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Studies of the Uptake of Nitrate in Barley[1]

IV. Electrophysiology

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ABSTRACT

Transmembrane electrical potential differences ($\Delta\psi$) of epidermal and cortical cells were measured in intact roots of barley (*Hordeum vulgare* L. cv Klondike). The effects of exogenous NO_3^- on $\Delta\psi$ (in the concentration range from 100 micromolar to 20 millimolar) were investigated to probe the mechanisms of nitrate uptake by the high-affinity (HATS) and low-affinity (LATS) transport systems for NO_3^- uptake. Both transport systems caused depolarization of $\Delta\psi$, demonstrating that the LATS (like the HATS) for NO_3^- uptake is probably mediated by an electrogenic cation (H^+?) cotransport system. Membrane depolarization by the HATS was "inducible" by NO_3^-, and saturable with respect to exogenous [NO_3^-]. By contrast, depolarization by the LATS was constitutive, and first-order in response to external [NO_3^-]. H^+ fluxes, measured in 200 micromolar and in 5 millimolar $Ca(NO_3)_2$ solutions, failed to alkalinize external media as anticipated for a 2 H^+:1 NO_3^- symport. However, switching from K_2SO_4 solutions (which were strongly acidifying) to KNO_3 solutions at the same K^+ concentration caused marked reductions in H^+ efflux. These observations are consistent with NO_3^- uptake by the HATS and the LATS via 2 H^+:1 NO_3^- symports. These observations establish that the HATS for nitrate uptake by barley roots is essentially similar to those reported for *Lemna* and *Zea mays* by earlier workers. There are, nevertheless, distinct differences between barley and corn in their quantitative responses to external NO_3^-.

Nitrate absorption by several plant species has been shown to be biphasic (see ref. 16 and references therein). At low external NO_3^- concentration ($[NO_3^-]_o$), net NO_3^- uptake and $^{13}NO_3^-$ influx are typically saturable (with K_m values in the range from approximately 10 to 100 μM), inducible by exogenous nitrate and thermodynamically active (1, 3, 12, 16, 17). The mechanism of energy coupling for active transport by HATS[2] for NO_3^- absorption has been investigated in a limited number of species by means of electrophysiological studies. Ullrich and Novacky (19) documented that NO_3^- absorption by *Lemna* was associated with depolarization of the $\Delta\psi$. Furthermore, the extent of depolarization of $\Delta\psi$ was increased ("induced") by prior exposure of NO_3^--deprived plants to NO_3^- solutions in the same way that NO_3^- uptake is enhanced ("induced") by exposure to NO_3^-. Depolarization of $\Delta\psi$ was followed by gradual repolarization of the electrical potential difference (in the presence of exogenous NO_3^-), presumably through activation of the proton pump. Removal of NO_3^- caused hyperpolarization of $\Delta\psi$. A mechanism for NO_3^- absorption involving a 2 H^+:1 NO_3^- symport was proposed to account for these observations, whereby the energy for active NO_3^- absorption is derived from the proton gradient ($\Delta\mu_{H^+}$) generated by the plasma membrane H^+-ATPase.

By contrast, Thibaud and Grignon (18) reported that NO_3^- absorption by corn roots was associated with hyperpolarization of $\Delta\psi$. Furthermore, the failure of diethylstilbestrol (an inhibitor of H^+-translocating ATPases) to inhibit this hyperpolarization led these authors to propose that NO_3^- absorption in corn roots occurred by means of a 2 NO_3^-:1 OH^- antiport. More recently, McClure *et al.* (9), also using corn roots, have established that hyperpolarization of $\Delta\psi$, associated with NO_3^- uptake, was preceded by a rapid and small (approximately 10 mV) depolarization. This depolarization was absent in NO_3^--starved roots, and was virtually independent of $[NO_3^-]_o$ even at [NO_3^-] as high as 20 mM. However, the extent of the hyperpolarization of $\Delta\psi$ that followed depolarization was a function of $[NO_3^-]_o$. Below 1 mM, hyperpolarization was saturable, with a half-saturation concentration (K_m) that was similar to the K_m for NO_3^- uptake. Beyond 1 mM, hyperpolarization was a first-order function of $[NO_3^-]_o$.

The kinetics of NO_3^- influx in barley roots have been studied extensively by use of $^{13}NO_3^-$ (5, 6, 15–17). Siddiqi *et al.* (16, 17) and Glass *et al.* (2) have recently characterized $^{13}NO_3^-$ influx in the high concentration range (>1 mM) by the LATS as constitutive, first-order with respect to $[NO_3^-]_o$, and relatively insensitive to metabolic inhibition. They suggested that NO_3^- uptake by LATS may occur via NO_3^--specific channels. The present studies were undertaken (a) to compare the electrical properties of the HATS for NO_3^- in barley to that of corn and *Lemna,* and (b) to investigate energy coupling in the LATS for nitrate by examining the

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[2] Abbreviations: HATS, high-affinity transport system; $\Delta\psi$, transmembrane electrical potential difference; LATS, low-affinity transport system.

effects of external [NO_3^-] on the electrical properties of this transport system.

MATERIALS AND METHODS

Growth of Plants

Seeds of barley (*Hordeum vulgare* L. cv Klondike) were germinated in moistened sand contained in polyethylene cups as described previously (9, 15). After 3 d at room temperature (22°C), roots had penetrated the plastic gauze that covered the lower end of the cups to a length of 2 to 3 cm. Adhering sand was washed from the roots with deionized water and the seedlings, supported by their polyethylene cups, were transferred to inorganic nutrient solutions contained in black polyethylene vessels (4). These contained 2.4 L of nutrient solution. Nutrient solutions consisted of 200 μM $CaSO_4$ or 1/80 strength modified Johnson's medium (15), plus or minus 100 μM NO_3^- (as the Ca salt). Each cup contained three or four seedlings and each vessel contained four cups. Hence, depletion of the inorganic nutrients was not a significant problem during the 3 to 4 d of hydroponic growth. Nevertheless, solutions in the polyethylene vessels were completely replaced on alternate days.

