Delineation of Transmembrane Domains of the Na\(^+\)/H\(^+\) Exchanger That Confer Sensitivity to Pharmacological Antagonists*

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Plasma membrane Na\(^+\)/H\(^+\) exchanger (NHE) isoforms NHE1 and NHE3 exhibit very different sensitivities to amiloride and its 5-amino-substituted analogues, benzoyl guanidinium derivatives (e.g., (3-methylsulfonyl-4-piperidinobenzoyle)guanidine methanesulfonate (HOE-694)), and cimetidine. To define structural domains that confer differential sensitivity to these antagonists, unique restriction endonuclease sites were engineered into cDNAs for each isoform near the regions that encode the putative membrane-spanning domains. These new sites did not modify their pharmacological properties and allowed several chimeric Na\(^+\)/H\(^+\) exchangers to be constructed by exchanging homologous segments. The modified parental (E1 and E3) and chimeric molecules were stably expressed in exchanger-deficient Chinese hamster ovary AP-1 cells and assayed for their sensitivities to amiloride, ethylisopropylamiloride, HOE694, and cimetidine. Most chimeras showed drug sensitivities corresponding to the dominant parental segment. However, interchanging a 66-amino acid segment containing the putative ninth transmembrane (M9) domain and its adjacent loops caused reciprocal alterations in the sensitivities of E1 and E3 to all antagonists. In addition, substituting the first five putative membrane-spanning domains of E3 with the corresponding region of E1 modestly reduced the transporter's sensitivity to cimetidine but not the other compounds. These data indicate that the protein segment between M8 and M10 may be a major site of interaction with these antagonists, although other regions modestly influence sensitivity to certain drugs.

The plasma membrane Na\(^+\)/H\(^+\) exchanger (NHE) participates in the control of intracellular pH (pH\(_i\)) and maintenance of cellular volume, transepithelial Na\(^+\) reabsorption, and the cell proliferation response to growth factors (1). To date, five distinct isoforms (NHE1 to NHE5) have been isolated (2–8). They share ~40–70% amino acid identity (M, ranging from ~81–93 kDa) and exhibit similar membrane topologies, with 10–12 predicted N-terminal membrane-spanning domains and a large C-terminal cytoplasmic region. They also show differences in their patterns of tissue expression, membrane localization, biochemical and pharmacological characteristics, and physiological functions (reviewed in Refs. 9 and 10).

The NHE is a known target for inhibition by the diuretic compound amiloride and its analogues (11). Amiloride analogues containing hydrophobic substituents on the 5-amino group of the pyrazine ring have higher affinity and specificity for NHE relative to other ion transporters. Using a heterologous expression system, the NHE isoforms exhibit a wide range of affinities for amiloride and its analogues that span over 2 orders of magnitude and show the following order of sensitivity: NHE1 > NHE2 > NHE3 (12, 13). Recently, new benzoyl guanidinium antagonists of NHE activity, (3-methylsulfonyl-4-piperidinobenzylo)guanidine methanesulfonate (HOE694) and its related compound HOE642, have also been found to inhibit the isoforms with a similar rank order as the amiloride compounds but over a larger concentration range (3–4 orders of magnitude) (14, 15). The more selective binding properties of these compounds have been exploited therapeutically as more effective agents in the treatment of cardiac ischemia and reperfusion injuries (15–17). Other pharmacological agents such as cimetidine, clonidine, and harmaline also exhibit differential affinities for the NHE isoforms (12, 13). While these compounds are chemically unrelated to amiloride or HOE694, they possess either an imidazoline or guanidinium moiety and hence bear some structural similarity to these compounds.

Biochemical analyses indicate that inhibition by amiloride compounds (18–21), cimetidine (22), and HOE694 (14) is reduced by high external Na\(^+\). This competitive inhibition indicates they bind near the external Na\(^+\) transport site and may also share a common site. However, under different anionic buffer conditions, amiloride and its derivatives also inhibit transport noncompetitively, suggesting that the external Na\(^+\) and amiloride binding sites may not be identical (23, 24). Furthermore, the extracellular Na\(^+\)- and amiloride-binding sites can be altered independently of each other using genetic selection techniques (25). Overall, these data indicate that amiloride and possibly other antagonists may bind to multiple regions of the exchanger.

