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Mechanism for the Activation of Plasma Membrane H^+-ATPase from Rice (*Oryza sativa* L.) Culture Cells by Molecular Species of a Phospholipid[1]

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ABSTRACT

The activation of H^+-ATPase solubilized from plasma membrane of rice (*Oryza sativa* L. var Nipponbare) culture cells was examined by the exogenous addition of various phospholipids, free fatty acids, glycerides, polar head groups of phospholipids and molecular species of phosphatidylcholine (PC). H^+-ATPase activity appeared to be stimulated by phospholipids in the following order: asolectin > phosphatidylserine > PC > lysophosphatidylcholine > phosphatidylglycerol, and maximal ATPase activation was noted at around 0.05 to 0.03% (w/v) of asolectin or molecular species of PC. Polar head groups such as glycerol, inositol, and serine only slightly activated ATPase activity or not at all, while ethanolamine and choline had no effect. Activation was dependent on the degree of saturation or unsaturation of the fatty acyl chain and its length. The activity decreased with increase in the length of fatty acyl chain from dimyristoryl(14:0)-PC to distearoyl(18:0)-PC and the degree of unsaturation from dioleoyl(18:1)-PC to dilinolenoyl(18:3)-PC. Maximum activation was observed when PC possessing 1-myristoyl(14:0)-2-oleoyl(18:1) or 1-oleoyl-2-myristoyl was added to the reaction mixture. These data show that the activation of plasma membrane H^+-ATPase by PC depends on a combination of saturated (myristic acid 14:0, palmitic acid 16:0, and stearic acid 18:0) and unsaturated (oleic acid 18:1, linoleic acid 18:2, and arachidonic acid 20:4) fatty acids at the *sn*-1 and *sn*-2 positions of the triglycerides.

The plasma membrane of plant cells contains a H^+-ATPase which produces a proton electrochemical gradient across the plasma membrane, thereby providing the driving force for transporting nutrients into cells (19, 20). Acidification stimulated by auxin may also cause the loosening required for cell enlargement, suggesting that the plasma membrane H^+-ATPase is involved in auxin action (13). The mechanism for regulation of the ATPase by auxin is unknown (17). In our previous paper (10) and other reports (2, 3, 18), plasma membrane H^+-ATPase was shown to require phospholipids for its activation, indicating that its activity may be regulated by interactions between this protein and the phospholipid environment in the plasma membrane. However, the effects of specific molecular species of each phospholipid have not yet been examined.

In the present study, we first examined the effects of adding phospholipids, and their constituents, *i.e.* polar head groups, glycerides and free fatty acids, on H^+-ATPase activity. Secondly, the effects of different molecular species of PC[2] on ATPase activity were examined for potential clues as to the mechanism of its activation.

MATERIALS AND METHODS

Plant Materials

Culture cells from rice (*Oryza sativa* L. var Nipponbare) were prepared as described previously (8).

Plasma Membrane Preparation

Plasma membranes were prepared from a microsomal fraction (10,000–80,000g pellet) using a dextran step gradient consisting of 8% (w/w) T-70 (mol wt 708,000) and 1% (w/w) dextran T-70, and then a sucrose density gradient of 30% and 42.9% as described previously (9).

Solubilization of Plasma Membrane H^+-ATPase

A two-step procedure using deoxycholate and zwittergent 3-14 was carried out to solubilize ATPase from the plasma membrane as described previously (7). The solubilized pellet was suspended in 1 mM DTT at 2 to 3 mg/mL and stored at −80°C until use.

ATPase Assay and Protein Determination

ATPase activity was measured at 38°C for 30 min using 2 to 10 μg of protein per assay (7). The reaction was carried out in a 0.5 mL reaction mixture containing 3 mM Tris-ATP (Boehringer), 3 mM $MgSO_4$, 50 mM KCl, 30 mM MES-Tris (pH 6.5), with or without 0.05% (w/v) asolectin (Associated Concentrates Inc.) or various concentrations of phospholipids. This reaction mixture contained 1 mM ammonium molybdate and 1 mM sodium azide to inhibit acid phosphatase

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[2] Abbreviations: PC, phosphatidylcholine; PS, phosphatidylserine; PI, phosphatidylinositol; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PA, phosphatidic acid; LPC, lysophosphatidylcholine.

Table I. *Effects of Phospholipids on the Activity of Plasma Membrane H^+-ATPase from Rice Culture Cells*

ATPase activity was assayed in the presence of 3 mM Tris-ATP, 3 mM $MgSO_4$, 50 mM KCl, 30 mM MES-Tris (pH 6.5), and 0.05% (w/v) phospholipid and expressed as percent of activity in the absence of added phospholipid.

