Macula densa Na\(^+\)/H\(^+\) exchange activities mediated by apical NHE2 and basolateral NHE4 isoforms

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Macula densa Na\(^+\)/H\(^+\) exchange activities mediated by apical NHE2 and basolateral NHE4 isoforms. Am. J. Physiol. Renal Physiol. 278: F452–F463, 2000.—Functional and immunohistochemical studies were performed to localize and identify Na\(^+\)/H\(^+\) exchange (NHE) isoforms in macula densa cells. By using the isolated perfused ascending limb with attached glomerulus preparation dissected from rabbit kidney, intracellular pH (pHi) was measured with fluorescence microscopy by using 2',7'-bis(2-carboxyethyl)-5-(and -6)-carboxyfluorescein. NHE activity was assayed by measuring the initial rate of Na\(^+\)-dependent pH recovery from an acid load imposed by prior lumen and bath Na\(^+\) removal. Removal of Na\(^+\) from the bath resulted in a significant, DIDS-insensitive, ethylisopropyl amiloride (EIPA)-inhibitable decrease in pHi. This basolateral transporter showed very low affinity for EIPA and Hoechst 694 (IC\(_50\) = 9.0 and 247 µM, respectively, consistent with NHE4). The recently reported apical NHE was more sensitive to inhibition by these drugs (IC\(_50\) = 0.86 and 7.6 µM, respectively, consistent with NHE2). Increasing osmolality, a known activator of NHE4, greatly stimulated basolateral NHE. Immunohistochemical studies using antibodies against NHE1–4 peptides demonstrated expression of NHE2 along the apical and NHE4 along the basolateral membrane, whereas NHE1 and NHE3 were not detected. These results suggest that macula densa cells functionally and immunologically express NHE2 at the apical membrane and NHE4 at the basolateral membrane. These two isoforms likely participate in Na\(^+\) transport, pHi, and cell volume regulation and may be involved in tubuloglomerular feedback signaling by these cells.

fluorescence microscopy; intracellular pH; amiloride analogs; Hoe-694; tubuloglomerular feedback

THE MACULA DENSA PLAQUE IS a unique group of 10–15 cells located within the cortical thick ascending limb (cTAL) and in close association with its own glomerulus. These cells play a pivotal role in signal transmission from tubular lumen to preglomerular vascular elements, thus participating in the regulation of glomerular blood flow and filtration rate (tubuloglomerular feedback) (3, 36). Generation of feedback signals is thought to involve, at least in part, changes in luminal fluid sodium chloride concentration ([NaCl]), which are detected by macula densa cells most likely through specific transport processes. Besides the well-known apical furosemide/bumetanide-sensitive Na\(^+\)-2Cl\(^-\)/K\(^+\) cotransporter (4, 5, 28), we have recently shown that macula densa possess an apical Na\(^+\)/H\(^+\) exchanger (NHE) (18) that may participate in Na\(^+\) transport and intracellular pH (pHi) regulation in these cells. In addition, this exchanger was upregulated by angiotensin II (34). Although these studies reported that the apical Na\(^+\)/H\(^+\) exchanger was relatively insensitive to the inhibitor amiloride, they did not define the isoform specificity of this exchanger. Also, at the present time, a detailed characterization and identity of a putative basolateral Na\(^+\)/H\(^+\) exchanger have not been performed. However, we did report that exchanger activity at the basal membrane is also enhanced by angiotensin II (6). Because different NHEs appear to have specialized roles in acid-base, Na\(^+\) transport, and cell volume and pH regulation, expression of specific NHE isoforms by macula densa cells might be important in terms of the osmolality- or [NaCl]-sensing and signal generation.

In the kidney, Na\(^+\)/H\(^+\) exchange activity is located at the apical and/or basolateral membranes in most nephron segments, including proximal tubule (19, 25, 26), descending and ascending limbs of the loop of Henle (23, 27), distal tubule (7), cortical collecting tubule (13), and outer and inner medullary collecting duct (20, 21).

To date, functional, immunologic, and molecular cloning experiments have identified six NHE isoforms, designated NHE1 to NHE6 (31, 33). NHE1, the so-
called “housekeeping” isoform, is restricted to the basolateral membrane of multiple nephron segments (9, 31, 33) and is involved in cell volume and pH\textsubscript{i} regulation. It is highly sensitive to amiloride analogs [ethylisopropyl amiloride (EIPA)] and the selective NHE1 inhibitor 3-methylsulfonyl-4-piperidinobenzoyl guanidine amiloride (EIPA) and the selective NHE1 inhibitor 3-methylsulfonyl-4-piperidinobenzoyl guanidine methanesulfonate (Hoe-694) (17, 31, 33). In contrast, NHE2 and NHE3 appear to have specific localization and physiological roles. The NHE2 isoform, which is relatively amiloride and Hoe-694 sensitive (17, 42), has been localized to apical membranes of distal nephron segments (16, 30, 33), NHE3 is an amiloride-resistant apical isoform in proximal tubules and medullary thick ascending limbs (9, 12, 31–33) and serves as a major pathway for Na\textsuperscript{+}, and HCO\textsubscript{3}– absorption (30). NHE4, a basolateral isoform, is highly expressed in the distal nephron (14, 33) and appears to be a functionally distinct member of the NHE family. This NHE isoform is thought to be primarily involved in the regulation of cell volume (11). In transfected cells, NHE4 is normally quiescent (11, 16); only under conditions of cell shrinkage (hyperosmolality, high concentrations of extracellular Na\textsuperscript{+}) or acidosis can NHE4 activity be demonstrated. When activated, it is highly resistant to amiloride (11, 14, 15, 33). Functional studies of the NHE5 and NHE6 isoforms are not yet available.

