Glutamate 346 of Human Na^+-H^+ Exchanger NHE1 Is Crucial for Modulating both the Affinity for Na^+ and the Interaction with Amiloride Derivatives†

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ABSTRACT: A NHE1 variant that exhibits very high resistance to (3-methyl sulfonyl-4-piperidinobenzoyl) guanidine methane sulfonate (HOE694), a potent inhibitor of Na^+-H^+ exchangers, was selected and characterized. Sequencing of the coding region corresponding to the N-terminal domain of this variant revealed the presence of only one mutation located within membrane-spanning segment 9 (M9). This base pair change replaces a glutamate (Glu) with an aspartate (Asp). We reproduced this amino acid change in wild-type NHE1 and found that this mutation alone is responsible for the huge decrease in sensitivity to the HOE694 compound and to ethylisopropylamiloride (EIPA). We found that the NHE1-Glu_{346}Asp mutant was more than 2000-fold more resistant to HOE694 and up to 300-fold more resistant to EIPA than wild-type NHE1, with the size, rather than the charge, of the amino acid in position 346 having the greatest effect. Interestingly, its affinity for Na^+ was at least 4-fold lower than that of wild-type NHE1. Mutation of amino acids in the vicinity of Glu_{346} did not change the sensitivity of mutated NHE1 proteins to inhibitors. We suggest there is a direct interaction of Glu_{346} or involvement of Glu_{346} in a coordination site with NHE inhibitors and with Na^+.

To date, nine members of the mammalian Na^+-H^+ exchanger family have been cloned [see recent publications for NHE7−9 (*1*−*3*) and reviews for NHE1−6 (*4*−*7*)]. Although they all catalyze an electroneutral and reversible exchange of one Na^+ for one H^+, they exhibit differences in cell membrane localization, tissue expression, regulation by extracellular agents, and sensitivity to amiloride and its analogues. It was the last property that first suggested to Clark and Limbird that more than one member of the Na^+-H^+ exchanger family is present in the kidneys and intestine (*8*), and this property is still used today to facilitate the identification of NHE[1] isoforms in new cell types.

By using molecular tools and heterologous systems, it has been clearly shown that NHEs exhibit marked differences in sensitivity to amiloride and its derivatives, as well as to HOECHST 694 (HOE694) and HOE642 (cariporide) (*4*, *9*−*12*), the relationship being as follows: NHE1 > NHE2 > NHE5 > NHE3 > NHE4. Other pharmacological agents such as cimetidine, clonidine, and harmaline also inhibit NHEs, with NHEs showing marked differences in sensitivity (*11*, *13*−*18*).

Upon characterization, a variant of hamster fibroblast NHE1 was found to be 5-fold more resistant to amiloride and 30-fold more resistant to MPA than wild-type NHE1. This resistance was found to be conferred by the Leu_{167}Phe mutation within membrane segment 4 (M4), which corresponds to the Leu_{163}Phe mutation of human NHE1 (*19*). Random mutagenesis followed by selection of resistant Na^+-H^+ exchangers revealed that mutation of Gly_{174} in human NHE1 to serine also induces resistance to amiloride, and that the Leu_{163}Phe and Gly_{174}Ser double mutation produces an even more resistant protein (*20*). Mutations of other amino acids of NHE1's M4, found to be present in NHE2, were also shown to modify amiloride sensitivity (*21*). Incorporating a phenylalanine, which is present in NHE3, in place of a leucine in position 163 of human NHE1 slightly decreases the sensitivity to amiloride and MPA but does not reproduce the typical pharmacological profile of NHE3 (*19*). These results suggest that although M4 is clearly involved in the interaction with inhibitors, other amino acids and possibly other membrane segments may also be involved in the interaction of NHEs with inhibitors (*19*, *21*). This hypothesis is supported by recent studies of spontaneous and revertant mutants showing that at least three other amino acids of human NHE1 M4, Phe_{162}, Ile_{169}, and Ile_{170}, are involved in sodium binding and in cariporide interactions (*22*). Furthermore, the functional analysis of conserved amino acids, and particularly polar residues of NHE1, revealed that Glu_{262} (*23*−*25*) and Asp_{267} (*25*) of M7 are critical to both Na^+-H^+ exchanger activity and 5-(*N*,*N*-hexamethylene)amiloride

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[1] Abbreviations: EIPA, ethylisopropylamiloride; HOE694, (3-methyl sulfonyl-4-piperidinobenzoyl) guanidine methanesulfonate; HR, HOE694-resistant; M, membrane segment; MPA, methylpropylamiloride; NHE, Na^+-H^+ exchanger; PS120, lung fibroblast cell line deficient in Na^+-H^+ exchange activity; VSVG, vesicular stomatitis virus glycoprotein.

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(HMA) interaction. Studies using site-directed mutagenesis (*25*−*27*) and NHE1−NHE3 chimeras (*15*) have demonstrated that transmembrane segment M9, particularly histidine in position 349 of human NHE1 (*26*) and glutamate 350 and glycine 356 of rat NHE1 (*27*), is involved in the interaction of NHE1 with inhibitors. The glutamate in position 391 of human NHE1 was also shown to be important (*25*), while adjacent amino acids did not seem to have an effect on NHE activity. This amino acid is part of the membrane-associated segment between M9 and M10, according to the topology proposed by Wakabayashi and co-workers (*6*). These observations point to the importance of many different residues within the membrane domain of NHE1 in promoting an optimal interaction of NHE1 with Na^+ and with inhibitors. In addition, different domains of NHE1 may be associated with a pore region of the transporting NHE1. Recent findings support this hypothesis; we (J. Noël, unpublished results) and others (*27*) found synergistic effects when amino acids of both M4 and M9 were mutated. These observations are consistent with the existence of two partial transmembrane domains between M4 and M5 (IL1), and between M8 and M9 (IL2) (*6*). Their exact role has yet to be determined, but some mutants within IL2 have been shown to inhibit exchange activity (*6*).

