Cloning and functional expression of rNBC, an electrogenic Na\(^{+}\)-HCO\(_3\)\(^{-}\) cotransporter from rat kidney

MICHAEL F. ROMERO,\(^1\) PEYING FONG,\(^1\) URS V. BERGER,\(^2\) MATTHIAS A. HEDIGER,\(^2\) AND WALTER F. BORON\(^1\)

\(^1\)Department of Cellular and Molecular Physiology, Yale University School of Medicine, New Haven, Connecticut 06520; and \(^2\)Renal Division, Department of Medicine, Brigham and Women’s Hospital and Harvard Medical School, Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, Massachusetts 02115

Rapid communication

Increased functional electrogenic sodium-bicarbonate cotransporter (NBC) in rat kidney.

METHODS

Cloning of Rat NBC cDNA

Library screening. The oligo(dT)-primed λgt10 cDNA library (28) was plated and grown until plaques were 1 mm in diameter, and filter lifts were screened at medium stringency (35°C for 14 h) with the EcoR1 fragment of Ambystoma NBC (aNBC, GenBank accession no. AF001958). The hybridization solution was 5× SSC (1× SSC is 0.15 M NaCl and 0.015 M sodium citrate, pH 7.0), 3× Denhardt's, 25 mM sodium-2-(N-morpholino)ethanesulfonic acid, 0.2% sodium dodecyl sulfate (SDS), 2.5 mM sodium pyrophosphate (pH 6.5), 10% dextran sulfate, 50% formamide, and 200 µg/ml yeast tRNA. Filters were washed with 1) three consecutive washes of 5× SSC, 0.05% sarcosyl, and 0.1% SDS at room temperature, 2) followed by several washes at 42°C over 1 h, and 3) several washes at 42°C with 0.1× SSC, 0.05% sarcosyl, and 0.1% SDS. Positive plaques were visualized by autoradiography, picked, and stored in SM buffer (25).

Isolation of rat NBC. Lambda phage DNA was isolated from plate lysates of pure plaques using the Lambda Midikit.
Cloning of Rat NBC

Table 1. Experimental solutions

<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na+, mM</td>
<td>96</td>
<td>100</td>
<td>0</td>
<td>96</td>
<td>0</td>
<td>96</td>
<td>96</td>
<td>0</td>
</tr>
<tr>
<td>Choline+, mM</td>
<td>0</td>
<td>0</td>
<td>96</td>
<td>0</td>
<td>96</td>
<td>0</td>
<td>96</td>
<td>0</td>
</tr>
<tr>
<td>K+, mM</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Mg2+, mM</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Ca2+, mM</td>
<td>1.8</td>
<td>0</td>
<td>1.8</td>
<td>1.8</td>
<td>1.8</td>
<td>1.8</td>
<td>1.8</td>
<td>1.8</td>
</tr>
<tr>
<td>Total cations, meq</td>
<td>100.8</td>
<td>103.0</td>
<td>100.8</td>
<td>100.8</td>
<td>100.8</td>
<td>100.8</td>
<td>100.8</td>
<td>100.8</td>
</tr>
<tr>
<td>Cl-, mM</td>
<td>100.8</td>
<td>103.0</td>
<td>100.8</td>
<td>76.8</td>
<td>76.8</td>
<td>0</td>
<td>90.8</td>
<td>90.8</td>
</tr>
<tr>
<td>Glucuronate, mM</td>
<td>0</td>
<td>0</td>
<td>76.8</td>
<td>76.8</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>HCO3-, mM</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>33</td>
<td>33</td>
<td>10</td>
<td>90.8</td>
<td>90.8</td>
</tr>
<tr>
<td>Total anions, meq</td>
<td>100.8</td>
<td>103.0</td>
<td>100.8</td>
<td>100.8</td>
<td>100.8</td>
<td>100.8</td>
<td>100.8</td>
<td>100.8</td>
</tr>
<tr>
<td>HEPES</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Pco3, % CO2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>1.5</td>
<td>1.5</td>
</tr>
<tr>
<td>pH</td>
<td>7.5</td>
<td>7.5</td>
<td>7.5</td>
<td>7.5</td>
<td>7.5</td>
<td>7.5</td>
<td>7.5</td>
<td>7.5</td>
</tr>
</tbody>
</table>

Choline solutions were titrated with choline-base. Glucuronate solutions were titrated with NaOH. CO2/HCO3- solutions were continuously bubbled with CO2-balanced O2 to maintain PCO2 and pH.

