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Expression of Na^+/H^+ Exchanger Isoforms in Inner Segment of Inner Medullary Collecting Duct ($IMCD_3$)

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Abstract. Na^+/H^+ exchangers (NHE) play a critical role in many cellular and transport processes in the inner medullary collecting duct (IMCD). Morphologically, the IMCD is divided into the outer ($IMCD_1$), middle ($IMCD_2$), and inner ($IMCD_3$) segments. The inner, $IMCD_3$ segment contains only one cell type, the IMCD cell, which is distinct in ultrastructure and in function from the principal and intercalated cells that are present in other portions of the IMCD. NHEs constitute a gene family containing several isoforms (NHE1, NHE2, NHE3, NHE4 and NHE5) which possess distinct characteristics and serve specialized functions. To understand the molecular basis of NHE-related processes in the IMCD, it is critical to know the molecular identity of the NHEs in this tubule segment. The purpose of the present study was to identify the NHE isoforms present and their polar distribution in $IMCD_3$. Applying the reverse transcription-polymerase chain reaction (RT-PCR) technique to $IMCD_3$ (obtained from distal 50% of inner medulla) of mouse and rat kidneys, we found that NHE1, NHE2 and NHE4, but not NHE3 were expressed in both species. The polar localization of NHE in $IMCD_3$ was examined in tubules isolated from rats and perfused in vitro with HEPES-buffered solutions under isotonic conditions. pHi was measured by BCECF fluorescence. Na^+-dependent, amiloride-inhibitable pHi recovery from cell acidification (consistent with NHE) was detected in the basolateral, but not the apical, membrane of $IMCD_3$. We conclude that NHE1, NHE2 and NHE4, but not NHE3, are present in both the mouse and rat $IMCD_3$. Functionally, NHE is limited to the basolateral membrane. Additional studies are needed to determine the physiological roles and regulation of basolateral NHE isoforms in this tubule segment.

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Key words: NHE — IMCD — $IMCD_3$ — RT-PCR — Hyperosmolality — Na^+/H^+ exchanger

Introduction

The inner medullary collecting duct (IMCD) serves as the final arbiter of urine composition. This nephron segment regulates urinary concentration, acid secretion and the final electrolyte composition of urine and the Na^+/H^+ exchanger (NHE) plays a critical role in these processes. In addition, Sun and Hebert [9] have demonstrated that basolateral NHE in the rat IMCD is involved in hypertonic cell volume regulation, which is critical for the survival and normal functioning of these cells in the hypertonic environment of the inner medulla. Morphologically, the IMCD has been divided arbitrarily into three segments: the outer segment ($IMCD_1$), middle segment ($IMCD_2$) and inner segment ($IMCD_3$). The $IMCD_1$ and outer portion of the $IMCD_2$ are similar in ultrastructure to the portion of outer medullary collecting duct ($OMCD_1$) contained within the inner stripe and are composed primarily of principal and intercalated cells [22]. In contrast, the inner portion of the $IMCD_2$ and the entire $IMCD_3$ contain a single distinct cell type, the IMCD cell, which is morphologically and functionally distinct from principal and intercalated cells [22].

To date, five NHE isoforms (NHE1, NHE2, NHE3, NHE4 and NHE5) have been identified and all, except NHE5, are expressed in the kidney [13, 16, 25]. These isoforms possess distinct kinetic, biochemical and pharmacological characteristics and serve specialized functions [16, 25]. Knowledge of the molecular identity of NHE in the IMCD is necessary to better understand the molecular basis of NHE-related processes in this tubule segment. As mentioned previously, principal and intercalated cells present in the initial portion of the IMCD are similar in ultrastructure (and most likely in function)

to cells in other portions of the collecting duct. To date, NHE activity has been observed in the basolateral, but not apical, membrane of principal and α- and β-intercalated cells [15, 23]. A recent immunohistochemical study by Biemesderfer et al. [2] showed that NHE1 staining was present in the basolateral membrane of a subpopulation of cells that were neither α- nor β-intercalated cells in the rabbit cortical collecting duct. Therefore, basolateral NHE in the principal cell is most likely encoded by NHE1 while the molecular identity of NHE in the α- and β-intercalated cells is not clear. Interestingly, recent studies showed that NHE4 is present in the collecting duct. Whether the basolateral NHE in the either α- and β-intercalated cells is encoded by NHE4 or other NHE isoforms remains to be determined. The molecular identity of NHE in IMCD cells is also not clear. Soleimani et al. [18] detected mRNA for NHE1 and NHE2, but not for NHE3 or NHE4, in a cell line developed from the $IMCD_3$ segment from a simian virus transgenic mouse which contains only IMCD cells. These findings have yet to be confirmed in studies of native tissue. Bookstein et al. [4] provided conflicting data reporting that NHE4 and mRNA was present in the rat IMCD, including the $IMCD_3$ segment. These discrepant data might result from species differences or from loss of NHE_4 expression by transformed, cultured cells in vitro. To overcome these limitations, we used the reverse transcription-polymerase chain reaction (RT-PCR) technique to examine NHE isoform mRNAs expressed in $IMCD_3$ segments in the rat and mouse. Based on previous studies indicating that the terminal 50% of IMCD contains only IMCD cells and represents $IMCD_3$ [22], segments were dissected from the inner 50% of the inner medulla [17]. We also examined the functional polarity of NHE in $IMCD_3$ using the in vitro tubule microperfusion technique. We found that both rat and mouse $IMCD_3$ contain NHE1, NHE2, and NHE4, but not NHE3 mRNA. Functionally, in the rat, NHE activity was limited to the basolateral membrane of the $IMCD_3$.