Plants were exposed to NO_3^- for up to 3 d prior to measurement of $\Delta\psi$ to induce the HATS for NO_3^- uptake. In our first experiments, we followed the protocol of McClure *et al.* (9), in which plants were pretreated with NO_3^- for 3 d. Subsequently, shorter exposures (18–24 h) were chosen because they result in larger NO_3^- fluxes (15) and larger depolarizing effects of applied NO_3^- on $\Delta\psi$.

Electrical Measurements

All impalements for the measurement of $\Delta\psi$ were in epidermal or cortical cells of roots of intact plants. Seedling plants were secured with a single seminal root positioned over platinum pins in a narrow Plexiglas chamber. The chamber was mounted to the stage of an Olympus BH-2 microscope (Spectra Services, Rochester, NY) as described previously (9). Roots targeted for impalement were held in place by small lengths of tygon tubing positioned roughly 5 mm on either side of the platinum pins that supported the root. Hence, during impalement, which was typically in a region approximately 3 to 4 cm from the root tip, displacement of the root was virtually impossible. Impalement of epidermal or cortical cells was achieved using a hydraulically driven Narashige micromanipulator (model M0–204; Narashige U.S.A., Greenvale, NY) mounted onto the microscope stage. Membrane potentials were measured using a WPI model KS-750 amplifier (World Precision Instruments, Inc., New Haven, CT), and microelectrodes (tip diameter approximately 0.5 μm) were made from a single-barreled borosilicate glass tube filled with 3 M KCl (adjusted to pH 2 to reduce tip potentials). Reference electrodes were made in an identical manner and placed within a chamber adjacent to and continuous with the root chamber to minimize any contamination of the solution bathing the root by K^+. Membrane potentials were recorded on a strip chart recorder.

Solutions bathing intact roots during impalement were supplied from 1-L reservoirs through ¼ inch tygon tubing. Compositions of these solutions are provided in the text but typically consisted of 200 μM $CaSO_4$ plus or minus various concentrations of $Ca(NO_3)_2$ or 1/80 strength Johnson's nutrient solution plus or minus $Ca(NO_3)_2$. The latter solution was chosen because all our previous $^{13}NO_3^-$ flux work (2, 16, 17) was undertaken in this solution. The protocol used in these experiments was to impale the roots in 200 μM $CaSO_4$ solution or 1/80 strength Johnson's solution (minus NO_3^-). When a stable reading of $\Delta\psi$ was obtained, the solution bathing the root was replaced by 200 μM $CaSO_4$ plus $Ca(NO_3)_2$ or by 1/80 strength Johnson's solution plus $Ca(NO_3)_2$.

In other experiments, a different protocol was adopted: roots were first impaled in $CaSO_4$ solution, which was replaced by $Ca(NO_3)_2$ solution at the same strength. The depolarizations obtained by the latter method were due solely to the differences in the electrical effects of NO_3^- and SO_4^{2-} without contributions from Ca^{2+}. A second method was used to examine NO_3^- effects in complete isolation from those due to Ca^{2+}. This involved titrating 15 mM Mes-Tris to pH 5.2 with HNO_3 and to pH 6 with $Ca(OH)_2$. For each ion (NO_3^- or Ca^{2+}), roots were first equilibrated in 15 mM Mes-Tris, before exposure at the same pH, to the particular ion. The buffers alone typically caused approximately 20 mV depolarization of $\Delta\psi$, which repolarized spontaneously.

Data shown in Figures 1, 3, 4, and 6 are representative traces from experiments repeated several times on a particular day with a series of roots and repeated on two or three separate days with different batches of roots. Data for the experiment shown in Figure 7 were obtained from a single experiment repeated three times with three separate roots to ensure that NO_3^- uptake by the HATS was not induced during the course of the experiment. The quantitative data shown in Figures 2 and 5 are means of three replicate measurements at each NO_3^- concentration using a single root. The data are representative of experiments repeated at least three times.

Measurement of H^+ Fluxes by Means of Ion-Selective Microelectrodes

Liquid membrane-type neutral carrier-based H^+-selective microelectrodes (tip diameter = 0.5 μm) were constructed as previously detailed (8) using Fluka H^+-selective cocktail (catalog No. 95291, Fluka Chemical Co., Ronkonkoma, NY).

The root and vertically positioned microelectrodes were viewed at 60 to 150× magnification with the Olympus microscope. To measure net H^+ fluxes, the appropriate experimental solution was passed through the chamber until the previous solution was displaced, and then flow was stopped to allow the development of ion gradients. Subsequently, H^+ activities in the unstirred layer were measured at 50 and 100 μm from the root surface and the net ionic fluxes at the root surface were determined from the following equation derived from diffusion analysis of the spatial symmetry of the H^+ activity gradients:

$$J_1 = \frac{2\pi D_1 (C_1 - C_2)}{ln\,(R_1/R_2)}$$

where J_1 is the net ionic flux (in μmol cm^{-1} s^{-1}), D_1 is the self-diffusion coefficient for the ion of interest (in cm^2 s^{-1}), C_1 and C_2 are the ion activities measured at the two radial

