Carbonic Anhydride II Increases the Activity of the Human Electrogenic Na\(^{+}\)/HCO\(_{3}^{-}\) Cotransporter*

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Several acid/base-coupled membrane transporters, such as the electrogenic sodium-bicarbonate cotransporter (NBCe1), have been shown to bind to different carbonic anhydrase isoforms to create a "transport metabolon." We have expressed NBCe1 derived from human kidney in oocytes of *Xenopus laevis* and determined its transport activity by recording the membrane current in voltage clamp, and the cytosolic H\(^{+}\) and Na\(^{+}\) concentrations using ion-selective microelectrodes. When carbonic anhydrase isoform II (CAII) had been injected into oocytes, the membrane current and the rate of cytosolic Na\(^{+}\) rise, indicative for NBCe1 activity, increased significantly with the amount of injected CAII (2–200 ng). The CAII inhibitor ethoxyzolamide reversed the effects of CAII on the NBCe1 activity. Co-expressing wild-type CAII or NH\(_{2}\)-terminal mutant CAII together with NBCe1 provided similar results, whereas co-expressing the catalytically inactive CAII mutant V143Y had no effect on NBCe1 activity. Mass spectrometric analysis and the rate of cytosolic H\(^{+}\) change following addition of CO\(_{2}/\)HCO\(_{3}^{-}\) confirmed the catalytic activity of injected and expressed CAII in oocytes. Our results show that the transport capacity of NBCe1 is enhanced by the catalytic activity of CAII, in line with the notion that CAII forms a transport metabolon with NBCe1.

Cytosolic proton buffering and proton regulation are essential properties of all cells, and regulation of H\(^{+}\) is mainly attributable to Na\(^{+}\) and/or H\(^{+}\)/HCO\(_{3}^{-}\)-dependent carriers, which can recover intracellular and extracellular pH from an acidification or alkalinization. These transporters include the SLC4 family of HCO\(_{3}^{-}\) transporters, divided into three Cl\(^{-}\}/HCO\(_{3}^{-}\) exchangers (AE1–3) and five Na\(^{+}\)-coupled HCO\(_{3}^{-}\) transporters (NBCe1, NBCe2, NBCn1, NDCBE, and NCBE) and the family of Na\(^{+}\)/H\(^{+}\) exchangers (NHEs) consisting of 9 known isoforms, NHE1-NHE9 (SLC9). The electrogenic sodium-bicarbonate cotransporter (NBCe1) is found in many epithelial cells, such as renal proximal tubule cells (1–3) and glial cells (4–7). One main source of intracellular HCO\(_{3}^{-}\) that serves as substrate for the NBC is CO\(_{2}\), the hydration and dehydration of which is catalyzed by the enzyme carbonic anhydrase (CA).

After the report on the binding of carbonic anhydrase isoform II (CAII) to the carboxyl terminus of the human anion exchanger AE family (8), evidence has accumulated for a direct interaction between various acid/base transporters with different isoforms of carbonic anhydrase (9, 10). Reports on interaction between NBC and CA goes back to the finding that application of the CA inhibitor acetazolamide inhibits the transport of bicarbonate across the basolateral cell membrane in renal proximal tubule of rabbits (11–13). By heterologous expression of the kidney NBCe1 in a mouse proximal tubule cell line, Gross et al. (14) found that CAII increases the short-circuit current through the transporter in an acetazolamide-sensitive manner, when the NBCe1 was operating in the 3 HCO\(_{3}^{-}:1\) Na\(^{+}\) mode, but not in the 2:1 mode. In the same study, isothermal titration calorimetry experiments showed a binding between the transporter and CAII. Pushkin et al. (15) then showed in the mouse proximal tubule cell line that CAII activity enhances the bicarbonate flux via the NBCe1 and is likely to form a "transport metabolon" with the cotransporter. Experiments with mutated CAII-binding sites on the NBCe1 indicated that activity of CAII can only enhance the NBCe1 current, when it is bound to the NBCe1, whereas unbound CAII had no effect on NBCe1-mediated transport. Binding and interaction was also shown for the electroneutral NBC3 with CAII (16), and for the NBCe1 with extracellular CAIV (17). Recently, however, Lu et al. (18), by measuring the slope conductance of NBCe1-expressing *Xenopus* oocytes, injected with CAII or co-expressing a fusion protein of NBCe1 and CAII, found no CAII-induced increase in NBCe1 activity. The authors concluded that CAII does not form a transport metabolon with NBCe1, and that the enzymatic activity of CAII in the vicinity of the NBC is unlikely to enhance HCO\(_{3}^{-}\) transport substantially, when CO\(_{2}\) is nearly in equilibrium across the cell membrane. Furthermore, by using solid-phase binding assays with enzyme-linked immunosorbent assay detection, the same group demonstrated that CAII cannot bind to pure SLC-Ct peptides, and can bind to GST-SLC4-Ct fusion proteins only when the CAII is immobilized and the fusion protein is soluble, but not vice versa (19). These results reject the idea of a physical and also functional metabolon formation between CAII and bicarbonate transporters. These results disagree with the findings of other groups (8, 15–17), reporting binding between CAII and transporters of the SLC4 family.
We have also addressed the question, if NBCe1 and CAII interact, by heterologously expressing human NBCe1 in *Xenopus* oocytes with and without injected or co-expressed CAII. We used two mutants of CAII, a catalytically inactive mutant (CAII-V143Y), in which the deletion of the hydrophobic pocket of the enzyme's active site significantly diminishes enzyme activity by 3 \( \times 10^{-9} \)-fold (20, 21), and a mutant with 6 exchanged amino acids in the NH\(_2\)-terminal tail, which prevents binding of the mutant to the anion exchanger AE1.\(^3\) We have measured the activity of both NBCe1 and CAII activity independently by recording cytosolic H\(^+\) and Na\(^+\), the membrane current in voltage clamp, and by using mass spectrometry. Our results clearly indicate that CAII enhances the transport activity of the NBCe1 under these conditions.

**EXPERIMENTAL PROCEDURES**

**Constructs, Oocytes, and Injection of cRNA and Carbonic Anhydrase**—The human CAII cDNA (CAII-WT) and the mutant CAII-HEX with mutations of the amino acids at positions H3P, H4Q, L9A, H10K, H15Q, and H17S was kindly provided by Dr. Reinhart Reithmeier. The catalytically inactive mutant CAII-V143Y was a gift from Dr. Carol Fierke (20, 21). All three constructs were subcloned into the oocyte expression vector pGemHejuel, which contains the 5' - and 3'-untranscribed regions of the *Xenopus* β-globulin flanking the multiple cloning site. The human NBCe1 cDNA (hNBCe1) cloned in oocyte expression vector pH19 was kindly provided by Dr. Walter Boron (22). Plasmid DNA was linearized with NotI and transcribed in *vitro* with T7 RNA polymerase in the presence of the cap analog m^G(5')p(5')G (mMessage mMachine, Ambion Inc.) to produce a capped RNA transcript. The cRNA was purified with the Qiagen RNeasy MinElute Cleanup Kit (Qiagen GmbH, Hilden, Germany) and stored at \(-80^\circ\)C in DEPC/H\(_2\)O. Integrity of the cRNA was checked by formaldehyde gel electrophoresis. *Xenopus laevis* females were purchased from bovine erythrocytes (C3934, Sigma), dissolved in 25 nl of DEPC/H\(_2\)O, were injected 20–24 h before electrophysiological measurement. Control oocytes were injected with 25 nl of DEPC/H\(_2\)O. For coexpression of CAII, 12 ng of CAII/cRNA were injected either alone or together with the NBCe1/cRNA.

