Regulation of the human NBC3 Na\(^{+}/\)HCO\(_3^{-}\) cotransporter by carbonic anhydrase II and PKA

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Loiselle, Frederick B., Patricio E. Morgan, Bernardo V. Alvarez, and Joseph R. Casey. Regulation of the human NBC3 Na\(^{+}/\)HCO\(_3^{-}\) cotransporter by carbonic anhydrase II and PKA. Am J Physiol Cell Physiol 286: C1423–C1433, 2004. First published January 21, 2004; 10.1152/ajpcell.00382.2003.—Human NBC3 is an electroneutral Na\(^{+}/\)HCO\(_3^{-}\) cotransporter expressed in heart, skeletal muscle, and kidney in which it plays an important role in HCO\(_3^{-}\) metabolism. Cytosolic enzyme carbonic anhydrase II (CAII) catalyzes the reaction CO\(_2\) + H\(_2\)O ⇌ HCO\(_3^{-}\) + H\(^{+}\) in many tissues. We investigated whether NBC3, like some Cl\(^{-}/\)HCO\(_3^{-}\) exchange proteins, could bind CAII and whether PKA could regulate NBC3 activity through modulation of CAII binding. CAII bound the COOH-terminal domain of NBC3 (NBC3Ct) with \(K_a = 101\) nM; the interaction was stronger at acid pH. Cotransfection of HEK-293 cells with NBC3 and CAII recruited CAII to the plasma membrane. Mutagenesis of consensus CAII binding sites revealed that the D1135-D1136 region of NBC3 is essential for CAII/NBC3 interaction and for optimal function, because the NBC3 D1135N/D1136N retained only 29 ± 12% of wild-type activity. Coexpression of the functionally dominant-negative CAII mutant V143Y with NBC3 or addition of 100 \(\mu\)M 8-bromoadenosine to NBC3 transfected cells reduced intracellular pH (pH\(_i\)) recovery rate by 31 ± 3%, or 38 ± 7%, respectively, relative to untreated NBC3 transfected cells. The effects were additive, together decreasing the pH\(_i\) recovery rate by 69 ± 12%, suggesting that PKA reduces transport activity by a mechanism independently of CAII. Measurements of PKA-dependent phosphorylation by mass spectroscopy and labeling with \([\gamma\text{-}3\text{P}]\)ATP showed that NBC3Ct was not a PKA substrate. These results demonstrate that NBC3 and CAII interact to maximize the HCO\(_3^{-}\) transport rate. Although PKA decreased NBC3 transport activity, it did so independently of the NBC3/CAII interaction and did not involve phosphorylation of NBC3Ct.

pH regulation; bicarbonate transport; metabolon

REGULATION OF INTRACELLULAR pH (pH\(_i\)) is critically important in all cells, because pH\(_i\) influences membrane transport, cell volume, metabolism, and intracellular messengers (3, 15, 21). Physiological changes in metabolic proton production and ambient CO\(_2\) make it necessary for cells to have a robust system to maintain pH\(_i\) homeostasis. Most cells experience transient alkalosis and acidosis, so that mechanisms for acid influx and efflux are required. NBC3 contributes to pH\(_i\) regulation in striated myocytes and HCO\(_3^{-}\) secreting epithelial cells (23, 43). NBC3, originally cloned from human skeletal muscle, was also localized by multiple-tissue Northern blot analysis of the heart (23). Subsequently, NBC3 has been identified at the apical surface of several epithelial cell types including renal outer medullary collecting duct type A intercalated cells and duct and acinar cells of parotid and submandibular glands (10, 17, 24). A recent report evaluated the contribution of Na\(^{+}\)-dependent transepithelial HCO\(_3^{-}\) flux in outer medullary collecting duct preparations and found that this transport mechanism provided only a minor contribution to the total transepithelial HCO\(_3^{-}\) flux (43). However, pH\(_i\) recovery in acid-secreting type A intercalated cells was inhibited by >50% in the absence of luminal Na\(^{+}\), which suggests that the major function of NBC3 in these cells is associated with pH\(_i\) regulation (43). In addition, NBC3 has a widespread distribution in salivary glands, yet HCO\(_3^{-}\) secretion from these glands is believed to be mediated by duct cells and not acinar cells, which further supports the role of pH\(_i\) regulation. By comparison, the rat orthologue of NBC3, NBCn1, localizes specifically to the basolateral membrane of duct cells, which leaves open the possibility that this protein may participate in transepithelial HCO\(_3^{-}\) flux (10, 40).

