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Communication

Rapid Induction of Na^+/H^+ Exchange Activity in Barley Root Tonoplast[1]

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ABSTRACT

Na^+/H^+ exchange activity in barley (*Hordeum vulgare* cv CM-72) root tonoplast was induced by Na^+ even in the presence of inhibitors of protein synthesis. Induction occurred with a half-time of only 15 minutes. When salt-treated roots were transferred to a nutrient solution containing no Na^+, the activity disappeared with a similar time course. The data suggest that Na^+/H^+ exchange was due to activation of an existing protein rather than to *de novo* protein synthesis.

Buildup of salts in the soil is a major problem on irrigated farmland and in semiarid regions where winter rainfall is not sufficient to flush accumulated salts from the top soil (18). To grow in saline soils, plants must maintain a much lower ratio of Na^+/K^+ in their cytoplasm than is present in their surroundings (10, 13, 14). Multiple mechanisms, including morphological and biochemical adaptations, are probably involved in maintaining this low cytoplasmic Na^+/K^+ ratio. In barley (*Hordeum vulgare*), two such mechanisms are extrusion of Na^+ into the vacuole across the tonoplast membrane and extrusion of Na^+ into the external medium across the plasma membrane (13, 16). The energy for Na^+ extrusion could be provided by the proton gradients generated by the plasma membrane or tonoplast H^+-ATPases. Recently, using the tonoplast ATPase to generate the H^+ gradient, we discovered a Na^+/H^+ exchange in vesicles from a tonoplast-enriched fraction isolated from barley roots (9).

Na^+/H^+ antiports have also been reported in tonoplast fractions from red beet (*Beta vulgaris*) (2) and sugar beet (3). The Na^+/H^+ exchange activity in cell suspension cultures of sugar beet, a halophyte, was partly constitutive (3). In contrast, that of barley, a relatively salt-tolerant glycophyte, was undetectable unless the plants were grown in the presence of Na^+. In this paper, induction of the tonoplast Na^+/H^+ exchange activity in barley is characterized in more detail.

MATERIALS AND METHODS

Plant Material

A salt-tolerant cultivar of barley, *Hordeum vulgare* L. cv CM-72 (1), was grown as described previously (7) above aerated nutrient solution (8) at 22°C for 7 d in the dark.

In Vivo Protein Synthesis

Protein synthesis was measured using [^{35}S]methionine labeling of intact plants as described in Hurkman and Tanaka (11). The plants were pretreated with the indicated compound for 1 h, then 50 μCi of [^{35}S]methionine (New England Nuclear,[2] 1100 Ci/mmol) was added and incubation continued for 2 h. After homogenization of the roots, aliquots were spotted onto Whatman 3MM discs. For determination of methionine uptake, the discs were dried and counted. For determination of protein synthesis, the discs were dried, washed in ice-cold 10% TCA for 20 min, and then in boiling 10% TCA for 15 min. Discs were rinsed again in 10% TCA followed by 95% ethanol and 100% ethanol; the discs were then dried and counted. Protein synthesis was calculated as percent of methionine uptake incorporated into protein (TCA precipitated counts/counts taken up).

Membrane Preparations

A tonoplast-enriched membrane fraction was isolated as described previously (6, 9). Roots (10 gm) were excised into ice water, rinsed, and then ground in homogenization buffer (67 mL) containing 250 mM sucrose, 50 mM Tris, 8 mM EDTA, and 4 mM DTT (pH 8.0). The fraction collected from the 22/30% interface of a sucrose step gradient was washed and resuspended in buffer containing 250 mM sorbitol, 1 mM DTT, and 5 mM Mes adjusted to pH 7.0 with Tris. Aliquots containing 0.5 to 1.5 mg protein/mL were stored at −70°C.

Enzyme Assays

The generation and dissipation of pH gradients across the membrane vesicles were followed by measuring quenching and recovery of acridine orange fluorescence as described previously (6, 9). The assay buffer contained 250 mM sorbitol, 50 mM choline-Cl, 1 mM $MgCl_2$, 1 mM EGTA, 2.5 μM acridine orange, and 5 mM Mes adjusted to pH 7.8 with Tris. The tonoplast H^+-ATPase was used to generate a pH gradient, acid inside the vesicles. Vesicles (approximately 10–25 μg protein) were added to 3 mL of the assay buffer and the reaction started by adding 1 mM Tris-ATP. After 2 min, 2 mM EDTA was added to stop the ATPase activity by chelating