MATERIALS AND METHODS

Tubule Microdissection for RT-PCR

Experiments were performed in male Spraque-Dawley rats (70–160 g) and male CD mice (25- to 35-day-old) that had free access to water and standard laboratory chow. The distal 50% of IMCD tubules ($IMCD_3$) were dissected for RT-PCR using a modified version of the protocol of Sands et al. [17]. Briefly, animals were anesthetized with Nembutal (50 mg/kg i.p.) and the left kidney was perfused *in situ* with 10-ml ice-cold bicarbonate-free Dulbecco's modified Eagle's medium (DMEM: GIBCO) containing 1-mg/ml type I collagenase (digestion solution). Coronal slices containing the entire corticomedullary axis were made and then inner medullary slices cut from the 50% of the papilla closest to tip. These slices were incubated in digestion solution at 37°C, bubbled with 100% O_2, for 45 min, and then washed three times in collagenase- and bicarbonate-free DMEM solution containing 0.1%-bovine serum albumin (Sigma) (dissection solution) and placed on ice. IMCD were microdissected and then transferred to a wash dish containing fresh dissection solution, captured on polylysine-coated glass microbeads (0.5-mm diameter, Thomas Scientific), and transferred to a 0.5-ml Eppendorf tube. Bead-tubules were rinsed three times with 10μl of dissection solution containing 2-U/μl RNase inhibitor (Boehringer-Mannheim) and solubilized with 10 μl of 2% Triton X-100 containing 2-U/μl RNase inhibitor.

Reverse Transcription (RT) and Polymerase Chain Reaction (PCR)

Detection of NHE isoforms by RT-PCR in microdissected renal tubules has been reported by us and other investigators [5, 21]. Samples were reversed transcribed *in situ* by adding to each tube an RT mix to make up a total volume of 20 μl. Each tube contained: 0.5 μg oligo (dT) primer, 200 U of Superscript MuMLV RT (GIBCO BRL), 0.5-mM dNTP mix, 10-mM dithiothreitol, 100-mM Tris-HCl (pH 8.4), 50-mM KCl and 2.5-mM $MgCl_2$. Tubes were incubated for 1 hr at 42°C and then the reaction was terminated by heating to 95°C for 5 min. After RT, each reaction tube was centrifuged briefly to pellet the beads and then the solution was transferred to a 0.2-ml thin-wall PCR tube.

For PCR, a PCR mix was added to the PCR tube to make up a total volume of 50 μl. Each PCR reaction tube contained: 10-mM Tris-HCl (pH 8.4), 50-mM KCl, 2.5 mM-$MgCl_2$, 0.5-mM dNTP, 2.5 U Taq DNA polymerase (Promega)/7-μM anti-Taq polymerase antibody (Clontech) mixture, 100 pmole of each NHE2 primer. Anti-Taq polymerase was used as an alternative to ''hot start'' PCR to optimize the PCR reaction. The tubes were placed in a DNA Thermal Cycler (Perkin Elmer GeneAmp PCR system 2400), which was programmed to execute the following protocol: 94°C 4 min (initial melt); 35 cycles of 94°C 1 min, 60°C 1 min, 72°C 1.5 min; and then 72°C 7 min (final extension). For the studies shown in Figs. 1–4, NHE1, NHE2, NHE3 and NHE4 primers for the PCR reactions were designed from the published rat sequences specific for each NHE isoform as described previously by Borensztein et al. [5]. These primers are (shown 5′ to 3′): NHE1 sense, TCTGCCGTCTCAACTGTCTCTA, NHE1 antisense, CCCTTCAACTCCTCATTCACCA; NHE2 sense, GCAGATGGTAATAGCAGCGA, NHE2 antisense, CCTTGGTGGGGGCTTGGGTG; NHE3 sense, GGAACAGAGGCGGAGGAGCAT, NHE3 antisense, GAAGTTGTGTGCCAGATTCTC; NHE4 sense, GGCTGGGATTGA-AGATGTATGT, NHE4 antisense, GCTGGCTGAGGATTCCTGTAA.

To confirm the identity of the PCR products, a nested PCR protocol was used in which the 35-cycle PCR products were diluted 50-fold, and then 2 μl of the diluted samples were reamplified in a second 50-μl reaction with a second set of primers internal to the first one. The NHE isoform primers for the nested PCR are shown in the Table. Nested PCR was performed under conditions of high stringency as follows 94°C 4 min (initial melt); 28 cycles of 94°C 1 min, 65°C 1 min, 72°C 1.5 min; and then 72°C 7 min (final extension).

