Cellular/Molecular

The Electrogenic Sodium Bicarbonate Cotransporter NBCe1 Is a High-Affinity Bicarbonate Carrier in Cortical Astrocytes

Shefeeq M. Theparambil,[1] Iván Ruminot,[1] Hans-Peter Schneider,[1] Gary E. Shull,[2] and Joachim W. Deitmer[1]

[1]Abteilung für Allgemeine Zoologie, FB Biologie, University of Kaiserslautern, P.B. 3049, D-67653 Kaiserslautern, Germany, and [2]Department of Molecular Genetics, Biochemistry and Microbiology, University of Cincinnati, Cincinnati, Ohio 45267

The electrogenic sodium bicarbonate cotransporter NBCe1 (*SLC4A4*) is a robust regulator of intracellular H^+ and a significant base carrier in many cell types. Using wild-type (WT) and NBCe1-deficient (NBC-KO) mice, we have studied the role of NBCe1 in cortical astrocytes in culture and *in situ* by monitoring intracellular H^+ using the H^+-sensitive dye BCECF [2′,7′-bis-(carboxyethyl)-5-(and-6)-carboxyfluorescein] in wide-field and confocal microscopy. Adding 0.1–3 mM HCO_3^- to an O_2-gassed, HEPES-buffered saline solution lowered the intracellular H^+ concentration with a K_m of 0.65 mM HCO_3^- in WT astrocytes, but slowly raised $[H^+]_i$ in NBCe1-KO astrocytes. Human NBCe1 heterologously expressed in *Xenopus* oocytes could be activated by adding 1–3 mM HCO_3^-, and even by residual HCO_3^- in a nominally CO_2/HCO_3^--free saline solution. Our results demonstrate a surprisingly high apparent bicarbonate sensitivity mediated by NBCe1 in cortical astrocytes, suggesting that NBCe1 may operate over a wide bicarbonate concentration in these cells.

Key words: **bicarbonate; glial cells; NBCe1-KO; pH; proton concentration; *Xenopus* oocytes**

Introduction

The electrogenic sodium bicarbonate cotransporter NBCe1 (*SLC4A4*) is one of the major regulators of intracellular proton concentration ($[H^+]_i$) in many cell types, but particularly in epithelial tissue and brain (Deitmer and Rose, 1996; Chesler, 2003). In the brain, NBCe1 is expressed in most cell types, with most prominent expression in astrocytes (Deitmer and Chesler, 2009; Majumdar and Bevensee, 2010), and the stoichiometry has been suggested to be one Na^+ cotransported with two HCO_3^- (Deitmer and Schlue, 1989; Romero and Boron, 1999). Depending on the ion distribution in these cells, the reversal potential of NBCe1 ranges between −50 and −90 mV, which would allow the carrier to function in both directions across the cell membrane. Thus, it would appear that NBCe1 activity can contribute to intracellular as well as extracellular acid/base homeostasis in the brain and could, therefore, have a significant impact on neuronal activity. Mutations in the *SLC4A4* gene are associated with pathophysiological states in several different organs. For example, defective expression of NBCe1 in astrocytes, due to homozygous mutation of the *SLC4A4* gene, has been shown to cause familial migraine (Suzuki et al. 2010; Russel and Ducros 2011), and chronic proximal renal tubular acidosis and ocular abnormality are also reported to be associated with *SLC4A4* mutation and NBCe1 dysfunction (Igarashi et al., 1999).

Here we have studied cortical astrocytes in culture and in acute tissue slices of wild-type (WT) and NBCe1-deficient (NBCe1-KO) mice, monitoring $[H^+]_i$ with the H^+-selective dye BCECF [2′,7′-bis-(carboxyethyl)-5-(and-6)-carboxyfluorescein]. Our results reveal that $[H^+]_i$ regulation was highly sensitive to external bicarbonate, and NBCe1-mediated $[H^+]_i$ regulation was even detected in saline solution without added HCO_3^- ("nominally HCO_3^--free"). Comparing astrocytes and tissue slices from WT and NBCe1-deficient mice indicated that this high bicarbonate sensitivity was attributable to the activity of NBCe1.

Materials and Methods

Chemicals and reagents. Standard chemicals, tissue culture reagents, EIPA (ethyl-isopropyl amiloride), DIDS (4,4′-diisothiocyano-2,2′-stilbenedisulfonic acid), and EZA (6-ethoxy-1,3-benzothiazole-2-sulfonamide) were purchased from Sigma. BCECF-AM was obtained from Invitrogen.

Animals, primary astrocyte culture, and acute slices from cerebral cortex. Astrocyte cultures from wild-type [C57BL/6, postnatal day 0 (P0) to P3] and NBCe1-KO (129S6/SvEv and Black Swiss background, P0–P3) mouse cerebral cortex were prepared as previously described in detail (Stridh et al., 2012). Astrocytes were plated on poly-D-lysine-coated glass coverslips and maintained in DMEM containing 5% fetal calf serum and 5% horse serum. The medium was renewed completely after 24 h of plating and subsequently every 3 d. Cells were used for experiments when they were 10–20 d old in culture.

Acute cortical slices were prepared from WT NMRI (New Medical Research Institute) mice, and from mice ("GFRT") that were genetically modified by expressing a monomeric red fluorescent protein (mRFP1) under control of the human glial fibrillary acidic protein promotor in astrocytes (Hirrlinger et al., 2005). Cortical slices from NBCe1-KO mice

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Correspondence should be addressed to Joachim W. Deitmer, Abteilung für Allgemeine Zoologie, FB Biologie, University of Kaiserslautern, P.B. 3049, D-67653 Kaiserslautern, Germany. E-mail: deitmer@biologie.uni-kl.de.

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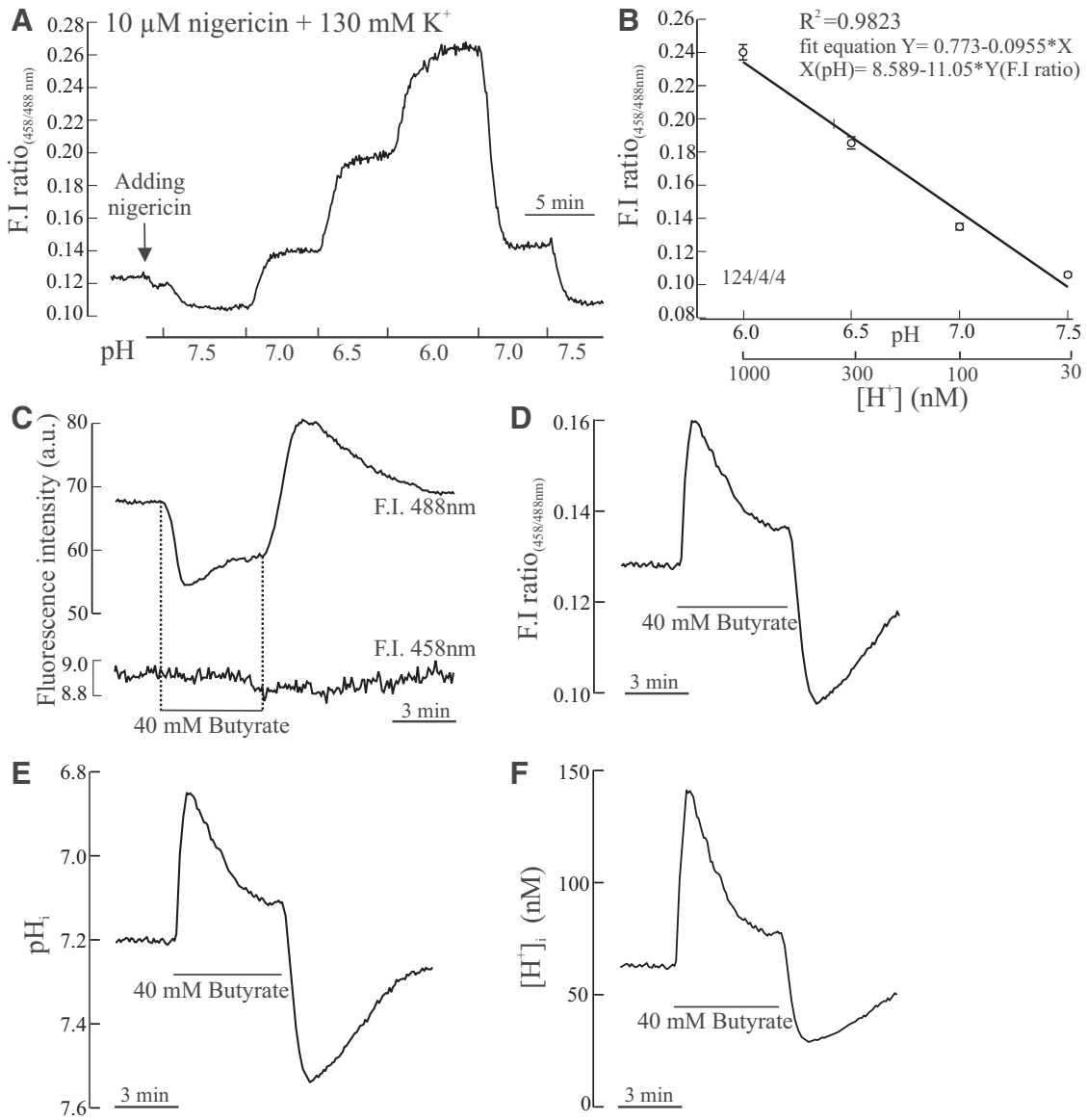

