Electrogenic Na/HCO₃ Cotransporter (NBCe1) Variants Expressed in Xenopus Oocytes: Functional Comparison and Roles of the Amino and Carboxy Termini

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Using pH- and voltage-sensitive microelectrodes, as well as the two-electrode voltage-clamp and macropatch techniques, we compared the functional properties of the three NBCe1 variants (NBCe1-A, -B, and -C) with different amino and/or carboxy termini expressed in Xenopus laevis oocytes. Oocytes expressing rat brain NBCe1-B and exposed to a CO₂/HCO₃⁻ solution displayed all the hallmarks of an electrogenic Na⁺/HCO₃⁻ cotransporter: (a) a DIDS-sensitive pHi recovery following the initial CO₂-induced acidification, (b) an instantaneous hyperpolarization, and (c) an instantaneous Na⁺-dependent outward current under voltage-clamp conditions (−60 mV). All three variants had similar external HCO₃⁻ dependencies (apparent Kₘ of 4–6 mM) and external Na⁺ dependencies (apparent Kₘ of 21–36 mM), as well as similar voltage dependencies. However, voltage-clamped oocytes (−60 mV) expressing NBCe1-A exhibited peak HCO₃−-stimulated NBC currents that were 4.3-fold larger than the currents seen in oocytes expressing the most dissimilar C variant. Larger NBCe1-A currents were also observed in current–voltage relationships. Plasma membrane expression levels as assessed by single oocyte chemiluminescence with hemagglutinin-tagged NBCs were similar for the three variants. In whole-cell experiments (Vₘ = −60 mV), removing the unique amino terminus of NBCe1-A reduced the mean HCO₃−-induced NBC current 55%, whereas removing the different amino terminus of NBCe1-C increased the mean NBC current 2.7-fold. A similar pattern was observed in macropatch experiments. Thus, the unique amino terminus of NBCe1-A stimulates transporter activity, whereas the different amino terminus of the B and C variants inhibits activity. One or more cytosolic factors may also contribute to NBCe1 activity based on discrepancies between macropatch and whole-cell currents. While the amino termini influence transporter function, the carboxy termini influence plasma membrane expression. Removing the entire cytosolic carboxy terminus of NBCe1-C, or the different carboxy terminus of the A/B variants, causes a loss of NBC activity due to low expression at the plasma membrane.

INTRODUCTION

Na/HCO₃ cotransporters (NBCs) are functionally diverse proteins that are involved in the regulation of intracellular pH (pHi), absorption or secretion of HCO₃⁻, and maintenance of ion homeostasis in many tissues. The first Na⁺/HCO₃⁻ cotransporter was identified by function in the proximal tubule of the kidney (Boron and Boulpaep, 1983), where the transporter has a 1:3 Na⁺:HCO₃⁻ stoichiometry (Soleimani et al., 1987) and is responsible for reabsorbing as much as ~90% of filtered bicarbonate (Boron et al., 1997). In glial cells of the central nervous system, an electrogenic Na⁺/HCO₃⁻ cotransporter with a 1:2 Na⁺:HCO₃⁻ stoichiometry (Deitmer and Schlue, 1989; O’Connor et al., 1994; Bevensee et al., 1997a,b) contributes to intracellular and extracellular pH changes that can influence neuronal activity (Chesler, 2003; McAlear and Bevensee, 2004).

At the molecular level, the first cDNA encoding an electrogenic NBC was identified by expression from salamander kidney (Romero et al., 1997). The cloning of salamander kidney NBC paved the way for homology cloning of both electrogenic and electroneutral NBCs, as well as other cation-coupled anion transporters (for review see Romero et al., 2004). Cation-coupled anion transporters in conjunction with anion exchangers (AEs) are members of a superfamily of bicarbonate transporters (BTs). Using the convention introduced by Choi et al. (2000) and expanded by Romero et al. (2004), we refer to the first cloned NBC as NBCe1-A, where “e” refers to electrogenic, “1” refers to the first gene cloned of this family, and “A” refers to the first splice variant identified. The putative membrane topology of NBCe1 shown in Fig. 1 is based on sequence alignment of NBCe1 and AE1 (Romero et al., 1998b). We mapped the NBCe1 sequence on a topology model

Abbreviations used in this paper: AE, anion exchanger; DIDS, 4,4′-disothiocyanatostilbene-2,2′-disulfonate; HA, hemagglutinin; HRP, horseradish peroxidase; NBC, Na/bicarbonate cotransporter; pHi, intracellular pH; PIP₂, phosphatidylinositol 4,5-bisphosphate; SOC, single-oocyte chemiluminescence.
of the related bicarbonate transporter anion exchanger 1 or AE1 (Taylor et al., 2001). As described by Taylor et al. (2001), the putative topology of AE1 is determined from proteolysis and cysteine accessibility data.

NBCe1 proteins arise from different splice variants of gene SLC4A4 (Abuladze et al., 2000). The electrogenic Na/HCO₃ cotransporters can be categorized into one of three groups based on differences at the amino and/or carboxy termini (Fig. 1): NBCe1-A (“kidney” clone), NBCe1-B (“heart,” “pancreas,” and rat-brain 1 NBC clone), and NBCe1-C (rat-brain 2 NBC clone). At the amino acid level, NBCe1-B is identical to NBCe1-A except at the amino terminus where 85 residues replace the 41 residues of NBCe1-A. NBCe1-C is identical to NBCe1-B except at the carboxy terminus where 61 residues replace the 46 residues of NBCe1-B. As reported for the A and B clones from human (Choi et al., 1999), the unique amino terminus of the rat A variant is only 6% identical and 13% homologous to that of the rat B and C variants. Furthermore, while both termini are hydrophilic, ~50% of the 85 amino-terminal residues of the B and C variants are charged residues in contrast to only ~22% of the 41 unique amino-terminal residues of the A variant. Although NBCe1-C has only been reported from rat brain, exon excision between introns at positions 23 and 24 in human SLC4A4 (Abuladze et al., 2000) would create the unique carboxy terminus. Indeed, using RTPCR techniques, we have recently identified the full-length cDNAs encoding both NBCe1-B and NBCe1-C from human brain (unpublished data).

Although the physiological significance of the three NBCe1 variants with different amino and/or carboxy termini is not fully known, several investigators have identified specific residues/regions within the cytoplasmic amino and carboxy termini of NBCe1 that contribute to protein function, regulation, and expression. For example, in Ussing-chamber experiments on transfected mouse renal proximal tubule cells, Gross et al. reported that PKA-dependent phosphorylation of the Ser at position 982 within the cytoplasmic carboxy terminus of the A variant (Gross et al., 2001), or position 1026 of the B variant (Gross et al., 2003), changes transporter stoichiometry from 1:3 to 1:2 Na⁺:HCO₃⁻. This Ser within the cytoplasmic carboxy terminus is common in all three variants. As proposed by the authors, the phosphorylation of the carboxy terminus may induce a conformation change that blocks a HCO₃⁻ binding site on the transporter. The stoichiometry of the A and B variants appears to be cell type dependent, and not due to the different amino termini (Gross et al., 2001). On the other hand, the amino terminus does contribute to the regulation of transporter activity. For example, Gross et al. (2003) report that the Thr at position 49 of human NBCe1-B is required for the cAMP-stimulated increase in transporter activity. This increase does not appear to involve a change in the phosphorylation of the Thr. Curiously, this Thr is one of two residues that are different in the homologous amino-terminal region of rat NBCe1-B and -C. The carboxy terminus of NBCe1 can also influence expression of the protein. For instance, removing the carboxy-terminal 23 residues of NBCe1-A causes a mistargeting of the transporter to the apical instead of the basolateral membrane when transfected into kidney epithelial cells (Li et al., 2004).

In addition to structure–function data, there is also genetic information that highlights the importance of the cytosolic amino terminus of NBCe1 before the first predicted transmembrane domain. There are human patients with mutations in SLC4A4 who present primarily with proximal renal tubular acidosis (pRTA) and ocular abnormalities (Igarashi et al., 1999; Dinour, D., A. Knecht, I. Serban, and E.J. Holtzman. 2000. J. Am. Soc. Neurol. 11:3A; Igarashi, T., J. Inatomi, T. Sekine, Y. Yakeshima, N. Yoshikawa, and H. Endou. 2000. J. Am. Soc. Nephrol. 11:106A; Dinour et al., 2004). One patient has an inactivating homozygous missense mutation in which a Ser replaces Arg at position 298 in the cytoplasmic amino terminus of NBCe1-A (Igarashi et al., 1999). In expression studies using ECV304 cells, the authors found that this mutant NBC displayed only ~50% of wild-type transporter activity. This substitution is found at position 342 in the B and C variants. A second patient has a homozygous missense mutation in which a Leu replaces Ser at position 427 at the beginning of the first predicted transmembrane domain of NBCe1-A (Dinour et al., 2004). When expressed in oocytes, the mutant NBC displayed only ~10% of wild-type transporter activity. In a separate study, this mutant NBC was mistargeted to the apical instead of the basolateral membrane of polarized Madin-Darby canine kidney (MDCK) cells (Li et al., 2005).

