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Calcium transport mediated by NhaA, a Na^+/H^+ antiporter from *Escherichia coli*

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In everted membrane vesicles of *E. coli* strain EP432/pGM42, which has only one Na^+/H^+ antiporter (NhaA), external $CaCl_2$ inhibits dissipation of the respiration-dependent ΔpH in response to the addition of NaCl at pH 7.5, and decreases equilibrium concentration of the intravesicular Na^+. In the NhaA proteoliposomes, imposition of an artificial ΔpH (acid inside) leads to the several-fold accumulation of calcium. The apparent K_m for this ΔpH-driven Ca^{2+} uptake at pH 8.5 is 2 mM, and the V_{max} is 1.79 μmol/min/mg of protein. Dissipation of ΔpH causes release of calcium from the vesicles. $CaCl_2$ was found to inhibit the ΔpH-driven Na^+ uptake mediated by reconstituted NhaA, and vice versa. Further, heterological Ca^{2+}/Na^+ exchange has been demonstrated in proteoliposomes containing NhaA. Transmembrane electric potential difference proved to drive this process. All these data are consistent with the assumption that NhaA can also catalyze Ca^{2+}/H^+ exchange.

Na^+/H^+ antiporter; Calcium transport; NhaA; *Escherichia coli*

1. INTRODUCTION

Membranes of *Escherichia coli* possess two Na^+/H^+ antiporters (NhaA and NhaB) playing an important role in homeostasis of intracellular pH and sodium (reviewed in [1–3]). One of them, NhaA, is electrogenic, exchanging two protons per each sodium ion [4]. Li^+, as well as Na^+, is a substrate for NhaA (see [2] for review). In contrast to NhaB, the activity of NhaA is strongly dependent on pH. Using purified protein reconstituted into proteoliposomes, it has been shown that at pH 8, NhaA is approximately 1,000-fold more active than at pH [5]. Recently, His-226 has been identified as a part of the pH sensor on NhaA [6]. Expression of *nhaA* is controlled by NhaR, a regulatory protein which can specifically bind to the promotor region of *nhaA*, increasing its expression [7]. At the physiological level, it has been demonstrated by the monitoring of the β-galactosidase activity of a chromosomal translation fusion of *nhaA'–'lacZ* that the *nhaA* gene may be induced by increased concentration of either Na^+ or Li^+; alkaline pH potentiates the inductory action of both cations [8]. All these biochemical features of NhaA account for its key role in the survival of *E. coli* in the presence of high concentrations of sodium or lithium, which is rather toxic to the cells because of its inhibitory influence upon pyruvate kinase [9].

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Abbreviations: $\Delta\psi$, transmembrane electric potential difference; ΔpH, ΔpNa, and ΔpCa, transmembrane H^+, Na^+, and Ca^{2+} concentration gradients; DTT, dithiothreitol.

In their pioneering study of 1978 [10], Rosen and co-workers identified two distinct systems exchanging protons for Na^+ (or Li^+) and for Ca^{2+} (or Mn^{2+}) in *E. coli*. By measuring changes in the fluorescence of quinacrine to monitor ΔpH across the membrane of everted vesicles, it has been shown that Ca^{2+} (as well as Mn^{2+}) does not prevent dissipation of respiration-dependent ΔpH caused by subsequent addition of Na^+ (or Li^+), and vice versa [10]. Later, applying a direct isotope uptake technique, the same group characterized the Ca^{2+} transport system of *E. coli* in detail [11–14]. In particular, a trypsin-insensitive calcium-phosphate co-transporter was found in everted vesicles in addition to the calcium-proton antiporter [12]. The Ca^{2+}/H^+ exchanging protein is sensitive to digestion with trypsin [12] and requires $\Delta\psi$ for its antiport activity [13]. Determination of kinetic parameters of the ΔpH-driven $^{45}Ca^{2+}$ uptake mediated by the Ca^{2+}/H^+ antiporter in proteoliposomes prepared with octyl glucoside extracts of inner membrane of *E. coli*, yielded a K_m for Ca^{2+} of about 1 mM, and a V_{max} of 170 nmol/mg/min [14]. Sodium or lithium ions do not affect Ca^{2+}/H^+ antiport in *E. coli* and at present no influence of divalent cations upon Na^+/H^+ exchange system of this bacterium has been reported.