The purpose of the present study was to determine the isoform specificity of Na\textsuperscript{+}/H\textsuperscript{+} exchangers in macula densa cells. Because different NHE isoforms have specific cellular functions, knowledge of the exact isoform expression may be important in understanding the unique physiological function of this cell plaque in transmission of signals between tubular fluid and vascular elements. Functional studies measuring pH\textsubscript{i} with fluorescent microscopy utilized pharmacological and well-known regulatory properties to identify and determine NHE isoform specificity of the basolateral exchanger and to determine the isoform of the previously reported apical antiporter. Identity of NHE isoforms at the apical and basolateral membrane of macula densa cells was further supported by immunohistochemistry by using antibodies against specific NHE isoforms in rat and rabbit kidney.

MATERIALS AND METHODS

cTAL Preparation

Individual cTALs with attached glomeruli were manually dissected from sagittal slices of kidneys obtained from female New Zealand White rabbits weighing ~0.5 kg. The dissection solution was a modified Ringer solution composed of (in mM) 148 NaCl, 5 KCl, 1 MgSO\textsubscript{4}, 1.6 Na\textsubscript{2}HPO\textsubscript{4}, 0.4 NaH\textsubscript{2}PO\textsubscript{4}, 1.5 CaCl\textsubscript{2}, and 5 d-glucose. Individual cTALs with glomeruli were transferred to a thermostated Leitz chamber mounted on a Leitz Fluorvert inverted microscope. The cTAL was cannulated and perfused with this same Ringer solution, except that [NaCl] was lowered to 25 mM by using N-methyl-d-glucamine cyclamate to maintain osmolality at 300 mosmol/kgH\textsubscript{2}O. The preparation was bathed in the Ringer solution ([NaCl] = 150 mM) continuously aerated with 100% O\textsubscript{2} and exchanged at a rate of 1 ml/min. Temperature was maintained at 37°C. For the experiments, solutions were modified by isosmotically replacing Na\textsuperscript{+} with N-methyl-d-glucamine and/or Cl\textsuperscript{–} with cyclamate to achieve a [Na\textsuperscript{+}] and/or [Cl\textsuperscript{–}] of between 0 and 150 mM. All solutions were adjusted to a pH of 7.4. In pharmacological experiments, 10\textsuperscript{–8} to 3 \times 10\textsuperscript{–4} M of EIPA, 10\textsuperscript{–8} to 10\textsuperscript{–3} M Hoe-694, or 250 µM DIDS were added to the perfusate or bath. Data were collected after an incubation of 2–3 min with these agents. In some experiments, hyperosmolality was achieved by addition of increasing amounts of mannitol. (All chemicals were from Sigma Chemical, St. Louis, MO, except Hoe-694, which was kindly provided by Dr. H. J. Lang, Hoechst Marion Russel, Frankfurt, Germany.)

Measurement of pH\textsubscript{i} and NHE Activity

pH\textsubscript{i} of macula densa cells was measured with dual-excitation wavelength fluorescence microscopy (Photol Technologies, Princeton, NJ) by using the fluorescent probe 2',7'-bis-(2-carboxyethyl)-5-(and -6) carboxyfluorescein (BCECF; Teflabs, Austin, TX). BCECF fluorescence was measured at an emission wavelength of 530 nm in response to excitation wavelengths of 500 and 440 nm, alternated at a rate of 25 Hz by a computer-controlled chopper assembly. An adjustable photometer sampling window (Fig. 1A) was positioned over the whole macula densa plaque (consisting of ~10–15 cells), and emitted photons were detected by a Leitz photometer that was modified for photon counting. Magnification was ~400 by using an Olympus x40 UVFL lens. Autofluorescence-corrected ratios (500 nm/440 nm) were calculated at a rate of 5 points/s by using PTI software. Macula densa cells were loaded with the dye (Fig. 1B) by adding BCECF-acetoxy-methyl ester (10 µM) dissolved in dimethyl sulfoxide, to the luminal perfusate. Loading required ~1 min, at which time counts per second for both wavelengths stabilized at values of at least one order of magnitude greater than background fluorescence. BCECF fluorescence was calibrated by using the nigericin/high K\textsuperscript{+} method (38), and fluorescence ratios were converted to pH units as described previously (18).

Using separate perfusion and bathing solutions allowed us to independently measure Na\textsuperscript{+}/H\textsuperscript{+} exchange activity at either the apical or basolateral membrane. Na\textsuperscript{+}/H\textsuperscript{+} exchange activity was assessed by monitoring Na\textsuperscript{+}-dependent pH\textsubscript{i} recovery from an acid load imposed by Na\textsuperscript{+} removal. Apical-side experiments consisted of altering luminal [Na\textsuperscript{+}] ([NaN\textsuperscript{+}], 25–0–25 mM) while keeping bath [Na\textsuperscript{+}] ([Na\textsuperscript{+}], 0 mM), whereas in basolateral-side experiments altering [Na\textsuperscript{+}] ([Na\textsuperscript{+}], 150–0–150 mM) while keeping [Na\textsuperscript{+}], 0 mM. Measurements consisted of the change in pH\textsubscript{i} from control (ΔpHi), and/or the initial rate of pH\textsubscript{i} recovery (ΔpHi/Δt, calculated from a linear fit, PTI software) in response to alterations in [Na\textsuperscript{+}], or [Na\textsuperscript{+}],. The use of nominally CO\textsubscript{2}/HCO\textsubscript{3}\textsuperscript{–}-free solutions minimized the likelihood that Na\textsuperscript{+}-dependent HCO\textsubscript{3}\textsuperscript{–} transport was responsible for the observed changes in pH\textsubscript{i}. The Na\textsuperscript{+}-dependent alkalinization in each experiment was always examined from the same starting pH\textsubscript{i} value because Na\textsuperscript{+}/H\textsuperscript{+} exchange activity is influenced by pH\textsubscript{i}.

