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Two Histidine Residues in the Juxta-Membrane Cytoplasmic Domain of Na^+/H^+ Exchanger Isoform 3 (NHE3) Determine the Set Point

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Abstract. Histidine residues in Na^+/H^+ exchangers are believed to participate in proton binding and influence the Na^+/H^+ exchanger activity. In the present study, the function of three highly conserved histidines in the juxtamembrane cytoplasmic domain of NHE3 was studied. His-479, His-485, and His-499 were mutated to Leu, Gln or Asp and expressed in an Na^+/H^+ exchanger null cell line and functional consequences on Na^+/H^+ exchange kinetics were characterized. None of the histidines were essential for NHE3 activity, with all mutated NHE3 resulting in functional exchangers. However, the mutation in His-475 and His-499 significantly lowered NHE3 transport activity, whereas the mutation in H485 showed no apparent effect. In addition, the pH profiles of the H479 and H499 mutants were shifted to a more acidic region, and lowered its set point, the intracellular pH value above which the Na^+/H^+ exchanger becomes inactive, by ~0.3–0.6 pH units. The changes in set point by the mutations were further shifted to more acidic values by ATP depletion, indicating that the mechanism by which the mutations on the histidine residues altered the NHE3 set point differs from that caused by ATP depletion. We suggest that His-479 and His-499 are part of the H^+ sensor, which is involved in determining the sensitivity to the intracellular H^+ concentration and Na^+/H^+ exchange rate.

Key words: Na^+/H^+ exchanger — Histidine — Set point — pH sensor

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Introduction

The Na^+/H^+ exchangers are ubiquitous integral plasma membrane proteins that regulate intracellular pH (pH_i) by exchanging extracellular Na^+ cell volume (Orlowski & Grinstein, 1997; Wakabayashi et al., 1997a; Fliegel et al., 1998). Mammalian Na^+/H^+ exchangers mediate the entry of one Na^+ into cells in exchange for one cytoplasmic H^+ in an electroneutral manner. The dependence of Na^+/H^+ exchange on intracellular H^+ concentration ($[H^+]_i$) is considerably steeper than can be accounted for by simple Michaelis-Menten kinetics, and the kinetics of the NHEs can be fit to an allosteric model represented by a Hill plot with Hill coefficients, $n \sim 2$–3. Aronson and coworkers suggested that Na^+/H^+ exchanger has an additional cytoplasmic H^+ binding site known as the H^+-modifier site, which allosterically activates Na^+/H^+ exchangers to enhance extrusion of H^+ (Aronson, Nee & Suhm, 1982; Kinsella, Heller & Frochlich, 1998). However, the location and the molecular nature of the H^+-modifier site remain elusive to date.

Na^+/H^+ exchangers have a characteristic pH_i dependence, which causes the exchanger to be inactive at pH_i values above a certain activity threshold, called the set point, and then to rapidly activate as pH_i falls below this set point. Due to the small amount of acid production by the cell, the set point is the pH_i at which the Na^+/H^+ exchanger just balances acid production, and at the set point, the Na^+/H^+ exchanger is transporting slowly rather than being shut off. This activity threshold or set point coincides with the normal physiological pH_i of many cells.

According to truncation studies of the NHE C-terminus, the transport activity and allosteric nature of the Na^+/H^+ exchanger are preserved until almost complete removal of the cytoplasmic C-terminus (Wakabayashi et al., 1992; Levine et al., 1995). Deletion of the NHE1 cytoplasmic domain shifted

the pK value of NHE activity to an acidic range (Wakabayashi et al., 1992; 1994; 1997b). It is suggested that a complex and specific interaction between the C-terminus and the N-terminal membrane domain is involved in modulating the H^+ binding sites and the set point of the exchanger (Yun, Tse & Donoritz, 1995).

It has been predicted that amino-acid residues that can be protonated and deprotonated near the physiological pH are important for translocation of H^+. The most likely candidate amino acid to accomplish this pH sensitivity is histidine, which has a pK value of around 6.0. His can be protonated at physiological pH 6.5–7, resulting in a positive charge at this residue. There is experimental evidence that histidine functions in the pH-dependent modulation of a number of ion transporters, including GLT-1, AE2, ROMK1, PEPT (Zhang, Pines & Kanner, 1994; Sekler, Kobayashi & Kopito, 1996; Fei et al., 1997; Teng & Grubmeyer, 1999; Chanchevalap et al., 2000). Consistently, histidine appears to play a similar role in Na^+/H^+ exchangers (Aronson et al., 1982; Wakabayashi et al., 1992; Gerchman et al., 1993; Rimon et al., 1995; Wang, Balkovetz & Warnock, 1995; Ikeda et al., 1997b). A histidine-specific inhibitor, diethyl procarbonate (DEPC), inhibits the maximal Na^+/H^+ exchange rate without affecting Na^+ binding affinity in rabbit brush border membrane vesicles and transfected cells (Grille & Aronson, 1986; Wang et al., 1995). In the bacterial Na^+/H^+ antiporter NhaA, His-226 has been proposed to be a part of the pH sensor, since mutation of His-226 changed the pH profile of the exchanger (Gerchman et al., 1993; Rimon et al., 1995). Mutation of His-226 of NhaA to Arg shifted the pH profile to acidic pH, whereas the substitution by Asp caused an alkaline shift (Rimon et al., 1995). In the mammalian Na^+/H^+ exchangers, the H^+-binding site was suggested as being located in the N-terminal transmembrane domain plus ~40 aa of the cytoplasmic C-terminus, since truncation mutants retained antiporter activity, although the contributing residues were not identified (Wakabayashi et al., 1992; Levine et al. 1995). However, mutation of several His (His-35, His-120 and His-349) of the N-terminal transmembrane domain of NHE1 failed to significantly alter the exchange rate or pH_i profiles of NHE1, indicating that the His residues in the N-terminal membrane domain might not be important in determining the pH_i profiles of NHE1 (Wang et al., 1995). On the other hand, studies by Wakabayashi and coworkers showed that the first ~90 amino acids of the NHE1 cytoplasmic domain have a pH_i maintenance function and are involved in the ATP sensitivity of the exchanger (Ikeda et al., 1997; Wakabayashi et al., 1997b). It is suggested that the transmembrane region contains the H^+-modifier site and that the C-terminal domain modulates the value of the set point.

