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Salt Stress-Induced Cytoplasmic Acidification and Vacuolar Alkalization in *Nitellopsis obtusa* Cells[1]

In Vivo ^{31}P-Nuclear Magnetic Resonance Study

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ABSTRACT

Time courses of cytoplasmic and vacuolar pH changes under salt stress were monitored by *in vivo* ^{31}P-nuclear magnetic resonance spectroscopy in intact cells of *Nitellopsis obtusa*. When cells were treated with 100 millimolar NaCl for 2 hours, the cytoplasmic pH deceased from 7.2 to 7.0, while the vacuolar pH increased from 4.9 to 5.2. This salt-induced breakdown of the pH gradient between the cytoplasm and the vacuole was also confirmed through direct measurements of change in vacuolar pH with a micro-pH electrode. We speculate that the intracellular pH changes induced by the salt stress mainly results from the inhibition of the H^+-translocating pyrophosphatase in the vacuolar membrane, since this H^+-translocating system is sensitive to salt-induced increase in the cytoplasmic [Na^+] and a simultaneous decrease in the cytoplasmic [K^+]. Since disturbance of the cytoplasmic pH value should have serious consequences on the homeostasis of living cells, we propose that the salt-induced intracellular pH changes are one of initial and important steps that lead to cell death.

Many plants including most crops are sensitive to salt stress (normally NaCl). The mechanisms of salt injury and salt tolerance of whole plants have been intensively studied (see 5 for review), whereas studies at the cellular, organellar, and molecular levels are limited (3, 10). A fresh water species of Characeae, *Nitellopsis obtusa*, grown in fresh water, is, like many higher plants, sensitive to salt stress (6). Treatment of internodal cells of *Nitellopsis* with 100 mM NaCl results in a rapid membrane depolarization amounting to 140 mV and a large increase in membrane conductance. After 20 min, about half of the cytoplasmic K^+ is exchanged for Na^+. Cell death ensues after 2 to 8 h. Although long-lasting abnormal ionic conditions in the cytoplasm may be critical for cell survival, changes following the abnormal ion distribution should be sought in order to understand the cellular events that lead to salt injury.

Intracellular compartmentation is important for metabolic regulation in living cells (8). It is possible that salt stress disturbs normal compartmentation of ions and low mol wt organic solutes. *In vivo* ^{31}P-NMR spectroscopy allows the noninvasive measurement of physiological information (pH, concentration of phosphorous compounds, etc.; 16) from different intracellular compartments, including the cytoplasm and vacuoles in higher plant cells (17) and algae (7). Recently Kuchitsu *et al.* (9) measured cytoplasmic and vacuolar pH in a salt-tolerant unicellular green alga *Dunaliella* with this technique. They reported that the salt-stress induced cytoplasmic alkalization in *Dunaliella*, and discussed that the cytoplasmic alkalization acts as one of the key factors in osmoregulation by regulating the metabolism of the osmoticum, glycerol. It is also of importance to monitor intracellular pH values after salt stress in a salt sensitive alga, *Nitellopsis*, in comparison with the salt tolerant alga. This technique was applied to *Nitellopsis* cells first by Mimura and Kirino (12). However, due to the low field strength of the magnet, resolution was not very good. In the present study, we succeeded in obtaining better NMR spectra with higher S/N ratio over a much shorter accumulation time. This allowed us to measure the intracellular pH values with high accuracy and to monitor the time courses of changes in cytoplasmic and vacuolar pH after the salt stress.

MATERIALS AND METHODS

Plant Materials and Cell Preparation for NMR Measurement

Young internodal cells of *Nitellopsis obtusa* growing in fresh water were prepared as described previously (6). Calcium carbonate deposits on the cell wall were removed by shaking the cells in an acidic medium, APW[2] supplemented with 5mM Mes-Tris (pH 5). APW contains 0.1 mM each of KCl, NaCl, and $CaCl_2$. The lengths of the cells used for NMR measurements were adjusted to 5 to 6 cm by ligating long cells with strips of polyester thread. Before the NMR measurement, ligated cells were incubated in APW supplemented with 10 mM Hepes-Tris (pH 7.5) overnight under continuous illumination (20 μmol m^{-2} s^{-1}) at 23°C. About 150 cells were placed

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[2] Abbreviations: APW, artificial pond water; MDP, methylene diphosphonic acid; ACyS, artificial cytoplasmic salt solution; AVS, artificial vacuolar sap; PPase, pyrophosphatase; S/N, signal to noise.

in an NMR tube (15 mm diameter, purchased from Wilmad Glass Co., Inc., Buena, NJ). Cells were washed several times with fresh APW (pH 7.5) and immersed in the same solution. The external solution was changed to APW (pH 7.5) containing 100 mM NaCl (termed Na-APW) to induce salt stress.

