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# Lysophosphatidylcholine Stimulates ATP Dependent Proton Accumulation in Isolated Oat Root Plasma Membrane Vesicles[1]

**Michael Gjedde Palmgren* and Marianne Sommarin**

*Department of Plant Physiology, University of Lund, P.O. Box 7007, S-220 07 Lund, Sweden*

## ABSTRACT

**Lysophosphatidylcholine at concentrations of 30 micromolar stimulated the rate of MgATP-dependent $H^+$-accumulation in oat (*Avena sativa* L. cv Rhiannon) root plasma membrane vesicles about 85% while the passive permeability of $H^+$ was unchanged. Activation was dependent on chain length, degree of saturation, and head group of the lysophospholipid. A $H^+$-ATPase assay was developed that allowed the simultaneous measurement of proton pumping and ATPase activity in the same sample. ATP hydrolysis was also stimulated by lysophospholipids and showed the same lipid specificity, but stimulation was only about 25% at 30 micromolar. At higher concentrations of lysophosphatidylcholine the ATPase activity in a latency-free system could be stimulated about 150%. The enzymic properties of proton pumping and ATP hydrolysis were otherwise identical with respect to vanadate sensitivity, $K_m$ for ATP and pH optimum. The stimulatory effect of lysophospholipids suggests that these compounds could be part of the regulatory system for plant plasma membrane $H^+$-ATPase activity *in vivo*.**

The plant plasma membrane $H^+$-ATPase is an electrogenic proton pump using ATP as an energy source to export protons from the cytoplasm across the plasma membrane to the apoplast. The activity of the $H^+$-ATPase builds up an electrochemical gradient, which in turn is believed to drive the transport of nutrients into the plant cell (6, 17). The acidification of the cell wall may also cause the loosening required for cell enlargement (11). In addition to controlling nutrient uptake and growth the plant plasma membrane $H^+$-ATPase itself is thought to be activated by plant hormones, but the mechanism of regulation is unknown (11, 14).

We have recently shown (8) that the plant plasma membrane $H^+$-ATPase is modulated by the cleavage products of an endogenous phospholipase A that hydrolyzes PC[2] to lyso-PC and free fatty acids in the plasma membrane. In the present communication, we demonstrate for the first time that lyso-PC also stimulates ATP dependent proton accumulation in plant plasma membrane vesicles. We suggest that the formation of an activating lipid is a possible mode for regulation of the plasma membrane $H^+$-ATPase.

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[2] Abbreviations: PC, phosphatidylcholine; lyso-PC, lysophosphatidylcholine; BTP, 1,3-bis(tris(hydroxymethyl)-methylamino)propane.

## MATERIALS AND METHODS

### Plant Material

Oat (*Avena sativa* L. cv Rhiannon) was grown hydroponically in the dark for 8 d (16).

### Plasma Membranes

Plasma membranes were purified from a microsomal fraction (10,000–30,000 g pellet) of oat roots in an aqueous polymer two-phase system (16) with minor modifications. The homogenization medium contained 0.25 M sucrose, 5 mM EDTA-BTP, 2 mM DTE, and 1 mM PMSF, 0.2% (w/v) bovine serum albumin (Sigma; protease free), 0.2% (w/v) casein (boiled enzymatic hydrolysate, Sigma type I), 25 mM Mops-BTP (pH 7.5). The final plasma membrane preparation was diluted in 0.25 M sucrose, 5 mM EDTA-BTP, 2 mM DTE, 10 mM Mops-BTP (pH 7.5), pelleted and resuspended in the same buffer. The washed plasma membranes were stored in liquid $N_2$ until use.

### Proton Pumping

Proton uptake into the vesicles was monitored as the absorbance decrease at 495 nm of the ΔpH probe acridine orange (18). The assay medium consisted of 20 μM acridine orange, 0.5 mM ATP-BTP, 2 mM $MgCl_2$, 10 mM Mops-BTP (pH 7.0), 140 mM KCl, 1 mM EDTA-BTP, 1 mM DTE, 50 μM PMSF, 85 μg $mL^{-1}$ casein, 2.5 μg $mL^{-1}$ valinomycin, and 50 μg $mL^{-1}$ membrane protein in a total volume of 1.2 mL. Lysophospholipids (30 μM, unless otherwise indicated), PC, and cholate were added to the reaction mixture after addition of the membranes. After 5 min preincubation at 20°C, the reaction was initiated by addition of $MgCl_2$. The rate of $H^+$-accumulation was estimated from the initial slope of absorbance quenching of acridine orange.