Recent molecular studies have shown that predicted membrane-associated domains of NHE1 are targets for interaction with amiloride and its analogues. Residues in the fourth (Phe\(^{105}\) and Leu\(^{167}\) and the ninth (His\(^{349}\)) transmembrane domains appear to contribute to amiloride sensitivity without affecting Na\(^+\) affinity (26, 27). However, mutations at these sites produce only modest changes in drug sensitivity and do not confer the full degree of resistance observed for NHE3, implicating additional residues in amiloride binding. Subsequent analysis of chimeric proteins of NHE1 and NHE3 has confirmed that all the molecular determinants for isoform-specific drug sensitivities reside within the N-terminal transmembranous regions, although their precise locations have yet to be defined (28).
The objective of this study was to conduct a more systematic analysis of putative membrane-spanning segments of the NHE that may contribute to drug recognition. To accomplish this, we exploited the striking pharmacological differences and extensive sequence homology in the N-terminal transmembranous regions between NHE1 and NHE3 to create chimeric exchangers. Unique restriction endonuclease sites were engineered into the N-terminal transmembranous region of each cDNA by site-directed mutagenesis, and chimeric cDNAs were constructed by exchanging homologous segments. The specific drug sensitivity profiles of each chimera were then determined in stably transfected cells and compared with their parental exchangers.

EXPERIMENTAL PROCEDURES

Materials—Carrier-free 22NaCl (radioactivity, 5 mCi/ml) was obtained from DuPont NEN. Amiloride, cimetidine, and ouabain were purchased from Sigma, and the amiloride derivative 5-(N-ethyl-N-isopropyl)amiloride (EIPA) was obtained from Molecular Probes (Eugene, OR). HOE694 was generously provided by Dr. Hans J. Lang (Hoechst AG, Frankfurt am Main, Germany). a-Ni minimal essential medium, fetal bovine serum, kanamycin sulfate, and trypsin-EDTA were purchased from Life Technologies, Inc. Cell culture dishes and flasks were purchased from Becton Dickinson and Co. (Montreal, Quebec). All other chemicals and reagents used in these experiments were purchased from British Drug House (St. Laurent, Quebec) or Fisher Scientific and were of the highest grade available.

Construction of Na+/H+ Exchanger Chimeras—Complementary DNA fragments of rat NHE1 (N-terminal-NsiI fragment, nucleotides -761 to +3820) and NHE3 (KpnI-Smal fragment, nucleotides -44 to +4288) were initially subcloned into the mammalian expression plasmid pCMV and called pCMV/NHE1 and pCMV/NHE3, respectively, as described previously (12). To facilitate construction of NHE chimeras, it was necessary to remove parts of the 5'- and 3'-untranslated regions of NHE1 and NHE3 in order to create a number of useful single restriction endonuclease sites. For NHE1, nucleotides -761 to -42 in the 5'-untranslated region were removed by polymerase chain reaction mutagenesis. As well, nucleotides +3454 to +3820 in the 3'-untranslated region and part of the pCMV polylinker region were eliminated by removing an Xbal-Xbal fragment. For NHE3, nucleotides -2950 to +4288 in the 3'-untranslated region and part of the polylinker were eliminated by removing an NsiI-NsiI fragment. Unique SpeI and BstBI sites were also eliminated from pCMV and NHE1, respectively, by site-directed mutagenesis (29). The removal of a unique BstBI site in NHE1 (nucleotide position +266 relative to the ATG start codon) did not change the amino acid sequence. The modifications resulted in both cDNAs being flanked by single HindIII and Xbal sites at the 5'- and 3'-ends, respectively. In addition, several unique restriction endonuclease sites (AgeI, BstBI, SpeI, NheI, and EagI) were engineered simultaneously into each cDNA at analogous positions in the N-terminal transmembranous regions by site-directed mutagenesis. In NHE1, the sites for AgeI, BstBI, SpeI, NheI, and EagI were created at amino acid positions 208, 281, 327, 391, and 504, respectively, whereas in NHE3, these sites were inserted at positions 155, 228, 276, 340, and 454, respectively. For the most part, generation of each site either preserved the native amino acid sequence or resulted in substitution of an amino acid present in one isoform with the homologous residue found in the other isoform (i.e. for NHE1, L208T (Agd) and S391A (Nhe), for NHE3, K277S (Spe) and K454R (Eag)). The modified cDNAs were completely sequenced to ensure that other random mutations were not introduced during the mutagenesis procedure. These modified plasmids were labeled pNHE1 (E1'), and pNHE3 (E3'), respectively. Chimeric NHEs were then constructed by exchanging homologous domains within the entire N-terminal transmembranous regions of E1' and E3'. The isoform composition of each chimera was further verified by either polymerase chain reaction analysis and Southern blotting using isoform-specific oligonucleotides or DNA sequencing.

