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Electrogenicity of the Na^+-ATPase from the marine microalga *Tetraselmis (Platymonas) viridis* and associated H^+ countertransport

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Abstract Sodium accumulation by the Na^+-ATPase in the plasma membrane (PM) vesicles isolated from the marine alga *Tetraselmis (Platymonas) viridis* was shown to be accompanied by $\Delta\Psi$ generation across the vesicle membrane (positive inside) and H^+ efflux from the vesicle lumen. Na^+ accumulation was assayed with $^{22}Na^+$; $\Delta\Psi$ generation was detected by recording absorption changes of oxonol VI; H^+ efflux was monitored as an increase in fluorescence intensity of the pH indicator pyranine loaded into the vesicles. Both ATP-dependent Na^+ uptake and H^+ ejection were increased by the H^+ ionophore carbonyl cyanide *m*-chlorophenylhydrazone (ClCCP) while $\Delta\Psi$ was collapsed. The lipophilic anion tetraphenylboron ion (TPB^-) inhibited H^+ ejection from the vesicles and abolished $\Delta\Psi$. Based on the effects of ClCCP and TPB^- on H^+ ejection and $\Delta\Psi$ generation, the conclusion was drawn that H^+ countertransport observed during Na^+-ATPase operation is a secondary event energized by the electric potential which is generated in the course of Na^+ translocation across the vesicle membrane. Increasing Na^+ concentrations stimulated H^+ efflux and caused the decrease in the $\Delta\Psi$ observed, thus indicating that Na^+ is likely a factor controlling H^+ permeability of the vesicle membrane.

Key words: Marine alga; Na^+-ATPase; Na^+/H^+ countertransport; Na^+ pump; Plasma membrane; *Tetraselmis (Platymonas) viridis*

1. Introduction

The functioning of the primary Na^+ pump in the plasma membrane (PM) of the marine green microalga *Tetraselmis (Platymonas) viridis* has been shown by us earlier [1,2]. The inverted PM vesicles isolated from this organism were able to transport $^{22}Na^+$ from outside into the vesicle lumen upon MgATP addition. The process occurred in the presence of the protonophore carbonyl cyanide *m*-chlorophenylhydrazone (ClCCP) and amiloride, an inhibitor of the Na^+/H^+ antiporters, thus indicating that ATP-dependent sodium uptake by the PM vesicles was performed by a proton-motive-force-independent fashion. The ATP-driven sodium uptake was sensitive to orthovanadate, and pH optimum of the process was 7.8–8.0. Recently, in the PM preparations isolated from *T. viridis*, a polypeptide with apparent molecular mass 100 kDa being phosphorylated in the presence of [γ-^{32}P]ATP was demonstrated by means of acid PAGE in combination with autoradiography [3]. The 100 kDa polypeptide phosphorylation was shown to require the presence of sodium ions in the reaction mixture. The phosphorylated polypeptide was sensitive to hydroxylamine treatment, thus indicating that an acyl-phosphate bond was formed during the phosphorylation reaction. The results of the above-mentioned experiments demonstrated that the primary Na^+ pump of *T. viridis* is a Na^+-transporting ATPase forming the phosphointermediate in the course of its catalytic cycle and, therefore, belonging to the family of the p-type ATPases.

An important problem related to the mechanism of ATP-dependent Na^+ transport is whether a counterion is utilized by the Na^+-ATPase from *T. viridis*. Many cation-translocating p-type ATPases are known to operate by cation exchange, for example Na^+,K^+-ATPase and H^+,K^+-ATPase from mammalian cells [4], Ca^{2+},H^+-ATPase from sarcoplasmic reticulum [5], Ca^{2+}-ATPase from plant plasma membrane that catalyzes Ca^{2+}/H^+ exchange as well [6]. Na^+-ATPase from the brown microalga *Heterosigma akashiwo* was suggested to be a Na^+,K^+-exchanging ATPase as judged by (i) synergistic stimulation of the PM ATPase activity by Na^+ and K^+ ions, and (ii) dephosphorylation of the phosphorylated intermediate of the ATPase by K^+ ions [7,8]. Since K^+ inhibited Na^+ translocation performed by the Na^+-ATPase from *T. viridis* [2] and there was no synergistic stimulation of the PM ATPase activity by K^+ and Na^+ [9], it is hardly possible to ascribe the role of the counterion to K^+ in the present case. Another possibility is that H^+ serves as a counterion in primary Na^+ transport in the *T. viridis* plasma membrane.