Whereas charged amino acids exhibited the expected special significance for Na^+ binding and transport, other hydrophobic amino acids have also been shown to be critical for optimal activity and inhibitor interaction (*22*). The Na^+ binding site and Na^+ coordination by the NHE1 protein probably involve many amino acids. Interaction of NHE inhibitors with NHEs is modified when the concentration of Na^+ increases, suggesting that the binding sites for Na^+ and NHE inhibitors are identical or close to each other (*28*). Accordingly, changes in the affinity of NHE1 for Na^+ were expected for the mutants identified above. However, only a few studies have in fact reported a modified affinity for Na^+. Indeed, apart from the spontaneous NHE1-Phe_{162}Ser mutant that shows a dramatic impairment in Na^+ affinity (248 mM vs 23 mM) (*22*), only the combination of the Leu_{163}Phe and Gly_{174}Ser mutations has been shown to slightly affect the affinity for Na^+ (K_m = 28 mM instead of 14 mM) (*20*).

Having selected variants of human NHE1 that exhibit a 1500−2000-fold decrease in sensitivity to HOE694 and a 200-fold decrease in sensitivity to MPA, the purpose of this study was to determine whether this high-resistance phenotype could be explained by the presence of mutated amino acid(s), and whether this highly amiloride-resistant mutant exhibits a modified affinity for Na^+. Sequencing of the entire transmembrane region of the NHE1 variant revealed the single Glu_{346}Asp mutation. We reproduced this mutation in wild-type NHE1 by site-directed mutagenesis, and then studied the sensitivity of this protein to HOE694 and to other amiloride analogues, as well as its affinity for extracellular Na^+, Li^+, H^+, and K^+. Our results indicate that the Glu_{346}Asp mutation induces a significant change in the interaction of amiloride derivatives with NHE1, and also a significant change in the affinity of NHE1 for Na^+. It is noteworthy that the fibroblast NHE1-transfected HR300 variant identified as NHE1-Glu_{346}Asp allowed us to identify glutamate in position 346 as a highly critical amino acid involved in NHE1 interaction with Na^+ and its NHE inhibitors. This very important role of glutamate 346 was further confirmed by demonstrating that mutation of amino acids in the proximity of Glu_{346} did not affect the sensitivity to inhibitors.

Data related to the identification of the NHE1-Glu_{346}Asp mutant were previously presented in abstract form at the American Society of Nephrology (*29*).

EXPERIMENTAL PROCEDURES

Material. ^{22}NaCl, [^{32}P]dCTP, and [α-^{32}S]dATP were purchased from DuPont NEN (Boston, MA). Hybond N and Hybond C extra were purchased from Amersham Corp./USB (Oakville, ON). The BioProbe RNA reagent kit and purified biotinylated-503 primer were purchased from Eurogentec (Seraing, ON). Restriction enzymes, heat-resistant Vent^R polymerase, and MuLV reverse transcriptase were obtained from New England Biolabs, Inc. (Beverly, MA). Streptavidin-linked magnetic beads were purchased from Dynal (Oslo, Norway). Amiloride, methylpropylamiloride (MPA), ethylisopropylamiloride (EIPA), trypsine-EDTA, ampicillin, cool calf serum (CS), ouabain, and cimetidine were obtained from Sigma-Aldrich Canada Ltd. (Oakville, ON). The (3-methyl sulfonyl-4-piperidinobenzoyl) guanidine methane sulfonate (HOE694) compound was kindly provided by H. J. Lang (Hoechst AG, Frankfurt am Main, Germany). We used the NHE1 polyclonal antibody (RPc28 antibody) that recognizes the last 157 amino acids at the C-terminus of human NHE1 (*30*) and the P4D5 antibody that recognizes the vesicular stomatitis virus glycoprotein (VSVG) epitope. Horseradish peroxidase-conjugated goat anti-rabbit IgG was obtained from Jackson Immuno Resarch Laboratories (West Grove, PA) and DMEM high glucose from Canadian Life Technologies, Inc. (Burlington, ON). The BCA kit for determining protein concentration was from Pierce (Rockford, IL). All other chemicals and reagents used in these experiments were purchased from Fisher Scientific (Nepean, ON), and were of the highest quality available.

Cell Lines and Culture. PS120 is a Chinese hamster lung fibroblast clone that lacks Na^+−H^+ exchange activity (*18*) and was derived from the CCL39 cell line (ATTC). These cells were transfected with wild-type human NHE1 or mutated NHE1 cDNAs (see below). Fibroblasts expressing the VSVG-tagged NHE3 isoform (*31*) were cultured and studied in parallel. Modified Eagle's medium (H21, Gibco) containing 25 mM $NaHCO_3$ was supplemented with penicillin (50 units/mL), streptomycin (50 μg/mL), and 7.5% cool calf serum (CS). Cells were grown at 37 °C in the presence of 5% CO_2.

Selection of HOE694-Resistant Variants. PS120 lung hamster fibroblasts expressing the wild-type human NHE1 were submitted to repeated acid loadings in the presence of 50 mM NH_4Cl at 37 °C, and in the absence of bicarbonate and CO_2, as reported previously (*19*). After incubation for 1 h, the cells were quickly washed in a sodium-free medium and incubated for 1 h at 37 °C in a recovery medium, in the absence of CO_2 and in the presence of 120 mM NaCl, with or without 30 μM HOE694, to inhibit NHE1 activity (*31*). After removal of this artificial medium, a bicarbonate (sodium)- and calf serum-containing DMEM medium (pH 7.4) was added and the cells were incubated at 37 °C in the presence of CO_2 to let them recover.

