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The Putative Na^+/H^+ Antiporter of *Vibrio cholerae*, Vc-NhaP2, Mediates the Specific K^+/H^+ Exchange in Vivo[†]

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ABSTRACT: The existence of bacterial K^+/H^+ antiporters that prevent the overaccumulation of potassium in the cytoplasm was predicted by Peter Mitchell almost 50 years ago. The importance of K^+/H^+ antiport for bacterial physiology is widely recognized, but its molecular mechanisms remain underinvestigated. Here, we demonstrate that a putative Na^+/H^+ antiporter, Vc-NhaP2, protects cells of *Vibrio cholerae* growing at pH 6.0 from high concentrations of external K^+. Resistance of *V. cholerae* to Na^+ was found to be independent of Vc-NhaP2. When assayed in inside-out membrane vesicles derived from antiporter-deficient *Escherichia coli*, Vc-NhaP2 catalyzed the electroneutral $K^+(Rb^+)/H^+$ exchange with a pH optimum of ~7.75 with an apparent K_m for K^+ of 1.62 mM. In the absence of K^+, it exhibited Na^+/H^+ antiport, albeit rather weakly. Interestingly, while Vc-NhaP2 cannot exchange Li^+ for protons, elimination of functional Vc-NhaP2 resulted in a significantly higher Li^+ resistance of *V. cholerae* cells growing at pH 6.0, suggesting the possibility of Vc-NhaP2-mediated Li^+/K^+ antiport. The peculiar cation specificity of Vc-NhaP2 and the presence of its two additional paralogues in the same genome make this transporter an attractive model for detailed analysis of the structural determinants of the substrate specificity in alkali cation exchangers.

Potassium is the major monovalent cation of the bacterial cytoplasm. It regulates internal pH, activates many intracellular enzymes, and functions as an important osmotic solute (*1*). However, excessive amounts of internal K^+ are detrimental (*2*−*4*). Therefore, bacteria tightly regulate their cytoplasmic K^+ through the activity of a number of different transport systems (reviewed in ref *1*). Kdp, Trk, and Kup systems import K^+ at the expense of ATP hydrolysis (TrkA and Kdp) or symport it with a proton (Kup) (*5*−*8*). In addition, tetracycline antiporters TetL in *Bacillus subtilis* and TetK in *Staphylococcus aureus* that are able to exchange monovalent cations may contribute to the net K^+ uptake (*9*−*11*). Export of K^+ can be mediated by (a) the glutathione adduct-activated "emergency" KefB/KefC systems of Gram-negative organisms (*12*), (b) mechanosensitive channels under severe hypoosmotic stress (*13*−*15*) [although they are thought to play only a minor role in overall K^+ homeostasis (*1*)], and (c) the MdfA multidrug resistance transporter, which at an external pH of >9.0 imports protons in exchange for extracellular Na^+ or K^+ (*16*).

All the potassium-expelling systems described above seem to be mobilized only in specific stressful situations. Paradoxically, the identity of the system(s) responsible for routine energy-dependent K^+ extrusion remains poorly understood. Almost 50 years ago, Peter Mitchell postulated the existence of "housekeeping" K^+/H^+ and Na^+/H^+ antiporters that can directly use the proton motive force to prevent the dangerous overaccumulation of alkali cations (*17*). Typically, growing bacteria employ a variety of primary proton pumps to maintain a high transmembrane electrical potential difference, $\Delta\psi$ (negative inside), over a wide range of external pH values. As a result, K^+ (or any other monovalent cation), if allowed to equilibrate with the $\Delta\psi$, would accumulate inside the cell at poisonous concentrations. At a $\Delta\psi$ of −120 mV and a moderate external K^+ concentration of 30 mM, at equilibrium the cell would accumulate as much as 3 M K^+, a concentration that clearly is beyond the physiological limit. A K^+/H^+ antiporter would allow H^+ expelled by the primary pumps to return to the cytoplasm in exchange for internal K^+, thus solving the problem.

Although several families of bacterial Na^+/H^+ antiporters have been identified and studied in great detail (*18*−*22*), identification of specific K^+/H^+ antiporters in bacteria remains elusive. K^+/H^+ antiport activity as such was demonstrated in everted membrane vesicles from *Escherichia coli* some time ago (*23*). Some Na^+/H^+ antiporters, exemplified by well-studied Ec-NhaA[1] and Ec-NhaB (*22*), strongly discriminate against K^+, while others exhibit more or less pronounced K^+/H^+ exchange as a concomitant activity, such as the multisubunit Vc-Mrp in *Vibrio cholerae* (*24*) or the alkali-activated Aa-NhaP from

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[1]Abbreviations: Aa-NhaP, Na^+/H^+ antiporter of the NhaP type from *Alkalimonas amylolytica*; BTP, Bis-Tris propane; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; DTT, 1,4-dithiothreitol; Ec-NhaA and Ec-NhaB, bacterial Na^+/H^+ antiporters of the NhaA type and NhaB type from *E. coli*, respectively; ORF, open reading frame; Vc-Mrp, Na^+/H^+ antiporter of the Mrp type from *V. cholerae*; Vc-NhaP2, Na^+/H^+ antiporter of the NhaP2 type from *V. cholerae*; pmf, proton motive force on the membrane; PMSF, phenylmethanesulfonyl fluoride; ΔpH, pH difference across the membrane; ΔpNa, chemical gradient of Na^+ across the membrane; $\Delta\psi$, transmembrane difference of electric potentials.

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Alkalimonas amylolytica that transports Na^+, K^+, and possibly NH_4^+, but not Li^+ (*25*).

Recently, Radchenko and co-workers reported that Vp-NhaP2 from *Vibrio parahemolyticus* might be a K^+-specific antiporter (*4*). If confirmed, this would set a valuable precedent, because in spite of the widely recognized importance of K^+/H^+ antiporters for bacterial ion and pH homeostasis (*1*), no transporter exclusively specific for K^+ has been identified thus far. The authors assayed inside-out vesicles obtained from antiporter-deficient *E. coli* overexpressing the cloned Vp-NhaP2. The antiporter displayed a rather modest activity with K^+ even at its pH optimum of 9.0; in the absence of K^+, Na^+ seemed to be a substrate as well, albeit a poorer substrate than K^+ (see Figure 5B of ref *4*). Unfortunately, the authors did not examine the effect of Na^+ concentration on the Na^+/H^+ antiport activity. Therefore, definitive conclusions about the specificity of Vp-NhaP2 are hard to make at the moment. Also, one more pressing question remained: would the chromosomal deletion of the *nhaP2* gene produce a potassium-sensitive phenotype in its native host, *V. parahemolyticus*?