In this work we demonstrate on isolated PM vesicles from *T. viridis* that ATP-dependent $^{22}Na^+$ uptake performed by the Na^+-ATPase is accompanied by $\Delta\Psi$ generation across the vesicle membrane and alkalinization of the vesicle lumen, i.e. H^+ efflux from the vesicles. The data obtained allowed us to conclude that the Na^+-ATPase from *T. viridis* PM operates as an electrogenic uniporter. H^+ is a counterion for Na^+ and movement of H^+ out of the vesicle lumen is energized by $\Delta\Psi$ generated in the course of Na^+ translocation by Na^+-ATPase.

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Abbreviations: BTP, 1,3-bis-(tris(hydroxymethyl)methylamino)propane; ClCCP, carbonyl cyanide *m*-chlorophenylhydrazone; EGTA, ethylene glycol-bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; HEPES, *N*-2-hydroxyethylpiperazine-*N′*-2-ethanesulfonic acid; MES, 2-[*N*-morpholino]ethanesulfonic acid; oxonol VI, bis-(3-propyl-5-oxo-isoxazol-4-yl)pentamethine oxonol; PM, plasma membrane; pyranine, 8-hydroxy-1,3,6-pyrene trisulfonate; TPB^-, tetraphenylboron ion; $\Delta\Psi$, membrane electric potential difference

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2. Materials and methods

2.1. Plant material and preparation of PM vesicles

Tetraselmis (Platymonas) viridis Rouch. cells were cultured in artificial sea water containing 0.45 M NaCl as described elsewhere [2].

The PM vesicles were prepared according to the method described earlier [9] with some minor modifications [2]. The final membrane pellet was suspended in medium containing 0.5 M mannitol, 10 mM 1,3-bis-(tris(hydroxymethyl)methylamino)propane–*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (BTP–HEPES), pH 7.8, 2 mM $MgSO_4$, 1 mM dithiothreitol, 0.4 mM ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA), and 10 μg/ml phenylmethylsulfonyl fluoride.

2.2. Na^+ uptake

Sodium uptake by the PM vesicles was monitored by a tracer technique using $^{22}Na^+$ as described in [1]. The assay mixture contained 0.5 M mannitol, 20 mM BTP–HEPES, pH 7.8, 25 mM NO_3–BTP, 1 mM $MgSO_4$, and ca. 600 μg of membrane protein. The reaction was initiated by successive addition of 10 mM $^{22}NaCl$ up to 0.5 MBq and 2 mM ATP (Tris salt). After defined time intervals, 60 μl aliquots of the suspension were taken, and vesicles were separated from the medium by filtering through a Synpore nitrocellulose filter with a pore size of 0.6 μm. Filters were washed three times with 1 ml of the assay solution free of the label and ATP, and radioactivity retained on the filters was counted with a scintillation counter.

2.3. Detection of transmembrane electric potential

Formation of transmembrane electric potential accompanying the Na^+ pump operation was detected by continuously recording the differential absorption change (621−582 nm) of the $\Delta\Psi$ indicator oxonol VI [10] with Hitachi 557, a dual wavelength spectrophotometer. The assay was performed in the reaction medium containing 0.5 M sucrose, 20 mM BTP–MES, pH 7.8, 1 mM $MgSO_4$, 5 mM Na_2SO_4, 1 mM EGTA, 3 μM oxonol VI, and ca. 100 μg of membrane protein in 2 ml. After 15 min incubation of the vesicles in the medium, 2 mM ATP (Tris salt) was added to initiate the ATP-driven $\Delta\Psi$ generation.