Northern Blot

A rat multiple tissue Northern blot was purchased from Clontech (Palo Alto, CA). The blot was probed with r715na, i.e., the entire 3.6 kb rat NBC (rNBC) cDNA in ExpressHyb (Clontech) at 65°C for 3 h, washed with 2× SSC-0.05% SDS at room temperature for 40 min, and finally washed with 1× SSC-0.1% SDS at 50°C for 40 min. Bound probe was visualized by autoradiography.

In Situ Hybridization

Digoxigenin-labeled antisense and sense runoff transcripts were synthesized from a polymerase chain reaction fragment, flanked by promoter sites for SP6 and T7 polymerases using a Genius Kit (Boehringer-Mannheim, Indianapolis, IN). Two different rNBC fragments were used as probes for in situ hybridization: nucleotides 143–2113 or nucleotides 2234–3495. After synthesis, the resulting RNAs were alkali- hydrolyzed to 200–400 nucleotide lengths. In situ hybridization was performed on cryosections (12 µm) of fresh-frozen rat kidney as previously described (26). We used a hybridization solution of 50% formamide, 5× SSC, 2% blocking reagent (Boehringer-Mannheim), 0.02% SDS, and 0.1% sarcosyl. The probe concentration was ~200 ng/ml. Sections were hybridized at 68°C for 18 h, washed three times in 2× SSC (68°C), and washed twice for 30 min each time in 0.2× SSC (68°C). The hybridized, labeled probes were visualized using anti-digoxigenin Fab fragments coupled to alkaline phosphatase (Boehringer-Mannheim) and 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium substrate (26). Sections were developed in substrate solution for 20 h, then rinsed in 10 mM tris(hydroxymethyl)aminomethane, 1 mM EDTA, pH 8.0, and covered with crystal mount (Fisher Scientific, Pittsburgh, PA). Adjacent sections were hybridized with a probe for the Na+-glucose transporter SGLT1, which hybridizes to the late S2 and S3 segments (15), to determine the tubule localization of rNBC mRNA.

Oocyte Experiments

Oocyte Experiments. Experimental solutions are detailed in Table 1. For oocyte preparation, a female Xenopus laevis was anesthetized with fresh 0.1–0.2% tricaine (3-aminobenzoic acid ethyl ester, methanesulphonate salt, catalog no. A-5040; Sigma, St. Louis, MO) in 5 mM N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (HEPES), pH 7.5, for 5 min and then packed in ice to cause hypothermia. After ovarian lobes were removed and placed in the 0 Ca2+-ND-96 solution, the skin incision was closed using 6-0 silk sutures. The frog was then placed in 0.1 M NaCl at room temperature until fully recovered from the anesthesia (usually 2–5 min); the frog remained in this NaCl solution for at least 12 h to facilitate healing. Oocytes in the pieces of ovary were enzymatically dissociated using 2 mg/ml type IA collagenase (catalog no. C-9891; Sigma) diluted in sterile-filtered (0.22-µm filter; Millipore, Bedford, MA) 0 Ca2+-ND-96 solution. After dissociating the oocytes, we selected stage V-VI oocytes and maintained them in sterile-filtered OR3 media at 18°C. Our OR3 media contained (in 2.0 liters) one pack of powdered Leibovitz L-15 media with l-glutamine (GIBCO-BRL no. 41300–039; Life Technologies, Gaithersburg, MD), 100 ml of 10,000 U penicillin and 10,000 U streptomycin solution in 0.9% NaCl (catalog no. P-0781; Sigma), 5 mM HEPES (final concentration). We titrated this solution to pH 7.5 at room temperature using NaOH. The solution had an osmolality of 195–200 mosmol/kg.

