Utilization of Amiloride Analogs for Characterization and Labeling of the Plasma Membrane Na^+/H^+ Antiporter from *Dunaliella salina*†

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ABSTRACT: The interactions of amiloride analogs with the Na^+/H^+ antiporter from plasma membrane of the halotolerant alga *Dunaliella salina* [Katz *et al.* (1989) *Biochem. Biophys. Acta 983*, 9–14] have been investigated. Analogs bearing hydrophobic substitutions at the guanidino moiety of amiloride, such as benzamil, are the most effective inhibitors of Na^+ uptake in plasma membrane vesicles, whereas substituents of the 5-amino group are less effective inhibitors than amiloride. This order of specificity is opposite to that found for most Na^+/H^+ antiporters. The photoaffinity amiloride analog 2′-methoxy-5′-nitrobenzamil (NMBA), a competitive inhibitor with respect to Na^+ with $K_i = 10\ \mu M$, photolabels upon illumination two polypeptides of apparent MW 30 and 50 kDa in purified plasma membrane vesicles. Similar labeling is obtained by immunodetection with antiamiloride antibodies and by incorporation of [^{125}I]NMBA. The specificity of the labeling was ascertained by competition with benzamil. Plasma membrane preparations from high-salt or ammonia-adapted cells, which have higher Na^+/H^+ antiporter activity [Katz *et al.* (1992) *Plant Physiol. 100*, 1224–1229], also show increased incorporation of NMBA into the 30- and 50-kDa polypeptides. It is suggested that: (1) the structure of the Na^+ binding site of the *D. salina* Na^+/H^+ antiporter differs from that of most Na^+/H^+ antiporters and (2) the 50- and/or 30-kDa polypeptides are subunits of the plasma membrane antiporter of this alga.

The diuretic drug amiloride is a potent inhibitor of several Na^+ transport systems in bacteria, animal, and plant cells (Kleyman & Cragoe, 1988), including Na^+ channels, Na^+/H^+ and Na^+/Ca^+ antiporters, and Na^+/amino acid transporters. A large number of amiloride analogs were synthesized aimed to elucidate structure–activity relationships and biochemical identification of the transporters by specific labeling.

Na^+/H^+ antiporters from animal and plant cells, are unique among the amiloride-sensitive transport systems in having increased sensitivity to hydrophobic substitutions at the 5-amino group, while substitutions at the guanidino group decrease the inhibitory ability. The sequence of inhibition is as follows: 5-amino-substituted analogs, such as EIPA[1] or MIA, > amiloride > guanidino-group-substituted analogs, such as benzamil (Kleyman & Cragoe, 1988; Simchowitz & Cragoe, 1986; Blumwald *et al.*, 1987). Photoreactive analogs have been utilized to label and identify polypeptide components of the Na^+/H^+ antiporter from renal brush border (Wu & Lever, 1989; Huot *et al.*, 1989) and from plant tonoplast (Barkla *et al.*, 1990).

While Na^+/H^+ antiporters from bacteria and plasma membranes of animal cells have been extensively characterized, little is known about antiporters in plasma membranes from plants. Plants possess a vacuolar Na^+/H^+ antiporter which is believed to have an important role in Na^+ compartmentalization (Blumwald & Poole, 1987). Only a few studies about plant nonvacuolar Na^+/H^+ antiporters have been reported (Braun *et al.*, 1988).

We have identified and characterized a Na^+/H^+ antiporter in the plasma membrane of the halotolerant alga *Dunaliella salina* (Katz *et al.*, 1986, 1989). It was demonstrated that the antiporter in *Dunaliella* is involved in regulation of intracellular pH and in adaptation to high salinity, similar to its function in animal cells (Katz *et al.*, 1992). The antiporter is highly specific for Na^+ and is competitively inhibited by Li^+ and amiloride.

In the present work, we describe the effects of several amiloride analogs on the Na^+/H^+ antiporter activity in plasma membrane vesicles isolated from the alga *D. salina*. Our results indicate that this antiporter differs from mammalian and plant vacuolar antiporters in the sequence of inhibition by amiloride analogs, suggesting a different structure of its Na^+ binding site.

Furthermore, we have utilized the photoreactive amiloride analog 2′-methoxy-5′-nitrobenzamil (NMBA) to identify two putative polypeptide components of the Na^+/H^+ antiporter in *D. salina* and have demonstrated that their relative abundance correlated with the activity of the Na^+/H^+ antiporter.