For PCR product analysis, the PCR samples were fractionated on 2% agarose gels and stained with ethidium bromide.

In vitro Tubule Perfusion

The basic techniques for dissecting and perfusing IMCD tubules have been previously described [19]. Briefly, 75- to 125-g pathogen-free Sprague-Dawley rats were given furosemide (5-mg/100g body weight i.p.) for 30 min before the kidneys were harvested. The inner medulla was freed from attached cortex and outer medulla and then $IMCD_3$

Table. NHE isoforms primers designed from rat sequences for nested PCR

		Sequence	Position
NHE1	sense	5′-ATAACAGACACACGCTGGTGGC-3′	2719–2740
	antisense	5′-CGATGGTGATGACAGGCAGGTC-3′	2910–2931
NHE2	sense	5′-CGGACATGGATGGAACCACT-3′	2276–2295
	antisense	5′-CCGTTGGTCTTTGGAGAGCA-3′	2486–2505
NHE3	sense	5′-ACACGTTGCAGCAGTACCTC-3′	1935–1954
	antisense	5′-GCCGACTTAAAGGACTCCAG-3′	2063–2082
NHE4	sense	5′-AGCCAAAGTCAAGCATTGTTTC-3′	2079–2100
	antisense	5′-TGGGGTTTGAGGTTGTATTTGT-3′	2298–2319

tubule segments were obtained from the distal 50% region of the inner medulla. The $IMCD_3$ tubule segments were then perfused at rates of 15–20 nl/min. The peritubular bath flowed at a rate of 15–20 ml/min, which is sufficient to exchange the bath in ~2 sec, and was maintained at 37°C. Control solutions contained (in mM): 140 NaCl, 5.0 KCl, 10 $CaCl_2$, 1.2 $MgCl_2$, and 3.0 N-2-hydroxyethyl-piperzaone-N/-2-ethanesulfonic acid (HEPES). Solutions were adjusted to an osmolality of 290 mosom/kg H_2O and a pH of 7.4 after equilibrating with 100% O_2. Na^+-free solutions were made by replacing Na^+ with N-methyl-D-glucamine (NMDG).

Intracellular pH (pHi) Measurement

The techniques for quantitative fluorescence measurement of pHi using the pH-sensitive dye BCECF (Molecular Probes, Eugene, OR) in in vitro perfused tubules have been described previously [20]. In brief, $IMCD_3$ tubules were loaded with BCECF by transient exposure (20 min) to the acetoxymethyl ester of BCECF (BCECF/AM) at 37°C. Fluorescence was alternatively measured from the output of a photomultiplier tube at excitation wavelengths of 495 nm and 440 nm (emission wavelength 530 nm) using a dual gating fluorometer (Deltascan, Photon Technology International, South Brunswick, NJ) connected to an inverted microscope (Diaphot 300, Nikon, Japan). Background fluorescence (1% of total) was subtracted from fluorescence intensity at each excitation wavelength to obtain intensities of intracellular fluorescence. The 495 nm/440 nm ratio was used as an indicator of pH and was calibrated using high K^+-nigericin standards.

Results

Detection of NHE mRNAs in the Rat $IMCD_3$

Shown in Fig. 1*A*, amplification products of the predicted size for NHE isoforms 1–4 were detected in rat kidneys. The specificity of the PCR product for each NHE isoform was assessed by nested PCR. As shown in Fig. 1*B*, the reamplified PCR products have expected sizes: 213 bp for NHE1, 230 bp for NHE2, 148 bp for NHE3 and 241 bp for NHE4. These results confirm that NHE1, NHE2, NHE3 and NHE4 mRNAs are present in the rat kidney, as previously shown by Borensztein et al. [5]. The data in whole kidney serves as a positive control demonstrating our ability to detect mRNA for all four NHE isoforms by these techniques.

As shown in Fig. 2*A*, amplification products of the predicted sizes for NHE1, NHE2 and NHE4 were detected in IMCD tubules dissected from distal 50% of rat IMCD ($IMCD_3$): NHE1 (422 bp), NHE2 (310 bp) and NHE4 (501 bp). No PCR products were obtained when reverse transcriptase was omitted from the RT reaction (negative control). The identity of the PCR products as specific for NHE1, NHE2 and NHE4 was assessed by nested PCR. As shown in Fig. 2*B*, the reamplified PCR products gave expected sizes for each of these three NHE isoforms. NHE3 mRNA was not detected by PCR (Fig. 2*A*) or nested PCR (Fig. 2*B*). These results indicate that NHE1, NHE2 and NHE4, but not NHE3 are expressed in the rat $IMCD_3$.

