Amiloride and the Na^+/H^+ exchanger protein: Mechanism and significance of inhibition of the Na^+/H^+ exchanger (Review)

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Received December 14, 1998; Accepted December 23, 1998

Abstract. Amiloride and its derivatives are important tools for studying NHE-1, the ubiquitous isoform of the sodium/hydrogen exchanger protein family. Three residues in putative transmembrane domains IV and IX have been implicated in amiloride binding and several models of the proposed amiloride-binding site have been reported. Though it has been shown that sodium ions and amiloride molecules interact at unique regions of the NHE-1 protein, physiological experiments reveal a competitive relationship between the two under some circumstances. The two binding sites are thus on closely related but distinct regions on the protein.

Contents

1. Introduction

The study of cellular Na^+/H^+ exchange began in 1961 when P. Mitchel suggested the importance of Na^+/H^+ and Na^+/K^+ antiport activities for cell function in the context of the chemiosmotic model (1). Six years following sodium/hydrogen antiport activity was first demonstrated by Mitchel and Moyle (2). The landmark finding that generated interest in this field was made by Johnson *et al* who discovered that fertilization of sea urchin eggs triggered intracellular alkalinization which in turn stimulated DNA synthesis leading to cell division (3). Five years later, Kinsella and Aronson demonstrated inhibition of the mammalian Na^+/H^+ exchanger by amiloride, the classic inhibitor of these proteins (4). Since that time, a great deal has been learned about the Na^+/H^+ antiporter, particularly by using pharmacological tools such as amiloride and its derivatives.

Members of the NHE family of proteins have many functions within the cell. These roles depend on cell type and include: intracellular pH control, regulation of cell volume, Na^+ reabsorption in epithelial cells, growth factor stimulated cell proliferation (5), bicarbonate reabsorption (6), and cell adhesion (7,8). The NHEs have also been implicated in hypertension and postischemic cell death (9,10). Mechanistically, the Na^+/H^+ antiporter catalyzes the non-electrogenic, reversible exchange of extracellular Na^+ and intracellular H^+ with 1:1 stoichiometry at low pH and is essentially inactive at neutral pH (11,12). Five isoforms of this family have been identified (11,13-18) sharing 40-70% homologous amino acid sequence (19) however each having unique pharmacology, tissue expression, and chromosomal location. These are summarized in Table I. Structurally, the Na^+/H^+ antiporter is an integral membrane protein consisting of an N-terminal and C-terminal domain. The N-terminal region traverses the membrane 10-12 times and is responsible for Na^+/H^+ transport and amiloride sensitivity (23,24). The C-terminal segment is most commonly believed to be exposed to the cytoplasm and has been implicated in exchanger regulation. It contains a number of phosphorylation sites (serine residues) and two calmodulin binding sites (high and low affinity) (11,13,23,25-27). The most current topological model of NHE is shown in Fig. 1.

3,5-diamino-6-chloro-N-(diaminomethylene)pyrazinecarboxamide, otherwise known as amiloride, is the classic inhibitor of the Na^+/H^+. Amiloride contains a guanidino group that has a pK' value of 8.4 at 37°C and so, at physiological pH, amiloride bears a net positive charge. Amiloride was derived as the end result of therapeutic diuretic research that sought a useful combination of diuretic, saluretic and antikaluretic features with appropriate mode of action, potency and limited side effects. This compound has been shown to affect a long list of cellular proteins including: Na^+/H^+ antiporter, Na^+/Ca^{2+} exchanger, some voltage-dependent Ca^{2+} channels, Na^+/K^+ ATPase, Na^+-coupled transporters, voltage-gated Na^+ channel, and many others. In addition to amiloride, derivatives have been synthesized with greater affinities for the Na^+/H^+ exchanger (Fig. 2). To illustrate, in cells treated with FMLP (N-formyl-methionyl-leucyl-phenylalanine, a compound known to activate NHE in resting cells), comparative K_i values were measured in the presence of 140 mM Na^+ at pH 7.4 and are as follows: amiloride = 84 μM; EIPA [5-(N-ethyl-N-isopropyl) amiloride] = 380 nM; MIBA [5-(N-methyl-N-isobutyl

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Key words: Na^+/H^+ exchanger, amiloride, NHE1, Hoe 694, cariporide, Na^+ binding

Table I. Summary of characteristics of NHE isoforms.