The carbonic anhydrase (CA) family of enzymes catalyze the reversible hydration of CO\(_2\) to yield HCO\(_3^{-}\) and a H\(^{+}\) by the following mechanism, CO\(_2\) + H\(_2\)O ⇌ H\(_2\)CO\(_3\) ⇌ H\(^{+}\) + HCO\(_3^{-}\) (6). The first reaction step is actively catalyzed and the second occurs spontaneously in aqueous solution. To date, 10 enzymatically active isoforms of CA have been identified (6). CA isoforms are involved in many physiological processes including H\(^{+}\)/HCO\(_3^{-}\) secretion, signal transduction, bone resorption, gluconeogenesis, cell proliferation, and oncogenesis. CAII is a cytosolic isoform that has been identified in skeletal muscle, kidney type A intercalated cells, and salivary glands, but not adult cardiomyocytes. CAII functionally interacts with all three members of the anion exchange (AE) family of bicarbonate transporters and physically via an acidic motif in the COOH-terminal region of these transporters (38, 39). The consensus interaction motif consists of a hydrophobic residue followed by at least two acidic residues within the next four amino acids (38). The reciprocal bicarbonate transporter-binding motif on CAII has been localized to a basic patch of amino acids within the first 18 residues of NH\(_2\) terminus of the enzyme (37). This interaction is essential for the full HCO\(_3^{-}\) transport activity of anion exchangers in transiently transfected HEK-293 cells as demonstrated by inhibition of transport by a dominant-negative mutant of CAII, presumably by displacement of wild-type CAII from binding sites on the intracellular surface of the anion exchangers (32).

Recently, members of two other families of pH regulating transporters have been shown to interact with CAII. Na\(^{+}/\)H\(^{+}\)...
exchanger I (NHE1) is a ubiquitously expressed electroneutral transporter, with a two-domain structure consisting of an NH₃-terminal ion translocation domain of ~500 amino acids and a COOH-terminal regulatory domain of ~300 amino acids. NHE1 reciprocally coimmunoprecipitates with CAII from CHO cells (19). The last 178 amino acids of NHE1 COOH-terminal domain are sufficient for the CAII interaction as shown by a microtiter binding assay (19). It is interesting that phosphorylation of NHE1 COOH-terminal domain enhanced CAII binding (CAB) (19). In the presence of a dominant-negative CAII mutant, NHE1 transport rate was reduced by ~50%. The kidney genetically Na⁺/HCO₃⁻ cotransporter NBC1a binds to CAII via its COOH-terminal domain (1, 13) and also interacts with the extracellular anchored CAIV isoform (1). The transport stoichiometry of NBC1a or pancreatic splice form NBC1b is shifted from 3:1 HCO₃⁻:Na⁺ to 2:1 by cAMP-dependent PKA phosphorylation at Ser982 or Ser1026, respectively (11). PKA-dependent phosphorylation of Thr99 of NBC1b increased transport activity without altering transport stoichiometry (11). It is interesting that the CAII inhibitor acetazolamide reduces short-circuit current through NBC1a by 65% when operating in the 3:1 (unphosphorylated) mode but has no effect on the 2:1 transport mode (13), suggesting a link between the effects of PKA and CAII on NBC1 transport. However, the effect of phosphorylation on CAB has not been investigated.

Recently, we characterized the structure of the COOH-terminal domain of NBC3 (NBC3Ct) and discovered that the region close to the membrane domain is proteolytically sensitive. It is interesting that the consensus PKA phosphorylation motif and CAB consensus motifs are present in this same region. With previous work on CA/ AE interactions, we decided to explore the possibility that NBC3 interacts with CAII and that the interaction is phosphorylation state dependent so that phosphorylation reduces the binding affinity. To examine the regulation of NBC3 activity by CAII and PKA, we first addressed the question of physical and functional interaction of CAII and NBC3, by performing microrack binding assays and dominant-negative competition assays in transiently transfected HEK-293 cells, respectively. We then addressed the physical and functional effects of PKA phosphorylation, using the microrack binding assay and two pulse pH recovery experiments in transiently transfected HEK-293 cells. Finally PKA and CAB motif mutants were constructed, and their effects on transport activity analyzed.

MATERIALS AND METHODS

Materials. Human NBC3 cDNA was a generous gift from Dr. Ira Kurtz (University of California Los Angeles). PCR primers were from Invitrogen (Burlington, ON, Canada). Recombinant expression vector pGEX-6p-1 enhanced chemiluminescence (ECL) reagent, and glutathione S-transferase (GST) fusion purification reagents were from Amersham Biosciences (Piscataway, NJ). Two DNA polymerase was from Roche (La Jolla, CA), and all other cloning enzymes, as well as the PKA catalytic subunit, were from New England Biolabs (Mississauga, ON, Canada). Escherichia coli recombinant expression strain BL21-CodonPlus was from Stratagene (La Jolla, CA). Protein concentrations were from Millipore (Billerica, MA). Protein quantification reagent was from Bio-Rad (Hercules, CA). Transfection and cell culture reagents were from Invitrogen (Burlington, ON, Canada). Forskolin, 5-(N-ethyl-N-isopropyl)amiloride, and PKA inhibitor H89 were from Sigma-Aldrich Canada (Oakville, Canada). The antibodies against NBC3, CAII, and GST, were from SynPrep (Dublin, CA), Serotec (Raleigh, NC), and Santa Cruz Biotechnology (Santa Cruz, CA), respectively.

