Na^+/H^+ antiport in isolated plasma membrane vesicles from the halotolerant alga *Dunaliella salina*

Adriana Katz*, H. Ronald Kaback and Mordhay Avron*†

Department of Biochemistry, Roche Institute of Molecular Biology, Roche Research Center, Nutley, NJ 07110, USA

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Plasma membrane vesicles isolated from the halotolerant alga *Dunaliella salina* catalyze Na^+/H^+ antiport in a manner that is highly specific for Na^+ (apparent $K_m \simeq 16$ mM). Li^+ and amiloride inhibit the process competitively with apparent K_i values of 38 and 37 μM, respectively. It is suggested that Na^+/H^+ antiport in this organism plays a major role in maintaining low intracellular Na^+ concentrations and may also function to drive Na^+/HCO_3^- symport, an important step in photosynthetic carbon fixation.

(Dunaliella) *Na^+/H^+ exchange* *Halotolerance* *HCO_3^- transport*

1. INTRODUCTION

Most living cells maintain high ratios of extracellular to intracellular Na^+. This is most pronounced in halotolerant species, such as the alga *Dunaliella*, where $[Na^+_{out}]/[Na^+_{in}]$ can exceed 100 [1]. In plant cells, where Na^+/K^+-ATPases are not operative [2], it has been suggested that sodium extrusion involves Na^+/H^+ antiport (i.e. exchange) across the plasma membrane. Such antiporters are widely distributed in bacteria and animal cells [3,4], and carrier-mediated Na^+/H^+ exchange has been demonstrated recently in isolated beet tonoplast vesicles [5].

Here we demonstrate that plasma membrane vesicles isolated from the halotolerant alga *Dunaliella salina* catalyze Na^+/H^+ antiport.

2. MATERIALS AND METHODS

D. salina was cultured in media containing 1 M NaCl, and plasma membrane vesicles were isolated by a modification of a recently described procedure [6]. About 4×10^{10} cells in late log phase were collected by centrifugation, washed once in 1 M NaCl, 10 mM Tris-Mops (pH 7.0) and once in 1.6 M glycerol, 10 mM KCl, 2 mM $MgCl_2$ and 10 mM Tris-Mops (pH 7.0). Cells were resuspended in the latter medium to about 4×10^8 cells/ml and mixed for 30 min at 0°C with 3 vols of the same medium lacking glycerol and containing 1 mM benzamidine and 5 mM ξ-aminocaproic acid. Debris was removed by centrifugation at $12000 \times g$ for 15 min, 4 mM K-EDTA (pH 7.0) was added to the supernatant which was then centrifuged at $150000 \times g$ for 90 min to collect plasma membrane vesicles. The pellet was hand-homogenized in 4 ml of 0.4 M glycerol, 10 mM KCl, 2 mM $MgCl_2$ and 10 mM Tris-Mops (pH 7.0) and recentrifuged at $2500 \times g$ for 5 min to remove residual thylakoid membranes. The supernatant was diluted to 25 ml with the same medium and centrifuged at $150000 \times g$ for 60 min. The pellet was hand-homogenized in 3–5 ml of the same medium and the turbid, light yellow suspension was frozen in liquid nitrogen and kept at −70°C until use.

Na^+/H^+ exchange activity in isolated membrane vesicles was assayed by monitoring changes in absorption of the aminoacridine probe, acridine

† To whom correspondence should be addressed
* On leave from the Biochemistry Department, Weizmann Institute of Science, Rehovot, Israel

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orange [7] in an Aminco DW-2C dual-wavelength spectrophotometer at 492–540 nm after imposition of a ΔpH (interior acid) across the vesicle membrane. Initial reaction mixtures contained 0.4 M glycerol, 10 mM $MgCl_2$, 2.5 μM acridine orange, 20 mM Tris-succinate (pH 5.2) and 1–10 μl membrane vesicles (15–150 μg protein). Samples were incubated for 10 min at 10°C to allow pH equilibration, and a premeasured amount of unbuffered 1 M Tris was added to create a transmembrane pH gradient of 2.5–3.0 units which was stable at 10°C ($t_{½}$ for decay >20 min). NaCl was then added to given concentrations, and the increase in the rate of acridine orange absorbance (i.e. decay of the pH gradient) was used as a measure of Na^+/H^+ antiport activity.

3. RESULTS

As shown in fig.1, imposition of a pH gradient (interior acid) of 2.5–3.0 units across the isolated plasma membrane vesicles of *D. salina* results in a marked decrease in acridine orange absorbance (~30%) which decays very slowly. Addition of valinomycin in the presence of K^+ does not affect the rate of decay (table 2), indicating that the membrane is relatively impermeable to protons. In contrast, addition of NaCl accelerates the rate of decay of the imposed ΔpH in a manner that is dependent on the concentration of NaCl added (fig.1). Moreover, the phenomenon exhibits saturation kinetics with an apparent K_m of 16 mM (fig.2). The anion does not appear to play a specific role in the reaction, as Na^+ is equally effective when Cl^- is completely replaced with SO_4^{2-} (table 1). Addition of 0.01% Triton X-100, 1 μM nigericin (in the presence of 30 mM KCl) or 1 mM NH_4Cl, before or after addition of Na^+, completely abolishes the transmembrane pH gradient.

