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Acidic residues of extracellular loop 3 of the Na^+/H^+ exchanger type 1 are important in cation transport

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Abstract

Mammalian Na^+/H^+ exchanger type I isoform (NHE1) is a ubiquitously expressed membrane protein that regulates intracellular pH (pHi) by removing one intracellular proton in exchange for one extracellular sodium ion. Abnormal activity of the protein occurs in cardiovascular disease and breast cancer. The purpose of this study is to examine the role of negatively charged amino acids of extracellular loop 3 (EL3) in the activity of the NHE protein. We mutated glutamic acid 217 and aspartic acid 226 to alanine, and to glutamine and asparagine, respectively. We examined effects on expression levels, cell surface targeting and activity of NHE1, and also characterized affinity for extracellular sodium and lithium ions. Individual mutation of these amino acids had little effect on protein function. However, mutation of both these amino acids together impaired transport, decreasing the Vmax for both Na^+ and Li^+ ions. We suggested that amino acids E217 and D226 form part of a negatively charged coordination sphere, which facilitates cation transport in the NHE1 protein.

Keywords Site-directed mutagenesis · Extracellular loop · Na^+/H^+ exchanger · Intracellular pH · Membrane protein · Cation transport

Introduction

The Na^+/H^+ exchanger isoform 1 (NHE1) is an integral membrane protein expressed ubiquitously in mammalian cells. As the first identified isoform, NHE1 has been the most studied and the best understood member of the SLC9A (SoLute Carrier family 9A) family. Studies have shown that it is the major plasma membrane pH regulatory protein in mammalian cells and has important physiological and pathological roles. NHE1 functions by removing one intracellular proton in exchange for one extracellular sodium ion, to protect cells from intracellular acidification. Physiologically, NHE1 promotes cell growth differentiation and mediates the programmed cell death through controlling cell volume and pH_i [1, 2]. NHE1 is the only identified plasma membrane Na^+/H^+ exchanger in the myocardium and thus it is key in maintaining the intracellular pH (pH_i) level and removing protons from within cardiomyocytes [3]. The activity of NHE1 is critical in several types of heart failure [4]. NHE1 is also important in breast cancer, where it acts as a trigger for metastasis. Increased NHE1 activity causes intracellular alkalinisation and extracellular acidification that facilitates tumorigenesis and tumour growth in breast cancer. Acidification of the extracellular tumour microenvironment also contributes to the development of chemotherapy resistance [5–7]. Current NHE1 inhibitors have significant off-target effects [8] so a deeper understanding of the structure and function of NHE1 is necessary for the design of inhibitors for breast cancer and cardiovascular disease.

NHE1 consists of a ~ 500 amino acid N-terminal domain responsible for pH sensing and ion transport, and an intracellular regulatory C-terminal tail of ~ 315 amino acids [1, 2]. An early study [9] used cysteine scanning accessibility and suggested that the N-terminal region is composed of twelve transmembrane (TM) segments linked through intracellular and extracellular loops. This was later confirmed [10]. More recently, we published a molecular model of the NHE1 protein based on the structure of MjNhaP1 of *Methanocaldococcus jannaschii* in combination with biochemical surface accessibility data [11]. However, directly observed structural and functional information on TM segments are still limited. To this day, there has been no study of the structure and function of the NHE1 extracellular loop 3 (EL3)

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comprising amino acids ~ 212–227. Residues of extracellular loops (ELs) of cation transporters are often critical to their function. For example, in the serotonin transporter, the glutamate transporter, and the glycine transporter [12–14], ELs play key roles in function. For Na^+, K^+ ATPase, the second EL is a major determinant of extracellular cation affinity [15, 16]. We recently demonstrated that negatively charged residues on an extracellular loop of the yeast Na^+/H^+ exchanger *Sp*NHE1 play an important role in that protein's function [17]. Previous studies have also suggested that amino acids of EL4 are important in expression and activity of NHE1 [18]. Similarly, the short EL2 loop was also shown to be important in NHE1 function [19]. Here, we examined EL3 of NHE1 with the hypothesis that acidic amino acids E217 and D226 are important in extracellular cation coordination, and that mutations E217A, E217Q, D226A, D226N of the extracellular loop 3 of NHE1 will affect NHE1 activity and cation transport, and thereby change the cell's response to intracellular acid loads. The results are the first analysis of these amino acids of EL3 and demonstrate an important role in the function of the protein.