**Intracellular \(pH\) and Na\(^+\) Measurements**—For measurement of intracellular pH (pH\(_i\)) and membrane potential double-layer, and for intracellular Na\(^+\) (Na\(^{+}\)), single-barreled microelectrodes were used; the manufacture and application have been described in detail previously (23). Briefly, for double-barreled microelectrodes, two borosilicate glass capillaries of 1.0 and 1.5 mm in diameter were twisted together and pulled to a micropipette. The ion-selective barrel was silanized with a drop of 5% tri-N-butylchlorosilane in 99.9% pure carbon tetrachloride, backfilled into the tip. The micropipette was baked for 4.5 min at 450 °C on a hot plate. The H\(^+\)-sensitive mixture (Fluka 95291, Fluka, Buchs, Switzerland) was backfilled into the tip of the silanized ion-selective barrel and filled up with 0.1 m sodium citrate (pH 6.0). The reference barrel was filled with 3 m KCl. To increase the opening of the electrode tip, it was beveled with a jet stream of aluminum powder suspended in H\(_2\)O. Calibration of the electrodes was carried out in oocyte saline by changing the pH by 0.6 units. The central and reference barrel of the electrodes were connected by chlorided silver wires to the head stages of an electrometer amplifier. Electrodes were accepted for use in the experiments, when their response exceeded 50 mV per unit change in pH; on average, they responded with 54 mV for unit change in pH. In the experimental chamber, they responded faster to a change in saline pH than the fastest reaction measured in the oocyte cytosol.

For single-barreled Na\(^+\)-sensitive microelectrodes, a 1.5-mm borosilicate glass capillary was silanized as described above and backfilled with a Na\(^+\)-sensitive mixture, made of 10 weight % sodium ionophore VI (Fluka 71739), 89.5 weight % 2-nitrophenylether (o-NPOE), and 0.5 weight % sodium tetraphenylborate. The pipette was filled up with 100 mM NaCl and 10 mM MOPS buffer (pH 7.0). Calibration of the electrodes was carried out in oocyte saline with Na\(^+\) concentrations of 5, 10, 15, and 84.5 mM; on average, the electrodes responded with 52 mV for a 10-fold change in the Na\(^+\) concentration.

As described previously (24), optimal pH changes were detected when the electrode was located near the inner surface of the plasma membrane. This was achieved by carefully rotating the oocyte with the impaled electrode. All experiments were carried out at room temperature (22–32 °C). Only oocytes with a membrane potential negative to \(-30\) mV were used for experiments.

**Calculation of \(H^+\)**—The measurements of pH\(_i\) were stored digitally using homemade PC software based on the program LabView (National Instruments Germany GmbH, München, Germany) and was routinely converted into intracellular H\(^+\) concentration, [\(H^+\)]. Thus, changes in the [\(H^+\)], are recorded, which take into account the different pH baseline, e.g. measured in HEPES- and CO\(_2\)/HCO\(_3\)-buffered salines (see also Ref. 25). Amplitude and rate of change of the measured pH\(_i\) or [\(H^+\)], were analyzed.

**Voltage Clamp Recording**—A borosilicate glass capillary, 1.5 mm in diameter, was pulled to a micropipette and backfilled with 3 m KCl. The resistance of the electrodes measured in oocyte saline was around 1 m\(\Omega\). For voltage clamp, both electrodes were connected to the head stages of an Axoclamp 2B.

\(^3\) Dr. R. Reithmeier, personal communication.
Call Increases NBCe1 Activity

Fluorescence staining of NBCe1 and Call in Xenopus oocytes. Surface view of oocytes expressing NBCe1 injected with Call (A, E, and J), or co-expressing NBCe1 together with Call-WT (B, F, and K), catalytically inactive mutant Call-V143Y (C, G, and L), and NH2-terminal mutant Call-HEX (D and H), respectively. Oocytes were incubated with a guinea pig antibody against NBCe1 and a rabbit antibody against human and bovine Call, respectively, and secondary antibodies against guinea pig and rabbit, respectively, linked with a fluorescent dye. A–D, signal of the Call. E–H, overlays of the signals for Call and NBCe1. I, exemplary image of NBCe1 staining. The staining for NBCe1 in oocytes co-expressing NBCe1 with Call-WT, Call-V143Y, and Call-HEX, respectively, gave similar results as shown in I, J–L, surface view on native control oocytes, incubated with a guinea pig antibody against NBCe1 (J) or a rabbit antibody against human Call (K) or bovine Call (L), and a secondary antibody against guinea pig or rabbit linked with a fluorescent dye. M–O, optical cross-sections of an oocyte co-expressing NBCe1 and Call-V143Y, showing the signal for Call-V143Y (M), the signal for NBCe1 (N), and an overlay of both signals (O). Staining of oocytes co-expressing NBCe1 with Call-WT and Call-HEX, respectively, as well as NBCe1-expressing oocytes injected with Call from bovine erythrocytes gave similar results as shown in M–O. P, cross-section through a Call-injected oocyte sectioned into 300-µm slices. Q and R, optical cross-section of native control oocytes, incubated with a guinea pig antibody against NBCe1 (Q) or a rabbit antibody against human Call (R), and a secondary antibody against guinea pig or rabbit linked to fluorescent dye.

Determination of Call Activity—Activity of Call was determined by monitoring the 18O depletion of doubly labeled 13C18O2 through several hydration and dehydration steps of CO2 and HCO3− at 25 °C (26, 27). The reaction sequence of 18O loss from 13C18O16O (m/z = 49) over the intermediate product 13C18O18O (m/z = 47) and the end product 13C16O16O (m/z = 45) was monitored with a quadrupole mass spectrometer (MSD 5970; Hewlett Packard, Waldbronn, Germany), with 50 ng of Call, as well as native control oocytes, were fixed in 4% paraformaldehyde in phosphate-buffered saline. Oocytes were treated with 100% methanol and permeabilized with 0.1% Triton X-100. Unspecific binding sites were blocked with 3% bovine serum albumin and 1% normal goat serum. The cells were incubated in phosphate-buffered saline containing the primary antibodies (guinea pig anti-Na+/HCO3− cotransporter polyclonal antibody (1:100) and rabbit anti-carbonic anhydrase II (human and bovine erythrocytes) and polyclonal antibody (1:1000, Chemicon International, Inc.) overnight at 4 °C. Oocytes were then incubated with the secondary antibodies (Alexa Fluor 488 goat anti-guinea pig IgG and Alexa Fluor 546 goat anti-rabbit IgG, Molecular Probes, Inc.). The stained oocytes were analyzed with a laser scanning microscope (LSM 510, Carl Zeiss GmbH, Oberkochem, Germany), using whole oocytes, through which cross-sectional optical planes were laid, or the cell surface was viewed. Additionally oocytes were embedded in 2% agarose and sectioned into 300-µm thick slices with a microtome (752M Vibroslice, Campden Instruments Ltd., United Kingdom).

