

Interaction between poly(L-lysine) and membranes inhibits proton pumping by corn root tonoplast H⁺-ATPase

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The influence of poly(L-lysine) binding on the coupled activities of nitrate-sensitive H⁺-ATPase in isolated corn (*Zea mays* L. cv. FRB73) root tonoplast vesicles was investigated. The addition of membrane-impermeable poly(L-lysine) caused a slow increase in light scattering of the tonoplast suspension. Electron microscopy showed that the increase was the result of an aggregation of the vesicles. In the presence of 75 mM KCl, a concentration sufficient to sustain near optimal ATP hydrolysis, poly(L-lysine) slightly enhanced the hydrolysis activity but significantly inhibited proton pumping of the H⁺-ATPase. Inhibition increased with the average molecular mass of poly(L-lysine) and reached a maximum at 58 kDa. When total osmolarity was kept constant, the replacement of sucrose by KCl enhanced both ATP hydrolysis and proton pumping activities. However, enhancement of proton pumping was significantly greater than that of ATP hydrolysis. An increase in KCl, but not K₂SO₄, significantly relieved poly(L-lysine)-induced inhibition of proton pumping. Kinetic analysis indicated that poly(L-lysine) did not significantly affect the proton leakage of the tonoplast membranes under different energetic conditions. These results suggest that the electrostatic interaction between poly(L-lysine) and the negative charges on the exterior surface of tonoplast vesicles could change the coupling ratio of ATP hydrolysis to proton pumping. Thus, the surface charge of the tonoplast membrane may be involved in the regulation of these two activities.

Key words – ATPase-coupled proton pumping, membrane electrostatic interaction, tonoplast H⁺-ATPase.

Introduction

Plant root cells contain tonoplast (v-type) H⁺-ATPase, which catalyzes the hydrolysis of Mg-ATP and utilizes released chemical free energy to support vectorial movements of protons across the vacuolar membranes (Sze 1984). The resulting transmembrane proton electrochemical potentials, $\Delta\mu_{H^+}$, may serve as the driving force for the redistribution of nutrients and metabolites between the cytoplasm and vacuole. Tonoplast H⁺-ATPase, an anion-sensitive enzyme, contains many different subunits and does not form a phosphorylated intermediate in its reaction pathway (Sze 1984, Nelson and Taiz 1989, Reinder et al. 1992). Qualitative analysis of the tonoplast phospholipid indicated that the membrane contains, in

addition to neutral phosphatidylcholine, significant amounts of both negatively charged (phosphatidylserine) and positively charged (phosphatidylethanolamine) phospholipids (Tu et al. 1990). The exact mechanism of coupling of ATP hydrolysis and H⁺-pumping in the tonoplast H⁺-ATPase system is unknown. However, to explain differential sensitivity of the two coupled activities to many treatments, we have proposed an indirect coupling mechanism (Tu et al. 1990, 1992).

Because of its ability to bind strongly with the negative surface charges, poly(L-lysine) has been used as a model peptide to study the influence of extrinsic proteins or charges on the properties of biomembranes (Davies et al. 1964, Clague and Cherry 1989, Singh et al. 1992). Upon binding, poly(L-lysine) may change its conforma-

tion from random coil to more ordered structures, such as α -helices (Carrier and Pezolet 1984) or β -sheets (Fukushima et al. 1988, Laroche et al. 1988). Although the binding induces lateral aggregation of membrane components having negative charges that are exposed to aqueous media (Laroche et al. 1988, Clague and Cherry 1989), it does not significantly affect the packing of hydrocarbon chains in the membrane bilayer (Takahashi et al. 1991). A complete neutralization of the surface negative charges of synthetic phosphatidyl acid vesicles may lead to a highly ordered multilamellar structure containing β -sheets of poly(L-lysine) sandwiched between the bilayer (Takahashi et al. 1991). For native biological membrane systems, poly(L-lysine) binding to band 3 protein (anion exchanger of red blood cell plasma membrane) may lead to an aggregation of vesicles as indicated by an increase in light scattering by erythrocyte ghosts (Clague and Cherry 1989). Poly(L-lysine) binding by itself does not significantly affect the leakage of solutes from erythrocytes or lipid vesicles. However, the binding does affect the insertion of membrane pore or channel peptides, e.g., melittin, gramicidin S and alamethicin (Portlock et al. 1990).

It has been proposed that the polar heads of phospholipids in biomembranes may be involved in the lateral movement of protons along the membrane surface (Tocanne and Teissié 1990). In an attempt to assess the possible involvement of the membrane surface charges in the regulation of the activities of tonoplast H^+ -ATPase, we studied the influence of poly(L-lysine) binding on the coupled activities. We found that the binding had only minimal effects on the ATP hydrolysis. The presence of poly(L-lysine) also did not significantly alter the proton leakage of the membrane under different energetic conditions. However, based on kinetic analyses, the initial proton pumping rate was significantly inhibited by poly(L-lysine). These results suggest that the arrangement of the membrane surface charges may be involved in regulating the ATP-driven proton pumping in corn root tonoplast vesicles.

Abbreviations – AO, acridine orange; BTP, bis-tris-propane; δ , absorbance change; δ_s , absorbance change at steady state; DPH, diphenyl hexatriene; k_1 , rate constant for measuring proton pumping retardation of energized (with ATP hydrolysis occurring) membranes; k_2 , rate constant for measuring proton leakage of de-energized (with ATP hydrolysis silent) membranes; R_{ATP} , initial rate of ATP hydrolysis; R_0 , initial proton pumping rate; t , time.