Materials and methods

Materials

PWO DNA polymerase was from Roche Molecular Biochemicals (Mannheim, Germany). Sulfo-NHS-SS-biotin was obtained from Pierce, Rockford, IL, USA. LIPOFECTAMINE™ 2000 Reagent was from Invitrogen Life Technologies (Carlsbad, CA, USA). Anti-hemagglutinin (HA) tag antibody was from Santa Cruz Biotechnology (Santa Cruz, CA). BCECF-AM was from Molecular Probes, Inc. (Eugene, OR). Synthetic oligonucleotides were from Integrated DNA Technologies (Coralville, Iowa, USA). All other chemicals were of analytical scale and were obtained commercially from Fisher Scientific (Ottawa, ON), Sigma or BDH (Toronto, ON). The AP-1 cell line was a generous gift of Dr. S. Grinstein (Hospital for Sick Children, Toronto, ON, Canada).

Site-directed mutagenesis

Mutations were introduced to an expression plasmid pYN4+, which contains the cDNA of the entire coding region of human NHE1. A HA tag is located at C terminus of the cDNA and previous studies have demonstrated that it has no effect on activity of NHE1 [20]. The Stratagene (LaJolla, CA, USA) QuikChange™ site-directed mutagenesis kit was used with synthetic DNA primers (Table 1) to mutate aspartic acid 217 or glutamic acid 226 to Ala. Mutations were designed to create or remove a restriction enzyme site for use in screening transformants. Additional mutations were made to change E217 to Q and D226 to N. Amplification of plasmid DNA was with PWO DNA polymerase (Roche Molecular Biochemicals, Mannheim, Germany) and mutations and fidelity of the polymerase were confirmed by DNA sequencing. Double mutations were made with sequential use of the appropriate pairs of primers.

Cell culture and stable transfection

AP-1 cells (a Chinese hamster ovary cell line that lack Na^+/H^+ exchanger) were incubated in a humidified atmosphere with 5% CO_2 in growth medium containing α-MEM supplemented with 10% (V/V) bovine growth serum, 25 mM HEPES, penicillin (100 U/mL) and streptomycin (100 µg/mL), pH 7.4 at 37 °C. Transfection was performed with LIPOFECTAMINE™ 2000 Reagent [21]. Briefly, 4.0×10^5 cells were grown in 35 mm Petri dishes containing 2 mL of media. Cells were transfected with 4 µg of wild-type or 4 µg of mutant plasmids when 90% confluent. Cells were treated with trypsin the second day after transfection, followed by 10- or 100-times dilution with α-MEM medium. Afterwards, cells were plated in 100 mm dishes containing 800 µg/mL geneticin (G418) to maintain selection pressure. Single clones surviving from the selection were picked as stable cell lines and they were collected and grown in α-MEM medium containing 400 µg/mL G418.

Table 1 Oligonucleotide primers for site-directed mutagenesis

Mutation	Sequence	Restriction Site
E217A	5′TGCCTGGTGGGCGGTcaGCAGATtAAtAACATCGGCCTCCTG3′	AseI
E217Q	5′TGCCTGGTGGGCGGTgcGCAGATtAAtAACATCGGCCTCCTG3′	AseI
D226A	5′GTGGGCGGTGAGCAGATtAAtAACATCGGCCTCCTGGcCAACCTGCTCTTCGGC3′	AseI
D226N	5′GTGGGCGGTGAGCAGATtAAtAACATCGGCCTCCTGaACAACCTGCTCTTCGGC3′	AseI

Underline indicates restriction site introduced. Lower case indicates changed base pair. Only the forward primer of each pair of primers is illustrated

SDS-PAGE and immunoblotting

Western blotting was used to compare NHE1 expression levels in samples collected from transfected AP-1 cells and wild-type cells [21]. 100 µg of total cell lysate was loaded onto 10% SDS-PAGE and transferred to nitrocellulose membranes. Afterwards, the Amersham™ enhanced chemiluminescence Western blotting and detection system was used to detect immunoreactive proteins using an anti-HA antibody tag. Densitometric analysis of X-ray film was performed using NIH Image software (National Institutes of Health, Bethesda, MD, USA).

