Carbonic anhydrase II binds to and increases the activity of the epithelial sodium-proton exchanger, NHE3

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KRISHNAN D, Liu L, Wiebe SA, Casey JR, Cordat E, Alexander RT. Carbonic anhydrase II binds to and increases the activity of the epithelial sodium/proton exchanger, NHE3. Am J Physiol Renal Physiol 309: F383–F392, 2015. First published June 3, 2015; doi:10.1152/ajprenal.00464.2014.—Two-thirds of sodium filtered by the renal glomerulus is reabsorbed from the proximal tubule via a sodium/proton exchanger isoform 3 (NHE3)-dependent mechanism. Since sodium and bicarbonate reabsorption are coupled, we postulated that the molecules involved in their reabsorption [NHE3 and carbonic anhydrase II (CAII)] might physically and functionally interact. Consistent with this, CAII and NHE3 were closely associated in a renal proximal tubular cell culture model as revealed by a proximity ligation assay. Direct physical interaction was confirmed in solid-phase binding assays with immobilized CAII and C-terminal NHE3 glutathione-S-transferase fusion constructs. To assess the effect of CAII on NHE3 function, we expressed NHE3 in a proximal tubule cell line and measured NHE3 activity as the rate of intracellular pH recovery, following an acid load. NHE3-expressing cells had a significantly greater rate of intracellular pH recovery than controls. Inhibition of endogenous CAII activity with acetazolamide significantly decreased NHE3 activity, indicating that CAII activates NHE3. To ascertain whether CAII binding per se activates NHE3, we expressed NHE3 with wild-type CAII, a catalytically inactive CAII mutant (CAII-V143Y), or a mutant unable to bind other transporters (CAII-HEX). NHE3 activity increased upon wild-type CAII coexpression, but not in the presence of the CAII V143Y or HEX mutant. Together these studies support an association between CAII and NHE3 that alters the transporter’s activity.

NHE3; carbonic anhydrase II; sodium; pH

INTRAVASCULAR VOLUME IS MAINTAINED via highly regulated control of sodium homeostasis, best observed along the course of the nephron, where the majority (at least 65%) of Na+ is reabsorbed from the proximal tubule. Transepithelial reabsorption of sodium from this nephron segment is determined by a series of transport events and enzyme-mediated catalysis. Apical Na+ influx occurs through the sodium/proton exchanger isoform 3 (NHE3) in exchange for a cytosolic H+. Cytosolic Na+ is excreted back into the blood via either the Na+-K+-ATPase or the sodium-dependent bicarbonate transporter (NBCe1). The rate-limiting step is apical Na+ influx via NHE3, whose activity depends on the presence of cytosolic protons. H+ is generated by the cytosolic carbonic anhydrase isoform II (CAII), an enzyme mediating the catalysis of CO2 and H2O into HCO3− and a H+. H2O and CO2 enter the cell from the tubular lumen, at least in part through the water channel aquaporin-1 (11, 23, 27). This process also drives HCO3− reabsorption, as glycosylphosphatidylinositol (GPI)-linked extracellular carbonic anhydrase IV mediates catalysis of effluxed H+ to convert HCO3− into H2O and CO2 (40).

NHE3 is one of nine isoforms of the Na+/H+ exchanger family (14). In mammals, NHE1-5 proteins are present in the plasma membrane, and NHE6-9 localize predominantly in endomembrane compartments (13, 14). NHE3 is located in the apical membrane of intestinal and renal epithelia (19). In the kidney, NHE3 is predominantly expressed in the proximal tubule and to a lesser extent in the thick ascending limb of the loop of Henle (9). NHE3 participates directly in Na+ reabsorption and indirectly in the reabsorption of bicarbonate, Ca2+, and the secretion of ammonium (10, 20, 25).

CAII both physically and functionally interacts with a number of transporters, including anion exchanger 1 (AE1), NBCe1, and MCT (6, 7, 24, 38, 39). The best characterized interaction is between AE1 and CAII. An LDADD motif in the cytosolic C terminus of AE1 binds to the histidine-rich N terminus of CAII (37, 39). This interaction augments AE1 transport, potentially via enzymatic provision of substrate to the transporter (38). Such an interaction has been dubbed a transport metabolon (33). NHE1 directly interacts with the cytosolic carbonic anhydrase CAII, through the NHE1 790RIQRCLSDPGHP802 motif in the carboxyl cytosolic terminus (16, 17). This interaction increases NHE1 activity (16). However, the existence of a direct AE1/CAII physical interaction has been questioned (1, 22).