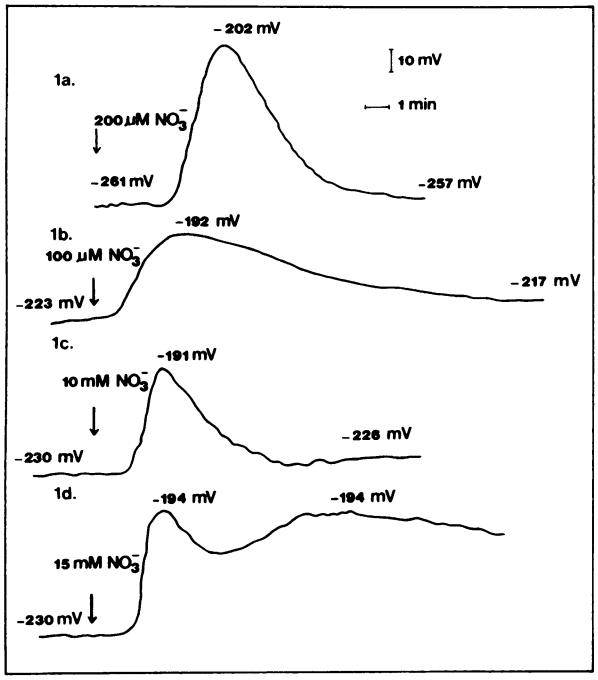

Figure 1. Depolarization and repolarization patterns at different concentrations of external NO_3^-. a, 200 μM NO_3^-; b, 100 μM NO_3^-; c, 10 mM NO_3^-; d, 15 mM NO_3^-. All plants had been induced by pretreatment with 100 μM NO_3^- for 18 h.

positions, and R_1 and R_2 are the respective distances from the positions where the ion activities were measured to the center of the root (10). The above flux equation yields a flux normalized for a 1-cm long root segment (thus the units of μmol cm^{-1} s^{-1}). Subsequently, the flux value calculated from the above equation was divided by the mass of the 1-cm segment of root (cross-sectional area of root in cm^2 × root density in g cm^{-3} × 1-cm root length) and multiplied by the appropriate unit's conversion factor (3600 s h^{-1}) to obtain a net flux in units of μmol g^{-1} h^{-1}. The net H fluxes determined in this study were measured approximately 2 cm back from the root apex.

RESULTS

HATS

$\Delta\psi$ in the range from −200 to −260 mV were recorded when roots were impaled in solutions containing 200 μM $CaSO_4$. Occasionally, values as low as −300 mV were recorded; these were less common and usually arrived at some time into a single impalement after several cycles of $CaSO_4$/$Ca(NO_3)_2$ treatment. Data shown in Figure 1a are for plants exposed to 100 μM NO_3^- for 24 h before brief equilibration in 200 μM $CaSO_4$ solution containing no NO_3^-. The provision of 200 μM NO_3^- [in the form of $Ca(NO_3)_2$] caused a rapid depolarization of $\Delta\psi$ from −261 to −202 mV, which was virtually complete within 2 min. The extent of depolarization due to external NO_3^- was found to vary from root to root and according to NO_3^- pretreatment, but was particularly reproducible for a single root or a single impalement. In this particular example (Fig. 1a), depolarization of $\Delta\psi$ in the presence of external NO_3^- was followed rapidly by repolarization to within a few mV of the original value of $\Delta\psi$.

During the course of greater than 100 impalements undertaken in this series of experiments, the patterns of repolarization, when evident, appeared to fall into four categories:

1. As in Figure 1a, depolarization by low $[NO_3^-]_o$ was followed rapidly by repolarization, restoring $\Delta\psi$ to within a few mV of its original value.
2. In many instances, particularly at low external $[NO_3^-]$, repolarization was slow and the original value of $\Delta\psi$ was not restored even after 5 to 10 min (Fig. 1b).
3. At high external $[NO_3^-]$, in the range beyond 5 mM, repolarization was extremely rapid, restoring $\Delta\psi$ to its original value within several minutes (Fig. 1c).
4. At the highest $[NO_3^-]$ investigated (20 mM), repolarization appeared to commence when depolarization reached its maximum value but was short-lived and only achieved a partial recovery (5–10 mV) before a second much smaller depolarization was evoked. This biphasic pattern of depolarization was not followed by repolarization (Fig. 1d).

In those cases where repolarization was incomplete in $Ca(NO_3)_2$, replacement of the $Ca(NO_3)_2$ solution by 200 μM $CaSO_4$ solution caused a rapid onset of full repolarization and, typically, a small hyperpolarization. Even when complete repolarization had occurred in $Ca(NO_3)_2$ solution, $CaSO_4$ solution still caused a small hyperpolarization, followed over a period of 2 to 5 min by a return to $\Delta\psi$ values typical of those recorded prior to $CaSO_4$ exposure.

The effect of NO_3^- concentration on $\Delta\psi$ is shown in Figure 2. The method used to obtain these results involved replacing 0.2 mM $CaSO_4$ solution by 0.2 mM $CaSO_4$ plus the appropriate concentration of $Ca(NO_3)_2$. The plot of depolarization *versus* $[NO_3^-]_o$ assumed the form of a rectangular hyperbola for roots that had been induced by NO_3^- pretreatment for 18 h

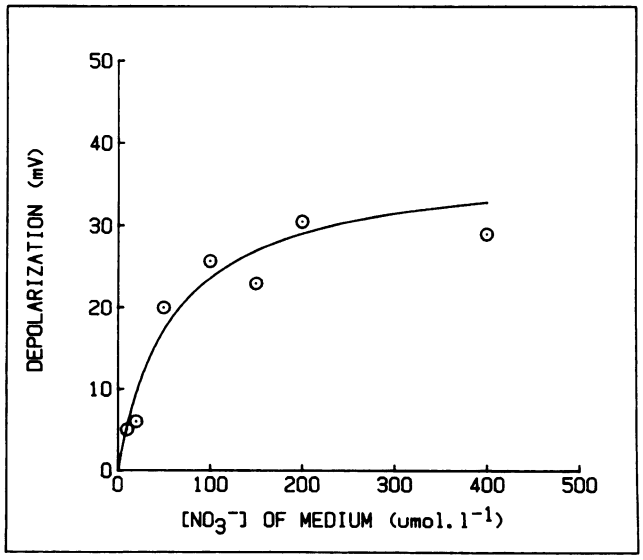

Figure 2. Concentration dependence of NO_3^--mediated depolarization of $\Delta\psi$ by the HATS in plants induced for NO_3^- uptake by pretreatment with 100 μM NO_3^- for 18 h.

