Characterization of an Anion Transporter in the Plasma Membrane of Barley Roots

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To examine the relationship between H⁺-ATPase and the transport of anions, we investigated the effects of various inhibitors on the activity of the H⁺-ATPase, the transport of protons, and the transport of Cl⁻ ions using plasma membrane vesicles prepared from barley roots. Some inhibitors, namely, 4,4-dilisothiocyanato-2,2-stilbene disulfonate (DIDS) and Zn²⁺ ions markedly inhibited H⁺-ATPase activity. Other compounds, such as phenylglyoxal (PGO) and niflumic acid (NIF), inhibited H⁺-ATPase activity by 20–30%, while anthracene-9-carboxylate (A-9-C) and tetraethylammonium chloride (TEA-C1) had little effect on this activity. The ATP-dependent acidification of the interior of vesicles was strongly dependent on the presence of permeant anions, such as chloride (Cl⁻) and nitrate (NO₃⁻), and it was completely inhibited by 0.2 mM NIF. Other compounds, namely, A-9-C of 0.1 mM and TEA-C1 of 10 mM, did not affect H⁺-transport activity. The inhibition of H⁺-transport activity by NIF was observed even when the activity was assayed in the presence of K⁺, KNO₃, or bis-tris-propane (BTP)-Cl. Using ³⁶Cl⁻, we quantified Cl⁻-transport activity by measuring the uptake of Cl⁻ ions into the plasma membrane vesicles. The uptake depended on the potential difference across the membrane that was generated by H⁺-ATPase; it was enhanced by an inside-positive potential gradient. At 0.1 mM, NIF completely blocked the voltage-dependent Cl⁻-transport activity. From these properties of the Cl⁻ transporter and the inhibition of H⁺-transport activity by NIF, we suggest that H⁺-transport activity across the plasma membrane might be modulated by the transport of anions via a NIF-sensitive anion-permeable transporter that acts to collapse the inside-positive potential generated by H⁺-ATPase.

Key words: Cl⁻ uptake — Hordeum vulgare L. — H⁺-transport activity — Niflumic acid — Plasma membrane.

The transport of the ions that are essential for plants growth is regulated mainly by the influx and efflux of ions across the plasma membrane of root cells. The fluxes of ions appear to be mediated by several systems, such as H⁺-ATPases, carriers, and channels associated with the plasma membrane. Anion-transport systems in plasma membranes and tonoplast membranes have been identified and characterized. In particular, many studies using the patch-clamp technique have shown that there are at least two types of anion channel associated with the plasma membrane. One type is active when the membrane is hyperpolarized (Tyrman et al. 1986). The other is activated by the elevation of the cytoplasmic level of Ca²⁺ ions (Schroeder and Hagiwara 1989, Okihara et al. 1991) and in the presence of ATP (Hedrich et al. 1990). These anion channels play roles in important processes, such as the regulation of turgor, the control of levels of anions, and maintenance of a membrane potential across the membrane (Tyrman 1992). The results of patch-clamp studies are, however, only applicable to protoplasts derived from suspension cultured-cells and leaf tissues. In root cells, there are few reports of the characteristics of anion currents across the plasma membrane (Skerrett and Tyrman 1994, Wegner and Raschke 1994). It is difficult to apply the patch-clamp technique to root cells because of their small size and it is also difficult to isolate protoplasts from root cells (Grabov 1990).

To examine the characteristics of ion-transport systems, it is often useful to prepare isolated membrane vesicles. Both the H⁺/NO₃⁻ symporter (Ruiz-Cristin and Briskin 1991) and the H⁺/sucrose symporter (Hecht et al. 1992) have been identified and characterized by studies of purified membrane vesicle. In such studies, the activity of the transporter can be quantified directly by measuring the uptake of an ion into the membrane vesicles with a radioisotope as a tracer. In addition, the activity of a vacuolar Na⁺/H⁺ antiporter has been demonstrated as the Na⁺-dependent influx of protons from vesicles using a fluorescent probe to monitor gradients in pH (Garbarino and DuPont 1988). The pH indicator, quinacrine, has also been used to demonstrate anion-transport activity in the tonoplast from estimations of the stimulation by anions of ATP- and PP⁻-dependent H⁺-transport activity (Pope and Leigh 1990), since H⁺-transport activity is limited by the transport of anions that acts to collapse the interior-positive potential established by the H⁺-ATPase (Vara and Serrano 1982). We demonstrated previously that plasma membrane H⁺-transport activity is enhanced by Cl⁻ ions but not by NO₃⁻ ions.