Inspired by the work of Radchenko and colleagues, we undertook a search for other possible antiporters exclusively transporting K^+. In the course of this search, we cloned, functionally expressed, and examined a homologue of Vp-NhaP2 from *V. cholerae* O395, Vc-NhaP2, encoded by open reading frame VC2703. We also engineered and characterized the Vp-NhaP2 chromosomal deletion mutant of *V. cholerae*. Data presented in this article define Vc-NhaP2 as an electroneutral K^+/H^+ antiporter, which in vitro is able to catalyze K^+/H^+, Rb^+/H^+, Na^+/H^+, and, possibly, Li^+/K^+ (but not Li^+/H^+) exchange but in situ operates as a Mitchellian K^+/H^+ antiporter, protecting *V. cholerae* cells growing at pH 6.0 from high concentrations of K^+. The peculiar behavior of Vc-NhaP2 in relation to the general problem of the search for specific K^+/H^+ antiporters in bacteria is discussed.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Culture Conditions. The Na^+/H^+ antiporter-deficient strain of *E. coli* TO114 [F l IN (*rrnD-rrnE*) *nhaA*::Km^R *nhaB*::Em^R *chaA*::Cm^R] was kindly provided by H. Kobayashi (Faculty of Pharmaceutical Sciences, Chiba University, Chiba, Japan) (*31*). For routine cloning and plasmid construction, DH5α (*supE*44 *hsdR*17 *recA*1 *endA*1, *gyrA*96 *thi*-1 *relA*1) (U.S. Biochemical Corp.) or TOP10 [F- *mcr*A Δ(*mrr-hsd*rms-*mcr*BC) φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*araleu*) 7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G] (Invitrogen) was used as the host. *V. cholerae* strain O395-N1 was used in this study (*26*), which is the classical Ogawa strain with partial deletion of the *ctx*AB operon (O1 classical biotype; Sm^R, Δ*ctxA*1). If not otherwise indicated, TO114 cells were grown aerobically at 37 °C in LBK medium [modified L broth in which NaCl was replaced with KCl (*27*)] supplemented with 100 μg/mL ampicillin, 30 μg/mL kanamycin, 34 μg/mL chloramphenicol, 100 μg/mL erythromycin, and 0.05% (w/v) arabinose. *V. cholerae* cells were grown aerobically at 37 °C in LB supplemented with 100 μg/mL carbenicillin, 100 μg/mL streptomycin, and 0.0002% (w/v) arabinose.

Cloning and Expression of Vc-NhaP2. Sequence data for *V. cholerae* were obtained from the Institute of Genomic Research (http://www.jcvi.org). The putative *Vc-nhaP2* ORF was amplified by high-fidelity polymerase chain reaction (PCR), using chromosomal DNA of *V. cholerae* O395-N1 as a template and directly cloned into the pBAD-TOPO vector (Invitrogen) under the arabinose-induced promoter (P_{BAD}), yielding pVc-NhaP2. The following primers were used for cloning: forward primer VcNhaP2expF, 5′-GAGGAATAATAAGTGGACGC-CGTTACGATTAAC-3′; and reverse primer VcNhaP2expR, 5′-TTACTCCGCGCCTTCTTGTAGCTC-3′. The forward primer was designed to achieve expression of the native enzyme without addition of the N-terminal leader sequence usually introduced by this vector. The primer contains an in-frame stop codon and a translation re-initiation sequence, which consists of a ribosome-binding site and the first GTG of the protein. In the reverse primer, the native stop codon of *Vc-nhaP2* was maintained.

PCR Conditions. Platinum PCR Supermix High Fidelity DNA polymerase (Invitrogen) was used to amplify the 1.75 kb fragment corresponding to *Vc-nhaP2*. A hanging adenine was added via incubation of the DNA in the presence of 1 unit of Taq DNA polymerase (Fermentas). A 5 μL aliquot of the PCR mixture was run on a gel to verify the product size, and the remaining PCR mixture was purified using the QIAquick PCR Purification Kit (Qiagen). The DNA was then introduced into the pBAD-TOPO vector using the manufacturer's protocol (Invitrogen). The ligation mixture was then used to transform TOP10 competent cells, which were then plated onto LB agar plates containing 100 μg/mL ampicillin and incubated overnight at 37 °C. Transformants were screened by PCR for the correct orientation by using a forward primer for the plasmid (pBAD Forward) and the 3′ expression primer for the gene. Transformants with the gene in the correct orientation were grown in LB broth containing 100 μg/mL ampicillin overnight at 37 °C, and plasmid DNA was isolated using the QIAprep Spin Miniprep Kit (Qiagen). The fidelity of the PCR was confirmed by DNA sequencing at the Oregon State University Center for Genome Research and Biocomputing core lab facility. The pVc-NhaP2 construct was then introduced into *E. coli* TO114 by chemical transformation and into *V. cholerae* ΔNhaP2 by electroporation as described in ref *28*.

Chromosomal Deletion of the Vc-nhaP2 Gene. Chromosomal deletion of the *Vc-nhaP2* gene was conducted by homologous recombination. The in-frame deletion construct was made using overlap extension PCR (*29*). A 1 kb fragment upstream of the start codon was amplified from genomic DNA by PCR using the following primer pair: 1, 5′-GGGGGACTAGTGGTTCTG GAGTAGTAACGATCTCCG-3′; and 2, 5′-GACTGACT-GACTGACTGACTGACTCACTCTACCTCCCAGTCTGCG-ATTAACG-3′. A 1 kb fragment downstream of the stop codon was amplified from genomic DNA by PCR using the following primer pair: 3, 5′-AGTCAGTCAGTCAGTCAGTCAGTCT-AACGATCGTTTGCGCCTTGACGTTGAGG-3′; and 4, 5′-GGGGGGAGCTCGGAACGCGCAAGGCGAGCCAGTAC-CG-3′. The 1 kb products of these two PCRs are able to anneal together due to complementary sequences engineered into the primers and were used as a template for a third PCR using primers 1 and 4, resulting in a 2 kb PCR product encompassing 1 kb upstream of the start codon and 1 kb downstream of the stop codon with the gene itself removed. This was cloned into suicide vector pWM91 (*30*) by restriction sites engineered into the primers and introduced into the chromosome of *V. cholerae* O395-N1 following sucrose selection as previously described (*30*). This process results in an in-frame deletion of 580 amino acids. This mutant strain (VcΔP2) along with its isogenic parent (VcWT) and VcΔP2/pVc-NhaP2 overexpressing Vc-NhaP2 in

trans were used to assess the possible physiological role of Vc-NhaP2 in *V. cholerae*.

