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Short Communication

A Sodium Pump in the Plasma Membrane of the Marine Alga *Heterosigma akashiwo*

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ATP-dependent transport of $^{22}Na^+$ into liposomes reconstituted from plasma membrane proteins of *Heterosigma akashiwo* was examined. The apparent K_m values for transport of Na^+ were 400 μM for ATP and 7 mM for Na^+. ATP-dependent transport of $^{22}Na^+$ was not inhibited by a protonophore or a membrane-permeable cation but was inhibited by an inhibitor of P-type ATPases.

Key words: *Heterosigma akashiwo* — Marine alga — Na^+ transport — Na^+-ATPase [EC 3.6.1.3] — Plasma membrane.

Most living cells maintain low concentrations of cytoplasmic Na^+ ions even when extracellular fluids, such as animal body fluids and seawater, contain high levels of Na^+ ions. The Na^+/H^+ anitporter is one of the systems that transports Na^+ ions across membranes. Bacteria and yeast cells can extrude Na^+ ions via Na^+/H^+ antiporters (Lolkema et al. 1994, Jia et al. 1992) that control the concentration of cytoplasmic Na^+ ions. Some terrestrial plants and marine algae also have Na^+/H^+ antiport activity in their plasma membranes (Blumwald and Poole 1985, Katz et al. 986, Popova and Balnokin 1992). The Na^+-transporting ATPase provides another system for the transport of Na^+ ions across membranes. Animal Na^+/K^+-ATPases are the best-known Na^+ pumps for the maintenance of a low cytoplasmic concentration of Na^+ ions. It has been suggested that bacterial (Lolkema et al. 1994), yeast (Haro et al. 1991) and algal (Balnokin and Popova 1994, Sullivan and Volcani 1974, Wada et al. 1989, Shono et al. 1995) cells also have Na^+-transporting ATPases in their plasma membranes.

Very little is known about the ATP-dependent efflux of Na^+ ions in marine algae. Balnokin and Popova (1994) demonstrated the ATP-dependent accumulation of $^{22}Na^+$ ions by plasma membrane vesicles from *Platymonas viridis*. Moreover, Na^+- and K^+-stimulated ATPase activities were found in plasma membrane fractions from *Nitzschia alba* (Sullivan and Volcani 1974) and *Heterosigma akashiwo* (Wada et al. 1989, Shono et al. 1995). These observations suggest that the concentration of cytoplasmic Na^+ ions in some algal cells might be controlled by Na^+-ATPases in the plasma membrane even though the molecular basis for the regulation of ATPase activity and of transport activity has not been clarified.

We have characterized the Na^+-ATPase and analyzed the kinetics of the corresponding reaction in a plasma membrane fraction of *H. akashiwo*, as described in previous reports (Wada et al. 1989, Shono et al. 1995). In this study, we examined the ATP-dependent Na^+-transport activity of a plasma membrane fraction from *H. akashiwo* cells. Our results suggest that the Na^+-ATPase might play a role in the extrusion of Na^+ ions from the cytoplasm of *H. akashiwo* by acting as a Na^+ pump.

An axenic clone of *Heterosigma akashiwo* (strain number OHE-1) was purchased from the National Institute for Environmental Studies (Tsukuba, Japan). Conditions for culture and components of the medium were the same as described previously (Shono et al. 1995). A plasma membrane fraction was obtained by discontinuous sucrose density gradient centrifugation after differential centrifugation, as described previously (Shono et al. 1995).

Liposome vesicles were prepared by the method of Karlish and Pick (1981), with some modifications (Hara and Nakao 1986). The plasma membrane fraction was diluted with 125 mM MES-Tris buffer (pH 7.0) that contained 1 mM EDTA and was centrifuged for 15 min at 430,000 × *g*. The pellet was resuspended in the same buffer at a concentration of protein of 10 mg ml^{-1} (determined as described by Lowry et al. (1951) with bovine serum albumin as the standard). The membrane fraction (500 μg of protein) was solubilized with 20 mM SM-1200 in the presence of 0.1 mM EDTA and 25 mM KCl, and the mixture was centrifuged at 430,000 × *g* for 2 min. Phosphatidylcholine was suspended at a concentration of 100 mg ml^{-1} in 100 mM KCl, and the mixture was dialyzed against a solution that contained 25 mM KCl and 0.1 mM EDTA for the replacement by K^+ ions of contaminating cations in