EXPERIMENTAL PROCEDURES

Materials. Amiloride and its analogs were a gift from Dr. E. Cragoe, Jr. (Nacogdoches, TX). Dilutions were made from stock solutions in DMSO, and the final DMSO concentration in the assay was less then 0.1%. Antiamiloride antibodies were raised as previously described (Kleyman *et al.*, 1986).

Cell Culture Conditions. D. salina algae were grown as previously described (Katz & Avron, 1985) under continuous illumination. The medium contained 1 M NaCl in control cells, 3.5 M NaCl in salt-adapted cells, and 1 M NaCl + 18 mM NH_4Cl in NH_3-adapted cells (Katz *et al.*, 1992).

Plasma Membrane Vesicle Preparation. Plasma membrane vesicles were prepared from algae adapted to different growth conditions, by osmotic lysis of the cells followed by differential centrifugation (Katz *et al.*, 1986).

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[1] Abbreviations: NMBA, 2′-methoxy-5′-nitrobenzamil; EIPA, 5-(*N*-ethyl-*N*-isopropyl)amiloride; MIA, 5-(*N*-methyl-*N*-isobutyl)amiloride.

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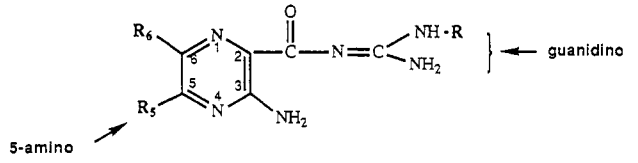
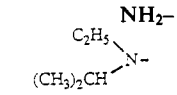
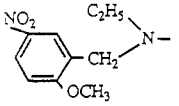
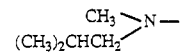
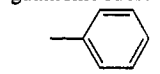
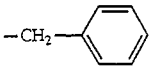
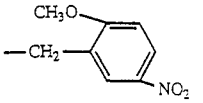
Table 1: The Structure of Amiloride Analogs and Their Inhibitory Potency on Na^+/H^+ Antiport Activity Was Measured by Monitoring the Uptake of $^{22}Na^+$ into Plasma Membrane Vesicles (see Experimental Procedures)[a]



compound	name	abbreviation	R_6	IC_{50} (μM)
R_5 = 5-amino subst				
1. 	5-(*N*-ethyl-*N*-isopropyl)amiloride	amiloride EIPA		15 80
2. 	5-[*N*-ethyl-*N*-(2-methoxy-5-nitrobenzyl)]amiloride		H Cl	50 45
3. 	5-(*N*-isobutyl-*N*-methyl)amiloride	MIA		82
R = guanidino subst				
4. 		phenamil		5
5. 		benzamil	Cl Br	3 2
6. 	2′-methoxy-5′-nitrobenzamil	NMBA I-NMBA H-NMBA	Cl I H	10 3 100

[a] The different analogs were added 10 min before addition of $^{22}Na^+$, during the preincubation in the acid stage (20 mM succinate, pH 5.2). The reaction was initiated by addition of Tris base (final pH 8.0) and 30 μM [^{22}Na]Cl.

Na^+/H^+ Antiporter Activity in Plasma Membrane Vesicles. The activity of the Na^+/H^+ antiporter was measured by imposition of a pH gradient (interior acid) across the plasma membrane and measuring the uptake of ^{22}Na into the vesicles (Katz *et al.*, 1989).

Photoaffinity Labeling. Plasma membrane vesicles were photolabeled as described by Kleyman *et al.* (1989). Plasma membrane vesicles were diluted into suspension buffer (0.4 M glycerol, 2 mM $MgCl_2$, 50 mM KCl, 10 mM Mops–Tris, pH 7) to a final protein concentration of 200 μg and preincubated with 0.2 μM NMBA (or as indicated) for 20 min on ice. Illumination was carried out for 2 min (or as indicated) at 4 °C with continuous stirring using a mercury lamp (HBO 200 W) filtered through a 313-nm narrow band filter. Treated vesicles were washed with suspension buffer and collected by centrifugation (30 min at 40 000 rpm). The vesicles were resuspended in a small volume of suspension buffer and assayed for Na^+/H^+ antiporter activity.