Analysis of Growth Phenotypes. For growth analysis of *V. cholerae* transformants, LBB medium (noncationic L broth) was supplemented with antibiotics, arabinose (see above), and varying concentrations of KCl, LiCl, or NaCl. The initial pH was adjusted to 6.0, 7.2, and 8.5 by the addition of 60 mM Bis-Tris propane (BTP) hydrochloride. Cells were inoculated at a starting optical density at 600 nm (OD_{600}) of 0.05 into 200 μL of liquid medium placed in 96-deep well plates (Whatman) and grown at 37 °C for 18 h with vigorous aeration. Growth was then measured as the OD of the bacterial suspension at 600 nm by scanning the plates on a Biotek Instruments plate reader using Gen5. All experiments were repeated at least three times in triplicate.

Isolation of Membrane Vesicles and Assays of Antiporter Activity. TO114/pVc-NhaP2 and TO114/pBAD24 transformants were grown in LBK medium supplemented with 100 μg/mL ampicillin, 30 μg/mL kanamycin, 34 μg/mL chloramphenicol, 100 μg/mL erythromycin, and 0.05% arabinose. Cells were harvested at an OD_{600} of 1.5−1.8 and immediately used for isolation of inside-out membrane vesicles essentially as described previously (*24*). Briefly, overnight cultures of TO114 transformants were grown in LBK medium containing the antibiotics listed above. These cultures were then used to inoculate the growth medium at a concentration of 1:100. For ΔpH measurements, after being harvested, the cells were washed three times in buffer containing 140 mM choline chloride, 10% (w/v) glycerol, and 20 mM Tris-HCl (pH 7.5). After the last wash, the bacterial pellet was resuspended in the same buffer containing 1 mM 1,4-dithiothreitol (DTT), 1 μg/mL pepstatin A, 0.1 mM phenylmethanesulfonyl fluoride (PMSF), and ~5 mg/L DNase. The bacterial suspension was then passed twice through a French press (Aminco); the unbroken cells were pelleted at 12000*g* for 10 min at 4 °C, and the supernatant was ultracentrifuged at 184000*g* for 90 min at 4 °C. The resulting membrane pellets were then resuspended and stored in the same buffer containing all the additions except DNase until the assay for cation/proton antiport activity. For $\Delta\psi$ measurements, vesicles were isolated in the buffer described above, but it was made Cl^--free by the elimination of choline chloride and replacement of it with 280 mM sorbitol. Membrane vesicles were isolated, and all experiments were performed at least two times.

Measurement of Transmembrane ΔpH. For ΔpH measurements, aliquots of vesicles (200 μg of protein) were added to 2 mL of buffer containing 140 mM choline chloride, 5 mM $MgCl_2$, 10% (w/v) glycerol, 4 μM acridine orange, and 50 mM BTP-HCl adjusted to the indicated pH. The cation/H^+ antiport activity was then registered using the acridine orange fluorescence quenching/dequenching assay. Respiration-dependent generation of ΔpH was initiated by the addition of 20 mM Tris-D-lactate, and the resulting quenching of acridine orange fluorescence was monitored in a Shimadzu RF-1501 spectrofluorophotometer (excitation at 492 nm and emission at 528 nm). Antiport activity was estimated on the basis of its ability to dissipate the established ΔpH in response to the addition of NaCl, LiCl, or KCl at the indicated concentrations. A concentration of 10 mM was used in the determination of the pH profile of activity, and concentrations of 0.05−100 mM were used in the determination of the half-maximal effective cation concentration (apparent K_m). The antiport activities are expressed as the percent restoration of lactate-induced fluorescence quenching. Each experiment was conducted in duplicate on at least two separate isolations of membrane vesicles. The traces shown in Figure 2 represent typical experimental results.

Measurement of Transmembrane Δψ. The $\Delta\psi$-sensitive dye, Oxonol V, was used to examine whether the Vc-NhaP2-mediated cation/proton antiport has any effect on the respiration-generated formation of $\Delta\psi$. In this case, vesicles were isolated in Cl^--free buffer as described above, resuspended in 2 mL of the same medium supplemented with 5 mM $MgSO_4$ as well as 20 mM diethanolamine (pH 7.5), and preincubated at room temperature for 5 min before the addition of 8.0 μM Oxonol V. Excitation and emission were at 595 and 630 nm, respectively. For some control experiments, vesicles were isolated from TO114 cells expressing Vc-NhaA (*38*). Each experiment was conducted in duplicate on at least two separate isolations of membrane vesicles. The traces shown in Figure 6 represent typical experimental results.

Protein Determination. The protein content in preparations of inside-out membrane vesicles was measured with the DC Protein Assay Kit (Bio-Rad) following the manufacturer's instructions for membrane proteins.

Materials. All chemicals were purchased from Sigma-Aldrich or Fisher Scientific. Restriction endonucleases and DNA-modifying enzymes were purchased from Invitrogen, MBI Fermentas, or New England Biolabs.

RESULTS

Cloning and Expression of Vc-NhaP2. A BLAST search of the *V. cholerae* O395 genome revealed three open reading frames homologous to *Vp-nhaP2*, namely, VC0389, VC0689, and VC2703. Of these paralogues, VC2703 exhibited the highest degree of identity and similarity to *Vp-nhaP2* at the level of translated sequence (76.7 and 83.4%, respectively); therefore, we designated it *Vc-nhaP2*. This putative *Vc-nhaP2* ORF was amplified by PCR and cloned into the pBAD-TOPO (Invitrogen) expression vector as described above. This construct was then introduced into *E. coli* TO114 and into *V. cholerae* O395-N1 after the chromosomal *Vc-nhaP2* gene had been deleted. Gene expression in pBAD-TOPO is under the control of the tightly regulated arabinose promoter (P_{BAD}). Expression of Vc-NhaP2 was induced by the addition of 0.05% arabinose to the growth medium in the case of *E. coli* TO114 during the middle of the log phase and in the case of *V. cholerae* by the addition of 0.0002% arabinose at the beginning of growth. These concentrations of arabinose did not have any toxic effect on the bacterial cells but allowed for adequate expression of Vc-NhaP2 as judged by activity in membrane vesicles and the results obtained from growth experiments performed in *V. cholerae* (see below).

