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Topical Review

Structure/Function Studies of the Epithelial Isoforms of the Mammalian Na^+/H^+ Exchanger Gene Family

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Introduction

Na^+/H^+ exchangers or antiporters are plasma membrane transport proteins, which in eukaryotes exchange extracellular Na^+ for intracellular H^+ with a stoichiometry of 1 : 1 [31, 56]. In intact cells, Na^+ enters down the Na-K-ATPase generated electrochemical Na^+ gradient. All eukaryotic cells studied have plasma membrane Na^+/H^+ exchangers, including yeast, *Caenorhabditis elegans* and crustaceans [1, 37, 49]. Prokaryotes have functionally similar Na^+/H^+ exchanger proteins which regulate the intracellular Na^+ ion concentration and pH [38, 60]. In contrast to eukaryotic Na^+/H^+ exchangers, prokaryotic Na^+/H^+ exchangers are electrogenic, exchanging two intracellular Na^+ for 1 H^+; usually utilizing the intracellular H^+ ion electromotive force.

In eukaryotic cells, the plasma membrane Na^+/H^+ exchangers have multiple functions, including pH homeostasis, volume regulation, cell proliferation, and transcellular Na^+ absorption [*reviewed in* 31]. In no cell is it the only mechanism for any one of these functions. For instance, multiple mechanisms of pH homeostasis are present in most eukaryotic cells including a Cl^-/HCO_3^- exchanger, a $NaHCO_3^-$ co-transporter, a Na^+-dependent Cl^-/HCO_3^- exchanger and multiple mechanisms of H^+ extrusion [*reviewed in* 41], including the H-K-ATPase pump.

In this review, we will focus on recent advances in identification and in understanding the structure/function relationships and regulation of the mammalian Na^+/H^+ exchanger gene family. The existence of multiple isoforms of mammalian Na^+/H^+ exchangers had been predicted on the basis of: (i) While all Na^+/H^+ exchangers are inhibited by the diuretic amiloride, they have widely different sensitivities to inhibition by amiloride from cell type to cell type and even between Na^+/H^+ exchangers on different plasma membrane domains (apical *vs.* basolateral in polarized epithelial cells) in the same cell. This has been recently reviewed by Clark and Limbird [16]. (ii) Protein kinases have different effects in regulating Na^+/H^+ exchangers depending not only on cell type, but also on different plasma membrane domains in the same cell [2, 16, 18, 20, 21, 35, 79]. With regard to the latter, Table 1 summarizes the effects of hormones, growth factors and protein kinases on intestinal and renal epithelial cells in which Na^+/H^+ exchangers are found on apical and/or basolateral surfaces. In most cases, apical membrane Na^+/H^+ exchangers are inhibited by C kinase [16], which stimulates the basolateral membrane Na^+/H^+ exchanger; cAMP inhibits both apical and basolateral membrane Na^+/H^+ exchangers. However, there are multiple exceptions to these generalizations. (iii) While it has been documented that regulation of Na^+/H^+ exchangers can occur by a mechanism that shifts the pK value for the intracellular H^+ of the exchangers, protein kinase regulation of some Na^+/H^+ exchanger isoforms involves a mechanism that changes the V_{max} of Na^+/H^+ exchange. Such a change in V_{max} may or may not be accompanied by a change in pH dependence of intracellular H^+ [35, 48, 51, 66, 76]. (iv) Na^+/H^+ ex-

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Table 1. Short-term kinase regulation of apical and basolateral Na^+/H^+ exchange in cultured intestinal or renal cell lines or intact intestinal or renal tissue

Cells/tissues	Apical Na^+/H^+ exchangers	Regulators				Basolateral Na^+/H^+ exchangers	Regulators			
		Hormones/growth factors		Protein kinase			Hormones/growth factors		Protein kinase	
LLC-PK/PE20 [11-13]	Yes	Calcitonin Vasopressin Phorbol Ester Forskolin 8-Br-cAMP	↓ ↓ ↓ ↓ ↓	A-Kinase C-Kinase	↓ ↓	Yes	8-Br-cAMP Forskolin Phorbol Ester Vasopressin	↓ ↓ ↑ ↑	A-Kinase C-Kinase	↓ ↑
A6 Cells [10]	No					Yes	Vasopressin Phorbol Ester Forskolin	↓ ↓ ↓	A-Kinase C-Kinase	↓ ↓
OK Cells [35, 53]	Yes	PTH Ca^{2+} Forskolin 8-Br-cAMP Phorbol Ester	↓ ? ↓ ↓ ↓	A-Kinase C-Kinase	↓ ↓	No				
MCT Cells [54]	Yes	PTH Forskolin 8-Br-cAMP Phorbol Ester	↓ ↓ ↓ ↓	A-Kinase C-Kinase	↓ ↓	Yes	PTH Phorbol Ester 8-Br-cAMP	↑ ↑ ↓	A-Kinase C-Kinase	↓ ↑
RKPC-2 Cells [55]	Yes	PTH 8-Br-cAMP Phorbol Ester	↓ ↓ ↑	A-Kinase C-Kinase	↓ ↑	Yes	PTH Phorbol Ester	↓ ↑	A-Kinase C-Kinase	↓ ↑
Caco-2 Cells [79]	No					Yes	Phorbol Ester Forskolin 8-Br-cAMP	0 0 0		
Rabbit Kidney [34, 53, 81–83]	Yes	8-Br-cAMP Phorbol Ester	↓ ↑	A-Kinase C-Kinase	↓ ↑	Yes				
Rabbit Small Intestine [17, 22–24, 58]	Yes	Ca^{2+}/Calmodulin Phorbol Ester Carbachol EGF	↓ ↓ ↓ ↑	A-Kinase C-Kinase CaM-Kinase Tyrosine Kinase	↓ ↓ ↓ ↑	Yes	Serotonin	?		
Rat Small Intestine or Colon [22, 64]	Yes	EGF cAMP Ca^{2+}	↑ ↓ ↓							

changers have been shown to have multiple physiologic roles, making it difficult to understand how a single transport protein could carry out so many functions. (v) By genomic Southern blot analysis, we demonstrated that the housekeeping Na^+/H^+ exchanger (NHE1) cDNA can hybridize to other closely related but not identical genes under low stringency hybridization and washing conditions [75].

Identification of Na^+/H^+ Exchanger Gene Family

Molecular identification of the mammalian Na^+/H^+ exchanger was pioneered by Pouyssegur, Sardet and co-workers who used genetic complementation [67, 69] with fibroblast cell lines that they had selected to lack all endogenous Na^+/H^+ exchangers (the Chinese hamster lung fibroblast derived cell line PS120 and the mouse fibroblast derived cell line LAPI [29, 61]). Since then additional members of this gene family have been identified, including an isoform that appears to be predominantly expressed in epithelial tissues [74] and an isoform expressed only in intestine, kidney, and stomach [59, 73]. Because there is no information concerning the total number of members of this gene family, we have named them in order of their molecular identification as NHE1 (standing for Na^+/H^+ exchanger), NHE2, etc. NHE1 is the isoform cloned initially by Pouyssegur et al. [69]; NHE2 is an isoform expressed predominantly in epithelia [74]; and NHE3 is the isoform expressed only in a subset of epithelia [59, 72]. The cloning, sequencing, and expression of several members of this gene family have been accomplished.