Preparation of Perchloric Acid Extract

About 5 g (fresh weight) of cells were frozen with liquid nitrogen and ground in perchloric acid (final 2 M) for 2 h on ice. The suspension was neutralized with $KHCO_3$, centrifuged at 8000 *g* for 10 min at 0°C to remove the $KClO_4$ precipitate, and the pH was adjusted to 6.5. To avoid the broadening of NMR signals by paramagnetic ions, EDTA (potassium salt, final 2 mM) was added to the extract before the NMR measurement.

^{31}P-NMR Measurements

^{31}P-NMR spectra were measured with a JEOL GX 400 spectrometer operating at 162.2 MHz in a pulsed Fourier transform mode with the NMR tube spinning, without proton decoupling and unlocked at 23°C (7, 9). The pulse angle was 45° and the pulse repetition time was 0.54 s. The digital resolution was 0.018 ppm at the measurement. The number of scans are shown in each figure legend. The chemical shift was expressed in ppm relative to 1.8% (w/v) MDP (free acid, purchased from Sigma) solution. It was sealed in a glass capillary tube and measured simultaneously with each sample (7, 9).

Intracellular pH Measurement with *in Vivo* ^{31}P-NMR

For obtaining pH calibration curves, chemical shifts of Pi signals under various pH values were measured in ACyS (12), Na-ACyS (Na in ACyS was substituted for K), and AVS (Table I). Na-ACyS mimics the cytoplasmic ion composition in salt-stressed cells (6). Ionic composition in the vacuole does not change within 1 h after salt stress (6). The pH values of the cytoplasm in control and salt-stressed cells, and that of the vacuole were determined by using the calibration curves with ACys, Na-ACyS and AVS, respectively.

Direct Measurement of Vacuolar pH with Micro-pH Electrode

Cells in APW (pH 7.5) and Na-APW were washed with distilled water and 200 mM sorbitol, respectively. Both cell ends were cut on a Plexiglas bench, and the vacuole was perfused with an unbuffered solution containing 25 mM K_2SO_4, 25 mM KCl, 5 mM $MgCl_2$, and 100 mM sorbitol using the vacuolar perfusion technique (26). The first 25 μL of exudate was collected as the cell sap sample with a glass microcapillary and directly measured with a micro-pH electrode (Micro combination pH probe, Microelectrodes Inc., Londonderry, NH).

Table I. *Composition of the Media Used for pH Titration of Pi*

pH was adjusted with either KOH (ACyS and AVS) or NaOH (Na-ACyS).

Medium	ACyS	Na-ACyS	AVS
		mM	
H_3PO_4	10	10	5
K_2SO_4	25	0	0
Na_2SO_4	0	25	0
KCl	25	0	80
NaCl	0	25	60
$MgCl_2$	5	5	5
$CaCl_2$	0	0	5

Extraction and Determination of ATP

Cells were frozen with liquid nitrogen before and after the salt stress. ATP was extracted from frozen cells by boiling for 5 min in a buffer solution containing 25 mM K-Hepes (pH 7.4), 10 mM EDTA, and 0.3% H_2O_2 (11). H_2O_2 was used as an inhibitor of adenylate kinase. The ATP assay medium contained, as final concentrations, 27.5 mM K-Hepes (pH 7.4), 25 mM $MgSO_4$, 10 mM K_2SO_4, and 2 mM EDTA. ATP was determined by the firefly-flash method with an ATP photometer (Chemglow photometer J4-7441, American Instrument Co., Silver Spring, MD). Since it is known that ATP is absent in the vacuole in Characean cells, we can calculate the cytoplasmic concentration of ATP if we know the cytoplasmic volume. The cytoplasmic volume was determined with a newly improved method using a membrane impermeable dye, Lucifer yellow CH (24). After the vacuolar perfusion of an internodal cell with AVS containing 2.5 mM Lucifer yellow CH, the cell content (the vacuolar sap and the cytoplasm) was squeezed out with a small Teflon bar. The dilution of Lucifer yellow CH was determined spectrophotometrically. The dilution index represents the relative volume of the vacuole to the total cell volume. The cytoplasmic volume can be calculated as the extravacuolar space.