Examination of the cytosolic C terminus of NHE3 revealed a potential CAII binding site (710IKEKDLELSD-TEE722), consistent with the motif established for NHE1 (16, 17). Given this, and the known dependence of CAII activity for what is currently appreciated as NHE3-mediated transepithelial Na+ transport (12, 26), we hypothesized that CAII and NHE3 physically and functionally interact. Consistent with this, a proximity ligation assay (PLA) revealed the close association of the two proteins; we could coimmunoprecipitate CAII with NHE3, and a microtiter plate assay confirmed that the glutathione-S-transferase (GST)-tagged NHE3 C-terminal domain, containing the putative CAII binding site, binds CAII. Moreover, in the presence of CO2 and bicarbonate, NHE3 activity is inhibited by acetazolamide, but is not when NHE3 activity is measured in the nominal absence of CO2 and HCO3−.
MATERIALS AND METHODS

Materials. Restriction enzymes EcoRI and Ncol were from New England Biolabs (Whitby, ON). BL21 Escherichia coli and pcDNA 3.1+ were from Invitrogen (Carlsbad, CA). pGEX 6P-1 and glutathione-Sepharose 4B were from GE Healthcare (Mississauga, ON). Recombinant human CAII, nigericin, acetazolamide, and EIPA were from Sigma-Aldrich Canada (Oakville, ON). Antibodies/stains used include rabbit anti-CAII polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA), mouse anti-hermiglutamin (HA; 16B12, Covance, Emeryville, CA), and phallloidin (Invitrogen). DMEM-F12 medium, geneticin, penicillin/streptomycin/glutamine (PSG), and neon transfection reagents were from Gibco Life Technologies (Burlington, ON). Fetal bovine serum was from VWR International (Mississauga, ON). Opossum kidney cells were from the American Type Culture Collection (ATCC, Manassas, VA). Fugene was from Promega (Madison, WI). Isopropyl β-D-thiogalactopyranoside (IPTG) was from Fermentas Canada, (Burlington, ON).

Cell culture. Opossum kidney (OK) cells were obtained from TAGC Applied Genomics core, University of Alberta. Opossum kidney cells were from the American Type Culture Collection (ATCC; Manassas, VA). Fugene was from Promega (Madison, WI). Isopropyl β-D-thiogalactopyranoside (IPTG) was from Fermentas Canada, (Burlington, ON).

Measurement of Na+/H+ exchange activity in the presence of CO2. NHE3 activity was measured in the absence of CO2 as previously (2). The rate of Na+/H+ exchange activity in the presence of CO2 was measured in the absence of CO2 as previously (2). After loading with BCECF-AM as above, the cells were rinsed with ison Na+ buffer (140 mM NaCl, 1 mM MgCl2, 1 mM CaCl2, 3 mM KCl, 10 mM glucose, and 5 mM HEPES, pH 7.4) to remove excess probe and then placed inside a fluorescence cuvette where the coverslip was held immobile with a holder device. Intracellular pH (pHi) was determined with a PTI-Fluorometer [LPS-220B, Photon Technology International (PTI), London, ON]. The NHE activity of OK cells overexpressing NHE3 or pcDNA3.1+ was measured as the rate of pH recovery after acidifying cells by switching them from iso Na+ -buffered medium to a HCO3- -buffered medium (130 mM NaCl, 1 mM MgCl2, 1 mM CaCl2, 3 mM KCl, 10 mM glucose, and 25 mM NaHCO3, pH 7.4), bubbled with 5% CO2-95% air; the perfusion rate was kept constant per experimental condition but varied between sets of experiments from 1.5–8 ml/min to ensure equivalent degree of acidification within experimental groups. Fluorescence measurements were made with dual excitation (440, 490 nm), and a single emission was measured (510 nm). Calibration was performed with high K+/containing buffer at 6.0, 6.5, 7.0, and 7.5 (140 mM KCl, 1 mM MgCl2, 1 mM CaCl2, 10 mM glucose, and 20 mM HEPES) containing 10 mM migericin for each individual coverslip (34). NHE3 activity was then calculated as the rate of change in pH over the first 30 s from maximal acidification.