Abbreviations: A-9-C, anthracene-9-carboxylate; BSA, bovine serum albumin; BTP, bis-tris-propane; DIDS, 4,4'-disothiocyanato-2,2'-stilbene disulfonate; DTT, dithiothreitol; EGTA, ethylene glycol bis (β-amino ethyl ether)-N,N'-tetraacetic acid; NIF, niflumic acid; PGO, phenylglyoxal; PMSF, Phenylmethylsulfonyl fluoride; TEA-C1, tetraethylammonium chloride. 

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in salt-stressed roots of barley, and we suggested that permeability to Cl\textsuperscript{−} ions might increase after salt treatment and also that the permeation of Cl\textsuperscript{−} and NO\textsubscript{3}\textsuperscript{−} ions might be mediated by different systems (Yamashita et al. 1994). More recently, Santi et al. (1995) reported that NO\textsubscript{3}\textsuperscript{−}-stimulated, ATP-dependent H\textsuperscript{+}-transport activity in plasma membrane vesicles was modified after exposure of maize roots to NO\textsubscript{3}\textsuperscript{−} ions. Such studies provided useful but only indirect information since anion-transport activity was not measured directly. Furthermore, it is impossible to manipulate separately the pH gradient and the membrane potential generated by H\textsuperscript{+}-ATPase, which are the probable driving forces for anion transport. Effective inhibitors of anion transporters might interfere with H\textsuperscript{+}-ATPase activity. Thus, it is difficult to characterize anion transport exactly from studies based on the effects of anions on H\textsuperscript{+}-transport activity.

In this report, we describe the characteristics of an anion transporter in plasma membrane vesicles prepared from barley roots. The activity of the anion transport was determined with \textsuperscript{36}Cl\textsuperscript{−} ions as a radiotracer. The anion transporter might serve as a regulator of H\textsuperscript{+}-transport activity in root plasma membrane.

Materials and Methods

Plant material—Seeds of barley (Hordeum vulgare L. cv. Kikaihadaka) were soaked in running tap water overnight. They were then placed in a plastic basket filled with deionized water for 3 days, and then 0.5 mM CaSO\textsubscript{4} was supplied to the plants for a further 3 days. Thereafter, 10 mM KNO\textsubscript{3} was supplied to the plants for 1 day. Plants were grown at 20°C under a fluorescent lamp (4,000 lux, 12 h of light and 12 h of darkness) with continuous aeration throughout all experiments.

Isolation of plasma membrane vesicles—Plasma membrane vesicles were purified from microsomes with an aqueous polymer two-phase system (Yamashita et al. 1994). The roots were ground in 0.3 M sucrose, 50 mM Tris, 8 mM EDTA, 5 mM DTT, 2 mM PMSF, 0.2% (w/v) BSA, and 1.5% (w/v) insoluble polyvinylpyrrolidone at a ratio of medium to tissue of 2 ml g\textsuperscript{−1}. After centrifugation of the homogenate at 10,000 \times g for 20 min, the supernatant was collected and centrifuged at 50,000 \times g for 35 min. The pelleted microsomes were suspended in 0.3 M sucrose, 5 mM potassium-phosphate (pH 7.8), 5 mM KCl, 0.1 mM EDTA, and 1 mM DTT. Microsomes were added to the two-phase system which had a final composition of 6.2% (w/v) dextran T 500 (Pharmacia, Sweden), 6.2% (w/v) polyethylene glycol 3350 (Sigma, MO, U.S.A.), 0.3 M sucrose, 5 mM potassium-phosphate (pH 7.8), 5 mM KCl, 0.1 mM EDTA, and 1 mM DTT. After shaking the contents of the first tube and centrifuging at 700 \times g for 5 min, the upper phase was transferred to the second tube. After second partitions, the resultant upper phase was diluted five-fold with a dilution buffer that contained 0.3 M sucrose, 5 mM HEPES-BTP (pH 7.0), and 1 mM DTT, and then it was centrifuged at 80,000 \times g for 40 min. The resultant pellet was resuspended in dilution buffer and stored at −80°C.