Examination of the amino-acid sequence of the carboxyl terminal region of the Na^+/H^+ exchanger isoforms reveals that there are three conserved His located in the juxtamembrane cytoplasmic region of NHEs at positions His-479, His-485, and His-499 in NHE3. In the present study, we investigated the role of these His in the activity of NHE3. We show that mutations of His-479 and His-499 significantly acid-shifted the set point of the Na^+/H^+ exchanger, NHE3.

Materials and Methods

Materials

Restriction enzymes and endonucleases were from Roche (Indianapolis, IN). The site-directed mutagenesis kit was from Stratagene (La Jolla, CA). Nigericin and 2′,7′-bis (2-carboxyethyl)-5 (6)-carboxylfluorescein (BCECF-AM) were purchased from Molecular Probes (Eugene, OR). Monoclonal anti-VSVG antibody was derived from the P5D4 hybridoma. Monoclonal GAPDH (Glyceraldehyde-3-phosphate dehydrogenase) antibody was purchased from US Biological (Swampscott, MA). EZ-Link Sulfo-NHS-SS-Biotin was from Pierce (Rockford, IL). Protease inhibitor cocktail (4-(2-aminoethyl) benzenesulfonyl fluoride (AEBSF), trans-epoxysuccinyl-L-leucylamido (4-guanidino) butane (E-64), bestatin, leupeptin, aprotinin and sodium EDTA), 2-deoxy-D-glucose, and oligomycin were purchased from Sigma (St. Louis, MO). Protein concentrations were determined by the method of Bradford.

Construction of Expression Vectors for NHE3 His Mutants

The mutation of His residues in NHE3 was accomplished using the Quick Change Site-Directed Mutagenesis Kit from Stratagen according to the manufacturer's protocol. The template for mutagenesis was pcDNA3.1/Hygro$^+$ vector (Invitrogen) carrying rabbit NHE3, which had an epitope tag derived from Vesicular Stomatitis Virus Glycoprotein (VSV G) in the COOH-terminal end. The sense oligonucleotides (5′-3′) used for introducing the mutations in pcDNA3.1/Hygro$^+$ NHE3V were: H479L, CTGAACGAGAAG CTGCTCGGCCGGGCTTTCGAC; H479D, CTGAACGAGAA GCTGGTCGGCCGGGCTTTCGAC; H485Q, GGCCGGGCTT TCGACCAAATCCTCTCGGCCATC; H485L, GGCCGGGCTT TCGACCTCATCCTCT-CGGCCATC; H499L, TCCGGGCAG ATTGGGCTCAATTATC-TCAGGGAC; and H499D, TCCGG GCAGATTGGGGTCAATTATCTCAGGGAC (16 cycles, 95°C, 30 sec; 55°C, 1 min; 68°C, 16 min). The altered nucleotides are underlined. The cDNAs were sequenced to confirm the presence of the mutations and to ensure that other random mutations were not introduced.

Cell Culture and Transfection of NHE3 His Mutants

The cDNAs of wild-type NHE3 and His mutants, H479L, H479D, H485Q, H485L, H499L and H499D with the VSVG epitope on the C-terminus in pcDNA3.1/Hygro+ vector (Invitrogen) were transfected into Na^+/H^+ exchanger-deficient PS 120 fibroblasts using Lipofectamine 2000 (Gibco BRL) according to the manufacturer's protocol. Transfected PS120 fibroblasts were maintained at 37°C in a humidified atmosphere with 5% CO_2 in Dulbecco's

modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum, penicillin (100 units/ml), and streptomycin (100 μg/ml). In addition to selection for resistance to hygromycin (600 unit/ml), cells were subjected to the H^+-killing procedure consisting of 50 mM NH_4Cl/saline solution for 1 hr, followed by an isotonic 2 mM Na^+ solution for 1 hr (Levine et al., 1993). Surviving cells were then placed in normal culture medium and allowed to reach 30–50% confluence.

Immunofluorescence

Cells were grown on tissue culture glass coverslips. Images were taken after cells were fixed with 3% paraformaldehyde at room temperature for 20 min. Primary antibody (1:50 dilution for monoclonal anti-VSVG antibody mP5D4) was incubated for 1 hr at room temperature in 10% goat serum, PBS, and 1% bovine serum albumin (BSA). Cells were then washed three times with PBS, 1% BSA, and 0.1% Tween-20 and incubated for 1 hr with fluorochrome-conjugated secondary antibodies. Cells were washed three times with PBS, 1% BSA, and 0.1% Tween-20 and mounted with Prolong Antifade (Molecular Probes). Nuclei were stained with Hoechst 33342 (Molecular Probes). Cells were examined with a confocal fluorescence microscope (Zeiss LSM 410) as previously described (Yun et al., 1998).

