Plant Physiol. (1987) 83, 63–68
0032-0889/87/83/0063/06/$01.00/0

Calcium- and Calmodulin-Regulated Breakdown of Phospholipid by Microsomal Membranes from Bean Cotyledons[1]

Received for publication March 19, 1986 and in revised form July 22, 1986

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ABSTRACT

Evidence for the involvement of Ca^{2+} and calmodulin in the regulation of phospholipid breakdown by microsomal membranes from bean cotyledons has been obtained by following the formation of radiolabeled degradation products from [U-^{14}C]phosphatidylcholine. Three membrane-associated enzymes were found to mediate the breakdown of [U-^{14}C] phosphatidylcholine, *viz.* phospholipase D (EC 3.1.4.4), phosphatidic acid phosphatase (EC 3.1.3.4), and lipolytic acyl hydrolase. Phospholipase D and phosphatidic acid phosphatase were both stimulated by physiological levels of free Ca^{2+}, whereas lipolytic acyl hydrolase proved to be insensitive to Ca^{2+}. Phospholipase D was unaffected by calmodulin, but the activity of phosphatidic acid phosphatase was additionally stimulated by nanomolar levels of calmodulin in the presence of 15 micromolar free Ca^{2+}. Calmidazolium, a calmodulin antagonist, inhibited phosphatidic acid phosphatase activity at IC_{50} values ranging from 10 to 15 micromolar. Thus the Ca^{2+}-induced stimulation of phosphatidic acid phosphatase appears to be mediated through calmodulin, whereas the effect of Ca^{2+} on phospholipase D is independent of calmodulin. The role of Ca^{2+} as a second messenger in the initiation of membrane lipid degradation is discussed.

A variety of enzymes are able to degrade phospholipids. Phospholipase A_2 and phospholipase C are the major phospholipid-degrading enzymes in animal tissue (11, 26), but the enzymes responsible for phospholipid breakdown in plants are less well characterized. Phospholipase A_2 and phospholipase C are apparently not present in plant tissues (13). Rather, the breakdown of plant phospholipids appears to be mediated by phospholipase D, which has been purified to apparent homogeneity (15), and nonspecific lipolytic acyl hydrolase (13, 17). Phosphatidic acid, the immediate product of phospholipase D activity, serves as an intermediate in phospholipid biosynthesis (22) but can also be converted to the corresponding diacylglycerol by phosphatidic acid phosphatase (4, 20). Indeed, studies with mung bean cotyledons have indicated that phospholipase D and phosphatidic acid phosphatase are both associated with protein bodies and degrade membrane phospholipids through autophagic catabolism (16).

In animal systems, phospholipid metabolism is strongly regulated by calcium, which serves as a second messenger. Mobilization of calcium is achieved, in part, by hormone-stimulated activation of phospholipase C and the ensuing release of phosphorylated inositol (3). The free calcium thus released is in turn able to regulate further metabolism of phospholipids such as the deesterification of arachidonic acid, a precursor of prostaglandin synthesis, by promoting phospholipase A_2-mediated hydrolysis of membrane phospholipids (11, 26). In the present study, we have examined the breakdown of phospholipids by microsomal membranes from senescing bean cotyledons and have obtained evidence that metabolism of phospholipid in plant membranes is also subject to regulation by calcium and calmodulin.

MATERIALS AND METHODS

Bean seeds (*Phaseolus vulgaris* L. cv Kinghorn wax, Ontario Seed Co., Waterloo, Ontario, Canada) were germinated in vermiculite in darkness at 29°C. Microsomal membranes were prepared from the cotyledons of 5-d-old seedlings. The tissue was suspended (0.5 g/ml) in buffer (50 mM Hepes, 2 mM EGTA, 150 mM KCl, 0.5 mM DTE, 0.5 mM phenylmethylsulfonyl fluoride, and 0.25 mM sucrose, pH 7.0) and homogenized at 4°C in a Sorvall Omnimixer for 30 s and again in a Polytron homogenizer for 40 s. The homogenate was filtered through four layers of cheesecloth and centrifuged at 10,000*g* for 20 min. The supernatant was recentrifuged at 105,000*g* for 60 min to yield a pellet of microsomal membranes. The microsomes were washed once by resuspension in wash buffer (50 mM Hepes, 0.2 mM EGTA, 150 mM KCl, and 0.25 M sucrose, pH 7.0) and centrifuged at 105,000*g* for 1 h.

