

Communication

Inhibition of Na⁺/H⁺ Antiport Activity in Sugar Beet Tonoplast by Analogs of Amiloride¹

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ABSTRACT

The effects of amiloride and a series of amiloride analogs have been tested on the Na⁺/H⁺ antiport activity in intact vacuoles and tonoplast vesicles isolated from sugar beet cell suspension cultures. There is a competitive interaction between amiloride analogs and sodium. Substitution of one or both H-atoms of the 5-amino group of amiloride (apparent K_i about 150 micromolar) resulted in a 3- to 200-fold increase in inhibitory potency of the antiport activity.

We have recently provided evidence of a Na⁺/H⁺ antiport in tonoplast vesicles isolated from storage tissue of red beet and sugar beet (3). Since the vacuolar accumulation of sodium is characteristic of salt-tolerant species (9, 10) this tonoplast Na⁺/H⁺ antiport may be one of the principal physiological factors conferring salt tolerance on the plant. Our studies have shown that the diuretic drug amiloride is a competitive inhibitor of the Na⁺/H⁺ antiport. Its reversibility limits the use of amiloride as a tool for the eventual identification and isolation of the antiport. Our objective is to design a radioactive affinity analog of amiloride suitable for this purpose.

Here we have studied the effects of a series of amiloride analogs on the Na⁺/H⁺ antiport activity in intact vacuoles and isolated tonoplast vesicles from sugar beet cell suspensions.

MATERIALS AND METHODS

Plant Material and Protoplast Preparation. Cell suspension cultures of sugar beet (*Beta vulgaris* L.) were grown as described previously (6). For the isolation of protoplasts, 50 g (fresh weight) of 6 d old cells were incubated at 25°C for 2.5 h with shaking at 75 rpm in 100 ml solution containing 0.5 M sorbitol, 0.5 mM CaCl₂, 0.1% BSA, 0.1% PVP (mol wt 10,000), 0.5 mM DTT, 2% Cellulase, 0.05% Pectolyase (Seishin Pharm. Co., Japan), and 25 mM Tris/Mes (pH 5.5). Protoplasts were washed three times by centrifugation at 500g and resuspension in wash buffer (0.5 M sorbitol, 0.2 mM CaCl₂, and 25 mM Tris/Mes, pH 5.5). Viability of protoplasts was 88% (by number) as measured by exclusion of Evan's blue dye (13), and 91% as measured by the fluorescence

of fluorescein diacetate (12). For further purification 20 ml protoplast suspension was mixed with 20 ml wash buffer containing 50% (v/v) Percoll (Pharmacia). Ten ml volumes were overlaid each with step gradients consisting of 10 ml of 10% (v/v) Percoll in wash buffer and 10 ml of wash buffer. After centrifugation at 500g for 20 min, the 0/10% Percoll interface was removed and the protoplasts were washed twice by centrifugation and resuspension in wash buffer.

Isolation of Vacuoles. Sugar beet protoplasts were subjected to changes in pH, calcium, and osmoticum to release vacuoles. Ten ml lysis buffer (4 mM EDTA, 2.5 mM DTT, 0.1% BSA, 100 mM KCl, and 20 mM Tris adjusted to pH 8.0 with concentrated HCl) were added to 5 ml purified protoplast suspension. After shaking at 40 rpm for 20 min at 4°C, 10 ml of stabilization buffer (1.0 M mannitol, 1 mM DTT, 20 mM Tris/Mes [pH 8.0] and 2.5% [w/v] Ficoll) were added. To separate intact vacuoles from other organelles and unlysed protoplasts, 6 ml volumes of the lysate were layered onto each of four step gradients consisting of 10 ml 5% (w/v) and 2.5% Ficoll containing 0.5 M sucrose, 1 mM Tris-EDTA (pH 8.0), 1 mM DTT, and 20 mM Tris/Mes (pH 8.0). The lysate was overlaid with 7 ml of 0.4 M mannitol, 1 mM DTT, and 20 mM Tris/Mes (pH 8.0). After centrifugation at 25,000g for 1.5 h, intact vacuoles, free of unlysed protoplasts, floated at the 0/1.25 Ficoll interface. Vacuoles were collected with a Pasteur pipette and counted in a haemocytometer before use. The yield of vacuoles ranged from 4 to 6% based on the initial number of protoplasts. Purity of the vacuoles was tested by staining procedure using fluorescein diacetate (1). The marker activities of some organelles were measured to further assess the purity of the isolated vacuoles. Vanadate-sensitive ATPase activity (plasma membranes) was hardly detectable. Glucose 6-P dehydrogenase (cytosol) and Cyt *c* oxidase (mitochondria) activities were 3 and 5%, respectively, as expressed on the basis of the number of vacuoles.