Under these conditions, more than 99% of the cells died after the first test. Few colonies nevertheless survived three

Table 1: List of Amino Acid Substitutions Engineered in Wild-Type Human NHE1

mutation	codon change	oligonucleotide (sense)
$Leu_{343}Val$	TTG ⇒ GTC	5′ GC TAC ATG GCC TAC GTC TCA GCC GAG CTC 3′
$Leu_{343}Trp$	TTG ⇒ TGG	5′ GC TAC ATG GCC TAC TGG TCA GCC GAG CTC 3′
$Ala_{345}Gly$	GCC ⇒ GGC	5′ G GCC TAC TTG TCA GGC GAG CTC TTC CAC CTG 3′
$Ala_{345}Trp$	GCC ⇒ TGG	5′ G GCC TAC TTG TCA TGG GAG CTC TTC CAC CTG 3′
$Glu_{346}Asp$	GAG ⇒ GAC	5′ TTG TCA GCC GAC CTC TTC CAC 3′
$Glu_{346}Gln$	GAG ⇒ CAG	5′ TTG TCA GCC CAG CTC TTC C 3′
$Leu_{347}Val$	CTC ⇒ GTC	5′ C TTG TCA GCC GAG GTC TTC CAC CTG TCA GG 3′
$Leu_{347}Trp$	CTC ⇒ TGG	5′ GCC TAC TTG TCA GCC GAG TGG TTC CAC CTG TCA GGC 3′

similar acid loading procedures performed every 2−4 days. Although obtaining more than one colony could appear to be surprising, these different colonies may come from identical cells issued from one original colony or represent cells expressing a large amount of wild-type NHE1 that is resistant to 30 μM HOE694 (*31*). Cells were then trypsinized and submitted to an acid load with recovery in the presence of 100 μM HOE694 to ensure that no sensitive (wild-type) cells were hidden by resistant ones and to allow a complete blockade of NHE1 exchange activity. Following two more acid loading procedures in the presence of 100 μM HOE694, many colonies resisted. They were again trypsinized and submitted to three acid loading procedures in the presence of 300 μM HOE694 in the recovery medium. This cell population following trypsination was stable, with no cells dying following repeated acid loading procedures. Finally, the same protocol was repeated in the presence of 1000 μM HOE694, with the overall procedure taking place over a period of 3 weeks. The different cell populations were named after their HOE694-resistant phenotype: HR100, HR300, and HR1000, respectively. After each step in the selection process, resistant cells were frozen at −80 °C until further analysis. A very stable resistance to very high inhibitor concentrations was observed for the HR100 and HR300 cell populations, but many cells within the HR1000 population lost their resistance and died during the selection process.

Northern Blot Analysis. Total RNA was obtained from cells expressing the NHE1 variants using the BioProbe kit, then submitted to agarose electrophoresis under denaturing formaldehyde conditions, transferred to Hybond N nitrocellulose membranes, and probed at 65 °C for 16 h with the [^{32}P]dCTP-labeled 570 bp *Pst*I−*Sac*I NHE1 insert or the [^{32}P]dCTP-labeled 1290 bp *Pst*I−*Eco*RI NHE3 insert. We loaded 30 μg of total RNA onto the gel.

RT-PCR. Total RNA (1 μg) extracted from the wild-type NHE1 and NHE1 variants was reverse transcribed at 42 °C in the presence of 2.5 mM $MgCl_2$ using a specific NHE1-encoding primer (310; 5′-TgggAgTATgCgCTCggAAgg), the Boehringer Mannheim cDNA synthesis kit, and 2 units of the MuLV reverse transcriptase. PCR was carried out on one-tenth of the cDNA reaction mixture using antisense NHE1 primer 310 and a biotinylated sense NHE1 primer (503-B; gggCTgCTgCCTgTTCTCAgg). The amplified 1785 bp biotinylated fragment was isolated with streptavidin-linked magnetic beads and denatured in 0.1 N NaOH, and the trapped biotinylated single-strand cDNA was sequenced according to the manufacturer's recommendations (Pharmacia kit) using specific human NHE1 primers covering the entire transmembrane domain (*32*).

Western Blot Analysis. Crude membranes were prepared in the presence of protease inhibitors from stable NHE1 variants or stable transfectants grown to 80−90% confluency, as described previously (*23*). A total of 20 μg of protein was loaded in each lane. Proteins were separated by SDS−PAGE (7.5%, w/v) and electrophoretically transferred to Hybond C extra-supported membranes. Membranes were blocked as described previously. The proteins were incubated with RPc28 antibodies overnight at 4 °C, and then revealed with horseradish peroxidase-conjugated goat anti-rabbit antibodies. The Western blots were developed using the enhanced chemiluminescence (ECL) detection system.

Site-Directed Mutagenesis. Site-directed PCR mutagenesis was performed with human NHE1, specific base pair oligonucleotides (Table 1), and high-fidelity temperature-resistant Vent polymerase**.** The amplified fragments were digested by the *Acc*I restriction enzyme and subcloned back in the eukaryote compatible pECE vector containing the full-length digested wild-type NHE1. The fragment junctions, as well as the entire PCR-generated cDNA fragments, were sequenced to ensure no random mutations had been introduced during the amplification procedure.

Transfection and Selection of Stable Na^+−H^+ Exchanger-Expressing Transfectants. PS120 fibroblasts (1×10^5 cells per 100 mm plate) deficient in Na^+−H^+ exchanger activity were plated and cultured in 7.5% CS containing DMEM for 20 h, and then transfected with 10 μg of plasmid containing wild-type or mutated NHE1 encoding cDNAs using the calcium phosphate−DNA coprecipitation technique (*33*). Forty-eight hours following transfection, a first acid loading test (see above and ref *34*) was performed to select only cells expressing an active membrane-bound Na^+−H^+ exchanger. Four other similar tests were performed over a period of 2 weeks to obtain cells homogeneously overexpressing the NHE1 proteins. Subsequently, an acid loading procedure was performed every 1−2 weeks to ensure the stability of NHE1 expression.

