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Biochimica et Biophysica Acta 1273 (1996) 207–216

BBA Biochimica et Biophysica Acta

K^+-dependent Na^+ transport driven by respiration in *Escherichia coli* cells and membrane vesicles

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Received 22 May 1995; revised 6 September 1995; accepted 17 October 1995

Abstract

Respiration-driven Na^+ transport from *Escherichia coli* cells and right-side-out membrane vesicles is strictly dependent on K^+. Cells from an *E. coli* mutant deficient in three major K^+ transport systems were incapable of accumulating K^+ or expelling Na^+ unless valinomycin was added. Membrane vesicles from an *E. coli* mutant from which the genes encoding the two known electrogenic Na^+/nH^+ antiporters *nha*A and *nha*B were deleted transported Na^+ as well as did vesicles from wild-type cells. Quantitative analysis of $\Delta\psi$ and ΔpH showed a high driving force for electrogenic Na^+/nH^+ antiport whether K^+ was present or not, although Na^+ transport occurred only in its presence. These results suggest that an Na^+/nH^+ antiporter is not responsible for the Na^+ transport. Respiration-driven efflux of Na^+ from vesicles was found to be accompanied by primary uphill efflux of K^+. Also, no respiration-dependent efflux of K^+ was observed in the absence of Na^+. Such coupling between Na^+ and K^+ fluxes may be explained by the operation of an Na^+, K^+/H^+ antiporter previously described in *E. coli* membrane vesicles (Verkhovskaya, M.L., Verkhovsky, M.I. and Wikström, M. (1995) FEBS Lett. 363, 46–48). Active Na^+ transport is abolished when $\Delta\overline{\mu}_{H^+}$ is eliminated by a protonophore, but at low concentrations the protonophore actually accelerated Na^+ transport. Such an effect may be expected if the Na^+, K^+/H^+ antiporter normally operates in tight conjunction with respiratory chain complexes, thus exhibiting some phenomenological properties of a primary redox-linked sodium pump.

Keywords: Sodium transport; Potassium transport; Energy transduction; (*E. coli*)

1. Introduction

It has been shown that *E. coli* cells maintain a sodium gradient over the entire physiological pH range [1]. Sodium transport is dependent on respiration but not on ATPase under aerobic conditions [2]. Because active Na^+ efflux from cells was found to be sensitive to protonophoric uncouplers, it was proposed to be a secondary process due to the operation of sodium/proton exchanger [2]. But when cells are suspended in neutral to alkaline medium, ΔpH (alkaline inside under acidic conditions) drops to zero and then becomes reversed [3]. Thus, it cannot be the driving force for Na^+/H^+ exchange. This led to the concept of an electrogenic Na^+/nH^+ antiporter with $n > 1$, driven by negative $\Delta\psi$ [4–7]. Subsequently, two proteins (products of the genes *nha*A and *nha*B) that catalyse electrogenic Na^+/nH^+ antiport were found in *E. coli* membranes (for a review, see [8]). However, several findings, as well as a simple thermodynamic argument (see Discussion) suggest that these may not be the only systems carrying out Na^+ transport in *E. coli*. It has been shown in the reconstituted system [9] that the activity of *nha*A is inhibited by lowering the pH to values normal for the *E. coli* cytoplasm [3]. Moreover, an *E. coli* mutant with deletion of the *nha*A gene and a defective *nha*B gene was

Abbreviations: CCCP, *m*-chlorocarbonyl cyanide phenylhydrazone; DEA, diethanolamine; DiS-C_3-(5), 3,3'-dipropylthiadicarbocyanine iodide; DTT, dithiothreitol; Mops, 4-morpholinepropanesulfonic acid; pyranine, 8-hydroxy-1,3,6-pyrenetrisulfonate; Q1, ubiquinone 1; $\Delta\psi$, transmembrane difference in electric potential; ΔpH, transmembrane chemical gradient of H^+; pH_{in}, internal pH; pH_{out}, external pH; ΔpNa, transmembrane chemical gradient of Na^+; ΔpK, transmembrane chemical gradient of K^+, $\Delta\overline{\mu}_{H^+}$, transmembrane electrochemical proton gradient.

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SSDI 0005-2728(95)00142-5

not capable of transporting Na^+ at alkaline pH, but only at neutral pH [10]. The *nha*B system was shown to be essential for intracellular pH regulation under alkaline conditions [11]. On the other hand, Skulachev and coworkers have reported that a primary redox-driven sodium pump is expressed in *E. coli* grown under alkaline conditions [12,13].

In the present work we have re-examined sodium transport in *E. coli* to find out whether the electrogenic Na^+/nH^+ antiporter is operating in cells and membrane vesicles, or whether the possibility of a primary redox-linked sodium pump should be considered. From our data we conclude that neither of these possibilities is likely, and that the sodium gradient generated by *E. coli* may be explained by the activity of an Na^+, K^+/H^+ exchanger that operates in tight conjunction with the respiratory chain quinol oxidases.

2. Materials and methods

2.1. Strains and growth conditions

The *E. coli* strain GR70N was a gift from Dr. R.B. Gennis, University of Illinois, strain TK2420 (F− *thi rha lac*Z *nag*A *Δ(kdp*ABC′*)5 trk*D1 *Δ(trk*A*))* was a gift from Dr. W. Epstein, University of Chicago, and strain EP432 (*mel*BLid, *Δnha*B1, *Δnha*A1, *Δlac*ZY, *thr*1) was a gift from Dr. E. Padan, Hebrew University, Jerusalem. GR70N cells were grown aerobically overnight at 37°C under strong aeration in moderately alkaline medium (pH 7.6–8.5) containing 50 mM sodium succinate [14]. TK2420 was grown in the same medium, but with potassium succinate, and EP432 was grown in LB medium with the addition of 500 mM KCl. The cells were harvested in the middle of the exponential growth phase.

Because the EP432 mutant has recently been shown to easily produce a second-site suppressor mutation in which the sensitivity to Na^+ is lost [15], the phenotype of EP432 was carefully checked after culturing the cells. A small part of an overnight EP432 culture, from which vesicles were prepared, was inoculated in LB medium with additions of different concentrations of alkali cations. The EP432 strain grew well in 300 mM KCl, but did not grow at all in the first 4 h in the presence of 150 or 300 mM NaCl; addition of 150 mM KCl to the 150 mM NaCl medium stimulated growth. These findings show that the EP432 phenotype was unchanged, i.e., the same as originally reported, but fundamentally different from the phenotype of the second-site suppressor mutant [15].

