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The ABC of Solute Carriers

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Diversity of the mammalian sodium/proton exchanger SLC9 gene family

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Abstract Sodium/proton antiporters or exchangers (NHE) are integral membrane proteins present in most, if not all, living organisms. In mammals, these transporters chiefly catalyze the electroneutral exchange of Na^+ and H^+ down their respective concentration gradients and are crucial for numerous physiological processes, ranging from the fine control of intracellular pH and cell volume to systemic electrolyte, acid-base and fluid volume homeostasis. NHE activity also facilitates the progression of other cellular events such as adhesion, migration, and proliferation. Thus far, eight distinct NHE genes (NHE1/SLC9A1–NHE8/SLC9A8) and several pseudogenes have been identified in the human genome. The functional genes encode proteins of varying primary sequence identity (25–70%), but share a common predicted secondary structure comprising 12 conserved membrane-spanning segments at the amino-terminus and a more divergent, cytoplasmically-oriented, carboxy-terminus. They show considerable heterogeneity in their patterns of tissue/cell expression and membrane localization. Functional studies have revealed further differences in their kinetic properties, sensitivity to pharmacological antagonists, and regulation by diverse hormonal and mechanical stimuli. Altered NHE activity has been linked to the pathogenesis of several diseases, including essential hypertension, congenital secretory diarrhea, diabetes, and tissue damage caused by ischemia/reperfusion. Further characterization of their functional properties should lead to a better understanding of their unique contributions to human health and disease.

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Introduction

Carrier-mediated transport of sodium in exchange for protons across biological membranes has been detected universally in organisms throughout the various phyla, from simple prokaryotes such as bacteria to more complex eukaryotes of the plant, fungi, and animal kingdoms. This cation flux is conducted by a family of polytopic membrane proteins commonly called Na^+/H^+ antiporters (Nha) or exchangers (NHE or NHX)[1]. These proteins are classified as secondary active transporters since the driving force for catalysis is not coupled directly to the hydrolysis of ATP, but instead is derived from the electrochemical gradient established for one of the solutes that drives countertransport of the other.

Throughout phylogeny, the NHEs have been co-opted to fulfill a diverse range of vital biological functions. For instance, unicellular organisms like the bacterium *Escherichia coli* contain two Na^+/H^+ antiporters (NhaA and NhaB) that utilize the inwardly-directed electrochemical H^+ gradient generated by inner membrane H^+-ATPase pumps to export Na^+ (or Li^+) electrogenically (the coupling stoichiometries for NhaA and NhaB are $1Na^+:2H^+$ and $2Na^+:3H^+$, respectively) (Fig. 1A). These antiporters exhibit considerable differences in their cation affinities and regulatory properties that, collectively, allow *E. coli* to flourish under adverse conditions, such as high saline and/or alkaline environments. The extrusion of Na^+ also helps to maintain an inwardly-directed Na^+ gradient that is coupled to the uptake of various other solutes and nutrients (reviewed in [101]).

[1] Historically, the term Na^+/H^+ antiporter (Nha or NHA) is often used when referring to those transporters found in unicellular organisms like bacteria and yeast, whereas in higher eukaryotes the term Na^+/H^+ exchangers is more commonly used and abbreviated as NHE or NHX, although this is not applied universally.

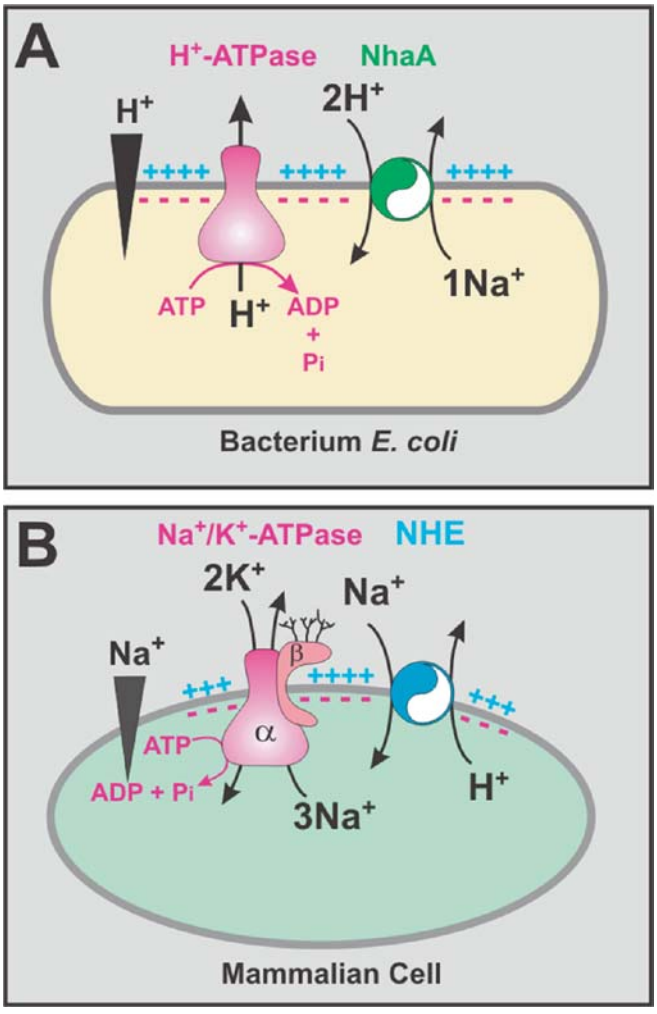

Fig. 1A, B Na^+/H^+ exchangers of bacteria and mammals. The diagram illustrates the cation pumps, F-type H^+-ATPase and Na^+/K^+-ATPase, that provide the respective driving force for (**A**) bacterial Na^+/H^+ antiporters (*Nha*) and (**B**) mammalian Na^+/H^+ exchangers (*NHE*)

By comparison, the source of energy that drives mammalian NHEs at the plasma membrane differs from their bacterial counterparts. Instead of an electrochemical H^+ gradient, an inward Na^+ gradient established by plasma membrane Na^+/K^+-ATPase pumps propels the countertransport of H^+ in an electroneutral manner ($1Na^+$:$1H^+$ stoichiometry) (Fig. 1B). Thus, under normal physiological conditions, plasmalemmal NHEs extrude excess acid accumulated by cellular metabolism and by various H^+ (acid equivalents) leak pathways, thereby playing an important role along side bicarbonate transporters in maintaining intracellular pH (pH_i) homeostasis. Since subtle fluctuations in steady-state pH_i modulate the activities of many biomolecules, and indeed may act as a regulatory stimulus, the fine control of pH_i is a fundamentally important mechanism to sustain a suitable cytoplasmic milieu for stable protein activity and interactions, and ultimately cell function and survival. Consistent with this notion, pH_i changes associated with NHE activity facilitate other biological processes, such as cellular adhesion, migration, and proliferation. In addition, the transmembrane influx of Na^+ contributes directly to the regulation of cell volume and to the absorption of salt and water across various epithelia (for earlier reviews, see [81, 84, 96, 112, 145]).

This review presents a current synopsis of the structural, functional and regulatory heterogeneity of mammalian NHE genes, emphasizing where possible their physiological roles and potential involvement in certain pathophysiological states.

Genetic diversity

Higher eukaryotes have evolved a diverse repertoire of NHEs. For example, genomes of the plant *Arabidopsis thaliana* and the nematode *Caenorhabditis elegans* encode for at least seven (AtSOS1, AtNHX1–6) [5, 124, 168] and nine (CeNHX1–9) [90] distinct NHE-like proteins, respectively, while mammals such as *Homo sapiens* possess a minimum of eight functional genes (HsNHE1/*SLC9A1*–HsNHE8/*SLC9A8*) (Fig. 2). In each case, these isoforms are expressed in a tissue/cell-specific manner and are localized differentially to discrete membrane compartments (cell surface or endomembrane organelles), implicating them in a variety of housekeeping and specialized cellular functions.