Figure 1. Calibration of the H^+-sensitive fluorescence signal in cultured cortical astrocytes. ***A***, ***B***, Calibration of the FI ratio signal (***A***) in the presence of nigericin and 130 mM K^+ to equilibrate K^+ and H^+ across the cell membrane, monitored at different extracellular pH values, and plotted against the pH value and the respective H^+ concentration (***B***). Numbers of cells/cultures/animals as used in the experiments are indicated in the plot, as well as the coefficient of correlation (R^2) and the fit equation (***B***). ***C–F***, The fluorescence recording during application of 40 mM butyrate, at pH 7.4, showing the two recorded wavelengths in arbitrary units (a.u.; ***C***), the ratio of the fluorescence at the two wavelengths (***D***), converted to the pH_i (***E***), and to $[H^+]_i$ (***F***).

showed the same BCECF loading pattern as WT mice. Astrocyte cultures and acute cortical slices from NBCe1-KO mice were prepared after animals were genotyped by PCR analysis (Gawenis et al., 2007).

Wild-type and NBCe1-KO animals from postnatal day 15–20 were decapitated and their brains were quickly transferred to an ice-cold, Ca^{2+}-reduced artificial CSF (aCSF), containing the following (in mM): NaCl 125, KCl 2.5, NaH_2PO_4 1.25, α-D-glucose 25, $MgCl_2$ 2.5, $CaCl_2$ 0.5, $NaHCO_3$ 26, aerated with 5% CO_2/95% O_2 to maintain the pH at 7.4. Coronal cortical slices of GRFT and NBC-KO animals were obtained at a thickness of 150 μm using a vibratome (VT 1000, Leica), and were stored in aCSF for 60 min at 30°C.

The animals were maintained on a 12 h day/night cycle at constant room temperature with *ad libitum* access to water and standard mouse fodder in the animal facility of the Technical University of Kaiserslautern. All procedures involving animals were approved by the Landesuntersuchungsamt Rheinland-Pfalz, Koblenz (23 177-07).

Protein extraction, immunoblot analysis, and immunocytochemistry. Immunoblot analysis was performed to compare the NBCe1 protein expression level in WT and NBCe1-KO murine astrocytes. Cultured cortical astrocytes from NBCe1-KO and littermate WT mice were collected separately, and lysed in 2% SDS solution containing protease inhibitors (Roche Diagnostics). Total protein content was determined using a BCA protein assay kit (Thermo Scientific). For immunoblotting, protein samples (50 μg) were loaded on to 4–20% Run Blue SDS Gel (Biozol Diagnostica Vertrieb) and electrotransferred onto nitrocellulose membranes. Blots were probed with anti-NBCe1 rabbit polyclonal antibody (Ab; diluted 1:500; anti-*SLC4A4*, Abcam) and visualized with a peroxidase-conjugated goat anti-rabbit IgG secondary antibody (diluted 1:2000; Santa Cruz Biotechnology). The signal was detected using a chemiluminescence kit (Lumi-Light, Roche) with a Versa Doc imaging system (Bio-Rad).

For marking cultured cortical astrocytes for expression of NBCe1, antibodies against NBCe1 attached to goat-anti-rabbit Alexa Fluor 488 antibody were used (Invitrogen/Molecular Probes). In brief, cells were fixed in 4% paraformaldehyde for 10 min at room temperature (RT). After washing the cells three times with PBS, cells were permeabilized with 0.1% Triton-X for 10 min at RT. Cells were then incubated with blocking solution containing 10% normal goat serum, 3% bovine serum

albumin and 0.1% Triton-X to reduce unspecific antibody binding, for 1 h at RT. Cells were then incubated with primary antibody against NBCe1 (rabbit anti-*SLC4A4* antibody, Abcam). After washing out the primary antibody with PBS, cells were incubated for 1 h with goat-anti rabbit antibody conjugated with Alexa Fluor 488 and Hoechst (5 μg/ml) for 15 min as cell nuclei marker. The fluorescence signals were detected by using a LSM-710 confocal microscope (Zeiss).

Intracellular H^+ imaging. To measure the intracellular H^+ concentration ($[H^+]_i$) changes in cultured cortical astrocytes, we used a confocal imaging system and acetoxymethyl ester of a proton-sensitive dye, BCECF-AM. The dye was loaded into the cells by incubating them with 3 μM BCECF-AM in HEPES-buffered saline solution for 15 min at room temperature. Cells were then mounted on a closed chamber of the confocal laser scanning microscope (LSM 510, Zeiss) and superfused continuously either with HEPES-buffered (in mM) NaCl 140, KCl 5, α-D-glucose 10, NaH_2PO_4 0.5, HEPES 10, $MgCl_2$ 1, and $CaCl_2$ 2, or the following CO_2/HCO_3^--buffered saline solution (in mM): NaCl 114, KCl 5, NaH_2PO_4 0.5, α-D-glucose 10, $NaHCO_3$ 26 (21 at 35°C), $MgCl_2$ 1, and $CaCl_2$ 2. BCECF was excited consecutively at 488 nm (proton-sensitive wavelength) and 458 nm (close to isosbestic point), and the changes in fluorescence emission were monitored at >505 nm (using LP 505 filter). Images were obtained every 5 s (0.2 Hz) with a 40× water-immersion objective. The fluorescence emission intensity of 488 nm excitation changes inversely with a change in $[H^+]_i$, whereas the fluorescence emission intensity of 458 nm excitation is largely pH insensitive. The changes in $[H^+]_i$ were monitored using the ratio $F_{(458)}/F_{(488)}$. The ratio was converted into pH and absolute intracellular proton concentrations ($[H^+]_i$) by using the nigericin-based calibration technique (Fig. 1*A*). Cells were perfused with calibration solutions, containing nigericin 10 μM, NaCl 15 mM, KCl 130 mM, HEPES 20 mM, $MgCl_2$ 1 mM and $CaCl_2$ 1 mM, at pH 6.0, 6.5, 7.0, and 7.5. The mean ratio values ($F_{(458)}/F_{(488)}$ were plotted as a function of pH to create the calibration curve (Fig. 1*B*). As a recording example, the changes in fluorescence intensity (FI) at the two wavelengths following application of 40 mM butyrate at a constant saline solution pH of 7.4 are shown in Figure 1*C*, and after forming the FI ratio (Fig. 1*D*). The ratio was then converted to intracellular pH (pH_i; Fig. 1*E*) and $[H^+]_i$ (Fig. 1*F*).

Acute cortical slices were loaded with 4 μM BCECF-AM in aCSF saline solution for 30 min at 30°C. The slices were mounted on a perfusion chamber of an upright microscope (BX50WI, Olympus) equipped with an epifluorescence unit (Polychrome IV, Till Photonics). The slices were superfused continuously with aCSF and subsequently changed to HEPES-buffered saline solution as follows (in mM): NaCl 136, KCl 3, α-D-glucose 2, L-lactate 1, HEPES 10, $MgSO_4$ 1,25 and $CaCl_2$ 1,25, pH 7.4. BCECF was excited at 440 and 490 nm for 5 ms at an interval of 5 s. The 535 nm fluorescence emission of the two excitation wavelengths was monitored through a 40× water-immersion objective with a Peltier-cooled CCD camera (Till Photonics). The ratio of BCECF emissions $F_{(440)}/F_{(490)}$ was converted into a proton concentration using nigericin-based calibration method as described above. Most measurements were performed at room temperature (22°C), while some experiments were performed at 35°C as indicated, with the $[HCO_3^-]$ being 21 mM at 5% CO_2 to maintain the pH value at 7.4 due to lower CO_2 solubility at higher temperature. It should be noted that at 35°C the saline solution pH value tended to destabilize, and the pH drifted more and faster after adding HCO_3^- at low concentration (1–3 mM) without CO_2 aeration, presumably due to faster spontaneous conversion of HCO_3^- to CO_2, which would leave the saline solution and cause an alkaline drift of the saline solution. Hence, the actual $[HCO_3^-]$ in these experiments may be lower than expected (by >10%). Therefore, only some protocols were performed at 35°C.