In some experiments, these washed microsomal membranes were used directly for measurements of phospholipid breakdown. However, for most experiments a partially purified enzyme system obtained by solubilizing the microsomal membranes with Triton X-100 (9) was used. The washed membranes were resuspended (2 mg protein/ml) in wash buffer containing 0.2% (v/v) Triton X-100. The mixture was stirred gently for 1 h at 4°C and then centrifuged at 105,000*g* for 60 min. The resulting pellet was resuspended in wash buffer (0.5 mg protein/ml) and used directly for enzyme assays.

Phospholipid breakdown was measured using [U-^{14}C]phosphatidylcholine essentially as described earlier (6). The basic assay mixture contained 50 mM Hepes (pH 7.0), 150 mM KCl, 0.2 mM EGTA, 1 mM $MgCl_2$, 0.01% (v/v) Triton X-100, 10 to 20 μg of membrane protein or partially purified enzyme preparation and 20,000 cpm of [U-^{14}C]phosphatidylcholine (New England Nuclear, 1.5 Ci/mmol) in a final volume of 0.5 ml. In some experiments, specified concentrations of calmodulin (Sigma) and calmidazolium (Boehringer Mannheim) were also included in the reaction mixture. The assay mixture was incubated at 30°C for up to 50 min and then terminated by adding 0.1 ml of 4 N HCl. Phospholipase D activity was measured by determining levels of radiolabeled choline released into the water-soluble fraction. Phosphatidic acid phosphatase activity was measured by determining levels of radiolabeled diacylglycerol, and acyl hydrolase by determining levels of radiolabeled free fatty acids. To obtain these measurements, the reaction mixture was extracted with 2 ml of 2:1 (v/v) chloroform:methanol. The aqueous

[1] Supported by the Natural Sciences and Engineering Research Council of Canada.

phase containing the radiolabeled choline was counted (0.5 ml of aqueous phase mixed with 5 ml of scintillation fluid, Scintiverse, Fisher) in a Beckman LS7500 Scintillation counter. Lipid components in the chloroform phase were separated by TLC and identified using authentic standards as described previously (6). An aliquot (0.8 ml) of the chloroform phase was transferred to a test tube and dried under N_2, dissolved in 70 μl of 2:1 (v/v) chloroform:methanol, and spotted on Whatman LKD plates. The plates were developed in chloroform:methanol:water (65:25:4). The diacylglycerols and free fatty acids, which run with the solvent front and just beneath the solvent front, respectively, were scraped off, and the plates were then developed again in chloroform:methanol:acetic acid:water (85:15:15:3.5) to obtain a better separation between phosphatidic acid and phosphatidylcholine. Scraped regions of the plate corresponding to diacylglycerols, free fatty acids and phosphatidic acid were placed in 5 ml of scintillation fluid (Scintiverse, Fisher) and counted.

Free calcium ion concentration was measured using a calcium-sensitive electrode (Model 93-20, Orion Research, Cambridge, MA). The calibration values for 0.1, 0.5, 1.0, 4.0, 6.0, 8.0, 10.0, 100.0, and 1000.0 μM free calcium were −48.0, −47.5, −40.5, −35.0, −30.5, −27.5, −25.5, −1.6, and 26.8 mV, respectively. Protein levels were determined as described by Bradford (7).

RESULTS

Microsomal membranes isolated from 5-d-old bean cotyledons proved capable of converting [U-^{14}C]phosphatidylcholine into free fatty acids, diacylglycerols, phosphatidic acid, and choline (Table I). The release of free fatty acids can be attributed to lipolytic acyl hydrolase, phosphatidic acid and choline to phospholipase D, and diacylglycerol to phosphatidic acid phosphatase. The specific radioactivities listed in Table I for phospholipase D, phosphatidic acid phosphatase, and acyl hydrolase cannot be used as a basis for comparing the relative activities of these enzymes in the microsomal membranes because the products of these reactions have different carbon numbers. However, estimations of this comparison can be made by correcting the values in Table I for these differences in carbon numbers. Using distearoylphosphatidyl choline as an example, choline contains 5/44 of the total number of carbons in the molecule, diacylglycerol 39/44, phosphatidic acid 39/44, and free fatty acid 18/44. If these proportions are used to normalize the data in Table I, one can calculate that phospholipase D and phosphatidic acid phosphatase are about 17 and 1.5 times, respectively, more active than acyl hydrolase in the microsomal membranes.

