Effect of Human Carbonic Anhydrase II on the Activity of the Human Electrogenic Na/HCO₃ Cotransporter NBCe1-A in Xenopus Oocytes*

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Others report that carbonic anhydrase II (CA II) binds to the C termini of the anion exchanger AE1 and the electrogenic Na/HCO₃ cotransporter NBCe1-A, enhancing transport. After injecting oocytes with NBCe1-A cRNA (Day 0), we measured NBC current (I\textsubscript{NBC}) by two-electrode voltage clamp (Day 3), injected CA II protein + Tris or just Tris (Day 3), measured I\textsubscript{NBC} or the initial rate at which the intracellular pH fell (dpH/dt) upon applying 5% CO₂ (Day 4), exposed oocytes to the permeant CA inhibitor ethoxzolamide (EZA), and measured I\textsubscript{NBC} or dpH/dt (Day 4). Because dpH/dt was greater in CA II than Tris oocytes, and EZA eliminated the difference, injected CA II was functional. I\textsubscript{NBC} slope conductance was unaffected by injecting oocytes, and EZA eliminated the difference, injected CA II was functional, and EZA eliminated the difference, injected CA II was functional. Thus, CA II does not enhance NBC activity. In a second protocol, we made a fusion protein with enhanced green fluorescent protein (EGFP) at the 5’ end of NBCe1-A and CA II at the 3’ end (EGFP-e1-CAII). We measured I\textsubscript{NBC} or dpH/dt (days 3–4), exposed oocytes to EZA, and measured I\textsubscript{NBC} or dpH/dt (Day 3–4). dpH/dt was greater in oocytes expressing EGFP-e1-CA II versus EGFP-e1, and EZA eliminated the difference. Thus, fused CA II was functional. Slope conductances of EGFP-e1-CAII versus EGFP-e1 oocytes were indistinguishable, and EZA had no effect. Thus, even when fused to NBCe1-A, CA II does not enhance NBCe1-A activity.

The electrogenic Na/bicarbonate cotransporter (NBCe1 or e1)² plays a central role in HCO₃⁻ reabsorption and regulation of intracellular pH (1). The kidney-specific splice variant NBCe1-A is localized at the basolateral membrane of renal proximal tubule cells (2), where it mediates efflux of HCO₃⁻ (and/or CO₃²⁻). Cytoplasmic HCO₃⁻ arises from the intracellular hydration of CO₂, which is catalyzed by the cytoplasmic enzyme carbonic anhydrase II (CA II) (3).

Because the reports of Vince and Reithmeier (4–6) that cytosolic CA II binds to the LDADD motif on the cytoplasmic C terminus of the CI-HCO₃ exchange AE1, Sterling et al. (7, 8) have measured rates of intracellular pH (pH\textsubscript{i}) change in HEK293 cells transiently transfected with AE1 and concluded that CA II enhances AE1-mediated HCO₃⁻ transport. The C terminus of all three NBCe1 splice variants, i.e. NBCe1-A as well as the more universally expressed variant NBCe1-B (9, 10) and the brain-specific NBCe1-C (11), have two motifs similar to LDADD in AE1. Moreover, isothermal titration calorimetry and pulldown assays suggest that, at least under non-reducing conditions, the common C terminus of NBCe1-A/B interacts with CA II \textit{in vitro} (12). Similarly, Pushkin and co-workers (12–15) working with a mouse proximal convoluted tubule (mPCT) cell line stably transfected with NBCe1-A, concluded that CA II enhances the current carried by NBCe1-A.

For three reasons, we set out to verify the hypothesis that CA II enhances the activity of NBCe1-A. First, a pH\textsubscript{i} measurement, as in the AE1 study, is an indirect index of the rate of HCO₃⁻ transport (J\textsubscript{HCO₃}). After transport of HCO₃⁻ into the cell, pH\textsubscript{i} does not increase until the reactions, HCO₃⁻ + H⁺ → H₂CO₃ → CO₂ + H₂O, consume cytosolic H⁺. Obviously, CA II will augment the rate of pH\textsubscript{i} increase even if J\textsubscript{HCO₃} is constant. Second, technical issues in the mPCT studies may make it difficult to measure NBC currents reproducibly and precisely. A goal of the mPCT approach is to control cytosolic composition by using amphotericin B to permeabilize the apical membrane of high-resistance monolayers in an Ussing chamber. Although the only data analyzed were those in which the current sensitive to dinitrostilbene disulfonate was at least 10-fold larger than that of the corresponding mock transfected cells, the dinitrostilbene disulfonate-sensitive current was in fact smaller than the background current (e.g. see Fig. 3C in Ref. 14). Third, preliminary data from our laboratory shows that in oocytes co-expressing NBCe1-A and the extracellular CA IV, inhibiting CA IV markedly increases the pH gradient near the surface of the cell but has little effect on the NBCe1-A current (16). If extracellular CA IV does not enhance NBCe1 activity, then we reasoned that cytosolic CA II should not enhance it either.

