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H^+-translocating ATPase and Na^+/H^+ antiport activities in the plasma membrane of the marine alga *Platymonas viridis*

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Proton transport by the vanadate-sensitive ATPase in plasma membrane (PM) vesicles from the marine unicellular microalga *Platymonas viridis* was investigated. The ATP-dependent generation of ΔpH across the membranes of PM vesicles was followed by the changes in the absorbance of the aminoacridine probe, Acridine orange. Na^+ caused the decay of ΔpH generated by the ATPase, the rate of the decay being dependent on the concentrations of Na^+ added. The phenomenon was specific for Na^+. Amiloride inhibited Na^+-dependent ΔpH decay. The experiments support the idea of a Na^+-extruding mechanism (H^+-translocating ATPase plus Na^+/H^+ antiporter) operating in the PM of marine alga *Pl. viridis*.

H^+-ATPase; Na^+/H^+ antiport; Marine alga; Plasmalemma; *Platymonas viridis*

1. INTRODUCTION

The plasma membrane (PM) in most of the unicellular algae of saline habitats plays a crucial role in the regulation of cytoplasmic Na^+ concentrations. Nevertheless, the membrane ion transport systems of the halotolerant algae have been investigated poorly. To date there has been no reason to doubt that the H^+-pump (H^+-ATPase) operates in the PM of glycophytes and fresh water algae [1]. It is generally accepted that the energy of $\Delta\tilde{\mu}H$ generated by the H^+-pump in the PM of these organisms is utilized for the extrusion of Na^+ from the cells by means of a Na^+/H^+ antiporter [2, 3]. Preliminary investigations on the ion transport and the properties of the PM ATPase from the halotolerant (marine) algae *Nitzschia* [4], *Halicistis* [5], and *Acetobularia* [6] have not demonstrated the H^+-pump (H^+-ATPase) in the PM of these organisms. A primary active Cl^--transporting system (Cl^--ATPase) has been demonstrated to operate in the PM of *Halicistis* [5] and *Acetobularia* [6]. An ATPase synergistically stimulated by Na^+ and K^+ was found in the PM-enriched membrane fractions from *Nitzschia [4]*. On the other hand, subsequent investigations of the ion transport carried out on intact cells of the halotolerant alga *Dunaliella* indicate an H^+-translocating system in the PM of this alga [7]. It has also been found in membrane fractions from algae of genus *Dunaliella* that the properties of the PM ATPase from these algae are similar to those of the common p-type ATPase from higher plants [8,9]. Nevertheless, there is no clear evidence as to whether this ATPase in the PM of the halotolerant algae is an H^+-translocating one, since all attempts to observe ATP-induced proton transport in PM vesicles from these algae were unsuccessful up to now [8,10]. At the same time, the Na^+/H^+ antiport activity has been found in the PM fractions isolated from *Dunaliella* cells [11].

We have previously reported the isolation of highly purified PM from the marine microalga *Platymonas viridis* and investigated the properties of its PM ATPase [12]. Here the ATP-dependent generation of the pH gradients across the plasma membrane vesicles and Na^+-dependent ΔpH decay in the PM vesicles from the marine alga *Pl. viridis* are demonstrated with an Acridine orange probe. These experiments have revealed that a Na^+ extrusion mechanism consisting of an H^+-translocating ATPase and a Na^+/H^+ antiporter operates in the PM of the marine alga *Pl. viridis*.

2. MATERIALS AND METHODS

Pl. viridis cells were cultured in artificial sea water at 0.46 M NaCl as described previously [12]. The highly purified PM vesicles were obtained according to a method presented by us and described in detail previously [12]. This method is based on the partial proteolysis of the glycoprotein cell wall followed by mild hypo-osmotic shock and subsequent membrane partitioning by differential centrifugation in a discontinuous sucrose gradient. Here, we employed this method with slight modifications, namely, mannitol was used instead of glycerol in all solutions. The final membrane pellet was resuspended in the medium consisting of 0.2 M mannitol, 5 mM Tris-MES (pH 7.2), 1 mM DTT, 2 mM $MgCl_2$ and 0.2 mM EGTA.