Stable Transfection and Expression of the Na+/H+ Exchanger Chimeric cDNAs—Chemically mutagenized Chinese hamster ovary cells (AP-1) devoid of endogenous NHE activity (30) were transfected with plasmids containing the various NHE constructs by the calcium phosphate/DNA coprecipitation technique of Davis and Olsen (11). Starting 48 h after transfection, the AP-1 cells were selected for survival in response to repeated (5–6 times over a 2-week period) acute NH4Cl-induced acid loads (i.e. H+ suicide technique) (12, 32) in order to discriminate between Na+/H+ exchanger positive and negative transfectants.

RESULTS

Structure and Function of Chimeric Rat Na+/H+ Exchangers—To systematically evaluate structural domains responsible for drug sensitivity, we took advantage of the strong sequence homology and large difference in affinities (2–3 orders of magnitude) between NHE1 and NHE3 for various pharmacological antagonists (summarized in Fig. 1) (12, 14). Nucleotides at homologous positions in the N-terminal transmembranous regions of the NHE1 and NHE3 cDNAs were substituted by site-directed mutagenesis to create several unique restriction endonuclease sites. These changes either preserved the native amino acid sequence or resulted in substitution of an existing amino acid with the corresponding residue found at the homologous position of the other isoform. These modified

![Fig. 1. The chemical structures and relative affinities of antagonists of the Na+/H+ exchanger. The chemical structures of amiloride, EIPA, HOE694, and cimetidine are illustrated. The values for half-maximal inhibition (K1/2) of wild type NHE1 and NHE3 by these compounds are listed and were obtained from references 12 and 14.](image-url)
exchangers were named E1 and E3 (illustrated in Fig. 2) to distinguish them from their respective NHE1 and NHE3 wild types.

Most E1 and E3 chimeras were constructed by substituting segments of E3 with homologous regions of E1 (Fig. 2), with the expectation that one or more of the resulting chimeras would exhibit increased drug sensitivity relative to E3. The acquisition of sensitivity by E3-dominant chimeras was considered more informative since a “gain of function” would more likely result from the transfer of structural components that directly contribute to the drug binding site than would a loss of drug sensitivity by E1, which could result from nonspecific alterations in protein structure. These chimeras were then transfected into Chinese hamster ovary cells devoid of endogenous transporter activity (AP-1) and stably selected for their ability to survive repeated intracellular acid loads. Of the 12 chimeras examined, only five (E3–1 HA, E3–1 BS, E3–1 SN, E3–1 BN, and E1–3 SN) were active. The other chimeras did not confer cell survival in response to repeated intracellular acidifications. The nonfunctional chimeras all contained foreign putative transmembrane (M) domains M6–M7 and/or M10–M12. Although speculative, these regions may be essential for homodimer assembly and targeting to the cell surface (34).