Fluorescent Staining of NBCe1 and Call in the Oocyte—Frog oocytes, either injected with cRNA for NBCe1 and Call, or the relative 18O enrichment was calculated from the measured 45, 47, and 49 abundance as a function of time according to: log enrichment = log(49 × 100/(49 + 47 + 45)). For calculation of the Call activity of the sample, the rate of 18O degradation was obtained from the linear slope of the log (enrichment) over the time, using the spreadsheet analyzing software Origin 6.0 (Microcal Software Inc.). The rate was compared with the corresponding rate of the non-catalyzed reaction. Enzyme activity
in units was calculated from these two values as defined by Badger and Price (28). From this definition, 1 unit corresponds to 100% stimulation of the non-catalyzed $^{18}$O depletion of doubly labeled $^{13}$C$^{18}$O$_2$. For the experiments, the cuvette was filled with 10 ml of oocyte saline with a pH of 7.35 according to the mean intracellular pH of CAII-injected oocytes at 25 °C. To determine the catalytic activity, CAII expressing oocytes were pipetted into the cuvette in a batch of 20. For calibration, 0.25, 0.5, 1, and 2 μg of CAII were directly added to the cuvette and compared with the activity of oocytes, which either expressed CAII or were injected with CAII.

**Calculation and Statistics**—Statistical values are presented as mean ± S.E. For calculation of significance in differences, Student’s $t$ test or, if possible, a paired $t$ test was used. In the figures shown, a significance level of $p \leq 0.05$ is marked with a single asterisk, $p \leq 0.01$ a double asterisk, and $p \leq 0.001$ with triple asterisks.

**RESULTS**

Expression of NBCe1—NBCe1-expressing oocytes either injected with CAII from bovine erythrocytes or co-expressing CAII-WT, CAII-V143Y, and CAII-HEX, respectively, were stained with Alexa dye-linked antibodies against an epitope of NBCe1 or CAII, respectively (see “Experimental Procedures”). Confocal images of the surface (Fig. 1, A–I) and in cross-sections (Fig. 1, M–O) of oocytes show staining for both NBCe1 (red, Fig. 1, I and N) and CAII (Fig. 1, A–D and M, green) in the plasma membrane of the oocyte, whereas no staining was evident in the cytoplasm. An overlay of both signals (Fig. 1, E–H and O) shows co-localization of the two proteins. Native oocytes treated the same way with the dye-linked antibodies showed no visible staining (Fig. 1, J–L, Q, and R). To confirm that cytosolic CAII was only localized in the plasma membrane, but not in the cytosol, and that the fluorescence was not quenched when optical slices were taken from intact oocytes, a CAII-injected oocyte was sectioned into 300-μm thick slices. The confocal image of the upper part of the slice shows that CAII is only found in the oocytes plasma membrane, but not in the cytosol (Fig. 1P).
same is true for oocytes expressing CAII (data not shown). We do not know what anchors the CAII in the membrane, but because this effect is not only observed in NBCe1-expressing oocytes, but also in native oocytes injected with, or expressing, CAII, CAII itself may bind to the plasma membrane of oocytes.

We determined the transport activity of the NBCe1 expressed in Xenopus oocytes by simultaneously measuring the membrane current, the intracellular sodium activity, Na\(^{+}\), and the intracellular proton activity, H\(^{+}\), of oocytes voltage-clamped to a membrane holding potential of −40 mV. The NBCe1 was challenged by changing from a HEPES-buffered to a 5% CO\(_2\), 24 mM HCO\(_3^-\)-buffered solution (Fig. 2A). In NBCe1-expressing oocytes, application of CO\(_2\)/HCO\(_3^-\) induced a membrane outward current and a rise in the intracellular H\(^{+}\) and Na\(^{+}\) concentrations. Expression of NBCe1 led to a significant reduction of the CO\(_2\)/HCO\(_3^-\)-induced intracellular acidification (ΔH\(^{+}\)) from 91.1 ± 0.9 to 31.0 ± 3.3 nM (p < 0.001; Fig. 2C). The rate of cytosolic H\(^{+}\) change following introduction of CO\(_2\)/HCO\(_3^-\) was also significantly changed by expressing NBCe1, being 33.0 ± 3.3 and 16.4 ± 1.6 nm/min in native and NBCe1-expressing oocytes, respectively (p < 0.001; Fig. 2D), presumably due to the dampening effect of HCO\(_3^-\) influx via the NBCe1 accompanying the diffusion of CO\(_2\) into the cells. The acidification was accompanied by a membrane outward current (ΔI\(_{\text{m}}\)) of 302 ± 30 nA (Fig. 2G) and an increase in cytosolic Na\(^+\) concentration (ΔNa\(^{+}\)) of 4.17 ± 0.56 nm (Fig. 2E) with a rate of 2.07 ± 0.32 nm/min (Fig. 2F), which was not observed in control oocytes injected with H\(_2\)O instead of NBCe1/cRNA (Fig. 2, B, F, and G). Furthermore, expression of the NBCe1 substantially increased the membrane conductance (G\(_{\text{m}}\)) of the oocytes in CO\(_2\)/HCO\(_3^-\)-buffered saline from 0.8 ± 0.1 to 6.1 ± 0.1 μS (p < 0.001; Fig. 3, A and C). Application of 0.5 mM of the anion exchange inhibitor DIDS increased the CO\(_2\)/HCO\(_3^-\)-induced acidification in NBCe1-expressing oocytes by 158%, whereas the membrane current and membrane conductance decreased to 20 and 38%, respectively (data not shown), suggesting reduced NBCe1 activity in the presence of DIDS. The reversal potential (E\(_{\text{rev}}\)) of the CO\(_2\)/HCO\(_3^-\)-induced current in NBCe1-expressing oocytes was −55.6 mV, suggesting a stoichiometry of 2 HCO\(_3^-\):1 Na\(^+\). If calculated from an intracellular Na\(^+\) concentration of 14.0 ± 3.5 mM and an intracellular HCO\(_3^-\) concentration of 21.0 ± 6.3 mM, reversal potentials of −52.6 and −28.0 mV are predicted for a stoichiometry of 2:1 and 3:1 for the NBCe1, respectively.

Effect of CAII Injection—Injection of CAII into native control oocytes resulted in a significant increase in the rate of rise of intracellular H\(^{+}\) concentration (ΔH\(^{+}\)/t) from 33.0 ± 3.3 to 128.0 ± 15.8 nm/min following introduction of CO\(_2\)/HCO\(_3^-\) (p < 0.001; Fig. 2D). The amplitude of the CO\(_2\)/HCO\(_3^-\)-induced acidification was, however, not changed by injection of CAII (92.5 ± 6.3 versus 91.1 ± 6.1 nm; Fig. 2C). Injection of CAII into native oocytes did not induce any membrane currents or changes in the cytosolic Na\(^+\) concentration (Fig. 2, B and E–G), and did not change the membrane conductance G\(_{\text{m}}\) of the oocytes (0.8 ± 0.1 versus 0.9 ± 0.1 μS; Fig. 3, A–C).