2.2. Cell treatment

E. coli cells were loaded with Na^+ using a procedure described by Nakamura et al. [16], which consists in repeated incubation of the cells of a solution containing 50 mM DEA (pH 9.3) and 150 mM NaCl (or KCl). During this treatment intracellular cations are replaced by DEA and when the cells are subsequently placed into the medium with desired cation content, exchange between intracellular DEA and alkali cation from the medium occurs [16]. This treatment, despite the drastic changes of the internal milieu, had no serious deleterious effects as judged by the internal ATP level. The measured ATP content in the untreated aerated *E. coli* cells (high K^+ concentration inside) was 6.2 nmol/mg cell protein. During DEA treatment it dropped to 5.5 nmol/mg, perhaps because of less aeration. After the treatment was complete and the cells were loaded with Na^+ the ATP level returned to the initial value of 6.2 nmol/mg protein in conditions of good aeration.

For destabilization of the outer membrane, the cells were suspended in 200 mM Tris-HCl (pH 8) containing 0.5 mM EDTA, and incubated for 10–15 min at room temperature, washed and suspended in a buffer with the appropriate cation content and 5 mM $MgCl_2$ (EDTA/Tris treated cells). After this treatment the wild-type cells retained, as a rule, enough endogenous respiratory substrates to perform fast Na^+ and K^+ transport against their concentration gradients. Mutant cells were partially depleted in endogenous substrates, and an artificial respiratory substrate couple, DTT and ubiquinone-1, was added to facilitate cation transport.

2.3. Measurements of Na^+ and K^+ content

Sodium and potassium inside the cells were determined by filtering the cell suspension (Millipore, 0.45 μm, 50–100 μg protein per sample), fast washing of the cells on the filter with 5 ml 400 mM sucrose solution (2–5 s), and measuring cation content by flame photometry.

2.4. Cytoplasmic pH and membrane potential in cells

The acidification inside the cells was measured by using a fluorescent pH probe 5-(and -6)-carboxy-4′,5′-dimethylfluorescein (CDMF) (λ_{ex} = 490 nm, λ_{em} = 530 nm). For loading the cells, CDMF diacetate (Molecular Probes) was used at 25 μM. EDTA/Tris-treated cells were used because CDMF diacetate apparently could not penetrate the outer membrane. No loading by CDMF could be obtained in intact cells even after incubation for 5–24 h. EDTA/Tris cells were incubated at room temperature for 1–2.5 h, washed once and kept on ice at a protein concentration of 40–50 mg/ml. For measurement of CDMF fluorescence the cells were diluted 500–1000-fold into aerated medium. As the EDTA/Tris-treated cell membrane is permeable to monensin, it was possible to calibrate the fluorescence response of the dye entrapped inside the cell as a function of ΔpH when Na^+-loaded cells were diluted into the medium with varying Na^+ concentrations in the presence of monensin. The experimental points were fitted by the

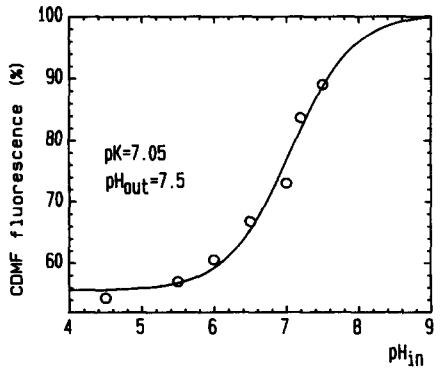

Fig. 1. Calibration curve of fluorescent response of the pH probe, CDMF, entrapped inside *E. coli* cells in dependence on cytoplasmic pH. Experimental points are fitted by theoretical titration curve with pK of CDMF equal to 7.05. Na^+-loaded cells containing CDMF were diluted 1000-fold into the medium with varied Na^+ concentration in the presence of 5 μM monensin to generate ΔpH across the membrane. Medium contained 200 mM choline chloride and 25 mM Mops-choline (pH 7.5). Concentration of cell protein was 40 μg/ml.

theoretical curve around the pK_a of CDMF, which is 7.05 (Fig. 1). The electric potential was measured in EDTA/Tris-treated cells using the tetraphenylphosphonium cation (TPP^+) and a TPP^+-sensitive electrode. The concentration of TPP^+ in the medium was 2 μM; cell protein concentration was 1–4 mg/ml. The membrane potential (negative inside) was calculated from the TPP^+ distribution with an assumed cell volume of 2 μl/mg protein. This value was obtained from cation transport experiments: untreated cells with low intracellular Na^+ were allowed to equilibrate with medium containing 200 mM NaCl in the presence of CCCP and CN^-. After approaching equilibrium, when the amount of intracellular Na^+ remained constant, the internal cell volume was calculated from the amount of measured Na^+ per mg protein on the assumption that the Na^+ concentration inside the cell was 200 mM. The same procedure was used for EDTA/Tris treated cells but monensin was added for faster equilibration, instead of CN^- and CCCP. Correction for probe binding was made by subtracting the amount of TPP^+ taken up by de-energized cells (CN^- and CCCP) from that of energized cells.

2.5. Preparation of membrane vesicles

Right-side-out membrane vesicles were prepared according to Kaback [17]. Lysozyme and sucrose were used at final concentrations of 50–100 μg/ml and 30%, respectively, for preparation of the spheroplasts. The process of spheroplast formation was controlled by the osmotic shock test described by Witholt et al. [18]. Several media were used for vesicle formation: (1) 100 mM Mops adjusted to pH 7.5 with NaOH; (2) 100 mM sodium gluconate, 10 mM Mops-NaOH (pH 7.5); (3) 150 mM Mops adjusted to pH 7.5 with equivalent amounts NaOH and KOH; (4) 25 mM K_2SO_4, 25 mM Na_2SO_4, 10 mM Mops-NaOH, KOH (pH 7.5). The vesicles were finally resuspended in the medium in which they were formed, stored on ice, and used on the day of preparation.

