Molecular and Functional Characterization of the Electroneutral Na/HCO3 Cotransporter NBCn1 in Rat Hippocampal Neurons*

Received for publication, July 29, 2004, and in revised form, February 17, 2005

Published, JBC Papers in Press, February 17, 2005, DOI 10.1074/jbc.M408646200

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We examined molecular and electrophysiological properties of the electroneutral sodium/bicarbonate cotransporter (NBCn1) that is present in rat hippocampal neurons. By PCR, a deletion variant (NBCn1-E) that lacks 123 amino acids in the cytoplasmic N-terminal domain was found in adult neurons. The previously characterized NBCn1-B, which does not have the deletion, was detected in embryonic neurons. In Xenopus oocytes, NBCn1-E raised the intracellular pH in the presence of HCO3 without significantly affecting the membrane potential. Despite this electroneutral cotransport activity, the transporter mediated a steady-state current that positively shifted the resting potential by almost 30 mV. The mean reversal potential of the steady-state current was −21.2 mV, close to the resting potential of −21.4 mV. The reversal potential shifted 26 mV in response to a 10-fold increase of external Na⁺ for concentrations above 10 mM. The current activity mediated by the transporter was unaffected by K⁺, Mg²⁺, Ca²⁺, or Cl⁻. Stable expression of NBCn1-E in human embryonic kidney cells also evoked an inward current that shifted the resting potentials more positive compared with the sham-transfected controls. In primary cultures of embryonic hippocampal neurons, the NBCn1 protein was localized in somatodendrites and synapses. NBCn1 protein was partially colocalized with the postsynaptic density protein PSD-95. Single-cell PCR showed that NBCn1 mRNA expression was present in both γ-aminobutyric acid (GABA)ergic and non-GABAergic neurons. We propose that NBCn1 in hippocampal neurons may affect neuronal activity by regulating local pH as well as steady-state inward currents at synapses.

The steady-state intracellular pH in a typical neuron in situ is 7.0–7.4, which is about 1 unit higher than the pH expected from the electrochemical equilibrium of hydrogen ions (for review see Ref. 1). Small changes in pH modulate the activity of many enzymes, ion channels and receptors and, therefore, can significantly influence neuronal function (2–5). An abrupt change in the intracellular pH (pHi) or extracellular pH (pHₒ) can be neurotoxic. Acidosis can lead to coma, whereas severe alkalosis can induce seizures (for review see Ref. 6). Therefore, neurons must have precise regulatory mechanisms to respond to changes in pHᵢ and/or pHₒ. The sodium/bicarbonate transporters are a subset of acid-base transporters specialized to move Na⁺ and HCO₃⁻. Except for the one present in the proximal tubules of the kidney, most sodium/bicarbonate transporters move Na⁺ and HCO₃⁻ into cells and raise the pHᵢ as HCO₃⁻ titrates H⁺. The principal function of these transporters is to extrude acid equivalents from cells, balance acid loads, and maintain the pHᵢ within a physiological range.

In hippocampal neurons, HCO₃⁻-dependent acid extrusion is thought to be governed by a Na⁺-driven Cl/HCO₃ exchanger (7–9). This transporter moves Na⁺ and HCO₃⁻ into cells in exchange for internal Cl⁻. In CA1 pyramidal neurons acutely isolated from 4- to 14-day-old rats, the Na⁺-driven Cl/HCO₃ exchanger dominates the recovery of pHᵢ from an acid load and can be inhibited by 4,4'-diisothiocyanato-2,2'-disulfonate stilbene (DIDS) (7). In hippocampal neurons, acid extrusion is mediated by the Na⁺-driven Cl/HCO₃ exchanger and the amiloride-insensitive Na/H exchanger (8, 9). However, nearly 80% of the HCO₃⁻-dependent pH recovery can be insensitive to DIDS in these embryonic neurons (9). These data suggest the presence of an additional acid extruder.

To date, five types of sodium/bicarbonate transporters have been cloned in mammals: NBCn1, NBCe1, NBCe2, NDCBE1, and NCBE (10–12). NBCn1 is an electroneutral Na/HCO₃ cotransporter. NBCe1 and NBCe2 are electroneutral Na/HCO₃ transporters with the stoichiometry of 1 Na⁺ and no less than 2 HCO₃⁻. NDCBE1 and NCBE are electroneutral Na⁺-driven Cl/HCO₃ exchangers. The genes for these transporters comprise the bicarbonate transporter superfamily SLC4A, which also contains three Cl/HCO₃ exchangers AE1–3, the functionally controversial AE4, and the uncharacterized BTR1 (13). NCBE and NBCe1 are known to be expressed in pyramidal neurons in adult rat hippocampus (14–16), but other transporters might also be present in these neurons. Knowledge of the exact localization and expression of these Na/HCO₃ transporters in primary cultures of embryonic or adult hippocampal neurons is presently incomplete.