Measurement of Na+/H+ exchange activity in the absence of CO2. NHE3 activity was assessed in the absence of CO2 as previously (2). After loading with BCECF-AM as above, the cells were rinsed with iso Na+ buffer (140 mM NaCl, 1 mM MgCl2, 1 mM CaCl2, 3 mM KCl, 10 mM glucose, and 5 mM HEPES, pH 7.4) to remove excess probe and then placed inside a fluorescence cuvette where the coverslip was held immobile with a holder device. pHi was determined with a PTI-Fluorometer (LPS-220B, PTI). The cells were perfused with iso Na+ buffer, containing 10 mM NH4Cl for 10 min before a switch to acidify the cells by perfusing with iso K+ buffer (140 mM KCl, 1 mM MgCl2, 1 mM CaCl2, 10 mM glucose, and 20 mM HEPES, pH 7.4) for 2 min. pH recovery in cells transfected with NHE3 or pcDNA3.1+ was then induced by perfusing cells with iso Na+ buffer. Calibration was performed with high K+/containing buffer with 10 mM nigericin for each individual coverslip as above (34). NHE3 activity was calculated as the change in pH over the first 30 s after iso Na+ readdition.

Generation of glutathione-S-transferase constructs and CAAI-myc. DNA encoding amino acids 630–730 or 730–830 of rat NHE3 was amplified by PCR. The former region contains a homologous region to the NHE1, CAII binding site (710IKEKDLELSDTEE722) (17). Rat full-length NHE3 was used as the template (2). Primers for the 630–730 region were forward: 5'-CCG CAC CAG CTG CTA GTT CGG GAC GGT GAG GCT ATG CCT CCA ATG TTC TTG T-3' and reverse: 5'-CG GGC GGC CGC TCA ATG CCT CCA ATG TCT TCT-3'. Primers for the 730–830 region were forward: 5'-CG GGC GGC GCC CTC CAT GTG TGT GGA CTC AGG G-3' and reverse: 5'-CG GGC GGC GCC CTC CAT GTG TGT GGA CTC AGG G-3'. Forward primers contained an EcoRI restriction site and reverse primers a NotI site. After amplification, the PCR fragment was ligated into the appropriate enzymes and then ligated into the pGEX 6P1 vector. The generated constructs are subsequently referred to as NHE3-Tail-1 (630–730) and NHE3-Tail-2 (730–830).

CAII-myc was subcloned from a CAII-GFP construct by PCR with forward primer 5'-CGG GAC GTC CCG GCC ACC ATC CAT CAC TGG GGC TAC G-3' and reverse primer 5'-AAGGAAAAAC GC GCG GCG TTA TAG GTC CTC CTC GGA CAT CAG CTG TTG TTG TTA GCA GGC AGG TTT GAT TTG CCT G-3'. EcoRI and NotI restriction sites were engineered into the primers, permitting digestion and ligation into the pcDNA 3.1+ mammalian expression vector. The construct was found to be functional by measuring the rate that lysate from cells overexpressing it acidified a solution bubbled with CO2 (5). All DNA sequences were confirmed by sequencing at the TAGC Applied Genomics core, University of Alberta.

Purification of glutathione-S-transferase fusion proteins. GST, NHE3-Tail-1, GST-NHE3-Tail-2, or pGEX 6P1 (empty vector) were
purified by methods used previously (16, 32). They were transformed into *E. coli* BL21 by electroporation (Eppendorf Electroporator 2510, Eppendorf North America, Westbury, NY) and then plated onto LB agar plates with 100 μg/ml ampicillin (Sigma-Aldrich Canada) and incubated at 37°C overnight. A single colony was used to inoculate LB broth, containing 100 μg/ml of ampicillin, which was then incubated overnight at 37°C in a shaker (Infors HT Ectron, Infors Canada, Anjou, Quebec). A 5-ml culture was subsequently inoculated into 250 ml of LB broth and grown at 37°C in a shaker until it reached A₆₀₀ 0.6–1.0. Isopropylthiogalactoside (1 mM final concentration) (Fermentas/Thermo Scientific, Ottawa, ON) was added, and the culture was subsequently incubated for 3 h further at 37°C while shaking. Cultures were then centrifuged at 7,500 g for 10 min in a Beckman-Avanti J-25 centrifuge (Beckmann Coulter, Mississauga, ON) at 4°C. The bacterial pellet was resuspended in 5 ml of 4°C PBS with benzamidine, PMSF, and protease inhibitor cocktail (Roche Diagnostics, Laval, Canada) with 75 μl of Triton X-100. Suspended cells were disrupted by sonicating (Braun-sonic 2000 sonicator) with four 45-s pulses at 20 kHz. Cells were then centrifuged at 12,000 g for 10 min at 4°C. The supernatant was transferred to glutathione Sepharose 4B resin (washed with PBS) and incubated at 20°C for 2 h. The fusion proteins were subsequently centrifuged at 1,500 g for 5 min and then washed with PBS. The fusion proteins were eluted with glutathione buffer (10 mM reduced glutathione in 50 mM Tris·HCl, pH 8.0). The concentration of protein was determined by measuring A₂₈₀ with a Nanodrop spectrophotometer, an extinction coefficient of 1 mg/ml (NanoDrop 2000c UV-Vis Spectrophotometers, Thermo Scientific) and BSA standards.

