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Photolabeling of Tonoplast from Sugar Beet Cell Suspensions by [^{3}H]5-(*N*-Methyl-*N*-Isobutyl)-Amiloride, an Inhibitor of the Vacuolar Na^+/H^+ Antiport[1]

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ABSTRACT

The effects of 5-(*N*-methyl-*N*-isobutyl)-amiloride (MIA), an amiloride analog, was tested on the Na^+/H^+ antiport activity of intact vacuoles and tonoplast vesicles isolated from sugar beet (*Beta vulgaris* L.) cell suspension cultures. MIA inhibited Na^+/H^+ exchange in a competitive manner with a K_i of 2.5 and 5.9 micromolar for ΔpH-dependent $^{22}Na^+$ influx in tonoplast vesicles and Na^+-dependent H^+ efflux in intact vacuoles, respectively. Scatchard analysis of the binding of [^{3}H]MIA to tonoplast membranes revealed a high affinity binding component with a K_d of 1.3 micromolar. The close relationship between the dissociation constant value obtained and the constants of inhibition for MIA obtained by fluorescence quenching and isotope exchange suggests that the high affinity component represents a class of sites associated with the tonoplast Na^+/H^+ antiport. Photolabeling of the tonoplast with [^{3}H]MIA revealed two sets of polypeptides with a different affinity to amiloride and its analog.

Plants growing in high salinity typically maintain a high K^+/Na^+ ratio in the cytoplasm. The transport of ions across the plasma membrane and tonoplast is thought to play an important role in maintaining low cytoplasmic sodium concentrations. Electrogenic H^+-translocating pumps located both at the plasma membrane (H^+-ATPase) and tonoplast (H^+-ATPase and H^+-PPase) of plant cells generate an electrochemical potential difference of protons (μH^+, protonmotive force) which drives the H^+-coupled Na^+ transport across the tonoplast into the vacuole, and across the plasma membrane out of the cell (4). The operation of H^+-coupled Na^+ transport systems has been reported for several species; in barley roots (23) and tobacco cells (26), the addition of Na^+ to the bathing medium increased the proton efflux across the plasma membrane. Braun *et al.* (5) presented evidence for the operation of an active Na^+/H^+ exchange across plasma membrane vesicles of *Atriplex* roots, and the presence of an Na^+/H^+ antiport was reported in plasma membrane vesicles from the halophyte *Dunaliella salina* (16).

Evidence for the active accumulation of Na^+ in vacuoles of halophytes and salt-tolerant glycophytes has been presented. In barley, a high ratio of Na^+/K^+ was reported in the vacuoles of cortical cells (14) and leaf cells (19). Short-term studies of exposure of barley roots to salt by *in vivo* NMR spectroscopy, revealed a Na^+-induced upward shift in vacuolar pH, consistent with the operation of a Na^+/H^+ antiport (8). In *Beta vulgaris,* Na^+/H^+ antiport exchange was demonstrated in intact vacuoles isolated from red beet roots (20), and sugar beet cell suspensions (3). In studies using isolated tonoplast vesicles, Na^+/H^+ antiport activity has been reported in membranes isolated from storage tissue of red beet (1) and sugar beet (3), and from barley roots (10). In barley, the antiport activity was almost completely induced by external NaCl (10), *de novo* protein synthesis was not detected and the activation by salt of an existing protein was suggested (11). In beet cell suspensions, a 200-fold increase in the external NaCl concentration resulted in only a 100% increase in antiport activity, suggesting that the tonoplast Na^+/H^+ antiport in these cells was consititutive (3).

Despite increasing evidence accumulating on the operation of the Na^+/H^+ antiport in membranes from different plant species, the biochemical identification of the antiport has not been accomplished. Our previous studies have shown that the diuretic drug amiloride acts as a competitive inhibitor of the plant vacuolar Na^+/H^+ antiport, similar to its effects in animal cells (1). However, amiloride is not a specific inhibitor of the Na^+/H^+ antiport and also inhibits a number of important membrane structures involved in Na^+ transport. These include Na^+ channels, the Na^+/Ca^{2+} antiport, Na^+/amino acid and Na^+/glucose transporter (17). Replacement of one or both hydrogen atoms of the 5-amino group of amiloride by alkyl or alkenyl groups resulted in a 10–500 fold increase in potency for inhibition of the Na^+/H^+ antiport, with a significant reduction in the inhibitory effect of other amiloride-sensitive transport systems (17, 25). We have studied the effect of a series of these 5-amino substituted amiloride analogs in intact vacuoles and isolated tonoplast vesicles from *B. vulgaris,* and found a 3- to 200-fold increase in the inhibitory effect on the Na^+/H^+ antiport activity without any detectable effect on the tonoplast H^+-ATPase or proton permeability (2).