Sardet and colleagues in 1989 [117] were the first to describe the primary structure of a mammalian Na^+/H^+ exchanger, termed NHE1, using an intricate genetic complementation approach. This isoform resides exclusively in the plasma membrane of most cell types and is considered the prototypical mammalian NHE. Following this pivotal study, other laboratories screened tissue cDNA libraries from various species by low-stringency hybridization—initially using the human NHE1 cDNA as a convenient probe—in search of other homologous genes that could account for their reported functional heterogeneity. Analyses of rat [8, 98, 150], rabbit [136, 137, 138] and human [9, 21, 86] libraries collectively yielded a total of five distinct plasmalemmal-type NHEs (NHE1–5). With the advent of the human and mouse genome sequencing projects, other more distantly related NHE genes (NHE6–8) have been identified and characterized partially (Table 1) [51, 92, 93]. In the case of NHE6 and NHE7, heterologous expression studies indicate that these isoforms accumulate predominantly in organellar compartments [22, 89, 92] (Table 1). Comparisons of their primary structures show considerable sequence divergence, ranging between 25 and 70% amino acid identity (Table 2). The human genome also appears to contain several putative NHE-like or NHE-pseudogenes, some of which have been characterized [66].

Additional molecular heterogeneity in the form of alternatively-spliced mRNAs has been reported for some isoforms. A recent study [116] has described the isolation of a purported splice-variant of NHE1 from a rat colon cDNA library that encoded the first 9 of 12 predicted

Table 1 SLC9—the sodium/proton exchanger gene family

Human gene name	Protein name	Aliases	Substrate	Transport type/coupling ions*	Tissue distribution and cellular/subcellular expression	Link to disease#	Human gene locus	Sequence accession ID	Splice variants and their specific features
SLC9A1	NHE1	APNH	Na^+, H^+, Li^+, NH_4^+	E: Na^+/H^+ (1:1)	Ubiquitous; (plasma membrane; basolateral surface of epithelia)	Ischemia/reperfusion injuries[A]; essential hypertension[A]; diabetes-associated vascular hypertrophy[A]	1p36.1-p35	NM_003047 XM_046881	
SLC9A2	NHE2		Na^+, H^+, Li^+, NH_4^+	E: Na^+/H^+ (1:1)	Stomach, intestinal tract>skeletal muscle >>>kidney, brain, uterus, testis>>heart, lung; (plasma membrane; apical surface of epithelia)		2q11.2	NM_003048	
SLC9A2L							Xq13.3	XM_066494	Limited homology in C-terminus
SLC9A3	NHE3		Na^+, H^+, Li^+, NH_4^+	E: Na^+/H^+ (1:1)	Intestinal tract, stomach >kidney, gall bladder, epididymis, >>>>brain; (apical surface and recycling endosomes of epithelia)	Congenital secretory diarrhea; hypertension	5p15.3	NM_004174	
SLC9A3P1							10q21.1	XM_171384	Pseudogene
SLC9A3P2							22q11.21	XM_066360	Pseudogene
SLC9A4	NHE4		Na^+, H^+; Li^+?; NH_4^+?	E: Na^+/H^+ (1:1)	Stomach>>>>kidney, brain; (plasma membrane; basolateral membrane of epithelia)		2q11-q12	XM_087199	Partial mRNA
SLC9A5	NHE5		Na^+, H^+, Li^+, NH_4^+?	E: Na^+/H^+ (1:1)	Brain (neurons); (plasma membrane and recycling endosomes/synaptic vesicles)		16q22.1	NM_004594	
SLC9A6	NHE6	KIAA0267	?	E: ?	Ubiquitous; (recycling endosomes)		Xq26.3	NM_006359	NHE6_v1 NHE6_v2
SLC9A7	NHE7		Na^+, K^+, H^+, Li^+, NH_4^+ ?	E: Na^+ $(K^+)/H^+$	Ubiquitous; (*trans*-Golgi network and endosomes)		Xp11.3	NM_032591	
SLC9A7P1							12q23.1	XM_062645	Partially processed pre-mRNA pseudogene
SLC9A7P2							3q24	XM_067158	Very limited homology; pseudogene?
SLC9A8	NHE8	KIAA0939	?	E: ?	Ubiquitous; (plasma membrane and possibly other endomembrane compartments)		20q13	XM_030524	

* *E* Exchanger, # *A* Acquired defect

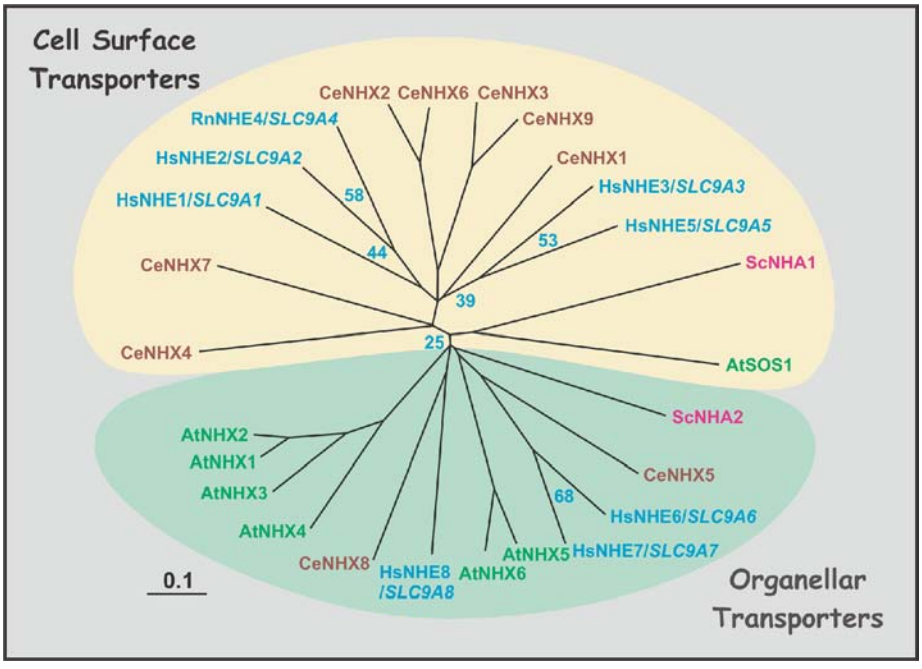

Fig. 2 Phylogenetic relationships of eukaryotic Na^+/H^+ exchangers. Phylogenetic relationships were determined by multiple sequence alignments using the CLUSTAL W algorithm [133] and the radial tree was drawn using TreeView [102]. The GenBank accession numbers for the various NHEs are as follows: *Saccharomyces cerevisiae* (*Sc*) NHA1 and NHA2/NHX1 (NC_001144 and NC_001136, respectively); *Arabidopsis thaliana* (*At*) SOS1 and NHX1–6 (AF256224, AF106324, AF490586, AAF08577, AF490588, AF490589, AF490590, respectively); *Caenorhab- ditis elegans* (*Ce*) NHX1–NHX9 (NM_078221, NM_063213, NM_072542, NM_171714, NM_171768, NM_061634, NM_077429, NM_170928, and NM_069858, respectively); *Homo sapiens* (*Hs*) or *Rattus norvegicus* (*Rn*) NHE1/*SLC9A1*-NHE8/*SLC9A8* (NM_003047, NM_003048, NM_004174, NM_173098/XM_087199, NM_004594, NM_006359, NM_032591, XM_030524, respectively). The *numbers* represent the percentage identity amongst the mammalian NHEs

