Immunolocalization of electroneutral Na-HCO$_3^-$ cotransporter in rat kidney

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bicarbonate handling; immunohistochemistry; immunoelectron microscopy; intracellular pH; ion transport

IN THE PROXIMAL TUBULES, reabsorption of HCO$_3^-$ by electronegenic Na-HCO$_3^-$ cotransport is well documented (4, 5). With the cloning of an electronegenic Na-HCO$_3^-$ cotransporter (NBC1) from the kidney (6, 27, 28), it was possible to produce antibodies to disclose the cellular and subcellular distribution of NBC1. Immunohistochemistry demonstrated the presence of NBC1 in the basolateral parts of proximal tubule segments S1 and S2 in rat (29) and the entire proximal tubule in rabbit (1), albeit less in segment S3. Immunoelectron microscopy has recently demonstrated that NBC1 immunogold labeling is exclusively found associated with basal and lateral plasma membranes of rat proximal tubule cells (21). However, there was no labeling in other segments of the rat kidney (21, 29). There is, however, HCO$_3^-$ reabsorption also in the distal part of the nephron (see, e.g., 10) and, although Na$^+$-H$^+$ and Cl$^-$/HCO$_3^-$ exchange are thought to be important (8), the precise role of these and, potentially, of Na-HCO$_3^-$ cotransport, is not clear (25). In this context, it is of interest that Na-HCO$_3^-$ cotransport activity has been suggested in the mouse medullary thick limb (14) and in rat inner medullary collecting duct cells (12) as well as in rat cortical thick ascending limb (16), whereas in rat outer medulla an electroneutral K$^+$-HCO$_3^-$ cotransport has been suggested in the thick ascending limb (17). Furthermore, HCO$_3^-$-dependent, DIDS-sensitive Na transport has recently been demonstrated in rat medullary thick ascending limb (18), consistent with Na-HCO$_3^-$ cotransport activity here. The situation with respect to a role for Na-coupled HCO$_3^-$ transport outside the proximal tubules is therefore confusing. Recently, several NBC1-like proteins have been identified, including NBC2 isolated from human retina (13) and NBC3 from human skeletal muscle (26). Also, a NBC-like partial clone was reported (3), which was also called NBC3. Recently, we (7) cloned a novel electroneutral Na-HCO$_3^-$ cotransporter (NBC$_{N1}$) from rat aorta (GenBank accession no. AF070475), which has a high degree of homology with the NBC3 cloned from a human muscle library (26). Northern blot analysis suggested a wide tissue distribution of NBC$_{N1}$, including its presence in the kidney. In the present study, we...
therefore wanted to establish the cellular and subcellular distribution of NBCN1 in rat kidney by using immunoblotting, immunohistochemistry, and immunoelectron microscopy as well as providing a functional assay for Na-HCO₃ cotransport activity.

**METHODS**

**NBCN1 Antibodies**

Rabbits were immunized with keyhole limpet hemocyanin conjugated with synthetic peptide, corresponding to the predicted COOH terminal of the NBCN1 (NH₂-EDEPSKKYM-DAETSL-COOH). Anti-NBCN1 IgG was affinity purified by a two-step strategy by using a protein A Sepharose CL-4B column (Pharmacia Biotech, Uppsala, Sweden) as the first step and an epoxy-activated amino hexane Sepharose 4B column (Pharmacia Biotech) as the second (31).

**Membrane Fractionation for Immunoblotting**

The kidneys from normal Munich-Wistar rats were divided into cortex, outer stripe and inner stripe of the outer medulla (ISOM), and inner medulla. These tissues were homogenized (0.3 M sucrose, 25 mM imidazole, 1 mM EDTA, pH 7.2, containing 8.5 μM leupeptin, 1 mM phenylmethylsulfonyl fluoride) by using an ultra-turrax T8 homogenizer (IKA Labortechnik) at maximum speed for 30 s, and the homogenate was centrifuged in an Eppendorf centrifuge at 4,000 g for 15 min at 4°C to remove whole cells, nuclei, and mitochondria. The supernatant was then centrifuged at 200,000 g for 1 h to produce a pellet containing membrane fractions enriched for both plasma membranes and intracellular vesicles (19). Gel samples (Laemmli sample buffer containing 2% SDS) were made of this pellet.