Among the sodium/bicarbonate transporters, NBCn1 (SLC4A7) is of particular interest and has been studied for its molecular and cellular properties in many tissues (17–22). This transporter has unique characteristics that distinguish it from other transporters. NBCn1 is largely insensitive to DIDS. In Xenopus oocytes expressing NBCn1 (18) and in human embryonic kidney (HEK) cells expressing NBC3 (human homolog of human embryonic kidney cells; NGS, normal goat serum; MAP2, microtubule-associated protein 2; GABA, γ-aminobutyric acid; NBCn1, electroneutral sodium/bicarbonate cotransporter.

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tentily induces a steady-state current that does not require cell Na potential of oocytes. The transporter-dependent inward current positively shifts the membrane potential and raises the intracellular Na by nearly 6-fold. Thus, in addition to its ability to regulate pH via the electrophoretic cotransport activity, NBCn1 may also affect the membrane potential via its ionic conductance. This dual function would be important particularly in neurons where changes in pH, membrane potentials, and Na+ play important roles in cellular activities.

Rat NBCn1 was initially reported to exist as multiple splice variants depending upon the amino acid cassettes in the cytoplasmic N- and C-terminal domains of the transporter (18). One N-terminal cassette containing 14 amino acids and one C-terminal cassette containing 36 amino acids are identified to produce at least three splice variants in rats. In addition, the first 10 amino acids in the N-terminal end are different in the human clone. Functional characterization of NBCn1 has been done with either NBCn1-B or NBC3. Recently, Odgaard et al. (23) has reported another cassette containing 123 amino acids in the N-terminal domain of the transporter in the thick ascending limb of rat kidney. We have also shown in an abstract that the transporter in adult rat neurons lacks this N-terminal cassette (17). The role of the 123 amino acids in the cassette remains unclear.

In this study, we characterized the molecular and electrophysiological properties of NBCn1 variants that are expressed in rat hippocampal neurons. We identified two variants from embryonic hippocampal cultures and adult neurons, examined the expression and localization of the one in primary cultures, and characterized the function of a previously unexamined variant by expressing it in Xenopus oocytes and HEK cells.

**Experimental Procedures**

**Identification of NBCn1 Variants**—Total RNA was extracted from adult rat (Sprague-Dawley) hippocampal slices or embryonic hippocampal neurons (E19) using TRIzol reagent (Invitrogen) according to the protocol provided by the manufacturer. In vitro transcription was made using Superscript Reverse Transcriptase (Invitrogen) primed with random primers annealed for PCR amplification. Rat NBCn1 (GenBank accession number NM_056521). Primers for the N-terminal domain are GACTTTTACAATGAAGTTTCAGCATC (3835–3864, reverse primer) and GACTCTTCTTACCGGAGAGAAGCT (3159–3188, reverse); GAD65 primers (GenBank accession number M72422) TGAGAACCCGAGGAGACGCCGAGAGAAGCT (1345–157, forward) and AGCCGGACATTCAACGGGAGGAGAAGCT (819–846, reverse) and GAD67 primers (GenBank accession number NM_017007) AGACGTTTGATCGCTCCAACTCCCAACCGAGAGAAGCT (1160–1183, forward) and TCTATGCCGCTGATTTGCTG (1343–1362, reverse). The PCR products were then diluted 100-fold and used as a template for PCR with the internal primers to nest amplification. The following primers were used for the nested PCR: NBCn1 primers used for distinguishing the presence of the cassette II (described above); GAD65 primers TGAGAACCCGAGGAGACGCCGAGAGAAGCT (3835–3864, reverse) and CTTTATGCCGCTGATTTGCTG (1343–1362, reverse).

**Expression of NBCn1 in Xenopus Oocytes**—A full-length cDNA encoding the deletion variant of rat NBCn1 (NBCn1-E) was constructed into the oocyte expression vector pH119 (25). The DNA was linearized by cacao and transcribed in vitro with T7 RNA polymerase. The mMessage mMachine kit (Ambion, TX). Defolinated Xenopus laevis oocytes (Stages V–VI) were injected with 20 ng of NBCn1 cRNA (0.4 µg/µl) or 50 nl of water and incubated in OR3 media (50% Leibovitz L-15 media with 1 g-trigaulem, 5 µm HEPES, pH 7.5) supplemented with 5 units/ml penicillin/streptomycin for 3–7 days at 18 °C before use. Voltage and pH microelectrodes were prepared as described previously (26). The electrode tip was filled with protonated 1 M mixture B (Sigma-Aldrich) and back-filled with a pH 7 phosphate buffer. Electrodes were connected to the high impedance electrometer FD-223 (WPI, Sarasota, FL), and were connected to the analogue-to-digital converter of an IBM computer. In electrophysiological experiments, the CO2/HCO3-free ND96 solution contained (in mM) 96 NaCl, 2 KCl, 1.8 CaCl2, 1 MgCl2, and 5 HEPES (pH 7.4); osmolality was adjusted to 300–315 mosmol/kg. Solutions equilibrated with 5% CO2, 25 mM NaHCO3 replaced an equivalent molarity of NaCl. In Na+-free solution, N-methyl-D-glucamine replaced Na+. All experiments were performed at room temperature (22 °C).