2.6. Alkali cation flux, ΔpH and $\Delta\psi$ in vesicles

For uphill respiration-dependent $Na^+(K^+)$ efflux $Na^+(K^+)$-loaded vesicles (5 μl, 50–80 μg protein) were diluted 10-fold into the same medium in which they had been formed, containing valinomycin or CCCP, where indicated. The reductant couple dithiothreitol (DTT) and Q1 were used as respiratory substrate. The suspension was stirred intensively at room temperature for various periods of time; then it was quickly transferred into 1 ml ice-cold 0.3 M sucrose and filtered immediately (Millipore 0.45 μm). The filter was washed once with 5 ml ice-cold 0.3 M sucrose. Sodium and potassium were extracted from the filter by washing it with IL-test solution containing detergent (Instrumental Lab. Co) and measured by flame photometry.

For ΔpH measurements 200 μM of the pH-sensitive fluorescence probe, pyranine, was added to the medium where the membrane vesicles were formed [19]. Membrane vesicles enclosing pyranine were washed once with medium without pyranine and suspended into this medium. The fluorescence experiments were performed by diluting 3–5 μl of the concentrated suspension of vesicles containing pyranine into an aerated and stirred medium of desired composition (1.6 ml) to a concentration 10–25 μg protein/ml. Pyranine fluorescence was measured at λ_{ex} = 460 nm and λ_{em} = 508 nm; slits 10 nm. Pyranine fluorescence changes as a function of ΔpH were calibrated using an imposed acetate gradient, as described by Damiano et al. [19].

A potassium diffusion potential, or $\Delta\psi$ (negative inside) generated by respiration, were monitored by following the fluorescence quenching of DiS-C_3-(5) [20] (λ_{ex} = 650 nm, λ_{em} = 670 nm). Membrane protein concentration was 10–15 μg/ml; the concentration of DiS-C_3-(5) was 0.25 μM. Fluorescence quenching as a function of $\Delta\psi$ was calibrated using imposed potassium diffusion potentials. The vesicles were loaded with K^+, and then diluted into K^+-free medium supplemented with 0.6 μg/ml valinomycin. Changes in fluorescence upon addition of small aliquots of K_2SO_4 were measured and plotted against the calculated diffusion potential. This calibration was used for determining $\Delta\psi$ created by respiration.

3. Results

3.1. Respiration-dependent active Na^+ transport in E. coli cells and right-side-out vesicles

To create an experimental model for monitoring sodium transport in *E. coli* cells, we loaded the cells with Na^+ at

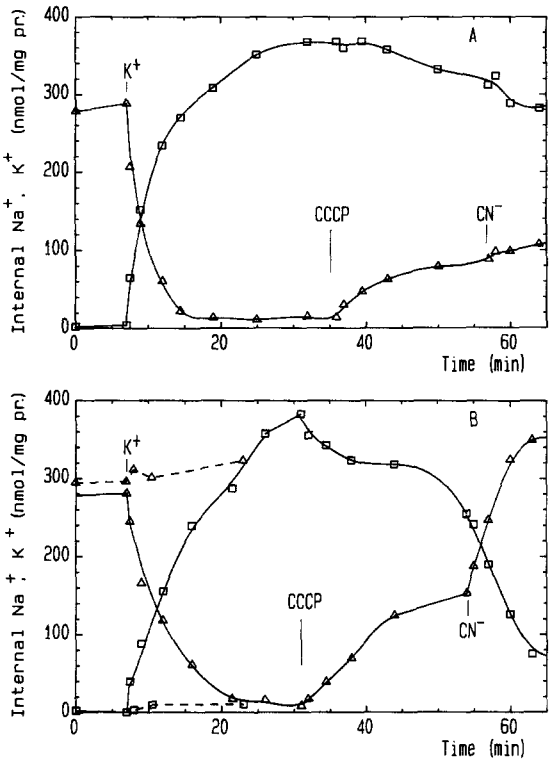

Fig. 2. Uphill sodium and potassium transport in *E. coli* cells loaded with Na^+ at pH 7.3 (A) and 8.5 (B). The cells were suspended in aerated and stirred medium containing 150 mM NaCl and 50 mM Mops-NaOH (a) or Tricine-NaOH (b). Additions: 4 mM KCl, 10 μM CCCP, 5 mM NaCN. Triangles: intracellular Na^+ content. Squares: intracellular K^+ content. Full line: no CCCP at zero time; dashed line: 10 μM CCCP was added at zero time.

high concentration. During the loading procedure the cells lost K^+ almost completely (see Materials and Methods), but retained endogenous respiratory substrates. The sodium concentration inside the cells was similar to that in the medium (170–200 mM). Under these conditions there was no Na^+ efflux driven by respiration, as long as the K^+ concentration in the medium remained low (< 25 μM). Only after addition of potassium did both K^+ and Na^+ transport commence against their concentration gradients, as shown in Fig. 2A and B at a medium pH of 7.3 or 8.5, respectively. Additional experiments showed that, under these conditions, respiring *E. coli* cells were able to create a large sodium gradient ($[Na^+]_{out}/[Na^+]_{in}$ approx. 50), which was independent of pH in the range 7.0–8.8 with respect to both the achieved cation concentration gradients and the initial rates of transport. This differs from the report by Pan and Macnab [1], who found that the $[Na^+]_{out}/[Na^+]_{in}$ decreased at alkaline pH, approaching 5 at pH 8.5. A possible reason for this discrepancy could be that the NMR method [1] requires high cell density, in which conditions it may be difficult to achieve proper aeration. We have found that the Na^+ gradient is very sensitive to the oxygen tension, especially at high pH. Even a small deficiency of oxygen decreases $\Delta p Na$, which is then stabilized at a new lower level (data not shown).

Potassium addition also resulted in a temporary increase of respiration rate. Comparison of the initial rate of Na^+ efflux with the acceleration of respiration by K^+ showed that Na^+ transport occurred with high efficiency, the Na^+/e^- ratio being close to unity.

Some dependence of Na^+ extrusion upon K^+ is expected because of the requirement of electroneutrality of the cytoplasm: under these conditions the total amount of Na^+ transported is too large to be counterbalanced purely by exchange for protons (the concentration of Na^+ in the cytoplasm decreases from 200 mM to less than 4 mM). The buffering capacity of the *E. coli* cytoplasm in the pH range 7–8.5 is close to 100 nmol H^+/pH unit/mg protein [21], i.e., 50 mM H^+/pH unit assuming an internal cell volume of 2 μl/mg protein. Thus, a cation other than H^+ is eventually required to replace Na^+ inside the cells. Such a dependence of Na^+ transport on K^+ has been observed by Borbolla and Rosen [2], who proposed the same explanation. The role of K^+ as a counter-ion is evident, but the possibility that K^+ may be specifically required for sodium transport still remains.