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[2] Mention of a specific product name by the United States Department of Agriculture does not constitute an endorsement and does not imply a recommendation over other suitable products.

magnesium. The background leak of protons from the vesicles was then observed as a gradual increase in fluorescence intensity. The background rate was subtracted from the rate obtained using EDTA plus 30 mM Na-gluconate to give the salt-dependent rate of recovery. The initial rate of recovery of fluorescence was measured using a PC-XT computer with the program Asystant+ (ASYST Software Technologies). The derivative was taken of the first 20 s of the recovery and the y-intercept of a line fitted through the derivative was used as the initial rate. Rates are expressed as $\%Q$/min, where Q is the difference between the fluorescence intensity when EDTA was added and the fluorescence intensity after the pH gradient was collapsed by the addition of 3 mM NH_4Cl.

Protein was measured by the method of Bradford (4) using the Bio-Rad microassay with bovine gamma globulin as the standard.

RESULTS AND DISCUSSION

Previously (9), we found high rates of Na^+/H^+ exchange in vesicles from a tonoplast-enriched fraction from salt-grown barley roots and little Na^+/H^+ exchange in vesicles from control (no salt) roots. The small amount of activity in vesicles from control roots could be abolished by the addition of K^+ and valinomycin to the assay mix and was attributed to passive H^+ movement. Active Na^+/H^+ exchange, possibly due to a Na^+/H^+ antiporter, was present only when the plants had been grown in NaCl (9). In the previous study, salt-grown plants were grown for 7 d in nutrient solution containing 100 mM NaCl. We have now measured the time course for induction of Na^+/H^+ exchange (Fig. 1). Plants were grown without Na^+ for 7 d, then 100 mM NaCl was added to the nutrient solution. The NaCl treatments were set up so that roots which had been treated with NaCl for different time periods were all harvested at the same time. Tonoplast vesicles were isolated and assayed for Na^+/H^+ exchange activity. The Na^+/H^+ exchange activity was detected after exposure of the roots to Na^+ for as little as 10 min, the shortest time tested. The half-time for induction was approximately 15 min, and maximum activity was attained within 0.5 to 1 h (Fig. 1). The maximum activity was as high as that previously observed when roots were grown continuously in 100 mM NaCl for 7 d. When barley roots were exposed to 100 mM NaCl for 1 h and then transferred into fresh nutrient solution without Na^+, the Na^+/H^+ exchange activity disappeared with a similar rapid time course (Fig. 2). These salt-shock and recovery treatments had no effect on the rate of formation of the pH gradients by the tonoplast ATPase *in vitro* (data not shown).

Na^+/H^+ exchange activity was induced even if the roots were pretreated with the protein synthesis inhibitor cycloheximide or with the amino acid analogs AZ[3] or FPA for 1 h before the addition of Na^+ (Table I). AZ reduced protein synthesis to 45% of the control level but did not significantly affect induction of the Na^+/H^+ exchange. At a concentration of FPA which reduced protein synthesis to 18% of the control level, the induction of Na^+/H^+ exchange activity was not affected. When protein synthesis was reduced to 5% of the control level by CHI, Na^+/H^+ exchange was still 60% of the control activity. This reduction of Na^+/H^+ exchange by CHI may not be due to a direct effect on protein synthesis since the tonoplast H^+-ATPase activity in vesicles from CHI-treated roots was also only 60% of the control activity (data not shown). Protein synthesis was measured as a fraction of methionine uptake. This adjusts for differences in methionine uptake caused by the different pretreatments. Methionine uptake was not significantly affected by CHI but was reduced to 69% of the control level by AZ and to 39% of the control level by FPA (Table I).

The effect of the addition of various other salts to the roots' nutrient solution was tested to determine if the induction of Na^+/H^+ exchange activity specifically required Na^+ (Table II). Addition of either 100 mM NaCl or 50 mM Na_2SO_4 to the