Injection of CAII into NBCe1-expressing oocytes increased the amplitude of the CO\(_2\)/HCO\(_3^-\)-induced acidification from 31.0 ± 3.3 to 51.0 ± 6.5 nm (p < 0.05; Fig. 2C), and, like in native, control oocytes, also increased the rate of rise of the intracellular H\(^{+}\) concentration following application of CO\(_2\)/HCO\(_3^-\) from 16.4 ± 1.6 to 145.1 ± 35.5 nm/min (p < 0.001; Fig. 2D). In contrast to control oocytes, however, injection of CAII into NBCe1-expressing oocytes increased the membrane current in CO\(_2\)/HCO\(_3^-\)-buffered solution from 302 ± 30 to 568 ± 37 nA (p < 0.001; Fig. 2G), and the rate of rise of Na\(^+\) concentration from 2.07 ± 0.32 to 8.20 ± 1.80 nm/min (p < 0.05; Fig. 2F). The amplitude of the cytosolic Na\(^+\) change was not significantly different, indicating an increase in transport rate, but not in absolute amount of transported substrates. Furthermore, injection of CAII added an additional membrane conductance of 2.5 ± 1.6 μS (Fig. 3, B–D), leading to a membrane conductance of 8.7 ± 0.6 μS in NBCe1-expressing oocytes (Fig. 3, A and C). The membrane current in NBCe1-expressing oocytes with CAII injected reversed at −53.5 mV, as compared with −55.6 mV in oocytes not injected with CAII (Fig. 3A). The membrane current attributable to CAII, isolated by subtraction of the
CAII Increases NBCe1 Activity

I/V curves, had a reversal potential of −46.2 mV, and a slope conductance of 2.5 ± 1.6 μS (Fig. 3, B and D). Thus, these results suggest that injection of CAII into NBCe1-expressing oocytes enhances the transport activity of NBCe1, as indicated by the increased \( \text{CO}_2/\text{HCO}_3^- \)-induced membrane current and conductance, as well as by the increased rate of rise of cytosolic Na\(^+\).

Effect of EZA on CAII-injected Oocytes—To check whether the increase in NBCe1 activity by CAII injection depends on the catalytic activity of the CAII, we used the potent enzyme inhibitor 6-ethoxy-2-benzothiazolesulfonamide (EZA, 10 μM). Application of EZA to NBCe1-expressing oocytes injected with CAII reduced the rate of rise of the intracellular H\(^+\) concentration from 216.2 ± 48.7 to 18.6 ± 3.0 nm/min (p ≤ 0.01; Fig. 4, A and C), the latter value being similar to the values of NBCe1-expressing oocytes not injected with CAII before (18.6 ± 3.0 nm/min) and after (22.2 ± 5.1 nm/min) application of EZA (Fig. 4C, n.s.). This also suggests that the CA activity in native Xenopus oocytes is negligible. EZA decreased the membrane currents in NBCe1-expressing oocytes injected with CAII from 513 ± 55 to 376 ± 48 nA (p ≤ 0.001), a value similar to those of currents in NBCe1-expressing cells not injected with CAII, being 282 ± 29 nA before, and 257 ± 36 nA after application of EZA (Fig. 4B, n.s.). The rate of the \( \text{CO}_2/\text{HCO}_3^- \)-induced rise in the cytosolic Na\(^+\) concentration was decreased in the presence of EZA from 8.20 ± 1.18 to 1.91 ± 0.60 mm/min (p ≤ 0.05), a value similar to those for oocytes not injected with CAII, being 2.07 ± 0.32 and 1.86 ± 0.30 mm/min in the absence and presence of EZA, respectively (Fig. 4D, n.s.). Application of 0.1 (v/v) ethanol, the solvent of EZA, alone had no effect on the \( \text{CO}_2/\text{HCO}_3^- \)-induced membrane current, rate of rise of intracellular H\(^+\) concentration, and membrane conductance (Fig. 4E), indicating that the observed effects of EZA were directly related to the blocker and not to the solvent. In summary, blocking the catalytic activity of CAII with EZA reversed the CAII-induced increase in the rate of rise of the cytosolic H\(^+\) and Na\(^+\) concentrations and in the membrane current, but had no significant effect on these parameters in oocytes not injected with CAII. This indicates that the CAII-mediated enhancement of NBCe1 transport activity is attributable to the catalytic activity of CAII.

Dose-response Curve of CAII—Whereas other studies working with CAII injected into Xenopus oocytes used an amount of 50 ng of CA/oocyte (29, 30), Lu et al. (18) injected an overall amount of 300 ng/oocyte. To determine the dependence of the CAII-mediated increase in NBCe1 activity on the concentration of CAII, we injected 2, 10, 50, or 200 ng of CAII into NBCe1 expressing oocytes and determined the NBCe1 activity by measuring membrane current and conductance during application of 5% \( \text{CO}_2 \), 24 mM \( \text{HCO}_3^- \)-buffered solution in the absence and presence of the CAII inhibitor EZA (10 μM). Both, the \( \text{CO}_2/\text{HCO}_3^- \)-induced membrane current and the membrane conductance showed a dependence on the concentration of injected CAII in the presence of \( \text{CO}_2/\text{HCO}_3^- \) (Fig. 5, A and C, filled symbols). In the presence of EZA, no dependence of membrane current and membrane conductance on CAII concentra-
Addition of 10 ng of bovine CAII catalyzed degradation of labeled CO$_2$. The difference of the non-catalyzed and the CAII-catalyzed change of CO$_2$/HCO$_3^-$ is shown in Fig. 6 from the currents and conductance shown in Fig. 7 during application of CO$_2$/HCO$_3^-$ via NBCe1. The EZA-sensitive membrane current and conductance as shown in Fig. 6B, EZA-sensitive membrane current as obtained by subtraction of the CO$_2$/HCO$_3^-$-induced currents in EZA from the control currents shown in Fig. 5, A black arrow indicates the addition of either oocytes or enzyme as indicated. The statistical analysis of the enzyme activity calculated from the difference of the non-catalyzed and the CAII-catalyzed change was observed (Fig. 5, A and C, open symbols). Subtracting the CO$_2$/HCO$_3^-$-induced membrane current and conductance in the presence of EZA from the currents and conductance observed before application of the CA inhibitor, gives the EZA-sensitive membrane current and conductance as shown in Fig. 5, B and D. Both, the EZA-sensitive membrane current and the EZA-sensitive membrane conductance show a clear dependence on the CAII concentration. The curves indicate that half-maximal effects on current and conductance of NBCe1 were obtained with a concentration of 20–30 ng CAII.

**CAII Activity in Oocytes Analyzed with Mass Spectrometry**

We also co-expressed wild-type CAII (CAII-WT) with the NBCe1 to see if, under these conditions, a similar interaction between the two proteins is observed. To compare the amount of expressed with the amount of injected CAII protein in the oocytes, we determined the enzymatic activity of CAII injected into, and CAII-WT expressed alone or together with the NBCe1, in oocytes by mass spectrometry. Original recordings of the log enrichment of either 20 oocytes injected with 50 ng of CAII, expressing CAII-WT alone or together with NBCe1, and of 1 µg of CAII directly added into the measuring cuvette, are shown in Fig. 6A. The first part of the curve gives the non-catalyzed degradation of labeled CO$_2$. The black arrow indicates the addition of either oocytes or enzyme as indicated. The statistical analysis of the enzyme activity calculated from the difference of the non-catalyzed and the CAII-catalyzed change in the log enrichment shows CAII activity in all probes (Fig. 6B). 20 oocytes injected with CAII showed an activity of 25.4 ± 1.7 units/ml, which was in the same range as CAII-WT expressed together with the NBCe1 with an activity of 25.3 ± 5.2 units/ml. CAII-WT expressed in native oocytes showed a slightly higher activity of 33.3 ± 1.7 units/ml (n.s.). We calibrated the CAII activity by measuring the enzymatic activity of defined amounts of CAII protein (0.25, 0.5, 1, and 2 µg) to generate a calibration curve (Fig. 6C). Using the linear regression of this relationship, the amount of protein was calculated from the measured enzyme activity (Fig. 6D). Native oocytes injected with cRNA for CAII-WT expressed an average amount of 64.5 ± 3.3 ng of CAII/oocyte, whereas oocytes additionally injected with NBCe1/cRNA expressed 49.3 ± 10.0 ng of CAII/oocyte, which corresponds well with the amount of CAII injected into each oocyte (50 ng). Calculating the amount of active CAII in oocytes injected with 50 ng of CAII each, resulted in an amount of 49.4 ± 3.2 ng of CAII/oocyte, indicating that virtually all CAII injected into the oocytes displayed catalytic activity.