Fig. 2B shows that uphill Na^+ and K^+ transport was completely prevented by the uncoupler CCCP (dashed line). Addition of CCCP after creation of the gradients caused their dissipation, and this was accelerated by cyanide (at alkaline pH; Fig. 2B). Cation concentration gradients in *E. coli* cells were more resistant to CCCP under neutral conditions, and thus a higher concentration of CCCP (up to 20 μM) was required. Such inhibition of Na^+ transport by a protonophore, observed earlier, has been interpreted to mean that Na^+ transport is not a primary redox-linked event, but due to the operation of an electrogenic Na^+/nH^+ antiporter [2].

A closer account of the situation, as well as a quantitative assessment of $\Delta\psi$ and ΔpH changes in *E. coli* cells might be obtained using ionophores, artificial permeable ions and fluorescent pH-indicators, but the outer membrane of intact *E. coli* cells prevents penetration of these compounds. However, treatment of the cells with Tris and EDTA has been shown to destabilize the outer membrane and to increase the permeability for many membrane-active compounds [22]. Hence, EDTA/Tris-treated cells were used in several subsequent experiments, and these could also be loaded with Na^+. As in intact cells, transport of Na^+ was found to start only after addition of K^+ and proceeded in parallel with accumulation of K^+.

As sodium transport was strictly dependent on potassium and the cations fluxes were equal, it was important to check whether there may be direct Na^+/K^+ exchange. Using a mutant strain, TK2420, that lacks the three major K^+ uptake systems (Trk, Kdp and Kup) [23], it could be shown that the mere presence of extracellular potassium was not enough to start sodium transport. As shown in Fig. 3, such respiring cells loaded with Na^+ and K^+ and

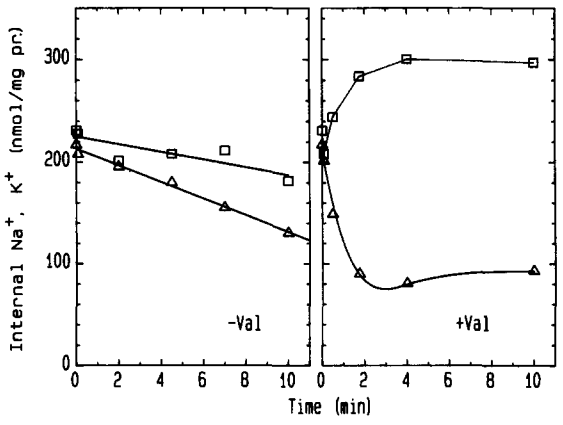

Fig. 3. Dependence of sodium and potassium transport on valinomycin in mutant *E. coli* TK2420 cells deficient in three major K^+ uptake systems. The EDTA/Tris-treated cells were loaded with 100 mM Na^+ and 100 mM K^+ and diluted at zero time into the medium containing 100 mM Na_2SO_4, 1 mM K_2SO_4, 25 mM Mops-NaOH (pH 7.5), 1 mM $MgSO_4$, 2 mM DTT, 50 μM Q1. 2 μg/ml valinomycin was present, when indicated. Triangles: intracellular Na^+ content. Squares: intracellular K^+ content.

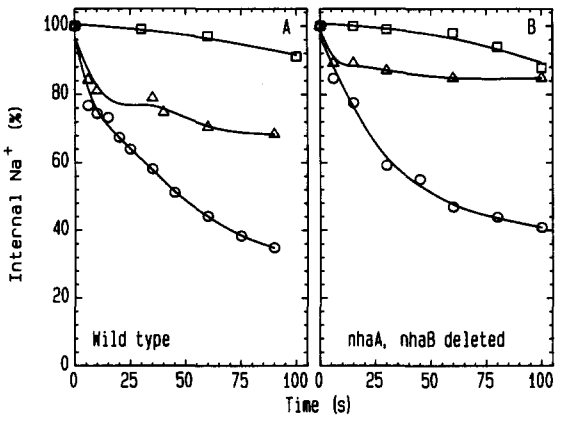

Fig. 4. Respiration-driven uphill Na^+ efflux from membrane vesicles obtained from wild-type and mutant EP432 (*ΔnhaA ΔnhaB*) *E. coli* cells. The vesicles loaded with 100 mM sodium gluconate and 20 mM Mops-NaOH (pH 7.5), were diluted at zero time into the same medium supplemented with 4 mM DTT and 100 μM Q1 and stirred. Squares: no additions. Triangles: in the presence of 4 mM K^+. Circles: in the presence of 4 mM K^+ and 1 μg/ml valinomycin.

diluted at zero time into the medium containing 200 mM Na^+ and 2 mM K^+, neither accumulated K^+ (squares), nor ejected Na^+ (triangles). However, valinomycin caused fast K^+ influx and Na^+ efflux as before (Fig. 3, right panel). Na^+ efflux also occurred upon K^+ addition with valinomycin, when the cells had been loaded with Na^+ alone (not shown). These findings show that there is no direct exchange of Na^+ for K^+ in *E. coli* cells, and suggest that $\Delta\psi$ or ΔpH may have to be changed to start Na^+ transport. However, the artificial membrane permeable cation, TPP^+, which like K^+ decreases $\Delta\psi$ and makes the inside more alkaline, caused only 30% of the K^+-stimulated Na^+ efflux (not shown). However, this does not answer the question of whether K^+ has a unique role in Na^+ transport, because of possible cell damage due to accumulation of the artificial cation.