$^{22}Na^+$ Uptake. $^{22}Na^+$ uptake was assessed on fibroblasts seeded in 24-well plates at a density of 1×10^5 cells/well and grown for 2 days to confluence. An acid load was generated by the NH_4^+-prepulse technique, as previously described (*23*, *31*, *34*, *35*). Cellular $^{22}Na^+$ accumulation was stopped, as required, after incubation at 37 °C for 30 s (for Na^+, H^+, and K^+ affinity experiments) or 3 min (for dose−response curves) by four rapid washes in ice-cold saline. Cells were solubilized with 0.1 N NaOH, and radioactivity was counted in a γ-spectrometer. Protein concentrations were measured using the BCA assay with albumin as the standard.

RESULTS AND DISCUSSION

Pharmacological Profiles of the Spontaneous NHE1 Variant. PS120 cells expressing the NHE1 isoform and that had, quite unexpectedly, survived the five acid loading procedures

performed in the presence of either 100, 300, or 1000 μM HOE694 in the recovery medium were studied for their transport activity in the presence of different concentrations of NHE inhibitors amiloride, MPA, and HOE694. For purposes of comparison, we also examined PS120 cells expressing wild-type NHE1.

Figure 1A presents the pharmacological profile for amiloride of wild-type NHE1 and NHE1 variants resistant to 100 μM HOE694 (HR100) and to 300 μM HOE694 (HR300). For clarity, the HR1000 data were not included since the inhibitory profiles were identical to those of HR300, in addition to the lack of stability of this population. Figure 1 shows that the HR300 variant was much more resistant to NHE inhibitors than wild-type NHE1 for all three inhibitors, and that the difference in sensitivity was specific to each inhibitor. The difference in sensitivity was especially astonishing for the HOE694 compound (Figure 1B), the IC_{50} being ~1000−2000-fold greater than that of wild-type NHE1. These results reflect differences in inhibitor conformation and, on the basis of previous studies (*19*−*21*), suggest differences in the interaction with specific amino acids of NHE1. This is the first time that such a significant difference between the sensitivity of mutants to NHE inhibitors and that of wild-type NHE1 has been observed. The IC_{50} of the HR300 variant for the HOE694 compound is in fact very close to the IC_{50} of the amiloride-resistant NHE3 for this inhibitor (*10*, *36*).

The HR100 population, which is resistant to 100 μM HOE694, tended to be less resistant than the HR300 population, and the flattened curve suggests a mixed population of Na^+-transporting molecules. This population was not analyzed further. Similarly, since the HR1000 population presented a less stable phenotype in the presence of the HOE694 compound, only the HR300 variant was further characterized.

mRNA and Protein Content of the HR Variant. Figure 2A shows that the acid selection pressure induced a progressive increase in the level of NHE1 variant mRNAs. Indeed, more NHE1 mRNAs were found in cells submitted to an acid loading and selected for 3 weeks in the presence of 300 μM HOE694 than in those selected with 100 μM HOE694 for 1.5 weeks. The same observation is true for the HR1000 variant. Similarly, the NHE1 content increased progressively for HR100, HR300, and HR1000 variants after the selection procedure (Figure 2B). Gene amplification is probably responsible for this phenomenon, as demonstrated previously (*19*). Since the sensitivity of the HR300 variant to NHE inhibitors is similar to that of NHE3-VSVG (*31*), RNA and proteins were extracted from HR300-expressing cells to confirm that this HOE694-resistant phenotype was not due to the presence of NHE3 mRNA and NHE3 proteins in the resistant cells, or to NHE3-expressing cells contaminating the HR300 cell population. The results presented in Figure 2 clearly show that this was not the case.

Identification of the Mutation Responsible for the HOE694-Resistant Phenotype in HR300 Cells. Only the HR300 variant was studied further. By sequencing the near-complete transmembrane domain of NHE1 corresponding to nucleotides 150−1600, we found only one nucleotide to be modified in the HR300 variant, when compared to the sequence of human wild-type NHE1 published by Sardet *et al.* (*37*), a cytosine replacing a guanosine in position 1038 from the ATG codon. This modification prevents a PCR fragment amplified by RT-PCR from total HR300 RNA from being cleaved by *Sac*I at position 1040 (data not shown). This base pair modification leads to only a minor amino acid change,

