Localization and functional characterization of Na\(^{+}/H\(^{+}\) exchanger isoform NHE4 in rat thick ascending limbs

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Molecular studies have demonstrated the existence of six distinct genes encoding the mammalian Na\(^{+}/H\(^{+}\) exchangers (NHEs). The first cloned gene, NHE1, encodes a widely expressed Na\(^{+}/H\(^{+}\) exchanger, which is believed to subserve housekeeping functions, including intracellular pH and cell volume regulation (34, 38, 43). Extensive studies revealed a basolateral localization of NHE1 in epithelial cells (5, 7, 44). The novel NHE6 gene is expressed in mitochondria-rich tissues (31). The other genes, NHE2, NHE3, NHE4, and NHE5, have a more restricted pattern of expression. NHE2, NHE3, and NHE4 are preferentially expressed in epithelia such as the gastrointestinal tract and the kidney (34, 41, 43, 49), whereas NHE5 resides in a selected number of nonepithelial tissues (4, 25). Immunologic and functional studies support the presence of both NHE3 and NHE2 isoforms in the apical membrane of most intestinal cells in various species (16, 22, 24, 50). NHE3 is probably the NHE isoform involved in basal and glucocorticoid-stimulated intestinal NaCl absorption (29). Regarding NHE2, a recent study demonstrated that, in proximal colon, this isoform is primarily responsible for the increase in Na\(^{+}/H\(^{+}\) exchange activity occurring in a physiological model of Na depletion (24). In contrast, renal expression sites of NHE2 are somewhat distinct from those of NHE3. Indeed, immunologic studies in the kidney have predominantly localized NHE3 to the apical domains of the proximal tubule and the thick ascending limb (TAL) (2, 5, 6, 8) whereas NHE2 seems to be predominantly expressed in the apical membrane of cortical TAL, macula densa, and distal convoluted tubule (14, 35). NHE3 is believed to play a significant role in NaCl and NaHCO\(_3\) absorption in the proximal tubule and HCO\(_3\) absorption in the TAL. Enhanced expression of NHE3 is involved in the adaptation of HCO\(_3\) absorption during chronic metabolic acidosis in these seg-
ments (1, 27, 51). Because the apical membrane Na\(^+\)/H\(^+\) exchange activity in the distal convoluted tubule is involved in bicarbonate and NaCl reabsorption, NHE2 could likely mediate these functions (47, 48).

In contrast, little is known about NHE4. NHE4 transcripts are highly expressed in the stomach, but they are also present in kidney (34). In situ hybridization studies suggested that mRNA for NHE4 is limited in the rat kidney to the inner medulla (11). In contrast, by indirect immunofluorescence of rat kidney cortex, we have recently localized the NHE4 isoform to the basolateral domains of cortical distal tubules (12), and immunoblot analysis of membrane fractions from rat kidney cortex has confirmed the basolateral expression of NHE4 (36). However, precise localization of this isoform in the mammalian kidney by immunostaining techniques has not been reported. In addition, because pharmacological properties have not yet been completely determined, no functional study has unambiguously assigned Na\(^+\)/H\(^+\) exchange activity in renal tubules to the NHE4 isoform. As a result, the physiological role of NHE4 remains speculative. In the present study, we used an affinity-purified antibody directed against a 10-amino acid peptide located in the cytosolic domain of the rat NHE4 protein to localize the Na\(^+\)/H\(^+\) exchanger isoform NHE4 along the rat nephron. We demonstrated that NHE4 is a basolateral membrane protein highly expressed in thick ascending limb and, to a lesser extent, in collecting ducts. We therefore used basolateral membrane vesicles isolated from rat medullary thick ascending limbs (MTAL) to describe the functional properties of NHE4 in its natural membranous environment.

**EXPERIMENTAL PROCEDURES**

**Materials.** Carrier-free \(^{22}\)Na was obtained from Amersham (Buckinghamshire, UK). 5-(N-ethyl-N-isopropyl)amiloride (EIPA) was purchased from Molecular Probes (Eugene, OR). 4-Isopropyl-3-methylsulfonylbenzoyl-guanidine methanesulfonate (HOE-642) was kindly provided by Dr. H. J. Lang (Hoechst Marion Russel). Stock solutions of EIPA and HOE-642 (100 mM) were prepared in DMSO. All other chemicals were of analytical grade.

