Modulation in the Activity of Purified Tonoplast H⁺-ATPase by Tonoplast Glycolipids Prepared from Cultured Rice (Oryza sativa L. var. Boro) Cells

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Glycolipids, phospholipids, and neutral lipids were extracted from the tonoplast fraction of cultured rice cells (Oryza sativa L. var. Boro). Acyl steryl glucoside (ASG) and glucocerebroside (GlcCer) were also prepared from this fraction. We determined the effects of these tonoplast lipids on the activity of H⁺-ATPase which was delipidated and purified from the tonoplast fraction. Exogenously added tonoplast phospholipids stimulated the activity of purified tonoplast H⁺-ATPase, but tonoplast glycolipids did not. When tonoplast glycolipids or tonoplast ASG was added in the presence of tonoplast phospholipids, they decreased the phospholipid-induced activation of the tonoplast H⁺-ATPase; tonoplast GlcCer only caused a small decrease. Steryl glucoside (SG) did not cause any decrease in this activation. Phospholipids, ASG, and GlcCer made up 35 mol%, 20 mol% and 7 mol% of the total lipids of the tonoplast fraction of cultured rice cells, respectively, and these glycolipid levels were enough to depress the phospholipid-induced activation of the tonoplast H⁺-ATPase. These results revealed that H⁺-ATPase activity in the tonoplast may be modulated toward activation and depression by tonoplast phospholipids and glycolipids, respectively. The acylation of SG would be responsible for the depression in the phospholipid-induced H⁺-ATPase activity.

Key words: Acyl steryl glucoside — Glucocerebroside — Oryza sativa L.— Tonoplast glycolipids — Tonoplast H⁺-ATPase (EC 3.6.3.14) — Tonoplast phospholipids.

Abbreviations: ASG, acyl steryl glucoside; DGDG, digalactosyl diacylglycerol; DTT, dithiothreitol; EGTA, ethyleneglycol-bis([1-aminoethylen]-N,N,N',N'-tetraacetic acid; GalCer, galactocerebroside; GlcCer, glucocerebroside; MGDG, monogalactosyl diacylglycerol; MOPS, 3-(N-morpholino)-propanesulfonic acid; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine; SG, steryl glucosides.

Introduction

Tonoplast H⁺-ATPase is known to require phospholipids to induce its activity (Manolson et al. 1985, Randall and Sze 1986, Kasamo 1988a, Matsuura-Endo et al. 1990, Yamanishi and Kasamo 1992). When this enzyme was delipidated and purified by anion-exchange chromatography, its activity almost disappeared, but this activity was restored by the exogenous addition of phospholipids. The activation of the tonoplast H⁺-ATPase was extensively studied using various classes of phospholipids (Yamanishi and Kasamo 1993), but glycolipids have been studied less extensively (Yamanishi and Kasamo 1994). All of these experiments were carried out using commercially available lipids, not lipids extracted from the plant materials used in the experiments. So far, there are no reports investigating the effect of lipids extracted from the tonoplast on the activity of purified tonoplast H⁺-ATPase.


Kasamo et al. (2000) showed that glycolipids prepared from chill-treated cells depressed proton pumping across the proteoliposomes with tonoplast H⁺-ATPase. These results imply that glycolipids may depress the H⁺-ATPase activity in the tonoplast.

In this study, we extracted phospholipids and individual glycolipids from the tonoplast fraction of cultured rice cells and examined the effects of these individual glycolipids on phospholipid-induced activation of highly delipidated tonoplast H⁺-ATPase from cultured rice cells. We further discussed the mechanism of the modulation of the activity of tonoplast H⁺-ATPase by glycolipids in the tonoplast.

Materials and Methods

Plant material

Suspension cultured cells of rice (Oryza sativa L. var. Boro) were prepared as previously described (Kasamo 1988b).

Chemicals

ATP was obtained from Boehringer Mannheim (Penzberg, Germany) as the disodium salt and converted to the Tris salt by ion exchange chromatography through a Dowex 50W H⁺ form (Muromachi Kagaku Kogyo, Ltd., Tokyo, Japan). Dextran T-70 was purchased from Pharmacia (Uppsala, Sweden). Lipids were obtained from the following sources: ASG and SG (soybean) from Larodan

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Fine Chemicals (Malmo, Sweden), and GalCer (bovine brain) and GlcCer (human spleen) from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Asolectin (soybean) from Nihon Pharm Co. (Tokyo, Japan) was partially purified and stored in a mixture of chloroform–methanol (3 : 1, v/v) at −20°C as described by Kagawa and Racker (1971). All the other chemicals were of analytical grade.