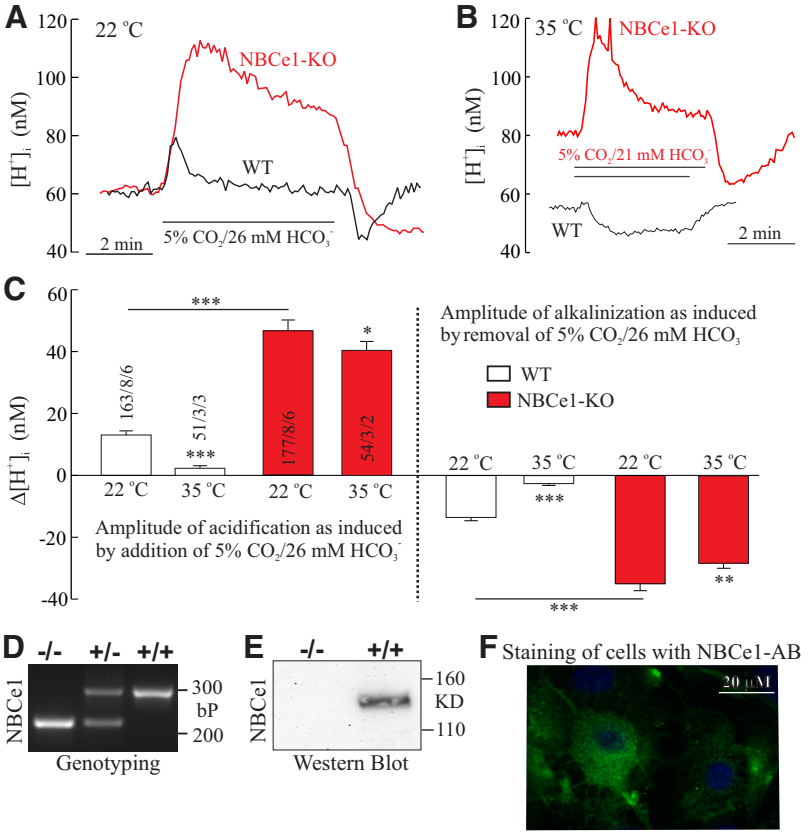

Figure 2. Intracellular H^+ shifts in cultured astrocytes of wild-type and NBCe1-KO mice. ***A–C***, Addition and removal of 5% CO_2/26 mM HCO_3^- at 22°C and 35°C resulted in only small H^+ transients in WT astrocytes (***A***, ***B***, black traces; ***C***, open bars), but large H^+ shifts in NBCe1-KO astrocytes (***A***, ***B***, red traces; ***C***, red bars). Both acid and alkaline transients upon addition and removal of CO_2/HCO_3^-, respectively, were significantly smaller in cells of WT and KO mice at higher temperature. ***D***, PCR genotyping of DNA from homozygous-null, heterozygous, and WT mice. ***E***, Western blots of astrocytes from KO (left) and from WT (right) mice. ***F***, Staining of cell culture with Abs against NBCe1 conjugated with Alexa Fluor 488 and Hoechst (5 μg/ml) as cell nuclei marker. Number of cells/cultures/animals as used in the experiments are indicated in or next to the bar plots.

Heterologous expression of NBCe1 and electrophysiology in Xenopus laevis *oocytes. Xenopus laevis* females were purchased from Xenopus Express. Frogs were anesthetized with 1 g/l 3-aminobenzoic acid ethylester (MS-222; Sigma-Aldrich), rendered hypothermic and segments of ovarian lobules were surgically removed under sterile conditions. The procedure was approved by the Landesuntersuchungsamt Rheinland-Pfalz (23 177-07/A07-2-003 §6) and has been described previously in detail (Becker and Deitmer, 2007). In brief, oocytes were isolated and singularized by collagenase treatment (Collagenase A, Roche) in Ca^{2+}-free oocyte saline solution at 28°C for 2 h. The singularized oocytes were left overnight in an incubator at 18°C in Ca^{2+}-containing oocyte saline solution, pH 7.8, to recover. The human NBCe1 cDNA was cloned in oocyte expression vector pGH19. Plasmid DNA was linearized with NotI and transcribed *in vitro* with T7 RNA-polymerase in the presence of the cap analogon m7G(5′)ppp(5′)G (mMessage mMachine, Ambion) to produce a capped RNA transcript. The cRNA was purified with the Qiagen RNeasy MinElute Cleanup Kit and stored at −80°C in diethylpyrocarbonate (DEPC)-H_2O. The oocyte saline solution had the following composition (in mM): NaCl 82.5; KCl 2.5; $CaCl_2$ 1; $MgCl_2$ 1, Na_2HPO_4 1; HEPES 5, titrated to pH 7.4. The bicarbonate-containing saline solution contained the following (in mM): NaCl 58.5; KCl 2.5; $CaCl_2$ 1; $MgCl_2$ 1;

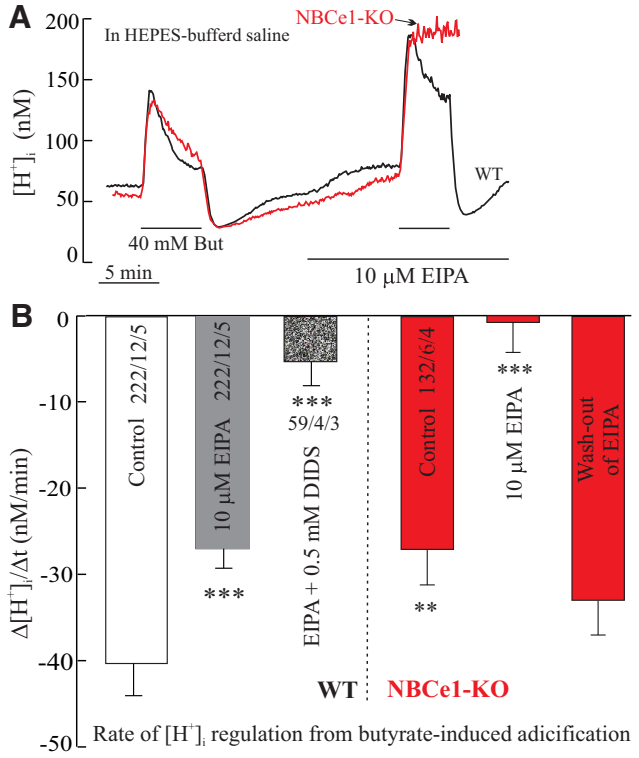

Figure 3. Intracellular H^+ regulation in nominally CO_2/HCO_3^--free saline solution. ***A***, Application of 40 mM butyrate to acidify the cytosol, and the subsequent recovery from acidification with and without EIPA to inhibit Na^+/H^+ exchange, in WT (black traces) and NBCe1-KO astrocytes (red traces). ***B***, Rate of recovery from intracellular acidification in WT astrocytes with EIPA, or with EIPA and DIDS, and in NBCe1-KO astrocytes treated with EIPA and after washout of EIPA. The number of cells/cultures/animals used in the experiments is indicated in or next to the bar plots.

Na_2HPO_4 1; $NaHCO_3$ 24, aerated with 5% CO_2 and HEPES 5, to stabilize the pH. Oocytes of the stages V and VI were selected and injected with 13.8 ng of NBCe1-cRNA dissolved in DEPC-H_2O using glass micropipettes and a microinjection device (Nanoliter 2000, World Precision Instruments).

For measurement of intracellular H^+ and membrane potential, double-barreled microelectrodes were used; the manufacture and application have been described in detail previously (Deitmer, 1991). The measurements of pH_i were stored digitally using custom-made PC software based on the program LabView (National Instruments Germany) and was routinely converted into $[H^+]_i$. Amplitude and rate of change of the measured $[H^+]_i$ were analyzed. For voltage-clamp, electrodes filled with 3 M KCl were connected to the head stages of an Axoclamp 2B amplifier (Molecular Devices). The experimental bath was grounded with a chlorided silver wire coated by agar dissolved in oocyte saline solution. Oocytes were clamped to a holding potential of −40 mV, and all experiments were performed at room temperature (22°C). For further details see Becker and Deitmer (2007).