Existence of a gene family of mammalian Na^+/H^+ exchangers was demonstrated by our group and by Orlowski and Shull by cloning—to date, four mammalian isoform Na^+/H^+ exchangers (NHE1, NHE2, NHE3 and NHE4) have been cloned and sequenced [59, 69, 72, 74]. NHE1, NHE2 and NHE3, [69, 72, 74] but as yet not NHE4, have been shown to function as Na^+/H^+ exchangers based on functional complementation in PS120 fi-

broblasts. Since NHE4 is structurally related, it will be discussed as if it were a Na^+/H^+ exchanger, although without having identified function this cannot be certain. Although NHE1, NHE2 and NHE3 are all inhibited by amiloride and 5'-amino substituted analogues, they exhibit a wide range of sensitivities to these drugs [16]. Additional members of the mammalian gene family almost certainly remain to be identified. At the least, these include a hippocampal isoform which is totally amiloride resistant [63], and perhaps some renal/intestinal forms. For instance, the OK (Opossum Kidney) cell line has a brush border Na^+/H^+ exchanger but appears not to have message for NHE1, NHE2 or NHE3 ([52]; J. Pouyssegur, H. Murer *unpublished*).

NHEs Are Independent Gene Products

NHEs are separate gene products as predicted from their primary structure, with differences being present throughout the entire amino acid sequence. There are as yet no examples of alternately spliced Na^+/H^+ exchanger isoforms. NHE1 and NHE3 have been mapped to separate chromosomes. NHE1 has been localized to human chromosome 1 p35–p36.1 by in situ hybridization of the NHE1 cDNA [50]. Genetic polymorphisms to restriction enzymes TaqI and MspI yielded 2 alleles for each enzyme with an observed heterozygosity of 47% each in unrelated individuals [50]. The NHE3 gene has been physically mapped to the distal portion of chromosome 5p 15.3 [9]. Probing EcoR1 digested human genomic DNAs detected three polymorphic sites containing a total of nine alleles. The observed heterozygosity for the NHE3 locus in unrelated individuals was 71%. Genetic mapping placed human NHE3 at chromosome 5p15.3, making NHE3 the most telomeric gene yet identified on this chromosome [9].

Tissue Distribution of Na^+/H^+ Exchanger Message and Protein

Based on Northern analysis and ribonuclease protection assays, NHE1 message is present in nearly all mammalian cells. The only mammalian cells studied in which NHE1 message was not identified are the OK renal proximal tubule cell line and rat proximal tubule cortical segments S_1 and S_2 [42]. All of these cells are known to lack basolateral Na^+/H^+ exchangers.

NHE2, NHE3 and NHE4 are more restricted in message distribution. NHE2 message is present in kidney, intestine, adrenal gland and much less in trachea and skeletal muscle [74] (Fig. 1). The message is most expressed in the kidney medulla exceeding that in the kidney cortex. In the gastrointestinal tract the ascending colon has the most message followed by jejunum > ileum > duodenum > descending colon.

In rabbit, NHE3 message is found exclusively in kidney, intestine, and stomach [72] (Fig. 1). The most message is present in the kidney cortex, exceeding the medulla. The area of second-most message is the rabbit ascending colon which is approximately equal to the ileum > the jejunum. NHE3 message is not present in the duodenum or descending colon. Orlowski characterized NHE3 message in rat [59] and found it was most present in the proximal colon exceeding the proximal small intestine = cecum = distal colon > kidney > stomach > duodenum $\gg$ heart and brain. NHE3 message distribution is found in tissues that have neutral NaCl absorption, which is present in rat distal colon but is absent from rabbit descending colon [*reviewed in* 22].

NHE4 message is present in largest amount in the rat stomach (maximum gastric antrum) > proximal small intestine = cecum = proximal colon with much smaller amounts in the uterus, brain, kidney, and skeletal muscle [59].

At the protein level, an antibody has only been used to localize NHE1. NHE1 is found in plasma membrane of fibroblasts. In the rabbit ileum it is restricted to the basolateral membrane of both the villus epithelial cell and the crypt epithelial cell, but appears to be diffusely present in the plasma membrane of goblet cells [75]. In addition, it is restricted to the basolateral membrane of the Cl^- secretory human colon cancer cell line, Caco-2 [79]. It appears to be restricted to the basolateral membrane of the porcine renal epithelial cell line, LLC-PK1 as well [65]. In rabbit kidney, NHE1 is on the basolateral membrane of proximal tubule cells, distal convoluted tubules, thick ascending limb, and the collecting duct but is absent from glomeruli, and the thin descending loop of Henle [6].

Na^+/H^+ Exchanger Gene Family—Structure

Topology of the Mammalian NHE Gene Family

The primary structure (amino acids) and secondary structure (hydrophobicity profile) of all four identified mammalian Na^+/H^+ exchanger gene family members are similar (Fig. 2). All isoforms appear to consist of a single type of subunit based on

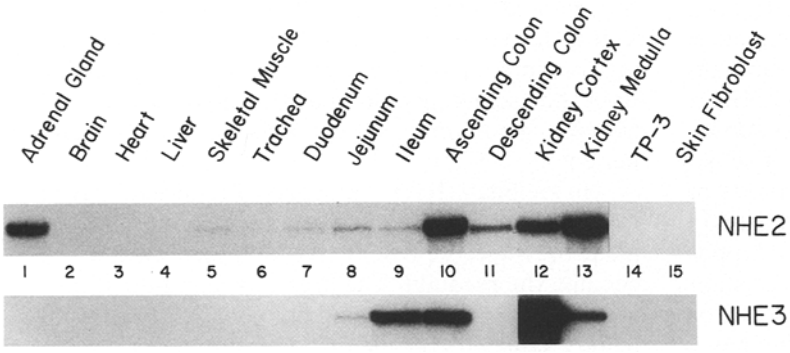

Fig. 1. Ribonuclease protection assay demonstrating tissue distribution of message for NHE-2 above and NHE-3 below [*reprinted from* 72, 74].

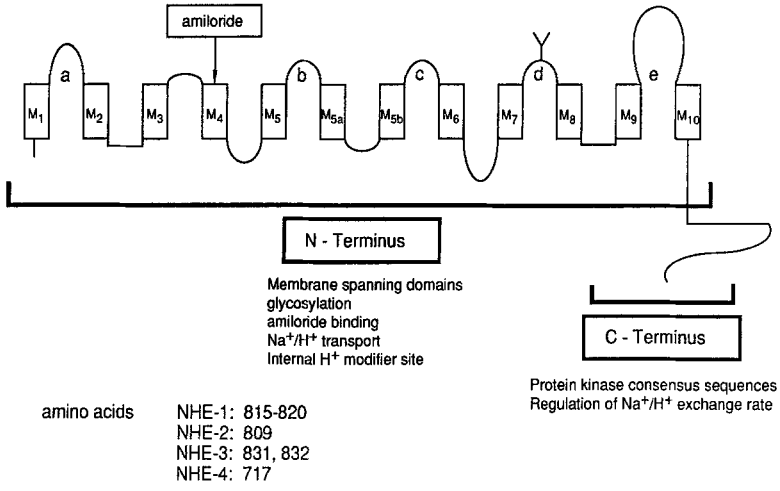

Fig. 2. Topology predicted from hydrophobicity analysis of gene family of mammalian Na^+/H^+ exchangers plus that determined by biochemical studies.

complementation of Na^+/H^+ exchange activity by a single cDNA in an exchanger deficient cell [59, 69, 72, 74]. At least NHE1 appears to exist as a dimer structurally [26, 78] and dimerization might require disulfide linkage. It is not yet known whether the functional unit of NHE1 is a monomer or an oligomer. NHE1 has been cloned from human, rabbit, rat, pig (LLC-PK1 cells) and hamster ([19, 27, 36, 59, 65, 69, 71] and D. Pearse, *personal communication*); and contains 815–820 amino acids (species variation). NHE2 has been cloned from rabbit and has 809 amino acids [74]. NHE3 has been cloned from rat and rabbit and has 831 and 832 amino acids, respectively [59, 62]. NHE4 has been cloned from rat and has 717 amino acids [57]. The corresponding predicted sizes of NHE1, 2 and 3 based on amino acid composition as predicted from cDNAs without considering glycosylation are ~91, ~91, and ~93 kD, respectively, while the predicted size of NHE4 is ~81 kD.