**Pharmacological Properties of Parental and Chimeric Rat Na+/H+ Exchangers**—To characterize the drug sensitivity of the functional chimeras, concentration-response experiments were conducted with amiloride, EIPA, HOE694, and cimetidine. The inhibitor concentration profiles for E3–1 SN, E3–1 BS, E3–1 SN, and E1–3 SN and E1 are shown in Fig. 3, and the values for apparent half-maximal inhibition \( K_{0.5} \) of \( H^+\)-activated \( ^{22}Na^+ \) influx are summarized in Table I. All four antagonists inhibited \( ^{22}Na^+ \) influx into cells expressing E1 to a significantly greater extent than those expressing E3, with the difference in potency being 90-, 445-, 7553-, and 170-fold for amiloride, EIPA, HOE694, and cimetidine, respectively. These results are similar to those previously reported for the unmodified wild type exchangers (see Fig. 1). Comparison of the chimeras revealed that only E3–1 SN, which contained a 66-amino acid segment of E1 (composed of the putative fourth cytoplasmic loop, M9 domain, and fifth extracellular loop), consistently showed an increase in its sensitivity to inhibition by these compounds. EIPA and HOE694 sensitivities were increased most (57-and 37-fold, respectively), whereas amiloride and cimetidine sensitivities were both increased about 2-fold. The E3–1 BS chimera (containing the M8 domain of E1) also showed a 2-fold increase in sensitivity to EIPA but not to the other compounds. The E3–1 BN chimera, which contained the M8 and M9 domains of E1, gave similar results to E3–1 SN (data not shown). In contrast, E3–1 HA showed a small decrease (2.5-fold) in its affinity for cimetidine, suggesting that the region encompassing membrane-spanning domains M1-M5 may also influence the sensitivity to certain drugs.

To verify the importance of the SN region containing M9 and adjacent loops to drug sensitivity, the reciprocal chimera was analyzed (E1–3 SN). Substitution of the SN segment of E1 with the homologous region of E3 drastically reduced the sensitivity.
of the chimera to inhibition by amiloride, EIPA, HOE694, and cimetidine by 108-, 179-, 3341-, and 42-fold, respectively, relative to E1* (Fig. 4 and Table I).

Kinetic Properties of Parental and Chimeric Na+/H+ Exchangers—The rat NHE1 isoform has a 2-fold lower affinity for Na+ than does NHE3 (10.0 ± 1.4 and 4.7 ± 0.6 mM, respectively) (12). Since the binding of these compounds can exhibit competitive or mixed competition for Na+ under particular conditions, we assessed whether the chimeras exhibited the appropriate kinetic behaviors. To this end, the initial rates of H+-activated 22Na+ influx were examined in cells expressing functional chimeras at varying extracellular Na+ (Na+o) concentrations. As illustrated in Fig. 5, A and B, the velocity of amiloride-inhibitable 22Na+ influx gradually approached saturation with increasing Na+o concentration for parental and chimeric exchangers, consistent with simple Michaelis-Menten kinetics. Analysis of the data using the algorithm of Eadie-Hofstee (V versus V/S) (Fig. 5, C and D) yielded straight lines, consistent with preservation of a single Na+ binding site in each case. Apparent Na+ affinity constants (KNa) and maximum velocities (Vmax) were estimated from the negative slope and y-intercept of these fits, respectively, and are given in Table II. These data show that the modified parental E1* and E3* exchangers retained a 2-fold difference in their apparent affinities for Na+ (27.0 ± 1.7 and 12.3 ± 0.9 mM, respectively), although the affinities are somewhat lower than those for unmodified NHE1 and NHE3. By contrast, all the chimeras exhibited a further 2-fold reduction in their Na+ affinity. In particular, the reciprocal chimeras E1–3SN and E3–1SN retained their normal Na+ transport properties; i.e., they did not show a reversal of their Na+ affinities compared with the parental exchangers. The E3–1SN chimera maintained a 2–3-fold higher Na+ affinity than the E1–3SN chimera, although both affinities were reduced.

**DISCUSSION**

The aim of the present study was to identify domains of the Na+/H+ exchanger that may be important for drug recognition and binding. In particular, we wished to delineate the segments responsible for the distinct drug sensitivity profiles exhibited by two members of the rat NHE family, NHE1 and NHE3. Previous comparisons of their drug sensitivity profiles demonstrated that NHE1 was substantially more sensitive to inhibition by amiloride, EIPA, HOE694, and cimetidine than NHE3 (12, 14). In the present study, we found that the differential potencies of amiloride, EIPA, HOE694, and cimetidine (90-, 445-, 7553-, and 170-fold, respectively) were preserved when unique restriction endonuclease sites were inserted at equivalent positions in the respective cDNAs. These modified parental exchangers (i.e., E1 and E3) served as the starting point when constructing chimeric proteins by exchange of homologous domains within the putative N-terminal transmembrane region, a region that contains all the elements necessary to confer isoform-specific responsiveness to various antagonists.