### ATPase Assay

ATPase activity was determined simultaneously with monitoring of proton pumping. Samples of 200 μL were withdrawn from the reaction mixture described above at 20, 140,

and 260 s after addition of $Mg^{2+}$, and released inorganic phosphate was determined (2).

### $H^+$-ATPase Assay

In some experiments the concentration of ATP in the proton pumping assay was kept constant by adding 0.25 mM NADH, 1 mM phospho*enol*pyruvate, 15 $\mu$g $mL^{-1}$ lactate dehydrogenase (Boehringer; solution in glycerol), and 30 $\mu$g $mL^{-1}$ pyruvate kinase (Boehringer, solution in glycerol) to the reaction mixture. In this coupled enzyme assay ATP hydrolysis is coupled to oxidation of NADH (7). Proton pumping and ATPase activity were monitored simultaneously in the same cuvette by plotting the absorbance at 495 and 340 nm, respectively, *versus* time.

### Lipid Preparations

All lipids were obtained from Sigma. Lysophospholipids were dissolved in 48% (w/v) ethanol, 10 mM Mops-BTP (pH 7.0), 1 mM DTE, 1 mM EDTA, and stored at −20°C. Prior to use, they were diluted 1:24 with water and added as 50 $\mu$L or diluted 1:200 with reaction mixture and added as 600 $\mu$L when indicated. Alternatively, lyso-PC or PC was solubilized to 1.5 mM with 7.5 mM sodium cholate-Mops (pH 7.0), and was added to the incubation mixture from this stock solution.

### Protein

Protein was measured essentially as in Bearden (1), with bovine serum albumin as standard.

## RESULTS AND DISCUSSION

### Characterization of $H^+$-ATPase Activity

Plasma membranes from oat roots isolated by two-phase partitioning exhibited MgATP-dependent intravesicular acidification as measured with absorbance quenching of acridine orange (Fig. 1). The acridine orange signal was abolished by nigericin (0.1 $\mu$g $mL^{-1}$) (Fig. 1), which catalyzes the electroneutral exchange of protons for potassium (9), the nonspecific ionophore gramicidin (1 $\mu$g $mL^{-1}$), and the permeant base imidazole (5 mM imidazole-BTP, pH 7.0). Proton pumping was stimulated by permeable anions ($NO_3^- > Cl^- \gg SO_4^{2-}$ at 50 mM $K^+$) and at 140 mM KCl intravesicular proton accumulation was stimulated three-fold by the $K^+$-ionophore valinomycin.

ATP hydrolysis was measured either by quantification of released inorganic phosphate or in a coupled assay where ATP hydrolysis was coupled to oxidation of NADH. Since the absorption spectra of acridine orange and NADH are not overlapping, it was possible to measure $H^+$-pumping and ATP hydrolysis simultaneously in the same cuvette (Fig. 2).

Proton pumping and the hydrolysis of ATP were catalyzed by the same enzyme as based on the following observations: (a) Both the proton pump and the ATPase were inhibited by $Na_3VO_4$ ($K_i$ = 10 $\mu$M at 100 $\mu$g protein $mL^{-1}$), an inhibitor of the plant plasma membrane $H^+$-ATPase (Fig. 3); (b) both reactions could be initiated by either $Mg^{2+}$ or ATP but required the presence of both for activity; (c) proton pumping and ATPase activity exhibited similar dependence on ATP