Na^+ transport in right-side-out membrane vesicles was quite similar to that in whole cells. The vesicles were formed in a medium containing Na^+ and diluted into the same aerated medium with the addition of a respiratory substrate (DTT and Q1, see Material and Methods). Under these conditions there was practically no active sodium efflux (Fig. 4A, squares). Addition of K^+ initiated some insignificant Na^+ transport (Fig. 4A, triangles), apparently due to low activity of the K^+ transport system in vesicles. The Trk system of K^+ transport requires TrkA, a peripheral protein, which is easily removed from the membrane upon cell disruption [24]. On the other hand, the Kdp system is an ATPase (for a review, see [23]), and cannot

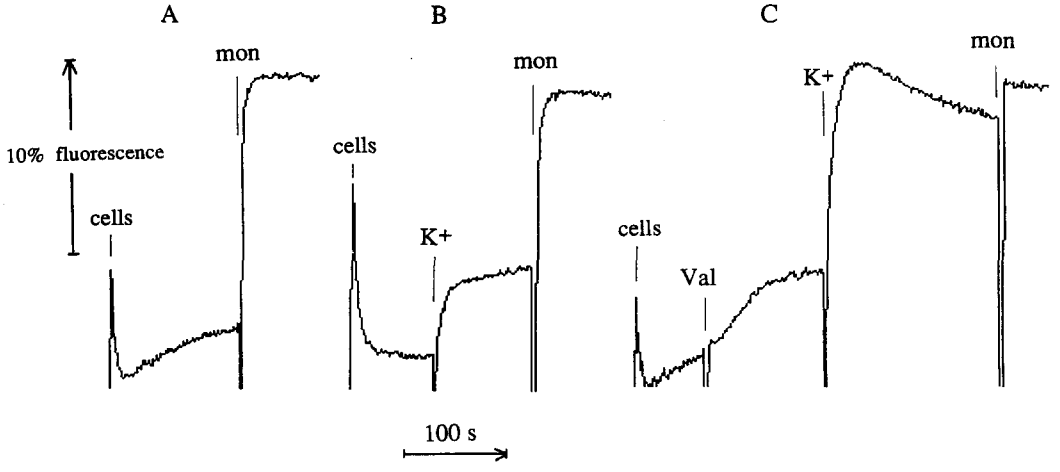

Fig. 5. Changes in cytoplasmic pH in EDTA/Tris-treated cells measured by fluorescence of entrapped CDMF. Na^+-loaded cells were diluted into the medium containing 100 mM Na_2SO_4, 25 mM Mops-NaOH (pH 7.5). 5 μM monensin, 2 mM K^+ and 2 μg/ml valinomycin were added as indicated.

operate in these vesicles due to the absence of ATP. In line with this, valinomycin strongly stimulated sodium efflux (Fig. 4A, circles). As in cells, TPP^+ at the same concentration as K^+ only resulted in approx. 25% of the K^+-stimulated Na^+ transport (not shown).

The *nha*A and *nha*B gene products have been shown to be electrogenic Na^+/nH^+ antiporters [25,26]. To determine whether they are responsible for redox-linked sodium transport we studied right-side-out membrane vesicles from the *E. coli* mutant, EP432, from which the *nha*A and *nha*B genes have been deleted [27]. Surprisingly, active sodium efflux from these vesicles did not differ significantly from wild-type vesicles under the same conditions (Fig. 4). Respiration-driven Na^+ efflux was again insignificant in the absence of K^+ (squares), a small stimulation could be seen after addition of K^+ (triangles), and Na^+ efflux was appreciably enhanced in the presence of valinomycin (circles). Thus, it is clear that the Na^+ transport observed here is not due to the activity of the *nha*A and *nha*B gene products. We could also exclude the possibility that the EP432 mutant cells used here had acquired the second site suppressor mutation described recently by Padan et al. [15] (see Materials and Methods).

3.2. Measurement of ΔpH and $\Delta\psi$ in whole cells and membrane vesicles

Despite the above, it is still possible that another not yet characterized electrogenic Na^+/nH^+ exchanger is functioning, however, because the most evident explanation for the above findings is that K^+ converts $\Delta\psi$ partially into ΔpH (alkaline inside), which drives sodium transport. Alternatively, addition of potassium may lead to dissipation of a pre-existing ΔpH, acid inside, created by an electrogenic antiporter, which could inhibit its function. In order to test these possibilities ΔpH and $\Delta\psi$ were measured in whole cells and membrane vesicles under the conditions of Na^+ transport.

A fluorescent pH-sensitive probe, CDMF, was used to measure pH in the cytoplasm of cells. Fig. 5 shows that respiring Na^+-loaded cells, diluted into the medium containing Na^+ at the same concentration, maintain pH_{in} lower than pH_{out}, because addition of monensin leads to increased CDMF fluorescence corresponding to cytoplasm alkalinization (monensin in the absence of a sodium concentration gradient, and at such a high sodium concentration, should drop any ΔpH to zero) (Fig. 5A). Using our calibration curve (see Materials and Methods), the initial pH_{in} was found to be close to 7.1–7.2 at $pH_{out} = 7.5$, and 7.5–7.6 at $pH_{out} = 8.5$. It is clear, however, that this cytoplasm acidification is not the result of electrogenic Na^+/nH^+ antiporter activity since Na^+-loaded cells suspended in Na^+-containing medium ($\Delta pNa = 0$) maintained a ΔpH which was dependent on pH_{out}: $\Delta pH \cong 1$ at $pH_{out} = 8.5$, $\Delta pH \cong 0.3$–0.4 at $pH_{out} = 7.5$, and $\Delta pH \cong 0$ at $pH_{out} = 6.7$ (not shown). The cytoplasmic acidification is rather due to a scalar metabolic process. This is consistent with measurements of respiration-dependent acidification of the cell suspension, as monitored by a pH electrode under the same conditions. Respiring Na^+-loaded cells acidified the medium at a rate that was not decreased but rather increased by CCCP. This acidification, which was Na^+-independent, is a clear indication of scalar acid production.

Addition of 2 mM K^+, which started Na^+ transport, indeed caused alkalinization of the cell interior, but this effect was small (pH_{in} approached the value of 7.3–7.4 at $pH_{out} = 7.5$ and 7.7–7.8 at $pH_{out} = 8.5$). Most importantly, the cytoplasm still remained more acidic than the medium, as is evident from the further effect of monensin (Fig. 5B). When K^+ was added in the presence of valinomycin, the alkalinization was faster and pH_{in} became 7.5–7.7 at $pH_{out} = 7.5$ (7.9–8.1 at $pH_{out} = 8.5$). However, this small alkalinization was transient, and was followed by slow acidification for several minutes, again making the cytoplasm more acidic than the medium (as shown by monensin addition; Fig. 5C). Notably, sodium transport proceeded during this entire time.