**Two-electrode Voltage Clamp**—Microelectrodes were filled with 3 M KCl and had resistances of 1–2 MΩ. The current recordings were done with an OC-725A voltage clamp (Warner Instrument, Hamden, CT), which was connected to the Digidata1222 interface (Axon Instruments, Union City, CA). For measurements of the steady-state current, oocytes were clamped to a holding potential of −60 mV and then stepped from −120 to +40 mV with 20-mV increments for 100 ms in ND96. The sensitivity of the current to external Na+ was examined by current-voltage recordings in solutions containing (in mM) 5, 10, 50, and 100, Na+ plus 1 MgCl2, 2 KCl, 1.8 CaCl2, 5 HEPES, and sufficient mannitol to maintain the osmolality. In other experiments, the concentration of KCl, MgCl2, or CaCl2 was doubled in the Na+-free ND96. For Cl− substitution, gluconate was used. All voltage clamp experiments were performed in the nominal absence of HCO3−. Data were acquired and analyzed using pClamp 8 (Axon Instruments).

**Single Cell PCR**—A glass capillary patch pipette was filled with a 10 × PCR buffer (Roche Applied Science) containing RNase inhibitor (20 units/ml), and the cytoplasm was aspirated into the patch pipette by applying backpressure and then expelled into a microcentrifuge tube containing the reaction mixture for reverse transcription (100 ng of random hexamer, 10 nm dithiothreitol, 0.5 µm dNTPs, and 1 unit of RNase inhibitor). The first strand cDNA was synthesized with Superscript II reverse transcriptase (Invitrogen) for 1 h at 42 °C in a total reaction volume of 20 µl. After reverse transcription, PCR was performed with the following primers: NBCn1 primers CACATTTTTCCACGACCTTTCACGGAGATG (532–561, forward) and CATTCCAGCTC-TACTTTTAAGCTGTTGAC (3159–3188, reverse); GAD65 primers (GenBank accession number M72422) TGAGAACCCGAGGAGACGCCGAGAGAAGCT (1345–157, forward) and AGCCGGACATTCAACGGGAGGAGAAGCT (819–846, reverse), and GAD67 primers (GenBank accession number NM_017007) AGACGTTTGATCGCTCCAACTCCCAACCGAGAGAAGCT (1160–1183, forward) and TCTATGCCGCTGATTTGCTG (1343–1362, reverse). The PCR products were then diluted 100-fold and used as a template for PCR with the internal primers to nest amplification. The following primers were used for the nested PCR: NBCn1 primers used for distinguishing the presence of the cassette II (described above); GAD65 primers TGAGAACCCGAGGAGACGCCGAGAGAAGCT (3835–3864, reverse) and CTTTATGCCGCTGATTTGCTG (1343–1362, reverse).
integration of the NBCn1/pCnA/FRT into the genome via the FRT site. Four hours after transfection, cells were washed with phosphate-buffered saline, and the culture medium was replaced. Twenty-four hours after transfection, cells were split into fresh medium such that they were no more than 25% confluent. After additional 2–3 h of incubation, the medium was changed with the one containing hygromycin. The cells were fed every 3–4 days with selective medium containing hygromycin (10–400 μg/ml) until foci were identified. After 1–2 weeks of selection, surviving cells were single colonized in a well and replated into 25-cm² flasks.

Electrophysiological Recordings of Transfected HEK293 Cells—Whole cell patch-clamp recording was performed using a List L/M EPC-7 amplifier (List Medical Systems, NY). The patch pipette solution contained (in mM) 90 potassium aspartate, 20 KCl, 10 NaCl, 2 MgCl₂, 1 CaCl₂, 10 EGTA, and 10 HEPES (pH 7.2). The external solution contained 140 NaCl, 5 KCl, 1.8 CaCl₂, 1 MglCl₂, 10 glucose, and 10 HEPES (pH 7.4). Whole cell currents were recorded at room temperature at a holding voltage of −30 mV, in response to voltage steps from −100 mV to +40 mV, in increments of 20 mV. Patch pipettes were fabricated from TW 150 glass (World Precision, Hamden, CT) and fire-polished to produce tips with an internal diameter of 1 μm or less. Cells were visualized using a Nikon Diaphot-TMD inverted microscope equipped with Hoffman Modulation Contrast. Whole cell currents were recorded using Clampex on an IBM-compatible personal computer for later computer analysis. The recordings were low-pass filtered (3 db at 2 kHz, 8-pole Bessel filter) before recording on Clampex. The current amplitude at the end of the pulse was measured using Clampfit.