**Electrophoresis and Immunoblotting**

Samples of membranes from rat kidney cortex, outer stripe and inner stripe of the outer medulla, and inner medulla were run on 6–16% gradient polyacrylamide minigels (Bio-Rad Mini Protein II). After transfer by electroelution to nitrocellulose membranes, blots were blocked with 5% milk in 0.1% Tween 20 (PBS-T), pH 7.5, for 1 h and incubated overnight at 4°C with anti-NBCN1 diluted 1:300. To control for nonspecific reactions, blocking experiments were performed by adding 10 μg of synthetic peptide to the anti-NBCN1 (~50-fold molar excess) and incubated overnight before immunoblotting. The labeling was visualized with horseradish peroxidase-conjugated secondary antibodies (P448 or P217, DAKO, Glostrup Denmark, diluted 1:3,000) by using the enhanced chemiluminescence system (Amersham International). The blots shown in Fig 1 represent one of three experiments, each with different rats.

**Deglycosylation**

For N-glycosidase F digestion, 100-μl membrane fractions from the inner stripe of the outer medulla were incubated at room temperature for 6 h in the presence of 5 μl of peptide-N-glycosidase F (PNGase F) obtained from Boehringer Mannheim (Mannheim, Germany). Boiling the suspensions in Laemmli sample buffer stopped the enzymatic reactions, and samples were analyzed by immunoblotting.

**Immunohistochemistry and Immunocytochemistry**

Kidneys from normal Munich-Wistar rats were fixed by retrograde perfusion via the aorta with periodate-lysine-paraformaldehyde (0.01 M NaIO₄, 0.075 M lysine, 2% paraformaldehyde, in 0.0375 M Na₂HPO₄ buffer, pH 6.2). Tissue blocks prepared from cortex, outer stripe of outer medulla and ISOM, and inner medulla were cryoprotected in 2.3 M sucrose containing 2% paraformaldehyde, mounted on holders, and rapidly frozen in liquid nitrogen (24). For preparation of cryostat sections, tissue was cryoprotected in 25% sucrose. Cryostat sections (10 μm) and semithin sections (0.8–1 μm, Reichert Ultracut S Cryoultramicrotome, Leica, Vienna) were incubated overnight at 4°C with anti-NBCN1 (diluted 1:150), and labeling was visualized with horseradish peroxidase-conjugated secondary antibody (P448, 1:100, DAKO) (33). The results, shown in Fig 2, represent the labeling seen in one of three rats.

**Immunoelectron Microscopy**

For immunoelectron microscopy, two different methods were used. The frozen samples were either used directly for cryosectioning (Reichert Ultracut S Cryoultramicrotome, Leica) or freeze-substituted in a Reichert AFS freeze-substitution unit (Leica) (20, 22, 23, 33). In brief, the samples for freeze substitution were sequentially equilibrated over 3 days in methanol containing 0.5% uranyl acetate at temperatures gradually raised from ~90°C to ~70°C, then rinsed in pure methanol for 24 h while the temperature was increased from ~70°C to ~45°C, and infiltrated with Lowicryl HM20 and methanol 1:1, 2:1, and, finally, pure Lowcryl HM20 before ultraviolet polymerization for 2 days at ~45°C and 2 days at 0°C. For immunoelectron microscopy, the ultrathin cryosections or ultrathin Lowicryl HM20 sections were first preincubated in PBS (10 mM sodium phosphate buffer containing 150 mM sodium chloride, pH 7.4) containing 0.1% skim milk powder and 50 mM glycine. Sections were rinsed and incubated overnight at 4°C with affinity-purified antibody (diluted 1:100) in 0.05 M Tris, pH 7.4, containing 0.2% milk for ultrathin sections or 0.1% Triton X-100 with 0.2% milk for...
Lowicryl HM20 sections. After the rinsing, sections were incubated for 1 h at room temperature with goat-anti-rabbit IgG conjugated to 10-nm colloidal gold particles (1:50, GAR.EM10, BioCell Research Laboratories, Cardiff, UK). The ultrathin cryosections were stained with 0.3% uranyl acetate in 1.8% methylcellulose, and Lowicryl HM20 sections were stained with uranyl acetate and lead citrate before examination in Philips 208 or CM100 electron microscopes.