Table 2 Identity of mammalian Na^+/H^+ exchangers. Pairwise comparison of the percentage similarity of human (h) or rat (r) Na^+/H^+ exchanger isoforms. The values were calculated by dividing the number of identical matches between pairs of isoforms by the length of the isoform listed in the leftmost column. This matrix yields two values for each pair of isoforms. The overall percentage identity between any pair of isoforms is calculated as the average of these two values and is presented below. The lengths of NHE1, -2,-3, -4, -5, -6, -7, -8 are 815, 812, 834, 717, 896, 669, 725, 581 amino acids, respectively

	hNHE1	hNHE2	hNHE3	rNHE4	hNHE5	hNHE6	hNHE7	hNHE8
hNHE1	100	46	39	42	39	25	27	25
hNHE2		100	42	58	40	27	27	24
hNHE3			100	38	53	27	28	26
rNHE4				100	35	27	26	26
hNHE5					100	25	25	27
hNHE6						100	68	26
hNHE7							100	27
hNHE8								100

membrane-spanning α-helices of NHE1 linked to a unique 63-amino acid segment at its C-terminus. However, alignment of this novel C-terminus with sequences in GenBank reveals homology to sequences located at human chromosome 9q21.32 (accession numbers NM_032307 and AL354733), whereas human NHE1 is located at chromosome 1p36.1-p35; suggesting that the former is part of another gene. Based on similar arrangements of syntenic genes amongst mammalian genomes, these two DNA segments are probably non-contiguous in rats as well. Since there is presently little evidence to support bona fide *trans*-splicing of pre-mRNAs in mammalian cells, further study will be required to verify the authenticity of this gene product.

On the other hand, there is suggestive evidence that the NHE6 pre-mRNA undergoes alternative splicing to form at least three gene products designated NHE6 (the original cDNA clone) [93], NHE6_v1 [89] and NHE6_v2 (M. Numata, I. Virdee, J. Orlowski, unpublished data). The latter two each contain an additional 32 amino acids that share ~85% identity to each other and are predicted to reside in the second exofacial loop (between original residues Leu^{143}-Val^{144}). Interestingly, these inserts are also highly homologous to an analogous segment of

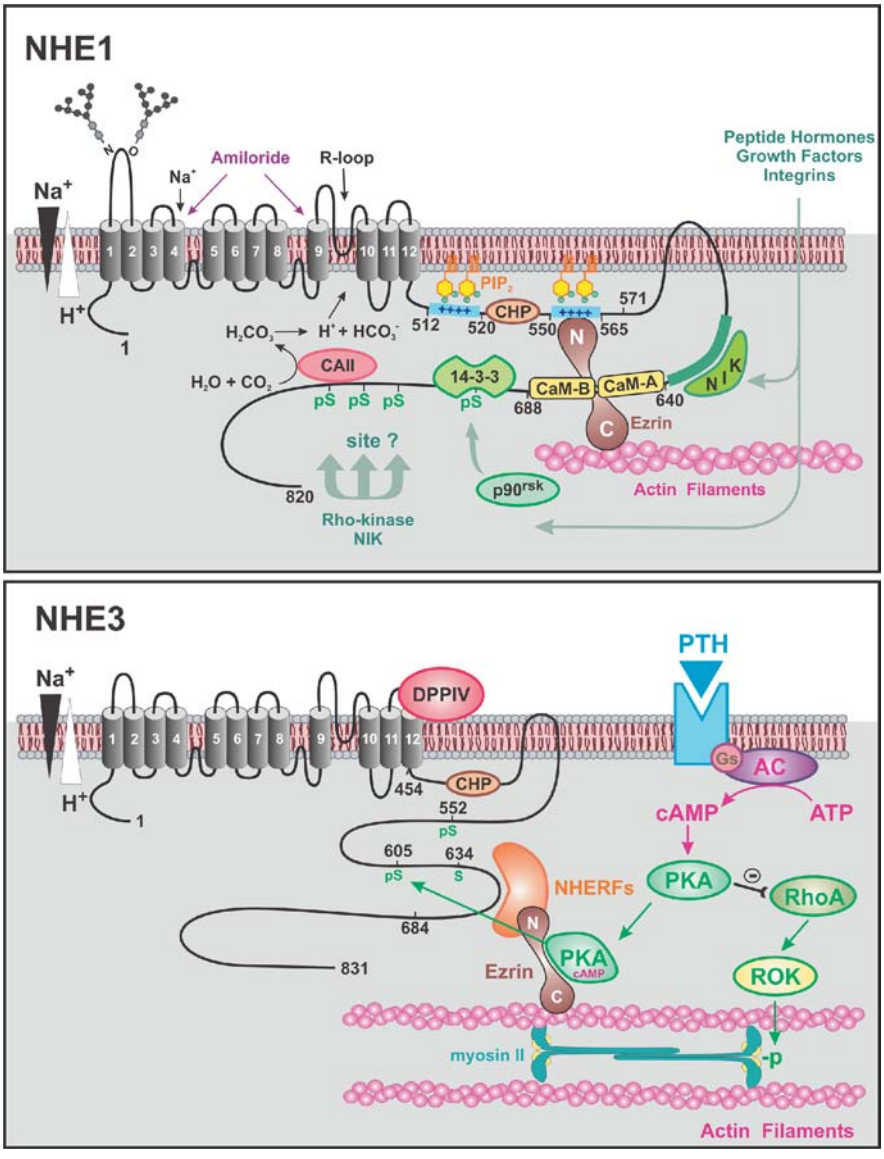

Fig. 3 Transmembrane organization and regulation of mammalian Na^+/H^+ exchangers NHE1 and NHE3 (*R-loop* reentrant loop, *PIP_2* phosphatidylinositol 4,5-bisphosphate, *CHP* calcineurin B homolog protein, *CaM* calcium-calmodulin, *NIK* Nck-interacting kinase, *CAII* carbonic anhydrase II, *PTH* parathyroid hormone, *DPPIV* dipeptidyl peptidase IV, *ROK* rho-associated kinase, *NHERF* NHE regulatory factor, AC adenylate cyclase)

NHE7, although the functional significance of this region, if any, has yet to be ascertained.

General structure-function correlates

Membrane topology

Computer modeling of the hydrophobic-hydrophilic nature and predicted secondary structures (i.e.; regions of α-helical and β-sheet structures) of the NHEs predicts a common membrane topology, with 12 relatively conserved membrane-spanning (M) segments at the N-terminus (~450–500 amino acids) and a more variable hydrophilic C-terminus that faces the cytoplasm (~130–450 amino acids depending on the isoform) and contains numerous canonical sites for phosphorylation by different protein kinases and for binding other ancillary factors, indicative of this region serving a regulatory function.

Empirical evidence generally supporting this model comes from a combination of approaches, including immunolocalization of epitopes [69, 162], delineation of glycosylation sites [32, 139], susceptibility to protease cleavage [125], functional measurements of C-terminal truncations [24, 75, 142] and accessibility of substituted cysteines [146]. The latter approach has provided the most detailed two-dimensional map of the organization of transmembrane helices and interconnecting hydrophilic loops of NHE1 (model illustrated in Fig. 3, upper panel). Of particular note is the presence of a large exofacial reentrant loop (R-loop) between M9 and M10 that resembles the pore (P)-loop structure identified in ion channels and pumps as part of the ion conduction pathway [119], and may represent an analogous structure in the NHEs. Likewise, intracellular loops IL2 and IL4 are accessible to thiol-modification by reagents placed on either side of the membrane, suggesting that they insert into the lipid bilayer and face an aqueous milieu, perhaps also consti-

tuting part of the ion translocation pathway. While it is widely accepted that the hydrophilic C-terminal region faces the cytoplasm, certain immunological analyses have suggested that ill-defined epitopes of the C-terminus of NHE1 [64] and NHE3 [15] may be exposed extracellularly.