Preparation of tonoplast fraction

The tonoplast fraction for the lipid extraction was prepared by sorbitol–sucrose step gradient centrifugation as previously described (Suzuki and Kasamo 1993) with some modifications. In brief, cells cultured for a total of 9 d at 25°C were homogenized with a grinding medium containing 0.25 M sorbitol, 50 mM MOPS-KOH (pH 7.6), 5 mM EGTA, 1 mM phenylmethylsulfonylfluoride (PMSF), 2.5 mM potassium metabisulfite, 1.5% polyvinylpyrrolidone, 0.1% bovine serum albumin, and 2 mM DTT. The homogenate was centrifuged at 3,600 g for 10 min. The supernatant was centrifuged at 156,000 g for 20 min. The precipitate was suspended in 15 ml of 0.25 M sorbitol solution containing 5 mM MOPS-KOH (pH 7.3), 1 mM EGTA, and 2 mM DTT. The suspension was layered over 15 ml of 10% (w/w) sucrose solution containing 5 mM MOPS-KOH (pH 7.3), 1 mM EGTA, and 2 mM DTT. After centrifugation at 89,000 g for 2 h, the interface between the sucrose and sorbitol solution was collected and diluted 5-fold with suspension buffer containing 0.25 M sorbitol, 10 mM MES-Tris (pH 7.3), and 2 mM DTT. The suspension was centrifuged at 156,000 g for 20 min, and the pellet (tonoplast fraction) was suspended in the suspension buffer. As an alternative method, the tonoplast fraction for purification of H+-ATPase was prepared by de- tran gradient centrifugation as previously described (Kasamo et al. 1991).

Solubilization and purification of the tonoplast H+-ATPase

The tonoplast H+-ATPase was solubilized and purified as previously described (Yamanishi and Kasamo 1993). In brief, a solution of 10% (w/v) Triton X-100 and 0.2 M KCl in a suspension buffer containing 0.25 M sorbitol, 10 mM MES-Tris (pH 7.3), and 2 mM DTT was added dropwise to the tonoplast fraction with stirring on ice. The final concentrations of Triton X-100 and KCl were 5% (w/v) and 0.1 M, respectively, and the protein was approximately 2 mg ml⁻¹. After an additional 30 min of stirring on ice, the suspensions were centrifuged at 150,000 g for 30 min. The resulting pellet was suspended in buffer A containing 20 mM Tris-acetate (pH 7.5), 20% glycerol (w/v), 1 mM EGTA, 1 mM DTT and 2 mM MgCl₂ at a protein concentration of 2 mg ml⁻¹. An equal volume of 4% lysophosphatidylcholine in buffer A was added dropwise to the suspension. The mixture was stirred for 10 min at room temperature and centrifuged at 150,000 g for 40 min at 8°C. The supernatant was applied to a Mono Q ion-exchange FPLC column (Pharmacia, Uppsala, Sweden) that had been pre-equilibrated with running buffer (buffer A containing 0.1 M, respectively, and the protein was approximately 2 mg ml⁻¹). After an additional 30 min of stirring on ice, the suspension was centrifuged at 150,000 g for 30 min. The resulting pellet was suspended in buffer A containing 20 mM Tris-acetate (pH 7.5), 20% glycerol (w/v), 1 mM EGTA, 1 mM DTT and 2 mM MgCl₂ at a protein concentration of 2 mg ml⁻¹. An equal volume of 4% lysophosphatidylcholine in buffer A was added dropwise to the suspension. The mixture was stirred for 10 min at room temperature and centrifuged at 150,000 g for 40 min at 8°C. The supernatant was applied to a Mono Q ion-exchange FPLC column (Pharmacia, Uppsala, Sweden) that had been pre-equilibrated with running buffer (buffer A containing 0.1% (w/v) Triton X-100). The sample was eluted with a stepwise gradient of NaCl as described previously (Yamanishi and Kasamo 1993). The tonoplast H+-ATPase was eluted between 0.20 and 0.25 M NaCl. The fractions with specific activities above 2.0 mol Pi (mg protein)⁻¹ min⁻¹ were pooled and used as purified tonoplast H+-ATPase, unless otherwise stated. A 10 μl aliquot of the ATPase fraction was assayed for ATPase activity. The ATPase purified by this method was almost free from phospholipids (Yamanishi and Kasamo 1993).