Preparation of inside-out plasma membrane vesicles—Inside-out plasma membrane vesicles were prepared by the method of Grouzis et al. (1987). Plasma membrane vesicles were incubated with 0.1% Triton X-100 for 20 min on ice. After centrifugation of this mixture at 100,000 \times g for 45 min, the pellet was resuspended in dilution buffer and stored at −80°C.

Assay of ATPase activity—The assay medium consisted of 30 mM HEPES-BTP (pH 7.0), 1 mM Na\textsubscript{3}MoO\textsubscript{4}, 50 mM KCl, 3 mM MgSO\textsubscript{4}, 3 mM ATP-BTP, with or without 0.01% (w/v) Brij 58. The reaction mixture was incubated for 30 min at 30°C, and the reaction was stopped by addition of 0.25 M H\textsubscript{2}SO\textsubscript{4}. The released P\textsubscript{i} was quantified by the method of Heinonen and Lahti (1981).

Determination of the transmembrane pH gradient and the inside-positive potential generated by H\textsuperscript{+}-ATPase—The transmembrane pH gradient and the inside-positive potential generated by H\textsuperscript{+}-ATPase were monitored with the fluorescence probes, quinacrine and Oxonol V, respectively. The assay medium consisted of 10 mM HEPES-BTP (pH 7.0), 1 mM EGTA-BTP, 3 mM MgSO\textsubscript{4}, 3 mM ATP-BTP, 150 mM KCl, and either 2 \mu M quinacrine (Nacalai Tesque, Kyoto, Japan) or 0.5 \mu M Oxonol V ( Molecular Probes Inc., OR, U.S.A.). The wavelengths for excitation of quinacrine and emission were 423 and 500 nm, respectively, and those for Oxonol V were 580 and 650 nm, respectively. After incubation of the reaction mixture for 5 min at 30°C, the reaction was started by the addition of ATP-BTP. The transmembrane pH gradient and the inside-positive potential were abolished by addition of 3 mM NH\textsubscript{4}Cl and 5 mM KSCN, respectively.

Assay of the Mg\textsubscript{2+}-ATP-driven transport of Cl\textsuperscript{−} ions—Plasma membrane vesicles were suspended in 0.3 M sorbitol, 10 mM HEPES-BTP (pH 7.0), 1 mM EGTA-BTP at a protein concentration of 0.5 mg ml\textsuperscript{−1}. After incubation for 5 min at 20°C, the uptake of Cl\textsuperscript{−} ions was initiated by adding 25 \mu l of the suspension of plasma membrane vesicles to 25 \mu l of the assay solution, which contained 0.3 M sorbitol, 10 mM HEPES-BTP (pH 7.0), 1 mM EGTA-BTP, 6 mM ATP-BTP, 6 mM MgSO\textsubscript{4}, and 20 mM Na\textsuperscript{36}Cl (592 GBq mg\textsuperscript{−1} Cl\textsuperscript{−}; Amersham International plc, U.K.). At indicated times, the uptake of Cl\textsuperscript{−} ions was stopped by addition of 1 ml of ice-cold stop buffer, which contained 0.3 M sorbitol and 10 mM HEPES-BTP (pH 7.0). The reaction mixture was then immediately filtered through a pre-wetted 0.45 \mu m Millipore filter (HAWP; Nikon Millipore Kogyo K.K., Japan), and the filter was then washed with 2 ml of stop buffer. The filter was dried and then radioactivity bound to the filter was determined by liquid scintillation spectroscopy in 5 ml of Clear-sol I (Nacalai Tesque Inc., Kyoto, Japan).