Physiological Role of Vc-NhaP2 in V. cholerae. The VcΔP2 mutant strain of *V. cholerae* bearing the chromosomal *Vc-nhaP2* deletion along with its isogenic parent, VcWT, and the VcΔP2/pVc-NhaP2 strain overexpressing Vc-NhaP2 in trans were used to assess the possible physiological role of Vc-NhaP2 in *V. cholerae*. Each strain was analyzed in LB-based medium (noncationic L broth), containing increasing concentrations of K^+ (Figure 1A), Na^+ (Figure 1B), and Li^+ (Figure 1C) at three different pH values (6.0, 7.2, and 8.5).

The presence of functional Vc-NhaP2 encoded by either chromosomal gene or plasmid-borne *Vc-nhaP2* is critical for the survival of *V. cholerae* at acidic pH in the presence of high

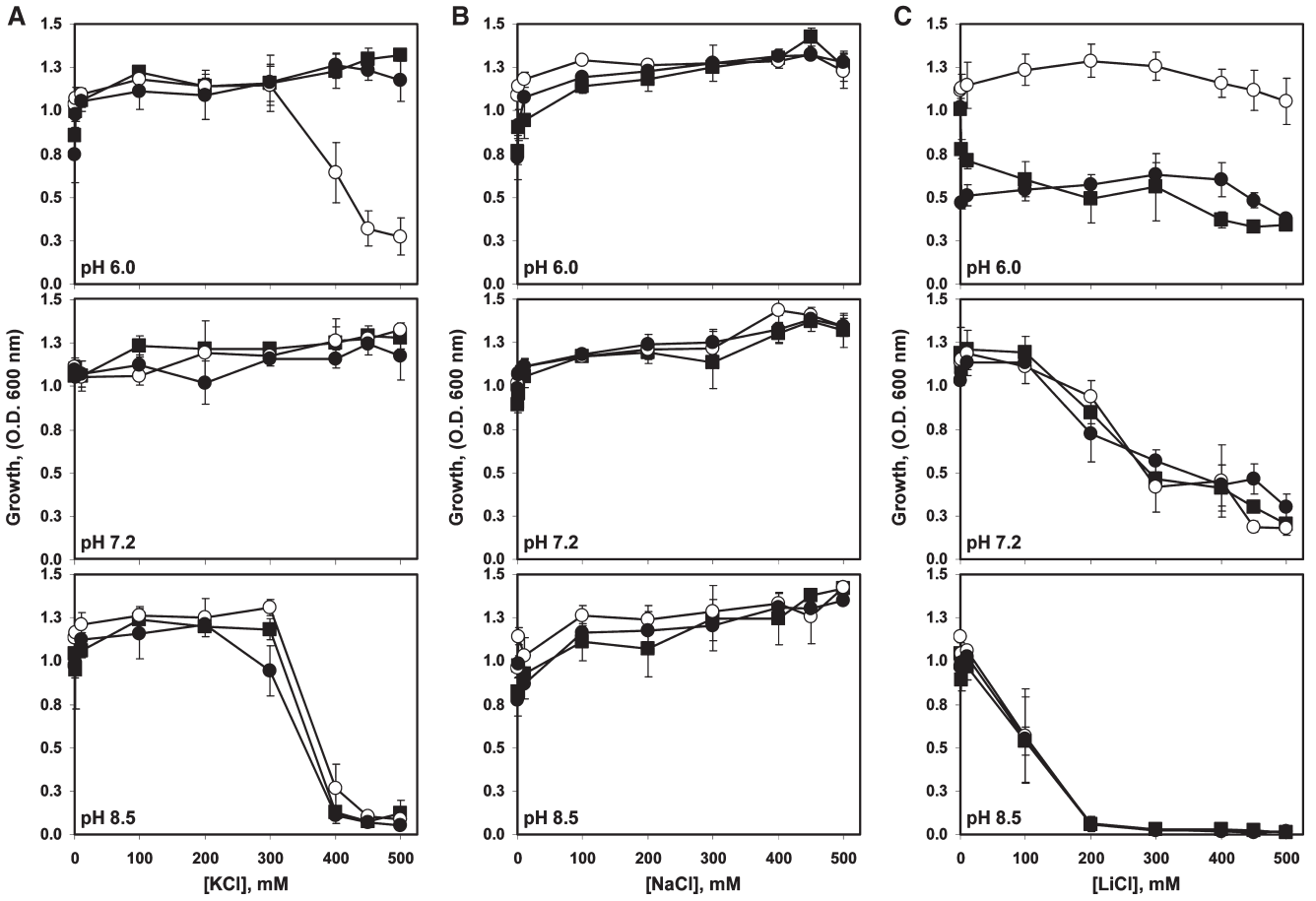

FIGURE 1: Vc-NhaP2 protects *V. cholerae* from high concentrations of external potassium at acidic pH. The pVc-NhaP2 plasmid, containing Vc-NhaP2, was used to transform *V. cholerae* strain VcΔNhaP2, including the deletion of the *Vc-nhaP2* gene (●). Wild-type cells (■) and VcΔNhaP2 (○) were transformed with empty pBAD24. Cells were grown aerobically for 18 h in 96-deep well plates as described in Experimental Procedures. In all cases, the LBB medium, adjusted to the desirable pH, was supplemented with 0.0002% (w/v) arabinose and the indicated concentrations of KCl (A), NaCl (B), or LiCl (C). Growth was measured as the OD_{600} of the bacterial suspension. The starting OD_{600} was approximately 0.05 in all cases. Plotted are the averages of three separate experiments, each performed in tripicate. Bars show the standard deviation.

concentrations of potassium (Figure 1A, top panel). In contrast, deletion of this antiporter does not affect the Na^+ resistance of *V. cholerae* (Figure 1B). This suggests that the actual physiological role of Vc-NhaP2 in *V. cholerae* is to maintain the internal concentration of potassium under toxic levels, by expelling cytoplasmic K^+ ions. This suggestion is further supported by the fact that the growth of the VcΔP2 strain is unimpeded in the presence of highly toxic Li^+ (which is a close chemical analogue of Na^+) compared to the wild-type strain and VcΔP2/pVc-NhaP2 at any pH tested (Figure 1C). Actually, at pH 6.0, deletion of Vc-NhaP2 had a rather beneficial effect on growth (Figure 1C, top panel).