In the present work, membrane vesicles isolated from EP432/pGM42 strain of *E. coli* (possessing NhaA as the sole Na^+/H^+ antiporter), as well as proteoliposomes containing purified NhaA, were used to study the effects of divalent cations on the sodium–proton exchange mediated by this protein. Data obtained reveal a new type of NhaA activity, namely, its ability to catalyze Ca^{2+}/H^+ antiport.

2. MATERIALS AND METHODS

The *E. coli* strain EP432 used in this study is K12 *mel*BLid, D*nha*B1, D*nha*A1, D*lac*ZY, thr1 [15]. The LBK growth medium consisted of standard LB medium supplemented with 70 mM KCl. Plasmid pGM42 is a pBR322 derivative bearing *nha*A [16].

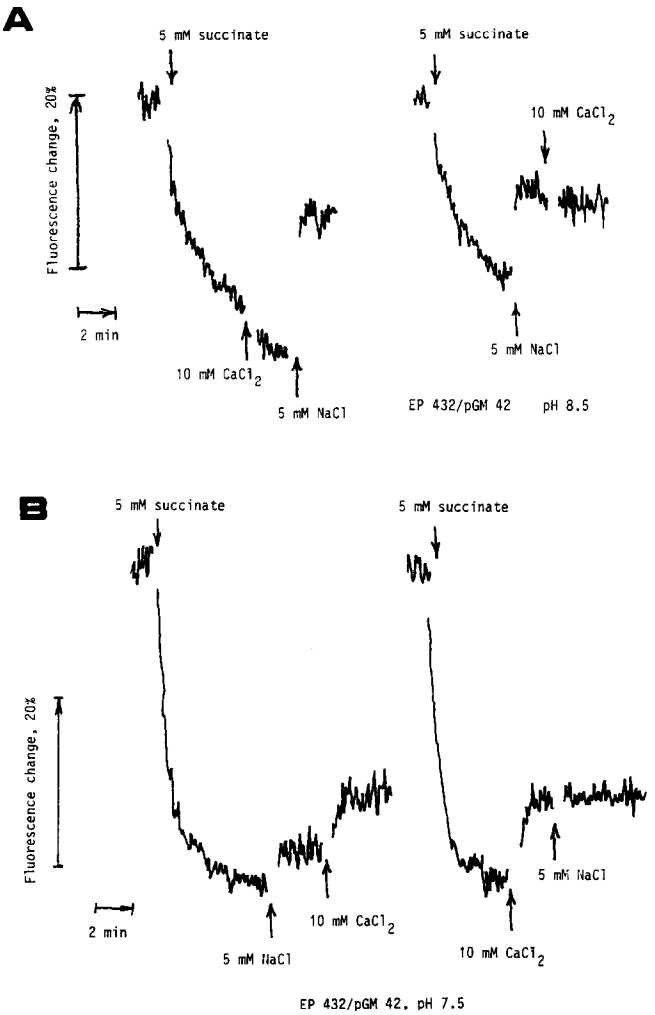
Everted sub-bacterial vesicles were prepared according to the protocol developed by Dr. M. Verkhovskaya (personal communication) with some modifications. 2 l of cells grown in LBK medium to the logarithmic phase (0.8 OD_{600}) were washed and resuspended in 400 ml of buffer containing 0.1 M KCl and 50 mM Tris-HCl, pH 7.5. Then cells were sedimented by centrifugation, resuspended in 40 ml of 0.2 M Tris-HCl (pH 8.0), and diluted twice with 1 M sucrose prepared in the same buffer. EDTA and lysozyme were added to this suspension to 0.5 mM and 400 μg/ml, respectively. After 30 min incubation at 37°C with gentle agitation, the suspension of formed sphaeroplasts was supplemented with 10 mM $MgCl_2$ and sedimented for 10 min at 12,000 × *g* (4°C). The pellet was washed and resuspended in 40 ml of ice-cold loading buffer containing 0.1 M KCl, 0.25 M sucrose, 50 mM Tricine (pH 7.5 or 8.5), 12 mM $MnCl_2$, 10 mM $MgCl_2$, and 0.5 mM DTT. After addition of ATP to 0.5 mM, the suspension was passed twice through French press at 4,000 psi (1 psi = 6.89 kPa) at 4°C. Unbroken cells were sedimented for 15 min at 12,000 × *g*. The supernatant was sedimented for 1 h at 100,000 × *g* 4°C to pellet sub-bacterial vesicles. The final pellet was washed once and resuspended in the same loading buffer at 50 mg of protein per ml, frozen with liquid nitrogen, and stored at −70°C.