While it is tempting to generalize this model to all NHEs, other in vitro analyses [175] have suggested that NHE3 contains a cleavable N-terminal signal peptide followed by 11 membrane-spanning segments, with the putative R-loop situated between the two most C-terminal helices, M10 and M11. However, it is unclear whether the apparent structural disparity between these two isoforms reflects true variations in their topologies, or methodological differences. The former model is more appealing since it relies on analyses of functional transporters.

Information regarding higher-ordered structures of the mammalian NHEs is limited to biochemical studies suggesting that they form homodimers through intermolecular interactions between transmembranous regions of the respective monomers [40]. Though limited, this rudimentary model compares favorably with recent higher resolution structures obtained for the *E. coli* Na^+/H^+ antiporter NhaA. Three-dimensional mapping of well-ordered, two-dimensional crystals of the NhaA protein analyzed by electron cryomicroscopy suggest that the antiporter assembles as a dimer, with 12 tilted membrane-spanning helices per monomer [161]. Thus, despite considerable differences in the primary structures of bacterial NhaA and mammalian NHEs, it is conceivable that their three-dimensional architectures may be conserved. There is also some limited kinetic [100] and biophysical [10] evidence to suggest that mammalian NHEs may exit as higher-order, tetramer complexes.

Cation selectivity

Numerous studies have shown uniformly that plasmalemmal NHEs exhibit a hyperbolic dependence on the extracellular Na^+ (Na^+_o) concentration (K_{Na}: 5–50 mM, depending on the isoform), indicative of a single binding site (see [127] and references therein). To date, only one residue located in transmembrane helix M4 (i.e., F^{162}) of human NHE1 has been implicated in Na^+ binding [135]. Other monovalent cations such as Li^+ and NH_4^+, but not K^+, can also be translocated by plasmalemmal-type NHEs in exchange for Na^+ or H^+, but usually at slower velocities [6, 7]. This contrasts with the organellar isoform NHE7 which is capable of transporting Na^+ or K^+ (and possibly other monovalent cations) in exchange for H^+, and probably functions primarily as a K^+/H^+ exchanger in vivo [92]. One possible exception to the apparent localization of "K^+-selective" NHE subtypes to intracellular compartments is the human erythrocyte, which manifests $K^+(Na^+)/H^+$ exchange on its surface when exposed to extracellular medium of low ionic strength [67]. However, it is unclear whether this transporter represents a novel plasmalemmal-type NHE or rather one of the known organellar NHEs such as NHE7, which, potentially, could accumulate on the cell surface following dissolution of the endomembrane compartments of this specialized cell-type.

In contrast to Na^+_o, plasmalemmal NHEs typically display a greater than first-order dependence on the H^+_i concentration, suggestive of an allosteric H^+_i-modifier site in addition to the H^+ transport site [6, 99]. Earlier structural studies have indicated that the transmembrane domain contains the "H^+_i-sensor", whereas the cytoplasmic region regulates the pH_i set-point of the exchanger [142]. This postulate is seemingly supported by recent mutational analyses showing that conserved residues within IL5 (R^{440}) and M11 (G^{445}, G^{446}) of human NHE1 decreased and increased, respectively, its sensitivity to pH_i [147]. However, the data did not resolve the question of whether mutations at these sites had altered interactions between H^+ and the allosteric modifier site or the transport site. Indeed, the positive cooperative effects of H^+_i on NHE activity are not observed universally. For instance, NHE5 shows a simple first-order dependence on the H^+_i concentration when expressed ectopically in fibroblastic cells [127], suggesting that allosteric regulation by H^+_i may be isoform-specific.

ATP dependence

It is well recognized that cation fluxes through the NHE are driven solely by the combined transmembrane chemical gradients of the substrates. Nevertheless, plasma membrane NHEs require physiological levels (i.e., millimolar) of ATP for optimal function, despite the fact that they neither bind nor consume ATP directly. Acute cellular depletion of this nucleotide markedly reduces the activities of NHE1 and NHE2, and almost completely abolishes that of NHE3 and NHE5, even in the presence of a large transmembrane H^+ gradient [59, 127, 142]. Kinetic analyses have indicated that inhibition of NHE1 and NHE2 mainly reflects reduced affinities for H^+_i, while alterations in both pH_i sensitivity and maximum velocity account for the drastic reductions in NHE3 and NHE5. The molecular mechanisms underlying these phenomenon are not fully resolved. A direct role for protein kinases or phosphatases have been excluded as contributing factors since the state of phosphorylation of NHE1 is unaffected during acute ATP depletion [50]. Instead, association of the plasmalemmal polyphosphoinositide, phosphatidylinositol 4,5-bisphosphate (PIP_2), with two positively-charged clusters in the cytoplasmic juxtamembrane region of NHE1 is critical for optimal exchange activity [1]. It has been proposed that net dephosphorylation of PIP_2 upon ATP depletion could account, at least in part, for the observed inhibitory effect on NHE activity. Other studies [55] have also shown that PIP_2 plays a significant role in regulating other ion carriers such as the cardiac Na^+/Ca^+ exchanger and K^+_{ATP} channels, highlighting a central role for this phosphoinositide in modulating ion fluxes across biological membranes.

Drug recognition

The NHEs isoforms are inhibited by several classes of pharmacological compounds, including the diuretic amiloride and its analogues, benzyolguanidinium-based derivatives (e.g., HOE642 or cariporide, HOE694), cimetidine, clonidine and harmaline [18, 25, 31, 92, 95, 120, 127, 170]. Though chemically distinct, each one of these inhibitors possesses either an imidazoline or guanidinium moiety and hence bears some structural similarity to the others. The affinity of these compounds for the NHEs is isoform-dependent and spans over two orders of magnitude, with NHE1 exhibiting the highest sensitivity (NHE1≥NHE2>NHE5>NHE3>NHE4). More recently, a preferential antagonist (S3226) of NHE3 has been synthesized that may also facilitate functional studies of this isoform [123].

Kinetic measurements have indicated that many of these compounds antagonize transport by competing with Na^+_o for binding to a common or nearby site, but also interact at sites not involved in cation binding [46, 83, 152]. This initial assessment has been corroborated largely by mutational analyses of NHE1 showing that residues spanning the second exofacial loop EL2 (G^{152}, P^{157} and P^{158}; positions numbered according to rat NHE1 sequence) and adjoining transmembrane helix M4 (F^{165}, F^{166}, L^{167} and G^{178}), and residues in M9 (E^{350}, H^{353} and G^{356}) are significant determinants of drug recognition [30, 33, 63, 97, 135, 148]. As mentioned above, only mutations of F^{166} (human F^{162}) in M4 reduce Na^+ affinity appreciably. In contrast, substitutions of E^{350} and G^{356} in M9 markedly decrease the catalytic turnover of the transporter without affecting the affinities of either substrate. Mutations at these latter sites are proposed to mimic potential conformational constraints imposed by antagonist binding, thereby compromising optimal substrate translocation. Thus, sites within and surrounding M4 and M9 are critical determinants of both drug recognition and cation translocation. Further detailed examination of these regions should aid in the rational design of more potent isoform-specific drugs with therapeutic potential.

Physiological aspects of the SLC9 isoforms

Members of the NHE gene family exhibit distinctive patterns of tissue/cell expression and membrane localization (Table 1). While their involvement in various cellular processes is well established, recent investigations of recognized plasma membrane-type NHEs and the discovery of several organellar isoforms have uncovered novel functions for members of this gene family in mammalian physiology.