Oocyte injections. The original rNBC-CRNA (clone r715na) was transcribed in vitro from the T7 promoter of pBluescript 1

1. pTLN is a modification of pSP6 (13, 17), with the multiple cloning sites between the 5‘- and 3‘-UTRs of Xenopus β-globin sequences. pTLN was cut at XbaI and XhoI and treated with calf intestinal phosphatase. The modified polylinker was constructed by annealing of primers designed to produce the following sites: 5’ SplI-RsrII-SstI-NotI/EagI-NdeI-AvuII-BglII-BstEI 3’. The XhoI site was not regenerated in pTLN2. Linearization sites (3‘ of the β-globin 3‘-UTR) were unaltered.
KS (−) using the mCap mRNA Capping Kit (Stratagene, La Jolla, CA). We also subcloned rNBC into the Xenopus expression vector pTLN2, which is a modified version of pTLN (16), and used the SP6 promoter to transcribe the rNBC-pTLN2 cRNA (mMessage mMachine; Ambion, Austin, TX). Each oocyte was injected with 10 ng of cRNA (50 nl of a solution containing 0.2 µg/µl). Oocytes were studied 3–10 days after injection of cRNA.

Electrophysiology experiments. An oocyte, visualized with a dissecting microscope, was held on a nylon mesh in a chamber having a volume of ~250 µl. The oocyte was continuously superfused with a saline solution (3–5 ml/min) that was delivered through Tygon tubing. Solutions were switched using a daisy-chain system of computer-actuated five-way valves with zero dead space. Solution changes in the chamber occurred within 15–20 s. Membrane voltage (V_m) and intracellular pH (pH_i) of X. laevis oocytes were measured simultaneously using microelectrodes, as described previously (23). Briefly, V_m electrodes were pulled from borosilicate fiber-capped glass (Warner Instruments, West Haven, CT); these were backfilled with 3 M KCl and had resistances of 3–5 MΩ. The pH electrodes were pulled in a similar manner, silanized by exposing them for 5–10 min to 40 µl of am-(methyl-dimethylsilane (Fluka Chemical, Ronkonkoma, NY), deposited in an enclosed container at 200°C, and then baked overnight. These pH microelectrodes were cooled under vacuum, deposited in an enclosed container at 200°C, and then baked overnight. These pH microelectrodes were cooled under vacuum, deposited in an enclosed container at 200°C, and then baked overnight.

RESULTS AND DISCUSSION
Sequence Features of rNBC

With the expression cloning and sequencing of aNBC (23), we realized that the renal, electrogenic Na⁺-HCO₃ cotransporter and the anion exchangers (i.e., AE1, AE2, AE3) are both part of a superfamly of HCO₃ transporters. Figure 1A is a multiple alignment of the deduced amino acid sequences of rat and Ambystoma NBC, as well as representative AEs. The sequences aligned in Fig. 1 are the AE sequences that were found to be most similar to the NBC sequences. Not only are the predicted membrane-spanning domains (MSDs) closely related, but the ~340-amino acid NH₂ terminal to the first MSD also exhibit a high degree of homology (only identity is shown in Fig. 1A). Regions in which several consecutive amino acids are identical among NBC and the AEs may represent the signature of superfamily members. rNBC and aNBC are 86% identical at the amino acid level. A dendrogram summarizing the divergence among the rNBC, aNBC, rat cardiac AE3, human AE2, and human AE1 sequences is shown in Fig. 1B. The rNBC sequence diverges from aNBC by 13% but diverges from AE1, AE2, and AE3 (isomers above) by 61%, 61%, and 58%, respectively. Thus the "percent divergence" for this group indicates that rNBC is most closely related to aNBC, more distantly related to rat cardiac AE3, and most distantly related to human AE1 and AE2.

The phylogenetic plot of rNBC is virtually identical to that of aNBC and is consistent with the presence of at least 10 MSDs and a large extracellular loop between MSDs 5 and 6. The major divergences between rNBC and aNBC amino acid sequences occur at the NH₂ terminus (first 20 amino acids) and at the 5–6 loop region. In this 5–6 loop, aNBC is predicted to have four N-linked glycosylation sites, whereas rNBC is predicted to have only three sites (boxes with dots in Fig. 1; sites in Table 2). Similarly, there are consensus sites for protein kinase A, protein kinase C, casein kinase II, and tyrosine phosphorylation (see Table 2). As is the case for aNBC, rNBC contains several predicted myristylation sites (Table 2).

