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A point mutation of the Na^+/H^+ exchanger gene (*NHE1*) and amplification of the mutated allele confer amiloride resistance upon chronic acidosis

(amiloride-binding site/Na^+/H^+ exchanger isoforms/pH regulation/gene amplification)

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ABSTRACT **The diuretic drug amiloride and its 5-amino substitute N^5-methyl-N^5-propylamiloride (MPA) are potent inhibitors of the growth factor-activatable Na^+/H^+ exchanger isoform 1 (NHE1). This inhibitor competes with Na^+, presumably by interacting with the ion-transport site of the NHE molecule. As an approach to identify this site, we previously reported the use of a specific H^+-killing selection technique for isolating amiloride-resistant variants of Chinese hamster lung fibroblasts. After long-term selection, two variants, AR40 and AR300, 100- and 1000-fold, respectively, resistant to MPA, were isolated. By comparing NHE1 cDNA sequences of parental and two variant cell lines, we show that the 1000-fold resistance to MPA results from two sequential genetic events. (*i*) In one AR40 allele a point mutation, Phe-167 → Leu, occurs in the middle of the fourth putative transmembrane segment of NHE1. Producing this mutant protein from human NHE1 cDNA by site-directed mutagenesis increased the K_i for MPA by 30-fold, as seen in AR300 cells. (*ii*) An ≈10-fold amplification of the mutated allele, which contributes to the acquired MPA resistance, accounts for the V_{max} increase. Mutating a close residue, Phe-165 → Tyr, increased by 40-fold the K_i for amiloride and reduced Na^+ transport rate 3- to 4-fold, indicating that we have identified a critical domain of the NHE molecule that controls amiloride binding and Na^+ transport. Interestingly, the epithelial amiloride-resistant NHE isoforms that occurred naturally possess some of the amino acid substitutions described here.**

Amiloride is an antihypertensive diuretic agent possessing saliuretic and antikaliuretic properties (1) that inhibits the eukaryotic Na^+/H^+ exchanger and epithelial Na^+ channel in the range of micromolar concentrations (2, 3). Amiloride and its derivatives have provided the pharmacological means to discriminate between various Na^+ transport systems that include several isoforms of Na^+/H^+ exchangers (NHE1–NHE4) (4–6), Na^+/Ca^{2+} exchanger (7), and the epithelial Na^+ channel (8). As far as NHE1 is concerned, amiloride inhibits Na^+ transport competitively, and substitution of alkyl groups at the 5-amino position of the molecule increases up to 100-fold the potency of inhibition (9, 10). In spite of this specificity and potency of inhibition, amiloride derivatives have not permitted isolation of the receptor or characterization of the corresponding site by standard biochemical techniques. Our approach to this question exploited the toxicity of intracellular protons and the selectivity of the amiloride analog N^5-methyl-N^5-propylamiloride (MPA) to isolate mutants impaired in their capacity to exchange Na^+ for H^+ and be inhibited by MPA (11). One class, devoid of exchanger activity, allowed us to clone a NHE isoform by gene complementation (4, 12). To isolate the second class of mutants impaired in their capacity to bind MPA, Chinese hamster lung fibroblasts (CCL39 diploid cell line) were submitted repeatedly to lethal intracellular acidifications in the presence of MPA. During this selection procedure, a functional Na^+/H^+ exchanger is absolutely required to protect cells against lethal acidosis. The MPA concentration was then steadily increased over a 6-mo selection period from 0.3 μM (half-lethal doses for CCL39 cells) up to 40 μM and 300 μM at which, respectively, AR40 and AR300 stable variants were isolated (13). Here we provide molecular evidence that this 1000-fold acquired MPA resistance to chronic lethal acidosis results from a single mutation within the structural NHE1-encoding gene that decreases 30-fold the affinity for MPA; the mutant allele was then amplified in AR300 cells.