Ion Specificity of Vc-NhaP2. To measure the activity of Vc-NhaP2 directly, we expressed it in trans in cells of the antiport-deficient strain of *E. coli*, TO114. Membranes of this triple deletion mutant are devoid of both specific Na^+/H^+ antiporters, Ec-NhaA and Ec-NhaB, and $Na^+(Ca^{2+},K^+)/H^+$ antiporter, Ec-ChaA (*31*, *32*). Consequently, the inside-out membrane vesicles derived from this strain display practically no Na^+/H^+ or Li^+/H^+ antiport activity at pH 6.0−7.75, and only at pH ≥8.0 is a minor background $Na^+(Li^+)/H^+$ exchange detectable (*4*). Importantly, the same seems to be true about K^+/H^+ exchange in TO114 vesicles, as well (*4*), which makes TO114 the host of choice for the heterologous expression and analysis of cation/proton antiporters. We therefore employed inside-out membrane vesicles prepared from TO114/pVc-NhaP2 and TO114/pBAD24 ("empty") cells. The cation/H^+ antiport activities were detected by the standard acridine orange fluorescence dequenching technique and expressed as the percent restoration of lactate-induced fluorescence quenching. The background activity mentioned above was measured at every pH tested in separate control experiments and subtracted from the levels obtained in Vc-NhaP2-containing vesicles to yield the data plotted in Figures 3−6. However, it must be noted that this activity was below 2% at pH 7.5 (where most of our measurements were taken), and even at pH 9.5 it did not exceed 8% when it was initiated by the standard addition of 10 mM substrate cation.

In accord with the growth data presented in Figure 1, it turned out that K^+ (Figure 2, trace a) and its analogue, Rb^+ (Figure 2, trace c), are preferable substrates of the Vc-NhaP2-mediated cation/proton exchange. In the absence of potassium, Vc-NhaP2 could also exchange Na^+ (Figure 2, trace b), but the overall antiport was much weaker (compare traces a and b in Figure 2). Noticeably, addition of 10 mM Li^+ failed to initiate H^+ translocation in the membranes of TO114 cells (Figure 2, trace d), and even at concentrations as high as 50−100 mM, Li^+ could not be exchanged with a proton (data not shown).

pH Profile of Vc-NhaP2 Activity. When assayed in inside-out membrane vesicles by the addition of 10 mM alkali cations, Vc-NhaP2 demonstrated a bell-shaped pH profile of K^+/H^+ antiport activity with a maximum at pH 7.5−7.75 [Figure 3 (■)].

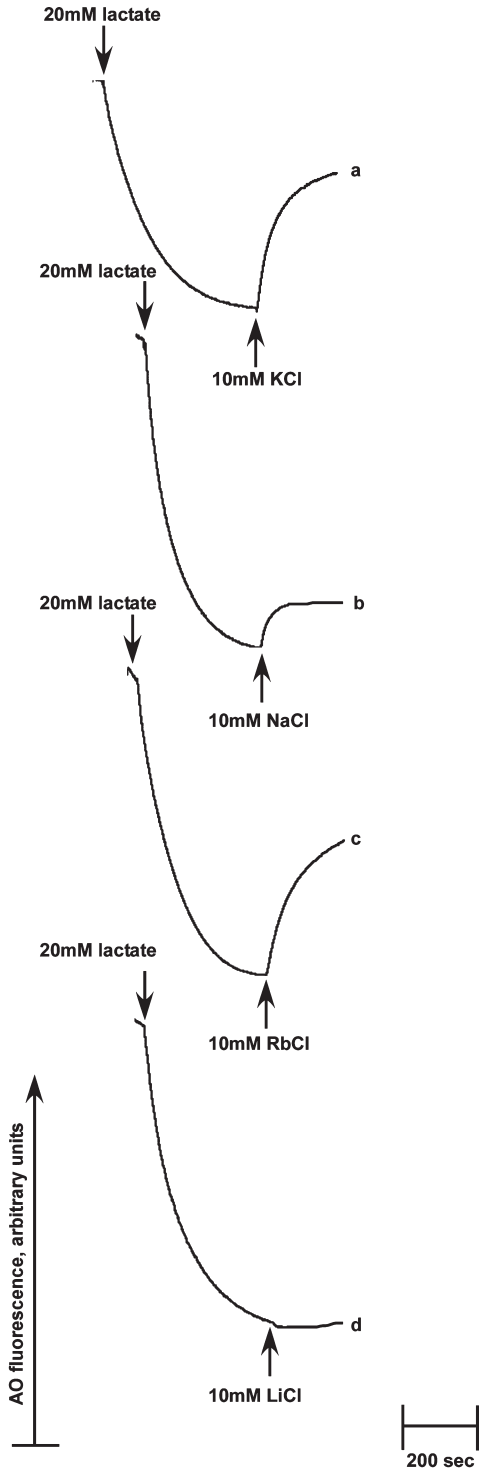

FIGURE 2: Vc-NhaP2 antiport activities in sub-bacterial vesicles. Inside-out membrane vesicles were isolated from TO114 cells transformed with pVc-NhaP2 and assayed with the specified salt at 10 mM in standard choline chloride buffer adjusted to pH 7.5. At the indicated time, respiration-dependent formation of the transmembrane pH gradient was initiated by the addition of 20 mM Tris-D-lactate. After the steady-state ΔpH had been reached, cation/H^+ antiporter activity was detected upon addition of 10 mM KCl (a), 10 mM NaCl (b), 10 mM RbCl (c), and 10 mM LiCl (d). Acridine orange fluorescence is shown in arbitrary units.