In the present study, we have used intact vacuoles and tonoplast vesicles isolated from sugar beet cell suspensions to further investigate the properties of the Na^+/H^+ antiport. We have studied the covalent incorporation of the 5-amino-sub-

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stituted amiloride analog [^{3}H]MIA[2] after photolysis to determine the membrane proteins containing binding sites for MIA.

MATERIALS AND METHODS

Plant Material

Cell suspension cultures of sugar beet (*Beta vulgaris* L.) were grown as described previously (3).

Isolation of Tonoplast Vesicles and Vacuoles

Purified tonoplast vesicles were prepared by homogenization of sugar beet cell suspensions followed by density gradient fractionation (2). Intact vacuoles were isolated by osmotic lysis of sugar beet protoplasts as described before (3).

ΔpH Dependent ^{22}Na Uptake

Tonoplast vesicles (200 μg protein) were added to 265 μl buffer containing 0.25 M mannitol, 5 mM Tris/Mes (pH 8.0), 2 mM DTT, 2 mM Tris/ATP, 5 mM $MgSO_4$, 5 mM glucose, and 60 mM tetramethylammonium chloride, to initiate proton translocation. When a steady state pH gradient, acidic inside, was formed (5 min), the ATP dependent H^+ transport activity was stopped by the addition of hexokinase (EC 2.7.1.1) and glucose (2). Radiolabeled $^{22}Na^+$ uptake was started by adding aliquots of 1 M NaCl plus ^{22}NaCl (2 μCi/mmol). After 1 min of incubation the vesicles were collected on 0.45 μm Millipore filters and washed twice with 1 mL of ice cold buffer containing 5 mM Tris/Mes (pH 8.0), 0.25 M mannitol, and 2 mM DTT. The radioactivity retained on the filter was measured in 8 mL of Aquasol-2 solution in a liquid scintillation counter. H^+-dependent Na^+ uptake is defined as Na^+ uptake in the presence of a pH gradient generated by the H^+-ATPase minus the uptake with membranes previously incubated with 2 mM NH_4Cl which collapsed the pH gradient across the tonoplast vesicles.

Fluorescence Assays

The fluorescence quenching of acridine orange was used to monitor the dissipation of inside-acidic pH gradients across the membranes of intact vacuoles. Vacuoles (1×10^5) were added to 1 ml buffer containing 0.4 M mannitol, 1 mM DTT, 10 mM Tris/Mes (pH 8.0), 5 mM glucose, 60 mM tetramethylammonium chloride, 1 mM Tris-ATP, and 5 μM acridine orange. Intravacuolar acidification was obtained by activation of the H^+-ATPase by addition of 2 mM $MgSO_4$, and the fluorescence changes with time were monitored in a thermostated cell at 25 °C with a Hitachi 4000 spectrofluorimeter at excitation and emission wavelengths of 495 and 540 nm, respectively, and a slit width of 3 nm for both excitation and emission. When a steady state pH gradient, acidic inside, was obtained the ATP-dependent H^+ transport was stopped by addition of hexokinase and the initial rate of fluorescence recovery after addition of increasing concentrations of Na^+ (2.5–75 mM) and MIA (0–8 μM) was measured as described previously (2).

[2] Abbreviations: MIA, 5-(*N*-methyl-*N*-isobutyl)-amiloride.

Measurement of [^{3}H]MIA Binding to Tonoplast Membranes

Tonoplast membranes (1 μg protein/μl) were incubated at 4 °C for 15 min in 60 μL of 100 mM mannitol, 10 mM Hepes/Tris (pH 7.6) containing various or fixed concentrations of [^{3}H]MIA in the presence or absence of 1 mM amiloride. Two 5 μL aliquots of the reaction mixture were withdrawn and counted for radioactivity to determine the total [^{3}H]MIA concentration. The remaining 50 μL sample was centrifuged for 1 min at 100,000*g* in a Beckman TLA-100 ultracentrifuge at 4 °C and two 5 μL aliquots of the supernatant were withdrawn and counted for radioactivity to determine the free [^{3}H]MIA concentration. All samples were counted for radioactivity using a Beckman LS 3801 scintillation counter. Bound [^{3}H]MIA was calculated by taking the difference between total and free [^{3}H]MIA measured before and after centrifugation of the samples.