Isoform[a]	1	2	3	Reference
Pharmacology[b]				
IC_{50} (Amiloride)	3 μM	3 μM	100 μM	(20)
IC_{50} (MPA)	0.08 μM	0.5 μM	10 μM	(20)
IC_{50} (EIPA)	0.02 μM	0.5 μM	8 μM	(21)
Tissue expression	Most tissues	Many tissues	Kidney, stomach, intestine	(22)
Chromosome location (Rat)	5	9	1	(22)

[a]NHE-4 and NHE-5 isoforms have been identified, however these proteins are not as well characterized and thus have been excluded from the table. [b]IC_{50} values obtain from exchanger-deficient fibroblasts transfected with the various isoforms. Also, NHE isoforms are inhibited by other amiloride derivatives and by cimetidine, clonidine, harmaline, and HOE694.

amiloride] = 240 nM; HMA [5-(*N*,*N*-hexamethylene) amiloride] = 160 nM (28). By comparing data from a large number of amiloride derivatives in various tissues, several general principles were postulated about amiloride analog structures and their relationships with NHE (28). First, the guanidino group must be unsubstituted in order to inhibit NHE. In fact, amiloride derivatives with hydrophobic groups (such as benzamil or phenamil) attached to the guanidino group are specific inhibitors of the epithelial Na^+ channel (28). Also, disubstituted molecules having 5 or 6 carbon alkyl groups at R2 and R3 positions have the greatest inhibitory strength (e.g. HMA). Amiloride and its derivatives have been crucial to arrival of the current understanding of cellular Na^+/H^+ exchange.

It is critical to note that in the heart, the Na^+/H^+ exchanger is important in the pathogenesis of tissue injury in myocardial ischemia and reperfusion. Inhibitors of the Na^+/H^+ exchanger such as the amiloride analogs have been found to effectively protect the myocardium from damage that may occur during ischemia and reperfusion. The mechanism by which this occurs is believed to involve excess accumulation of intracellular protons during ischemia. The activity of the Na^+/H^+ exchanger during ischemia and reperfusion causes removal of the intracellular protons and results in accumulation of intracellular Na^+. The increased levels of intracellular Na^+ have been suggested to be able to alter the reversal potential of the Na^+/Ca^{2+} exchanger. Extrusion of Ca^{2+} by the Na^+/Ca^{2+} exchanger may be inhibited and further Ca^{2+} entry may even occur by this bi-directional exchanger. Excess calcium may therefore accumulate and is believed to cause detrimental effects including cell necrosis, contracture and arrhythmias. Amiloride derivatives have been shown to be successful in