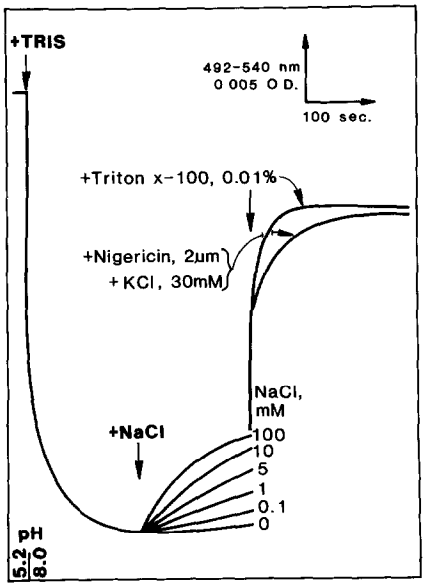

Fig.1. Na^+/H^+ antiport activity in isolated plasma membrane vesicles from *D. salina*. The assay was carried out as described in section 2. Reaction mixtures contained 8 μl plasma membrane vesicles (105 μg protein). Total absorbance of acridine orange was 0.061 at pH 5.2 and 0.054 at pH 8. Unbuffered 1 M Tris (30 μl) was added at the first arrow, which changed the medium pH from 5.2 to 8.0. At the second arrow NaCl was added to given final concentrations. At the third arrow, 0.01% Triton X-100 or 1 μM nigericin/30 mM KCl were added.

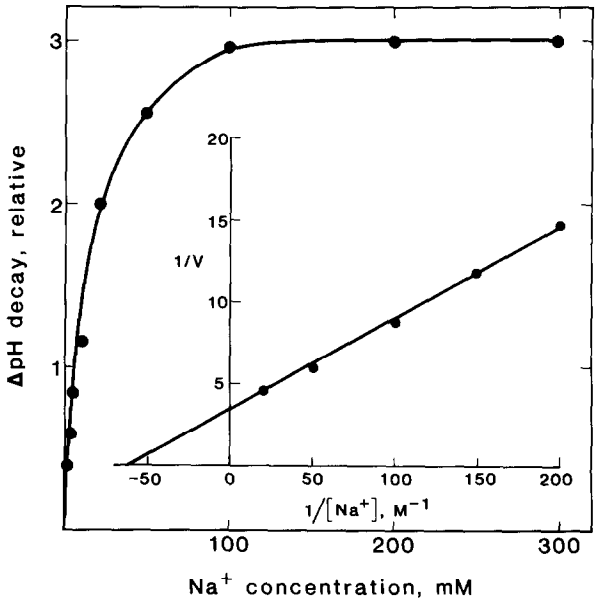

Fig.2. Na^+ concentration dependence. Assays were performed as described in fig.1, except that the NaCl concentration was varied as indicated. The relative initial rate of the NaCl-induced increase in acridine orange absorption was normalized to the total absorption change induced by the acid-base transition (i.e. the final Triton X-100 induced value minus the minimal absorption reached after Tris addition). This value was then corrected for the absorption increase observed in the absence of NaCl (cf. fig.1) and taken as a measure of Na^+/H^+ exchange activity.

The antiporter is highly specific for Na^+ (table 1). No other ion tested [K^+, Li^+, Cs^+, $(CH_3)_4N^+$, choline$^+$, Tris$^+$ or divalent cations such as Mg^{2+} or Mn^{2+}] causes significant acceleration in the rate of decay of the imposed ΔpH. Strikingly, Li^+, a better substrate than Na^+ for certain Na^+/H^+ antiporters [3,4], is a potent competitive inhibitor (table 2), and kinetic analyses reveal an apparent K_i of 38 μM (not shown). Although a number of other monovalent ions do not inhibit Na^+-induced collapse of ΔpH in the assay described, amiloride, a specific inhibitor of Na^+/H^+ antiport in animal systems [8], is a potent competitive inhibitor with an apparent K_i of 37 μM (table 2; kinetic data not shown). Finally, sulfhydryl reagents, such as *p*-chloromercuribenzenesulfonate or $HgCl_2$, or the H^+-ATPase inhibitor, *N',N'*-dicyclohexylcarbodiimide, have no significant effect on antiport activity.