Statistical analysis. All statistical analyses were performed using the software SigmaPlot 11.0 and ClampFit 10.2. Statistical values are presented as the mean ± SEM. For calculation of significance in differences, a Student's *t* test was used. In the figures shown, significance levels are as follows: $*p \leq 0.05$, $**p \leq 0.01$ and $***p \leq 0.001$.

Results

NBCe1 is a major acid/base regulator in cortical astrocytes

Intracellular pH was monitored in cultured cortical astrocytes loaded with the H^+-selective dye BCECF, calibrated, and converted to $[H^+]_i$ (Fig. 1; for details, see Materials and Methods). In HEPES-buffered saline solution, pH 7.4, the mean $[H^+]_i$ values measured in cortical astrocytes from WT and NBCe1-KO mice at room temperature (22°C) were 67 ± 1.6 nM ($n = 332$) and 58 ± 1.8 nM ($n = 242$), respectively. These values are significantly different ($p < 0.01$), suggesting that at steady state, NBCe1 extrudes net HCO_3^-. When the bathing solution is switched from a HEPES-buffered, nominally CO_2/HCO_3^--free saline solution, to saline solution buffered by 5% CO_2/26 mM HCO_3^-, changes in $[H^+]_i$ are attributable primarily to CO_2 diffusion across the cell membrane, conversion of CO_2 to H^+ and HCO_3^-, and subsequent $[H^+]_i$ regulation. These changes differed greatly in cultured cortical astrocytes from WT or NBCe1-KO mice (Fig. 2*A*,*C*). The acid $[H^+]_i$ transient was almost three times as large, while the rate of recovery from acidification was greatly reduced in cells from NBCe1-KO mice compared with WT mice (Fig. 2*A*). The steady-state $[H^+]_i$ in saline solution buffered with 5% CO_2/26 mM HCO_3^- was 63 ± 2.3 nM ($n = 253$) in WT astrocytes and 62 ± 2 nM ($n = 280$) in astrocytes from NBCe1-KO mice. These values are not significantly different from each other and from those measured in HEPES-buffered saline solution. From the average $[H^+]_i$ in CO_2/HCO_3^--buffered saline solution, a mean intracellular HCO_3^- concentration of 14.7 mM was calculated in astrocytes from WT mice and 14.9 mM in astrocytes from NBCe1-KO mice. From these values and the extracellular $[HCO_3^-]$ of 26 mM, H^+/HCO_3^- equilibrium potentials of −14.5 and −14.1 mV in WT and NBCe1-KO, respectively, is obtained by the Nernst equation.

When the experimental temperature was raised to 35°C, the mean $[H^+]_i$ in cortical astrocytes from WT and NBCe1-KO mice was 66 ± 3.0 nM ($n = 51$) and 75 ± 3.4 nM ($n = 54$), respectively. The value for NBCe1-KO mice at 35°C was significantly greater than that at 22°C and was also greater than that measured in WT cells at 35°C (both $p < 0.001$), while the $[HCO_3^-]$ was calculated to be 10.5 mM in astrocytes at 35°C in NBCe1-KO mice. Changing from HEPES-buffered, nominally CO_2/HCO_3^--free saline solution, to saline solution buffered by 5% CO_2/21 mM HCO_3^- at 35°C induced a very small, brief acidification followed by a partially transient $[H^+]_i$ shift by approximately −10 nM in cells from WT mice, while a robust acidification was recorded in cells from NBCe1-KO mice, followed by a recovery (Fig. 2*B*,*C*). From the rate of alkalinization, which followed the acidification upon addition of 5% CO_2/26 mM HCO_3^-, at 22°C and 35°C (Fig. 2*A*,*B*), a Q_{10} temperature coefficient of 1.8 was calculated. The transient alkalinization upon removal of CO_2/HCO_3^- was smaller and faster in cells from WT mice compared with those from NBCe1-KO mice (Fig. 2*A*–*C*).

In these and other studies described below, genotyping of DNA from NBCe1-KO mice revealed only the smaller PCR product characteristic of the null allele; in WT mice, only the larger PCR product characteristic of the WT allele was observed, and bands for both alleles were observed in heterozygous mice (Fig. 2*D*). Accordingly, no expression of NBCe1 protein was detected in astrocytes of homozygous NBCe1-KO mice, in contrast to WT mice, as revealed by Western blotting (Fig. 2*E*). Antibody directed against NBCe1 showed intense staining of the cultured cortical astrocytes from WT mice (Fig. 2*F*).

We used butyric acid (40 mM butyrate) in HEPES-buffered saline solution, with a constant pH of 7.4, as another method for acidifying the cytosol. Butyric acid was chosen as the weak acid to acidify the cells, because the $[H^+]_i$ completely returned to its initial value after removal of butyric acid after inhibiting H_i^+ regulation (in the absence of CO_2/HCO_3^- and in the presence of EIPA; Fig. 4*A*,*C*; see also Fig. 8*A*,*B*), indicating that there was little or no metabolic consumption of butyrate by the cells during

the exposure of butyric acid for 5–30 min. In addition, the uptake of butyric acid in *Xenopus* oocytes was the same when oocytes were injected with cRNA for monocarboxylate transporter 1 (MCT1) or water, indicating that the contribution of MCT1-mediated transport of butyric acid into oocytes was insignificant, and the uptake of butyrate occurred as undissociated acid (Bröer et al., 1998).

Blocking sodium/hydrogen exchange (NHE) with EIPA (10 μM) in HEPES-buffered, nominally CO_2/HCO_3^--free saline solution considerably slowed $[H^+]_i$ regulation in WT cells and reversibly suppressed $[H^+]_i$ regulation in NBCe1-KO mouse astrocytes (Fig. 3*A*,*B*). In WT astrocytes, the rate of H^+_i recovery was reduced to 67% by EIPA, while the anion transport antagonist DIDS (0.5 mM), applied together with EIPA, reduced this rate of recovery to ~10% of the control. These results suggest that (1) NHE and NBCe1 both make major contributions to $[H^+]_i$ regulation, and (2) after blocking NHE, NBCe1 can still sustain robust $[H^+]_i$ regulation even in the nominal absence of CO_2/HCO_3^- in cortical astrocytes.

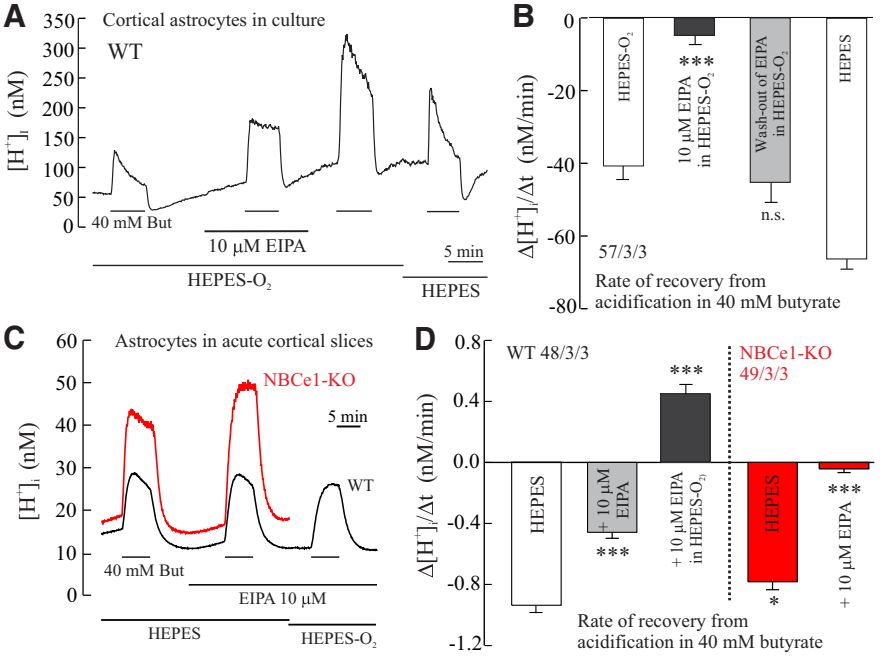

Figure 4. Intracellular H^+ regulation is still maintained by residual bicarbonate in nominally CO_2/HCO_3^--free saline solution. ***A***, ***B***, Intracellular H^+ recovery from butyrate-induced acidification in astrocytes from WT mice is still significant when residual HCO_3^- is lowered by aerating the HEPES-buffered, nominally CO_2/HCO_3^--free saline solution with 100% O_2 (HEPES-O_2), but is greatly reduced after inhibiting NHE with EIPA. The H^+ regulation increased again after removing EIPA, and still further after removal of O_2 aeration. ***C***, ***D***, Recording of intracellular $[H^+]$ in astrocytes of acute cortical slices obtained from WT (black traces) and from NBCe1-KO (red traces) mice during the application of 40 mM butyrate in HEPES-buffered and in HEPES-O_2 saline solution in the absence and presence of EIPA (***C***), and a plot of the rates of intracellular H^+ recovery from butyrate-induced acidification in the different solutions in WT (open, gray and black bars) and in NBCe1-KO (red bars) mice (***D***). The number of cells/cultures/animals used in the experiments is indicated in ***B*** and ***D***.