**Antibodies.** Rabbit polyclonal anti-rat NHE4 antibody was generated against a unique 10-amino acid synthetic peptide corresponding to a region near the NH\(_2\) terminus of NHE4 (sequence NH\(_2\)-SYNKYNLKPQ-COOH; amino acids 609–618) as previously described (12). Goat antiserum to human uromodulin (Tamm-Horsfall glycoprotein) was obtained from Cappel (ICN Pharmaceuticals, Aurora, OH). Antibodies to aquaporin-2, aquaporin-3, and the Na-K-2Cl cotransporter BSC1 were gifts of Dr. M. A. Knepper (National Institutes of Health, Bethesda, MD). Anti-Na-K-ATPase polyclonal antibodies and monoclonal antibodies to NHE3 were from Chemicon International (Temecula, CA).

**Crude membrane preparation.** Membranes were prepared from various organs from Sprague-Dawley rats, euthanized by an intraperitoneal injection of pentobarbital sodium, as follows. The inner stripes of the outer medulla from the kidneys of two rats were excised under a dissecting microscope and placed into ice-cold isolation buffer (250 mM sucrose, 20 mM Tris-HEPES, pH 7.4) containing protease inhibitors as follows (in \(\mu\)g/ml): 4 aprotinin, 4 leupeptin, 1.5 pepstatin A, and 28 4-(2-aminoethyl)-benzenesulfonyl fluoride. Minced tissues were homogenized in a Dounce homogenizer rotating at 1,000 rpm. The homogenate was centrifuged at 1,000 \(g\) for 10 min, and the supernatant was centrifuged at 100,000 \(g\) for 1 h at 4°C. The pellet was resuspended in a small amount of isolation buffer. Samples were solubilized in loading buffer, incubated at 20°C for 30 min, and then frozen at −20°C until used.

**Preparation of MTAL tubules.** The tubule isolation procedure was similar to that described by Attmane-Elakeb et al. (5). Male Sprague-Dawley rats, weighing 250–300 g, were anesthetized with pentobarbital sodium. Kidneys were removed quickly, decapsulated, and sliced sagittally. Slices were transferred in Hanks’ modified medium, and the inner stripe of the outer medulla was carefully excised under stereomicroscopic control. The resulting tissue was subjected to collagenase treatment. In the final suspensions, most of the tubules (>95%) proved to be of MTAL origin, on the basis of immunofluorescent staining with Tamm-Horsfall, a specific marker for the TAL.

**Isolation of basolateral plasma membrane vesicles from MTAL.** Typically, the preparation began with 15–20 mg of protein from MTAL tubules obtained from 10 rats. Basolateral membrane vesicles (BLMV) were isolated simultaneously by a combination of Ca\(^2+\) aggregation and differential and density gradient centrifugations, as described previously (5). Compared with the homogenate, the basolateral membrane fractions were enriched ninefold in the basolateral marker, Na\(^+\)-K\(^+\)-ATPase activity, and only twofold in the apical membrane marker, γ-glutamyltranspeptidase activity, whereas luminal membrane fractions were deenriched in Na\(^+\)-K\(^+\)-ATPase (enrichment factor of 0.8) and markedly enriched in γ-glutamyltranspeptidase (enrichment factor of 21). These enzymatic assays are consistent with immunoblot analysis of apical membrane proteins such as H\(^+\)-ATPase and NHE3 and basolateral membrane proteins such as α- and β-subunits of the Na-K-ATPase and NHE1 (5, 18, 19). In eight separate plasma membrane preparations, apical and basolateral Na/H exchange activities measured by H\(^+\)-stimulated \(^{22}\)Na uptake sensitive to a high dose of EIPA were 18.34 ± 3.92 and 21.74 ± 7.63 pmol·mg protein\(^{-1}\)·s\(^{-1}\) respectively. On the basis of these data, it could be assumed that only a marginal fraction (8%) of the basolateral Na\(^+\)/H\(^+\) exchange activity could be of apical membrane origin. Consistently, although a small number of apical membrane proteins could be detected in basolateral membrane vesicles, previous studies demonstrated that MTAL basolateral and luminal membrane transport properties are largely separated from each other. Indeed, polarized functional expression of several plasma membrane transporters of the MTAL, such as the apical Na\(^+\)-K\(^+\)-2Cl\(^-\) cotransporter BSC1 (37), the basolateral H\(^+\)-lactate cotransporter MCT2 (19), and the basolateral Cl\(^-\)/HCO\(_3\)\(^-\) exchanger AE2 (18), has been demonstrated.