Immunoblots. The photolabeled proteins were precipitated in 80% acetone at −20 °C overnight, solubilized in sample buffer, and subjected to 10% tricine–SDS PAGE (Schagger & Von Jagow, 1987). Proteins were then transferred to nitrocellulose (Towbin *et al.*, 1979) followed by incubation of the blot overnight at 4 °C with PBS, 0.05% Tween, and 10% low-fat milk (T-PBS milk buffer). The blot was incubated for 1 h at room temperature with antiamiloride antibodies (Kleyman *et al.*, 1986) at a dilution of 1:4000 in T-PBS buffer. The blot was extensively washed with T-PBS milk buffer and then incubated at room temperature for 1 h with a 1:2000 dilution of antimouse HRP in T-PBS followed by washing in the same buffer. Bound antibodies were then detected with the ECL (enhanced chemiluminescence) detection system.

Photolabeling Using [^{125}I]NMBA. The synthesis of [^{125}I]-NMBA was performed according to Cassel *et al.* (1988). Radioactive 6-I-NMBA was prepared from the corresponding 6-H derivative by reaction with [^{125}I]Cl. The labeled derivative was purified by TLC, eluted from the silica with ethanol, and used within 48 h. Plasma membrane vesicles were photolabeled with [^{125}I]NMBA under the same conditions as described for NMBA. The labeled proteins were separated on tricine–SDS PAGE, and the gels were stained with Coomasie brilliant blue, destained, and dried under vacuum. The dried gels were exposed to a photographic film for 3–5 days at −70 °C.

RESULTS

Structure–Activity Relations for Amiloride Analogs on the Na^+/H^+ Antiporter. We have shown previously that amiloride is a competitive inhibitor of the Na^+/H^+ antiporter in plasma membrane vesicles isolated from *D. salina* (Katz *et al.*, 1989).

Table 1 summarizes the relative inhibitory potency (expressed as IC_{50}) on the Na^+/H^+ antiporter activity in plasma membrane vesicles of *D. salina*. The analogs are grouped according to the site and type of substitution as follows: (a) 5-Amino (R_5) substituents (analog nos. 1–3) which exhibit substantial loss in potency. Therefore, the unsubstituted 5-amino group seems to be required for optimal inhibition of the Na^+/H^+ antiporter. (b) Guanidino group substitutents (R): substitution with hydrophobic groups enhanced the inhibitory potency (analog nos. 4–6). Benzamil was the most potent inhibitor. (c) 6-Position substituents (R_6): substitution of position 6 on the pyrazine ring with different halogens can change the inhibitory potency of the analog. 6-Cl, 6-Br, and

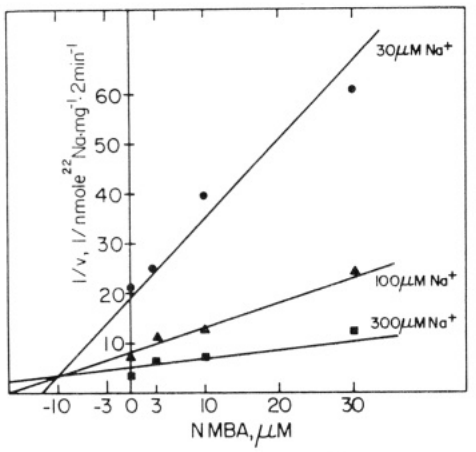


FIGURE 1: Kinetic mechanism of inhibition of ^{22}Na uptake by NMBA in plasma membrane vesicles; Dixon plots of the initial rate of ^{22}Na uptake in reaction medium containing: (●) 30 μM NaCl, (▲) 100 μM NaCl, (■) 300 μM NaCl. Measuring conditions were as described in Table 1. Each point represents the average of three independent experiments.

6-I analogs show increasing inhibitory potency, respectively. In contrast, 6-H analogs are very poor inhibitors. Similar results have been reported with the mammalian Na^+/H^+ antiporter (L'Allemain *et al.*, 1984).