MATERIALS AND METHODS

Cell Culture. Chinese hamster lung fibroblast (CCL39 cell line), the CCL39-derived PS120 variant, and PS120 cells transfected with the Na^+/H^+ antiporter cDNA constructs were maintained in Dulbecco's modified Eagle's medium/7.5% fetal calf serum/penicillin at 50 units/ml/streptomycin at 50 μg/ml in a humidified atmosphere of 5% CO_2/95% air at 37°C.

cDNA Cloning. Total RNA was extracted from AR300 cells by using the LiCl precipitation method. Poly(A)$^+$ RNAs were selected on oligo(dT) columns (Pharmacia, type 7) and were reverse transcribed by using oligo(dT) priming (Amersham and Boehringer Mannheim cDNA synthesis kits). Double-stranded cDNAs over 2 kb were size-selected on an agarose gel and cloned into λgt10 bacteriophage (Amersham λgt10 cDNA cloning kit), yielding a library containing 4×10^5 independent clones. After three rounds of screening with the ^{32}P-labeled human NHE1 cDNA as a probe (Amersham random-priming labeling kit), followed by sequencing and restriction map analysis, isolated cDNA clones were found to be highly homologous to the human cDNA. The largest clone was missing 750 bp at the 5′ extremity of the coding region. A second library was built into λgt10 from AR300 poly(A)$^+$ RNA, with random primers, to increase the probability of reverse-transcribing 5′ regions of the mRNA. Clones from this library bearing 2×10^5 independent recombinants of >700-bp length provided sequences containing the translation initiation region (14), which allowed the design of oligonucleotides for PCR amplification of the missing fragment. The 5′ oligonucleotide contained an *Eco*RI restriction site followed by a translation initiation sequence, whereas the 3′ oligonucleotide was situated immediately after the first *Sac* I restriction site of the AR26 cDNA (Fig. 1). PCR was done on ≈10 ng of randomly primed first-stranded cDNA (Cetus *Taq* polymerase). Complete cDNAs could be reconstituted by

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Abbreviations: NHE1, Na^+/H^+ exchanger isoform 1; MPA, N^5-methyl-N^5-propylamiloride.

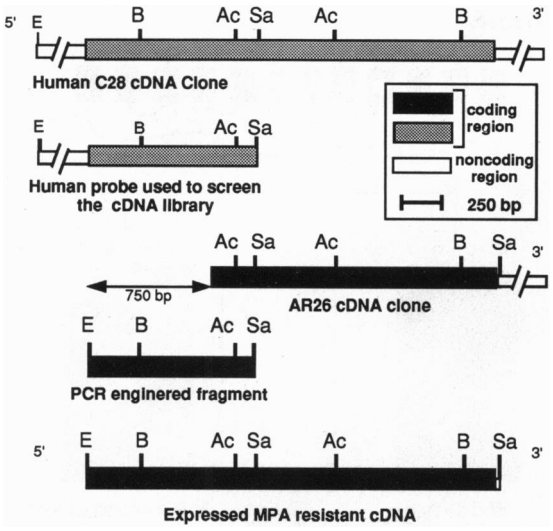

FIG. 1. Human probe and organization of the cDNA fragments isolated during cloning of AR300 NHE1 cDNA. Restriction enzymes: Ac, *Acc* I; B, *Bam*HI; E, *Eco*RI; Sa, *Sac* I.

using the biggest clone obtained from the oligo(dT)-primed library and the fragment synthesized by PCR.

Site-Directed Mutagenesis. An *Hin*dIII–*Sac* I restriction fragment isolated from plasmid pEAP-Δ5′ (15) cloned into pECE mammalian expression vector (16) was subcloned into the pTZ18 vector. Site-directed mutagenesis was done on single-stranded plasmid, according to the method described by Kunkel (17) (Bio-Rad kit). Mutagenized cDNA fragments were reintroduced in pEAP-Δ5′ cloned in pECE by enzymatic restriction cutting and subsequent ligation.

Expression of Na^+/H^+ Antiporters. NHE1 cDNAs from AR300, CCL39, and human were subcloned between *Eco*RI and *Sac* I restriction sites of the pECE expression vector polylinker. The cDNA coding for the NHE2 isoform was provided by M. Donowitz (Johns Hopkins University, Baltimore). The cDNAs coding for NHE3 and NHE4 isoforms were provided by J. Orlowski (McGill University, Montreal). PS120 cells were transfected with the calcium phosphate precipitation method (18), and positive transfectants were selected by their ability to survive a 1-hr acidification induced by NH_4^+ prepulse acid loading, as described (4).

Other Methods. $^{22}Na^+$-uptake studies, Southern blot, and immunoblot analyses were done as described (12, 15, 19). Protein concentrations were determined by the method of Lowry *et al.* (20).