Abbreviations: FCCP, carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone; SM-1200, sucrose monolaurate; TBA^+, tetra-*n*-butylammonium.

the preparation of phosphatidylcholine (Sigma, St. Lowis, U.S.A.). The dialyzed mixture was combined with the supernatant of the solubilized plasma membrane fraciton. The final concentrations of protein, SM-1200, EDTA, KCl and phosphatidylcholine were 1 mg ml^{-1}, 9 mM, 0.1 mM, 25 mM and 25–30 mg ml^{-1}, respectively. The mixture was frozen in liquid nitrogen and thawed at room temperature, and then it was sonicated briefly to yield small liposome vesicles. The resulting suspension of vesicles was then passed through a small column of Sephadex G50 (Pharmacia, Uppsala, Sweden) that had been equilibrated with 125 mM MES-Tris buffer (pH 7.0) for removal of detergent and cations.

Liposome vesicles (75 μg of protein) were suspended at pH 7.0 in 116 mM MES-Tris, 3 mM $MgCl_2$, 10 mM NaCl and 1.3 kBq μl^{-1} ^{22}Na ions (Dupont, Boston, U.S.A.). The suspension was incubated at 30°C for 3 min. The reaction was started by the addition of 3 mM ATP and stopped by cooling the reaction mixture on ice and the addition of 50 mM NaCl. The reaction mixture was passed through a small column of Sephadex G50 that had been equilibrated with 125 mM Tris-MES buffer (pH 7.0) to remove external isotope, and the radioactivity in the recovered vesicles was determined in a scintillation counter. All the assays were performed with triplicate samples. The ATP-dependent transport of Na^+ ions was quantitated by subtracting the radioactivity of control vesicles incubated without ATP from that of vesicles incubated with ATP.

The transport of Na^+ ions into liposomes prepared with phosphatidylcholine and proteins that had been solubilized from the plasma membrane was measured. $^{22}Na^+$ ions accumulated in the liposomes after the addition of ATP, and the accumulation reached saturation within about 5 min (Fig. 1). These saturation kinetics strongly suggest the presence of an ATP-dependent Na^+-pump in the plasma membrane fraction of *H. akashiwo*.

If the Na^+-ATPase were to function as a Na^+ pump, we would expect the kinetics of $^{22}Na^+$-transport activity to be very similar to those of the Na^+-ATPase activity. The apparent K_m for transport of Na^+ ions was about 400 μM for ATP in the presence of 100 mM K^+, 10 mM Na^+ and 3 mM Mg^{2+} ions (Fig. 2). This value is close to the K_m of 880 μM for the Na^+-ATPase activity (Shono et al. 1995), even though the experiments in which these values were determined differed slightly in terms of ionic conditions and pH. The conditions for the Na^+-transport assay were not identical to those for the assay of Na^+-ATPase activity because of the instability of liposomes. Although the uptake of Na^+ ions into the liposomes reached equilibrium at high concentrations of Na^+ ions, the apparent K_m for the Na^+-transport activity was estimated to be about 7 mM for Na^+ ions (Fig. 3). This value is also very close to that of the Na^+-ATPase activity in the plasma membrane of *H. akashiwo* (Shono et al. 1995). The K_m for Na^+ ions of the Na^+-transport activity was also similar to K_m values determined for Na^+/K^+-ATPases of animal origin (Karlish and Pick 1981, Cornelins 1991).