2.4. Detection of H^+ ejection from the vesicles

The formation of the interior-alkaline pH gradient across the vesicle membranes in the course of Na^+ pump operation (lumenal alkalinization) was detected by monitoring the changes in fluorescence intensity of the pH indicator pyranine loaded into the vesicles. To load pyranine into the vesicles, isolation of the PMs was carried out in the presence of 100 μM pyranine added to the solutions used. To eliminate pyranine outside the vesicles, 0.5 ml final vesicle suspension was passed through a 1×10 cm column with G-50 Sephadex.

The fluorescence of pyranine loaded into the PM vesicles was recorded with a Hitachi 850 fluorescence spectrophotometer set at 450 nm (excitation) and 510 nm (emission). The assay was performed in 1 ml of the reaction medium containing 0.5 M mannitol, 20 mM BTP–HEPES, pH 7.8, 50 mM NO_3^-–BTP, 1 mM $MgSO_4$, and ca. 40 μg of membrane protein. After 15 min incubation of the vesicles in the medium, the reaction was initiated by successive addition of 10 mM Na_2SO_4 and 2 mM ATP (Tris salt).

2.5. Protein determination

Protein content was determined by the micromethod of Simpson and Sonne [11] with bovine serum albumin as a standard.

3. Results

The PM vesicles isolated from *T. viridis* accumulated $^{22}Na^+$ in the presence of $^{22}NaCl$ (Fig. 1A). Sodium uptake was sig-

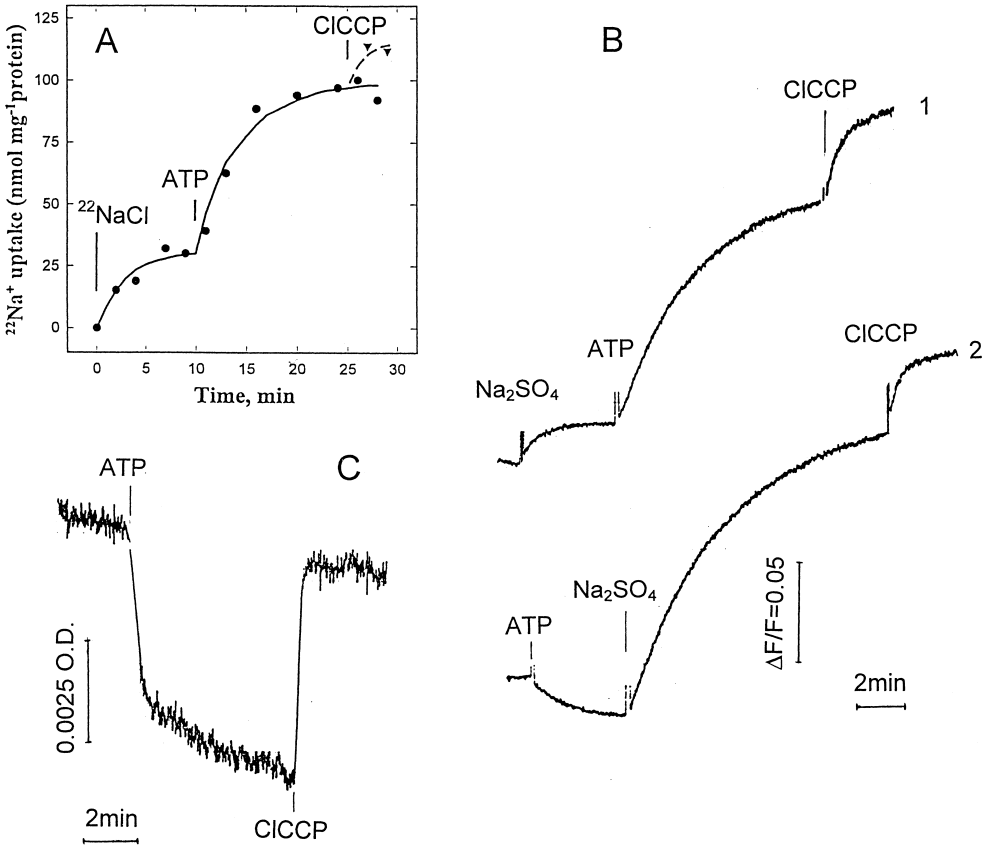

Fig. 1. $^{22}Na^+$ translocation into the PM vesicles isolated from *T. viridis* (A) and associated processes: lumenal alkalinization (B) and generation of membrane potential (C). Additions made: 10 mM Na_2SO_4, 2 mM ATP (Tris salt), 12 μM ClCCP. The conditions of the experiments are described in Section 2.