Assay of the K\textsuperscript{+}/valinomycin-driven transport of Cl\textsuperscript{−} ions—The uptake of Cl\textsuperscript{−} ions was initiated by the addition of plasma membrane vesicles (5 \mu g of protein) to the reaction mixture, which contained 150 mM potassium-glucose, 10 mM HEPES-BTP (pH 7.0), 1 mM EGTA-BTP, 10 mM Na\textsuperscript{36}Cl, with or without 0.5 \mu M valinomycin (total volume, 50 \mu l). The Cl\textsuperscript{−}-transport activity was determined by filtration on a 0.45-\mu m filter as described above.

Protein was quantified by the method of Bradford (1976) with BSA as the standard.

Results

The effects of various inhibitors on the H\textsuperscript{+}-ATPase activity of the plasma membrane—There are numerous reports that some inhibitors developed in animals are also effective in blocking anion transporters in higher plants (for review, see Tyerman 1992). However, it has not yet been determined whether the inhibitors are specific to the
activities of other enzymes also in higher plants. Therefore, we selected some inhibitors whose actions have been described in animal and plant cells, and we examined the effects of those inhibitors on the activity of the plasma membrane H^+-ATPase.

As shown in Table 1, blockers of anion channels, DIDS (Falke and Chan 1986, Hedrich and Kurkdjian 1988, Skerrett and Tyerman 1994) and Zn^{2+} ions (Schauf and Wilson 1987, Skerrett and Tyerman 1994), caused quite large (70-80%) reductions in H^+-ATPase activity. The arginine-modifying reagent, PGO, which inhibits the activity of the H^+/NO_3^- symporter (Ruiz-Cristin and Briskin 1991), and an anion-channel blocker, NIF (Schwartz et al. 1995), inhibited H^+-ATPase activity by 20-30%. A-9-C, characterized as an anion-channel blocker (Tyerman et al. 1986, Shiina and Tazawa 1987, Schwartz et al. 1995), and TEA-Cl, characterized as potassium-channel blocker, had no effects on the H^+-ATPase activity.

The effects of inhibitors on H^+-transport activity—To investigate the specificity of inhibitors, we examined the effects of inhibitors on H^+-transport activity. The acidification of plasma membrane vesicles was monitored in terms of the quenching of quinacrine fluorescence. As shown in Figure 1, the initial rate of acidification of vesicles caused by H^+-ATPase was strongly dependent upon the nature of anions present. H^+-transport activity was stimulated by permeant anions, such as NO_3^- and Cl^- ions. In particular, NO_3^- ions allowed the formation of a greater transmembrane pH gradient than Cl^- ions when assayed at lower concentrations. The rates of transport of protons in the presence of different anions reflect the relative permeability of the membrane to these anions, and they indicate a greater permeability to NO_3^- than to Cl^- ions.

The effects of NIF, A-9-C, and TEA-Cl on the H^+-transport activity of the plasma membrane were investigated as illustrated in Figure 2. The presence of KCl doubled the H^+-transport activity, as compared with that of BTP-Cl. In 150 mM KCl, the formation of a transmembrane pH gradient was markedly inhibited by NIF of 0.1 mM. By contrast, 10 mM TEA-Cl and 0.1 mM A-9-C had no effect on H^+-transport activity. The sensitivities to NIF, TEA-Cl, and A-9-C of the H^+-transport activity were unchanged even when assays were performed in the presence of BTP-Cl instead of KCl.