Cell surface expression

We examined the level of cell surface targeting to ensure that NHE1 mutants were correctly targeted to the plasma membrane. Mutants were incubated with 3 mL of 0.5 mg/ml Sulpho-NHS-SS-Biotin in borate buffer at 4 ℃ for 30 min [21] to label the cell surface expressed membrane proteins. Cells were then washed three times with cold quenching buffer (192 mM glycine/25 mM Tris, pH 8.3) and then solubilized on ice in 0.5 mL of immunoprecipitation buffer [1% (w/v) deoxycholic acid/ 1% (w/v) Triton X-100/ 0.1% (w/v) SDS/ 150 mM NaCl/ 1 mM EDTA/ 10 mM Tris/ HCl, pH 7.5/ 0.1 mM benzamidine/ 0.1 mM PMSF/ 0.1% (v/v) protease inhibitor cocktail]. Biotin-labelled proteins were captured by incubating lysed cells with immobilized streptavidin resin. To determine cell surface targeting, equal amounts of total cell lysate and unbound lysate extracted with immobilized streptavidin resin proteins were analysed by 10% SDS-PAGE, followed by western blotting against the HA tag present on the NHE1 protein. The amount of 110 kDa and 90 kDa immunoreactive forms of NHE1 was compared between the unbound (extracted lysate) and the total fractions of all four mutants and the wild type. These ratios reflect the relative cell surface expression level of the different cell lines.

Na^+/H^+ exchange activity

Measurement of NHE1 activity was with a PTI Deltascan spectrofluorometer, as described previously [21]. Stably transfected mutant cells and wild type were plated onto glass coverslips (2×10^5 cells per coverslip) and grown to 80–90% confluence. Cells were then incubated with 2.5 µg/mL 2′,7-bis- (2-carboxyethyl)-5-carboxyfluorescein-AM (BCECF-AM) for 20 min, followed by addition of "Normal buffer" (135 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, 5.5 mM glucose, and 10 mM HEPES, pH 7.3) for 3 min. Induction of intracellular acidosis was achieved by NH_3/NH_4^+ pre-pulse (injection of 2.5 M NH_4Cl to a final concentration of 50 mM NH_4Cl) and then incubated for 3 min, followed by 30 s in "Na^+ free buffer" (135 mM N-methyl-D-glucamine, 5 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, 5.5 mM glucose, and 10 mM HEPES, pH 7.4). Afterwards, cells were placed in "Normal buffer" containing 135 mM NaCl for at least 3 min to allow for intracellular pH recovery. Raw data collected directly from the spectrofluorometer were further processed based on a three-point pH calibration curve using the K^+/nigericin method with Na^+ free calibration buffers (135 mM N-methyl-D-glucamine, 5 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, 5.5 mM glucose, and 10 mM HEPES, pH 6, 7, 8) and 10 µM nigericin [21]. For measurement of cation affinity varying concentrations of cations were added with N-methyl-D-glucamine being used to maintain osmotic strength. Results are shown as mean ± standard error. Statistical significance was determined using the Wilcoxon–Mann–Whitney test. The determination of kinetic parameters was essentially as described earlier [22, 23]. Na^+ and Li^+ concentrations were varied while maintaining osmolarity with N-methyl-D-glucamine.

Results

In this study, we examine the effect of mutagenesis of two negatively charged amino acids of extracellular loop 3 of the NHE1 isoform of the human Na^+/H^+ exchanger. Figure 1a illustrates the putative two-dimensional topology of the protein. It is an integral membrane protein with 12 transmembrane segments and a large, cytosolic tail. The right panel is an expanded view of the amino acids of EL3. Figure 1b, c present a three-dimensional model we recently made [11] of human NHE1 based on structure of *Methanocaldococcus jannaschii* in combination with biochemical surface accessibility data. It is interesting to note that despite the relatively large linear distance between E217 and D226, in the molecular model, they are relatively close to one another and project into a putative cation channel.