Photolabeling of Sugar Beet Tonoplast Membranes with [^{3}H]MIA

Sixty μL samples of sugar beet tonoplast membranes (1 μg protein/μL) were incubated with increasing [^{3}H]MIA concentrations in 100 mM mannitol, 10 mM Hepes (pH 7.6) in the presence or absence of 1 mM amiloride for 15 min on ice. Membranes were centrifuged at 4 °C in a Beckman TLA-100 ultracentrifuge for 1 min at 100,000*g*. Supernatants were aspirated completely and the pellets photo-irradiated for 5 min on ice with high intensity light from a 100 W mercury

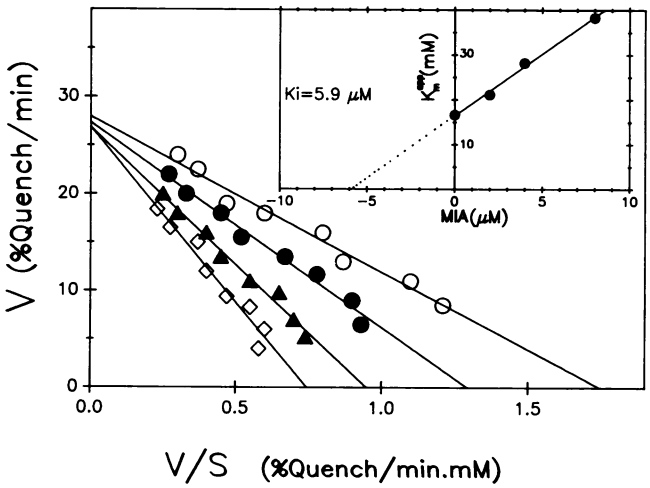

Figure 1. Effects of MIA on Na^+-dependent H^+ fluxes in vacuoles isolated from sugar beet cell suspensions. Eadie-Hofstee plot of the initial rate of change of fluorescence quench on addition of Na^+. Conditions as described previously (2) and in "Materials and Methods." (○) Control, (●) 2 μM, (▲) 4 μM, (□) 8 μM MIA. Each point represents the average of three independent experiments. The lines are those of the best fit by the least square method. *Inset*: Secondary plot of apparent K_m *versus* MIA concentration.

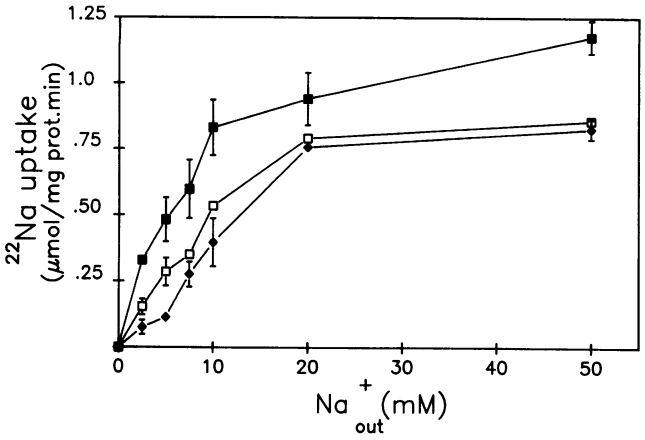

Figure 2. Effect of MIA on ΔpH-dependent $^{22}Na^+$ fluxes in isolated tonoplast vesicles. $^{22}Na^+$-uptake was measured as described in "Materials and Methods." Values are mean ± SD (n = 6). (■) Control, (□) 10 μM, (♦) 25 μM MIA.

arc lamp and M303 power supply (Photochemical Research Associates). A <300 nm UV cutoff filter was employed to reduce radiation damage to the membranes.

SDS-PAGE and Autoradiography

Samples were prepared as described by Parry *et al.* (22). Protein samples were diluted 50 times in 1:1 ethanol/acetone and stored at −20 °C for 3 h followed by centrifugation at 4 °C at 13,000*g* for 7 min. Supernatants were completely aspirated and pellets air dried at 4 °C before resuspension in Laemmli sample buffer (18). Samples were then heated at 60 °C for 2 min before loading on gels. SDS-PAGE was carried out using a 7.5% to 15% gradient gel by the method of Laemmli (18). Molecular weight markers (BRL) included, lysozyme (14,300), β-lactoglobulin (18,400), carbonic anhydrase (29,000), ovalbumin (43,000) BSA (68,000), phosphorylase *b* (97,400), and myosin-H chain (200,000). Following electrophoresis, the gels were stained with Coomassie brilliant blue R250 (0.25% in 50% methanol, 7% acetic acid) and destained, then impregnated with Fluor using Amplify (Amersham) and dried under vacuum. The dried gels were exposed to Kodak X-Omat AR film for 4 to 8 weeks at −70 °C.