The viability of the macula densa cells, and function of the Na\textsuperscript{+}/H\textsuperscript{+} exchangers, was well preserved for the duration of these studies. Time control experiments for both the apical- and basolateral-side experimental models demonstrated that the Na\textsuperscript{+}-dependent pH\textsubscript{i} changes were reproducible over time, baseline pH\textsubscript{i} was stable, and the initial rates of pH\textsubscript{i} changes (dph\textsubscript{i}/dt) did not vary during the time interval of these studies. These observations validate that the pH\textsubscript{i} changes we measured were due to the effects of various maneuvers and drugs on the Na\textsuperscript{+}/H\textsuperscript{+} exchanger activity.
Immunohistochemistry

Light microscopic immunohistochemistry of NHE2 and NHE3. FIXATION AND PREPARATION OF TISSUE FOR IMMUNOHISTOCHEMISTRY. Female New Zealand White rabbits (500 g) purchased from Charles River (Cloon, France) were prepared for retrograde vascular perfusion under intravenous pentobarbital sodium anesthesia. The abdominal aorta was clamped above and below the renal arteries and perfused with freshly made cold 4% paraformaldehyde in Dulbecco's modified Eagle/F12 medium. The kidneys were then removed, and coronal kidney sections were incubated overnight at 4°C in 4% paraformaldehyde and then embedded in paraffin.

SECTIONING AND IMMUNOLABELING. Subsequently, 4-µm-thick sections of the paraffin block were deparaffinized with toluene, washed in graded ethanol, and rehydrated in PBS, then labeled by using a three-layer method that utilizes a biotinylated secondary IgG in conjunction with horseradish peroxidase-conjugated streptavidin. To reduce nonspecific binding, sections were treated with background-reducing buffer from Dako (Copenhagen, Denmark), followed by overnight incubation with a 1:200 dilution of anti-NHE2 antibodies. Sections were then washed in PBS and incubated with 1:200 dilution of goat biotinylated anti-rabbit IgG (Vector Laboratories, Burlingame, CA), followed by the Immunopure peroxidase suppressor (Pierce Chemicals, Rockford, IL) to block endogenous peroxidase activity. After washing in PBS, sections were incubated with a 1:400 dilution of horseradish peroxidase-conjugated streptavidin (Amersham, Arlington Heights, IL), and peroxidase activity was revealed with 3-amino-9-ethylcarbazole and H2O2. After a distilled-water wash, sections were counterstained with hematoxylin, washed, and mounted after liquid phase glycergel solution (Dako, Copenhagen, Denmark) was applied to the tissue sections. Immunostaining for NHE3 was performed as for NHE2, except that sections were subjected to microwave antigen retrieval before staining. Dilution of the monoclonal antibody to NHE3, done 2B9 (1:100, Ref. 10, marketed by Chemicon International, Temecula, CA), and 1:200 dilution of goat biotinylated anti-mouse IgG (Vector Laboratories) were used as primary and secondary antibodies, respectively. Sections were examined with a Zeiss photomicroscope and photographed by using Kodak T64 film. Sections in which the anti-NHE2 was preadsorbed with the monomeric NHE2 peptide antigen served as controls. Production and character-
Immunofluorescence of NHE4. Using similar methods described above, kidneys were removed from Sprague-Dawley rats and New-Zealand rabbits and frozen in PolyFrezet tissue freezing medium (Polysciences) on dry ice. Because earlier studies of NHE4 localization determined that aldehyde fixation destroyed epitope reactivity (14), we utilized immunofluorescence of methanol-fixed tissue for these studies. Five-micrometer-thick sections were cut on a cryostat, fixed at −20°C in methanol for 10 min, and then air dried. After 5 min of rehydration in Dulbecco’s PBS (pH 7.4), sections were treated with the blocking buffer containing nonimmune swine serum diluted 1:25 in Dulbecco’s PBS. Rabbit kidney sections were also blocked with an affinity-purified goat anti-rabbit IgG Fab fragment (Jackson Laboratories) to lower background when a rabbit polyclonal antibody was used as primary antibody. After subsequent washings in Dulbecco’s PBS, sections were treated with 30 μg/ml affinity-purified rabbit anti-NHE4 polyclonal antibody for 1 h at room temperature. After washing in PBS, sections were incubated for 40 min with rhodamine-conjugated swine anti-rabbit IgG (Dako), washed, and mounted with Vectashield media, containing 4,6-diamino-2-phenylindole for nuclear staining (Vector Laboratories). Tissue sections were examined by using an Olympus IX70 inverted epifluorescence microscope at 623-nm (red) or 488-nm (green) excitation wavelengths by using a UApo340×40 objective. Images were captured by using a SenSys digital camera and IPLab Spectrum software equipped with power microtome (Signal Analytics). To test the specificity of the anti-NHE4 polyclonal antibody, 50 μl of the antibody (30 μg/ml) were preincubated with 50 μl of the synthetic NHE4 oligopeptides (ICN Pharmaceuticals). Monoclonal antibodies to the 1-subunit of rabbit a 1-subunit of rabbit Na+/K+-ATPase (Upstate Biotechnology, Lake Placid, NY). Slides were then incubated with a mixture of rhodamine-conjugated anti-rabbit IgG (ICN Pharmaceuticals, Costa Mesa, CA) and fluorescein-conjugated goat anti-mouse IgG (ICN Pharmaceuticals).