Extraction and Determination of PPi

Extraction and determination of PPi were described elsewhere in detail (24). Internodal cells (0.6–0.8 g) were frozen in liquid nitrogen and ground into a powder in a mortar. The powder was transferred into 20% (w/v) TCA solution to denature enzymes. The extract was then centrifuged at 15,000*g* for 10 min at 0°C. The pellet was resuspended in water and centrifuged again. The first and second supernatants were combined and the pH of the solution was carefully adjusted to between 10.0 and 10.5 with Tris and KOH. Then 1 M $CaCl_2$ and 0.25 M K_2CO_3 solutions were added. The resulting precipitate of $CaCO_3$ functions as a coprecipitant for $Ca_2P_2O_7$. The solution was kept on ice for 15 to 30 min and centrifuged at 15,000*g* for 10 min at 0°C. The supernatant was discarded and the pellet was washed twice with water and dissolved into a small amount of 1 N HCl.

The PPi present in the samples was determined enzymically using a commercial pyrophosphate assay kit (Sigma Chemical Co., P7275). In addition, 400 mM Hepes-KOH (pH 7.5), 15 mM ascorbate, and 25 mM EGTA were added to the assay mixture. The reaction was initiated by adding an aliquot of sample, and the oxidation of NADH was measured spectrophotometrically at 340 nm using a spectrophotometer (Hitachi 220A).

RESULTS

Calibration Curve for Intracellular pH Measurement

Figure 1 shows the calibration curve for pH *versus* the chemical shift between the Pi signal and the MDP signal. There was no difference between the curves obtained using ACyS and Na-ACyS. Thus, the substitution of Na^+ for K^+ in the cytoplasm in salt-stressed cells was assumed to have no influence on the measurement of intracellular pH. There was some difference between the curves generated using ACyS and AVS. This may be a result of the difference in their ionic strength (18).

NMR Spectra of Intact Cells and Perchloric Acid Extract

Figure 2a shows a typical NMR spectrum of intact cells. There are two signals in the Pi region. The spectrum of the perchloric acid extract (Fig. 2b) shows a single signal in the Pi region, while the pattern for the other signals are similar, indicating that the two Pi signals represent Pi in two distinctive intracellular compartments. Considering that the vacuole occupies more than 90% of the total cell volume, we conclude that the large Pi signal, which is in a position approximately equivalent to pH 5, comes from the Pi in the vacuole, and we assigned the smaller signal (pH 7.2) to cytoplasmic Pi. The ratio of the signal intensity between the cytoplasmic and vacuolar Pi in *Nitellopsis* was about 1:10. This reflects the large relative volume of the vacuole compared to the cytoplasm in *Nitellopsis* in contrast to the case in microalgae with smaller vacuoles (7, 9). In addition to the Pi signals, we also recognized small signals for probable sugar-phosphates (SP in Fig. 2) and nucleotide triphosphates, where no polyphosphate signals were detected (data not shown).

The spectrum of cells treated with Na-APW for a long time (6 h) showed only one signal in the Pi region, indicating the destruction of intracellular compartmentation or loss of Pi from the cytoplasm (data not shown).