Determination of Vacuolar Membrane Protein

Vacuoles were counted using a Spencer Bright Line Hemocytometer, diluted in buffer containing 0.25 M mannitol and 5 mM Tris-Mes (pH 8.0), and sedimented at 100,000*g* for 30 min at 4 °C. The supernatant was aspirated and tonoplast pellets resuspended in dilution buffer. Protein content was measured as described previously (1).

Chemicals

Carrier-free $^{22}NaCl$ was obtained from Amersham (Ontario, Canada). Amiloride and MIA were synthesized as previously described (2). [^{3}H]MIA (28 Ci/mmole) was purchased from New England Nuclear (Dupont, Canada).

RESULTS

Inhibition of Na^+/H^+ Exchange by MIA

The effect of MIA on the Na^+/H^+ antiport activity was determined using tonoplast vesicles and intact vacuoles isolated from sugar beet cell suspensions. The effect of MIA on Na^+ driven proton fluxes was measured in intact vacuoles. A proton gradient, acidic inside, was generated in vacuoles by activation of the ATP driven H^+ transport. Following the removal of ATP, sodium aliquots (2.5–75 mM) were added and the initial rates of proton efflux were determined in the absence and presence of increasing MIA concentrations. The initial rates of proton efflux displayed a Michaelis-Menten relationship to Na^+ concentration, with an apparent K_m of 16.5 mM (Fig. 1). Addition of MIA to the external medium inhibited the Na^+-dependent H^+ fluxes. Figure 1 shows an Eadie-Hofstee plot of the kinetic data obtained by measuring Na^+-dependent H^+ fluxes in the presence of increasing concentrations of MIA. In the experimental range, 0 to 8 μM, MIA inhibited Na^+/H^+ antiport activity in a competitive manner. A secondary plot of apparent K_m *versus* MIA concentration yielded an apparent K_i of inhibition of 5.9 μM (Fig. 1, inset).

The effect of MIA on $^{22}Na^+$ influx was measured in isolated tonoplast vesicles. Tonoplast vesicles and not intact vacuoles were used to measure H^+-dependent $^{22}Na^+$ fluxes due to the fragile nature of the vacuoles and their bursting during the filtration procedure used to stop the uptake reaction. The pH gradient generated by activation of the ATP driven H^+ pump was used to drive $^{22}Na^+$ uptake by the tonoplast vesicles. Following the removal of ATP by hexokinase, sodium aliquots (2.5–50 mM) were added and ^{22}Na accumulation rates were determined in the absence and presence of increasing concentrations of MIA (Fig. 2). H^+-dependent ^{22}Na uptake displayed saturation kinetics with respect to extravesicular Na^+ concentrations with an apparent K_m of 6 mM. Addition of increasing MIA concentrations to the assay medium reduced H^+-dependent $^{22}Na^+$ influx (Fig. 2). Figure 3 shows a double reciprocal plot of the kinetic data obtained in Figure

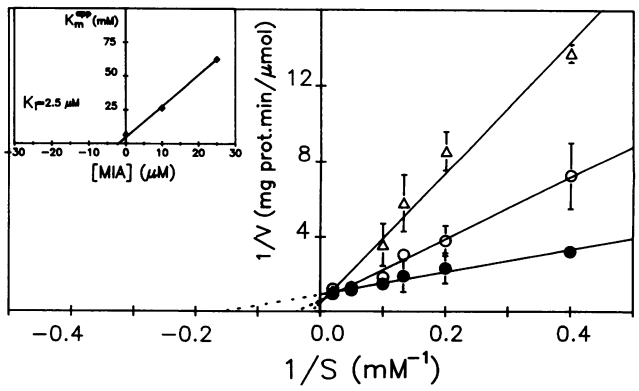

Figure 3. Double reciprocal plot of the values shown in Figure 2. (●) Control, (○) 10 μM, (△) 25 μM. The line is that of best fit by the least square method. *Inset*: Secondary plot of apparent K_m *versus* MIA concentrations.