Immunofluorescence of NHE1 and NHE3. Tissue preparation for immunocytochemistry. Production, characterization of the guinea pig anti-FP347A (NHE1) polyclonal antibody, and the monoclonal 2B9 anti-NHE3 antibody and their immunofluorescence labeling in rabbit and rat kidney have been described earlier (9, 10). Briefly, New Zealand White rabbits (for NHE1) and Sprague-Dawley rats (for NHE3; Gabrielle Farms, Woodstock, CT) were anesthetized with pentobarbital sodium, and the kidneys were perfusion-fixed by first inserting a cannula into the descending aorta distal to the renal arteries. The kidneys were then perfused retrograde first with PBS, pH 7.4 at 37°C, to remove blood, followed by PLP fixative containing 2% paraformaldehyde, 75 mM lysine, and 10 mM sodium periodate in phosphate buffer, pH 7.4 (29).

Identification of the Basolateral Na+/H+ Exchanger

In the presence of 0 mM [Na+]L and 150 mM [NaCl]B, control macula densa pHi for all experiments averaged 7.32 ± 0.06 (n = 30). The first series of experiments were performed to determine the effects of NaCl removal from the bathing solution in the absence of Na+L. As shown in the tracing (Fig. 2), removal of NaCl from the bath caused a rapid intracellular acidification (75% of the total reduction in pHi occurred in <50 s), whereas readdition of NaCl caused a rapid recovery of pHi. The effects of changes in either [Na+]L or [NaCl]m on macula densa pHi were summarized in Fig. 3. There was no difference in the magnitude of Na+L-dependent acidification or realkalinization in the presence or absence of Cl−. Further studies examined whether this intracellular acidification in response to [Na+]L removal was due

Fig. 2. Representative tracing of bath NaCl-dependent changes in macula densa intracellular pH (pHi). Removal of bath NaCl resulted in a rapid and sustained intracellular acidification. This response was reversible (pHi recovery) on return of bath NaCl concentration ([NaCl]) to 150 mM. We measured magnitude of bath NaCl-dependent acidification (ΔpHi) and initial rate of pHi recovery (ΔpHi/Δt), following readdition of bath NaCl.
to Na+/H+ exchange or was influenced by a HCO₃⁻ transport mechanism (Cl⁻/HCO₃ exchange or Na⁺-HCO₃ cotransport). In these experiments, [Na⁺]₀ was removed in the presence of the Na⁺/H⁺ exchange blocker DIDS, or in the presence of the HCO₃⁻ transport blocker DIDS. As shown in Fig. 3, addition of 250 µM DIDS to the bath had no effect on the magnitude of decrease in pHᵢ with reductions in [Na⁺]₀, whereas addition of 300 µM EIPA to the bath nearly abolished Na⁺-induced acidification (P < 0.01). From these experiments we concluded that macula densa cells exhibit basolateral Na⁺/H⁺ exchange activity.

Apical Na⁺/H⁺ Exchange Activity

In a previous study (18) we reported evidence for apical Na⁺/H⁺ exchange in macula densa cells. To further elucidate the nature of this Na⁺/H⁺ exchanger, we utilized an experimental procedure similar to the basolateral experiments shown in Fig. 2. As illustrated in Fig. 4, [Na⁺]₀ was kept constant at 0 mM, and macula densa cells were acidified by removing [Na⁺]₀, under Cl⁻-free (bath and lumen) conditions. In these experiments, macula densa pHᵢ was 7.29 ± 0.05 (n = 5), and removal of [Na⁺]₀ reduced pHᵢ by 0.37 ± 0.01 pH units. Readdition of [Na⁺]₀ resulted in a complete recovery in pHᵢ, and the initial rate of this pHᵢ recovery was inhibited 99.5 ± 0.2% by 100 µM EIPA (n = 5).

Pharmacological Properties of the Na⁺/H⁺ Exchangers

To functionally identify isoform specificity of the apical and basolateral Na⁺/H⁺ exchangers, their relative sensitivity to well-characterized inhibitors was measured. All known Na⁺/H⁺ exchanger isoforms are inhibited by amiloride and its derivatives, but the various NHEs differ in their sensitivities to these compounds. Thus pharmacological properties were used as one basis for isoform identification by using the experimental procedures shown in Fig. 2 (for the basolateral NHE) and Fig. 4 (for the apical NHE). Because it has been shown that Cl⁻ can modify EIPA sensitivity of the Na⁺/H⁺ exchanger (41), these experiments were performed under Cl⁻-free conditions.

Inhibition by EIPA. Macula densa cells were acidified by Na⁺ removal, and after 2-min incubation at various concentrations of EIPA (0.01, 0.1, 1, 10, 100, 300 µM) the effect on Na⁺-dependent pHᵢ recovery was measured. Figure 5A shows the dose-response curves for inhibition of apical and basolateral Na⁺/H⁺ exchange activity by EIPA. Inhibition curves indicated a single Na⁺/H⁺ exchange activity at both the apical and basolateral membranes with an IC₅₀ of 0.86 and 9 µM, respectively. These data are consistent with reported values (Table 1) for an amiloride-sensitive (most probably NHE2) isoform at the apical, and a highly amiloride-resistant (most likely NHE4) isoform at the basolateral membrane.