Statistical Analysis—Data were reported as means ± S.E. Levels of significance were assessed using the unpaired, two-tailed Student’s t test. The p value of <0.05 was considered significant. Rates of pH change (d(pH)/dt) were fitted by a line using a least-squares method.

RESULTS

Two NBCn1 Variants Exist in Embryonic and Adult Hippocampal Neurons—Alternative splicing results in at least three variants of rat NBCn1 (NBCn1-B/C/D), which have different combinations of amino acid cassettes in the cytoplasmic N- and C-terminal domains of the protein (Fig. 1A) (18). NBCn1-B lacks cassette III (36 amino acids), NBCn1-C lacks cassette I (14 amino acids), and NBCn1-D has no deletion. Human homolog NBC3 (i.e. NBCn1-A) is similar to NBCn1-B with the exception of the first 10 amino acids at the N-terminal domain (20). In addition, two other variants without cassette II (123 amino acids) were identified in rat kidney (23). These renal variants vary with or without cassette I.

We performed RT-PCR to determine which variants exist in rat hippocampal neurons. The primers corresponding to the flanking region of cassettes I and II were used so that they would amplify either ~1.5 kb products for variants containing these two cassettes or ~1.1 kb products for variants lacking them. We found that PCR generated a 1.5-kb product in hippocampal neurons isolated from embryonic (E19) rats and a 1.1-kb fragment in adult hippocampal slices (Fig. 1B). Sequencing these PCR products revealed that cassettes II was present in embryonic neurons but absent from adult neurons. Both samples had cassette I, thus indicating that the difference between two PCR products is due to the splicing event of cassette II. To identify the full-length variant(s) in hippocampal neurons, another set of primers was used to amplify the entire coding region of the NBCn1 sequence. A PCR product with 3.2 kb in size was amplified in adults (Fig. 1B, far right lane). Sequencing this product confirmed the absence of both N-terminal cassette II and C-terminal cassette III. Thus, the splice variant of NBCn1 in adult hippocampal neurons is identical to the previously characterized NBCn1-B except for the deletion of cassette II (hereafter, this variant is NBCn1-E). NBCn1-E is also different from the variants in the medullary thick ascending limb (23). The variant in embryonic neurons is NBCn1-B (data not shown).

NBCn1-E Is an Electroneutral Na/HCO₃ Cotransporter with an Ionic Conductance—Because the function of NBCn1-B has been previously reported (18), we focused our study on characterizing the functional significance of the 123 amino acids in cassette II that are deleted in NBCn1-E. NBCn1-E was expressed in Xenopus oocytes, and its electrophysiological properties were examined using ion-selective microelectrodes. We measured the cotransport activity by examining pH recovery under acidic conditions and effects on membrane potential (V_m) during the pH recovery. Fig. 2A shows typical pH and V_m traces in oocytes expressing the transporter. Applying 1.5% CO₂/10 mM HCO₃⁻ to oocytes caused the pH to slowly rise after an initial rapid acidification by CO₂. This rise is due to HCO₃⁻ entry via the transporter. Water-injected control oocytes showed no pH recovery (Fig. 2B). Applying CO₂/HCO₃⁻ to oocytes expressing NBCn1-E did not affect the V_m as would have been expected if the transporter mediated an unequal ratio of Na⁺ and HCO₃⁻ transport. A slow depolarization is likely due to nonspecific CO₂ effect, because it also occurred in controls. These results indicate that the pH recovery by NBCn1-E is electroneutral. Removing Na⁺ from the bath (N-methyl-D-glucamine substitution) in the continued presence of CO₂/HCO₃⁻ blunted the pH change. Na⁺ removal also caused a hyperpolarization, which implies an associated ionic conductance of the transporter as discussed below. The pH recovery rate presented by the pH change per second (d(pH)/dt) was 19 ± 2 × 10⁻⁵ (n = 4) in the forward direction (i.e. Na⁺ and HCO₃⁻ moving into cells), significantly higher than 0.9 ± 0.6 × 10⁻⁵ (n = 10) in controls (Fig. 2C). The d(pH)/dt in the reverse direction (i.e. Na⁺ and HCO₃⁻ moving out of cells) was −3 ± 1 × 10⁻⁵ in NBCn1-E oocytes and −1.8 ± 0.7 × 10⁻⁵ in controls.