The significance of residual bicarbonate in nominally CO_2/HCO_3^--free saline solution for $[H^+]_i$

Our experiments showed that NBCe1 activity is maintained even in the nominal absence of CO_2/HCO_3^- in HEPES-buffered saline solution, suggesting that residual HCO_3^- in the saline solution, derived from equilibration of air CO_2 and calculated to be ~150–200 μM at pH 7.4, is sufficient to maintain NBCe1 activity. We tested this hypothesis by aerating HEPES-buffered saline solution with 100% O_2 to displace CO_2 from the saline solution and hence reduce the $[HCO_3^-]$ further. In this solution (HEPES-O_2), EIPA reversibly reduced $[H^+]_i$ regulation by >90% in WT astrocytes when they were challenged by application of 40 mM butyrate. Subsequent removal of O_2 aeration accelerated $[H^+]_i$ regulation substantially (Fig. 4*A*,*B*).

A similar experiment in acute cortical brain slices from mice showed that in astrocytes, $[H^+]_i$ recovery from a 40 mM butyrate-induced acidification was reduced, but was not abolished, by EIPA in HEPES-buffered, nominally CO_2/HCO_3^--free saline solution, but completely suppressed when the saline solution was aerated with O_2 (Fig. 4*C*,*D*). In acute slices of NBCe1-KO mice, $[H^+]_i$ regulation was fully blocked by EIPA alone. Similar results were obtained in slices from mRFP/NBC-KO mice. These results strongly suggest that residual bicarbonate in nominally CO_2/HCO_3^--free saline solution is sufficient to maintain significant NBCe1 activity and hence $[H^+]_i$ regulation in cortical astrocytes *in vitro* and *in situ*, as measured in the presence of EIPA to block NHE.

Membrane potential-dependent activation of NBCe1

Since NBCe1 is electrogenic, it can be activated by changes in the membrane potential (Deitmer and Szatkowski, 1990; Brookes and Turner, 1994). To confirm the molecular identity responsible for the bicarbonate-dependent $[H^+]_i$ regulation, cultured cortical astrocytes were depolarized by elevating the external K^+ concentration from 5 to 15 mM (Fig. 5). In WT astrocytes, 15 mM K^+ elicited a fall in $[H^+]_i$ in HEPES-buffered saline solution; this fall was augmented in CO_2/HCO_3^--buffered saline solution, and decreased in HEPES-O_2 saline solution with EZA (10 μM), which was added to lower the effective $[HCO_3^-]$, both in amplitude and rate (Fig. 5*A*,*C*,*D*). In astrocytes from NBCe1-KO mice, 15 mM K^+ elicited a small rise in $[H^+]_i$ both in HEPES-buffered and in CO_2/HCO_3^--buffered saline solution (Fig. 5*B*–*D*). These results indicate that (1) the intracellular alkalinization in 15 mM K^+ observed in WT cells was due to activation of NBCe1; (2) the high K^+-dependent alkalinization was still robust in HEPES-buffered, nominally CO_2/HCO_3^--free saline solution; and (3) lowering the $[HCO_3^-]$ by O_2 aeration in HEPES-buffered, nominally CO_2/HCO_3^--free saline solution reduced the amplitude and rate of alkalinization, as predicted by experiments shown in Figure 4.

Cortical astrocytes respond to low bicarbonate concentrations

The high sensitivity for bicarbonate in cortical astrocytes that appeared to be due to NBCe1 was further studied by adding low concentrations of bicarbonate to a HEPES-buffered saline solution aerated with 100% O_2 (Fig. 6). Exposing the cells to 3 mM, 1 mM, and 0.3 mM HCO_3^- evoked a concentration-dependent fall of the $[H^+]_i$ in astrocytes of WT mice (Fig. 6*A*,*B*). In cells from NBCe1-KO mice, a rise of 10–20 nM $[H^+]_i$ was observed at 3 mM $[HCO_3^-]$ (Fig. 6*A*, red trace), presumably due to diffusion of CO_2 formed from the added HCO_3^-. It should be noted here that

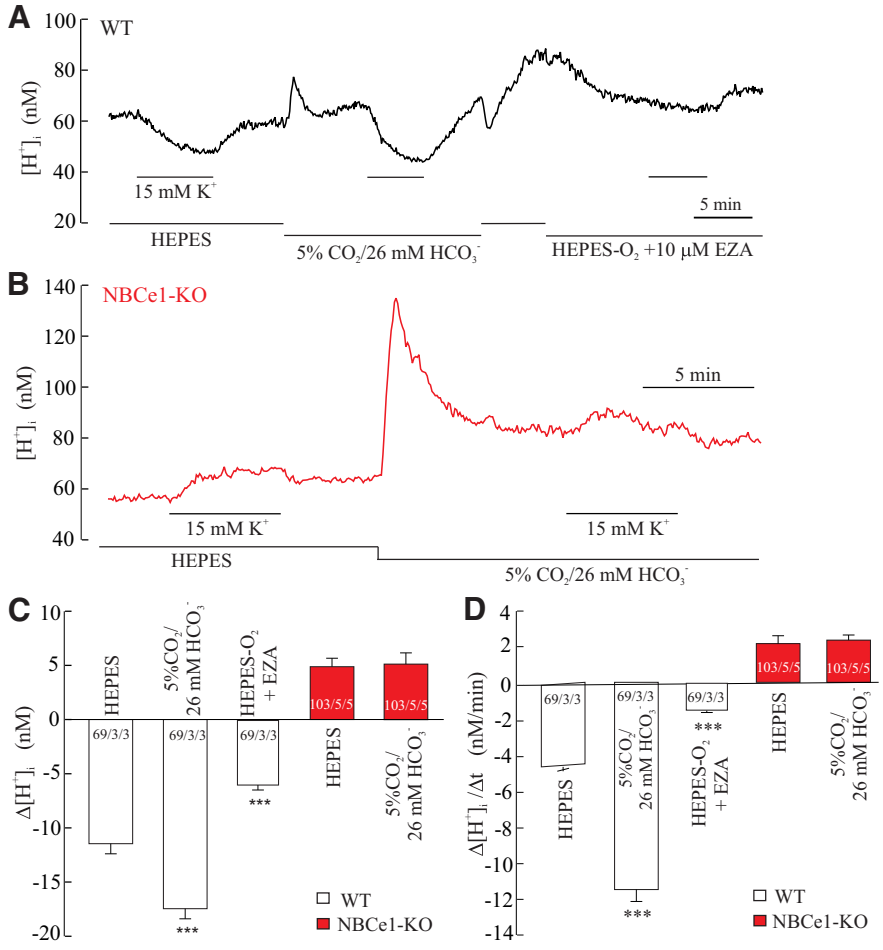

Figure 5. Membrane potential-dependent changes in intracellular H^+ concentration in saline solution with different potassium concentrations. ***A***, ***B***, Intracellular H^+ changes in WT (***A***) and in NBCe1-KO (***B***) cortical astrocytes during exposure to 15 mM K^+ (from normal 5 mM K^+) in a HEPES-buffered, nominally CO_2/HCO_3^--free saline solution, in a 5% CO_2/26 mM HCO_3^--buffered saline solution, and in a HEPES-O_2 saline solution containing EZA (10 μM) to block carbonic anhydrase activity (only in ***A***). ***C***, ***D***, Changes in the intracellular $[H^+]$ (***C***) and the rate of $[H^+]_i$ changes (***D***) in the different saline solutions, in WT (open bars) and NBCe1-KO (red bars) astrocytes. The number of cells/cultures/animals used in the experiments is indicated in the bars.

adding HCO_3^- to a solution not aerated with CO_2, will lead to continuous loss of HCO_3^- from the saline solution as CO_2, while the saline solution pH would accordingly rise. From this rise in saline solution pH, we calculated that the loss of HCO_3^- might be up to 10% at 22°C, within the duration of our experiments of 15–30 min. Therefore, the effective HCO_3^- concentration would be lower than the amount added at the beginning of the experiments (e.g., at 22°C by up to 0.1 mM when 1 mM HCO_3^- was added, and up to 0.3 mM, when 3 mM HCO_3^- was added). In addition, some of the CO_2 formed from the added HCO_3^- would diffuse into the cells and acidify the cytosol, as observed in cells of NBCe1-KO mice (see above), and would counteract the NBCe1-mediated alkalinization in WT cells. To minimize the loss of added HCO_3^- from these saline solutions, the low $[HCO_3^-]$ solutions were always freshly prepared within 3–6 min before use.