Lipid extraction and separation by silica Sep-Pak cartridges

Total lipids were extracted from the tonoplast fraction by the method of Brown and DuPont (1989). In brief, isopropanol (2.12 ml) and chloroform (0.6 ml) were mixed with 0.8 ml of tonoplast fraction. Insoluble proteins were sedimented by centrifugation at 1,000 g for 3 min, and the supernatant was decanted. Chloroform (3.66 ml) and 0.1 M KCl (0.8 ml) were added to the supernatant. After thorough mixing, the two phases were separated by centrifugation. The lower phase was washed three times with 1.5 ml aliquots of 0.1 M KCl saturated with chloroform. The interface and the upper phase were removed and discarded. The lower phase was dried under a stream of nitrogen gas, and the lipids were dissolved in 0.5 ml chloroform. The lipid extract was stored at −80°C.

The lipid extract of the tonoplast fraction was partitioned into the neutral lipids, glycolipid G₁, glycolipid G₂, and total phospholipids successively on silica Sep-Pak cartridges (Millipore, Milford, MA, U.S.A.) according to Sandstorm and Cleland (1989) with slight modifications. In brief, the total lipid extract of the tonoplast fraction was concentrated under a stream of nitrogen gas and brought to a volume of 2 ml with chloroform : acetic acid (100 : 1, v/v). The sample was transferred to a Sep-Pak cartridge attached to a 10-ml glass syringe. After the lipid sample entered the cartridge, 2 ml of chloroform : acetic acid (100 : 1, v/v) were used to wash the residual lipid from the original container. The total neutral lipid was eluted first with 10 ml of chloroform : acetic acid (100 : 1, v/v). The glycolipids (mainly containing ASG, SG and GlcCer) were eluted next with 35 ml of chloroform : acetone : water (4 : 6, v/v) and 10 ml of chloroform : acetone (3 : 7, v/v) (ASG and SG were eluted with 10 ml of chloroform : acetone (4 : 6, v/v)). GlcCer was eluted with 25 ml of chloroform : acetone (4 : 6, v/v) and 10 ml of chloroform : acetone (3 : 7, v/v). This fraction was designated the glycolipid G₁ fraction and was used as the tonoplast glycolipids. Glycolipids (DGDG, as major component) were eluted, followed by 8 ml of acetone and successive 20 ml of acetone : acetic acid (100 : 1, v/v). This fraction was designated the glycolipid G₂ fraction. The total phospholipid was eluted last with 10 ml of methanol : chloroform : water (100 : 50 : 40 by volume) and recovered by effecting a phase separation with 1 ml of chloroform and 2 ml of water and transferring the chloroform phase. This fraction contained phospholipids as the major component. This fraction was used as the tonoplast phospholipids.

TLC separation of each lipid class and quantification

Individual lipids were separated by TLC (silica gel 60, 0.25-mm layer thickness, Merck Darmstadt, Germany). TLC plates were prerun with chloroform : methanol (2 : 1, v/v), dried at room temperature, and activated at 130°C for 1 h. The glycolipid G₁ and glycolipid G₂ were separated by TLC with a solvent mixture of chloroform : methanol (85 : 15, v/v) and chloroform : acetone : methanol : acetic acid : water (100 : 40 : 20 : 30 : 10 by volume), respectively. The phospholipids were separated on two-dimensional TLC with solvent mixtures of acetonitrile : benzene : methanol : water (80 : 30 : 20 : 10 by volume) in the first dimension and chloroform : acetone : methanol : acetic acid : water (100 : 40 : 20 : 30 : 10 by volume) in the second. Lipids were identified by cochromatography with standards and the use of specific reagents; molybdenum blue reagent for phosphorus, ninydrin for free amino groups, and α-naphtol or anthrone for the detection of glycolipids. Glycolipids were quantified by the method of Rousier and Batt (1968) after hydrolyzation in 1 M H₂SO₄ at 100°C for 1 h. Galactose was used as the standard for DGDG and MGDG quantification and glucose for ASG, SG and GlcCer. The sterol contents of ASG and SG were also quantified by the method of Zlatkis and Zak (1969) using cholesterol as a standard. The contents of ASG and SG assayed by these two methods were in good agreement. The fatty acid content of DGDG was also quantified by gas liquid chromatography according to Hayashi et al. (1992). The contents of DGDG assayed by these two methods were in good agreement. Phospholipids were quantified according to Rouser et al. (1970).
H+-ATPase modulation by tonoplast lipids