The membrane potential generated by respiration was measured under the same conditions. Addition of K^+ to the cells loaded with Na^+ and suspended in Na^+-containing medium, caused a decrease of $\Delta\psi$ by 20 mV at pH 7.5, and by 25 mV at pH 8.5, which occurred faster but to the same value in the presence of valinomycin (not shown).

As in cells, respiring membrane vesicles maintained a high membrane potential (up to -190 mV) before addition of K^+ (Fig. 6A, curve 1). There was no acidification of the vesicle interior (Fig. 6B, curve 1) that would be expected if electrogenic Na^+/nH^+ antiporters were functioning. Addition of potassium lead to a strong decrease of membrane potential (to less than 40 mV, negative inside; Fig. 6A, curve 2), and to an increase of ΔpH (Fig. 6B, curve 2), which approached a maximal value 0.6 pH units at 30 mM K^+. Therefore, the driving force for an $Na^+/2H^+$ antiporter did not increase on K^+ addition; it actually decreased somewhat while the activity of Na^+ transport changed from zero to maximum rate. Taken together with the ΔpH measurements, these data are difficult to reconcile on the basis of a functional Na^+/nH^+ antiporter.

3.3. Respiration-driven Na^+ efflux is coupled with primary K^+ efflux from membrane vesicles

In the previous experiments we added K^+ into the outer medium to initiate Na^+ transport. However, it seems possible that K^+ may not *only* be a counter-ion, but that it may also have another role, possibly having to be present in the cytoplasm for effective Na^+ transport to occur. Indeed, when the vesicles were loaded with both K^+ and Na^+, and diluted into the same medium containing respiratory substrate, efflux of *both* cations was observed primar-

ily (Fig. 7). Even in the presence of valinomycin, when there should be electrophoretic influx of K^+ driven by $\Delta\psi$, K^+ was nevertheless initially co-transported outwards with Na^+. Subsequently, this was followed by slower influx. If true co-transport occurs, there should be two oppositely directed K^+ fluxes: efflux due to putative Na^+, K^+ symport and influx due to electrophoretic K^+ movement, which could explain our observation. Moreover, there was insignificant efflux of Na^+ in the absence of K^+, and practically no K^+ efflux without Na^+. Thus, the observed uphill Na^+ efflux is clearly coupled to efflux of K^+. Hence, we conclude that it could be catalysed by an Na^+, K^+/H^+ antiporter activity, such as that reported previously for passive Na^+ flux in membrane vesicles [28].

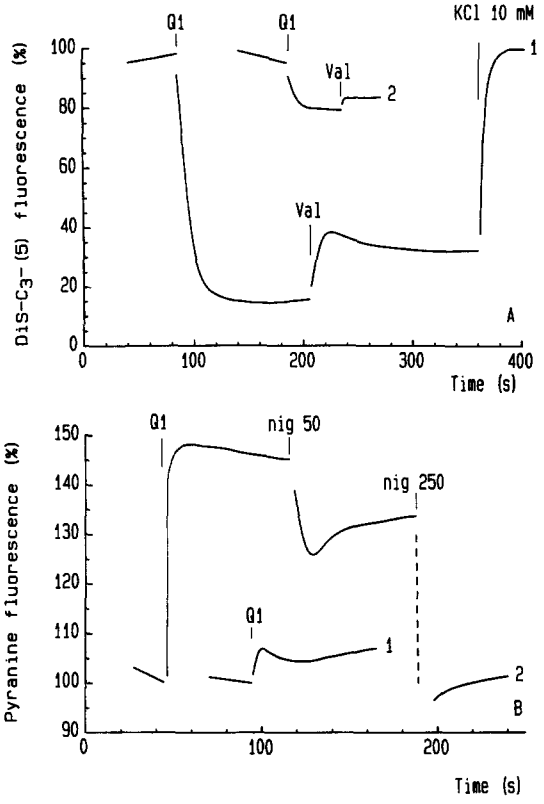

Fig. 6. $\Delta\psi$ and ΔpH generated by respiration in membrane vesicles in the presence and absence of K^+. (A) Negative electric potential was measured using DiS-C_3-(5). The vesicles were loaded with 50 mM Na_2SO_4 and 20 mM Mops-NaOH (pH 7.5), and diluted into the same medium (curve 1) or into medium containing 50 mM K_2SO_4 and 20 mM Mops-KOH (pH 7.5) (curve 2). In both cases the dilution media were supplemented with 2 mM DTT and 0.25 μM DiS-C_3-(5), stirred and aerated. Concentration of membrane protein was 12 μg/ml. Additions: 50 μM Q1 and 0.5 μg/ml valinomycin. (B) ΔpH, alkaline inside, was measured using pyranine. The vesicles loaded with 100 mM sodium gluconate, 20 mM Mops-NaOH (pH 7.5), and 200 μM pyranine were diluted into the same aerated medium without pyranine. When indicated 50 μM Q1 (together with 2 mM DTT), 50 and 250 ng/ml nigerycin were added. Concentration of membrane protein was 15 μg/ml. Curve 1, potassium was not added; curve 2, 27 mM K^+.

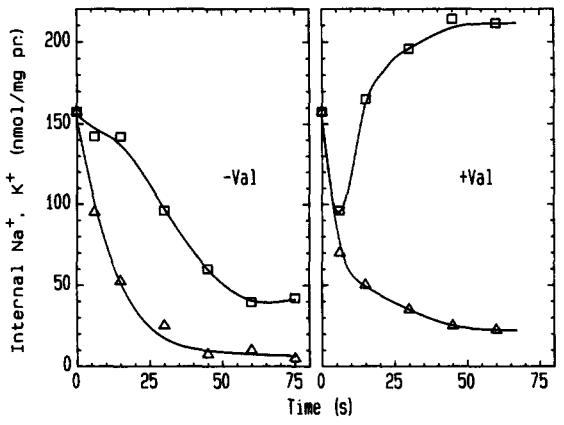

Fig. 7. Respiration-driven uphill efflux of Na^+ and K^+ from membrane vesicles in the absence and presence of valinomycin. The vesicles were loaded with 25 mM Na_2SO_4, 25 mM K_2SO_4 and 20 mM Mops-NaOH, KOH (pH 7.5), and diluted at zero time into the same medium aerated and supplemented with 4 mM DTT and 100 μM Q1. 5 μg/ml valinomycin was added to medium where indicated. Triangles: internal Na^+. Squares: internal K^+.