**Intracellular pH Measurements**

Kidneys from 13-wk-old male Wistar rats were quickly removed after the rats were killed with CO₂. An ~1-mm slice of the kidney (sliced perpendicular to the polar axis) was fixed above a coverslip, which formed the floor in a 10-ml organ bath. The bathing solution was physiological salt solution (PSS; for composition, see below). The slice was incubated for ~40 min with 5 μM of the acetoxymethyl form of 2,7-bis-carboxyethyl-5,6-caboxyfluorescein (BCECF-AM) at 37°C. The temperature was thereafter reduced to ~21°C, and the organ bath was placed on the stage of an inverted microscope for either time-resolved fluorescence measurements or confocal fluorescence measurements. For time-resolved measurements, the preparation was excited alternately via a monochromator with 435- and 488-nm light. The emission from the preparation was collected through a band-pass filter (520–560 nm), and the ratio of the emissions at the two excitation wavelengths was determined after subtraction of the background fluorescence, which was always <10% of the signal. The equipment used was a PTI Deltascan fitted to a Leica DM IRB microscope with a Leica 340/0.55 objective. The confocal fluorescence measurements were made with a laser scanning confocal microscope (Odyssey XL, Noran), with excitation set at 488 nm (Ar/Kr laser), and emission was collected through a 520-nm-long pass filter. Sixteen frames (640 × 480 pixels) collected at 33 Hz were averaged to produce one image. To reduce dye bleaching to a minimum, an image was generally only obtained immediately before and after a solution change. With this protocol, little dye
bleaching was seen. For image analysis, the fluorescent images were represented as 256 intensity levels by using the programs Intervision (Noran) and Imagespace (Molecular Dynamics). The PSS contained (in mM) 119 NaCl, 4.7 KCl, 1.8 KH2PO4, 1.17 MgSO4, 25 NaHCO3, 1.6 CaCl2, 0.026 EDTA, 10 HEPES, and 5.5 glucose. The solution was gassed with 5% CO2 in air, and pH was 7.45–7.5. In Na-free PSS, NaCl was substituted with N-methyl-D-glucamine (NMDG) and NaHCO3 with cholinebicarbonate on an equimolar basis, and the pH was titrated to 7.45 with HCl. In HCO3−-free solution, NaHCO3 was substituted with NaCl, and, in the Na- and HCO3−-free solutions, the NaCl of the HCO3−-free solution was substituted with NMDG. The HCO3−-free solutions were gassed with air.

RESULTS

Anti-NBCN1

After purification of the antibody, immunoblotting was performed by using membrane fractions from different regions of rat kidney. As demonstrated in Fig. 1A, a strong band of NBCN1 was observed, corresponding to ~180 kDa. The strongest signal was obtained in membranes from the outer stripe of the outer medulla and ISOM, whereas no specific labeling was encountered in membranes from kidney cortex (Fig. 1A). By using higher membrane loading, an anti-NBCN1 signal was also observed in the inner medulla (Fig. 1B). Immunolabeling controls performed by using affinity-purified antibody preabsorbed with the immunizing peptide exhibited no labeling (Fig. 1C).

To determine whether the protein was extensively glycosylated, we determined its sensitivity to PNGase F treatment, which cleaves N-linked saccharides. As demonstrated in Fig. 1D, treatment of membranes from kidney with PNGase F caused a significant reduction in the molecular mass to ~140 kDa.