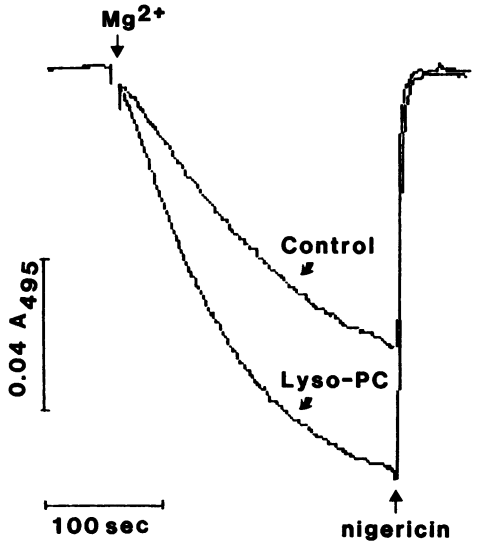


**Figure 1.** Stimulatory effect of 30 $\mu$M palmitoyl lyso-PC on ATP-dependent $H^+$-accumulation in oat root plasma membrane vesicles. Proton uptake into the plasma membrane vesicles (50 $\mu$g membrane protein $mL^{-1}$) was measured as the absorbance decrease at 495 nm of the ΔpH probe acridine orange. $Mg^{2+}$ and nigericin (0.1 $\mu$g $mL^{-1}$) were added as indicated by arrows.

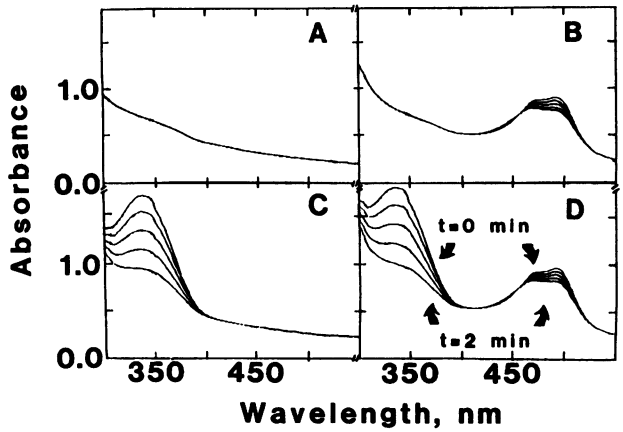


**Figure 2.** Simultaneous measurement of MgATP dependent proton pumping and of MgATPase activity in oat root plasma membrane vesicles. Proton transport was measured by the quenching of acridine orange absorbance, and the ATPase activity was coupled to NADH oxidation. The $H^+$-ATPase assay mixture described in "Materials and Methods" was used except that acridine orange and NADH were initially omitted. After addition of $MgCl_2$ (2 mM) spectra were obtained at 30 s intervals. A, Without any further additions. In subsequent experiments the following reagents had been added to the reaction mixture: B, 20 $\mu$M acridine orange; C, 0.25 mM NADH; D, 20 $\mu$M acridine orange and 0.25 mM NADH.

concentration and an apparent $K_m$ of 0.2 mM at 140 mM KCl could be extrapolated for both reactions (Fig. 4); (d) an acidic pH optimum of pH 6.5 was observed for both the proton pump (Fig. 5) and the ATPase (8). These data are consistent with the previous findings that inside-out vesicles of plant plasma membranes accumulate $H^+$ ions through an electrogenic $H^+$-ATPase (17). Inside-out vesicles constitute ≥30% of the plasma membrane vesicles isolated from oat roots by two-phase partitioning (5), and equals 50% after

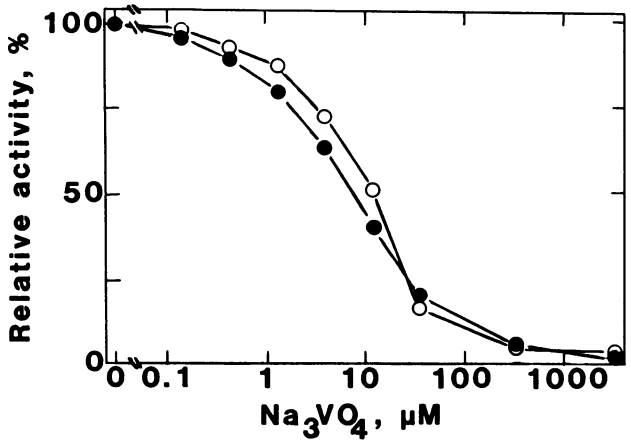


**Figure 3.** Vanadate inhibition of $H^+$-accumulation (○) and ATPase activity (●). The reaction mixture contained plasma membrane protein 100 μg $mL^{-1}$, indicated final concentrations of vanadate, 3 mM $MgCl_2$, no EDTA, and the reaction was started with 2 mM ATP-BTP, otherwise as described in "Materials and Methods." One hundred % corresponds to a specific activity of 0.67 $A_{495}$ (mg protein)$^{-1}$ $min^{-1}$ (○) and 0.89 μmol Pi (mg protein)$^{-1}$ $min^{-1}$ (●). Data are from one representative experiment.