Cell Surface Biotinylation

Transfected PS120 cells were grown to 70–80% confluence in 10-cm Petri dishes. The cells were then serum-starved for ~4 hr. All subsequent manipulations were performed at 4°C. For surface labeling of NHE3, cells were incubated with 1 mM NHS-SS-biotin (biotinylation solution; Pierce) for 20 min (and this was repeated), solubilized with the lysis buffer and then incubated for 1 hr with streptavidin-agarose beads as described (Lee-Kwon et al., 2001). Western analysis was performed using mP5D4 as the primary antibody and horseradish peroxidase-conjugated anti-mouse antibody as the secondary antibody. Bands were then visualized by Renaissance Enhanced Lumimol Reagent (NEN™ Life Science) on Hyperfilm™ MP film (Amersham) under developing conditions, in which the dilutions gave linear signals as determined empirically. The volume of each band was determined in arbitrary densitometry units by using a scanning densitometer and ImageQuant software from Molecular Dynamics (Sunnyvale, CA). Values for each band were plotted as densitometry units versus sample volume. Curves for each fraction (total, surface, and intracellular) were analyzed by linear analysis using Origin 6.0 (Microcal Software), and each fraction was quantitated as previously described (Lee-Kwon et al., 2001).

Measurement of Na^+/H^+ Exchange Activity

The transfected PS 120 cells grown to 70–80% confluence on glass coverslips were placed in serum-free medium for ~4 hr. The Na^+/H^+ exchanger activities of transfected PS120 cells were measured using the intracellular pH-sensitive dye BCECF-AM, as previously described (Levine et al., 1993, 1995). The cells were pulsed with 30 mM NH_4Cl in TMA-Cl for 3–5 min, followed by washing TMA^+ medium resulting in acidification. The perfusion chamber was then perfused with Na^+ medium, and Na^+-dependent pH recovery was measured. The product of Na^+-dependent change in pH_i times the buffering capacity at each pH_i was analyzed, using a nonlinear regression data analysis program, Origin 6, that allowed fitting of data to a general allosteric model described by the Hill equation ($V = V_{max}[H^+]^n/(K^n + [H^+]^n)$), with estimates for V_{max} and $K[H^+]_i$ and calculation of the pH_i at which the NHE3 mutants had ~ zero activity. To determine the steady-state pH_i or the set point of the transfected PS120 cells, the cells were loaded with BCECF and perfused with Na^+ medium without acidification.

ATP Depletion

For ATP depletion studies, cells were incubated with 2 μg/ml oligomycin and 5 mM 2-deoxy-D-glucose for 30 min at 37°C. This protocol has been shown to reduce ATP concentrations in the PS120 and other fibroblast cell lines by >90% (Levine et al., 1993; Wakabayashi et al., 1992).

Results

Conserved Histidines in the Juxta-Membrane Cytoplasmic Domain of NHEs

Primary sequence comparisons of NHEs of human, rat, and rabbit reveals that there are three His residues (His-479, His-485, and His-499), which are highly conserved in the cytoplasmic domain of NHEs, as shown in Fig. 1. His-479 and His-485 are strictly conserved in NHE1-5. His-499 is conserved in NHE1-4 but replaced by a Tyr in NHE5. There is a His cluster located in NHE1 at amino acids 540–545 (equivalent to aa 496–501 in NHE3), which is present only in NHE1. To investigate the possible roles of the imidazolium ring of histidine, the following mutations were made: His-475 to Leu (H475L) and Asp (H475D), His-485 to Gln (H485Q) and Leu (H485L), and His-499 to Leu (H499L) and Asp (H499D).

Protein Expression

All the NHE3 constructs with mutations at one of the His residues enabled the Na^+/H^+ exchanger-null PS 120 fibroblasts to survive "acid-killing" treatment, suggesting that these mutants retained their Na^+/H^+ exchange transport activity. We initially determined whether the His mutations affected expression of NHE3 protein in PS120 cells by Western immunoblot analysis using mP5D4 (Fig. 2*A*). Mutated NHE3s were all expressed well, although at different levels relative to GAPDH expression, as shown in Fig. 2*A* and quantitated in Fig. 2*B*. Equal loading of the samples was checked by determining GAPDH protein expression level as a control (Fig. 2*A*: lower panel). The relative total protein expression of H479L, H479D, H485Q, H485L, H499L and H499D normalized to that of wild-type NHE3 expression were 51.6 ± 6.5%, 91.4 ± 11.2%, 113.4 ± 14.3%, 114.7 ± 16.9%, 65.7 ± 8.0%, and 88 ± 11.0%, respectively (Fig. 2*B*). The substitution at His-479 and His-499 led to significant decrease in protein expression level, although a similar mutation at His-485 did not affect protein expression.

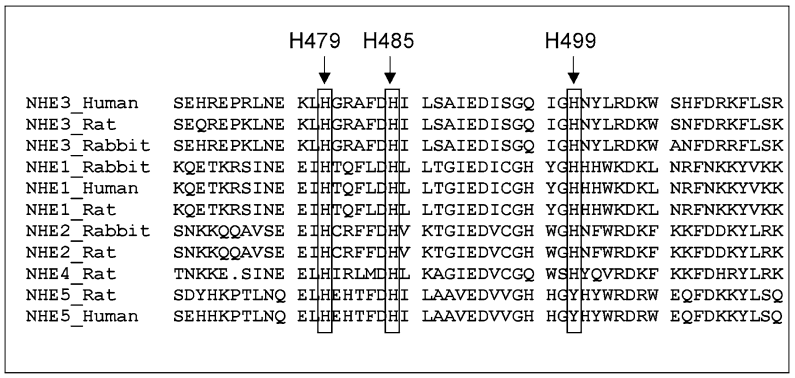

Fig. 1. Amino acid sequence alignment of conserved His in the juxtamembrane region of the mammalian NHE gene family 1–6 cytoplasmic domain. The conserved Histidines (His-479, His-485, and His-499 using NHE3 aa numbering) are highlighted. Accession numbers for the sequences, top to bottom, are P48764, P26433, P26432, P23791, P19634, P26431, P50482, P48763, P26434, Q9Z0X2, and Q14940.