Purification of ASG and GlcCer

ASG and GlcCer were purified using silica Sep-Pak cartridges and successive TLC. The lipid extract of the tonoplast fraction was transferred to a Sep-Pak cartridge as described in lipid separation. After dialysis of neutral lipids, ASG was eluted with 10 ml of chloroform:acetone (9 : 1, v/v) and 10 ml of chloroform:acetone (8 : 2, v/v). After SG was eluted with 20 ml of chloroform:acetone (6 : 4, v/v), GlcCer was eluted with 20 ml of chloroform:acetone (4 : 6, v/v). The ASG and GlcCer fractions were further purified by TLC. The ASG fraction was separated by TLC developed with chloroform:methanol (95 : 10, v/v) as the mobile phase. The GlcCer fraction was separated by TLC developed with chloroform:methanol (85 : 10, v/v) as the mobile phase. The purity of each individual glycolipid was evaluated by TLC as a single spot.

Assay of ATPase activity in the presence of lipids

The lipid was dried under a gentle stream of nitrogen gas and suspended in distilled water; the suspension was sonicated in a bath sonicator (Taitec, Inc., Saitama, Japan) to near clarity prior to use. In the assays of the glycolipid in the presence of phospholipid, the glycolipid and phospholipid in organic solvent were combined at the indicated ratio. The lipid suspension was added to the reaction mixture to give the indicated amounts of lipids. The ATPase activity was measured at 38°C as previously described (Kasamo 1986). The reaction was initiated by the addition of purified ATPase to a 0.5 ml reaction mixture that contained 3 mM Tris-ATP, 3 mM MgSO4, 50 mM KCl, 1 mM ammonium molybdate, and 30 mM MES-Tris (pH 7.5) with or without lipids at various concentrations and terminated after 30 min by the addition of 0.1 ml 50% (w/w) TCA. Phosphate released from ATP was determined as previously described (Kasamo 1979).

Quantification of protein

Proteins were quantified by the method of Lowry et al. as modified by Peterson (1977) using bovine serum albumin as the standard.

Results

Lipid composition of tonoplast fraction

The lipid composition of the tonoplast fraction prepared from O. sativa L. cells is listed in Table 1. One of the major glycolipids in the tonoplast fraction of cultured rice cells is ASG, which accounted for 20 mol% of the total lipids. ASG is a prominent glycolipid of the tonoplast in some plants (Haschke et al. 1990, Tavernier et al. 1993). GlcCer is also a major glycolipid in the tonoplast fraction amounting to 7 mol% of the total. The content of SG is small, 1.3 mol% of the total. Free sterol accounted for 6 mol% of the total. DGDG, which is abundant in this tonoplast fraction, is known to be a glycolipid characteristic of the envelope and thylakoid membrane of plastids (discussed later). Therefore, we excluded this lipid from other glycolipids.

Table 1 Lipid composition of the tonoplast fraction from cultured rice cells

<table>
<thead>
<tr>
<th>Lipid class</th>
<th>Lipid content (nmol (mg protein)–1)</th>
<th>Glycolipid : Phospholipid (mol : mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phospholipid</td>
<td>0.523</td>
<td>38.0</td>
</tr>
<tr>
<td>GlcCer a</td>
<td>0.097</td>
<td>7.0</td>
</tr>
<tr>
<td>ASG a,b</td>
<td>0.276</td>
<td>20.0</td>
</tr>
<tr>
<td>SG a,b</td>
<td>0.018</td>
<td>1.3</td>
</tr>
<tr>
<td>MGDG</td>
<td>trace</td>
<td>trace</td>
</tr>
<tr>
<td>DGDG</td>
<td>0.429</td>
<td>31.1</td>
</tr>
<tr>
<td>Free sterols b</td>
<td>0.083</td>
<td>6.0</td>
</tr>
<tr>
<td>Total</td>
<td>1.378</td>
<td>100</td>
</tr>
</tbody>
</table>

The results show the representative data of the two independent experiments; two assays were run for each experiment. Errors of analysis were within 15% of the values indicated.

a (GlcCer + ASG + SG) : phospholipid = 43 : 57.
b Sterol distribution (mol% of total sterols): SG (4.8), ASG (73.2), Free sterols (22.0).