Figure 2 shows the predicted NHE topology. The molecule has two parts: an approximately 500 amino acid N-terminus and an approximately 300 amino acid C-terminus. As discussed below, these two domains are involved in different functional aspects of Na^+/H^+ exchange. Hydropathy analysis using the method of Engelman et al. [25] or Kyte and Doolittle [43] suggests that the N-terminus is made up of 10 or 12 membrane spanning α-helices, respectively, and contains five extracellular hydrophilic loops (Fig. 2). Antibody studies indicate that the C-terminus for NHE1 is intracellular [69], based on the requirement for membrane permeabilization to visualize the epitope.

The most highly conserved portions of the molecule among the identified isoforms are the membrane spanning domains (Figs. 2, 4); and of these, membrane spanning domains 5A and 5B are the most conserved. Each protein is predicted to contain a signal peptide sequence at the N-terminus and the first membrane spanning domain may be cleaved off in the intact protein [28]. A single putative N-linked glycosylation consensus sequence is present in extracytoplasmic loop D in all isoforms [59, 69, 72, 74]. There are other putative N-linked glycosylation consensus sequences present in some isoforms which are not conserved, including one in the first extracytoplasmic loop in NHE1 [69]. The areas least

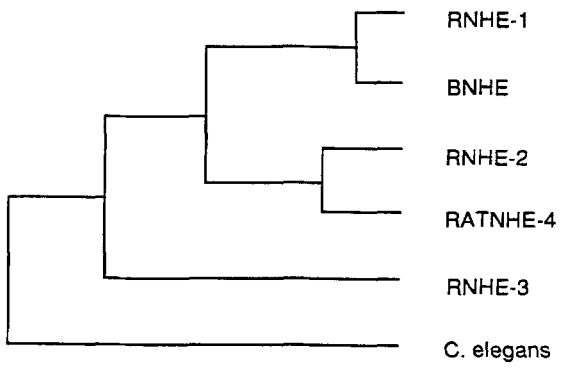

Fig. 3. Relationship among members of the eukaryotic Na^+/H^+ exchanger gene family cloned to date based on amino acid identity using PC gene subprogram Clustal dendogram (R, rabbit; B, trout red blood cell; rat, rat).

related among the Na^+/H^+ exchanger isoforms, include: (i) the first membrane spanning domain and extracellular loop A; and (ii) the intracytoplasmic C-terminal domain. There is even less relationship among the isoforms as the C-terminus is approached. The C-terminus for all isoforms contains multiple putative protein kinase consensus sequences. The individual putative protein kinase consensus sequences in the C-terminus are extremely variable among members of the gene family.

Based on comparison of the amino acid makeup of the individual NHE gene family members, NHE3 is the isoform which is most different from the others (Fig. 3). NHE2 and NHE4 most resemble each other. NHE1 is remarkably conserved across multiple species (human, rabbit, rat, hamster and pig), having at least 90% amino acid identity.

NHE1 Is a Glycosylated Protein

Deglycosylation by neuraminidase and endoglycosidase F but not endoglycosidase H reduced the size of NHE1 from Mr 110,000 to 90,000 [65]. Thus NHE1 is N-glycosylated. NHE1 has two putative N-linked glycosylation consensus sequences in extracytoplasmic loops A and D [67]. It is not known whether both sites are glycosylated or whether glycosylation always is restricted to one and not the other. The functional consequences of deglycosylation of NHE1 are not yet described, although similar deglycosylation of rat renal brush border membranes with endoglycosidase F reduced the V_{max} of the Na^+/H^+ exchanger without causing a change in the apparent $K_m Na^+$ [86]. It is assumed but not yet demonstrated that NHE2, NHE3 and NHE4 are glycosylated.

Phylogenetic Relationships of Eukaryotic NHEs

No detailed phylogenetic studies of Na^+/H^+ exchangers have been carried out. In addition to the four mammalian isoforms, Na^+/H^+ exchanger proteins have been identified at a molecular level in *Escherichia coli* [38, 60], the worm *C. elegans* [49], the yeast *Schizosaccharomyces pombe* [37], the trout [7], and multiple mammalian species including, to date, human, rat, rabbit, hamster and pig [19, 27, 59, 65, 71]. The *E. coli* Na^+/H^+ exchanger physiologically acts to remove intracellular Na^+ in exchange for external H^+ using an intracellular alkaline pH gradient as the driving force. At least two *E. coli* exchangers have been identified [38, 60]. Neither appears to have significant homology with the eukaryotic Na^+/H^+ exchangers at an amino acid level. They have 10–12 putative membrane spanning domains but a very short C-terminal domain and carry out electrogenic exchange of Na^+ and H^+. The identified yeast Na^+/H^+ exchanger has 12 putative membrane spanning domains, with four of the putative membrane domains having between 27 and 37% identity with the cloned mammalian exchangers (the most related portions of the molecule are the membrane spanning domains, 2, 5A, 5B and 10). The yeast exchanger also has some limited amiloride sensitivity. The yeast exchanger, however, does not have a long C-terminal domain as occurs in the mammalian exchangers. The *C. elegans* exchanger is much more closely related to mammalian Na^+/H^+ exchangers in the partial clones so far obtained (Figs. 3, 4), having a similar hydrophobicity profile, 52% amino acid identity predominantly in the membrane spanning domains, and a cytoplasmic 220 amino acid C-terminus which contains several putative protein kinase consensus sequences. Thus, whereas the *E. coli* Na^+/H^+ exchangers do not appear to be related to the eukaryotic isoforms, the yeast Na^+/H^+ exchanger is related, although in a more primitive way, having some similarity in the transmembrane domains, while the *C. elegans* exchanger is a clearly related predecessor. Trout red blood cell Na^+/H^+ exchanger has been cloned and shown to be closely related to NHE1 (it is also called B-NHE1 because of this similarity), but has differences in the C-terminus which allow cAMP to regulate its transport rate when expressed in PS120 cells [7].

Functional Studies

All cloned isoform Na^+/H^+ exchangers functionally evaluated when expressed in fibroblasts (NHE1, NHE2 and NHE3) demonstrate an intracellular H^+

