The Expanding Family of Eucaryotic Na\(^+\)/H\(^+\) Exchangers*

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Maintaining intracellular pH values close to neutrality is a crucial task for a wide variety of cells. Hence, various mechanisms for pH regulation have been selected early in evolution and are ubiquitously distributed. Among the actors in this scene, the members of the Na\(^+\)/H\(^+\) exchanger gene family (NHE isoforms) are widely expressed and constitute extremely efficient systems for protecting cells against internal acidification. To date, at least six genes have been shown to be located in the cytoplasm, at least for the NHE family members and highlight the most characterized isoforms. In this short review, we will update our current knowledge on these NHE family members and discuss important aspects of the basic function of these transporters. Then, in a broader physiological context, we will present what we think are the most prominent features of the different NHE isoforms.

**Structural and Functional Domains of NHEs**

The first cDNA encoding the NHE-1 isoform was cloned using an expression strategy based on the ability of Na\(^+\)/H\(^+\) exchangers to protect antiporter-deficient cells (1) against otherwise lethal intracellular acidification (2). Variations in the hormonal regulation and pharmacological features of Na\(^+\)/H\(^+\) exchange were the first indications that a large family of Na\(^+\)/H\(^+\) exchange molecules existed (3). Therefore, using the NHE-1 cDNA as a probe led to the molecular identification of the NHE-2, -3, and -4 (4–7) isoforms. In addition, non-epithelial isoforms such as NHE-5 (8–10) and NHE-6 have been cloned recently. By contrast to the other Na\(^+\)/H\(^+\) exchangers, NHE-6 is not expressed at the plasma membrane but in the mitochondria (11).

**Topological Features and Sequence Conservation**

The highly hydrophobic N-terminal region of the protein is predicted to span the membrane 10–12 times depending on the algorithm used to calculate the hydropathy plot of the protein. In particular, the region situated in the central part of the transmembrane domain (residues 226–281 in the human NHE-1) is quite hydrophobic but contains several negatively charged residues. By contrast the C-terminal region of the Na\(^+\)/H\(^+\) exchangers is hydrophilic and has been shown to be located in the cell cytoplasm, at least for the NHE-1 isoform (12, 13). Interestingly, recent experiments on the NHE-3 isoform seem to indicate that epitopes within the C-terminal region of this protein are extracellularly exposed (14). For NHE-1 and -2, the loop between the putative transmembrane segment 1 and 2 is glycosylated and therefore extracellular (15, 16). Methods such as scanning N-glycosylation mutagenesis (17) will be necessary to gain further topological information.

**Sequence Comparison between NHE Isoforms**

The transmembrane domain exhibits from 45 to 65% amino acid identity, although this score drops to about 25–35% for the cytoplasmic domain. A more detailed analysis of the sequence homology clusters reveals the presence of two subfamilies of isoforms that have probably diverged later in evolution: NHE-2 and -4 as well as NHE-3 and -5. The central part of the domain (putative transmembrane segments 5a and 5b between residues 226 and 281 in human NHE-1) is nearly identical between all the NHE isoforms. This part of the polypeptide possesses negatively charged residues (aspartates 226, 238, and 267 and glutamates 247, 248, 253, and 262 in the human NHE-1) included in a highly hydrophobic stretch of sequence, and the substitution of Glu-262 in the NHE-1 isoform results in the inactivation of the transporter (18). Although it is not possible to rule out an indirect effect of this mutation, this result is in association with the extreme sequence conservation of this region among the NHE members strongly suggests that these two transmembrane segments of the exchangers constitute the catalytic core of the Na\(^+\)/H\(^+\) exchangers.

By contrast, the first putative transmembrane segment of the Na\(^+\)/H\(^+\) exchangers and the first extracellular loop are not well conserved in the NHE family. These N-terminal sequences are divergent even in the same isoform cloned from various mammalian species (19), indicating that sequence conservation in the first extracellular loop is not crucial for the function of the protein. A closer analysis of the first stretch of hydrophobic residues using the von Heijne rules (20, 21) reveals that this first putative transmembrane segment has the features of a signal peptide, including a positively charged N-terminal end and a relatively short hydrophobic stretch.