Inhibition by Hoe-694. Recently, the benzoylguanidine derivative, selective NHE1 inhibitor Hoe-694 has been used to discriminate between NHE isoforms, because there is a two orders of magnitude difference in Hoe-694 sensitivity among NHE1, NHE2, and NHE3 (Table 1) (17). Figure 5B shows the dose-response curves for inhibition of apical and basolateral Na⁺/H⁺ exchange activity at various concentrations of Hoe-694 (0.01, 0.1, 1, 100, 500, 1,000 µM). Inhibition curves indicated a single Na⁺/H⁺ exchange activity at both the apical and basolateral membranes with an IC₅₀ value of 7.6 and 247 µM, respectively. The value obtained for the apical exchanger is consistent with that reported for NHE2 activity (Table 1). To date, there is no information on the sensitivity of NHE4 isoform for Hoe-694. Therefore, the data obtained for the basolateral exchanger cannot specify which drug-resistant isoform (NHE3 or NHE4) accounts for the basolateral Na⁺/H⁺ exchange. However, it is clearly very resistant to inhibition by Hoe-694.

Effects of Hyperosmolality on Basolateral Na⁺/H⁺ Exchange

Hyperosmolality-induced cell shrinkage has been shown to stimulate NHE4 activity in contrast to NHE3, which is inhibited by hyperosmolality (11, 37). This regulatory characteristic, therefore, serves as a means of distinguishing NHE4 from NHE3 activity. Figure 6 shows macula densa basolateral Na⁺/H⁺ exchange

![Fig. 3. Acidification of macula densa cells by removal of bath NaCl. Under Cl⁻-free conditions cyclamate was used to substitute for Cl. In some experiments, changes in macula densa pHᵢ was measured in presence of 250 µM DIDS or 300 µM ethylisopropyl amiloride (EIPA) after a 2-min preincubation with these drugs added to bath. n = 7 Each: Ctrl, control. *P < 0.01.](http://ajprenal.physiology.org/)

![Fig. 4. Representative recording of luminal Na⁺-dependent changes in macula densa pHᵢ. Removal of luminal Na⁺ resulted in a rapid and sustained intracellular acidification. This response was reversible (pHᵢ recovery) on return of luminal [Na⁺] to 25 mM. We measured initial rate of pHᵢ recovery following readdition of luminal Na⁺.](http://ajprenal.physiology.org/)
activity at osmolalities ranging from 125 to 710 mosmol/kgH₂O. As observed visually, increasing basolateral osmolality resulted in marked shrinkage of macula densa cells. There was a highly significant increase in the initial rate of Na⁺/H⁺ exchange activity as bath osmolality was increased from 125 to 230, 280, 400, and 520 mosmol/kgH₂O. Peak activity occurred between 400 and 500 mosmol/kgH₂O. At higher osmolalities (>500 mosmol/kgH₂O) there was some reduction in activity. Under hypertonic conditions (500 mosmol/kgH₂O bathing solution) low-dose (1 µM) EIPA caused a 9.3 ± 5.1% inhibition of the basolateral NHE activity, whereas high-dose (300 µM) EIPA almost completely abolished Na⁺/H⁺ exchange activity (99.6 ± 0.2% inhibition, n = 5 each). This finding shows that the increased initial rate of Na⁺/H⁺ exchange recovery with hyperosmolality was due to the stimulation of the basolateral Na⁺/H⁺ exchange activity, which was very resistant to EIPA. Overall, these results are consistent with the presence of an osmolality-sensitive basolateral NHE4 isoform.

Immunolocalization of NHE Isoforms

In an effort to confirm our functional evidence that macula densa apical and basolateral Na⁺/H⁺ exchange is mediated by NHE2 and NHE4, respectively, specific antibodies raised against NHE1–4 isoforms were used to carry out immunohistochemistry on rat and rabbit kidney sections.

Table 1. Sensitivity of NHE isoforms to EIPA and Hoe-694

<table>
<thead>
<tr>
<th>Isoform</th>
<th>EIPA (µM)</th>
<th>Hoe-694 (µM)</th>
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<tbody>
<tr>
<td>NHE1</td>
<td>0.02</td>
<td>0.16</td>
</tr>
<tr>
<td>NHE2</td>
<td>0.66</td>
<td>5</td>
</tr>
<tr>
<td>NHE3</td>
<td>2–8</td>
<td>650</td>
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<tr>
<td>NHE4</td>
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IC₅₀ values are from Refs. 15, 17, and 31. NA, not available; EIPA, ethylisopropyl amiloride; Hoe-694, (3-methylsulphonyl-4-piperidinobenzoyl)-guanidine methanesulfonate.

Apical Membrane

Light microscopic immunoperoxidase labeling of NHE2 and NHE3 in rabbit kidney. As illustrated in Fig. 7B, macula densa and cTAL cells were positive for the anti-NHE2 antibody. Labeling was restricted to the apical aspects of these cells, and there was no evidence for basolateral staining. Preincubation of primary antibody with the NHE2 oligopeptide used for immunization revealed an absence of labeling, which indicates specific recognition (Fig. 7A). However, anti-NHE3 antibody was not detected in macula densa cells, whereas proximal tubule cells were positive at the apical membrane (Fig. 8A).

Immunofluorescence labeling of NHE3 in rat kidney. Because the general staining intensity of the anti-NHE3 antibody used in these studies is much greater in the rat kidney than in the rabbit (10), we performed immunofluorescence on rat tissue sections. As shown in Fig. 8B, in rat tissue, cTAL cells adjacent to the macula...
densa were positive at the apical membrane for the same anti-NHE3 antibody. However, there was no labeling in macula densa cells.