**Immunoblot analysis.** Proteins were separated by SDS-PAGE (7.5%), then transferred to nitrocellulose (Amersham, Arlington Heights, IL). Immunoblots were performed as follows: strips of nitrocellulose were first incubated in 10% nonfat dry milk in PBS (pH 7.4) for 1 h at room temperature to block nonspecific binding of antibody, followed by an overnight incubation at 4°C with different primary antibodies diluted in PBS containing 1% nonfat dry milk. Membranes were then washed four times with PBS containing 0.1% Tween 20 for 5 min each before incubation (1:3,000 dilution) of goat anti-rabbit IgG conjugated to horseradish peroxidase (Bio-Rad, Hercules, CA). Blots were washed as above, and
luminol-enhanced chemiluminescence (Amersham) was used to visualize bound antibodies on Polaroid film. Photographs of immunoblots were numerized under National Institutes of Health Image software. For peptide inhibition experiments, the monomeric peptide antigen or cognate NHE peptides (100 μg/ml) were incubated with 3.4 μg/ml of the affinity-purified rabbit polyclonal anti-rat NHE4 peptide antibody (3.4 μg/ml) for 8 h at 4°C, and the resulting mixture was used in place of the antibody.

**Immunohistochemistry.** Kidneys were removed from adult Sprague-Dawley rats and frozen (without fixation) in 2-methylbutane chilled in a dry-ice acetone bath. Serial frozen sections, 5 μm thick, were air-dried and then incubated with 30 μg/ml affinity-purified rabbit anti-NHE4 oligopeptide IgG, goat anti-Tamm-Horsfall glycoprotein (100 μg/ml), or rabbit anti-aquaporin-2 or -3 (15 μg/ml) for 15 min at room temperature. Sections were washed with PBS and then labeled with rhodamine-conjugated goat anti-rabbit IgG or rabbit anti-goat IgG (Organon Teknika-Cappel, Durham, NC), diluted 1:100 in PBS, as appropriate. Slides were then examined by epifluorescence, and the same areas from adjacent sections were photographed. For peptide inhibition experiments, the anti-NHE4 antibody (at 30 μg/ml) was preincubated with 100 μg/ml of the synthetic NHE4 peptide antigen or cognate NHE1 and NHE2 peptides for 1 h at room temperature. This mixture was then added to the sections, and the same labeling conditions described above were then followed.

**Transport measurements.** Na\(^+/\)H\(^-\) exchange activity was assayed by measurement of \(^{22}\)Na uptake at 20–25°C by a rapid filtration technique. BLMV were preincubated for 2 h with a pH 6.0 medium consisting of (in mM) 100 mannitol, 60 2-N-methyl-D-glucamine (NMG) nitrate, 40 NMG gluconate, 3 EGTA, and 100 MES/Tris. In general, a 10-μl aliquot of BLMV (20–40 μg protein) was added to 200 μl of a pH 8.0 buffer containing 1.5 μCi/ml of \(^{22}\)Na and (in mM) 100 NMG nitrate, 40 NMG gluconate, 3 EGTA, and 100 Tris/HEPES as well as the indicated concentrations of EIPA. Incubation periods of 9 s were used to estimate initial rates. The assay medium was K\(^+\) and ATP free to prevent the transport of \(^{22}\)Na by the Na-K-ATPase. Uptakes were terminated by adding 1.4 ml of an ice-cold stop solution containing 20 mM Tris/HEPES, pH 7.4, and the desired potassium-D-glucurate concentration to maintain isosmolarity (equilibration medium, incubation medium, and stopping medium were always kept isosmotic). This suspension was rapidly filtered on the center of a 0.45-μm prewetted Millipore cellulose filter (HAWP) and washed with an additional 18 ml of the same ice-cold stop solution. For all experiments, nonspecific isotopic binding to the filter was measured with appropriate blanks and subtracted from values of the incubated samples. The filters were dissolved in 3 ml of scintillant (Filter-count, Packard), and radioactivity was determined using a scintillation counter.

