The Na\(^{+}\)-driven Cl\(^{-}\)/HCO\(_3\)\(^{-}\) Exchanger

**CLONING, TISSUE DISTRIBUTION, AND FUNCTIONAL CHARACTERIZATION**

Chang-Zheng Wang, Hideki Yano, Kazuaki Nagashima, and Susumu Seino

From the Department of Molecular Medicine, Chiba University Graduate School of Medicine, 1-8-1 Inohana, Chuo-ku, Chiba 260-8670, Japan

The Na\(^{+}\)-driven Cl\(^{-}\)/HCO\(_3\)\(^{-}\) exchanger is an important regulator of intracellular pH in various cells, but its molecular basis has not been determined. We show here the primary structure, tissue distribution, and functional characterization of Na\(^{+}\)-driven chloride/bicarbonate exchanger (designated NCBE) cloned from the insulin-secreting cell line MIN6 cDNA library. The NCBE protein consists of 1088 amino acids having 74, 72, and 55\% amino acid identity to the human skeletal muscle, rat smooth muscle, and human kidney sodium bicarbonate cotransporter, respectively. The protein has 10 putative membrane-spanning regions. NCBE mRNA is expressed at high levels in the brain and the mouse insulinoma cell line MIN6 and at low levels in the pituitary, testis, kidney, and ileum. Functional analyses of the NCBE protein expressed in Xenopus laevis oocytes and HEK293 cells demonstrate that it transports extracellular Na\(^{+}\) and HCO\(_3\)\(^{-}\) into cells in exchange for intracellular Cl\(^{-}\) and H\(^{+}\), thus raising the intracellular pH. Thus, we conclude that NCBE is a Na\(^{+}\)-driven Cl\(^{-}\)/HCO\(_3\)\(^{-}\) exchanger that regulates intracellular pH in native cells.

Regulation of intracellular pH (pH\(_i\))\(^{1}\) in response to various stimuli is critical in many cellular functions (1–4). A family of bicarbonate transporters is the major pH\(_i\) regulator under physiological conditions in animal cells (5). Bicarbonate transporters are divided functionally into four groups (5): the Na\(^{+}\)-independent Cl\(^{-}\)/HCO\(_3\)\(^{-}\) exchanger (alternatively called an anion exchanger (AE)); the Na\(^{+}\)-HCO\(_3\)\(^{-}\) cotransporter (NBC); the K\(^{+}\)-HCO\(_3\)\(^{-}\) cotransporter; and the Na\(^{+}\)-driven Cl\(^{-}\)/HCO\(_3\)\(^{-}\) exchanger. Three AEs (3) and three NBCs (6–10) have been cloned and functionally characterized, but the molecular structure of the K\(^{+}\)-HCO\(_3\)\(^{-}\) cotransporter and the Na\(^{+}\)-driven Cl\(^{-}\)/HCO\(_3\)\(^{-}\) exchanger has remained unknown.

The Na\(^{+}\)-driven Cl\(^{-}\)/HCO\(_3\)\(^{-}\) exchanger was first discovered in invertebrate neurons (11) and was found later in vertebrate neurons and non-neuronal cells, including the brain (12), vascular endothelial cells (13), sperm (14), kidney (15), and pancreatic \(\beta\)-cells (16). The Na\(^{+}\)-driven Cl\(^{-}\)/HCO\(_3\)\(^{-}\) exchanger is an intracellular pH regulator that transports extracellular Na\(^{+}\) and HCO\(_3\)\(^{-}\) into cells in exchange for intracellular Cl\(^{-}\) and H\(^{+}\) playing an important role in cellular alkalinization (5, 11).