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Abbreviations: $\Delta\tilde{\mu}H$, electrochemical H^+ potential difference; CCCP, *m*-chlorocarbonylcyanide phenylhydruzone; DTT, dithiothreitol; EGTA, ethyleneglycol-bis(β-aminoethyl ether) *N,N,N',N'*-tetraacetic acid.

The ATP-dependent formation and the Na^+-induced dissipation of the interior-acid pH gradient across the membranes in isolated PM vesicles of *Pl. viridis* (H^+-pump activity and Na^+/H^+ exchange activity, accordingly) were assayed by monitoring the changes in absorbance of the amino-acridine probe, Acridine orange, recorded with a Hitachi 557 dual-wavelength spectrophotometer at 492–540 nm [13]. The assay was performed in 2 ml of reaction mixture containing 0.2 M mannitol, 10 mM Tris-MES (pH 7.0), 1 mM DTT, 0.2 mM EGTA, 100 mM NO_3^- (as Tris salt), 10 mM $MgSO_4$, 8 μM Acridine orange, 20–30 μg protein. The vesicles were equilibrated with the medium for 15–20 min before adding ATP. ATP (buffered with Tris to pH 7.0) at a final concentration of 1 mM was added to initiate the formation of ΔpH.

Various concentrations of Na^+ (as indicated in the figure legends) were added to initiate Na^+/H^+ exchange. The initial rates of absorbance changes due to antiport activity were determined by drawing the tangents of the recorded traces obtained in the first 10 s after salt addition.

Protein content was determined by the method of Simpson and Somme [14] with bovine serum albumin as a standard.

3. RESULTS

As shown in Fig. 1, the addition of ATP to the reaction mixture containing the *Pl. viridis* PM vesicles resulted in an appreciable decrease in Acridine orange absorbance. The protonophorous uncoupler CCCP completely abolished the ATP-induced decrease in absorbance indicating a ΔpH generation across the membranes of PM vesicles in the presence of ATP. A spontaneous decay of the generated ΔpH was slow, showing a relatively low permeability of the vesicles to protons. The additions of vanadate, an inhibitor of PM ATPase of the plant and fungi cells, decreased the generated ΔpH to a considerable degree (Fig. 1). This indicates the involvement of the vanadate-sensitive ATPase in the ΔpH generation across the *Pl. viridis* PM.

Na^+ (either as chloride or as sulfate) caused the decay of ΔpH generated by the ATPase (Fig. 2). The rate of

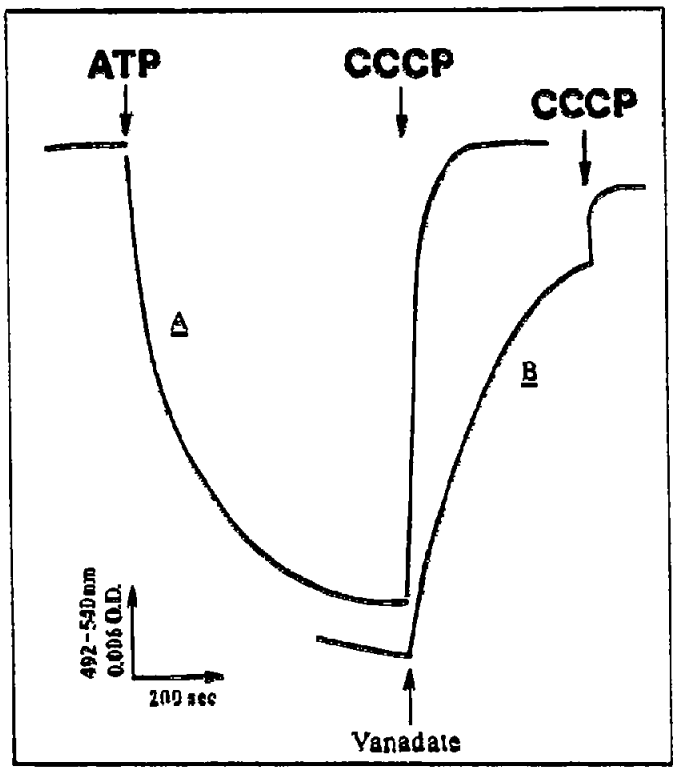

Fig. 1. ATP-dependent intravesicular acidification in isolated plasma membrane vesicles from *Pl. viridis* monitoring by changes in the absorbance of Acridine orange. The assay was carried out as described in section 2. ATP, buffered with Tris to pH 7.0, was added at the first arrow. At the second arrow 12 µM CCCP was added to dissipate proton gradient. Trace (A), standard assay; trace (B), standard assay with addition of 0.1 mM sodium vanadate.