Immunohistochemical Localization of NBCN1

To determine the cellular localization of NBCN1, we performed immunohistochemistry by using cryostat sections from perfusion-fixed rat kidneys (Fig. 2). In ISOM strong labeling was associated with thick ascending limbs (Fig. 2B), and the labeling was exclusively present in the basolateral domains. In contrast, apical domains were unlabeled, which is especially apparent in supranuclear regions, which are completely devoid of NBCN1 labeling (arrowheads in Fig. 2B). In the outer stripe of outer medulla, only sparse labeling was associated with thick ascending limbs (not shown), whereas in cortex no detectable labeling was found (Fig. 2A). In addition to the labeling of thick ascending limb cells, distinct labeling was also observed of intercalated cells in the inner medulla (Fig. 2, C and D). Parallel sections immunolabeled for aquaporin-2, known to be present in collecting duct principal cells, confirmed the presence of NBCN1 in intercalated cells and not in principal cells (Fig. 2D). Immunoelectron microscopy documented this labeling pattern (see below). Only weak NBCN1 labeling was associated with intercalated cells in the ISOM (arrows in Fig. 2B). As demonstrated in Fig. 2, C and D, the NBCN1 immunolabeling was exclusively associated with basolateral plasma membrane domains (arrows in Fig. 2, C and D). Immunolabeling controls, by using peptide-preabsorbed anti-NBCN1, revealed no labeling (not shown). At the immunolabeling conditions used, no detectable immunolabeling was seen associated with glomeruli (Fig. 2A), proximal tubules (Fig. 2A), collecting duct principal cells (Fig. 2, B-D), descending or ascending thin limbs, or vascular structures (Fig. 2, B-D). Thus NBCN1 immunolabeling in rat kidney is associated with basolateral domains of thick ascending limb cells and medullary collecting duct intercalated cells.

Immunoelectron Microscopic Localization of NBCN1

Immunoelectron microscopy using ultrathin cryosections of rat kidney confirmed the presence of NBCN1 in thick ascending limb cells. As demonstrated in Fig. 3, NBCN1 labeling was exclusively associated with the basolateral plasma membrane of thick ascending limb cells (arrows in Fig. 3). Figure 4A shows a survey ultrathin cryosection of a collecting duct from kidney inner medulla. Figure 4B shows a higher magnification of the area indicated by the rectangle in Fig. 4A. Immunogold labeling of NBCN1 was exclusively associated with the basolateral plasma membranes of intercalated cells (arrows in Fig. 4B), whereas no labeling was seen of the apical plasma membrane domains or of collecting duct principal cells. Identical labeling patterns were also observed in ultrathin Lowicryl HM20 sections (not shown).

Intracellular pH Measurements

Time-resolved measurements. The recovery from an intracellular acidosis induced by washout of 20 mM NH4Cl is demonstrated Fig. 5, and mean values are shown in Table 1. In slices from ISOM (Fig. 5A), the recovery in the presence of HCO3− and 1 mM amiloride was inhibited by removal of Na [difference in recovery rate with and without Na (Δrate): 62 ± 6 ratio units/s]. In the absence of HCO3−, there was no recovery from acidosis in the presence of amiloride (Fig. 5A), and omission of Na had little effect (Δrate: 4.5 ± 1.8 ratio units/s). Even though buffering power was not determined, these findings strongly suggest the presence of net influx of HCO3− coupled to Na in ISOM. Figure 5A also shows that 1 mM amiloride affected intracellular pH in a way consistent with the presence of Na+/H+ exchange in ISOM. To further substantiate the presence of HCO3− transport under these conditions, the effect of anion transport inhibitor DIDS was evaluated. In these experiments, the tissue was preincubated with DIDS for ~30 min. In the last 15 min, 20 mM NH4Cl was present. DIDS (which washes out from the tissue very slowly) and NH4Cl were then washed out, and 1 mM amiloride was washed in (if DIDS and amiloride are present in solution simultaneously, precipitation forms). The rate of recovery from the acidosis in four experiments was inhibited 76 ± 7% in the presence of amiloride and DIDS relative to the recov-
ery rate in the absence of inhibitors. Without preincubation with DIDS (only amiloride present), the recovery from the acidosis was inhibited \(33 \pm 9\%\) relative to the recovery rate in the absence of amiloride in four experiments. The inhibition in the presence of DIDS was significantly \((P < 0.01)\) larger than in the absence of DIDS.

In contrast to these findings, there was no recovery from acidosis in the presence of amiloride and \(\text{HCO}_3^-\) in slices from mid-inner medulla (Fig. 5B). Furthermore, removal of Na in the presence of amiloride had little effect. In the absence of \(\text{HCO}_3^-\), the pattern was the same (Fig. 5B). This is consistent with little or no Na-coupled \(\text{HCO}_3^-\) transport in the cells of the midportion of the inner medulla.

