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Freezing Injury and Phospholipid Degradation *in Vivo* in Woody Plant Cells

II. REGULATORY EFFECTS OF DIVALENT CATIONS ON ACTIVITY OF MEMBRANE-BOUND PHOSPHOLIPASE D[1]

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ABSTRACT

Activity of membrane-bound phospholipase D in microsomes from bark tissues of black locust tree (*Robina pseudoacacia* L.) was demonstrated to be regulated by a competitive binding of divalent cations. Binding of Ca^{2+} at high concentrations (1 to 50 millimolar) modified the pH activity profile, shifting the optimum pH by 0.5 unit toward neutral and increasing the activity in the neutral pH. Mg^{2+}, on the other hand, inhibited the reaction of membrane-bound phospholipase D without added Ca^{2+}, and competitively inhibited the Ca^{2+} stimulation. The regulatory effects of those ions were dependent on pH. Reduction in pH resulted in a decrease in the apparent dissociation constant for Ca^{2+} and an increase in that for Mg^{2+}. From Lineweaver-Burk double reciprocal plots of Ca^{2+} and the initial velocity, it was suggested that the binding of Ca^{2+} in the higher concentration resulted in nearly the same conformational change of enzyme as reduction in pH. Mg^{2+}, on the other hand, counteracted those effects of Ca^{2+} and lower pH on the enzyme conformation in such a manner as to inactivate. The membrane-bound phospholipase D became more sensitive to Ca^{2+} and less sensitive to Mg^{2+} as the hardiness of the tissues decreased. This fact may indicate that some qualitative changes in membranes are involved in the hardiness changes and also in the susceptibility of phospholipid to degradation by phospholipase D in plant cells.

Although phospholipase D is widely distributed in plants (13), little information with respect to its physiological functions and regulation has yet been reported. When less hardy plant cells are frozen at a critical temperature, a drastic degradation of phospholipids by phospholipase D is brought about *in vivo* in the frozen state even at −25 C (18, 22). Freezing injury might change either the compartmentation of soluble phospholipase D resulting in contact between membranes and enzyme, or alter the regulatory properties of the membrane-bound enzyme *per se*.

As is well known (6, 13), phospholipase D activity requires Ca^{2+} *in vitro*, and its optimum pH ranges between 5 and 6. Thus, a change in the locus of Ca^{2+} and/or reduction in the cytosol pH may have important roles in the *in vivo* reaction of phospholipase D in plant cells.

The present results refer to studies on the possible involvement of divalent cations, in particular Ca^{2+} and Mg^{2+}, in the regulation of activity of membrane-bound phospholipase D in living bark cells of black locust trees. A preliminary account of this study has been presented elsewhere (19).

[1] Contribution 2028 from The Institute of Low Temperature Science.

MATERIALS AND METHODS

Preparation of EGTA-washed Microsome-enriched Fraction. The living bark tissues of the black locust tree (*Robinia pseudoacacia* L.) were fractionated as in the preceding paper (20). Pellets obtained by centrifugation from 14,000 to 189,000*g* were designated as the microsome-enriched fraction. The microsome-enriched pellets were resuspended in 5 mM Tris-HCl (pH 7.6), containing 1 mM EGTA by gentle homogenization in a loosely fitted Teflon homogenizer and then recentrifuged. The EW-Ms,[2] suspended in 5 mM Tris-HCl (pH 7.6), contained no detectable amount of soluble phospholipase D.

Measurement of Activity of Membrane-bound Phospholipase D. The reaction mixture for the determination of activity of membrane-bound phospholipase D using endogenous substrate consisted of 0.5 ml of the suspension of the EW-Ms (0.6–0.8 mg protein), 60 mM of buffers varied in pH, and divalent cations as required in a final volume of 1 ml. The buffer systems were glycine-HCl (pH 2.5–3.5), Na-acetate (pH 4.0–6.5), and Tris-HCl (pH 7.0–8.5). Incubation was carried out at 25 C for 5 to 10 min and then the reaction was terminated by the addition of 0.2 ml of 1 N $HClO_4$. The liberated choline in the supernatant was analyzed according to the iodine-potassium iodide method (6). The phospholipase D activity was usually expressed as the rate of choline-liberation per 10 min in the reaction. In every experiment, a blank value at the zero time of the reaction was subtracted.