Lipid separation

Fig. 1 shows the thin layer chromatogram of the lipids separated by the silica Sep-Pak cartridges. The neutral lipids contained free sterols (FS) as major components and unidentified neutral lipids (N), which may contain some amount of diacylglycerols, triacylglycerols, and free fatty acids (<0.1 nmol (μg protein)–1) (Fig. 1, lane 1). Because we did not determine the amount of these lipids completely, the data in Table 1 do not take into account these glicerides and free fatty acids in assessing the neutral lipids. The glycolipid fraction containing ASG, SG and MGDG (lane 2) and the glycolipid fraction containing GlcCer (lane 3) were free from DGDG but contained a negligible amount of MGDG (<0.016 nmol (μg protein)–1). These glycolipid fractions (lanes 2 and 3) were combined and used as the tonoplast glycolipid G1. The composition of this fraction was ASG (67.8 mol%), SG (4.4 mol%), GlcCer (23.8 mol%), and a small amount of MGDG (<4.0 mol%). DGDG is the major component of the glycolipid G2 fraction (lane 4). The phospholipid fraction (lane 5) contained a small amount of DGDG (0.008 nmol (μg protein)–1), which accounted for 1.6 mol% of the tonoplast phospholipid; this was negligible compared to the tonoplast phospholipid and glycolipid. There were unidentified lipids in lanes 2, 4 and 5 (Fig. 1). These were separated from identified lipids on two-dimen-
H+-ATPase modulation by tonoplast lipids

The phospholipids, glycolipid G1 and neutral lipids were used as tonoplast lipids in the following experiments.

Effects of various lipids extracted from the tonoplast fraction on the activity of purified tonoplast H+-ATPase

Table 2 shows the effects of exogeneously added tonoplast lipids and asolectin on the activity of the purified tonoplast H+-ATPase. Asolectin is a phospholipid mixture derived from soybean. Its composition was PC (26.7 mol%), PE (34.5 mol%), PI (20.9 mol%), PG (2.3 mol%), PA (14.2 mol%) and PS (1.3 mol%). The tonoplast phospholipids stimulated the activity of tonoplast H+-ATPase as well as asolectin, but tonoplast glycolipids and neutral lipids induced little or no stimulation.

Effects of tonoplast glycolipids on phospholipid-induced activation of purified tonoplast H+-ATPase

Fig. 2 shows the effects of various amounts of a mixture of tonoplast glycolipids and phospholipids on the purified tonoplast H+-ATPase. The ratios of tonoplast glycolipids to phospholipids and ASG to phospholipids were 43 : 57 (mol/mol) and 34 : 66 (mol/mol), respectively (see Table 1). The tonoplast H+-ATPase activity due to the glycolipid–phospholipid mixture was dependent on lipid amounts added. The phospholipid-induced activation of tonoplast H+-ATPase was markedly decreased by the addition of tonoplast glycolipids and tonoplast ASG (Fig. 2). In all cases, the H+-ATPase activity reached a plateau around 25 nmol of phospholipid/assay.

We also assayed the purified tonoplast H+-ATPase activity using commercially available lipids. Fig. 3 shows the effects

**Table 2** Effects of tonoplast lipids and asolectin on the activity of purified tonoplast H+-ATPase

<table>
<thead>
<tr>
<th>Lipid added</th>
<th>ATPase activity (μmol Pi (mg protein)⁻¹ min⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asolectin</td>
<td>4.13</td>
</tr>
<tr>
<td>Phospholipid</td>
<td>2.85</td>
</tr>
<tr>
<td>Glycolipid G₁</td>
<td>0.01</td>
</tr>
<tr>
<td>Neutral lipid</td>
<td>0.00</td>
</tr>
</tbody>
</table>

ATPase activity was assayed in a 0.5 ml reaction mixture as described in Materials and Methods. 25 nmol each of tonoplast lipid and asolectin were added to the mixture; the amount of neutral lipid was represented by the free sterol content. Reactions were started by addition of 10 μl (0.65 μg) of purified tonoplast H+-ATPase. The results show the representative data of the two independent experiments; three assays were run for each experiment.