Upon Na⁺ removal, oocyte membranes hyperpolarized. The change in V_m in oocytes expressing NBCn1-E (27.7 ± 2.7 mV) was significantly greater than that in control oocytes (5.9 ± 1.0 mV, p < 0.01). The robust hyperpolarization in NBCn1-E oocytes suggests that the transporter has a Na⁺-sensitive ionic conductance. We measured the ionic current corresponding to this conductance in oocytes expressing the transporter in two-electrode voltage clamp. In current-voltage (I-V) recordings (Fig. 3A), the
in the absence and presence of 25 mM HCO₃⁻ conductance was measured between NBCn1-B (n = 7, open circle). The steady-state current was acquired in ND96 by voltage-clamp at −60 mV holding and then steps (100 ms) from −120 to +60 mV with 20-mV increments. B, comparison of the slope conductance in NBCn1-B (n = 5), NBCn1-E (n = 6), and controls (n = 7). The slope conductance was measured between −80 and +20 mV. C, conductance in the absence and presence of 25 mM HCO₃⁻. The HCO₃⁻ solution was made by substituting 25 mM NaCl with an equivolality of NaHCO₃ (pH 7.4). Values of the conductance were obtained from oocytes expressing NBCn1-E (n = 7) and controls (n = 5).

reversal potential of the steady-state current was −21.2 ± 1.2 mV (n = 6), close to the resting Vm of −21.4 ± 1.5 mV. A significant amount of inward current (−400 nA) was generated at −51 mV, which corresponds to the average resting potential of control oocytes. The slope conductance between −80 to +20 mV was 13.9 ± 3.2 μS (n = 6), which was significantly greater than 1.3 ± 0.1 μS (n = 7, p < 0.01) in controls. In I-V recordings from oocytes expressing the previously characterized NBCn1-B, the conductance was 16.1 ± 3.3 μS (n = 5) measured on the same recording day (~3 days after RNA injection) (Fig. 3B). The magnitude of the conductance in NBCn1-E was not significantly different from that in NBCn1-B (p > 0.05). Although the NBCn1-E-mediated conductance was Na⁺-dependent, it was not affected by HCO₃⁻ in the bath (Fig. 3C).

The Associated Conductance Is Mostly Na⁺-dependent—To analyze the contribution of Na⁺ to the NBCn1-mediated current, we recorded the currents in normal ND96 solution containing 96 mM Na⁺ and then in Na⁺-free solution (Fig. 4A). We then subtracted the amplitudes of the currents measured in Na⁺-free solution from those in ND96. The resulting difference in current was inwardly directed and did not reverse up to +40 mV (Fig. 4B). The Na⁺ sensitivity of the current was examined by plotting the logarithmic change of the reversal potential as a function of extracellular Na⁺ concentration ([Na⁺]₀). In the range of 10–100 mM [Na⁺]₀, the slope was 26 mV per decade change in [Na⁺]₀. This represents 45% of the expected potential change based on the Nernst equation (Fig. 4C). The reversal potential was unchanged below 10 mM [Na⁺]₀.

The lack of changes in the reversal potential at low [Na⁺]₀ may be due to other ions passing through the conductive pathway in NBCn1-E protein when Na⁺ is not available. To determine if other ions contribute to the NBCn1-E-mediated current, we measured the effect of K⁺, Ca²⁺, and Mg²⁺ in the bath solution on the reversal potential. Removing these cations from ND96 (with Na⁺ substitution) did not affect the reversal potential of the NBCn1-E current or slope conductance (data not shown). Nonetheless, because these cations are within the range of 1–2 mM in ND96, removal of such ions might not dramatically affect the current activity mediated by the transporter in the presence of full [Na⁺]. Thus, we took another approach in which the amplitude of the NBCn1-E-mediated current was examined in solutions containing a 10-fold increase in K⁺, Ca²⁺, and Mg²⁺ ions. For these experiments, oocytes were incubated for 24 h in the Na⁺-free media 2 days after RNA injection, and then recordings were done in the Na⁺-free solution. Under this condition, the slope conductance was 2.1 ± 0.3 μS (n = 5) (Fig. 5A). This value is comparable to the baseline value in control oocytes shown in Fig. 3. Not shown in Fig. 5A is the mean conductance (1.1 μS, n = 4) in Na⁺-depleted control oocytes. The difference in conductance between Na⁺-depleted NBCn1 oocytes and Na⁺-depleted control oocytes is 1.0 μS, which is estimated to be ~6% of the NBCn1 conductance in Fig. 3. The nature of this residual current is
unclear: it might represent the Na"-independent portion of the conductance associated with NBCn1 or other cellular effects owing to Na" depletion. Longer incubation of oocytes in the Na"-free media tends to decrease cell viability. The low conductance in Na"-depleted oocytes is, however, not due to poor expression of the transporter, because the same batch of oocytes incubated in normal ND96 had significant current amplitudes (data not shown). Increasing K" , Mg" , or Ca" in the bath solution by 10-fold did not significantly affect the reversal potential of the current nor the slope (Fig. 5A). These data suggest that the current activity mediated by NBCn1-E is not due to the tested external cations. We also incubated oocytes in Cl"-free media after RNA injection to examine the effect of the outwardly directed Cl" on the NBCn1-E-mediated current. In ND96, the reversal potential and the conductance were -18 ± 3 mV and 6.2 ± 1.0 µS (n = 6), respectively (Fig. 5B). These values are similar to those in oocytes incubated in normal ND96 containing 100 mM Cl" (-23 ± 1 mV and 6.5 ± 0.5, p > 0.05 for both values). These data show that internal Cl" does not affect the current mediated by NBCn1-E and that the conductance associated with the transporter is primarily Na"-dependent.