We performed site-directed mutagenesis of the wild-type NHE1 protein. We mutated the two polar and negatively charged residues E217 and D226 independently to the small, non-polar amino acid alanine. In addition, E217 and D226 were mutated to glutamine and asparagine, respectively, which are uncharged amino acids of similar size. Later, mutation of both residues was done to determine the cumulative effects of the mutations on NHE1 protein activity. To characterize the effects of the mutation of extracellular loop 3, we measured the expression levels, surface targeting and activity of mutant NHE1 proteins expressed in AP-1 cells relative to that of wild-type NHE1 protein. Figure 2 illustrates the effects of individual mutations (a) and mutation of both residues simultaneously (b). All sample types were composed of a glycosylated (110 kDa) and a de-glycosylated (95 kDa) protein [18]. Most of the mutations had little effect

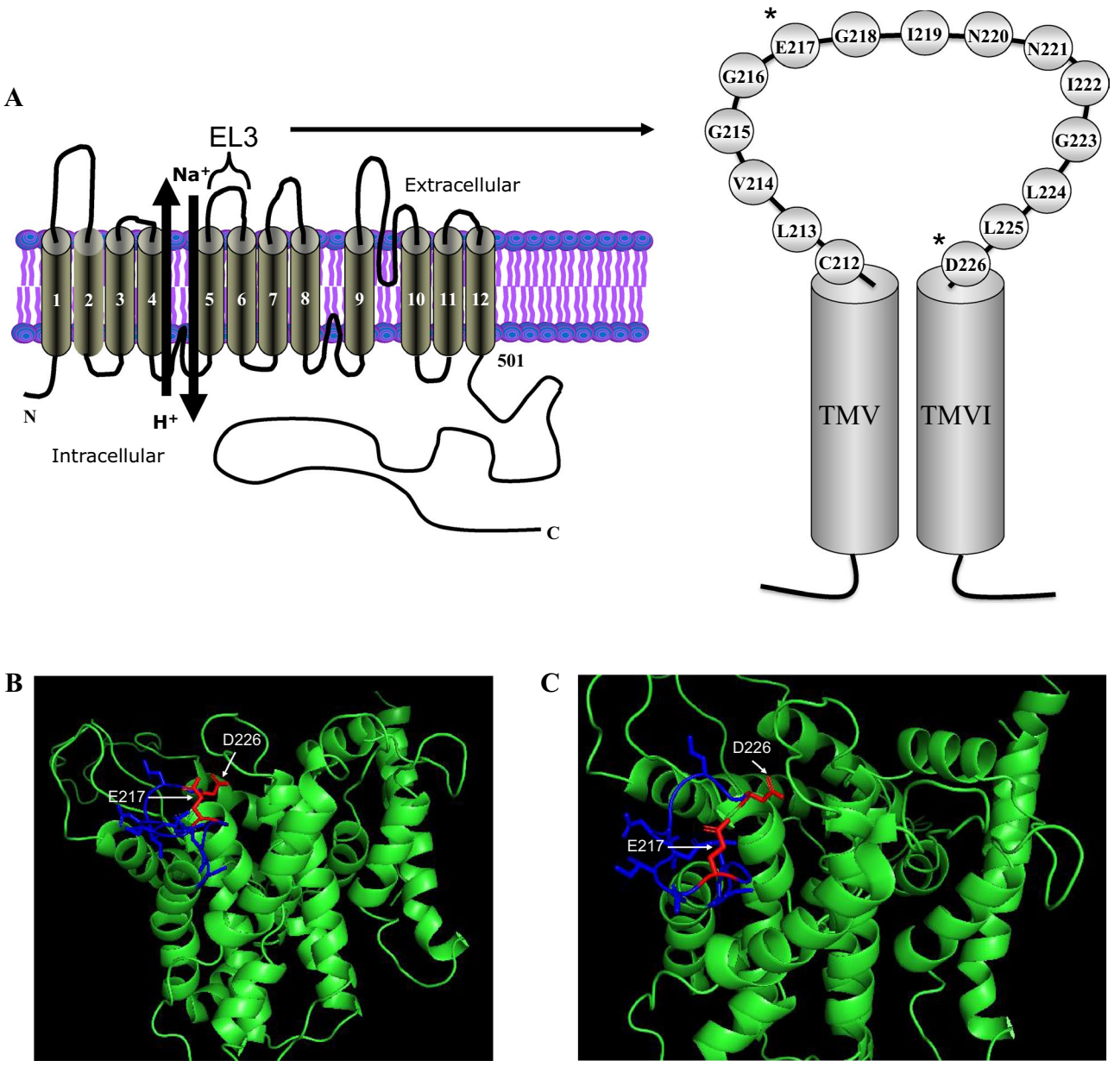

Fig. 1 Molecular models of human NHE1 and extracellular loop 3. **a** Left panel, two-dimensional molecular model of human NHE1 topology, based on [10] and [9]. EL3, extracellular loop 3. Right panel, expanded two-dimensional model of EL3 and TMV and TMVI. *Indicates negatively charged amino acids mutated in this study. **b**, **c** Three-dimensional model of human NHE1 as described in [11]. **b** Lateral view of the model from the lipid bilayer. EL3 with side chains is shown in blue, amino acids E217 and D226 are in red. **c** View from the extracellular face of the membrane. Labelled as in "B"

