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$\Delta\psi$-dependent gating of Na^+/H^+ exchange in *Halobacterium halobium*: a $\Delta\tilde{\mu}H^+$-driven Na^+ pump

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Na^+/H^+ antiporter-mediated $^{22}Na^+$ transport was studied in envelope vesicles from *Halobacterium halobium* by manipulating the size of each $\Delta\tilde{\mu}H^+$ component, ΔpH and $\Delta\psi$, in the dark. Neither inside alkaline ΔpH nor outwardly directed ΔpNa^+, nor a combination could facilitate $^{22}Na^+$ extrusion from the vesicles. Likewise, $\Delta\psi$ up to 144 mV (inside negative) was not capable of initiating $^{22}Na^+$ extrusion unless ΔpH existed. This extrusion was facilitated only when approx. 100 mV $\Delta\psi$ (gating potential) was superimposed on ΔpH (either 1 or 2). On the other hand, no uptake of $^{22}Na^+$ took place even when both inside acidic ΔpH and inwardly directed Na^+ gradient were imposed with or without $\Delta\psi$. Under these conditions, monensin mediated the rapid uptake of $^{22}Na^+$. The present results indicate that halobacterial Na^+/H^+ exchange is regulated not only by a $\Delta\psi$-dependent gate but also by a certain mechanism to restrict the back flux of Na^+, this making the antiporter capable of functioning as an efficient $\Delta\tilde{\mu}H^+$-driven pump for Na^+ in a high saline environment.

Na^+/H^+ antiporter; Proton electrochemical-driven Na^+ pump; Membrane potential-dependent gate; (*Halobacterium halobium*)

1. INTRODUCTION

Na^+ plays an important role in bioenergetics in halotolerant or halophilic organisms [1–4], where the electrochemical gradient of Na^+ ($\Delta\tilde{\mu}Na^+$) is established primarily by respiration [1] or ATPase [2]. In *Halobacterium halobium* which lives in an almost saturated saline environment, Na^+ is also essential for physiological reactions such as nutrient uptake [5], but no primary pump for Na^+ has so far been identified in this bacterium, even though bacteriorhodopsin and halorhodopsin function as the primary light-driven pumps for H^+ and Cl^- [7]. From the early studies of Lanyi and MacDonald [8], of Eisenbach et al. [9] and also from our recent studies [10] the H^+-electrochemical potential ($\Delta\tilde{\mu}H^+$) coupled, dicyclohexylcarbodiimide-sensitive Na^+/H^+ antiporter has been found to function as a device for extruding Na^+ from the cell [10a]. From studies on light-induced Na^+ extrusion in envelope vesicles, Lanyi and Silverman [11] proposed that the halobacterial antiporter is electrogenic and gated at the threshold $\Delta\tilde{\mu}H^+$ of approx. -140 mV. Our results [10b], however, indicate that the gating is regulated by membrane potential ($\Delta\psi$) rather than $\Delta\tilde{\mu}H^+$. Since the size of transmembrane pH gradient (ΔpH) and $\Delta\psi$ in the $\Delta\tilde{\mu}H^+$ is difficult to control by steady-state illumination, even with ionophores, we studied the effects of each $\Delta\tilde{\mu}H^+$ component imposed artificially in the dark on the extrusion and uptake of $^{22}Na^+$ in envelope vesicles from *H. halobium*. The results show that the halobacterial Na^+/H^+ exchange system is a $\Delta\tilde{\mu}H^+$-driven secondary pump for Na^+ with a $\Delta\psi$-dependent gate.

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2. MATERIALS AND METHODS

The envelope vesicles of *H. halobium* R_1M_1 (bR+,hR+) were prepared by the freeze-thaw method described [10] and were more than 95% right-side out oriented as determined by the menadione reductase assay [12]. Extrusion or uptake of $^{22}Na^+$ was determined by the filtration method described in [10b] and also briefly in the figure legends.

