Minireview

Na⁺/H⁺ Exchangers of Mammalian Cells*

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Ejection of intracellular H⁺ in exchange for external Na⁺ is the most effective means of eliminating excess acid from actively metabolizing cells. Na⁺/H⁺ exchange is also crucial for the regulation of the cellular volume and for the reabsorption of NaCl across renal, intestinal, and other epithelia. This remarkable array of essential functions is carried out by a family of antiporters, known generically as Na⁺/H⁺ exchangers (NHEs). These are highly regulated (glyco)phosphoproteins present in virtually all mammalian tissues and species studied to date. The intent of this review is to provide a concise update of the structure, distribution, and regulation of the activity of the known members of the mammalian NHE family.

The Na⁺/H⁺ Exchanger Gene Family

In mammalian cells, NHE activity is localized to both the plasma membrane (1, 2) and the mitochondrial inner membrane (3). To date, five NHE isoforms (NHE1–NHE5) have been identified (4–8). In addition, a novel isoform (NHE6) was recently isolated and is still incompletely characterized. These transporters are derived from distinct genes that are dispersed throughout the mammalian genome, with no clear evidence of alternative splicing of single mRNA transcripts (reviewed in Ref. 9). A variant rat NHE2 cDNA lacking the coding sequences for the N-terminal 116 amino acids was isolated and postulated to be derived from an alternatively spliced transcript (10). However, the possibility remains that this cDNA represents a partially processed RNA transcript retaining an intron sequence at its 5’ end. Overall, NHE1–NHE5 share ~34–60% amino acid identity, their predicted molecular mass ranging from ~81 to 93 kDa. The recently identified NHE6 shows only ~20% identity to the other isoforms.

Structural Features

Membrane Topology—Based on their primary structure, a similar membrane topology can be predicted for all isoforms, with 10–12 membrane-spanning (M) regions at the N terminus and a large cytoplasmic region at the C terminus. Although the precise topological organization of the NHEs remains uncertain, a tentative model showing 12 transmembrane segments is illustrated in Fig. 1. The N-terminal residues resemble a putative signal peptide sequence that may be cleaved during protein maturation. The membrane-spanning segments M3–M12 share a great deal of identity among the various isoforms. Of these M6 and M7 are most highly conserved (95% identity), suggesting that this region participates in the transport of Na⁺ and H⁺ across the membrane. By contrast, the highly hydrophilic C-terminal region exhibits a lower degree of similarity among isoforms (~24–56% identity). This entire domain is seemingly oriented toward the cytosol (Fig. 1), since it is inaccessible to extracellular antibodies or proteases (9). Less is known about the tertiary or quaternary structure of the NHEs, although recent evidence suggests that they exist in the membrane as homodimers (11, 12). While the precise location of the contact sites is yet to be defined, the monomers appear to interact at the level of the putative transmembrane region (12) and may be linked by disulfide bonding (13).

Glycosylation—Examination of the primary structures of the NHE isoforms reveals several potential glycosylation sites. NHE1 contains both N- and O-glycosylated residues, and mutation of Asn-75 abolishes the N-linked glycosylation (13). In contrast, rabbit NHE2 exhibits only O-linked glycosylation (14) and NHE3 appears not to be glycosylated (13, 15) (Fig. 1). The state of glycosylation of NHE4–NHE6 is unknown.

Glycosylation has been implicated in the proper biosynthetic processing and transit of ion transport proteins to the membrane surface (16), but its role in the case of the NHEs is not evident. Removal of the carbohydrate moieties of NHE1 and NHE2 had no apparent effect on the rate of ion exchange in either membrane vesicles (17) or transfected cells (13, 14).