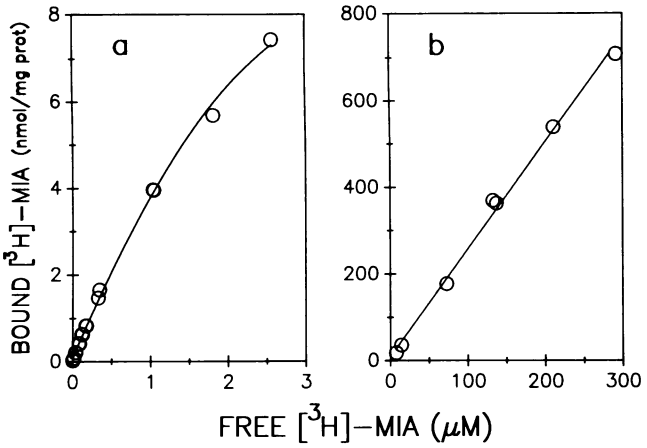

Figure 4. Equilibrium binding of [^{3}H]MIA to tonoplast membranes. Binding assays were performed as described in "Materials and Methods." [^{3}H]MIA concentrations: (a) 0 to 3 μM, (b) 0 to 300 μM. Values are the average of three independent experiments.

2. In the experimental range, MIA inhibited Na^+/H^+ antiport activity in a competitive manner. A secondary plot of apparent K_m *versus* MIA concentration yielded an apparent K_i of inhibition of 2.5 μM (Fig. 3, inset).

Binding of [^{3}H]MIA to Tonoplast Membranes

Equilibrium binding of [^{3}H]MIA was assayed by incubation of the tonoplast membranes with increasing concentrations of [^{3}H]MIA. Binding of [^{3}H]MIA to tonoplast membranes displayed a saturable component at lower concentrations (0–3 μM) (Fig. 4a), and a large, linear, nonsaturable component at high concentrations (10–300 μM) of [^{3}H]MIA (Fig. 4b). Scatchard analysis of the binding data yielded a nonlinear plot (Fig. 5), suggesting that more than one class of [^{3}H]MIA binding sites are present in tonoplast membranes. The nonlinear Scatchard plot could be resolved graphically (24, 28) into two components: a high affinity [^{3}H]MIA binding site (dissociation constant, K_d = 1.3 μM and a maximum binding activity, B_{max} = 4.9 nmol [^{3}H]MIA/mg protein), and a low affinity [^{3}H]MIA binding site (K_d = 205 μM and a B_{max} = 410 nmol [^{3}H]MIA/mg protein). The K_d value of the high affinity site (1.3 μM) correlates favorably with the K_i values observed for MIA inhibition of Na^+-dependent H^+ fluxes (5.9 μM, Fig. 1) and for ΔpH-dependent Na^+ uptake (2.5 μM, Fig. 3) at the same experimental conditions which suggest that this binding component is associated with the Na^+/H^+ antiport. The low affinity binding component of [^{3}H]MIA may represent hydrophobic interactions with membrane phospholipids. To test this possibility, the proportion of lipid-bound [^{3}H]MIA was estimated. The amount of bound-[^{3}H]MIA to membranes (conditions as described in Materials and Methods for the binding experiments) was compared with the amount of bound-[^{3}H]MIA to pellets after delipidation with 1:1 ethanol/acetone. The results indicated that only 2% of the total [^{3}H]MIA was associated with the protein pellet (data not shown). This suggests that the low affinity binding component reflects the interaction between MIA, a highly hydrophobic compound (25), and the membrane lipids.

In order to distinguish between nonspecific binding of [^{3}H]MIA to tonoplast proteins and the specific binding to the antiport polypeptides, binding experiments were performed in the presence and absence of excess amiloride (1 mM). Binding of [^{3}H]MIA could be resolved into two components: a nonsaturable component which was not displaced by excess amiloride, and a saturable component which was displaced by 1 mM amiloride (Fig. 6). Scatchard analysis of the specific component was consistent with binding of [^{3}H]MIA to a single class of high affinity binding sites (K_d = 0.46 μM, B_{max} = 1.12 nmol/mg protein).

Photolabeling of Tonoplast Membranes with [^{3}H]MIA

To identify protein components which may be associated with the Na^+/H^+ antiport, tonoplast membranes were labeled by photo-irradiation in the presence of [^{3}H]MIA. Following photolysis, labeled tonoplast polypeptides were resolved by SDS-PAGE and autoradiography as described in "Materials and Methods." Figure 7 is a representative autoradigram (seven independent experiments) showing the pattern of [^{3}H]MIA incorporation by tonoplast membrane proteins. When tonoplast membranes were incubated with increasing [^{3}H]MIA concentrations and exposed to high intensity UV light from a mercury arc lamp, the label was covalently incorporated into a large number of polypeptides (Fig. 7B). Photolysis of [^{3}H]MIA was also carried out in the presence of 1 mM amiloride (Fig. 7A). Amiloride was included to minimize specific labeling, thus allowing the distinction between specific and nonspecific labeling. The incorporation of label into three polypeptides of apparent molecular masses 223, 123, and 32 kD was totally blocked by 1 mM amiloride at all concentrations of [^{3}H]MIA tested. At low [^{3}H]MIA concentrations, the incorporation of label into three polypeptides, 174, 38, and 35 kD, was totally blocked, and only partially blocked by 1 mM amiloride at [^{3}H]MIA concentrations higher than 500