Measurement of Activity of Phospholipase D Using Added Substrate. Egg phosphatidylcholine purified through silicic acid column chromatography was used as the exogenous substrate. The reaction mixture consisted of 3 μmol of egg phosphatidylcholine, 60 μmol of buffer solutions varied in pH, 10 mM of $CaCl_2$, 1.5 mM of SDS or 1/2 volume of ethyl ether as the activators, and 0.1 to 0.2 ml of the EGTA-washed microsomal suspension in a final volume of 1 ml. The buffer systems used were glycine-HCl (pH 2.0–2.5), Na-acetate (pH 4.0–6.5), and Tris-HCl (pH 7.0–8.0). The reaction was carried out at 30 C and then terminated by the addition of 0.2 ml of 1 N $HClO_4$. When SDS was used as the activator, liberated free choline was analyzed directly with an aliquot of the supernatant as described above. When ethyl ether was utilized as the activator, the aqueous layer was repeatedly washed with ethyl ether and then centrifuged. After removing ethyl ether by evaporation, the liberated free choline in the supernatant was determined as described above.

Protein was determined by the method of Lowry *et al.* (8) after precipitation with trichloroacetic acid. BSA was used as the standard.

[2] Abbreviation: EW-Ms: EGTA-washed microsome-enriched membranes.

RESULTS

Reaction Using Endogenous Substrate. Figure 1 shows the effect of pH upon the activity of phospholipase D in EW-Ms in the presence or absence of divalent cations. In this experiment EW-Ms prepared from winter hardy tissues were used. When the pH was reduced to near 4, degradation of endogenous phosphatidylcholine was observed without addition of Ca^{2+}. However, little or no degradation occurred above pH 5.5. When 50 mM of $CaCl_2$ was added to the reaction mixture, the optimum pH was shifted by 0.5 unit toward the neutral pH and a remarkable increase in activity was noted near the neutral pH region. Considerable reduction in activity was observed below pH 4.5.

Mg^{2+} inhibited the reaction of phospholipase D on endogenous substrate strongly at all pH values tested.

Table I shows the effects of various divalent cations on the activity of membrane-bound phospholipase D. Sr^{2+} had nearly the same stimulatory effect as Ca^{2+}. Heavy metal ions such as Zn^{2+}, Mn^{2+}, Co^{2+}, and Cu^{2+} were found to be inhibitory.

Concentration curves for the effect of Ca^{2+} upon the reaction of membrane-bound phospholipase D were dependent on pH in the reaction mixture (Fig. 2). The lower the pH, the greater the stimulatory effect of Ca^{2+} on the reaction was noted. The affinity for Ca^{2+} decreased at higher pH values. Reciprocal plots (not shown) of data in Figure 2A gave straight lines, permitting calculation of apparent dissociation constants (Table II).

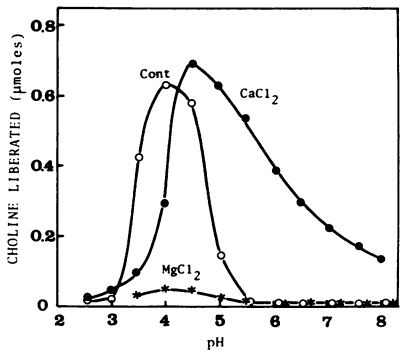

FIG. 1. pH activity profiles of reaction of membrane-bound phospholipase D in EW-Ms on endogenous substrate in the presence or absence of divalent cations. Membranes were prepared from winter hardy tissues. Reaction mixture contained 0.5 ml of the membrane suspension (0.8 mg protein), 60 mM of buffers varied in pH, and 50 mM of $CaCl_2$ or $MgCl_2$ in a final volume of 1 ml. Reaction was carried out at 25 C. Phospholipase D activity is expressed as rate of choline liberation per 10 min. Blank value at zero time of incubation was subtracted. Cont: control without addition of divalent cations.

Table I. *Effect of Divalent Cations on Activity of Membrane-bound Phospholipase D on Endogenous Substrate in EGTA-washed Microsome-enriched Membranes*

The reaction mixture contained 0.5 ml of the membrane suspension (0.8 mg protein) prepared from winter hardy tissues, 60 mM Na-acetate and 25 mM of each cation in a final volume of 1 ml. Reactions were carried out at 25 C. The experimental details are described in Figure 1.