When the microsomal membranes were treated with 0.2% (v/v) Triton X-100, a treatment that has been used to solubilize Ca^{2+}-ATPase (9), phospholipase D and phosphatidic acid phosphatase were enriched in the insoluble fraction that was pelletable after the detergent treatment. On a specific acitivity basis, phospholipase D showed enrichments of about 2.5-fold relative to intact microsomal membranes, and phosphatidic acid phosphatase showed enrichments of 4- to 5-fold (Table I). Phospholipase D was also slightly enriched in the supernatant derived from the Triton X-100 solubilization (Table I). This may reflect activation of the solubilized enzyme. In contrast, acyl hydrolase was not enriched in the pellet obtained after Triton X-100 treatment (Table I). This selective enrichment of phosphatidic acid phosphatase and phospholipase D was also reflected in the relative proportions of these enzyme activities in the partially purified preparation obtained after Triton X-100 solubilization. Using the correction factors identified above to approximate these relative proportions, phospholipase D and phosphatidic acid phosphatase proved to be ≅37 and ≅6 times more active than acyl hydrolase in the purified pellet.

Further characterization of these lipid-degrading enzymes was conducted using the pellets obtained after Triton X-100 solubilization of the microsomal membranes. The time-course for formation of phosphatidic acid, diacylglycerols, water-soluble products and free fatty acids from [U-^{14}C]phosphatidylcholine is illustrated in Figure 1. Phospholipase D activity reflected by the formation of radiolabeled water-soluble product reached a plateau after about 20 min. This was also observed in a summation plot of diacylglycerols and phosphatidic acid, which again represents phospholipase D activity. When protein levels in the reaction mixture were increased, phospholipase D showed a linear increase in activity initially and then began to plateau as the protein concentration was raised to higher levels (Fig. 2).

Table I. *Phospholipase D, Phosphatidic Acid Phosphatase and Acyl Hydrolase Activities of Triton X-100 Solubilized Microsomal Membranes*

Phospholipase D activity was determined by measuring choline, phosphatidic acid phosphatase by measuring diacylglycerols, and acyl hydrolase by measuring free fatty acids. Enrichments relative to intact microsomal membranes are indicated in parentheses.

Fraction	Phospholipase D	Phosphatidic Acid Phosphatase	Acyl Hydrolase
	cpm × 10^{-4} (mg protein·10 min)$^{-1}$		
Microsomal membranes	1.57	1.10	0.33
Supernatant of Triton X-100 solubilization	2.48 (1.58)	0.85 (0.77)	0.06 (0.18)
Pellet of Triton X-100 solubilization	4.03 (2.57)	4.77 (4.34)	0.39 (1.18)

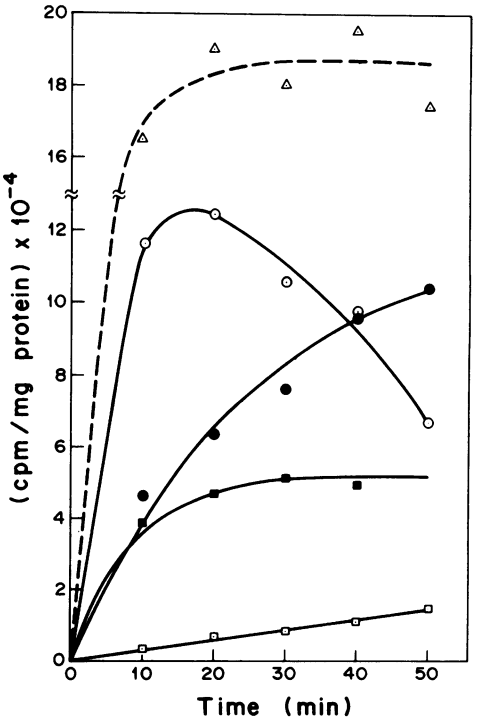

FIG. 1. Formation of phosphatidic acid (○), diacylglycerol (●), free fatty acids (□) and water-soluble product (■) from [U-^{14}C]phosphatidylcholine over time by the pellet obtained after Triton X-100 solubilization of microsomal membranes. [(△---△) shows the summation of phosphatidic acid and diacylglycerol levels]. Values are from one of three separate experiments all showing the same trends. The reaction mixture contained 12.5 μg of protein.

