Action and Inhibition of Endogenous Phospholipases during Isolation of Plant Membranes

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ABSTRACT

Endogenous phospholipase D and phosphatidylic acid phosphatase activities were demonstrated in membrane fractions isolated from soybean (Glycine max L) hypocotyls. Phospholipase D activity was distributed widely among different membrane fractions while phosphatidylic acid phosphatase was found predominantly in membranes equilibrating in lower sucrose densities. Phospholipase D action was unaffected by ethylenediamine-N,N'-tetraacetic acid, sodium salt or ethylene glycol-bis(β-aminoethyl ether)-N,N'-tetraacetic acid but was prevented by a mixture of 4% choline and 4% ethanolamine. Phosphatidylic acid phosphatase was inhibited by 10 millimolar glycerol 1-phosphate or by homogenization media prepared with coconut milk as a solvent instead of water. Under fully protected conditions the phospholipid composition of soybean membrane fractions remained unchanged for at least 1 hour at 20°C. Membranes prepared under protected conditions had low phosphatidylic acid contents and the phospholipid compositions closely resembled those of corresponding animal membranes.

Quantitative comparisons of the phospholipid compositions of different membrane fractions have been complicated by poor recoveries of individual phospholipids and degradative alterations (22, 31). As a part of ongoing investigations of the in vitro interactions of plant membranes with plant growth hormones, we were prompted to undertake a detailed investigation of the stability of isolated plant membranes in cell homogenates. We found that phospholipids of membranes from soybean hypocotyls under investigation were surprisingly susceptible to degradation. Both phospholipase D and phosphatidylic acid phosphatase appeared to contribute. Subsequently, a procedure was developed to inhibit phospholipid degradation by these enzymes. The procedure kept membranes stable even at room temperature so that physiologically effective concentrations of hormones could be studied with less drastically altered membrane preparations.

The phenomenon of rapid phospholipid degradation we describe here is probably common for isolated plant membranes with some clear exceptions (9, 10). Fast growing plant tissues generally have high phospholipase D activities (33). Thus the procedures described to protect plant membranes against phospholipid degradation should be of general interest.

MATERIALS AND METHODS

Plant Material. Fungicide-treated (Orthocide 75) soybean seeds (Glycine max L. cv. Wayne) were soaked in water overnight and planted in moist Vermiculite. After 4 days growing in the dark, the hypocotyls (about 1 cm long) were used for membrane isolation.

Membrane Isolations. The isolation medium contained 0.1 M K-phosphate (pH 6.5), 20 mM EDTA2 or EGTA, and 0.5 mM sucrose in freshly prepared and filtered coconut milk. Hypocotyls were homogenized for 45 sec with a Polytron 20 ST (Kinematica, Lucerne, Switzerland) operating at about 5,000 rpm. Membranes from about 80-g bean hypocotyls were isolated as described (40). Four membrane fractions were collected finally from a discontinuous coconut milk–sucrose gradient and designated A, B, C, and D (40). For protection against lipid degradation 4% choline (w/v), 4% ethanolamine (v/v), and 10 mM glycerol-1-P were added to all solutions.

Assays. Membrane pellets of the individual gradient fractions were resuspended with an all-glass homogenizer of the Potter-Elvehjem type in either 6 ml (experiment in Fig. 1) or 18 ml (all other experiments) of assay buffer. The standard incubation buffer was 50 mM Na-acetate (pH 5.5) with or without 4% choline (w/v) and ethanolamine (v/v). The final assay volume for a single determination was 1 ml or 3 ml, respectively. Glycerol-1-P and EDTA or EGTA was added to an equal volume of resuspended membranes in the same buffer as used for incubation so that the final concentration was 10 mM for each compound. In some earlier experiments glycerol-1-P was replaced by 10 mM ATP. In assays containing coconut milk, the coconut milk was buffered and added to an equal volume of resuspended membranes. The incubation was started by transferring the membrane suspensions from an ice-water bath to a 20°C water bath. The assays were stopped by addition of 1 ml of chloroform to each tube with rapid mixing. Then 2 ml of chloroform-methanol (1:1, v/v) were added for the first extraction step.