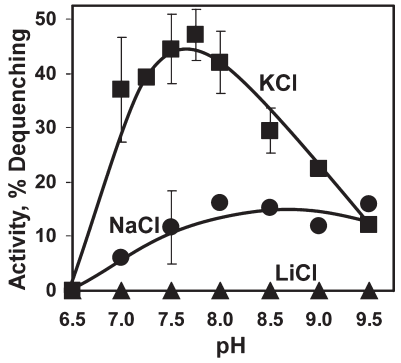

FIGURE 3: pH profile of Vc-NhaP2 activity. Inside-out membrane vesicles were isolated from TO114 cells transformed with pVc-NhaP2 or empty pBAD24 and assayed with the specified salt in standard choline chloride buffer adjusted to the indicated pH with 50 mM BTP-HCl. In each case, residual nonspecific activity measured in empty vesicles was subtracted from that registered in Vc-NhaP2-containing vesicles, and the resulting Vc-NhaP2-dependent activity was plotted as a function of pH. All other conditions were as described in the legend of Figure 2. Plotted are the averages of six measurements (taken in duplicate with three separate isolations of vesicles).

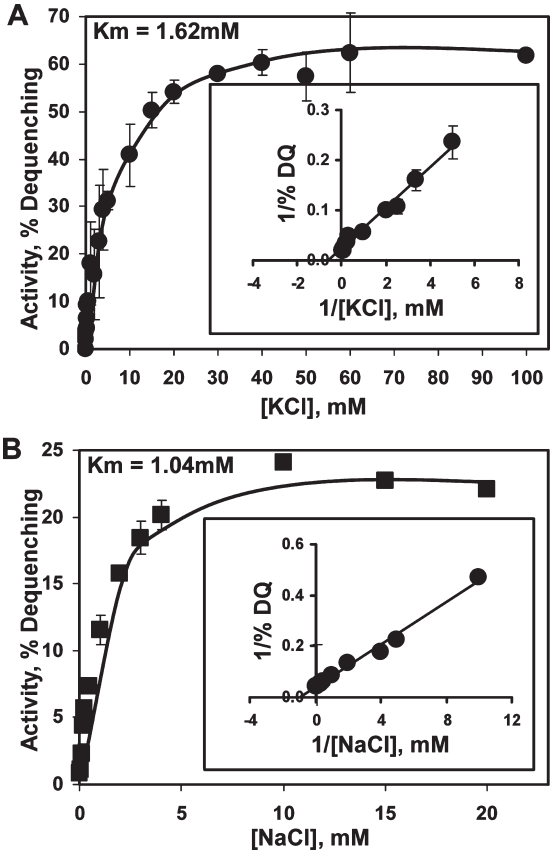

FIGURE 4: Determination of kinetic parameters of Vc-NhaP2 in inside-out membrane vesicles isolated from TO114 transformants (inset, double-reciprocal plot). Measurements were taken in standard choline chloride buffer adjusted to pH 7.5 with the final concentration of added KCl (A) and NaCl (B) varying from 0.05 to 50 mM. Each point represents the average of four measurements (taken in duplicate with two separate isolations of vesicles). Bars show the standard deviation.

At pH 7.75, the activity of Vc-NhaP2 with 10 mM potassium reached almost 50% of dequenching [Figure 3 (■)]. No activity was detected at pH ≤6.5 with any of the probed cations. When probed with 10 mM sodium, the antiporter exhibited a gradual increase of activity up to pH 8.0, reaching a plateau at approximately 15% of dequenching [Figure 3 (●)]. With lithium, Vc-NhaP2 did not exhibit any activity at all the pH values tested [Figure 3 (▲)], even at Li^+ concentrations as high as 50−100 mM

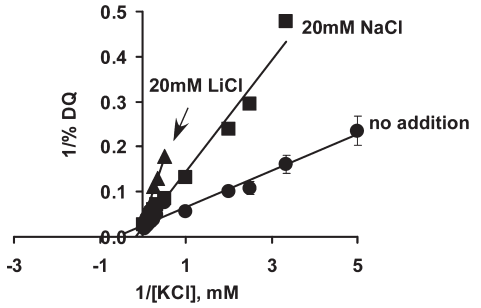

FIGURE 5: Na^+ and Li^+ compete with K^+ ions for the Vc-NhaP2 antiporter. Dequenching of acridine orange in response to varying concentrations of KCl was monitored at pH 7.5 in standard choline chloride buffer with or without the indicated concentrations of NaCl or LiCl. Each point represents the average of four measurements (taken in duplicate with two separate isolations of vesicles). Data are shown in reciprocal coordinates. Bars show the standard deviation.

(not shown). As expected, the behavior of Rb^+ was identical to that of K^+ (data not shown). Thus, K^+/H^+ antiport seems to be a major mode of activity of Vc-NhaP2.

Affinity of Vc-NhaP2 for Alkali Cations. To assess the affinity of Vc-NhaP2 for transported potassium and sodium, we initiated the dequenching response in membrane vesicles isolated from TO114/pVc-NhaP2 by varying the concentration of K^+ or Na^+ at pH 7.5. These measurements yielded the concentrations of K^+ and Na^+ required for the half-maximal response (Figure 4). Although only indirectly related to the actual K_m values of the antiport, these easily assessable parameters are by convention used as a measure of affinity of cation/proton antiporters and termed the apparent K_m for the corresponding substrate (see, for example, refs (*33*−*35*) and references cited therein). For Vc-NhaP2, $[K^+]_{1/2}$ and $[Na^+]_{1/2}$ are 1.62 mM (Figure 4A) and 1.04 mM (Figure 4B), respectively. Therefore, it is not the poor affinity for sodium as such that is responsible for the relative kinetic incompetence of Vc-NhaP2 in Na^+/H^+ antiport compared to K^+/H^+ antiport.

In the next series of experiments, the K^+/H^+ antiport activity was analyzed in the presence of 20 mM NaCl or 20 mM LiCl at pH 7.5 (Figure 5). It turned out that both Na^+ and Li^+ weakened the affinity of Vc-NhaP2 for potassium ions: when 20 mM NaCl was added to the experimental buffer, its K_m for potassium changed from 1.62 to 5.95 mM, and 20 mM LiCl affected the K_m even more, increasing it to 9.00 mM (Figure 5). These competition assays clearly show that Li^+ ions, as well as Na^+, indeed compete with K^+ for binding to the antiporter, affecting its apparent K_m for K^+, despite the fact that Vc-NhaP2, evident from Figures 2 and 3, does not catalyze Li^+/H^+ antiport per se (see Discussion).