**Co-expression of NBCe1 with CAII-WT**

The NBCe1 activity was measured in oocytes, in which NBCe1 was co-expressed with CAII-WT, by simultaneously recording the membrane current, Na$^+$, and H$^+$, of voltage-clamped oocytes during application of 5% CO$_2$, 24 mM HCO$_3^-$ before (Fig. 7A, black traces) and after the addition of the CAII inhibitor EZA (10 µM; Fig. 7A, gray traces). Application of CO$_2$/HCO$_3^-$ to NBCe1 and CAII-WT co-expressing oocytes led to an intracellular acidification of 13.1 ± 2.0 nM, whereas application of CO$_2$/HCO$_3^-$ to oocytes expressing NBCe1 alone induced an alkalinization of 8.3 ± 5.0 nM (Fig. 7, C and D). Application of EZA to NBCe1 + CAII-WT co-expressing oocytes resulted in an alkalinization during application of CO$_2$/HCO$_3^-$ of 4.6 ± 4.6 nM. The alkalinization is likely to be due to the import of HCO$_3^-$ via the NBCe1. Expression of CAII-WT accelerates the hydration of CO$_2$ to an extent that the formation of H$^+$ is faster than the import of HCO$_3^-$ via the NBCe1, resulting in an intracellular acidification. Blocking CAII-WT with EZA reduces the rate of CO$_2$ hydration leading to an alkalinization during application of CO$_2$/HCO$_3^-$, presumably due to the fast inward transport of HCO$_3^-$ via NBCe1.

Co-expression of CAII together with the NBCe1 lead to a significant increase in the membrane current during application of CO$_2$/HCO$_3^-$ from 720 ± 40 nA in oocytes expressing
NBCe1 alone, to 1024 ± 52 nA (p ≤ 0.001). This increase in current was largely reversed by EZA, which decreased the current to 763 ± 40 nA (p = 0.001; Fig. 7B). EZA had no effect on the membrane current in oocytes expressing NBCe1 alone.

The rate of rise of the cytosolic Na⁺ concentration in oocytes expressing NBCe1 + CAII-WT was 7.24 ± 1.29 mmol/min, which was significantly larger than in oocytes expressing NBCe1 alone (1.69 ± 0.31 mmol/min; p ≤ 0.01). EZA reduced the rate of Na⁺ rise significantly to 2.75 ± 0.60 mmol/min (p ≤ 0.001). In oocytes expressing NBCe1 alone, EZA had no effect on the rate of Na⁺ rise, being 1.69 ± 0.31 mmol/min before, and 1.39 ± 0.39 mmol/min during application of EZA (Fig. 7F). The amplitude of the increase in intracellular Na⁺ concentration mediated by the NBCe1 was neither changed by co-expression with CAII-WT nor by application of EZA (Fig. 7E).

The current-voltage relationships of oocytes expressing NBCe1 alone and together with CAII-WT, in the presence and absence of EZA, were very similar (Fig. 8A). It must be noted that in these batches of oocytes, the background currents and conductance were relatively large, being around 1 µA at 0 mV with a slope conductance of 16–18 µS, respectively (Fig. 8, A and E). The reversal potential was not changed by the expression of CAII-WT or EZA, and values between −55.2 and −56.6 mV in the different types of oocytes were measured (Fig. 8A). By subtracting the currents of oocytes expressing NBCe1 with and without CAII-WT, a current attributable to CAII-WT could be isolated (Fig. 8B), which amounted to 150 nA at 0 mV, very similar to that obtained for injected CAII (see Fig. 3B).

In 5% CO₂, 24 mM HCO₃⁻-buffered solution, the membrane conductance of oocytes expressing NBCe1 + CAII-WT was 18.2 ± 0.7 µS before and 16.3 ± 0.8 µS after addition of EZA, respectively (p ≤ 0.001; Fig. 8, A and E). NBCe1-expressing oocytes without CAII-WT had a membrane conductance of 15.7 ± 0.7 µS before and 16.8 ± 0.8 µS during application of EZA (p ≤ 0.01; Fig. 8, A and E). Co-expression of CAII-WT together with NBCe1 added an additional membrane conductance of 2.2 ± 0.1 µS in the presence of CO₂/HCO₃⁻ (Fig. 8, B and F), whereas expression of CAII-WT without NBCe1 had no effect on the membrane conductance. Application of 10 µM EZA in CO₂/HCO₃⁻-buffered solution increased the membrane conductance of NBCe1-expressing oocytes without CAII by 1.1 ± 0.1 µS, and with CAII-WT, decreased Gₘ by 1.2 ± 0.2 µS (Fig. 8C). Subtracting the EZA-sensitive currents of NBCe1 + CAII-WT-expressing oocytes from the EZA-sensitive currents of oocytes expressing NBCe1 alone revealed the effect of EZA on the CAII-induced current (Fig. 8D), amounting to 130 nA at 0 mV. The slope of the linear regression line gives a conductance of 2.4 ± 0.2 µS, a value close to the 2.2 ± 0.1-µS increased mediated by CAII-WT (Fig. 8D) as indicated by the dotted line.

**Co-expression of NBCe1 with CAII Mutants**—To specify the role of the catalytic activity of CAII as well as a possible binding of the enzyme for the augmentation of NBCe1 activity, we co-expressed the NBCe1 with the catalytically inactive mutant CAII-V143Y, and the NH₂-terminal mutant CAII-HEX. The activity of the NBCe1 was measured in oocytes, in which NBCe1 was co-expressed with CAII-HEX or CAII-V143Y, by simultaneously recording the membrane current, Na⁺, and H⁺, of voltage-clamped oocytes during application of 5% CO₂, 24 mM HCO₃⁻ before (Fig. 9A, black traces) and after the addition of the CAII inhibitor EZA (10 µM; Fig. 9A, gray traces; Table 1). Similar to oocytes co-expressing NBCe1 with CAII-WT, application of 5% CO₂, 24 mM HCO₃⁻ resulted in NBCe1 + CAII-HEX co-expressing oocytes in a robust acidification, whereas application of CO₂/HCO₃⁻ in the presence of 10 µM EZA induced a slight alkalization (Fig. 9, A and C). Like in CAII-WT expressing oocytes, co-expression of NBCe1 with CAII-HEX resulted in a CO₂/HCO₃⁻-induced membrane current of 983 ± 21 nA (Fig. 9, A and B), an increase in intracellular Na⁺ with a rate of rise of 5.29 ± 0.98 mmol/min (Fig. 9, A and D),
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and a membrane conductance of 17.7 ± 0.3 μS (Fig. 9, E and F). All three indicators of NBCe1 activity were significantly decreased by the application of the CA inhibitor EZA. The membrane current fell to 718 ± 28 nA (p = 0.001), the rate of rise in Na⁺, was reduced to 2.71 ± 0.57 nm/min (p = 0.05), and the membrane conductance decreased to 13.3 ± 0.3 μS (p = 0.01).