Detection of NHE mRNAs in the Mouse $IMCD_3$

To assess the pattern of NHE expression in native mouse $IMCD_3$, RT-PCR was performed on RNA extracted from mouse kidney (as a positive control) and tubules obtained from the distal 50% of the mouse IMCD. As shown in Fig. 3*A*, amplification products for NHE1, NHE2, NHE3 and NHE4 were obtained from the mouse kidney that were similar in size to those found in the rat (Fig. 1*A*). Shown in Fig. 3*B*, each mouse NHE isoform product reamplified by nested PCR was also similar in size to those of the rat (Fig. 1*B*). To further characterize and compare the PCR products from the mouse and the rat, the product for each NHE isoform from both rat and mouse were digested by restriction enzymes: ApaI for NHE1, MboI for NHE2, BstEII for NHE3 and Sau3A for NHE4. Not shown, the digested fragments for each NHE isoform were similar in size in the mouse and rat. These results are consistent with previous reports indicating that the sequences of the various NHE isoforms are highly conserved across species [16, 25]. Furthermore, these data strongly suggest that the mouse kidney, like the rat kidney, expresses NHE1, NHE2, NHE3 and NHE4.

We also studies NHE mRNA levels in the mouse $IMCD_3$. Shown in Fig. 4*A*, amplification products for NHE1, NHE2, and NHE4 were observed in $IMCD_3$ seg-

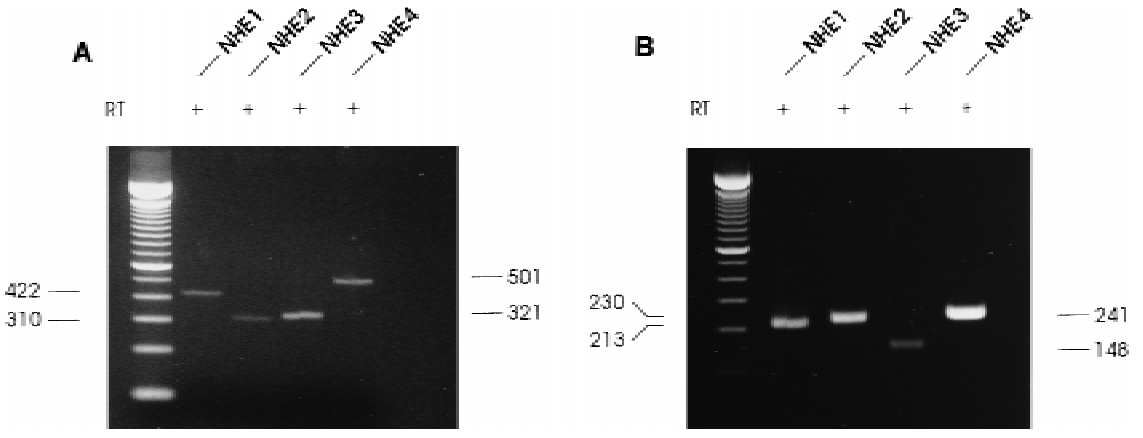

Fig. 1. RT-PCR demonstrating NHE isoform mRNA expression in rat kidney. PCR products were electrophoresed on 2% agarose gel and stained with ethidium bromide. (*A*) NHE1, NHE2, NHE3 and NHE4 RT-PCR products. Each reaction was performed with 1-μg total RNA extracted from whole rat kidney homogenate. (*B*) Nested PCR products of NHE1, NHE2, NHE3 and NHE4. Each reaction was performed in a final 1:1250 dilution of first round PCR products of NHE isoforms from *A*. Expected sizes of PCR products were marked. Molecular weight standards were 100-bp ladder from GIBCO-BRL. *See* text for abbreviations.

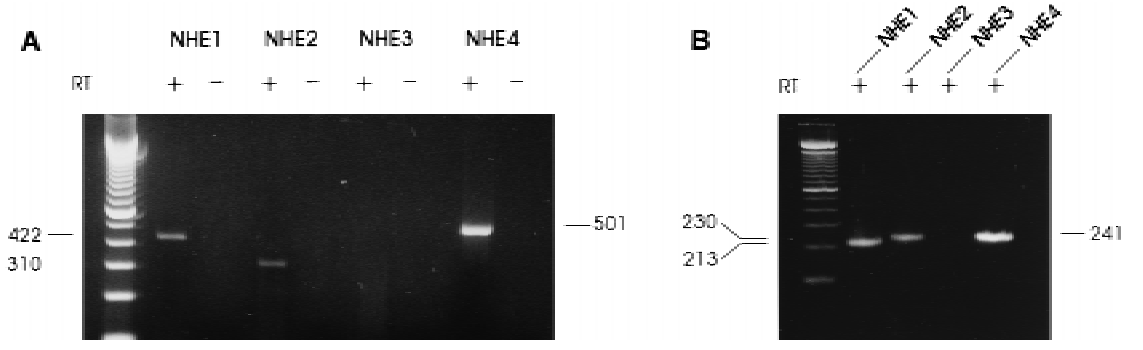

Fig. 2. RT-PCR demonstrating NHE isoform mRNA expression in rat $IMCD_3$. PCR products were electrophoresed on 2% agarose gel and stained with ethidium bromide. (*A*) NHE1, NHE2, NHE3 and NHE4 PCR products amplified from microdissected rat $IMCD_t$ tubules. Each reaction was performed in the presence (+) and absence (−) of RT using total RNAs isolated from 1 to 1.5-mm of tubules. (*B*) Nested PCR products of NHE1, NHE2, NHE3 and NHE4. Each reaction was performed in a final 1:1250 dilution of first round PCR product (+ RT) of NHE isoforms from *A*. Expected sizes of PCR products were marked. Molecular weight standards were 100 bp ladder from GIBCO-BRL. *See* text for abbreviations.