Tissue and Subcellular Distribution

NHE1 mRNA is expressed in virtually all tissues and cells, where it most likely fulfills “housekeeping” functions including the maintenance of the cytosolic pH (pH₇) and of cellular volume (see Refs. 9 and 18 for reviews). In epithelial cells, NHE1 is largely restricted to the basolateral domain. NHE2, NHE3, and NHE4 mRNAs show a more limited pattern of expression. They are preferentially found in the gastrointestinal tract and in the kidney (5, 6, 8). The targeting of NHE2 in polarized renal and intestinal epithelial cells is controversial, with some studies reporting basolateral (19) and others apical (brush border) localization (20). Its precise physiological roles are unclear, but when transfected into mutagenized cells devoid of endogenous NHE activity, NHE2 is capable of regulating pH₇, cellular volume, and proliferation in a manner resembling NHE1 (21). Immunological studies have localized NHE3 to the apical membranes of renal proximal tubule (15) and intestinal (20, 22) epithelia, implicating this isoform in Na⁺ absorption. The accompanying luminal secretion of H⁺ is essential for HCO₃⁻ reabsorption in renal tubules. NHE4 is highly abundant in the stomach (5) and is also found in the collecting tubule of the renal inner medulla (23). This latter region is normally exposed to high osmolarity, and NHE4 may therefore play a specialized role in the volume homeostasis of these cells (23). Unlike the other isoforms, NHE5 resides in a selected number of nonepithelial tissues (brain > spleen > testis > skeletal muscle) (7) and may represent the amiloride-insensitive NHE variant reported in hippocampal neurons (24). NHE6 is expressed in several human tissues examined, with the highest levels found in brain and skeletal muscle (25).

Immunolocalization and subcellular fractionation studies have provided initial indications that the antipoters are not distributed homogeneously or even exclusively within the plasma membrane. Though present throughout the surface membrane of adherent fibroblasts, NHE1 was found to accumulate along the border of lamellipodia (25). Vinculin, talin, and F-actin were concentrated at sites of NHE1 accumulation, suggesting that the antipoters can be sequestered in specialized regions by interacting with the cytoskeleton. In this context, it is noteworthy that NHE is activated by engagement of integrins (26). Cross-linking of these adherence receptors may mediate not only the activation but also the redistribution of the antipoters.

Functional analysis of subcellular fractions initially suggested

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¶ The abbreviations used are: NHE, Na⁺/H⁺ exchanger; PKA, protein kinase A; PKC, protein kinase C; CaM, calmodulin; Ca²⁺, calcineurin B homolog protein.

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3 L. D. Shrode and S. Grinstein, unpublished observations.

4 J. Orlowski, unpublished data.
that Na\(^+\)/H\(^+\) exchangers are present in endomembrane compartments as well. Exchange activity was detected in renal (27) and hepatic (28) endosomes, although the hallmark sensitivity of NHE to amiloride (see below) was not evident. This could be an indication that NHE3, a highly amiloride-resistant isofrom, was the species internalized, at least in the kidney. Accordingly, recent immunohistochemical determinations using antibodies specific for NHE3 detected antiporters not only on the microvillar membrane of renal tubular cells but also in a population of subapical vesicles (Fig. 2).\(^5\) Interestingly, pronounced intracellular staining is also observed when NHE3 is heterologously transfected into antiporter-deficient non-epithelial cells. Staining is predominant in a juxtanuclear cluster of vesicles that co-localize with transferrin receptors and with cellubrevin, markers of recycling endosomes (Fig. 2). Internalization motifs present in the cytosolic tail of NHE3 may target this isoform to endosomes, where it could serve as a functional reservoir of spare transporters.

Its wide tissue distribution and greater structural divergence make NHE6 a good candidate for the mitochondrial inner membrane NHE. This mitochondrial exchanger is responsible for extruding Na\(^+\) from the alkaline matrix of respiring mitochondria (3, 29) and, as such, may contribute to organellar volume homeostasis. Mitochondrial NHE is also indirectly involved in facilitating the efflux of Ca\(^{2+}\) and NH\(_4\)\(^+\) from the matrix.