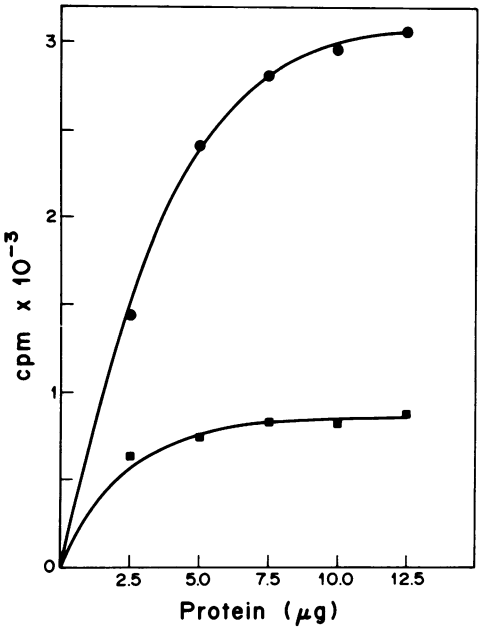

FIG. 2. Effect of increasing protein on the activity of phospholipase D in the pellet from Triton X-100 solubilized microsomal membranes as measured by the summation of phosphatidic acid and diacylglycerol (●) and water-soluble product (■) formation from [U-^{14}C]phosphatidylcholine. The reaction was allowed to proceed for 50 min. Values are from one of three separate experiments all showing the same effect.

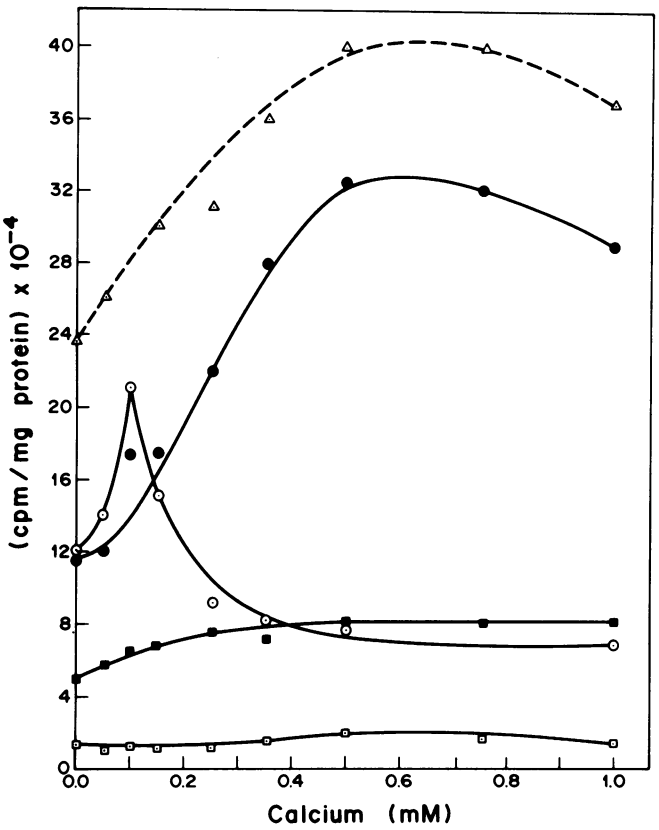

FIG. 3. Effect of calcium on the formation of phosphatidic acid (○) diacylglycerol (●), free fatty acids (□) and water-soluble product (■) from [U-^{14}C]phosphatidylcholine by the pellet from Triton X-100 solubilized microsomal membranes. The reaction mixture contained 12.5 μg of protein and was allowed to proceed for 50 min. Free calcium concentrations for 100, 150, 200, and 250 μM added calcium were <1, 1, 15, and 40 μM, respectively. At higher additions, the free calcium concentration was equivalent to the amount added. Values are from one of five separate experiments all showing the same trend except that the peak in phosphatidic acid levels was not always apparent. (△---△ shows the summation of phosphatidic acid and diacylglycerol.)