The phosphatidic acid phosphatase assay was as described (26) but in 50 mM Na-acetate (pH 5.5) at 20°C for 2 hr. A 50-ml volume of membrane suspension was added to a final reaction volume of 250 ml. The substrate concentration was 2 mM. Protein was estimated by the Lowry et al. procedure (25).

Lipids. Lipids were first extracted with an equal volume of chloroform-methanol (2:1). Then 0.2 ml 2 M KCl was added for each 3-ml original water phase and the mixture was extracted twice with two-thirds of the initial volume chloroform-methanol (2:1, v/v). The procedure was convenient that large volumes of solvent were avoided and could be carried out in a test tube by taking up the lower phase with long tip Pasteur pipette. The recovery for phospholipids was greater than 95% and 70 to 75% for lysophospholipids. Lipids were dried in vacuo and redissolved

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2 Abbreviations: PA: phosphatidic acid; PC: phosphatidylcholine; PE: phosphatidylethanolamine; PG: phosphatidylglycerol; PI: phosphatidylinositol; LPC: lysophosphatidylcholine; LPE: lysophosphatidylethanolamine; EDTA: ethylenediaminetetraacetic acid, sodium salt; EGTA: ethylene glycol-bis(β-aminoethyl ether)-N,N'-tetraacetic acid; acetate: 50 mM sodium acetate (pH 5.5); t: time.
Table I. Phospholipid Composition of Membrane Fractions from Soybean Hypocotyls

Membranes were isolated in coconut milk-sucrose medium and gradients (37) with no additions

<table>
<thead>
<tr>
<th>Gradient Fraction</th>
<th>Experiment</th>
<th>Phospholipid</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>% of total phospholipid phosphorous</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>PC</td>
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<td>27.4</td>
<td>14.1</td>
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<td>PE</td>
<td>17.6</td>
<td>16.4</td>
<td>22.4</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>PI</td>
<td>7.1</td>
<td>5.2</td>
<td>6.8</td>
<td>5.1</td>
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</tr>
<tr>
<td></td>
<td>PG</td>
<td>4.7</td>
<td>4.8</td>
<td>6.6</td>
<td>10.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>PA</td>
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<td>42.6</td>
<td>46.3</td>
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<td></td>
</tr>
<tr>
<td>LPC + LPE</td>
<td>Trace</td>
<td>Trace</td>
<td>1.0</td>
<td>1.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PS</td>
<td>Present</td>
<td>Present</td>
<td>Present</td>
<td>Present</td>
<td>Present</td>
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</tr>
<tr>
<td>2</td>
<td>PC</td>
<td>54.3</td>
<td>46.6</td>
<td>40.7</td>
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<tr>
<td></td>
<td>PE</td>
<td>28.3</td>
<td>29.3</td>
<td>33.4</td>
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<tr>
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<td>11.2</td>
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<tr>
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<td>2.1</td>
<td>5.1</td>
<td>5.9</td>
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<tr>
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<tr>
<td>LPC + LPE</td>
<td>Trace</td>
<td>Trace</td>
<td>1.0</td>
<td>1.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PS</td>
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<td>Present</td>
<td>Present</td>
<td>Present</td>
<td>Present</td>
<td></td>
</tr>
</tbody>
</table>

in a small volume of chloroform for TLC.

For TLC Silica Gel HR plates were activated for at least 2 hr at 100 C. The first dimension was chromatographed in either chloroform-methanol-ammonium hydroxide-water (60:35:7:2.8, v/v) (3) or chloroform-methanol-ammonium hydroxide-water (60:35:0.5:8, v/v) (31). These solvent systems were also used for the one-dimensional separations in the experiments shown in Figures 1 and 2. Lipid standards were included. For two-dimensional separations, the second solvent was chloroform-methanol-acetone-acetic acid-water (100:20:40:20:10, v/v) (31, 38). The air-dried plates were stained with iodine vapor. Phospholipid-containing spots were removed by scraping and transferred to tubes for determination of lipid phosphorous as described (36).