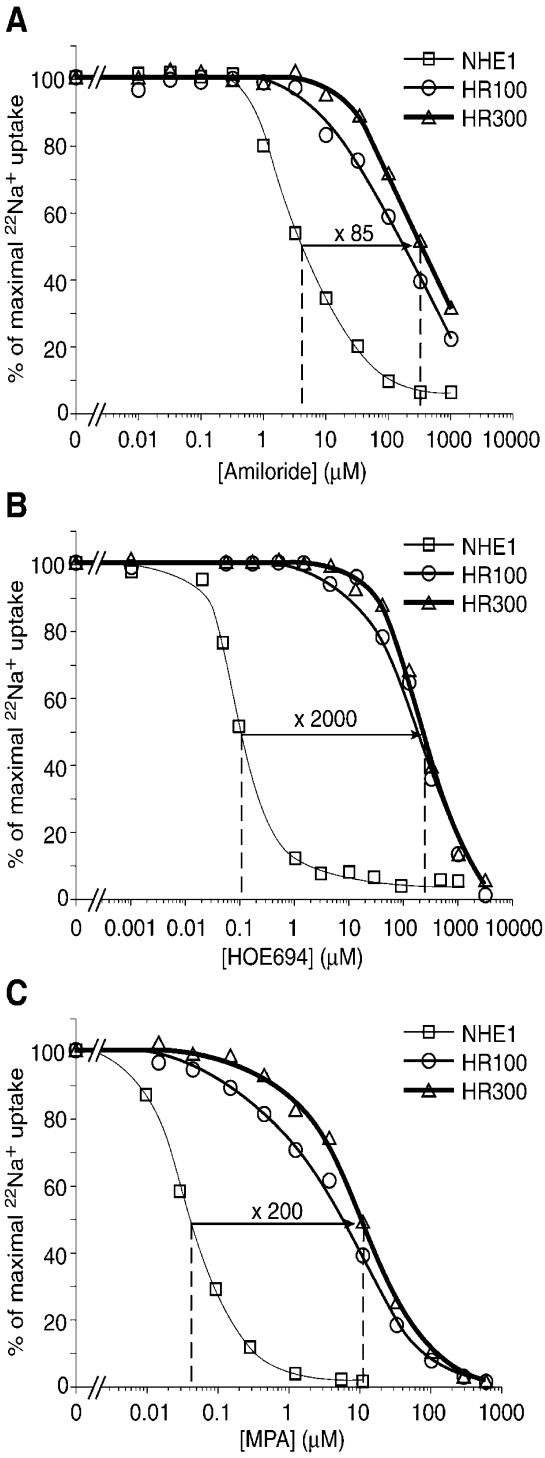

FIGURE 1: Pharmacological profiles of HOE694-resistant NHE1 variants to NHE inhibitors, compared to wild-type NHE1. NHE1-expressing PS12O cells (NHE1, □) and selected cell populations resistant to 100 μM (HR100, ○) and to 300 μM HOE694 (HR300, △) in the Na^+-containing recovery medium were analyzed for their Na^+-H^+ exchange sensitivity to amiloride (A), HOE694 (B), and MPA (C). The maximal Na^+-H^+ exchanger activity was measured in the near absence of cold external Na^+ and is expressed here as the percent of $^{22}Na^+$ accumulation in the absence of inhibitors. The data are means of values obtained from two independent experiments performed in triplicate.

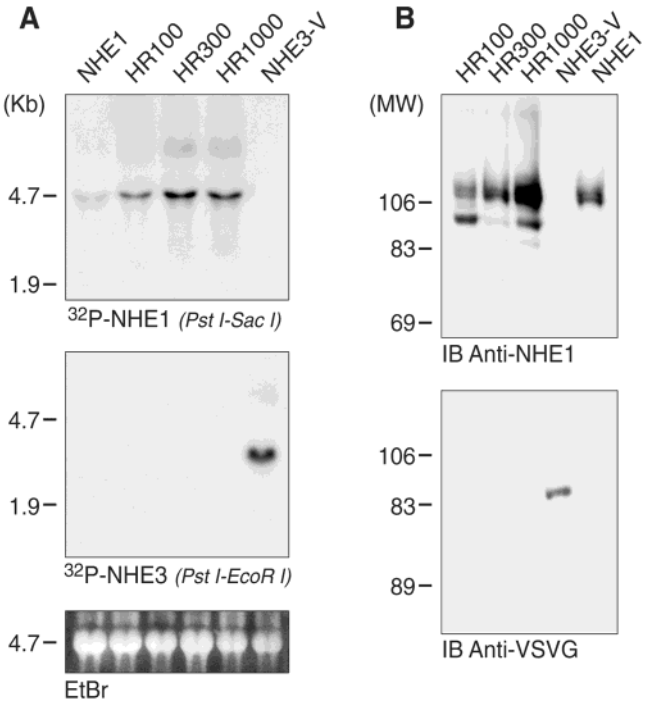

FIGURE 2: RNA and protein content analysis of HR100, HR300, and HR1000 cell variants and of cells expressing wild-type NHE1 and NHE3-VSVG. (A) Northern blot analysis of total RNA isolated from HR100, HR300, and HR1000 mutants probed with NHE1 (top panel) and NHE3 (middle panel)-specific probes, compared with wild-type NHE1. Ethidium bromide coloration (bottom panel) is presented as a loading control. (B) Western Blot analysis of crude membrane proteins isolated from stable HR100, HR300, and HR1000 mutants probed with antibodies recognizing the NHE1 epitope or the VSVG tag grafted to NHE3, compared with wild-type NHE1. The data are representative results from three similar experiments.

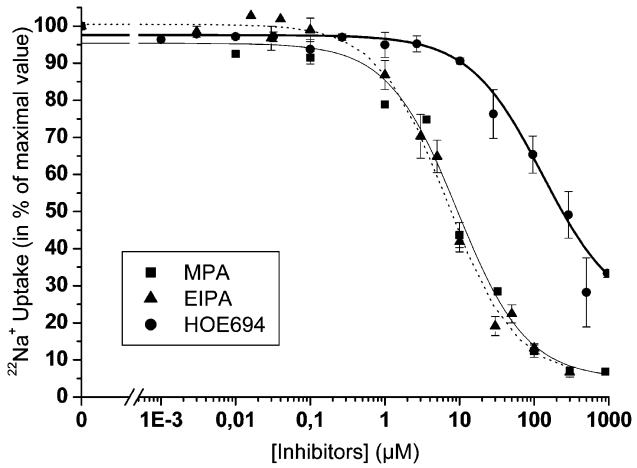

FIGURE 3: Pharmacological profiles of the NHE1-Glu_{346}Asp mutant to NHE inhibitors. PS120 cells transfected with the mutated NHE1-Glu_{346}Asp cDNA and selected for their Na^+-H^+ exchanger activity were analyzed for their transport activity in the presence of different concentrations of MPA (■), EIPA (▲, ⋯), and HOE694 (●). The activity is expressed as the percent of $^{22}Na^+$ accumulation in the absence of inhibitors. The data are presented as the average of values obtained from three to eight independent experiments performed in triplicate. The normalized intrinsic activity of NHE1-Glu_{346}Asp is quite similar to that of wild-type NHE1 (data not shown).

an aspartic acid replacing a glutamic acid, which is, according to the recently adopted nomenclature (*6*), located in position 346 of segment M9. This amino acid change is very conservative relative to its molar volume (91 vs 109 Å), negative charge, and pK_a values (4.5 vs 4.6). This result is surprising; it means that the highly resistant phenotype is due to an aspartate replacing the glutamate in position 346 of human NHE1. A similar IC_{50} shift was found upon mutating, by site-directed mutagenesis, the glutamate in position 350 of rat NHE1 (*27*).