The current activity mediated by NBCn1-E was slowly enhanced by a stilbene derivative DIDS (500 µM, n = 9) (Fig. 6), consistent with the previous observation of the stimulatory effect of this drug on the NBCn1-B current (18). Stimulation was gradual over 15 min and then saturated. When normalized to the initial current in ND96, the current at -100 mV increased by ~20%. DIDS decreased the basal currents in water-injected controls (n = 6). The current activity mediated by the transporter was not inhibited by amiloride, tetrodotoxin, ouabain, furosemide, or niflumic acid (data not shown).

**Fig. 5. Effects of single ion substitution on the I-V relationship.** A, effects on a 10-fold increase in bath cations. Recordings were made with oocytes that were preincubated for 24 h in Na"-free media 2 days after RNA injection. Recordings were made in Na"-free solution (closed diamond) and then in solutions containing 20 mM K" (open circle), 18 mM Ca" (open triangle), or 10 mM Mg" (open square). Data were obtained from 3-7 oocytes for each maneuver. B, effects of internal Cl" on the NBCn1-E current. Recordings were made in normal ND96 with oocytes (n = 6 for each group) that were preincubated in Cl"-free (open circle) or normal ND96 (closed circle).

**Fig. 4. Na" dependence of the current mediated by NBCn1-E.** A, I-V plots before and after Na" removal. Recordings were obtained from oocytes expressing NBCn1-E (n = 5) in ND96 containing 96 mM Na" (closed circle) or 0 mM Na" (open circle). Controls (n = 5) were also recorded in 96 or 0 mM Na" (closed and open triangles), N-Methyl-D-glucamine substitutes Na". B, Na" component of the NBCn1-mediated current. The Na" components were measured by subtracting the current amplitudes in nominally Na"-free solution from the amplitudes in 96 mM Na" media shown in A. C, dependence of the reversal potential (Vrev) on extracellular Na" concentration ([Na" ])o. 

*Fig. 7A.* Whole cell recordings, cells stably transfected with the transporter showed an inwardly directed current that was distinguishable from a basal current in sham-transfected controls (Fig. 7B). The reversal potential of the current averaged -3.4 ± 2.0 mV (n = 7), which was about 17 mV more positive than controls. The current was progressively stronger at more negative voltages. At positive voltages, the current in transfected cells became small and eventually reduced to the basal current shown in sham-transfected controls. The nature of the outward current at positive voltages in both controls and NBCn1-E expressing cells is unclear but most likely due to K" currents that are further away from the equilibrium potential for K". A significant amount of the current (~100 pA) was generated at ~20 mV that corresponds to the average resting potential in untransfected cells.

To examine the contribution of Na" to the currents in stable cells, we performed whole cell recordings in normal media containing 140 mM Na" and then in Na"-free media. A typical I-V plot in stable cells is shown in Fig. 7C. Removing bath Na"...
characterized NBCn1-B, NBCn1-E is an electroneutral Na/HCO$_3$-cotransporter with an associated ionic conductance. Because mRNAs for the clones are detected in hippocampal neurons, we then performed immunocytochemistry to localize NBCn1 protein in primary cultures of embryonic hippocampal neurons. Immunofluorescence was achieved with the anti-NBCn1 antibody that recognizes the C-terminal 14 amino acids of rat NBCn1 (22). NBCn1 staining was prominent on the plasma membrane of cell soma and in dendrites of most neurons (Fig. 8A). The NBCn1 staining colocalized with that for microtubule-associated protein 2 (MAP2), which serves as a marker for dendrites (Fig. 8, B and E). NBCn1 staining was also highly punctuate and along MAP2-labeled dendrites, with a subcellular distribution resembling pre- or postsynaptic boutons (Fig. 8, D–F). Double labeling with the antibody against the postsynaptic density protein PSD-95, which labels postsynaptic components of excitatory synapses, revealed partial colocalization with NBCn1 (Fig. 8, G–I). Controls without primary antibodies showed no immunofluorescence (data not shown).