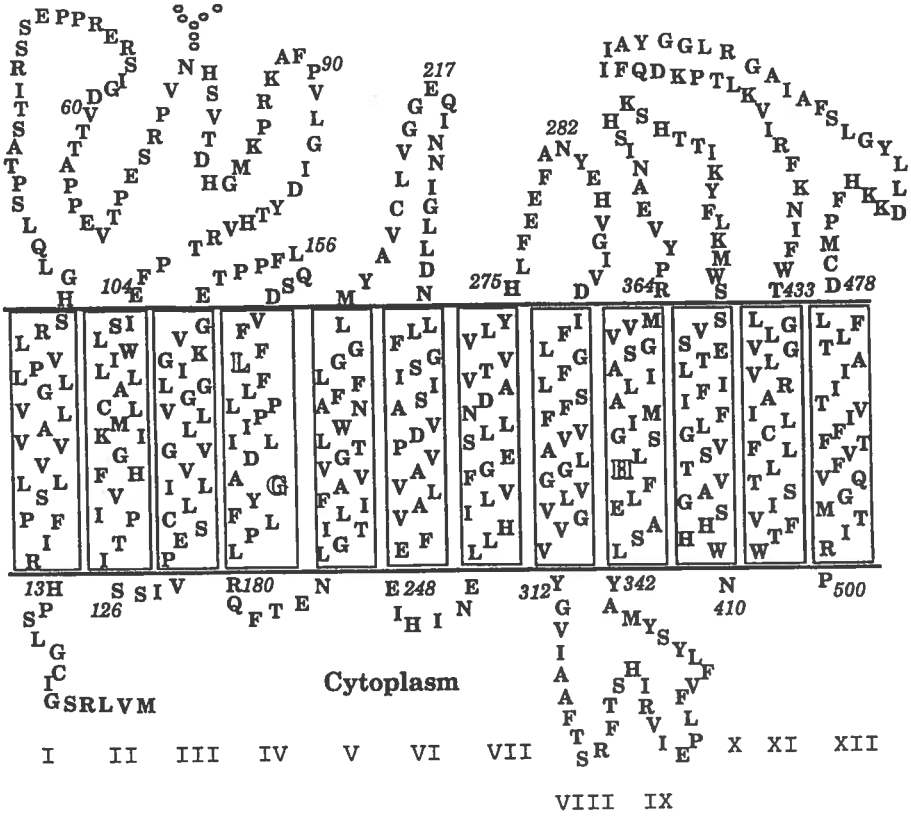

Figure 1. Model of the transmembrane domain of the NHE-1 isoform of the human Na^+/H^+ exchanger. Transmembrane domains and topology were estimated as described earlier (22). Leu 163, Gly 174 and His 349 are highlighted.

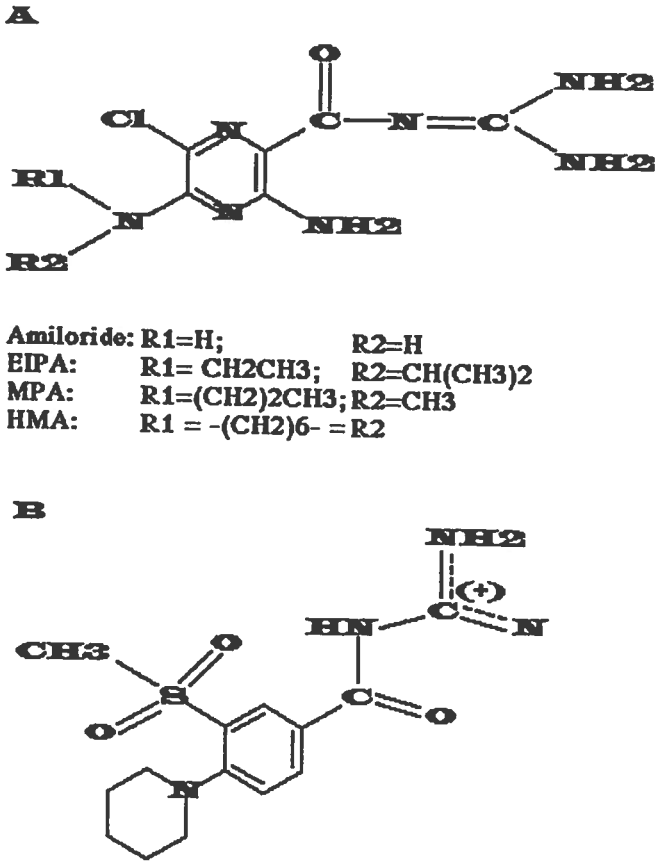

Figure 2. Molecular structure of amiloride, selected derivatives of amiloride and HOE 694.

blocking this cycle of damage to the myocardium. In addition, a new class of anti-ischemic guanidinium derivative compounds have proven useful for this purpose. Members of this new family include Hoe 694 and Hoe 642 (cariporide) which is currently in clinical trials to assess its effectiveness in high risk cardiac patients (29-31).