**Fig. 1** Thin layer chromatogram of tonoplast lipids separated by the silica Sep-Pak cartridges. The solvent mixture was chloroform : acetone : methanol : acetic acid : water (100 : 40 : 20 : 30 : 10 by volume). Spots were developed by spraying with 30% H₂SO₄ and subsequent heating. Neutral lipid fraction (lane 1), glycolipid fraction containing ASG, SG, and MGDG (lane 2), glycolipid fraction containing GlcCer (lane 3), glycolipid fraction containing DGDG (lane 4), and phospholipids (lane 5) were separated by the silica Sep-Pak cartridges as described in Materials and Methods. Lane 2 and lane 3 were combined and designated glycolipid G₁. Lane 4 was designated glycolipid G₂. Spots in lanes 1 to 4 were identified according to the note on the left side of the chromatogram. Spots in lane 5 were identified according to the note on the right side. N, neutral lipids containing glycerides and free fatty acids; FS, free sterols; Arrow, unidentified lipids.

**Fig. 2** Effects of various amounts of lipid mixture containing tonoplast phospholipids and glycolipids on the activity of the purified tonoplast H+-ATPase. ATPase activity was assayed as described in Table 2. Various amounts of lipid mixture containing tonoplast phospholipids and glycolipids were added to the reaction mixture. The ratios were as follows; tonoplast phospholipids : tonoplast glycolipids = 57 : 43 (mol/mol), tonoplast phospholipids : tonoplast ASG = 66 : 34 (mol/mol) or 100 : 0 (mol/mol) as control. Reactions were started by addition of 10 μl (0.51 μg) of purified tonoplast H+-ATPase. Activity is expressed as a percentage of the activity in the presence of 25 nmol tonoplast phospholipids. Values are means of two independent experiments (two assays were run for each experiment), and the vertical bars represent SE.
H+-ATPase modulation by tonoplast lipids

of various amounts of the lipid mixture containing glycolipid and asolectin on the purified tonoplast H+-ATPase activity. The glycolipid and asolectin were combined in equal moles and used as the lipid mixture. The tonoplast H+-ATPase activity due to asolectin was dependent on lipid amounts added as well as the tonoplast phospholipids. Asolectin induced the activity of tonoplast H+-ATPase, which reached a plateau around 25 nmol/assay. Activity is expressed as a percentage of the activity in the presence of 25 nmol asolectin. Values are means of two independent experiments (two assays were run for each experiment), and the vertical bars represent SE.

In the next experiment, the tonoplast glycolipids were added to 25 nmol of phospholipid/assay to give the indicated glycolipid content (Fig. 4). Phospholipid-induced activation of the tonoplast H+-ATPase decreased with the increasing content of the tonoplast glycolipids, ASG, and GlcCer. The activities at 10 mol% and 50 mol% of the tonoplast glycolipids were 87% and 47% of those of the tonoplast phospholipids, respectively, and the activities at 10 mol% and 50 mol% of ASG were 90% and 55% of those of the tonoplast phospholipids, respectively. The depression of the phospholipid-induced activation of the tonoplast H+-ATPase increased with the increasing ratio of GlcCer but to a smaller extent than it did with the tonoplast glycolipids or ASG. The activities at 10 mol% and 50 mol% of the GlcCer were 96% and 85% of those of the tonoplast phospholipids, respectively.

We also assayed the purified tonoplast H+-ATPase activity using commercially available lipids to examine the effect of SG and to compare the effect of glycolipids of other origins. Almost the same effect was observed with the commercially available lipids with respect to ASG and GlcCer (Fig. 5). When SG or GalCer was mixed with asolectin, the tonoplast H+-ATPase activity was constant or slightly stimulated in the range between 0 and 50 mol%. When GlcCer was mixed with asolectin, the H+-ATPase activity was slightly stimulated at 10 mol% but declined in the range between 10 and 50 mol%, and the activity at 50 mol% was 84% of that of asolectin. In case of ASG, the tonoplast H+-ATPase activity linearly declined in the range between 0 and 50 mol%, and the activity at 50 mol% was 46% of that of asolectin.