Phospholipids Added	ATPase Activity
	μmol Pi/mg protein·min
None	0.404 (100)
Soybean phospholipid extract	0.606 (150.0)
Asolectin	0.820 (203.0)
LPC	0.504 (124.8)
PS	0.580 (143.6)
PI	0.340 (84.2)
PE	0.390 (96.5)
PC	0.544 (134.7)
PG	0.492 (121.8)
PA	0.264 (65.3)
Ergosterol	0.302 (74.8)

and mitochondrial ATPase activities. Phosphate released from ATP was determined by the method described previously (5). Protein content was determined by the method of Bradford (1), using BSA as the standard.

Lipid Preparations

All phospholipids and molecular species of PC were obtained from Avatipolar Lipids Inc., Sigma, or Serdary Research Laboratories (Ontario, Canada). Each lipid was dissolved in distilled water at 5% (w/v) and was sonicated in a bath sonicator (Kokusai Denki Co., Type UA-100, Tokyo) prior to use and added to the reaction mixture.

RESULTS

Effects of Phospholipids on Plasma Membrane H^+-ATPase Activity

Table I shows the effects of one concentration (0.05%, w/v) of various phospholipids on the activation of plasma membrane H^+-ATPase. Asolectin, a phospholipid mixture derived from soybean, caused the greatest activation. Activation of ATPase activity was dependent on the individual purified phospholipid exogenously added, in the following order: asolectin > PS > PC > PLC > PG. Activity tended to be inhibited by PI, PA, PE, and ergosterol. VO_4 inhibited phospholipid-stimulated ATPase activity more strongly than basal ATPase activity (data not shown).

The effects of various concentrations of asolectin, 1-palmitoyl(16:0) 2-oleoyl(18:1)-PC, glycerol, and stearic acid (18:0) on activation of ATPase activity were also examined (Fig. 1). Maximum activations were noted when either 0.05% (w/v) asolectin or 0.03% (w/v) 1-palmitoyl 2-oleoyl-PC was added. Neither glycerol nor stearic acid had any effect. Stearic acid somewhat inhibited the activity as its concentration was increased.

Effects of Free Fatty Acids, Glycerides, and Polar Head Groups of Phospholipid on Plasma Membrane H^+-ATPase Activity

A phospholipid is comprised of polar head groups, glycerides, and two fatty acyl chains. To determine which of these components is required for activation, the effects of free fatty acids on ATPase activity were first examined. ATPase activity was dependent on the length of the fatty acyl chain and was apparently inhibited with increasing acyl chain length, in proceeding from palmitic acid (16:0) to stearic acid (18:0) (Table II). It was further inhibited on increasing the degree of unsaturation from oleic acid (18:1) to linolenic acid (18:3) (Table II). Glycerol, diglycerol, and triglycerol only slightly activated ATPase activity or not at all. Polar head groups of phospholipid such as glycerol, inositol, and serine slightly activated ATPase activity, while ethanolamine and choline had no effect (Table III).

Effects of Molecular Species of PC on Plasma Membrane H^+-ATPase Activity

The effects of various molecular species of PC containing different fatty acyl chains were examined (Table IV). In the case of molecular species of PC possessing two saturated acyl chains, activity decreased with an increase in the number of