Measurements of \(^{22}\)Na influx specific to the Na\(^+\)/H\(^-\) exchanger were determined as the difference between the initial rates of H\(^-\)-activated \(^{22}\)Na influx in the absence and presence of 500 μM EIPA and expressed as the NHE-mediated \(^{22}\)Na uptake. The background levels of \(^{22}\)Na influx that were not attributable to the Na/H exchanger were <10%.

**Statistical methods.** All data are represented as means ± SE. NHE-mediated \(^{22}\)Na uptake values determined at intravesicular pH 5.5–7.5 were fitted to a four-parameter logistic sigmoidal equation using Prism 3.0 (GraphPad Software). NHE-mediated \(^{22}\)Na uptake values determined at extravesicular Na\(^+\) concentrations of 2.5–40 mM were fitted using Prism 3.0 (GraphPad Software).

**RESULTS**

**Immunofluorescence localization of NHE4 along the rat nephron.** The affinity-purified anti-rat NHE4 peptide was used to localize NHE4 in adult rat kidney by indirect immunofluorescence. Strong staining for NHE4 was found in cortical (Fig. 1A) and MTAL (Fig. 2A), which were identified by their characteristic immunostaining for Tamm-Horsfall glycoprotein (Figs. 1C and 2B). As previously described (23, 32, 40), staining for Tamm-Horsfall glycoprotein of TAL cells was diffuse; luminal surface fluoresced brilliantly whereas cell interior (except nuclei) and basolateral membrane were only faintly stained. Staining for NHE4 was also seen in tubules exhibiting less intense staining for Tamm-Horsfall glycoprotein (Fig. 1, A and C) that could correspond to distal convoluted tubules (23, 32).

The absence of colocalization of the NHE4 protein with the luminal Tamm-Horsfall glycoprotein confirmed basolateral rather than apical expression of NHE4 in TAL, as reported in a previous study based on colocalization of NHE4 with the basolateral Na-K-ATPase (12). Because faint staining for NHE4 does appear in Tamm-Horsfall-negative tubules with anatomic features of collecting ducts, antibodies to collecting duct water channels were used to provide complete identification of the NHE4-positive segments. Collecting ducts from cortex to inner medulla, which were identified by immunostaining for aquaporin-2 or -3, showed weak staining for NHE4 (Figs. 1, A and B, and 3, A and B). There was close correspondence between labels for NHE4 and aquaporin-3 on basolateral membranes of these tubules (17). When an antibody to aquaporin-2, which is found in both intracellular and apical membranes of collecting duct cells (30), was used, the basolateral distribution of NHE4 was confirmed. Very weak staining for NHE4 of basolateral membrane of proximal tubules within the cortex and the outer stripe of the outer medulla was also seen (Figs. 1–3).

Specificity of the labeling was demonstrated by the absence of signal when anti-NHE4 antibodies were incubated with the synthetic monomeric peptide antigen (SYNKYNLKPQ) before application to sections (Fig. 4C). When amino acid sequences of rat NHE1, NHE2, NHE3, and NHE4 are compared, it appears that three consecutive amino acids of the rat NHE4 peptide antigen (S-Y-N) are identical to the corresponding residues in rat NHE2 and NHE1. Incubations were therefore carried out in the presence of synthetic NHE1 (SYNRHTLVAD) and NHE2 (SYNRHNLTAD) decapeptides related to the corresponding sequence of the rat NHE4 peptide antigen (Fig. 4, A and B). In contrast to the NHE4 peptide antigen, cognate NHE1 and NHE2 peptides were ineffective in blocking the binding of the anti-NHE4 antibody.

**Plasma membrane localization of NHE4.** Two independent preparations of crude membranes from the renal inner stripe of the outer medulla and basolateral membrane fractions were assessed by immunoblot for the presence of NHE4. As shown in Fig. 5A, the affinity-purified anti-NHE4 peptide antibody recognized a...
A major band at a relative molecular mass ($M_r$) of 95 kDa that was enriched in basolateral membranes (lanes 2 and 4) compared with crude membranes (lanes 1 and 3). In addition to the 95-kDa protein, smaller bands at $M_r$ of 80–75 kDa were also detected with the anti-NHE4 antibody in crude membranes (lanes 1 and 3) but not in basolateral membranes (lanes 2 and 4).