With rNBC added to the superfamily, two sequence features become more apparent. 1) The signature sequences for the superfamily are confirmed. 2) An additional site is suggested at which 4,4'-disothiocya-nostilbene-2,2'-disulfonic acid (DIDS) might covalently react with the protein. Biochemical experiments identified two lysine residues in AE1 with which DIDS reacts (2, 7, 19). The alignment of AE1, AE2, and AE3, as well as the knowledge of the first of the two DIDS reaction sites (human K539 or mouse K558), suggested a consensus amino acid sequence K(L/K)(X)K (X = I, V, Y) that might be characteristic of a DIDS reaction site (10). The cognate sequence in both Ambystoma and rat NBC is KM1K (558–561). In addition, at a more COOH-terminal site, both NBC clones have the original AE consensus motif sequence KL(K/K) (X = M, L and X = I, V, Y).

mRNA Distribution of rNBC

Northern analysis. Using high-stringency Northern analysis, we observed rNBC-mRNA at ~7.5 kb (Fig. 2). rNBC message is abundant in kidney (source of rNBC-cDNA) but also present in substantial amounts in liver and brain and at lower levels in lung, spleen, and heart. The r715na-cDNA that encodes the rNBC protein is ~3.6 kb, indicating that the message has additional UTRs of more than 4 kb. In Ambystoma, not only was the message smaller, but there was no reactivity in the liver, lung, and spleen, and an even smaller message (~2 kb) was in the heart (23).
Table 2. Putative modification sites of rNBC

<table>
<thead>
<tr>
<th>Predicted Type</th>
<th>Consensus Site</th>
<th>Sites</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-linked glycosylation</td>
<td>-P-[ST]-[P]</td>
<td>S92, S97, 617</td>
</tr>
<tr>
<td>PKA</td>
<td>[RK]-[2]-[X][ST]</td>
<td>S982</td>
</tr>
<tr>
<td>Tyrosine phosphorylation</td>
<td>[RK]-[X][2,3]-[DE]</td>
<td>Y897</td>
</tr>
</tbody>
</table>

Square brackets ([ ]) indicate that one of the listed amino acids is present; braces ({} ) indicate one of the optional amino acids may be present; "X" indicates that any amino acid may be present; and the number in parentheses is the number of the intervening amino acids, e.g., (2,3) means two or three intervening amino acids. rNBC, rat Na⁺-bicarbonate cotransporter; PKA and PKC, protein kinases A and C, respectively.

In situ hybridization. In the kidney, two antisense rNBC probes showed identical hybridization patterns. With sense probes, no hybridization was evident (data not shown). rNBC mRNA is strongly expressed in PTs at the corticomedullary junction (Fig. 3A). Comparison of the in situ hybridization patterns of rNBC (Fig. 3B) and SGLT1 (Fig. 3C), which localizes to the straight portion of the S2 as well as to the S3 segment of the PT (15), suggests that rNBC localizes to the straight portion of the S2 segment. rNBC mRNA expression was not detected in any other tubule segment.

The in situ localization of NBC is more restricted spatially than the well-documented Na⁺-HCO₃⁻ cotransporter, which has been detected in S1 and convoluted segments of S2 (1, 3, 8, 9, 12, 20, 30, 31), as well as S3 (8, 14, 18). Several polyclonal antibodies developed in one of our laboratories (W. F. Boron) demonstrate strong and specific basolateral immunoreactivity in S1 and S2 segments of rabbit and rat kidneys (Ref. 27; M. O. Bevensee and B. M. Schmitt, unpublished observations). The differences between the in situ hybridization and immunocytochemistry data could reflect differences in the sensitivities of the methods, real differences in the relative abundances of NBC mRNA vs. NBC protein, or differences in detected NBC isoforms.

Functional Properties of rNBC in Xenopus Oocytes

Expression of “native” rNBC. To confirm that the isolated rat kidney clone encodes an electronegatic Na⁺-HCO₃⁻ cotransporter, we injected cRNA from the clones into Xenopus oocytes to evaluate cotransporter activity (see METHODS). Figure 4A shows that, unlike the salamander clone, expression of rNBC subcloned into pBlue-script was weak, as judged by the responses to the expression assay (METHODS). First, adding 5% CO₂/33 mM HCO₃⁻ (pH 7.5) caused the rNBC-expressing oocyte to hyperpolarize by only 15–20 mV (due to Na⁺ and HCO₃⁻ influx). By contrast, in oocytes expressing the salamander clone, one-third of the [HCO₃⁻], 1.5% CO₂/10 mM HCO₃⁻ typically caused a hyperpolarization of ~50 mV. In water-injected controls, addition of 5% CO₂/33 mM HCO₃⁻ caused a depolarization of about +20 mV, rather than a hyperpolarization. Sec-
ond, removing Na\(^+\) (choline replacement) in the continued presence of 5% CO\(_2\)/33 mM HCO\(_3\) depolarized rNBC-expressing oocytes (due to electrogenic Na\(^+\)/HCO\(_3\) efflux), but only by 5–10 mV. Once again, expression of salamander NBC was more robust than that of rNBC. Removing extracellular Na\(^+\) from the 1.5% CO\(_2\)/10 mM HCO\(_3\) solution bathing oocytes expressing the salamander NBC elicited a 50-mV depolarization. In water-injected control oocytes, removing Na\(^+\) from even the 5% CO\(_2\)/33 mM HCO\(_3\) solution hyperpolarized the oocytes by 3–5 mV, rather than depolarizing them. Removing Cl\(^-\) (gluconate replacement) did not alter pH\(_i\) or V\(_m\) in oocytes expressing rNBC.