Co-expression of NBCe1 together with the catalytically inactive mutant CAII-V143Y resulted in a small CO₂/HCO₃⁻-induced acidification before, and a small alkalization during, application of CAZ (Fig. 9, A and C). Membrane current, rate of rise in intracellular Na⁺ concentration, and membrane conductance of NBCe1 + CAII-V143Y co-expressing oocytes were not significantly different from the values of oocytes expressing NBCe1 alone as shown in Fig. 7, none of these parameters showed any sensitivity to EZA. The CO₂/HCO₃⁻-induced membrane current was 644 ± 61 nA before and 696 ± 58 nA also at −80 mV, where the NBCe1 is less active (25), and compared the effect of injected CAII on NBCe1 activity at different holding potentials. Fig. 10A shows an original recording of a NBCe1-expressing oocyte injected with 50 ng of CAII, clamped to a membrane potential of either −40 or −80 mV. With CAII injected, the CO₂/HCO₃⁻-induced membrane currents in NBCe1-expressing oocytes were 1105 ± 47 and 774 ± 42 nA at −40 and −80 mV, respectively (p = 0.001; Fig. 10B).

Both values were significantly larger than in oocytes not injected with CAII, being 609 ± 39 nA at −40 mV (p = 0.001) and 485 ± 50 nA at −80 mV (p = 0.001). The rate of change of the intracellular H⁺ concentration increased by changing the membrane potential from −40 to −80 mV from 35.9 ± 10.5 to 76.7 ± 16.9 nm/min in NBCe1-expressing oocytes with CAII (p = 0.05), which was significantly larger than the rate of change in NBCe1-expressing cells without injected CAII with 12.6 ± 2.4 nm/min at −40 mV (p = 0.01) and 18.1 ± 4.6 nm/min at −80 mV.

During application of EZA (Fig. 9B, n.s.), the rate of rise in Na⁺, was 1.78 ± 0.29 nm/min before and 1.72 ± 0.48 nm/min during application of EZA (Fig. 9D, n.s.), and the membrane conductance was 14.0 ± 0.2 μS before and 14.3 ± 0.2 μS during application of EZA (Fig. 9, E and F, n.s.).

We also measured the enzymatic activity of the expressed CAII mutants by mass spectrometry. 20 oocytes expressing CAII-HEX alone showed a catalytic activity of 41.2 ± 1.9 units/ml, whereas 20 oocytes co-expressing CAII-HEX together with NBCe1 had an activity of 36.7 ± 0.7 units/ml (Fig. 9G). Using the calibration curve shown in Fig. 6C, an amount of CAII-HEX of 79.7 ± 6.2 and 71.0 ± 1.8 ng/oocyte was calculated for oocytes expressing CAII alone and together with NBCe1, respectively (Fig. 9H). Oocytes expressing the catalytically inactive mutant CAII-V143Y alone or together with NBCe1 showed a low catalytic activity of 2.6 ± 0.5 and 1.9 ± 0.8 units/ml, respectively (Fig. 9G), indicating the significant catalytic deficit of the mutant CAII-V143Y.

**Dependence of the CAII-induced Activation of NBCe1 on the Membrane Potential**—It was studied if the increase in NBCe1 activity as induced by CAII was due to voltage clamping the oocytes to −40 mV during the application of CO₂/HCO₃⁻, which lead to a strong activation of the transporter. We therefore measured the NBCe1 activity during application of EZA (Fig. 9B, n.s.), the rate of rise in Na⁺, was 1.78 ± 0.29 nm/min before and 1.72 ± 0.48 nm/min during application of EZA (Fig. 9D, n.s.), and the membrane conductance was 14.0 ± 0.2 μS before and 14.3 ± 0.2 μS during application of EZA (Fig. 9, E and F, n.s.).
The CAII-induced activation of NBCe1 was also measured, when CO$_2$/HCO$_3^-$ was applied under current-clamp conditions (Fig. 11A), and compared with that when the oocyte membrane was held in voltage clamp (Fig. 11B, VC). Because application of CO$_2$/HCO$_3^-$ activates the NBCe1, a large voltage gradient would be created with the oocyte membrane held at -40 mV. On the other hand, with the oocyte membrane not voltage-clamped, a substantial hyperpolarization is observed, which would be expected to decrease the driving force of NBCe1, and hence reduce NBCe1 activity. The membrane hyperpolarization was not significantly larger, when CAII had been injected into the oocytes (Fig. 11C), although the I/V relationships (Fig. 11D) indicated a larger slope conductance (Fig. 11G), being 19.9 ± 0.2 μS with CAII and 15.7 ± 0.4 μS without CAII, when the cell was in current clamp (p ≤ 0.05; n = 6), and only briefly (<1 min) held in voltage clamp to obtain the I/V curve. The CO$_2$/HCO$_3^-$-induced membrane current in voltage clamp increased when CAII had been injected (Fig. 11D), and the I/V relationships (Fig. 11F) indicated a slope conductance of 21.2 ± 0.6 μS with CAII and 18.2 ± 0.5 μS without CAII, when the oocyte was in voltage clamp throughout (p ≤ 0.05; n = 6; Fig. 11G).

**DISCUSSION**

The present study has analyzed the activity of the human kidney NBCe1 expressed in *Xenopus* oocytes with and without injected or co-expressed CAII. The results indicate that CAII enhances transport activity of the NBCe1, and supports the conclusion of others that NBCe1 forms a transport metabolon with CAII (14, 15), but is in contrast to a recent study, which did not find evidence for functional interaction between NBCe1 and CAII (18). Our conclusion is based on recording membrane current and cytosolic Na$^+$ as measures for NBCe1 transport activity. Both current and the rate of cytosolic Na$^+$ rise following addition of CO$_2$/HCO$_3^-$ are increased in oocytes, which had been injected or co-expressed CAII. An increase in the membrane slope conductance, indicative for enhancement of NBCe1 transport activity, could also be attributed to injected or co-expressed CAII. All these effects of CAII on the NBCe1

**FIGURE 8.** Membrane currents and conductance of oocytes expressing NBCe1 with and without co-expressed CAII-WT. A, I/V relationships of oocytes in 5% CO$_2$, 24 mM HCO$_3^-$-buffered solution before (filled symbols) and after (open symbols) addition of 10 μM EZA. B, I/V relationship of current attributable to co-expressed CAII-WT, as obtained by subtraction of the currents in oocytes co-expressing NBCe1 and CAII-WT and oocytes expressing NBCe1 alone. In the presence of CO$_2$/HCO$_3^-$, the co-expression of CAII adds an additional membrane conductance of 2.2 μS. C, I/V relationships of EZA-sensitive current in oocytes, obtained by subtraction of currents as indicated. D, I/V relationship of the isolated, EZA-sensitive current (filled circles), and the CAII-WT-induced current (dotted line from B). From the I/V relationships, the slope conductance of the currents in the different oocytes as obtained in A were plotted (E), as well as those from the subtracted currents (F) as shown in B and D.

mV (p ≤ 0.01; Fig 10C). The rate of rise of the intracellular Na$^+$ concentration decreased with a more negative membrane potential; injection of CAII induced an increase in the rate of rise from 2.55 ± 1.55 to 7.51 ± 0.83 ms/min at -40 mV (p ≤ 0.05) and from 2.04 ± 0.69 to 5.00 ± 0.48 ms/min at -80 mV (p ≤ 0.05; Fig. 10D). The membrane conductance as calculated from the slope of the I/V curves (Fig. 10E) was not affected by changing the membrane holding potential from -40 to -80 mV (Fig. 10F). At both holding potentials, the conductance of NBCe1-expressing cells in CO$_2$/HCO$_3^-$-buffered solution was larger, when CAII had been injected (19.7 ± 0.4 μS as compared with 15.3 ± 0.3 μS at -40 mV, p ≤ 0.01, and 19.2 ± 0.2 μS as compared with 15.4 ± 0.4 μS at -80 mV, p ≤ 0.01).
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activity could be reversed by blocking the catalytic activity of CAII by EZA. Dependence of the CAII-induced augmentation of NBCe1 activity on the enzyme’s catalytic activity was also confirmed by a catalytically inactive mutant of CAII (CAII-V143Y). Co-expression of CAII-V143Y with NBCe1 failed to increase NBCe1 activity. The activity of injected and expressed CAII in oocytes was demonstrated by the substantial increase in the rate of change following addition of CO$_2$/HCO$_3$⁻, as compared with oocytes without CAII, and by measuring and calibrating the catalytic activity of CAII by mass spectrometry.