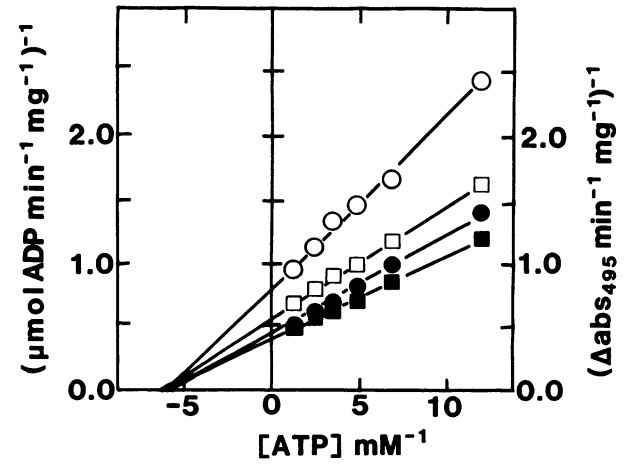


**Figure 4.** Effect of lyso-PC on the apparent $K_m$ for ATP and on the $V_{max}$ of oat root plasma membrane $H^+$-ATPase activity as estimated by Lineweaver-Burk plots. The $H^+$-ATPase assay described in "Materials and Methods" was used. Plasma membranes were preincubated with lyso-PC for 5 min at 20°C prior to the addition of $MgCl_2$. The reaction mixture contained either no additions (○, □) or 50 μM lyso-PC (●, ■). Proton pumping (○, ●) and ATPase activity (□, ■) were measured simultaneously in the same cuvette. The $V_{max}$ values were 2.44 (●) and 1.24 (○) Δabs$_{495}$ $min^{-1}$ mg protein$^{-1}$ for proton pumping, and 2.61 (■) and 1.71 (□) μmol ADP released from ATP $min^{-1}$ mg protein$^{-1}$. The observed $K_m$ values were all 0.17 in this experiment but varied from 0.15 to 0.21 between different experiments.

freezing and thawing (MG Palmgren, M Sommarin, unpublished observations).

### Effect of lyso-PC

Preincubation of the membranes for 1 h at 20°C with sodium dodecyl sulfate resulted in a plasma membrane preparation where no latent ATPase activity could be demasked by the detergents digitonin, Triton X-100, or SDS (Fig. 6). Incubation of membranes with SDS has been reported to cause fragmentation and disappearance of the vesicular structure of the plasma membranes (4). Addition of the natural detergent lyso-PC to such a latency-free system produced about 150% activation of the ATPase activity (Fig. 6).

Addition of 30 μM palmitoyl 16:0 lyso-PC to plasma membrane vesicles markedly increased (nearly twofold) the rate of MgATP dependent acridine orange absorbance quenching (Fig. 1). Since the absorbance change was also sensitive to

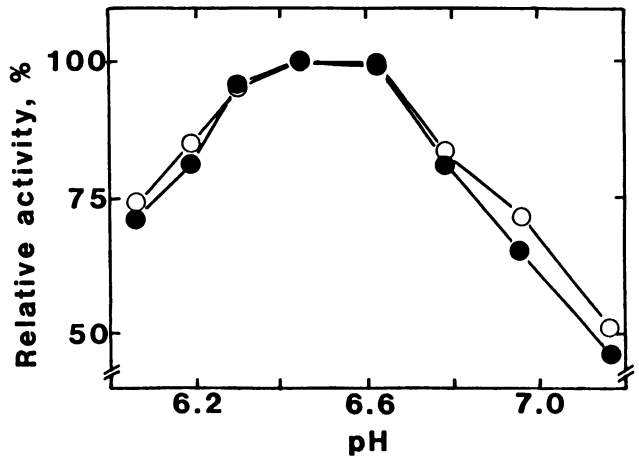


**Figure 5.** Effect of lyso-PC on pH optimum of plasma membrane proton pumping activity. (○), No additions; (●) 30 μM lyso-PC. Plasma membranes were incubated with lyso-PC for 5 min at 20°C prior to addition of $MgCl_2$ to the proton pumping assay mixture described in "Materials and Methods." The final pH was measured in the reaction medium after addition of all reagents. Maximal activities were 1.09 (○) and 2.25 (●) Δabs$_{495}$ $min^{-1}$ mg protein$^{-1}$.