Enzyme Assays. Vanadate-sensitive ATPase activity and Cyt *c* oxidase were measured as described previously (6). Glucose 6-P dehydrogenase was determined according to Brulfert *et al.* (7).

Reagents. Amiloride and its analogs were synthesized as previously described (8). Dilutions were made from freshly prepared stock solution in DMSO, and the final DMSO concentration, in the absence or presence of amiloride and analogs, was 0.02% in all assays.

Isolation of Tonoplast Vesicles. Purified tonoplast vesicles were isolated as described previously (6). The vesicles were preloaded with a buffer of the desired ionic composition by suspension and sedimentation at 100,000g for 30 min. Final membrane pellets were resuspended in the same loading medium to 6 mg/ml and incubated at 4°C for 2 h before use.

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Protein Determination. Protein was estimated by a dye-binding method (Bio-Rad) as previously described (4).

Fluorescence Assays. The fluorescence quenching of acridine orange was used to monitor the dissipation of inside-acid pH gradients across the membranes of the tonoplast vesicles (3, 5) and intact vacuoles. Na⁺-dependent H⁺ fluxes were measured in isolated tonoplast vesicles as described previously (6). Vacuoles (1.5×10^5) were added to 2 ml buffer containing 0.4 M mannitol, 1 mM DTT, 20 mM Tris/Mes (pH 8.0), 1 mM Tris-EDTA (pH 8.0), 5 mM glucose, 100 mM tetamethylammonium chloride, 1 mM Tris-ATP and 5 μ M acridine orange. Proton translocation was initiated with the addition of 2 mM MgSO₄, and the fluorescence decrease with time was monitored in a thermostated cell at 25°C with a Perkin-Elmer spectrofluorimeter model LS-3 at excitation and emission wavelengths of 495 and 540 nm, respectively, and a slit width of 10 nm for both excitation and emission. During the measurement, the samples were continuously stirred. When a steady state pH gradient, acidic inside, was formed, the ATP dependent H⁺ transport activity was stopped by the addition of hexokinase (EC 2.7.1.1) (ATP + glucose \rightarrow glucose 6-P + ADP). After a constant rate of fluorescence recovery (H⁺ leak) was obtained, aliquots of 1 M Na₂SO₄ solutions were injected during continuous fluorescence recording, and the initial change in rate of Na⁺-dependent fluorescence recovery was determined. The rate of fluorescence quench is proportional to proton flux, at least when comparing initial rates of quench starting at the same Δ pH (2). The initial rates were determined by drawing the tangents of the recorded traces obtained in the first 2 min following the addition of sodium. The initial rates of dissipation of pH gradient by Na⁺ were corrected by subtraction of those obtained after addition of hexokinase and expressed as rate of change in fluorescence per min.