RESULTS

Cloning the cDNA of the "MPA-Resistant" Exchanger. Genomic DNA, from the MPA-resistant variant AR300, transfected into NHE-deficient mouse fibroblasts, restored Na^+/H^+ exchange activity with the corresponding lower affinity for MPA (12). This finding was important because it provided strong evidence for the existence of a mutated NHE1-encoding gene in AR300 cells. We therefore decided to clone the MPA-resistant exchanger by screening cDNA libraries prepared from the hamster AR300 cell line with the human NHE1 cDNA as probe.

A cDNA containing the entire AR300 NHE-coding sequence was cloned, as judged by restriction enzyme pattern analysis, partial DNA sequencing, and hybridization with 5′ and 3′ probes of the corresponding human NHE1 cDNA. Fig. 1 outlines the two-step cloning procedure used, and details are described in *Materials and Methods*. When this cDNA, deleted of most of its 5′- and 3′-noncoding regions (Fig. 1), was stably transfected into NHE-deficient PS120 cells (21), it could restore Na^+-dependent intracellular pH regulation. Most important, $^{22}Na^+$-uptake kinetic studies revealed that this cDNA conferred an identical MPA pharmacological profile as that seen in the AR300 parental cell line: K_i for MPA = 1.5 μM instead of 0.05 μM observed in CCL39 cells or in PS120 cells transfected with the human NHE1 cDNA (Fig. 2). This result validates the hypothesis that the MPA-resistant phenotype seen in AR300 cells was due to a mutation(s) within the gene for the Na^+/H^+ exchanger and demonstrates that the cDNA coding for this mutated antiporter has been cloned.

To identify the mutation(s) by sequence comparison, we cloned the corresponding cDNA of the parent Chinese hamster cell line CCL39 (22). Like the human counterpart, it contains 12 putative transmembrane segments and two conserved glycosylation sites, and it displays 92.5% homology at the amino acid level with the human NHE1 (4, 22).

Identification of the Mutation Responsible for MPA Resistance. Before sequencing the complete AR300-derived cDNA, we first constructed chimeric antiporters by using corresponding wild-type and mutant cDNA restriction fragments (see Fig. 3). By comparing the MPA-sensitivity in the H^+-killing test, as well as the amiloride sensitivity of Na^+ uptake, the area containing the mutation(s) has clearly been narrowed to a 700-bp cDNA fragment encoding transmembrane segments 2–7 in the putative topological model deduced from the NHE1 primary sequence (4, 23).

The comparative nucleotide sequence of this 700-bp fragment revealed only a single point mutation (C → T) that generated a Leu-167 → Phe substitution in the middle of the fourth putative transmembrane segment (Fig. 4, *Upper*). Subsequent analysis of the entire sequence revealed that this Leu-167 → Phe substitution was the only mutation seen.

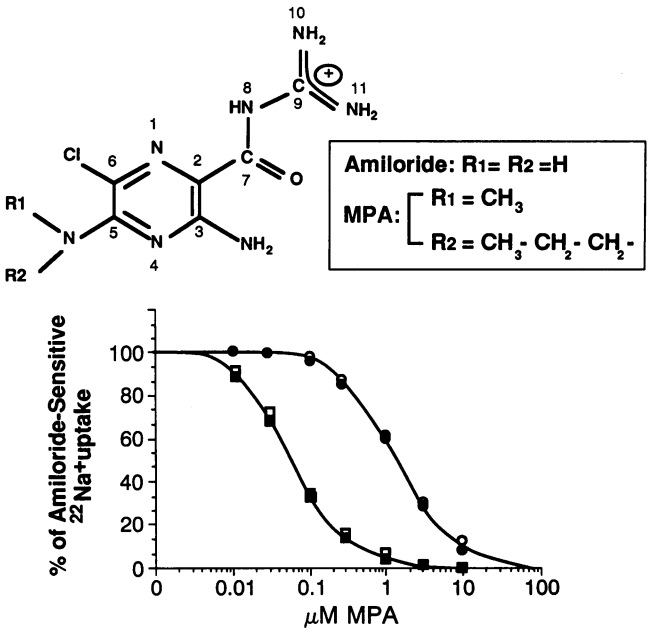

FIG. 2. Amiloride structure and MPA pharmacological profile of CCL39 and AR300 cells. (*Upper*) Structure of protonated form of amiloride and of analog MPA. (*Lower*) Concentration–response curves for inhibition of $^{22}Na^+$ influx by MPA in CCL39 cells (□) and AR300 cells (○). NH_4^+-loaded cells were incubated for 6 min in a medium (pH 7.4) containing $^{22}Na^+$ (carrier-free), 1 mM ouabain, and various MPA concentrations. Initial rates of $^{22}Na^+$ uptake were determined, as described (12, 13). Dose–response curves clearly show that PS120 cells expressing NHE1 cDNAs cloned from CCL39 (■) and from AR300 cells (●) exhibit the same pharmacological profile as the "parental" CCL39 and AR300 cell lines, respectively.