Although the cytosolic domain sequence seems to be more poorly conserved, alignment methods based on the presence of hydrophobic secondary structures (hydrophobic score analysis) (22, 23) show that these C-terminal domains clearly exhibit structural similarities.** Recently, circular dichroism measurements performed on the Escherichia coli-expressed NHE-1 C-terminal region confirmed that this part of the protein possesses a high degree of structural organization (24).

**Biochemical Features of Na\(^+\)/H\(^+\) Exchangers**

**Transported Cations**—Under physiological conditions, the Na\(^+\)/H\(^+\) exchanger mediates the electroneutral exchange of one extracellular sodium ion for one intracellular proton (for review, see Ref. 25). For the NHE-1, -2, and -3 isoforms, extracellular sodium binding occurs at a site that cannot be distinguished from the Na\(^+\) transport site (26), the saturation function following simple Michaelis-Menten kinetics (27). Hence, the simplest model for Na\(^+\) transport predicts that sodium binds to a unique external site and is then translocated. For most isoforms, Na\(^+\) binding at its external site on Na\(^+\)/H\(^+\) exchangers occurs with a measured \(K_m\), which is about 3 times below the physiological extracellular Na\(^+\) concentration. The Na\(^+\)/H\(^+\) exchangers are hence close to saturation by extracellular sodium, and moderate variations of this Na\(^+\) concentration have virtually no effect on the activity of these antiporters. It has been documented that hypertonic medium moderately activates NHE-1 (28) but has an inhibitory effect on NHE-2 and -3 isoforms (29, 30). Interestingly, the NHE-4 isoform exhibits a radically different behavior with respect to extracellular sodium. This antiporter is directly activated with cooperative kinetics for external sodium (31).

The extracellular cation binding site is not totally selective for sodium, because it can also accommodate H\(^+\) and Li\(^+\), which can compete with sodium on its extracellular site (26, 32). By contrast, K\(^+\) ions, which are larger than Na\(^+\) ions, are not transported by the antiporter but inhibit NHE-1, having no effect on NHE-2 and -3 (33).

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¶The abbreviations used are: NHE, Na\(^+\)/H\(^+\) exchanger; RT-PCR, reverse transcription-polymerase chain reaction; MAPK, mitogen-activated protein kinase; NHE-RF, NHE regulatory factor.

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Competitive Inhibitors—Dose-response curves of initial rates of Na\(^+\)/H\(^+\) exchange inhibition by guanidinium exhibit a simple competitive behavior, suggesting that sodium and guanidinium are interacting with the same extracellular sodium site (34). Because of the poor affinity of this molecule for the Na\(^+\)/H\(^+\) exchangers, acyl guanidinium derivatives have been widely developed and tested for the inhibition of Na\(^+\)/H\(^+\) exchange. This led to the discovery of amiloride, a diuretic compound that is about 10,000 times more potent than guanidinium itself for the inhibition of the NHE-1 isoform (35). However, because of the lack of specificity and the side effects of this molecule (36, 37), other inhibitors possessing a greater specificity and selectivity for Na\(^+\)/H\(^+\) exchangers have been designed. Basically, all the alkyl 5-N-substituted derivatives of amiloride such as 5-N-dimethyl amiloride or 5-N-ethyl isopropyl amiloride possess a high specificity for NHE-1, inhibiting this isoform with 10–100-fold higher \(K_i\) values than amiloride itself (38, 39), while poorly interacting with the other NHE isoforms, which are often referred to in the literature as “amiloride-resistant” Na\(^+\)/H\(^+\) exchangers. A new class of NHE-1 competitive inhibitors (HOE694 and -642) derived from phenylacyl guanidinium derivatives has been shown to be about 4000 times more efficient for NHE-1 inhibition than for NHE-3 (40). These compounds represent promising clinical tools because they possess extremely potent anti-arrhythmic properties (41–43) and exhibit very limited side effects, their target being the NHE-1 isoform, which is predominantly expressed in heart (44, 45). Additionally, other compounds unrelated to substituted guanidinium such as cimetidine, clonidine, or harmaline have been shown to inhibit Na\(^+\)/H\(^+\) exchange and can also moderately discriminate between the NHE isoforms (33, 46).