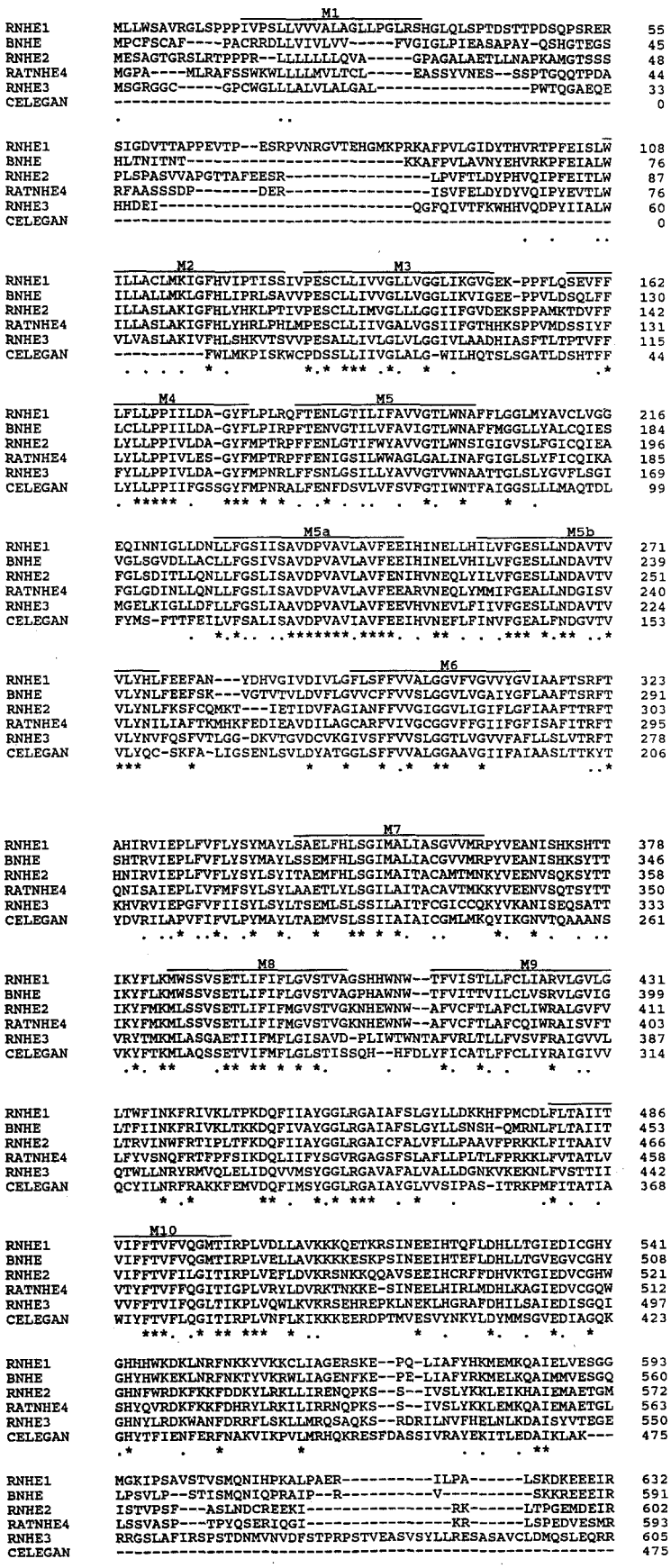

Fig. 4. *Legend on next page.*

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RNHE1       K------------ILRNNLQKTRQRLRS--YNRHTLVADPYEEAWNQMLLRRQKA   673
BNHE        R------------ILRANLQNNKQKMRSRSYSRHTLFDADEEDNVSEVRLRKTKN   634
RNHE2       E------------ILSRNLYQIRQ--RTLSYNRHNLTADTSERQAKEILIRRRHS   643
RATNHE4     D------------ILTRNMYQVRQ--RTLSYNKYNLKPQTSEKQAKEILIRRQNT   634
RNHE3       RSVRDAEDVITHHTLQQYLVKPRQEYKHL-YSRHVLSPSEDEKQDKEIFHRTMRK   659
CELEGAN     ---------VKNNIQNKRLERIKSKGRV-----------------APILPDKISN   504
                              .   .       .

RNHE1       RQ---------LEQ------KINNYLTVP-AHKLDS-PTMSRARIGSDPLAYEPK   711
BNHE        EME--RRVS-VMER------RNSHYLTVP-ANRESPRPGVRRVRFESDNQVFSAD   679
RNHE2       LRESIRKDN-SLNRERRASTSTSRYLSLPKNTKLPEKLQKRKNISNADGDSSDSE   697
RATNHE4     LRESLRKGQ-SLPWVKPAGTKNFRYLSFPYSNPQPARRGAR---------AAEST   679
RNHE3       RLESFKSAKLGLGQSKKATKH----------KRERERAQKRRNSSVPNGKL---P   701
CELEGAN     QKTMTRKD-----------------------------------------------   512

RNHE1       ADLPVITID----------PASPQSPESVDLV-NEELKGKVLGLSREPRVAEEA   754
BNHE        S-FPTVHFE----------QPSPPSTPDAVSL----------------------   700
RNHE2       ADAGTTVLNLQPRARRFLPEPFSKKASQAYKMEWKNEVDAGSGQGQPSPPAAPRS   752
RATNHE4     GNPCCWLLH-------FL-------------------------------------   690
RNHE3       LDSPRYGLTLKERE---LELSDPEEAPDYYE---AEKMSGGIEFLASVTKVSTSD   750
CELEGAN     -------LQLK----RFME---SGENIDSLYTLFSDLLDRKLHEMNRPSVQITDV   553

RNHE1       AEEDEDGGI-----VMRPK-EPSSP---GTDDVFSPAPSDSPS-------SQRMQ   793
BNHE        --EEEEEEV-----PKRPSLKADIE---GPRGNASDNHQGELD-------YQRLA   738
RNHE2       KEGGTQTPA-----VLRQPLLSKDQ---GREDSLTEGGRPKPP--------PRLV   791
RATNHE4     --------------LCRAMVEKIW---GP-------GGQETQ--------PRLL   712
RNHE3       SPAGIDNPVFSPDEDLAPSLLARVPPWLSPGEAVVPSQRARVQIPYSPGNFRRLA   805
CELEGAN     DGQDDIQDDYMA-EVSRSNL----------SAMFRSTEQLPSETPFHSGRRQSTG   597

RNHE1       R-CLSDPGPHP---EPGEGEPFIPKGQ   816
BNHE        R-CLSDPGPNK---DKEDDDPFMSC--   759
RNHE2       R-RASEPGNRK---SRLGSDK-----P   809
RATNHE4     C-RNLN---------------------   717
RNHE3       PFRLSNKSVDSFLLAEDGAEHPESTHM   832
CELEGAN     DLNATRRA--DF-------------NV   609
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Fig. 4. Deduced amino acid composition and alignment of amino acid sequences of several members of the eukaryotic Na^+/H^+ exchanger gene family. (*) Indicates identically conserved residues among the sequences shown. Residues that are conserved in five of the isoforms shown are indicated by a dot (·). Sequences shown are rabbit NHE1 (RNHE1, ref 75), rabbit NHE2 (RNHE2, ref 74), rabbit NHE3 (RNHE3, ref 73), rat NHE4 (RATNHE4, ref 59), trout red blood cell BNHE (BHNE, ref 7), and Na^+/H^+ exchanger from *C. elegans* (CELEGAN, ref 49). Note that the entire N-terminus of *C. elegans* has not been cloned.

modifier site, are regulated by growth factors and protein kinases, and are inhibited by the diuretic amiloride. Differences as well as similarities in regulation have been identified.

Kinetics

NHE1, NHE2, and NHE3 have been studied when stably transfected [47, 67, 73, 75, 77]. The expression system used for these functional studies has primarily been the PS120 fibroblast cell line. The cloned exchangers show similar kinetic characteristics when undergoing Na^+-dependent pH recovery following an acid load [3, 8], although the exchangers differ in their response to growth factors and phorbol esters. For all three expressed exchangers, Na^+/H^+ exchange [46] is entirely inhibited by amiloride and/or 5′-amino substituted amiloride analogues. The kinetics for external Na^+ follows a classical Michaelis-Menten model with a $K_m^{Na^+} \sim 16$ mM and with a Hill coefficient of 1, suggesting that there is a single binding site for external Na^+ [46].