on the level of protein expression, though the E217A mutant cell line had decreased expression of the NHE1 protein.

Figure 3 illustrates the effects of the mutations on surface targeting of the wild-type and mutant NHE1 proteins. Approximately, 68–76% of the NHE1 proteins were targeted to the cell surface. This did not vary significantly between wild-type NHE1 and the mutant NHE1 proteins.

To examine the effect of these mutations on the initial rate of NHE1 activity after a large acid load, we treated the cell lines with a large acute acid load and then examined the effect on the initial rate of recovery. The results are shown in Fig. 4. We measured the activity directly and also corrected for minor changes in surface targeting and expression levels. Uncorrected activity of the E217A, D226N and the double mutant were significantly reduced compared to the wild-type activity. For the E217A, this difference disappeared when correcting for the expression levels of the protein. In the case of D226N and the combined mutant with both the E217Q

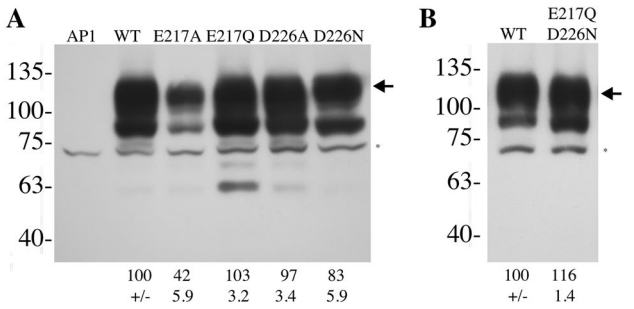

Fig. 2 Expression and targeting of wild-type NHE1 and mutants. **a** Western blot (anti-HA antibody) of whole cell lysates of stably transfected AP1 cells containing the indicated plasmids WT, wild-type NHE1, E217A, E217Q, D226A and D226N. Asterisk indicates non-specific immunoreactive band present in all cell lysates. AP1, mock transfected AP1 cells. The level of expression relative to the wild type is indicated below each lane, mean ± SE, $n \geq 3$. **b** As in A except illustrating wild-type NHE1 protein and double mutant with E217Q and D226N mutation