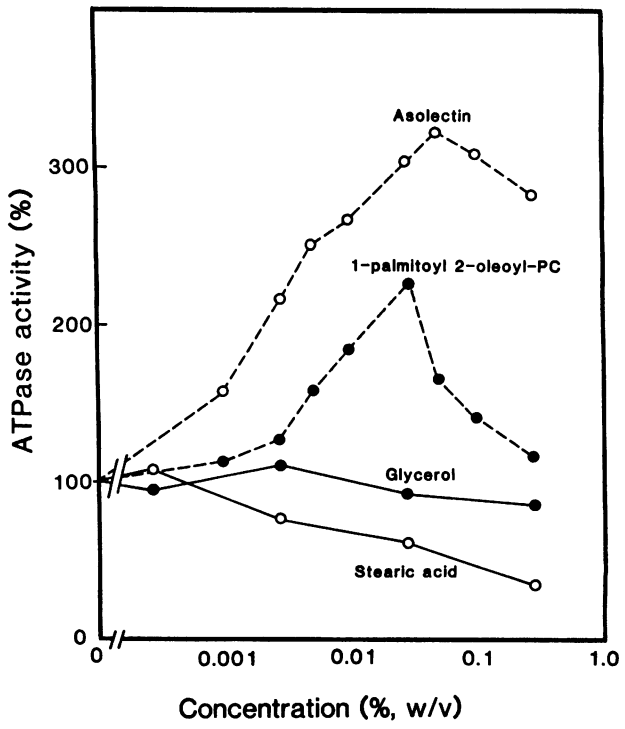

Figure 1. Stimulation of the activity of plasma membrane ATPases from rice culture cells by asolectin, 1-palmitoyl 2-oleoyl-PC, glycerol, and stearic acid. ATPase activity was assayed in the presence of 3 mM $MgSO_4$, 3 mM Tris-ATP, 50 mM KCl, 30 mM MES-Tris (pH 6.5), and various concentrations as indicated. Control activity without each phospholipid, glycerol, and fatty acid was 0.967 μmol Pi/mg protein·min.

Table II. *Effects of Fatty Acids on Activity of Plasma Membrane H+-ATPase from Rice Culture Cells*

Each acid (0.05%, w/v) was added to 4.7 μg of partially purified ATPase in a total volume of 0.5 mL. ATPase activity was assayed in the presence of 3 mM Tris-ATP, 3 mM $MgSO_4$, 50 mM KCl, and 30 mM MES-Tris (pH 6.5), and expressed as percent of activity in the absence of added phospholipid.

Fatty Acid Added	ATPase Activity
	μmol Pi/mg protein·min
None	0.506 (100)
16:0	0.496 (98.0)
18:0	0.426 (84.2)
18:1	0.308 (60.9)
18:2	0.228 (45.0)
18:3	0.216 (42.7)
Asolectin	1.118 (220.9)

carbon atoms in the acyl chain from 14 to 20. Thus, 1,2-dimyristoyl(14:0)-PC and 1,2-dispalmitoyl(16:0)-PC stimulated ATPase activity while 1,2-distearic(18:0)-PC and 1,2-diarachidoyl(20:0)-PC markedly inhibited ATPase activity. Molecular species of PC possessing two unsaturated acyl chains caused the activity to decrease with increase in the degree of unsaturation in their acyl chains. 1,2-Dilinoleoyl(18:3)-PC in particular markedly inhibited ATPase activity when there were 18 carbon atoms in its acyl chain, and 1,2-dioleoyl(18:1)-PC stimulated ATPase activity to a greater extent than 1,2-distearoyl (18:0)-PC.

An examination was then conducted on the effects of molecular species of PC possessing a 1-saturated and a 2-unsaturated acyl chain on ATPase activity. Activity was maximum following the addition of 1-palmitoyl(16:0) 2-oleoyl(18:1)-PC, the stimulation being 2.4-fold. 1-Palmitoyl(16:0) 2-stearoyl(18:0)-PC failed to have any effect on the activity.

An examination of the effects of various types of PC possessing a 1-unsaturated and a 2-saturated acyl chain or a 1-saturated and a 2-unsaturated acyl chain indicated that either combination increased ATPase activity. Furthermore, the ATPase activity was enhanced on decreasing the length of the saturated acyl chain at position *sn*-1 or *sn*-2. In either case, activation was observed to be three- to four fold and was maximum following the addition of 1-oleoyl(18:1)-2-myristoyl(14:0)-PC.

DISCUSSION

When plant plasma membrane vesicles are delipidated *in vitro* with detergents, the H+-ATPase is inactivated and partial restoration of activity can be achieved by the addition of exogenous phospholipids. Specifically, PC is the major phospholipid component (*ca.* 30–35%) in the plasma membrane (10, 14) and activated ATPase activity (Table I) (10). Although previous studies have examined the requirement of phospholipids to induce the ATPase activity using delipidated membrane vesicles (2), in this study we have completely solubilized the ATPase with a two-step procedure using deoxycholate and zwittergent prior to lipid additions. Serrano *et al.* (18) performed a less extensive examination of the lipid requirements of a cholate-extracted preparation of the plant plasma membrane H+-ATPase and the protein content of this preparation was unclear. In the present study we have extended the earlier observations of lipid activation by examining, for the first time, the effect of different fatty acyl components of the solubilized enzyme. We have found that the type of fatty acyl side chain greatly affects the stimulatory effect of PC. For example, stimulation was found to depend on the degree of saturation of the acyl chain and its length (Table IV). Similar results were observed by Palmgren and Sommarin (11) but these investigators used an intact membrane vesicle preparation to examine ATPase activation by the detergent LPC. It is not clear whether this 'detergent' activation at 50- to 100-fold lower concentrations of LPC is related to the present study of activity restoration with delipidated enzyme.