According to the aforementioned studies, regions within the cytoplasmic amino and carboxy termini can influence the function, regulation, and expression of NBCe1 variants. However, a detailed comparison of the biophysical properties of all three variants with different amino and/or carboxy termini has yet to be performed. In the present manuscript, we used pH- and voltage-sensitive microelectrodes to characterize, for the first time, the function of rat brain NBCe1-B expressed in Xenopus oocytes. Subsequently, we used the two-electrode, voltage-clamp technique to compare the activities, as well as the ion and voltage dependencies of all three NBCe1 variants. Although all three variants have similar external ion and voltage dependencies, as well as plasma membrane expression levels as assessed by single oocyte chemiluminescence, the activity of the A variant is greater than the activities of the other two variants. According to structure–function analyses using whole-cell and macropatch recording techniques, the higher activity of A is due to its unique amino terminus. While the different amino termini influence function, one or more regions within the different carboxy termini contribute(s) to plasma membrane expression.

MATERIALS AND METHODS

NBC Constructs and Mutagenesis

Wild-type NBCe1 Variants. We used cDNAs encoding NBCe1-A from rat kidney (Romero et al., 1998b), and NBCe1-C from rat brain (Bevensee et al., 2000) subcloned into the oocyte expression vector pTLNII as previously described. Full-length NBCe1-B, which was also previously identified from rat brain by RT-PCR (Bevensee et al., 2000), was constructed using convenient restriction enzymes to swap the unique carboxy terminus of full-length variant B from a partial-length construct in pTLNII. For both wild-type and mutant constructs (see below), NBC expression was optimized by introducing a Kozak sequence (Kozak, 1986) at the initiator codon using PCR techniques and a Peltier thermal cycler (PTC-220 DNA Engine Dyad, MJ Research, Inc.). Sequence analysis and primer design were performed using either DNAsis (Hitachi Software) or Vector NTI Advance 9.0 (InforMax, Invitrogen), and all constructs were confirmed by bidirectional DNA sequencing (DNA Sequencing Core, Center for AIDS Research and the Genomics Core Facility, Heflin Center for Human Genetics, both at the University of Alabama at Birmingham).

HA-tagged NBCe1 Constructs. To insert the nine-residue hemagglutinin (HA) epitope (YPYDVPDYA) into NBCe1-C, we used the QuikChange PCR-based mutagenesis kit (Stratagene) to create a unique restriction site (Bsa36I) at base pair 1941 (residue 647) within the extracellular loop between transmembrane domains 5 and 6. A double-stranded primer pair was then ligated into the engineered Bsa36I site in NBCe1-C. Because of the introduced Bsa36I cut sites, the inserted HA epitope in the protein was flanked by Asp and Gly. HA-tagged NBCe1-A and -B were constructed from the tagged NBCe1-C using convenient restriction enzymes.

Truncated NBCe1 Variants. Truncated NBCe1 constructs (see Fig. 1) were generated using PCR techniques and HA-tagged NBCe1 variants subcloned into pTLNII as templates. In generating amino-terminal truncations, we targeted residue 43 of the A variant and the homologous residue 87 of the C variant to optimize the Kozak sequence. NBCe1 constructs truncated at the carboxy terminus were generated by introducing a targeted stop codon using site-directed mutagenesis (QuikChange kit, Stratagene).

Functional Studies on NBCs

Generation of cRNA. pTLNII plasmids containing NBCe1 constructs were linearized with the restriction enzyme MluI. The linearized cDNA was then transcribed from the SP6 promoter using the SP6 transcription kit (Ambion), and the resulting cRNA was purified using the RNeasy kit (Qiagen).

Isolation and Injection of Oocytes. Oocytes were harvested from female *Xenopus laevis* frogs using an approach very similar to that previously described (Romero et al., 1998b; Bevensee et al., 2000). A small incision was made in the abdominal cavity of the frog, and oocyte-containing segments of the ovarian lobe were removed. The segments were teased apart into small pieces and digested for 1.5–2 h in sterile Ca2+-free ND96 containing 2 mg ml−1 collagenase A (Roche Applied Science). Subsequently, the dissociated oocytes were first washed in Ca2+-free ND96, and then in Ca2+-containing ND96 before healthy-looking stage V/VI oocytes were separated under a dissecting microscope (GZ6, Leica). The oocytes were incubated at 18°C in sterile ND96 containing 10 mM Na+/pyruvate and 10 mg ml−1 gentamycin (Mediatech Inc.).

Oocytes were injected with 48 nl of either RNase-free H2O or a cRNA solution using a “Nanoject II” microinjector (Drummond Scientific). Injected cells were incubated at 18°C in the aforementioned
oocyte media, and experiments were performed at room temperature at least 2 d after injection.

pH, and Vm Experiments. Injected oocytes were placed in a flowthrough chamber connected to a custom-designed, dual-bank, solution delivery system. Main solution lines from two banks, which are each connected to six solution lines via a six-way rotary manifold, were directed to a two-position, Eagle four-way miniature solenoid valve with five ports (Clippard Instrument Laboratory). During experiments, solution from one bank was directed to the chamber, and solution from the other bank was directed to waste for priming purposes. Changing solutions delivered to the chamber occurred by pneumatically alternating between the two valve positions.

For simultaneous pH, and voltage recordings, microelectrodes were pulled from borosilicate glass capillaries (G200F-4, Warner Instruments) with a Brown-Flaming micropipette puller (P-80 or P-97, Sutter Instruments). For pH electrodes, pulled acid-washed capillaries were subsequently baked at 200°C and silanized with bis-(methylamino)dimethylsilane (Fluka). Electrode tips were filled with hydrogen ionophore I-cocktail B (Fluka), and then the electrodes were backfilled with a pH 7.0 solution containing (in mM): 150 NaCl, 40 KH2PO4, and 23 NaOH. pH electrodes were then connected to one channel of a high-impedance electrometer (FD223, WPI). Voltage electrodes were filled with a saturated KCl solution and connected to a second channel. The microelectrodes typically had resistances of 1–3 MΩ. The pH signal was obtained with a four-channel electrometer (Biomedical Instrumentation Laboratory, Department of Cellular and Molecular Physiology, Yale University, New Haven, CT) that subtracts the potential of the voltage electrode from the potential of the pH electrode. A miniature calomel electrode (Accumet, Fisher Scientific) filled with saturated KCl served as the reference for the voltage electrode. Data were acquired and plotted using custom-designed software written by Mr. Duncan Wong for the Boron laboratory (Department of Cellular and Molecular Physiology, Yale University).

Two-electrode, Voltage-clamp Experiments. Voltage-sensitive and current-passing microelectrodes were pulled from borosilicate glass capillaries (G200F-4 or G83165T-4, Warner Instruments) with a micropipette puller (P-80 or P-97, Sutter Instruments or PC-10, Narishige, Tokyo, Japan). The electrodes were filled with saturated KCl and attached to the OC-725C voltage-clamp apparatus (Warner Instruments). Electrode resistances were typically 1-3 MΩ for the voltage electrodes, and 0.1–0.6 MΩ for the current electrodes. For experiments at a fixed holding potential of −60 mV, data were obtained at a filtering frequency of either 8–10 or 800 Hz with an 8-pole Bessel filter (LFP-8, Warner Instruments) and a sampling frequency of 30 Hz or 2 kHz, respectively. For the current-voltage (IV) relationships shown, the data were obtained at a filtering frequency of 800 Hz and a sampling frequency of 2 kHz. The voltage-step protocol for the IV plots shown included 12 sweeps in which the voltage was held at −60 mV for 60 ms, then stepped to one of 12 voltages (−200 mV to 20 mV in increments of 20 mV) for 20 ms, and finally returned to −60 mV for 20 ms before the next sweep. After obtaining each IV plot, we confirmed that the oocyte was electrically tight by turning off the voltage clamp and verifying that the spontaneous Vm was close to the acquired reversal potential (Virkki et al., 2002). Data acquired with the ClampEx software (Axon Instruments pClamp 8.2, Molecular Devices, San Jose, CA) were digitized with an 1322A interface (Axon Instruments), and then analyzed with ClampFit software (pClamp 8.2, Axon Instruments).

Inside-out macropatch experiments. Macropatch studies were performed on oocytes using a modification of the technique described by Hilgemann (1995). Patch pipettes were pulled from N-51-A borosilicate glass capillaries (O.D. 0.084 in. or 0.064 in., Drummond) using a PC-10 micropipette puller (Narishige). Tips gently broken to 10–12 μm were plunged into a bead of melted 8161 Corning glass (G86165T-4, Warner Instruments) fixed to 30-gauge, MF-9 platinum wire (Technical Products International Inc.), which was transiently heated by passing current. A break at the tip resulted from the wire retracting as it cooled. The process of heating the glass bead, plunging the tip, and cooling the wire was repeated until a satisfactory jagged-free tip of ~14 μm in diameter was obtained. Pipette resistances were ~4 MΩ.

Experiments were performed at room temperature (~23°C) in a flowthrough chamber on the stage of an inverted microscope (DMIRB, Leica). All solutions contained low Cl− (2 mM) to minimize contaminating endogenous Cl− currents in the oocyte (Machaca et al., 2002; Weber, 2002). Immediately before experiments, pipettes were backfilled with solution containing 5% CO2/33 mM HCO3−. Access to the plasma membrane was obtained by shrinking the oocyte in a hypertonic solution (see below) and then removing the vitelline membrane with fine forceps. GJ seals were obtained at a negative holding potential (Vm = −50 mV), and solution flow was initiated after patch excision. NBC currents were similar from patches obtained from either the animal or vegetal pole of the oocyte.