In further experiments, the effects of NIF on the ATPase and H^+-transport activities were analyzed as a function of the concentration of NIF (Fig. 3). In 150 mM KCl, H^+-transport activity was completely inhibited by 0.2 mM NIF. Inhibition of H^+-transport activity by 50% was observed with NIF at approximately 40 \mu M. No significant change in the extent of inhibition of H^+-transport activity by NIF was observed when assays were conducted with KNO_3 instead of KCl. By contrast, the ATPase activity of the plasma membrane was reduced by NIF at concentrations above 10 \mu M when the activity was determined in the absence of Brij 58. In the absence of Brij 58, 0.2 mM NIF inhibited the ATPase activity by 20-30%. At concentration, Brij 58 abolished the inhibition of the ATPase activity by NIF.

Effects of NIF on the membrane potential generated by H^+-ATPase—The interior-positive potential generated by H^+-ATPase was monitored with Oxonol V. In the pres-

Table 1: Effects of inhibitors on the plasma membrane H^+-ATPase activity

<table>
<thead>
<tr>
<th>Inhibitor (mM)</th>
<th>H^+-ATPase activity (μmol P_i (mg protein)^{-1} min^{-1})</th>
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<tbody>
<tr>
<td>None</td>
<td>0.92 (100%)</td>
</tr>
<tr>
<td>A-9-C (0.1)</td>
<td>0.94 (102%)</td>
</tr>
<tr>
<td>DIDS (0.01)</td>
<td>0.23 (25%)</td>
</tr>
<tr>
<td>NIF (0.1)</td>
<td>0.73 (79%)</td>
</tr>
<tr>
<td>PGO (5.0)</td>
<td>0.63 (68%)</td>
</tr>
<tr>
<td>TEA-Cl (10)</td>
<td>1.04 (113%)</td>
</tr>
<tr>
<td>ZnSO_4 (0.1)</td>
<td>0.26 (28%)</td>
</tr>
</tbody>
</table>

Plasma membrane vesicles were prepared from barley roots, and ATPase activity was determined in the presence of the indicated inhibitors at the indicated concentrations. Assays were performed in the absence of Brij 58. H^+-ATPase activity is represented by the ATPase activity that was inhibited by 0.1 mM Na_2VO_4. Numbers in parentheses show percentages relative to the ATPase activity assayed in the absence of inhibitors, which was taken as 100%. Results are the means of replicate measurements from one experiment.

Fig. 1: Effects of anions on the H^+-transport activity of the plasma membrane. Plasma membrane vesicles were prepared from barley roots, and H^+-transport activity was determined in the presence of either Cl^- or NO_3^- ions. Anions at indicated concentrations were added as the potassium salt, and the final concentration of K^+ ions was adjusted to 130 mM with potassium-MES. The initial rate of H^+-transport is represented by the rate of quenching of fluorescence from 30 to 60 s after the addition of ATP-BTP.
MgATP-driven uptake of Cl\textsuperscript{−} ions—The proportion of inside-out plasma membrane vesicles was approximately 50% in our preparation (Yamashita et al. 1994). To investigate Cl\textsuperscript{−}-transport systems, it is best to use vesicles with the same orientation. We prepared our inside-out vesicles using detergent, as described by Grouzis et al. (1987). After plasma membrane vesicles had been treated with 0.1% Triton X-100, the H\textsuperscript{+}-transport activity increased approximately 4-fold, while the ATPase activity was stimulated by less than 20%, as compared with the values for the native plasma membrane vesicles (data not shown). To determine the orientation of vesicles, the latency of ATPase activity was assessed using detergent Brij 58. A latency of 50–60% was obtained with the ATPase in native plasma membrane vesicles, whereas plasma membrane vesicles washed with Triton X-100 showed a latency of 10–20% (data not shown). These results indicate that plasma membrane vesicles have a high proportion of inside-out vesicles after treatment with Triton X-100. The stimulation of H\textsuperscript{+}-transport activity that we observed after washing vesicles with Triton X-100 might have been due to the increased proportion of inside-out vesicles, to a decrease in the permeability to protons, or to both (Grouzis et al. 1987). Furthermore, H\textsuperscript{+}-transport activity was markedly inhibited by 0.1 mM NIF even after washing with Triton X-100 (data not shown). For studies of the characteristics of the Cl\textsuperscript{−}-transporter, all experiments were performed with Triton X-100-treated vesicles.