In pancreatic islet \(\beta\)-cells, glucose is physiologically the most important regulator of insulin secretion. It has been shown that glucose metabolism induces an increase in pH\(_i\) in pancreatic \(\beta\)-cells (17–21) and that this glucose-induced rise in pH\(_i\) is evoked primarily by the action of the Na\(^{+}\)-driven Cl\(^{-}\)/HCO\(_3\)\(^{-}\) exchanger (16). To determine the structure and functional roles of the Na\(^{+}\)-driven Cl\(^{-}\)/HCO\(_3\)\(^{-}\) exchanger, we attempted to clone the Na\(^{+}\)-driven Cl\(^{-}\)/HCO\(_3\)\(^{-}\) exchanger from the mouse insulin-secreting cell line, MIN6. We describe here the primary structure, tissue distribution, and functional properties of a Na\(^{+}\)-driven Cl\(^{-}\)/HCO\(_3\)\(^{-}\) exchanger, designated NCBE.

**EXPERIMENTAL PROCEDURES**

cDNA Cloning and RNA Blot Analysis—A partial cDNA fragment of human kidney NBC cDNA (7) was amplified by polymerase chain reaction (PCR) using a human kidney cDNA as a template. The sense and antisense primers used were 5\' -TTTGAGAAAACCCCTGTT-3\' (nt 2323–2350) and 5\' -GAGCATCCAGGAGACTCTG-3\' (nt 2912–2931). PCR was performed for 40 cycles under the following conditions: denaturation at 94°C for 15 s, annealing at 60°C for 30 s, and extension at 72°C for 45 s in a thermal cycle GeneAmp PCR system 9600 (Applied Biosystems, Foster City, CA). The 700-base pair PCR product was cloned into Toyobo pGEM5Z vector and the sequence confirmed by a DNA sequencer.

RNA blot analysis was performed using 10 µg of total RNA from the various tissues and cells. The RNAs were denatured with formaldehyde, electrophoresed on a 1% agarose gel, and transferred to a nylon membrane. The blot was probed with NCBE cDNA (1.1–840) under the standard conditions described previously (24). The blots were washed in 0.1\% SSC and 0.1\% SDS at room temperature for 1 h and then at 50°C for 1 h before autoradiography.

Reverse Transcription (RT)-PCR—Total RNA was prepared with TRIZOL reagent (Life Technologies, Inc.) from isolated mouse pancreatic islets. First-strand cDNA (10 ng) was generated using Superscript\textsuperscript{TM} II reverse transcriptase (Life Technologies) with random primers. PCR was performed with the Expand high fidelity PCR system (Roche Diagnostics, Mannheim, Germany) using about 1 ng of template DNA in a 20-µl reaction volume under standard conditions. The sense and antisense primers used were 5\' -TTTGGTATAGCGACACT-3\' (nt 4320–4326) and 5\' -TGGTTAAACGGACACTCTG-3\' (nt 4911–4928). The product was resolved on a 1% agarose gel and confirmed by DNA sequences in the on-line version of this article (available at http://www.jbc.org).
Cloning and Characterization of a Na⁺-driven Cl⁻/HCO₃⁻ Exchanger

TABLE I
Composition of experimental solutions

Solutions A, B, C, D, E, and F for experiments with oocytes were used as standard solution, Na⁻-free solution, Cl⁻-free solution, HCO₃⁻-free solution, and HCO₃⁻-free washing solution, respectively. The pH of all solutions was adjusted to 7.4. HCO₃⁻ solutions were bubbled with 100% O₂ to remove trace CO₂ and HCO₃⁻. Solutions G, H, I, J, K, L, M, N, and O for experiments with HEK293 cells were used as Na⁻-free solution, NH₄Cl-containing Na⁻-free solution, Na⁺-containing solution, Na⁺- and HCO₃⁻-free solution, Na⁺- and Cl⁻-free solution, Na⁺- and HCO₃⁻-free solution, respectively. All solutions were bubbled with 95% O₂ and 5% CO₂ and adjusted to pH 7.4.