Expression of rNBC in a Xenopus expression vector. Despite the 86% identity to the aNBC coding region, oocytes expressed the rNBC clone poorly when compared with oocytes expressing aNBC (23). We entertained the hypothesis that Xenopus oocytes would express rNBC better if the 5'- and 3'-UTRs were more typical of those present in Xenopus. Therefore, we subcloned the original r715na cDNA, which included the coding sequence and 500 bp of the rat 3'-UTR, into the Xenopus expression vector, pTLN2. As shown in Fig. 4, B and C, expression is increased substantially when this r715na cRNA is flanked by the Xenopus \(\beta\)-globin UTRs. Thus, in an oocyte expressing rNBC in the pTLN2 vector, adding one-third the [HCO\(_3\)] elicited a hyperpolarization three- to fourfold greater (60 mV with 10 mM HCO\(_3\) rather than 15–20 mV obtained with 33 mM HCO\(_3\) and the other vector). After the initial CO\(_2\)-induced acidification, the experiment in Fig. 4C shows a pH\(_i\) recovery, due to continued Na\(^+\)/HCO\(_3\) influx into the oocyte. Subsequently removing Na\(^+\) elicited a depolarization of some 60 mV and rapidly decreased pH\(_i\) (Fig. 4C). This depolarization...
was far larger than the 5- to 10-mV depolarization observed with the r715na-pBluescript clone, obtained with a threefold higher [HCO₃⁻] (Fig. 4, A and B).

In light of our results with rNBC in two different vectors, it is interesting to note that we had found it impossible to expression clone rNBC from rabbit kidney (23). It may be that the expression in the Xenopus oocyte system of mammalian NBC depends critically on the presence of 5' and/or 3' amphibian UTRs, which may enhance the initiation of translation and/or the stability of the rRNA.

In summary, we have cloned an electrogenic Na⁺-HCO₃⁻ cotransporter from the rat kidney, rNBC. Not only is rNBC 86% identical to our original Ambystoma NBC, nNBC, but the mRNA is predominantly expressed in the kidney. Functional expression of rNBC in Xenopus oocytes illustrates that rNBC is electrogenic, dependent on Na⁺, and dependent on HCO₃⁻.

We thank Dr. Chairat Shayakul for providing the rat kidney cortex cDNA library. We thank Prof. Thomas Jentsch for the gift of the pTLN vector. We also thank Drs. Mark O. Bevensee and Bernhard M. Schmitt for sharing unpublished antibody data and for helpful comments on the manuscript.

This work was supported by National Institute of Diabetes and Digestive and Kidney Diseases Grants DK-30344 (to W. F. Boron), DK-40163 (to M. A. Hediger), and DK-09342 (to M. F. Romero) and by the American Heart Association (to M. F. Romero). This work was supported by National Institute of Diabetes and Digestive and Kidney Diseases Grants DK-30344 (to W. F. Boron), DK-40163 (to M. A. Hediger), and DK-09342 (to M. F. Romero) and by the American Heart Association (to M. F. Romero) and by Digestive and Kidney Diseases Grants DK-30344 (to W. F. Boron), DK-40163 (to M. A. Hediger), and DK-09342 (to M. F. Romero) and by

REFERENCES


14. Kurtz, I. Basolateral membrane Na\textsuperscript{+}/H\textsuperscript{+} antiport, Na\textsuperscript{+} cotransport, and Na\textsuperscript{+}-independent Cl\textsuperscript{-}/base exchange in the rabbit S3 proximal tubule. J. Clin. Invest. 83: 616–622, 1989.