In primary cultures, we found that most, but not all, neurons were labeled with the NBCn1 antibody. To determine if NBCn1 expression correlates with a specific neuronal cell type (excitatory or inhibitory), we performed single cell PCR. For this analysis, single neurons were randomly selected with a patch pipette and assessed for PCR with either primers for NBCn1 or glutamic acid dehydrogenase (GAD) 65 or 67. GAD65 and 67 serve as markers for GABAergic neurons (27, 28). We found that most neurons selected for the assay were NBCn1-positive. The size of NBCn1 PCR products was 1.5 kb, consistent with the presence of NBCn1-B splice variant containing cassette II in embryonic neurons. The NBCn1-positive neurons were either GAD65/67-positive or not (Fig. 9). GAD65/67-positive neurons constituted about 15% of neurons selected for the assay. The relatively low level of GAD65/67-positive neurons in the samples is in agreement with the low level of GABAergic neurons in cultures (29). These data support that NBCn1 expression does not correlate solely with GABAergic neurons.
that is activated by NBCn1 expression, it is less likely that this would be seen in two very different expression systems. Channel-like properties are also found in other bicarbonate transporters, although the magnitude of the conductance is relatively minor compared with that seen with NBCn1. Oocytes expressing the Drosophila Na/HCO₃ transporter NDÆE1 elicit a small Cl⁻ current (31). The AE protein in erythrocytes is also thought to mediate a small conductive anion flux (32). Regarding the nature of the current, our data lead us to the conclusion that Na⁺ is the major ion responsible for generating the current. We tested if the reversal potential of the transporter current could be altered by bath cations or internal Cl⁻ and found only Na⁺ affecting the reversal potential. This implies either that the current is strictly Na⁺-dependent or that our attempt to identify the ions responsible for the extra current was unsuccessful. The reversal potential at low [Na⁺], was unchanged by any other ions we tested. Why then does the reversal potential at the range above 10 mM Na⁺ change in a less than Nernstian manner? This suggests that the mechanism for the dependence of the reversal potential on [Na⁺] is more complex than we anticipate from a single channel model. It is possible that the conductance mediated by the transporter does not occur through a pore-forming tunnel (channel) but instead via an electrotonic transport pathway with multiple binding sites. If this is true, the partial change in the reversal potential at higher range of [Na⁺] could be accounted for by assuming multiple Na⁺ sites per ion translocation step. Nevertheless, it will be necessary to perform more sophisticated electrophysiological analyses to distinguish a carrier pathway from a channel pathway as the source of this Na⁺ current.

Would NBCn1 also elicit the Na⁺ current in hippocampal neurons? The appearance of the Na⁺ currents in both frog oocytes and HEK cells strongly suggests that the same protein would also induce Na⁺ currents in neurons. It is, however, difficult to predict whether the conductance properties may behave similarly in neurons, where the transporter could interact with other native proteins. Furthermore, because the conductance does not require HCO₃, it is technically difficult to monitor the transporter-dependent Na⁺ current in neurons. Lack of proper blockers is a major problem in identifying the transporter-dependent current. An alternative approach would be to use DIDS, which enhances the magnitude of the current. However, DIDS has a broad spectrum of inhibitor effects on other anion channels and transporters, and thus caution would be needed in analysis of such experiments.

What would be the physiological role of NBCn1 in neurons? An obvious answer is to regulate local pH at synapses. The pH is closely associated with neuronal firing at synapses (33–35). An action potential promotes electrogenic Na/HCO₃ cotransport in astrocytes (36–38) and lowers local pHo. The low pHo inhibits Na⁺-driven Cl/HCO₃ exchange in presynaptic neurons and thus acidifies neuronal pH. Acidification then causes feedback inhibition of the action potential. This feedback inhibition would require a cooperative and global activation of multiple Na/HCO₃ transporters at multiple synapses. The synaptic localization of NBCn1 provides support for a potential role of Na/HCO₃ transport in neuronal modulation. Any change in the pHo at synapses would likely affect NBCn1-E activity, which could then change the pre or postsynaptic pH. Because many neurotransmitter receptors and ion channels, including N-methyl-D-aspartate receptors (2, 3), γ-aminobutyric acid (GABA) A receptors (5), and voltage-gated Ca²⁺ channels (4) are sensitive to pH, such an NBCn1-mediated pH, and/or pH change would significantly affect synaptic activities. Moreover, in addition to this pH effect, NBCn1 would constitutively pass an inward current at the resting membrane potentials of neu-

FIG. 9. Single cell PCR. Individual neurons in cultures were randomly selected and subjected to PCR with primers for NBCn1, GAD65, or GAD67. The results shown here are representative neurons that are positive to GAD65 (GAD65) or GAD67 primers (GAD67), or negative to both (none). GAD65 or GAD67-positive neurons are GABAergic, whereas GAD65/67-negative neurons are non-GABAergic.