ments dissected from the mouse and were similar in size to those found in the rat (Fig. 1*A*) and mouse (Fig. 3*A*) kidney and the rat $IMCD_3$(Fig. 2*A*. In addition, the re-amplified PCR products of all three NHE isoforms in the mouse were similar in size to those obtained from rat (Fig. 1*B* and mouse (Fig. 3*B*) kidney and rat $IMCD_3$ (Fig. 2*B*). As in the rat, NHE3 mRNA was not detected by PCR (Fig. 4*A*) or nested PCR (Fig. 4*B*) in the mouse $IMCD_3$. Taken together, these results indicate that NHE1, NHE2 and NHE4, but not NHE3, are expressed in the mouse $IMCD_3$.

Functional Localization of NHE in the In Vitro Perfused Rat $IMCD_3$

Figure 5 presents data from a representative experiment assessing the effect of removal of external Na^+ on pHi recovery from NH_4Cl-induced cell acidification in a single perfused $IMCD_3$ tubule dissected from the distal 50% of the rat IMCD. In HEPES-buffered solution (pH 7.4), the steady-state intracellular pH (pHi) was 7.36 ± 0.17 (n = 4). Rapid addition of 20-mM NH_4Cl to both the apical (Ap) and peritubular (Bl) bathing solutions induced cell alkalization followed by gradual cell acidification (*data not shown*). Fifteen to 20 min after incibation in NH_4Cl-containing solutions, NH_4Cl and Na^+ were rapidly removed from both the Ap and Bl solutions (point a), which led to rapid cell acidification (segment ab) to a pH value of 6.33 ± 0.21 (NH_4Cl prepulse). There was no significant pHi recovery in the absence of external Na^+ and CO_2/HCO_3^- (segment bc). When 140-mM Na^+ was added to both the Ap and Bl solutions, pHi promptly recovered to baseline (segment cd). Figure 6 shows one of four experiments designed to assess the

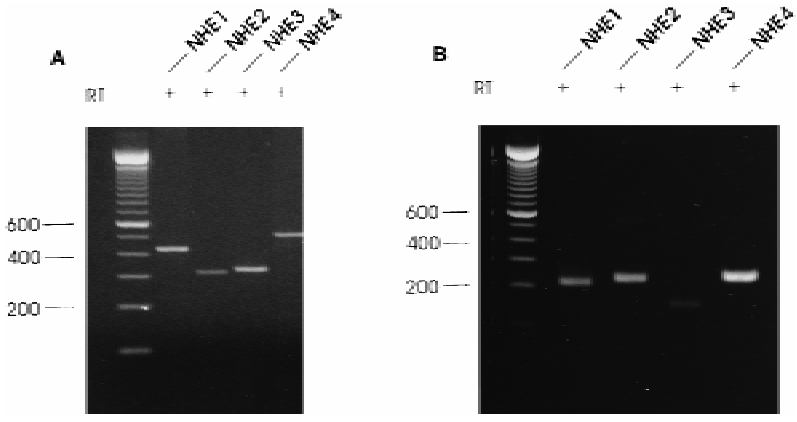

Fig. 3. RT-PCR demonstrating NHE isoform mRNA expression in mouse whole kidney. PCR products were electrophoresed on 2% agarose gel and stained with ethidium bromide. (*A*) NHE1, NHE2, NHE3 and NHE4 RT-PCR products. Each reaction was performed from 1-μg total RNA extracted from whole mouse kidney homogenate. (*B*) Nested PCR products of NHE1, NHE2, NHE3 and NHE4. Each reaction was performed in a final 1:1250 dilution of first round PCR product of NHE isoforms from *A.* Molecular weight standards were 100-bp ladder from GIBCO-BRL. *See* text for abbreviations.