Confocal Measurements

With the confocal microscope it was possible to visualize the individual segments of the nephron in the ISOM (Fig. 6). It was therefore possible to analyze the intracellular pH transients in the individual segments by choosing appropriate regions of interest. With this approach, it was confirmed that the \(\text{NH}_4\text{Cl}\)-induced intracellular pH transients seen in the time-resolved measurements were indeed reflecting changes in thick tubules in the inner stripe (Fig. 6, A-C). The data further confirmed that the Na-dependent recovery from acidosis in the presence of \(\text{HCO}_3^-\) and amiloride was present in the thick ascending limbs (Fig. 6E). Finally, by having a thin and a thick tubular structure in the same frame (Fig. 6D), it was possible to show that no Na- and \(\text{HCO}_3^-\)-dependent recovery from acidosis is present in the thin tubular structures in the presence of amiloride (Fig. 6E).

DISCUSSION

With the cloning of the kidney form of NBC1 (6, 27, 28), the tissue distribution of electrogenic Na-\(\text{HCO}_3^-\)
cotransport activity (4) could be determined at the cellular and subcellular level. These experiments confirmed the presence of NBC1 in the proximal tubule and demonstrated that NBC1 is present in the basolateral plasma membranes of the rat proximal tubule segments S1 and S2 (21, 29). Bicarbonate transport is also of significant importance in other nephron and collecting duct segments of the rat kidney where Cl/HCO_3^- exchange is known to play a significant role (2, 18, 30). The role of Na-dependent HCO_3^- transport outside the proximal tubule is, however, uncertain (25). In the present study we determined the cellular localization in the kidney of an electroneutral Na-HCO_3^- cotransporter. Our results demonstrate that NBC_N1 is present in the basolateral plasma membranes of thick ascending limb cells and medullary collecting duct intercalated cells. Thus the results suggest that NBC_N1 may participate in electroneutral Na-dependent HCO_3^- transport in these cells.

Immunoblot analyses with anti-NBC_N1 revealed an ~180-kDa protein in membrane fractions from rat kidney. The labeling was ablated after preabsorption of the antibody with the immunizing peptide, indicating specific labeling. The expected molecular mass of NBC_N1 is ~136 kDa (7). The 180 kDa seen here is therefore higher than expected. However, deglycosylation by PNGase F treatment reduced the molecular size to ~140 kDa, which is close to the predicted size for the gene product. Thus the immunoblotting data strongly support the view that the antibody recognizes NBC_N1.

Immunoblotting demonstrated that NBC_N1 is abundant in ISOM and less abundant in the outer stripe of the outer medulla and inner medulla. Very little label-
ing was observed in membrane fractions from cortex. Consistent with immunoblotting, immunohistochemistry demonstrated that NBCN1 is present in thick ascending limb cells. The labeling was exclusively associated with basolateral domains. This finding was substantiated by high-resolution immunoelectron microscopy.

Surprisingly, NBCN1 labeling was also found associated with intercalated cells of the inner medulla and faintly in collecting ducts in the ISOM. The cell type was documented by the use of immunoelectron microscopy. The labeling was exclusively present in the basolateral plasma membranes.

The relatively strong labeling of NBCN1 in thick ascending limbs suggests that Na-dependent Na-HCO$_3^-$ cotransport activity should be detectable in ISOM. To test this, we measured time-resolved intra-

Table 1. Recovery rates of pH, measured between 2 and 4 min after washout of NH$_4$Cl in the presence of 1 mM amiloride

<table>
<thead>
<tr>
<th>Condition</th>
<th>ISOM ($n = 5$)</th>
<th>IM ($n = 5$)</th>
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<tbody>
<tr>
<td>HCO$_3^-$</td>
<td>51 ± 6</td>
<td>-8 ± 9</td>
</tr>
<tr>
<td>Na$^+$-free HCO$_3^-$</td>
<td>-11 ± 5</td>
<td>-26 ± 10</td>
</tr>
<tr>
<td>No HCO$_3^-$</td>
<td>-7 ± 8</td>
<td>-37 ± 21</td>
</tr>
<tr>
<td>Na$^+$-free, no HCO$_3^-$</td>
<td>-13 ± 10</td>
<td>-56 ± 11</td>
</tr>
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Values are means ± SE of change in fluorescence ratio units/s. *n*, No. of experiments. Data were obtained in slices from both the inner stripe of the outer medulla (ISOM) and the midportion of the inner medulla (IM).