Vc-NhaP2 Is Not Electrogenic. The kinetic data presented above do not determine the overall Vc-NhaP2 stoichiometry (i.e., the number of protons exchanged per each alkali cation). The *V. cholerae* growth results, however, indicate that Vc-NhaP2 probably requires ΔpH to extrude K^+. Indeed, Vc-NhaP2 protects the growth from K^+ in acidic (pH 6.0) but not in alkaline (pH 8.5) medium (in Figure 1A, compare the top and bottom panels). This implies that it may catalyze electroneutral exchange of one K^+ per H^+, because while at low external pH values ΔpH is the major component of the proton motive force on the bacterial membrane, at pH 8.5 it is close to zero or even may have an inverted polarity (cytoplasm more acidic than the culture medium) (*36*).

To probe the stoichiometry of Vc-NhaP2, inside-out membrane vesicles were isolated from TO114 transformants and

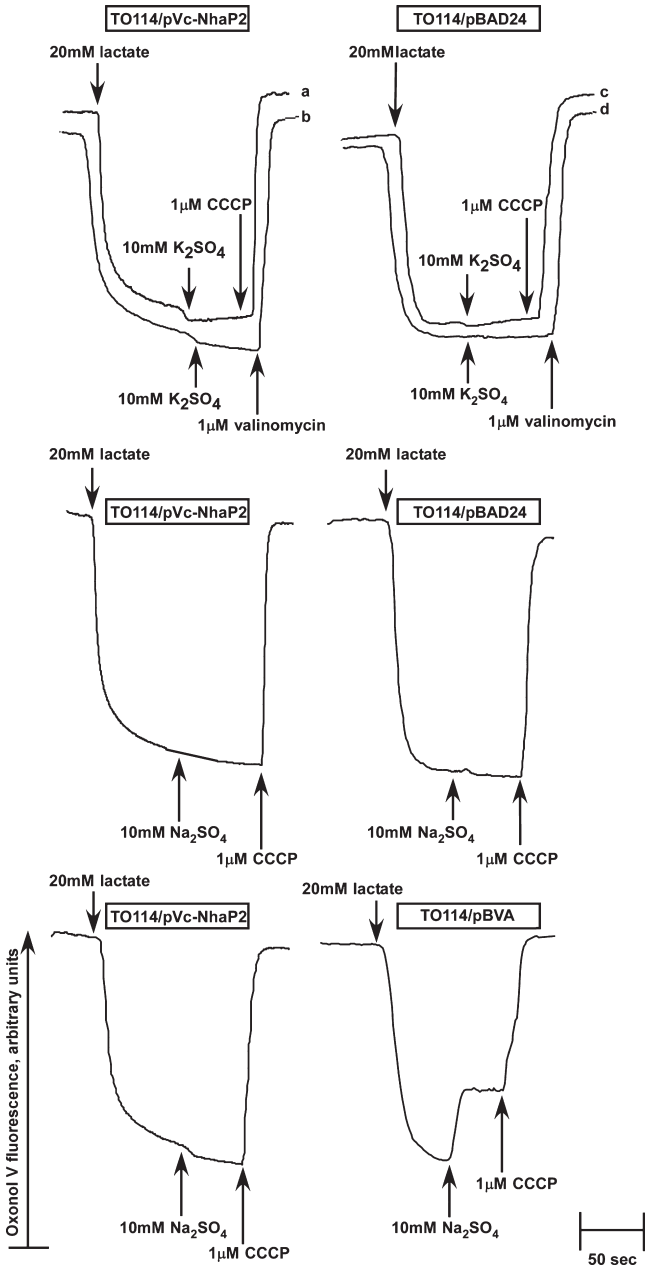

FIGURE 6: Probing the stoichiometry of Vc-NhaP2. Inside-out membrane vesicles were isolated from TO114 transformants and assayed for $\Delta\psi$ at pH 7.5 in sorbitol-based medium devoid of K^+ and Cl^-. Diethanolamine at 20 mM was added to the experimental mixture 5 min prior to the addition of Oxonol V. At the indicated time, respiration-dependent formation of $\Delta\psi$ was initiated by the addition of 20 mM Tris-D-lactate. After the steady-state $\Delta\psi$ had been reached, cation/H^+ antiport was initiated by the addition of 10 mM K_2SO_4 and 10 mM Na_2SO_4 (as indicated). The protonophore CCCP (traces a and c) or valinomycin in the presence of K^+ (traces b and d) was added at the end of each measurement to collapse the generated $\Delta\psi$ for the control. The control experiment shown in the two bottom panels compares the side-by-side behavior of the electrogenic antiporter, Vc-NhaA (bottom right panel, TO114/pBVA vesicles) and Vc-NhaP2 (bottom left panel). The fluorescence of Oxonol V is shown in arbitrary units.

assayed for $\Delta\psi$ in chloride-free, potassium-free (sorbitol-based) buffer. To maximize the respiration-generated $\Delta\psi$, 20 mM diethanolamine was added to the vesicle suspension 5 min prior to addition of the $\Delta\psi$-sensitive dye, Oxonol V. As a control, empty (TO114/pBAD24 without the *Vc-nhaP2* insert) vesicles were isolated and assayed in the same way (Figure 6). Energization

by lactate led to the rapid generation of respiratory $\Delta\psi$ in both empty and TO114/pVc-NhaP2 vesicles (Figure 6), yet the addition of K^+ (Figure 6, top panels) or Na^+ (Figure 6, middle panels) resulted in no depolarization, clearly indicating the electroneutral nature of the cation/H^+ exchange mediated by Vc-NhaP2, i.e., exchanging one alkali cation per proton. Addition of the protonophore CCCP or valinomycin in the presence of potassium completely dissipated the respiratory $\Delta\psi$ (the last addition in each trace of Figure 6). As a positive control in these experiments, vesicles were isolated from TO114/pBVA cells expressing the electrogenic Vc-NhaA, which we cloned and functionally expressed in *E. coli* previously (*38*). Depolarization in response to the addition of its substrate cation differentiates this electrogenic antiporter from Vc-NhaP2 (in Figure 6, compare the two bottom panels). As another positive control, we pretreated empty TO114 vesicles with very high (2.0−5.0 μM) concentrations of nigericin, an artificial ion exchanger which at submicromolar concentrations catalyzes the electroneutral K^+/H^+ antiport but at concentrations exceeding 1.0 μM acts as an electrogenic antiporter (*9*, *37*). As expected, addition of potassium to the nigericin-treated vesicles energized by lactate resulted in considerable depolarization (not shown).