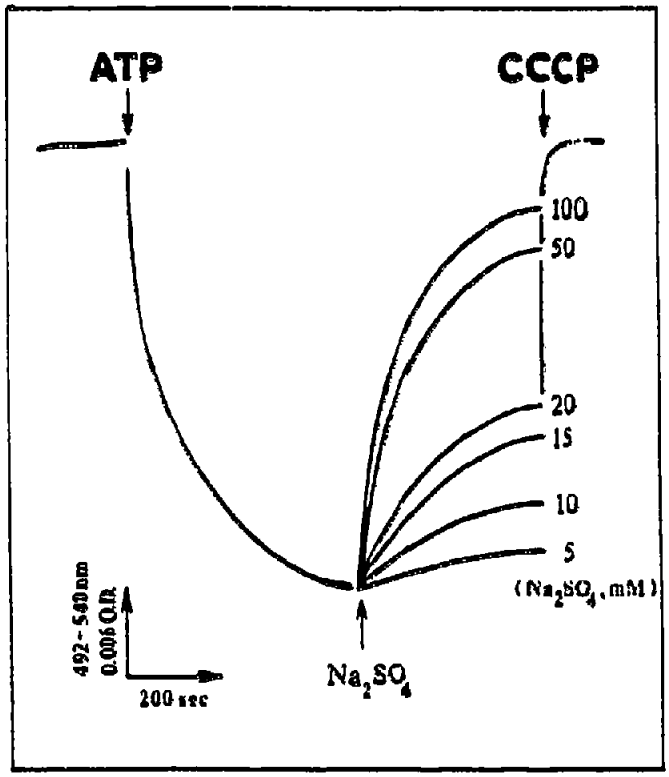

Fig. 2. Na^+/H^+ antiport activity in isolated plasma membrane vesicles from *Pl. viridis*. ΔpH generated by the addition of ATP to the reaction mixture contained PM vesicles (first arrow) was dissipated by Na^+ (second arrow, indicated concentrations). CCCP (12 µM, third arrow) abolished the remaining proton gradient.

Table 1

Cation specificity of Na^+/H^+ antiport in *Pl. viridis* plasma membrane vesicles

Addition	Activity (relative)
NaCl, 100 mM	100
Na_2SO_4, 50 mM	98
KCl, 100 mM	22
CsCl, 100 mM	10
LiCl, 100 mM	0

Assays were performed as described in Fig. 2. The activities were determined as initial rates of absorbance changes (as described in section 2).