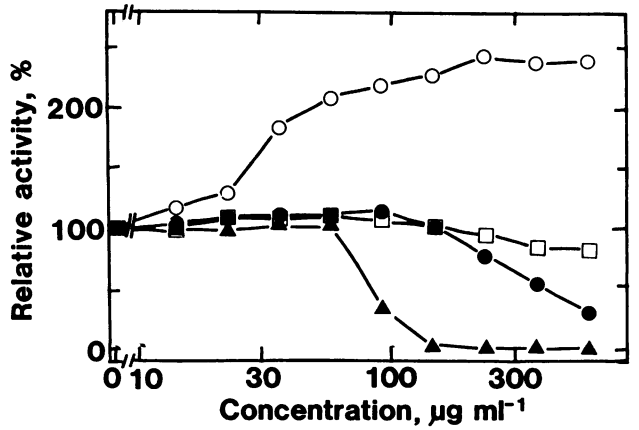


**Figure 6.** Effect of lyso-PC on the ATPase activity in a latency free membrane system. Oat root plasma membranes (180 μg $mL^{-1}$) were incubated with SDS (220 μg $mL^{-1}$) in 10 mM Mops-BTP (pH 7.5), 3 mM ATP-BTP, 1 mM EDTA-BTP, 1 mM DTE. After incubation for 60 min at 20°C, aliquots of 10 μl (1.8 μg) were transferred to a ATPase reaction mixture with the indicated final concentrations of egg lysophosphatidylcholine (Sigma type I) (○), digitonin (□), Triton X-100 (●), SDS (▲) and 10 mM Mops-BTP (pH 7.0), 3 mM ATP-BTP, 5 mM $MgCl_2$, 1 mM EDTA-BTP, 1 mM DTE, 50 mM KCl in a total volume of 400 μL. One hundred % corresponds to an activity of 0.9 μmol Pi $min^{-1}$ mg protein$^{-1}$.

nigericin, the lysophospholipid apparently stimulated $H^+$-uptake through $H^+$-ATPase. The rate of ATP-hydrolysis, measured as Pi released (Fig. 7A) or with a coupled enzyme assay (Fig. 8B), was stimulated to a lesser degree by 30 $\mu$M palmitoyl lyso-PC but further addition of lyso-PC continued to stimulate the activity (Fig. 7A). These data suggest that the apparent inhibition of proton pumping above the optimal concentration 30 $\mu$M (Fig. 7) is not due to inhibition of $H^+$-ATPase activity, but is due to induced leakage of the plasma membranes to protons. At assay conditions where the stimulation of the ATPase activity by lyso-PC are maximal the $K_m$ for ATP and the pH optimum of the $H^+$-ATPase are altered (8), but at 30 $\mu$M lyso-PC the affinity for ATP (Fig. 4) and the pH dependency (Fig. 5) for both proton pumping and ATP hydrolysis were not found to be significantly changed. This suggests that the lipid effect at a low lysophospholipid to protein ratio is only an effect on $V_{max}$.

### Lipid Specificity

Various lysophospholipids were tested for their ability to stimulate the rate of MgATP-dependent $H^+$-uptake in the oat plasma membranes using the $H^+$-ATPase assay. The stimulation was dependent on the length of the fatty acyl chain, with maximum stimulation obtained with palmitoyl 16:0 lyso-PC (84 ± 7%) and oleoyl 18:1 lyso-PC (85 ± 1%), whereas short-chain lyso-PC produced minor effects (Fig. 8A). Alteration of the head group of oleoyl 18:1 lyso-PC to phosphatidylethanolamine or phosphate (lysophosphatidic acid) reduced its effect by 28 and 43%, respectively (Fig. 8A). The stimulation of ATPase activity by lysophospholipids showed the same relative dependency on chain length and head group, but stimulation was always about one-third of the stimulation of proton accumulation (Fig. 8B).