ΔpH was estimated from Acridine orange fluorescence in a Perkin-Elmer fluorimeter with exiting light of 420 nm. Emission was measured at 500 nm. The reaction mixture contained, in 2.5 ml: 100–170 μg of membrane protein, 1 μM Acridine orange, 1 mM $MgCl_2$, 0.1 M KCl, 0.25 M sucrose, 50 mM Tricine (pH 7.5 or 8.5), and 5 mM potassium succinate.

In the $^{22}Na^+$ transport experiments, 100 μl of freshly thawed vesicles (5 mg of membrane protein) were diluted twice with buffer containing 0.1 M KCl, 0.1 mM NaCl, 0.25 M sucrose, 50 mM Tricine (pH 7.5 or 8.5), 1 mM $MgCl_2$, and $^{22}NaCl$ (1.0 μCi/ml). Experiments were carried out at room temperature. The process was terminated at appropriate time points by rapid dilution of 5 μl aliquots with 2 ml of the same ice-cold buffer without isotope. After filtration on 0.2 μm filters, probes were washed with an additional 2 ml. Radioactivity retained on the filters was determined on a γ-counter.

Methods for the over-production and purification of NhaA, as well as preparation of the NhaA-containing proteoliposomes by dilution of detergent, were described previously [5]. Proteoliposomes were formed in the reconstitution buffer containing 0.2 M NH_4Cl, 15 mM Tris-HCl (pH 7.5), and 1 mM DTT (NH_4Cl-loaded proteoliposomes), or in the same buffer containing 0.2 M KCl instead of NH_4Cl (KCl-loaded proteoliposomes), or in buffer containing 0.1 M potassium acetate, 10 mM sodium acetate, 15 mM Tris (pH 8.5), and 1 mM DTT (Na–K-loaded proteoliposomes). For the control experiments, 'empty' NH_4Cl-loaded liposomes without protein were prepared.

ΔpH-driven calcium and sodium uptake in the NhaA proteoliposomes was monitored as follows. To produce ammonium-dependent ΔpH on the membrane, 4.5 ml of NH_4Cl-loaded proteo- liposomes containing 170–220 ng of protein was diluted with 0.5 ml of medium consisting of 0.2 M choline chloride, 1 mM $MgCl_2$, 15 mM Tris-HCl (pH 8.5), 0.5 mM $CaCl_2$, and $^{45}CaCl_2$ to 4 μCi/ml (for calcium uptake measurements). When sodium transport was measured, $CaCl_2$ was replaced by 0.1 mM NaCl, and $^{45}CaCl_2$ by $^{22}NaCl$ (1 μCi/ml). The reaction was stopped at different times by the rapid addition of 2 ml of the same ice-cold isotope-free mixture, filtered on 0.2 μm filters, and washed with an additional 2 ml. Radioactivity on the filters was measured with a γ-counter (sodium uptake) or by liquid scintillation



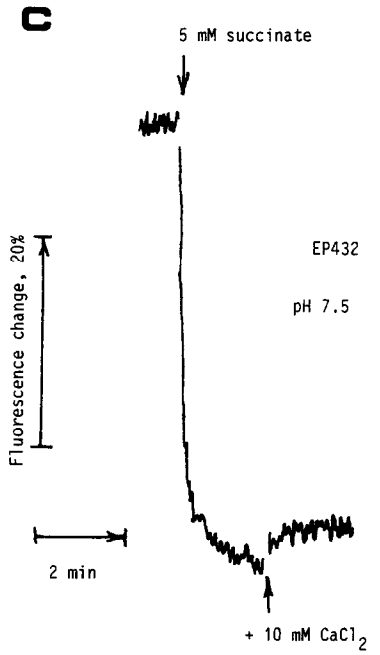


Fig. 1. Ca^{2+}/H^+ and Na^+/H^+ antiport in everted membrane vesicles isolated from EP432/pGM42 (A,B) and EP432 (C) at pH 8.5 (A) or 7.5 (B,C). Vesicles were prepared and assayed as described in section 2. Traces shown start from the steady-state level of fluorescence reached after the addition of 1 μM Acridine orange and 100–170 μg of membrane protein into the experimental medium.