The kinetics with respect to internal H^+ are also similar for the three exchangers with all three deviating from the hyperbolic response expected with Michaelis-Menten kinetics and all having a Hill coefficient of between 2–3 [46]. The data describing the effect of intracellular H^+ best fit an allosteric model with at least two independent binding sites for H^+ [46]. In addition to the internal H^+ transport site, there is thought to be an internal modifier site for intracellular H^+, which can regulate the activity of the exchanger [3]. This modifier site is located in the N-terminal domain as its effects are still seen in truncated antiporters in which almost the entire C-terminal cytoplasmic domain has been deleted (this has been determined for NHE1, NHE2, and NHE3) ([77]; S. Levine, C. Yun, M. Donowitz, M. Tse, *unpublished data*). Of note is that this contradicts some plasma membrane vesicle transport studies which claimed that several intestinal Na^+/H^+ exchangers lacked an internal modifier site, based on the demonstration of a nonallosteric, Michaelis-Menten relationship between internal H^+ and rate of Na^+/H^+ exchange in colonic brush border and ileal brush border and basolateral membranes [39, 62]. While these results may predict the presence of additional epithelial specific NHE isoforms, they also may represent an artifact of either the method of preparing the vesicles or be due to the difficulty of performing vesicle studies with a sufficient number of intracellular pHs to adequately define the relationship between pH and rate of Na^+/H^+ exchange. Of note is that a modifier site was not demonstrated in rabbit ileal basolateral membranes which are known to contain NHE1 [39]. This supports the dif-

ficulty of demonstrating a modifier site using vesicles; and a modifier site has been demonstrated with slightly different techniques in human small intestinal apical membranes (K. Ramaswamy, *privileged communication*).

Functional Domains: Role of the N-Terminal Domain and C-terminal Cytoplasmic Domain

To obtain insight into molecular mechanisms of second messenger regulation of the Na^+/H^+ exchanger, Wakabayashi et al. [77] constructed a set of deletions within the cytoplasmic carboxyl-terminus of human NHE1 and stably expressed the truncated cDNAs in PS120 fibroblasts. A number of conclusions can be drawn from their studies: (i) almost complete deletion of the C-terminus (approximately 15 amino acids from the end of the putative last membrane spanning domain) retains amiloride-sensitive Na^+/H^+ exchange activity, indicating that the cytoplasmic domain is not essential for ion transport and that the N-terminal portion can be inserted into the plasma membrane and carry out transport. However, this truncation transported at a much slower rate than did the wild type exchangers. Not yet known is the relative amount of each exchanger expressed in the plasma membrane. (ii) The H^+ modifier site must be located within the N-terminal domain since the allosteric activation of the exchanger by internal H^+ is preserved after almost complete removal of the cytoplasmic C-terminus. Of note, the truncated NHE1 is turned off at a lower pH than is the wild type indicating a change in set point of the exchanger. (iii) Presence of a negative element that "downregulates" the exchanger at the C-terminal portion of the long intracytoplasmic-end is suggested. The latter is based on the observation that a small C-terminal deletion of NHE1 (removing the last 117 amino acids) exhibited higher activity than the wild type, whereas further deletions led to a decrease in activity. However, these measurements were done using transiently transfected cells and activity was normalized to the expression of a reporter gene and not to the amount of Na^+/H^+ exchanger protein expressed. (iv) In NHE1 the cytoplasmic region between amino acid 566 and 635 is required for second messenger regulation since deletion of this region abolished response to growth factors, thrombin, and second messengers. There are eight serines in this region. Deletion of each individually failed to eliminate C kinase regulation of the Na^+/H^+ exchange rate. Thus, it is likely that multiple serines are phosphorylated [68].

The separation of the Na^+/H^+ exchanger into a part of the molecule which has multiple membrane spanning domains and a portion of the molecule which is cytoplasmic is found in another epithelial neutral ion exchange protein, the Cl^-/HCO_3^- exchange gene family, which is related to the red blood cell Band 3 [40]. The anion exchanger gene family also has a conserved domain which is hydrophobic and is thought to be the part of the protein involved in ion movement and a long intracellular domain. The order is reversed in comparison with the Na^+/H^+ exchanger gene family—in the anion exchanger, the C-terminus contains the hydrophobic domains. Of note, it has not yet been established that the anion exchanger is a phosphoprotein or is regulated by phosphorylation and thus it is not clear if there is also a functional analogy with the parts of the Na^+/H^+ exchanger.

Functional Domains: Amiloride Binding Site

The diuretic amiloride and its 5′-amino substituted analogues are potent inhibitors of the Na^+/H^+ exchanger and block the exchanger by competing with Na^+ for the external Na^+ binding site [*reviewed in* 4]. There are multiple types of data which support that amiloride binds to the outside of the Na^+/H^+ exchanger, including that amiloride inside red blood cell plasma membrane (ghosts) does not duplicate the effect of extracellular amiloride [32]. Extracellular amiloride is a competitive inhibitor of external Na^+ [4]. Amiloride seems to act by binding to the N-terminal part of the protein. Almost complete removal of the C-terminal 300 amino acids of NHE1 produces a protein, which is amiloride sensitive and has similar sensitivity to amiloride and a 5′-amino substituted amiloride analogue compared to the wild type Na^+/H^+ exchanger [19, 77]. Of note is that deglycosylating NHE1 did not alter sensitivity to methylpropyl amiloride, indicating an independence of amiloride binding from glycosylation in NHE1. The cloned NHE isoforms have different sensitivities to amiloride and its 5′-amino substituted analogues. NHE1 is sensitive to amiloride and to 5′-amino substituted analogues; NHE3 is resistant to both; while NHE2 is sensitive to amiloride and resistant to 5′-amino substituted analogues.

The first hints of which part of the Na^+/H^+ exchanger contains the amiloride binding site implicate the fourth transmembrane helix (Table 2). The basis for this conclusion is the result of detection of amiloride-resistant Na^+/H^+ exchanger clones [28]; sequencing their cDNAs [19]; comparing their amino acid composition and amiloride and methylpropylamiloride sensitivity with those of several

Table 2. Comparison of amino acid composition of NHE1 and NHE2 in the area of the putative fourth membrane spanning domain which appears to be involved in determining sensitivity of the Na^+/H^+ exchanger isoform to amiloride and 5'-amino substituted amiloride.

cDNA	Amino acid sequence	IC_{50} (Amiloride) μM	IC_{50} (MPA) μM	IC_{50} (EIPA) μM
NHE1	V F F L F L	3	0.05	
NHE1 → AR300	V F F F F L	15	1.5	
NHE1 → NHE2	V F F L Y L	3	0.05	
NHE1 → NHE3	V F F F Y L	15	1	
NHE1 → NHE4	V Y F L Y L	100	1	
NHE2	V F F L Y L	1		0.5
NHE2 → AR300	V F F F F L	10		5
NHE2 → NHE1	V F F L F L	1		0.3
NHE2 → NHE3	V F F F Y L	4		10

Point mutations were made in NHE1 (top) and NHE2 (bottom) to mimic the amino acid composition in comparable areas in NHE1, 2, 3, 4, and AR300. Underlined amino acids show which amino acid was mutated in NHE1 or NHE2 to the amino acid present in the isoform shown at the tip of the arrow.

Na^+/H^+ exchange isoforms which differ in amiloride sensitivity; and construction of point mutations in the various isoform Na^+/H^+ exchangers to test the significance concerning amiloride sensitivity of several amino acids which are predicted as being important for amiloride binding [19, 84]. Franchi et al. [28, 29] used acid exposure in the presence of amiloride analogues as a selection criterion and succeeded in selecting two Chinese hamster lung fibroblast mutants, AR40 and AR300, that overexpressed a mutated Na^+/H^+ exchanger. AR40 and AR300 were 10- and 30-fold less sensitive, respectively, to the 5'-amino substituted methylpropylamiloride (MPA) compared to the wild type (NHE1) control, but only 2–3.5-fold less sensitive to amiloride. Subsequent cloning and transfection of a cDNA coding the mutant exchanger from AR300 in PS120 cells expressed a Na^+/H^+ exchanger with the same lower affinity to MPA that AR300 had compared to the wild type [19]. Identification of the mutation in AR40 and AR300 by complementation showed that the MPA resistance in both was conferred by a single basepair mutation at the codon for amino acid 167 (amino acid number refers to NHE1), which converted Leu to Phe (Table 2). The difference in AR40 and AR300 was related to gene amplification with more copies of the mutant gene expressed in AR300 and the fact that AR40 has co-existence of the mutated and wild type alleles [19]. Amino acid 167 is located in the fourth putative transmembrane helix.