Materials and methods

Isolation of tonoplast vesicles

Corn (*Zea mays* L. cv. FRB 73) seeds were germinated on filter paper moistened with 0.1 mM $CaCl_2$ for 3 days at 28°C and harvested as previously described (Tu et al. 1987). Approximately 60 g of excised roots were homogenized with a mortar and pestle at 4°C in isolation medium containing 0.3 M sucrose, 5 mM ethyleneglycol-bis(β -aminoethylether-N,N'-tetraacetic acid) (EGTA), 5

mM β -mercaptoethanol, 5 mM dithiothreitol (DTT), and 0.1 M N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), pH 7.7 (adjusted at 4°C). After being filtered through four layers of cheesecloth, the homogenate was subjected to differential centrifugation, 6 000 g for 20 min and 80 000 g for 40 min, to obtain a microsomal pellet. The microsomal pellet was suspended in isolation medium and centrifuged again at 80 000 g for 40 min. The washed microsomes were suspended in isolation medium and layered over a 15 to 45% (w/w) linear sucrose gradient buffered with 5 mM HEPES (titrated to pH 7.7 with 2-[N-morpholino]ethanesulfonic acid [MES]) plus 1 mM DTT and centrifuged at 4°C for 18 h at 84 000 g. The tonoplast fractions between 19 and 23% sucrose were pooled and used for experiments. The protein concentration was determined by a modified Lowry method using bovine serum albumin (BSA) as the standard (Bensadoun and Weinstein 1976).

Media used for the measurement of coupled activities

The coupled activities of the tonoplast H^+ -ATPase were measured in media of different compositions. The standard assay medium contained 75 mM KCl, 17.5 mM MES (titrated to pH 6.45 with bis-tris-propane [BTP]), 2.5 mM $MgSO_4$, 1 mM ammonium molybdate, 0.1 mM sodium vanadate, 7.5 μ M acridine orange and 5 mM glucose. To determine the sensitivity of coupled activities to nitrate, the KCl in the standard assay medium was replaced by KNO_3 . To test the effects of changing K-salt concentration, isotonic solutions were used. The solutions contained the same components described for the standard assay medium except the 150 mM KCl was replaced by various concentrations of sucrose and K-salts. The total osmolarity of the replacement solutes was kept at 300 mM. Since osmotic pressure is determined by the number of particles in solution, K_2SO_4 and KCl are three and two times, respectively, more effective than sucrose in producing the pressure. The exact composition of the assay solutions in different experiments is mentioned in the figure legends.

ATP hydrolysis measurement

ATP hydrolysis catalyzed by the H^+ -ATPase was assayed by the direct measurement of inorganic phosphate released. After the tonoplast vesicles were incubated in 2.2 ml of assay solutions for 10 to 20 min at room temperature, the reaction was initiated by the addition of 20 μ l of 0.2 M ATP. At least 90% of the total ATP hydrolysis was catalyzed by the tonoplast H^+ -ATPase based on the sensitivity to nitrate, bafilomycin A_1 , vanadate, azide and molybdate. The amount of phosphate released was determined by the malachite green-molybdate assay as previously described (Tu et al. 1987). The ATP hydrolysis rate remained constant for about 15 to 20 min under the experimental conditions. Thus, the average rate over a time period of 10 min was used to represent the initial

hydrolysis rate (R_{ATP}) of tonoplast H^+ -ATPase after subtracting the ATP hydrolysis in the same assay media with the addition of 10 nM bafilomycin A_1 or with 50 mM KCl replaced by 50 mM KNO_3 . We found no significant quantitative difference in the hydrolysis activity of the tonoplast H^+ -ATPase determined by either KNO_3 or bafilomycin inhibition.

Proton pumping and membrane leakage measurement

ATP-driven H^+ -transport was followed by changes in absorbance of acridine orange (AO) at 492.5 nm. Typically, 200 μ l vesicles were diluted to 2.2 ml in the same solutions used for ATP hydrolysis measurements. Assays were conducted with a Beckman (Irvine, CA, USA) DU-70 spectrophotometer interfaced to a personal computer. The digitized data (sampling time = 1 s) were then analyzed according to a steady-state kinetic model developed in this laboratory (Tu et al. 1987). Briefly, the model assumes that the build-up of a gradient induces a proton leakage and gradually decreases the net ATP-hydrolysis-induced proton transport. When the net transport rate approaches zero, i.e. when leakage equals initial pumping rate, a steady-state condition is reached. Mathematically, the ATP-supported proton pumping can be represented by the following equations:

$$\text{net proton pumping rate} \quad d\delta/dt = R_0 - k_1\delta \quad (1)$$

$$\text{steady-state approximation} \quad R_0 = k_1\delta_s \quad (2)$$

$$\text{time-course of pumping} \quad \ln(1 - \delta/\delta_s) = -k_1t \quad (3)$$

where δ , δ_s , R_0 and k_1 represent the extent of proton transport, the extent of transport at steady state, the initial proton pumping rate, and pumping inhibition constant (proton leakage with ATPase activity on), respectively. The initial ATP hydrolysis rate (R_{ATP}) and initial pumping rate (R_0) are related by:

$$R_{ATP} = m R_0 \quad (4)$$

in which m is the stoichiometric ratio or the extent of coupling between proton pumping and ATP hydrolysis. A decrease in the value of m reflects a decrease in the coupling between the ATP hydrolysis and proton pumping resulting in a decrease in net proton uptake by the tonoplast vesicles. We have previously suggested that this decrease reflects an altered and weakened conformational coupling between the ATP hydrolytic domain and proton pumping domain in the enzyme structure (Tu et al. 1990).

A rapid quenching of ATP hydrolysis can discharge the proton gradient δ_s by leakage through the de-energized membrane. Hexokinase-induced phosphorylation of glucose may be utilized to rapidly exhaust added ATP. The leakage of protons follows a simple first-order decay:

$$\ln(\delta/\delta_s) = -k_2t \quad (5)$$

Here, δ represents the residual gradient at time t after the quenching. It should be noted that k_2 represents the proton leakage constant of de-energized (ATP hydrolysis silent) membrane. Because the structural features of the membrane need not be the same under different energetic conditions, it should not be surprising that the k_1 and k_2 may have different values.