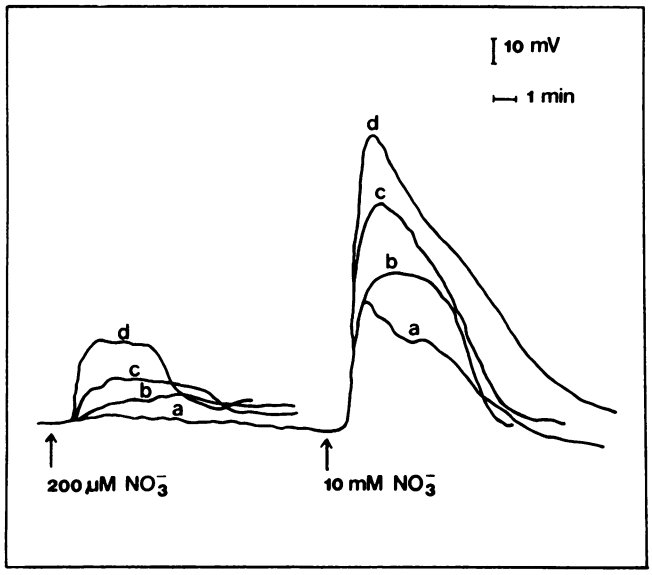

Figure 3. Depolarization of $\Delta\psi$ by 200 μM and 10 mM NO_3^- as a function of time in plants that had (initially) received no NO_3^- exposure. a, 0 h; b, +1 h; c, +2.5 h; d, +5 h of exposure to NO_3^-.

(Fig. 2). The half-saturation value for this depolarization (analogous to a K_m value) was 60 μM, whereas the maximum depolarization (analogous to a V_{max}) was 38 mV. These values were obtained by a direct fit of the data to the Michaelis-Menten equation by an iterative computer program. The r^2 for regression was 0.96. The effect of NO_3^- concentration was also evaluated by switching from a given concentration of $CaSO_4$ to the identical concentration of $Ca(NO_3)_2$. The recorded depolarization under these conditions eliminated any effect of Ca^{2+}. The results of these experiments (data not shown) were virtually indistinguishable from those shown in Figure 2.

The electrical effects of NO_3^- exposure were also examined in a complete inorganic nutrient solution (1/80 strength Johnson's modified medium) rather than in 200 μM $CaSO_4$. The depolarizing effects of NO_3^- in Johnson's solution were virtually identical to those observed in $CaSO_4$ solution. However, membrane potentials were typically less negative in the Johnson's solution.

Time Course of Induction of NO_3^- Response

Roots that had received no prior exposure to NO_3^- ("uninduced plants") gave little or no electrical response to $Ca(NO_3)_2$ in the low concentration range (approximately 5–100 μM). In an experiment designed to compare the electrical responses of uninduced roots to NO_3^- exposures in the low (200 μM) to those in the high (10 mM) concentration range, as a function of duration of NO_3^- exposure, plants were alternately exposed to these two concentrations of NO_3^- for a period of 5 h. It is evident from Figure 3 that, although 200 μM NO_3^- caused no significant depolarization at first exposure, the 10-mM NO_3^- solution caused a strong depolarization only minutes later. Over the 5-h period, the depolarizing effect of 200 μM NO_3^- increased gradually. Likewise, depolarization by 10 mM NO_3^- increased to an extent that corresponded with the gradually increasing depolarization due to the HATS (at 200 μM) (Fig. 4). It is apparent that depolarizations due to the two transport systems were additive, resulting in a very large depolarization by 10 mM NO_3^- by 5 h.

The effect of NO_3^- pretreatment over longer durations was examined by exposing plants to 100 μM NO_3^- for 1, 2, or 3 d. The depolarizations caused by 200 μM NO_3^- actually diminished when plants were induced for longer than 24 h, declining from 37 ± 4 mV after 24 h of NO_3^- exposure to 23 ± 4 mV after 48 h and 20 ± 0.9 mV after 72 h.

LATS

Unlike the depolarization due to the high-affinity system for NO_3^- uptake, depolarization by $[NO_3^-]$ values beyond 0.5 mM (the concentration range in which the LATS becomes evident) appeared to be constitutive (Figs. 3 and 4). Moreover, depolarization in this concentration range showed no indication of saturation, even at 10 to 20 mM NO_3^- (Fig. 5). It should be emphasized that NO_3^- was provided throughout these experiments as the Ca salt.

Because cations such as K^+ or Ca^{2+} may themselves be depolarizing at quite moderate concentrations, it was important to distinguish between Ca^{2+} and NO_3^- as the source of the large depolarizations observed in the presence of high $[NO_3^-]_o$. Two methods were used to investigate this question. In the first, uninduced roots were exposed to $CaSO_4$ solutions in the high concentration range (>1 mM); these solutions typically caused significant depolarizations (Fig. 6) that were not accompanied by repolarization. When depolarization by $CaSO_4$ had caused $\Delta\psi$ to reach a new steady value, the solution bathing the root was replaced by an identical concentration of $Ca(NO_3)_2$. Thus, the Ca^{2+} concentration remained unchanged, but NO_3^- replaced SO_4^{2-}. The effect of this treat-