This review is divided into two sections. The first discusses the identification of the putative amiloride binding site of NHE-1 and its hypothesized structure. In the second part, the question of shared or unique binding sites for sodium ions and amiloride molecules is addressed. We concentrate on the mechanism of inhibition of amiloride and its analogs since most of the literature available is on these compounds rather than the newer guanidinium derivatives.

2. Identification of the amiloride binding site of NHE-1

In the context of the NHE protein there are two specific reasons for defining the binding site for amiloride and its derivatives. First, this knowledge is crucial to design more specific inhibitors to control various functions attributed to the anti-porter or to create even more discriminating agents to target particular isoforms. Secondly, since several published sources describe an apparent competition between sodium and amiloride, it is hypothesized that knowledge of the amiloride-binding site may provide insight into sodium transport. Consequently, several groups have studied the amiloride-binding site and this section will describe their results and ideas.

Chimeras. Two separate studies using chimera constructs have identified putative transmembrane (TM) domains IV and IX as critical for amiloride inhibition of NHE-1. The first group isolated a variant, AR300, that is 1000-fold resistant to MPA [5-(N-methyl, N-propyl)amiloride] (32) by progressive exposure to increasing MPA concentrations during acid loading. As a preliminary identification of the domain conferring MPA resistance, chimeric antiporter proteins were constructed with segments of wild-type and AR300 (33). By this method, the mutation conferring MPA resistance was localized to TMII-VII of the putative NHE-1 topological model and a point mutation was identified in TMIV that will be discussed below. Another group constructed chimeras of modified NHE-1 and NHE-3 (19). Modifications introduced restriction enzyme sites to facilitate construction of the chimeras and either did not change the amino acid sequence or replaced an amino acid with the corresponding residue in the other isoform. The chimera E3-1SN, which was composed primarily of NHE-3 but had putative transmembrane domain IX and framing cytoplasmic loops 4 and 5 of NHE-1, was found to be two-fold more sensitive to amiloride, 57-fold more sensitive to EIPA and 37-fold more sensitive to HOE694 than the wholly NHE-3 construct. Since NHE-3 is quite resistant to these drugs relative to NHE-1, the increased sensitivities are derived from the NHE-1 portion of the chimera thus localizing a drug interaction domain in TM1X. Taken together, results obtained from these chimera constructs suggest that regions of membrane-spanning domains IV and IX may be responsible for amiloride binding and inhibition.

Transmembrane domain IV. Looking more closely at the putative transmembrane domain IV of NHE-1 (Fig. 1), the literature has identified two key residues involved in amiloride inhibition: L163 and G174 (numbers refer to the human protein sequence).

Chimeras of Chinese hamster lung fibroblast wild-type NHE-1 and 1000-fold MPA resistant NHE-1 from AR300 cells identified that putative transmembrane domains II-VII may be responsible for amiloride inhibition. Sequencing of the 700 bp cDNA fragment encoding these domains revealed the point mutation L167 to F in domain IV (corresponds to L163 in human sequence) (30). Introduction of this mutation into the wild-type human NHE-1 gene gave the resulting protein the same decreased affinity for amiloride and MPA as the AR300 variant thus this residue appeared to be very important for inhibitor interaction. As a result, the investigators next considered the amino acid sequence surrounding L167 in NHE-1 and the corresponding regions in NHE-2, NHE-3, and NHE-4 isoforms. This amino acid neighborhood, consisting of residues 164-173 in NHE-1, is quite conserved among the isoforms, however NHE-2 differs from the NHE-1 sequence by one amino acid while NHE-3 and NHE-4 differ at two sites. Since the isoforms vary in IC_{50} values for amiloride (NHE-1 = NHE-2 < NHE-3) and MPA (NHE-1 < NHE-2 < NHE-3), site-directed mutagenesis was used to introduce the putative amiloride

binding sequence of NHE-2, 3 or 4 in to NHE-1 cDNA. Although NHE-1 and NHE-2 have the same IC_{50} for amiloride, NHE-2 is 10-fold more resistant to MPA. Mutating F168 to Y in NHE-1 to create the putative amiloride binding site sequence found in NHE-2, however, did not change its IC_{50} for MPA. Similarly, mutating the proposed amiloride binding site of NHE-1 into that of NHE-3 conferred increased IC_{50} values for both amiloride and MPA. The relative resistance that is characteristic of NHE-3 was not observed. Thus, although L167 does appear to be involved in NHE-1 inhibition by amiloride and derivatives, other sites must be involved in interaction with these drugs since the differing pharmacological profiles of the isoforms could not be localized to the 164-173 stretch.