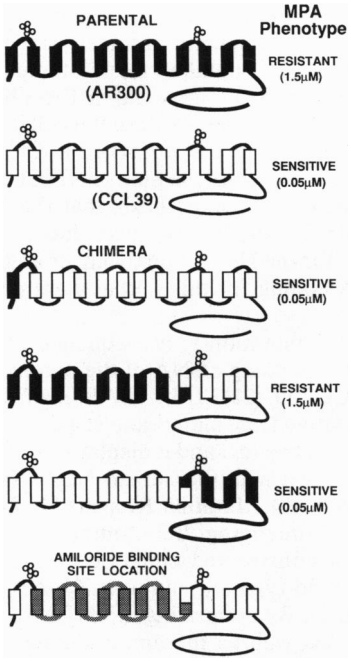

FIG. 3. MPA phenotype of different NHE1 cDNA chimera constructs. The chimeras used to map a piece of DNA containing the mutation(s) responsible for the MPA-resistance phenotype were constructed by enzymatic restriction cutting and subsequent religation of wild-type and mutant AR300 cDNAs. Resulting chimeric cDNAs have been inserted in the pECE expression vector and expressed in PS120 cells. The black areas of the putative topological model of the Na^+/H^+ exchanger correspond to regions of the molecule encoded by the AR300 mutant cDNA. The corresponding phenotype is indicated beside each chimera with the inhibition constants in parenthesis.

Accordingly, this mutation introduced by site-directed mutagenesis in the human NHE1 cDNA shifted the corresponding MPA-sensitive phenotype to an MPA-resistant one with the corresponding 30-fold increase in K_i value for MPA (Fig. 4, *Lower*). This result shows that this sole mutation is responsible for the observed altered pharmacological profile of the antiporter expressed by AR300 cells. Also, this result strongly suggests that Leu-167 interacts with amiloride, presumably by hydrophobic interaction with the pyrazine ring of the molecule and, to a greater extent, with the 5-amino substituents because affinity for MPA is much more affected than affinity for amiloride when this leucine is changed to a phenylalanine.

Elucidation of the Molecular Mechanisms Responsible for Acquisition of Resistance. During the continuous selection that led to the emergence of the MPA-resistant phenotype, AR40 cells, which display an "intermediate phenotype," emerged quickly (≈1 mo), whereas an additional 5 mo were required to yield the highly resistant AR300 cells. It was, therefore, of interest to elucidate the mechanism of evolution of the fibroblast population exposed to this chronic acidosis. (*i*) A marked difference was noticed between AR40 and AR300 at the *NHE1* gene copy level. Fig. 5 shows that AR300 has amplified the *NHE1* gene ≈10-fold with no sign of rearrangement. This genetic event could easily account for overexpression of the Na^+/H^+ exchange activity reported (13). We then analyzed by PCR the corresponding "amiloride-binding" region of the AR40 NHE1-encoding gene. Sequence analysis of 11 cDNA clones, prepared by using AR40 mRNA, revealed that 5 of them were wild type, whereas the others possessed the same mutation as the AR300 cells. This distribution of cDNA sequences clearly indicates that AR40 cells possess both wild-type and mutant