Cations	pH 4.5	Cation Effect[1]	pH 5.5	% of Activity at pH 4.5
	Choline, μmol	*%*	*Choline, μmol*	
None	0.44	0	0	
$MgCl_2$	0.23	−46	0	
$CaCl_2$	0.59	+33	0.36	81.8
$SrCl_2$	0.57	+30	0.23	52.2
$ZnCl_2$	0.15	−66	0	
$MnCl_2$	0.24	−45	0	
$CoCl_2$	0.23	−46	0	
$CuCl_2$	0.05	−89	0	

[1] (−) indicates inhibition compared to rate with no cation added; (+) indicates promotion of activity.

The effect of Ca^{2+} was also observed to be dependent on the hardiness of the tissues. In EW-Ms from winter hardy tissues (hardy membranes) activity with Ca^{2+} was small at pH values higher than 6.0 (Fig. 2A). In EW-Ms from summer tissues (less hardy membranes), the rate of degradation of endogenous phosphatidylcholine was high even at pH 7.0 as the concentration of Ca^{2+} increased. It appeared that the reaction of phospholipase D on endogenous substrate became very sensitive to Ca^{2+} as the hardiness of the tissues decreased.

The inhibitory effect of Mg^{2+} was also dependent on pH (Fig. 3). The higher the pH, the greater the inhibitory effect. The inhibitory effect of Mg^{2+} on the degradation of phosphatidylcholine was also observed to be dependent on the hardiness of the tissues from which the EW-Ms were prepared. At pH 4.5, the addition of 10 mM $MgCl_2$ resulted in about 55% inhibition in hardy EW-Ms, while only 17% in less hardy membranes. Mg^{2+} inhibition of the endogenous activity of hardy EW-Ms was reduced at lower pH and was competitive with Ca^{2+} (Fig. 4).

The calculated apparent dissociation constants of hypothetical enzyme-cation complexes as a function of pH are summarized in Table II. The lower the pH, the smaller the dissociation constant for Ca^{2+}. The inverse relation was noted with Mg^{2+}. EW-Ms from summer tissues gave much smaller dissociation constants for Ca^{2+} and inversely much greater dissociation constants for Mg^{2+}, when a comparison was made at the same pH value. There seems to be a great difference in the properties of membrane-bound phospholipase D between hardy and less hardy microsome-enriched membranes. These results may indicate that the degradative activity of membrane-bound phospholipase D can be regulated by the presence of divalent cations and by pH changes.

As noted earlier (13), ethyl ether stimulated the endogenous reaction of phospholipase D. We also noted that it broadens the pH curve, much as Ca^{2+} does (data not shown). As noted earlier (14), basic proteins were inhibitory. We also found strong inhibition by the cationic detergent cetyl trimethylammonium bromide (data not shown). The anionic detergent SDS stimulated the

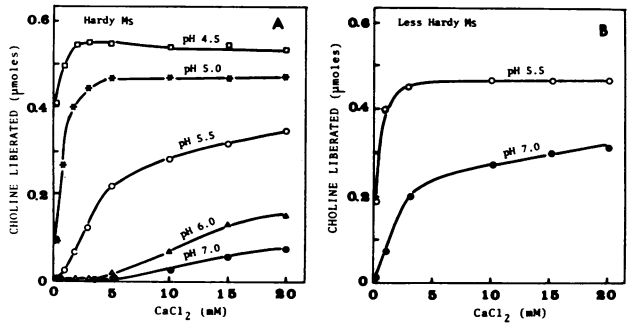

FIG. 2. Effect of Ca^{2+} on reaction of membrane-bound phospholipase D in EW-Ms on endogenous substrate depending on pH. Membranes were prepared both from winter hardy (A) and summer less hardy (B) tissues. Experimental details are described in Figure 1.

Table II. *Apparent Dissociation Constants for Ca^{2+} and Mg^{2+}*

pH	Microsome-enriched Membranes from Winter Hardy Tissues		Microsome-enriched Membranes from Summer Less Hardy Tissues	
	Ca^{2+}	Mg^{2+}	Ca^{2+}	Mg^{2+}
	mM	*mM*	*mM*	*mM*
4.5	0.17	16.1		
5.0	2.04	2.9		
5.5	13.50	0.9	0.19	15.5
6.0	70.00			
7.0			4.78	

reaction slightly at pH 4.5, then inhibited it at higher levels. At pH 5.5 a slight stimulation was seen as the concentration rose to 3 mM (data not shown).