RESULTS AND DISCUSSION

The effect of Na⁺ on the dissipation of a transmembrane pH gradient was tested in isolated tonoplast vesicles (Fig. 1) and intact vacuoles (Fig. 2) from sugar beet cell suspensions. The addition of tonoplast vesicles equilibrated at pH 5.8 to a pH 8.0 buffer caused an immediate quench of acridine orange fluorescence (Fig. 1). After fluorescence stabilized, various concentrations of Na⁺ were added, and the initial rate of H⁺ efflux was measured in terms of change in fluorescence quench (Q) per min. In the presence of equimolar K⁺ concentrations (15 mM) across the membranes plus valinomycin (to clamp the membrane potential) the initial rate of Na⁺/H⁺ exchange (fluorescence recovery) displayed saturation kinetics with an apparent K_m of 11.5 mM (Fig. 1, inset). H⁺ efflux showed a simple Michaelis relationship to external Na⁺ concentrations when the potential was clamped with K⁺ and valinomycin, but when the potential was not controlled, there was an additional linear component of Na⁺/H⁺ exchange (3). Thus, there was a component of Na⁺/H⁺ exchange mediated by the membrane potential, indicating a significant Na⁺ permeability. This Na⁺ permeability may not be a natural property of the tonoplast of *Beta* but may be due to a separate population of leaky (damaged) vesicles (14). Vesicles leaky to Na⁺ should hold a pH gradient as long as there is no counter-ion to balance H⁺ movement. Valinomycin and K⁺ collapse the pH gradient in this population, which accounts for the rapid partial recovery of fluorescence seen in the first few seconds after the pH jump in Figure 1. This interpretation is supported by experiments with intact vacuoles (below).

A proton gradient, acidic inside, was generated in intact vacuoles by activation of the ATP driven H⁺ transport by addition of Mg²⁺, and the H⁺ transport was subsequently stopped by removal of ATP from the assay by the addition of a hexokinase ATP trap. As in the case of tonoplast vesicles, addition of Na⁺ resulted in the recovery of fluorescence (H⁺ efflux) and the initial

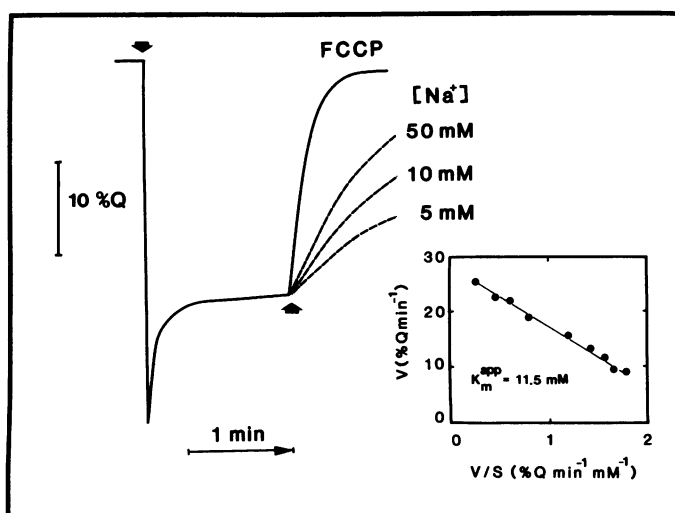


FIG. 1. H⁺-gradient-dependent quenching of acridine orange fluorescence and its reversal by Na⁺ in isolated tonoplast vesicles from cultured sugar beet cells. At the indicated time (♣), 10 μ l of isolated vesicles loaded with a solution containing 250 mM mannitol, 15 mM K-gluconate, 1 mM DTT, 250 mM glycerol, and 10 mM Tris/Mes (pH 5.8) were added to 2 ml buffer containing 250 mM mannitol, 15 mM K-gluconate, 1 mM DTT, 250 mM glycerol, 10 mM Tris/Mes (pH 8.0), 1 μ M valinomycin and 5 μ M acridine orange at 25°C. At the indicated time (♣) Na⁺ or FCCP were added. Inset, Eadie-Hofstee plot of initial rate of change of fluorescence quench on addition of Na⁺. Each point represents the average of three independent experiments.