The same K^+ co-transport phenomenon might indeed occur also in whole cells. The first measurement point in Fig. 3B suggests the presence of a primary K^+ efflux phase here, too, but the time resolution in this case is much less than in the vesicle experiment of Fig. 7B. Moreover, the inward K^+ gradient is much larger in the cell experiment (Fig. 3B) so that electrophoretic K^+ influx probably largely outweighs primary efflux in this case.

The experiments of Fig. 7 raise the question of what the counter-ion might be for the substantial efflux of both Na^+ and K^+. This function could be shared by H^+ and anions. In the net process Na^+, K^+ and anions leave the vesicles, which would shrink as they are large and osmotically active.

3.4. Dependence of respiration-driven Na^+ transport on a protonophoric uncoupler

Active sodium transport in whole cells, which maintain an acid-inside ΔpH under alkaline conditions, can obviously not be explained by an Na^+, K^+/H^+ antiporter, unless its function were somehow tightly coupled with the complexes of respiratory chain. Such an idea is somewhat reminiscent of the functioning of a 'primary sodium pump', which has been suggested to occur in *E. coli* under certain conditions [12,13]. However, as already concluded, the inhibition of Na^+ transport by protonophoric uncouplers contradicts this (Fig. 2B). But since an independent Na^+, K^+/H^+ antiporter also cannot explain our findings, we decided to study the effect of protonophore more carefully by titrating respiration-driven Na^+ efflux over a wide range of CCCP concentrations. Membrane vesicles were loaded with Na^+ and K^+ and diluted into medium with the same Na^+ and a low K^+ concentration. Under these

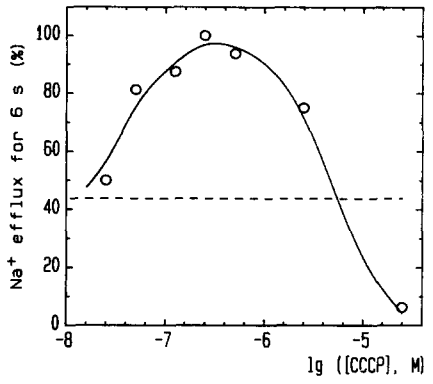

Fig. 8. Dependence of initial rate of respiration-driven uphill Na^+ efflux from membrane vesicles on concentration of CCCP. The vesicles loaded with 25 mM Na_2SO_4, 25 mM K_2SO_4 and 20 mM Mops-NaOH, KOH (pH 7.5), were diluted into medium containing 25 mM Na_2SO_4, 50 mM Mops-choline (pH 7.5), and 25 mM sucrose. The dilution medium was supplemented with 4 mM DTT, 100 mM Q1 and CCCP at different concentrations. Na^+ efflux for first 6 s was measured. Dotted line shows Na^+ efflux in the absence of CCCP.

conditions, where $\Delta\psi$ is generated by respiration, CCCP was found to actually stimulate Na^+ transport more than 2-fold at low concentrations, whereas higher concentrations inhibited transport as before (Fig. 8). Separate experiments showed that CCCP significantly decreased $\Delta\bar{\mu}_{H^+}$ already at concentrations of 50 nM.

4. Discussion

There is a basic problem associated with the widely supported explanation of active redox-linked Na^+ transport in *E. coli* by the functioning of an electrogenic $Na/2H^+$ antiporter [25]. Assuming that respiring cells attain a high membrane potential (say, -180 mV; [1]; this work), the lack of Na^+ transport (without K^+) would mean that the cytoplasmic pH would have to be some three pH units more acidic than the medium, at equilibrium of the antiporter. Since this seems unlikely (see below), an antiporter must instead be mechanistically switched off in the absence of K^+. Since we also found that the artificial membrane-permeable cation TPP^+ could not efficiently replace K^+ in stimulating Na^+ transport, a closer study of the K^+ requirement seemed warranted.

So far, two well-characterized systems, *nha*A and *nha*B, have been been suggested to be responsible for Na^+ transport in *E. coli* (for a review see [8]), and shown to catalyse electrogenic sodium/proton exchange [25,26]. However, we found that respiring vesicles from the mutant EP432, which lacks both *nhaA* and *nhaB* genes, transported Na^+ as well as did vesicles from wild type *E. coli*. This agrees with the data of Ohyama et al. [10] that the *nha*A and *nha*B systems are not required for Na^+ efflux from *E. coli* cells at pH 7.5. In contrast, Padan et al. [15] have recently reported that the EP432 mutant is deficient in Na^+ transport, but that this activity can be regained by a second-site suppressor mutation. However, in our hands the EP432 mutant showed the original EP432 phenotype after culturing (see Materials and Methods). Hence, this second-site mutation could not have occurred in our case. We believe that the reasons for this apparent discrepancy must be sought in the physiological functions of the *nha*A and *nha*B gene products. Deletion of these genes causes failure of the cells to grow in media containing high concentrations of Na^+ in the absence of K^+ ([15]; not shown). It is possible that the *nha* gene products are involved in Na^+-linked pH-regulation of the cytoplasm (cf. Ref. [11]).

An electrogenic Na^+/nH^+ antiporter, other than those encoded by the *nha* genes, could still be responsible for redox-linked Na^+ transport, although reasons for some doubt were already discussed above. A key parameter to test is the driving force for such an exchanger. Our experiments with both cells and membrane vesicles showed clearly, as expected (see above), that the driving force for Na^+/nH^+ exchange was considerable before K^+ addition when no Na^+ transport occurred. Addition of K^+ certainly did not increase this driving force and yet Na^+ transport commenced. We must thus conclude that, if an electrogenic Na^+/nH^+ is operating, it must be switched off kinetically before K^+ addition. How, then, could K^+ provide a signal for switching on the antiporter? K^+ addition causes (i) an alkaline shift of pH_{in}, (ii) a decrease of $\Delta\psi$, and (iii) an increase of the K^+ concentration inside.