The effects of ASG on the activity of tonoplast H+-ATPase in the assay containing 25 nmol lipid (25 nmol (ASG + asolectin)) or ASG with 25 nmol asolectin (ASG + 25 nmol asolectin) were also examined (data not shown). The depressive effect by ASG in the presence of 25 nmol asolectin/assay was almost the same as that by 25 nmol (ASG + asolectin)/assay.

Discussion

This is the first paper disclosing that glycolipid, especially ASG extracted from the tonoplast fraction, depresses the...
phospholipid-induced activation of purified tonoplast H⁺-ATPase.

The lipid composition of the tonoplast has been reported in some plant sources (Verhoek et al. 1983, Yoshida and Uemura 1986, Haschke et al. 1990, Tavernier et al. 1993). In the present study, the tonoplast phospholipid composition of cultured rice cells was essentially similar; however, the glycolipid composition slightly differed from these reports. An especially high amount of ASG (ca. 20 mol% of the total lipids) was characteristic of the tonoplast fraction (Table 1). The ASG content in the tonoplast differs among plant species (Yoshida and Uemura 1986, Haschke et al. 1990, Tavernier et al. 1993). Genetic variability in the ASG content has also been reported in the plasma membrane, and ASG is related to the cryostability of the plasma membrane (Palta et al. 1993, Uemura and Steponkus 1994).

We detected DGDG in the tonoplast fraction of cultured rice cells. DGDG is known to be the glycolipid characteristic of the envelope and thylakoid membrane of plastids. We tried several methods for preparing the tonoplast: a dextran-gradient system (Kasamo et al. 1991), a sucrose linear gradient system (Yoshida et al. 1986), a floating method described by Matsura-Endo et al. (1990), and a vacuole isolation described by Martinioa et al. (1981). We found DGDG in the tonoplast fraction prepared from cultured rice cells in every case. The tonoplast vesicles prepared from mung bean by the method described in this study contain only a small amount of DGDG as described by Yoshida and Uemura (1986) (data not shown). Thus, there does not seem to be any technical problem. Unfortunately, the presence of DGDG in the tonoplast itself is still unclear (Verhoek et al. 1983). So, we separated DGDG from other glycolipids in this study.

It is reported that purified plasma membrane ATPase binds exogenously added lipids (Dufour and Goffeau 1980), so a similar binding of purified tonoplast H⁺-ATPase with lipids is to be expected. Yamanishi and Kasamo (1993) showed that phospholipids in a liquid-crystalline phase are essential for the activation of tonoplast H⁺-ATPase. This can be explained by the hypothesis that the lipids interact with tonoplast H⁺-ATPase hydrophobically and that the phospholipids in a liquid-crystalline phase around the tonoplast H⁺-ATPase help the conformational change of the enzyme, possibly at a Vₒ-sector like the F₁Fₒ-ATPase c subunit (Rastogi and Girvin 1999). Tonoplast H⁺-ATPase may be incorporated into the lipid bilayer of exogenously added lipids. In any case, the lipids probably bind around the Vₒ sector of tonoplast H⁺-ATPase and interact with the subunits, causing the modulation of the rotation of the V₁ sector (Junge et al. 1997).

In this study, ASG depressed the phospholipid-induced activation of the tonoplast H⁺-ATPase, but commercially available SG did not. The content of SG in the tonoplast glycolipids is so small that we could not run experiments using SG extracted from the tonoplast fraction. The only difference in structure between ASG and SG is that 6-hydroxyl group of the glucose of the SG is acylated (Fig. 6). Therefore, the acylation of SG must be responsible for the decline in the tonoplast H⁺-ATPase activity. We suggest three possible mechanisms for the depression of the phospholipid-induced activation of the tonoplast H⁺-ATPase by ASG. First, ASG could change the lipid phase around the tonoplast H⁺-ATPase to a gel-like state, and the conformational change of the enzyme would be restricted by the gel-like state induced by ASG; as is often reported, the
acyl composition of ASG is mainly saturated fatty acids (Wojciechowski 1992). Secondly, the acyl chain of ASG might interfere with the interaction between the acyl chain of phospholipids and the tonoplast H^+-ATPase. The relationship between the acyl chain of ASG and tonoplast H^+-ATPase will differ from the interaction between phospholipid acyl group and H^+-ATPase because ASG has another hydrophobic region, the sterol group. Kasamo and Yamanishi (1997) reported that free fatty acids depress the asolectin-induced activation of tonoplast H^+-ATPase. Therefore, it is also possible that the acyl chain of ASG directly interferes with the interaction between the acyl chain of phospholipids and tonoplast H^+-ATPase. Finally, the inverted hexagonal phase (or an inverted hexagonal-like structure) might inhibit the interaction between the phospholipid and H^+-ATPase, because ASG has the propensity to form the inverted hexagonal phase when it is mixed with phospholipids (Webb et al. 1995). In any case, the acylation itself and the physicochemical change derived from the acylation of SG must be responsible for the decline in the tonoplast H^+-ATPase activity.