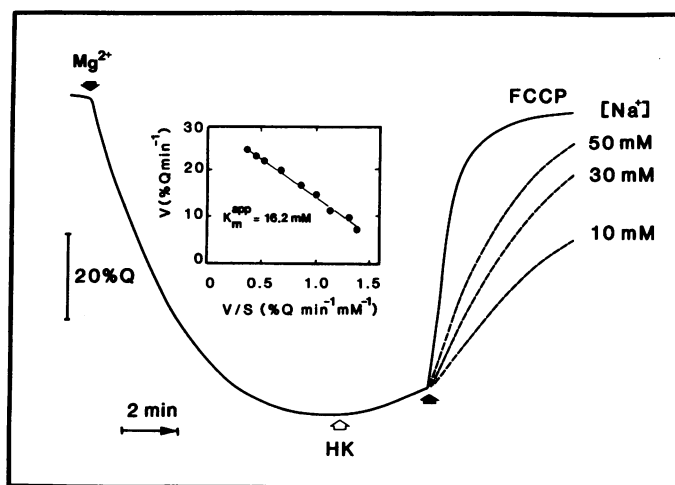


FIG. 2. ATP-dependent-quenching of acridine orange fluorescence and its reversal by Na⁺ in isolated vacuoles, obtained from protoplasts of sugar beet suspension culture cells. Conditions as described in "Materials and Methods." At the indicated time (♣) the H⁺-ATPase was activated by the addition of Mg²⁺. At the indicated times hexokinase (HK) (♣) and Na⁺ or FCCP (♣) were added. Inset, Eadie-Hofstee plot of the initial rate of change of fluorescence quench on addition of Na⁺. Each point represents the average of three independent experiments.

rates of proton efflux displayed a Michaelis-Menten relationship to Na⁺ concentration, with an apparent K_m of 16.2 mM, without any additional linear component (Fig. 2, inset).

The similarity between the vesicle preparation and the intact vacuoles confirms that transport is being measured primarily in tonoplast vesicles. It also suggests that the properties of the Na⁺/H⁺ antiport are not much affected by the unknown factors in vesicle preparations, such as the possible presence of a population of inside-out vesicles, or vesicles differing greatly in size, or

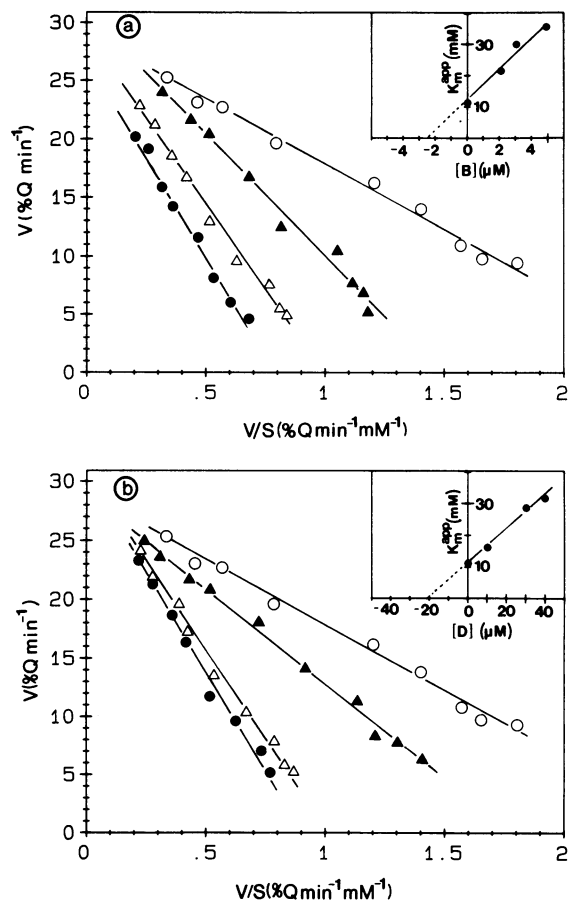


FIG. 3. Effects of amiloride analogs on Na^+ -dependent H^+ fluxes in isolated tonoplast vesicles. Eadie-Hofstee plots of the initial rate of change of fluorescence quench on addition of Na^+ . Conditions as in Figure 1. a, Analog B: (O), control; (\blacktriangle), 2 μM ; (\triangle), 3 μM ; (\bullet), 5 μM . Each point represents the average of three independent experiments. The lines are those of best fit by the least square method. Inset, secondary plot of apparent K_m versus analog B concentration. b, Analog D: (O), control; (\blacktriangle), 10 μM ; (\triangle), 30 μM ; (\bullet), 40 μM . Each point represents the average of three independent experiments. The lines are those of best fit by the least square method. Inset: secondary plot of apparent K_m versus analog D concentration.