Phosphatidic acid levels rose quickly during the first 20 min of the reaction and then declined in accordance with a corresponding increase in diacylglycerols (Fig. 1). These temporal changes in phosphatidic acid and diacylglycerols are consistent with the contention that phosphatidic acid formed by phospholipase D is converted to diacylglycerols by phosphatidic acid phosphatase. Levels of free fatty acids increased in an essentially linear fashion throughout the time-course of the reaction (Fig. 1).

The effects of calcium on the formation of phosphatidic acid, diacylglycerols, water-soluble product, and free fatty acids from [U-^{14}C]phosphatidylcholine by the partially purified enzyme preparation are illustrated in Figure 3. The basic reaction mixture contained 0.2 mM EGTA, which in the absence of added calcium reduced the free calcium ion concentration below 1 μM. At 15 μM free calcium (200 μM added calcium), which approximates the level of cytoplasmic calcium in animal cells after its release in response to external stimuli (8), phospholipase D activity as measured either by the summation of diacylglycerols and phosphatidic acid or the formation of water-soluble product showed an increase of 25 to 30%, and even further stimulation was achieved at higher nonphysiological Ca^{2+} levels (Fig. 3). Phosphatidic acid phosphatase activity as reflected by diacylglycerol formation showed an increase of ≅65% at 15 μM free calcium (200 μM added calcium), and saturation was attained at an added calcium concentration of 500 μM. Still higher concentrations of Ca^{2+} tended to inhibit the formation of diacylglycerols (Fig. 3). With increasing Ca^{2+}, levels of diacylglycerols rose in the reaction mixture, and there was a corresponding decrease in phosphatidic acid (Fig. 3). These patterns of change are consistent with the contention that diacylglycerol is formed from phosphatidic acid by phosphatidic acid phosphatase. Phosphatidic acid consistently declined with increasing Ca^{2+}, and the sharp peak in phosphatidic acid preceding the steep rise in diacylglycerols (Fig. 3) was seen in only one of five experiments performed. This peak presumably reflects the fact that phosphatidic acid phosphatase tends to be more strongly stimulated by calcium than phospholipase D. By contrast, the liberation of free fatty acids was not promoted by physiological concentrations of free calcium, although a slight stimulation was noted at high concentrations (500–600 μM) of added calcium (Fig. 3). The stimulation of phospholipase D and phosphatidic acid phosphatase activities by physiological levels of calcium (40 μM free calcium) is also apparent from measurements of the enzymes over time in the presence and absence of added calcium (Figs. 4 and 5).

Calmodulin, a calcium-binding protein of ubiquitous occurrence in living systems, considerably enhanced the formation of diacylglycerols from phosphatidic acid, indicating that phosphatidic acid phosphatase is Ca^{2+}-calmodulin promoted (Fig. 6). In five different experiments, diacylglycerol accumulation was promoted by 30 to 55% at calmodulin concentrations ranging from 0.075 to 0.15 nM. Stimulation by exogenous calmodulin relative to the activity measured in the absence of calmodulin and calcium was ≅2.7-fold (Fig. 6), indicating that endogenous calmodulin remains associated with the microsomal preparations. Indeed, in two other experiments, calmodulin had a negligible effect on diacylglycerol accumulation, presumably because native calmodulin had not been effectively removed from the enzyme during the isolation procedure. In the absence of calcium, calmodulin had no effect. The increase in diacylglycerol in response to added calmodulin was accompanied by a corresponding decrease in phosphatidic acid (Fig. 6) indicating that

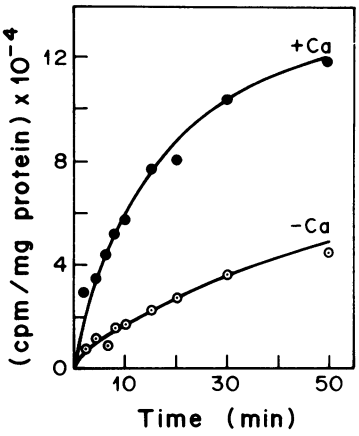

FIG. 4. Liberation of diacylglycerols from [U-^{14}C]phosphatidylcholine in the presence and absence of 40 μM free calcium by the pellet from Triton X-100 solubilized microsomal membranes. Values are from one of two separate experiments showing the same effect. The reaction mixture contained 12.5 μg of protein.