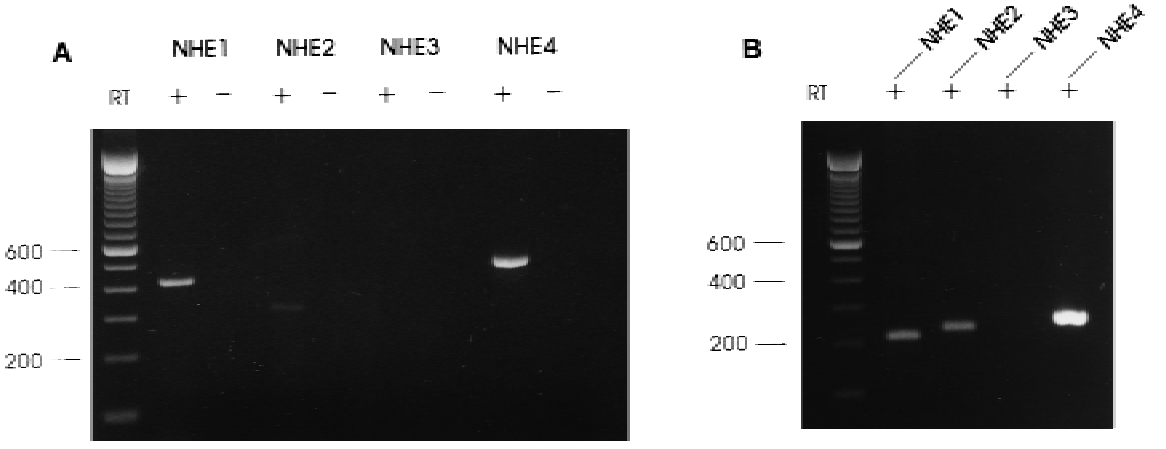

Fig. 4. RT-PCR demonstrating NHE isoform mRNA expression in mouse $IMCD_3$. PCR products were electrophoresed on 2% agarose gel and stained with ethidium bromide. (*A*) NHE1, NHE2, NHE3 and NHE4 PCR products amplified from microdissected mouse $IMCD_3$ tubules. Each reaction was performed in the presence (+) and absence (−) of RT using total RNAs isolated from 1 to 1.5 mm of tubules. (*B*) Nested PCR products of NHE1, NHE2, NHE3 and NHE4. Each reaction was performed in a final 1:1250 dilution of first round PCR product (+ RT) of NHE isoforms from *A.* Molecular weight standards were 100-bp ladder from GIBCO-BRL. *See* text for abbreviations.

effect of amiloride (Na^+/H^+ exchanger inhibitor) on pHi recovery from NH_4Cl prepulse-induced cell acidification in the absence of CO_2/HCO_3^- in rat $IMCD_3$. Na^+ of 140 mM was present in both the Ap and Bl solutions throughout the experiments. After a 15–20-min incubation in NH_4Cl containing solutions, rapid removal of external NH_4Cl-induced cell acidification (NH_4Cl prepulse) (segment ab) which was followed by spontaneous pHi recovery (segment bc). This pHi recovery was not affected by addition of 0.5-mM amiloride to the Ap solution at point c (segment cd). In contrast, addition of 0.5-mM amiloride to the Bl solution at point d was associated with significant inhibition (85 ± 3%, $n = 4$) of pHi recovery (segment de). Thus, we show that in the absence of external CO_2/HCO_3^-, there is a Na^+-dependent H^+-extruding mechanism in the rat $IMCD_3$ (Fig. 5) which is inhibited by basolateral, but not apical, amiloride (Fig. 6). These results are consistent with presence of basolateral, but not apical, NHE in the rat $IMCD_3$.

Discussion

This study provides the first comprehensive examination of the pattern of NHE isoform expression in native $IMCD_3$ segments. In the rat, rabbit, human, and, most likely the mouse, this tubule segment contains only one cell type, the IMCD cell. IMCD cells are distinct in ultrastructure and function from the principal and intercalated cells that are found in more proximal portions of the IMCD [22]. We demonstrate that NHE1, NHE2 and NHE4, but not NHE3, mRNAs are present in the rat (Fig. 2) and the mouse (Fig. 4) $IMCD_3$. The absence of NHE3 mRNA in the present study is consistent with previous reports that failed to detect NHE3 antigen in either rat [1] or rabbit [3] IMCD by immunohistochemistry. The presence of NHE1 and NHE2 mRNAs in native cells is also consistent with previously described patterns of NHE expression in a mouse $IMCD_3$ cell line [18]. However, although our data in native rat $IMCD_3$ confirm previous