In the present study, we expressed human NBCe1-A in \textit{Xenopus} oocytes and demonstrated by two-electrode voltage

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3 The abbreviations used are: NBCe1/e1, the electrogenic Na/bicarbonate cotransporter; CA, carbonic anhydrase; dpH/dt, the initial rate at which intracellular pH falls; EZA, ethoxzolamide; EGFP, enhanced green fluorescent protein; pH\textsubscript{i}, intracellular pH; HEK, human embryonic kidney; AE, anion exchanger; mPCT, mouse proximal convoluted tubule; J\textsubscript{HCO₃}, the rate of HCO₃⁻ transport; TBS, Tris-buffered saline; BSA, bovine serum albumin; D\textsubscript{HCO₃}, diffusion constant of HCO₃⁻; D\textsubscript{CO₂}, diffusion constant of CO₂.
Effect of CA II on NBCe1-A

clamp that the NBCe1-A current ($I_{NBCe1-A}$) was 10–20-fold greater than the background currents. We found that injecting a recombinant human CA II protein into oocytes markedly accelerated hydration of intracellular CO$_2$ but had no effect on the slope conductance of $I_{NBCe1}$. Moreover, exposing CA II-injected oocytes to the membrane-permeant CA II inhibitor ethoxzolamide (EZA) reversed the effect of CA II on CO$_2$ hydration without affecting the slope conductance of $I_{NBCe1}$. Finally, we created a double fusion protein consisting of enhanced green fluorescent protein (EGFP) fused to the N terminus of NBCe1-A and human CA II fused to the C terminus of NBCe1-A. This EGFP-e1-CAII construct accelerated hydration of intracellular CO$_2$, an effect blocked by EZA. However, the current of EGFP-e1-CAII was insensitive to EZA and was no different from the current of an EGFP-e1 fusion protein that lacked CA II.

**EXPERIMENTAL PROCEDURES**

**Expression of NBCe1-A and Co-injection with CA II in Xenopus Oocytes**

cDNA encoding human NBCe1-A had been cloned into the pGH19 expression vector (17) by Dr. Inyeong Choi (10). Capped mRNA was synthesized in vitro with the T7 message Machine kit (Ambion, Austin, TX). The RNA concentration was determined by ultraviolet absorbance, and its quality was assessed by gel electrophoresis.

Stage V-VI oocytes from *Xenopus laevis* were isolated as described previously (19). One day later, oocytes were injected with 50 nl of deionized water (Ambion) containing 0.5 ng/nl of mRNA encoding hNBCe1-A. Three days after injecting the cRNA, we injected oocytes with 50 nl of Tris H$_2$O.

The expression of EGFP-e1 and e1-CAII have one unique NcoI site in the EGFP-e1 and e1-CAII constructs.

- The current of EGFP-e1-CAII was insensitive to EZA and was no different from the current of an EGFP-e1 fusion protein that lacked CA II.

**Expression of EGFP-e1 and EGFP-e1-CAII Constructs in Xenopus Oocytes**

**EGFP-e1**—The e1-EGFP cDNA construct was a gift from Dr. Leila Virkki, consisting of human NBCe1-A fused in-frame at its 3’ end to EGFP (Clontech), and subcloned into pGH19 *Xenopus* expression vector (17). The EGFP was flanked by AgeI sites. We obtained the EGFP-e1 construct as follows. (i) We used QuikChange (Stratagene) to introduce a silent mutation (C$_{GATGG}$ → C$_{GATGG}$) that eliminated the NcoI site in EGFP of the e1-EGFP construct. (ii) In parallel, we used QuikChange to create in the 5’ untranslated region of NBCe1-A an AgeI site that was separated by 60 nucleotides from the ATG of the start methionine of NBCe1-A. (iii) We used AgeI to cut the silently mutated EGFP from the aforementioned vector and ligated it non-directionally into the newly created AgeI site in the 5’ untranslated region of NBCe1-A. Thus, when translated, this construct would consist of EGFP that has its NcoI site silently mutated, followed by a 20-amino acid linker (QLWQINSP-SEAEGGLGLAGK), which in turn would be followed by the start methionine of NBCe1-A.

**EGFP-e1-CAII**—We made the e1-CAII construct as follows. (i) We used PCR with the aforementioned CA II construct to synthesize CA II cDNA flanked at both ends by AgeI sites (underlined). The oligonucleotide primers were synthesized by the Keck Oligonucleotide Synthesis Facility at Yale University (upstream sense, 5’-cgaagaccgacctgctccgacctgctccgacctgc-3’; downstream antisense, 5’-cgaagaccgacctgctccgacctgctccgacctgc-3’). (ii) Meanwhile, we used AgeI to excise the EGFP sequence from e1-EGFP (see above). (iii) We used AgeI to cut the PCR product in step i, which we then ligated non-directionally into the AgeI site at the 3’ end of NBCe1-A, obtained in step ii. (iv) We performed another round of QuikChange (upstream sense, 5’-cctggaaacgccacactctgctccgacctgctccgacctgc-3’; downstream antisense, 5’-cctggaaacgccacactctgctccgacctgctccgacctgc-3’). Thus, when translated, the construct would consist of NBCe1-A, followed by an 8-amino acid linker (GLTGGGSAG), and the start methionine of CA II.

**EGFP-e1-CAII**—We obtained the EGFP-e1-CAII construct from the EGFP-e1 and e1-CAII constructs as follows. (i) Both EGFP-e1 and e1-CAII have one unique NcoI site in the NBCe1-A coding sequence as well as one unique NotI site in the pGH19 vector. Therefore, we used NcoI/NotI to liberate NotI-EGFP-e1-NcoI from one construct and NcoI-CAII-NotI from the other. (ii) We ligated these fragments to generate EGFP-e1-CAII.

**Solutions**

- **$pH$ Experiments on Injected CA II and All Voltage-clamp Experiments**—The nominally CO$_2$/HCO$_3^-$free ND96 solution contained (in mM) 96 NaCl, 2 KC1, 1 MgCl$_2$, 1.8 CaCl$_2$, and 5 HEPES at a pH of 7.50. CO$_2$/HCO$_3^-$-containing solutions were prepared by replacing 33 mM NaCl with 33 mM NaHCO$_3$ in ND96 and equilibrating the solution with 5% CO$_2$-balanced oxygen. Osmolality of all solutions was ~200 mosmol/kg of H$_2$O.