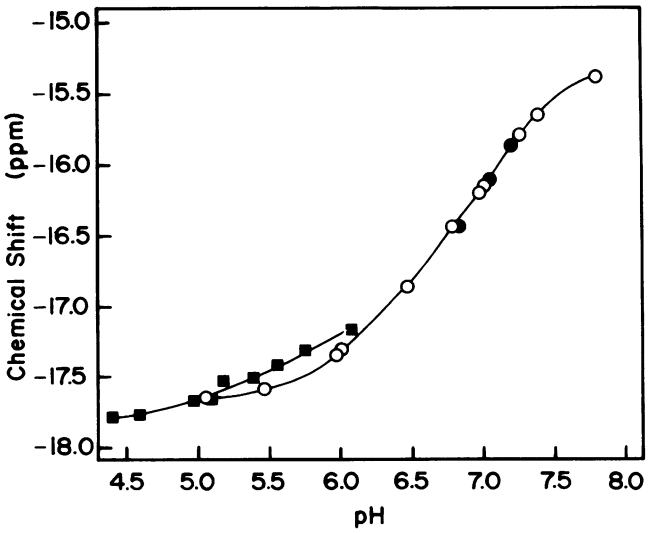

Figure 1. Titration of Pi with pH changes in the presence of artificial cytoplasmic salt solution (ACyS, ○), ACyS substituting Na^+ for K^+ (Na-ACyS, ●), or artificial vacuolar sap (AVS, ■). The chemical shift relative to 1.8% (w/v) MDP signal was expressed in ppm.

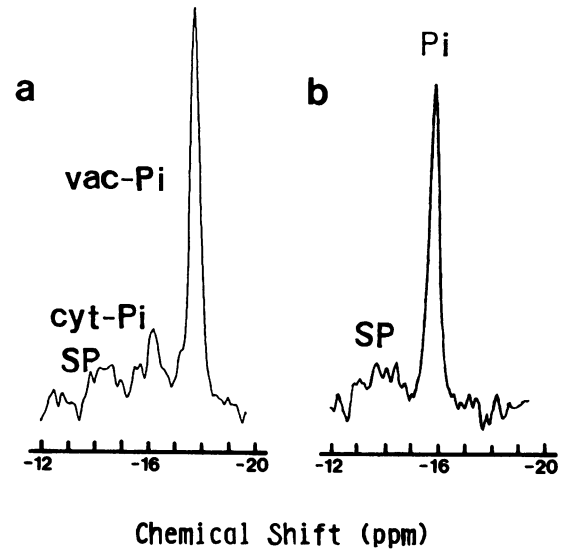

Figure 2. ^{31}P-NMR spectra of intact *Nitellopsis* cells in APW (pH 7.5) after 10,000 scans (a) and the perchloric acid extract after 1,000 scans (b). Chemical shift was expressed in ppm relative to the MDP signal.

Effects of Salt Stress on Intracellular pH

The chemical shift of the cytoplasmic Pi decreased and that of the vacuolar Pi increased after the salt stress, indicating that a cytoplasmic acidification and a vacuolar alkalization occurred. Figure 3 shows a typical time course of the change in the cytoplasmic pH. The pH value dropped from 7.19 to 7.01 after 1 h of the salt stress. By 2 h after the stress, the cytoplasmic pH approached 6.9. By contrast, the vacuolar pH increased. The effects of the salt stress on the intracellular pH values measured by ^{31}P-NMR are summarized in Table II. The salt stress induced both a cytoplasmic acidification and a vacuolar alkalization. The cytoplasmic and vacuolar pH values of control cells showed no significant difference between just after cells were placed in an NMR tube (5000 scans) and after 1 or 2 h. After more than 2 h, the cytoplasmic pH decreased slightly by about 0.10 pH unit in some control cells. Significant decrease in the cytoplasmic pH in the salt-stressed cells for both 1 and 2 h in comparison with the control cells were statistically confirmed with *t*-test ($P < 0.05$). As for the alkalization of the vacuole, *t*-test showed that probability of difference was $0.05 < P < 0.1$.

In measuring the vacuolar pH with a micro-pH electrode, we avoided contamination by the cytoplasm while collecting the cell sap, since such contamination would cause an alkaline shift of the vacuolar pH. The vacuolar pH in control cells was 5.0 ± 0.2 (SD, $n = 8$). It increased to 5.5 ± 0.3 ($n = 5$) 4 h after the salt stress. Significant difference between those pH values were statistically confirmed with *t*-test ($P < 0.05$).