Inhibition of Na^+/H^+ Antiporter by NMBA. Since the *Dunaliella* Na^+/H^+ antiporter is specifically inhibited by analogs substituted at the guanidino group (see Table 1), NMBA was chosen as the photoaffinity label for the antiporter. NMBA is a photoreactive analog of benzamil bearing 2-methoxy and 5-nitro groups on the benzene ring and has been previously used to identify the amiloride-binding components of the epithelial Na^+ channel (Kleyman *et al.*, 1989). To evaluate the potential of NMBA as a good label, we first examined the kinetics of inhibition of the Na^+/H^+ antiporter activity (Figure 1). NMBA inhibits Na^+ uptake competitively with a K_i of 10 μM. In order to check whether NMBA can be covalently bound to the Na^+ site of the antiporter, plasma membrane vesicles were photoactivated in the presence of NMBA, washed from unbound probe, and assayed for Na^+ uptake activity (see Experimental Procedures). Figure 2a shows that within 2 min of illumination 50% of the activity was inhibited. Thus, under these conditions, it seems that 50% of the sites are occupied by the analog. Inhibition of 50% of the activity is obtained by 10 μM NMBA (Figure 2b). These results fit well with the calculated K_i (Figure 1). Photolysis of vesicles in the presence of amiloride instead of NMBA showed no effect on the activity (data not shown), indicating that the labeling is specific.

Photoaffinity Labeling. Identification of the protein components which incorporated NMBA was achieved by two methods: (a) immunodetection using antiamiloride antibodies (Kleyman *et al.*, 1986, 1989); these antibodies are directed against the substituted pyrazinoyl moiety of amiloride, which remains intact in NMBA and therefore can be recognized; and (b) incorporation of radioactivity, detection using [^{125}I]-NMBA for photolabeling.

Identification of protein components which incorporate NMBA was achieved by irradiation of vesicles in the presence of 0.2 μM NMBA for 2 min at varying light intensities. The photolabeled proteins were precipitated, separated on tricine–SDS PAGE, and immunoblotted. As demonstrated in Figure 3, the label is incorporated into two polypeptides with an apparent MW of 50 000 and 30 000 in correlation with the light intensity. The specificity of photoincorporation was

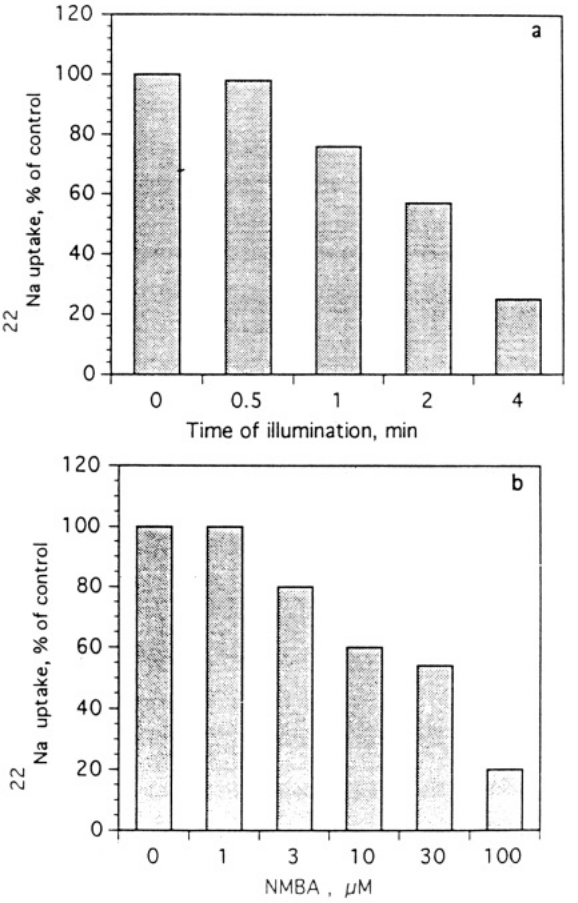


FIGURE 2: Irreversible inhibition of Na^+/H^+ antiporter activity in plasma membrane vesicles by photolabeling with NMBA. Plasma membrane vesicles were photolabeled, as described under Experimental Procedures, washed, and concentrated, and ΔpH-dependent Na^{22} uptake was measured: (a) the effect of illumination time, NMBA concentration during photolabeling was 10 μM, and (b) the effect of NMBA concentration during illumination, photolabeling during 3 min.