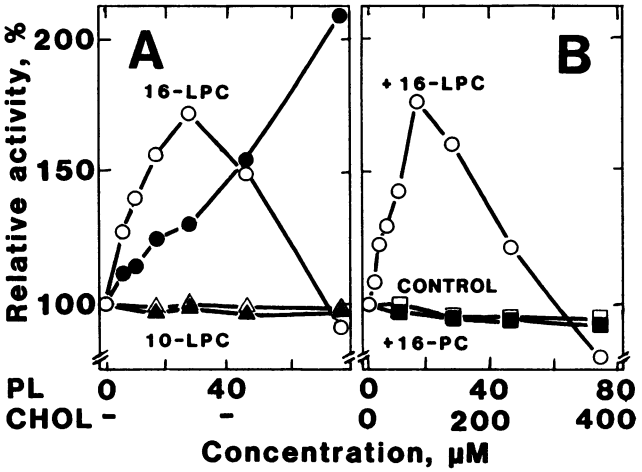


**Figure 7.** Dose-response profiles for palmitoyl 16:0 lyso-PC (○, ●), decanoyl 10:0 lyso-PC (△, ▲), dipalmitoyl 16:0 PC (■) and cholate (□) effects on $H^+$-accumulation (○, △, ■, □) and ATPase activity (●, ▲) in oat root plasma membrane vesicles. A, Phospholipids were added to the plasma membranes in a 1:1 volume ratio, to prevent high local concentrations of detergent; B, phospholipids were added together with cholate. PL, Phospholipids; CHOL, cholate; 10-LPC, decanoly 10:0 lyso-PC; 16-LPC, palmitoyl 16:0 lyso-PC; 16-PC, dipalmitoyl 16:0 PC.

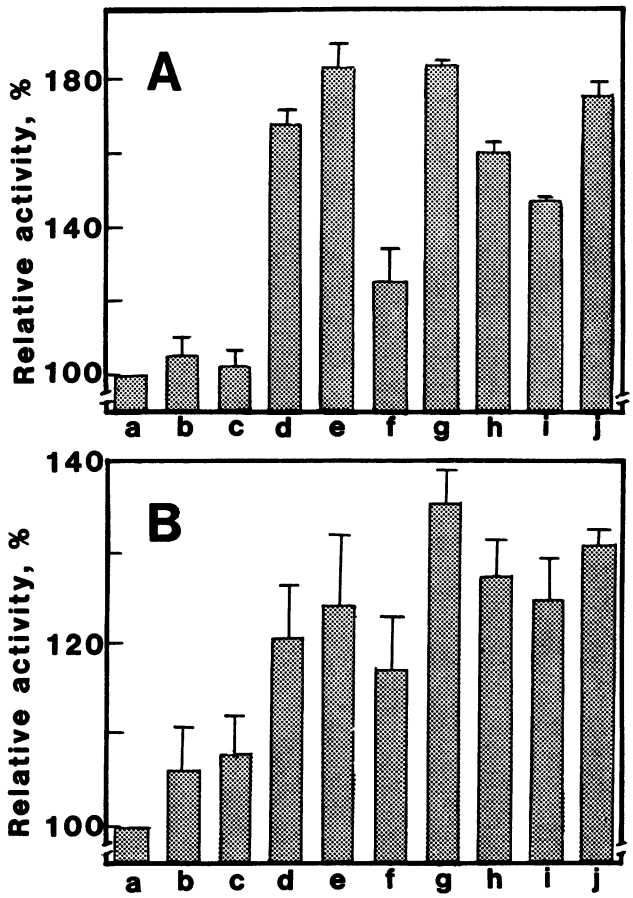


**Figure 8.** Effect of various lysophospholipids on $H^+$-accumulation (A) and ATPase activity (B) in oat root plasma membrane vesciles using the $H^+$-ATPase assay. a, control; b, decanoyl 10:0 lyso-PC, c, lauroyl 12:0 lyso-PC; d, myristoyl 14:0 lyso-PC, e, palmitoyl 16:0 lyso-PC; f, stearoyl 18:0 lyso-PC; g, oleoyl 18:1 lyso-PC; h, oleoyl 18:1 lysophosphatidylethanolamine; i, oleoyl 18:1 lysophosphatidic acid-BTP (pH 7.0); j, 1-O-alkyl lyso-PC (15 $\mu$g $mL^{-1}$). The standard deviation ($n$ = 3) using material from different membrane preparations is indicated.