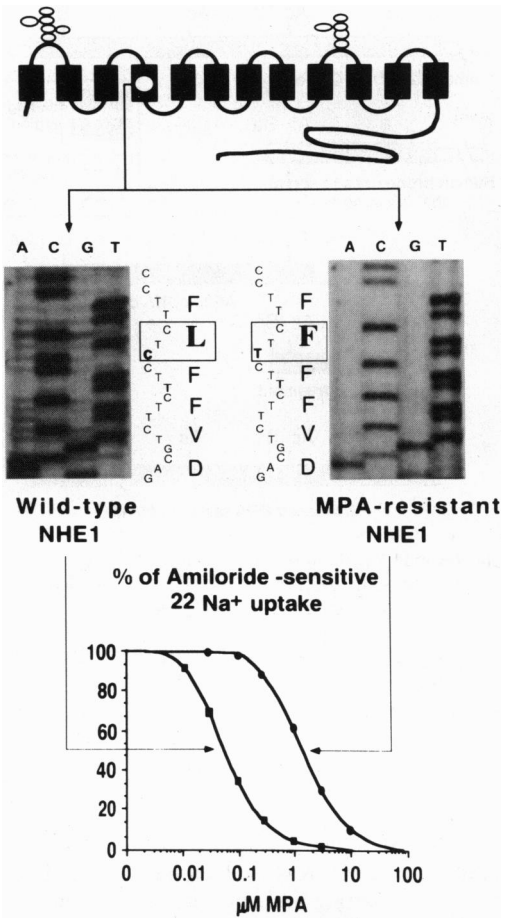

FIG. 4. Identification of NHE1 mutation responsible for MPA-resistant phenotype of AR300 variant by nucleotide sequence analysis and MPA pharmacological profiles. (*Upper*) Portion of DNA sequencing gel showing single nucleotide change (C → T) in codon 167 resulting in Leu → Phe. (*Lower*) Dose–response curves for inhibition of $^{22}Na^+$ influx by MPA in PS120 cells transfected with human NHE1 cDNA (■) and with human NHE1 cDNA containing the Leu-167 → Phe mutation (●). The human NHE1 cDNA was mutagenized according to the method of Kunkel (17).

NHE1 alleles, and therefore we conclude that the observed pharmacological profile results from the coexpression in equal amounts of both MPA-resistant and -sensitive NHE1 molecules.

Investigation of Other Amino Acids Involved in Amiloride Binding. In contrast to NHE1, Na^+/H^+ exchanger isoforms expressed in epithelial cells have been reported to display relative resistance to amiloride and its analogs (24, 25). With the recent availability of the corresponding sequences for

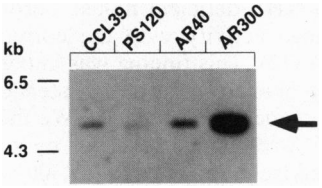

FIG. 5. Genomic DNA analysis of Chinese hamster fibroblasts and derived null and MPA-resistant variants. Southern blots of the CCL39, PS120, AR40, and AR300 cell lines. Twenty micrograms of *Eco*RI-cut genomic DNA was electrophoresed, blotted onto Nylon filter, and hybridized with ^{32}P-labeled human NHE1 probe [H18-*Pst* I; (4)]. The arrow indicates the NHE1-specific *Eco*RI fragment detected by the probe. Note the marked amplification in AR300 cells over wild-type (CCL39) and AR40 cells.

NHE2, NHE3, and NHE4 isoforms (5, 6), it was of particular interest to examine the amino acid region proposed as the "amiloride-binding site." The sequence of amino acids ^{164}VFFLFLLPPI^{173} is rather well conserved among the four isoforms; boldface letters correspond to complete conservation among these forms (Table 1). Some differences, however, are noteworthy. The pharmacological sensitivity of the NHE isoforms was tested after expression in PS120 cells. The *NHE2* and *NHE3* genes could be expressed, whereas we were unable to express *NHE4*. NHE2 expressed in PS120 cells displays a decreased affinity for MPA but does not show decreased affinity for amiloride. Its K_i value for MPA is 10 times higher than it is for NHE1, and its amino acid sequence in the 164–173 region varies by only one conservative substitution (Phe-168 → Tyr) (Table 1). Introduction of this substitution into NHE1 does not affect the K_i for either MPA or amiloride. This result clearly indicates that the pharmacology for 5-amino substitutes of amiloride is also dictated by additional residues, outside the 164–173 amino acid stretch. NHE3, however, possesses the same Leu-167 → Phe substitution as that found in AR40/AR300 mutants. In addition, NHE3 has also the "neutral" conservative Phe-168 → Tyr change found in NHE2. When both substitutions, Leu-167 → Phe and Phe-168 → Tyr, are introduced into NHE1 and the mutated molecule is expressed in PS120 cells, we observed exactly the pharmacology of AR300 but did not observe that of NHE3 (Table 1). Indeed, NHE3 expressed in PS120 cells displays a notable amiloride-resistant phenotype with a 50-fold and 200-fold increase in the K_i values for amiloride and MPA, respectively (Table 1). Here also we must conclude that besides the key region where the Leu-167 → Phe mutation defines an "amiloride hot spot," other residues outside the 164- to 173-amino acid stretch contribute to the recognition of amiloride and its derivatives. NHE4 possesses the "neutral" substitution Phe-168 → Tyr and another conservative change, Phe-165 → Tyr. Introduction of these mutations into NHE1 profoundly altered the amiloride pharmacology: it increased K_i values for amiloride and MPA ≈30- to 40-fold. This result is particularly important, as it independently shows that the hydrophobic pocket ^{164}VFFLFL^{169} contributes to the recognition of amiloride and of its 5-amino substitutes. Unfortunately, at this stage it was not possible to express the NHE-4 isoform in PS120 cells, and therefore its pharmacological profile with the corresponding NHE1 site-directed mutated cDNA could not be compared. Nevertheless, we could easily predict that NHE4 will be one of the very amiloride-resistant isoforms. In addition, the NHE1 molecule bearing the Phe-165 → Tyr and Phe-168 → Tyr substitutions of NHE4 showed a 3- to 4-fold decrease in Na^+ transport rate when compared with NHE1 and all the previously characterized mutated antiporters (Fig. 6 *Left*). As the affinity for external Na^+ is apparently not modified (results not shown) and the quantity of antiport protein expressed in membranes is not decreased (Fig. 6 *Right*), we conclude that the Phe-165 → Tyr substitution affects both amiloride binding and the V_{max} for Na^+ transport.