SLC9A1/NHE1

Tissue distribution and subcellular location

NHE1 is present in most cell types and is the most extensively characterized member of this family. It resides exclusively on the cell surface, but accumulates preferentially in discrete microdomains of the plasma membrane depending on the cell type. For instance, NHE1 concentrates along the border of lamellipodia in fibroblasts [52], the basolateral membrane of epithelia [12], and the intercalated disks and t-tubules of cardiac myocytes [107].

Physiological roles

NHE1 is believed widely to fulfill two fundamental functions. First, it serves as the principal alkalinizing mechanism in many cell types to guard against the damaging effects of excess acidification that would arise if metabolic acid generation and/or electrically driven H^+ accumulation through various leak pathways remained unchecked. Thus, it plays a crucial role—together with bicarbonate-transporting systems (i.e., Na^+-HCO_3^- co-transporters, Na^+-dependent HCO_3^-/Cl^- exchangers and Cl^-/HCO_3^- exchangers)—in maintaining cytoplasmic acid-base balance (Fig. 4A). Second, it provides a major conduit for Na^+ influx, coupled to Cl^- and H_2O uptake, which is required to restore cell volume to steady-state levels following cell shrinkage induced by acute elevations in external osmolality [115] (Fig. 4B). In more specialized secretory cell types, such as the acinar cells of the parotid and sublingual glands, NHE1 activity is also essential for secretagogue-induced fluid secretion [91, 105].

The cell type-specific localization of NHE1 to distinct subdomains of the plasma membrane also suggests that it may play more subtle, specialized roles in cell function. In cardiac myocytes, the localization of NHE1 to intercalated disks and t-tubules, but not the peripheral sarcolemma, suggests a potential role in controlling the activities of neighboring pH-sensitive proteins, such as the gap-junction protein connexin43 [160] and the ryanodine-sensitive Ca^{2+} release channel [163] that resides near the cytoplasmic surface of the sarcoplasmic reticulum cisternae, thereby influencing impulse conduction and excitation-contraction coupling. Consistent with this notion, inhibition of NHE1 activity by amiloride decreases the conductance of gap junctions of paired cardiomyocytes in culture [43].

Recent evidence also suggests that NHE1 expression may be a significant factor in regulating cell morphology, adhesion and migration. Denker and colleagues [37] uncovered a novel structural role for NHE1 in remodeling the cortical actin cytoskeleton and cell shape of fibroblasts through its association with the cytoskeletal-associated proteins ezrin, radixin and moesin (ERMs) that is seemingly independent of cation translocation [37].

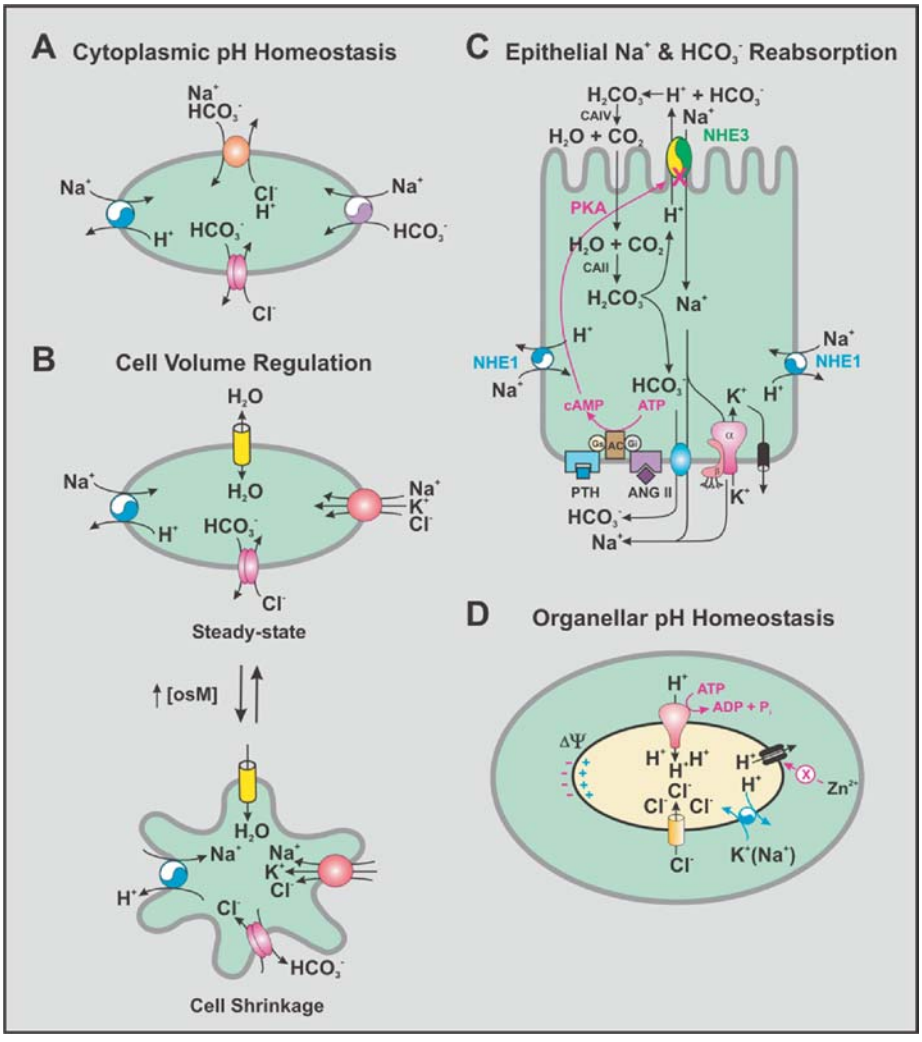

Fig. 4A–D Schematic representation of major physiological roles fulfilled by mammalian Na^+/H^+ exchangers. **A** Cytoplasmic pH homeostasis, **B** cell volume regulation, **C** epithelial Na^+, HCO_3^- reabsorption, **D** organelle pH homeostasis)

However, both cation translocation and anchorage to the cytoskeleton are required for remodeling focal adhesions at the front and trailing edges of the cell necessary for guided movement [38] and may account, at least in part, for the observed reduction in proliferation of NHE1-null fibroblastic cells [59, 110]. Similar conclusions have also been reached using viral-transformed invasive renal epithelial MDCK tumor cells where pharmacological inhibition of NHE1 activity induced disassembly of filamentous actin, retraction of pseudopodia, and reduced cell adhesion and motility [71]. Thus, in at least two cell types, NHE1 appears to influence some cellular functions through its physical linkage to the cytoskeleton. However, the extent to which this phenomenon applies to other cell types is uncertain, as mice with null mutations of *Nhe1* are viable at birth [11, 34], suggesting that in vivo most cells have redundant or compensatory mechanisms that allow embryogenesis to proceed normally.

Regulation

Diverse stimuli that activate numerous receptor tyrosine kinases and G protein-coupled receptors enhance NHE1 activity; thereby alkalinizing the cell and facilitating the progression of certain biological processes. Ultimately, many growth factors and peptide hormones (e.g., serum, angiotensin II, thrombin, vasopressin, α-adrenergic receptors agonists) that activate NHE1 are thought to transduce their signals through a common mitogen-activated protein kinase (MAPK) pathway involving mitogen-activated, extracellular signal-related kinase (MEK-ERK)-$p90^{rsk}$. The $p90^{rsk}$ kinase phosphorylates S^{703} of human NHE1 directly, which enables binding of the multifunctional scaffolding protein 14-3-3 to that site [74, 132] (Fig. 3). In turn, 14-3-3 could serve as focal point for the assembly of other signaling molecules [42]. NHE1 is also a substrate for other kinases, including p160-Rho-associated kinase (termed p160ROCK or ROK), which mediates signals from the integrin receptors that modulate cell adhesion and spreading [134], and Nck-interacting kinase (NIK) which transduces signals

from receptor tyrosine kinases, such as platelet-derived growth factor, independently of the ERK-p90^{rsk} pathway [164]. In the case of NIK, it directly binds to the central portion of the NHE1 C-terminus, but phosphorylates a downstream site that has yet to be mapped precisely.