| Compound | Solutions for oocytes | Solutions for HEK293 cells |
|----------|----------------------|--------------------------|
|          | A  B  C  D  E  F  G  H  I  J  K  L  M  N  O |
| NaCl     | 100  70  80  96  115  75  90  115  75  25  10  10  10  10  10  10 |
| TMA-Cl   | 100  115  75  90  115  75  25 |
| KCl      | 2   2   2   2   1   1   1   1   1   1   1   1   1   1   1   1   1   1 |
| MgCl₂    | 1   1   1   1   1   1   1   1   1   1   1   1   1   1   1   1   1   1 |
| CaCl₂    | 115  115  75  75  25  25  25  25  25  25  25  25 |
| NaHCO₃   | 8   8   10  5   5   5   10  10  10  10  10  10  10  10  10  10  10 |
| Heps     | 8   16  16  8   16  16  8   8   8   8   8   8   8   8   8   8   8   8 |
| Choline-HCO₃ | 100  100  10 |
| Sodium butyrate | 10 |
| NH₄Cl    | 25  25  25  25  25  25  25 |
| KClO₃    | 40  40  40  40 |
| MgCl₂    | 1   1   1   1   1   1   1   1   1   1   1   1   1   1   1   1   1   1 |
| Calcium gluconate | 1 |
| Glutamine | 1 |
| Potassium gluconate | 2 |
| Magnesium gluconate | 1 |
| Calcium gluconate | 1 |

sequencing. The coding sequence of NCBE in pBDs was linearized by digestion with FspI and in vitro transcribed with SP6 RNA polymerase as described previously (24). Defolliculated oocytes were injected with NCBE cRNA (50 nl, 0.5 μg/μl) or water and incubated for 3–5 days at 18 °C before the studies. For the study of 22Na⁺ activity, solutions H, K, and N were used, and the results were compared with control and NCBE-transfected cells. All solutions were bubbled with 95% O₂, 5% CO₂, and 100% N₂, adjusted to pH 7.4. The osmolarity of each solution was adjusted with sucrose. The assays were carried out at 37 °C.

Statistics—The results are expressed as means ± S.E. The statistical significance between each experiment was determined by Student’s t test.

RESULTS AND DISCUSSION

Cloning of the NCBE cDNA and Predicted Protein—Using a partial human kidney NBC (7) cDNA as a probe, we cloned a cDNA encoding NCBE by screening a MIN6 cdNA library. The composite nucleotide sequence of 5385 base pairs contains an open reading frame following an in-frame termination signal upstream of the ATG, which encodes a protein of 1088 amino acids with a predicted molecular mass of 122 kDa (GenBank™/EBI/DDBJ data bank with accession no. AB033759, available on-line as Fig. 1S). A hydropathy analysis suggests that NCBE has 10 putative membrane-spanning segments (26). There are three potential N-linked glycosylation sites in the extracellular loops between the third (TM3) and fourth (TM4) membrane-spanning region (Asn-647, Asn-657, and Asn-667). Comparison of the amino acid sequences between NCBE and other bicarbonate transporters shows that NCBE has 74, 72, 55, 49, 38, and 34% amino acid identity to human skeletal muscle NBC...
(9), rat smooth muscle NBC (8), human kidney NBC (7), Drosophila Na⁺-driven anion exchanger (NDAE1) (30), mouse brain AE3 (31), and mouse erythrocyte AE1 (32), respectively, indicating that NCBE represents a novel bicarbonate transporter. NCBE is also homologous to several members of a bicarbonate transporter superfamily, the functional properties of which have not yet been characterized. NCBE has 76, 74, 52, and 49% amino acid identity to an NBC-related clone from human brain (SLC4A8, GenBank™ accession no. NM004858), putative human retinal NBC (33), Drosophila gene product alt 2 (GenBank™ accession no. AAF52497), and Drosophila gene product alt 2 (GenBank accession no. AAF52497), respectively. The amino acid sequence in the putative transmembrane regions is well conserved between NCBE and members of the bicarbonate transporter superfamily, whereas the intracellular amino- and carboxyl-terminal regions and a large extracellular loop between the third and fourth membrane-spanning region are rather divergent.