The temperature dependence of the reaction of membrane-bound phospholipase D on endogenous substrate is shown in Figure 5. EW-Ms prepared from winter hardy tissues were utilized in this experiment. Little or no activity was observed below 10 C. Above 10 C, the activity increased with increasing temperature. Without addition of Ca^{2+}, a slight decrease in the activity was noted above 40 C. With the presence of 5 mM Ca^{2+}, no decline in the activity was observed above 40 C.

Reaction with Added Substrate. Some experiments were carried out to clarify the effect of divalent cations upon the activity of membrane-bound phospholipase D on added substrate. Figure 6A shows the pH activity profile of the reaction with added phosphatidylcholine, 10 mM $CaCl_2$, ethyl ether, and EW-Ms as the enzyme source. In this reaction system, addition of Ca^{2+} was obligatory for the enzyme activity. As indicated in Figure 6B, the inhibitory effect of Mg^{2+} was also observed in this reaction system. The pH dependency in the inhibitory effect of Mg^{2+} was also confirmed with the reaction system using added substrate. Competition between Ca^{2+} and Mg^{2+} was also observed in the reaction system (data not shown). On the other hand, no inhibitory effect of Mg^{2+} was observed in the reaction system when SDS was utilized as the activator (Fig. 7). The inhibitory effect of Mg^{2+} was found to be greatly dependent on the reaction conditions *in vitro*, *i.e.* the differences in the activators. Nearly the same results as described above were obtained with soluble phospholipase D which was partially purified from bark tissues of black locust tree on a DEAE-cellulose column and with commercially available soluble phospholipase D from cabbage leaves (purchased from Boehringer Mannheim).

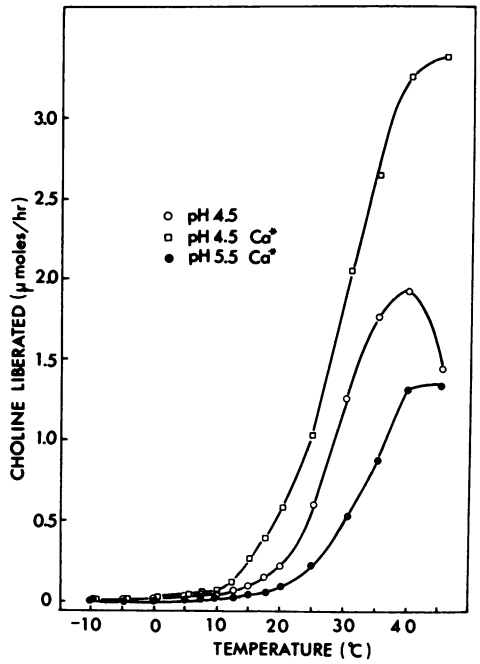

FIG. 5. Temperature dependencies of reaction of membrane-bound phospholipase D in EW-Ms with endogenous substrate. $CaCl_2$ was added in a final concentration of 5 mM. Membranes were prepared from winter hardy tissues. Activity of phospholipase D is expressed as rate of choline-liberation per h. Experimental details are described in Figure 1.

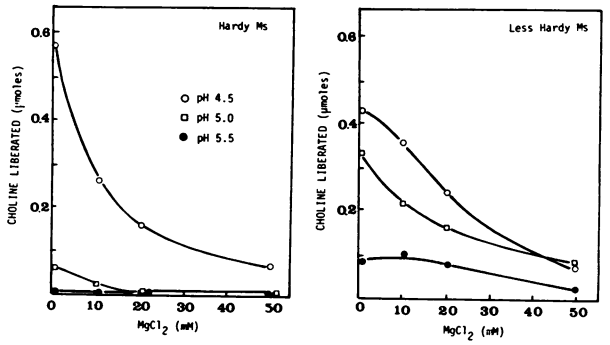

FIG. 3. Inhibitory effect of Mg^{2+} on reaction of membrane-bound phospholipase D in EW-Ms on endogenous substrate depending on pH. Membranes were prepared from winter hardy (A) and summer less hardy (B) tissues. Experimental details are described in Figure 1.