The mechanisms by which protein phosphorylation enhances cation exchange remain obscure. One tantalizing possibility comes from a recent study showing that serum-induced phosphorylation of the extreme C-terminus of NHE1 (last 178 amino acids) facilitates binding of carbonic anhydrase II (CAII), which catalyses the hydration of CO_2 to form HCO_3^- and H^+ [76]. Ectopic co-expression of both proteins in NHE-deficient Chinese hamster ovary AP-1 cells increases NHE1 activity significantly above cells expressing only NHE1; an effect that is abrogated by acetazolamide, a CAII antagonist. These data support the notion that regulated binding of CAII to NHE1 may stimulate transport activity and cellular alkalinization transiently by increasing the local production of protons, which are extruded, while simultaneously elevating cellular bicarbonate levels. At present, it is unclear whether CAII and 14-3-3 compete for binding to the same phosphorylated site or interact independently of each other.

NHE1 activity is also modulated by interactions with other ancillary regulatory factors that may themselves be targets for phosphorylation by protein kinases. Ca^{2+}/calmodulin binds to a high (CaM-A, K_d ~20 nM; residues 637–656) or low (CaM-B, K_d ~350 nM; residues 657–700) affinity, positively-charged cluster in its C-terminal regulatory domain [143]. The association of Ca^{2+}/calmodulin to the high affinity CaM-A domain is thought to activate NHE1 by relieving an autoinhibitory intramolecular interaction that increases the transporter's affinity for H^+_i, thereby contributing to its regulation by agonists that mobilize cytoplasmic Ca^{2+} [144].

Three other Ca^{2+}-binding (phospho)proteins that are closely related to each other (~34–50% identity), called calcineurin B homologous protein-1 and -2 (CHP1 and CHP2) and tescalcin (renamed CHP3 to indicate its relatedness), also associate with NHE1 in vitro and in vivo [78, 85, 103, 104]. CHP1 and CHP2 are equally capable of interacting with other plasmalemmal-type NHEs [103, 104], suggesting a broad role in NHE function. The binding of CHP3 to other NHEs has yet to be tested. Distinctions between these proteins emerge when comparing their patterns of tissue expression and effects on NHE activity. CHP1 is abundantly expressed in all adult tissues [78], whereas CHP2 is undetectable in most normal tissues but is significantly up-regulated in malignant cells [104]. In contrast, CHP3 is expressed in the developing testis, but is found exclusively in cardiac tissue of adult animals [85]. With respect to CHP1 and CHP2, biochemical and functional studies show that residues in the juxtamembrane region of the NHE cytoplasmic C-terminus (amino acids 520–535 of rat NHE1) are critical for binding and optimal basal transport activity [103, 104]. However CHP2, but not CHP1, constitutively activates NHE1 and raises the steady-state pH_i under serum-free conditions that seemingly protects against serum deprivation-induced cell death [104]. The mechanistic basis for this intriguing phenomenon, however, remains unknown. Interestingly, the CHP-interacting region is flanked by the two positively-charged clusters that bind PIP_2 in vitro and that are also critical for basal NHE1 activity [1]. The distal site can also associate with the related cytoskeletal-associated ERM proteins in vitro and in vivo [37]. Thus, this juxtamembrane region of the cytoplasmic C-terminus appears to be a crucial domain for regulation of NHE activity.

Involvement in pathophysiological states

Despite the apparent normal fetal development of *Nhe1*$^{-/-}$ mice, postnatally these animals gradually develop a severe neurodegenerative disorder characterized by locomotor ataxia, epileptic-like seizures, and significant mortality prior to weaning [11, 34]. The molecular basis for this phenotype is largely ill defined, but correlates in part with reduced steady-state pH_i and attenuated pH_i recovery from cell acidification, and hyperexcitability of hippocampal CA1 neurons [53, 165].

In contrast to the phenotype resulting from loss of NHE1 function, acute over-activation of NHE1 activity is a common occurrence in cardiac and neural tissues during episodes of ischemia-reperfusion, which results in a dramatic increase in Na^+_i that, in turn, causes an increase in Ca^{2+}_i. This Na^+-induced Ca^{2+}_i overload triggers a cascade of deleterious events that lead to tissue dysfunction (e.g., cardiac arrhythmias, altered synaptic transmission) and ultimately tissue damage, including free radical toxicity, cellular edema, apoptosis and necrosis (reviewed in [61, 111, 159]). Persuasive evidence for NHE1 involvement is provided by numerous studies using selective NHE1 antagonists that significantly reduce Na^+_i and Ca^{2+}_i overloads and effectively mitigate cardiac [23, 61] and neural [4, 57] injuries associated with ischemia-reperfusion both in vitro and in vivo.

Chronic activation of NHE1 activity in several tissues, also in association with altered Ca^{2+}_i regulation, is often considered an intermediate phenotype for essential or primary hypertension (reviewed in [94]). Current evidence argues against a genetic defect in NHE1 being a primary cause of essential hypertension in humans [77]. Rather, hypertension appears to correlate with enhanced activity of the MAPK pathway and phosphorylation of NHE1 [108, 126], suggesting that alterations in protein kinase signaling may be responsible for altered cation homeostasis leading to hypertension. Pharmacological inhibition of NHE1 activity also appears to be beneficial in attenuating cardiac hypertrophy and failure in response to biomechanical stress [60].

SLC9A2, SLC9A3 and SLC9A4/NHE2–4

Tissue distribution and subcellular location

NHE2–4 are expressed predominantly in the epithelia of the kidney and gastrointestinal tract, but are also detected at low levels in other tissues [21, 86, 98, 137, 138, 150]. In renal epithelia, NHE4 is located in the basolateral membrane [27, 106, 109] and may fulfill similar or overlapping functions with NHE1, particularly in the macula densa [106] and intercalated cells of the cortical collecting duct [12, 155] that lack detectable levels of NHE1.

In contrast, NHE2 and NHE3 reside in the luminal membranes of discrete nephron and intestinal segments [13, 56, 106]. In the kidney, NHE2 is enriched in the cortical thick ascending limb, macula densa, distal convoluted tubules and connecting tubules [26, 106], whereas NHE3 is located predominantly in the proximal tubule and, to a lesser extent, in the medullary thick ascending limb [3, 13]. In addition to the microvillus, NHE3 [14, 28], but not NHE2 [29], is detected in a discrete population of clathrin-associated subapical endosomes, where it could serve as a reservoir of functional transporters that shuttles to and from the brush-border in response to hormonal cues. NHE3 also contributes to acute pH regulation of this endomembrane compartment [2, 35]; a process that seemingly facilitates receptor-mediated endocytosis [48].

Physiological roles

NHE3 is the major contributor to bulk Na^+ and fluid reabsorption by the proximal tubule [140]. The associated secretion of H^+ by NHE3 into the lumen of renal tubules is also essential for approximately two-thirds of renal HCO_3^- reabsorption [149] (illustrated in Fig. 4C). Proton secretion by NHE2 also regulates HCO_3^- reabsorption in the distal convoluted tubule, although its contribution to plasma HCO_3^- levels is less evident [149]. NHE3, but not NHE2, regulates basal as well as meal-stimulated ileal Na^+ absorption in vivo [82, 166]. However, the physiological distinction between these two apical transporters is best revealed by studies of mice with targeted disruptions of the *Nhe2* and *Nhe3* loci. NHE3-null mice exhibit slight diarrhea and alkalinization of the intestinal luminal contents, sharply decreased HCO_3^- and fluid absorption in proximal convoluted tubules, mild acidosis, reduced blood pressure, elevated serum aldosterone and higher renal renin mRNA expression, consistent with the volume-contracted state of the animals [73, 122]. This phenotype differs considerably from that observed for NHE2-null mice, which show severe degeneration of gastric parietal and zymogenic cells and significantly decreased parotid gland fluid secretion, but no apparent intestinal or renal absorptive defects [73, 105, 121]. Taken together, these studies highlight the central importance of NHE3 in absorptive functions that profoundly influence systemic electrolyte, acid-base, and blood pressure homeostasis, whereas NHE2 appears to function primarily in secretory processes of certain glands.