**Microtiter plate binding assay.** A microtiter plate binding assay was used to assess a physical interaction between NHE3 and CAII, as per other transporter-CAII interactions (16, 38). Human recombinant CAII (25 nM) was coated on 96-well microtiter plates (96-well EIA/RIA plate, Costar 3590, Corning, Corning, NY) for 30 min at room temperature in ELISA buffer (150 mM NaCl, and 100 mM Na₂HPO₄, pH 6.0) with 1.25 mg/ml of N-cyclohexyl-N-carbodiimide metho-p-toluene sulfonate (Sigma-Aldrich Canada). Plates were washed with PBS before blocking with 2% bovine serum albumin in PBS for 2 h at 20°C. Plates were then washed with PBS and subsequently overlaid with either NHE3-Tail-1, NHE3-Tail-2, or GST alone (50–400 nM) in antibody buffer (100 mM NaCl, 5 mM EDTA, 0.25% gelatin, 0.05% Triton X-100) at 20°C for 16 h. Note that for experiments...
examining the effect of pH, the amount of NaCl or pH of solution was altered to the stated content. Plates were next washed with PBS, and the samples were incubated with a rabbit polyclonal anti-GST antibody (1:5,000; Santa Cruz Biotechnology) for 2 h at 20°C. After washing with PBS, plates were incubated with a donkey anti-rabbit IgG polyclonal antibody (1:5,000; Santa Cruz Biotechnology), washed again with PBS, and finally incubated with streptavidin conjugated to HRP (Pierce Biotechnology, Rockford, IL) for 1 h at 20°C. Plates were developed with TMB substrate reagent (San Jose, CA) and then stopped by adding 200 µl/well of 5 M H2SO4. The relative amount of GST-fusion protein binding was finally measured at A450 nm in a Synergy Biotech microplate reader.

PLA. An association between NHE3 and CAII was assessed by PLA using a previously established methodology (30, 36). OK cells were transfected with CDNA encoding either NHE3 (2), human AE1 (37, 39), or human concentrative nucleoside transporter (hCNT3) (41) and myc-tagged CAII. Two days after transfection, the cells were washed with PBS, lysed in IPB buffer (1% NP-40, 5 mM EDTA, 0.15 M NaCl, 0.5% deoxycholate, and 10 mM Tris·HCl, pH 7.5) containing protease inhibitors, washed twice in PBS, and then fixed (3.5% paraformaldehyde, 1 mM CaCl2, 1 mM MgCl2 in PBS; pH 7.4 for 20 min). They were then washed twice with PBS and finally quenched with 50 mM NH4Cl for 10 min. Fixed cells were permeabilized with 0.1% Triton X-100 in PBS for 1 min at 20°C and then washed again with PBS. Coverslips were mounted in the provided mounting medium and observed through a 60×/1.42 PlanApo oil-immersion objective of a Z-stack confocal image capture was performed with a C9100-13 EM-CCD Digital Camera (Hamamatsu, Hamamatsu City, Japan), using Volocity software (PerkinElmer, Mississauga ON).