Effects of Salt Stress on Intracellular ATP and PPi Levels

The cytoplasm occupied 7.9% of the total cell volume. Using this value, the concentration of cytoplasmic ATP was

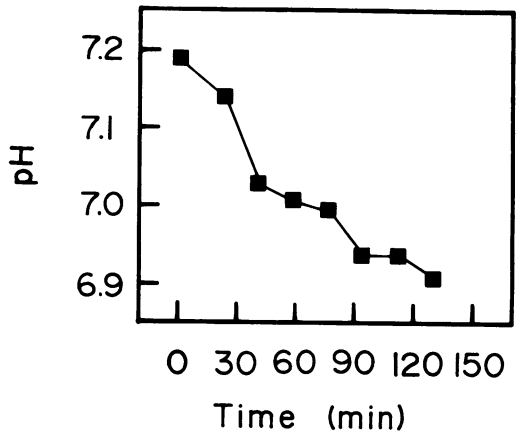

Figure 3. A typical time course of the cytoplasmic pH change in salt-stressed cells of *Nitellopsis* measured by *in vivo* ^{31}P-NMR. The external medium was changed from APW (pH 7.5) to Na-APW (see "Materials and Methods") at time 0. Each point represents the pH value estimated after 5000 scans by the method as follows: Elemental 1000 scans were subsequently measured. After the whole measurement, five successive elemental scans were stacked and analyzed by an operational computer. By shifting the beginning of five successive elemental scans every one or two elemental scans, we monitored the pH changes in shorter interval. The datum at time 0 in salt-stressed cells shows the pH after 5000 scans in the same sample in APW (pH 7.5), before the salt stress.

Table II. *Effects of Salt Stress on Cytoplasmic and Vacuolar pH Values in Internodal Cells of Nitellopsis*

The pH values were determined from ^{31}P-NMR spectra after 5000 scans. Each value represents the mean of three experiments ± SE. Control cells were bathed in APW (pH 7.5) in NMR tubes for 1 or 2 h. Salt stressed-cells were treated with Na-APW in NMR tubes for 1 or 2 h.

	Time	Control	Salt Stress
	h	*pH ± SE*	
Cytoplasmic pH	1	7.23 ± 0.05	7.10 ± 0.06
	2	7.20 ± 0.03	7.04 ± 0.08
Vacuolar pH	1	4.85 ± 0.08	5.00 ± 0.09
	2	5.01 ± 0.11	5.21 ± 0.11

calculated. It remained almost constant after 1 h of the salt stress, but decreased to 64% of the control after 2 and 3 h (Table III). Thus, the ATP decrease occurred later than the changes in cytoplasmic and vacuolar pH which were observed soon after the salt stress (Fig. 3; Table II).

The amount of PPi as well as ATP in the vacuole was much less than that in the cytoplasm (24). The cytoplasmic PPi level remained constant at around 100 to 150 μM after 3 h of the salt stress (Fig. 4).

DISCUSSION

When internodal cells of *Nitellopsis* were treated with 100 mM NaCl, cytoplasmic acidification and vacuolar alkalization were observed with *in vivo* ^{31}P-NMR spectroscopy. The latter was also confirmed by the direct measurement with a micro-pH electrode.

The ratio of the Pi content between the cytoplasm and the

Table III. *Effect of Salt Stress on Cytoplasmic ATP Level ([ATP]$_c$) in Internodal Cells of Nitellopsis*

[ATP]$_c$			
Control[a]	Duration of salt stress (h)		
	1	2	3
	mM ± SE		
2.5 ± 0.2[b] (100%)	2.4 ± 0.2 (96%)	1.6 ± 0.2 (64%)	1.6 ± 0.1 (64%)

[a] Control cells were incubated in APW (pH 7.5). [b] The values represent the mean of six cells ± SE.