Currents were obtained using an Axopatch 200B patch-clamp amplifier (Axon Instruments). Low-pass (1 kHz, internal filter) currents were digitized with a Digitdata-1322A interface (Axon Instruments) at a sampling frequency of 5 kHz. Clampex software (pClamp 8.2, Axon Instruments) was used for data acquisition and analysis. Seal stability was routinely monitored throughout the experiment by measuring both membrane capacitance (using the membrane test function of Clampex) and seal resistance. For the figures shown, current recordings were further filtered at 100 Hz (8-pole Bessel filter), and subjected to data reduction (substitute average by a factor of four) using Clampfit (pClamp 8.2, Axon Instruments). Data were exported to Microsoft Excel 2002 for analysis and Origin 7.5 (OriginLab Corporation) for graphing.

Solutions

Two-electrode, Voltage-clamp Experiments. The standard ND96 solution (pH 7.5) contained (in mM): 96 NaCl, 2 KCl, 1 MgCl2, 1.8 CaCl2, 5 HEPES, 2.5 NaOH. In the standard 5% CO2/33 mM HCO3− solution, 33 mM NaCl was replaced with an equimolar amount of NaHCO3, and the solution was equilibrated with 5% CO2/95% O2 at pH 7.5. In the low Cl− (7.6 mM)-containing ND96 solution contained (in mM) 96 sodium cyclamate, 2 mM KOH, 0.25 sodium aspartate, 2.5 sodium cyclamate, 2.5 HEPES. These solutions were used to remove the vitelline membrane contained (in mM): 220 NMDG+, 220 aspartic acid, 2 MgCl2, 10 EGTA, and 10 HEPES. The solution was titrated to pH 7.2 with NMDG−.

Inside-out Macropatch Experiments. The hyperosmotic solution used to remove the vitelline membrane contained (in mM): 220 NMDG+, 220 aspartic acid, 2 MgCl2, 10 EGTA, and 10 HEPES. The solution was titrated to pH 7.2 with NMDG−.

For inside-out macropatch experiments, the 2 mM Cl−, ND96 solution contained (in mM) 96 sodium cyclamate, 2 mM KOH, 2 mM cyclamic acid, 1 mM MgCl2, 1.8 mM calcium cyclamate, 5 mM HEPES, and was adjusted to pH 7.5 with NaOH. In the 2 mM Cl− solution containing 5% CO2/33 mM HCO3−, 33 mM sodium cyclamate was replaced with an equimolar amount of NaHCO3, and the solution was equilibrated with 5% CO2/95% O2.

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Functional Expression of Rat Brain NBCe1-B in Oocytes

At the time the cDNA encoding rat brain NBCe1-C was cloned by homology and the protein characterized as an electronegatic NBC when expressed in oocytes, rat brain NBCe1-B was also identified (Bevensee et al., 2000). The amino acid sequence of rat brain NBCe1-B is 96% identical to that of human heart NBCe1-B, which has been characterized as a stilbene-sensitive, electronegatic NBC when expressed in oocytes (Choi et al., 1999). In the present manuscript, we expressed the rat brain version of NBCe1-B in oocytes and evaluated NBC function using pH- and voltage-sensitive microelectrodes.

At the onset of the experiment shown in Fig. 2A, an oocyte injected with rat brain NBCe1-B and bathed in a nominally CO₂/HCO₃⁻-free, HEPES-buffered solution (ND96, pH 7.5) had a resting pHᵢ of ~7.32 (top trace) and a Vₑ of ~−55 mV (bottom trace). Exposing the oocyte to a solution containing 1.5% CO₂/10 mM HCO₃⁻ (pH 7.5) elicited an initial decrease in pHᵢ (ab) due to CO₂ entry and subsequent formation of H⁺ in the cell. The pHᵢ then increased (bc) due to NBC-mediated HCO₃⁻ transport into the oocyte. The mean pHᵢ recovery rate at a pHᵢ of 7.12 ± 0.02 (after the initial CO₂-induced acidification) was 7.1 ± 0.8 × 10⁻³ pH units s⁻¹ (n = 6). This pHᵢ recovery was blocked (cd) by 200 μM DIDS, an inhibitor of bicarbonate transporters. As shown in Table I, stilbenes reduced the rate of pHᵢ recovery under different CO₂/HCO₃⁻ conditions by a mean of 76 ± 7.5% (range: 58–100%). Inhibition of the pHᵢ recovery was at least partially reversible (de) when DIDS was removed. Returning the oocyte to ND96 caused pHᵢ to increase rapidly (ef) due to the net conversion of intracellular H⁺ and HCO₃⁻ to H₂O and CO₂, which exited the cell. The final resting pHᵢ was higher than the initial value, an observation consistent with net NBC-mediated HCO₃⁻ influx during segment be.

The voltage changes in the NBC-expressing oocyte exposed to CO₂/HCO₃⁻ and then DIDS (Fig. 2A, bottom trace) demonstrate that NBCe1-B is electronegatic. Applying the CO₂/HCO₃⁻ solution elicited an ~17-mV hyperpolarization (a’b’) that is consistent with NBC transporting more bicarbonate than sodium into the oocyte. In the same six experiments summarized above, the mean hyperpolarization elicited by 1.5% CO₂/10 mM HCO₃⁻ was 25 ± 8 mV. Also consistent with electronegatic Na/HCO₃⁻ cotransport activity was the ~10-mV depolarization seen when the oocyte in CO₂/HCO₃⁻ was exposed to 200 μM DIDS (c’).

The experimental maneuvers shown in Fig. 2A had markedly different effects on pHᵢ and Vₑ. The H₂O-injected control oocyte shown in Fig. 2B. As shown in the top trace, the 1.5% CO₂/10 mM HCO₃⁻ solution elicited the initial pHᵢ decrease (ab), but no subsequent pHᵢ recovery (bc). At a mean pHᵢ of 7.08 ± 0.07, the
mean pHr recovery rate of $2.1 \pm 1.2 \times 10^{-5}$ pH units s$^{-1}$ ($n = 4$) was 3.4-fold less ($P < 0.01$) than the rate seen in NBCe1-B-expressing oocytes. Furthermore, 200 μM DIDS in the presence of CO$_2$/HCO$_3$ had little effect on the low, sustained pHr (cd). Returning the oocyte to ND96 caused pHr to increase and return to approximately the initial resting value (ef). The voltage changes in the control oocyte were equally unimpressive (bottom trace). CO$_2$/HCO$_3$ had no effect on Vm (a’b’), and DIDS only caused a small hyperpolarization (c’), consistent with inhibition of an endogenous Cl$^-$ conductance. In the same four experiments summarized above, the mean HCO$_3$-induced hyperpolarization of 1.5 ± 0.3 mV was 17-fold less than the hyperpolarization seen in NBCe1-B–expressing oocytes.

To increase the activity of NBCe1-B, we performed additional pHr/Vm experiments using solutions containing 33 mM HCO$_3$ and equilibrated with 5% CO$_2$ to maintain pH at 7.5. The mean pHr-recovery rate at a pHr of 6.87 ± 0.03 was 12.0 ± 2.5 × 10$^{-5}$ pH units s$^{-1}$ ($n = 11$). At a similar pHr (6.84 ± 0.03) in H$_2$O-injected oocytes, the mean pHr recovery rate of 4.3 ± 1.9 × 10$^{-5}$ pH units s$^{-1}$ ($n = 6$) was 2.8-fold less ($P = 0.01$). In these same experiments, the mean hyperpolarization elicited by 5% CO$_2$/33 mM HCO$_3$ was 53 ± 4.8 mV in NBC-expressing oocytes, and only 0.5 ± 0.5 mV in H$_2$O-injected oocytes. In summary, rat brain NBCe1-B expressed in oocytes is electrogenic, stilbene-sensitive, and activated by CO$_2$/HCO$_3$. As described below in voltage-clamp experiments, the transporter is also Na$^+$ dependent. All these characteristics are hallmarks of an electrogenic Na/HCO$_3$ cotransporter (see Romero et al., 2004).

### Voltage Dependencies of the NBCe1 Variants
To study the electrogenicity of the NBCe1 variants in more detail, we used the two-electrode, voltage-clamp technique to examine current–voltage (I-V) relationships of all three NBCe1 variants. In each experiment, I-V relationships were obtained from oocytes first bathed in a low Cl$^-$, ND96 solution, and then after 10 min in 5% CO$_2$/33 mM HCO$_3$ (to allow for intracellular equilibration of the physiological buffer). In some experiments, I-V plots were subsequently obtained after 2 min in the CO$_2$/HCO$_3$ solution containing 200 μM DIDS. The HCO$_3$-dependent I-V plot for an NBC is the

### Table 1

<table>
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<tr>
<th>Experiment</th>
<th>CO$_2$ / HCO$_3$</th>
<th>Stilbene</th>
<th>pHr</th>
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DIDS, 4,4’-dinitrostilbene-2,2’ disulfonate.

*Intracellular pH at the time of stilbene inhibition.
difference between the I-V plots obtained in the presence and absence of CO2/HCO3−, after subtracting the corresponding HCO3−-dependent I-V plot obtained from batch-matched H2O-injected eggs. An experiment performed on an oocyte expressing NBCe1-C is shown in Fig. 3 A. NBC currents obtained at potentials from −200 to +20 mV were larger in the presence of 5% CO2/33 mM HCO3− (squares) than in ND96 (diamonds). The larger currents were particularly evident at voltages more positive than −80 mV. In the presence of DIDS, the I-V plot (triangles) reverted back to that seen with ND96. DIDS-sensitive HCO3− currents were not observed in an H2O-injected control oocyte (Fig. 3 B). I-V plots looked similar for the control oocyte exposed to ND96 and CO2/HCO3− ± DIDS.