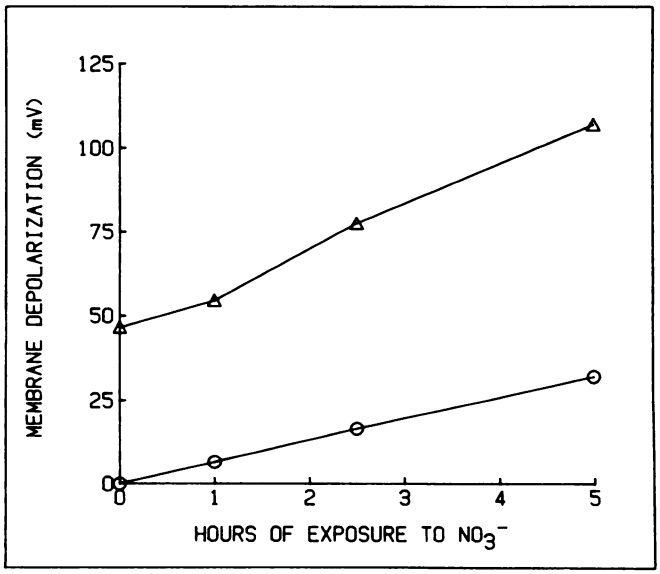

Figure 4. A plot of depolarization of $\Delta\psi$ by 200 μM (O) and 10 mM NO_3^- (Δ) as a function of duration of NO_3^- exposure. Plants were initially uninduced for NO_3^- uptake.

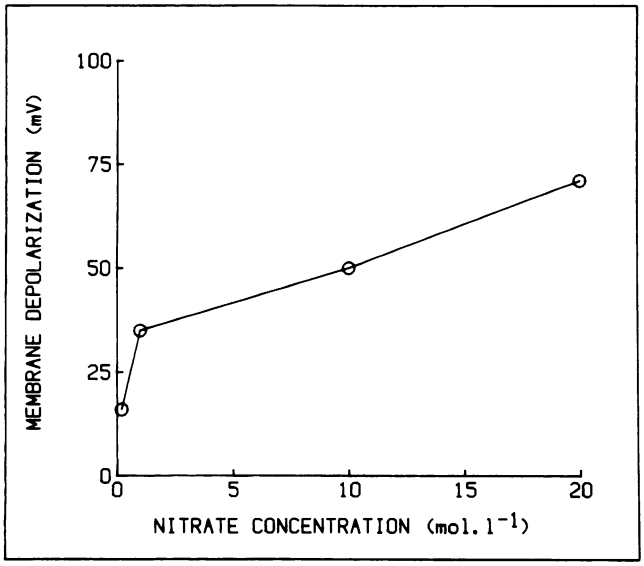

Figure 5. A plot of NO_3^--mediated depolarization of $\Delta\psi$ by the LATS *versus* [NO_3^-]. Plants had been induced for NO_3^- uptake by pretreatment with 100 μM NO_3^- for 18 h.

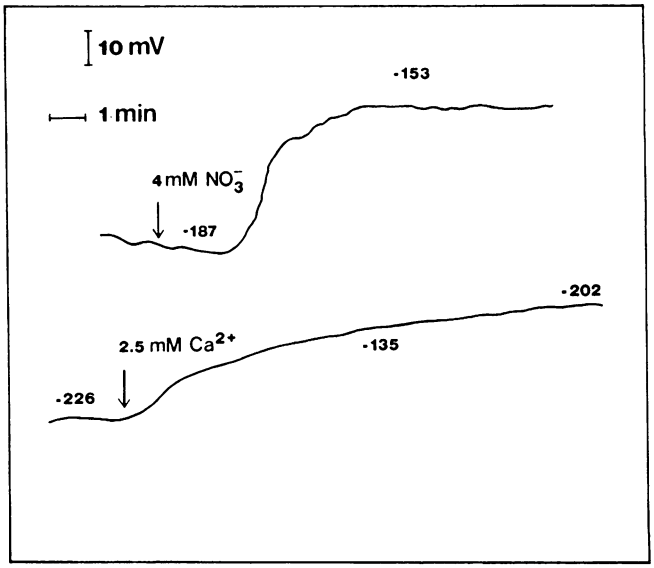

Figure 7. Depolarization of $\Delta\psi$ by NO_3^- or Ca^{2+}. In each case, the conjugate acid or base had been titrated with 15 mM Mes-Tris. Plants were uninduced for NO_3^- uptake.

ment was to cause a further large depolarization. In the second method, buffered solutions (15 mM Mes-Tris) were employed to titrate $Ca(OH)_2$ or HNO_3 to a desired pH. By this method, it was possible, in a background of Mes-Tris (which itself caused approximately 20 mV depolarization), to evaluate the effect of Ca^{2+} and NO_3^- independently. Figure 7 demonstrates that the NO_3^- anion was strongly depolarizing; Ca^{2+} also caused a substantial (28 mV) depolarization. The pattern of the depolarizing effect of Ca^{2+}, however, was distinctly different from that caused by NO_3^-. The Ca-induced depolarization was smaller than that due to NO_3 (28 mV compared with 42 mV) and took much longer to develop (approximately 16 min to reach the 28 mV reported compared to 5 min for NO_3^-). In neither case was spontaneous repolarization evident, but upon returning roots to the buffered solutions minus Ca^{2+} or NO_3^-, strong repolarizations brought $\Delta\psi$ back to their original values.