When acute cortical slices were used, the $[H^+]_i$ responded with a small decrease in WT astrocytes *in situ* and a small increase in NBCe1-KO astrocytes, when 1 and 3 mM HCO_3^- was added to the bathing solution at 35°C (Fig. 6*C*,*D*). Changing from a HEPES-buffered, O_2-aerated saline solution, to a saline solution buffered by 5% CO_2/21 mM HCO_3^- at 35°C, pH 7.4, induced a small, brief acidification followed by a $[H^+]_i$ shift by 5–10 nM in cells from WT mice, while a robust acidification was recorded in cells from NBCe1-KO mice, followed by a recovery (Fig. 6*C*,*D*), similar to that described for cultured cortical astrocytes (Fig. 2*B*,*C*). A direct comparison of NBCe1 transport activity at 22°C and 35°C in the same cells turned out to be difficult *in situ*, as the cells in the slice preparation appeared to lose viability during the rapid (~1°C/min) warming process.

Activation by low $[HCO_3^-]$ of human NBCe1 heterologously expressed in *Xenopus* oocytes

To estimate the sensitivity of NBCe1 activity to HEPES-buffered saline solution with and without O_2 aeration, we heterologously expressed human NBCe1 in *Xenopus* oocytes (Becker and Deitmer, 2007) and recorded the membrane current during intracellular acidification induced by 20 mM butyrate in HEPES-buffered saline solution in the nominal absence of CO_2/HCO_3^- (Fig. 7*A*,*B*). The NBCe1 current was reduced by ~50% in HEPES-O_2 saline solution, while in native oocytes no current change was recorded in 20 mM butyrate, suggesting that there is a substantial reduction of NBCe1 activity during O_2 aeration.

To confirm that NBCe1 could be responsible for the $[H^+]_i$ fall evoked by low extracellular $[HCO_3^-]$, human NBCe1 was expressed in *Xenopus* oocytes and exposed to low $[HCO_3^-]$ in HEPES-buffered, nominal CO_2/HCO_3^--free saline solution. The addition of 1 and 3 mM $[HCO_3^-]$ evoked a fall in $[H^+]_i$ (Fig. 7*C*,*D*) and a robust outward current (Fig. 7*D*,*E*) in NBCe1-expressing oocytes, while in native oocytes a slow rise in $[H^+]_i$ and no membrane current was observed (Fig. 7*C–E*), even when 5% CO_2/25 mM HCO_3^- was added (Fig. 7*E*). The rate of $[H^+]_i$ fall and the outward current in NBCe1-expressing oocytes show that NBCe1 can be activated by low-millimolar $[HCO_3^-]$. Again, the effective concentrations of HCO_3^- would be somewhat lower than the amount added, as stated above.

Determination of NBCe1 transport activity at different $[HCO_3^-]$ and $[H^+]_i$

To challenge NBCe1 activity by acidifying the cytosol, we exposed cultured astrocytes to 40 mM butyrate in HEPES-buffered saline solution aerated with O_2 and with EIPA to block NHE. This increased $[H^+]_i$ from 20 to 90 nM up to >100 nM with the saline solution pH set at constant pH 7.4 (Fig. 8*A*,*B*,*D*). Under these conditions, the addition of a low $[HCO_3^-]$ between 0.1 and 3 mM evoked a concentration-dependent fall of the $[H^+]_i$ in astrocytes from WT mice (Fig. 8*A*,*C*). In contrast, in cells from NBCe1-KO mice, the low $[HCO_3^-]$ elicited a slow acidification, which only turned into a moderate alkalinization at 3 mM HCO_3^- (Fig. 8*B*,*C*). The rate of fall in $[H^+]_i$ in WT cells showed a dependence on the $[HCO_3^-]$ with a K_m of 0.65 mM, as calculated from the fit through the data points (Fig. 8*C*, control). Removal of external

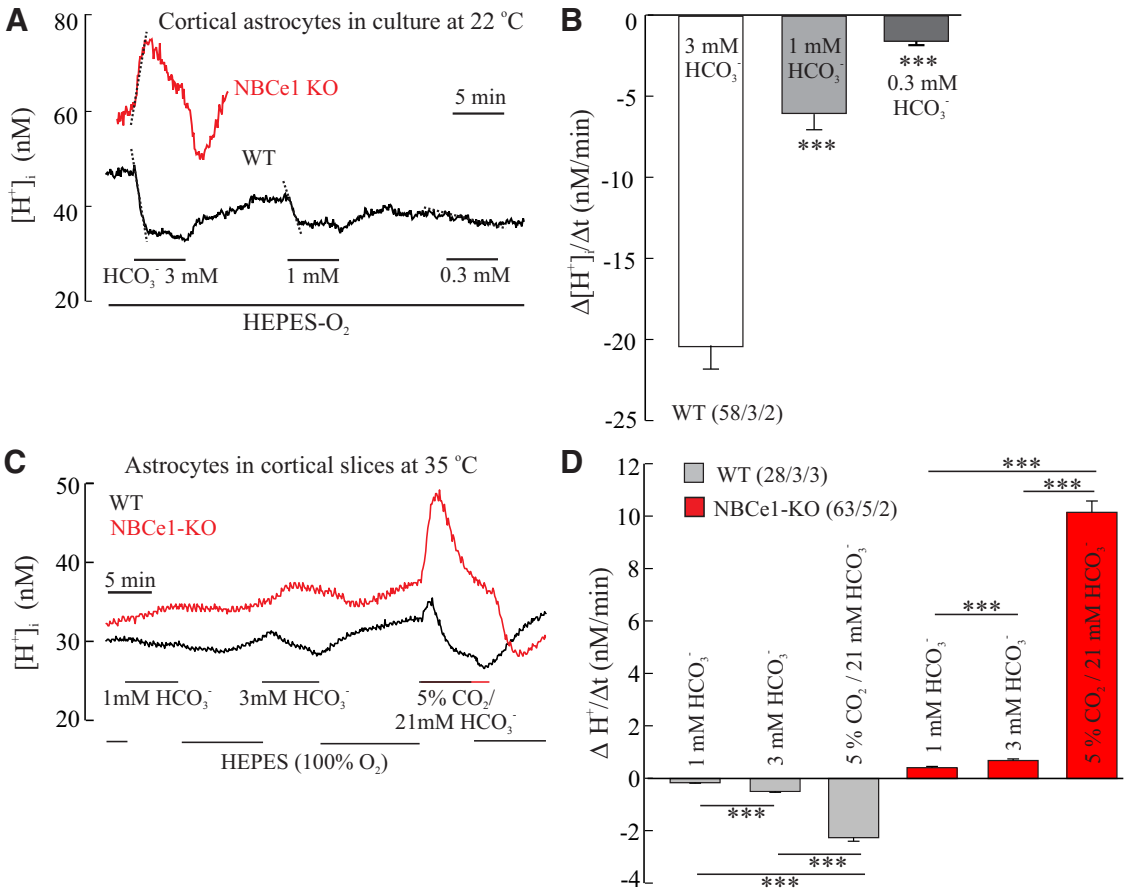

Figure 6. Bicarbonate sensitivity of wild-type and NBCe1-KO astrocytes. ***A–D***, Changes in intracellular [H^+] of cultured astrocytes (***A***, ***B***) during the addition of low concentrations of HCO_3^- in HEPES-O_2 saline solution in cells of WT (***A***, black trace) and of NBC-KO (***A***, red trace) mice at 22°C and corresponding rates of $[H^+]_i$ changes (***A***, dotted lines) in WT cells (***B***). Changes in intracellular [H^+] in astrocytes of acute cortical slices (***C***, ***D***) during the addition of low concentrations of HCO_3^- in HEPES-O_2 saline solution in cells of WT (***C***, black trace) and NBC-KO (***C***, red trace) mice at 35°C, and corresponding rates of $[H^+]_i$ changes (***D***).

chloride (replaced by gluconate) had little effect on the rate of bicarbonate-dependent $[H^+]_i$ fall, while in the presence of EZA (10 μM), an effective inhibitor of carbonic anhydrase activity, this rate was reduced by >70%, both in the presence and absence of external chloride (K_m values 0.54 mM in EZA, and 1 mM in EZA/0 Cl^-, as calculated from the fits through the data). Due to loss of some HCO_3^- from these O_2-aerated saline solutions (see above), the K_m values would likely be even lower. These experiments also suggest that at very low extracellular CO_2/HCO_3^- concentrations, rapid conversion of CO_2 to and from HCO_3^-, catalyzed by carbonic anhydrase activity, is essential to maintain the effective $[HCO_3^-]$ and hence HCO_3^- transport via NBCe1, while the absence of chloride makes little difference to the rate of bicarbonate-dependent $[H^+]_i$ fall.