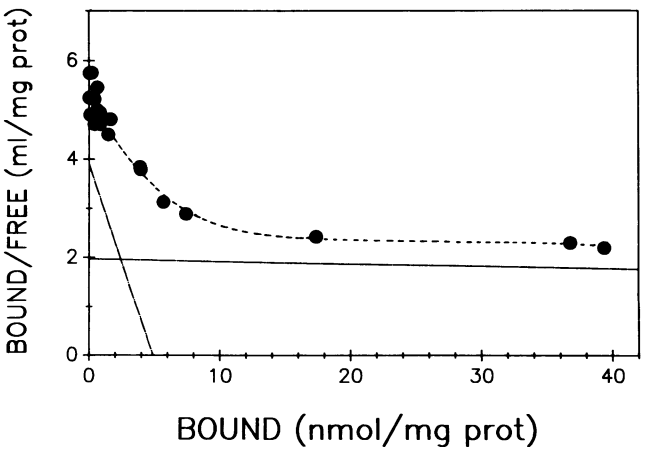

Figure 5. Scatchard analysis of the equilibrium binding data from Figure 4. The nonlinear plot was resolved into two linear components by a graphic method described by Rosenthal (24).

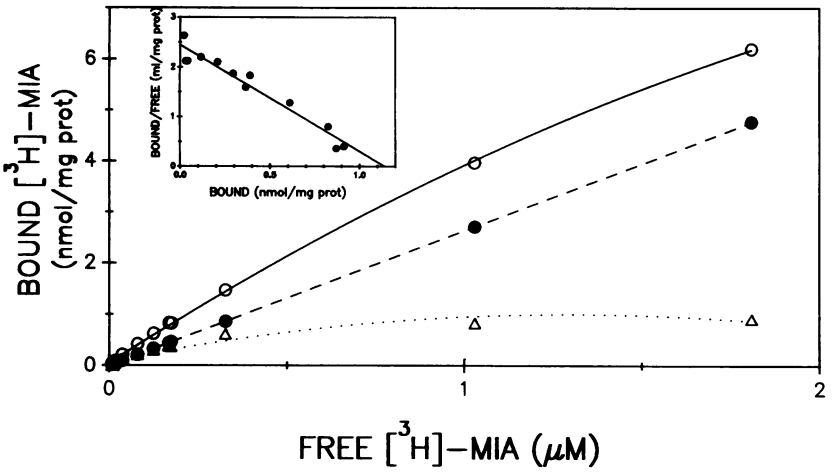

Figure 6. Amiloride-sensitive [^{3}H]MIA binding activity in tonoplast membranes. Conditions as described in "Materials and Methods." Membranes were photo-irradiated in the presence of increasing [^{3}H]MIA concentrations, with (●) or without (○) 1 mM amiloride. (△) Amiloride-sensitive component shown after subtraction of amiloride-insensitive nonspecific binding. *Inset*: Scatchard plot of the amiloride-sensitive component. The line is that of best fit by the least square method (correlation coefficient = −0.98).

μM. Incorporation of label into one polypeptide of molecular mass 158 kD was enhanced by 1 mM amiloride. Although an equal amount of protein was loaded onto each lane (data not shown), some differences in background intensity were observed in different experiments. This is most likely due to nonspecific labeling of membrane phospholipids not totally removed by the ethanol/acetone wash procedure.

DISCUSSION

In the presence of a pH gradient, addition of Na^+ induced a rapid H^+ efflux in tonoplast vesicles and intact vacuoles from sugar beet cell suspensions (2), and ^{22}Na influx in tonoplast vesicles (Fig. 2). In the absence of a pH gradient (collapsed by the addition of NH_4Cl), ^{22}Na influx was less than 5% of that in the presence of a pH gradient (not shown). In the absence of sodium, H^+ efflux was very slow (2, 3). Both H^+ efflux and Na^+ influx displayed saturation kinetics and a similar dependence on external sodium. Na^+-dependent H^+ fluxes displayed an apparent K_m for external sodium of 11.5 and 16.2 mM for tonoplast vesicles and vacuoles, respectively (2). pH gradient-dependent Na^+ influx displayed an apparent K_m for external sodium of 6 mM (Fig. 2). With a vesicular volume of 5 to 10 μL/protein and a pH gradient of 1.9 units (our unpublished results), tonoplast vesicles were able to accumulate Na^+ about 15-fold when the external sodium concentration was 7.5 mM. Thus, the generally assumed pH gradient of 2 units across the tonoplast of most plant cells would clearly be capable of energizing vacuolar sodium accumulation.