Site-Directed Mutagenesis of NHE1-E_{346} and Its Surrounding Amino Acids in M9. To confirm that the Glu_{346}Asp mutation alone is responsible for the phenotype of high resistance to amiloride analogues observed in the HR300 variant, we introduced (by PCR) the Glu_{346}Asp mutation into wild-type human NHE1 by replacing only the guanosine at position 1038 with a cytosine. Using the calcium phosphate precipitation procedure, cDNA encoding this NHE1-Glu_{346}Asp mutant was transfected in PS120 fibroblasts deficient in membrane-bound Na^+-H^+ exchange activity (*33*), and stable cell transfectants overexpressing NHE1 were selected by using the acid loading selection procedure described in Experimental Procedures.

The mutagenized NHE1-Glu_{346}Asp mutant exhibits resistance to MPA and HOE694 (Figure 3) equal to that of the HR300 variant shown in Figure 1. A high resistance to EIPA was also observed. For this latter inhibitor, the IC_{50} ratio relative to that of wild-type NHE1 (IC_{50} = 0.027 ± 0.002 μM) increased 300-fold. Together, these results unequivocally confirm that the highly resistant phenotype observed for the HR300, and most probably for the other variants, was a consequence of a single point mutation substituting the glutamate in position 346 of NHE1 with an aspartate.

When the amino acid sequences of M9 of NHE1 and NHE3 were compared, a glutamate was found with all the other NHE isoforms, and not an aspartate, as could be expected from our observations. These results thus indicate that despite the similar sensitivity of NHE1-Glu_{346}Asp and NHE3 proteins to inhibitors, the glutamate in position 346 of NHE3 cannot alone be responsible for this highly resistant phenotype. Indeed, chimera analysis (*15*) and the initial results of site-directed mutagenesis (*19*) suggest that the high resistance to NHE inhibitors must be due to more than one domain or amino acid of NHE3 being involved in interactions with inhibitors. A combination of amino acids within the same membrane segment or between different segments may be responsible for creating a specific conformation.

To determine if the glutamate in position 346 of NHE1 interacts directly with inhibitors or Na^+ or is part of a conformational architecture that favors interaction with the inhibitors, we introduced mutations at the sites closest to Glu_{346}, i.e., Leu_{343}, Ala_{345}, and Leu_{347}. Two amino acids were chosen for each site: the structurally similar glycine and the bulky tryptophan for Ala_{345}, and valine and tryptophan in place of both Leu_{343} and Leu_{347}. The mutated NHE1s were selected as described above, and dose−response curves were determined for EIPA and HOE694. As shown in Table 2, none of the mutations induced a shift in the IC_{50}. These results were expected for mutations that induce a slight structural change (Ala_{345}Gly, Leu_{343}Val, and Leu_{347}Val), but were surprising for the Ala_{345}Trp, Leu_{343}Trp, and Leu_{347}Trp mutations. Indeed, tryptophan is a large amino acid with a molar volume of 163 Å, compared to volumes of 67 and

Table 2: IC_{50} Values of NHE1 Mutants for EIPA

	IC_{50} for EIPA (μM) (n)
wild-type NHE1	0.03 ± 0.003 (8)
$Leu_{343}Val$	0.03 ± 0.001 (3)
$Leu_{343}Trp$	0.04 ± 0.002 (3)
$Ala_{345}Gly$	0.07 ± 0.005 (3)
$Ala_{345}Trp$	0.04 ± 0.006 (3)
$Glu_{346}Asp$	7.52 ± 1.937 (8)
$Glu_{346}Gln$	0.35 ± 0.17 (3)
$Leu_{347}Trp$	0.03 ± 0.003 (3)
$Leu_{347}Val$	0.04 ± 0.004 (3)

124 Å for Ala and Leu, respectively. We therefore expected that introducing tryptophan into the primary sequence would, by evoking a conformational change, interfere with the interaction of NHE1 with inhibitors, especially in the proximity of Glu_{346}. The fact that the mutations of amino acids adjacent to Glu_{346} had no effect on transport activity and on sensitivity to inhibitors confirms that Glu_{346} is critical for the interaction of NHE1 with inhibitors. Furthermore, these results suggest that the decreased affinity of inhibitors for NHE1 is due to an altered interaction between the $Glu_{346}Asp$ mutant and inhibitors. Indeed, if this mutation has induced a conformational change in the structure, the mutated amino acids near Glu_{346} should also have induced such a change; however, this was not the case, so a direct interaction should be considered. Our results are in agreement with those of Murtazina *et al*. (*25*) who, by modifying the amino acids closest to amino acid 391, found no effect of these mutations on NHE activity.

To determine if the charge of the amino acid in position 346 plays a significant role in interaction with inhibitors or Na^+, the charge ($pK_a = 4.6$) was neutralized by introducing a glutamine (Gln). This mutant [exhibiting an IC_{50} of 0.35 ± 0.17 μM for EIPA (Table 2)] was 10 times more resistant than wild-type NHE1 but 20 times more sensitive than the $Glu_{346}Asp$ mutant. We conclude from this mutant that the charge and size of the amino acid in position 346 are critical for the interaction of NHE1 with inhibitors. Surprisingly, these results indicate that since the pK_a values of Glu and Asp are similar, the size of the amino acid is more influential than the charge in ensuring an optimal interaction with inhibitors. These results are in accord with the findings of Khadilkar *et al*. (*28*) for rat NHE1, which show that mutating Glu_{350} in glutamine significantly reduces the sensitivity of the protein for inhibitors but does not interfere with its affinity for Na^+.