Fig. 5. The ratio of fluorescence of 2,7-bis-carboxyethyl-5,6-carboxyfluorescein (BCECF) when excited at 488 and 435 nm as a measure of intracellular pH (pH$_i$) transients in the ISOM (A) and IM (B) of rat kidney. Na$^+$ and HCO$_3^-$ were present as indicated, and amiloride was always present (added with NH$_4$Cl), except where indicated for trace 1 when amiloride was washed in and out. For reasons of presentation, the addition of NH$_4$Cl is only shown for trace 1. The other traces start immediately before NH$_4$Cl washout, which is indicated by arrows. The experiments were made at room temperature. A and B were obtained with different settings of the bandwidth of the excitation light, so the ratios between A and B are not comparable.

Fig. 6. Confocal images (A, B, and D) of BCECF-loaded structures in ISOM of rat kidney and time-resolved fluorescence emission measured from the marked structures (C and E). In A and B, 4 thick structures are seen running in parallel. The areas within the hatched rectangles in A and B correspond to the points A and B in C. In C, the fluorescence is seen during exposure to 20 mM NH$_4$Cl and Na-free conditions as indicated (typical of 4 experiments). In D, a thin structure (i) and a thick structure (ii) are seen. The fluorescence from these 2 structures during exposure to Na-free solution with 1 mM amiloride and Na-containing solution with 1 mM amiloride as indicated is seen (typical of 5 experiments). The experiments were made at room temperature and in the presence of 25 mM HCO$_3^-$, PSS, physiological salt solution.
cellular pH transients with BCECF in kidney slices obtained from ISOM and in slices from mid-inner medulla. The observation that a DIDS-sensitive and Na- and HCO₃⁻-dependent recovery from acidosis was seen in slices from the ISOM is consistent with the presence of a Na-HCO₃ cotransporter. In time-resolved measurements it is not possible to know from which cells the fluorescence is obtained, which weakens the interpretation. We therefore obtained measurements of spatially resolved intracellular pH transients by using confocal microscopy. The dominating structures in the images were the relatively thick tubular structures, of which the majority are thick ascending limbs with thick walls. It was also possible to recognize thin structures, which are likely to be thin limbs, although we cannot exclude that they could be vascular structures. When imaging the structures, we took great care to focus them so they had the greatest possible diameter. The dye did not distribute in a uniform way. A patchy distribution of BCECF has previously been reported for rabbit isolated cortical collecting tubules (32) under certain loading conditions. In the study by Weiner and Hamm (32), this dye distribution was reflecting uptake into different cell populations. Whether this is the case here or whether it reflects a nonuniform subcellular distribution of BCECF cannot be determined. In a preliminary analysis we therefore analyzed the bright spots and the more weakly stained areas separately. We found no qualitative differences in this analysis, and the basis for the data in Fig. 6 is therefore an analysis of the entire tubular structure. One concern with the use of the NH₄Cl technique to acid load the cells of the thick ascending limb is that the effect of NH₄Cl depends on whether luminal or basolateral application is used (15). Because we found alkalinization on addition of NH₄Cl and acidification with washout of NH₄Cl, it seems likely that the application in this setup is predominantly basolateral, which is consistent with lumen of the tubules running parallel with the tissue surface and being at least partly collapsed. The confocal data therefore strongly support the time-resolved measurements and are furthermore consistent with the lack of labeling of the thin limbs in the immunohistochemical experiments. The functional data thus provide support for the immunohistochemical observation that the electroneutral Na-dependent Na-HCO₃ cotransporter NBCN1 is present in thick ascending limb cells in ISOM.¹

In the inner medulla no Na- and HCO₃⁻-dependent recovery from acidosis could be demonstrated. This also supports the immunohistochemical data, which showed no NBCN1 immunolabeling in this part of the kidney (mid-inner medulla). Immunolabeling of NBCN1 was only present in the collecting duct intercalated cells, which are present only in the proximal part of the kidney inner medulla.

¹The effect of amiloride on the intracellular pH in slices from ISOM (in both the absence and presence of HCO₃⁻) is consistent with the presence also of an amiloride-sensitive Na/H exchange here.