Fluorescence polarization measurements

The effects of poly(L-lysine) on the lipid structure of the tonoplast membrane were qualitatively estimated from changes in fluorescence properties of 1,6-diphenyl-1,3,5-hexatriene (DPH) incorporated into the membrane (Tu et al. 1989). The tonoplast vesicles in the specified media were incubated with 2.0 μ M DPH for 20 min at 22°C with or without poly(L-lysine). The suspension was then illuminated with vertically polarized light (370 nm) and the emission intensities at the parallel (I_1) and perpendicular (I_2) directions at 424 nm were measured with a Perkin-Elmer (Norwalk, CT, USA) LS-5B spectrofluorometer. The polarization (p) was calculated by the ratios of $(I_1 - I_2) / (I_1 + I_2)$.

Electron microscopy

Aliquots of 50 μ l of the vesicles treated with various concentrations of poly(L-lysine) in the standard proton pumping medium were applied to gold-coated glass coverslips and allowed to stand in a moist chamber for 10 min before immersion in 1% (w/v) glutaraldehyde in 0.1 M imidazole chloride solution, pH 6.8. The adsorbed vesicles and aggregates on coverslips were dehydrated in a graded series of ethanol solutions, critical point dried with carbon dioxide and coated with a thin layer of gold by DC sputtering. Vesicles and aggregates were examined at a magnification of 10 000 in the secondary-electron imaging mode using a JSM 840A (JEOL USA, Peabody, MA, USA) scanning electron microscope (SEM).

Chemicals and other methods

Synthetic poly(L-lysine), bafilomycin A_1 , ATP, DPH, hexokinase, BSA, AO, glucose, malachite green and sucrose were purchased from Sigma. All other chemicals used were of analytical grade.

Results

Interactions between poly(L-lysine) and tonoplast vesicles

Because of its size and charge, poly(L-lysine) is not expected to be membrane permeable. Thus, the interactions with sealed tonoplast vesicles should occur only on the exterior surface. The addition of a small amount of poly(L-lysine) to a suspension of tonoplast vesicles

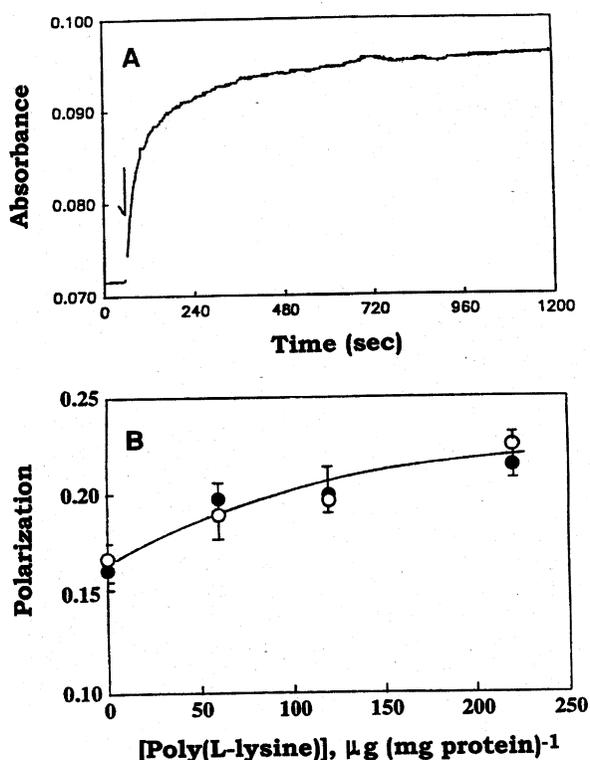


Fig. 1. Interactions between poly(L-lysine) and tonoplast vesicles. A. Light scattering of the tonoplast vesicles. Tonoplast vesicles ($\sim 70 \mu\text{g protein}$) were incubated in 2.0 ml of the standard proton pumping solution at room temperature for 10 min before the addition of $4 \mu\text{g}$ of poly(L-lysine) at indicated time (down arrow). The light scattering of the vesicles was monitored by the absorbance change at 600 nm. B. Fluorescence polarization of DPH in tonoplast membrane. Tonoplast vesicles ($\sim 100 \mu\text{g protein}$) in 2 ml of the same solution in (A) were incubated in the presence of $2 \mu\text{M}$ of DPH and various amounts of poly(L-lysine) for 10 min at room temperature before polarized fluorescence intensities were measured at 429 nm with an excitation wavelength of 396 nm (filled circles). Ten μl of 0.2 M ATP was then added to the vesicle suspension and the polarized fluorescence intensities were measured again after 10 min (open circles). The data shown represent the average ($\pm\text{SD}$) of two independent experiments with triplicated measurements.

caused an increase in turbidity (Fig. 1A) indicating an increase in size/volume of light-scattering entities in the solution. The increase in light scattering could result from enlargement of vesicles due to solute/solvent uptake, formation of large vesicles by membrane fusion mediated by poly(L-lysine), or aggregation of vesicles due to minimized electrostatic repulsion. These possibilities were examined by SEM experiments with the results being described later in the text.

The electrostatic binding of poly(L-lysine) may only slightly affect the packing of the membrane lipid region (Takahashi et al. 1991). We have examined the influence of poly(L-lysine) binding on the mobility of a lipophilic fluorophore in the lipid phase of the membranes. The polarization of DPH fluorescence in the tonoplast mem-

brane increased slightly as the concentration of poly(L-lysine) increased (Fig. 1B). The change in DPH mobility appeared to be independent of the energetic status of the membrane since the increases were observed in the absence and presence of ATP. Thus, the interactions between the surface charges and poly(L-lysine) moderately increased the order (decreased fluidity) of the membrane.

Greenfield and Fasman (1969) reported that poly(L-lysine) assumes a random coil conformation in solutions of neutral pH. The radius and the cross-sectional area of the coil may be estimated from the apparent molecular weight and the specific volume of the synthetic peptide. The radius and the cross-sectional area of a 58 kDa poly(L-lysine) molecule (assuming specific volume as $0.72 \text{ cm}^3 \text{ g}^{-1}$) were calculated as 3.17 nm and 315 nm^2 , respectively. In the absence of poly(L-lysine), the transmission electron microscope imaging revealed, in agreement with our previous observation (Tu et al. 1987), that the tonoplast vesicles assumed normal spherical shapes with an average radius around 150 nm (Fig. 2A) and a surface area of $2.8 \times 10^5 \text{ nm}^2$. Thus, the vesicle surface has enough space to accommodate the binding of approximately 8800 poly(L-lysine) molecules. The addition of minimal amounts of poly(L-lysine) induced some aggregation of vesicles (Fig. 2B). At higher concentrations of poly(L-lysine), only vesicle aggregates were observed (Fig. 2C). The aggregates appeared to be random in shape and contained many spherical elements with sizes similar to those of the individual vesicles.