Coimmunoprecipitation. Coimmunoprecipitation (IP) was performed essentially as previously (4). OK cells were cotransfected with NHE338HA3 and CAII-myc by neon transfection as per the PLA (see above). The cells were grown in 10-cm tissue culture dishes for 2 days in DMEM-F12 media in the presence of 5% CO2 at 37°C. Once they were 100% confluent, the cells were washed with PBS then collected with 200 µl of elution buffer (1% NP-40, 5 mM EDTA, 0.15 M NaCl, and 10 mM Tris·HCl, pH 8.1) containing protease ligation cocktail (1:100, Calbiochem) and PMSF (1:1,000). The homogenized cell lysate was placed on ice for 30 min, then centrifuged at 12,000 rpm for 10 min at 4°C. The supernatant was subsequently collected, and the concentration of protein was determined by measuring A280 with a Nanodrop spectrophotometer (NanoDrop 2000c UV-Vis Spectrophotometers, Thermo Scientific), using an extinction coefficient of 1 mg/ml and BSA standards. Two separate 50-µl aliquots of Dynabeads protein G (Thermo Fischer) were precleared and then rinsed with 100 µl of elution buffer. To one sample of beads, 2 µg of goat anti-HA antibody was added; to the other sample, 2 µg of rabbit serum was added. Both were then incubated on ice for 20–30 min. Protein cell lysate (300 µg) was added with elution buffer to make the total volume 250 µl, and the samples were incubated overnight at 4°C. The next day the beads, were washed three times with elution buffer containing protease inhibitor cocktail and PMSF. Finally, to elute the bound protein from the beads, 40 µl of sample buffer was added to either the CO-IP, or control sample and to a total protein lysate sample (50 µg), and incubated at 37°C for 20 min. The samples were then subjected to SDS-PAGE and immunoblotting with anti-myc as above.

Statistical analysis. Data are presented as means ± SE. Paired or unpaired Student’s t-tests or ANOVA was carried out to determine statistical significance as appropriate. Tests were performed using Excel software (Microsoft, Santa Monica, CA), and P values < 0.05 were considered statistically significant.

RESULTS

Acetazolamide inhibits NHE3 activity in the presence of CO2 and HCO3-. To assess whether a functional interaction between NHE3 and CAII exists, we stably expressed NHE3 with three exofacial HA epitopes in OK cells (OK-NHE338HA3) (2). This both increased NHE3 expression and activity in this cell line.
but also provided an epitope tag greatly facilitating NHE3 detection and manipulation (3, 20). Cell lysate immunoblotted for the HA epitope revealed a single band of the appropriate molecular weight in cells expressing NHE338HA3, but not cells expressing the vector alone (Fig. 1A). Immunoblotting of the same cell lysate revealed endogenous expression of CAII (Fig. 1B, bottom band). Immunostaining of OK cells with HA antibody and 4,6-diamidino-2-phenylindole to stain nuclei found NHE3 predominantly present in the apical membrane of OK cells (Fig. 1C), consistent with its localization in vivo.

NHE3 activity was then assessed as the rate of recovery of pH induced by an acid load and measured with the fluorescent radiometric pH sensitive dye BCECF-AM. The cells were acidified by switching them from a bicarbonate-free medium to one containing bicarbonate and bubbled with 5% CO2. OK-NHE338HA3 cells demonstrate significantly greater Na+-dependent recovery of pH than vector-transfected controls (Fig. 1, D–G). Recovery from an acid load under these conditions was both Na+-dependent (Fig. 1, D and E) and inhibited by 100 μM EIPA (Fig. 1, F and G), a dose sufficient to block NHE3 activity (35). Recovery from a CO2-induced acid load was also prevented when a choline-HCO3-containing buffer was used instead of the NaHCO3 (dpH/dT = 0.0014 ± 0.0005 in the presence of Na+ vs. −0.0004 ± 0.0002 in the absence). This is consistent with NHE3 activity and not the activity of either a bicarbonate transporter or an H+-ATPase. To examine the possibility of a functional interaction between NHE3 and CAII, we inhibited endogenous CAII with acetazolamide and then repeated the measurement. This greatly attenuated NHE3 activity in the overexpressing cells (Fig. 2), consistent with a requirement for CAII activity to significantly augment NHE3 activity.

Acetazolamide does not inhibit NHE3 activity in the absence of CO2. To ascertain whether the effect of acetazolamide on NHE3 activity was via inhibition of CAII per se and not by blocking NHE3 directly, we repeated the assay in the absence of bicarbonate and CO2, in cells acidified by an ammonium chloride prepulse. Under these conditions, cells stably expressing NHE3 also demonstrated significant recovery from an acid load, which was Na+-dependent and inhibited by 100 μM EIPA (Fig. 3, A and B). However, under these conditions, acetazolamide had no effect on the rate of recovery from an acid load in NHE3-expressing cells (Fig. 3, C and D). These results are consistent with CAII increasing NHE3 activity and not with acetazolamide directly inhibiting NHE3.