- **$pH$ Experiments on EGFP-e1-CAII and EGFP-e1**—The nominally CO$_2$/HCO$_3^-$-free solution contained (in mM) 42.4 NaCl, 2 KCl, 1 MgCl$_2$, 1.8 CaCl$_2$, 32.5 HEPES, and 33 sodium gluconate at a pH of 7.50. We generated the out of equilibrium 5% “pure” CO$_2$ solution, which contained 5% CO$_2$, nominally no HCO$_3^-$, and had a pH of 7.50, by rapidly mixing equal volumes of solutions A and B (22). Solution A (in mM) 80 NaCl, 2 MgCl$_2$, and 3.6 CaCl$_2$, bubbled with 10% CO$_2$, and with pH

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4 We injected 10$^{-11}$ mol of CA II per oocyte. Assuming an H$_2$O content of 37% (24), [CA II] = 24 μM, about 20% higher than the level in red blood cells.
adjusted to 5.4. Solution B contained (in mM) 0.7 NaCl, 4 KCl, 65 HEPES, and 66 sodium gluconate with pH adjusted to 7.65. A stock of the CA II inhibitor, EZA (Sigma), was prepared in 0.05 N NaOH to a final concentration of 50 mM. For experiments, we diluted this stock to prepare 400 μM EZA in ND96, and pH adjusted to 7.5 with 5 M HCl.

In experiments in which we blocked NBCe1-A with tenidap (5-chloro-2,3-dihydro-3-(hydroxy-2-thienylmethylene)-2-oxo-1H-indole-1-carboxamide, a gift of Pfizer Inc., Groton, CT), we made a fresh 1 M stock solution of tenidap powder in Me2SO, and then diluted this 1:1000 into a CO2/HCO3− solution. Parallel experiments (not shown) proved that 1:1000 Me2SO does not affect the NBCe1-A current.

Electrophysiological Measurements

We placed an oocyte in a chamber and constantly superfused at a flow of 4 ml/min. We delivered bath solutions using syringe pumps (Harvard Apparatus, South Natick, MA), and switched among solutions with pneumatically operated valves (Clippard Instrument Laboratory, Cincinnati, OH).

Measurement of Intracellular pH—We impaled the oocyte with two microelectrodes, one for sensing membrane potential (V_m) and the other for sensing pH_i. A calomel electrode served as the reference in the bath. We fabricated and used the electrodes as described previously (1, 23). Microelectrodes were pulled from thin-walled borosilicate glass (catalog number 30-0077, Harvard Apparatus, Holliston, MA) and had resistances ~0.3 megaohm when filled with 3 M KCl. The tip of the pH electrode contained a liquid, pH-sensitive membrane (Hydrogen Ionometer 1, mixture B, Fluka Chemical Corp., Ronkonkoma, NY). The V_m electrode was filled with 3 M KCl. We obtained the voltage due to pH_i by subtracting the signal of the V_m electrode from that of the pH electrode. We obtained V_m by subtracting the signal of the calomel electrode from that of the V_m electrode. Voltages were measured with an FD-223 electrometer (World Precision Instruments, Sarasota, FL), and data were acquired with software written in-house. The pH electrodes were calibrated with buffered pH standards at pH 6.0 and 8.0. An additional single-point calibration was performed and voltage-clamp measurements, each of which lasted 60 ms. We used the Clampfit module of pCLAMP to analyze the data. All pH and voltage-clamp data were acquired with software written in-house. The pH electrode contained a liquid, pH-sensitive membrane (Hydrogen Ionometer 1, mixture B, Fluka Chemical Corp., Ronkonkoma, NY). We held oocytes at a voltage close to the holding potential from 160 to 20 mV in 20-mV increments.

Current-voltage (I-V) relationships were generated by stepping the holding potential from −160 to +20 mV in 20-mV increments, each of which lasted 60 ms. We used the Clampfit module of pCLAMP to analyze the data. All pH, and voltage-clamp experiments were performed at room temperature (~22 °C).

Preparation of Oocytes for Immunocytochemistry

Fixing—Single oocytes were fixed at room temperature by washing twice for 10 min in 1 ml of phosphate-buffered saline (pH 7.4), once for 60 min in 1 ml of phosphate-buffered saline containing 1% paraformaldehyde, and three times for 10 min in 1 ml of phosphate-buffered saline. We prepared phosphate-buffered saline from tablets (catalog number P-4417, Sigma).

Dehydration—We dehydrated fixed oocytes by consecutive washes for 10 min in 1 ml of each of the following: 70% EtOH, 95% EtOH, 100% EtOH, and polypropylene oxide.

Permeation and Embedding—We prepared embedding medium using an Embed 812 Kit (Electron Microscopy Science, Hatfield, PA). Fixed, dehydrated oocytes were permeated by incubation for 60 min in a 1:1 mixture of embedding medium and polypropylene oxide. We subsequently transferred the oocytes to individual Easy Mold™ embedding capsules (Size “00,” Electron Microscopy Science) that were filled with 100% embedding medium. Finally, the resin was cured overnight in an oven at 60 °C.

Mounting—We sectioned oocytes to a thickness of 3 μm using a Leica Ultracut UCT (Leica-Microsystems, Milton Keynes, UK). We mounted slices on Superfrost™ Plus microscope slides (Electron Microscopy Science), layering each slice onto a water drop and heating the slide on a Lab-Line™ Slide Warmer (Barnstead Instruments, Dubuque, IA) to evaporate the H2O.

Immunocytochemistry on Oocytes

Etching—We immersed slides in a 2:1 mixture of 20% (w/v) NaOH/methanol and polypropylene oxide for 5 min, then the slides were washed in methanol (2 times for 5 min) and Tris-buffered saline (TBS; once for 5 min). TBS consisted of 100 mM Tris base and 149 mM NaCl.

Antigen Retrieval—We preheated 500 ml of citrate buffer (pH 6.0, prepared by mixing 1/9 (v/v) 0.1 M citric acid and 0.1 M sodium citrate) for 4 min at 100% power in a 700-watt microwave. Slides placed in a glass rack were immersed in this solution and boiled at 40% power in the microwave twice for 10 min, followed by cooling in the solution at room temperature.