These data indicated that the light-scattering increase was not a result of the fusion of smaller vesicles to form spherical vesicles with a larger radius. Also, the presence of moderate amounts of poly(L-lysine) did not significantly increase the dimensions of nonaggregated vesicles (cf. Fig. 2A and B). Thus, solute/solvent uptake by individual vesicles is probably not an important contributor to the observed increase in light scattering. It should be mentioned that a poly(L-lysine)-induced increase in light scattering by red blood cell ghosts was previously reported by Clague and Cherry (1989). Without detailed experimental evidence, they proposed that the increase originated from an aggregation of the ghosts.

Effects of poly(L-lysine) on coupled activities

The results presented in Figs 1 and 2 indicate that the interaction between poly(L-lysine) and the membrane causes an aggregation of the vesicles but only slightly affects the structure of the lipid phase. The biochemical effects of this binding on the activities of tonoplast H^+ -ATPase in the vesicles were examined (Fig. 3). The presence of $13.7 \mu\text{M}$ ($2 \mu\text{g ml}^{-1}$) of lysine did not change either the ATP hydrolysis or the initial proton pumping rate of the tonoplast vesicles in the standard proton pumping medium mentioned in Materials and methods (data not shown). When lysine was replaced by the same monomer equivalent concentration of poly(L-lysine) ($2 \mu\text{g ml}^{-1}$),

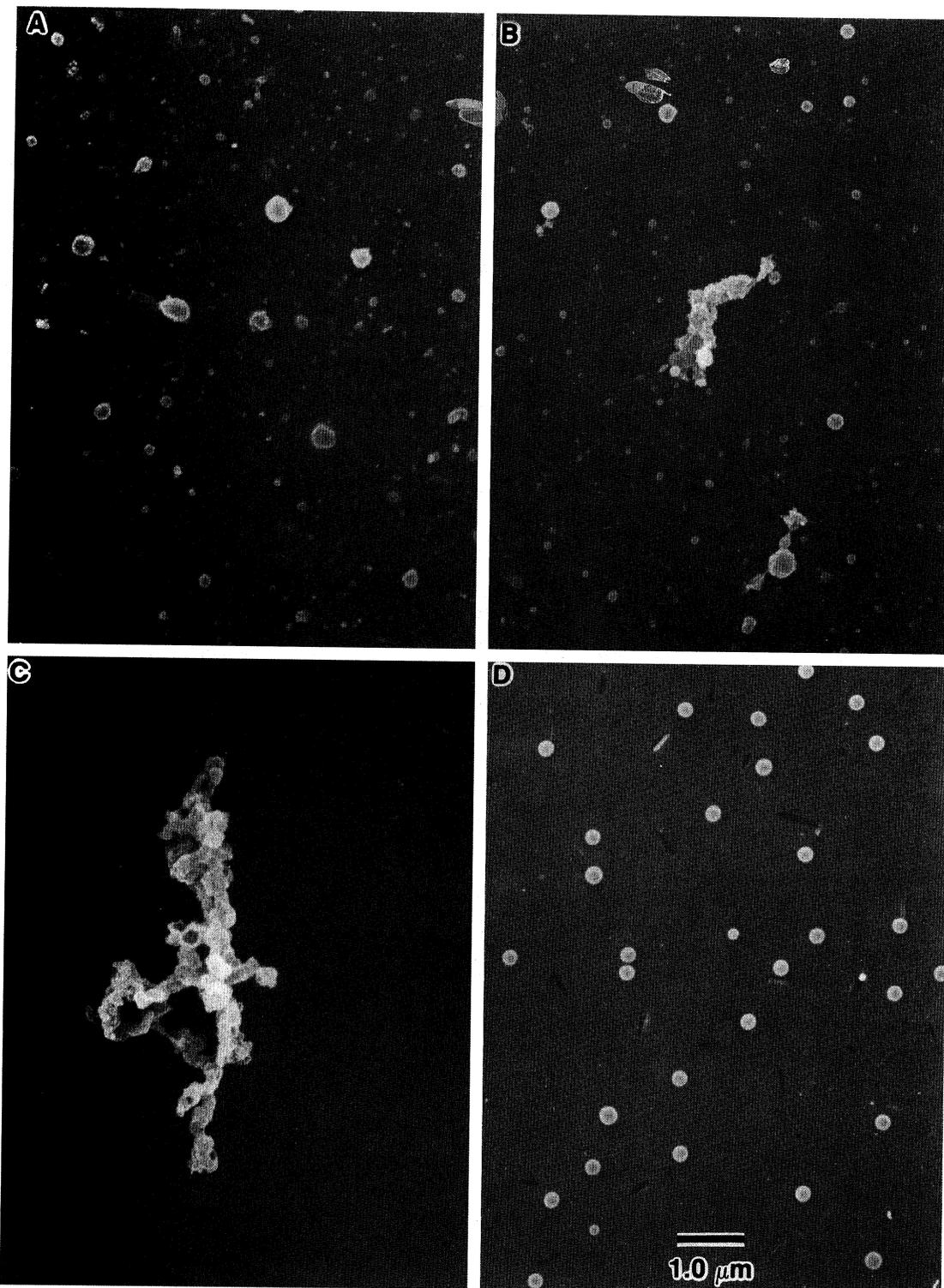


Fig. 2. SEM determinations of poly(L-lysine)-induced vesicle aggregation. The tonoplast vesicles ($11.16 \text{ mg protein ml}^{-1}$) were incubated with different concentrations of poly(L-lysine) in the standard proton pumping medium for 15 to 20 min at 22°C . The incubation time period was sufficient to essentially complete the light-scattering increase of the vesicles. The vesicle suspensions were then prepared and analyzed by SEM as described in Materials and methods. The size and shape of the suspending particles obtained in the presence of 0, 0.2 and $2.0 \text{ } \mu\text{g}$ poly(L-lysine) per ml, or 0.0, 0.027 and $0.10 \text{ } \mu\text{g}$ of poly(L-lysine) per μg of tonoplast protein, are shown in A, B and C, respectively. Standard polystyrene beads ($0.11 \text{ } \mu\text{m}$ in radius) were treated in the same manner and are shown in D for comparison.