In a later study, this group introduced three other amino acids at position 163 of human NHE-1 in order to elucidate the mechanism that allowed F to disrupt amiloride interaction with the antiporter (34). It was concluded that high affinity binding of amiloride could only be achieved with L as residue 163 since neither substitution by the smaller amino acid, A, the larger residue, W, or the more polar amino acid, R, were able to produce a lower IC_{50} value than the wild-type for amiloride, MPA and HOE694.

To further underscore the involvement of L163 in amiloride inhibition of sodium/proton antiport, it is worthwhile to briefly discuss a study using site-directed mutagenesis of the corresponding region of the NHE-2 isoform (21). Transmembrane segment IV of NHE-2 is homologous to transmembrane segment IV of NHE-1 and L143 of NHE-2 is equivalent in position to L163 of NHE-1. Yun *et al* mutated NHE-2 in the putative amiloride-binding region of transmembrane domain IV to create homologues of NHE-1, NHE-3, and AR300 by mutation of Y144F, L143F or both L143F and Y144F, respectively. In $^{22}Na^+$ uptake experiments, the NHE-1 homologue showed a small increase in sensitivity to both amiloride and EIPA however these increases were not significant. The homologue of NHE-3 displayed an IC_{50} for EIPA very close to that of the NHE-3 isoform but it was approximately 8-fold more sensitive to amiloride than wild-type NHE-3. In contrast, IC_{50} for amiloride of AR300 and its homologue were found to be the same. Thus, although other residues must be involved in binding to amiloride as suggested by the unchanged pharmacology of the NHE-1 homologue and the only partial mimicking of NHE-3 by its homologue, L143 in NHE-2 (thus L163 in NHE-1) does play a critical role in the pharmacological profiles of these isoforms.

A second residue involved in inhibitor interaction was discovered when Counilon *et al* identified decreased amiloride binding of an NHE-1 mutant bearing a G174 to S substitution (34). These investigators used random chemical mutagenesis to obtain altered NHE-1 cDNA followed by transfection of the products in to PS120 fibroblasts and isolation of clones that exhibited amiloride resistance. Clones that demonstrated amiloride resistance by simply overexpressing NHE-1 were disqualified from further analysis. Using this method, three identical mutants were obtained which were resistant to amiloride, MPA and HOE694 (3.3, 3.0, and 2.7-fold, respectively, compared to the wild-type phenotype). Sequencing revealed the point mutation G174 to S in the putative transmembrane domain IV. To confirm that this mutation alone was responsible for the change in pharmacological characteristics, G174 was replaced with S in NHE-1 cDNA by site-directed mutagenesis and PS120 fibroblasts transfected with this construct did exhibit the same resistance to amiloride, MPA and HOE694 as the original mutant. After illustrating the significance of G174 to amiloride binding, investigators went on to replace this residue with A and D. The G174A NHE-1 exhibited the same amiloride sensitivity as the wild-type while the G174D mutant was slightly more resistant to amiloride inhibition than the G174S mutant. These results suggest that a polar amino acid at position 174 has a detrimental effect on amiloride binding. Furthermore, since the G174S mutation increased both amiloride and MPA resistance by approximately 3-fold, residue 174 may be interacting with a region of the inhibitor molecule common to both amiloride and the MPA derivative and not with the N5 substituents of MPA.