CAII binding to various acid/base transporters, such as the anion exchanger AE1 or the sodium/hydrogen exchanger NHE, has been shown and can result in considerable augmentation of transport activity of these proteins (8, 9, 31). Similarly, several studies have reported interaction of NBC isoforms with carbonic anhydrases, in particular NBCe1 with CAII (14, 15), and NBC1 and NBC3 with CAIV (16, 17). These studies were either performed in the transfected mouse proximal convoluted tubule cell line (14, 15) or in transfected HEK293 cells. In the only other study using *Xenopus* oocytes as the heterologous expression system, Lu et al. (18) measured the slope conductance of oocytes expressing NBCe1 with and without injected CAII, and employed a double fusion protein with NBCe1 and CAII attached to enhanced green fluorescent protein to evaluate the effect of CAII on NBCe1 activity. Lu et al. (18) found that injecting CAII into oocytes markedly accelerated hydration of intracellular CO$_2$, but had no effect on the slope conductance of the NBCe1 current, and that the conductance of oocytes expressing EGFP-NBCe1-CAII was insensitive to the CAII inhibitor EZA. Hence, our study comes to an opposite conclusion as the studies by Lu et al. (18) and Piermarini et al. (19).

**FIGURE 9.** Effect of EZA on oocytes co-expressing NBCe1 with a NH$_2$-terminal mutant of CAII (CAII-HEX) and a catalytically inactive mutant of CAII (CAII-V143Y). A, original recordings from oocytes either co-expressing NBCe1 and the NH$_2$-terminal mutant CAII-HEX (left traces) or the catalytically inactive mutant CAII-V143Y (right traces). The upper traces show the changes in membrane current, the middle traces the intracellular H$^+$ concentration, and the lower traces the intracellular Na$^+$ concentration during application of 5% CO$_2$, 24 mM HCO$_3$⁻ before (black traces) and after (gray traces) addition of the CAII inhibitor EZA (10 μM). B–F, statistical summary of the membrane current (B), the rate of change in the intracellular H$^+$ concentration (C), the rate of change in the intracellular Na$^+$ concentration (D), and the membrane conductance (F) derived from the current-voltage relationships as shown in E in oocytes co-expressing NBCe1 and CAII-HEX or CAII-V143Y. G, enzymatic activity of CAII-HEX and CAII-V143Y in units/ml expressed either alone or together with NBCe1 as determined by mass spectrometry, and H, amount of expressed CAII-HEX in nanograms/oocyte as calculated from the enzymatic activity in G.
TABLE 1
Summary of the CO₂/HCO₃⁻-induced changes in membrane current, rate of rise in Na⁺ concentration and membrane conductance of NBCe1-expressing oocytes either injected with CAII or co-expressing CAII-WT, CAII-V143Y, and CAII-HEX, respectively, before and during application of the CA inhibitor EZA (10 μM)

| Injected CAII | \(\Delta I_m\) | \(\Delta I_m\) | \(G_m\) |
|---------------|---------------|---------------|--------|
| NBCe1         | \(-EZA\)      | \(+EZA\)      | \(-EZA\) | \(+EZA\) |
|               | \(\text{nm}\) | \(\text{nm}\) | \(\mu S\) | \(\mu S\) |
| NBCe1 + CAII  | 282 ± 29      | 257 ± 36      | 2.07 ± 0.32 | 1.86 ± 0.30 | 6.1 ± 0.1 |
| NBCe1 + CAII  | 513 ± 55      | 376 ± 48      | 8.20 ± 1.18 | 1.91 ± 0.60 | 8.7 ± 0.6 |

| Expressed CAII | \(\Delta I_m\) | \(\Delta I_m\) | \(G_m\) |
|----------------|---------------|---------------|--------|
| NBCe1          | \(-EZA\)      | \(+EZA\)      | \(-EZA\) | \(+EZA\) |
| NBCe1 + CAII-WT | 720 ± 40      | 755 ± 56      | 1.69 ± 0.31 | 1.39 ± 0.39 | 15.7 ± 0.7 |
| NBCe1 + CAII-V143Y | 1024 ± 52 | 763 ± 40 | 7.24 ± 1.29 | 2.75 ± 0.60 | 18.2 ± 0.7 |
| NBCe1 + CAII-HEX | 644 ± 61      | 696 ± 58      | 1.78 ± 0.29 | 1.72 ± 0.48 | 14.0 ± 0.2 |
| NBCe1 + CAII-HEX | 983 ± 21      | 718 ± 28      | 5.29 ± 0.98 | 2.71 ± 0.57 | 17.7 ± 0.3 |

In the present study, we investigated a possible interaction between NBCe1 and CAII by expressing the transporter in Xenopus oocytes either injected with CAII or co-expressing CAII-WT and CAII mutants. One problem with determining the flux of HCO₃⁻ by the NBCe1, as done in different studies, is the determination of the rate of pH change, which is directly affected by the activity of CAII, making it difficult to discriminate between a change in HCO₃⁻ flux through the NBCe1 and the direct effect of CAII on the changes in pH. To check for possible interaction between the two proteins, we therefore measured the changes in membrane current and cytosolic Na⁺ concentration, two parameters that are directly and exclusively related to the NBCe1 transport activity (25, 32). Both parameters did not change in native oocytes or in oocytes only injected with CAII, showing that they are both attributable to the expression of NBCe1. Another indicator for NBCe1 activity, also used by Lu et al. (18), was the membrane slope conductance of the oocytes, which was increased in NBCe1-expressing oocytes in the presence of CO₂/HCO₃⁻, but remained largely unchanged in the nominal absence of CO₂, when the NBCe1 is not active (32).