Several inhibitor-resistant mutants were isolated (47), and two amino acid changes (L163F and G174S in the human NHE-1) affecting the interaction of amiloride and 5-N-substituted derivatives with NHE-1 were mapped (48, 49). These two mutations obtained by totally independent approaches are both situated in highly flexible regions of the fourth transmembrane segment of the antipporter. More recently, Noël et al.\(^3\) selected HOE694-resistant mutants exhibiting amino acid substitutions in the ninth transmembrane region indicating that this region is also involved in the interaction with NHE-competitive inhibitors, a result confirmed by Orlowska and Kandasamy (50). Similarly, Wang et al. (51) have systematically mutated all the transmembrane histidine residues of NHE-1 to identify crucial residues involved in cation binding. Although these substitutions did not result in any loss of function, the mutation of His-349 to Leu or Gly in the same putative transmembrane segment 9 resulted in a moderate loss in affinity for amiloride. Interestingly the H349F and H349Y are more sensitive to amiloride than the wild-type NHE-1 isoform.

The inhibition of Na\(^+\)/H\(^+\) transport by amiloride is not the only specific inhibitory effect. As mentioned above, 5-N-substituted guanidinium derivatives have been widely developed and tested for their inhibitory effect on Na\(^+\)/H\(^+\) exchange. However, whereas the presence of NHE-2 in the intestine has been confirmed by independent investigations (see for example Ref. 66), the expression of this protein in the kidney is somewhat controversial (59, 64, 65).

NHE-4 mRNA can be found in the stomach, intestine, kidney, and in the cavi ammoni fields of the hippocampus. In the kidney, NHE-4 is mostly present in the inner medulla collecting duct and has also been found heterogeneously distributed on the basolateral membrane of cortical tubule cells (65).

The recently cloned NHE-5 isoform has been detected predominantly in testis, spleen, and skeletal muscle by Northern blot (8), whereas NHE-6, which is expressed in mitochondria, has a wide tissue distribution.

The common mechanism by which intracellular signaling pathways modulate the Na\(^+\)/H\(^+\) exchangers involves the C-terminal region of these proteins, as shown in a series of key experiments. For example, the expression of a chimera consisting of the transmembrane region of the cAMP-insensitive human NHE-1 and the cytosolic region of the cAMP-activable β-NHE-1 of trout red cells results in a protein which is activated by cAMP, identical to the way the complete β-NHE-1 isoform behaves (66, 67). Conversely, NHE-3 is inhibited by cAMP in epithelial cells, and a chimeric construct between the transmembrane region of NHE-1 and the cytosolic region of NHE-3 becomes inhibited by cAMP (68). These key findings indicate that the C-terminal domain dictates the type of hormonal regulation in a given cell.

Molecular Dissection of NHE-1 Activation—NHE-1 activation by an extreme variety of extracellular stimuli, including hormones, integrins, and virtually all growth factors results from an increase in affinity of the transporter for intracellular protons. The simplest model that has been proposed is that the cytoplasmic tail cooperates with the central pH\(_i\) sensor to decrease the pH\(_i\) threshold value of NHE-1. In this regard, the cytoplasmic tail is seen as a signal integrator capable of transmitting hormonal signals to the transmembrane built-in pH\(_i\) sensor (69). Therefore, as for a promoter region of a regulated gene, it is not surprising to see that the NHE-1 cytoplasmic tail has “collected” regulatory boxes that convey specific extracellular signals. For example, all growth factors have been shown to induce a very rapid and transient activation of cytoplasmic calcium as well as a more or less sustained activation of the p42/p44 MAPK cascade. Interestingly and as presented below, the NHE-1 cytoplasmic domain intercepts these distinct signals for transmission into a cytoplasmic alkalinization.