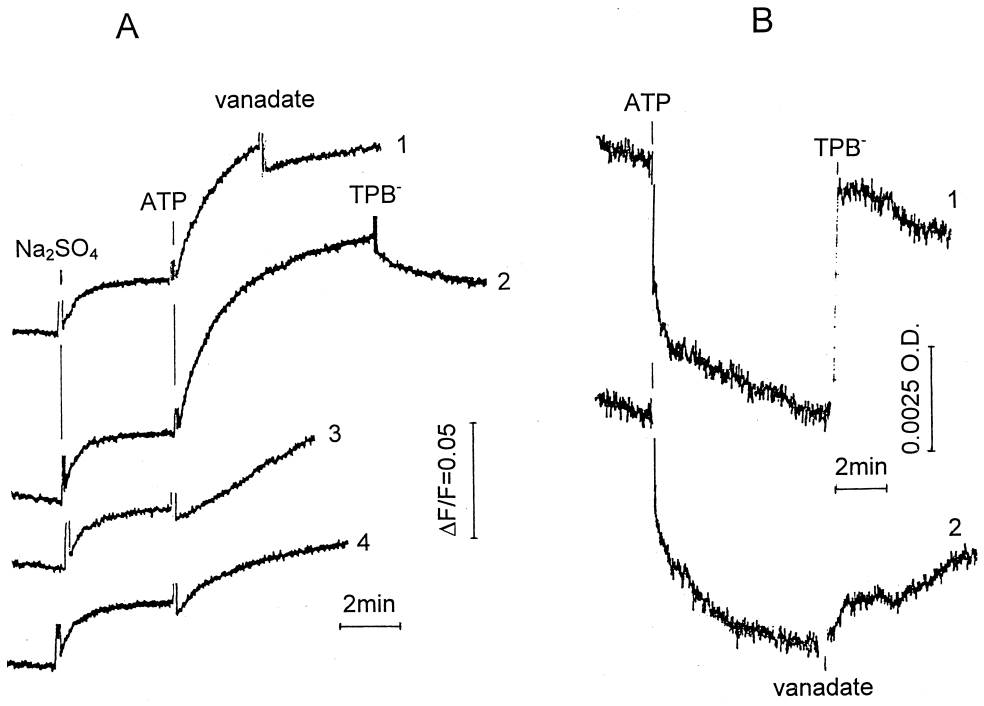

Fig. 2. Effects of vanadate and TPB^- on lumenal alkalinization (A) and membrane potential generation (B) in the PM vesicles from *T. viridis*. Additions made: 10 mM Na_2SO_4, 2 mM ATP (Tris salt), 500 μM vanadate, 100 μM TPB^-. The conditions of the experiments were as described in Section 2, except for traces 3 and 4 in A, where vanadate (trace 3) and TPB^- (trace 4) were present in the reaction mixture initially, before additions of Na_2SO_4 and ATP.

nificantly accelerated in response to ATP addition when the reaction medium was supplied with Mg^{2+} ions and the reaction was performed in the weakly alkaline medium. The protonophore ClCCP added to the medium following ATP enhanced Na^+ uptake. These results are consistent with our previous data on sodium transport in *T. viridis* PM vesicles [2] and show that ATP-linked Na^+ translocation across the PM is mediated by a primary Na^+ pump (Na^+-ATPase) in this organism.

