A Unique Sodium-Hydrogen Exchange Isoform (NHE-4) of the Inner Medulla of the Rat Kidney Is Induced by Hyperosmolarity*

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Membrane sodium-hydrogen exchangers (NHEs), found in virtually all cell types, appear to have diverse and essential roles in regulating cellular pH and mediating vectorial transport by epithelial cells. However, the functional and physiological role of the recently cloned isoform NHE-4 remains unknown. Unlike other Na-H exchanger isoforms, NHE-4 transfected into NHE-deficient mutant fibroblasts demonstrated no amiloride-inhibitable sodium uptake, under basal or acid-loaded isoosmotic conditions. By immunoblot analysis, only the NHE-4 transfectants synthesized a 100-kDa protein, which cross-reacted to polyclonal antibody made to an NHE-4 fusion protein. However, when cells were subjected to acute hyperosmolar cell shrinkage conditions, amiloride-sensitive NHE activity was readily detected at 420 mosm, exhibiting maximal activity at 490 mosm. By in situ hybridization, NHE-4 expression in the rat kidney was found to be limited to the inner renal medullary collecting tubules, the region of highest tissue osmolarity fluctuations in the body. We conclude that NHE-4 is an unusual isoform of sodium-hydrogen exchangers that may play a specialized supplementary role in cell volume regulation.

Na-H exchange activity can be identified in virtually all cell types and is characterized by being amiloride-inhibitable (particularly by N-substituted analogs), electroneutral and, in many cell types, allosterically sensitive to intracellular proton concentrations (1, 2). To date, four major NHE isoforms, designated NHE-1, NHE-2, NHE-3, and NHE-4, have been identified and their expression is tissue- and cell-specific (1, 3-7). NHE-1 was cloned first by genetic complementation of mutant fibroblasts devoid of NHE activity with human genomic DNA (3). This isoform proved to be the "housekeeping," growth factor-activated, amiloride-sensitive NHE found in most cells and localized to the basolateral membranes of polarized epithelial cells (8). The related isoforms, NHE-2, NHE-3, and NHE-4, were subsequently cloned by cross-hybridization from various cDNA and plasmid libraries (4-7). Based on predicted amino acid sequences, these isoforms have approximately 40% amino acid identity and, by hydropathy profiles, appear to have similar transmembrane organization (4). However, considerable differences appear in the non-membranous C-terminal domains.

In contrast to NHE-1, the other isoforms remain incompletely characterized and have undefined physiological roles. Preliminary studies have suggested that NHE-2 and NHE-3 are selectively, but not exclusively, expressed by epithelial cells of tissues such as intestine and kidney (4-7). NHE-3 is localized on the apical membrane of the small intestine (9). Thus, these three isoforms are potential isoformshome the major vectors of vectorial sodium transport, a major function of such cells.

Far less is known about NHE-4. By Northern blot analyses, it appears to be most highly expressed in stomach, intestine, kidney, brain, uterus, and skeletal muscle (4), but its functional characteristics, membrane localization, and physiological role(s) are unknown. In this report, we provide evidence that expression of NHE-4 in the kidney is restricted to the collecting tubules of the inner medulla, the region of highest tissue osmolarity of the body. We also demonstrate that transfected full-length NHE-4 is quiescent in exchanger-deficient fibroblasts when acid-loaded at isoosmolarity but is activated when acid-loaded under hyperosmolar conditions. We speculate that this isoform may play a specialized role in the kidney in rectifying cell volume in response to extreme fluctuations of hyperosmolar-stimulated cell shrinkage.

EXPERIMENTAL PROCEDURES

Construction of Recombinant DNA—Rat full-length NHE-4 cDNA (pSN4) was subcloned by ligation of the partial cDNA fragment BamHII-BslIII from pRSNHE4(4-1) to the overlapping NHE-4 fragment BslIII-PstII from pRSNHE4(10-2) (generously provided by G. Shull; Ref. 4) into pAlter (Promega, Madison, WI). The EcoRI-HindIII 3.1-kilobase NHE-4 cDNA from pSN4 was then ligated into the shuttle vector pCB6+ (courtesy of M. Stinski, University of Iowa), so that the cytomegalovirus promoter directed expression of the NHE-4 message. This DNA (pCN4) was transfected into the NHE-deficient PS120 fibroblasts.

The cRNA riboprobe (pG7N4A) was constructed by ligating the unique BamHII-EcoRVos NHE-4 cDNA fragment into BamHI/Smal-cut pGEM7Z (Promega). SP6 and T7 promoters were used to generate, respectively, sense and antisense cRNA probes. This construct generated a probe that included 428 bases of 5' non-coding region and 166 bases of coding region corresponding to amino acids 1-56 of NHE-4.