The NHE4 bands (Fig. 5, B and C, lane 1) were not detected when the antibody was preincubated in the presence of excess synthetic monomeric NHE4 peptide antigen before immunoblotting of crude membrane proteins (Fig. 5B, lane 2) and basolateral membranes (Fig. 5C, lane 2). The antibody was also preincubated before immunoblotting of membrane proteins in the presence of an excess of either synthetic NHE2 peptide or synthetic NHE1 peptide related to the corresponding sequence of the rat NHE4 peptide antigen. As illustrated in Fig. 5, B and C, NHE4 bands could still be detected in the presence of excess NHE1 (lane 3) or NHE2 (lane 4)-related peptides, demonstrating the specificity of these bands for NHE4 over NHE1 and NHE2 proteins.

Basolateral membranes were appropriately enriched in Na-K-ATPase compared with crude membranes (Fig. 5D), whereas the apical Na-K-2Cl cotransporter...
BSC1, identified in crude membrane as a 160-kDa protein, was absent from basolateral membrane fractions (Fig. 5E). As shown in Fig. 5F, the apical Na⁺/H⁺ exchanger NHE3 identified as the 85-kDa protein, was poorly detected in basolateral membranes. Thus NHE4 was identified as a 95-kDa protein expressed in basolateral membrane. The smaller NHE4 polypeptides (80–75 kDa) that were not enriched in basolateral membrane fractions could represent intracellular forms of NHE4. This distribution coincided with immunofluorescence studies shown above.

**Transport properties of NHE4 in native membrane preparations.** To determine whether, beyond the ubiquitous Na/H exchanger NHE1, NHE4 was also functionally present, we measured NHE-mediated ²²Na uptake into BLMV of MTAL. Because NHE1 and NHE4 are respectively of high and low sensitivity to inhibition by the amiloride derivative EIPA (13), this study was undertaken in the presence of increasing concentrations of EIPA, from 10 nM to 500 µM. The inhibition curve for EIPA depicted in Fig. 6 shows that there were two components of Na⁺/H⁺ exchange with different EIPA sensitivities. The major component, which accounted for ~80% of NHE-mediated ²²Na uptake, exhibited relative resistance to EIPA with an IC₅₀ of 1.90 ± 0.12 µM. The minor component, accounting for the remaining 20% of NHE-mediated ²²Na uptake, was highly sensitive to EIPA inhibition. The first five data points of the inhibition curve (point 1 being 100%) were used to estimate the IC₅₀ of the minor
component, whereas data points 5–11 were used to determine the IC$_{50}$ of the major component: the IC$_{50}$ values were 11 nM and 2.5 mM, respectively. These results are consistent with the existence of two NHE activities showing different sensitivities to inhibition by EIPA in BLMV of MTAL, where NHE1 and NHE4 could account for the EIPA high-affinity and EIPA low-affinity NHE activities, respectively.

To characterize NHE4, studies were next performed in the presence of 200 nM EIPA, a concentration that should have completely inhibited NHE1 activity and inhibited NHE4 activity only marginally. Recently, the benzoylguanidine derivative HOE-642 has been used to discriminate among NHE1, NHE2, and NHE3 isoforms (39). To assess its effect on NHE4, dose-response experiments for HOE-642 were next performed. Very high HOE-642 concentrations were required to inhibit NHE4 activity; the IC$_{50}$ value was 545 ± 33 mM (Fig. 7).

To establish the kinetic characteristics of NHE4, the initial rates of the H$^+$/Na$^+$ activated $^{22}$Na influx as a function of the extravesicular Na$^+$ concentration were examined in BLMV. As illustrated in Fig. 8, the velocity of NHE4-mediated $^{22}$Na uptake gradually approached saturation with increasing extravesicular Na$^+$ concentration and conformed to simple Michaelis-Menten kinetics. Analysis of the data gave an apparent Na$^+$ affinity constant of 12.1 ± 1.32 mM.

NHE4 activity was also measured as a function of the intravesicular pH over a range of 5.5–7.5. The results are presented in Fig. 9. Values fitted a sigmoidal curve, suggesting an allosteric H$^+$-binding site. The half-maximum intravesicular H$^+$ activation value was pH 6.21 ± 0.15.