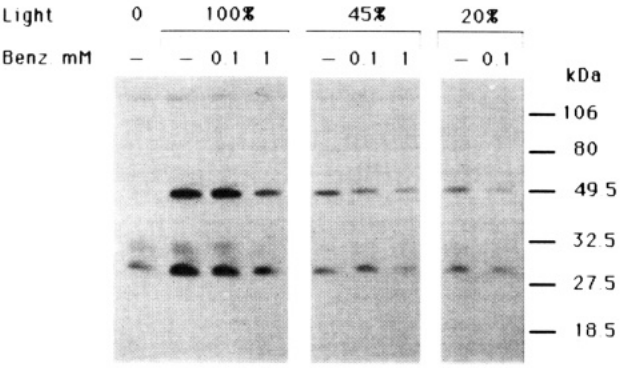


FIGURE 3: Immunoblots of photoaffinity labeled plasma membrane vesicles. Photoaffinity labeling of *Dunaliella* plasma membrane vesicles (control cells) was performed for 2 min of exposure to varying light intensities with 0.2 μM NMBA. The indicated concentrations of benzamil were added for competition. Photolabeled proteins were analyzed by tricine–SDS PAGE (10% acrylamide) and immunoblotting using antiamiloride monoclonal antibodies. 100% light intensity = 0.7 W m^{-2}. Light intensity was varied with neutral density filters.

tested by comparing the labeling of NMBA in the presence and absence of varying concentrations of benzamil, a competitive inhibitor of the antiporter (see Table 1) which is not photoreactive. Therefore, excess of this analog is expected to compete with NMBA for binding on the antiport polypeptides. As shown in Figure 3, a large excess of benzamil over NMBA

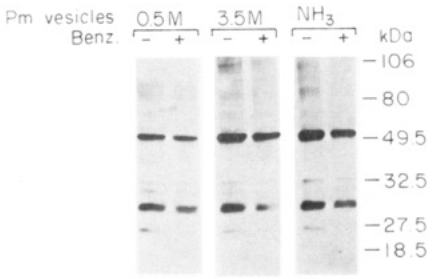


FIGURE 4: Immunoblots of photolabeled vesicles isolated from salt-adapted and NH_3-adapted cells. Plasma membrane vesicles isolated from cells grown in 0.5 and 3.5 M NaCl and NH_3-adapted cells (see Experimental Procedures) were photolabeled with 0.1 μM NMBA in the absence and presence of 100 μM benzamil. The analysis of the proteins was as described in Figure 3.

is needed in order to compete for the labeling.

To evaluate whether incorporation of NMBA into the 50- and 30-kDa proteins is correlated with the Na^+/H^+ antiporter activity, we compared the photolabeling of vesicles derived from *Dunaliella* cells that were induced to overproduce the Na^+/H^+ antiporter. We have previously shown that adaptation of *D. salina* algae to ammonia at alkaline pH or to high salinity was associated with a pronounced increase in the plasma membrane Na^+/H^+ antiport activity (Katz *et al.*, 1992). The enhanced activity was shown to result from an increase in the V_{max}. Equal amounts of these different membrane preparations were photolabeled, separated on tricine–gel, and immunoblotted. Figure 4 demonstrates that the incorporation of the label into the 50- and 30-kDa proteins is significantly enhanced in plasma membranes derived from cells adapted to high salinity (3.5 M NaCl) or to NH_3 (pH stress) with respect to control (0.5 M NaCl) cells. As previously shown, benzamil decreased the incorporation of the label into the two polypeptides in all the plasma membrane preparations, indicating that the label was specific. This increased incorporation is correlated with the increased Na^+/H^+ antiporter activity in the high-salt and NH_3-adapted preparations (Katz *et al.*, 1992).

Photolabeling with [^{125}I]NMBA. As presented in Table 1, substitution of NMBA at position 6 (R_6) with iodine increases the inhibitory potency for Na^+/H^+ antiporter activity. In contrast, substitution with H (H-NMBA) shows very low inhibitory potencies. ^{125}I was incorporated into H-NMBA using the method discribed by Cassel *et al.* (1988) and utilized for photolabeling and identification of the proteins.

We photolabeled plasma membrane vesicles with 0.1 μM [^{125}I]NMBA (0.25 mCi) for varying periods of time (Figure 5) and also compared the labeling of ammonia-adapted with that of control plasma membrane vesicle preparations (Figure 6). The photolabeled proteins were analyzed by tricine–SDS PAGE and autoradiography. Maximal labeling of two major bands of 50 and 30 kDa was achieved within 2 min. The labeling of these two bands is higher in NH_3-adapted than in control plasma membrane vesicles, in correlation with the activity of the Na^+/H^+ antiporter in these preparations. In conclusion, photoaffinity labeling with both methods, immunodetection and radioactive I-NMBA, led to similar results.