**Basic Functional Properties**

**Transport Kinetics**—In native systems, the rate of Na\(^+\)/H\(^+\) exchange has generally been found to have a hyperbolic dependence on the external Na\(^+\) concentration ([Na\(^+\)]\(_{in}\)) at a simple Michaelis-Menten kinetics (1). A similar kinetic profile is observed for NHE1, NHE2, and NHE3 when expressed heterologously in fibroblasts (30, 31). In contrast, NHE4 exhibits a sigmoidal dependence on [Na\(^+\)]\(_{in}\), although the functional significance of this is unclear (32). The affinity of the NHE isoforms for Na\(^+\) ranges between 5 and 50 mM.

One of the identifying features of the NHE is its exquisite sensitivity to the intracellular pH. The exchangers are allosterically activated by cytosolic H\(^+\), promoting the rapid extrusion of acid once intracellular pH drops below a threshold level (33). This feature is conserved in the NHE isoforms examined to date (NHE1–NHE3), although the apparent H\(^+\) sensitivity, which determines the “set point” for activation, varies between isoforms (30, 31). Deletion mutagenesis studies suggested that the N-terminal transmembranous region of human NHE1 contains the H\(^+\) sensor site, whereas the C-terminal cytoplasmic domain modulates the value of the set point (34). However, other data indicate that this delineation of structural and functional domains may be simplistic (35).

**Electroneutrality**—Kinetic as well as thermodynamic considerations indicate that the activity of the mammalian exchangers is electroneutral (1:1 stoichiometry) (see Ref. 36 for review). Nevertheless, the notion of electroneutrality was recently challenged by the observation that antiport activation in the colon was associated with sizable transepithelial currents (37). However, detailed analysis of similar currents in cultured mammalian cells revealed that they were mediated by a separate proton conductance that is very sensitive to local changes in [H\(^+\)], which can result from activation of the exchangers (38).

**Drug Sensitivity and Binding Sites**—The NHE is a known target

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\(^5\) D. Biemesderfer and P. S. Aronson, unpublished observations.
for inhibition by the diuretic compound amiloride and its analogs (39) and by benzoyl guanidinium compounds (e.g. HOE694) (40, 41). The NHE isoforms vary greatly in their sensitivity to these drugs. The apparent affinities of the plasma membrane isoforms for a defined inhibitor can span up to 4 orders of magnitude, generally following the order: NHE1 > NHE2 > NHE3 > NHE4 (30, 32, 41). In contrast, the mitochondrial NHE is relatively insensitive to amiloride but is effectively inhibited by its analog, benzamid (29). Other pharmacological agents such as cimetidine, clonidine, and harmaline also exhibit differential affinities for the NHE isoforms (30). While these compounds are chemically unrelated to amiloride or HOE694, they possess either an imidazoline or guanidinium moiety and hence bear some structural similarity.

Inhibition by amiloride derivatives, cimetidine, and HOE694 is reduced by high external Na\textsuperscript{+}, suggesting that these compounds bind near the external (Na\textsuperscript{+}) transport site (36, 40). However, other kinetic (42) and genetic selection (43) studies suggest that the Na\textsuperscript{+} and amiloride-binding sites may not be completely identical. Indeed, recent site-directed mutagenesis studies (44, 45) and analysis of NHE chimeras (41) have shown that residues in the predicted M4 and M9 segments contribute to drug sensitivity (Fig. 1) without affecting Na\textsuperscript{+} affinity.

**ATP Dependence—**Fluxes through the antiporter are driven by the combined chemical gradients of Na\textsuperscript{+} and H\textsuperscript{+} and hence do not directly consume metabolic energy. Nevertheless, ATP appears to be required for optimal Na\textsuperscript{+}/H\textsuperscript{+} exchange. Procedures that reduce intracellular ATP levels drastically inhibit exchange in a variety of native systems and in antiport-deficient cells transfected with either NHE1, -2, or -3 (9, 21, 46). Metabolic depletion appears to depress the rate of transport at least in part by reducing the affinity of the exchangers for intracellular H\textsuperscript{+} (9), without altering the number of plasmalemmal transporters.