Upon identification of L163 and G174 involvement in amiloride binding, Counillon *et al* constructed a double mutant, L167F/G174S, to study the joint effect of these substitutions on pharmacology and activity (34). They constructed dose-response curves displaying $^{22}Na^+$ uptake by PS120 cells transfected with the double mutant NHE-1. They were unable to show an experimentally significant increase in the IC_{50} for amiloride and MPA by the double mutant [though L163F/G174S does have slightly higher IC_{50} values for both amiloride and MPA (17 μM and 2 μM) compared to G174S (10 μM and 0.12 μM) or L163F (12 μM and 1.5 μM)]. However, using the inhibitor HOE694, investigators have found a significant increase in the IC_{50} value for the double mutant compared to the single mutants. This compound is structurally related to amiloride and is thus hypothesized to act mechanistically similar upon interaction with the antiporter. The advantage of HOE694 is that it has very distinct inhibition constants for each isoform (NHE-1 = 0.16 μM; NHE-2 = 5 μM; NHE-3 = 650 μM) compared to amiloride (NHE-1 = 3 μM; NHE-2 = 3 μM; NHE-3 = 100 μM) (5). Though HOE694 will not be discussed further, its sensitivity is key to recognizing the decreased ability of inhibitors to bind the double mutant. Thus, pharmacological properties of the L167F/G174S NHE-1 mutant re-enforce the importance of these particular residues for inhibitor interaction.

Transmembrane domain IX. In addition to L163 and G174 in putative transmembrane domain IV, H349 in membrane-spanning domain IX has been implicated in amiloride binding of NHE-1 (35). Since the transport of protons across the cell membrane involves histidines in other systems (such as the Lac permease of *E. coli*) (36), Wang *et al* chose to modify and analyze NHE-1 H residues. To do this, mouse LAP-cells transfected with human NHE-1 cDNA were treated with DEPC (diethyl pyrocarbonate) and the altered exchanger was characterized. NHE-1 activity was inhibited by 70% when treated with 50 μM DEPC however functionality was partially restored to 27% of untreated Na^+-dependant pH recovery by hydroxylamine (within experimental error, treatment of cells with hydroxylamine alone shows this decrease in exchange activity). Since DEPC can modify C, Y, K and H residues (though some sources suggest DEPC is His-specific at pH 5.5-7.5) (37,38) but hydroxylamine only reverses DEPC alterations of H residues, the changes in NHE-1 activity was

localized to histidines and confirmed their role in protein function. Next these investigators showed that DEPC NHE-1 inactivation could be partially blocked by pretreatment of the cells with amiloride which suggested that H residues may be involved in amiloride binding. Subsequently, conserved putative transmembrane histidines 35, 120 and 349 were replaced with G by site-directed mutagenesis and characterized. Though these mutants were as sensitive to DEPC as wild-type NHE (suggesting different H residues are involved in H^+ translocation), it was found that the H349G single mutant was 2.4-fold less sensitive to amiloride and 4.5-fold more resistant to the amiloride derivative EIPA. These results suggested that H349 is involved in the interaction of amiloride with NHE-1.

To understand how amiloride could interact with H349, various amino acids were introduced at this position by site-directed mutagenesis. The ability of amiloride to inhibit initial pH recovery following acid loading was measured for each mutant. It was found that replacing H349 with G, L or S decreased amiloride sensitivity while introduction of V, Q and N yielded wild-type amiloride susceptibility and F and Y substitutions created a more amiloride sensitive exchanger. From these results the authors drew the following conclusions. First, amiloride does not bind to residue 349 via hydrogen bonding because both V and F are incapable of participating in hydrogen bonds at their side chains yet these mutant exchangers had the same or greater amiloride sensitivity than the wild-type protein. Second, it is unlikely that these mutations induced a conformational change in the amiloride binding site located in a distant region of the protein because there is no relationship between the side chain length of the replacing residue and the resulting amiloride sensitivity (both short chain, G, and long chain, L, decreased amiloride inhibition yet intermediate side chain, V, had the same amiloride sensitivity as wild-type). Also, aromatic substitutions should have caused a conformational change that discouraged inhibitor binding to a distant site however both F and Y mutants were more sensitive to amiloride. Therefore, these investigators postulated that the amiloride binding site includes H349 or a region in close proximity to this residue.