In Fig. 4 A, the average I-V relationships are shown for NBCe1-A (closed diamonds), -B (open squares), and -C (closed triangles). There are four noteworthy observations. First, the currents for the A variant are the largest. Second, all three I-V plots display a slight outward rectification. Third, the I-V plots for the B and C variants are nearly identical. The final observation is that the Erev is ~−85 mV for A (Fig. 4 A), but markedly more negative for B (~−155 mV) and C (~−170 mV) (Fig. 4 A, boxed inset). Although the more negative Erev values are consistent with different Na:HCO3− transport stoichiometries, a more likely explanation is different transmembrane Na+ and HCO3− gradients established by the NBCs. For example, compared to A, the less active B and C variants generate a smaller increase in [Na+] and [HCO3−] on the cytosolic side of the membrane, and therefore a more negative Vm for B and C is required to drive transport in the reverse direction out of the cell. We tested this possible explanation by injecting a smaller amount of NBCe1-A cRNA to reduce the magnitude of the whole-cell A current. Indeed, oocytes exhibiting smaller A currents displayed a negative shift in Erev (Fig. 4 B, open diamonds). For comparison, the I-V relationship for NBCe1-A shown in A is replotted in Fig. 4 B (closed diamonds). To minimize the influence of different transmembrane gradients, we compared the voltage dependencies of the three variants under similar transporter-mediated whole-cell currents. NBCe1-B and -C currents were increased by injecting oocytes with NBC cRNAs containing a modified Kozak sequence, whereas NBCe1-A currents were decreased by injecting oocytes with less cRNA. As shown in Fig. 4 C, the voltage dependencies of the three variants exhibiting comparable NBC-mediated currents are very similar.

Expression of the NBCe1 Variants at the Plasma Membrane of the Oocyte

Activity of Hemagglutinin-tagged NBCe1 Variants. The higher activity of NBCe1-A compared to the B and C variants shown in Fig. 4 A could be due to differences in plasma membrane expression. We therefore used the SOC technique with HA-tagged NBCe1 constructs to assess surface expression. We first tested the function of the tagged constructs expressed in oocytes. As shown in Fig. 4 B, there is no difference between the voltage dependencies of the untagged NBCe1-A (closed diamonds) redrawn from Fig. 4 A, and the HA-tagged transporter (NBCe1-AHA, closed squares). We performed additional studies to compare the function of tagged NBCe1-C. In pHi/Vm and voltage-clamp studies in which oocytes expressing either tagged or untagged NBCe1-C were exposed to 5% CO2/33 mM HCO3−, both groups displayed similar NBC-mediated hyperpolarizations, pHi recovery rates following the initial CO2-induced acidification, HCO3−-induced outward currents under voltage-clamp conditions, and I-V relationships. In conclusion, the introduced HA epitope does not alter NBCe1 activity.

Comparing Function and Surface Expression of NBCe1 Variants. We expressed HA-tagged NBCe1-A and NBCe1-C in oocytes, and then evaluated both transporter function with the two-electrode, voltage-clamp technique, and expression with the SOC technique. In our functional
assay, we monitored NBC-mediated outward currents in oocytes voltage clamped at −60 mV and exposed to 5% CO2/33 mM HCO3−. In contrast to an oocyte injected with H2O, an oocyte injected with either the A or C variant displayed an outward current when exposed to 5% CO2/33 mM HCO3− (Fig. 5 A). The A and C currents differed in the following three ways. First, the A-mediated current was 4.5-fold larger than the C-mediated current. Second, the A-mediated current decayed faster after its peak. The current decay is consistent with reduced transport in response to the buildup of substrate or a pHi increase at the inner surface of the oocyte membrane (and opposite changes at the outer surface of the membrane). According to subsequent data presented, the more active the transporter (as judged by the magnitude of the peak HCO3−-induced current), the faster the decay. As summarized in Fig. 5 B on two batches of day-matched oocytes, the mean H2O-subtracted, peak CO2/HCO3−-induced current was 4.3-fold larger in oocytes expressing NBCe1-A (874 ± 82 nA, n = 8) compared to NBCe1-C (201 ± 4 nA, n = 7). The larger NBC-mediated current seen with NBCe1-A compared to C is consistent with the I-V plot data shown in Fig. 4 A.

We used the same two batches of oocytes to examine plasma membrane NBC expression with the SOC technique. For each batch, the luminescence for each oocyte was normalized to the mean luminescence of oocytes expressing NBCe1-A. As expected, the mean normalized luminescence (Norm. Lum.) for A- or C-expressing oocytes was considerably higher than the mean value for H2O-injected oocytes (Fig. 5 C). These readings are specific for the anti-HA antibody because in a separate experiment (not depicted), luminescence was not observed when the anti-HA antibody was preabsorbed with an equal amount (1 mg ml−1) of the HA peptide. As shown by the second and third bars in Fig. 5 C, the mean Norm. Lum. was the same for the two NBCe1 variants (P > 0.3).

Similar functional and expression studies were performed on the HA-tagged NBCe1-B variant. The mean CO2/HCO3−-induced current was threefold smaller in oocytes expressing NBCe1-B (258 ± 10 nA, n = 6) compared to NBCe1-A (787 ± 57 nA, n = 7). According to SOC analysis, the mean Norm. Lum. was identical for oocytes expressing the B variant (1.01 ± 0.07, n = 5) and A variant (1.00 ± 0.09, n = 5). Therefore, the lower activity of B or C vs. A cannot be explained by lower plasma membrane expression.

**External Ion Dependencies of the NBCe1 Variants**

The increased activity of NBCe1-A compared to the B and C variants may be due to a higher affinity for HCO3− and/or Na+. In the following two sections, we compare –for each NBCe1 variant– the external HCO3− and Na+ dependencies.

**External HCO3− Dependencies.** We used an approach introduced by Grichtchenko et al. (2000) to determine the HCO3− dependencies of NBCe1s expressed in oocytes voltage clamped at −60 mV. NBC-mediated outward currents were recorded when oocytes were exposed to either 5% CO2/33 mM HCO3− (standard HCO3−),
or solutions containing different HCO$_3^-$ concentrations and equilibrated with appropriate CO$_2$/O$_2$ mixtures to maintain a pH of 7.5. All solutions contained 7.6 mM Cl$^-$ as described above.

Results from an oocyte expressing NBCe1-C are shown in Fig. 6 A. The oocyte was initially bathed in the normal Cl$^-$ (103.6 mM), HEPES-buffered solution and voltage clamped at $-60$ mV. Exposing the oocyte to a solution containing 7.6 mM Cl$^-$ generated a slow outward current (ab), possibly due to a small gluconate conductance. Exposing the oocyte to solutions containing different [HCO$_3^-$]s elicited rapid NBC-mediated outward currents (c-i). Currents were larger at progressively higher [HCO$_3^-$]s (h vs. d vs. e vs. f). Each non-33 mM HCO$_3^-$ exposure was flanked by standard HCO$_3^-$ exposures. A similar experiment performed on an H$_2$O-injected oocyte is shown in Fig. 6 B. The CO$_2$/HCO$_3^-$ solutions elicited only slow inward currents (c-i).

Using data from experiments similar to those shown in Fig. 6 A, we plotted the HCO$_3^-$ dependencies of NBCe1-B and -C. To correct for current drifts during experiments, as well as any differences in NBC expression among oocytes, we normalized the current elicited by each HCO$_3^-$ exposure to the mean current elicited by the flanking standard HCO$_3^-$ exposures. Corresponding currents obtained from H$_2$O-injected oocytes were subtracted before normalization. The external HCO$_3^-$ dependencies are shown for NBCe1-B (Fig. 6 C) and NBCe1-C (Fig. 6 D). The data for both variants are well fit using a model that combines a Michaelis-Menten mechanism with a linear component, as has been reported for rat kidney NBCe1-A (Grichchenko et al., 2000). The external HCO$_3^-$ dependencies of the B and C variants are nearly identical. For NBCe1-B, the apparent $K_M$ for HCO$_3^-$ is $4.68 \pm 0.47$ mM and the normalized $V_{max}$ is $0.92 \pm 0.03$ ($n = 24$ from six oocytes). For NBCe1-C, the apparent $K_M$ for HCO$_3^-$ is $4.31 \pm 0.46$ mM and the normalized $V_{max}$ is $0.88 \pm 0.03$ ($n = 24$ from six oocytes). These values for the B and C variants are very similar to our values of apparent $K_M$ (6.90 $\pm$ 3.04 mM) and normalized $V_{max}$ (0.96 $\pm$ 0.12) ($n = 10$ from three oocytes) obtained for the A variant. Furthermore, our values for NBCe1-A are nearly identical to the values of apparent $K_M$ (6.5 mM) and normalized $V_{max}$ (0.97) previously reported for NBCe1-A (Grichchenko et al., 2000).

We also examined the bicarbonate dependence of an NBCe1-A/C chimera that contains the 41 amino-terminal residues of the A variant and the 61 carboxy-terminal residues of the C variant. In these studies, the A/C chimera exhibited a mean outward current (1240 $\pm$ 103 nA, $n = 5$) similar to that of wild-type NBCe1-A (1096 $\pm$ 296 nA, $n = 3$) when the oocytes were exposed to 5% CO$_2$/33 mM HCO$_3^-$.