The experiments carried out on the PM vesicles loaded with the pH indicator pyranine demonstrated that Na^+ uptake into the vesicles is accompanied by alkalinization of the vesicle lumen indicating H^+ efflux from the vesicles to the outer medium (Fig. 1B, trace 1). Addition of Na^+ ions to the vesicle suspension initiated H^+ efflux from the vesicle interior. This H^+ efflux coupled with Na^+ uptake obviously reflects the functioning of the Na^+/H^+ antiporter in the vesicular membrane. ATP accelerated H^+ efflux to a great extent, implying the involvement of the ATPase reaction in the H^+ translocation. Na^+ ions were found to be a prerequisite of ATP-dependent lumenal alkalinization. In the absence of Na^+, ATP added to the reaction mixture resulted in a slight H^+ uptake by the vesicles, thus indicating some residual activity of the H^+ pump at pH 7.8 (Fig. 1B, trace 2). Proton-translocating ATPase with optimum activity at pH 6.5–7.0 was demonstrated in *T. viridis* PM earlier [1,12]. Subsequent addition of Na^+ to the suspension turned H^+ flux to the opposite direction and led to lumenal alkalinization so that intravesicular pH reached a much higher value than the initial one observed before ATP addition. In this case, Na^+ ions not only led to dissipation of the ΔpH (acid inside) generated by the H^+ pump, but also initiated operation of the Na^+ pump accompanied by H^+ translocation from the vesicle lumen to the outside.

The protonophore ClCCP stimulated ATP-dependent lumenal alkalinization just as observed for the ATP-dependent Na^+ uptake. Stimulatory effects of ClCCP on ATP-dependent sodium uptake and lumenal alkalinization (Fig. 1A,B) indicate that an overall excess of positive charge inhibiting subsequent transfer of Na^+ ions to the vesicle interior is produced

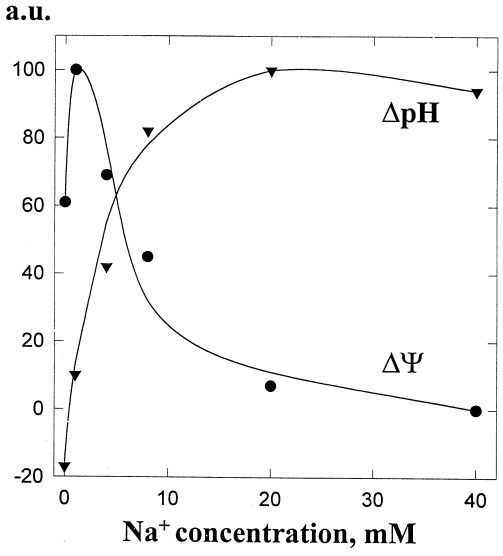

Fig. 3. Effects of increasing Na^+ concentrations on ATP-dependent lumenal alkalinization (ΔpH) and membrane potential generation ($\Delta\Psi$) in the PM vesicles from *T. viridis*.

in the vesicle lumen due to the Na^+-ATPase operation. It may be assumed that ClCCP-facilitated H^+ diffusion out of the vesicle lumen converts electric potential difference (positive inside) into ΔpH (alkali inside) and thus stimulates ATP-dependent Na^+ uptake. To confirm the electrogenic nature of H^+ transfer out of the vesicles, the effect of lipophilic anion TPB^- on vesicle alkalinization was tested. TPB^-, known to induce negative electric potential at the vesicular membrane, inhibited the ATP-dependent lumenal alkalinization when added following ATP or presented initially in the reaction mixture (Fig. 2A, traces 2 and 4). These results suggest that the ATP-dependent lumenal alkalinization is mediated by the $\Delta\Psi$ (positive inside) generated across the vesicle membrane during Na^+ translocation by Na^+-ATPase. It should be noted that TPB^- did not affect Na^+-induced H^+ efflux from the vesicles observed before ATP addition (Fig. 2A, trace 4).

The monitoring of electric potential difference across the vesicle membrane with oxonol VI, a $\Delta\Psi$ indicator, demonstrated that ATP added to the vesicles suspended in the reaction mixture permitting Na^+ uptake resulted in $\Delta\Psi$ generation (positive inside vesicle lumen) (Fig. 1C). ClCCP collapsed $\Delta\Psi$ generated upon ATP addition (Fig. 1C) as well as TPB^- (Fig. 2B, trace 1).