Regulation

Numerous hormones and physical parameters such as osmolality also influence NHE3 function, thereby contributing to the fine control of electrolyte and fluid homeostasis [96]. For example, hormones that activate cAMP-dependent protein kinase (PKA), such as parathyroid hormone, reduce renal Na^+ and HCO_3^- reabsorption in part by inhibiting apical NHE3 activity through a reduction in the transporter's maximum velocity and apparent affinity for H^+_i [88] (Fig. 4C). Exposure of renal epithelial cells to hyperosmotic conditions also causes a similar decrease in NHE3 activity, but mainly by decreasing its sensitivity to H^+_i [153], whereas hyposmolality elicits an increase in renal NHE3 activity by raising the maximal velocity without altering H^+_i affinity [154]. Like NHE1, multiple molecular mechanisms are involved in regulating NHE3 activity in response to these stimuli, including direct phosphorylation of the exchanger, interactions with ancillary factors, and trafficking of endomembrane vesicles containing NHE3 to and from the cell surface (illustrated in Fig. 3, lower panel).

Phosphorylation of serine residues (Ser^{552} and Ser^{605}) in the carboxy-terminal cytoplasmic region of NHE3 has been implicated in its responsiveness to PKA [68, 174]. Notably, mutation of Ser^{605} ($RRRS^{605}IR$) blocks PKA-mediated phosphorylation of the transporter, but only reduces the acute inhibitory effect on transport activity by ~50% [68]. Mutation of an additional nearby serine ($YS^{634}RHEL$) is required to abolish the effect of PKA, even though it is not phosphorylated upon activation by PKA. This finding implicates the existence of other modes of action of PKA that indirectly regulate NHE3 activity (discussed below).

Recent studies have indicated that NHE3 forms macromolecular complexes with other proteins in the plasma membrane of both non-epithelial and epithelial cells, some of which modulate its function. These include: (1) CHP1 and CHP2, which are essential for optimal transport activity [103, 104]; (2) megalin, a scavenger receptor that interacts with NHE3 in intermicrovillar clathrin-coated pits of the renal brush border, where it renders the transporter inactive [16, 17]; (3) dipeptidyl peptidase IV (DPPIV), a protease present on the outer membrane leaflet of many cell types, especially microvilli of epithelial cells, but its role in NHE3 function is unknown [49]; and, finally, (4) two homologous PDZ-domain scaffolding proteins (class I) called Na^+/H^+ exchanger regulatory factor 1 (NHERF1) (also termed EBP50) [156] and NHE3 kinase A regulatory protein (E3KARP) (also called NHERF2) [171]. These latter two proteins bind to the cytoplasmic C-terminus of NHE3 and have been postulated to mediate indirectly the effect of

PKA [157]. Class I PDZ-binding sequences consist of a short tri-peptide sequence (S/T-*X*-Ø, where Ø represents a hydrophobic residue) at the very C-terminus. Interestingly, NHE3 contains a closely related sequence THM at its C-terminus. However, the NHERFs are believed to interact at an internal region (residues 585–660) of the cytoplasmic domain of NHE3 which includes two of the serines (Ser^{605} and Ser^{634}) implicated in responsiveness to PKA [172]. Both NHERF1 and NHERF2 also bind to the N-terminal domain of ezrin [113, 172], an ERM protein abundant in apical microvilli of epithelial cells and actin-containing structures of non-epithelial cells, and serves as a link between membrane-associated proteins and actin filaments [169]. Ezrin is also capable of functioning as a PKA-anchoring protein or AKAP [39], which could physically position PKA in close proximity to NHE3 and promote the phosphorylation of the exchanger and/or cytoskeletal proteins.

These observations raised the possibility that the activity of NHE3 may be controlled in some manner by its state of association with the actin cytoskeleton, which may facilitate regulation by PKA. Indeed, a fraction of total cellular NHE3 co-sediments with F-actin, suggestive of an interaction between NHE3 and the cytoskeleton [70]. Moreover, disruption of normal actin structure by pharmacological means (i.e., using cytochalasins or latruculin) or by expression of dominant-negative constructs of RhoA or its downstream target ROK—signaling effectors that control the organization of the actin cytoskeleton—impair NHE3 activity in stably transfected Chinese hamster ovary cells without altering its abundance at the plasma membrane [70, 129]. Interestingly, the inhibition of NHE3 by cytoskeleton-disrupting agents is conferred by C-terminal residues 638–684 and bears structural and functional similarities with that induced by elevation of PKA activity, which also dramatically alters cell morphology by antagonizing the activity of the RhoA [72], thereby disrupting the actin microfilament network. Consistent with this notion, constitutively-active forms of RhoA and ROK interfere with actin disruption caused by activated PKA and also attenuate PKA-mediated inhibition of NHE3 [130]. Other stimuli, such as hyperosmotic-induced cell shrinkage, also disrupt the actin cytoskeleton and may account for the associated decrease in NHE3 activity [128]. Thus, linkage of NHE3 to the actin cytoskeleton appears to be important for both basal and regulated transport activity.

As mentioned above, recent evidence has shown that NHE3, in addition to being present at the surface membrane, is also detectable in intracellular vesicles of epithelia and transfected fibroblasts. In these cells, plasmalemmal transporters undergo constitutive uptake into clathrin-coated vesicles that are recycled back to the plasma membrane in a phosphatidylinositol 3-kinase-dependent manner [69]. Gradual redistribution of renal NHE3 to a vesicular compartment is observed following chronic in vivo treatment of rats with parathyroid hormone [41, 173] or after induction of hypertension [167]. Taken together, these observations are consistent with an intracellular redistribution of the exchangers following treatment with certain agents that modify the rate of transport.

Involvement in pathophysiological states

Defective NHE3 function has been linked to the pathogenesis of human congenital secretory diarrhea; a rare autosomal recessive disorder characterized by the absence of intestinal brush-border Na^+/H^+ exchange, severe diarrhea typified by Na^+-enriched alkaline stools, hyponatremia and metabolic acidosis [19], and which closely mimics the phenotype of *Nhe3*$^{-/-}$ mice.

Aberrant NHE3 activity has been implicated as a potential contributing factor in the pathophysiology of other disease states, including hypertension and renal ischemia-reperfusion injuries. Isolated ileum [141] and renal proximal tubules [62] of spontaneously hypertensive rats (SHR) show increased levels of NHE3 activity and protein in brush-border membranes compared with control Wistar-Kyoto (WKY) animals, which may contribute to increased Na^+ reabsorption and the pathogenesis of hypertension. Similarly, induction of diabetes mellitus in rats—a disease associated with hypertension and renal dysfunction—increases renal brush-border NHE3 activity, which reverses upon insulin treatment [54]. In contrast, ischemic-reperfusion injury in the kidney leads to drastic and prolonged reductions in NHE3 as well as other apical ion transporters, and could contribute to the increased fractional excretion of NaCl and water that occurs during recovery from ischemic acute renal failure [151].