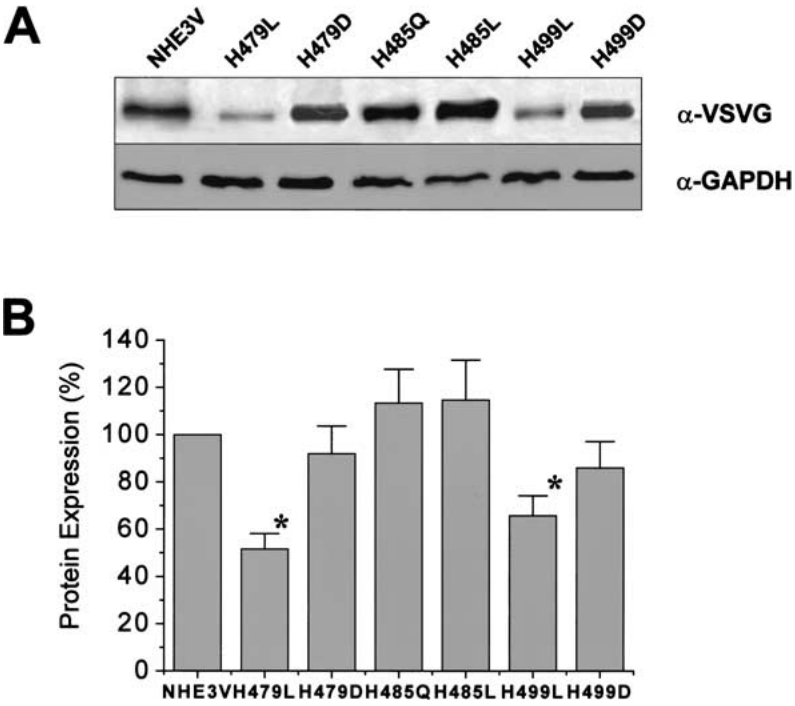

Fig. 2. Relative protein expression levels of wild-type NHE3 and His mutants. (*A*) Western blot analysis was performed on total cell lysates of PS120 cells transfected with wild-type NHE3V and mutated NHE3V cDNAs. The total protein expression was evaluated by Western blot analysis on 20 μg of total lysate from transfected PS120 cells using mP5D4 (upper panel). A representative figure from three experiments is shown. Relative loading was determined by reblotting the blot using α-GAPDH antibody (lower panel). (*B*) Expression levels of NHE3V and mutants were quantitated by densitometric scanning. The protein expression levels from three separate experiments are normalized against wild-type NHE3V protein level. Results are presented as the mean ± SE, $n \geq 3$. *$P < 0.05$ *vs.* NHE3V.

To determine whether the decreased expression in H479L and H499L is due to aberrant cellular expression of NHE3 protein, confocal microscopy was used to determine cellular distribution of NHE3V and the His mutants expressed in PS120 fibroblasts. Figure 3 shows the immunofluorescence images of NHE3 expression in PS120 cells. Figure 3*A* shows wild-type NHE3V, which is localized to the juxtanuclear region and the plasma membrane, as previously determined (D'Souza et al., 1998; Janecki et al., 2000). H479L shows a similar pattern of expression although it is expressed at lower level than wild type (Fig. 3*B*). Similarly, H485Q mutant showed the typical juxtanuclear and plasma membrane staining (Fig. 3*C*). H479D, H485L, H499L and H499D mutants also showed similar NHE3 distributions (*data not shown*). Figure 3*D* shows the lack of immunofluorescence in untransfected PS120 cells in comparison. These results qualitatively show that the expression pattern of the His mutants is similar to that of wild-type NHE3V.

Effect of His Mutants on NHE3 Activity

In order to investigate the specific effect of mutation on the His residues, the Na^+/H^+ exchange rates were determined over a range of intracellular pH_i values by measuring Na^+-induced recovery from an acid load for PS120/NHE3V and the NHE3 His mutants. The relative maximum activity, V_{max}, of H479L, H479D (Fig 4*A*), H499L and H499D (Fig. 4*C*) mutants was significantly decreased compared to wild-type NHE3V, whereas the transport rates were not affected by the H485Q and H485L mutations (Fig. 4*B*). The V_{max} was decreased by approximately 70% in H479L, 50% in H479D, 50–60% in H499L and H499D, relative to wild type NHE3V.

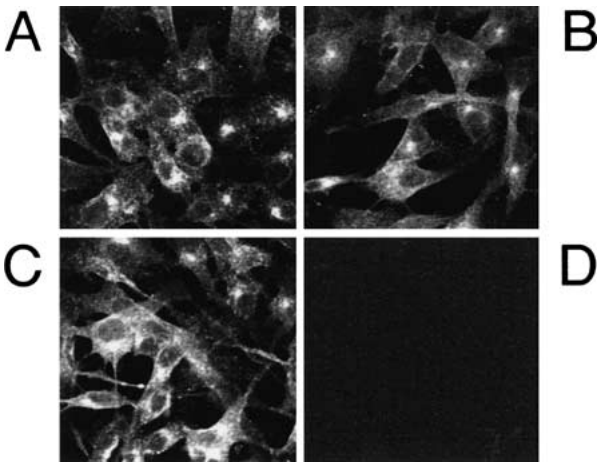

Fig. 3. Immunofluorescence images of wild-type NHE3V and its His mutations. (*A*) Wild-type NHE3V as a positive control was localized to the juxtanuclear region and to the plasma membrane. (*B*) H479L mutant cells showed a lower expression level than the control NHE3V but had a similar distribution. (*C*) H485Q mutant cells yielded slightly stronger fluorescence signals than the control NHE3V but had a similar distribution. (*D*) Untransfected PS120 cells as a negative control show only a very weak background.