Tissue Expression of NCBE—RNA blot analysis revealed a 5.5-kilobase NCBE mRNA expressed at high levels in brain and insulin-secreting cell line MIN6 cells and expressed at low levels in the pituitary, testis, kidney, and ileum (Fig. 1). RT-PCR analysis shows that NCBE mRNA is also expressed in pancreatic islets (Fig. 1b).

Functional Expression of NCBE in Xenopus Oocytes and HEK293 Cells—We first examined the functional properties of NCBE using Xenopus oocyte system. 22Na⁺ and 36Cl⁻ uptake or efflux were measured 3–5 days after injection of the cRNAs into oocytes injected with water (open columns) or NCBE cRNA (filled columns) 3–5 days after injection was measured in standard solution (solution A), Na⁺-free solution (solution B), Cl⁻-free solution (solution C), HCO₃⁻-containing solution (solution D), HCO₃⁻-free solution (solution E) and standard solution in the presence of 500 μM DIDS (solution A + DIDS). a, effects of different ions on 22Na⁺ uptake in oocytes injected with NCBE cRNA or water. b, effects of different ions on 36Cl⁻ uptake in oocytes injected with NCBE cRNA or water. c, effects of different ions on 36Cl⁻ efflux in oocytes injected with NCBE cRNA. The percent Cl⁻ efflux is shown. d, functional properties of NCBE during the period of decrease in pH. The direction of ion movement through NCBE under various conditions is shown: 1, physiological; 2, extracellular Cl⁻-free; 3, extracellular Na⁺-free; 4, HCO₃⁻-free. Thin arrows indicate decreased activity. Note that solutions A, B, and C were bubbled with 1.5% CO₂, but solutions D and E were without CO₂. Results were obtained from 2 to 3 independent experiments. The values represent the mean ± S.E. of 7–22 oocytes from each experiment.

In contrast, the 22Na⁺ uptake in NCBE-expressing oocytes was almost zero (n = 10) in Na⁺-free solution (solution B), but it increased in an extracellular Na⁺ concentration-dependent manner (data not shown), indicating that the activity of NCBE depends on extracellular Na⁺. When oocytes were acidified with butyric acid rather than CO₂ (8), the 22Na⁺ uptake in NCBE-expressing oocytes in HCO₃⁻-containing solution (solution D) was 19 ± 2.8 nmol/oocyte/h (n = 8). Thus, acidification with butyric acid is also effective on uptake of 22Na⁺. To determine the effect of HCO₃⁻, we used HCO₃⁻-free solution (solution E) with butyric acid without CO₂. Under this condition, 22Na⁺ uptake was significantly decreased (3.1 ± 0.5 nmol/oocyte/h, n = 8, p < 0.05), indicating that extracellular HCO₃⁻ is required to transport Na⁺ into cells. We then examined the effect of Cl⁻ on 22Na⁺ uptake. 22Na⁺ uptake in NCBE-expressing oocytes in solution C was 18.6 ± 1.6 nmol/oocyte/h (n = 16), decreased to 50% of that in standard solution, indicating that extracellular Cl⁻ accelerates NCBE activity and that there is 22Na⁺ uptake even under extracellular Cl⁻-free conditions (also see on-line supplemental information, Table II). To determine whether extracellular Cl⁻ accelerates NCBE activity and whether intracellular Cl⁻ is exported by NCBE, we measured the uptake and efflux of 36Cl⁻. 36Cl⁻ uptake was measured in NCBE-expressing oocytes or control oocytes injected with water in solution A, B, C, or E. Although control oocytes showed no increase in 36Cl⁻ uptake, NCBE-expressing oocytes showed a significant increase in 36Cl⁻ uptake in all solutions.

Fig. 1. Expression of NCBE mRNA. a, tissue distribution of NCBE mRNA. RNA blot analysis of NCBE mRNA in various rat tissues and hormone-secreting cell lines is shown. The size of the hybridized transcripts is indicated. Sk., skeletal; Pan., pancreatic; kb, kilobases. b, RT-PCR detection of NCBE mRNA in mouse pancreatic islets. DNA length markers and RT-PCR products are shown in lanes 1 and 2, respectively.