The present study shows maximum activity of H+-ATPase to be induced by the exogenous addition of PC possessing one saturated and one unsaturated acyl chain. Plasma membrane ATPase has an amphipathic structure (4) and its activity may thus be regulated by direct interactions between active site(s) of the enzyme moiety and the hydrophobic environment of the plasma membrane (6) and particularly by interactions between two fatty acyl chains of a phospholipid (Table IV). It is impossible to be certain that the effects we observe *in vitro* are related to regulation of the enzyme *in vivo*. It is useful to note that all solubilized and purified preparations of the plant plasma membrane H+-ATPase obtained to date are highly denatured. Thus, the maximal specific activities obtained with the purified enzyme are slightly higher than that observed in the original membranes even though the protein concentration is reduced at least 10-fold. This denaturation may be related to the loss of an essential kind of lipid (15) but it is irreversible since even the best lipid combinations do not completely restore the activity.

In conclusion, this study demonstrates that the length and degree of saturation of fatty acyl chains in phospholipids are

Table III. *Effects of Glycerides and the Polar Head Groups of Phospholipids*

Glyceride or polar head group (0.05%, w/v) was added to 3 μg of partially purified ATPase in a total volume of 0.5 mL. ATPase activity was assayed in the presence of 3 mM Tris-ATP, 3 mM $MgSO_4$, 50 mM KCl, and 30 mM MES-Tris (pH 6.5), and expressed as percent of activity in the absence of added phospholipid.

Glycerols and Polar Head Groups Added	ATPase Activity
	μmol Pi/mg protein·min
None	0.460 (100)
Triglycerol	0.522 (113.5)
Diglycerol	0.588 (127.8)
Glycerol	0.536 (116.5)
Ethanolamine	0.358 (77.8)
Choline	0.382 (83.0)
Inositol	0.626 (136.0)
Serine	0.612 (133.0)
Asolectin	0.996 (216.5)

Table IV. *Effects of Molecular Species of PC on Activity of the Plasma Membrane H^+-ATPase from Rice Culture Cells*

Each molecular species of PC (0.05%, w/v) was added to 4 μg of partially purified ATPase in a total volume of 0.5 mL. ATPase activity was assayed in the presence of 3 mM Tris-ATP, 3 mM $MgSO_4$, 50 mM KCl, and 30 mM MES-Tris (pH 6.5), and expressed as percent of activity in the absence of added phospholipid.

Molecular Species of PC Added	ATPase Activity
	μmol Pi/mg protein·min
None	0.460 (100)
14:0 (*sn*-1,*sn*-2)	0.792 (172.2)
16:0 (*sn*-1,*sn*-2)	0.710 (154.3)
18:0 (*sn*-1,*sn*-2)	0.288 (62.6)
20:0 (*sn*-1,*sn*-2)	0.266 (57.8)
18:1 (*sn*-1,*sn*-2)	0.726 (157.8)
18:2 (*sn*-1,*sn*-2)	0.478 (103.9)
18:3 (*sn*-1,*sn*-2)	0.292 (63.4)
16:0 (*sn*-1) 18:0 (*sn*-2)	0.434 (94.3)
16:0 (*sn*-1) 18:1 (*sn*-2)	1.098 (238.7)
16:0 (*sn*-1) 18:2 (*sn*-2)	0.510 (110.9)
16:0 (*sn*-1) 20:4 (*sn*-2)	0.562 (129.5)
Asolectin	0.996 (215.6)
None	0.292 (100)
18:1 (*sn*-1) 14:0 (*sn*-2)	1.214 (415.8)
18:1 (*sn*-1) 16:0 (*sn*-2)	1.102 (377.4)
18:1 (*sn*-1) 18:0 (*sn*-2)	1.030 (352.7)
14:0 (*sn*-1) 18:1 (*sn*-2)	1.168 (400.0)
16:0 (*sn*-1) 18:1 (*sn*-2)	1.102 (377.4)
18:0 (*sn*-1) 18:1 (*sn*-2)	1.000 (342.5)
Asolectin	1.090 (373.3)

clearly possible candidates for a mechanism for regulating ATPase activity *in vivo*. This suggestion is supported by observations which suggest that changes in phospholipase A_2 may be involved in the *in vivo* activation of ATPase (12), although other studies have suggested the involvement of protein phosphorylation (16). Further experiments are needed to clarify the precise mechanism(s) used for regulating the plasma membrane H^+-ATPase.

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