DISCUSSION

The most potent inhibitors of the Na^+/H^+ antiporter in *D. salina* were found to be amiloride analogs bearing a hydrophobic substitution on the guanidino moiety, such as benzamil and phenamil (Table 1). Amiloride analogs bearing substitution on the 5-amino group are very poor inhibitors and less

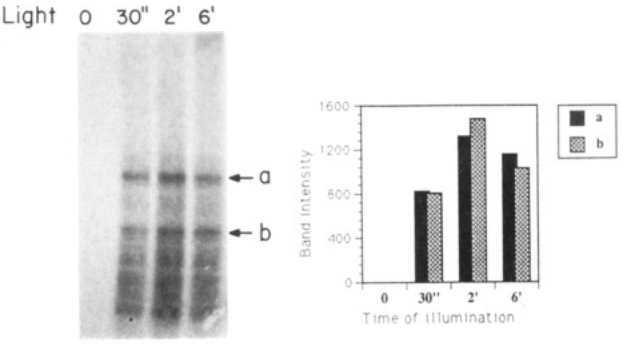


FIGURE 5: Incorporation of [^{125}I]NMBA into plasma membrane vesicles by photoirradiation. Vesicles were photoirradiated for the indicated periods of time in the presence of 0.25 mCi [^{125}I]NMBA. The vesicles were washed and precipitated, and the proteins were separated on SDS PAGE, analyzed by autoradiography: (left) autoradiograph and (right) bar graph representing densitometric quantitation of the bands.

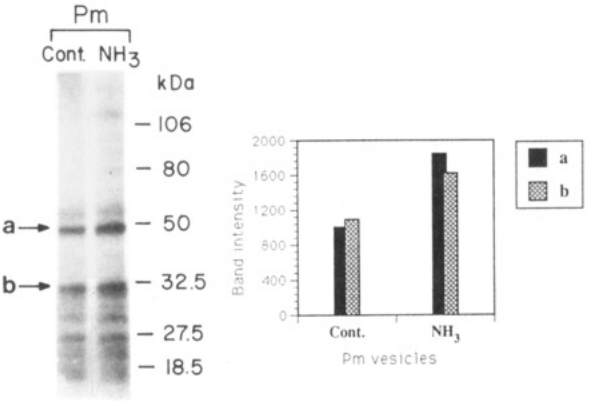


FIGURE 6: Incorporation of [^{125}I]NMBA into plasma membrane vesicles isolated from control and NH_3-adapted cells. Plasma membrane vesicles isolated from control and NH_3-adapted cells were photolabeled for 2 min under the same conditions as described in Figure 5.

effective than amiloride. The inhibitory order of potency is therefore: benzamil > amiloride > MIA. This order of inhibition is exactly opposite to that reported for the mammalian (L'Allemain *et al.*, 1984) and plant vacuolar (Blumwald *et al.*, 1987) Na^+/H^+ antiporters (see the introduction). These findings suggest that the molecular structure of the amiloride binding site of this antiporter is different from other known Na^+/H^+ antiporters.

Another characteristic of the *Dunaliella* Na^+/H^+ antiporter is its very high specificity for Na^+. Even Li^+, which is closest in size to Na^+, is a competitive inhibitor but is not transported by the antiporter (Katz *et al.*, 1986). All other known Na^+/H^+ antiporters (mammalian, plant vacuolar, and bacterial) are less specific to Na^+ and transport Li^+ as well as NH_4^+ cations. The high specificity for Na^+ and the order of inhibition of amiloride analogs that we found for the *Dunaliella* Na^+/H^+ antiporter resemble the characteristics of the epithelial Na^+ channel (Kleyman & Cragoe, 1988; Cuthbert & Fanelli, 1978). The epithelial Na^+ channel is known to be very specific for Na^+. It excludes all the big cations except Na^+, Li^+, and H^+ (Smith & Benos, 1991). Thus, the similar inhibition by the amiloride analogs of the *Dunaliella* Na^+/H^+ antiporter and epithelial Na^+ channel suggests a similarity of their Na^+ binding site.

The labeling experiments with the photoreactive analog NMBA suggest that the 50- and/or 30-kDa polypeptides may be polypeptide subunits of the plasma membrane Na^+/H^+ antiporter for the following reasons: (1) Benzamil, a com-

petitive inhibitor of the antiporter, reduced the labeling of these polypeptides, indicating that the labeling is specific. (2) The increased NMBA labeling of the 50- and 30-kDa polypeptides correlates with enhanced Na^+/H^+ antiporter activity in plasma membrane preparations from high-salt or NH_3-adapted cells. These results support the idea that higher activity of the Na^+/H^+ antiporter in the adapted cells is due to overproduction of the protein.