Fig. 2. Functional analysis of NCBE in Xenopus laevis oocytes. a, effects of different ions on 22Na⁺ uptake in oocytes injected with NCBE cRNA or water. 22Na⁺ uptake (expressed as nmol/oocyte/h) in oocytes injected with water (open column) or NCBE cRNA (filled column) 3–5 days after injection was measured in standard solution (solution A), Na⁺-free solution (solution B), Cl⁻-free solution (solution C), HCO₃⁻-containing solution (solution D), HCO₃⁻-free solution (solution E) and standard solution in the presence of 500 μM DIDS (solution A + DIDS).
versus 32 cyttes in solution A was 41 mmol/oocyte/h (n = 7) in solutions A, B, C, D, and E, respectively. The increase in $^{36}\text{Cl}^-$ uptake of NCBE-expressing oocytes in these solutions indicates that NCBE transports extracellular Cl$^-$ into the cell and that the importing activity of Cl$^-$ is significantly increased in the absence of extracellular Na$^+$ (solution A versus solution B, p < 0.05) or HCO$_3^-$ (solution D versus solution E, p < 0.05). We then measured the Cl$^-$ efflux of the NCBE-expressing oocytes (Fig. 2c). The rate (%) of $^{36}\text{Cl}^-$ efflux was 74.7 ± 2.8 (n = 8), 43.0 ± 2.0 (n = 9), 42.3 ± 1.3 (n = 17), 48.0 ± 4.0 (n = 8), and 17.0 ± 4.0% (n = 8) in solutions A, B, C, D, and E, respectively (solution A versus other solutions, p < 0.05). These results indicate that: 1) 75% of intracellular $^{36}\text{Cl}^-$ is exported out of the cell in standard solution, 2) extracellular Na$^+$ is necessary for exporting intracellular Cl$^-$, 3) extracellular HCO$_3^-$ is essential for exporting intracellular Cl$^-$, and 4) extracellular Cl$^-$ accelerates Na$^+$ uptake and Cl$^-$ efflux. Because Cl$^-$ can be transported into and out of the cell when NCBE-expressing oocytes are acidified, the transporting activity appears to be bidirectional. The functional properties of NCBE under the various conditions were measured in HEK293 cells transiently transfected with NCBE. All experiments were carried out under the physiological condition (Fig. 2d-1) or the extracellular Cl$^-$-free condition (Fig. 2d-2) during the period of decrease in pH$_i$ is forward, whereas the ion movement through NCBE under the extracellular Na$^+$-free (Fig. 2d-3) or HCO$_3^-$-free (Fig. 2d-4) condition is reverse. Taken together, these findings suggest that NCBE exchanges extracellular Na$^+$ and HCO$_3^-$ with intracellular Cl$^-$ under the physiological condition, that is, in the presence of extracellular Na$^+$, HCO$_3^-$, and Cl$^-$, when the cells are acidified. We also examined the effect of DIDS on $^{22}\text{Na}^+$ uptake and $^{36}\text{Cl}^-$ efflux. $^{22}\text{Na}^+$ uptake in NCBE-expressing oocytes in standard solution was 6.0 ± 0.7 nmol/oocyte/h (n = 14) in the presence of 300 μM DIDS, indicating that DIDS decreased $^{22}\text{Na}^+$ uptake to 20% of that in solution A (Fig. 2a). The $^{36}\text{Cl}^-$ efflux in NCBE-expressing oocytes in solution A was 41 ± 2% (n = 9) in the presence of 300 μM DIDS. Although DIDS decreased the $^{36}\text{Cl}^-$ efflux to 55%, the value of $^{36}\text{Cl}^-$ efflux was at almost the same level as in solution C (Fig. 2c).