$^{22}Na^+$ was purchased from CEA, France and monensin was generously given by Dr H. Kobayashi (Chiba University).

3. RESULTS AND DISCUSSION

When the vesicles loaded with 2.9 M K^+ and 0.1 M Na^+ containing $^{22}Na^+$ were diluted 200-fold with 3 M choline-Cl medium at pH 7 in the dark, the passive extrusion of $^{22}Na^+$ was quite slow (~1 nmol/mg protein per min). However, the addition of monensin, an equimolar Na^+/H^+ exchanger, caused rapid extrusion of $^{22}Na^+$ from the vesicles (fig.1). Accordingly, a certain regulatory mechanism may be involved in Na^+ extrusion mediated by an intrinsic Na^+/H^+ antiporter in *H. halobium*. To find which $\Delta\tilde{\mu}H^+$ component is involved in the antiporter activation, $^{22}Na^+$ extrusion from the vesicles was measured by individually modulating the ΔpH or $\Delta\psi$ imposed by the pH jump and valinomycin + K^+. Typical experimental results are given in fig.2. $^{22}Na^+$ extrusion was quite small when pH 6 vesicles were injected into 3 M choline-Cl (pH 6); only ΔpNa^+ was present. $^{22}Na^+$ extrusion was not accelerated significantly even when the pH 6 vesicles were injected into pH 4 medium; i.e., $\Delta pH = 2$ was imposed. Neither was $^{22}Na^+$ extrusion accelerated when pH 7 vesicles were injected into the pH 4 medium to impose $\Delta pH = 3$ (not shown). Thus, neither ΔpH nor ΔpNa^+ nor even a combination is capable of initiating antiporter-mediated Na^+/H^+ exchange in the envelope vesicles of *H. halobium*. This feature of the halobacterial Na^+/H^+ exchange system is quite different from that in other cell types [13] in which the antiporters are driven by either ΔpH or ΔpNa^+.

The effect of $\Delta\psi$ was also examined. At $\Delta pH = 0$, no significant acceleration of $^{22}Na^+$ extrusion occurred even when $\Delta\psi$ at approx. 146 mV (inside negative) was imposed by valinomycin + K^+. This indicates that $\Delta\psi$ alone, like ΔpH, is not sufficient to drive Na^+/H^+ exchange.

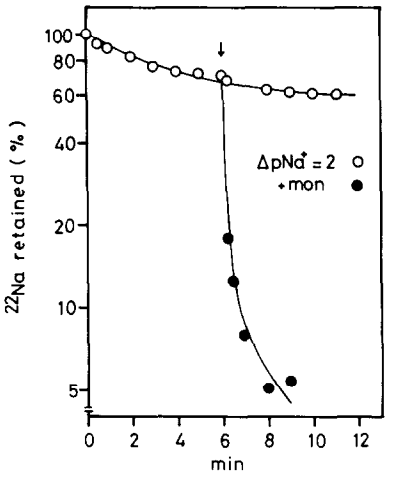

Fig.1. $^{22}Na^+$ extrusion from membrane vesicles of *H. halobium* along the Na^+ gradient. The membrane vesicles were equilibrated in 2.9 M KCl, 0.1 M NaCl and 10 mM Pipes (pH 7) containing ^{22}Na (spec. act. 1.7×10^4 cpm/μmol Na^+) in the dark before use. To initiate the Na^+ efflux from the vesicles, a 100 μl portion of the vesicle suspension (50 mg protein/ml) was diluted with a 200-fold excess salt medium consisting of 3 M choline-Cl, 20 mM KCl, and 10 mM Pipes (pH 7) at 25°C. At a specified time, an aliquot of the reaction solution was taken and filtrated on a nitrocellulose filter (Toyo Roshi, pore size 0.45 μm) under reduced pressure. The radioactivity on the filter was measured by a liquid scintillation counter (Aloka, LSC-700). Monensin was added as an ethanol solution to a final concentration of 1 μM.