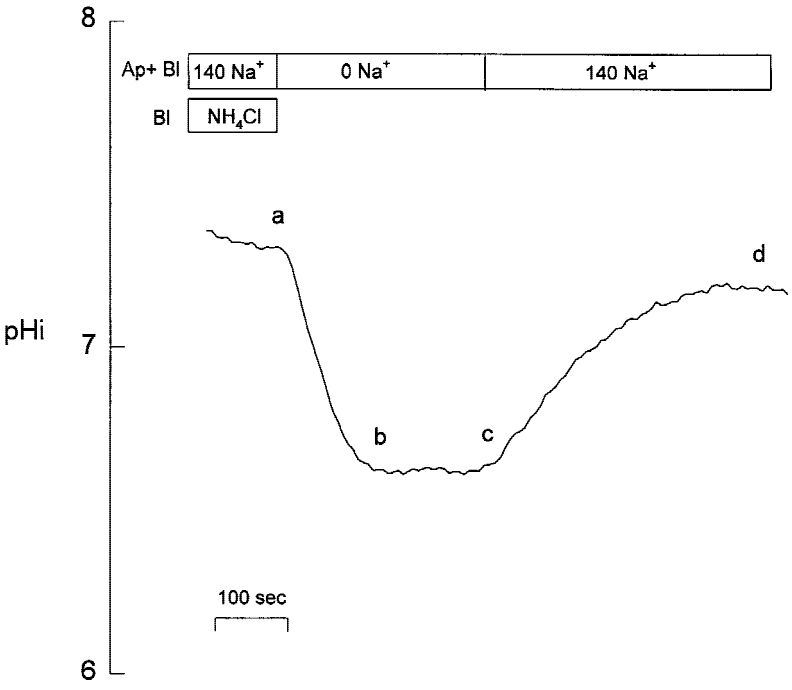

Fig. 5. Representative experiment in an in vitro perfused rat $IMCD_3$ tubule showing the effect of removal of external Na^+ on pHi recovery following NH_4Cl-induced cell acidification. After 15-min incubation in NH_4Cl-containing solution, removal of NH_4Cl (point a) resulted in rapid cell acidification (NH_4^+ prepulse), which recovered in the presence (segment cd), but not absence (segment bc) of external Na^+. Similar results were obtained in 4 tubules.

findings using the *in situ* hybridization technique indicating that NHE4 is expressed in the rat $IMCD_3$, our data in the mouse $IMCD_3$ conflict with that of Soleimani et al. [18] who reported that NHE4 mRNA was not present in a SV40-transformed mouse $IMCD_3$ cell line. One possible explanation for these divergent results is that NHE4 expression was lost in the cultured cells secondary to SV40 transformation and/or other cell culture procedures. Alternatively, it may be that stable expression of NHE4 only occurs in a hypertonic environment. Support for this hypothesis was provided by Bookstein et al. [4] who found that NHE4 mRNA expression in the rat kidney was limited to the collecting duct and the IMCD, which are exposed to a hyperosmotic environment in vivo. Furthermore, unlike other NHE isoforms, when NHE4 was transfected into PS120 cells (an NHE-deficient cell line), activity was found under hypertonic, but not isotonic, conditions [4]. These findings suggest that long-term culture of $IMCD_3$ cells under isotonic conditions may lead to loss of NHE4 expression.

In the present study, we demonstrate in the rat that functional expression of NHE in $IMCD_3$ is limited to the basolateral membrane (Figs. 5 and 6). Our data are consistent with studies performed in cultured rat [8] and rabbit [11] IMCD cells grown on permeabilized membranes and with data from in vitro perfused hamster IMCD tubules [14] that also display NHE activity only in the basolateral membrane. We did not examine the polarity of NHE function in the mouse $IMCD_3$. However, as discussed above, NHE function in IMCD has been limited to the basolateral membrane under all conditions studied to date. Given that mouse and rat $IMCD_3$

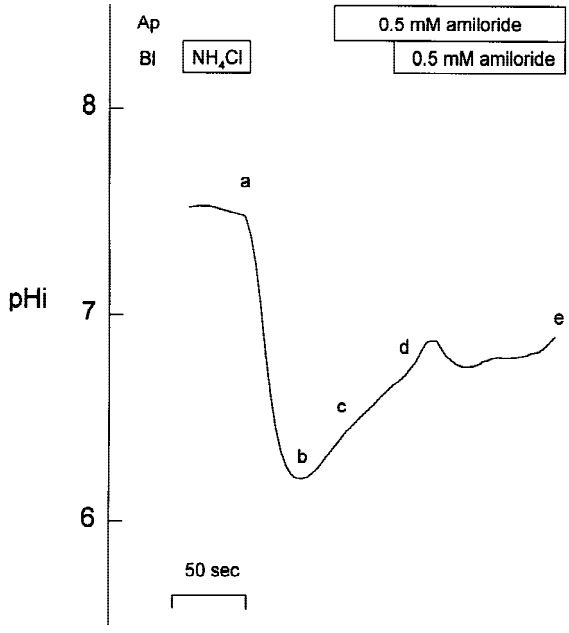

Fig. 6. Representative experiment in an in vitro perfused rat IMCDt tubule showing the effect of apical (Ap) *vs.* peritubular (Bl) amiloride on pHi recovery following NH_4Cl-induced cell acidification. Sodium (140 mM) was present throughout the experiment. NH_4^+-prepulse induced cell acidification followed by spontaneous pHi recovery (segment bc). Addition of 0.5-mM amiloride to the peritubular (Bl) (point d), but not the apical (Ap) (point c) bathing solution, inhibited pHi recovery from cell acidification. Similar results were obtained in 4 tubules.

express the same NHE isoforms, it is likely that the functional expression of NHE in the mouse native $IMCD_3$ tubule is also limited to the basolateral membrane.