The rate of bicarbonate-dependent fall in $[H^+]_i$ was also plotted versus the initial, steady-state $[H^+]_i$, as measured just before adding bicarbonate, from experiments as shown in Figures 6*A* and 8, *A* and *D*. There was only a small dependence of this rate at an initial concentration of <100 nM $[H^+]_i$, and the linear fit had a slope of 36.9 ± 16.5 min^{-1}; however, at an initial $[H^+]_i$ of >100 nM, as achieved in the presence of 40 mM butyrate, this dependence was robust. Between 200 and 800 nM $[H^+]_i$, pH_i 6.7–6.1, the linear fits had slopes of 142.8 ± 9.6 and 384 ± 59.5 min^{-1}, respectively, in 0.3 mM HCO_3^- and 3 mM HCO_3^- ($p < 0.0001$). These results show that the rate of $[H^+]_i$ change was dependent on the extracellular $[HCO_3^-]$ and the intracellular $[H^+]$.

Discussion

Using WT and NBCe1-deficient mice, we have shown that the electrogenic sodium bicarbonate cotransporter is an apparently high-affinity bicarbonate transporter in mouse cortical astrocytes. NBCe1 can be activated by submillimolar $[HCO_3^-]$ during moderate or severe acidosis, and is hence—to our knowledge—the bicarbonate transporter/protein with the highest apparent sensitivity for bicarbonate that has been described so far. As a consequence of its high bicarbonate sensitivity, NBCe1 may function as a sensor for low bicarbonate concentrations, such as those that occur during severe acidosis. The NBCe1 activity and bicarbonate sensitivity were even increased at a physiological temperature of 35°C compared with room temperature. We show that NBCe1 in cortical astrocytes and in cRNA-injected frog oocytes transports a significant amount of substrate even in nominally CO_2/HCO_3^--free saline solution (i.e., with no CO_2/HCO_3^- added), which contain ~150–200 μM HCO_3^- due to air CO_2 equilibrated in saline solutions. The results are significant because (1) bicarbonate can still be transported across the cell membrane, even if the $[HCO_3^-]$ on either side of the membrane is low; (2) saline solutions, nominally free of CO_2/HCO_3^-, as has been used in numerous physiological and biochemical experiments, and is assumed to be

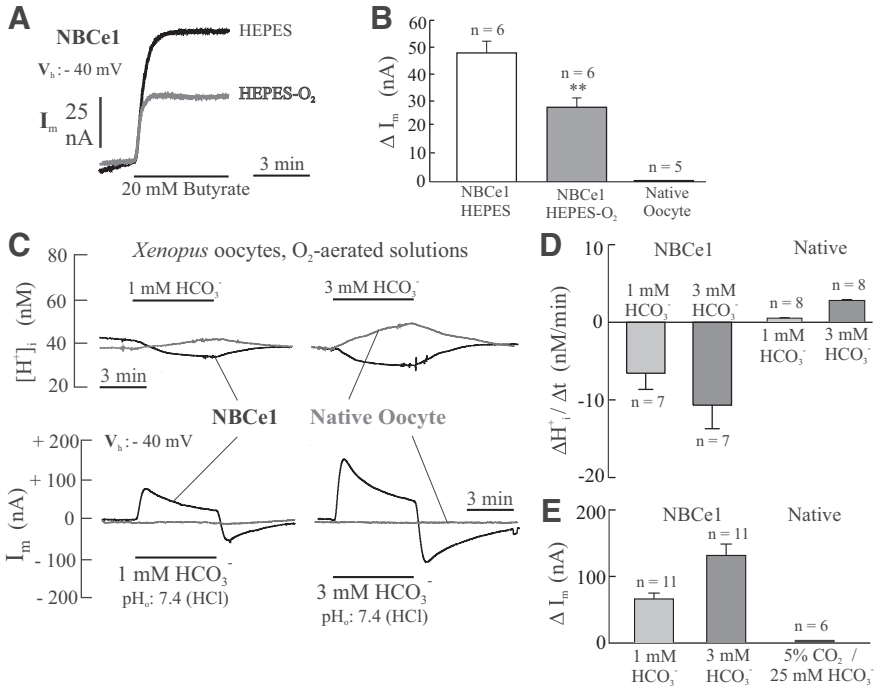

Figure 7. Characteristics of human NBCe1 heterologously expressed in *Xenopus* oocytes. ***A***, ***B***, Membrane current in NBCe1-expressing *Xenopus* oocytes in HEPES-buffered, nominally CO_2/HCO_3^--free saline solution, challenged by intracellular acidification induced by exposure to 20 mM butyric acid, with and without 100% O_2 aeration (i.e., HEPES-O_2). Lowering residual bicarbonate by O_2 aeration reduced this membrane current significantly ($p < 0.01$, $n = 6$). ***C***, ***D***, Changes in the intracellular $[H^+]_i$ (***C***, upper traces) and in the membrane current I_m (***C***, lower traces, ***E***), and plot of the rate of $[H^+]_i$ change (***D***) in NBCe1-expressing and native oocytes to show that NBCe1 is activated by low extracellular $[HCO_3^-]$ in this heterologous expression system. The number of experiments is indicated above or below the bar plots in ***B***, ***D***, and ***E***.

HCO_3^--free, may contain enough bicarbonate (150–200 μM) to activate or maintain significant transport; and (3) NBCe1 has been identified as an apparently high-affinity bicarbonate transporter in cortical astrocytes.

NBCe1 is activated by low bicarbonate concentrations in both astrocytes and oocytes

The following evidence indicates that the apparently high bicarbonate transport sensitivity is mediated by NBCe1: (1) the activity was greatly reduced or absent in cultured cells, and acute tissue slices were obtained from NBCe1-KO mice; (2) an intracellular alkalinization could be elicited by high-K^+ saline solution in WT astrocytes, consistent with previously demonstrated depolarization-induced bicarbonate inward transport (Deitmer and Szatkowski, 1990; Brookes and Turner, 1994; Pappas and Ransom, 1994), but not in astrocytes from NBCe1-KO mice (see also Svichar et al., 2011); (3) reduction of the residual $[HCO_3^-]$ by O_2 aeration of the saline solution suppressed both alkalinization in high-K^+ saline solutions and intracellular H^+ regulation following acidification with concurrent NHE inhibition; and (4) NBCe1 heterologously expressed in *Xenopus* oocytes was shown to be activated by the residual $[HCO_3^-]$ in HEPES-buffered, nominally CO_2/HCO_3^--free saline solution, and by the addition of 1 and 3 mM HCO_3^-.

A high bicarbonate sensitivity of the NBC in glial cells had previously been suggested for cerebral astrocytes (Brookes and Turner, 1994) and for leech neuropil glial cells (Deitmer and Schneider, 1998). In both studies, residual CO_2/HCO_3^- was shown to maintain NBC activity, which was stimulated by raising the extracellular $[K^+]$, although the precise molecular identity of the NBC could not be presented then (the first mammalian NBC cloned was reported by Romero et al., 1998). We postulate that the NBCe1 in cortical astrocytes, and possibly in astrocytes of other brain regions and in other cell types, might be a sensor for low $[HCO_3^-]$.