After plasma membrane vesicles had been treated with Triton X-100, the influx of \textsuperscript{36}Cl\textsuperscript{−} ions into the vesicles was slightly stimulated as compared to that into native vesicles (Fig. 4). In addition, the uptake of Cl\textsuperscript{−} ions into both native and Triton X-100-treated vesicles was 2-fold higher in the presence of MgATP than in the absence of ATP. This result suggests that the driving force for transport of Cl\textsuperscript{−} ions might be an electrochemical potential gradient of protons (pH gradient and interior-positive potential) generated by H\textsuperscript{+}-ATPase. To determine whether the driving
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Fig. 4 MgATP-dependent transport of Cl⁻ ions into membrane vesicles. Plasma membrane vesicles (PM), prepared from barley roots, were washed with 0.1% Triton X-100 (TXP), and Cl⁻-transport activity was determined using ³⁶Cl⁻ ions. Cl⁻-transport activity was determined with (••) or without (○○) ATP-BTP. Each point represents the mean of replicate measurements from two independent preparations. Error bars represent ±SE (n=4).

force for the transport of Cl⁻ ions is attributable to the pH gradient or the membrane potential, we examined the effects of NH₄⁺ and SCN⁻ ions on the MgATP-dependent uptake of Cl⁻ ions. NH₄⁺ and SCN⁻ ions dissipated the pH gradient and the membrane potential, respectively, that had been generated by H⁺-ATPase. As shown in Figure 5, 5 mM SCN⁻ ions inhibited the MgATP-dependent uptake of Cl⁻ ions to the level observed without ATP, while 5 mM NH₄⁺ ions stimulated the uptake of Cl⁻ ions by 30-40%.

These results suggest that Cl⁻ ions are driven electrically into the interior of vesicles in response to the inside-positive potential generated by H⁺-ATPase.

The K⁺/valinomycin-driven uptake of Cl⁻ ions — The voltage-dependence of the Cl⁻-transport activity was investigated. An interior-positive potential across membrane vesicles was generated by a K⁺ diffusion potential, which was

Fig. 5 Effects of SCN⁻ and NH₄⁺ ions on the MgATP-dependent transport of Cl⁻ ions. Plasma membrane vesicles were washed with 0.1% Triton X-100, and Cl⁻-transport activity was determined with or without ATP. Cl⁻-transport activity was assayed in the presence of either 5 mM KSCN or 2.5 mM (NH₄)₂SO₄ in the presence of ATP. Relative activity is the percentage of the Cl⁻-transport activity in the presence of ATP, which was taken as 100%. Each point represents the mean of replicate measurements from two independent preparations. Error bars represent ±SE (n=4).

Fig. 6 Stimulation of Cl⁻-transport activity by valinomycin. Plasma membrane vesicles were washed with 0.1% Triton X-100, and Cl⁻-transport activity was determined in the presence of 150 mM potassium-gluconate with (•) or without (○) 0.5 μM valinomycin. Each point represents the mean of replicate measurements from two independent preparations. Error bars represent ±SE (n=4).

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Fig. 7 The K⁺-stimulated Cl⁻-transport activity and its inhibition by NIF. Plasma membrane vesicles were washed with 0.1% Triton X-100, and Cl⁻-transport activity was determined with 0.5 μM valinomycin in the presence of potassium-gluconate at indicated concentrations. The Cl⁻-transport activity was assayed with or without 0.1 mM NIF. The final concentration of gluconate was adjusted to 150 mM with sodium-gluconate. Each point represents the mean of replicate measurements from two independent preparations.
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The formation of a transmembrane pH gradient by the H⁺-ATPase of the plasma membrane was stimulated by impermeant anions such as Cl⁻ and NO₃⁻ ions (Fig. 1). The inward movement of anions minimizes the inside-positive potential generated by the H⁺-ATPase and, thereby, maximizes the formation of a transmembrane pH gradient, as expected from the chemiosmotic hypothesis (Vara and Serrano 1982). However, in root cells, little is known about the mechanism of transport of Cl⁻ ions across the plasma membrane. It has been demonstrated that anions have little effect on H⁺-transport activity in liposomes reconstituted with the purified H⁺-ATPase from the plasma membrane (Gibrat et al. 1990, Seto-Young and Perlin 1991), indicating the relatively low permeability of membrane lipids to anions. In clathrin-coated vesicles, acidification of the interior of vesicles, mediated by H⁺-ATPase, depends on uptake of Cl⁻ ions via a Cl⁻ transporter (Xie et al. 1989).