A fusion protein to glutathione S-transferase consisted of the fragment BstIII-PstII from the subcloned BamHI-cutter pGEM7Z (Pharmacia Biotech Inc.). This generated a fusion encompassing amino acids 396-625 and was verified by Sanger dideoxy sequencing.

Cell Culture—PS120 (NHE-deficient) and PS127 (human NHE-1-transfected) fibroblast cells, Chinese hamster lung CCO39 derivatives (10) were provided to M. Villereal by J. Pouyssegur. Cells were maintained at 37°C under an atmosphere of 5% CO₂, 95% air in Dulbecco's...
modified Eagle's medium (high glucose) supplemented with 10% fetal calf serum (Life Technologies, Inc.), 5 units/ml penicillin, and 5 μg/ml streptomycin. RNA and protein was prepared from pre- and post-confluent cells according to previously described methods. Transfections into the PS120 cell line were done with Lipofectin (Life Technologies, Inc.), according to manufacturer's directions. All NHE-4 transfectants were selected by resistance to the antibiotic G418 (Life Technologies, Inc.), a gene carried on the shuttle vector pCB6+. G418-resistant cells were clonally selected, NHE-4 expression was verified by Northern and Western analysis, and four clones were chosen for further analysis. The clone used for expression studies here, PSCN4-4, represents these.

Preparation of RNA and Ribonuclease Protection Assay—Isolation and purification of RNA were as described previously (9). Ribonuclease protection was done as described in the Promega Biotech Technical Bulletin. For the antisense strand probe, pG7N4A was linearized by BamHI (Life Technologies, Inc.) and the 607-base cRNA probe was generated by T7 polymerase (Promega). SP6 polymerase transcribed the 652-base sense probe from EcoRI-linearized pG7N4A. [α-35S]UTP or [α-32P]UTP, respectively, were used to label internally the cRNA for ribonuclease protection assays and in situ hybridizations.

In Situ Hybridizations—Male Sprague-Dawley rats were deeply anesthetized by an intraperitoneal injection of 7% chloral hydrate and were then transcardially perfused with a solution of 4% paraformaldehyde (initially at a pH of 6.5, then changed to the same solution at pH 9.5). Tissues were resected and placed in a solution of 15% sucrose and 4% paraformaldehyde overnight. Cryostat sections (12 μm) were mounted on gelatin- and poly-L-lysine-coated slides. The slides were treated with a solution of 0.001% proteinase K in 0.1 M Tris-HCl, pH 8, and 0.05 mM EDTA, pH 8, for 30 min at 37 °C and then with 0.05% acetic anhydride for 10 min. They were rinsed in 2x SSC and dehydrated in increasing concentrations of ethanol. The tissue sections were hybridized with cRNA probes internally labeled with [32P] for 16 h at 55 °C in a solution of 50% formamide, 10% dextran sulfate, 0.5 mM NaCl, 1 x Denhardt's solution, 10 μg/ml Tris-HCl, pH 8, and 0.001 mM EDTA for 30 min at 37 °C. The slides were further washed with decreasing concentrations of SSC for 1 h at room temperature, and unhybridized probe was removed by digestion with a solution of 20 μg/ml ribonuclease A in 0.5 mM NaCl, 0.01 x Tris-HCl, pH 8, and 0.001 mM EDTA for 30 min at 37 °C. The slides were further washed with decreasing concentrations of SSC for 1 h with a final wash in 0.1 x SSC at 60 °C for 30 min and then dehydrated in increasing concentrations of ethanol. Slides were initially exposed to x-ray film (Hyperfilm-max, Amersham Corp.) for 4 days to provide an indication of the intensity of the hybridization signal. They were then dipped in Kodak NTB-2 liquid autoradiography emulsion. After 10 days, the slides were developed with D-19 developer and fixed with Kodak rapid fixer.

Construction of NHE-4 Fusion Protein Antibody—A glutathione S-transferase fusion to the carboxyl amino acids 393-625 of NHE-4 was expressed in bacteria (9). The pGEM7Z. This sequence of NHE-4 cDNA consists of 428 base pairs derived from the 5' end of NHE-4 cDNA, was constructed by subcloning the BamHIter from EcoRV restriction fragment into the vector pGEM7Z. This sequence of NHE-4 cDNA consists of 428 base pairs from the 5' non-coding region and 166 base pairs that encode amino acids 1-56. There is no homology between pG7N4A and any of the other cloned NHE isoforms (sequence analyses by Pustell matrix analysis). This fact was of particular importance in defining an entirely unique region of NHE-4 that would not hybridize to NHE-2, its most closely related isoform. Even at low stringency, matrix analyses could find no regions of identity with NHE-2 cDNA longer than 6 contiguous bases, a size well below the detection capabilities of the conditions used in in situ hybridization.