These HCO$_3^-$ exposures elicited rapid NBC-mediated outward currents. A similar approach we used a similar approach to the one described above to determine the external Na$^+$ dependencies of the NBCe1 variants. In our assay, we examined the magnitudes of the NBC-mediated outward currents elicited by 5% CO$_2$/33 mM HCO$_3^-$ solutions containing various [Na$^+$]s. Our experimental protocol minimized changes in intracellular [Na$^+$] ([Na$^{i+}$]) by minimizing the time oocytes spent in reduced [Na$^+$]s.

Results from an oocyte expressing NBCe1-C are shown in Fig. 7 A. The voltage-clamped oocyte was initially bathed in ND96 and then exposed to 5% CO$_2$/33 mM HCO$_3^-$ solutions containing different amounts of Na$^+$. These HCO$_3^-$ exposures elicited rapid NBC-mediated outward currents (a-i), which were smaller at progressively lower concentrations of Na$^+$ (b vs. d vs. f vs. h).
Using an approach similar to that described above for our bicarbonate-dependence experiments, each low-Na⁺, HCO₃⁻ exposure was flanked by full-Na⁺ (98.5 mM) HCO₃⁻ exposures. A similar experiment on an H₂O-injected oocyte subjected to the same experimental protocol is shown in Fig. 7 B. The CO₂/HCO₃⁻ solutions of different Na⁺ concentrations elicited only small outward currents.

One noteworthy observation (unpublished data) with oocytes expressing NBCe1-A was the presence of a Na⁺-independent, outward current elicited by CO₂/HCO₃⁻ that was not seen in H₂O-injected oocytes. In the absence of external Na⁺, the 33 mM HCO₃⁻ solution elicited a mean outward current in A-expressing oocytes (153 ± 48 nA, n = 4) that was 15-fold larger (P = 0.03) than that seen in H₂O-injected oocytes (12 ± 3 nA, n = 4), and 18 ± 5% of the total HCO₃⁻ current in "full" 98.5 mM Na⁺. This Na⁺-independent HCO₃⁻ current, which was not seen in oocytes expressing NBCe1-B (12 ± 3 nA, n = 6) or NBCe1-C (19 ± 3 nA, n = 10), may be due to transporter slippage as described for H⁺ pumps, neurotransmitter transporters, and metal ion transporters (Nelson et al., 2002). Alternatively, a small NBC-mediated conductance (e.g., HCO₃⁻) that is independent of Na⁺-coupled transport may be responsible. A Na⁺ conductance has been described for the electroneutral NBC, NBCn1 (Choi et al., 2000).

We used data from experiments similar to that shown in Fig. 7 A to determine the Na⁺ dependencies of NBCe1-A, -B, and -C. For each experiment, we normalized the current elicited by 5% CO₂/33 mM HCO₃⁻ solutions containing different [Na⁺]s to the mean current elicited by a flanking CO₂/HCO₃⁻ solution containing full, 98.5 mM Na⁺. HCO₃⁻ currents obtained in the absence of Na⁺ were subtracted before normalization. We plot the external Na⁺ dependencies for the two most dissimilar variants: NBCe1-A in Fig. 7 C and NBCe1-C in Fig. 7 D. The apparent KM values are similar for both the A and C variants. The apparent KM values are 20.6 ± 1.7 mM for NBCe1-A (n = 33 from five oocytes) and 31.7 ± 2.6 mM for NBCe1-C (n = 71 from 12 oocytes). Sciortino and Romero (1999) reported a similar apparent KM of 30 mM for NBCe1-A. Our corresponding normalized Vmax values are 1.21 ± 0.03 for NBCe1-A and 1.31 ± 0.04 for NBCe1-C. In additional studies, we also examined the external Na⁺ dependence of NBCe1-B and obtained an apparent KM of 35.5 ± 3.7 mM and normalized Vmax of 1.35 ± 0.05 (n = 23 from five oocytes). In conclusion, all three NBCe1 variants have similar external Na⁺ dependencies.

Role of the Amino Termini on the Function and Expression of NBCe1 Variants in Oocytes

According to the aforementioned HCO₃⁻ and Na⁺ dependence data, the higher NBC current seen with NBCe1-A vs. -B and -C (Figs. 4 and 5) is not due to
higher substrate affinities. Therefore, the A variant must have a higher transport velocity compared to the B and C variants. As shown in Fig. 1, the differences in amino acid sequence among these three variants reside only at the amino and/or carboxy termini. The amino terminus is likely responsible for differences in NBC activity because the B and C variants (with low activity) share the same amino terminus, which is different than the unique amino terminus of the A variant (with high activity). Furthermore, the A/C chimera containing the unique carboxy terminus of C attached to the A variant displays NBC currents similar to those of the wildtype A variant.

Activity and Surface Expression of NBCe1 Variants Truncated before the First Transmembrane Domain. We used the HCO$_3^-$-induced, outward-current assay shown in Fig. 5 A to examine the function of NBCe1 constructs with amino-terminal truncations. The three experimental traces shown in Fig. 8 A are from voltage-clamped oocytes injected with cRNA encoding wild-type NBCe1-C (CWT), NBCe1-C missing the first 426 residues before the first predicted transmembrane domain (CΔN426), or NBCe1-C missing the first 213 residues (CΔN213). Oocytes exposed to a solution containing 5% CO$_2$/33 mM HCO$_3^-$ elicited an outward current of ~250 nA in the oocyte expressing CWT, but little/no current in the oocyte expressing either CΔN426 or CΔN213.

From experiments similar to those shown in A, the mean CO$_2$/HCO$_3^-$-induced currents from oocytes expressing CΔN426 or CΔN213 were similar to those seen in day-matched, H$_2$O-injected oocytes, and more than 90% less than those in day-matched, CWT-expressing eggs (Fig. 8 B). (Because we often examined several constructs on a given day, we necessarily used current and SOC data obtained from oocytes injected with H$_2$O and/or wild-type NBC constructs in more than one analysis.) A similar loss of NBC activity was observed with the homologous truncation of NBCe1-A before the first transmembrane domain, AΔN382 ($n = 2$, not depicted).

SOC analysis was performed on the same two oocyte batches, and luminescence for each oocyte was normalized to the mean luminescence of oocytes expressing wild-type CWT. Compared with the mean Norm. Lum. for CWT, the mean values were actually 50% higher ($P = 0.002$) for CΔN426 and similar ($P = 0.14$) for CΔN213 (Fig. 8 C). In summary, the lost NBC activity of CΔN426 and CΔN213 is not due to the absence of expression at the plasma membrane.

In further structure–function studies, we examined the effects on transporter activity and plasma membrane expression of removing the NH$_2$-terminal 43 residues of NBCe1-A (AΔN43) and 87 residues of NBCe1-C (CΔN87). Because the amino terminus of the B and C variants is identical, studies on CΔN87 provide NH$_2$-terminal information on the B variant.
Activity and Surface Expression of NBCe1-A Lacking its Unique Amino Terminus. At a holding potential of −60 mV, an oocyte expressing A\(_{\Delta N87}\) (Fig. 9 A, left) displayed an HCO\(_3^-\)-induced outward current that was ∼50% smaller than the current seen in the oocyte expressing wild-type NBCe1-A (A\(_{WT}\)). Therefore, removing the amino-terminal 43 residues of the A variant decreases transporter activity. The summary data of HCO\(_3^-\)-mediated outward currents are shown in Fig. 9 B (left).

From five batches of oocytes, the mean HCO\(_3^-\)-induced current for A\(_{\Delta N87}\) (443 ± 31 nA, n = 18) was 55% smaller (P < 0.001) than the mean current for A\(_{WT}\) (990 ± 81 nA, n = 15). The mean HCO\(_3^-\)-induced current from batch-matched, H\(_2\)O-injected oocytes was only 0.8% of the mean current from A\(_{WT}\)-expressing oocytes.

In four of these five batches in which SOC data were obtained, the mean Norm. Lum. was 10% lower (marginal significance; P = 0.05) for oocytes injected with A\(_{\Delta N87}\) compared to those injected with A\(_{WT}\) (Fig. 9 C, left). A 10% lower surface expression would not explain the 55% decrease in transporter activity of A\(_{\Delta N87}\) compared to A\(_{WT}\). In addition, in one of these four batches in which the surface expression of A\(_{\Delta N87}\) (n = 5) was 96 ± 14% of A\(_{WT}\) (n = 5), the mean NBC-mediated current for A\(_{\Delta N87}\) (656 ± 105 nA, n = 5) was 46% smaller (P = 0.04) than for A\(_{WT}\) (1220 ± 192 nA, n = 3). Therefore, the lower activity of A\(_{\Delta N87}\) compared to A\(_{WT}\) cannot be explained by a difference in surface expression.

Activity and Surface Expression of NBCe1-C Lacking the Different Amino Terminus. Different results were obtained with the homologous NH\(_2\)-terminal truncation of NBCe1-C (C\(_{AN87}\)). At a holding potential of −60 mV, an oocyte expressing C\(_{AN87}\) (Fig. 9 A, right) displayed an HCO\(_3^-\)-induced outward current that was 3.4-fold larger than the current seen in the oocyte expressing C\(_{WT}\). Therefore, removing the amino-terminal 87 residues of C\(_{WT}\) increases transporter activity. The summary data of HCO\(_3^-\)-mediated outward currents from experiments similar to those shown in Fig. 9 A (right) are shown in B (right).