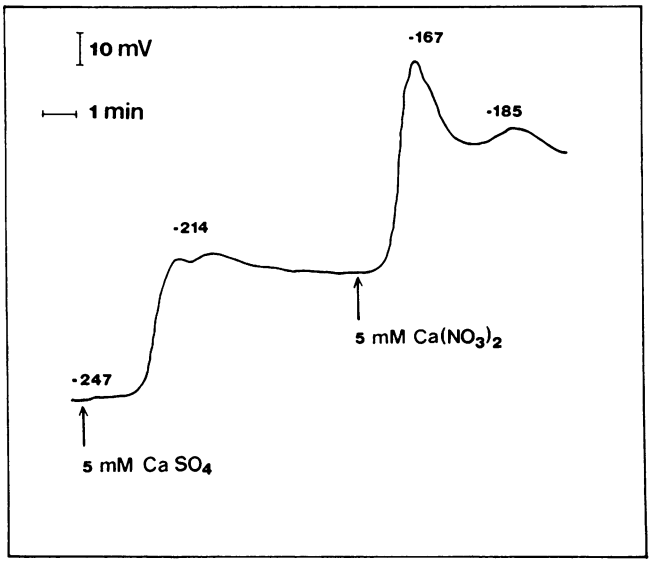

Figure 6. Depolarization of $\Delta\psi$ by the LATS. Roots were first exposed to 5 mM $CaSO_4$. When a steady value of $\Delta\psi$ had been achieved, the $CaSO_4$ solution was replaced by an identical concentration of $Ca(NO_3)_2$. Plants were uninduced for NO_3^- uptake.

To evaluate the hypothesis that a NO_3^-/nH^+ symporter was responsible for the depolarizing effects of NO_3^-, H^+ fluxes were measured close to the root surface during exposure to different salt solutions (Table I). It is evident that only when the K^+ fluxes had been down-regulated by 3-d exposure to K^+ was NO_3^- exposure accompanied by alkalinization of external media (Table IB). Nevertheless, whenever NO_3^- was provided to the roots of induced plants, the extent of acidification caused by K^+ salts was substantially diminished. This was particularly true at high external [K^+]. This response was evident in uninduced plants only at high external [NO_3^-].

DISCUSSION

The experiments described above demonstrate that exposure to $Ca(NO_3)_2$ solutions may cause large depolarizations of $\Delta\psi$ in barley roots. At low $Ca(NO_3)_2$ concentrations, the reported values for depolarization are almost exclusively due to the absorption of NO_3^-. This is demonstrated by the fact that 200 μM $Ca(NO_3)_2$ caused virtually no depolarization in uninduced plants (*e.g.* Fig. 3). Yet, within 1 to 5 h (which corresponds to the time scale for the induction of increased NO_3^- uptake), exposure to the same concentration of $Ca(NO_3)_2$ caused >20 mV depolarization. Also, the extent of depolarization diminished after 24 h, corresponding to the reduction of $^{13}NO_3^-$ influx, which has been reported for prolonged exposure to NO_3^- in barley roots (15). Two other arguments support this conclusion. (a) In switching experi-

Table I. *Fluxes of H^+ Associated with Various Ion Treatments*

A, Plants had been exposed to 100 μM NO_3^- for 24 h. B, Plants were grown for 3 d on 100 μM K_2SO_4 to down-regulate the K^+ flux. On the third day, plants also received 50 μM $Ca(NO_3)_2$ to induce NO_3^- uptake. C, Plants had been grown without exposure to NO_3^- so that the HATS for NO_3^- uptake was uninduced. Negative values of H^+ fluxes indicate H^+ efflux.

Treatment	H^+ Fluxes[a]
	$\mu mol\ g^{-1}\ h^{-1}$
A. 200 μM $CaSO_4$	−0.41 ± 0.05
100 μM $Ca(NO_3)_2$	−0.42 ± 0.06
100 μM K_2SO_4	−2.05 ± 0.2
200 μM KNO_3	−1.24 ± 0.1
200 μM $CaSO_4$	−0.39 ± 0.06
5 mM $Ca(NO_3)_2$	−0.07 ± 0.01
100 μM K_2SO_4	−2.74 ± 0.2
100 μM K_2SO_4 + 5 mM $Ca(NO_3)_2$	−0.43 ± 0.06
B. 200 μM $CaSO_4$	−4.8 ± 0.3
100 μM $CaSO_4$ + 100 μM $Ca(NO_3)_2$	+0.1 ± 0.02
100 μM $CaSO_4$ + 100 μM K_2SO_4	−7.35 ± 0.25
100 μM $CaSO_4$ + 200 μM KNO_3	−3.64 ± 0.13
C. 200 μM $CaSO_4$	−0.61 ± 0.06
200 μM $CaSO_4$ + 100 μM K_2SO_4	−6.68 ± 0.19
200 μM $CaSO_4$ + 200 μM KNO_3	−8.75 ± 0.19
200 μM $CaSO_4$ + 5 mM K_2SO_4	−14.06 ± 0.60
200 μM $CaSO_4$ + 10 mM KNO_3	−10.54 ± 1.75

[a] H^+ fluxes reported are the means of five separate measurements.

ments where $CaSO_4$ solution was replaced by the same concentration of $Ca(NO_3)_2$, the results were virtually identical to those in which $Ca(NO_3)_2$ was added in a background of $CaSO_4$ solution (see "Materials and Methods"). (b) In most of the experiments reported here, plants had been grown for 3 or 4 d in 200 μM $CaSO_4$. This treatment produces Ca^{2+}- and SO_4^{2-}-rich plants that would be expected to express low rates of Ca^{2+} and SO_4^{2-} uptake.

Thus, the HATS for NO_3^- influx in barley roots, like those of *Lemna* and corn roots, operate with concomitant depolarization of $\Delta\psi$ (9, 19). Like *Lemna* and corn, depolarization was commonly followed by repolarization and, sometimes, by a modest hyperpolarization. However, unlike corn, depolarization in barley was $[NO_3^-]$-dependent and saturable in a manner similar to the uptake of NO_3^-. Indeed, the K_m for depolarization in the presence of NO_3^- was 60 μM. Siddiqi *et al.* (16) found that the influx of $^{13}NO_3^-$ was also saturable below 1 mM, and the K_m for influx varied between 30 and 80 μM, depending on NO_3^- pretreatment. According to McClure *et al.* (9), corn, by contrast, showed only a small depolarization of approximately 10 mV in response to NO_3^-. This was independent of $[NO_3^-]$ even at concentrations as high as 10 mM. In this regard, barley more closely resembles *Lemna*. Like *Lemna* and corn, depolarization by low $[NO_3^-]_o$ is evident only after induction by NO_3^- pretreatment. As shown in Figure 3, the extent of depolarization by the HATS increased rapidly over a period of 5 h. This corresponds well with the induction of $^{13}NO_3^-$ influx in this barley variety (15) as well as the induction of net NO_3^- uptake (our unpublished results). Likewise, the reduced depolarization of $\Delta\psi$ caused by 200 μM NO_3^- after 2 and 3 d of NO_3^- pretreatment (compared with 1 d of NO_3^- pretreatment) mirrors the reduction of $^{13}NO_3^-$ influx that occurs between 24 and 96 h after the first exposure to NO_3^- (15). This effect has been interpreted as the result of negative feedback from internal NO_3^- or reduced N (7).