However, at ΔpH 2 and $\Delta\psi$ 146 mV (simultaneously), a marked extrusion of $^{22}Na^+$ occurred. The $\Delta\psi$-dependent feature of $^{22}Na^+$ extrusion was more clearly demonstrated when $\Delta\psi$ was superimposed on ΔpH, by adding valinomycin 5 min after vesicle dilution (fig.2). The $^{22}Na^+$ extrusion occurred when the dilution medium was replaced by 3 M NaCl (not shown). Also in the 3 M NaCl medium, no significant decline of $^{22}Na^+$ inside the vesicles was observed with valinomycin addition unless ΔpH was imposed. Thus, under these conditions, Na^+-$^{22}Na^+$ exchange reaction may be quite slow.

Two explanations are possible for the above $^{22}Na^+$ extrusion profiles: (i) neither applied ΔpH

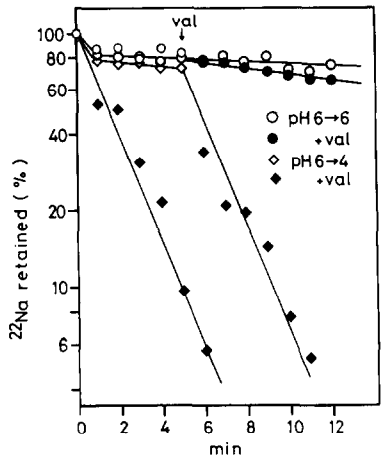

Fig.2. Effects of artificially imposed pH gradient and membrane potential on the $^{22}Na^+$ efflux from the vesicles. A 200 μl portion (10 mg protein) of the membrane vesicles loaded with 2.5 M KCl, 0.5 M ^{22}NaCl (4000 cpm/μmol Na^+) and 20 mM Mes (pH 6) was injected into a 300-fold excess of a dilution buffer consisting of 3 M choline-Cl, 10 mM Mes, Pipes, Hepes, Epps and 50 mM NaCl (ΔpNa = 1). The pH of the dilution buffer was adjusted to 6 and 4 with HCl. Valinomycin was added as an ethanol solution to a final concentration of 1 μM prior to the dilution or at the time indicated (arrow). Conditions: $\Delta pH = 0$, $\Delta\psi = 0$ (○); $\Delta pH = 0$, $\Delta\psi = 146$ mV (inside negative) (●); $\Delta pH = 2$, $\Delta\psi = 0$ (◇); $\Delta pH = 2$, $\Delta\psi = 146$ mV (inside negative) (◆).

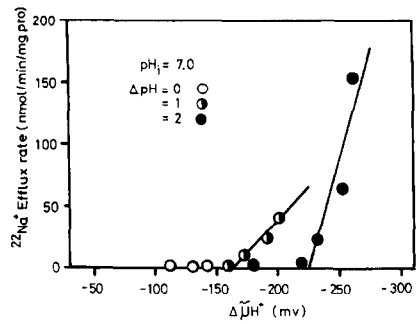

Fig.3. $^{22}Na^+$ efflux as a function of total $\Delta\tilde{\mu}H^+$. Membrane vesicles were equilibrated in 2.5 M KCl, 0.5 M NaCl and 20 mM Good buffers containing ^{22}Na (4000 cpm/μmol Na^+). pH of the vesicles suspension was originally adjusted to pH 7 (○) with Pipes. 200 μl of the vesicle suspension (10 mg protein) were diluted in 3 M choline-Cl buffered with a combination of 10 mM of Mes, Pipes, Hepes and Epps to initiate the reaction. The pH of the dilution solution was adjusted to 7 (○), 6 (◑) and 5 (●), respectively, by NaOH and HCl. The final Na^+ concentration was kept at 50 mM. The magnitude of K^+ diffusion potential was adjusted by substituting choline with KCl in the dilution medium.