From seven batches of oocytes, the mean HCO\(_3^-\)-induced current for C\(_{AN87}\) (654 ± 48 nA, n = 23) was 2.7-fold larger (P < 0.001) than the mean current for C\(_{WT}\) (242 ± 12 nA, n = 19). As expected from Fig. 5 data, the mean current for C\(_{WT}\) was ∼25% of the mean current for A\(_{WT}\).

In five of the seven batches above in which SOC data were obtained, the mean Norm. Lum. was 33% higher (P < 0.001) for oocytes injected with C\(_{AN87}\) compared to those injected with C\(_{WT}\) (Fig. 9 C, right). A 33% increase in surface expression would not explain the 170% increase in transporter activity of C\(_{AN87}\) compared with C\(_{WT}\). Moreover, in two of these five batches in which the surface expression of C\(_{AN87}\) (n = 9) was 102 ± 4% of C\(_{WT}\) (n = 10), the mean NBC-mediated current for C\(_{AN87}\) (713 ± 137 nA, n = 6) was also 2.7-fold larger (P = 0.01) than for C\(_{WT}\) (264 ± 20 nA, n = 5). Therefore, the higher activity of C\(_{AN87}\) compared to C\(_{WT}\) cannot be explained by a difference in surface expression. Data from Fig. 9 are consistent with the amino-terminal residues of NBCe1-A stimulating transporter activity, and the amino-terminal residues of NBCe1-B and C inhibiting transporter activity.

If the amino termini of the NBCe1 variants are solely responsible for the higher transporter activity seen with A compared to B and C, then the activities of A\(_{\Delta N43}\) and A\(_{\Delta N213}\) should be identical. However, as shown in Fig. 9, the mean C\(_{AN213}\) current (654 nA) is ∼50% larger (P < 0.001) than the mean A\(_{\Delta N43}\) current (444 nA). The larger mean C\(_{AN213}\) current could be explained by the slightly higher surface expression of C\(_{AN213}\) vs. A\(_{\Delta N43}\) as revealed in the SOC analysis shown in Fig. 9 C. However, a difference in surface expression does not appear to be the only explanation according to current–voltage (I-V) analyses (Fig. 10).

**Figure 8.** Inhibited NBCe1 activity elicited by removing regions of the cytosolic amino terminus. (A) Exposing oocytes to a solution containing 5% CO\(_2\)/33 mM HCO\(_3^-\) elicited an outward current in the oocyte expressing wild-type NBCe1-C (C\(_{WT}\)), but little/no current in the oocyte expressing either C\(_{\Delta N426}\) or C\(_{\Delta N213}\). (B) Summary of HCO\(_3^-\)-induced outward currents from experiments similar to those shown in A. For each bar, n ≥ 3 from two batches of oocytes. SEM values for the C\(_{\Delta N426}\) or C\(_{\Delta N213}\) data are small. (C) Compared to the mean normalized luminescence (Norm. Lum.) for C\(_{WT}\), mean Norm. Lum. was 1.5-fold greater for C\(_{\Delta N426}\) and similar for C\(_{\Delta N213}\). In oocytes from the same two batches in B, n ≥ 10 for each bar.

I-V Relationships of Wild-type and Mutant NBCe1-A and C Constructs. We next examined the I-V relationships of
AWT, CWT, AΔN43, and CΔN87. A representative experiment on an oocyte expressing CΔN87 is shown in Fig. 10 A. I-V relationships were obtained from the oocyte first bathed in ND96 (diamonds), then after 1 min (circles) and 10 min (squares) in 5% CO2/33 mM HCO3−, and finally after 2 min in the physiological buffer containing 200 μM DIDS (triangles). The larger currents obtained with the oocyte in CO2/HCO3− were inhibited by the presence of 200 μM DIDS. The left-shifted I-V plot obtained after a 1 vs. 10 min exposure to CO2/HCO3− was due to the incomplete equilibration of CO2/HCO3− across the membrane, and consequently, a larger extracellular-to-intracellular HCO3− gradient.

From experiments similar to that shown in Fig. 10 A, mean HCO3−-dependent I-V plots were obtained from oocytes injected with wild-type NBCe1-A and -C, and the truncated constructs AΔN43 and CΔN87 (Fig. 10 B). The data were from day-matched experiments, and mean I-V data from H2O-injected oocytes were subtracted. Similar to the Fig. 9 results obtained at a fixed potential of −60 mV, the voltage-dependent AΔN43 currents (Fig. 10 B, open diamonds) were smaller than the corresponding wild-type A currents (closed diamonds), and the CΔN87 currents (open triangles) were larger than the corresponding wild-type C currents (closed triangles) at all holding potentials from −200 to +20 mV (except at −80 mV). For each construct, the NBC conductance (gNBC) was determined from the slope of a linear fit to either the mean inward currents (Vh from −200 to −100 mV), or the mean outward currents (Vh from −80 to +20 mV) plotted in Fig. 10 B. For the inward currents, the gNBC of AΔN43 was 64% lower than that of AWT, and the gNBC of CΔN87 was 2.3-fold higher than that of CWT. Similar gNBC results were obtained for the outward currents.
C_{ΔC97} currents were also found to be consistently larger than the A_{ΔN43} currents. The mean C_{ΔN87} current was approximately twofold larger than the mean A_{ΔN43} current at −200 and +20 mV. Furthermore, g_{NBC} of C_{ΔN87} was 1.8-fold higher than the g_{NBC} of A_{ΔN43}. These data are consistent with the unique carboxy terminus of the C variant vs. the carboxy terminus of the A/B variant contributing to higher NBC activity in the absence of the amino terminus.

Role of the Carboxy Termini on the Function and Expression of NBCe1 Variants in Oocytes

Activity and Surface Expression of NBCe1 Truncated after the Last Transmembrane Domain. To determine the importance of the cytosolic carboxy terminus of NBCe1, we removed the 97 residues after the last predicted transmembrane domain of NBCe1-C (C_{ΔC97}). This construct is identical to the homologous truncation of NBCe1-B. After expressing C_{ΔC97} in oocytes, we used pH-sensitive microelectrodes and the voltage-clamp technique to measure simultaneously pH_{i} and membrane current of oocytes voltage clamped at −60 mV.

Records from simultaneous pH_{i} and voltage-clamp experiments on oocytes expressing wild-type NBCe1-C (C_{WT}) and C_{ΔC97} are shown in Fig. 11. After stable pH_{i} and V_{m} values were obtained, oocytes were voltage clamped at −60 mV and exposed to 5% CO_{2}/33 mM HCO_{3}−. As expected from Fig. 2 and Fig. 5 data, the C_{WT}-expressing oocyte (panel A) exhibited an instantaneous HCO_{3}−-stimulated outward current (point a′, bottom trace), as well as a pH_{i} recovery following the initial CO_{2}−-induced acidification (abc, top trace). Removing external Na^{+} reversed the outward current (c′), and blocked the pH_{i} recovery (cd). In contrast, the C_{ΔC97}-expressing oocyte (panel B) exposed to CO_{2}/HCO_{3}− exhibited neither a HCO_{3}−-stimulated outward current (point a′, bottom trace), nor any appreciable pH_{i} recovery after the CO_{2}-induced acidification (abc, upper trace). Stars in the voltage traces represent time points during the experiment when I-V plots were obtained (unpublished data).

The summary data from experiments similar to those shown in Fig. 11 (A and B) are shown in Fig. 12 (A and B). Data were obtained from two batches of oocytes. The mean rate of pH_{i} recovery (dP_{i}/dt) during the segment bc pH_{i} recovery in oocytes expressing C_{ΔC97} was fourfold less than the pH_{i} recovery rate in oocytes expressing C_{WT}, and similar (P > 0.14) to the rate in oocytes injected with H_{2}O (panel A). The mean CO_{2}/HCO_{3}−-induced outward current (a′) was 14-fold smaller in oocytes expressing C_{ΔC97} compared to C_{WT}, and no different (P > 0.18) than the mean current in H_{2}O-injected eggs (panel B).

To identify the explanation for the lost transporter activity of C_{ΔC97}, we used immunoblotting and SOC techniques to examine protein expression. According to the immunoblot data shown in Fig. 12 C, a polyclonal antibody to the amino terminus of NBCe1 recognized the expected-size protein from total microsomal protein isolated from an oocyte injected with either C_{ΔC97} (lane 1) or C_{WT} (lane 3). No labeling was observed in protein from an H_{2}O-injected oocyte (lane 2). Thus, the injected C_{ΔC97} RNA is translated and the protein is expressed in a microsomal fraction. However, according to the SOC data (normalized to the mean luminescence of C_{WT}-expressing oocytes), the normalized mean luminescence of C_{ΔC97}-injected oocytes is approximately threefold lower than that of C_{WT}, and even slightly lower than that of H_{2}O-injected oocytes (P < 0.01). Therefore, C_{ΔC97} is not expressed at the plasma membrane of oocytes, a result that was confirmed by immunocytochemistry with the anti-HA antibody.
(unpublished data). In summary, the loss of C∆C97 activity in oocytes is due to the absence of C∆C97 protein at the plasma membrane.

Activity and Surface Expression of NBCe1 Variants Truncated at the Different Carboxy Termini. To explore the contribution of the different carboxy termini of the NBCe1 variants to plasma membrane expression, and possibly function, we performed additional studies comparing the expression and function of wild-type and truncated variants. More specifically, we created two additional constructs: NBCe1-A truncated 46 residues from the carboxy terminus (A∆C46) and NBCe1-C truncated 61 residues from the carboxy terminus (C∆C61). The C∆C61 construct is identical to the homologous truncation of NBCe1-B.