To summarize, it appears that both putative membrane spanning domains IV and IX play a role in the amiloride binding site. In light of the significant evidence for L163 in NHE interaction with amiloride, however, the current literature refers to 160-VFFLFLL-166 as the amiloride binding domain when comparing the Na^+/H^+ antiporter to related proteins (33). Thus, although much is now understood about how amiloride interacts with NHE-1, much remains to be learned.

Amiloride binding site structure. Ultimately, acquisition of the three dimensional structure of NHE-1 would provide the most complete understanding of the amiloride binding site. The route to achieving such a goal is incredibly difficult and essentially impossible at the present time. However, Kleyman and colleagues have hypothesized how transmembrane domain IV of NHE-1 may bind amiloride using an anti-amiloride monoclonal antibody, BA7.1, and the concept of molecular mimicry (39). Since both NHE-1 and BA7.1 can bind amiloride, small regions of homologous sequence are expected where these complexes contact the ligand hence the antibody is expected to mimic the antiporter. This group made monoclonal antibodies against amiloride, sequenced the heavy and light chains, deduced the amino acid sequence and used two computer programs (Peptide Structure from GCG software package 7.1 and Iditis version 2.0 by Oxford Molecular Ltd.) to elucidate a plausible secondary structure. Based on this structure and sequence common to NHE-1 transmembrane domain IV and BA7.1, a 3-D model for amiloride binding was proposed wherein the pyrazine ring of amiloride stacks with F166 and the alkyl groups of MPA contact L167 or the drug ring stacks with F168 while alkyl groups touch V164 and L167. Since no other favorable interactions for stabilizing the remainder of the amiloride molecule could be identified, Kleyman *et al* suggested that a second helix must be involved in forming the amiloride binding pocket of NHE-1. Though this proposed structure is very intriguing and may indeed be correct, further evidence is required to confirm that amiloride does bind NHE-1 as suggested.

A second group has also reported on a model of putative transmembrane domain IV based on fitting the primary sequence to an α-helix, the secondary structure most commonly found to traverse the membrane bilayer (34). Of particular interest are the highly conserved proline residues at positions 167, 168, and 178 that produce a kink in the backbone of the α-helix. Thus, in addition to being unavailable to hydrogen bond with residue O_{i-4} (since the cyclic side chain structure of proline is bonded to its amide group leaving it unable to be a hydrogen bond donor), this kink prevents hydrogen bonds from forming between N_{i+1} and O_{i-3} residues. As a result, the carbonyl moieties of L163, F164, L165, G174, and Y175, do not participate in hydrogen bonding leaving the structure highly flexible and these residues free to potentially bind sodium or amiloride. However, much like the structure proposed above using antibodies, ultimate verification of these ideas awaits determination of NHE-1 three dimensional structure.

3. Do sodium and amiloride bind the same site of NHE-1?

By considering molecular structures, Counillon *et al* pointed out that the guanidinium group of amiloride and a hydrated Na^+ ion are of similar size and shape (20) thus it is plausible that amiloride blocks cation transport by binding directly at the Na^+ site of the NHE protein. To this end, competitive inhibition of NHE-1 activity by amiloride and its derivatives has been demonstrated by measuring H^+ uptake or release by CCL39 cells (Chinese hamster lung fibroblast line) with various intracellular and extracellular ionic environments (12). More recently, Counillon and colleagues have discovered that in two double mutants of NHE-1, a change in amiloride sensitivity is accompanied by a detectable difference in Na^+ transport kinetics (23,31). The first mutant, F165Y/F168Y, was generated in a series of constructs that created homologues of isoforms 2, 3, and 4, in the amiloride binding stretch (164-VFFLFLLPPI-173) of NHE-1 cDNA (23). While NHE-2 and -3 homologues were less sensitive to amiloride but Na^+ movement was unchanged relative to wild-type, the double mutant F165Y/F168Y (reflecting the NHE-4 isoform) had a decreased V_{max} for Na^+ transport in addition to being 33-fold more resistant to amiloride than wild-type NHE-1. The second mutant, L163F/G174S, was constructed following the discovery of the importance of these particular residues in

amiloride binding and was found to have decreased affinity for both amiloride (5.6-fold less than wild-type) and Na^+ (2-fold less than wild-type) (34).