**DISCUSSION**

In the present study, we have investigated the regional and cellular localization of the NHE4 isoform in the rat kidney, using immunoblotting and immunofluorescence techniques, as well as its functional properties, using purified BLMV isolated from rat MTAL. Our principal findings are, first, that NHE4 is highly ex-

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*Fig. 5. Immunoblot analysis of NHE4 peptide in 2 independent preparations of outer medullary crude membranes and basolateral membranes vesicles (BLMV) isolated from rat medullary thick ascending limbs. A: affinity-purified anti-rat NHE4 peptide antibody detects predominant 95-kDa and less abundant 80- to 75-kDa polypeptides in crude membranes (lanes 1 and 3). The 95-kDa polypeptide is markedly enriched in BLMV (lanes 2 and 4). B and C: all bands detected by the affinity-purified anti-NHE4 peptide antibody (lane 1) in crude membranes (B) and BLMV (C) are competed by peptide antigen (lane 2) but not by related NHE1 (lane 3) or NHE2 peptides (lane 4). D: Na-K-ATPase α- and β-subunits identified, respectively, as 100- and 50-kDa proteins are appropriately enriched in BLMV (lane 2) compared with crude membranes (lane 1). E: Na-K-2Cl cotransporter BSC1 is detected in crude membranes (lane 1) but not in BLMV (lane 2). F: apical Na/H exchanger NHE3 identified as a 85-kDa protein is barely detectable in BLMV (lane 2). Each lane was loaded with 7 µg of membrane protein except in B, where 15 µg of membrane protein were separated. Left: molecular mass expressed as 10$^{-3}$ × Mr.

*Fig. 6. 5-(N-ethyl-N-isopropyl)amiloride (EIPA) sensitivity of Na$^+$/H$^+$ exchange activity in BLMV isolated from rat medullary thick ascending limbs. Because NHE1 and NHE4 are respectively of high and low sensitivity to EIPA, initial rates of H$^+$-activated $^{22}$Na uptake (50 µM) were measured in BLMV from rat medullary thick ascending limbs over a range of EIPA concentrations (denoted by brackets) from 10 nM to 500 µM. Na$^+$/H$^+$ exchange activity was obtained indirectly by subtracting rates of $^{22}$Na uptake that were not inhibitable by 500 µM EIPA from the total uptake. Data were normalized as a percentage of the maximal rate of NHE-mediated $^{22}$Na uptake in the absence of inhibitor. Values represent the average of 3 experiments in 6 separate BLMV preparations.*
pressed in basolateral membrane of TAL as well as distal convoluted tubules, and to a lesser extent in collecting ducts and proximal tubules; second, NHE4 is constitutively active in basolateral membrane of MTAL in the basal condition; and third, significant differences of affinity for EIPA and HOE-642 as well as intracellular H$^+$ clearly distinguish NHE4 from NHE1, the other basolateral Na$^+$/H$^+$ exchanger.

The results of our immunohistochemical study showed that, like NHE1, NHE4 is localized within the kidney to the basolateral membrane of the same tubules. NHE4 was found in multiple nephron segments, including proximal tubules, MTAL, cortical TAL, distal convoluted tubules, and cortical and medullary collecting ducts. The epithelium of the collecting duct is composed of the segment-specific collecting duct cells (principal cells) and intercalated cells. Anti-NHE4 antibody labeled most cells of the collecting ducts, consistent with the presence of NHE4 in collecting tubule cells. Although breaks in NHE4 labeling are seen in Fig. 2, whether intercalated cells are labeled or not could not be clearly established from the present immunofluorescent photomicrographs. For comparison, rat NHE1 protein, as previously reported, was found in the basolateral membrane of distal convoluted tubule cells, connecting tubule cells, cells of TAL, and principal cells of the collecting duct (7). In microdissection studies of nephron segments isolated from rat cortex, NHE1 transcripts were additionally detected by RT-PCR reactions in S1 and S2 proximal segments of juxtamedullary nephrons (26). Although there is a considerable overlap in the distribution of NHE1 and NHE4, differences in the levels of expression of these two basolateral transporters in nephron segments may exist. However, because labeling experiments were not done simultaneously, direct comparison of the distribution of NHE1 and NHE4 along the nephron axis could not be accomplished.