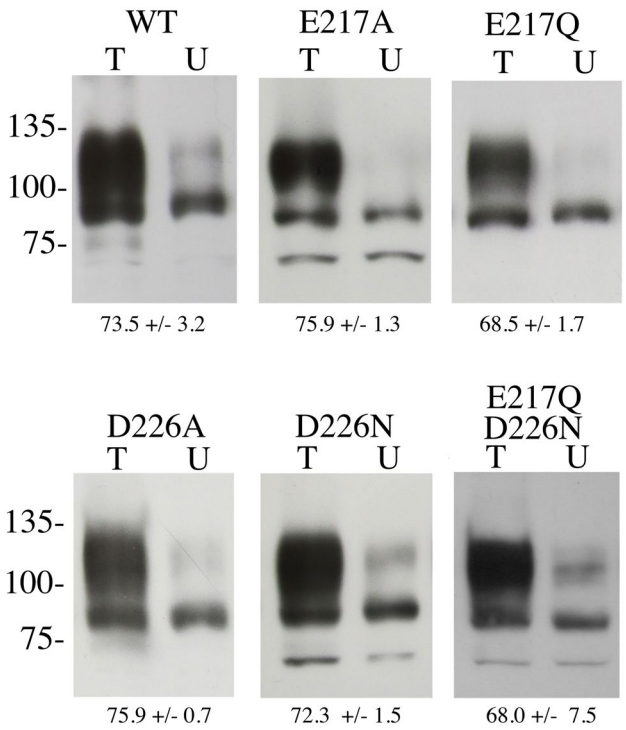

Fig. 3 Surface localization of wild-type (WT) and mutant NHE1 proteins in AP1 cells. Examples of results and mean ± the S.E. $n \geq 3$ determinations. The % targeted to the cell surface is indicated. After external cell surface biotinylation, equal amounts of total cell lysates (T) and unbound intracellular lysates (U) were examined by western blotting with anti-HA antibody to identify NHE1 protein. WT and mutant cell lines are indicated. Lysates were from cell lines stably expressing wild-type NHE1 and mutant NHE1 proteins, respectively. The level of surface targeting is indicated below each lane, mean ± SE, $n \geq 3$

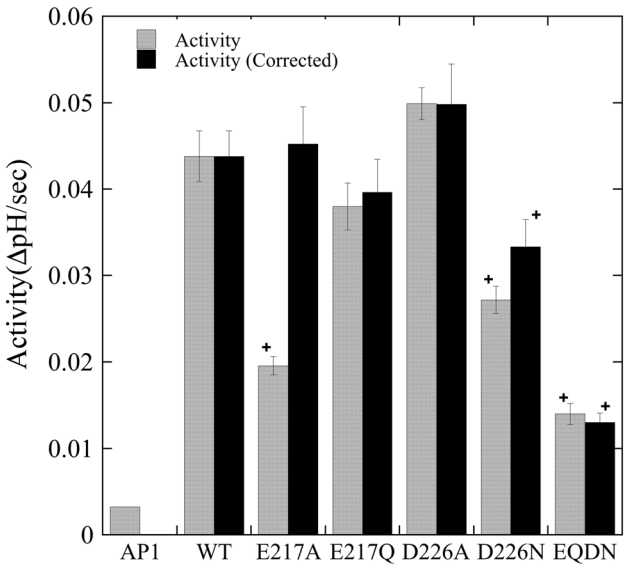

Fig. 4 Summary of Na^+/H^+ exchanger activity of wild-type (WT) and mutant NHE1 proteins before and after correction for surface targeting and expression levels. Cells containing wild-type or mutant proteins were acidified with ammonium chloride as described in the "Materials and Methods". The initial rate of recovery was measured and recorded as Δ pH/s. $n \geq 6$, mean ± SE, $^+P < 0.001$ compared with wild-type protein

and D226N mutations, the inhibition of activity occurred when correcting for expression and surface targeting levels. The E217Q mutation had a minor effect on activity that was not significant.

Figure 5 and Table 2 illustrate the effects of varying Na^+ and Li^+ ions on activity of wild-type NHE1 protein and NHE1 D226N mutant and the double mutant with both the E217Q and D226N mutations. The D226N mutant showed both a reduced Vmax and Km for Na^+ and a slightly reduced Vmax and a reduced Km for Li^+. The double mutant showed a greater reduction in the Vmax for both ions and a reduction in the Km for both ions also.

Discussion

The mechanism of Na^+/H^+ exchange is of great interest from a scientific point of view and from an applied standpoint. Scientifically, there has been some progress in understanding the structure and function of non-mammalian Na^+/H^+ exchangers such as that of *E. coli* sodium transporter NhaA [24], *Thermos thermophilus* (NapA) [25], *Pyrococcus abyssi* (PaNhaP) [26] and *Methanocaldococcus jannaschii* (MjNhaP1) [27]. However, details of the transport mechanism of other kinds of Na^+/H^+ exchangers such as human Na^+/H^+ exchangers have not been complete. Negatively charged amino acids have been suggested to be of importance in