Na^+/H^+ antiporters constitute a highly heterogeneous group of proteins with respect to size and sequence homology: Na^+/H^+ antiporter subunits were identified by different methods in animals (Wu & Lever, 1989; Huot *et al.*, 1989), plant vacuoles (Barkla *et al.*, 1990), and bacteria (Karpel *et al.*, 1991). However, the molecular weight of the reported subunits varies greatly (25–170 kDa). Similarly, cloning and sequencing of the mammalian Na^+/H^+ antiporter (Sardet *et al.*, 1989), the bacterial Nha A (Karpel *et al.*, 1991), and yeast sod2 (Jia *et al.*, 1992) reveal very little sequence homology between them, indicating convergent evolution from different ancestor proteins.

The unique kinetic properties and specificity for amiloride analogs of the Na^+/H^+ antiporter from plasma membrane of *Dunaliella* suggest that it may also be unique in its structure with respect to known antiporters. The successful labeling with NMBA will hopefully lead to the identification and purification of this Na^+/H^+ antiporter protein.

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REFERENCES

Barkla, B. J., Charuk, J. H. M., Cragoe, E. J., Jr., & Blumwald, E. (1990) *Plant Physiol. 93*, 924–930.

Blumwald, E., & Poole, R. J. (1987) *Plant Physiol. 83*, 884–887.

Blumwald, E., Cragoe, E. J., Jr., & Poole, R. J. (1987) *Plant Physiol. 85*, 30–33.

Braun, Y., Hassidim, M., Lerner, H. R., & Reinhold, L. (1988) *Plant Physiol. 87*, 104–108.

Cassel, D., Rotman, M., Cragoe, E. J., Jr., & Igarashi, P. (1988) *Anal. Biochem. 170*, 63–67.

Cuthbert, A. W., & Fanelli, G. M. (1978) *Br. J. Pharmacol. 63*, 139–149.

Huott, S. J., Cassell, D., Igarashi, P., Cragoe, J. E., Jr., Slayman, C. W., & Aronson, P. S. (1989) *J. Biol. Chem. 264*, 683–686.

Jia, Z. P., McCullough, N., Martel, R., Hemmingsen, S., & Young, P. G. (1992) *EMBO J. 11*, 1631–1640.

Karpel, R., Alon, T., Glaser, G., Shuldiner, S., & Padan, E. (1991) *J. Biol. Chem. 266*, 21753–21759.

Katz, A., & Avron, M. (1985) *Plant Physiol. 78*, 817–820.

Katz, A., Kaback, H. R., & Avron, M. (1986) *FEBS Lett. 202*, 141–144.

Katz, A., Pick, U., & Avron, M. (1989) *Biochim. Biophys. Acta 983*, 9–14.

Katz, A., Pick, U., & Avron, M. (1992) *Plant Physiol. 100*, 1224–1229.

Kleyman, T. R., & Cragoe, E. J., Jr. (1988) *J. Membr. Biol. 105*, 1–21.

Kleyman, T. R., Rajagopalan, R., Cragoe, E., Jr., Erlanger, B. F., & Al-Awqati, Q. (1986) *Am. J. Physiol. 250*, C165–C170.

Kleyman, T. R., Cragoe, E. J., Jr., & Kraehenbuhl, J. P. (1989) *J. Biol. Chem. 264*, 11995–12000.

L'Allemain, G., Franchi, A., Cragoe, E. J., Jr., & Poussegur, J. (1984) *J. Biol. Chem. 259*, 4313–4319.

Sardet, C., Franchi, A., & Pousségur, J. (1989) *Cell 56*, 271–280.

Schagger, H., & Von Jagow, G. (1987) *Anal. Biochem. 166*, 368–379.

Simchowitz, L., & Cragoe, E. J., Jr. (1986) *Mol. Pharmacol. 30*, 112–120.

Smith, P. R., & Benos, O. J. (1991) *Annu. Rev. Physiol. 53*, 509–530.

Towbin, H., Staehelin, T., & Gordon, J. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 4350–4354.

Wu, J. S. R., & Lever, J. E. (1989) *Biochemistry 28*, 2980–2984.