Table 1

Cation specificity of Na^+/H^+ antiport in *Dunaliella* membrane vesicles

Addition	Activity (relative)
NaCl, 50 mM	(100)
Na_2SO_4, 25 mM	99
KCl, 50 mM	0
KCl, 50 mM + valinomycin, 5 μM	0
CsCl, 50 mM	4
Tetramethylammonium chloride, 50 mM	0
LiCl, 50 mM	0
Choline chloride, 50 mM	6

Assays were performed as described in fig.1

Table 2

Inhibition of Na^+/H^+ antiport in *Dunaliella* membrane vesicles

Addition	Activity (relative)
NaCl, 50 mM	(100)
+ LiCl, 50 mM	5
+ LiCl, 10 mM	30
+ amiloride, 0.25 mM	10
+ amiloride, 0.05 mM	45
+ KCl, 50 mM	97
+ KCl, 50 mM + valinomycin, 1 μM	98
+ CsCl, 50 mM	91
+ choline chloride, 50 mM	94
+ tetramethylammonium chloride, 50 mM	100
+ *p*-chloromercuriphenylsulfonic acid, 50 μM	85
+ $HgCl_2$, 100 μM	90
+ *N',N'*-dicyclohexylcarbodiimide, 1 mM	100

Assays were performed as described in fig.1

4. DISCUSSION

Development of a technique for preparation of thylakoid-free plasma membrane vesicles from the halotolerant alga *D. salina* [6] allows transport studies to be carried out in a relatively defined system. In this communication, we demonstrate that these preparations catalyze Na^+/H^+ antiport by a mechanism that exhibits saturation kinetics and is highly specific for Na^+. The existence of such an antiporter in plant plasma membranes has been postulated frequently, and evidence consistent with its operation has been obtained with intact cells (e.g. [9,10]). However, this is apparently the first demonstration of Na^+/H^+ antiport activity in plasma membrane vesicles from algae. Contrary to most of the Na^+/H^+ antiporters described thus far [3–5,10], the *Dunaliella* antiporter is extremely specific for Na^+ and strongly inhibited by Li^+ in a competitive fashion. Nevertheless, the antiporter described here resembles the animal Na^+/H^+ antiporters in that it is also inhibited by amiloride. In addition, it is noteworthy that valinomycin in the presence of K^+ has no significant effect on Na^+/H^+ antiport activity in this system which is consistent with the notion that the antiporter translocates Na^+ and H^+ with a stoichiometry of 1:1.

The Na^+/H^+ antiporter may play dual roles in *Dunaliella*: (i) By functioning in conjunction with a H^+-ATPase, it may remove Na^+ from the cell interior, thereby allowing the cell to grow in saturating concentrations of NaCl while maintaining low intracellular Na^+ concentrations [1,11]. In this regard, it is interesting that a vanadate-sensitive ATPase has been identified recently as a component of the plasma membrane of *Dunaliella* [6,12]. (ii) By functioning in conjunction with an

Na^+/HCO_3^- symporter [13,14], the Na^+/H^+ antiporter may play a critical role in driving HCO_3^- uptake. Photosynthetic carbon fixation consumes CO_2 from incoming HCO_3^-, leaving one OH^- intracellularly for each molecule of CO_2 fixed. Thus, a transmembrane pH gradient (interior alkaline) is created. This pH gradient would then drive Na^+/H^+ exchange via the antiporter, balancing the excess OH^- with incoming H^+ and simultaneously removing Na^+ from the internal compartment. Na^+ would then return with another HCO_3^- via the Na^+/HCO_3^- symporter, and the cycle would be maintained.

REFERENCES

[1] Pick, U., Karni, L. and Avron, M. (1986) Plant Physiol. 81, in press.
[2] Poole, R.J. (1978) Annu. Rev. Plant Physiol. 29, 437–460.
[3] Krulwich, T.A. (1983) Biochim. Biophys. Acta 726, 245–264.
[4] Aronson, P.S. (1985) Annu. Rev. Physiol. 47, 545–560.
[5] Blumwald, E. and Poole, R.J. (1985) Plant Physiol. 78, 163–167.
[6] Sheffer, M. and Avron, M. (1986) Biochim. Biophys. Acta 857, in press.
[7] Schuldiner, S., Rottenberg, H. and Avron, M. (1972) Eur. J. Biochem. 25, 64–70.
[8] L'Allemain, G., Franchi, E., Cragoe, E. jr nd Pouyssegur, J. (1984) J. Biol. Chem. 259, 4313–4319.
[9] Latorella, A.H. and Vadas, R.L. (1973) J. Phycol. 9, 273–277.
[10] Blumwald, W., Wolosin, J.M. and Packer, L. (1984) Biochem. Biophys. Res. Commun. 122, 452–459.
[11] Avron, M. (1986) Trends Biochem. Sci. 11, 5–6.
[12] Gilmour, D.J., Kaaden, R. and Gimmler, H. (1985) J. Plant Physiol. 118, 111–126.
[13] Kaplan, A., Volokita, M., Zenvirth, D. and Reinhold, L. (1984) FEBS Lett. 176, 166–168.
[14] Miller, A.G. and Canvin, D.T. (1985) FEBS Lett. 187, 29–32.