Thus, although the experimental approach used here is a qualitative one, it provides means for reliably distinguishing an electroneutral process from an electrogenic one. Definitively, to measure the stoichiometry of electrogenic antiport, a more refined experimental model of reconstituted proteoliposomes would be required, which has been done for Ec-NhaA (*39*) and Ec-NhaB (*40*).

DISCUSSION

The data presented above define Vc-NhaP2 as a nonelectrogenic antiporter (Figure 6) exchanging internal alkali cations for extracellular protons with clear maximum of activity at neutral pH (Figure 3). Not only K^+ and Rb^+ but also Na^+ ions were found to be the substrates (Figures 2 and 3), binding to the antiporter with similar affinities (Figures 4 and 5), displaying K_m values in the low millimolar range that are typical for bacterial Na^+/H^+ antiporters (*35*, *41*, *42*). Although Li^+ was not a substrate of cation/proton exchange (Figures 2 and 3), Vc-NhaP2 obviously provided the route of entry for toxic Li^+ ions into *V. cholerae* cells growing at pH 6.0: the Vc-NhaP2 deletion mutant exhibited much better Li^+ resistance compared to both its wild-type parent and the mutant expressing Vc-NhaP2 in trans (Figure 1C, top panel). What could account for such apparent discrepancy?

In addition to antiport with proton, Vc-NhaP2 also must be able to catalyze homo-ion (i.e., Na^+/Na^+, K^+/K^+, or Li^+/Li^+) and hetero-ion exchange (i.e., K^+/Na^+, Li^+/Na^+, and Li^+/K^+). When lithium is added to the growth medium, the latter of these processes will inevitably result in Li^+ entry, thus explaining the Vc-NhaP2-dependent Li^+ sensitivity shown in the top panel of Figure 1C. The effect is only evident in acidic medium, where the major Li^+ (Na^+) extruding system, electrogenic antiporter Vc-NhaA, is virtually inactive (J. L. Winogrodzki and P. Dibrov, unpublished observations; also see ref *43*). Higher pH values cause a steep activation of NhaA, so it is not surprising that the presence of Vc-NhaP2 does not affect overall Li^+ sensitivity (Figure 1C, middle and bottom panels). As the external pH increases, the toxicity of Li^+ ions increases dramatically (Figure 1C, empty symbols in all three panels). This phenomenon has been observed in several species and is thought to reflect the higher permeability of the membrane for Li^+ ions in more alkaline media (*18*−*20*). As Figure 1C shows, however, Vc-NhaP2 is not responsible for this massive alkali-stimulated Li^+ leakage.

The inability of Vc-NhaP2 to catalyze Li^+/H^+ exchange (Figures 2 and 3), despite the fact that the exchange of Li^+ for other alkali cations is apparently in place (Figure 1C), is one of the most curious features of this antiporter. It is widely accepted that in Na^+/H^+ antiporters all substrate alkali cations and protons share the same cation-binding site of the protein. This could be demonstrated by kinetic analysis (see ref *24* for a recent example) as well as inferred from structural data (*22*). Of course, in a general case, translocated ions may use different subsets of ligands available at the cation-binding site; thus, while H^+ requires only one electronegative atom to which to bind, the optimal number for coordination of Na^+ ion by polypeptides is six (see ref *44* and references cited therein for a detailed discussion). Therefore, if in Vc-NhaP2 Li^+ ion happens to bind, in addition to its other ligands, to a group normally employed by H^+, it would outcompete the proton, thus preventing "normal" Li^+/H^+ exchange without affecting the exchange of Li^+ with other alkali cations. X-ray diffraction-based structural data (cocrystallization with different substrates) could provide an ultimate test of this supposition, but kinetic analysis of mutant forms of Vc-NhaP2 appears to be a viable complementing, if not alternative, approach. On a more general note, the broad substrate specificity together with the peculiar behavior of Li^+ makes Vc-NhaP2 a promising experimental subject for detailed studies of structural determinants of cation specificity in ion exchangers.

The alkali cation exchange via Vc-NhaP2 in the cells of *V. cholerae* bearing functional Vc-NhaP2 should lead to the net uptake of not only Li^+ but also Na^+ ions. Nevertheless, Vc-NhaP2 does not weaken the Na^+ resistance even at pH 6.0, where NhaA is silenced: *V. cholerae* cells grow well in Na^+-rich media irrespective of the presence of Vc-NhaP2 at any pH (Figure 1B). It should be noted in this regard that one of the *V. cholerae* primary Na^+ pumps, namely, the Na^+-translocating NADH: ubiquinone oxidoreductase, NQR (*45*), could contribute to the net export of Na^+. Indeed, despite the presence of the array of different Na^+/H^+ antiporters in the membrane, elimination of functional NQR renders growth of *V. cholerae* somewhat more sensitive to Na^+ (C. C. Häse et al., unpublished observations; also J. L. Winogrodzki and P. Dibrov, unpublished results). Furthermore, in the presence of HQNO, a potent inhibitor of NQR, the growth of cells possessing Vc-NhaP2, but not the deletion Vc-NhaP2 mutant, also became severely compromised at high NaCl concentrations (data not shown). It is also worth mentioning that, in contrast to NhaA, Li^+ is not a substrate for NQR.

Perhaps the most interesting result of this work is the direct demonstration of an ion antiporter with a potentially broad substrate specificity acting as a specific K^+/H^+ antiporter expelling K^+ in vivo. It is evident from the potassium-sensitive but not sodium-sensitive phenotype of the Vc-NhaP2 deletion mutant (Figure 1A,B) as well as the kinetic data obtained in the in vitro system. Despite comparable affinities for alkali cations (Figures 4 and 5), the much lower levels of Na^+/H^+ antiport compared to K^+/H^+ antiport and the lack of Li^+/H^+ antiport mediated by

Vc-NhaP2 in inside-out membrane vesicles (Figures 2 and 3) clearly point toward K^+/H^+ exchange as the only physiological function of this antiporter.

In a sense, this finding may effectually end the ongoing quest for "specific" K^+/H^+ antiporters. Indeed, on the basis of the ion radii of K^+ (1.38 Å) and Na^+ (1.02 Å), one may argue that only Na^+/H^+ antiporters could efficiently discriminate K^+ ions by simple size exclusion; however, K^+/H^+ antiporters can hardly avoid binding smaller Na^+ as a substrate.

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