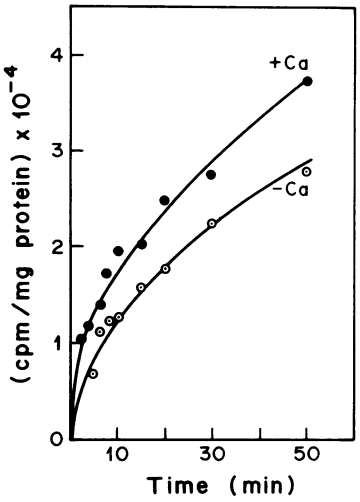

FIG. 5. Liberation of water soluble products from [U-^{14}C]phosphatidylcholine in the presence and absence of 40 μM free calcium by the pellet from Triton X-100 solubilized microsomal membranes. Values are from one of two separate experiments showing the same effect. The reaction mixture contained 12.5 μg of protein.

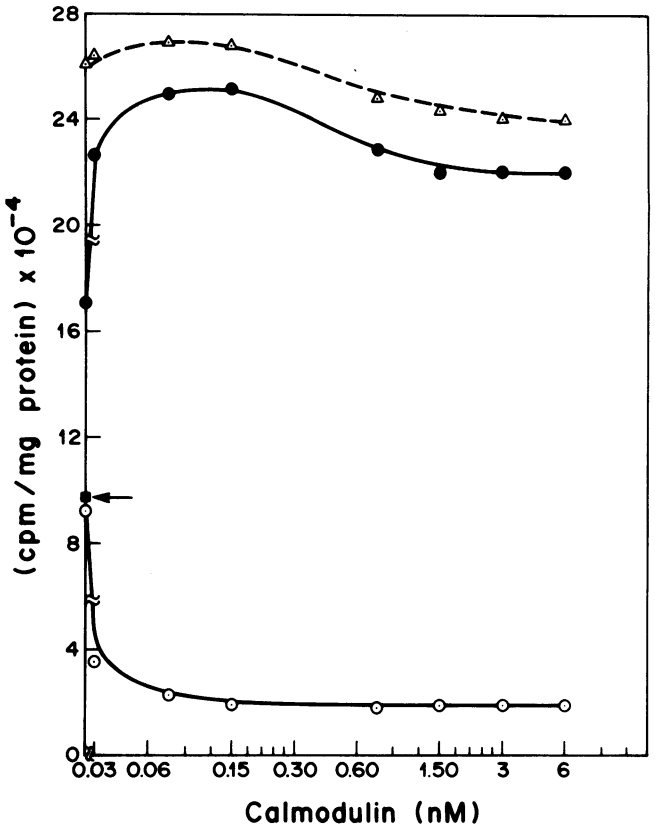

FIG. 6. Effect of bovine brain calmodulin on the formation of phosphatidic acid (○) and diacylglycerol (●) from [U-^{14}C]phosphatidylcholine by the pellet from Triton X-100 solubilized microsomal membranes. The reaction mixture contained 25 μg of protein, 40 μM free calcium (250 μM added calcium) and was allowed to proceed for 50 min. The molarity of calmodulin was calculated assuming a mol wt of 16,500. Values are from one of four separate experiments all showing the same trend. (△---△ shows the summation of phosphatidic acid and diacylglycerol levels.) The arrow indicates the level of diacylglycerols in the absence of calcium and calmodulin.

calmodulin has no effect on phospholipase D. As well, the liberation of water-soluble product was not stimulated above the calcium-promoted level by increasing calmodulin (data not shown).

Calmodulin promotion of diacylglycerol formation was further confirmed by using calmidazolium, an inhibitor of calmodulin action (14). Calmidazolium inhibited diacylglycerol formation reflecting phosphatidic acid phosphatase activity with an IC_{50} of approximately 15 μM, and the activity was fully inhibited to the $-Ca^{2+}$-level by 30 μM calmidazolium (Fig. 7). In addition, although phospholipase D activity was not promoted by calmodulin, the formation of water-soluble product was also reduced to the $-Ca^{2+}$-level by calmidazolium (Fig. 7). In contrast, the liberation of free fatty acids was unaffected by calmidazolium (Fig. 7).