Bertrand et al. (70) demonstrated that calmodulin physically interacts with a particular subdomain of the NHE-1 cytosolic region (71) releasing a negative constraint, thus resulting in the activation of NHE-1 by increases in intracellular Ca\(^{2+}\). Therefore, this calmodulin-binding regulatory box is sufficient to account for the rapid and transient activation of NHE-1 in response to growth factors.
and other Ca\(^{2+}\)-mobilizing agonists. By contrast, a similar sequence is not found in NHE-3, which is also regulated by calmodulin, both in a calmodulin kinase-dependent and -independent manner (72).

Direct phosphorylation of NHE1 and/or phosphorylation of ancillary proteins could account for more robust and sustained activation of NHE-1. Both mechanisms have been well documented. It was shown that NHE-1 is a phosphorylated protein and that its level of phosphorylation is increased in mitogen-stimulated cells when compared with unstimulated controls (13, 73–75). Phosphopeptide mapping carried out on wild-type and deletion mutants revealed that the phosphorylation sites are located in the C-terminal cytoplasmic region of the protein. Ser-703 was recently demonstrated to be phosphorylated in vivo by the p42/p44 MAPK-activated target, p90\(^{RKP}\) (77), and to represent a major site for serum activation. This result is in agreement with our demonstration, using a Raf-activatable construct, that p42/p44 MAPK plays a key role in NHE1 activation (78). Besides the MAPK pathway, NHE-1 has been shown to be phosphorylated by p160 ROCK (79), a Rho effector associated with the assembly of stress fibers and focal adhesions. However, p42/p44 MAPK-mediated NHE-1 activation cannot be entirely explained by the direct phosphorylation of NHE-1. First, deletion of the distal cytoplasmic tail containing Ser-703 and other major phosphorylation sites attenuates but does not abolish growth factor activation. The residual activation (about 50%) remains sensitive to the MEK inhibitor PD98059 (78). The simplest working model taking into account this set of results is that additional ancillary regulatory proteins, which themselves be phosphorylated, interact with various domains of the cytosolic region of the exchanger. Candidate proteins have been identified, such as p24 NHE-1 (80), HSP70 (81), CHP (82), and other proteins obtained by double hybrid screening, such as myosin light chain phosphatase. Additionally, NHE-1 can be activated by different mechanical stimuli such as osmotic stress or cell spreading. Grinstein et al. (83) have demonstrated that the mechanism of this activation is phosphorylation-independent. In view of their finding that NHE-1 is associated with the actin cytoskeleton in focal adhesion plaques (84), these results suggest that this activation might be mediated by direct contact with cytoskeletal proteins. This hypothesis is reinforced by the fact that the presence of relatively high concentrations of ATP is required for optimal NHE-1 activation (85) and that ATP depletion results in a more homogeneous plasma membrane distribution of NHE-1. As the NHE-1 phosphorylation level is not changed upon cellular ATP depletion, NHE-1 might interact in an ATP-dependent manner with an ancillary protein mediating NHE-1 interaction with cytoskeletal elements (86). Bianchini et al. (78) demonstrated that the p42/44 MAPK is a major pathway for NHE-1 activation by many growth stimuli and is a stress-activated phosphoprotein. In the latter pathways (JNK and p38 MAPK) are not involved in the activation of NHE-1 by hypertonicity. Thus the C-terminal domain of NHE-1 can be viewed as a series of regulatory cassettes, where upon phosphorylation or binding of regulatory proteins, the affinity of the transporter for intracellular protons is modulated.