All mutants studies retained the allosteric activation, as shown in Fig. 4*A–C*. In all cases but one, the Hill coefficients were between 2 and 3, indicating that these His residues are not necessary for the function of the H^+-modifier site, and probably do not constitute the major part of the H^+-modifier site. In H499D, the pH-dependent Na/H exchange profile was steeper than the NHE3V control of other His mutants.

The set point is defined as the pH_i value above which the exchanger is inactive, and often correlates with the steady-state pH. At this pH, H^+ efflux from the cells is thought to be balanced by H^+ influx plus H^+ production within the cell. As pH_i falls below this value, Na^+/H^+ exchanger gradually becomes activated. Therefore, the set points can be obtained from pH_i ($V = 0$) when the rate of transport of Na^+/H^+ exchange equals zero. The set point of wild-type NHE3 is about 7.4–7.5 (Fig. 4*A*) (Levine et al., 1993, 1995; Tse et al., 1993). The set points of the H485Q and H485L mutants were not significantly affected (~7.2–7.4) by the mutations (Fig. 4*B*), whereas the set points of H479L, H479D, H499L and H499D were shifted to more acid values (Fig. 4*A*, *C*).

The set points often correlate with the steady-state pH_i. The shift in the set point observed above was confirmed by determining the steady-state pH_i without acidification. The steady-state pH_i was determined by exposing cells to Na^+ medium for 20 min without initial acidification. The results obtained this way are consistent with the acid shift by the substitutions in His-479 and His-499 in Fig. 4. Interestingly, the steady-state pH of H485Q was marginally lower than the control.

Cell Surface Biotinylation

Substitution of His-479 and His-499 to Leu significantly decreased the Na^+/H^+ exchange rate and the total NHE3 expression level. We sought to determine whether the decreased Na^+/H^+ transport could be attributed to decreased surface expression of NHE3 protein. The surface NHE3 expression was measured by cell surface biotinylation and representative results are shown in Fig. 5. Qualitative comparison of the surface fractions against the total fractions suggests that the surface expression of H479L and H499L is significantly lower compared to the control NHE3V. In Fig. 5*C*, NHE3V protein in the surface fraction is represented as percentage of the total NHE3V. The surface membrane fraction of wild-type NHE3V was estimated as 9.8 ± 1.0%, which is in agreement with previous results (Lee-Kwon et al., 2001). The surface membrane fractions of H479L, H485Q, H485L, and H499L were estimated as 4.6 ± 1.3%, 16.9 ± 2.2%, 14.2 ± 2.8% and 6.9 ± 1.7%, respectively. These results show that H479L and H499L mutation significa ntly lowers the surface expression ($P < 0.05$ compared to wild-type NHE3V). However, the small increase in the surface fraction in H485L and H485Q is unexpected, since both the transport rate and the total expression level were comparable to those of the NHE3V control. The reason for this increase is not known.

ATP Depletion

Previous studies have shown that ATP depletion results in a shift in the set point of Na/H exchangers. We therefore determined whether the shift in the set point by the mutations H479L and H499L shares a common mechanism with ATP depletion. Consistent with the previous results (Wakabayashi et al., 1992; Levine et al., 1993; Demaurex et al., 1997), ATP depletion resulted in a drastic decrease in Na^+/H^+ exchange activity in PS120/NHE3V cells (Fig. 6*A*). The basal NHE3 transport rate in the absence of ATP was decreased by 87% at pH_i 6.2. The set point also shifted by ~0.6 pH units to a more acidic region in the absence of ATP (Fig. 6*C*).

ATP depletion exhibited similar drastic effects on the H479L mutant by decreasing the transport rate by 91% at pH_i 6.2 (Fig. 6*B*) and shifting the set point by ~0.4 pH unit to a more acidic region (Fig. 6*D*). Other His mutants (H479D, H485Q/L and H499L/D) also showed similar shifts by ATP depletion (*data not shown*), suggesting that the mutation of H479 and H499 affects the set point by a mechanism different from ATP depletion.

Discussion

We describe here our initial study to identify amino-acid residues involved in determining the pH_i sensi-