DISCUSSION

Three enzymes appear to be involved in the breakdown of phospholipid in microsomal membranes from bean cotyledons: lipolytic acyl hydrolase, which liberates free fatty acids; phospholipase D, which catalyzes the formation of phosphatidic acid; and phosphatidic acid phosphatase, which dephosphorylates phosphatidic acid to generate diacylglycerol. Estimates of comparative activities obtained by normalizing the number of carbons in the various products released from [U-^{14}C]phosphatidylcholine indicate that at concentrations of free calcium below 1 μM phospholipase D is more active in the native isolated membranes than phosphatidic acid phosphatase or acyl hydrolase. Moreover, each of the enzymes appears to be intimately associated with the membranes and not simply a cytosolic contaminant that has been adsorbed to the membrane surface. This is demonstrated by the finding that the enzymes were present in the pellet derived after Triton X-100 solubilization. Phospholipase D and phosphatidic acid phosphatase were enriched in the pellet obtained after Triton X-100 solubilization by approximately 2-fold and 4- to 5-fold, respectively, relative to the native microsomal membranes. There is also a strongly active lipolytic acyl hydrolase in the cytosol of this tissue, and this enzyme has been shown capable of acting on a variety of lipid species (JB Brown, JE Thompson, unpublished data) and thus exhibits the feature of nonspecificity that is characteristic of plant lipolytic acyl hydrolases (13).

The temporal pattern in which the products of phospholipase D and phosphatidic acid phosphatase appear during the reaction

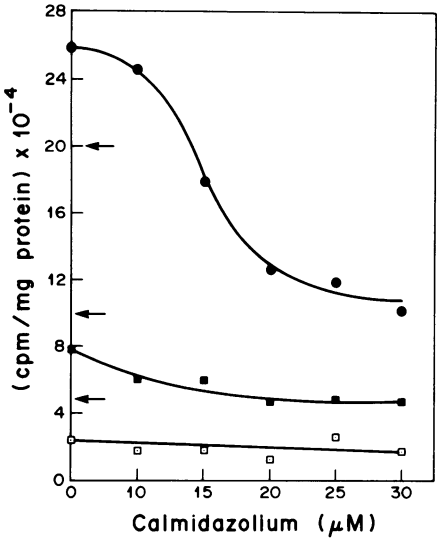

FIG. 7. Inhibition by calmidazolium of the formation of diacylglycerol (●), free fatty acids (□), and water-soluble product (■) from [U-^{14}C]phosphatidylcholine by the pellet from Triton X-100 solubilized microsomal membranes. The reaction mixture contained 12.5 μg of protein, 15 μM free calcium (200 μM added calcium), 0.075 nM calmodulin, and was allowed to proceed for 50 min. Upper arrow: calcium-promoted activity for the formation of diacylglycerols; middle arrow: basal activity for the formation of diacylglycerols; lower arrow: basal activity for the formation of water-soluble product. Values are from one of three separate experiments all showing the same trend.

indicates that these enzymes function in tandem in microsomal membranes. In particular, the decline in phosphatidic acid over time could be accounted for by a corresponding rise in diacylglycerol, indicating that the phosphatidic acid formed by phospholipase D is in turn dephosphorylated by phosphatidic acid phosphatase. There are also indications from the time-course of the reactions that phospholipase D exhibits molecular species specificity. Over a 50-min reaction period, 10 to 15% of the [U-^{14}C] phosphatidylcholine was broken down. Yet phospholipase D activity as measured both by the release of water-soluble product and the summation of phosphatidic acid and diacylglycerol began to plateau within approximately 20 min of the reaction being initiated. These observations raise the interesting possibility that the various molecular species of phosphatidylcholine are not uniformly acted upon by the enzyme.

Herman and Chrispeels (16) have reported that in mung bean cotyledons there are two distinguishable phosphatidic acid phosphatase activities, one associated with endoplasmic reticulum membranes, which has a pH optimum of 7.5, and another in protein bodies with a pH optimum of 5.0. There are also reports of soluble and membrane-bound forms of phospholipase D (24, 27). In the living bark tissue of black locust, the membrane-bound enzyme is tightly associated with microsomal membranes and can be solubilized only after treatment with detergent, whereas the soluble enzyme is thought to be compartmentalized in vacuoles (27). Thus the soluble enzyme in homogenates of black locust bark tissue may well be analogous to the phospholipase D activity found in protein bodies in mung bean, compartments that are thought to serve a lytic function in storage parenchyma cells (16). Indeed, Herman and Chrispeels (16) have proposed that the phospholipase D and phosphatidic acid phosphatase in protein bodies mediate the breakdown of membrane phospholipids through autophagy. However, the finding in the present study and in previous studies (16, 24, 27) that there are also phospholipase D and phosphatidic acid phosphatase activities tightly associated with microsomal membranes suggests that there may be an alternative pathway for phospholipid catabolism that is mediated by enzymes associated directly with the membrane and does not require a lytic compartment. This contention is in part supported by a previous finding that the addition of partially purified soluble phospholipase D from living bark tissue of black locust to microsomal membranes from the same tissue caused only a slight (8%) stimulation of the degradation of endogenous microsomal phospholipid (28). This observation was interpreted as indicating that degradation of microsomal phospholipid is more effectively catalyzed by the membrane-associated enzyme than by the soluble enzyme (28).