Repolarization and hyperpolarization following depolarization is thought to reflect stimulation of H^+ pumping by the plasma membrane ATPase. Clearly, the extent of the depolarizing current (due to the proposed 2 H^+:1 NO_3^- symport), balanced or exceeded by the activity of the H^+ pump, will determine, at any instant, the measured value of $\Delta\psi$. The small depolarization of $\Delta\psi$, independent of $[NO_3^-]$, observed in corn may indicate narrow tolerance limits for depolarization in this species. Hence, a small depolarization may result in activation of the H^+ pump. In barley, by contrast, large depolarizations (up to 60 mV) appear to be tolerated before the H^+ pump is sufficiently activated to produce repolarization. As discussed above, the pattern of repolarization was variable and sometimes complex in barley roots. The clear concentration dependence of hyperpolarization observed in corn was rarely evident in barley. Generally, repolarization was sufficient to restore $\Delta\psi$ to values close to the predepolarized value. Hyperpolarization was usually small, a matter of 5 to 10 mV.

The depolarizing effect of NO_3^- in the high concentration range (>1 mM), especially in roots of plants that had received no previous NO_3^- exposure, is particularly important. It demonstrates that NO_3^- absorption by the LATS, which has been examined in some detail by means of $^{13}NO_3^-$ studies (2, 16, 17), is not a diffusive flux, as has been proposed by various other workers (11, 14, 19). A diffusive NO_3^- flux would not be depolarizing. Moreover, a major difficulty with a diffusive model, as indeed with a channel-mediated flux, which we proposed earlier (16, 17), is that according to the Nernst potential, with $\Delta\psi$ values as low as −200 to −300 mV, cytoplasmic $[NO_3^-]$ would have to be in the nanomolar range for the NO_3^- flux to be diffusive.

We have recently suggested (17) that in cells actively engaged in the reduction of nitrate, particularly epidermal cells, $[NO_3^-]$ values may be much lower than in inner cortical cells, which have been reported to contain but a small proportion of root nitrate reductase activity (13). However, even if $[NO_3^-]$ values were sufficiently low to accommodate a channel-mediated flux in thermodynamic terms, the depolarizing effect of high NO_3^- still remains to be accounted for. The latter observation suggests that the LATS system for transport is a cation (possibly H^+) cotransport system. Considering the importance of this finding, it was critical to be certain that the depolarizing effects of high concentrations of $Ca(NO_3)_2$ could be ascribed to NO_3^- influx and not to effects of high $[Ca^{2+}]$. Moreover, because treatments involving high $[NO_3^-]$ might include the depolarizing effects of the HATS, it was important to evaluate the depolarizing effects of the LATS using uninduced plants where the HATS for NO_3^- is virtually absent.

The depolarizing effect of HNO_3 presented in 15 mM Mes-Tris (pH 5.2) to uninduced roots (Fig. 7) effectively addresses the concerns described above. The extent of depolarization shown (42 mV) was typical of three independent determinations (mean = 39 ± 2.5 mV). To check that exposure to NO_3^-

during the course of the experiment had not induced the HATS, the $Ca(NO_3)_2$ solution was displaced by flowing 200 μM $CaSO_4$ solution through the root chamber for 5 min. Roots were then exposed to 200 μM $CaSO_4$ plus 100 μM $Ca(NO_3)_2$. The very small depolarizations (2–4 mV) demonstrated that the roots had not been induced by the short exposure to high concentrations of NO_3^-.

$CaSO_4/Ca(NO_3)_2$ switching experiments with uninduced roots (*e.g.* Fig. 6) also demonstrated that high $[NO_3^-]$ caused a large depolarization of $\Delta\psi$, over and above that caused by $CaSO_4$. Because the $[Ca^{2+}]$ was held constant in this protocol, the depolarization arising from a switch from $CaSO_4$ to an identical concentration of $Ca(NO_3)_2$ represents the extent to which the NO_3^- depolarization exceeded that due to SO_4^{2-}. The observed depolarization (Fig. 6) is therefore an underestimate of the NO_3^- effect because SO_4^{2-} itself may cause a significant depolarization.

Therefore, it was decided to investigate the concentration dependence of the depolarization of $\Delta\psi$ by the LATS by supplying $Ca(NO_3)_2$ in a background of 200 μM $CaSO_4$. The reported depolarizations (Figs. 3–5) are therefore the sum of the Ca^{2+} and NO_3^- effects on $\Delta\psi$. This method suffers from this shortcoming, but because the Ca^{2+} effect was relatively small by comparison with the NO_3^- effect and relatively slow to reach its maximum effect (Fig. 7), it was considered to be a satisfactory compromise. By contrast, the switching method, although useful at low $Ca(NO_3)_2$ concentration where SO_4^{2-} has little depolarizing effect, suffers a greater disadvantage at high $CaSO_4$ because of the potential depolarizing effects of SO_4.