Affinity for Na^+. In contrast to previous observations concerning $L_{163}F$, $G_{174}S$, and $H_{349}G$ mutations that showed no change in affinity for Na^+ despite significant changes in the sensitivity to NHE inhibitors, we found that the single $Glu_{346}Asp$ mutation significantly affects the affinity of the NHE1 protein for Na^+ (Figure 4). Indeed, the K_m of the NHE1-$Glu_{346}Asp$ mutant for Na^+ is 4-fold higher (~68 mM) than that of wild-type NHE1 (~18 mM). In contrast, no significant change in Li^+ affinity was observed for the NHE1-$Glu_{346}Asp$ mutant (data not shown). Touret *et al*. (*22*) made a similar observation for the extremely resistant cariporide revertants. These results, along with those presented in this study, suggest that amino acid Glu_{346} within the M9 domain appears to be sufficient to modify on a very local basis the conformation of the protein, or at least its

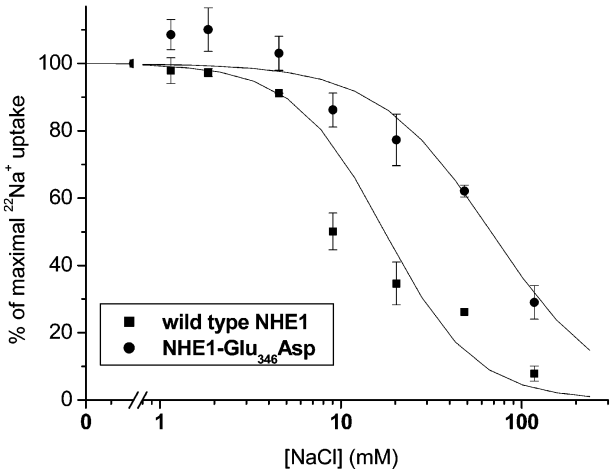

FIGURE 4: Determination of the affinity of the NHE1-$Glu_{346}Asp$ mutant for Na^+. Wild-type (■) and NHE1-$Glu_{346}Asp$ (●) cells were analyzed for their transport activity in the presence of different concentrations of external NaCl. The $^{22}Na^+$ accumulation was performed in the presence of the tracer for only 30 s. The transport activity is expressed as the percent of counts per minute accumulated in cells in the absence of cold NaCl, as a function of NaCl concentration expressed on a logarithmic scale. With this method, the concentration of NaCl corresponding to 50% of the maximal Na^+-H^+ exchange activity (K_{mNa^+}) can be directly determined in the figure or obtained from curve fitting analysis. $K_m = 17.5 \pm 3.4$ ($n = 4$) for wild-type NHE1 and 67.6 ± 6.3 ($n = 4$) for NHE1-$Glu_{346}Asp$.

direct interaction with Na^+ and its competitive inhibitors. These results again confirm the importance of Glu_{346}. Furthermore, these results correlate with the idea that Na^+ and inhibitors share the same binding site or at least are located in some overlapping binding sites (*28*). Indeed, considering that the hydrated Na^+ and the guanidinium group of amiloride are very similar in their shape and size, Counillon *et al*. proposed a competitive behavior between Na^+ and inhibitors (*10*). Later we identified a NHE1-$L_{163}F/G_{174}S$ double mutant presenting a 22-fold decreased affinity for HOE694 and a 2-fold decrease in Na^+ affinity (*20*). More recently, Touret *et al*. (*22*) found a NHE1-$F_{162}S$ mutant in which both the cariporide and Na^+ affinities are impaired. In fact, in this work, two revertants, $F_{162}S/I_{169}S$ and $F_{162}S/I_{170}T$, restored the Na^+ affinity but not the affinity for cariporide. These results sustained the idea of a shared binding site, whereas the results provided by the revertants rather suggest a distinct binding site. Furthermore, some works have reported results which suggest that the binding sites for Na^+ and inhibitors should be distinct. Among these studies, Wang *et al*. (*26*) found a NHE1-$H_{349}G$ mutant which presents 2.4- and 4.5-fold decreases in amiloride and EIPA sensitivity, respectively, whereas its affinity for Na^+ was unaltered. For an integrated view of these studies, see the review by Harris *et al*. (*28*). These results are corroborated by studies using the radiolabeled amiloride derivatives methylisobutylamiloride (MIA), which revealed that the Na^+ does not reduce the level of binding of the labeled inhibitors to the NHE (*38*). So, although it is more and more accepted that the Na^+ and inhibitor binding sites are physically distinct, some clues support an overlapping binding site.

Affinity for H^+. As previously demonstrated, Na^+-H^+ exchange is a reversible transport event (*34*, *39*−*43*). H^+ in the uptake medium (out) could interact or compete with the extracellular site for Na^+ and modify $^{22}Na^+$ transport activity.