β-counting (calcium uptake). In some of the experiments, choline was replaced by KCl in the experimental mixture. To create nigericin-dependent ΔpH, KCl-loaded proteoliposomes were used, and the choline chloride experimental mixture was supplemented with 1 μM nigericin.

To monitor Ca^{2+}/Na^+ exchange in the NhaA proteoliposomes, 5 μl of Na–K-loaded vesicles (180 ng of protein) were diluted into 0.5 ml of experimental buffer containing 0.1 M choline acetate, 15 mM Tris (pH 8.5), 1 μM valinomycin, 1 mM $MgCl_2$, 0.5 mM $CaCl_2$, and $^{45}CaCl_2$ (2 μCi/ml), and accumulation of radioactive calcium inside the vesicles was registered as described above.

2.1. *Materials*

$^{22}NaCl$ (carrier-free) was from Du Pont-New England Nuclear; $^{45}CaCl_2$ was from Amersham International; 0.2 μm filters were from Schleicher & Schuell. Valinomycin, nigericin, and Ca^{2+} ionophore A23187 ('calcimycin') were from Sigma.

3. RESULTS AND DISCUSSION

Experiments with *E. coli* strain EP432/pGM42, which has only one sodium-proton antiporter (NhaA), revealed inhibitory effects of Ca^{2+} on Na^+/H^+ exchange. Activity was measured by the dissipation of respiration-dependent ΔpH across the membrane of everted vesicles in response to the addition of Na^+ at neutral pH (Fig. 1B). At pH 8.5 this effect was absent (Fig. 1A). For some unknown reason, no ΔpH changes were observed at this pH in response to the addition of as much as 10 mM of $CaCl_2$. Addition of Na^+ did not prevent changes in ΔpH caused by Ca^{2+}/H^+ exchange (Fig. 1B). The latter observation is in accordance with the conclusion of Rosen and his colleagues that the calcium-proton antiporter of *E. coli* does not recognize sodium ion as a substrate [10]. The activity of the sole Ca^{2+}/H^+ antiporter may be visualized in membrane vesicles of parental EP432 strain devoid of both Na^+/H^+ antiporters

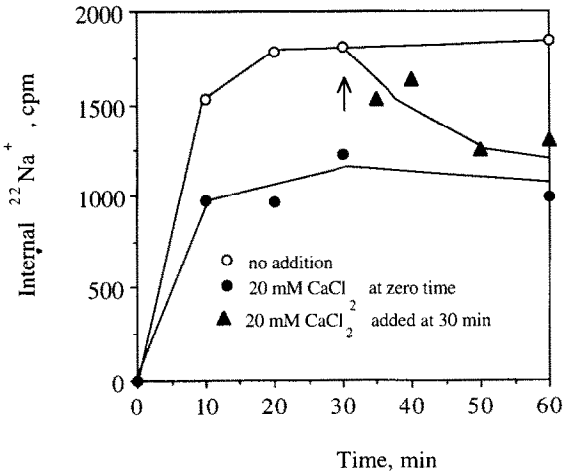
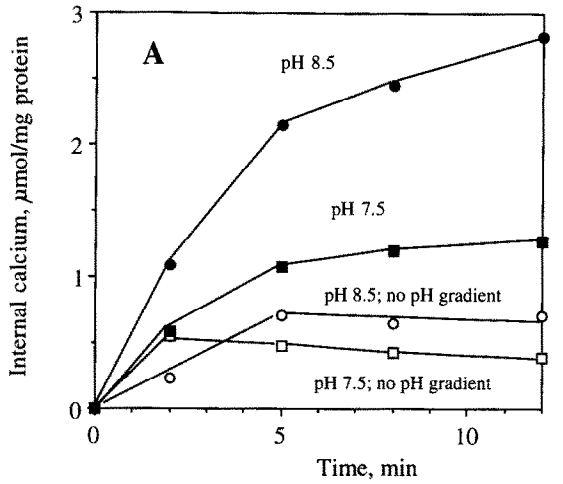


Fig. 2. Effect of external $CaCl_2$ on the passive $^{22}Na^+$ transport in everted membrane vesicles isolated from EP432/pGM42, at pH 7.5. At zero time, radioactive sodium (1.0 μCi/ml; 0.1 mM) was added to the experimental mixture containing 25 mg of membrane protein per ml. At the time point indicated by the arrow, $CaCl_2$ was added up to 20 mM. All experimental points are from duplicates. ○, control (no calcium added); ▲, $CaCl_2$ added at 30 min; ●, $CaCl_2$ added just before zero time.