We have shown previously that the diuretic drug amiloride is a competitive inhibitor of the Na^+/H^+ antiport in tonoplast vesicles from storage roots of *B. vulgaris* (1). Its reversibility, low specificity, and relatively low inhibitory potency (17) limits its use as a tool for the identification and isolation of the antiport. The substitution of the H-atoms of the 5-amino groups of amiloride by alkyl or alkenyl groups results in an increase in the inhibitory effect on the Na^+/H^+ antiport, with a significant reduction in the inhibitory effect on other amiloride sensitive transport systems (25). In previous studies we tested the effects of a series of 5-amino substituted amiloride analogs on the Na^+/H^+ antiport activity of isolated tonoplast vesicles and intact vacuoles of sugar beet cell suspensions. An increase in the inhibitory potency (3–200-fold) of these analogs was found without any detectable effect on tonoplast H^+-ATPase or proton permeability (2). In this paper, the amiloride analog MIA inhibited Na^+/H^+ exchange in a competitive manner with K_i of 2.5 and 5.9 μM for ΔpH dependent Na^+ influx in tonoplast vesicles and Na^+ dependent H^+ efflux in intact vacuoles, respectively (Figs. 1 and 3). The higher inhibitory potency displayed by MIA, compared with that of amiloride itself (140 μM) (2), suggests that MIA would be a suitable inhibitor for the design of a radioactive photo-labile probe for the identification of the Na^+/H^+ antiport of sugar beet tonoplast.

Several radioactive 5-*N*-substituted amiloride derivatives have been used in different animal systems to characterize and identify the Na^+/H^+ antiport (12, and references therein). [^{3}H]MIA was used to estimate the density and turnover number of the Na^+/H^+ antiport from lymphocytes (7) and to identify antiport subunits in brush border membranes (6, 27). In this work we describe the use of [^{3}H]MIA for the biochemical identification of the tonoplast Na^+/H^+ antiport of sugar beet cell suspensions. [^{3}H]MIA binding to tonoplast membranes displayed a saturable component at low [^{3}H]MIA concentrations and a large nonsaturable component at high [^{3}H]MIA concentrations. Scatchard analysis revealed at least two classes of binding sites (Fig. 5). The large amount of bound [^{3}H]MIA to tonoplast lipids and the high lipid partition coefficient (16.9) of MIA (25) indicates that the low affinity binding component represents the interaction of [^{3}H]MIA with the membrane lipids. The close relationship between the dissociation constant value obtained for the high affinity binding component (Fig. 5) and the constant of inhibition for MIA obtained by fluorescence quenching (Fig. 1) and by isotope exchange (Fig. 3) strongly suggests that the high affinity component represents a class of sites associated with the tonoplast Na^+/H^+ antiport. From the Scatchard analysis, the maximum number of binding sites for [^{3}H]MIA is in the order of 4.7 nmol/mg protein (Fig. 6). The membrane associated proteins were estimated at 0.8 pg/vacuole, thus the

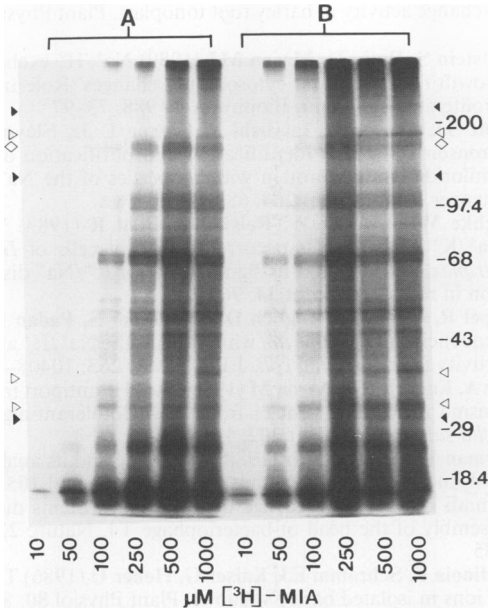

Figure 7. Incorporation of [^{3}H]MIA into tonoplast membrane proteins after photo-irradiation. Tonoplast membranes were incubated with increasing concentrations of [^{3}H]MIA in the presence or absence of amiloride. Membranes were photo-irradiated for 5 min with high intensity light from a mercury lamp. A <300 nm cutoff filter was used to reduce radiation damage to the membranes. Before SDS-PAGE membranes were exposed to UV light. Assay conditions, SDS-PAGE, and autoradiography as described in "Materials and Methods," (A) with 1 mM amiloride, (B) without amiloride. [^{3}H]MIA incorporation: (◀) completely blocked by 1 mM amiloride, (◁) partially blocked by 1 mM amiloride, (◇) enhanced.

maximum number of [^{3}H]MIA binding sites is estimated at 4.5×10^4 sites per vacuole.