Twelve chimeras were constructed, of which only five had functional activity. While the reasons for the lack of activity of some chimeras are uncertain, we noted that all of those with a predominantly E3* primary structure contained E1 segments spanning either M6–M7 (i.e., AB fragment) or M10–M12 (i.e., NE fragment). All of our attempts to study the function of chimeras containing various combinations of these segments were unsuccessful. Moreover, the reciprocal chimeras (i.e., those with predominantly E1* primary structure) were also inactive (data not shown). These data suggest that exchanging either one, or both, of these segments disrupts structural elements that are critical for isoform-specific function. An intriguing possibility is that these regions contain structural elements involved in homodimer assembly and function of the exchanger (34). Construction of additional chimeras through creation of additional unique restriction endonuclease sites may help delineate these elements more precisely and restore function.

Analysis of the active chimeras revealed that all of the molecular determinants responsible for the relative drug insensitivity of E3* compared with E1* are confined to the distal portion of the N-terminal transmembrane region (i.e., M6–M12). This was clearly demonstrated by the chimeric transporter E3–1HA, in which the region encompassing M1–M5 of E3* was replaced with the homologous region of E1*. Despite the large substitution, the drug sensitivity of E3–1HA was unaltered relative to E3*, except for cimetidine, which showed a minor reduction (2.5-fold) in its potency. Further analysis of chimeras constructed by interchanging segments between M6 and M12 revealed that the SN fragment, which spans 66 amino acids and contains the putative fourth cytoplasmic loop, the M9 domain, and the fifth extracellular loop, conferred drug sensitivity that was intermediate between the two parental exchangers. More specifically, substitution of the SN fragment of E3* with the homologous domain of E1* increased the chimera’s (E3–1SN) affinity for EIPA and HOE694 by 57- and 37-fold and its affinity for amiloride and cimetidine by 2-fold. The more substantial changes for EIPA compared with amiloride suggest that the 5-amino-substituted moiety of EIPA may interact strongly with amino acid determinants within the SN fragment. This would also explain the enhanced sensitivity to HOE694, which shares closest structural similarity to EIPA (see Fig. 1A). In contrast, the affinities of reciprocal chimera (E1–3SN) for amiloride, EIPA, HOE694, and cimetidine were
Comparison of the inhibition constants of parental and chimeric rat Na\(^+/\)H\(^+\) exchangers for various pharmacological antagonists

Values for half-maximal inhibition (K\(_{0.5}\)) were determined from the logit transformation of the sigmoidal inhibition data presented in Figs. 3 and 4. The transformation involved plotting the ln(P/(100 - P)) (where P represents the percentage of inhibition) as a function of the log(inhibitor). The K\(_{0.5}\) is the concentration when logit = 0. Values represent the mean ± S.D.

| Chimeras | Inhibition constants (K\(_{0.5}\)) |
|----------|----------------------------------|
|          | Amiloride | EIPA | HOE694 | Cimetidine |
| E1\(^*\) | 1.3 ± 0.1 \times 10^{-4} | 8.5 ± 0.1 \times 10^{-6} | 6.4 ± 0.3 \times 10^{-4} | 5.6 ± 0.5 \times 10^{-3} |
| E3-1HA  | 1.5 ± 0.1 \times 10^{-4} | 8.8 ± 0.3 \times 10^{-6} | 8.5 ± 0.7 \times 10^{-4} | 1.4 ± 0.1 \times 10^{-2} |
| E3-1BS  | 3.7 ± 0.2 \times 10^{-4} | 8.9 ± 0.4 \times 10^{-4} | 3.5 ± 0.2 \times 10^{-5} | 2.7 ± 0.2 \times 10^{-5} |
| E3-1SN  | 1.5 ± 0.1 \times 10^{-4} | 1.5 ± 0.1 \times 10^{-7} | 1.8 ± 0.3 \times 10^{-5} | 1.4 ± 0.1 \times 10^{-3} |
| E1-1SN  | 1.7 ± 0.1 \times 10^{-4} | 3.4 ± 0.3 \times 10^{-6} | 2.8 ± 0.2 \times 10^{-4} | 3.3 ± 0.4 \times 10^{-5} |
| E1\(^*\) | 1.6 ± 0.1 \times 10^{-6} | 1.9 ± 0.1 \times 10^{-8} | 8.5 ± 0.2 \times 10^{-8} | 3.3 ± 0.4 \times 10^{-5} |

