion is supported by results from density centrifugation studies of the membrane pellets. With both the 6,000*g* and 90,000*g* pellet, the PLase D activity tended to be recovered at the higher density interface with the greatest specific activity at the 38/45% (w/w) sucrose interface (Fig. 4). Additionally, there was a strong association between the enrichment and depletion of CCO activity, a marker for mitochondrial membranes, and PLase D among fractions from the sucrose gradient. Although there are reports of membrane-bound PLase D from plant sources, subcellular localization has been attempted infrequently. Scherer and Morre (23) found that PLase D activity from soybean hypocotyls was present in most membrane fractions from discontinuous sucrose gradients. On the other hand, Herman and Chrispeels (4) found that PLase D was localized almost exclusively in protein bodies from castor bean cotyledons. Therefore, PLase D from different tissue types may have widely different subcellular localization depending on its function in the particular cell type. Localization of acyl hydrolase on

mitochondrial membrane has been reported (3), possibly indicating a role for mitochondrial membranes in phospholipid metabolism.

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