A method previously reported in studies on the Na^+/H^+ antiport of lymphocytes (7) and brush border membranes (27) was used to distinguish between specific and nonspecific [^{3}H]MIA binding sites to tonoplast membranes. Excess amiloride was used to displace [^{3}H]MIA binding and the total binding resolved into two components: specific binding which was displaced by 1 mM amiloride and nonspecific binding that was not displaced by amiloride. Scatchard analysis of the specific binding component was consistent with a single class of saturable binding sites (Fig. 6). From the B_{max} and the tonoplast associated protein values we estimated the maximum specific [^{3}H]MIA binding sites at 10,000 per vacuole. However, these results should be considered carefully. In addition to the potential for amiloride to displace [^{3}H]MIA from nonspecific as well as specific binding sites, its affinity for the tonoplast Na^+/H^+ antiport is much lower than that of MIA (2). Therefore high amiloride concentrations are required to effectively block probe binding, especially in the present studies where up to 300 μM of [^{3}H]MIA was used to saturate tonoplast receptors. Moreover, amiloride concentrations higher than 1 mM precipitated tonoplast vesicles (not shown) and in one case actually increased [^{3}H]MIA binding (Fig. 7).

Photo-irradiation of tonoplast membranes in the presence of increasing [^{3}H]MIA concentrations resulted in incorporation of label by several polypeptides. The high level of nonspecific labeling of tonoplast polypeptides can be explained by the hydrophobicity of MIA. MIA could infiltrate the lipid bilayer and become associated with proteins through nonspecific hydrophobic interactions. Amiloride either prevented or reduced labeling of six polypeptides: The incorporation of the label by the 223, 123, and 32 kD polypeptides was completely blocked. On the other hand, label incorporation by 35, 38 and 174 kD polypeptides was inhibited at low [^{3}H]MIA concentrations, but only partially inhibited at high [^{3}H]MIA concentrations. The differential sensitivity to 1 mM amiloride suggests the presence of two groups of receptors in tonoplast membranes which differ in their affinity for MIA.

Table I presents some of the Na^+/H^+ antiport related subunits identified in plants, bacteria and animal systems. The variability observed in reports on the brush border membrane may be an indication that the antiport is composed of multiple subunits of different molecular weights.

The difference observed with the protection of amiloride against MIA labeling would suggest the presence of two different sets of polypeptides differing in their affinity to MIA in tonoplast membranes from sugar beet cell suspensions. A voltage-dependent cation channel has been recently described in these membranes. This channel was not selective between K^+ and Na^+ (21), and amiloride is an inhibitor of the channel activity (O Pantoja, E Blumwald, in preparation). It is possible that the two groups of polypeptides, *i.e.* 223, 123, 32 kD, and 174, 38, 35 kD may correspond to Na^+ channel and Na^+/H^+ antiport subunits, respectively. To confirm this hypothesis,

Table I. *Na^+/H^+ Antiport-Related Subunits in Plants, Bacteria, and Animal Systems*

Source	Method	Estimated Mass	Reference
		kD	
Rat—renal brush border	[^{14}C]-Br-EIPA[a] photolabeling	65.2	(8)
Rabbit–renal brush border	Affinity chromatography; analog A35 coupled to Sepharose CL-4B	25	(12)
Pig—renal brush border	[^{3}H]MIA photolabeling	81–107	(26)
Dog—renal brush border	[^{3}H]MIA photolabeling	40–60	(5)
Escherichia coli		35	(14)
Beta vulgaris tonoplast	[^{3}H]MIA photolabeling	174–38–35	This report

[a] 5-(*N*-Ethyl-*N*-isopropyl-bromoamiloride).

we are currently studying the effect of amiloride analogues with distinct affinities for the Na^+/H^+ antiport and the Na^+ channel. These analogues are being used to protect differentially against labeling of tonoplast proteins by photo-irradiation of [^{3}H]MIA.

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