Although the demonstration of competition and the preceding citations provide evidence for a shared sodium and amiloride binding site on NHE-1, there are several examples from the literature that suggest the two molecules may contact unique regions of the exchanger protein. In each of the three studies discussed here, investigators found that amiloride sensitivity could be changed without a detectable parallel alteration of Na^+ transport activity. First, using homologues of NHE-1 and NHE-3 (generated by mutation of the putative amiloride binding site of NHE-2), the same Na^+ kinetics were found for all isoforms despite the fact that NHE-1 was amiloride sensitive, NHE-2 was EIPA resistant and NHE-3 was resistant to both amiloride and EIPA (21). Secondly, mutation of H349 to G in NHE-1 decreased amiloride sensitivity by 2.4-fold and EIPA inhibition by 5.7-fold yet Na^+ transport was unaltered (35). Finally, chimeras in which putative transmembrane domain IX were switched between NHE-1 and NHE-3 (i.e. each chimera contains the whole NHE molecule of one isoform except that transmembrane segment IX has been replaced by the equivalent region from the opposite isoform) had amiloride inhibition constants that were characteristic of the grafted domain IX but Na^+ transport kinetics were typical of the dominant isoform of the chimera (19).

In all the experimental work defining a common Na^+ and amiloride binding region on the NHE-1 protein described above, data was obtained by a very similar procedure. Briefly, wild-type NHE-1 cDNA was altered by site-directed mutagenesis or chimera construction, mutants were transfected in to a cell line lacking endogenous Na^+/H^+ exchange and protein activity of the transfectants was measured predominantly via $^{22}Na^+$ uptake analysis in the presence and absence of amiloride and its derivatives. This approach demonstrates amiloride or sodium binding and competition as a function of protein activity. Thus, since the strongest evidence resulting from these experiments supports a common Na^+ and amiloride interaction site on NHE-1, it could be said that sodium and amiloride have the same functional binding site. However, such a definition does not necessitate a physically identical binding region since none of the experiments described above make structural measurements of the NHE-1 protein. To this end, several other groups have used radiolabelled amiloride derivatives such as [^{3}H]-EPA (5-(N-ethyl-N[^{3}H] propyl) amiloride and [^{3}H]-MIBA [^{3}H]5-(N-methyl-N-isobutyl) amiloride (28) to quantitate binding site density and protein activity and have shown that the presence of Na^+ ions does not reduce the specific binding of these labels to the antiporter. Taken together, the results suggest a competitive relationship between amiloride and sodium for physically distinct binding sites that function cooperatively with respect to NHE function such that competitive behavior is observed.

4. Conclusion

In conclusion, this review has described the current understanding of the amiloride binding site of NHE-1 and the relationship between sodium ions and amiloride molecules with respect to their interaction with NHE-1. Solid evidence has been presented to show the importance of transmembrane domains IV (specifically L163 and G174) and IX (in particular, H349) for binding of amiloride and its derivatives. Based on this, several models of the NHE-1 amiloride binding pocket have been published. Also, it appears that Na^+ and amiloride do bind unique sites of the Na^+/H^+ antiporter however these binding regions may be functionally related and an apparent competition is found in assays that measure exchanger activity. Finally, since Mitchel's postulates of 1961, much is now understood about the Na^+/H^+ antiporter and the NHE protein family through the use of amiloride and its derivatives. The future promises a more detailed picture of how amiloride binds NHE-1 that will facilitate the design of more specific inhibitors and provide greater understanding of the antiporter itself.

Acknowledgments

Work by LF was supported by grants from the Heart and Stroke Foundation and by the Medical Research Council of Canada. We thank Pavel Dibrov and Christine Wiebe for their review of the manuscript. LF is a Senior Scholar of the Alberta Heritage Foundation for Medical Research.

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