Studies of truncation mutants led to the suggestion that constitutive phosphorylation of the cytosolic domain of NHE1 is essential for optimal function (9), thereby accounting for the continued requirement for ATP. Subsequent analysis revealed, however, that the activity changes in metabolically depleted cells are not accompanied by detectable alterations in the phosphorylation pattern of the antiporter (47). Moreover, more detailed mutagenesis studies indicate that at least part of the responsiveness to ATP persists following elimination of all the identified phosphorylation sites of NHE1 (9, 47). Comparable studies have not been reported for other isoforms, but NHE3 remains sensitive to ATP even after truncation of a large part of its cytosolic domain (48). Thus the ATP specificity of the antiporters involves direct phosphorylation of the antiporter, an ancillary regulatory protein has been invoked. In fact, preliminary experiments indicate that the ATP sensitivity of the exchange is absent in resealed ghosts prepared by direct phosphorylation of the antiporters. Indeed, pepsin of their primary sequence reveals the existence of consensus sites for phosphorylation by PKA and/or PKC, as well as multiple sites that are suitable substrates for CaM kinase and for proline-directed Ser/Thr kinases (see Ref. 1 and Ref. 55). The latter include the mitogen-activated protein kinases, which have recently been implicated in the activation of the antiporter (50, 51).

Tyrosine phosphorylation of the exchangers has not been detected, but the anticipated phosphorylation on Ser residues was borne out experimentally (9, 47, 51). NHE1 was found to be constitutively phosphorylated in resting cells, and further phosphorylation occurred upon addition of growth factors, phorbol esters, or phosphatase inhibitors (see Ref. 9 for review). Multiple phosphorylation sites were detected, all localized to the region of the cytosolic tail distal to residue 635. Similarly, NHE3 was reported to be phosphorylated in untreated cells, and additional phosphorylation occurred following elevation of camp (52).

However attractive, the notion that the activity of the antiporters is modulated exclusively by their direct phosphorylation appears simplistic, as it fails to account for the following observations: (i) differential responses have been reported for individual isoforms depending on the cellular expression context (cf. Refs. 30 and 31); (ii) in the case of NHE1 at least part of the regulation by growth factors persists in truncated mutants lacking all the known phosphorylation sites; and (iii) some stimuli activate the antiporter without detectable changes in phosphorylation. One must therefore consider the possibility that regulation results from interaction with other cellular components and that constitutive phosphorylation of the antiporters may facilitate this interaction. Indeed, a variety of proteins capable of associating with different isoforms of the NHE have been identified in recent years. These are illustrated in Fig. 1 and are discussed below.

**Associated Proteins—**The cytosolic tail of NHE1 contains two domains capable of binding calmodulin with high (CaM-A, K\textsubscript{D} ~20 nM) or low (CaM-B, K\textsubscript{D} ~350 nM) affinity. These are amphipathic regions rich in basic side chains that likely assume a-helical structure. The high affinity CaM-A domain (residues 636–656) is thought to be important in transport regulation. Deletion of this domain segment renders the exchanger constitutively stimulated, as if cytosolic [Ca\textsuperscript{2+}] was continuously elevated. It has therefore been suggested that, at basal [Ca\textsuperscript{2+}] levels, the unoccupied CaM-A-binding domain exerts on the exchanger an autoinhibitory effect that is relieved upon ligand binding (53).

Only NHE1 has been convincingly shown to be regulated by CaM. Nevertheless, the transmembrane regions of other isoforms can respond to conformational changes of the tail induced by CaM, since insertion of the CaM-binding domain of NHE1 conferred [Ca\textsuperscript{2+}] sensitivity to NHE3 (54). A second Ca\textsuperscript{2+}-binding protein was recently found to interact with NHE1. A calcineurin B homolog protein (CHP) can associate with the cytosolic tail of the antiporter near its site of emergence from the transmembrane region (residues 636–655; see Fig. 1 and Ref. 55). Binding of CHP exerts an inhibitory effect on NHE1, although it remains unclear whether Ca\textsuperscript{2+} is required for this interaction. CHP appears to be constitutively phosphorylated, and stimulation of transport is accompanied by its dephosphorylation. This prompted the suggestion that this phosphoprotein is normally associated with the antiporter, thereby exerting a suppressive effect, and that this dissociation upon dephosphorylation may lead to activation of Na\textsuperscript{+}/H\textsuperscript{+} exchange (55). Independent experiments detected a polypeptide of ~24 kDa, the approximate size of CHP, constitutively associated with NHE1 in several cell types (56).