It should be pointed out that while the molecular identity and functional polarity of NHE in $IMCD_3$ were demonstrated by the present study, the physiological roles and relative levels of expression of the three NHE isoforms (NHE1, NHE2 and NHE4) present in this tubule segment remain to be determined. However, it is interesting to consider the possible physiological implications of recent investigations examining the effects of hypertonicity on the levels of expression of these NHE isoforms, since the $IMCD_3$, unlike most other tissues, exists in a hypertonic environment.

In most other tubule segments, the majority of the basolateral NHE activity is encoded by NHE1, however, recent studies suggest that this may not be true for $IMCD_3$. In many cell types including renal medullary cells (*see below*), acute hyperosmolality stimulates NHE1 activity which then contributes to hypertonic cell volume regulation. However, Soleimani et al. [18] reported that chronic hyperosmolality suppressed NHE1 mRNA expression in cultured mouse $IMCD_3$ cells. Therefore, because $IMCD_3$ cells normally exist in a hyperosmolar environment, it is likely that basal NHE1 expression in vivo in this tubule segment may be very low. Consistent with this view, *in situ* hybridization studies in rat kidney reveal that NHE1 expression is extremely low in IMCD as compared to other tubule segments (Bookstein, *personal communication*). In fact, as discussed below, this biphasic response of NHE1 expression to hyperosmolality (acute = stimulation, chronic = suppression) may be a critical adaptive response of the $IMCD_3$ to changes in osmolality in vivo.

It is well known that medullary osmolality increases during the transition from water diuresis to antidiuresis. High interstitial osmolality induces cell shrinkage, which activates a salt uptake mechanism followed by osmotic water flow tending to restore cell volume. This phenomenon has been termed the acute hypertonic regulatory volume increase (RVI) response. Evidence suggests that, in medullary thick ascending limb (MTAL) [10] and IMCD [19] cells, NHE in the basolateral membrane mediates sodium uptake during the acute hypertonic RVI response. Furthermore, although other cell types manifest an acute RVI response, the response in the renal medullary cells is unique in that it is strictly vasopressin (AVP)-dependent [9, 19]. In the renal medullary cells, it is likely that NHE1 accounts for basolateral NHE during the RVI response. This is supported by recent studies showing that only NHE1 is present in the basolateral cell membrane of MTAL. In addition, Sun and Hebert [19] demonstrated that the acute hypertonic RVI response in the IMCD resembles that in the MTAL, with regard to basolateral NHE and vasopressin-dependence. This suggests that NHE1 also accounts for the acute vasopressin-dependent RVI response in the IMCD.

Acutely, the cellular defense against hypertonicity depends on salt accumulation, however if sustained, increases in intracellular salt are toxic to cells [24]. Instead, chronic hyperosmolality induces compatible organic osmolyte accumulation in renal medullary cells [7]. Therefore, with prolonged hyperosmolality, it is hypothesized that NHE1-mediated salt uptake is inhibited and accumulated salt is replaced by nonperturbing organic osmolytes to maintain cell volume. The data demonstrating inhibition of NHE1 by chronic hyperosmolality in IMCD cells [18] is consistent with this hypothesis.

Based on the discussion above, it seems likely that tonic basolateral NHE activity in $IMCD_3$ is encoded predominantly by NHE2 and/or NHE4. However, the relative levels of expression and physiological roles of NHE2 and NHE4 in the $IMCD_3$ are also unclear. As is the case for NHE1, acute hyperosmolality stimulates functional expression of NHE4, however the effects of chronic hyperosmolality have not been assessed. Soleimani et al. [18] reported that chronic hyperosmolality increases NHE2 mRNA expression in cultured $IMCD_3$ cells concomitant with an increase in NHE activity. In addition, NHE2 transfected into a NHE-deficient cell line is stimulated by acute hyperosmolality [12]. Taken together, these data suggest that NHE activity in $IMCD_3$ is attributable to NHE2. However, we (*unpublished observations*) and others [6] have failed to detect NHE2 in $IMCD_3$ by immunohistochemistry, suggesting that protein levels of this isoform may be very low. Finally, it is interesting to note that while NHE is present only in the basolateral membrane of $IMCD_3$, we and other investigators have shown that NHE2 is localized to the apical membrane of several other renal tubule segments, including the thick ascending limb and the distal convoluted tubule([6], and our *unpublished observation*). Thus, if NHE2 is present in $IMCD_3$, its polarity in this segment would be opposite to other renal tubules. Although these studies do not rule out a significant contribution of NHE2 to total NHE activity in $IMCD_3$, the question needs to be further examined.

In summary, NHE1, NHE2 and NHE4, but not NHE3 are expressed in both the mouse and the rat $IMCD_3$. Functional studies show that NHE is limited to the basolateral membrane in this segment. These three isoforms have distinct kinetic, biochemical and pharmacological characteristics as well as different responses to external hyperosmolality. Basolateral NHE participates in the acute hypertonic RVI response and it is likely that different NHE isoforms account for NHE activity in the $IMCD_3$ depending on the level and duration of medullary tonicity. Further studies are required to precisely determine the physiological roles and regulation of basolateral NHE isoforms in this tubule segment.

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