CAII association and activity are necessary to enhance NHE3 activity. To confirm that CAII activity is required to augment NHE3-mediated transport, we overexpressed NHE338HA3 with either wild-type CAII or the catalytically inactive CAII mutant (CAII-V143Y) (33) and then repeated the functional assay in the presence of bicarbonate and CO2. The rate of pH recovery was significantly higher in cells expressing NHE3 than in those expressing CAII alone (Fig. 4, A and B). NHE3 activity was further increased by coexpression with wild-type CAII. However, this increase in activity was not observed when the catalytically inactive CAII-V143Y was coexpressed with NHE3 (Fig. 4, A and B). These studies revealed an increase of NHE3 activity induced by CAII but not catalytically inactive CAII (Fig. 4, A and B).
To assess whether CAII activity was sufficient to enhance NHE3 activity, we overexpressed NHE3 with a CAII mutant that fails to bind other transporters due to elimination of an N-terminal polybasic sequence, CAII-HEX (7, 37), and repeated the functional studies in the presence of bicarbonate and CO2. Coexpression with CAII-HEX did not increase NHE3 activity beyond coexpression with the empty vector alone (Fig. 4, A and B). Importantly, when we immunoblotted the remaining cells for NHE3 or CAII we did not observe a significant difference in expression of either protein (Fig. 4, C and D). Taken together, these results demonstrate that CAII activity and physical interaction are required to increase NHE3 activity.

NHE3 and CAII associate closely. NHE3 and CAII are both expressed in the brush-border membrane of the proximal tubule (9, 36); however, given the resolution of the light microscope, these two proteins could still be 250 nm away from one another. To assess a closer physical interaction, we cotransfected myc-tagged CAII and either NHE3, hCNT3, or AE1 and then performed a PLA (30). hCNT3 is a plasma membrane nucleoside transport protein, not known to interact with CAII, while a CAII-AE1 coassociation has been reported previously, including by PLA (36, 38). We observed a PLA signal when AE1 and CAII were coexpressed, indicating the ability of the PLA to detect a protein-protein interaction. The absence of a PLA signal in CAII- and hCNT3-coexpressing cells illustrates the specificity of the PLA technique (Fig. 5, A and B). The coexpression of NHE3 and CAII also generated a significant PLA signal consistent with a close association of the two proteins (Fig. 5C). Importantly, we were able to detect all transfected proteins on immunoblots, indicating that the lack of a PLA signal in CAII- and hCNT3-coexpressing cells did not arise from the absence of protein expression (Fig. 5D).

CAII binds the C terminus of NHE3. To determine whether NHE3 and CAII physically associate, we coexpressed NHE338HA3 and CAII-myc in OK cells. We then immunoprecipitated NHE3 with an anti-HA antibody and immunoblotted with anti-myc. We used total protein cell lysate as a positive control and immunoprecipitated with rabbit serum as a negative control. CAII was immunoprecipitated by NHE3, but not rabbit serum (Fig. 6A). To examine whether there is a direct physical interaction between the cytosolic carboxyl terminus of NHE3 and CAII, we expressed amino acids 630–730 (NHE3-Tail-1) or 730–831 (NHE3-Tail-2) of rat NHE3 as GST fusion proteins (Fig. 6B). NHE3-Tail-1 contains the putative CAII binding site (710IKEKDLELSDTEE722). We then performed a microtiter plate binding assay with these two constructs (16). Recombinant human CAII was fixed to the plate and then overlaid with different concentrations of the GST-fusion protein (50–400 nM). The degree of binding was then assessed by...
applying an anti-GST antibody followed by a HRP secondary
and measuring the development of a colorimetric reaction. The
assay was also performed with GST alone, and the results were
subtracted from both Tail constructs. We observed signifi-
cantly greater binding of NHE3-Tail-1 to CAII than NHE3-
Tail-2 (Fig. 6C). These data infer a direct interaction between
the cytosolic terminus of NHE3 and CAII.

Finally, we tested the effects of varying pH and ionic
strength on the interaction between CAII and NHE3, using
the microtiter plate assay. Binding of GST alone was mea-
sured and subtracted from all values. Maximal binding
between NHE3-Tail-1 and CAII occurred at 100 mM NaCl;
this was set to 100% (Fig. 7A). Increasing ionic strength past
this decreased the interaction, and above 400 mM NaCl
completely eliminated the interaction (Fig. 7A). We found
that the NHE3/CAII interaction was strongly pH dependent.
There was increased interaction at an alkaline pH (>8.0),
and the interaction was prevented at an acidic pH, i.e., <6.0
(Fig. 7B). Half-maximal binding was observed at pH ~7.2.
Therefore, the interaction between NHE3 and CAII is de-
pendent on both ionic strength and pH, consistent with an
electrostatic interaction.