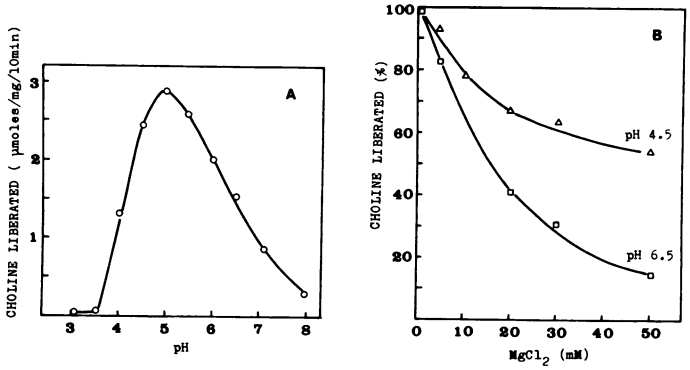

FIG. 6. pH activity profile (A) and effect of Mg^{2+} on activity of membrane-bound phospholipase D on added substrate (B) with presence of ethyl ether as activator. Reaction mixture contained 3 μmol of egg phosphatidylcholine, 60 mM of a buffer solution, 10 mM $CaCl_2$, 0.5 ml of ethyl ether, and 0.2 ml of EW-Ms (0.8 mg protein) as the enzyme source in a final volume of 1 ml. Buffer systems were glycine-HCl (pH 3.0–3.5), Na-acetate (pH 4.0–6.5), and Tris-HCl (pH 7.0–8.0). Activity is expressed as the rate of choline liberation per mg protein (A) or as per cent of choline liberation in control reactions without addition of $MgCl_2$.

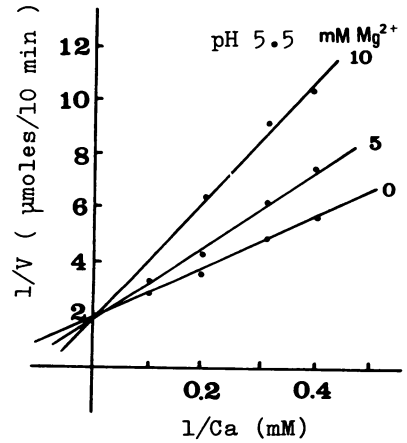

FIG. 4. Plot of reciprocal of initial velocity of reaction of phospholipase D with endogenous substrate at pH 5.5 (V) *versus* reciprocal of concentration of Ca^{2+} as variable concentration of Mg^{2+}. The EW-Ms were prepared from winter hardy tissues. Experimental details are described in Figure 1.

DISCUSSION

EW-Ms, containing no detectable amount of soluble phospholipase D, were used throughout this study. They were obtained by thoroughly washing the 189,000*g* pellets with 5 mM Tris-HCl containing 1 mM EGTA (pH 7.6). The results of this study suggest that membrane-bound phospholipase D in EW-Ms is regulated by the ratio of Ca^{2+} to Mg^{2+} and by changes in pH. In the EW-

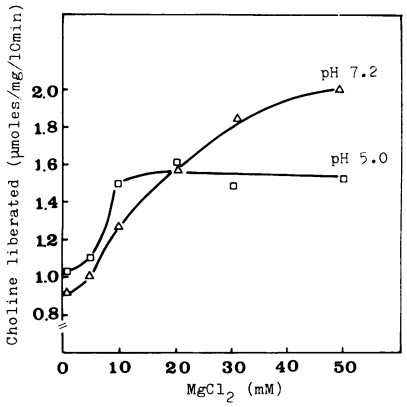

FIG. 7. Effect of Mg^{2+} on activity of membrane-bound phospholipase D on added substrate with presence of SDS as activator. Phospholipase D activity was assayed in the same reaction mixture as described in Figure 6, except for activator. SDS (2 mM) was substituted for ethyl ether. Experimental details are described in Figure 6.

Ms, merely a reduction in pH of the reaction system resulted in a marked degradation of the endogenous phospholipids. The reaction was reversibly inhibited by chelators, such as EGTA and EDTA. These results may indicate that a minor amount of Ca^{2+} required for the enzyme activity has been tightly bound to membranes. In fact, 11 to 12 natoms of Ca^{2+} per mg protein (equivalent to 6–8 μM in each reaction), were found to be bound to the EW-Ms (21). Exogenously added Ca^{2+} ranging from 1 to 50 mM showed a modulating effect on membrane-bound phospholipase D activity against endogenous substrates, shifting the pH optimum toward neutral. The regulation of the activity of membrane-bound phospholipase D by divalent cations and pH may be brought about through either conformational changes of enzyme or changes in the physical state of membrane phospholipids.