We reported that the glycolipid from tonoplast of chill-treated cells decreased the proton pumping across the proteoliposomes with tonoplast H^+-ATPase (Kasamo et al. 2000). Kasamo et al. (2000) also showed that phospholipid liposomes containing GlcCer decrease the fluidity of the surface region of the lipid bilayer of the membrane and that the H^+-ATPase activity in the proteoliposomes is related to the fluidity of the surface region of the membrane. Thus, the effect of ASG on membrane fluidity of the surface region might be a cause of depression of the phospholipid-induced activation of the tonoplast H^+-ATPase. The examination of the relation between ASG and membrane fluidity of the surface region is now in progress.

In this study, the amounts of tonoplast glycolipids, phospholipids, ASG, and GlcCer in the tonoplast fraction of cultured rice cells were estimated. The ratio of GlcCer to phospholipid was 16 : 84 (mol/mol), that of ASG to phospholipids was 34 : 66 (mol/mol), and that of tonoplast glycolipids to phospholipids was 43 : 57 (mol/mol) (Table 1). These ratios are sufficient to depress the phospholipid-induced activation of tonoplast H^+-ATPase (Fig. 2, 4). Thus, tonoplast glycolipids and ASG are effective in vivo in decreasing the activation of tonoplast H^+-ATPase. As shown in this study, phospholipids activate the tonoplast H^+-ATPase, ASG depresses its activation, and they coexist in the tonoplast in an effective ratio. Thus, phospholipids and ASG might be expected to modulate the ATPase activity in the tonoplast. It has also been reported that the ratio of the ASG to phospholipids is 20 : 80 (mol/mol) and 22 : 78 (mol/mol) in the tonoplast of Acer pseudoplatanus cultured cells (Tavernier et al. 1993) and Kalanchoe (Haschke et al. 1990), respectively. In these plants, the ASG content will also be sufficient to depress the tonoplast H^+-ATPase activity.

ASG is synthesized from SG and the contribution of SG to the formation of GlcCer was recently revealed (Lynch et al. 1997). Since the turn-over of the glucosyl residue in SG and of the fatty acid ester of ASG are reported to be rapid (Wojciechowski et al. 1976), Axelos and Peaud-Lenoel (1982) postulated that SG and ASG might act as modulators of membrane proteins. From these results, we postulate that these glycolipids modulate the activity of the tonoplast H^+-ATPase by maintaining their ratios in the tonoplast, which is caused by acylation of SG, deacylation of ASG and GlcCer formation from glucose of SG.

The plant GlcCer has mainly hydroxyl fatty acids (Cahoon and Lynch 1990, Norberg et al. 1996). The effect of tonoplast GlcCer on the activity of tonoplast H^+-ATPase was almost the same as the effect of commercially available GlcCer, which has exclusively non-hydroxyl fatty acids (Fig. 4, 5). Thus, the activity of tonoplast H^+-ATPase seems not to be affected by the hydroxyl group of the GlcCer acyl chain. In the GlcCer–phospholipid mixture, a liquid-crystalline and gel phase should coexist under our experimental conditions (38°C) regardless of the GlcCer fatty acid hydroxylation (Freire et al. 1980, Curatolo et al. 1986, Norberg et al. 1996). Thus, no differences in the effect of tonoplast H^+-ATPase by GlcCer with or without hydroxyl fatty acids on phospholipid-induced activation should be observed.

In summary, the tonoplast glycolipids decreased the activation of tonoplast H^+-ATPase induced by the tonoplast phospholipids. Tonoplast H^+-ATPase activity may be modulated toward activation and depression by tonoplast phospholipids and glycolipids, respectively. The acylation of SG may depress the phospholipid-induced activation of the tonoplast H^+-ATPase.

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References