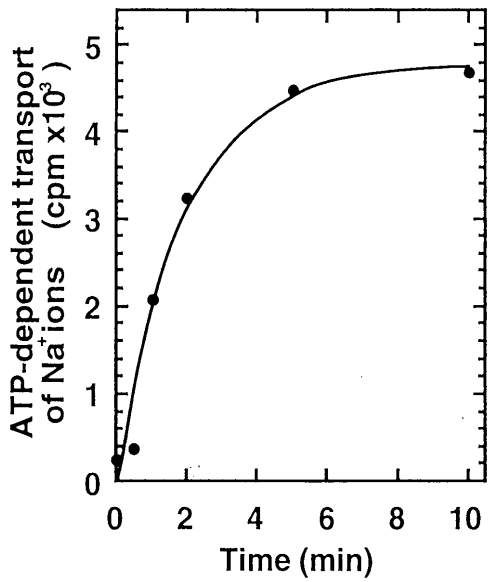

Fig. 1 The ATP-dependent transport of $^{22}Na^+$ ions into liposomes prepared with phosphatidylcholine and plasma membrane proteins solubilized from *Heterosigma akashiwo*. Liposome vesicles (75 μg of protein) were suspended in 116 mM MES-Tris, 3 mM $MgCl_2$, 10 mM NaCl and 1.3 kBq μl^{-1} ^{22}Na ions (pH 7.0). The reaction was started by the addition of 3 mM ATP, incubated at 30°C and then stopped by the addition of 50 mM NaCl at 0°C.

The plasma membranes of various eukaryotes have Na^+/H^+ antiporter activity. We could not ignore the possibility that the uptake of Na^+ ions into the liposomes was

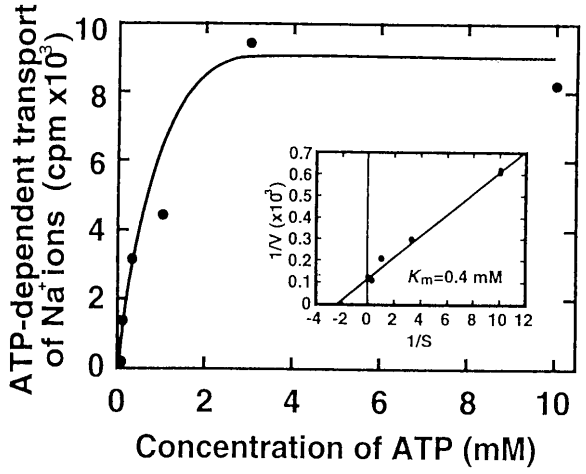

Fig. 2 Effects of the concentration of ATP on the transport of $^{22}Na^+$ ions into liposomes. The reaction was allowed to proceed for 3 min in the presence of ATP at various concentrations. Other conditions were the same as mentioned in the legend to Fig. 1. Inset: a Lineweaver-Burk plot of the data gives a K_m of 400 μM.

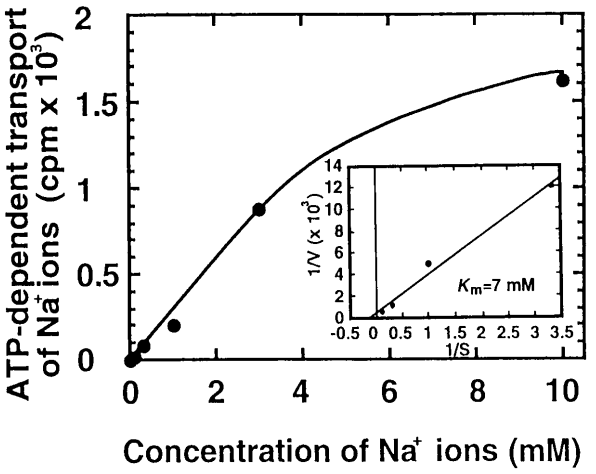

Fig. 3 Effects of the concentration of Na^+ ions on the transport of $^{22}Na^+$ ions into liposomes. The reaction was allowed to proceed for 3 min in the presence of extravesicular NaCl at various concentrations. Other conditions were the same as mentioned in the legend to Fig. 1. Inset: a Lineweaver-Burk plot of the data gives a K_m of 7 mM.

due to a Na^+/H^+ antiporter driven by a pH gradient that was generated by the H^+-ATPase, even though we failed to detect any H^+-pumping activity in the plasma membrane fraction (data not shown).