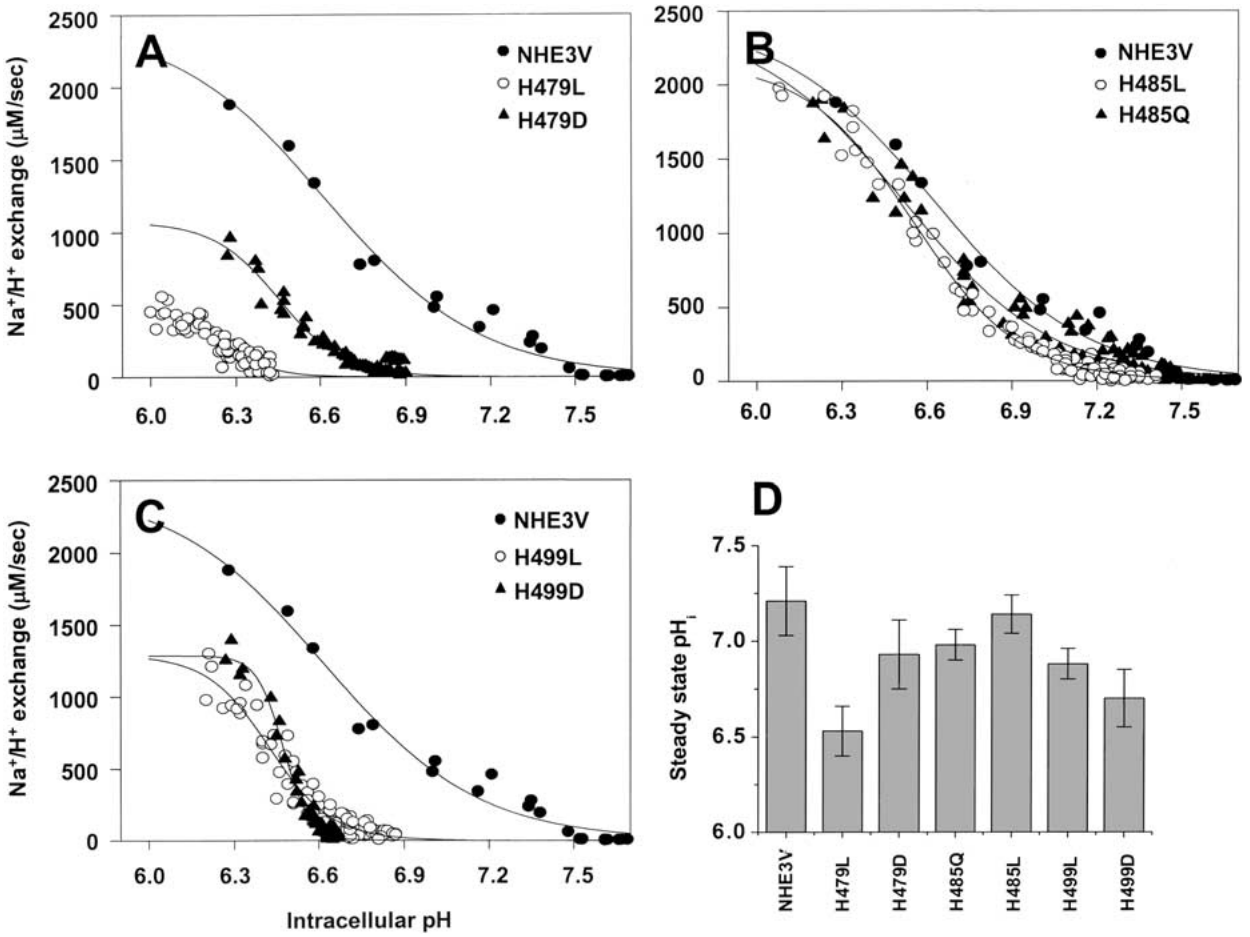

Fig. 4. NHE3 exchanger activity of the control NHE3V and His-mutants in PS120 cells. PS120 fibroblasts expressing NHE3V control or mutant NHE3V were loaded with BCECF, and the Na-dependent pH recovery rate was determined as described in Materials and Methods. Na^+/H^+ exchange rate *vs.* pH_i of control NHE3V and the His mutants is shown. (*A*) H479L and H499L. (*B*) H485L and H485Q. (*C*) H499L and H499D. (*D*) The steady-state pH_i wild-type NHE3V and the His mutants. Values are calculated from at least five runs for each exchanger and are shown as mean ± SE. $^*P < 0.01$ *vs.* wild type NHE3V.

tivity and H^+-modifier site in the Na^+/H^+ exchanger. This study reveals two important results: (1) His-475 and His-499 but not His-485 located in the juxtamembrane domain are important in determining the pH_i sensitivity of NHE3. (2) Mutations of His-479 and His-499 failed to alter the H^+-modifier site or allosteric activation of NHE3, suggesting independent determinants for pH_i sensitivity and H^+-modifier site, which is thought to be the hallmark of the allosteric activation of Na^+/H^+ exchangers.

In this study, we mutated three highly conserved histidine residues (His-479, His-485, and His-499) in the cytoplasmic domain of NHE3 and determined their effects on NHE3 transport activity. All the mutants studied expressed functional Na^+/H^+ exchangers as evident by their survival of acid-killing procedures. Confocal immunofluorescence microscopy showed that all the mutant NHE3s exhibited a cellular distribution similar to wild-type NHE3. However, quantitative Western immunoblot revealed that the H475L and H499L mutants were expressed at a significantly lower level than wild-type NHE3. We do not know the reason for the lowered expression levels of H479L and H499L. The decreased expression level in H479L and H499L is probably a specific effect of the replacement by Leu, since the substitution with Asp did not affect the expression level. In contrast, the H485L/Q mutations did not appear to affect NHE3 activity or expression.

Consistent with the lowered expression levels, the transport rates were lowered with the H479L and H499L mutations. The effect on individual protein molecules could not accurately be determined in the present studies, given the potential error for both immunoblot analysis and the surface biotinylation. However, when the surface expression levels were taken into account, the normalized V_{max} per surface mutant NHE3 was similar to that of NHE3V control. For example, both the total expression and percent surface expression of the H475L mutant were about 50% of the control. The amount of surface H475L per mg of total protein is hence one quarter of wild-type