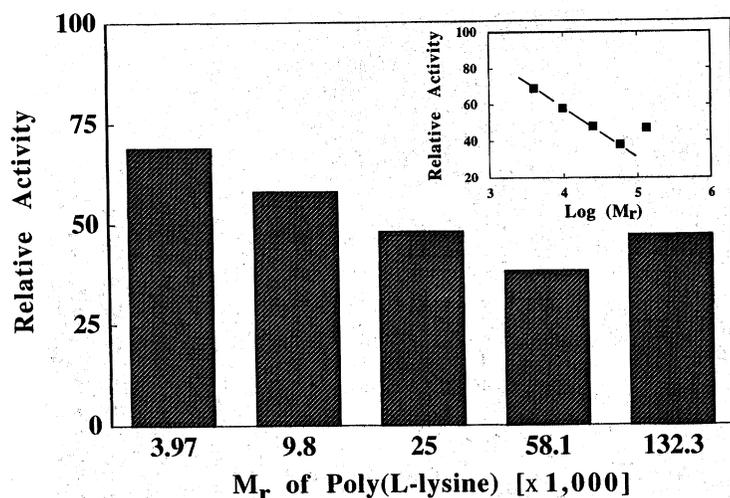


Fig. 3. Effects of the size of poly(L-lysine) on proton pumping in corn root tonoplast vesicles. The tonoplast vesicles (67 μg of protein) were incubated with 4 μg of poly(L-lysine) with molecular weights ranging from 3 970 to 132 300 in 2 ml of the standard proton pumping solution for 20 min at room temperature before the addition of 20 μl of 0.2 M ATP. The proton uptake was monitored by the absorbance change of AO at 492.5 nm. Data are plotted relative to the activities of vesicles without poly(L-lysine) treatment, which had an initial rate of 0.0352 ΔA ($\text{mg protein}^{-1} \text{min}^{-1}$). The data represent the average of 3 independent experiments with SD of $\pm 10\%$.

the proton pumping rate decreased as the apparent molecular mass of the poly(L-lysine) increased. However, the ATP hydrolysis activity was only slightly affected, showing an increase of about 10% (details described below). This result suggests that the binding of negative charges within a larger surface area is more effective in inhibiting the proton pumping activity. The inhibition reached a maximum with a molecular mass of poly(L-lysine) close to 58 kDa. The inhibition decreased for the poly(L-lysine) with a molecular mass of 132 kDa. It should be mentioned that the choice of logarithms of molecular mass as the x-axis values in Fig. 3 was primarily for convenience. Thus, no effort was made to define the physical significance of the linear relationship observed within the molecular mass range of 146 (lysine monomer) to 58 000 Da.

Two sets of control experiments were performed to assure that the responses of AO to proton gradients across the membrane were not significantly affected by the presence of poly(L-lysine). First, in the absence of tonoplast vesicles, the absorption properties of AO were not altered by the presence of poly(L-lysine) at different pHs. Second, the absorbance changes of the indicator to imposed pH gradients across tonoplast membrane vesicles (ΔA vs ΔpH) were not significantly affected by the presence of poly(L-lysine). This conclusion was obtained from experiments in which vesicles were incubated in the standard proton pumping assay solution at pH 5.3 for 15 min before the addition of calculated amounts of KOH to rapidly increase the pH values of the suspension. The induced rapid absorbance changes of AO were then compared.

Concentration dependence of the poly(L-lysine) inhibition

The inhibition of proton pumping increased as the concentration of the 58 kDa poly(L-lysine) increased (Fig. 4A). In Fig. 4A, the concentration of poly(L-lysine) is expressed as μg poly(L-lysine) per μg of tonoplast pro-

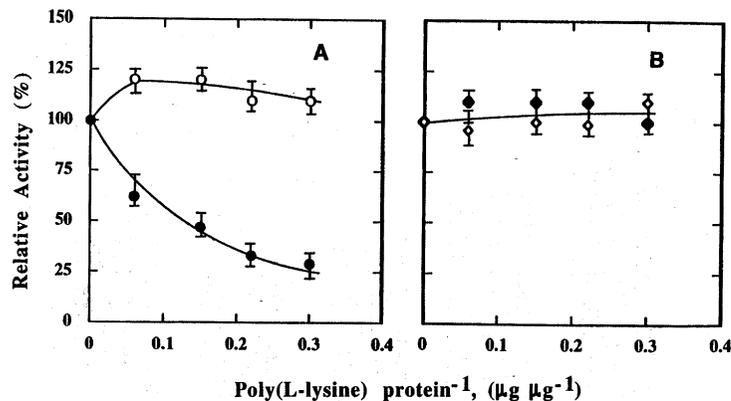
tein. The equivalent concentration of added poly(L-lysine) for experiments described in Fig. 4 may be calculated by multiplying the x-axis value by 33.33 to obtain $\mu\text{g ml}^{-1}$ of poly(L-lysine). Thus, the first data point 0.06 μg ($\mu\text{g protein}^{-1}$) is equivalent to 2 $\mu\text{g ml}^{-1}$. The data indicated that the proton pumping rate was decreased by 50% with the concentration of added poly(L-lysine) at 0.15 mg ($\mu\text{g membrane protein}^{-1}$). Within the concentration range, however, the ATP hydrolysis activity was stimulated slightly (10 to 20%). Thus, the aggregation of the vesicles did not affect the hydrolysis activity of the ATPase, implying that the enzyme did not itself directly bind poly(L-lysine).