RESULTS

Soybean membranes isolated by methods used routinely in our laboratory (40) vary considerably in their phospholipid composition (Table I). Especially conspicuous is the variable content of PA.

Freshly isolated membranes were incubated under conditions employed for investigations of the mechanism of action of auxin. A buffer with an organic anion (acetic acid) and low pH (5.5) was chosen as being optimal for auxin binding (34). This pH value was also optimal for phospholipase D activity (7, 18). A rapid degradation of phospholipids, especially PC and PE, was observed in a self-digestion experiment carried out under these conditions (Fig. 1).

The concomitant increase in the phosphatidic acid containing fractions of silica gel matches the decrease in intact phospholipids only in membrane fractions C and D. Thus, a second activity is suggested to be present in addition to that of phospholipase D. To test this possibility, we monitored phosphatidic acid phosphatase which has been described to be most active in the ER but is active also in other membrane compartments (26). Table II shows that phosphatidic acid phosphatase activity is mainly located in our fractions A and B to account for the additional degradative changes in these fractions.

Studies were designed to generate procedures to prevent self-digestion. EDTA or EGTA was already present in the isolation media. Even though phospholipase D has been described to be

FIG. 1. Phospholipid degradation of membrane fractions from soybean hypocotyls. Membranes were isolated in unmodified coconut milk-sucrose medium (13, 40). Each membrane fraction was assayed individually in 50 mm Na-acetate (pH 5.5) without additions. A: mixed fraction of mostly smooth membranes; B: Golgi apparatus and plasma membrane; C: plasma membrane, mitochondria, and tonoplast; D: mitochondria, plasma membrane and tonoplast. Predominant organelles in each fraction are named first. For example, fraction C is about 50% plasma membrane, while fraction D is >80% mitochondria. Values are for 100-g hypocotyls.
Ca$^{2+}$-dependent (6, 18, 20) neither EDTA nor EGTA inhibited the phospholipase D activity sufficiently to be useful alone as a membrane protective agent (Table III).

Free bases such as choline and ethanolamine which are constituents of phospholipids have been described as inhibitors of phospholipase D (8, 42). For reasons discussed below, the combined effect of 4% choline and 4% ethanolamine was therefore tested. This mixture kept the phospholipid composition of the membranes stable at 20°C for at least 1 hr (Fig. 2 and Table IV).

Media prepared in coconut milk as solvent preserve the morphology of plant dictysomes (28, 29) so that coconut milk might influence phospholipid degradation. The results of a comparison of the action of coconut milk, glycerol-1-P, and choline plus ethanolamine singly and combined are shown in Table IV. The comparison with the control and with choline and ethanolamine alone shows that the effects of glycerol-1-P and coconut milk are similar. Both increase phospholipid degradation but appear to inhibit degradation of PA.

The combination of choline, ethanolamine, coconut milk, and glycerol-1-P was most effective. The phospholipid composition of a membrane preparation obtained using an isolation medium and gradient containing coconut milk, 10 mM glycerol-1-P, 20 mM EGTA, 4% choline, and 4% ethanolamine is shown in Table V. We obtained very similar results with membranes isolated in media and gradients without coconut milk containing 20 mM EGTA, 4% choline, 4% ethanolamine, and 0.15 mM nupercaine (ref. 37 and unpublished data).

**DISCUSSION**

Preparations of plant membranes are altered by enzymes which degrade phospholipids and other constituents (12, 21). Three major phospholipid-degrading enzymes have been identified as membrane-associated: phospholipase D, phospholipase A, and phosphatidic acid phosphatase.

Phospholipase D has been obtained as a soluble enzyme (7, 16, 18) and from organelle-rich fractions (19, 20); the enzyme from peanut seeds is found in a high speed supernatant (16). Carefully isolated chloroplasts and plant mitochondria had no phospholipase D activity (4). These same fractions prepared by differential centrifugation showed contamination by phospholipase D (4). Other studies show an association of this enzyme with microsomes (2, 4, 17, 22, 31) which is demonstrated either by high PA content of membranes or by direct assay. We find phospholipase D activity in all fractions which might indicate that the enzyme is not associated with any single compartment (Table II).