The amino acid residues near Leu 167 are highly conserved among the Na^+/H^+ exchanger isoform gene family (Fig. 4). To further understand the molecular basis of amiloride resistance among the known Na^+/H^+ exchanger isoforms, both the Pouyssegur and Donowitz/Tse laboratories introduced mutational substitutions into either human NHE1 or rabbit NHE2, respectively, to mimic the other isoforms [19, 84] (Table 2). Using the amino acid numbering of NHE1, mutating Leu 167 to Phe increased the K_i for MPA/EIPA and amiloride, with a greater effect on sensitivity to MPA than to amiloride. Also Counillion and Pouyssegur showed that mutating Phe 165 to Tyr increased the K_i for MPA and amiloride, with a greater effect on sensitivity to amiloride than to MPA. The ability to affect the magnitude of the K_is for amiloride or its 5'-amino substituted analogues suggests that the putative fourth transmembrane helix is likely to be part of the amiloride binding domain. In addition, based on differences in sensitivity to amiloride *vs.* MPA, amino acid Leu 167 likely interacts with the 5'-amino substituted part of amiloride, while the amino acid Tyr 165 probably interacts with a conserved part of amiloride, most likely the guanidinium group or the pyrazine ring [19, 84]. This suggests at least two attachment points for amiloride in the fourth membrane spanning domain. In addition, failure to reproduce amiloride K_i values of native Na^+/H^+ exchangers by the mutational substitutions,

particularly in NHE2, suggests the presence of additional amiloride binding domains elsewhere or additional parts of the exchanger which affect amiloride binding. Of interest was the lack of change in $K_m^{Na^+}$ by the point mutations in NHE2 [84]. This suggested at least a second site of amiloride binding and/or that Na^+ and amiloride do not bind at the identical site, as initially suggested by Pouyssegur [28].

Regulation

(1) *Short-Term Regulation*

Protein kinases regulate the rate of Na^+/H^+ exchange, although how that occurs is not yet understood at a molecular level. In native fibroblasts, Pouyssegur et al. demonstrated that the Na^+/H^+ exchanger was stimulated by thrombin and EGF, with thrombin acting via phosphatidylinositol turnover, while EGF acted by affecting tyrosine phosphorylation [14, 44–46]. NHE1 is a phosphoprotein [67, 68] and the amount of phosphorylation on serine of the same specific phosphopeptide in NHE1 changes with exposure to EGF and thrombin in parallel with the change in intracellular pH, likely due to a change in Na^+/H^+ exchange rate. Also the phosphatase 1 and 2A inhibitor okadaic acid, which would be expected to increase phosphorylation on the assumption that phosphorylation is present under basal conditions, increases phosphorylation of NHE1 and increases the basal intracellular pH. It also adds to the stimulatory effect of EGF and thrombin [67]. Nonetheless, it is unknown if changes in the phosphorylation of the Na^+/H^+ exchanger or in an associated protein lead to changes in the Na^+/H^+ exchange rate.

Phosphopeptide mapping demonstrated that NHE1 had changes in phosphorylation in response to both thrombin and EGF but that these changes occurred only on serine residues with no tyrosine phosphorylation identified [67]. This indicated that an intermediate kinase was involved in EGF regulation. This has been postulated to involve MAP kinase (mitogen activate protein kinase) [67]. In addition, it has been postulated that NHE1 may be more directly regulated by a kinase associated with the exchanger, perhaps similar to the regulation of the β-adrenergic receptor by a receptor-related kinase [68, 78].

Concerning mechanisms of regulation, it is hypothesized that the phosphorylated NHE C-terminus is coupled to the internal H^+ modifier site and that phosphorylation is crucial to allow interaction of the two parts of the exchanger, since depleting intracellular ATP alters the H^+ modifier site functionally [46, 77].

There are multiple unanswered questions concerning regulation of Na^+/H^+ exchange rate by phosphorylation including: while nearly all recognized putative protein kinase consensus sequences are in the C-terminal portion of the NHE, is it only the C-terminal portion that is phosphorylated? Are the epithelial isoform NHEs only phosphorylated on serine or are they also phosphorylated on tyrosine or threonine? Are the intermediates postulated as being involved in regulation of NHE1, which includes MAP kinase, also involved in regulation of the epithelial isoforms? Does location in the plasma membrane domain in an epithelial cell change the mechanism of regulation via phosphorylation? Is a specific serine residue responsible for regulation by a specific kinase or is there redundancy in the serines, as is suggested to occur for regulation of CFTR by cAMP [5]?

Not all functional changes in Na^+/H^+ exchange rate appear to be carried out via phosphorylation. Specifically, NHE1 involvement in cell swelling following osmotic shrinkage is dependent on ATP, but is not associated with a change in the amount of phosphorylation of NHE1 [33]. Thus, Grinstein et al. proposed dual control mechanisms of NHE1 by phosphorylation-dependent and phosphorylation-independent mechanisms [33].

Regulation of the three stably expressed Na^+/H^+ exchanger isoforms, NHE1, NHE2 and NHE3, by external signals and second messengers differs both in mechanism and in direction of regulation. It is thought that second messenger regulation of NHE1 is mediated through reversible phosphorylation-dependent coupling of the C-terminal cytoplasmic domain with the H^+ modifier site [67, 69, 78]. NHE1 activity is stimulated by growth factors, including, insulin, and also by thrombin and phorbol esters [46, 67, 69, 73] (Fig. 5). In the presence of these factors, the exchanger shows increased affinity for $[H^+]_i$, with no change in V_{max}. It has been hypothesized that this increased affinity occurs at the H^+ modifier site, thus enhancing the allosteric properties of the exchanger.

NHE2 activity is also stimulated by growth factors, thrombin and phorbol esters, but with different kinetic characteristics [46]. These agents induce an increase in V_{max} with no apparent change in affinity for H^+ [46]. NHE3 exhibits yet another difference in regulation, with stimulation by growth factors, serum and thrombin, but with inhibition by phorbol esters [46, 73] (Fig. 5). This is similar to the C kinase regulation seen in the rabbit ileal villus cells and rat colonic brush border Na^+/H^+ exchangers involved in electroneutral NaCl absorption [18, 20, 21], and