Using the kinetic model described in Materials and methods, the proton leakage constants of both energized tonoplast membrane (k_1) and de-energized membrane (k_2) were found to be relatively insensitive to poly(L-lysine) treatment (Fig. 4B). As described, the interaction between poly(L-lysine) and the tonoplast membrane should be restricted only to the exterior surface. The observed insensitivity of the membrane proton leakage to poly(L-lysine) treatment seems to indicate that the possible lateral movement of the negatively charged membrane components (Clague and Cherry 1989) and the aggregation of the vesicles (Fig. 1) were not severe enough to change the nonspecific and passive proton leakage through the membrane. Thus, according to Eq. 4, the observed decrease in the initial proton pumping rate (Fig. 4A) should be the result of lowered values in m which is a measure on the extent of coupling between ATP hydrolysis and proton pumping of the H^+ -ATPase.

Effects of increasing KCl concentration on coupled activities

The effects of poly(L-lysine) on the tonoplast H^+ -ATPase presumably are due to its electrostatic binding to the vesicles. It is of interest to determine whether any specific binding is involved. If the observed poly(L-lysine) effects derive mainly from a nonspecific electrostatic in-

Fig. 4. Concentration dependence of poly(L-lysine) inhibition of ATP hydrolysis and proton pumping activities. A. Effects on the initial rates. Tonoplast vesicles (67 μg) were incubated with various amounts of 58 kDa poly(L-lysine) for 20 min at room temperature in 2 ml of the standard proton pumping solution. The reaction was initiated by the addition of 2 mM ATP. The proton uptake was monitored by the absorbance change of AO at 492.5 nm. The hydrolysis was stopped by the addition of 500 μl of ice-cold 25% (w/v) trichloroacetic acid. The amount of inorganic phosphate released was determined by the formation of the malachite green-molybdate complex. The proton pumping (filled circles) and ATP hydrolysis (open circles) activities obtained in the presence of poly(L-lysine) were plotted relative to the activities (assigned as 100) of untreated vesicles. The control values for proton pumping and ATP hydrolysis were 0.0219 ΔA (mg protein) $^{-1}$ min $^{-1}$ and 79.5 nmol (mg protein) $^{-1}$ min $^{-1}$, respectively. B. Effects on membrane proton leakage. The time courses of proton pumping in A were used to determine the proton leakage of both energized membrane, k_1 (open diamonds), and de-energized membrane, k_2 (filled diamonds). The proton pumping solution mentioned above was the same except for the addition of 5 mM glucose. Once the reaction reached steady state, hexokinase was added to determine the H^+ leakage rate of de-energized membrane, k_2 . The data were plotted relative to the activities of nontreated vesicles which exhibited values for k_1 and k_2 as 0.597 and 1.067 min $^{-1}$, respectively. The data represent the average \pm SD of 3 independent experiments with triplicated measurements.



teraction, then an increase in ionic strength should decrease the interaction and thus reduce the effects. On the other hand, an increase might be expected if the effects were derived from the strong binding of poly(L-lysine) to some specific sites. An increase in ionic strength could minimize the nonspecific weak binding and thus might increase the availability of poly(L-lysine) to the specific sites. In order to differentiate between these two possibilities, the effects of KCl on coupled activities in the absence of poly(L-lysine) were first analyzed.

It has been shown that chloride anions interact directly with plant root tonoplast H^+ -ATPase to activate ATP hydrolysis and proton pumping activities (Bennett and Spanswick 1983, Churchill and Sze 1984, Ward and Sze 1992). The enhancement in the pumping activity is not a result of converting the membrane potential portion of the $\Delta\mu_{\text{H}^+}$ into the proton gradient (Churchill and Sze 1984). The dependence of the two activities on KCl concentration in isotonic solutions is shown in Fig. 5. Under these experimental conditions, the ATP hydrolysis activity increased as the concentration of KCl increased, according to a simple saturation dependence, leveling off at about 75 mM KCl. The proton pumping activity also increased as the concentration of KCl increased. However, a more complex relationship between the pumping activity and the KCl concentration was observed. Furthermore, the extent of stimulation of the proton pumping was much greater than that found with the ATP hydrolysis. More than 90% of the total ATP hydrolyzing activity in our membrane preparations was bafilomycin A_1 or nitrate-sensitive indicating a minimal contamination of nontonoplast origin. Thus, this low level of nontonoplast ATPase contamination could not account for the lower stimulation of ATP hydrolysis activity by KCl. This difference in the response to KCl (Fig.

5) suggests that proton pumping and ATP hydrolysis are indirectly coupled.

It has been reported that the absorption of AO at 492.5 nm decreases by about 10% as the concentration

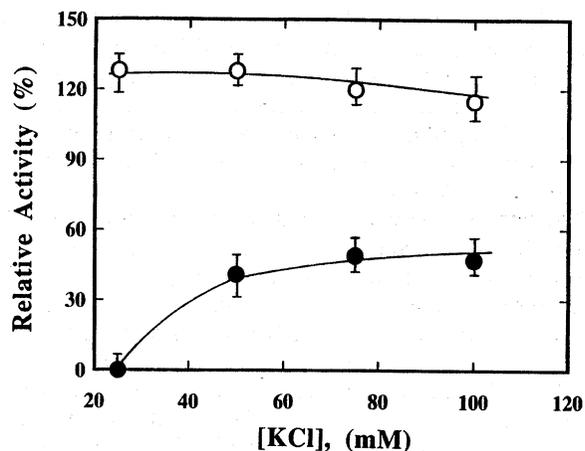


Fig. 5. Salt concentration dependence on polyelectrolyte inhibition of coupled activities. Tonoplast vesicles (67 μg) were treated with 6 μg of poly(L-lysine) (58 kDa) in 2 ml assay solution containing 20 mM MES-BTP, pH 6.45, 1 mM EGTA, 1.25 mM MgSO_4 , 10 μM AO, 0 to 100 mM KCl and 0 to 0.3 M sucrose for 20 min before the addition of 2 mM ATP, and assayed for proton pumping (filled circles) and ATP hydrolysis (open circles). The osmotic pressure was maintained constant in the assay media by adjusting the sucrose concentration. The conditions for measuring ATP hydrolysis and proton pumping were the same as in Fig. 3 unless specified otherwise. Data are plotted relative to the activities of untreated vesicles at 25, 50, 75 and 100 mM KCl which exhibited initial rates for proton pumping and ATP hydrolysis of 0.0089, 0.0149, 0.0261 and 0.0354 ΔA (mg protein) $^{-1}$ min $^{-1}$ and 45.6, 62.2, 83.1 and 77.4 nmol (mg protein) $^{-1}$ min $^{-1}$, respectively. The data represent the average (\pm SE) of 3 independent experiments with duplicated measurements.