Immunofluorescence

Prior to antibody staining, we washed slides in each of the following: TBS (three times for 5 min), 0.5 M NH4Cl (once for 15 min), TBS (once for 5 min), TBS + 1% SDS (once for 5 min), and TBS (once for 5 min). Slides were blocked by washing (once for 15 min) in TBS + 0.1% BSA + 10% normal goat serum (Vector Laboratories, Burlingame, CA). We incubated the preparations with mouse monoclonal anti-EGFP (BD Biosciences; 1:100 in TBS + 0.1% BSA + 10% goat serum) and rabbit polyclonal anti-human CA II (Rockland, Gilbertville, PA; 1:1000 in TBS + 0.1% BSA + 10% goat serum) overnight. The preparations were then washed three times for 5 min with high-salt TBS (100 mM Tris base + 427 mM NaCl) + 0.1% BSA, and then incubated once in 1 L with the secondary antibody: goat anti-mouse conjugated to Alexa Fluor® 488 (1:200 in TBS + 0.1% BSA + 10% goat serum) and goat anti-rabbit conjugated to Alexa Fluor® 594 (1:200 in TBS + 0.1% BSA + 10% goat serum). Finally, the slides were washed twice for 5 min in high-salt TBS + 0.1% BSA and once for 5 min in TBS before mounting in Vectashield (Vector Laboratories).

Confocal Microscopy on Oocytes

We qualitatively localized EGFP-e1-CA II and EGFP-e1 by obtaining confocal images of fixed oocyte slices using a Zeiss
**Effect of CA II on NBCe1-A**

LSM 510 confocal laser-scanning microscope (Carl Zeiss AG, Gottingen, Germany) equipped with Argon 2 and HeNe1 laser units. For the images labeled with Alexa Fluor 488, excitation/emission filters were set at band pass 488 nm/long pass 505 nm. For the images labeled with Alexa Fluor 594, excitation/emission filters were set at band pass 543 nm/long pass 560 nm. Images are the result of 8-fold scan averaging, and assembled using LSM Image Browser (version 3.5).

**Statistics**

We obtained the initial rate of pH change (dpH/dt) from the slope of a linear regression line fitted to a portion of the pH versus time record that represented the maximal rate of pH decline soon after the addition of CO2/HCO3−. We obtained the slope conductance of the NBC current by subtracting, at each voltage, the current in ND96 from the total current in CO2/HCO3−. Data are reported as mean ± S.E. Means were compared using paired Student’s t tests (two tails), considering p < 0.05 as significant.

**RESULTS**

**Effect of Injected CA II on CO2-induced pH Changes**—In Fig. 1, we outline our experimental design for studies in which we examined the effect of injected, recombinant, human CA II protein or the effect of CA II fused to the C terminus of NBCe1-A.

The following narrative applies to the pH experiments done for studies in which we examined the effect of injected CA II protein. After injecting the cRNA encoding NBCe1-A into *Xenopus* oocytes (Day 0/Step 1), we waited 3 days and randomized the oocytes into the “CA II + Tris” group and the “Tris” group. Later on the same day, we injected either CA II protein in Tris buffer or just Tris buffer (Day 3/Step 3). We then used pH measurements (Day 4/Step 4) to verify that the CA II could catalyze the reaction CO2 + H2O → HCO3− + H+. Exposing oocytes to 5% CO2/33 mM HCO3− (pH 7.50) caused the pH to decrease much faster in a CA II + Tris oocyte (Fig. 2A, Pre-EZA) than in a Tris oocyte (Fig. 2B, Pre-EZA). The solid bars in Fig. 2C summarize the initial, CO2-induced dpH/dt values for a total of 8–10 oocytes in each group, and confirm that CA II-injected oocytes acidify far more rapidly than the controls. After obtaining these pre-EZA data, we exposed the same oocytes from each group for ~3 h to 400 μM EZA, a permeant CA II inhibitor (Day 4/Step 5) and then repeated the pH measurements (Day 4/Step 6). For the CA II + Tris cell, EZA reduced the initial CO2-induced dpH/dt (Fig. 2A, Post-3h EZA) to about the value observed in the Tris oocyte, pre-EZA (Fig. 2B, Pre-EZA). However, EZA had no effect on the Tris oocyte (Fig. 2B, Post-3h EZA). The hatched bars in Fig. 2C summarize the initial, CO2-induced dpH/dt values for 8–10 oocytes in each group, and shows that EZA treatment blocked the CA II effect by 96%. Thus, the injected CA II markedly enhances the conversion of CO2 + OH− to HCO3−.

**Effect of Injected CA II on NBCe1-A Currents**—Fig. 3 summarizes data obtained on an oocyte expressing NBCe1-A, but before injection of either CA II + Tris or Tris. In our experiments using a two-electrode voltage-clamp, we define the current through NBCe1-A (I NBC) as the difference in currents measured in the presence of CO2/HCO3− (Fig. 3A, open diamond curve) versus the absence of CO2/HCO3− (i.e. ND96 solution, Fig. 3A, black square curve). In separate experiments, we found that in the presence of CO2/HCO3−, the current measured in the presence of near-saturating levels (1 mM) of tenidap5 (Fig. 3A, open triangle curve), an inhibitor of NBCe1-A (25), is virtually the same as the current measured in the absence of CO2/HCO3− (Fig. 3A, black square curve). However, because tenidap is not totally reversible, we did not routinely use this inhibitor in this study. The I NBC-V relationship in Fig. 3B (open diamond curve) is the difference between the

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5 Under the conditions of these experiments, the apparent K of tenidap is ~20 μM (J. Lu and W. F. Boron, unpublished observations).
open diamond and black square curves in Fig. 3A. The dashed line in Fig. 3B represents a linear fit to the $I_{\text{NBC}}-V$ relationship, and has a slope of 14 $\mu$S. A comparison of the two plots shows that the $I_{\text{NBC}}-V$ relationship is slightly concave upward. However, the deviation from linearity is very slight, so that we regarded the slope of the dashed lines as a reasonable indicator of the slope conductance of NBCe1-A throughout our study.