SLC9A5/NHE5

Tissue distribution and subcellular location

NHE5 is distinguished from other NHEs by its concentrated expression in neuronal-enriched regions of the central nervous system [8, 9]. It is most closely related to NHE3 (~50% amino acid identity, Table 2) and shares similar properties; namely its localization in the plasma membrane and its internalization by clathrin-mediated endocytosis into recycling endosomes when stably transfected into Chinese hamster ovary cells [131]. A comparable subcellular distribution is also observed when transiently over-expressed in differentiated neuroendocrine PC12 cells and primary cultures of rat hippocampal neurons, in which NHE5 accumulates in somatodendritic vesicles, but also in synaptic or synaptic-like microvesicles along the axons or neurite processes [131].

Physiological roles

The role of NHE5 in neuronal function is presently unknown. However, it is tempting to speculate that it may modulate the acidity of synaptic vesicles, which is an

important determinant of neurotransmitter concentration, and ultimately of synaptic transmission [79]. In this regard, it is intriguing to note that genetic mapping studies have localized NHE5 to a region of chromosome 16q22.1 [65] that coincides with a locus linked to a late-onset (20–60 years of age) form of autosomal dominant spinocerebellar ataxia (*SCA4*) [44], implicating NHE5 as a possible candidate gene in the development of this neurodegenerative disease.

Regulation

Little is known about the regulation of NHE5. Pharmacological activation of protein kinase A or C, as well as hyperosmolality, reduce rat NHE5 activity when the latter is over-expressed in Chinese hamster lung fibroblast PS120 cells, similar to that observed for NHE3 [7]. Likewise, the trafficking of NHE5-containing vesicles is also dynamically regulated by phosphatidylinositol 3′-kinase and by the state of F-actin assembly [131].

SLC9A6, SLC9A7 and SLC9A8/NHE6–8

Tissue distribution and subcellular location

Sequence comparisons show that NHE6–8 have diverged considerably from the plasmalemmal-type NHEs (~25% overall amino acid identity, Table 2), clustering with homologous genes from diverse eukaryotic species in a separate branch of the NHE phylogenetic tree (Fig. 2). These isoforms are expressed ubiquitously, suggesting that they probably perform housekeeping functions. NHE6 and NHE7 are closely related to each other (~70% identity, Table 2), differing largely at their extreme N- and C-termini. By comparison, NHE8 shares only 25% identity with NHE6 and NHE7, and therefore represents the most evolutionarily distant isoform amongst the mammalian NHEs.

Initial transfection studies using HeLa cells showed that an NHE6-green fluorescent protein (GFP) chimera localized to intracellular vesicles that closely overlapped MitoTracker Red-stained mitochondria [93], and was thought to partly account for earlier descriptions of monovalent cation/H^+ exchangers in this organelle [47]. However, other reports [22, 89] have since provided more compelling data that both NHE6 and its splice variant NHE6_v1 are processed preferentially through the secretory pathway and accumulate chiefly in endosomal vesicles when transiently over-expressed in mammalian cells. Hence, the molecular entity/entities that ostensibly mediate Na^+/H^+ exchange in mitochondria remain elusive.

By comparison, NHE7 localizes predominantly to the *trans*-Golgi network and associated endosomes [92]. As mentioned earlier, NHE7 is distinguished from other cloned mammalian NHEs by its ability to transport either Na^+ or K^+ in exchange for H^+. Since K^+ is the major intracellular cation, it is likely that NHE7 catalyzes chiefly K^+/H^+ exchange. The cation specificities of NHE6 have yet to be determined empirically, but are likely to be similar, given its close relatedness to NHE7.

Considerably less is known about NHE8. Northern blot analyses have shown NHE8 mRNA to be expressed widely in adult mouse [51] and human (M. Numata, I. Virdee, S. Grinstein, J. Orlowski, unpublished data) tissues. Goyal and colleagues [51] have demonstrated further that the corresponding protein can be detected in isolated brush-border membrane vesicles of renal proximal tubule epithelia using an isoform-specific polyclonal antibody, although other tissue preparations were not examined. The antibody, however, was ineffective in immunocytochemical analyses, precluding precise determination of its subcellular distribution. By contrast, ectopic expression of an epitope-tagged form of human NHE8 in HeLa cells shows a diffuse punctate distribution throughout the cell, suggesting that it may reside primarily in endomembrane compartments (M. Numata, I. Virdee, S. Grinstein, J. Orlowski, unpublished data). Similarly, the orthologous isoform from *C. elegans*, CeNHX8-GFP, accumulates in punctate, perinuclear vesicles of cells in transgenic nematodes [90]. A more accurate description of the subcellular distribution of NHE8 awaits the development of more versatile isoform-specific antibodies.

Physiological roles

What may be the roles of NHEs in organellar function? It is well established that the luminal pH of organelles along the secretory and endocytic pathways of eukaryotic cells is acidic and tightly regulated, with the [H^+] differing up to 100-fold between compartments [158]. This acidification is important for proper post-translational processing and sorting of newly synthesized proteins, and for the redistribution and degradation of internalized membrane proteins such as ligand-receptor complexes and fluid-phase solutes [87]. The precise determinants of steady-state pH within the lumen of different endomembrane compartments are incompletely understood. However, current evidence supports a complex interplay between the rates of H^+ pumping by the vacuolar H^+-ATPase [45], counterion conductances of anions such as Cl^- [58], and ill-defined H^+ (or acid equivalent) leak pathways that are readily manifested by the rapid dissipation of the transmembrane H^+ chemical gradient upon inhibition of the vacuolar H^+-ATPase with bafilomycin or concanamycin [36, 80, 118] (illustrated in Fig. 4D). A component of this H^+ leak in the Golgi complex has been identified recently as a Zn^{2+}-inhibitable H^+ conductance [118], but can not account fully for H^+ turnover. The presence of NHE7 in organelles of the secretory pathway may account for part of this sizable H^+ leak [36, 80]. Likewise, NHE6 may contribute to pH regulation of organelles of the endocytic pathway.

In addition to serving as organellar H^+ efflux pathways, isoforms such as NHE7 may also regulate the

luminal K^+ concentration, and consequently volume homeostasis, of these compartments. Indeed, the $[K^+]$ of the Golgi complex was recently determined to be close (~107 mM) to that of the cytoplasm (~140 mM), implicating the existence of K^+ influx pathways [118]. Recent intriguing findings also suggest that the luminal $[K^+]$ may function as a meaningful allosteric regulator of the Kex2/furin family of endoproteases located in the secretory pathway; thereby influencing the processing of newly synthesized protein and polypeptide precursors [114]. Consistent with the importance of NHEs in organellar function, *Saccharomyces cerevisae* containing null or functionally-inactive mutants of the organellar ScNha2/Nhx1 isoform show aberrant processing and missorting of proteins along the secretory pathway [20].

Conclusions

In summary, the mammalian NHE gene family has shown itself to be more diverse that previously anticipated from earlier functional studies. Much progress has been made over the last few years in characterizing the kinetic, regulatory, and physiological properties of the plasma membrane-type NHEs. Studies of the organellar-type NHEs are still in their infancy, but undoubtedly will provide exciting new insights into their contributions to cellular physiology. Are there additional NHEs yet to be identified molecularly? Functional studies suggest that two distinct types of monovalent cation/H^+ exchangers are present in mammalian mitochondria; a Na^+-selective and a non-selective monovalent cation/H^+ exchanger (reviewed in [47]). However, the mitochondria-enriched preparations used in many of these studies have been generated by differential gravity centrifugation, and hence are relatively crude in nature. Further biochemical studies using more highly-purified mitochondria and isoform-specific antibodies will be required to distinguish whether these activities represent truly novel NHEs or are known isoforms resident in contaminating endomembrane vesicles.

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