The significance of NBCe1 for H^+ regulation

There are multiple processes in the nervous system, which are critically dependent upon extracellular and intracellular pH, including ion channel activation, neuronal excitability, neurotransmitter release, anion-dependent synaptic inhibition, and cerebral blood flow (for review, see Deitmer and Rose, 1996; Chesler, 2003; Attwell et al., 2010). The expression of NBCe1 primarily in astrocytes, but also in oligodendrocytes and some neurons, suggests that NBCe1 (*SLC4A4*), in addition to NHE, is a major regulator of pH in the brain (Deitmer and Chesler, 2009; Majumdar and Bevensee, 2010). Due to its electrogenic operation, NBCe1 activity depends on the membrane potential, with depolarization promoting inward transport, and hyperpolarization promoting outward transport, of Na^+ and HCO_3^-. Therefore, NBCe1 is capable of operating as either a base loader or an acid loader, and may also help to buffer the extracellular space (Deitmer, 1991; Rose and Deitmer, 1994). This may be significant for controlling neuronal activity via pH-dependent channel gating and efficacy of synaptic inhibition, and hence may contribute to suppression of epileptiform hyperactivity (Ransom, 2000; Choi et al., 2012). Consistent with this view, homozygous mutations in *SLC4A4* were reported to be associated with familial migraine, possibly through dysregulation of synaptic pH (Suzuki et al., 2010). Our present study suggests that the high apparent bicarbonate transport sensitivity in cortical astrocytes might be specialized for acidotic conditions, when the $[HCO_3^-]$ in the tissue may fall considerably, as occurs during neuronal activity-induced acidosis and during pathophysiological conditions such as ischemia.

Synaptic clefts may acidify considerably, dependent on the rate of presynaptic activity and synaptic morphology, during exocytosis of acidic neurotransmitter vesicles (Miesenböck et al., 1998), which in turn suppresses presynaptic calcium current and hence modulates synaptic transmitter release as has been shown in mammalian cone photoreceptors (DeVries, 2001). Extracellular acidification may cause substantial inhibition of NMDA glutamate receptors in cerebellar neurons (Traynelis and Cull-Candy, 1990), which may be aggravated or counteracted by HCO_3^- transport into or out of neighboring astrocytes, and thus may function as either negative- or positive-feedback signals for neuronal activity.

In ischemia, the extracellular pH may drop to as low as 6.7 (Rossi et al., 2007), which can aggravate pathological conditions by activating acid-sensing cation channels (Wemmie et al., 2006), and would reduce the extracellular bicarbonate concentration to as low as 4.8 mM. Though the apparent K_m value of NBCe1, which we report here to be 0.65 mM, the membrane depolarization as-

sociated with ischemia can alter the V_{max} of NBCe1 and enable inward bicarbonate transport from an acidic extracellular space. Traumatic brain injury, for example, may cause ischemia, impaired cerebral blood flow, edema, and excitotoxicity, all of which can be associated with brain acidosis (Gupta et al., 2004; Clausen et al., 2005). Bicarbonate administration, on the other hand, has been shown to attenuate the severity of traumatic brain injury in a lateral fluid percussion injury model in mice (Yin et al., 2013).

Under isocapnic conditions (5% CO_2), the extracellular [HCO_3^-] would drop to near 1 mM when external pH decreases to 6.0; on the other hand, a similarly low [HCO_3^-] could be achieved at a pH of 6.7 under hypocapnic conditions (1% CO_2). Although these low pH/CO_2/HCO_3^- levels are expected to occur only transiently in cytosolic and extracellular compartments of the normal brain, NBCe1, with the properties described here, would be expected to allow significant transport of HCO_3^- into or out of astrocytes under these conditions.

The high HCO_3^- sensitivity of NBCe1 may have functional relevance for a number of reasons, including the following: (1) NBCe1 could mediate inwardly directed bicarbonate transport, even when extracellular [HCO_3^-] is low during acidosis (under physiological and pathophysiological conditions, as described above); (2) bicarbonate transported into astrocytes may activate energy metabolism by increasing the rate of glycolysis (Ruminot et al., 2011; Choi et al. 2012), either by lowering intracellular [H^+] or by activating bicarbonate-dependent cyclic adenylyl cyclase (Chen et al., 2000; Zippin et al., 2001); and (3) the transporter may also be activated at low intracellular bicarbonate concentration and hence reduce or prevent extracellular acidification, which remains to be shown experimentally.

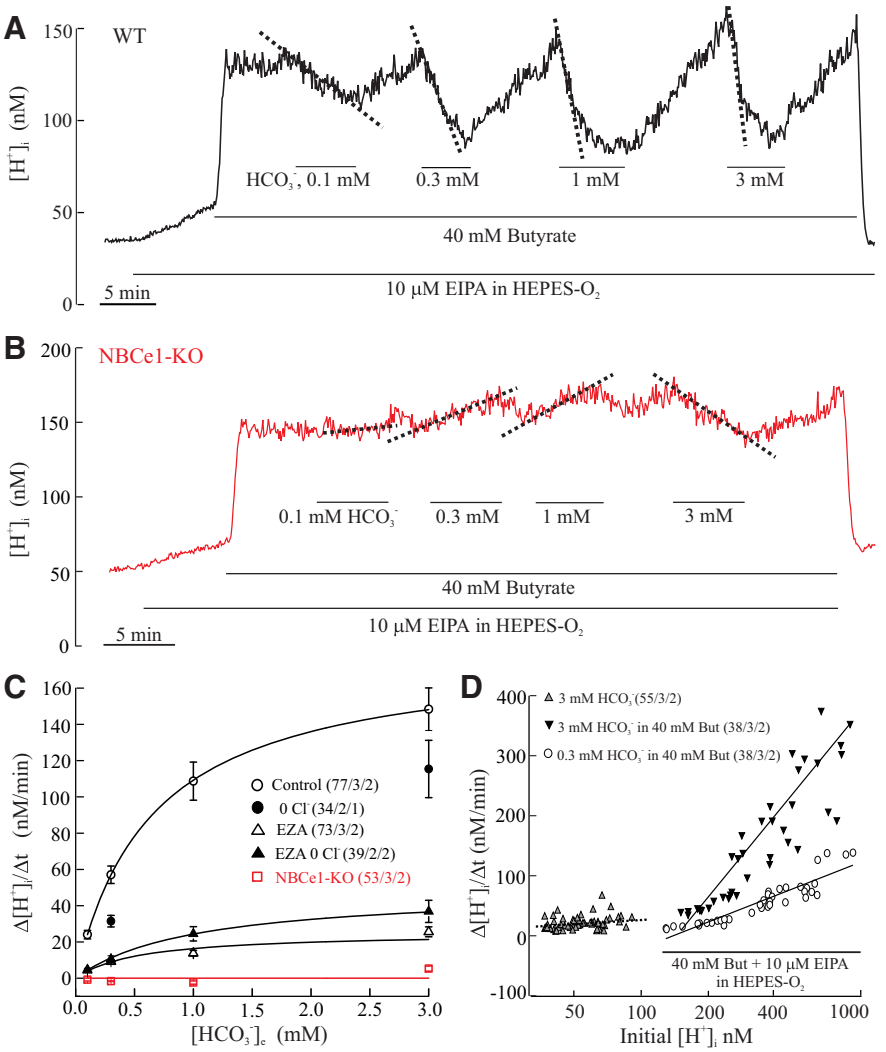

Figure 8. Bicarbonate sensitivity of the intracellular H^+ concentration at elevated intracellular H^+ concentration in the presence of butyrate and EIPA. ***A***, ***B***, Addition of different low concentrations of HCO_3^- in HEPES-O_2 saline solution containing butyrate (40 mM) and EIPA (10 μM) in WT (***A***) and NBCe1-KO (***B***) astrocytes. ***C***, Kinetic analysis of the intracellular [H^+] changes versus the added [HCO_3^-] analyzed from experiments, as shown in ***A***, and in saline solution containing no chloride (replaced by gluconate) with and without EZA (10 μM) in astrocytes from WT mice (black traces), and in astrocytes from NBCe1-KO mice (red trace), indicating a K_m value for HCO_3^- transport rate of 0.65 mM (Control). ***D***, The rates of $[H^+]_i$ change elicited by 3 mM HCO_3^- at low (resting) initial $[H^+]_i$ (<100 nM, gray triangles), and at higher initial $[H^+]_i$ (>100 nM) elicited by 0.3 mM HCO_3^- (open circles) and by 3 mM HCO_3^- (black triangles), showing that these rates are dependent on the $[H^+]_i$.

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