Yet another protein, hsp70, has been reported to interact with NHE1 (57). Because hsp70 is a molecular chaperone, this interaction may reflect mainly an intermediate stage in the biosynthesis of NHE1. On the other hand, because the association is reversed by MgATP (57), it is tempting to speculate that hsp70 may bind also to mature NHE1 in metabolically depleted cells, perhaps accounting for the ATP dependence of the exchanger.

Proteins associating with NHE2 have not been identified to date. However, two proline-rich domains (743PPSVTPAP750 and 787PPK-789PPD) that resemble SH3-binding domains are present in the C-terminal region. Proline-rich SH3-binding domains have been found to mediate the apical targeting of epithelial Na\textsuperscript{+} channels (58) and may perform a similar function in NHE2.

The apical exchanger of renal epithelial cells, most likely NHE3, also interacts with at least one protein (RPF in Fig. 1). Fractionation and reconstitution experiments suggested that a distinct dissociable cofactor is essential for PKA to inhibit the exchanger (60). Subsequent studies identified this cofactor as a phosphoprotein of 42–44 kDa, which is a substrate for PKA phosphorylation (60). It is

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not clear whether PKA-mediated phosphorylation of NHE3 itself is required for the inhibitory interaction. In addition, NHE3 contains a potential PDZ binding motif (THM) at its very C-terminal end. A related consensus sequence (Thr/Ser-X-Val-COO⁻) is recognized by PDZ domains present in submembrane complexes that mediate the clustering of ion channels or junctional proteins (61).

**GTP-binding Proteins**—Transfection as well as microinjection experiments provided evidence that NHE activity is regulated by both heterotrimeric and small GTP-binding proteins. Activated forms of Goqα, G12α, and G13α have been shown to activate Na⁺/H⁺ exchange (62, 63). In the case of G12, the effect is mediated by RhoA and/or Cdc42, which in turn activate MEKK-1 (64). Accordingly, transfection of activated (GTPase-deficient) forms of these Rho family members, or of Rac1, recapitulate the stimulation of the antiport observed in cells stimulated by hormones or growth factors.

Members of other families of small GTP-binding proteins also induce activation of transport through NHE. In particular, oncogenic forms of Ras greatly enhance the intracellular [H⁺] sensitivity of the antiporter (65). This seemingly results from downstream action with growth factor receptors.

Regulation by Lipids—The role of amino phospholipids in antiport function was explored recently (49). This study was triggered by a report that loss of lipid asymmetry drastically inhibited a related transporter, the Na⁺/Ca²⁺ exchanger. Nevertheless, inhibition of the "flipase" that maintains amino phospholipid asymmetry from endomembrane stores to the plasmalemma. This attractive possibility is regulated in various tissues by recruitment of transporters related transporter, the Na⁺/Ca²⁺ antiporter by growth factors (66, 67). Stimulation of NHE was reduced or eliminated not only by pharmacological inhibition of the antiporter by growth factors (66, 67). Stimulation of NHE was reduced or eliminated not only by pharmacological inhibition of the kinase but also by point mutations specifically abolishing its interaction with growth factor receptors.

Vesicular Traffic—Fluxes of sugar and H⁺ pumping are effectively regulated in various tissues by recruitment of transporters from endomembrane stores to the plasmalemma. This attractive paradigm may apply not only to the regulation of NHE, particularly in the case of NHE3, which is believed to reside in intracellular vesicles (see above). Indeed, agents and conditions that induce or modulate NHE activity are known to alter the rates of endocytosis or effect redistribution of vesicles within cells. These include CAMP, the products of phosphatidylinositol 3'-kinase, and cytosolic acidification. The model of regulation of NHE by vesicular traffic is being tested by ongoing work, but no experimental support can be offered at present.

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