NBC-mediated outward currents in response to CO2/HCO3− are shown in Fig. 13 A for two voltage-clamped oocytes; one expressing AWT and the other expressing A∆C46. The HCO3−-induced outward current of ~0.43 pA in the A∆C46-expressing oocyte was 71% smaller than the current of ~1.45 pA in the AWT-expressing oocyte. The summary data from similar experiments performed on AWT and A∆C46, as well as CWT and C∆C61, are plotted in Fig. 13 B. The mean currents obtained from H2O-injected oocytes were subtracted from the corresponding currents seen in the NBC-injected oocytes. The mean NBC-mediated currents of the truncated NBCs were ~30% of the corresponding wild-type NBCs. As shown in Fig. 13 C, both AWT and A∆C46 are expressed in a microsomal membrane fraction. Similar results were obtained with CWT and C∆C61 (not depicted). However, according to summary SOC data (Fig. 13 D) from the same batches of oocytes, A∆C46 and C∆C61 are poorly expressed at the plasma membrane compared to the corresponding wild-type NBCs. Therefore, the reduced activity of A∆C46 and C∆C61 in oocytes is predominately due to low surface expression.

Transporter Activity of NBCe1 Variants in the Inside-out Macropatch
To examine the contribution of the amino termini to NBCe1 transporter activity in the absence of cytosolic factors, we next examined the function of CWT, AWT, C∆N87, and A∆N15 in excised macropatches in the inside-out configuration from oocytes. In our experimental protocol (Fig. 14 A), we excised patches into a low-Cl− solution containing 200 µM DIDS and then activated NBCs by exposing the patch to the low-Cl− solution containing 5% CO2/33 mM HCO3−. NBC-mediated inward currents were obtained at a holding pipette potential (Vp or −Vm) of +60 mV.

CWT and C∆N87. As shown in Fig. 14 B, there was no appreciable change in current when a macropatch from an H2O-injected oocyte was exposed first to CO2/HCO3−, and then to the HCO3− solution containing 200 µM DIDS. In contrast, exposing a macropatch from a AWT-expressing oocyte to CO2/HCO3− elicited an inward current of ~5 pA (Fig. 14 C). This inward current is consistent with NBC-mediated net-negative charge moving from the bath to the pipette held at +60 mV. Switching back to the ND96 solution caused the current to return to the original level. In many experiments with either the C or A variant, we could reactivate this HCO3−-induced inward current by reexposing the patch to the CO2/HCO3− solution (unpublished data). However, the current change elicited by the second HCO3− exposure was often smaller—a result consistent with “rundown” of transporter activity. The phenomenon of rundown is observed with channels such as ROMK1 (McNicholas et al., 1994), and reflects events such as protein dephosphorylation (McNicholas et al., 1994) or loss of protein interaction with membrane phospholipids such as phosphatidylinositol 4,5-bisphosphate (PIP2) (Huang et al., 1998; Suh and Hille, 2005).

As shown in Fig. 14 D, exposing a macropatch from an oocyte expressing C∆N87 to the low-Cl−, CO2/HCO3− solution elicited a large inward current of ~28 pA. The current was ~5.5-fold larger than the HCO3−-induced current seen with CWT (Fig. 14 C). If these HCO3−-induced inward currents are NBC mediated, then they should be inhibited by stilbene derivatives such as DIDS. Indeed, the inward current seen with C∆N87 was
completely reversed by exposing the patch to 200 μM DIDS in the continued presence of CO₂/HCO₃⁻ (Fig. 14 D). DIDS inhibits NBCe1 activity from either the extracellular or intracellular side of an oocyte macropatch (Heyer et al., 1999). In a total of five experiments similar to those shown in A, n ≥ 6 for each bar from two batches of oocytes. (C) Immunoblot analysis of total microsomal protein from single oocytes injected with H₂O, AWT cRNA, or AΔC₄₆ cRNA. An NBCe1 antibody labeled bands of the expected sizes: ~130 kDa for AWT and ~120 kDa for AΔC₄₆. (D) The mean normalized luminescence (Norm. Lum.) for oocytes expressing the truncated NBCs were markedly less than the Norm. Lum. for oocytes expressing the corresponding wild-type NBCs. n ≥ 11 for each bar from two batches of oocytes.

Figure 13. Reduced activity and low surface expression of AΔC₄₆ and CΔC₆₁ expressed in oocytes. (A) 5% CO₂/33 mM HCO₃⁻ elicited an outward current in an oocyte expressing NBCe1-A (ΔWT) that was markedly larger than the current in the oocyte expressing AΔC₄₆. (B) Summary of H₂O-subtracted, HCO₃⁻-induced currents from experiments similar to those shown in A, n ≥ 6 for each bar from two batches of oocytes. (C) Immunoblot analysis of total microsomal protein from single oocytes injected with H₂O, AWT cRNA, or AΔC₄₆ cRNA. An NBCe1 antibody labeled bands of the expected sizes: ~130 kDa for AWT and ~120 kDa for AΔC₄₆. (D) The mean normalized luminescence (Norm. Lum.) for oocytes expressing the truncated NBCs were markedly less than the Norm. Lum. for oocytes expressing the corresponding wild-type NBCs. n ≥ 11 for each bar from two batches of oocytes.

Figure 14. NBCe1 activity from inside-out macropatches excised from oocytes. (A) NBC-mediated inward currents were elicited by exposing patches to a low-Cl⁻, 5% CO₂/33 mM HCO₃⁻ solution ± 200 μM DIDS with the patch pipette (−Vₑ = −60 mV) containing the same low-Cl⁻, 33 mM HCO₃⁻ solution. (B) H₂O-injected oocyte. The HCO₃⁻ solution without or with DIDS did not elicit any change in current. (C) CWT-injected oocyte. The HCO₃⁻ solution elicited a small inward current that was reversible when the patch was returned to ND96. (D) CΔN₈₇-injected oocyte. The inward current elicited by the HCO₃⁻ solution was larger than the current seen in C, and completely inhibited by DIDS. (E) AWT-injected oocyte. The HCO₃⁻ solution elicited a small, reversible inward current. (F) AΔN₄₃-injected oocyte. No change in current was observed when the patch was exposed to HCO₃⁻.

AWT and AΔN₄₃. We performed macropatch experiments on AWT and AΔN₄₃ using the aforementioned protocol. Based on the finding that macropatch currents for CΔN₈₇ were larger than those of CWT, an observation that paralleled the whole-cell data, we anticipated AWT to exhibit the largest macropatch currents of all the NBC constructs in the present study. As shown in Fig. 14 E, an HCO₃⁻-induced, NBC-mediated current of ~7 pA was indeed obtained in a macropatch from an oocyte expressing AWT. Unexpectedly however, the mean AWT current was of similar magnitude to the CWT current (Fig. 14 C), and considerably less than the CΔN₈₇ current (D). In additional studies (not depicted), we confirmed that this AWT-mediated HCO₃⁻-induced current was eliminated with 200 μM DIDS. The inward current elicited by CO₂/HCO₃⁻ in a macropatch expressing AΔN₄₃ was absent (Fig. 14 F). The NBC-mediated current rose much faster for the AWT (Fig. 14 E) variant than the CWT variant (C)—an observation also made in whole-cell experiments (see Fig. 5). Therefore, the activation kinetics of A appear faster than those of C, even in the absence of the cytosol.
Voltage and Ion Dependencies of NBCe1 Variants in Xenopus Oocytes

While all three variants exhibited similar voltage and external ion dependencies, the reversal potential ($E_{\text{rev}}$) of NBCe1-A was more positive ($\sim -85$ mV) than the $E_{\text{rev}}$ values of the B and C variants ($\sim -160$ mV) (Fig. 4 A). Working on rat kidney NBCe1-A expressed in oocytes, Sciortino and Romero (1999) obtained an $E_{\text{rev}}$ similar to ours, and calculated a corresponding 1:2 Na$^+$:HCO$_3^-$ stoichiometry for the transporter. As mentioned in Results, the more negative $E_{\text{rev}}$ values for the B and C variants are unlikely to reflect different Na$^+$:HCO$_3^-$ stoichiometries; but rather, different substrate and pH gradients established immediately across the plasma membrane by the variants. Indeed, we observed a similar negative shift of $E_{\text{rev}}$ in oocytes displaying smaller NBCe1-A currents in response to less cRNA injected (Fig. 4 B). Furthermore, the I-V relationships for the three variants looked similar when we adjusted expression levels and matched the magnitude of the whole-cell, transporter currents (Fig. 4 C).

For the NBCe1 variants, we calculated transporter stoichiometry using $E_{\text{rev}}$ values and the extracellular Na$^+$ and HCO$_3^-$ concentrations used in the study. The equilibrium potential of an electrogenic NBC ($E_{\text{NBC}}$) is described by the following equation (Boron and Boulpaep, 1983; Deitmer and Schlue, 1989):

$$E_{\text{NBC}} = \frac{RT}{nF} \ln \left( \frac{[\text{HCO}_3^-]^n}{[\text{Na}^+]^x} \right),$$

where $R$, $T$, and $F$ have their usual meanings, and $n$ is the HCO$_3^-$:Na$^+$ stoichiometry. Solving Eq. 1 for $n$ yields the transformed equation:

$$n = \frac{E_{\text{NBC}} - RT \ln x}{E_{\text{NBC}} F + RT \ln y},$$

where $x = \frac{[\text{Na}^+]}{[\text{HCO}_3^-]}$ and $y = \frac{[\text{HCO}_3^-]}{[\text{Na}^+]}$.