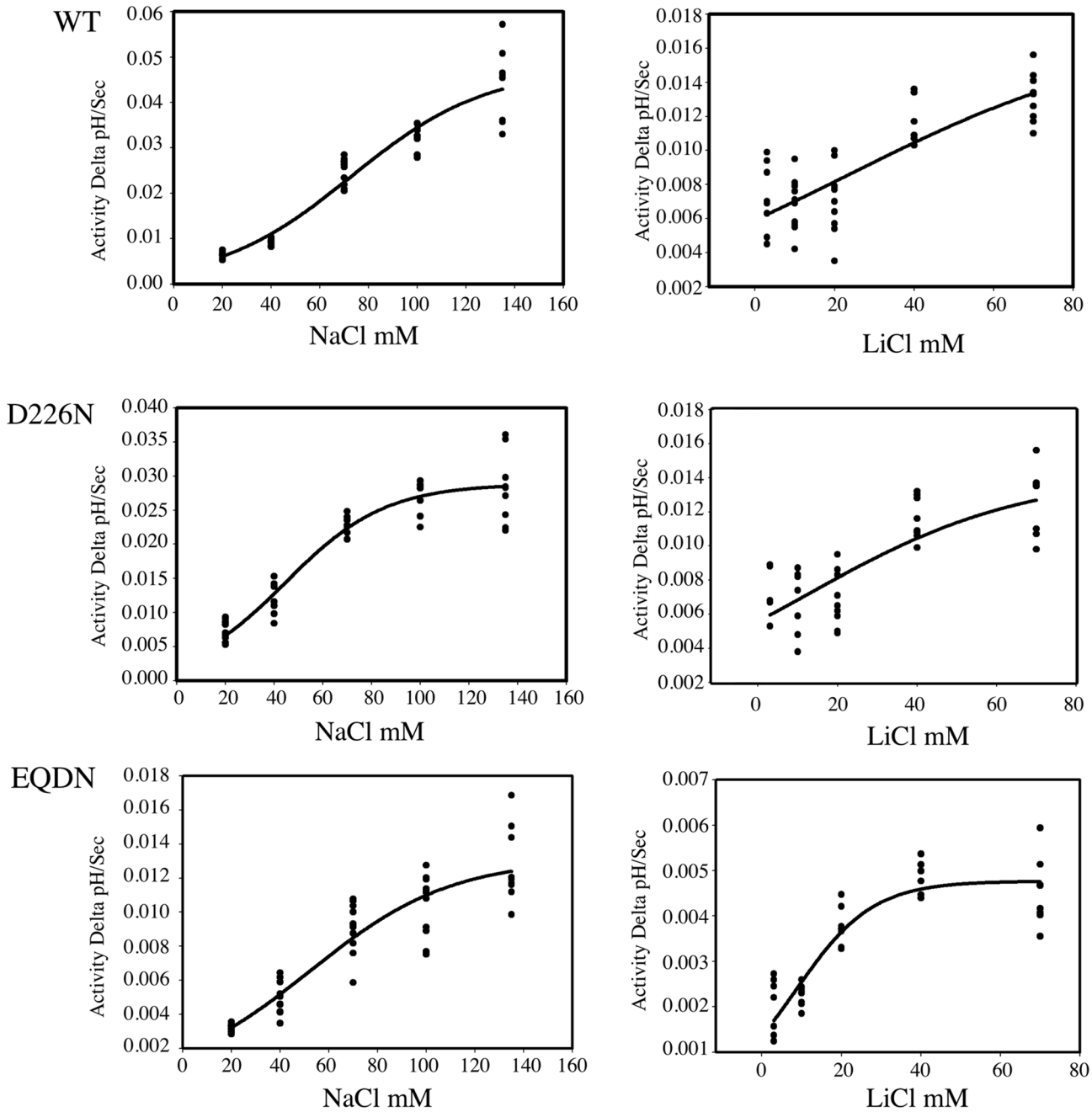

Fig. 5 Effects of varying Na^+ and Li^+ concentrations on Na^+/H^+ exchanger activity of wild-type (WT) and mutant NHE1 proteins D226N and E217QD226N double mutant protein. NHE1 activity was determined as described in the "Materials and Methods"

Table 2 Kinetic parameters of wild-type and mutant NHE1 proteins

Mutant	Km NaCl mM	Vmax NaCl ΔpH/S	Km LiCl mM	Vmax LiCl ΔpH/S
Wild-type	73.0	0.047	23.3	0.017
D226N	44.7	0.029	12.1	0.014
E217QD226N	52.4	0.013	8.7	0.0048

coordination of cations [28]. Earlier studies have examined negatively charged amino acids of the transmembrane domain of NHE1. The negative charges within the membrane are sometimes, but not always, important in protein function [22, 29, 30].