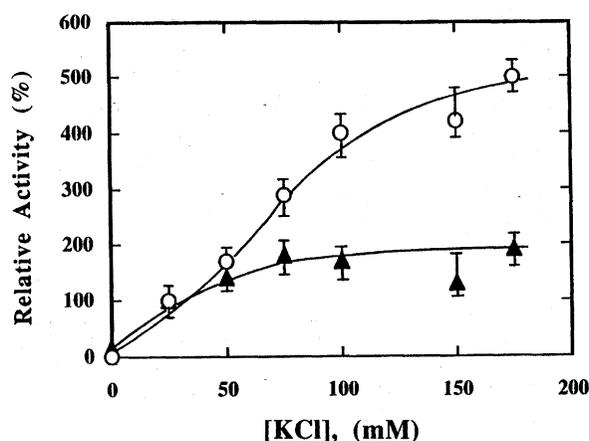


Fig. 6. The effect of KCl concentration on tonoplast ATPase activities. Vesicles (67 μg protein) were assayed for proton pumping (open circles) and ATP hydrolysis (filled triangles) at different concentrations of KCl from 0 to 175 mM. The assay conditions were the same as those for Fig. 4. Data are plotted relative to the initial rates obtained at 25 mM KCl (for proton pumping and ATP hydrolysis (0.0089 ΔA [mg protein] $^{-1}$ min $^{-1}$ and 45.6 nmol [mg protein] $^{-1}$ min $^{-1}$, respectively). The data represent the average (\pm SD) of 2 independent experiments with triplicated measurements.

of KCl increases from 0 to 200 mM (Palmgren 1991) in an unbuffered solution. We have measured the absorption spectra of the indicator in the solutions mentioned in Fig. 5 (without the addition of ATP). The results indicated that the absorbance at 492.5 nm essentially remained unchanged (data not shown). To maintain the electric neutrality, the positive charges of pumped protons may be neutralized, in part, by a co-transport of chloride ions. Thus, it is possible that the accumulation of chloride and protons in the internal aqueous space of the vesicles led to the decrease of the absorption of AO after the initiation of the ATPase activity. However, the pH gradient generated by pumping in plant tonoplast

vesicles is normally not greater than 2 pH units (Sze 1985). Under the experimental conditions of Fig. 5, a change in internal pH from 6.45 to 4.45 would represent a change of 0.036 mM of protons. If chloride is the only anion to neutralize the protons, then the internal chloride concentration should also increase by 0.036 mM. Such a small change in the anion concentration should not appreciably change the absorption of the indicator. Thus, the observed absorption changes of AO mentioned in Fig. 5 reflect mainly changes in internal pH.

Effects of salt concentration on poly(L-lysine)

When KCl was used to increase the ionic content of the medium, the sucrose concentration was reduced to keep the osmolarity of the solution constant. The choice of isotonic conditions minimized the possible complications arising from changes in vesicle volume and water movement on the measurement of proton pumping. The effects of a fixed concentration of 58 kDa poly(L-lysine) (2 μg ml $^{-1}$) on the tonoplast H $^{+}$ -ATPase activities were determined (Fig. 6). In the figure, the activity observed in the presence of poly(L-lysine) was expressed relative to the activity determined in its absence. The ability of poly(L-lysine) to inhibit the proton pumping decreased as the concentration of KCl increased. The ATP hydrolysis activity exhibited a 20% stimulation independent of the salt concentration in the medium.

The results of Fig. 5 demonstrated that the tonoplast H $^{+}$ -ATPase was activated by KCl. However, the increase in KCl concentration increased both the ionic strength and chloride concentration of the media. To test the potential effects of ionic strength, experiments in Fig. 7 were performed. With the chloride concentration kept constant at 75 mM, the ionic strength of the isotonic solution was increased by substituting K $_2$ SO $_4$ for sucrose. The increase in K $_2$ SO $_4$ induced a gradual and equal decrease in both activities (Fig. 7A). This result suggested that the increase in K $_2$ SO $_4$ was likely to slow down ATP hydrolysis without affecting the coupling between ATP

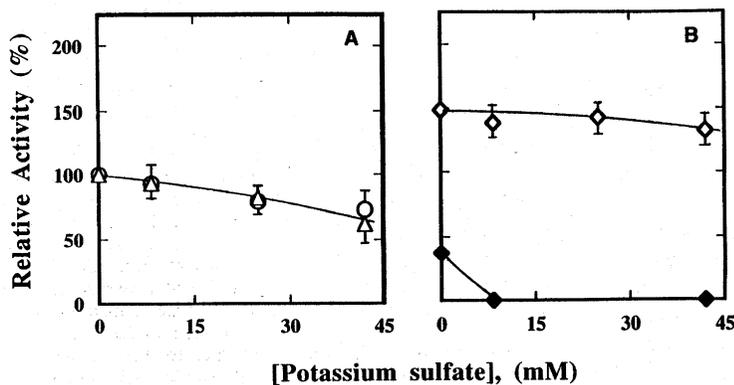


Fig. 7. The effect of K $_2$ SO $_4$ concentration on tonoplast coupled activities. A. Untreated vesicles (67 μg protein) were incubated in 2 ml of assay solution containing 20 mM MES-BTP, pH 6.45, 1 mM EGTA, 1.25 mM MgSO $_4$, 10 μM AO, 75 mM KCl, 0 to 42 mM K $_2$ SO $_4$, and 0 to 0.3 M sucrose for 20 min before the addition of 2 mM ATP. The osmotic pressure was maintained constant in the assay media by adjusting the sucrose concentration. Vesicles were assayed for proton pumping (open triangles) and ATP hydrolysis (open circles). B. Tonoplast vesicles (67 μg protein) were treated with 6 μg of 58 kDa poly(L-lysine) and assayed for proton pumping (filled diamonds) and ATP hydrolysis (open diamonds) under the same conditions as mentioned above. Data are plotted relative to the untreated tonoplast vesicles in 75 mM KCl. The data represent the average (\pm SE) of 2 independent experiments with triplicated measurements.