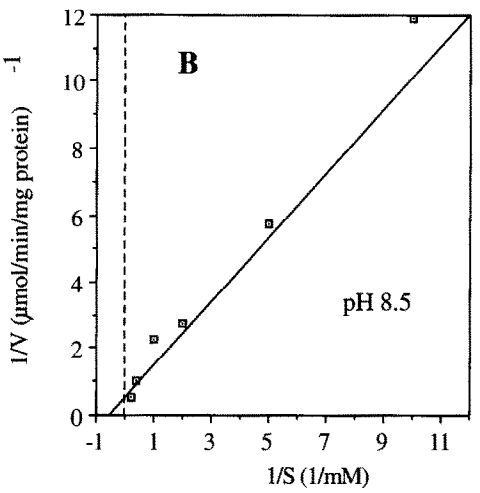


Fig. 3. (A) ΔpH-driven calcium uptake in the NhaA proteoliposomes loaded with NH_4Cl, at pH 7.5 and 8.5, 4.5 μl aliquots of proteoliposomes were diluted into 0.5 ml of choline chloride experimental buffer (filled symbols), pH 7.5 (squares) or 8.5 (circles), and $^{45}Ca^{2+}$ transport was measured. In the control experiment (without applied ΔpH) the corresponding reconstitution buffer supplemented with 0.5 mM $CaCl_2$ and radioactive calcium was used as an experimental medium (open symbols) to evaluate passive calcium transport into vesicles. All points are duplicates. (B) Initial ΔpH-driven uptake rate (5 min) was determined in duplicate at $CaCl_2$ concentrations of 0.1 to 5 mM, pH 8.5. K_m and V_{max} values were extracted from a Lineweaver–Burk regression.

[15], if the experiment is carried out in the absence of P_i (Fig. 1C). In this case, addition of NaCl or LiCl up to 10 mM was necessary before $CaCl_2$ no longer affected the process (data not shown).

Fig. 2 shows the effect of external calcium on passive sodium transport in EP432/pGM42 vesicles at pH 7.5. When added after reaching a plateau, Ca^{2+} caused efflux and re-equilibration to a lower level of intravesicular Na^+. The same equilibrium concentration of sodium