Like ATP-dependent Na^+ uptake by the PM vesicles from *T. viridis* [2], ATP-dependent lumenal alkalinization and $\Delta\Psi$ generation across the vesicle membrane are related to p-type ATPase functioning as indicated by the sensitivity of both processes to orthovanadate. Orthovanadate inhibited lumenal alkalinization (Fig. 2A, trace 1) and $\Delta\Psi$ generation (Fig. 2B, trace 2), although higher concentrations of orthovanadate were necessary than usually used for inhibition of plant PM ATPases [13]. The inhibition of ATP-dependent lumenal alkalinization was observed when orthovanadate was introduced into the reaction mixture in the course of H^+ translocation (Fig. 2A, trace 1) as well as when it was added initially, prior to Na^+ and ATP (Fig. 2A, trace 3).

Fig. 3 shows the effects of various Na^+ concentrations on the ATP-dependent lumenal alkalinization and $\Delta\Psi$ formation. The initial rate and final level of lumenal alkalinization increased as the Na^+ concentration increased. The dependence of the initial rate of lumenal alkalinization on Na^+ concentrations demonstrates a saturable kinetics with an apparent K_m of about 5 mM. This value is appreciated as the sodium concentration at which a semi-maximal rate of intravesicle alkalinization is achieved. Opposite to ATP-dependent lumenal alkalinization, the $\Delta\Psi$ generated upon ATP addition declined as the Na^+ concentration increased. This result suggests the rise in H^+ permeability of the vesicle membrane as Na^+ concentrations increased and, thus, stimulated conversion of $\Delta\Psi$ (positive inside) into ΔpH (alkaline inside). ATP-dependent $\Delta\Psi$ generation observed in the absence of Na^+ in the reaction mixture (Fig. 3) is likely mediated by a residual activity of the H^+ pump.

4. Discussion

The results of the present work demonstrate clearly that (i) Na^+ translocation across *T. viridis* PM by Na^+-ATPase is an electrogenic process, and (ii) H^+ is a counterion for Na^+. Electrogenicity of the Na^+-ATPase from *T. viridis* PM has been demonstrated in the experiments with the $\Delta\Psi$ indicator, oxonol VI, by direct recording of the electric potential difference generated across the vesicle membrane in response to ATP addition in the presence of Na^+ ions. H^+ countertransport from the vesicle lumen to the exterior was detected by recording the ATP-dependent lumenal alkalinization in the experiments with the pH indicator pyranine entrapped in the PM vesicles.

We found that the observed ATP-dependent processes, such as Na^+ uptake, $\Delta\Psi$ generation and lumenal alkalinization, are interrelated and reflect the operation of the electrogenic Na^+-ATPase in *T. viridis* PM. The following facts allowed us to draw such a conclusion:

1. the requirement for Na^+ ions, substrate of the Na^+-ATPase for the ATP-dependent lumenal alkalinization (Fig. 1B, trace 2);
2. the sensitivity of both ATP-dependent $\Delta\Psi$ generation and lumenal alkalinization to orthovanadate (Fig. 2), the inhibitor of the *T. viridis* PM Na^+-ATPase [2,3];
3. the similar kinetics of Na^+ uptake and lumenal alkalinization (Fig. 1A,B);
4. the occurrence of all three processes at the same pH 7.8–8.0.

The abolition of the $\Delta\Psi$ (positive inside) generated across the vesicular membrane upon ATP addition (Fig. 2B) and the inhibition of ATP-dependent lumenal alkalinization (Fig. 2A, traces 2,4) by the lipophilic anion TPB^- not only confirm the electrogenicity of the Na^+-ATPase but also suggest that ATP-dependent H^+ efflux from the vesicle lumen to the outside is a secondary event with respect to the electrogenic Na^+ uptake. The $\Delta\Psi$, positive inside, generated in the course of ATP-dependent Na^+ translocation produces a moving force for H^+ transport to the exterior. TPB^- diminishing the $\Delta\Psi$ across the vesicle membrane abolishes the driving force for H^+ translocation.