composed of different tonoplast domains with heterogeneous properties. These experiments also show that similar results are obtained whether the pH gradient is generated by ATPase activity or by transferring vesicles to a different buffer solution.

We have shown previously that the diuretic drug amiloride, a specific inhibitor of Na^+/H^+ exchange in various animal membranes, is a competitive inhibitor of the Na^+/H^+ antiport in tonoplast vesicles isolated from storage tissue of red and sugar beet (3). However, the reversibility of the inhibition limits the use of amiloride as a tool for the identification and isolation of the antiport. Several groups have recently studied the structure-activity relationships of various amiloride derivatives in myocytes (16), epidermoid carcinoma cells (17), kidney epithelial cells (11), and human neutrophils (15). In all these cases the substitution of one or both H-atoms of the 5-amino group of amiloride resulted in an increase in potency for inhibition of Na^+/H^+ exchange activity. None of the analogs inhibit the Na^+/H^+ exchange irreversibly, and as such are not useful for the identification of antiport polypeptides. In the present work we have tested the effect of several of these derivatives on the Na^+/H^+ antiport activity in tonoplast vesicles and intact vacuoles isolated from sugar beet cell suspension cultures.

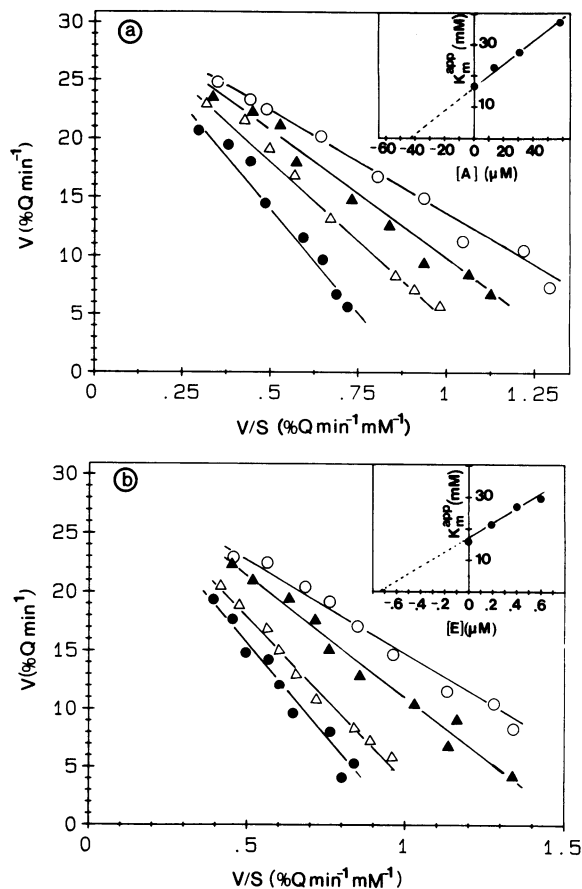


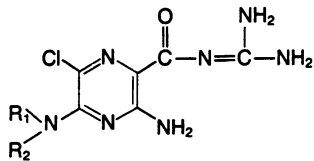
FIG. 4. Effects of amiloride analog on Na^+ -dependent H^+ fluxes in intact vacuoles. Eadie-Hofstee plots of the initial rate of change of fluorescence quench on addition of Na^+ . Conditions as in Figure 2. a, Analog A: (O), control; (\blacktriangle), 15 μM ; (\triangle), 30 μM ; (\bullet), 60 μM . Each point represents the average of three independent experiments. The lines are those of best fit by the least square method. Inset: secondary plot of apparent K_m versus analog A concentration. b, Analog E: (O), control; (\blacktriangle), 0.2 μM ; (\triangle), 0.4 μM ; (\bullet), 0.6 μM . Each point represents the average of three independent experiments. The lines are those of best fit by the least square method. Inset: secondary plot of apparent K_m versus analog E concentration.