DISCUSSION

NHE3 has an important role in renal function by reabsorbing
Na\(^+\), HCO\(^{-}_{3}\), and H\(_{2}\)O from the proximal tubule (28). We
provide several lines of evidence that NHE3 and CAII physi-
cally interact. A PLA confirmed a close association of the two
proteins when expressed in a proximal tubular epithelial cell
culture model. CO-IP confirmed a physical association be-
tween NHE3 and CAII and microtiter plate assays identified a
region in the cytosolic terminus of NHE3 required for CAII
binding. We also provide evidence that NHE3 and CAII
functionally interact. Coexpression of NHE3 with CAII in-
creased NHE3 activity, which depended on CAII catalysis,
since augmented NHE3 activity was only prevented by acet-
azolamide in the presence of CAII substrate. Moreover, coex-
pression of NHE3 and a catalytically dead CAII did not
increase NHE3 activity. Finally, a NHE3-CAII physical inter-
action is required to increase NHE3 activity, as a CAII con-
struct lacking the putative transporter-binding motif failed to
increase NHE3 activity. Together, these data support a physical
and functional interaction between CAII and NHE3 in the
proximal tubule.

We examined the interaction between NHE3 and CAII in a
proximal tubule model, as this is a site of prodigious sodium
and water reabsorption facilitated in large part by NHE3
activity (28). NHE3-CAII cooperation likely helps to max-
imize NHE3 function, permitting this process. NHE3 is also
expressed throughout the intestine, where it plays a similar role
in sodium and water reabsorption (15, 28). CAII is also
expressed throughout the small and large bowel (18, 21, 29),
there are also data suggesting a direct physical interaction is not required (1, 22). NHE1 and CAII also physically and functionally interact (16). Using similar experimental approaches, we found that both the putative transporter binding site in CAII and CAII catalysis are necessary to increase NHE3 activity. The interaction between NHE3 and CAII is different from the interaction between CAII and the monocarboxylate transporters, as CAII activity is required for increased NHE3 activity (7, 8). Our observations therefore support a mechanism whereby association of CAII with NHE3 increases transporter activity.

We found that the region between amino acids 630 and 730 in the cytosolic carboxy terminus of NHE3 binds CAII. We found this interaction to be pH sensitive. Consistent with a role for amino-terminal CAII histidine residues in mediating the interaction with NHE3, the interaction was titrated with a \( pK_a \) of 7.2, and enhanced at alkaline pH. This \( pK_a \) is nearly identical to the \( pK_a \) established for the AE1-CAII interaction, which is mediated through amino-terminal histidines in CAII, as mutation of histidine residues in the CAII N-terminal region prevents binding to AE1 (37, 38). Increasing ionic strength inhibited the NHE3-CAII interaction, suggesting that it is potentially an ionic interaction. Although the interaction was also inhibited at lower ionic strength, perhaps due to another factor such as altered protein conformation, however, this is purely speculative.

We observed maximal interaction between CAII and the cytosolic C terminus of NHE3 at pH 8 and above. Although there was some binding at the lower pH of our functional assays, the effect of an NHE3-CAII interaction would nonetheless be attenuated in our studies, underestimating its effect. Previous studies of sodium/proton exchangers found maximal transport activity when the cytosol was acidic. This makes intuitive sense for housekeeping transporters such as NHE1, as the role of this exchanger is to rid the cytosol of protons generated by metabolism. In this case, reduced activity at a physiological pH would prevent alkalinization of the cell. However, NHE3 does not appear to play a housekeeping role in volume regulation (28). Consequently, there may be a need to maintain NHE3 activity at physiological pH or even an alkaline pH. Perhaps the binding of CAII to the cytosolic terminus of NHE3 mediates this? NHE3 is activated by an acidic pH. However, NHE3 does not appear to play a housekeeping role such as this; instead, it is implicit to the reabsorption of sodium from intestinal and renal epithelia and therefore plays a central role in volume regulation (31). Transport metabolons are a functional coupling of an enzyme to a transporter that increases flux through the transporter. The AE1 transport metabolon appears to require a direct physical interaction between the enzyme and the transporter (33), although

We thus speculate that a physical and functional interaction between NHE3 and CAII in the mucosal membrane of the intestine may also serve to increase sodium and water absorption there.