Fig. 4 summarizes the effect of injected CA II protein on NBCe1-A activity. As outlined in Fig. 1, we serially measured NBCe1-A currents as many as three times for each oocyte: (i) Day 3/Step 2, 3 days after injecting the NBCe1-A cRNA, but immediately before injecting CA II or Tris; (ii) Day 4/Step 4, 1 day after i; and (iii) Day 4/Step 6, on the same day.

FIGURE 2. Effect of EZA on initial, CO$_2$-induced acidification rate ($dpH/dt$) in oocytes expressing hNBCe1-A and injected with CA II + Tris or Tris. A, representative pH$_i$ records from an oocyte injected 3 days earlier with cRNA encoding NBCe1-A and 1 day earlier with CA II + Tris. The solid record (Pre-EZA) and the dotted record (Post-3 h EZA) were obtained from the same oocyte, separated by a 3-h exposure of the oocyte to the ND96 solution supplemented with 400 $\mu$M EZA. The extracellular solution was ND96 at the beginning of the experiment and, during the indicated time, 5% CO$_2$/33 mM HCO$_3$ (pH 7.50). B, representative pH$_i$ records from an oocyte injected 3 days earlier with cRNA encoding NBCe1-A and 1 day earlier with Tris buffer. C, summary of data from experiments like those in panels A and B. Values are mean ± S.E., with numbers of oocytes as indicated. The statistical comparisons between adjacent bars were made using a paired, two-tailed $t$ test (**, $p < 0.001$). The left arrow represents the average effect of EZA on CA II + Tris oocytes. The right arrow represents the difference between the solid bar for the CA II + Tris oocytes and the average of the solid and hatched bars for the Tris oocytes. The ratio of the two arrows is an inhibition of 96%.
random differences in NBCe1-A expression in oocytes selected from the same population, was not statistically significant. The dark gray bars show that, 24 h after injection, the NBCe1-A slope conductance was 12% lower than before injection for both CA II + Tris and Tris oocytes, presumably reflecting a small decrease in NBCe1-A expression in both subpopulations. The difference between the two dark gray bars was not statistically significant, presumably reflecting the same random differences responsible for the difference between the two subpopulations. Thus, the presence of injected CA II protein does not significantly affect NBCe1-A activity.

The solid bars in Fig. 5 summarize the NBCe1-A slope conductance 24 h after injection of CA II + Tris into 27 oocytes or Tris into 20 oocytes (Day 4/Step 4). The 27 oocytes in Fig. 5, left, are a subset of the 41 in Fig. 4, left, the ones that survived the subsequent exposure to EZA. Similarly, the 20 oocytes in Fig. 5, right, are a subset of the 27 in Fig. 4, right. The 3-h treatment with EZA caused a minute but statistically significant increase in the NBCe1-A slope conductance (Day 4/Step 6), which amounted to 4% for both CA II + Tris and Tris oocytes. This increase may represent a small, nonspecific effect of EZA on NBCe1-A activity in both subpopulations. Thus, the addition of the CA inhibitor did not have a differential effect on the NBCe1-A slope conductance in oocytes that contained versus those that did not contain CA II protein.

Effect of Fused CA II on CO2-induced pH Changes—The above data show that injected CA II does not increase the activity of NBCe1-A. However, it is possible that the amount of CA II protein in the oocyte was insufficient to bind to all NBCe1-A molecules at the plasma membrane. Alternatively, it is possible that CA II does not bind to NBCe1-A at all. To test these possibilities, our next step was to fuse NBCe1-A at its 5′ end to EGFP and at its 3′ end to human CA II to create EGFP-e1-CAII. In addition, as a control, we generated the construct EGFP-e1, which lacks a fused CA II. To verify delivery of the fusion proteins to oocytes as ii but 3 h after exposing the oocyte to EZA. The two light gray bars in Fig. 4 summarize the average NBCe1-A slope conductances of oocytes just before injection of CA II + Tris into 41 oocytes or Tris into 27 oocytes. As expected, the difference in these mean values, which presumably reflect

![Graph](image-url)
the plasma membrane, we performed immunocytochemistry on sections from oocytes expressing either EGFP-e1-CAII or EGFP-e1. The leftmost column of Fig. 6 shows anti-EGFP laser-scanning fluorescence micrographs from oocytes expressing EGFP-e1-CAII (Fig. 6A), expressing EGFP-e1 (Fig. 6B), or injected with water (Fig. 6C). The second column shows similar data, but with a CA II antibody (Fig. 6, D–F). The third column shows the merges (Fig. 6, G–I). Finally, the rightmost column shows Nomarski (light) micrographs of the same sections (Fig. 6, J–L). These data confirm that both EGFP-e1-CAII (Fig. 6A) and EGFP-e1 (Fig. 6B) traffic to the plasma membrane. Moreover, in oocytes expressing EGFP-e1-CAII, CA II colocalizes with EGFP (Fig. 6C). In H2O-injected oocytes, the signals for both EGFP (Fig. 6C) and CA II (Fig. 6F) were negligible.