nor $\Delta\psi$ provided the significantly large $\Delta\tilde{\mu}H^+$ required for gating of the Na^+/H^+ antiporter; (ii) each component of $\Delta\tilde{\mu}H^+$ contributed independently to the different regulatory steps of the antiporter function. To answer this question, the rate of Na^+ extrusion was determined at different $\Delta\psi$values imposed by changing the K^+ concentration in the dilution medium with a constant ΔpH of 0, 1 or 2. The data plotted as a function of total $\Delta\tilde{\mu}H^+$ applied (fig.3) clearly show that in the absence of ΔpH, no significant extrusion of Na^+ occurs even when increasing $\Delta\psi$ up to 146 mV, a value comparable to the gating potential reported previously by Lanyi and Silverman [11]. However, in the presence of ΔpH of 1 and 2, $^{22}Na^+$ extrusion was initiated at the different $\Delta\tilde{\mu}H^+$ values at approx. 160 and 220 mV, respectively, and the extrusion rates increased with $\Delta\tilde{\mu}H^+$. At these threshold $\Delta\tilde{\mu}H^+$values, the $\Delta\psi$ value superimposed on the preformed ΔpH was approx. 100 mV (inside negative) in both cases. The $^{22}Na^+$ extrusion rate was also varied depending on ΔpH when $\Delta\psi$ was kept constant above the gating voltage. These results strongly indicate that each component of $\Delta\tilde{\mu}H^+$ contributes independently to the different regulatory steps of Na^+/H^+ exchange: the gating of the antiporter is regulated by $\Delta\psi$ and the Na^+/H^+ exchange is driven by $\Delta\tilde{\mu}H^+$ (or ΔpH). This $\Delta\psi$-dependent gating is interesting because light-induced ATP synthesis is also triggered by $\Delta\psi$ of comparable magnitude in living cells [14].

To find whether the $\Delta\tilde{\mu}H^+$ coupled transport of Na^+ is manipulated only by the $\Delta\psi$-dependent gating mechanism, the downhill uptake of $^{22}Na^+$ in the vesicle was studied under conditions of inside-acidic ΔpH and an inwardly directed Na^+ gradient. However, no significant uptake of $^{22}Na^+$ occurred in either case (fig.4). Na^+ uptake did not take place even when additional $\Delta\psi$ (inside negative) was applied to the gating. Under these conditions, monensin facilitated the rapid uptake of $^{22}Na^+$. Thus, Na^+ back flux is highly restricted even when an outwardly directed $\Delta\tilde{\mu}H^+$ is established. This suggests that the halobacterial antiporter preferentially mediates the unidirectional transport of Na^+

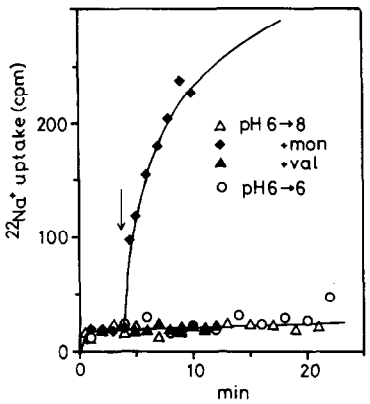

Fig.4. $^{22}Na^+$ uptake in the membrane vesicles imposed on inside acidic ΔpH. 50 μl of the membrane vesicles loaded with 3 M KCl and 20 mM Mes (pH 6) (50 mg protein/ml) were taken into 20 ml of 3 M choline-Cl, 30 mM NaCl buffered with 20 mM Mes (pH 6) or 20 mM Epps (pH 8) containing ^{22}Na (6.48×10^4 cpm/μmol Na^+) to initiate the reaction. At a specified time, 1 ml of the reaction mixture was taken and filtrated for measuring the radioactivity. Final concentrations of valinomycin and monensin were 1 and 0.6 μM, respectively. Conditions: (○) ΔpH = 0; (△) ΔpH = 2 (inside acidic); (▲) ΔpH = 2 (inside acidic) and $\Delta\psi$ = 150 mV (inside negative); (◆) ΔpH = 2 (inside acidic) and monensin.