The inhibitory effect of Mg^{2+} was also demonstrated in the reaction systems with added substrate both of membrane-bound and soluble enzymes with the presence of ethyl ether as the activator and egg phosphatidylcholine as the exogenous substrate. Little or no inhibitory effect of Mg^{2+} was observed in the reaction system using added substrate, when 1.5 mM of SDS replaced ethyl ether as the activator.

According to Quarles and Dawson (14), the conformation of phospholipase D is affected by anionic detergents, such as SDS and acidic phospholipids, resulting in a marked shift of the optimal pH toward neutral in reactions using added substrate. According to Heller *et al.* (4), highly purified soluble phospholipase D from peanut seeds has a mol wt of 200,000, which dissociates into smaller subunits with mol wt of 50,000 in the presence of SDS or urea.

As the hardiness changes, the regulatory properties of membrane-bound phospholipase D also changed. The apparent dissociation constants of Ca^{2+} decreased as the hardiness decreased, when comparison was made at the same pH. The inverse relation was obtained in the apparent dissociation constants for Mg. This fact suggests that the regulatory properties of membrane-bound phospholipase D are also dependent on the nature of membranes and/or the interaction between the enzyme and membranes as well. The increased sensitivity to Ca^{2+} and the decreased sensitivity of Mg^{2+} in the *in situ* activity of phospholipase D in membranes (EW-Ms) from summer less hardy cells may have a quite important role in the susceptibility of cells to freezing and subsequent degradation of phospholipids *in vivo* during freezing and after thawing. In such less hardy cells, a slight increase in Ca^{2+} concentration and a slight decrease in pH in cytosol may bring about a drastic change in the activity of membrane-bound phospholipase D.

From experiments on hydrolysis of a monolayer of phosphatidylcholine at the air/water interface by a surface radioactivity technique (15), it has been demonstrated that phospholipase D can penetrate into the monolayer within a certain range of the surface pressure and degrade phospholipid into phosphatidic acid. This fact may suggest that a hydrophobic association between enzyme and substrate is prerequisite for the full activity of phospholipase D with endogenous substrate. The difference in the sensitivity of the activity of membrane-bound phospholipase D with endogenous substrate to divalent cations between hardy and less hardy microsomes may be attributed to the changes in the binding mode of the enzyme in membranes. In support of this idea, the activity of phospholipase D in membranes (EW-Ms) from summer less hardy cells showed more resistance to a pronase treatment than those from winter hardy cells (unpublished data). This fact may imply that phospholipase D in less hardy membranes is more deeply intercalated into the hydrophobic interior of the membranes than in hardy membranes, and thus protected from the proteolytic enzyme.

It has recently been reported that acidic phospholipids such as phosphatidylserine, phosphatidylglycerol, and phosphatidic acid in synthetic bilayers of phospholipid mixtures are induced to phase separation by the addition of Ca^{2+} (5, 11, 12) and by lowering in pH (16). Mg^{2+}, on the other hand, induces little or no phase separation, but competitively inhibits the phase separation produced by Ca^{2+} (12). A binding specificity between Ca^{2+} and Mg^{2+} to phosphatidylserine molecule has been also shown (11). Thus, a similarity exists between the lateral phase separation of acidic phospholipids in synthetic membranes and the properties in the reaction of phospholipase D in microsome-enriched membranes with endogenous substrate.

The physiological functions of soluble and membrane-bound phospholipase D in plant cells are obscure as yet. However, the soluble enzyme compartmentalized in vacuoles may take part in the physiological catabolic processes of cell organelles during processes such as senescence (1), aging, germination, and the cell differentiation and development (9). In fact, a lysosomal function in vacuoles similar to that in animal cells has been observed in plant cells (3, 9, 17).

There is no evidence to show that phospholipase D takes part in lipid turnover; however, the turnover of polar head groups in phospholipids has recently been reported to occur at a significantly high rate in plant cells (10). It is possible that the membrane-bound phospholipase D has some physiological functions in lipid turnover somewhat different from those of the soluble enzyme. Controlled changes in the concentration of Ca^{2+} in the cytosol (2, 7) as well as the loss of the regulatory properties of membrane-bound phospholipase D may induce an irreversible dysfunction of membranes caused by a rapid degradation of phospholipids.

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