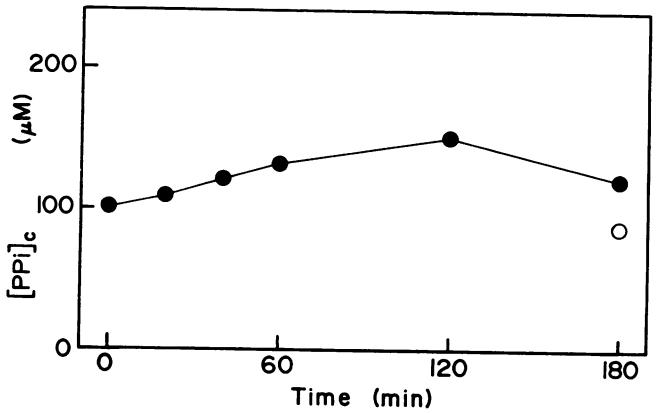

Figure 4. Time course of the change in cytoplasmic PPi concentration. Each point represents the mean of duplicate samples. The external medium was changed from APW (pH 7.5) to Na-APW (●) or APW containing 180 mM sorbitol (○) at time 0.

vacuole (about 1:10) remained constant for 2 h during the salt stress. This value agrees with the ratio calculated from previous data; the volume ratio is about 1:20 and the ratio of Pi concentration is about 2:1 between the cytoplasm and the vacuole (12).

Although pH changes are induced by anaerobiosis in many plant cells (7), Characean cells in an NMR tube seemed not to be hypoxic because of their low metabolic activity. Active cytoplasmic streaming showed no changes during the 2 h NMR measurement, indicating that enough ATP remained in the cytoplasm. Furthermore, Characean cells are tolerant against the low oxygen condition as demonstrated by the fact that the electrogenic H^+ pump activity remains even when most dissolved oxygen is driven out by bubbling with N_2-CO_2 gas (4). Actually, the cytoplasmic pH showed almost no changes during the 2 h treatment in APW (pH 7.5) (control) (Table II). These facts, as well as the statistical analysis of the data shown in Table II, indicate that the salt-induced cytoplasmic acidification is not due to anaerobiosis.

The deviation of the pH values shown in Table II implies both the errors implicit in the techniques and the variations between culture batches. Digital resolution of the signal in this system did not limit accuracy of pH measurement. The deviation of the estimated cytoplasmic pH values in the same cells was ±0.04. In every experiment, a rapid cytoplasmic acidification was induced by the salt stress with smooth curve with a little deviation of the data as shown in Figure 3. Its

time courses, however, varied between the culture batches because of the variation in their salt sensitivity. This resulted in larger deviations when the period of the salt stress was longer (Table II). In spite of the high S/N ratio of the vacuolar Pi, the measurement error of the vacuolar pH is larger than that of the cytoplasmic pH (Table II) because of the gentle slope of the pH titration curve in the low pH region (Fig. 1). However, it is to be noted that the vacuolar alkalization under the salt stress was observed without exception in all experiments with *in vivo* NMR as well as the direct pH measurement.

In contrast to Characean cells, Ben-Hayyim and Navon (1) found that the cytoplasmic and the vacuolar pH values in both wild-type and NaCl-tolerant *Citrus* cultured cells were rather constant under salt stress conditions. The difference between our results and theirs may be due to differences in the salt sensitivity between the two genera. Under our experimental conditions, *Nitellopsis* cells die within a day after application of salt stress. By contrast, *Citrus* cells continue to grow, although at a reduced rate under a similar stress.