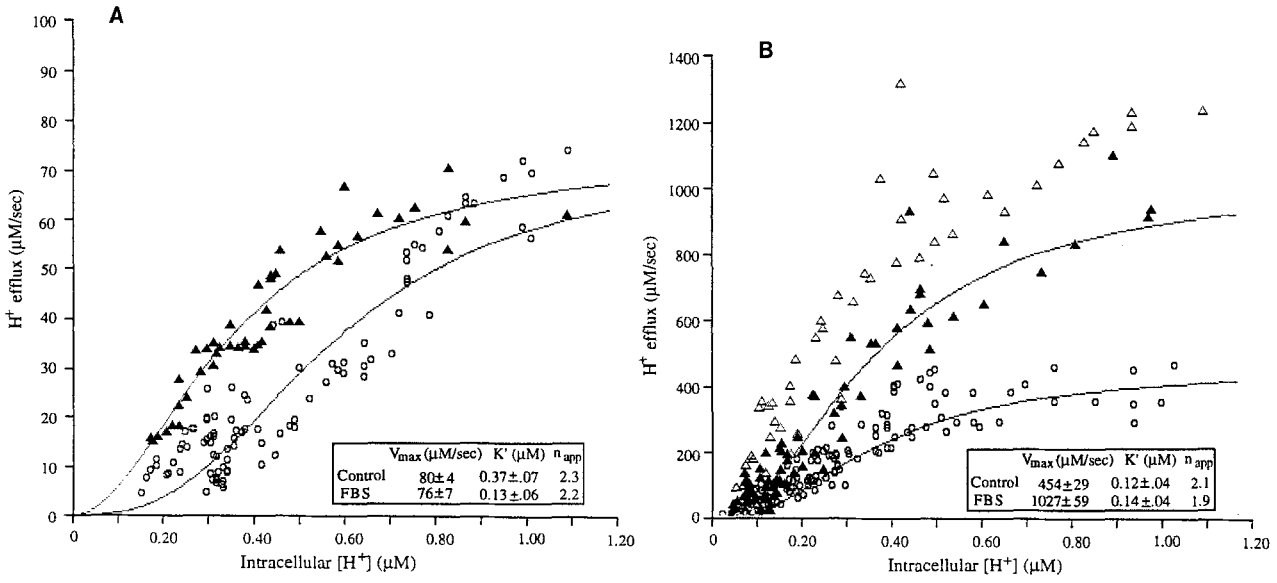

Fig. 5. Effect of serum on Na^+/H^+ exchange rate of NHE1 and NHE3, stably expressed in PS120 fibroblasts. FBS stimulated Na^+/H^+ exchange rate in PS120/NHE1 and NHE3 cells when added at the beginning of the Na^+-dependent pH recovery. In this figure, control cells (○) were acidified with an NH_4Cl prepulse and allowed to recover in Na^+ medium, while treated cells (▲) were similarly acidified, then perfused with Na^+ medium containing 10% FBS. (*A*) For PS120/NHE1 cells, the stimulation in exchanger activity was not reflected in an elevated V_{max}, rather a decrease in $K'H_i^+$ was seen. In contrast, for PS120/NHE3 (*B*) cells, there was an increase in V_{max} with addition of FBS. When PS120/NHE3 cells (△) were incubated with the PKC inhibitor H7 (65 μM) for 10 min prior to addition of Na^+ medium with 10% FBS, there was a greater stimulation of exchanger activity. Incubation with H7 alone did not change the exchanger activity compared with control cells (*data not shown*).

also to the C kinase response of the Na^+/H^+ exchanger present on the apical surface of the OK proximal tubule cell line [35]. The effect of all these factors on the kinetics of NHE3 activity is similar to that seen with NHE2; a change in V_{max} with no apparent change in affinity for H^+.

Studies of the effect of cAMP on the cloned Na^+/H^+ exchangers are difficult to interpret, as variable results have been reported for the different isoforms when expressed in various cell types. Of note, there is no consensus sequence for cAMP in the cytoplasmic C-terminus of NHE1 suggesting indirect regulation, if it occurs [69]. In the OK cell line, the apical Na^+/H^+ exchanger is inhibited by cAMP [35]. OK cells lack basolateral Na^+/H^+ exchange and also lack NHE1 normally. However, when NHE1 is transfected into OK cells a basolateral Na^+/H^+ exchanger appears and it is inhibited by cAMP (H. Murer, J. Pouyssegur, *unpublished observations*). In a SV-40 transformed rabbit kidney proximal tubule cell line (RKPC-2), NHE2 appears to be present on the apical surface and NHE1 on the basolateral membrane, with both exchangers inhibited in the presence of cAMP [55]. In the PS120 transfected fibroblasts, there was no effect of cAMP on the activity of any of the exchangers [46], and in the Caco-2 cell line there is no effect of cAMP on the activity of the endogenous NHE1 antiporter, which is present only on the basolateral membrane [79]. Only one antiporter expressed in PS120 cells (β-NHE1 cloned from trout red blood cells) is regulated cAMP; this exchanger is stimulated by cAMP, but only under conditions of high external pH (7.8) [7]. This isoform has two cAMP-dependent protein kinase consensus sequences in the cytoplasmic C-terminus which are separated by only four amino acids [6]. Thus, cAMP effects are widely variable, which is explained partially but not entirely by the putative protein kinase consensus sequences in the individual Na^+/H^+ exchanger isoform C-terminus, and at least partially seems to be dependent on the cell type which contains the NHE.

C kinase is involved in regulation of NHEs. The amino acid composition of the NHE appears to determine the C kinase regulation. C kinase stimulates NHE1 and NHE2 and inhibits NHE3 all expressed in PS120 cells. C kinase increases Na^+/H^+ exchange by NHE1 expressed in fibroblasts and in the basolateral membranes of the SV-40 transfected cell line of rabbit S2 proximal tubules (RKPT-2) as well as in that normally occurring in the proximal tubule cell line LLC-PK_1. The only exception is Caco-2 cells in which C kinase has no effect on NHE1 in the basolateral membrane.

Another way to study the role of phosphorylation in regulation of Na^+/H^+ exchange is via studying the effects of ATP depletion. ATP-depletion studies of NHE1 have shown that depleting ATP eliminates regulation of Na^+/H^+ exchange rate by growth factors and protein kinases [77]. In addition, while the ATP-depleted exchanger maintains almost full activity at low pH and there is still evidence of a H^+ modifier site, the affinity for H^+ is reduced and the exchanger is inactivated at a lower pH than in ATP-replete cells [77]. Not all studies support that NHE1 is affected by ATP depletion with a decrease only in H^+ affinity and not in V_{max}. In rat aortic smooth muscle, which is thought to contain NHE1, ATP depletion altered the affinity but also decreased the V_{max} [48]. It must be emphasized that it is not known if aortic smooth muscle contains other Na^+/H^+ exchanger isoforms. In ATP-depletion studies of NHE2 and NHE3, there are similar findings relating to the modifier site, with persistence of the modifier site and with a reduced H^+ affinity; in addition, ATP depletion reduces V_{max} of the exchangers, even at high intracellular H^+ concentrations (low pH) [46]. This implies that although in NHE2 and NHE3 the H^+ modifier site does not appear to be influenced by changes in second messengers above conditions of basal phosphorylation, the basal level of phosphorylation influences the internal modifier site function, as it does for NHE1. In addition, these studies show that the internal modifier site functions in the dephosphorylated exchanger and in the absence of ATP. Also consistent with the observations that regulation of NHE2 and NHE3 involves changes in V_{max} is the demonstration that reducing ATP lowers their V_{max}.

(2) *Long-Term Regulation—Effects of Glucocorticoids on Ileal Brush Border Na^+/H^+ Exchange*

It had been previously shown that glucocorticoids stimulate intestinal water and NaCl absorption [15], which in the rabbit takes approximately 18 hr to reach a maximum effect. Methylprednisolone stimulates rabbit ileal neutral NaCl absorption, whereas induction of glucocorticoid deficiency with aminoglutethimide inhibits NaCl absorption [70]. Studies were done to determine whether the mechanism of these longer term effects involved stimulation of ileal villus cell brush border Na^+/H^+ exchange and if glucocorticoid regulation potentially involves a change in transcription of any NHE isoform [85]. Rabbits treated with methylprednisolone for 24 and 72 hr had increased ileal brush border Na^+/H^+ exchange ~100%, whereas aminogluthethimide treatment led to a 50% decrease in Na^+/H^+ exchange (Fig. 6*A*). The effects on Na^+/H^+ exchange were specific to the extent that diffusive Na^+ uptake (no pH gradient), glucose-dependent Na^+ uptake and Na^+ equilibrium volumes were not affected. Quantitation of message of NHE1, NHE2 and NHE3 showed that methylprednisolone stimulated NHE3 mRNA level by 4–6 fold (Fig. 6*B*). In contrast, messages for NHE1 and NHE2 were not affected by methylprednisolone. These results demonstrate that glucocorticoids regulate ileal Na^+ uptake by an effect on the brush border Na^+/H^+ exchanger. They are analogous to the earlier findings of Freiberg et al. [30] that the glucocorticoid dexamethasone, but not the mineralocorticoid aldosterone, increased rat proximal tubule brush border Na^+/H^+ exchange, but had no effect on Na^+-dependent glucose uptake or Na^+ uptake without a pH gradient. More importantly, this study suggests, although it does not prove, that NHE3 is the Na^+/H^+ exchanger isoform involved in ileal NaCl absorption and in brush border Na^+/H^+ exchange; that it is under basal control of glucocorticoids; and probably can be stimulated at the level of transcription by glucocorticoids (regulation by changes in mRNA stability is also possible).