Phospholipase D usually has been described to be dependent on Ca$^{2+}$ or other activators in the assay (6, 20). We find that the addition of EDTA or EGTA is not sufficient to inhibit the enzyme action (Table III). Heller and Arad (16), and Kates (20) found that desaturated phospholipids are slowly converted to PA without activating agents. In soybean phospholipids, linoleic and linolenic acid are the prevalent fatty acids (14, 32, 41). Our data support the suggestion that highly unsaturated fatty acids can replace the requirement for other activators.

Protection of membranes against phospholipase D is difficult. General enzyme poisons might reduce the value of the preparations for further experimentation. The enzyme catalyzed two types of reactions, a hydrolyase activity of the general type PX → PA + X or a transferase activity of the type PX + Y → PY + X, and Y being alcohols like choline or ethanolamine (8, 42). The possibility of either or both enzyme activities must be considered. The relative velocity of both the hydrolyase and the transferase activity of the enzyme decreases in the order PC > PE > PG > PI (16,
The transferase activity of the enzyme (8, 42) is inhibited by 4% ethanolamine but only by about 50% (42). Addition of choline alone would favor conversion of PE to PC. Addition of ethanolamine alone would favor conversion to PC to PE. We applied a mixture of choline and ethanolamine both of which inhibit the hydrolase activity and also might “balance” a residual transferase activity. Both the hydrolase and transferase activities are much slower toward other phospholipids (16, 42). Isolation of aleurone grains has been achieved in glycerol but not in sucrose media (17).

Glycerol at high concentrations is an effective inhibitor of phospholipase D (8).

Phospholipase A is considered to be associated predominantly with mitochondria in plants (27). Activity was also observed in nuclear envelope (31) and is indicated in other microsomal fractions by high lysophospholipid contents (22, 39). Our data indicate a low activity in fractions C and D (Tables I and V). These fractions contain all of the mitochondria present on our gradients (13). Phospholipase A is also observed in other fractions during incubation at 20 C (compare Table IV). Phospholipase A is inhibited by nupercaine and other anesthetics (27, 37).

Phosphatic acid phosphatase has been reported to be located predominantly in ER but may also be found in all other fractions (26). It is usually not discussed as a potential contributor to misleading phospholipid data of plant membranes although a sequential action of phospholipase D and phosphatic acid phosphatase is well known (19). With both enzymes present, compositions of membranes isolated without protection against degradation reflect the balanced action of both enzymes (compare experiment 1 with experiment 2 in Table I and also Table V). This makes an interpretation of the validity of phospholipid determinations for plant membranes especially dangerous because even a low PA content does not necessarily indicate low degradation of phospholipids. A low PA content may also result from PA degradation by phosphatic acid phosphatase.

The inhibitions of phosphatic acid phosphatase and phospholipase A by glycerol-1-P may be analogous (30). Coconut milk inhibited phosphatidic acid phosphatase (Table IV). We do not know what compound of the coconut milk inhibits this enzyme. The prevention of the total breakdown of the phospholipid backbone could contribute to the structural preservation of plant dictyosomes isolated in coconut milk media (28, 29).

Phospholipase C seems not to be typical for plant membranes (12). This activity may be so low that it was masked by the sequential action of phospholipase D and phosphatic acid phosphatase.