Experiments with the K^+ uptake-deficient mutant showed that the presence of potassium in the medium was a necessary but insufficient condition for sodium transport. pH_{in} changes with medium pH, whereas Na^+ transport proceeds at any medium pH in the physiological range. Therefore, the observed small alkaline shift of internal pH could hardly regulate Na^+ transport. Moreover, the changes of pH_{in} due to variations of medium pH are higher than those caused by K^+ addition. The same could be said about the membrane potential: it decreases when medium pH is lowered, and the magnitude of these deviations is higher than upon K^+ addition. The only remaining possibility, in our view, is that the $\Delta\psi$ has a gating effect on the putative antiporter, i.e., it is blocked by high electric potential. However, if this is the case, the antiporter should have an extremely steep threshold (a change of 20–30 mV when $\Delta\psi$ is close to -170 to -200 mV should switch it on). Moreover, such a threshold potential should be higher at high medium pH (-150 mV at 7.5 and -180 mV at 8.5). Finally, the extensive decrease of $\Delta\psi$ by TPP^+ in cells and vesicles did not result in sodium transport comparable to that with K^+. We conclude that gating of an Na^+/nH^+ antiporter by $\Delta\psi$ is an unlikely explanation for the effect of K^+. On the basis of the above, it is clearly difficult to ascribe the Na^+ transport to an electrogenic Na^+/nH^+ antiporter at all, which is obviously in line with

our observation of undiminished transport in the *ΔnhaA*; *ΔnhaB* mutant.

Lombardi et al. [29] showed that the permeable dibenzyldimethylammonium cation did not stimulate active respiration-driven Na^+ efflux from *E. coli* membrane vesicles, while Rb^+ was active. Our finding that K^+ could not be replaced by TPP^+ is in agreement with these results, and we have also found that Rb^+ can replace K^+ (not shown). Thus, the role of K^+ may indeed be unique, and it should be considered whether redox-linked Na^+ transport might be catalysed by the Na^+, K^+/H^+ antiporter previously shown to be responsible for downhill Na^+ efflux in *E. coli* vesicles [28]. The following four findings are in accordance with this proposal: (i) Na^+ efflux is K^+-dependent, (ii) K^+ cannot be completely replaced by TPP^+, (iii) Na^+ transport is blocked by high $\Delta\psi$, (iv) there is primary energy-dependent efflux of K^+ against concentration and electric gradients, but only when vesicles are loaded with Na^+ as well as with K^+.

On the other hand, an independent Na^+, K^+/H^+ antiporter activity cannot explain Na^+ transport in whole cells, because it cannot be driven by $\Delta\psi$, or by ΔpH, which is zero or reversed at pH > 7.5. ΔpK, maintained by respiration, could be the driving force but uphill Na^+ efflux in intact cells does not depend on a potassium concentration gradient.

We are thus faced with a dilemma. On the one hand, our evidence suggests that redox-linked Na^+ transport is not catalysed by an Na^+/nH^+ antiporter as previously thought. On the other, whilst many properties of active Na^+ transport indicate that it could be catalysed by an Na^+, K^+/H^+ antiporter, measurements of $\Delta\psi$ and concentration gradients that could drive such a device fail to support such functioning in *E. coli* cells.

Skulachev and co-workers have proposed that a primary sodium pump is specifically expressed in *E. coli* during growth under low $\Delta\bar{\mu}_{H^+}$ conditions, e.g., alkaline pH [12,13]. We did not find any difference in Na^+ transport in cells grown at neutral or alkaline pH. Moreover, we found that Na^+ transport was completely blocked by 10 μM of the uncoupler CCCP, in agreement with work by others [2,5,6], which contrasts with the expected *stimulation* of a primary sodium pump by protonophores. However, careful titrations of Na^+ transport did reveal significant stimulation at low concentrations of CCCP, concomitant with a decrease in $\Delta\psi$. Hence, we find that Na^+ transport does indeed exhibit some of the expected properties of a primary sodium pump.

A primary sodium pump would imply, in *E. coli*, that one or both of the respiratory quinol oxidases (cytochromes bo_3 and *bd*) would be able to function as redox-linked Na^+ pumps. We have found that redox-linked Na^+ extrusion operates well in *E. coli* mutants from which either the cytochrome bo_3 or the cytochrome *bd* quinol oxidase has been genetically deleted (not shown). Cytochrome bo_3 is a proton pump and member of the large heme-copper oxidase family, whereas cytochrome *bd* is unrelated structurally and does not translocate protons [30]. It is difficult to imagine that *both* could function as primary sodium pumps. However, both enzymes consume the protons required for the reduction of O_2 from the cytoplasmic phase, and both create $\Delta\psi$ albeit with different efficiencies [30].

We thus arrive at a scenario where Na^+ transport shows several properties consistent with being catalysed by an Na^+, K^+/H^+ antiporter, but where the measured bulk phase electrical and concentration gradients cannot explain such transport. On the other hand, Na^+ transport shows some properties of a primary sodium pump, but others that contradict it. As a possible synthesis from these findings we offer the possibility that the Na^+, K^+/H^+ antiporter entity might normally operate in tight linkage with the respiratory cytochrome bo_3 and *bd* complexes, by analogy to the proposed linkage of such complexes with the transhydrogenase in *E. coli* [31] and the F_0 sector of the ATP synthase in extreme alkalophiles [32]. Such coupling may be accomplished if the inward flux of H^+ on the antiporter would be directed to a secluded phase within the redox complex on the cytoplasmic side of the membrane, from which the protons are consumed in O_2 reduction. Protons in this phase would not be in equilibrium with the bulk cytoplasm during turnover. Such a mechanism would give the system some of the properties expected from a primary sodium pump, e.g., its cessation at high $\Delta\psi$ and its stimulation by low concentrations of protonophores. Yet, it would also exhibit properties of an independent Na^+, K^+/H^+ antiporter in other conditions. The electrochemical proton gradient between the extracellular phase and the secluded protonic phase near the redox complex would be the force that drives the antiporter. It is therefore not difficult to envisage how its functioning would be impaired at high protonophore concentrations. Though our proposal is clearly hypothetical, it is a reasonable working model that we hope will stimulate further investigation.

Acknowledgements

This work was supported by grants from the Sigrid Juselius Foundation and the Academy of Finland (M.R.C.). We are grateful to Dr. R.B. Gennis, Dr. E. Padan and Dr. W. Epstein, who kindly supplied us with mutant *E. coli* strains.

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