We used an experimental design similar to that outlined above for the CA II protein experiments. As outlined in Fig. 1, but omitting the steps on the gray background, we randomized the oocytes into two groups, one group injected with cRNA encoding EGFP-e1-CAII, the other group injected with cRNA encoding EGFP-e1 (Day 0/Step 1). Using an approach similar to that in Fig. 2, we used pH measurements to verify that the CA II in the EGFP-e1-CAII fusion protein could catalyze the reaction CO2 + H2O -> HCO3^- + H^+ (pH 7.50), the initial, CO2-induced acidification was only modestly faster for EGFP-e1-CAII (~40 x 10^-4 pH units/s, data not shown) than for EGFP-e1 (~30 x 10^-4 pH units/s, data not shown). Of course, dpH/dt depends on the balance between the acidifying effect of CO2 influx across the entire plasma membrane versus the alkalinizing effect of HCO3^- (or CO3^-) influx via the NBCe1-A constructs.

We reasoned that, when physically coupled to the NBCe1-A, CA II preferentially magnifies the alkalinizing effect of NBCe1-A, and dwarfs the acidifying effect of CO2 influx, because the uncatalyzed reaction CO2 + H2O -> HCO3^- + H^+ can occur at sites distant from EGFP-e1-CAII. To minimize the alkalinizing effect of NBCe1-A, we assayed the CA II activity of EGFP-e1-CAII by exposing oocytes to a pH 7.50 out of equilibrium solution (22) containing 5% CO2, but virtually no HCO3^- (pure CO2). Three to 4 days after injecting the cRNA encoding either EGFP-e1-CAII or EGFP-e1, 5% pure CO2 caused pHi to decrease faster in a cell expressing EGFP-e1-CAII (Fig. 7A, Pre-EZA) than in a control cell expressing EGFP-e1 (Fig. 7B, Pre-EZA). The solid bars in Fig. 7C summarize the initial, 5% pure CO2-induced dpH/dt values in each group.

After obtaining these pre-EZA data, we exposed both groups of oocytes to 400 μM EZA for ~3 h (Day 3–4/Step 5). For an oocyte expressing EGFP-e1-CAII (Day 3–4/Step 6), EZA reduced the initial 5% pure CO2-induced dpH/dt (Fig. 7A, Post-3h EZA) nearly to the value observed in an oocyte expressing EGFP-e1 before the EZA treatment (Fig. 7B, Pre-EZA). EZA had no effect on EGFP-e1 oocytes (Fig. 7B, Post-3h EZA). The hatched bars in Fig. 7C summarize the initial, 5% pure CO2-induced dpH/dt values in each group. Thus, the fused CA II markedly enhances the conversion of CO2 + OH^- to HCO3^-, and we can completely block this CA II activity.

Effect of Fused CA II on NBCe1-A Currents—Before incubation in EZA (Day 3–4/Step 4), cells expressing EGFP-e1-CAII and cells expressing EGFP-e1 had identical HCO3^- induced NBCe1-A currents and slope conductances (Fig. 8, solid bars). Moreover, the 3-h treatment with EZA (Day 3–4/Step 5) had no effect on the slope conductance (Day 3–4/Step 6) of either...
Effect of CA II on NBCe1-A

FIGURE 8. Effect of EZA on NBCe1-A currents in oocytes expressing EGFP-e1-CAII versus oocytes expressing EGFP-e1. The solid bars on the left in each pair summarize the NBCe1-A slope conductances in oocytes expressing EGFP-e1-CAII versus oocytes expressing EGFP-e1, with the number of experiments as indicated. The hatched bars on the right in each pair summarize the NBCe1-A slope conductances after a 3-h treatment with EZA in each group. The statistical comparisons between adjacent bars were made using a paired, two-tailed t test, and between two solid or two hatched bars were made using an unpaired, two-tailed t test. n.s. means not statistically significant (p > 0.05).

oocytes expressing EGFP-e1-CAII or oocytes expressing EGFP-e1 (Fig. 8, hatched bars). Thus, even when physically coupled to the NBCe1-A, CA II does not significantly affect the current carried by the cotransporter.

DISCUSSION

General Comments

In the present study, we examined the effect of CA II on the activity of NBCe1-A. Our experimental approach, expressing an electrogenic transporter in oocytes, offers several advantages not available with electroneutral transporters or cultured mammalian cells. First, our measure of NBCe1 activity was direct (i.e. the current carried by the transporter), whereas the measurement of a rate of pH change is one step removed from the transporter. Second, our measured parameter (i.e. the NBCe1-A current) is not intrinsically sensitive to CA II, whereas rates of pH change are extremely sensitive to CA II in a CO₂/HCO₃⁻ environment. Third, the NBCe1 current was extremely large, represented an overwhelming fraction of total current, and could be measured with precision (Fig. 3), which is not the case for rates of pH change. Finally, the approach allows one to control the extracellular composition easily and precisely. Thus, the combination of oocytes, the heterologous expression of NBCe1, and the two-electrode voltage-clamp provides an easy and reliable system for evaluating whether CA II enhances the flux through the transporter.

We approached the problem from two directions. First, we injected large amounts of recombinant, human CA II protein into oocytes expressing NBCe1-A. The reason for microinjecting the protein, rather than co-injecting it along with NBCe1-A, was to avoid the possibility that the translation of CA II mRNA might reduce the translation of NBCe1-A mRNA. After demonstrating that the injected CA II was functional via pH assays (Fig. 2), we compared NBCe1-A slope conductances in the same oocyte before and after injecting CA II + Tris. The results indicated that, compared with controls injected only with Tris, CA II protein does not significantly affect the NBCe1-A activity (Fig. 4). After demonstrating that the membrane-permeant CA II inhibitor EZA (400 µM) blocks the enzymatic activity of CA II (Fig. 2), we analyzed NBCe1-A currents in the same oocyte before and after a 3-h exposure to EZA. Comparing oocytes injected with CA II + Tris versus Tris, we found that EZA has no effect on I₉⁺₁ (Fig. 5).