CAII forms a transport metabolon with AE1, enhancing its activity (33). A metabolon is a complex of enzymes from one metabolic pathway coupled together so as to facilitate substrate movement from one active site to the next (31). Transport metabolons are a functional coupling of an enzyme to a transporter that increases flux through the transporter. The AE1 transport metabolon appears to require a direct physical interaction between the enzyme and the transporter (33), although

We thus speculate that a physical and functional interaction between NHE3 and CAII in the mucosal membrane of the intestine may also serve to increase sodium and water absorption there.

CAII forms a transport metabolon with AE1, enhancing its activity (33). A metabolon is a complex of enzymes from one metabolic pathway coupled together so as to facilitate substrate movement from one active site to the next (31). Transport metabolons are a functional coupling of an enzyme to a transporter that increases flux through the transporter. The AE1 transport metabolon appears to require a direct physical interaction between the enzyme and the transporter (33), although

There are also data suggesting a direct physical interaction is not required (1, 22). NHE1 and CAII also physically and functionally interact (16). Using similar experimental approaches, we found that both the putative transporter binding site in CAII and CAII catalysis are necessary to increase NHE3 activity. The interaction between NHE3 and CAII is different from the interaction between CAII and the monocarboxylate transporters, as CAII activity is required for increased NHE3 activity (7, 8). Our observations therefore support a mechanism whereby association of CAII with NHE3 increases transporter activity. We found that the region between amino acids 630 and 730 in the cytosolic carboxy terminus of NHE3 binds CAII. We found this interaction to be pH sensitive. Consistent with a role for amino-terminal CAII histidine residues in mediating the interaction with NHE3, the interaction was titrated with a \( pK_a \) of 7.2, and enhanced at alkaline pH. This \( pK_a \) is nearly identical to the \( pK_a \) established for the AE1-CAII interaction, which is mediated through amino-terminal histidines in CAII, as mutation of histidine residues in the CAII N-terminal region prevents binding to AE1 (37, 38). Increasing ionic strength inhibited the NHE3-CAII interaction, suggesting that it is potentially an ionic interaction. Although the interaction was also inhibited at lower ionic strength, perhaps due to another factor such as altered protein conformation, however, this is purely speculative.

We observed maximal interaction between CAII and the cytosolic C terminus of NHE3 at pH 8 and above. Although there was some binding at the lower pH of our functional assays, the effect of an NHE3-CAII interaction would nonetheless be attenuated in our studies, underestimating its effect. Previous studies of sodium/proton exchangers found maximal transport activity when the cytosol was acidic. This makes intuitive sense for housekeeping transporters such as NHE1, as the role of this exchanger is to rid the cytosol of protons generated by metabolism. In this case, reduced activity at a physiological pH would prevent alkalinization of the cell. However, NHE3 does not appear to play a housekeeping role such as this; instead, it is implicit to the reabsorption of sodium from intestinal and renal epithelia and therefore plays a central role in volume regulation (28). Consequently, there may be a need to maintain NHE3 activity at physiological pH or even an alkaline pH. Perhaps the binding of CAII to the cytosolic terminus of NHE3 mediates this? NHE3 is activated by an acidic pH. However, NHE3 does not appear to play a housekeeping role such as this; instead, it is implicit to the reabsorption of sodium from intestinal and renal epithelia and therefore plays a central role in volume regulation (28). Consequently, there may be a need to maintain NHE3 activity at physiological pH or even an alkaline pH. Perhaps the binding of CAII to the cytosolic terminus of NHE3 mediates this? NHE3 is activated by an acidic pH. However, functional studies have been performed predominantly in the absence of \( \text{HCO}_3^- \) and \( \text{CO}_2^- \). Hence the activating effect of CAII on NHE3 activity would not have been recognized.
In summary, we show CO-IP of NHE3 with CAII, close association in renal epithelial cells, and direct interaction by GST-binding studies. This physical interaction between NHE3 and CAII contributes to increased NHE3 activity. However, a physical interaction is insufficient to increase NHE3 activity as CAII catalysis is also required. Thus NHE3 and CAII both physically and functionally interact, an association likely facilitating the vast amount of salt and water reabsorption from the proximal tubule and intestine.

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

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