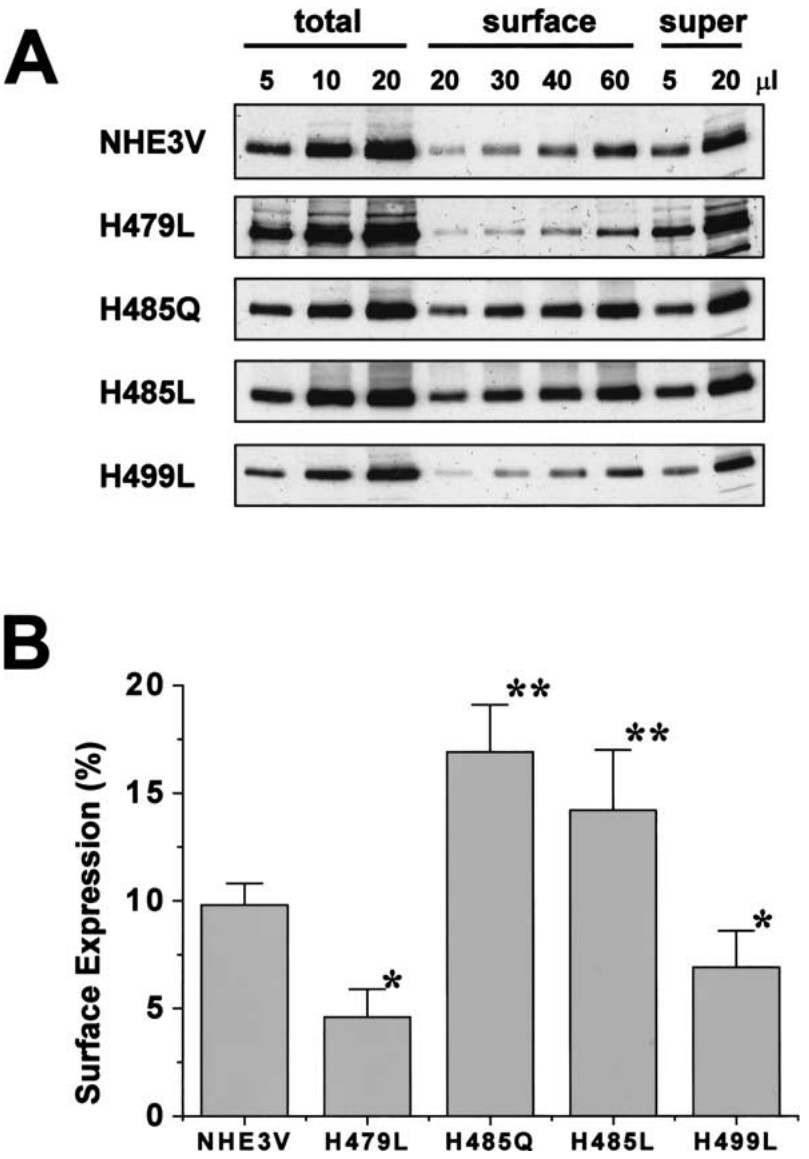

Fig. 5. Cell surface biotinylation of wild-type NHE3V and its His-mutants. (*A*) Representative Western blots of control NHE3V and His mutants using mP5D4 are shown. Quantification was achieved by running diluted fractions of total, surface, and intracellular fractions to give a linear range of signals, as described in Materials and Methods. (*B*) Summary of surface expression levels (%) of the control NHE3V and its His mutants, H479L, H485Q, H485L, and H499L. Experiments were repeated at least three times for each exchanger and data are shown as mean ± SD. $*P < 0.05$ for H479L and H499L and $**P < 0.1$ for H485Q and H485L.

control. Consistently, the V_{max} of the H475L mutant was about 25–30% of wild-type control, and the estimated transport rates per the surface NHE3 are then similar in the control and H479L. Similarly for the H499L mutant, the 40% decrease in V_{max} was accompanied by concomitant decrease in the percent surface expression by 30% and total NHE3V expression by 33%. Therefore the normalized V_{max} per surface protein of the H479L and H499L mutants was similar to NHE3V control within the experimental limitation.

The substitution of His-479 and His-499 shifted the pH profile to a more acidic region. In contrast, replacing His-485 with Leu or Gln did not exhibit any significant effect on the pH profile of the mutant NHE3. Given the proximity of His-485 to His-479 and H499, it is unlikely that the effects of the mutations at H479 and H499 stem from gross conformational aberration caused by the amino-acid substitutions. The specific effects of the H479 and H499 are also supported by the recent studies, in which the His-rich cluster of amino acids 540–545 (HYGHHH) of NHE1 was mutated to HHHHHH, HYGAAA, and HYGRRR. The mutation in NHE1 did not affect the set point of NHE1 but resulted in a decreased maximal transport rate (Dibrov et al., 2000).

These results suggest that His-479 and His-499 are important in determining the pH sensitivity and that this is probably achieved by altering H^+ binding by NHE3. However, the allosteric activation of the exchanger by intracellular H^+ was still preserved in His mutants, indicating that His-479 and His-499 probably do not constitute the H^+-modifier site. This preservation of the allosteric nature of the exchanger