The expression of NHE4 in the whole distal nephron is apparently at variance with in situ hybridization studies reporting the presence of NHE4 mRNA in collecting ducts only (11). Of note, the cRNA probe hybridized to tubules in inner and outer medulla, and to a subset of tubules dispersed throughout the cortex. Although this pattern of labeling is consistent with NHE4 message in collecting ducts, one cannot rule out other tubules expressing NHE4 mRNA, such as TAL, which extends through the outer medulla (inner stripe...
and outer stripe) and the cortex. It is worth noting, however, that, in the latter study, labeled tubules were identified only by their anatomic features (location in the kidney), whereas in our study well-characterized antibodies were used to unambiguously identify tubular segments that expressed NHE4. First, to identify TAL, an antibody to Tamm-Horsfall glycoprotein was employed. This protein is specifically expressed in TAL cells (23). Although Tamm-Horsfall glycoprotein staining can also be seen to extend to distal convoluted tubules, the less intense staining along the distal convoluted tubule permits simple differentiation of distal convoluted tubule from TAL (23, 32, 40). Second, to identify collecting ducts, antibodies to water channels aquaporin-2 and -3 were used.

Immunoblot analysis of renal membrane preparations with our anti-NHE4 peptide antibody revealed apparent Mr of 95 and 80–75 kDa. Only the 95-kDa protein was enriched in basolateral membranes compared with crude membranes, suggesting that the 95-kDa form is preferentially expressed at the basolateral surface of the renal cells whereas the smaller proteins could represent immature intracellular forms of NHE4. These apparent Mr values correspond to previously reported Mr of NHE4. Indeed, a 100-kDa protein was detected by Bookstein et al. (10) in a NHE4-transfected cell line but not in the parent NHE-deficient cell line or in the cell line transfected with NHE1 cDNA using antibodies against the fusion protein of glutathione-S-transferase and amino acids 395–625 of rat NHE4. In another study, Anderie et al. (3) have reported a 80-kDa protein in rat pancreatic zymogen granule membranes using antibodies against the fusion protein of glutathione-S-transferase and the NH2-terminal 40 amino acids of NHE4. Regarding apparent Mr by SDS-PAGE, it should be pointed out that, in our hands, the 95-kDa protein was no longer detectable when crude membrane proteins were solubilized under heating at 95°C (data not shown). It also appears from our immunoblots of basolateral membrane proteins that the 95-kDa protein aggregated when heated (data not shown). Pizzonia et al. (36) identified NHE4 as a 65- to 70-kDa protein in renal cortical crude membrane and cortical basolateral membrane fractions. Interestingly, besides the major band at 65–70 kDa, Pizzonia et al. also detected a minor, larger band at 75 kDa, which is very close to the Mr reported here. Although the existence of alternatively spliced gene products for NHE4 have not been determined as yet, these two forms could represent NHE4 splice variants, as tissue Northern blot analysis revealed a broad band (34). Pizzonia et al. (36), using their monoclonal antibody, may have detected both of the two putative variants of NHE4 whereas our anti-NHE4 peptide antibody specifically recognized one of them.

Heterologous mammalian expression systems have been widely used to study intrinsic kinetic and pharmacological properties of the individual NHE1, NHE2, and NHE3 isoforms (15, 28, 33, 42, 43, 52). In contrast, heterologous expression of NHE4 in an active form has proved to be difficult. Bookstein et al. (13) reported that NHE4-transfected PS120 cells exhibit no NHE activity at isosmolarity (i.e., 280 mosmol/l) and a very modest level of activity at hyperosmolarity (i.e., 490 mosmol/l) (10). In another study, DIDS was used to activate NHE4 stably expressed in a mutant mouse fibroblast cell line and define its functional properties (13). However, one cannot assume whether these characteristics determined in the presence of DIDS reflect the functional properties of NHE4 activity in vivo. Because our immunofluorescence study revealed a high level of expression of NHE4 in basolateral membranes of TAL, we have used purified BLMV isolated from the rat MTAL to investigate the functional properties of the native NHE4 isoform. As expected, the dose-response profile for inhibition of the basolateral Na+/H+ exchange activity by EIPA is consistent with the presence of two distinct Na+/H+ exchangers. The IC50 value of the EIPA high-affinity component (0.011 μM) is similar to that previously reported for NHE1 (33). Existence of NHE1 in basolateral membranes of TAL is in agreement with previous immunoblot and immunohistochemical data (5, 7). The IC50 value of the EIPA low-affinity component (2.5 μM) clearly indicates that the basolateral membrane also contains an amiloride-resistant NHE type that could correspond to NHE4, which is known for its amiloride resistance. Although this IC50 value is also consistent with that reported for NHE3 (33), it is unlikely that the apical NHE3 isoform could account for the EIPA low-affinity NHE activity observed in BLMV that rather represents NHE4 activity. Indeed, BLMV are largely purified away from luminal membrane vesicles (LMV) (5, 18, 19). We have previously shown that the LMV and not the BLMV preparations exhibit KCl-dependent, bumetamide-sensitive Na+ uptake, demonstrating activity of the apical Na-K-2Cl cotransporter in appropriate (apical) membrane fractions but not in basolateral membrane fractions (37). In addition, dose-response EIPA experiments (Houillier P, unpublished observations) on in vitro microperfused MTAL showed that some basolateral Na+/H+ exchange activity persisted in the presence of 1 μM EIPA, a concentration that completely inhibited NHE1 in the presence of 140 mM extracellular Na+ whereas complete inhibition was obtained with 100 μM EIPA. These results confirm our finding in membrane vesicles of the presence of two Na+/H+ exchangers with different sensitivities to EIPA in the basolateral membrane of MTAL cells in basal conditions.