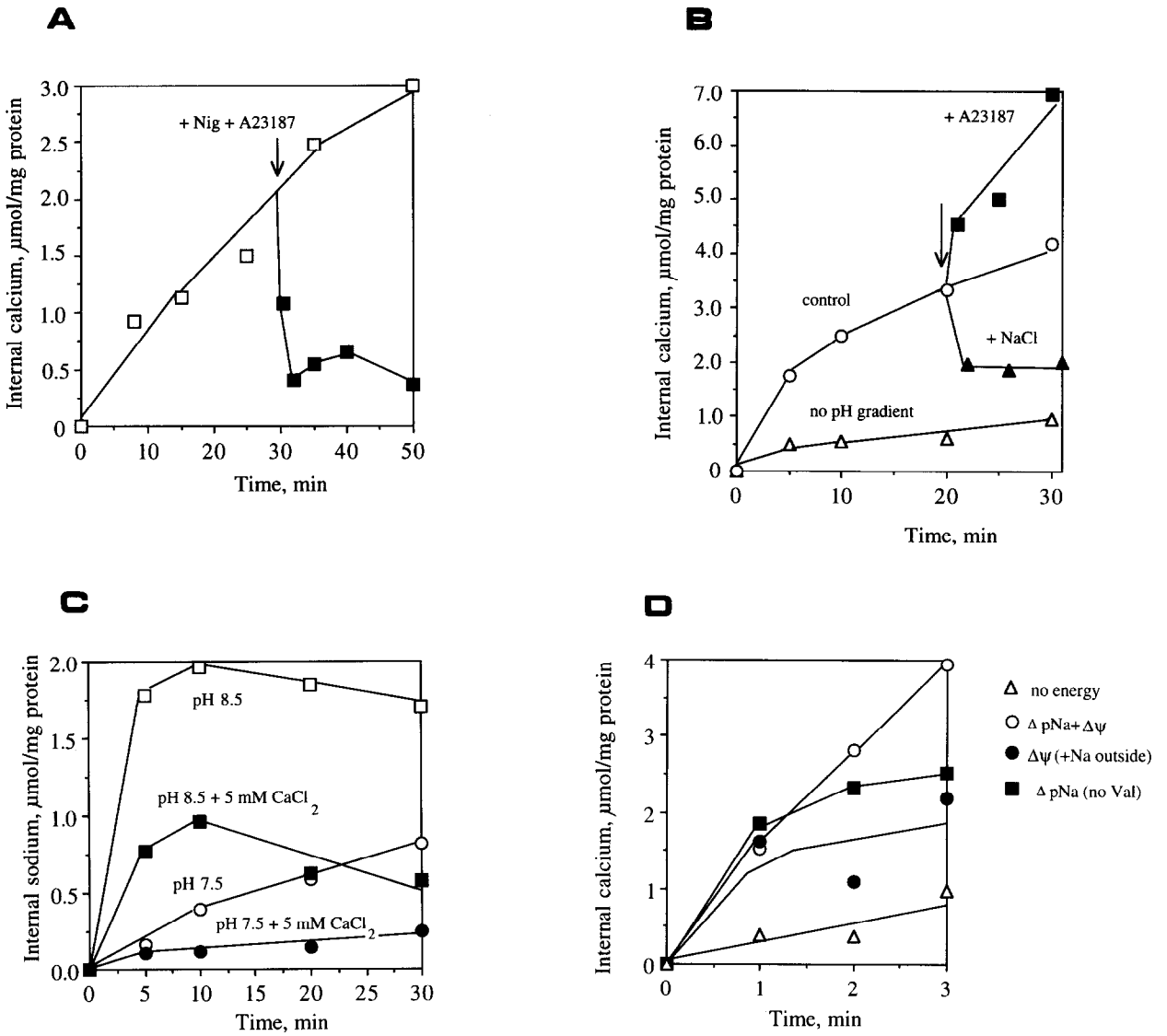


Fig. 4. (A) Combination of an artificial Ca^{2+}/H^+ exchanger A23187 ('calcimycin') and nigericin reverses the ΔpH-driven calcium accumulation into the NhaA proteoliposomes. NH_4Cl-loaded proteoliposomes were diluted with KCl medium (pH 8.5). Final concentrations of ionophores were the following: 1 μM for nigericin, and 10 μM for A23187. Other experimental conditions as in Fig. 3. (B) Effects of external NaCl and A23187 on the ΔpH-driven $^{45}Ca^{2+}$ uptake in NhaA proteoliposomes at pH 8.5. KCl-loaded proteoliposomes were diluted into choline chloride medium supplemented with 0.1 μM nigericin to create ΔpH (acid inside the vesicles). At the time indicated by the arrow, A23187 to 20 μM (■) or NaCl to 10 mM (▲), were added to the experimental mixture. △, passive equilibration with added $^{45}Ca^{2+}$ was monitored in proteoliposomes diluted into the loading buffer. (C) External calcium inhibits the ΔpH-driven sodium uptake in proteoliposomes at pH 7.5 and 8.5. NH_4Cl-loaded proteoliposomes were diluted with choline chloride experimental buffer at pH 7.5 (circles) or pH 8.5 (squares) with (filled symbols) or without (open symbols) 5 mM $CaCl_2$. Sodium uptake was monitored as described in section 2. All points are duplicates. (D) Calcium/sodium exchange mediated by NhaA incorporated into proteoliposomes at pH 8.5. Na-K loaded proteoliposomes were diluted 100-fold with choline acetate experimental buffer in the presence of 1 μM valinomycin and calcium uptake was measured as described in section 2. See the text for further details.

inside the vesicles was observed when calcium was added 10 s before sodium. In accordance with the data of ΔpH measurements (Fig. 1A), the effect of Ca^{2+} on the energy-independent $^{22}Na^+$ influx was practically absent at pH 8.5 (not shown).

Based on the above findings, the ability of purified NhaA reconstituted into proteoliposomes to catalyze ΔpH-driven calcium transport was examined in the next series of experiments. As one can see from Fig. 3A, ammonium-created ΔpH (acid inside the vesicles) leads to several-fold accumulation of Ca^{2+} in the NhaA proteoliposomes compared to its passive influx registered in the absence of a pH gradient, either at pH 7.5 or 8.5. However, both the initial rate and total accumulation of calcium were markedly higher at pH 8.5.