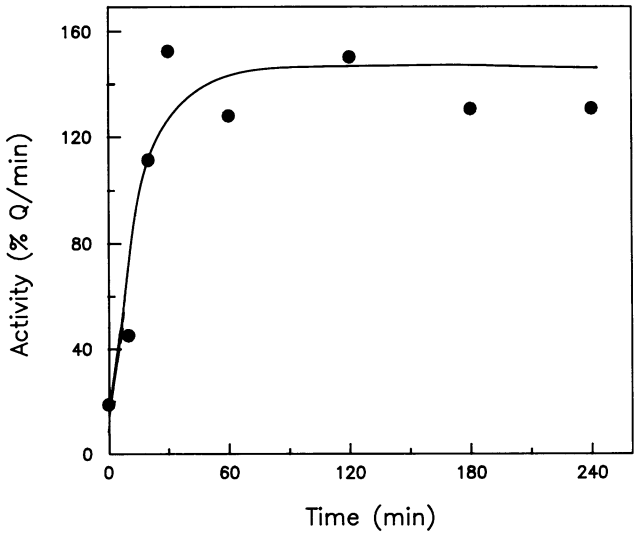

Figure 1. Induction of Na^+/H^+ exchange as a function of time. Roots were exposed to 100 mM NaCl for the indicated time. Tonoplast vesicles were isolated and assayed for Na^+/H^+ exchange. The figure shows the average of two experiments.

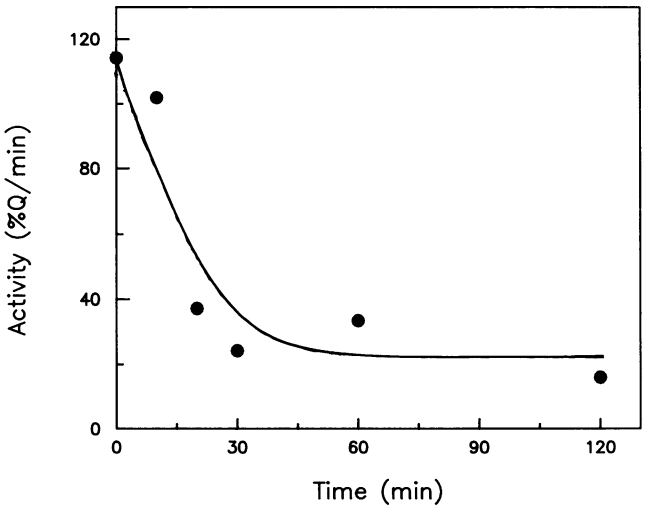

Figure 2. Disappearance of Na^+/H^+ exchange as a function of time. After exposure to 100 mM NaCl for 1 h, roots were transferred to fresh nutrient solution lacking NaCl for the indicated time. Tonoplast vesicles were isolated and assayed for Na^+/H^+ exchange. The figure shows the average of two experiments.

[3] Abbreviations: AZ, L-azetidine carboxylic acid; CHI, cycloheximide; FPA, *p*-fluorophenylalanine.

Table I. *Treatment of Barley Roots with Protein Synthesis Inhibitors*

Plants were treated for 1 h with the indicated compound and then protein synthesis was measured. Protein synthesis was calculated as the percentage of [^{35}S]methionine taken up that was incorporated into protein. For measurements of induction of Na^+/H^+ exchange, the indicated compound was added to the nutrient solution. After 1 h, 100 mM NaCl was added. Roots were harvested after an additional hour. Control: [^{35}S]Met uptake was 3.6 μCi/gm fresh weight, 35% of the methionine taken up was incorporated into protein, and Na^+-dependent H^+ efflux was 119 %*Q*/min.

Treatment	[^{35}S]Met Uptake	Protein Synthesis	Na^+-Dependent H^+ Efflux
		% of control	
0.40 mM AZ	69	45	90
0.40 mM FPA	39	18	97
0.36 mM CHI	120	5	60

Table II. *Na^+/H^+ Exchange Activity after Treatment of Roots with Various Salts or Mannitol*

Roots were treated for 1 h. Vesicles were isolated and Na^+/H^+ exchange measured. Results are the average of 3 assays ± SD.

Treatment	Rate of Recovery
	% Q/min
100 mM NaCl	102.2 ± 10.4
100 mM KCl	5.3 ± 5.3
50 mM Na_2SO_4	83.4 ± 6.6
100 mM NaCl + 5 mM $CaSO_4$	109.1 ± 7.0
3% Mannitol	14.3 ± 17.0

nutrient solution induced the exchange, indicating that the accompanying anion was not critical. The addition of 100 mM KCl did not induce the activity, indicating that Na^+ was required. Mannitol (3%) also gave no significant induction, indicating that induction was not due to the increase in the osmotic potential of the nutrient solution. Calcium has been shown to mitigate the effects of high Na^+ (5), but the addition of 5 mM $CaCl_2$ to the nutrient solution along with 100 mM NaCl did not affect the induction of the Na^+/H^+ exchange. Different concentrations of Na^+ were tested using an incubation period of 0.5 h. The best induction of Na^+/H^+ exchange was obtained using 100 mM NaCl (Fig. 3). The decrease in activity at higher concentrations of NaCl approximately correlates with a reduction of growth of CM-72 at NaCl concentrations above 100 mM (11).