Basolateral Membrane

Immunofluorescence labeling of NHE1 and NHE4. Figure 7, C-F, shows the distribution of NHE4 labeling within the cTAL and macula densa. In both rat and rabbit kidney (Fig. 7, D and F, respectively), basolateral plasma membrane domains of macula densa cells were heavily labeled. The apical membrane and cytoplasm exhibited virtually no labeling. In contrast, cTAL cells were positive across the entire cytoplasm, with significant distribution in small intracellular vesicles (Fig. 7D). Control sections, in which the primary antibody was preincubated with the NHE4 oligopeptide used for immunization, revealed no specific labeling (Fig. 7C). As illustrated in Fig. 9, A and B, cTAL and distal tubule cells were positive for the anti-NHE1 antibody. Labeling was restricted to the basolateral aspects of these cells. However, macula densa cells were devoid of staining for NHE1. Positive staining was completely blocked by preincubation of the serum with the FP347A affinity matrix (NHE1 fusion protein, not shown).

To further localize NHE4 to the basolateral membrane of macula densa cells, double-labeling experiments (Fig. 7E) were carried out in which cryostat...
sections were labeled with anti-NHE4 antibody (red) and antibodies against the α1-subunit of Na⁺-K⁺-ATPase (green), a protein that is localized in basolateral membranes of renal epithelia. NHE4 was colocalized (yellow) with Na⁺-K⁺-ATPase at the basolateral membrane, with the highest degree of colocalization occurring at the base of the cells.

Fig. 8. Immunohistochemical localization of Na⁺/H⁺ exchanger isoform NHE3 in rabbit (A) and rat (B and C) kidney. Magnification: ×400. A: light microscopic immunoperoxidase labeling of NHE3 in rabbit kidney. Proximal tubule (PT) cells are labeled with NHE3 (brown), and labeling is present at apical membranes of these cells. Macula densa (arrowhead) and cTAL cells are devoid of staining. B and C: indirect immunofluorescence and phase-contrast microscopy showing distribution of NHE3 in rat kidney. cTAL cells adjacent to macula densa (arrowheads) are positive, but macula densa cells are not labeled.

DISCUSSION

This study is a detailed description of the localization and isoform identification of Na⁺/H⁺ exchangers in macula densa cells. The present work was initiated by our previous studies (18) in which we found evidence for apical Na⁺/H⁺ exchange in macula densa cells. This previous work, however, did not address the isoform specificity of this apical exchanger nor did it seek to determine whether these cells expressed an exchanger at the basolateral membrane. Many other renal epithelia express both apical and basolateral NHE isoforms (31, 33). Therefore, it was of interest to establish the existence and isoform specificity of a macula densa basolateral membrane Na⁺/H⁺ exchanger, because the specific processes controlled by Na⁺/H⁺ exchangers (Na⁺ transport, intracellular volume, pH regulation, and intracellular signaling) may be involved in the sensor function of the macula densa.

Na⁺/H⁺ Exchanger Activities

pHᵢ of macula densa cells was measured by using the fluorescent probe BCECF. Because [NaCl]ₒ at the macula densa is thought to be in the range of 20 to 40 mM (3–5), [NaCl]ₒ was initially set at 25 mM. [NaCl]ᵢ was initially set at 150 mM, consistent with normal extracellular fluid electrolyte composition. However, it
should be stated that there is no information concerning \([\text{NaCl}]\) or osmolarity at the interface of the extraglomerular mesangium and the basolateral membrane of macula densa cells, at least in mammalian species. Thus setting \([\text{NaCl}]_B\) at 150 mM may or may not represent the physiological interstitial conditions that normally exist at the macula densa.

To initially characterize basolateral \(\text{Na}^+/-\text{H}^+\) exchange, \([\text{Na}]_L\) was reduced to 0 mM. This was done to avoid the complications of apical \(\text{Na}^+/-\text{H}^+\) exchange activity as the result of changes in intracellular [\(\text{Na}^+\)] during alterations in [\(\text{Na}]_B\). In response to a decrease in \([\text{NaCl}]_B\) from 150 to 0 mM, there was a prompt decrease in \(p\text{Hi}\), that was sustained and reversible on return to the 150 mM \(\text{NaCl}\) bathing solution. In addition, nearly identical intracellular acidification was obtained when \(\text{Na}^+\) alone was removed. The most likely explanation for these data is that, like other renal epithelia, the macula densa cells possess a basolateral \(\text{Na}^+\)-coupled acid/base transporter. Three such transporters have been described so far and include the \(\text{Na}^+/-\text{H}^+\) exchanger, \(\text{Na}-\text{HCO}_3\) cotransporter and the \(\text{Na}^+/-\text{Cl}^\text{HCO}_3\) exchanger (19, 20, 26). Although these studies were performed in nominally \(\text{HCO}_3\)-free solutions, it is possible that \(\text{HCO}_3\) generation by macula densa cells could provide substrate for these \(\text{HCO}_3\)-dependent transporters. To identify, which transporters might be involved in \(\text{Na}^+\)-dependent changes in \(p\text{Hi}\) across the basolateral membrane, we used two antagonists of these electrolyte transport processes. We found that EIPA, an inhibitor of \(\text{Na}^+/-\text{H}^+\) exchange, blocked the \(\text{Na}^+\)-induced acidification, whereas DIDS, an inhibitor of \(\text{HCO}_3\) transport systems, had no effect on this response even at a relatively high concentration. These results are consistent with the existence of a basolateral \(\text{Na}^+/-\text{H}^+\) exchanger in macula densa cells. However, they do not eliminate the possibility that macula densa cells also possess \(\text{HCO}_3\)-dependent transport systems that might be active in the presence of normal extracellular fluid \(\text{HCO}_3\) levels.