To determine the kinetic parameters of the process, the initial uptake rate was measured at $CaCl_2$ concentrations from 0.1 to 5.0 mM. The apparent K_m for ΔpH-dependent calcium uptake at pH 8.5 is 2 mM, and the V_{max} is 1.79 μmol/min/mg of protein (Fig. 3B).

To demonstrate that the Ca^{2+} uptake is specifically mediated by NhaA, NH_4Cl-loaded liposomes formed without NhaA were assayed in analogous experiments (choline chloride was replaced by KCl in the experimental buffer). No detectable Ca^{2+} transport was observed unless 20 μM of an artificial Ca^{2+}/H^+ exchanger, A23187 ('calcimycin') was added, which caused more than 100-fold accumulation of isotope in 2 min. Subsequent addition of 1 μM nigericin (exchanging intraliposomal protons for external potassium ions) provoked the immediate release of all accumulated Ca^{2+} from the liposomes (data not shown). The latter effect proves that no irreversible Ca^{2+} binding occurs in the liposome interior. A combination of A23187 with nigericin effectively reverses the ΔpH-driven calcium accumulation via NhaA, as well (Fig. 4A). Addition of nigericin and A23187 or nigericin alone before Ca^{2+} prevents the uptake of isotope (not shown). Therefore, the observed accumulation of calcium ion into the NhaA proteoliposomes can be attributed to a ΔpH-driven process. In addition, no irreversible binding of Ca^{2+}, either to NhaA protein itself or to the components of liposomal membrane, has been detected.

Nigericin may also be used to create ΔpH (acid inside) in KCl-loaded proteoliposomes resuspended in potassium-free medium. This ΔpH drives Na^+ accumulation to a steady-state level. In this kind of experiment, A23187 causes an additional uptake of Ca^{2+} (Fig. 4B). External NaCl added at 20 min of transport, evoked the efflux of calcium from the vesicles (Fig. 4B). In a reciprocal manner, $CaCl_2$ depresses the ΔpH-driven Na^+ uptake mediated by reconstituted NhaA (Fig. 4B). This mutual repression of transport against the concentration gradient indicates possible competition between Na^+ and Ca^{2+} for the common cation-binding site of the antiporter. Such competition would be not surprising if we compare the ionic radii, R_i, of both cations (0.95 Å for Na^+, and 0.99 Å for Ca^{2+} [17]). It should be also pointed out here that Mn^{2+} (R_i = 0.80 Å) but not Mg^{2+} (R_i = 0.66 Å) [17]) showed the same effect as Ca^{2+} in experiments with EP432/pGM42 membrane vesicles (data not shown).

Reconstituted NhaA can also catalyze Ca^{2+}/Na^+ exchange (Fig. 4D). Proteoliposomes preloaded with NaCl and KCl were diluted with a buffer devoid of both cations. In some of the experiments carried out in the presence of 1 μM valinomycin, both ΔpNa ($[Na^+]_{in} > [Na^+]_{out}$) and $\Delta\psi$ (positive outside) were the driving forces for the cation exchange (Fig. 4D, open circles). In the absence of a Na^+ gradient, $\Delta\psi$ can drive Ca^{2+} accumulation (Fig. 4D, closed circles). To monitor the process driven solely by ΔpNa, valinomycin was excluded from the buffer (Fig. 4D, closed squares). When neither a Na^+ gradient nor $\Delta\psi$ were generated across the proteoliposomal membrane, very little uptake was detected (Fig. 4D, open triangles). As one can see from Fig. 4D, $\Delta\psi$ stimulated ΔpNa-dependent Ca^{2+} accumulation. Moreover, it can drive uphill calcium transport in the absence of a sodium concentration gradient, as well.

Taken together, the data presented above allow us to conclude that NhaA can exchange calcium ions for protons. Ca^{2+} apparently competes with Na^+ for the common binding site. In the eukaryotic world, sharing of the same exchanger by sodium and calcium has been documented for the electrogenic Na^+/H^+ antiporter from antennal glands of *Homarus americanus* [18]. However, NhaA is the first bacterial Na^+/H^+ antiporter which can also use calcium ion as a substrate. The possible physiological significance of this type of NhaA activity has still to be evaluated.

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