To verify the specificity of our pG7N4A probe, we analyzed total RNA from whole kidney and NHE-4 cDNA-transfected fibroblasts by ribonuclease protection assay. This assay is particularly sensitive to any base pair mismatches. Antisense T7-generated pG7N4A cRNA was incubated with total RNA from three clonal NHE-4/PS120 transfectants and from rat kidney (Fig. 1). The RNA-cRNA hybrids were incubated and washed at high stringency, and then all single-stranded RNA was digested by RNase A and T1 (same conditions of stringency as used in the in situ hybridization analyses). A single base pair mismatch will be recognized and deleted by these RNases. The protected duplexes were visualized after separation on urea-PAGE. The 607-base probe, alone and undigested (Fig. 1, lane 1), includes 13 bases of vector DNA. A perfect hybrid match to NHE-4 mRNA would be 594 base pairs in size. Controls for this experiment included digested probe alone to prove that reaction conditions were sufficient to completely digest all single-stranded nucleotide, the SP6-generated sense strand probe, and total RNA from the non-transfected parental PS120 cell line. Both unhybridized probe and sense probe were completely digested, and no hybrid was detected by the antisense probe in the parental, nontransfected cells' RNA (data not shown). In those clones stably expressing NHE-4, the predicted 594-base region was protected by the NHE-4 cRNA antisense probe (Fig. 1). The same 594-base region was protected in samples of total

![Fig. 1](image-url)
FIG. 2. Whole kidney *in situ* hybridization to rat kidney by the antisense strand G7N4A riboprobe. *A,* stable hybrids with NHE-4 mRNA were evident in the inner medulla, continuing into the outer medulla and scattered throughout the cortex. *IM* is inner medulla, *OM* is outer medulla, and *C* is cortex. *B,* sense strand N4A riboprobe *in situ* hybridization to section from the same rat kidney shown in *A.* *C,* detailed view of...
RNA from rat kidney, indicating that the NHE-4 transcript was endogeneously expressed.

In situ hybridizations of frozen rat kidney sections with [³²P]UTP internally labeled pG7N4A antisense riboprobe revealed NHE-4 transcript primarily in the collecting ducts of the inner medulla (Fig. 2A). Tissues probed with the sense strand were clear of any hybridization signal (Fig. 2B). For both antisense and sense probes, the same conditions of high stringency hybridization and washing were used, so that all non-duplexed nucleotides were completely digested. Both sense and antisense probes were hybridized on adjacent cyrossections of the same tissue. These experiments were repeated on three separate occasions on tissue freshly harvested and prepared from different animals. Fig. 2A shows distinct signals of hybridized antisense probe concentrated in the inner medulla and continuing into the outer medulla. The specificity of this probe for well delineated tubules in the inner medulla is shown in Fig. 2C. Fig. 2D (inner and outer medulla at a higher magnification) shows NHE-4 message restricted to a subset of tubules, which appeared to be continuous with the collecting ducts of the inner medulla. NHE-4 message also appeared in tubules dispersed throughout the cortex (Fig. 2E and F). These probably represent cortical collecting duct segments rather than proximal tubules, the major component of the cortex, although we cannot rule out another specialized tubule expressing NHE-4 mRNA.

Expression of NHE-4 in Na/H Exchange-deficient Fibroblasts—To examine the physiological role of this isoform, we transfected a mutant NHE-deficient fibroblast cell line (Chinese hamster lung CCL39 derivative, called PS120) (10) with pCN4 (full-length rat NHE-4 cDNA expressed constitutively by the cytomegalovirus promoter). This strain was mutagenized to destroy all NHE activity and has been used by many investigators to study NHE isoforms because it is one of two readily available cell lines with no endogenous NHE activity. G418-resistant cells were clonally selected for genomic incorporation of the CN4 cDNA construct, and, after determining the other clones transfected, we used the more extensive studies presented here.

To confirm successful transfection of functional NHE-4 cDNA, we used both Northern and Western blot analyses of the fibroblasts. By Northern analysis (Fig. 3A), NHE-4 transcript was found only in the transfectants and not in the parent PS120 mutant fibroblasts. This indicated that the transfected NHE-4 cDNA construct was being stably transcribed into full-length mRNA.