At the apical membrane, we found that macula densa \(p\text{Hi}\) was sensitive to changes in [\(\text{Na}^+\)], in the presence of 0 mM [\(\text{Na}^+\)]. In this series of experiments [\(\text{Na}^+\)] was removed to eliminate any contribution of the basolateral \(\text{Na}^+/-\text{H}^+\) exchanger to changes in \(p\text{Hi}\). In addition, these studies were performed in the absence of [\(\text{Cl}^-\)], to prevent \(\text{Na}^+/-2\text{Cl}^-/-\text{K}^+\) cotransporter-induced changes in intracellular [\(\text{Na}^+\)]. In response to a decrease in [\(\text{Na}^+\)] from 25 to 0 mM, there was a prompt, reversible decrease in \(p\text{Hi}\), similar to what was found in the basolateral experiments. \(\text{Na}^+\)-dependent \(p\text{Hi}\) recovery was completely abolished by 100 µM EIPA, which confirmed that \(\text{Na}^+\)-dependent \(p\text{Hi}\) changes are due to the function of apical \(\text{Na}^+/-\text{H}^+\) exchange.

Functional and Pharmacological Properties of the \(\text{Na}^+/-\text{H}^+\) Exchangers

Amiloride and its analogs, including EIPA and the benzoylguanidine derivative Hoe-694, have been widely used for pharmacological characterization of distinct \(\text{Na}^+/-\text{H}^+\) antiporter isoforms (17, 31, 33). Table 1 shows the \(IC_{50}\) values for EIPA and Hoe-694 of the NHE1–4 isoforms. In our experiments, \(IC_{50}\) values of the macula densa apical and basolateral \(\text{Na}^+/-\text{H}^+\) exchanger for EIPA (0.86 and 9.0 µM, respectively) suggest that the exchanger present in the apical membrane is an amiloride-sensitive isoform (perhaps NHE2), whereas the basolateral membrane isoform is a highly amiloride-resistant type (NHE3 or NHE4). Recently, the compound Hoe-694 has been used to discriminate between NHE isoforms, because there are large differences in the sensitivity of the various isoforms for this compound (Table 1). \(IC_{50}\) values of the apical and basolateral exchangers for Hoe-694 (7.6 and 247 µM, respectively) clearly suggest that the apical exchanger is NHE2 whereas the basolateral isoform is either NHE3 or NHE4. Furthermore, it should be noted that dose-response curves with each inhibitor at both apical and basolateral membranes are consistent with the presence of only one form of the \(\text{Na}^+/-\text{H}^+\) exchanger. Although other NHE isoforms might be present at either the apical or basolateral membranes, our results are consistent with the inhibition of a single population of exchangers at each membrane. These findings are somewhat unique because most epithelial cells express NHE-1 at the basolateral membrane (9, 31, 33); however, our results clearly do not support the existence of a highly amiloride-sensitive NHE isoform at the basolateral membrane of macula densa cells.

Further studies were performed in an effort to discriminate between the drug-resistant isoforms, NHE3 and NHE4, at the basolateral membrane. Inhibition of NHE3 activity in response to hyperosmotic conditions is very well documented (37), whereas NHE4 is activated by cell shrinkage (11). In our experiments, hyperosmolality-induced cell volume contraction greatly stimulated basolateral \(\text{Na}^+/-\text{H}^+\) exchange activity.

One might argue that in hypertonc medium, the ubiquitously expressed NHE1 may become activated and contribute to basolateral NHE activity, because this isoform has also been shown to be activated by hyperosmolality (11, 31). However, under hypotonic conditions, addition of EIPA at a concentration that should completely inhibit NHE1 activity, resulted in a very slight inhibition of basolateral NHE activity. Considering the very low \(IC_{50}\) value (0.02 µM, Table 1) of NHE1 for EIPA, we conclude that NHE1 does not contribute to the basolateral NHE activity under either isosmotic or hypotonic conditions. Also, if we compare the EIPA data under hyperosmolality to those in Fig. 5, the dose-response profile for inhibition of the basolateral NHE activity by EIPA is exactly the same under isosmotic and hypotonic conditions. This functional finding strongly suggests that the drug-resistant NHE4 is the isoform responsible for basolateral NHE activity in macula densa cells. According to earlier NHE4 transfection studies (11, 15), this isoform, unlike other members of the NHE family, requires a stimulus for activity (e.g., cell shrinkage, preacidification, high extracellular [\(\text{Na}^+\)]) and is rapidly inhibited by actin cytoskel-
eral Na\textsuperscript{+} exchanger at the basolateral membrane of macula densa cells is provided by localization studies by using a specific antibody against NHE4 (see below).