from the cytoplasmic side to the external medium only when an inside alkaline ΔpH or inwardly directed $\Delta\tilde{\mu}H^+$ is established. Further, not even a marginal Na^+-$^{22}Na^+$ exchange reaction occurred across the vesicle membrane under the above experimental conditions. Thus, a simple channel or ion exchanger may be excluded for the mechanism of Na^+ efflux. A more complicated regulatory mechanism is expected to be involved in directing Na^+ flux. Since *H. halobium* lives in highly saline environment, this kind of mechanism is likely to be essential for the efficient coupling of Na^+ extrusion with $\Delta\tilde{\mu}H^+$ to create and maintain an appropriate $\Delta\tilde{\mu}Na^+$ under extreme conditions. Thus the antiporter may behave as an efficient $\Delta\tilde{\mu}H^+$-driven pump for Na^+.

The present data show halobacterial Na^+/H^+ antiporter to have a quite unique characteristic not possessed by ordinary H^+-coupled antiporters, especially non-halophilic organisms such as *E. coli* [15] or eukaryotic cells [16]. In non-halophiles, many of the processes driven by $\Delta\tilde{\mu}H^+$ are reversible. In the presence of $\Delta\tilde{\mu}H^+$, H^+ moves down its electrochemical gradient and drives the uphill translocation of the substrate. Conversely, under nonenergized conditions, the downhill movement of a substrate along a concentration gradient drives the uphill transport of H^+, to generate $\Delta\tilde{\mu}H^+$, whose polarity is determined by the direction of the substrate concentration gradient. Therefore, ΔpNa^+-driven ΔpH formation or vice versa is routinely used for assaying Na^+/H^+ antiporter activity in these cell types using fluorescent ΔpH probes or a $^{22}Na^+$ tracer [17]. However, this is not the case in *H. halobium*. So far no one has succeeded in showing clearly the existence of Na^+/H^+ antiporter activity in the vesicles from *H. halobium* in the dark by the present approach. The present study may be the first to do so, since it has been demonstrated that not only ΔpH but also $\Delta\psi$ are necessary for the activation of the Na^+/H^+ antiporter in *H. halobium*.

Since hR, originally proposed as a primary light driven Na^+ pump, was reidentified as an electrogenic Cl^- pump [7], it is expected that *H. halobium* may lack a primary Na^+ extrusion system, thus differing from other halotolerant or halophilic bacteria such as Ba1 [4], *V. alginolyticus* [1] and *Streptococcus faecalis* [2]. It is thus reasonable to say that this unidirectional Na^+/H^+ exchange system is a unique pump for the creation and maintenance of $\Delta\tilde{\mu}Na^+$ in the form of ΔpNa^+ and $\Delta\psi$. Both are essential for the regulation and driving of physiological reactions such as amino acid symport [5] and ATP synthesis [14] in an extremely high saline environment. Further, that the halobacterial Na^+/H^+ antiporter acts as a Na^+ pump provides strong support for our previous claim that the primary role of this antiporter is a ΔpH-$\Delta\psi$ (ΔpNa^+) converter that functions as a secondary energy transducer [10]; in a highly saline medium, the chemical component (ΔpNa^+) in $\Delta\tilde{\mu}Na^+$ is not large (usually 1 M NaCl inside vs 4 M outside), thus, the electrical one ($\Delta\psi$) is dominant in the $\Delta\tilde{\mu}H^+$ established by Na^+/H^+ exchange.

The $\Delta\psi$-gating event may represent the movement of a charged group in an electric field or orientation of a molecular dipole. Further study is in progress on the molecular mechanism of activation and regulation of this unique Na^+/H^+ exchange system.

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