Kinase/growth factor regulation of NHE2 and NHE3 is by changes in V_{max}. V_{max} changes have been postulated as being due to changes in turnover number of a transporter or in the number of active membrane proteins. Attempts were made to inhibit protein kinase regulation of NHE1, NHE2 and NHE3 expressed in PS120 fibroblasts with the microfilament inhibitor cytochalasin D; the microtubule inhibitor colchicine; and the golgi-golgi golgi-endoplasmic reticulum vesicle trafficking inhibitor brefeldin A. None of these agents altered Na^+/H^+ exchange.

The only hint of actin involvement in regulation of Na^+/H^+ exchange has come from studies of serum regulation of basolateral Na^+/H^+ exchange (NHE1) in the polarized colon cancer cell line Caco-2 [80]. Removing serum reduces Na^+/H^+ exchange rate, an effect which occurs in 2 hr, reaches a maximum in 4 hr and can be returned to full activity by adding serum back for 4 hr [80]. The removal and return of Na^+/H^+ exchange is temperature dependent with both processes inhibited by reducing temperature to 13°C. The removal process but not the return process was inhibited by cytochalasin D, implicating actin in the process of lowering Na^+/H^+ exchange following serum removal. Whether vesicle trafficking of NHE1 occurs is unknown.

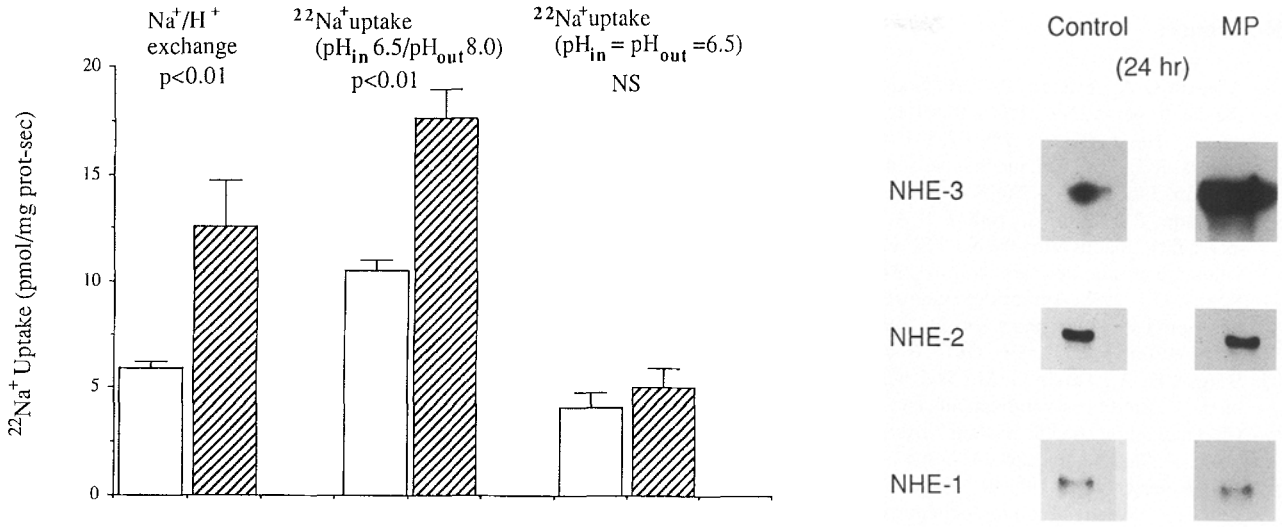

Fig. 6. (*Left*) Methylprednisolone increases ileal brush border Na^+/H^+ exchange. Control animals were injected with saline and killed 24 hr later, whereas methylprednisolone-treated animals were injected once (40 mg daily) and also killed 24 hr later. Brush border vesicles were prepared from villus cells by magnesium precipitation. Initial rates of $^{22}Na^+$ uptake were determined in the presence (pH_{in} 6.5/pH_{out} 8.0) (middle panel) or absence of an acid inside pH gradient (pH_{in} = pH_{out} = 6.5) (right panel) and Na^+/H^+ exchange was determined as the difference (left panel). Final Na^+ concentration in the transport buffer was 1 mM. Both membrane and transport buffers contained 60 mM TMA/nitrate as a voltage clamp. Membrane buffer (15 μl) and 30 μl of transport buffer were mixed, and $^{22}Na^+$ uptake was studied over 3, 5, and 8 sec at 25°C. Data represent slopes of the rate of uptake over time, and Na^+/H^+ exchange rate was determined by subtracting Na^+ uptake with a pH gradient minus Na^+ uptake with no pH gradient at each time point and using those data to determine the rate of Na^+/H^+ exchange in each experiment. Each data point represents the mean of quadruplicate determinations. Results are mean ± SE of the slopes representing Na^+ uptake from *N* separate experiments. *P* values are a comparison of slopes from paired individual experiments (paired *t*-tests). NS, not significant [*reprinted from* 85]. (□) Control; ▨ methylprednisolone, n = 3. (*Right*) Methylprednisolone treatment for 24 hr increases expression of Na^+/H^+ exchanger message for NHE3 but not NHE2 or NHE1. A single representative experiment of ribonuclease protection assay of ileal villus cell RNAs from control and methylprednisolone-treated animals is shown. Total RNA was isolated, and ribonuclease protection assay was performed; 30 μg of total RNAs for NHE1 and NHE2 or 15 μg for NHE3 was loaded in each lane and separated on a 6% polyacrylamide gel [*reprinted from* 85].

Effect of Cell Type on NHE Plasma Membrane Location and Protein Kinase Regulation

There is evidence that the cell type (epithelial or nonepithelial) can influence second messenger regulation of Na^+/H^+ exchange, and that the different exchanger isoforms may be targeted to different membrane domains in polarized cells. Caco-2, a human intestinal epithelial cell line derived from a colon carcinoma, when grown in 10% serum normally expresses only NHE1, which is present only on the basolateral surface [79, 80]. Under these conditions, Caco-2 has no brush border Na^+/H^+ exchanger, at least in the variant studied in our laboratory [79, 80]. Unlike in the PS120 fibroblasts, the Caco-2 cell endogenous NHE1 is not regulated acutely by serum, phorbol esters or growth factors [79]. However, the kinetic parameters for Na^+ and H^+ are similar for NHE1 in both PS120 cell and in Caco-2 cells, with evidence of an internal H^+ modifier site and Hill coefficient > 2 with respect to H^+. Thus, cell type or location on a specific plasma membrane domain in a polarized epithelial cell influences protein kinase regulation of NHE1. Since NHE1 in the basolateral membrane of other epithelial cells is regulated by second messengers makes it less likely that it is the membrane location and more likely that it is cell type specific regulation which is involved.

When Caco-2 cells are transfected with NHE3 they express it predominantly, if not entirely, on the apical surface, suggesting that there is specific targeting to the brush border [47, 73]. NHE2 is also expressed on the Caco-2 apical surface, although it is not known if it also appears on the basolateral surface [47, 74]. In addition, second messenger regulation of NH3 in these transfected Caco-2 cells is similar to the regulation seen in transfected PS120 fibroblasts. PMA inhibits apical NHE3. This implies that these regulatory pathways are intact in the Caco-2 cells and that the lack of regulation of the endogenous NHE1 is due to a difference in the isoform subtype or to some cell specific aspect of Caco-2 cells.

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