As discussed above, evaluation of phospholipid compositions of plant membranes must be made with caution. High contents of PA and lysophospholipids are evidence of lipolytic activities. Equal amounts of PC and PE in plant membranes (or PE > PC) could originate from the preferred degradation of PC by phospholipase D (1, 16, 22, 35). Low amounts of PA might not truly indicate the absence of phospholipase D but could result from the combined actions of phosphatic acid phosphatase and phospholipase D (see for instance, ref. 1). The phospholipid composition of our membrane fractions isolated under protected conditions (Table V) agrees well with data from rat liver and other animal sources (5, 23, 24) and the castor bean endosperm system where

Fig. 2. Protection of phospholipids in membrane fractions from soybean hypocotyls. Membranes were isolated in coconut milk-sucrose medium with 4% choline, 4% ethanolamine, and 10 mM glycerol-1-P. Membrane fractions were incubated in 50 mM Na-acetate (pH 5.5) with 4% choline and 4% ethanolamine. A–D refer to corresponding gradient fractions. Values are for 100-g hypocotyls.

Table IV. Effectiveness of Protecting Agents Against Phospholipid Degradation

<table>
<thead>
<tr>
<th>Phospholipid</th>
<th>Acetate t=0 (no additive)</th>
<th>Acetate + 10 mM Glycerol 1-P</th>
<th>Acetate + 50% Coconut Milk</th>
<th>Acetate + 10 mM Glycerol 1-P</th>
<th>4% Choline + 4% Ethanolamine</th>
<th>4% Choline + 4% Ethanolamine</th>
</tr>
</thead>
<tbody>
<tr>
<td>PC</td>
<td>5.88</td>
<td>1.93</td>
<td>1.65</td>
<td>0.37</td>
<td>4.70</td>
<td>6.42</td>
</tr>
<tr>
<td>PE</td>
<td>4.32</td>
<td>1.67</td>
<td>1.08</td>
<td>0.52</td>
<td>4.64</td>
<td>3.52</td>
</tr>
<tr>
<td>PI</td>
<td>1.43</td>
<td>0.50</td>
<td>0.42</td>
<td>0.07</td>
<td>1.19</td>
<td>1.58</td>
</tr>
<tr>
<td>PG</td>
<td>1.28</td>
<td>0.44</td>
<td>0.39</td>
<td>n.d.</td>
<td>0.77</td>
<td>0.96</td>
</tr>
<tr>
<td>PA</td>
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<td>3.09</td>
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</tr>
<tr>
<td>LPC + LPE</td>
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<td>n.d.</td>
<td>n.d.</td>
<td>0.43</td>
<td>0.69</td>
</tr>
<tr>
<td>Other</td>
<td>Trace</td>
<td>1.11</td>
<td>1.23</td>
<td>0.72</td>
<td>0.43</td>
<td>Trace</td>
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<tr>
<td>Total</td>
<td>14.46</td>
<td>9.17</td>
<td>9.45</td>
<td>10.94</td>
<td>15.19</td>
<td>15.12</td>
</tr>
</tbody>
</table>

*Table* III. Effectiveness of Protecting Agents Against Phospholipid Degradation

The use of stachydrine (stachydrine) as a protecting agent was described in the text (see also ref. 30). The data indicate that glycerol-1-P is not an effective protector against degradation by phospholipase D.

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such phospholipid degradation is not encountered (9, 10). Even under protective conditions, some phospholipid degradation may occur in individual experiments, especially during the pelleting step. Centrifugation of the collected membrane fractions against a sucrose cushion as an alternative procedure is not always practical.

Because our fractions are enriched only in certain components and not as pure as animal membrane preparations, it is premature to assign phospholipid compositions to specific membrane compartments (23). Only the influence of mitochondria in fractions C and D is evident from the increase in proportions of PE and to a lesser extent of PI (1, 11, 27). The level of PA is still relatively high in all fractions. Depending on the tissue used for membrane isolation it might not be possible to overcome completely the very rapid initial degradation of phospholipids (35) during the disruption of cells. Very probably, the in vivo membrane has a low PA content. PS is present only in trace amounts and cannot be separated easily from the large amounts of PC present which tend to streak into the region of the thin layer plates where PS migrates. It seems most likely that except for the low PS content and the absence of sphingomyelin, compositions of plant membranes are very similar to those of animal membranes (see refs. 9, 10, and 31). Major deviations previously reported in our work and that of others may be the result of membrane degradation.

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