DISCUSSION

In this study, we examined the molecular and cellular physiology of NBCn1 from hippocampal neurons. NBCn1 is localized in somatodendrites and synapses in primary cultures, and NBCn1 expression is not limited to only inhibitory (or excitatory) synapses. We have shown two splicing variants with or without the cassette II to exist differentially in embryonic and adult hippocampal neurons. Like the previously reported NBCn1-B, the deletion variant NBCn1-E has characteristics of the electroneutral Na/HCO₃ transport with an additional associated conductance. We propose that this dual function not only enables the transporter to regulate local pHo, but it may also modulate the Vm (or even [Na⁺]) at synapses. Our data provide the first evidence for an electroneutral Na/HCO₃ transporter in hippocampal neurons where the Na⁺-driven Cl/HCO₃ exchange had been previously considered the major HCO₃-dependent acid extruder (7–9).

The functional properties of NBCn1-E shown in this study are comparable to those of NBCn1-B that we previously identified from rat vascular smooth muscle. The similarity between two variants indicates that cassette II does not significantly affect the intrinsic properties of Na/HCO₃ cotransport or ionic conductance of the transporter. Despite this functional similarity, the two splice variants are expressed in hippocampal neurons at different developmental stages. It is possible that cassette II is associated with protein trafficking, insertion into plasma membranes, or other cellular event(s) related to NBCn1 function in embryonic versus adult tissue. The Cl/HCO₃ exchanger AE1, which is another member of the SLC4A gene family, has 40 amino acids at the region corresponding to cassette II with highly homologous flanking regions (30). These amino acids in AE1 contain a binding motif for ankyrin, a cytoskeleton protein. It is thus tempting to postulate that the amino acids at the corresponding cassette II in NBCn1 would play a role in interacting with other cellular proteins. We have a preliminary observation (17) in which expression of NBCn1-E, but not NBCn1-B, increases ouabain-sensitive Na/K-ATPase activity in mammalian kidney cell line. It is possible that association of the amino acids in cassette II with Na/K-ATPase could inhibit the activity of the Na pump.

The currents produced by NBCn1 in both frog oocytes and HEK cells support the conclusion that the associated ionic conductance is intrinsic to the transporter protein itself. Although the conductance could come from a separate molecule...
rons. One might expect that the depolarizing current causes the postsynaptic potential to be less excitable, probably due to Na\(^+\) channel inactivation. The significance of this depolarization would depend upon the extent of protein expression and the magnitude of the currents generated by the transporter.

We also note the potential role of the Na\(^+\) conductance in brain pathology. The relative Na\(^+\) movement via the conductive pathway versus the carrier pathway in NBCn1 is estimated to be 1/300, calculated from charge movement and \(dpH/dt\) in NBCn1 oocytes. Thus, the direct contribution of the Na\(^+\) conductance to the [Na\(^+\)] would be minimal in neurons, although it could have significant local effects on the membrane potential. The effect on [Na\(^+\)] may be seriously augmented under certain conditions when this transporter expression is overstimulated and the Na/K-ATPase is inactivated. In the kidney, NBCn1 expression is dramatically stimulated by chronic metabolic acidosis (39). Whether such stimulatory effect can also occur in neurons is not known, but it will certainly be interesting to investigate NBCn1 expression stimulated by cerebral acidosis.

Knock-out mice deficient in NBCn1 show prolonged degeneration of sensory neurons in the eye and inner ear (40), resulting in blindness and auditory impairment. The cause for these phenotypic defects is suggested to be an altered acidic pH\(_i\) that induces Ca\(^{2+}\) overload and subsequent apoptosis. Knock-out mice appear to have normal brain, although a detailed examination on neurological disorder has not been performed. Taken together, these pathological studies and the knock-out mice model strongly suggest that NBCn1 is an important pH regulator and that altered activity of the transporter closely associates with acid/base imbalance.

In conclusion, our data show that the electroneutral Na/HCO\(_3\) cotransporter NBCn1 is present in rat hippocampal neurons and that it could play an important role in regulating synaptic pH as well as activities.

Acknowledgments—We acknowledge Ronald Abercrombie for helpful discussion, Joseph Kippen for ion sensitivity experiments, and Delia Cucoraru for PCR data.

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J. Biol. Chem. 2005, 280:17823-17830.
doi: 10.1074/jbc.M408646200 originally published online February 17, 2005

Access the most updated version of this article at doi: 10.1074/jbc.M408646200

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