The effects of various reagents on the Na^+-transport activity are summarized in Table 1. The accumulation of $^{22}Na^+$ ions in the liposomes was not inhibited by the addition of the protonophore FCCP (10 μM). However, an inhibitor of P-type ATPases, orthovanadate (100 μM), inhibited the accumulation of $^{22}Na^+$ ions in the liposomes. Thus, the accumulation of $^{22}Na^+$ ions was caused by the Na^+-ATPase in the plasma membrane fraction and not by a Na^+/H^+ antiporter.

The possibility that the transport of Na^+ ions might be related to the membrane potential was eliminated since TBA^+ (3 mM), a membrane-permeating cation, did not inhibit the accumulation of $^{22}Na^+$ ions in the liposomes (Table 1).

Table 1 Effects of various inhibitors on the ATP-dependent transport of $^{22}Na^+$ ions into liposomes

Addition	Activity
Control (no addition)	100±25%
+orthovanadate (100 μM)	56±15%
+TBA^+ (3 mM)	88±16%
+FCCP (10 μM)	92±35%

Reactions were carried out as described in the legend to Figure 1. Data are means±SE of results from two independent experiments with triplicate assays.
TBA^+, tetra-n-butylammonium chloride. FCCP, carbonyl cyanide *p*-(trifluoromethoxy)phenyl-hydrazone.

Because Na^+-ATPase activity was considerably diminished during the chromatographic steps required for its purification, perhaps as a consequence of the delipidation of ATPase molecules, we could not demonstrate the transport of Na^+ ions across the plasma membrane using the chromatographically purified protein. The similarity of the K_m values for ATP and Na^+ ions between the Na^+-ATPase activity and the Na^+-transport activity, and the features of the Na^+-ATPase activity and Na^+-transport activity indicate that the Na^+-ATPase in the plasma membrane of *H. akashiwo* is indeed a Na^+ pump, as is the Na^+/K^+-ATPase in animal cells and, moreover, that the Na^+-ATPase plays a role in the removal of Na^+ ions from the cytoplasm of *H. akashiwo*. Because the Na^+-ATPase requires K^+ ions for maximum activity and because a phosphorylated intermediate of Na^+-ATPase is dephosphorylated by the subsequent addition of K^+ ions (Shono et al. 1995), K^+ ions are likely to be the counter ions for the transport of Na^+ ions, as is the case for the Na^+/K^+-ATPase in animal cells.

There are some strong similarities between the Na^+-ATPase of *H. akashiwo* and the Na^+/K^+-ATPases of animal cells, such as ionic requirements of ATPase activity, formation of a phosphorylated intermediate and multiple Na^+-binding sites (Shono et al. 1995). The K_m for Na^+ ions of the Na^+-transport activity is also similar to that of animal Na^+/K^+-ATPases. However, some differences, such as relative molecular mass, subunit composition (Shono et al. 1995), and sensitivity to ouabain (Wada et al. 1989) clearly indicate that these two ATPases are different molecules that, nonetheless, have very similar activity. Direct evidence for their differences requires cloning of the respective genes.

It has been suggested that Na^+/H^+ antiporters on the plasma membrane of marine algae act as Na^+-extrusion systems (Katz et al. 1986, Popova and Balnokin 1992). However, the Na^+/H^+ antiporter of *Dunaliella salina* plays a role in the regulation of intracellular pH via transient influxes of Na^+ ions (Weiss and Pick 1990, Katz et al. 1991), and no studies have proven that the Na^+/H^+ antiporters on the plasma membranes of algae are actually responsible for the efflux of Na^+ in vivo. Using plasma membrane vesicles derived from *Platymonas viridis*, Balnokin and Popova (1994) demonstrated the ATP-dependent accumulation of $^{22}Na^+$ ions in membrane vesicles. In this study, we demonstrated the ATP-dependent transport of Na^+ ions into proteoliposomes prepared with solubilized plasma membrane proteins of *H. akashiwo*. It is possible that Na^+-ATPases that act as Na^+-transporting enzymes are widely distributed in marine algae and function as primary extruders of Na^+ ions from the algal cells.

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