hydrolysis and proton pumping. Thus, the stimulation described in Fig. 5 was mainly due to the increase in chloride concentration. The efficacy of poly(L-lysine) in isotonic solutions containing 75 mM, chloride but different concentrations of K_2SO_4 was determined (Fig. 7B). The inhibition of proton pumping by poly(L-lysine) was greatly increased by K_2SO_4 (see Fig. 4 for comparison). The slight enhancement of ATP hydrolysis by poly(L-lysine) in KCl media was significantly increased by the addition of K_2SO_4 (from no more than 30% to almost 50%). The amplification of the effects of poly(L-lysine) on proton pumping by increasing K_2SO_4 suggests that the strong binding of poly(L-lysine) to specific sites on the surface of tonoplast vesicles may be the basis for the observed effects on this H^+ -ATPase activity. Alternatively, K_2SO_4 may expose additional sites for poly(L-lysine) binding.

Discussion

In the present work, we investigated the effects of poly(L-lysine) binding on the activities of the tonoplast H^+ -ATPase. While the main objective of the study was to investigate the coupling mechanism of the ATPase, the observed effects on the membrane may be useful for elucidating the physiological roles of naturally occurring polyamines like spermine and spermidine in plants. As described in our earlier report (Tu et al. 1987), tonoplast vesicles capable of pumping protons should retain the *in vivo* membrane orientation. Thus, poly(L-lysine) exerts its effects by interacting with the charged groups residing on the cytoplasmic surface of the tonoplast membrane. The electron microscope investigation showed that the interaction led to an aggregation but not a fusion of the membrane vesicles. The aggregation of the vesicles slightly enhanced the ATP hydrolysis activity of the tonoplast H^+ -ATPase suggesting that the accessibility of the active site(s) was not hindered by the treatment. As proposed by Clague and Cherry (1989) for a similar observation with red blood cells, the aggregation may involve the lateral clustering of negatively charged membrane proteins and phospholipids. Like those found in other eukaryotic systems (Forgac 1989), the multimeric plant tonoplast H^+ -ATPase is composed of a peripheral (cytoplasmic) sector containing the ATP hydrolytic site and a membrane integral sector that forms the proton transport pathways (Sze et al. 1992). If the poly(L-lysine) binding and clustering involve a subunit which is responsible for a conformational coupling between ATP hydrolysis and H^+ -pumping, then the coupling between these two activities of the enzyme may be disrupted. The indirect coupling concept (Tu et al. 1992) could easily accommodate the decoupling described in Fig. 4. The charge neutralization may also increase the accessibility of negatively charged ATP to its catalytic site(s) and thus enhance ATP hydrolysis by the tonoplast ATPase.

While the decoupling effect allows the occurrence of

ATP hydrolysis without activating the proton pumping, other possibilities also exist. The involvement of charged groups in the architecture of the proton conduction pathway has been suggested for bacteriorhodopsin, a transmembrane proton pump (Henderson et al. 1990, Chou 1993). The arrangement of charged groups may also influence the proton movement along the membrane surface (Tocanne and Teissié 1990). Thus, if some of these components either are directly involved in the proton pumping or indirectly affect the integrity of the specific pumping pathway, then the cluster formation and charge neutralization caused by poly(L-lysine) binding would be expected to perturb the proton pumping efficiency. The interaction between poly(L-lysine) and the membrane negative charges could result in the formation of an extensive network of salt bridges, e.g. linkage of $-NH_3^+-OOC^-$. The salt bridges could provide alternative pathways or short-circuit the normal energized transmembrane proton flow supported by ATP hydrolysis. In a sense, the binding of poly(L-lysine) may be considered as creating localized protonophoretic pathways on the external surface of the membrane vesicles. Since protons never really enter the internal aqueous space, a change in the transmembrane proton leakage characteristics (k_1 and k_2) is not expected.

The effects of poly(L-lysine) on the tonoplast H^+ -ATPase activities appear to be enhanced by an increase in ionic strength (Fig. 7). This suggests that the functionally effective binding of poly(L-lysine) occurs at certain specific molecular sites on the cytoplasmic surface of the tonoplast membrane. The positive charges carried by poly(L-lysine) allow the peptide to interact with multiple negative charges; the interactions may include some weak and nonspecific attractions. An increase in ionic strength by the addition of K_2SO_4 can screen out these weak electrostatic interactions and thus increase the effective concentration of poly(L-lysine) for the specific sites.

The influence of KCl concentration on the coupled activities of corn root tonoplast H^+ -ATPase confirms the notion that the activation of tonoplast H^+ -ATPase is dependent on the availability of chloride anion (Bennett and Spanswick 1983, Churchill and Sze 1984, Ward and Sze 1992). The increase in ionic strength alone brought by higher concentrations of KCl does not explain the activation since K_2SO_4 substitution did not mimic the effect of KCl. The fact that chloride activation decreases the extent of inhibition of proton pumping by poly(L-lysine) (Fig. 5A) suggests that the anion may induce rearrangements of certain surface negative charges that are involved in regulating the proton pumping process. In conclusion, the present study indicates that the coupled activities of corn root tonoplast H^+ -ATPase may be differentially affected by poly(L-lysine) and chloride anion. These results are consistent with the hypothesis that a rigid and direct coupling between ATP hydrolysis and proton pumping may not be an essential feature of the tonoplast H^+ -ATPase from corn roots (Tu et al. 1990).

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