However, these NHE-4 transfectants expressed no amiloride-inhibitable Na-H exchange activity after acid loading. The next step was to determine if the lack of NHE activity was due to a block of efficient translation or instability of the NHE-4 protein. Membrane proteins were purified from the parent cell line, PS120; PS127, a cell line expressing NHE-1; and the NHE-4 transfectants previously identified by Northern blot analysis. Immunoblot analysis (Fig. 3B) determined that only the NHE-4-transfected cell lines synthesized a 100-kDa protein that cross-reacted to the NHE-4 fusion protein antibody. Neither the parent strain (not shown) nor the PS127 transfectants expressed this protein. The apparent size by SDS-PAGE (100 kDa) is greater than the 81 kDa predicted by sequence alone, possibly due to glycosylation. NHE-4 exhibits a mobility differential similar to NHE-1, whose sequence predicted size is 91.6 kDa, but by SDS-PAGE appears to be 110 kDa. However, as shown in Fig. 3B, there is no cross-reactivity between NHE-1 and the NHE-4 antibody. This antibody also showed no cross-reactivity with PS120 transfectants expressing rat NHE-1 or NHE-3 proteins (data not shown).

Effect of Hypertonicity on NHE-4 Activity—Next, linear influx rates of [²²Na] into subconfluent PS127 (NHE-1-transfected) and PSCN4-4 (NHE-4-transfected) fibroblasts were measured over 4 min, a period where influx was determined to be linear (data not shown). Because [²²Na] influxes under basal conditions were barely measurable in all cell groups, all studies were performed under acid-loaded conditions, established by 60 min of incubation with 50 mM NH₄Cl, followed by a rapid washout, as described previously (12). [²²Na] influxes were determined in media containing 20 mM sodium, in the presence or absence of the amiloride analog, dimethyl amiloride (DMA) (500 μM, gift from T. Kleyman, University of Pennsylvania). As shown in Fig. 4A, the NHE-deficient PS120 cells demonstrated no amiloride-inhibitable [²²Na] uptake. In contrast, PS127 cells expressing NHE-1 exhibited significant [²²Na] uptake, much of which was DMA-sensitive. Under the same conditions, no amiloride-sensitive [²²Na] uptake could be demonstrated in the NHE-4 transfectant, PSCN4-4. These data could be consistent with PSCN4-4 cells expressing a functionally defective NHE-4 protein. However, Western analysis (Fig. 3B) indicated that a full-size NHE-4 protein was being synthesized in these cells. Given the location of NHE-4 transcript in the hyperosmolar milieu of the renal inner medullary collecting tubules, we investigated the possibility that the activation of NHE-4 might be observable only under specialized conditions. A similar Na/H exchange mechanism in barnacle muscle, which is only activated by osmolarity changes, has been described extensively (14).

We measured linear [²²Na] uptake by PSCN4-4 cells acutely exposed to a wide range of extracellular osmolalities. In all
and in human endothelial cells (16), it was not known which isoform was being affected. Based on our observations that NHE-1 is activated in acid-loaded isoosmolar and hyperosmolar conditions, we speculate these studies involved NHE-1. In contrast, we believe our observations with NHE-4 may be analogous to previous findings reporting unique activation of NHE activity in the barnacle muscle (17). NHE activity in these cells cannot be detected under basal or acid-loaded conditions, but only under conditions such as hyperosmolar fluid-induced cell shrinkage or G-protein activation. Thus, we speculate that the barnacle muscle may express an NHE-4-like exchanger. Na-H exchange in this organism may be important for cell volume regulation after hyperosmolar-induced cell shrinkage, which can occur when these cells are exposed to fluctuations in fluid tonicity encountered in sea and brackish waters. Since it appears to have little or no activity under basal or acid-loaded conditions, NHE-4, like the barnacle muscle NHE, may have a relatively minor role in cellular pH regulation.

In the PS120-transfected hamster fibroblasts, NHE-4 did not show any activity above 700 mosm, while NHE-1 exhibited a more gradual drop in fluxes after peaking at 490 mosm. Collecting tubules can incur external osmolarities of >1000 mosm. If NHE-4 represents a dominant factor in regulating cell volume in the extreme conditions that occur in the kidney, we would have expected to see a much greater DMA-inhibited response in the transfected cells. Absolute flux values were also relatively low when compared to NHE-1. This discrepancy between what would be required in the in vivo milieu and what we observe in the in vitro system may be because fibroblasts do not have the regulatory or accessory proteins that are present in the renal cells and are required for maximal and sustained activation of this isoform. In contrast, NHE-1 is an ubiquitously expressed isoform, and regulatory proteins that it requires should exist in most cell types. Differences in turnover rates or post-synthetic modifications could also account for differing transport rates.