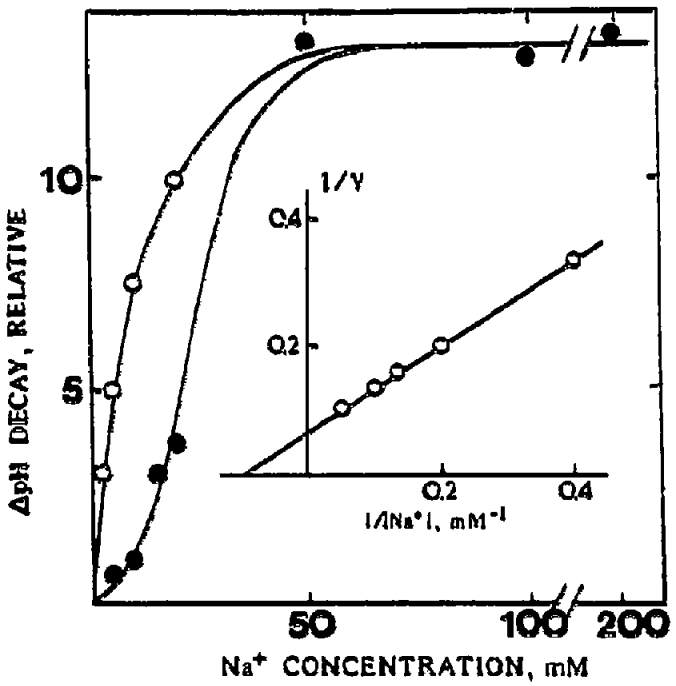

Fig. 3. Na^+ concentration dependence of ΔpH decay. Data from Fig. 2. The initial relative rates of absorbance changes were determined in section 2. (●), experimental curve; (○), theoretical Michaelis-Menten curve. Insert: Lineweaver-Burk plot of the theoretical curve gives the theoretical K_m = 10 mM.

the decay depended on the concentrations of Na^+ added. The effect of Na^+ displayed a sigmoidal curve with increasing Na^+ concentrations (Fig. 3). It can be supposed that the sigmoidal nature of Na^+ effect is due to the superposition of two independent processes: (1) an acceleration of intra-vesicular acidification due to the stimulation of the ATPase by low Na^+ concentrations [12], and (2) ΔpH decay due to Na^+/H^+ antiport. Assuming there is no stimulation of the ATPase by Na^+, this sigmoidal curve could be approximated by a Michaelis- Menten curve with apparent K_M = 10 mM (Fig. 3). The Na^+/H^+ antiport under consideration is specific for Na^+. Other cations tested (K^+, Cs^+ and Li^+) resulted in ΔpH decay at significantly lower rates (Table I). Accordingly, these cations are the potent competitive inhibitors of Na^+/H^+ exchange (Table II). Amiloride, a specific inhibitor of the Na^+/H^+ antiport in various animal membranes [15] added prior to Na^+ also delayed ΔpH decay induced by Na^+ (Table II).

Table II

Inhibition of Na^+/H^+ antiport in *Pl. viridis* plasma membrane vesicles

Addition		Activity (relative)
NaCl,	100 mM	100
+KCl,	100 mM	28
+LiCl,	100 mM	37
+CsCl,	100 mM	20
+amiloride,	0.2 mM	10

Assays were performed as described in Fig. 2. The cations were added before addition of Na^+.

4. DISCUSSION

The vanadate-sensitive ATPase activity has been identified recently as a component of the PM of *Dunaliella* [8–10] and *Platymonas* [12] cells. Here we have shown the translocation of protons in the PM vesicles from *Pl. viridis* to be initiated by addition of the ATP and sensitive to vanadate. This is apparently the first direct demonstration of a H^+-translocating ATPase in the PM of the alga living under high salinity conditions.

The dissipation of ΔpH generated by the ATPase in response to an addition of Na^+ by a mechanism that exhibits saturation kinetics is specific for Na^+ and sensitive to amiloride, and indicates the functioning of the Na^+/H^+ antiporter in the PM of *Pl. viridis*.

H^+-ATPase and Na^+/H^+ antiporter in the PM of *Pl. viridis* probably form a mechanism extruding Na^+ from the cell interior and maintaining low intracellular concentrations of Na^+ under high salinity conditions.

It remains to be elucidated whether this system (H^+-ATPase plus Na^+/H^+ antiporter) is the only ion transporting system in the PM of salt-tolerant algae extruding Na^+ from the cells. In this respect the earlier data on the unusual PM ATPases in *Nitzschia* [4], *Halicystis* [5], and *Acetabularia* [6] are extremely interesting. It is worth mentioning here the organization of ion transport in the cytoplasmic membrane of the halo- and alkalotolerant bacterium *Vibrio alginolyticus*, in which the ATP-driven primary Na^+ pump was discovered [16]. A similar enzyme may function in the plasmalemma of halotolerant algae. So, one should study the possibility of ATP-driven transport of Na^+ in PM vesicles from marina algae. Positive results would demonstrate the operation of a Na^+-translocating ATPase in the plasmalemma of marine algae.

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