Table 1. IC_{50} of MPA and amiloride for the various NHE isoforms and the mutated NHE1 cDNAs

cDNA	Amiloride binding site	Mutation	IC_{50} for MPA, μM	IC_{50} for amiloride, μM
NHE1	^{164}VFFLFLLPPI^{173}		0.05	3
AR300	VFF**F**FLLPPI		1.5	15
NHE1		L167F	1.5	15
NHE2	VFFL**Y**LLPPI		0.5	3
NHE1		F168Y	0.05	3
NHE3	VFF**FY**LLPPI		10	150
NHE1		L167F F168Y	1	15
NHE4	V**Y**FL**Y**LLPPI		Unknown	Unknown
NHE1		F165Y F168Y	1	100

IC_{50} values have been measured by inhibition of initial rates of amiloride-sensitive $^{22}Na^+$ influx. Measurements were made in duplicate, as described (12, 13). The variations between duplicate experiments did not exceed 20%. Boldface letters indicate amino acid conservation. Underlined amino acids indicate variations from the NHE1 sequence.

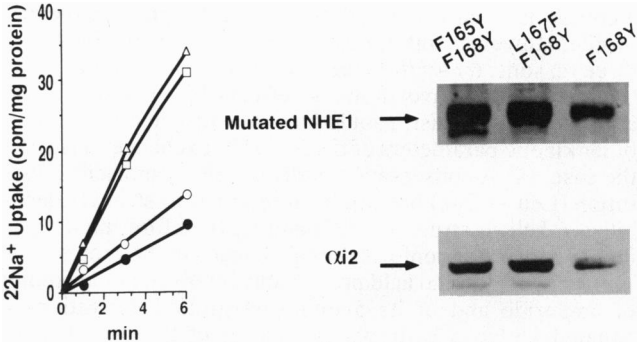

FIG. 6. Na^+/H^+ exchange activity of various mutated NHE1 cDNAs expressed in hamster lung fibroblasts. (*Left*) Comparison of initial rates of amiloride-sensitive $^{22}Na^+$ influx in CCL39 cells (●) and in PS120 cells transfected with human NHE1-mutagenized cDNAs F168Y (△), L167F-F168Y (□), and F165Y-F168Y (○). The CCL39 cells exhibit a lower antiporter activity (*i*) because of the lower transcriptional activity of the endogenous promoter when compared with the simian virus 40 promoter of the pECE vector used to express the mutated cDNAs and (*ii*) because deletion of the 5′ untranslated region of the mutated cDNAs enhances their expression (15). (*Right*) Immunoblot analysis of the expression level of the 3 site-directed mutated NHE1 cDNAs. About 50 μg of crude membrane proteins was separated on SDS/PAGE and blotted onto a nitrocellulose membrane. The blot was incubated with RP1-C28 anti-NHE1 antibody (19) and revealed by enhanced chemiluminescence technique, as described (15). Protein concentration in each lane is normalized by quantitation of $G_i\alpha 2$ subunit (α12) protein using a $G_i\alpha 2$ antibody on the same blot.