Addition of up to 100 $\mu$M PC did not affect MgATP-dependent $H^+$-accumulation in the oat plasma membranes when added as liposomes (data not shown). The $H^+$-ATPase may interact in a more facilitated way with the detergent lyso-PC than with the PC vesicles. This difference in accessibility could explain the efficiency of lyso-PC over PC in stimulating $H^+$-accumulation (15). Therefore, proton pumping activity with PC or lyso-PC was measured by inclusion of another detergent, cholate. The lipids were solubilized in cholate and incorporated into the membrane vesicles by a dilution procedure (10). In the presence of cholate only lyso-PC, and not PC, stimulated $H^+$-accumulation (Fig. 7B). The final concentrations of cholate were well below the critical micelle concentration, which is in the millimolar range, and cholate itself only slightly reduced the rate of acridine orange absorbance quenching (Fig. 7B).

ATP dependent proton accumulation was almost doubled in zucchini microsomes when 1-O-alkyl-2-acetyl PC, platelet activating factor, and a supernatant fraction were included in the assay (13). The plasma membranes used in our experi-

ments were extensively washed and only a minor effect of 1-O-alkyl-2-acetyl PC was observed (data not shown), while 1-O-alkyl lyso-PC, consisting of a mixture of chain lengths, stimulated proton accumulation to almost the same degree as palmitoyl lyso-PC (Fig. 8). This suggests the possible involvement of a lipase in the supernatant dependent action of 1-O-alkyl-2-acetyl PC.

### Effect of Proteases or Lipases

Homogenization in a medium devoid of PMSF, EDTA, albumin, and casein resulted in a plasma membrane fraction in which ATP-dependent proton accumulation could not be reproducibly stimulated by lyso-PC. When plasma membranes were isolated in the presence of the above mentioned protease protectants and when PMSF, EDTA, and casein were present in the $H^+$-ATPase assay, a consistent 80 to 100% activation of the proton pumping activity by long-chain lysophospholipids was observed. In addition to inhibit metalloproteinases EDTA may also have an inhibiting effect on lipases. Albumin binds fatty acids which may act as uncouplers. Both a phospholipase A, which generates lyso-PC and fatty acids, and an lysophospholipase, which deacylates lyso-PC, are known to be present in the oat plasma membranes (8).

### Mechanism Behind the Lipid Effect

In principle, proton accumulation in membrane vesicles could be stimulated in several ways. The influx of protons could be increased by either (a) direct stimulation of proton transport systems, (b) indirectly by depolarization of the membrane potential by influx of anions or efflux of cations which in turn would facilitate $H^+$-influx, or (c) the passive efflux of protons from the vesicles could be reduced; alternatively, (d) the proportion of inside-out vesicles capable of accumulating protons could be increased by inversion of right-side-out vesicles.

Stimulation by palmitoyl lyso-PC was seen whether KCl, choline-Cl, or $KNO_3$ was present in the reaction medium (data not shown) indicating that the effect is not dependent on any specific cation or anion. Furthermore, electrical balance between the interior and the exterior of the membrane vesicles was obtained during the assay since the potassium ionophore valinomycin was present. The passive efflux of protons from the vesicles was followed after terminating the MgATP catalyzed reaction by chelating $Mg^{2+}$ with EDTA (Fig. 9A) or by depletion of ATP by addition of hexokinase and glucose (Fig. 9B). No influence of 30 $\mu$M palmitoyl lyso-PC on the apparent passive permeability to protons could be detected (Fig. 9). Inversion of right-side-out plasma membrane vesicles with latent ATPase activity to proton accumulating inside-out vesicles cannot explain the stimulating effect of lysophosphatidylcholine that is seen in a latency-free system (Fig. 6). Taken together, these results suggest that lyso-PC stimulates the active $H^+$-influx.