The cytoplasmic buffering capacity of Characean cells is about 12 mmol H^+/pH unit/L cytoplasm at pH 7 (25). The acidification of the cytoplasm by 0.16 pH units after 2 h salt stress (Table II) means that H^+ in the cytoplasm increased by 1.5×10^{-9} mol (assuming that the cell is 0.5 mm in diameter and 50 mm in length, and that the cytoplasm occupies 7.9% of the total cell volume). The vacuolar sap of *Chara corallina* has a buffer capacity of 0.7 mmol H^+/pH unit/L vacuolar sap (25). Assuming the same buffer capacity for *Nitellopsis*, an increase in 0.20 pH unit during salt stress is equivalent to a decrease in 1.3×10^{-9} mol H^+ from the vacuole. These results suggest that the component generating the pH gradient across the vacuolar membrane (tonoplast) is inhibited under salt stress. Two distinct H^+-pumps are known to be present in the tonoplast of both higher plants (2, 15, 27) and Characean cells (19, 22). One is the H^+-ATPase driven by ATP (for review, see ref. 21) and the other is the H^+-PPase driven by PPi. The PPi-dependent H^+-translocating activity is comparable to the ATP-dependent activity in both higher plants (2, 15) and *Chara* (23). In tonoplast vesicles, the PPi-dependent H^+-transport is stimulated by KCl with a K_m of 20 mM, and inhibited by 80% by 80 mM NaCl in the presence of 50 mM KCl (22). Treating cells with 100 mM NaCl causes a drastic decrease in cytoplasmic K^+ from 80 to 30 mM and a marked increase in cytoplasmic Na^+ from 12 to 90 mM (6). Thus, the PPi-dependent H^+-transport across the tonoplast is probably inhibited by the changes in ion distribution induced by the salt treatment. On the other hand, the ATP-dependent H^+-transport in tonoplast vesicles from *Chara* is relatively insensitive to cations: the substitution of 50 mM of NaCl for 50 mM of KCl inhibits the ATP-dependent H^+-transport activity only by 12% (22). K_m value for the ATP-dependent H^+-transport into tonoplast vesicles is less than 1 mM *in vitro* (K Takeshige, unpublished data). Although we do not know the value of K_m *in vivo*, the intracellular ATP concentration during the salt stress (Table III) seemed to be much higher than the K_m value discussed above. So ATP-dependent H^+-transport across the tonoplast would hardly be inhibited during salt stress. Therefore, we suggest that a breakdown of the pH gradient between the cytoplasm and the vacuole depends mainly on the inhibition of PPi-dependent H^+-transport. Since the cytoplasmic PPi level during the salt stress did not decrease (Fig. 4), the substrate level is not the limiting factor for the PPi-dependent H^+-transport under salt stress. The present results support the idea that the H^+-translocating PPase in the tonoplast is functioning and essential in keeping the H^+-gradient in addition to the H^+-ATPase *in vivo*.

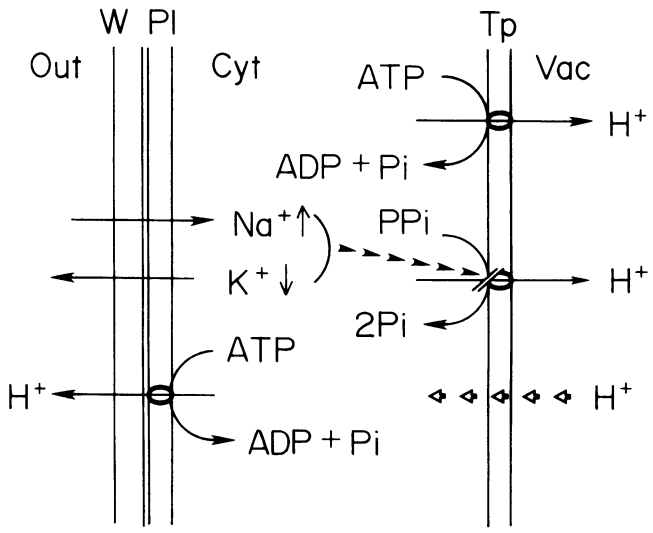

Figure 5. A possible mechanism of the salt-induced intracellular pH changes in *Nitellopsis* cells. After salt stress, [Na^+] increases and [K^+] decreases in the cytosol. The H^+-translocating pyrophosphatase is inhibited under such ionic conditions. W, Cell wall; Pl, plasmamembrane; Tp, tonoplast. ⇐ represents passive H^+ movement.

Figure 5 summarizes the above considerations. Moriyasu *et al.* (13) estimated the passive H^+ efflux rate from the vacuole to the cytoplasm to be 3×10^{-7} mol m^{-2} s^{-1}. This means that the salt-induced increase in cytoplasmic H^+ represents only 1% of the passive proton leakage. In other words, even under salt-stressed condition, 99% of the H^+ leaked out of the vacuole are transported back into the vacuole by the H^+-translocating ATPase.

The cytoplasmic acidification caused by the salt stress may also be partially explained by an inhibition of the biochemical pH-stat (20). For example, malate dehydrogenase, which may play an important role in pH homeostasis, is inhibited by high concentrations of NaCl (14).

Since disturbances of the cytoplasmic and vacuolar pH values should have serious consequences on the metabolic regulation and the homeostasis of living cells, we propose that the salt-induced intracellular pH changes are one of initial and critical steps that lead to cell injury.

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