Residues of extracellular loops (ELs) of cation transporters are often critical to their function. For Na^+, K^+ ATPase, the second EL is a determinant of extracellular

cation affinity and that loop is thought to be functionally linked to a gating mechanism that controls access of K^+ to its binding site [15, 16]. ELs also play key roles in function in the serotonin transporter, the glutamate transporter, and the glycine transporter [12–14]. Previous studies have also suggested that ELs of NHE1 are important in function. Mutation of several residues of EL4 causes defects in expression and activity of NHE1 [18]. The structure of EL4 indicated that it acted as a flexible link between transmembrane segments. EL2 of NHE1 is much smaller in size than either EL4 or EL3, but mutation of its residues also showed it to be important in NHE1 function [19].

In this contribution, we used the AP-1 cell line. AP-1 cells are a CHO-derived cell line that have their own NHE1 protein deleted. They have no detectable NHE activity of any kind with deletion of this protein. They have been used in many studies from different laboratories to study both the importance of amino acids in the function of NHE1 and to study regulation of NHE1 (i.e. [22, 31–36].) and have proven to be an excellent model. In the present study, we used these cells to express full length human NHE1 successfully as described earlier [31, 32]. Expression in these cells allowed unambiguous characterization of activity of the protein, without the presence of background endogenous NHE1. The NHE1 protein was expressed and targeted successfully to examine the importance of acidic residues of EL3. While in linear models of the protein, these acidic residues do not appear to be near to each other; our most recent molecular model [11] of NHE1 suggests that they are not distant from one another (Fig. 1). Individual mutation of amino acids E217 and D226 to Ala had little effect on function. Similarly, mutation of these amino acids individually to similar sized but unchanged amino acids had no effect in the case of the E217Q mutation, and only a partial effect on function of the D226N mutation. However, when both these amino acids were mutated simultaneously to neutral amino acids, there was a large effect on function. The activity decreased greatly, and notably, this occurred through a changed in the Vmax of transport of the protein for both Na^+ and Li^+ ions. It appeared as though accumulated deletion of two negative charges was needed to have a more significant effect. Curiously, the double mutation caused a decrease in the Km for both Na^+ and Li^+ indicating that less cation was required for activation of the protein. At present the cause of this effect is unknown. We speculate that if these amino acids function in cation attraction, there is sufficient attraction by other surrounding amino acids and their elimination may facilitate release of cations to the membrane domain. Further experiments or computer modelling are necessary to confirm this hypothesis. It is, however, clear that, the maximum velocity of the protein was greatly reduced. As extracellular Na^+ is approximately 140 mM, it seems unlikely that these mutations would change the activity of the protein if these mutations occurred in vivo.

Our results in this study are strikingly similar to those found in a recent study on an extracellular loop of the yeast Na^+/H^+ exchanger *Sp*NHE1 [17]. EL6 of *Sp*NHE1 contains several acidic residues. Mutation of four acidic residues was required to affect function of the protein and it was thought that elimination of a significant part of a cation coordination sphere was necessary to affect function. Earlier we suggested that binding of cations could be based on a crown ether-like cluster of polar amino acids [28]. It is unclear if this could be the case in the extracellular region. It is of note that the periplasmic surface of the *E. coli* transporter NhaA is negatively charged (in contrast to the cytoplasmic surface that is positively charged). The negatively charged funnel of the protein is thought to function to attract cations and to increase their local concentration over that of anions [24]. We suggest that amino acids E217 and D226 serve a similar function.

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Compliance with ethical standard

Conflict of interest The authors declare that they have no conflicts of interest.

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