The effects of the protonophore ClCCP on the Na^+ and H^+ transport and, accordingly, on the electrogenesis in the PM vesicles from *T. viridis* are in agreement with the effects exerted by TPB^-. ClCCP stimulated ATP-dependent Na^+ uptake by the vesicles (Fig. 1A) and lumenal alkalinization (Fig. 1B), and led to dissipation of the ATP-induced $\Delta\Psi$ generated on the vesicular membrane (Fig. 1C). These findings can also be explained by assuming that the Na^+-ATPase of *T. viridis* is an electrogenic uniporter, and generation of $\Delta\Psi$, positive inside vesicles, is necessary for H^+ efflux to the exterior. A low H^+ permeability of the PM most likely limits the H^+ translocation rate and, hence, the Na^+ influx into vesicles. Addition of ClCCP results in facilitation of H^+ transport and, thereby, induces conversion of $\Delta\Psi$, positive inside, into ΔpH, alkaline inside, thus favoring the operation of the electrogenic Na^+ pump and acceleration of Na^+ uptake.

The dependences of the ATP-induced lumenal alkalinization and $\Delta\Psi$ generation on Na^+ concentration (Fig. 3) show that Na^+ not only is the substrate for the Na^+-ATPase but also appears to be a regulator of H^+ efflux from the vesicles, i.e. Na^+ is a factor controlling H^+ permeability of the vesicle membranes. This conclusion follows from the observed reciprocal correlation between the $\Delta\Psi$ and ΔpH generated during Na^+ pump operation at increasing Na^+ concentration. At a low Na^+ concentration, when the H^+ permeability of PM is low, the $\Delta\bar{\mu}H$ is presented mainly in the form of $\Delta\Psi$. Increasing Na^+ concentrations provide enhancement of H^+ conduc-

tivity, as may be suggested, by opening some specific H^+ channels, thus promoting conversion of $\Delta\Psi$ into ΔpH.

The obtained results do not agree with the model of Na^+/H^+ antiport catalyzed by the Na^+-ATPase directly, but provide strong evidence that this enzyme is an electrogenic uniporter which translocates only Na^+ ions while H^+ movement is electrophoretically driven in response to generation of $\Delta\Psi$ across the vesicle membrane.

Another Na^+-transporting system displayed in the experiments on isolated PM vesicles from *T. viridis* is the Na^+/H^+ antiporter. Addition of Na^+ to the vesicle suspension in the absence of ATP resulted in Na^+ uptake and lumenal alkalinization (Fig. 1A,B). In this case, Na^+-induced lumenal alkalinization was not affected by the lipophilic anion TPB^-, indicating that the Na^+/H^+ antiporter in the PM of *T. viridis* is an electroneutral system exchanging Na^+ and H^+ with a 1:1 stoichiometry.

In conclusion, one point should be commented upon concerning the effects of NO_3^- ions used as a component of the reaction mixtures in our experiments on sodium uptake and lumenal alkalinization. NO_3^- is an anion that generally penetrates easily across biological membranes. It is often used as a charge-compensating anion to abolish $\Delta\Psi$ (positive inside) on the vesicular membranes during the measurements of ATP-driven H^+-pumping activity accomplished with optical pH indicators. Hence, one should expect NO_3^- ions will cause a decay of $\Delta\Psi$ generated on the vesicular membrane from *T. viridis* as well. As a result of dissipation of the membrane potential difference, stimulation of ATP-dependent sodium uptake and inhibition of associated $\Delta\Psi$-driven lumenal alkalinization should be predicted. Indeed, NO_3^- ions stimulated ATP-dependent sodium uptake by the PM vesicles from *T. viridis* [2], but, unexpectedly, did not inhibit the intravesicular alkalinization under investigation. On the contrary, they stimulated the latter process (not shown). Therefore, we suggest that the stimulatory effect of NO_3^- ions on ATP-driven Na^+ uptake by the PM vesicles from *T. viridis* is not related directly to the action of nitrate as a permeant anion but rather is conditioned by increased H^+ conductivity of the membrane in the presence of nitrate and/or direct activation of the Na^+-ATPase by this ion.

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