The observed depolarizing effect of high $[NO_3^-]$ in uninduced plants is also important because it indicates that the NO_3^- flux due to the LATS traverses the plasma membranes of epidermal or cortical cells. It is not represented by NO_3^- entering the stele through undifferentiated endodermal regions, passage cells, or breaks of the endodermis associated with lateral roots. The existence of the LATS in unicellular organisms (11, 14) also provides evidence that this flux does not depend upon a complex tissue organization. The constitutive LATS for NO_3^- should be acknowledged as a physiologically meaningful transport system at the cellular level. The use of higher concentrations of NO_3^- to observe the nature of the concentration dependence or to obtain stronger (more definitive) NO_3^- responses does not deny the expression of the LATS at much lower concentrations. In *Skeletonema,* a linear dependence of NO_3^- uptake was apparent at $[NO_3^-]$ <20 μM (14). In higher plants, the system becomes apparent between 200 and 500 μM $[NO_3^-]_o$ (16).

The existence of a constitutive LATS, given our knowledge of an inducible HATS, nevertheless is perplexing. However, our failure to comprehend the biological significance of this system should not lead to its dismissal as irrelevant. Indeed, in microbial systems, constitutive LATS that operate at high external ion concentration, together with derepressible HATS at low ion concentration, are typical. One explanation for its existence may be to facilitate very rapid induction of the HATS during seasonal flushes of NO_3^-. These are well documented in marine as well as in terrestrial environments. As $[NO_3^-]_o$ falls due to initial (constitutive) absorption and further uptake associated with growth stimulation, the presence of a fully induced HATS might confer significant advantages in competition for the diminishing resource.

The concentration dependence of the depolarization by high $[NO_3^-]$ is entirely consistent with the reported concentration dependence of $^{13}NO_3^-$ influx at high $[NO_3^-]_o$ in barley (16). Moreover, as shown in Figure 4, the depolarizations due to the HATS and LATS are apparently additive. The experiments in which $CaSO_4$ solution was replaced by $Ca(NO_3)_2$ at the same Ca^{2+} concentration, as well as the experiments with $Ca(OH)_2$- or HNO_3-buffered solutions, clearly establish that, notwithstanding a depolarizing effect of Ca^{2+}, there is an even more pronounced effect of NO_3^-.

The most likely explanation for the depolarization of $\Delta\psi$ by high $[NO_3^-]$ is that NO_3^- uptake by the LATS is thermodynamically uphill even in those (epidermal) cells that, by virtue of the localized nitrate reductase activity (13), may have lower cytoplasmic $[NO_3^-]$ than inner cortical cells. As a consequence, cotransport with H^+ may provide the necessary driving force for this flux. This explanation, in light of the present electrophysiological data, clearly demands that the earlier proposal of a channel-mediated NO_3^- influx localized in epidermal cells (17) be rejected.

Attempts to demonstrate H^+ influx associated with provision of NO_3^- were complicated by the net H^+ efflux in $CaSO_4$ solution and results were not entirely unequivocal (Table I). However, when H^+ efflux was stimulated by application of K_2SO_4 solution, the extent of this efflux was strongly reduced by transfer to equimolar KNO_3. The simplest interpretation of this observation is that NO_3^- uptake by the HATS is mediated by a 2 H^+:1 NO_3^- symport. When plants were pretreated with 100 μM K^+ for 3 d to reduce cation uptake, substitution of 200 μM $CaSO_4$ by 100 μM $Ca(NO_3)_2$ plus 100 μM $CaSO_4$ caused a net influx of H^+ (Table IB). Similarly, when 100 μM K_2SO_4 plus 100 μM $CaSO_4$ was replaced by 200 μM KNO_3 plus 100 μM $CaSO_4$, H^+ efflux declined from 7.35 to 3.64 μmol g^{-1} h^{-1} (Table I).

These experiments were undertaken with plants that had been induced for NO_3^- uptake by pretreatment with 100 or 200 μM NO_3^-. Higher $[NO_3^-]$, typical of the $[NO_3^-]$ for absorption by the LATS, caused a much stronger reduction of H^+ efflux. For example, Table I indicates that the substitution of 100 μM K_2SO_4 by 200 μM KNO_3 reduced H^+ efflux from 2.05 to 1.24 μmol g^{-1} h^{-1}. When roots acidifying in 100 μM K_2SO_4 were treated with 5 mM $Ca(NO_3)_2$ in addition to the 100 μM K_2SO_4, H^+ efflux declined from 2.74 to 0.43 μmol g^{-1} h^{-1}.

Table IC shows data for H^+ fluxes by uninduced plants. It is evident that exposure to 200 μM NO_3^- failed to reduce the extent of H^+ efflux in these plants, consistent with the low level of expression of the HATS for nitrate uptake in NO_3^--deprived plants (16). By contrast, 10 mM NO_3^- was able to bring about significant reduction of H^+ fluxes (from 14.06 to 10.54 μmol g^{-1} h^{-1}). This observation is consistent with the documented constitutive character of the LATS for nitrate influx (16) and for depolarization of $\Delta\psi$ reported in the present paper. These results indicate that NO_3^- uptake by the LATS, like HATS, is probably mediated by a 2 H^+:1 NO_3^- symport.

In summary, the electrophysiological experiments described above establish that both the high- and low-affinity NO_3^- transport systems of barley roots are electrically depo-

larizing. The electrical properties of these transport systems conform in all aspects examined (concentration dependence, negative feedback effects, inducibility) to the transport properties examined earlier by means of $^{13}NO_3^-$ (15, 16). The use of uninduced plants enabled us to isolate the LATS and to demonstrate unequivocally its constitutive capacity for NO_3^- absorption and plasma membrane depolarization. The measurement of H^+ fluxes associated with NO_3^- absorption by both the HATS and LATS suggest that 2 H^+:1 NO_3^- symports may represent the means for driving NO_3^- "uphill" against a sizable electrochemical potential gradient.

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