We found that EIPA inhibited NHE4 with an IC50 value of ~2 μM. This is in good agreement with previous observations using macula densa cells where only NHE4 is expressed at the basolateral membrane, although the IC50 value reported for EIPA was slightly higher (9 μM) (35). Of importance, our data show that NHE1 exhibited a higher affinity for EIPA than NHE4, with a 150-fold difference in sensitivity. Because NHE1 is completely inhibited at 100 nM EIPA whereas NHE4 is sensitive to EIPA starting at 300 nM, EIPA provides a means to specifically inhibit NHE1 in BLMV preparations and thus unambiguously charac-
terize NHE4, which cannot be readily expressed in an active form in any heterologous expression system. This approach allowed us to specifically determine affinity of NHE4 for the new Na⁺/H⁺ exchanger inhibitor HOE-642 as well as extracellular Na⁺ and intracellular H⁺. Comparison of our results with those of a previous study reveals that NHE1 and NHE2 are 11,000- and 180-fold more sensitive to inhibition by the compound HOE-642 than is NHE4, which exhibits a low sensitivity to HOE-642 similar to that of NHE3 (with IC₅₀ values of 0.5, 3, 250, and 1,000 μM, respectively) (39). The rate of activation of NHE1, NHE2, and NHE3 by extracellular Na⁺ followed a rectangular hyperbola, consistent with simple, saturating Michaelis-Menten kinetics. A similar Na⁺ dependence was also obtained for NHE4 in BLMV. In addition, the Na⁺ affinity constant of 12 mM for NHE4 does not differ from the published values for NHE1 (33). This result is at variance with that of Bookstein et al. (10), who, using NHE4-expressing fibroblasts under hyperosmolar conditions, reported a sigmoidal response of NHE4 to increasing concentrations of extracellular Na⁺. However, in this latter study, it should be noted that, at an extracellular Na⁺ concentration of 20 mM, only 30% of the total ²²Na uptake occurs via the 5-(N,N-dimethyl) amiloride-sensitive Na⁺/H⁺ exchanger, rendering difficult an accurate estimation of kinetic and pharmacological properties. Interestingly, our study reveals that NHE4 exhibits low sensitivity to intravesicular H⁺, with an apparent pK value of 6.21. Of note, a higher pK value of 6.6 was reported for the Na⁺/H⁺ exchange activity in a BLMV preparation from MTAL, where both NHE1 and NHE4 were functionally present (9), consistent with the higher intravesicular H⁺ sensitivity of NHE1 (i.e., pK 6.75) determined in NHE1-transfected fibroblasts (33).

The basolateral Na/H exchanger in renal epithelial cells has been shown to be involved in homeostatic functions such as intracellular pH and cell volume regulation. In a preliminary report, Good and Knepper (21) showed that, in isolated microperfused MTAL, the addition of 1 mM amiloride to the basolateral solution (21) showed that, in isolated microperfused MTAL, the expression of NHE1 and NHE4 in the apical membrane of rat renal proximal tubule and thick ascending limb. Kidney Int 48: 1206–1215, 1995.