Because epithelial cells of inner medulla of the kidney are frequently subjected to significant fluctuations in luminal and interstitial osmolarity, it is possible that NHE-4 functions as a rectifying mechanism for hyperosmolar fluid-induced cell shrinkage. Its physiological role may differ substantially from NHE-1, which appears to be ubiquitous in the kidney and other tissues and has a primary role in cell pH regulation (18). NHE-1 is sensitive to and allosterically modified by increasing osmolarity, while NHE-4 appears to have little or no activity under basal or acid-loaded conditions, but shows any activity above 700 mosm. If NHE-4 represents a dominant factor in regulating cell volume under conditions of osmolar-induced cell shrinkage. Its physiological role may differ substantially from NHE-1, which appears to be ubiquitous in the kidney and other tissues and has a primary role in cell pH regulation (18). NHE-1 is sensitive to and allosterically modified by increasing osmolarity, while NHE-4 appears to have little or no activity under basal or acid-loaded conditions, but shows any activity above 700 mosm. If NHE-4 represents a dominant factor in regulating cell volume under conditions of osmolar-induced cell shrinkage.

This report provides the first description of the specific sites of expression for NHE-4 in the kidney and the first report of conditions under which this isoform can be activated in vitro. The location of NHE-4 transcript corresponds to a specialized region of the kidney where interstitial osmolarity is high, allowing collecting duct cells to reabsorb sodium and other solutes.

Although osmotic shrinkage has been reported to stimulate NHE activity in protein kinase C-depleted lymphocytes (15), instances, [22Na] uptake was measured in the presence of 20 mM extracellular sodium and after acid loading, as described above. Osmolarity was adjusted by varying mannitol concentrations and verified by osmometry. Table I shows that there was minimal DMA-sensitive activity in NHE-4 transfected cells at osmolarities greater than 350 mosm. However, as osmolarity increased, DMA-inhibitable [22Na] uptake became demonstrable, peaking at ~490 mosm, and declining thereafter (Fig. 4B). Similarly, there was an increase in NHE-1 activity in PS127 cells with increasing osmolarity, also peaking at 490 mosm. In contrast, no DMA-inhibitable activity could be measured in NHE-deficient PS120 fibroblasts at any osmolarity. Similarly, no DMA-inhibitable activity could be observed in PSCN4-4 in the absence of acidification.

**DISCUSSION**

This report provides the first description of the specific sites of expression for NHE-4 in the kidney and the first report of conditions under which this isoform can be activated in vitro. The location of NHE-4 transcript corresponds to a specialized region of the kidney where interstitial osmolarity is high, allowing collecting duct cells to reabsorb sodium and other solutes.

Although osmotic shrinkage has been reported to stimulate NHE activity in protein kinase C-depleted lymphocytes (15),
tissues in which it has been detected. This isoform may have a number of supplementary and highly specialized roles to play in the various tissues in which it appears. Here, we have demonstrated only one mechanism that activates this isoform in fibroblasts. This protein may require cell-specific auxiliary factors that only occur in cells normally expressing NHE-4. We can conjecture that NHE-4 serves highly specialized functions in the various cell types in which it appears. NHE-1 responds to a variety of stimulatory conditions (e.g., changes in cell volume or pH,, mitotic agents, protein kinase C activation, etc.). Not all conditions of activation may be functional in any one cell type. Takaichi et al. (19) demonstrated that protein kinase C stimulation of the endogenous human NHE-1 in human fibroblasts was barely detected in human NHE-1-transfected mouse fibroblasts. With further study (in more appropriate model systems), we may find that NHE-4 exhibits similar versatility and that it responds to one set of conditions in the kidney medulla, whereas different conditions stimulate activation in other tissues.

The location of NHE-4 transcript differs substantially from that of NHE-1 and NHE-3, the former being diffusely expressed throughout the kidney, whereas the latter appears predominantly in the proximal tubules of the outer medulla, to a lesser extent in the cortex with none expressed in the inner medulla (20). This further supports our hypothesis that NHE-4 plays a highly specialized role in the kidney.

In summary, we find NHE-4 to be a unique isoform of sodium-hydrogen exchangers, which is predominantly expressed in inner medullary collecting ducts in the rat kidney. Its activation profile in transfected NHE-deficient PS120 fibroblasts differs from other isoforms such as NHE-1, having no demonstrable activity under acid-loaded isoosmolar conditions. However, it can be activated under conditions of hyperosmolar-induced cell shrinkage. We conclude NHE-4 may play an important role in the kidney, possibly for volume rectification of collecting duct cells subjected to the hyperosmolar milieu of the inner medulla.

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