Immunolocalization of the NHE1 isoforms

Our immunohistochemical studies clearly demonstrate that macula densa cells express NHE2 protein in the apical, and NHE4 in the basolateral, membrane, whereas NHE3 and NHE1 isoforms were not detected. With regard to NHE2, apical membrane localization is in agreement with our recent observation (16) of apical localization of this isoform in cTAL cells. Although Na\textsuperscript{+}/H\textsuperscript{+} exchange activity mediated by NHE3 has been described in the apical membrane of other nephron segments, including proximal tubule and TAL (8, 23, 31, 33), recent NHE3 immunolocalization studies have produced equivocal results depending on the antibody used. Amemiya and coworkers (1) showed very good staining in both mTAL and cTAL. More recently, three monoclonal antibodies [MAB 2B9, 4F5, and 19F5 marketed by Chemicon (10)] have been raised to a COOH-terminal fusion protein of NHE3, which recognizes at least two distinct epitopes. Interestingly, MAB 4F5 and 19F5 do not stain the TAL at all although they do stain the thin limb (10). MAB 2B9 stains the cortical and medullary TAL very well (10); therefore, this MAB was used in the present study. Also, it has been shown (10) that for some unknown reason the MABs worked much better in rat kidney than in rabbit even though the fusion protein was derived from the rabbit sequence. However, in both rat and rabbit kidney, this well-defined MAB against NHE3 failed to detect this isoform in macula densa cells in the present study (Fig. 8). These findings further support our functional data identifying a single apical exchange activity mediated by NHE2.

Immunofluorescence studies in both rat and rabbit kidney sections demonstrated extensive labeling of NHE4 in macula densa and neighboring cTAL cells. However, the distribution of NHE4 staining was quite different in these cell types. The apical membrane and the cytosol of macula densa cells were negative, whereas there appeared to be intensive staining of the basolateral membrane domains. In addition, using double-labeling, NHE4 colocalized with Na\textsuperscript{+}-K\textsuperscript{+}-ATPase, thereby strongly supporting the localization of this exchanger at the basolateral membrane of macula densa cells. This finding supports the functional studies that demonstrated constitutive activity of basolateral Na\textsuperscript{+}/H\textsuperscript{+} exchange even in the presence of hypo- and osmotic solutions. In neighboring cTAL cells, NHE4 labeling was predominant in the cytosol and appeared to be located in intracellular vesicles and was much less intense in the basolateral membrane. This finding is consistent with other studies that have reported an intense homogenous cytosolic staining for NHE4 in distal tubular cells (14). In addition, our functional studies have found (unpublished observations) a much lower activity of the basolateral Na\textsuperscript{+}/H\textsuperscript{+} exchange in adjacent cTAL cells than in the macula densa. This finding further suggests that these cells are morphologically and functionally distinct from macula densa cells. Furthermore, our work (Fig. 9) and other immunohistochemical studies (9) of kidney cortex demonstrated the existence of NHE1 isoform in basolateral membranes of distal convoluted tubule and cTAL, i.e., those nephron segments proximal and distal to the macula densa. However, macula densa cells were not labeled, suggesting that the level of NHE1 protein is below the limits of detection in these cells. This finding confirms the results of functional studies that the drug-resistant NHE4 is the dominant NHE isoform at the basolateral membrane in macula densa cells.

As indicated, there is no information concerning [NaCl] or osmolality of the interstitial space beneath the macula densa cells. As suggested by previous work (24), macula densa cells are impermeable to water, and electron micrographs (22, 35) indicate that, under normal conditions, water flows from macula densa cells into the extraglomerular mesangial compartment. Because luminal fluid is normally hypotonic, water flowing from lumen to the extraglomerular interstitial spaces could potentially dilute electrolytes and the total solute of this space. Also, it has been suggested by others that the interstitial space beneath the macula densa plaque is poorly drained because few lymphatics and capillaries are present in this area (2). Therefore, under normal conditions where luminal fluid at the macula densa has a low [Na\textsuperscript{+}] and osmolality, [Na\textsuperscript{+}] and osmolality in the space beneath the macula densa cells may be relatively low. Increases in [Na\textsuperscript{+}] and osmolality should be reflected by increases in interstitial [Na\textsuperscript{+}] and osmolality, which should shrink these cells and increase basolateral Na\textsuperscript{+}/H\textsuperscript{+} exchange.

In terms of overall Na\textsuperscript{+}/H\textsuperscript{+} exchange activity in macula densa, our present and previous work (18) demonstrate that increases in [Na\textsuperscript{+}] result in increased activity of the apical Na\textsuperscript{+}/H\textsuperscript{+} exchanger NHE2, thus leading to Na\textsuperscript{+} reabsorption and alkalization of macula densa cells. The increase in luminal fluid composition would also result in macula densa cell shrinkage and activation of the basolateral Na\textsuperscript{+}/H\textsuperscript{+} exchanger NHE4, an isoform that might contribute to cell volume regulation. Both of these processes could play an important role in the regulation of macula densa cell function and its role as the sensor element for tubuloglomerular feedback. In addition, the previously reported enhancement of exchange activities by angiotensin II, which is a specific modulator of feedback activity, further supports the physiological importance of these Na\textsuperscript{+}/H\textsuperscript{+} exchangers in TGF (6, 34).
Finally it should be emphasized that this polarized NHE2/NHE4 configuration in the macula densa is clearly distinct from the usual NHE3/NHE1 arrangement in other nephron segments. In fact, the function of NHE2 and NHE4 in the nephron has been somewhat of an enigma. In this regard, our studies are the first to demonstrate constitutively active, functional apical NHE2 and basolateral NHE4.

We thank Clay Isbell for technical support and Martha Yeager for secretarial assistance.

This work was supported by National Institute of Diabetes and Digestive and Kidney Diseases Grants DK-32032 (P. D. Bell), DK-34972 (D. R. Abrahamson), and DK-19407 (D. G. Warnock). J. Peti-Peterdi is a National Kidney Foundation/American Society of Nephrology Research Fellow.

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Received 4 November 1998; accepted in final form 12 October 1999.

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