Of particular interest is the finding in the present study that the activities of microsomal phospholipase D and phosphatidic acid phosphatase are stimulated by calcium. Both enzymes showed substantive stimulations at levels of free calcium that are well within the physiological calcium levels observed in the cytoplasm when calcium is released under stimulated conditions (8). Acyl hydrolase activity proved to be insensitive to calcium. Phosphatidic acid phosphatase was also stimulated by exogenous calmodulin, a calcium-binding protein of ubiquitous occurrence in living systems that serves to mediate physiological responses to an increase in cytoplasmic free calcium (8). The apparent dissociation constant (K_d) for calmodulin stimulation of phosphatidic acid phosphatase (*i.e.* concentration of calmodulin giving half-maximal stimulation of activity) was approximately 3×10^{-11} M. This value is low by comparison with the dissociation constant reported for calmodulin stimulation of cyclic-nucleotide phosphodiesterase (10^{-9} M) (18). It is apparent, therefore, that the phosphatidic acid phosphatase of these microsomal membranes exhibits a high affinity for calmodulin, and hence the removal of endogenous calmodulin bound to the enzyme by treatment with chelators is not likely to be fully effective. This presumably explains why it proved possible to completely inhibit both calcium- and calmodulin-promoted phosphatidic acid phosphatase activity with calmidazolium. Neither calmodulin nor calmidazolium had any detectable effect on acyl hydrolase activity, which is to be expected in view of its apparent insensitivity to Ca^{2+}. However, calmidazolium did inhibit the Ca^{2+}-promotion of phospholipase D activity despite the fact that this enzyme was not stimulated by calmodulin. Such inhibition of Ca^{2+}-promoted activity that is insensitive to external addition of calmodulin has been noted previously for other enzymes (25).

These observations collectively indicate that calcium plays a key role in mediating membrane lipid deterioration. The presumed trigger that releases compartmentalized calcium remains unknown. However, there is some evidence that triggering mechanisms for calcium release analogous to the mechanism involving hormonally mediated turnover of phosphorylated phosphatidylinositol, now well characterized in animal systems (3, 11), may also be operative in plants. For example, auxin-induced release of Ca^{2+} in soybean microsomal membranes has been reported to involve turnover of phosphatidylinositol (21), and inositol triphosphate has been shown capable of releasing calcium from plant microsomal membranes (10). Only the phospholipase D/phosphatidic acid phosphatase sequence is responsive to calcium, but inasmuch as diacylglycerols do not form stable lamellar configurations in biomembranes, this could, nonetheless, induce rapid and extensive loss of membrane lipid components. For example, increasing the intracellular calcium concentration of erythrocytes, which activates phospholipase C and results in extensive diacylglycerol formation, has been shown to cause budding of microvesicles enriched in diacylglycerol away from the plasma membrane and a consequent change in cell shape (1). Similar microvesiculation has been observed after treatment of intact red blood cells and isolated muscle microsomes with exogenous phospholipase C (2, 12). By EM it is apparent that these diacylglycerol-enriched microvesicles tend to remain associated with the membrane (2, 12). As plant tissues senesce, there

is extensive breakdown of membrane phospholipid (5, 19), and electron-dense deposits in close association with the plasmalemma, which could be manifestations of diacylglycerol-enriched microvesicles, have been observed in senescing cowpea cotyledons (23). Thus release of compartmentalized Ca^{2+} would stimulate the production of diacylglycerols from membrane phospholipids, which would then presumably be released from the membrane and further metabolized by cytosolic lipolytic acyl hydrolase.

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