Comparison of the kinetic parameters of parental and chimeric Na\(^+/\)H\(^+\) exchangers in AP-1 cells as a function of the extracellular Na\(^+\) concentration. A and B, AP-1 cells expressing rat parental (E1\(^*\) and E3\(^*\)) exchangers and the E1-3SN chimera were grown to confluence in 24-well plates. Transport activity was measured as described in the legend to Fig. 3. Values represent the average of two or three experiments, each performed in duplicate.

TABLE II

Comparison of the kinetic parameters of parental and chimeric rat Na\(^+/\)H\(^+\) exchangers

Values represent the mean ± S.D.

| Chimeras | K\(_{\text{Na}}\) | V\(_{\text{max}}\) |
|----------|----------------|----------------|
|          | mmol min/mg | mmol min/mg |
| E1\(^*\) | 12.3 ± 0.9 | 444 ± 32 |
| E3-1HA   | 28.8 ± 4.7 | 208 ± 34 |
| E3-1BS   | 32.0 ± 4.5 | 280 ± 39 |
| E3-1SN   | 24.2 ± 4.3 | 50 ± 10 |
| E1-3SN   | 60.8 ± 12.8 | 415 ± 87 |
| E1\(^*\) | 27.0 ± 1.7 | 621 ± 38 |

Comparison of the kinetic parameters of parental and chimeric Na\(^+/\)H\(^+\) exchangers

Values represent the mean ± S.D.
demonstrated that a single amino acid substitution, Leu \textsuperscript{167} → Phe, in the putative M4 domain of NHE1 could account for the loss of amiloride sensitivity and probably binding. Likewise, mutagenesis of the equivalent residue in rabbit NHE2 (Leu \textsuperscript{143} → Phe) also reduced its sensitivity to amiloride compounds (35). However, mutations at the above mentioned sites did not confer the level of amiloride resistance observed for the NHE3 isoform (12, 14, 26); therefore, it is likely that other residues help determine drug sensitivity. Since phenylalanine is also present at the equivalent position in M4 of native NHE3 (i.e. Phe \textsuperscript{144} in rat NHE3), this site was presumed to be responsible, at least in part, for its weak sensitivity to amiloride. However, the region around Phe \textsuperscript{144} did not seem important for isoform-specific drug sensitivity, since exchanging the first five putative transmembrane domains of E3 with the homologous domain of E3 (i.e. E3-1HA) did not affect amiloride, EIPA, or HOE694 sensitivity. These differences can be reconciled by assuming that other molecular determinants within the M1–M5 region of E3 can compensate for any destabilizing effect of Phe \textsuperscript{144} in M4 for drug binding. Thus, while this site in M4 may participate directly or indirectly in drug sensitivity and possibly binding, it does not appear to be responsible for the observed pharmacological differences between NHE1 and NHE3.

In addition to Leu \textsuperscript{167}, Wang et al. (27) recently reported that mutation of His \textsuperscript{349} in the putative M9 domain of human NHE1 to Tyr, Phe, Gly, or Leu produced a modest 2-fold increase (Tyr and Phe) or decrease (Gly and Leu) in amiloride sensitivity, although other amino acid substitutions examined had no effect. These data nicely complement our results with the E3–E1–3 and E3–C1–2, i.e. E3–C1–2 and E3–E1–3 did not affect amiloride, EIPA, or HOE694 sensitivity, and possibly binding, between NHE1 and NHE3. Future studies aimed at identifying the precise structural elements involved in sensitivity to these antagonists could enable rational drug design. Compounds that inhibit NHE activity in an isoform-specific manner would permit the selective modulation of the appropriate Na\textsuperscript{+}/H\textsuperscript{+} exchanger implicated in diseases such as hypertension (36, 37) and cardiac ischemia and reperfusion injury (15, 16).

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