Since proton accumulation is stimulated to a higher degree by lyso-PC than ATP-hydrolysis, lyso-PC may also affect other $H^+$-transport systems that the plasma membrane $H^+$-ATPase; more than one vanadate-sensitive ATP-hydrolyzing

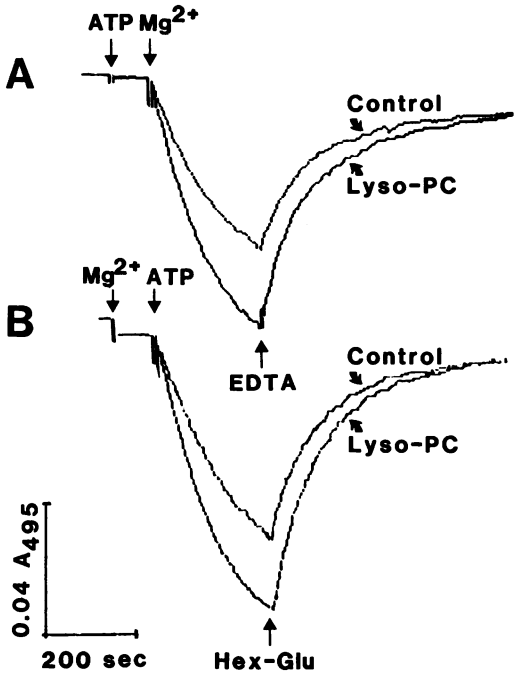


**Figure 9.** Effect of palmitoyl lyso-PC (30 $\mu$M) on the apparent passive permeability of the plasma membranes to protons. The reactions were initiated in the absence of EDTA either by 0.6 mM $Mg^{2+}$ (A) or 0.5 mM ATP (B). After a pH gradient was developed between the vesicles and the exterior, 3 mM EDTA-BTP (pH 7.0), was added to chelate $Mg^{2+}$ (A) or 15 IU $mL^{-1}$ of hexokinase (Sigma; sulfate-free) and 1.5 mM glucose were added to remove ATP (B), and the passive efflux of protons was recorded. Membranes in (A) and (B) were from different preparations. $T_{½}$ for the increase in absorbance in A and B was approximately 85 s in the absence and in the presence of lyso-PC.

enzyme could be present, of which one may not be sensitive to lyso-PC, or lyso-PC may alter the $H^+$/ATP stoichiometry of the plasma membrane $H^+$-ATPase. Alternatively, the acridine orange signal is not linearly related to changes in the ATPase activity. This is less likely since the acridine orange absorbance change is closely related to changes in ATPase activity when altering the vanadate concentration (Fig. 3), ATP concentration (Fig. 4), and pH (Fig. 5).

## CONCLUSION

This study demonstrates that long chain lyso-PC modulates the $H^+$-transport activity in plant plasma membranes. The stimulating effect of lyso-PC appears to be in line with previous reports on the action of lysophospholipids on membrane ion-transport in animal systems. For instance, lyso-PC stimulates the rate of $H^+$ and $K^+$ transport across rat heavy gastric membranes by the $H^+$-$K^+$-ATPase (3) and the rate of $Ca^{2+}$-transport exhibited by the $Ca^{2+}$-ATPase of red blood cell plasma membranes (12). Since the plasma membrane $H^+$-ATPase might be regulated by protein kinase-mediated phosphorylation (13), the stimulatory effect of lyso-PC could also be indirect; that is, through activation of an endogenous protein kinase that in turn activates the $H^+$-ATPase. The action of lyso-PC indicates the importance of the lipid envi-

ronment for the plasma membrane $H^+$-ATPase. A possible explanation of the lyso-PC effect could be that alteration of the lipid environment stabilizes the $H^+$-ATPase in a more reactive conformation (8). Oat roots possess an enzyme that hydrolyzes PC to lyso-PC and a free fatty acid (8). This raises the question of whether the endogenous phospholipase activity in the oat roots may be part of the system responsible for physiological modulation of proton transport across the plant plasma membrane in response to the action of growth-controlling substances.

## ACKNOWLEDGMENTS

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