In this study, using ⁸⁶Cl⁻ ions, we identified and characterized Cl⁻-transport activity in plasma membrane vesicles isolated from barley roots. The Cl⁻-transport activity was enhanced with increasing interior-positive potential and it was blocked by NIF, a blocker of anion channels (Fig. 4-7). These results suggest that the transport of Cl⁻ ions is mediated by anion channels, as has been shown in the case of plasma membranes of other tissues by patch-clamp studies. Furthermore, NIF also inhibited generation of a transmembrane pH gradient by H⁺-ATPase (Fig. 2-3). Thus, the formation of a pH gradient by H⁺-ATPase is stimulated by anion transport via a NIF-sensitive anion transporter that acts to bring about for charge compensation.

The opening of anion channels is voltage-dependent, and it is activated at membrane potentials below —100 mV (Schwartz et al. 1995). The activation is very rapid and can usually be detected within 1 s after a voltage pulse (Skerrett and Tyerman 1994, Schwartz et al. 1995). If an inside-positive potential is generated by the action of the plasma membrane H⁺-ATPase, anion channels should be rapidly activated in response to the positive potential. An influx of anions occurs and the inside-positive potential is abolished. Therefore, we would expect that the presence of NIF would generate formation of a large positive potential by the plasma membrane H⁺-ATPase since NIF inhibited the inward movement of anions (Fig. 7). However, NIF had little effect on the membrane potential developed by the H⁺-ATPase when the assay was performed in the presence of KCl (data not shown). The explanation of the effect of NIF is not clear at present, but it might involve the dependence on voltage of the plasma membrane H⁺-ATPase activity. In yeast, it has been demonstrated that the activity of the plasma membrane H⁺-ATPase and its sensitivity to vanadate depend on the membrane voltage, and the activity is rapidly (within 1 min) inactivated upon formation of an inside-positive potential (Seto-Young and Perlin 1991). It has also been demonstrated, using a planar bilayer system, that the H⁺ current due to a plasma membrane H⁺-ATPase from red beet exhibits voltage-dependence and decreases when the membrane is hyperpolarized (Briskin et al. 1995). Furthermore, we observed that NIF inhibited the H⁺-ATPase activity of the plasma membrane by approximately 20% only assays were performed in the absence of Brij 58 which permeabilizes the vesicles (Fig. 3). This result suggests that the plasma membrane H⁺-ATPase activity might be regulated by membrane potential, and it might be rapidly inactivated upon formation of an inside-positive potential. However, in the present study, we could not determine whether NIF rapidly modulated the membrane potential generated...
by H⁺-ATPase. At present it is unclear whether H⁺-ATPase activity recovers rapidly after such activity has disappeared upon generation of an inside-positive potential.

The presence of K⁺ ions doubled the H⁺-transport activity, as compared with that in the presence of BTP-Cl⁻ (Fig. 2). There are several possibilities that might explain the mechanism of stimulation of H⁺-transport activity by K⁺ ions. K⁺ ions might have an effect on the membrane potential generated by the H⁺-ATPase through passive H⁺/K⁺ exchange (Gibrat et al. 1990). Although little is known about the properties of such a passive H⁺/K⁺ exchange, the K⁺-channel blocker TEA had no effect on H⁺-transport activity (Fig. 2), a result suggests that movement of ions through TEA-sensitive K⁺ channels is unlikely to participate in the ATP-dependent acidification of the interior of vesicles. Alternatively, K⁺ ions might directly activate the plasma membrane H⁺-ATPase and, thus, stimulate the H⁺-transport activity (Vara and Serrano 1982).