DISCUSSION

To gain some insight into residues involved in the Na^+ transport site or nearby the NHE1 transporter, we exploited the competitive interaction of the amiloride analog with this site. Previously, we showed that a long-term adaptation of cells in culture to chronic acidosis with increased MPA concentrations led to the isolation of a highly MPA-resistant variant, AR300 (13). Here we showed that this 1000-fold acquired resistance resulted from two genetic events: (*i*) A point mutation that occurred spontaneously in AR40 and was selected because it reduced the affinity of MPA by 30-fold. However, in AR40 the coexistence of the wild-type and mutated *NHE1* alleles gave only an intermediate MPA-resistant phenotype. (*ii*) At this stage, increased stringency of selection for an additional 5 mo could have generated either the emergence of secondary mutations or amplification of the mutated *NHE* gene. Molecular analysis of AR300 has clearly shown that gene amplification of the mutated allele prevailed, as seen for other examples of drug resistance in eukaryotic cells (26).

This study has identified an amino acid, Leu-167, of the NHE1 protein, which is likely to contact the amiloride molecule. Indeed, that this substitution induces a much weaker effect on the K_i for amiloride than for MPA suggests that Leu-167 contacts the 5-amino substituents of the MPA molecule. We cannot exclude, however, that Leu-167 is, in fact, not involved in amiloride binding but, instead, induces

a conformational change, thus altering the pharmacological profile. However, this explanation seems unlikely for at least three reasons: (*i*) An important conformational change might be expected to have dramatic effects for function of the antiporter or, at least, should have pleiotropic effects on the other kinetic parameters of the Na^+/H^+ exchange; this is not the case. (*ii*) A conservative hydrophobic amino acid substitution (Leu → Phe) has not been reported to strongly destabilize α-helical structures (27) and is, therefore, unlikely to induce important conformational changes in the protein. (*iii*) This nonpolar amino acid presumably involved in the binding of amiloride and of its 5-amino-substituted derivatives is situated within a hydrophobic pocket of the Na^+/H^+ exchanger, a result in good agreement with data from the structure–activity relationship studies in the amiloride series (28).

Leu-167 → Phe substitution of AR300 has highlighted a hydrophobic region of the fourth transmembrane α-helix that we refer to here as a putative amiloride-binding site. This notion is reinforced by two additional findings. (*i*) We have found that this substitution occurred naturally in the sequence of NHE3, an epithelial isoform recently cloned that displays an amiloride-resistant phenotype (5, 6). Although we have shown that this substitution cannot by itself account for the very amiloride-resistant phenotype of NHE3, it is probably involved. (*ii*) Examination of the corresponding NHE4 sequence has outlined a nearby conservative substitution, Phe-165 → Tyr. Here we showed that this mutation introduced into NHE1 reduced severely not only the affinity for MPA (20-fold) but also that of amiloride (30- to 40-fold) (Table 1). Therefore, Phe-165 probably contacts the pyrazine ring and/or the guanidinium moiety of the amiloride molecule.

The putative fourth transmembrane domain, where the above-mentioned interaction sites occur, exhibits, indeed, very interesting structural features. The stretch of sequence ^{164}VFFLFLLPPI^{173}, previously identified as the amiloride-binding site, contains a very unusual doublet of prolines. Although single proline residues have often been reported to appear in transmembrane domains of ion transporters, this is not so for proline doublets, which exert an important torsion constraint on α-helices. This rare motif, which is absolutely conserved among all NHE isoforms, should, therefore, play a critical structural role in folding of the transmembrane domains of Na^+/H^+ exchangers. In addition, two conserved charged amino acids, Asp-163 and Asp-176, appear in this putative fourth transmembrane domain. The presence of two negative charges in an otherwise highly hydrophobic region suggests involvement of these two residues in maintaining protein structure or in ion translocation.

Another potential interest of our study is the clinical implication. Amiloride is currently used for treating hypertension, and Na^+/H^+ exchange activity has been hypothesized as important in postischemic myocardial arythmia and fibrillation (29), one of the major causes of death after cardiac infarction. Elucidation of the structural binding site for amiloride and its analogs should facilitate the design of more specific Na^+/H^+ antiporter inhibitors, taking the specificity of the various NHE isoforms into account.

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