To clarify the role of NCBE in regulating pH$_i$, pH changes under the various conditions were measured in HEK293 cells transiently transfected with NCBE. All experiments were carried out in acidified pH$_i$ conditions with NH$_4$-prepulse (with solutions H, K, or N). To determine whether the change in pH$_i$ is dependent on extracellular Na$^+$, the environment of the cells was changed from Na$^+$-free solution (solution G) to Na$^+$-containing solution (solution I). In the presence of 1 mM 5-(N-ethyl-N-isopropyl)amiloride (EIPA), a specific inhibitor of the Na$^+$/H$^+$ exchanger, rapid pH$_i$ recovery ($\Delta$H$_i$) was observed only in the NCBE-transfected cells (ΔpH$_i$ was 0.238 ± 0.028 in NCBE-transfected cells (n = 97) and 0.003 ± 0.015 in control (n = 70), p < 0.05) (Fig. 3a). This pH$_i$ recovery was partially inhibited by 300 μM DIDS (ΔpH$_i$ was 0.023 ± 0.042 (n = 89), p < 0.05) (Fig. 3a). To determine whether the change in pH$_i$ is bicarbonate-dependent, the environment of NCBE-transfected cells was changed from HCO$_3^-$-free and Na$^+$-free solution (solution J) to HCO$_3^-$-free but Na$^+$-containing solution (solution L) in the presence of 1 mM EIPA. No pH$_i$ recovery was detected (ΔpH$_i$ was 0.002 ± 0.014 (n = 71)) (Fig. 3b). We also examined Cl$^-$ dependence. NCBE-transfected cells were kept in Cl$^-$-free solution (under the intracellular Cl$^-$-depleted condition) throughout the experiments. Under this condition, the environment of the cells was changed from Na$^+$-free (solution M) to Na$^+$-containing solution (solution O). In the presence of 1 mM EIPA, pH$_i$ recovery was not detected (ΔpH$_i$ was 0.067 ± 0.012 (n = 95)) (Fig. 3c). These results show that pH$_i$ recovery from intracellular acidification is detected only in the presence of extracellular Na$^+$, HCO$_3^-$, and intracellular Cl$^-$.

Functional studies of NCBE heterologously expressed in Xenopus oocytes and HEK293 cells show that NCBE causes pH$_i$ recovery from acute intracellular acidification by transporting extracellular Na$^+$ and HCO$_3^-$ in exchange for intracellular Cl$^-$ in the presence of extracellular Na$^+$, HCO$_3^-$, and Cl$^-$. NCBE is functionally distinct from the anion exchangers (9, 10), because NCBE-expressing oocytes show an increase in $^{22}\text{Na}^+$ uptake that is dependent on Cl$^-$ and HCO$_3^-$, and NCBE-expressing HEK293 cells show an increase in pH$_i$ that is dependent on extracellular Na$^+$, HCO$_3^-$, and Cl$^-$. These properties are similar to those of the Na$^+$-driven Cl$^-$/HCO$_3^-$ exchanger. The functional properties of NCBE are different from those of the recently identified Drosophila NDAE1, which does not require HCO$_3^-$ for transport activity (30).

Expression of NCBE mRNA in insulin-secreting cell line MIN6 and pancreatic islets implies its physiological relevance.
It has been shown that glucose-induced insulin secretion is accompanied by a rise in $pH_i$ in pancreatic islet $\beta$-cells (17–21). Although several $pH_i$ regulators have been suggested to be present in pancreatic $\beta$-cells (16, 21), the molecular basis of these regulators is not known. NCBE is the first $pH_i$-regulating exchanger of which the primary structure and functional properties have been determined. NCBE mRNA also is present in the testis, although its level is low. It has been shown that $pH_i$ regulates many sperm functions including sperm capacitation (1, 2, 14). Because sperm capacitation results in $pH_i$ acidification by glucose metabolism. NCBE mRNA also is present in hippocampal neurons and astrocytes, but its physiological significance in such cells is not known at present. Further investigation is necessary to clarify the structure and function relationships of NCBE and its physiological roles in various tissues.

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