Although inhibitors are useful in attempts to understand the characteristics of anion transporters, the specificity of such reagents has not been established in higher plants. In the present study, DIDS, Zn²⁺ ions, NIF, A-9-C, PGO, and TEA-CI were used to investigate whether such inhibitors might be effective against the plasma membrane H⁺-ATPase, the transport of protons, and the uptake of Cl⁻ ions. Some of inhibitors tested seemed not to be specific for anion transporters; the plasma membrane H⁺-ATPase activity was inhibited by DIDS, Zn²⁺ ions, and PGO (Table 1). At high concentrations, DIDS is known to react with lysine residues in proteins and to have nonspecific effects (Falke and Chan 1986). DIDS and PGO might interfere directly with the H⁺-ATPase since the lysine and arginine moieties in the plasma membrane H⁺-ATPase are known to be essential for its activity (Kasamo 1988, Briskin 1990). A-9-C had no effect on the activity of H⁺-ATPase, or on the transport of protons and Cl⁻ ions (Table 1), even though it is well known as an antagonist of anion channels (Tyerman et al. 1986, Shina and Tazawa 1987, Schwartz et al. 1995). Recently, Schwartz et al. (1995) suggested that A-9-C might block anion currents from the extracellular side of the plasma membrane. Therefore, A-9-C seems unlikely to be able to inhibit H⁺-transport and anion-transport activities under our experimental conditions because of the inside-out orientation of many of the plasma membrane vesicles.

Of all inhibitors tested in the present study, NIF was the most specific antagonist of the transport of Cl⁻ ions across root plasma membranes. In the tonoplast membrane, NIF blocks the voltage-dependent Cl⁻-transport activity, as determined with the chloride-sensitive fluorescent probe, 6-methoxy-1-(3-sulfonatopropyl) quinalinium (SPQ; Pope and Leigh 1990). In patch-clamp studies, NIF was found to be effective in blocking slow anion currents across the plasma membrane (Schwartz et al. 1995). Ryan et al. (1995) found that NIF inhibited the aluminum-induced efflux of malate from root cells, and they suggested that the efflux of malate might occur through the action of NIF-sensitive anion channels. There is, however, evidence for at least two types of anion channel in plant plasma membranes (Tyerman 1992). We speculated previously that permeability to Cl⁻ and NO₃⁻ ions might be mediated by different systems (Yamashita et al. 1994). However, NIF inhibited H⁺-transport activity when it was assayed not only with Cl⁻ but also with NO₃⁻ ions (Fig. 3), suggesting that NIF is not useful for distinguishing between the permeability to Cl⁻ and that to NO₃⁻ ions. It seems that NIF is not a specific antagonist for one type of anion channel among the various anion channels in the plasma membrane and tonoplast.

Although relatively little is known about the function of anion channels in higher plants, they are thought to allow the efflux of anions with subsequent depolarization of the membrane (Tyerman 1992). Okihara et al. (1995) reported recently that A-9-C inhibited the extrusion of protons from Catharanthus roseus cells. Thus, anion channels might be important modulators of the activities of ion-transport systems, such as the H⁺-ATPase and outward-rectifying K⁺ channels.

Finally, although the Cl⁻-transporter might be functionally independent of the plasma membrane H⁺-ATPase, anion transport via the Cl⁻-transporter collapses the inside-positive potential generated by H⁺-ATPase, thereby modulating H⁺-ATPase activity and the H⁺-pumping activity. In the present study, we found that the transport of Cl⁻ ions across root plasma membrane is mediated by NIF-sensitive anion channels. Further studies are needed if we are to understand the characteristics of anion transporters in the plasma membrane of barley roots, for example by solubilization of component and their incorporation into liposomes.

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References


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