Lu et al. (18) did not measure cytosolic Na⁺, and analyzed the membrane current only in terms of its slope conductance, which was not changed by injection of 300 ng of CAII. The slope conductance of their oocytes expressing NBCe1 was

FIGURE 10. Effect of the membrane potential on the CAII-induced activation of NBCe1. A, original recordings from a NBCe1-expressing oocyte injected with 50 ng of CAII at a membrane potential of −40 and −80 mV. The upper trace shows the membrane current, the middle trace the intracellular H⁺ concentration, and the lower trace the intracellular Na⁺ concentration during application of 5% CO₂/24 mM HCO₃⁻. B–F, statistical summary of the parameters as measured in A, the changes in the membrane current (B), the rate of change of the intracellular H⁺ concentration (C), and the intracellular Na⁺ concentration (D) in NBCe1-expressing oocytes, either injected with CAII or H₂O, during application of 5% CO₂/24 mM HCO₃⁻. B–F, statistical summary of the parameters as measured in A, the changes in the membrane current (B), the rate of change of the intracellular H⁺ concentration (C), and the intracellular Na⁺ concentration (D) in NBCe1-expressing oocytes, either injected with CAII or H₂O, during application of 5% CO₂/24 mM HCO₃⁻. B–F, statistical summary of the parameters as measured in A, the changes in the membrane current (B), the rate of change of the intracellular H⁺ concentration (C), and the intracellular Na⁺ concentration (D) in NBCe1-expressing oocytes, either injected with CAII or H₂O, during application of 5% CO₂/24 mM HCO₃⁻. B–F, statistical summary of the parameters as measured in A, the changes in the membrane current (B), the rate of change of the intracellular H⁺ concentration (C), and the intracellular Na⁺ concentration (D) in NBCe1-expressing oocytes, either injected with CAII or H₂O, during application of 5% CO₂/24 mM HCO₃⁻. B–F, statistical summary of the parameters as measured in A, the changes in the membrane current (B), the rate of change of the intracellular H⁺ concentration (C), and the intracellular Na⁺ concentration (D) in NBCe1-expressing oocytes, either injected with CAII or H₂O, during application of 5% CO₂/24 mM HCO₃⁻. B–F, statistical summary of the parameters as measured in A, the changes in the membrane current (B), the rate of change of the intracellular H⁺ concentration (C), and the intracellular Na⁺ concentration (D) in NBCe1-expressing oocytes, either injected with CAII or H₂O, during application of 5% CO₂/24 mM HCO₃⁻.
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FIGURE 11. Changes in membrane potential and membrane current in NBCe1-expressing oocytes with and without injected CAII during application of CO2/HCO3−. Original recordings from a NBCe1-expressing oocyte injected with 50 ng of CAII in current clamp (CC; A) and in voltage clamp (VC; B). The upper traces show the changes in the membrane current, the lower traces the changes in membrane potential. To generate an I/V relationship the oocyte in A was taken into voltage clamp for a short period (<1 min) as indicated by the VC bar in A and the membrane potential was changed stepwise between −120 and +20 mV. C–G, statistical summary of the parameters as measured in A and B; changes in membrane potential (C) of NBCe1-expressing or H2O-injected oocytes injected with 50 ng of CAII or H2O during application of CO2/HCO3− as shown in protocol A; changes in membrane current (D) in NBCe1-expressing or H2O-injected oocytes, injected with 50 ng of CAII or H2O during application of CO2/HCO3− in voltage clamp (−40 mV) as shown in protocol B; I/V relationships (E) of NBCe1-expressing oocytes either injected with CAII (filled symbols) or H2O (open symbols) in CO2/HCO3−-buffered solution as shown in protocol A; I/V relationships (F) of NBCe1-expressing oocytes either injected with CAII (filled symbols) or H2O (open symbols) in CO2/HCO3−-buffered solution as shown in protocol B. G, slope conductance as calculated from the I/V relationships shown in E and F.

between 14 and 18 μS, and they found a significant decrease of the slope conductance by injecting Tris buffer with and without CAII (Fig. 4 of Ref. 18). In our oocytes with NBCe1 expressed, the slope conductance ranged between 6 μS and nearly 21 μS, and we could easily isolate a CAII-attributable current and conductance (Figs. 3 and 8–11). The expression level of NBCe1 in the oocytes might have been higher in the study by Lu et al. (18), because they injected 25 ng of NBCe1/cRNA, whereas in our study, 14 ng of NBCe1/cRNA was injected. This may have resulted in a significantly higher membrane conductance of the oocytes in their study (18). The amount of CAII in the oocytes also differed in the two studies; we injected 50 ng of CAII/oocyte, as previously also used by Boron’s group (29) and by us and because they only used the slope conductance, a conductance of 2–3 μS could have easily been missed, the more, as EZA appears to increase the membrane conductance on its own in NBCe1-expressing oocytes, as both Lu et al. (18) and we have found in the present study.

In a discussion with Dr. Walter Boron following the presentation of their results (18) and our study in a meeting, we performed additional experiments (Figs. 10 and 11). Because the membrane of NBCe1-expressing oocytes hyperpolarizes up to about −120 mV upon the introduction of CO2/HCO3− due to the strong activation of NBCe1, voltage clamping of the oocyte membrane to −40 mV keeps the NBCe1 at a high activity. Lu et al. (18) had taken their I/V relationship near the zero current...
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potential; this might have occluded an effect of CAII on the NBCe1, because the activity of the cotransporter was too small. When voltage clamping the oocyte membrane at \(-80\) mV, where the electrochemical gradient for NBCe1 activity upon application of CO\(_2\)/HCO\(_3^-\) was much smaller, CAII still enhanced NBCe1 activity. Even if we reproduced the experiments of Lu et al. (18), recording the free oocyte membrane potential during application of CO\(_2\)/HCO\(_3^-\), and then took the I/V relationship keeping the oocyte membrane only briefly in voltage clamp, the NBCe1 activity was still increased in the presence of CAII, as measured by the membrane current and the slope conductance of the I/V relationships.

It has been shown that phosphorylation of kNBC1-Ser\(^{982}\) by cAMP-protein kinase A shifts the stoichiometry of the NBC from 3:1 to 2:1 and that this phosphorylation might lower binding of CAII (14, 15). However, expressed in Xenopus oocytes, the NBCe1 operates with a stoichiometry of 2:1 (25, 33, 34). With a measured reversal potential of NBCe1-expressing oocytes in CO\(_2\)/HCO\(_3^-\)-buffered saline of \(-55.6\) mV, the present study also supports a stoichiometry of 2:1 (with a predicted reversal potential of \(-54.4\) mV for 2:1 and \(-27.8\) mV for 3:1). Injection or co-expression of CAII did not change the reversal potential (\(-54\) and 57 mV, respectively), indicating that CAII still operates in the 2:1 mode when interacting with CAII. In contrast to the studies of Gross et al. (14) and Pushkin et al. (15), our data give no evidence about whether binding and interaction of CAII with NBCe1 depends on the transporter stoichiometry regulated by protein kinase A.

Pushkin et al. (15) identified two clusters of acidic amino acids (\(^{958}\)LDDV and \(^{986}\)DNDD) as putative CAII binding sites in wild type kNBC1. These clusters show homology to the CAII binding site of the anion exchanger AE1 \(^{886}\)LDADD (35). Therefore, we also tested a CAII mutant with changes in the NH\(_2\)-terminal (CAII-HEX), which is unable to bind to AE1,\(^3\) on the NBCe1. CAII-HEX increased the activity of NBCe1 similar to CAII-WT, suggesting that CAII-HEX can still interact with NBCe1. If binding of CAII to NBCe1 is not obligatory for augmentation of transport activity or CAII-HEX binds to NBCe1 at different binding sites than AE1 remains to be studied.

In summary, our study does not contribute to the question, if binding of CAII to NBCe1 is necessary for this interaction, as Gross et al. (14) suggested, but Lu et al. (18) and Piermarini et al. (19) refute. However, by the membrane currents attributable to NBCe1 and by the rate of cytosolic Na\(^+\) rise, our results clearly indicate that CAII enhances NBCe1 activity, in line with a transport metabolon as suggested by previous studies (10, 14, 15).

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