**NHE-3 Regulation**—The NHE-3 regulation mechanism is completely different from the NHE-1 activation because the changes in activity reflect modifications of the V_{max} of the transporter instead of changes in its apparent affinity for intracellular protons. Using immunofluorescence techniques and confocal microscopy, it is possible to detect NHE-3 accumulation in recycling endosomes (87). Weinman and Shenolikar (88) have described the cloning and characterization of a 538-amino acid NHE regulatory factor (NHE-RF) (for review, see Ref. 88). This protein, which can be either cytoplasmic or membrane-bound, negatively regulates NHE-3 activity by direct binding. When coexpressed in PS120 cells, NHE-RP is able to confer cAMP inhibition to NHE-3. This protein is present in various sections of both the intestine and the kidney tubule. Further, it has been detected in the liver where NHE-3 is not detected, indicating that it could mediate cAMP regulation of proteins other than the NHE-3 isoform. Indeed, NHE-RP-like proteins, such as E3KARP, have been shown to regulate other transmembrane transporters, including the Na-HCO\(_3\) or Na-P\(_{i}\) cotransports. E3KARP can also confer cAMP regulation upon NHE-3, indicating that the NHE regulatory proteins might be multiple (89). Recently Hall et al. (90) demonstrated that the \(\beta\)-adrenergic receptor can directly associate with NHE-RP through its PDZ domain, providing a direct regulation of NHE-3 activity. Similarly, Yun et al. (91) have demonstrated that E3KARP binds directly to an intracellular region within the C-terminal domain of NHE-3 and defined more precisely the protein regions participating in this interaction. Taken together, these results suggest that E3KARP or NHE-RF have a scaffolding function, permitting the co-localization of NHE-3 and kinase A.

Similar to NHE-1, the NHE-3 cytosolic region therefore appears to consist of various regulatory cassettes, which seem to integrate activating and inhibitory signals from various signaling pathways (72). In an independent work Cabado and co-workers (68) showed that the region situated between residues 579 and 684 mediates cAMP inhibition. In this region, which contains 6 Ser, only Ser-605 and -634 are crucial for cAMP inhibition (92). Interestingly, although mutation of Ser-634 affects forskolin response, only Ser-605 is indeed phosphorylated.

### Concluding Remarks

Gene knockout is an elegant approach to obtain new insights into the physiological role of proteins belonging to multiple gene families. Results from this approach are highly relevant to NHE isoforms. Schultheis et al. (93) have constructed knockout mice for NHE-3, an isoform which was expected to largely contribute intestinal sodium absorption as well as kidney sodium reabsorption coupled to bicarbonate reabsorption. As expected, these homozygous knockout mice exhibit a decrease in blood pressure, they are mildly acidic, and they present absorptive defects both in kidney and intestine. This important result confirms the predicted physiological role of NHE-3 and shows that this isoform, when compared with NHE-1, -2, and -4, mediates the great majority of the absorptive process in kidney and intestine.

By contrast, Cox et al. (94) have reported the molecular characterization of the genetic defect present in epileptic and ataxic (SWE) mice. They discovered that these mice possess a point mutation, which introduces a stop codon in the coding sequence of NHE-1, resulting in the production of a truncated, inactive transporter. The homozygous inactivation of NHE-1 gene function reported thereafter (95) confirmed the non-lethality of this mutation, a finding which was anticipated in light of the ubiquitous expression of compensatory pH regulating systems such as sodium-dependent Cl-/HCO\(_3\) exchangers. Interestingly, these mice have no apparent defects in their acid-base homeostatic balance or in their kidney or intestine function. Rather, they show defects in brain function; this organ, which is highly sensitive to pH, was not initially hypothesized as the main target of the NHE-1 gene disruption. It is therefore tempting to predict that, as for NHE-1, the disruption of the other isoforms may have surprising physiological consequences. Hence, the recent knockout of the NHE-2 isoform in mouse did not result in detectable modifications in intestinal function but in a modified gastric mucosa, resulting in deficient acid secretion in the stomach (96). Therefore, as has been reported for many gene disruptions, the interpretation of the resulting phenotypes might be confusing because of the possible involvement of the NHE isoforms during critical steps of the embryonic development and the presence of compensatory mechanisms in adult mice. In this case, the recent development of tissue-specific gene disruption and of inducible knockout techniques should prove to be very useful for the future physiological studies of Na/H\(^{+}\) exchange.

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