Our second approach was to fuse NBCe1-A at its 5’ end to EGFP and at its 3’ end to CA II to create EGFP-e1-CAII. The reason for generating this construct was to circumvent the possibilities that: (i) the amount of injected CA II protein was insufficient to saturate the NBCe1-A, (ii) the oocyte degraded the CA II protein before our assay, or (iii) the CA II protein was incapable of binding to NBCe1-A, at least under the conditions of our experiments. As a precautionary measure, we confirmed the delivery of the EGFP-e1-CAII fusion protein to the oocyte plasma membrane by immunocytochemistry (Fig. 6). Moreover, we demonstrated the colocalization of EGFP and CA II, proving that the fusion protein was intact. After demonstrating that, as expressed in oocytes, the CA II moiety of the EGFP-e1-CAII fusion protein was functional and blocked by EZA via pH assays (Fig. 7), we compared NBCe1-A currents in the same oocyte before and after exposing it to EZA. We found that oocytes expressing EGFP-e1-CAII and EGFP-e1 had indistinguishable NBCe1-A slope conductances and, furthermore, that EZA had no effect on slope conductances in either group of oocytes (Fig. 8). Thus, even when we forced CA II to be immediately adjacent to the C terminus of NBCe1-A, CA II still does not enhance the NBCe1-A activity.

NBCe1-A Stoichiometry

A major difference between the oocyte expression system and the renal proximal tubule is that NBCe1-A in the intact tubule cell is believed to operate with an apparent Na⁺:HCO₃⁻ stoichiometry of 1:3 (26), whereas the cotransporter in oocytes operates with an apparent stoichiometry of 1:2 (27). One might argue that the interaction between NBCe1-A and CA II requires that the cotransporter be operating with a 1:3 stoichiometry. The evidence on this point is not consistent. Thus, on the one hand, with NBCe1-A expressed in mPCT cells, the phosphorylation of Ser-982 (in the second of two putative CA-II-binding motifs in the cytoplasmic C terminus) shifts the stoichiometry from 1:3 to 1:2 (13) and lowers CA II binding (12). On the other hand, in the same experimental preparation, phosphorylation of Ser-982 (which presumably generates the 1:2 stoichiometry) does not decrease but rather increases the binding of NBCe1-A to CA II (15). Thus, it is not clear that the phosphorylation state or the stoichiometry should have any impact on the binding of NBCe1-A to CA II. Finally, it is worth noting that the NBCe1-B splice variant (which shares the same Ct as the renal NBCe1-A) operates with a 1:2 stoichiometry in pancreatic ducts, and the NBCe1-C splice variant operates with a 1:2 stoichiometry in the brain.
Potential Mechanisms by Which CA II Might Enhance NBCE1-A Activity

The HCO$_3^-$ metabolon hypothesis (4–6), as extended by later authors (7, 8), predicts that the binding of CA II to a HCO$_3^-$ transporter should increase the transport rate. Our data indicate that, at least under the conditions of our experiments, CA II does not enhance the activity of NBCE1. How in principle might CA II ever enhance NBCE1 activity?

Binding of CA II—One possibility is that CA II binding could alter the conformation of NBCE1 and thereby increase cotransport. According to this view, it might not make any difference whether the CA II were enzymatically active. Although such an interaction does not appear to occur in oocytes heterologously expressing NBCE1-A, for three reasons, we cannot rule out such an interaction in cells that naturally express NBCE1-A. (i) Perhaps the binding or critical conformational changes require a temperature of 37 °C. (ii) The appropriate interaction between human NBCE1-A and human CA II may require that two proteins be post-translationally modified in a mammalian cell at 37 °C. On the other hand, at least some of the binding data of Vince et al. (4–6) were obtained with recombinant peptides and CA II, and all of the binding data were obtained at room temperature. Therefore, we know of no a priori reason that our expression system or the temperature should have prevented the predicted acceleration of HCO$_3^-$ transport by CA II. (iii) An intermediary protein(s) not present in oocytes may be required for the interaction between CA II and NBCE1-A.

Enzymatic Activity of CA II—The alternative mechanism for how CA II might enhance transport, and the only one explicitly considered by recent proponents of the HCO$_3^-$ metabolon hypothesis, is that the enzymatic activity of CA II per se is key. However, for this model, we can rule out all three potential complications discussed above for CA II binding. (i) Temperature should not be a critical factor in testing the model, inasmuch as we demonstrated that CA II is active in oocytes. (ii) Post-translational modifications should not be critical inasmuch as both the enzyme and cotransporter are active in oocytes. Moreover, the EGFP-e1-CAII fusion protein ensured that the two were adjacent to one another. Finally, (iii) intermediary proteins are irrelevant, once again because of our experiments with the EGFP-e1-CAII fusion protein.

Molecular Models

If CA II were to enhance NBCE1-A activity by an enzymatic mechanism (see above), the role of CA II would depend critically on whether NBCE1, operating with a stoichiometry of 1:2, were transporting one Na$^+$ plus two HCO$_3^-$ ions as in Fig. 9A or one Na$^+$ and one CO$_3^{2-}$ ion as in Fig. 9B.

In Fig. 9A, with every cycle of NBCE1, one Na$^+$ and two HCO$_3^-$ ions from the bulk extracellular fluid would diffuse to the outer surface of the cell membrane, where NBCE1 would transfer them to the inner surface of the cell membrane, raising the local [Na$^+$] and [HCO$_3^-$] by small amounts. This rise in local concentrations would drive the diffusion of Na$^+$ and HCO$_3^-$ to the bulk intracellular fluid, where the HCO$_3^-$ would neutralize bulk H$^+$ and thereby raise bulk pH$_i$. If CA II were present in the immediate vicinity of NBCE1, it would convert some of the incoming HCO$_3^-$ (as well as local H$^+$) to CO$_2$ and H$_2$O. The CO$_2$ could then diffuse either into the bulk cytoplasm or across the membrane and out of the cell. It is reasonable to assume that the binding of HCO$_3^-$ to the inner face of NBCE1 would inhibit net influx via the cotransporter. The key question is whether the action of local CA II to consume local HCO$_3^-$ would lower local [HCO$_3^-$] enough to stimulate NBCE1.