Figure 3 shows an Eadie-Hofstee plot of the kinetic data obtained by measuring Na^+ -dependent H^+ fluxes in isolated tonoplast vesicles in the presence of increasing concentrations of amiloride analogs in the extravesicular medium. Analog concentrations were in the range of 0 to 5 μM for analog B (Fig. 3a) and 0 to 40 μM for analog D (Fig. 3b). External sodium concentrations were in the range of 5 to 75 mM. Both analogs inhibited Na^+/H^+ exchange activity in a competitive manner. A secondary plot of apparent K_m versus analog concentrations yielded an apparent K_i of 2.8 and 21 μM for analogs B and D, respectively (inset of Fig. 3, a and b).

The effects of amiloride analogs on Na^+ -dependent H^+ fluxes were also tested in intact vacuoles. A proton gradient, acidic inside, was generated in intact vacuoles by activation of the H^+ -ATPase in the presence of increasing concentrations of amiloride analogs in the assay medium. Following the removal of ATP from the assay by addition of hexokinase, sodium aliquots were added (range 5–60 mM) and the initial rates of proton efflux were determined as described in "Materials and Methods." Figure 4 shows an Eadie-Hofstee plot for the kinetic data obtained with analogs A (Fig. 4a) and E (Fig. 4b). In the experimental concentration range, 0 to 60 μM for analog A and 0 to 0.6 μM for analog

Table I. Effects of Substituents on the 5-amino Group on the Na⁺-dependent H⁺ Efflux in Tonoplast Vesicles and Intact Vacuoles

The apparent K_i values were calculated from secondary plots of apparent K_m versus analog concentration as described in Figures 3 and 4.



Analog Code	R ₁	R ₂	Apparent K_i	
			Vesicles	Vacuoles
			μM	
Amiloride	H	H	127	148
A	—CH ₃	—CH ₃	37	42
B	—C ₂ H ₅	—CH(CH ₃) ₂	2.6	2.8
C	—CH ₃	—CH ₂ CH(CH ₃) ₂	7.8	8.5
D	H	—C(CH ₃) ₃	21	27
E*		—(CH ₂) ₆ —	0.64	0.74

* Two alkyl substituents on the 5-amino group were joined to form a hexamethyl ring.

E, both analogs inhibited Na⁺/H⁺ exchange activity in a competitive manner. A secondary plot of apparent K_m versus analog concentrations yielded an apparent K_i of 42 and 0.74 μM , for analogs A and E, respectively (inset of Fig. 4, a and b).

Table I summarizes apparent K_i values for amiloride and five of its analogs, all bearing substitutions on the 5-amino group. The effect of the analogs ranged from 3- to 200-fold more inhibitory than amiloride itself.

In summary, comparison of the Na⁺/H⁺ antiport activity and the inhibitory effect of amiloride analogs in tonoplast vesicles versus intact vacuoles suggests that the properties of the antiport are not much affected by the unknown factors in vesicle preparations. The order of potency of the amiloride analogs (Table I) corresponds with that reported in studies of the Na⁺/H⁺ antiport in animal plasma membranes (11, 15). Thus the data, as far as they go, suggest similarities in the molecular structure of the tonoplast antiporter and those of animal plasma membranes.

The high affinity derivatives do not have any detectable effect on proton pumping or proton leakage through the tonoplast in the absence of Na⁺, and should therefore be useful, especially

the 5-*N*-ethyl-*N*-isopropyl (analog B) and the 5-*N,N*-hexamethylene (analog E) derivatives, both as physiological tools and for the design of an affinity label for identification of the antiport protein.

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