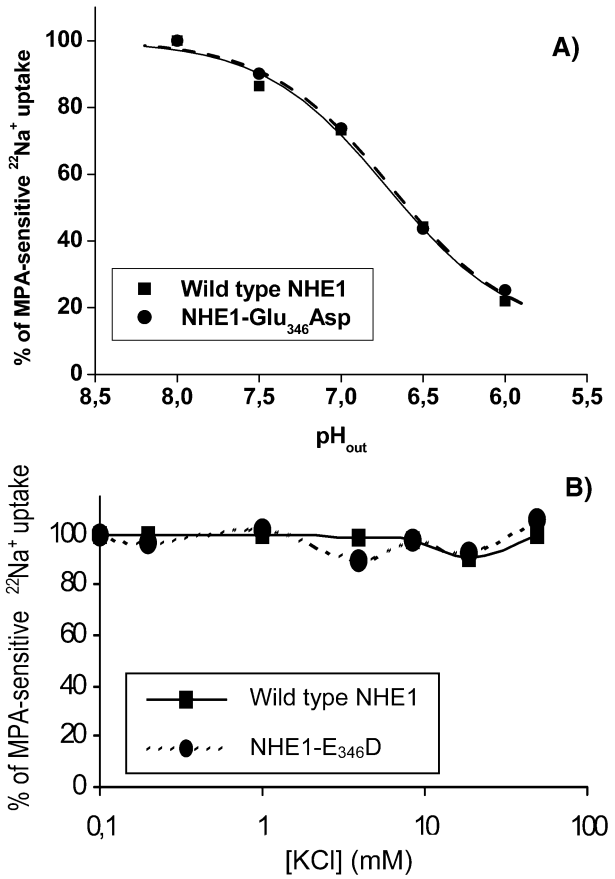

FIGURE 5: Effects of varying extracellular H^+ and K^+ concentrations on Na^+-H^+ exchange activity of wild-type and NHE1 proteins. Wild-type NHE1 (■, —) and NHE1-Glu_{346}Asp (●, — — —) cells were analyzed for their transport activity in the presence of different concentrations of external H^+ (varying pH out) (A) and KCl (B). The $^{22}Na^+$ accumulation was performed in the presence of the tracer only for 30 s. The amount of $^{22}Na^+$ accumulated in cells and sensitive to 1 mM MPA was expressed as the percent of maximal $^{22}Na^+$ accumulation at pH 8.0 (A) or in the absence of external KCl (B). The data are means of values obtained from two to four independent experiments performed in triplicate.

Figure 5A shows that, in accord with previous findings (*13*, *22*, *34*), an acidic extracellular pH decreased the Na^+-H^+ exchange activity of NHE1 expressed in PS120 cells. Since Paris *et al*. (*39*) showed that extracellular H^+ competitively inhibits the extracellular Na^+ stimulation of H^+ release, a mutation such as the Glu_{346}Asp mutation decreasing the affinity for Na^+ should likely impair the affinity for extracellular H^+. The response of the Glu_{346}Asp mutant to a modification of the external pH was very similar to that of wild-type NHE1 in the pH range that was studied (6.0−8.4). Our observations and results previously published (*13*, *22*) thus support the idea that the binding sites for extracellular Na^+ and H^+ are not necessarily the same but could overlap like those of Na^+ and inhibitors. Alternatively, because of their respective sizes, Na^+ and H^+ may react differently to a change in the amino acid at that position. As the H^+ ion is smaller than the Na^+ ion, H^+ could lodge more easily within the Na^+ binding site of the NHE1-Glu_{346}Asp mutant than Na^+. The observations of Touret *et al*. (*22*) support this interpretation. The NHE1-F_{162}S mutant presents a K_m for Na^+ that is impaired by a factor of 10 compared to that of wild-type NHE1, while the K_m for both Li^+ and H^+ remained unchanged.

Affinity for K^+. It has been reported that the amiloride-sensitive NHE1 and the amiloride-resistant isoform NHE3 differ in their sensitivity to potassium; the activity of NHE1 is inhibited at high concentrations of potassium, while that of NHE3 is unaffected (*15*). K^+ ions were reported to compete with Na^+ for the same binding site on NHE1 but are not transported. We wanted to determine whether the Glu_{346}Asp mutation, modifying the interaction with Na^+, could also change the interaction of NHE1 with K^+. We examined how varying the concentration of potassium in the uptake medium affected the activity of wild-type NHE1 and the Glu_{346}Asp mutant expressed in PS120 fibroblasts (Figure 5B). The response of these two stable transfectants to modification of the potassium concentration was identical (Figure 5B). No change in the activity was observed up to 50 mM potassium for both mutant and wild-type NHE1. Our results for wild-type NHE1 contrast with those of Orlowski *et al*. (*15*), even though we followed the same protocol (bumetanide to inhibit a potential $Na^+,K^+,2Cl^-$ transporter). A species difference may, however, explain these differences since we used human NHE1 cDNA while these authors used rat NHE1 cDNAs. Small differences in amino acid sequences between rat and human NHE1 could modify some interactions between amino acids and explain these differences. Structure−function studies of NHEs from different species, particularly from human NHEs, are therefore crucial in providing new insights into structural relationships between NHEs and inhibitors that will foster drug development.

In summary, a spontaneous variant of NHE1 that exhibits a huge increase in resistance to amiloride and its derivatives (up to 2000-fold) has been isolated and characterized. The amino acid responsible for the phenotype of high resistance has been identified, an aspartate replacing a glutamate in position 346. Since our research shows that mutations of Leu_{343}, Ala_{345}, and Leu_{347} with bulky amino acids do not affect the sensitivity of mutated NHE1 proteins for EIPA, the hypothesis of a major conformation change is not valid. Together with the observation that the NHE1-Glu_{346}Asp mutant presents a significant difference in affinity for Na^+, but not for H^+ and K^+, compared to the wild-type protein, our results support the idea that competitive inhibitors and Na^+ interact with the same binding site(s) or with separated binding sites in the proximity of each other. Amino acid Glu_{346} of M9 takes part of this binding site. A detailed mutagenesis study, by introduction of amino acids with different physical characteristics, is in progress in an effort to understand how Glu_{346} is involved in the interaction of NHE1 with inhibitors and/or Na^+. The results presented above, by identifying amino acid Glu_{346} within M9 as a crucial amino acid involved in interaction with both inhibitors and Na^+, should contribute to the development of more specific and more potent drugs for therapeutic use.

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