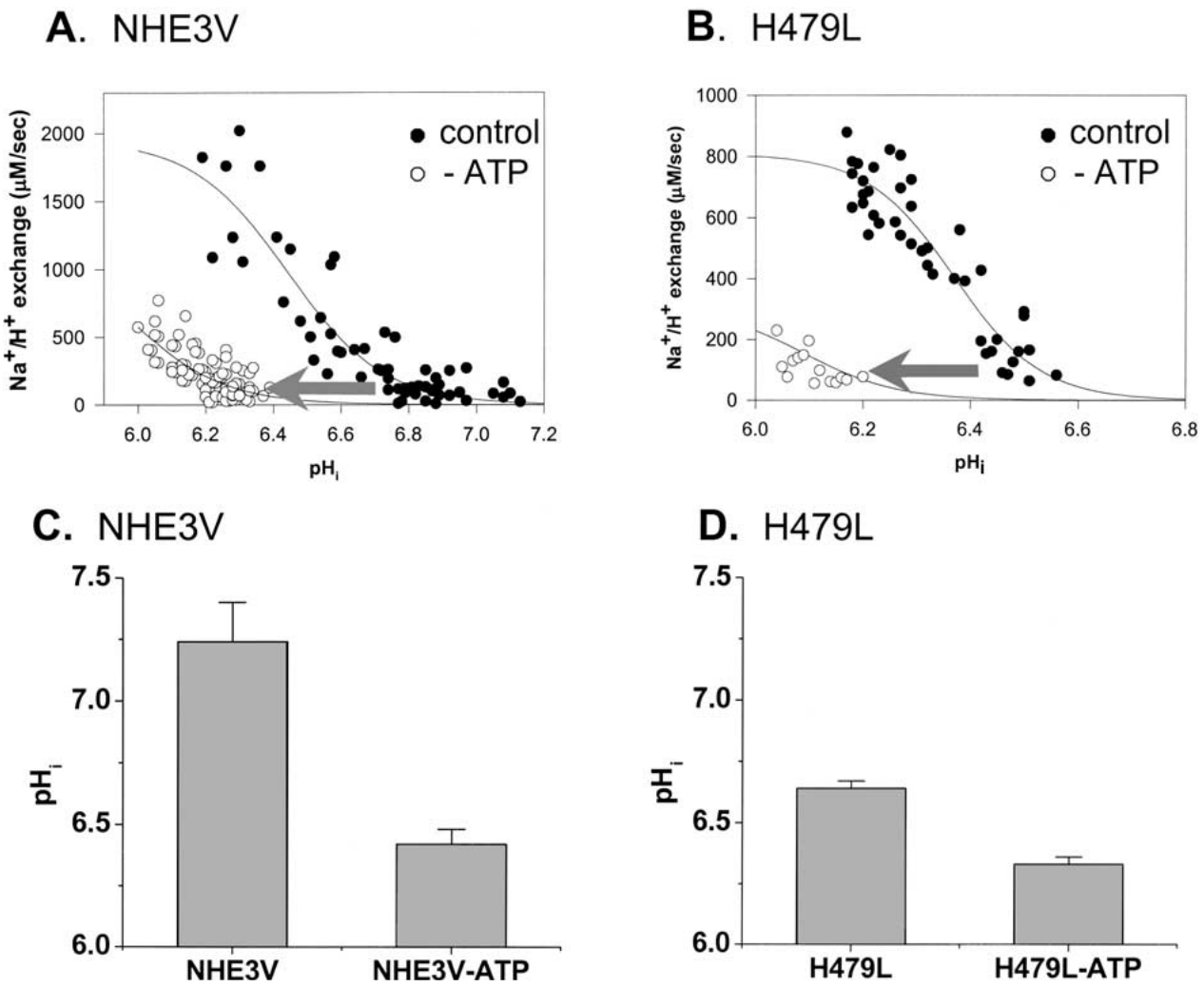

Fig. 6. Effect of ATP depeletion on Na^+/H^+ exchange rates in PS120 cells transfected with the control NHE3V and its H479L mutants. The rate of NHE3 activity was studied in PS120 cells transfected with (*A*) wild-type NHE3V or (*B*) H479L mutant either untreated (*control*) or subjected to ATP-depletion (*−ATP*). The set-point change by ATP depletion was determined in (*C*) wild type and in (*D*) H479L. Values were determined from at least five measurements and are shown as mean ± SE.

was not surprising, based on previous reports that the allosteric behavior was retained after almost complete removal of the cytoplasmic C-terminus (Wakabayashi et al., 1992a; Levine et al., 1995). In these previous works, deletion of amino acids 516–595 (aa 472–551 in NHE3) in the cytoplasmic domain of NHE1 led to an acidic shift of resting pH_i by ~0.8 pH units (Wakabayashi et al., 1992a; Levine et al., 1995; Ikeda et al., 1997a).

We also compared effects of mutation at His-479 and His-499 with ATP depletion, which was previously shown to inhibit Na^+/H^+ exchange and cause similar acid shifts (Levine et al., 1993; Wakabayashi et al., 1992; Demaurex et al., 1997). The mutations at His-475 and His-499 failed to prevent the effect of ATP depletion, suggesting that the His mutation and ATP depletion affect the set point by different mechanisms. Although mechanisms underlying ATP depletion are not fully resolved, hydrolysis of ATP is needed for the synthesis of phosphotidylinositol 4,5-biphosphate (PIP_2), which directly interacts with Na^+/H^+ exchangers (Aharonovitz et al., 2000). Based on the current findings, it seems unlikely that His-475 and His-499 are involved in interaction with PIP_2.

It is logical to assume that the protonation state of the imidazole ring of His contributes to the pH sensitivity of NHE3. However, in addition to PIP_2, NHE3 interacts with other associated proteins, including NHERF, calmodulin, CHP (Yun et al., 1998; Nath et al., 1999; Aharonovitz et al., 2000; Pang et al., 2001). Because this region is highly α-helical, His-479 and His-499 may play a direct role in interaction

with associated proteins. In particular, CHP interacts with Na^+/H^+ exchangers via a hydrophobic domain overlapping His-479 and His-485, and disruption of CHP binding drastically reduces Na^+/H^+ exchange activity (Pang et al., 2001). Because the α-helices in the region are highly amphipathic, the α-helices may have a dual role, one interacting with CHP or other associated proteins and the other interacting with the membrane domain of Na^+/H^+ exchangers. Further studies will be necessary to determine the molecular basis of the pH sensitivity by the His-479 and His-499.

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