We determined the mean intracellular HCO$_3^-$ concentration of $\sim$8.3 mM from simultaneous pHi and voltage-clamp experiments on NBCe1-C–expressing oocytes similar to those shown in Fig. 11 A. For the intracellular Na$^+$ concentration, we used a measured value of 10.4 mM previously reported by Sciortino and Romero (1999) for unclamped oocytes expressing NBCe1-A. Our calculated Na$^+$:HCO$_3^-$ stoichiometries are 1:1.8 for the B variant and 1:1.7 for the C variant. These values are similar to the 1:2 Na$^+$:HCO$_3^-$ stoichiometry previously calculated for the A variant (Sciortino and Romero, 1999). Moreover, we used the $E_{\text{rev}}$ of $-120/-140$ mV from the I-V relationship shown in Fig. 4 (B and C) to calculate a similar Na$^+$:HCO$_3^-$ stoichiometry of $\sim$1:2 for the A variant. We used the lower-current I-V

**DISCUSSION**

**Activity of NBCe1 Variants**

In this study, we compared the function and expression of the three NBCe1 variants (A, B, and C) heterologously expressed in *Xenopus* oocytes. In whole-cell experiments, the A variant displayed larger NBC currents than the B and C variants; an observation not due to differences in plasma membrane expression, or differences in voltage or external ion dependencies. The higher transport velocity of the A variant is due to its unique amino terminus. In both whole-cell and macropatch studies, removing the unique amino terminus of the A variant decreases activity, whereas removing the different amino terminus of the C variant increases activity.
relationships for the analysis to minimize the complication of a large NBC-mediated substrate/pH gradient across the membrane (see above). In summary, all three variants expressed in oocytes appear to have the same 1:2 Na\(^+\):HCO\(_3\)^\(-\) stoichiometry.

Contribution of the Carboxy Termini to NBCe1 Activity

The carboxy-terminal truncations of NBCe1 variants examined in this study express poorly at the plasma membrane of oocytes. Based on our results, one or more regions within the different carboxy termini among the variants is/are required for proper plasma membrane expression in oocytes. Similarly, Cordat et al. (2003) reported that an AE1 construct truncated 11 residues from the carboxy terminus displayed reduced plasma membrane expression when expressed in HEK293 cells. Furthermore, Li et al. (2004) reported that GFP-tagged, human NBCe1-A mutants missing either the carboxy-terminal 26 or 50 residues retarget to the apical membrane of MDCK cells, and the latter construct displays significant intracytoplasmic expression. Our data with both A\(_{\Delta C46}\) and C\(_{\Delta C46}\) are consistent with this finding that the carboxy termini can influence trafficking. However, the effect of truncating the different carboxy termini found in A and C on plasma membrane expression is more dramatic in oocytes. The reduced surface expression could be due to the loss of a residue/region required for protein expression/stability, or an altered conformation that leads to organellar retention.

Contribution of the Amino Terminus to NBCe1 Activity

The following three general models would explain the contribution(s) of the different amino termini to NBC activity: (1) the unique amino terminus of A (A\(_{\text{N41}}\)) is stimulatory for an otherwise low-activity transporter, (2) the amino terminus of B/C is inhibitory (C\(_{\text{N87}}\)) for an otherwise high-activity transporter, or (3) both termini can influence function of an otherwise intermediate-activity transporter. Our observation that both whole-cell and macropatch NBC currents were inhibited to an intermediate level by A\(_{\text{N41}}\) and stimulated to an intermediate level by C\(_{\text{N87}}\) are consistent with the third possibility. The amino-terminal truncations likely lead to changes in transporter velocity. Indeed, the external Na\(^+\) and HCO\(_3\)^\(-\) dependencies for C\(_{\text{N87}}\) were similar to those for C\(_{\text{WT}}\) (unpublished data). Therefore, the different amino termini of the variants can influence transporter activity two to threefold even though they only comprise a small fraction of the entire NBCe1 sequence (4% for A and 8% for C). While the different amino termini stimulate or inhibit transporter activity, one or more regions of the cytosolic amino terminus downstream of the variable region are required for NBC function. Removing nearly all of the cytosolic amino terminus of NBCe1-A or -C, or approximately half of this region of NBCe1-C, leads to a complete loss of transporter function without affecting plasma membrane expression.

Potential Mechanism(s) for the Amino-terminal Effects.

There are several possible mechanisms by which the amino terminus of an NBCe1 could affect transporter activity. An amino terminus may bind to a region of the same NBC protein and either elicit a change in protein conformation or directly interact with the ion translocation pathway. However, a specific carboxy terminus does not appear to be essential in light of the observation that the A variant and the A variant containing the carboxy terminus of C (A/C chimera) display a similar level of activity. Interaction with the translocation pathway may be similar to the “ball and chain” mechanism of inactivation of potassium channels (Zagotta et al., 1990; Tseng-Crank et al., 1993). Alternatively, the amino terminus of one NBC may exert its effect by binding to another NBC protein. Evidence for multimerization of NBCe1 has been reported in preliminary form (Espiritu, D.J.D., A.A. Bernardo, and J.A.L. Arruda. 2003. FASEB J. 17:A462; Espiritu, D.J.D., A.A. Bernardo, and J.A.L. Arruda. 2004. J. Am. Soc. Nephrol. 15:F-PO016.). Another mechanism for the amino-terminal effects may involve regulatory proteins/factors that are either cytosolic or membrane bound (see below). Regulatory proteins may serve as intermediate proteins that couple the amino termini to regions of an NBC protein.

NBCe1 Macropatch Currents

NBCe1-mediated HCO\(_3\)^\(-\) Transport across a patch of Oocyte Membrane. We used the macropatch technique in the inside-out configuration to examine the potential involvement of a cytosolic component on the activity of NBCe1 constructs expressed in oocytes. The magnitudes of our NBC macropatch currents were similar to those previously reported for rat NBCe1-A (Heyer et al., 1999). In our studies, it was not uncommon to record from patches with little or no NBC-mediated currents. For all NBC groups, there was a broad range of HCO\(_3\)^\(-\) mediated currents. For C\(_{\text{WT}}\), current responses ranged from an outward current of 4.2 pA to an inward current of −14 pA. The range of responses may reflect different plasma membrane expression levels of NBCs among patches. Indeed, A\(_{\text{WT}}\) currents from three vegetal-pole patches obtained from each of two oocytes also exhibited a range of currents: 0.4, −5.1, and −3.3 pA for one oocyte, and −7.8, −3.3, and −7.4 pA for the other oocyte. NBCe1 expression at the oocyte surface may therefore be somewhat heterogeneous and reflect clustering of transporter proteins.

Influence of the Amino Termini on NBCe1 Activity.

There are three noteworthy observations regarding the macropatch results and the influence of the amino termini on NBC activity. First, the mean macropatch current for
$C_{AN87}$ is 3.2-fold larger than the mean current for $C_{WT}$. A similar 2.7-fold larger current for $C_{AN87}$ was observed in the whole-cell experiments. Second, the mean current for $A_{AN43}$ is less than that of $A_{WT}$. Similar results were obtained in the whole-cell experiments: the mean $A_{AN43}$ current was 55% less than that of $A_{WT}$. Both of these observations corroborate the whole-cell data regarding the functional impact of the different amino termini.

The third observation in the macropatch studies, which deviates from the whole-cell data, is the observation that the mean macropatch current for $A_{WT}$ is similar to the mean current for $C_{WT}$. Furthermore, the mean current for $A_{AN43}$ is not only lower than for $A_{WT}$, but is nonexistent and similar to that seen in patches from $H_2O$-injected oocytes. These deviations from the whole-cell data may be due to different intracellular ion dependencies or sensitivities among the variants, and the fact that the macropatch experiments were performed with a symmetrical solution containing extracellular ion concentrations. High $Na^+$ and $HCO_3^-$ concentrations on the cytosolic side were used to maximize the transport currents. Another explanation is the involvement of cytosolic factors in the whole-cell experiments that are absent in the macropatch experiments. While such factors could either stimulate A or inhibit C, the $A_{AN43}$ current observed in whole-cell but not macropatch experiments is consistent with a cytosolic factor stimulating the A variant.

Potential cytosolic factors include cytoskeletal elements such as actin or enzymes. Indeed, the amino-terminal domain of AE1 interacts with actin, protein 4.1, protein 4.2, as well as glycolytic enzymes (Reithmeier et al., 1996). Classic regulatory factors include cyclic AMP and ATP. Heyer et al. (1999) reported that 2 mM ATP increases the activity of rat NBCel-A from inside-out macropatches by twofold. The effect of ATP on NBCel-A activity may be mediated by ATP-dependent lipid kinases and PIP$_2$, as previously reported for the cardiac Na-Ca exchanger and $K_{ATP}$ potassium channels (Hilgemann and Ball, 1996). There is considerable evidence highlighting the regulatory effects of membrane phospholipids such as PIP$_2$ on ion channels and transporters (Suh and Hille, 2005). Future studies will be necessary to characterize potential regulatory factors such as ATP, PIP$_3$, and kinases/phosphatases that may differentially modulate the NBCel-A variants.

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REFERENCES