In order to understand the role of the tonoplast Na^+/H^+ exchange in salt tolerance and to identify the protein(s) involved, an important question is whether induction of the activity by Na^+ is due to synthesis of the transport protein or to activation of a transporter that is already present. The induction of the activity in the presence of cycloheximide or amino acid analogs suggests that synthesis of new proteins was not necessary. Although there is little information on the time needed for synthesis of membrane proteins in plants, the rapidity of the induction also suggests that synthesis of new proteins was not required. When pea epicotyls were treated with auxin, the earliest induced mRNAs appeared after 10 to 15 min (15, 19). One auxin-induced protein in soybean epicotyl was reported to be present after 1 h (23) but most take longer to appear (22, 23).

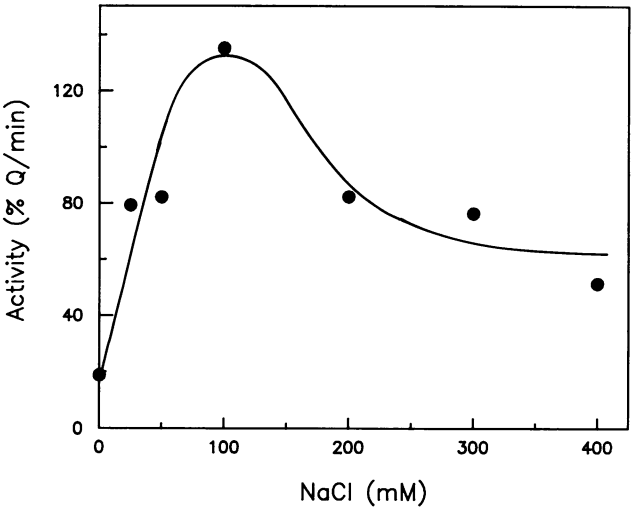

Figure 3. Induction of Na^+/H^+ exchange as a function of concentration. Roots were exposed to the indicated NaCl concentration for 0.5 h. Tonoplast vesicles were isolated and assayed for Na^+/H^+ exchange. The figure shows the average of two experiments.

Some work has been done measuring rates of protein synthesis and transport to the plasma membrane in animal cells. When [^{35}S]methionine was added to cells from the human hepatoma cell line HepG2, labeled albumin began appearing in the medium after about 50 min (20). Synthesis and transport of two related histocompatability glycoproteins to the plasma membrane of B cell lymphoma AKTB-1b cells also has been measured (21). The faster of the two proteins began to arrive at the cell surface after about 30 min of chase following a 10 min labeling period. Comparison of these data with the rate of incubation of the Na^+/H^+ exchange in barley suggests that the induction was more rapid than can be accounted for by synthesis of a new protein. The polypeptides of tonoplast fractions from control and salt-grown barley roots have also been compared on two-dimensional gels (12). Plants grown with 100 mM NaCl showed no large increase in any integral membrane polypeptide that might correspond to the Na^+/H^+ antiporter.

A means of regulation that is more rapid than synthesis of new protein is the modification of existing proteins, for instance by phosphorylation and dephosphorylation. Phosphorylation of membrane proteins from zucchini hypocotyls occurred in 2 to 4 min at 37°C (17). Other chemical modifications such as acylation or reduction of disulfide bonds could also be involved in regulation. The data also do not rule out regulation of Na^+/H^+ transport at an intermediate step such as assembly or insertion of the transporter into the tonoplast membrane.

Our results suggest that the induction of Na^+/H^+ exchange activity in barley root tonoplast is a rapid response to Na^+, which may be achieved by activation of an existing protein. The accumulation of Na^+ in the vacuole may be an emergency response. Slower adjustments may also occur, such as more efficient exclusion of Na^+ at the plasma membrane and the synthesis of sugars and glycinebetaine as osmotica. The rapid response reported here of an intracellular, membrane-bound enzyme to addition of Na^+ to the external medium may prove

to be a useful system for studying how plants sense and respond to chemical stimuli.

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