We will consider two extreme cases, one in which all of the incoming HCO$_3^-$ remains HCO$_3^-$ and one in which all of incoming HCO$_3^-$ is converted locally to CO$_2$. In the first case, local [HCO$_3^-$] depends only on how fast the HCO$_3^-$ can diffuse into the intracellular bulk solution, governed by a diffusion constant ($D_{HCO_3^-}$) of ~0.8 × 10$^{-7}$ cm$^2$/s (28). In the second case, local [HCO$_3^-$] depends on how fast CA II can convert the incoming HCO$_3^-$ to CO$_2$, and how fast this CO$_2$ can diffuse into the bulk intracellular fluid, governed by a $D_{CO_2}$ of ~1.1 × 10$^{-5}$ cm$^2$/s (Table H-7 of Ref. 29), or out of the cell across the cell membrane. Because (i) $D_{CO_2}$ is not much greater than $D_{HCO_3^-}$, (ii) CA II might encounter some delay as incoming HCO$_3^-$ diffuses to the enzyme, and (iii) the cell membrane may offer some resistance to CO$_2$ exit, local CA II activity would likely have a minimal effect on local [HCO$_3^-$].

The influence of local [HCO$_3^-$] on the NBCE1-mediated HCO$_3^-$ influx is unknown. However, it is reasonable to presume that the stimulatory effect of lowering local [HCO$_3^-$] would be greatest at an initial concentration of near zero, and would fall off at higher concentrations. Thus, if NBCE1 transports HCO$_3^-$, we do not think it is reasonable, given the above assumptions, to expect CA II to enhance NBCE1 activity substantially under physiological conditions. Finally, we note that, by consuming
local H\(^+\), the CA II would tend to exaggerate the local alkalinity, which could have a deleterious effect on NBCe1 or other membrane proteins.

In Fig. 9B, with every cycle of NBCe1, one Na\(^+\) and two HCO\(_3^-\) ions from the bulk extracellular fluid would diffuse to the outer surface of the cell membrane, where they would undergo a disproportionation reaction in which one HCO\(_3^-\) would dissociate to form CO\(_3^{2-}\) and H\(^+\) and the other would consume the H\(^+\) to form CO\(_2\) and H\(_2\)O. The NBCe1 would transfer the Na\(^+\) and CO\(_3^{2-}\) to the inner surface of the cell membrane, raising the local [Na\(^+\)] and [CO\(_3^{2-}\)] by small amounts. In parallel, CO\(_2\) and H\(_2\)O would also diffuse into the cell. The rise in local [Na\(^+\)] and [CO\(_3^{2-}\)] would favor the diffusion of Na\(^+\) and CO\(_3^{2-}\) to the bulk intracellular fluid. However, local H\(^+\), derived in part from protonated buffers (HBuf\(^{\alpha^+}\)), would rapidly convert the incoming CO\(_3^{2-}\) to HCO\(_3^-\), which would then diffuse to the bulk intracellular fluid, where it would consume H\(^+\) and thereby raise bulk pH. In parallel, the incoming CO\(_2\) and H\(_2\)O would slowly form H\(^+\) (which would help titrate incoming CO\(_3^{2-}\) to HCO\(_3^-\)) and HCO\(_3^-\), which would diffuse into the bulk intracellular fluid.

If CA II were present in the immediate vicinity of NBCe1, it would enhance the conversion of CO\(_2\) to HCO\(_3^-\) and thereby replenish the H\(^+\) that had been consumed by CO\(_3^{2-}\), thus minimizing local alkalinity. In this scenario, the main role of CA II would be to stabilize local pH. CA II would only slightly lower local [CO\(_3^{2-}\)] and only slightly enhance NBCe1 activity. Indeed, preliminary data on the effects of (extracellular) CA IV activity on extracellular surface pH and NBC currents suggest that NBCe1 actually carries CO\(_3^{2-}\) rather than HCO\(_3^-\) (16), and thus favors the model in Fig. 9B. Specifically, these data show that inhibiting CA IV, as predicted by the preceding discussion, has only a very small effect on NBCe1 currents, but markedly increases the pH difference between the extracellular surface and extracellular bulk fluid.

The above analyses indicate that the enzymatic activity of CA in the vicinity of a transporter is unlikely to enhance substantially either HCO\(_3^-\) or CO\(_3^{2-}\) transport when CO\(_2\) is nearly in equilibrium across the cell membrane. However, our analysis does not detract from the very important roles that CA II and other carbonic anhydrases play in certain other processes. For example, CA II plays a central role in accelerating the fall in pH caused by the influx of CO\(_2\) (Figs. 2 and 7), and presumably in enhancing the actual influx of CO\(_2\). Thus, in the red blood cell, CA is critically important for promoting CO\(_2\) fluxes across the cell membrane. In the renal proximal tubule, tubule cells secrete H\(^+\), that, under the influence of apical CA IV, titrates filtered HCO\(_3^-\) in the lumen to CO\(_2\) and H\(_2\)O. Thus, blockade of CA IV would greatly slow this process and thus inhibit renal HCO\(_3^-\) reabsorption. Once formed in the tubule lumen, CO\(_2\) and H\(_2\)O enter the proximal-tubule cell and, under the influence of CA II (not bound to NBCe1), regenerate H\(^+\) and HCO\(_3^-\) in the bulk intracellular fluid. Thus, blockade of CA II would inhibit renal HCO\(_3^-\) reabsorption.

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