DNA constructs. Expression constructs for NBC3, NBC3Ct, CAII, and CAII V143Y have been described previously (20, 32). PKA phosphorylation site mutants, NBC3-S1132A and CAB site mutants, NBC3-CAB1 (D1135N and D1136N), and NBC3-CAB2 (D1163N and D1165N) were constructed by using the mega-primer mutagenesis strategy (27). First-round PCR forward primer for all mutants was 5’-gctagctgctgcctagtgg-3’. First round PCR reverse primers were 5’-cggcatacattatactggaagagcgccttcg-3’ and 5’-gcacatatgtaggtttggatcagc-3’ for NBC3-CAB1 and NBC3-CAB2, respectively. Amino acid sequences of consensus CAB motifs were therefore mutated to L₁₁³⁴NLM₁₁³⁸ and L₁₁⁶²QNNN₁₁⁶⁶, for CAB1 and CAB2, respectively. The products from the first round of PCR were used as forward mega-primers for the second round of PCR, and 5’-tagaagcagctgtag-3’ was used for the reverse primer. Second-round products were subcloned back into the NBC3 cDNA (pNBC3) using EcoRI and NotI at 5’ and 3’ ends, respectively. GST fusion protein constructs GST.NBC3CtCAB1, GST.NBC3CtCAB2, and GST.NBC3CtS1132A were engineered by using the respective expression constructs as PCR templates. The subcloning strategy has been previously described (20).

Protein expression in mammalian cells. NBC3, NBC3-CAB1, NBC3-CAB2, CAII, and CAII V143Y proteins were expressed by transient transfection of HEK-293 cells (7, 32) using the calcium phosphate method (26). All experiments with transfected cells were carried out 48-h posttransfection. Cells were grown at 37°C in an CO₂-air (1:19) environment in DMEM, supplemented with 5% (vol/vol) fetal bovine serum, (vol/vol) calf serum, and penicillin-streptomycin-glutamine.

NBC3 transport assay. The transport activity of NBC3 was monitored by using a fluorescence assay, as previously described (14). Briefly, HEK-293 cells grown on poly-L-lysine-coated coverslips were transiently transfected as described in the previous section. Forty-eight hours posttransfection, coverslips were rinsed in serum-free DMEM and incubated in 4 ml serum-free medium, containing 2 μM BCECF-AM (37°C, 20 min). Coverslips were then mounted in a fluorescence cuvette and perfused with Ringer buffer (in mM: 5 glucose, 5 K gluconate, 1 Ca gluconate, 1 MgSO₄, 140 NaCl, 2.5 NaH₂PO₄, 25 NaHCO₃, 10 HEPES, pH 7.4), equilibrated with 5% CO₂-air. The pH recovery activity of HEK-293 cells transfected with NBC3, NBC3-CAB1, NBC3-CAB2, or NBC3 plus CAII V143Y was measured during the recovery from transient intracellular acidification. Acid loading was accomplished by using the NH₄Cl pulse technique (4). Cells were transiently perfused with Ringer buffer, containing 40 mM NH₄Cl for 5 min, followed by the washout of NH₄Cl with Ringer buffer. All perfusion was performed with flow rate 3.5 ml/min. All experiments were performed in the presence of 5 μM 5-(N-ethyl-N-isopropyl)amiloride (Sigma) to block endogenous NHE activity. Fluorescence was monitored by using a Photon Technologies International CR fluorimeter at excitation wavelengths of 440 and 500 nm and an emission wavelength of 530 nm. After calibration using the nigericin/high-potassium technique (33) at three pH values between 6.5 and 7.5, fluorescence ratios were converted to pH. The initial rate of pH recovery from an acid load was calculated by linear regression of the first 1–3 min of the initial linear phase of pH recovery after maximum acidosis. In all cases, the pH recovery of cells transfected with empty vector was subtracted from the total recovery to ensure that these recoveries were only due to the transiently expressed proteins.

Measurement of intrinsic buffer capacity and proton flux. Intracellular buffering capacity measurements were made by the ammonium pulse method (4). HEK-293 cells grown on coverslips were sham transfected as described in Protein expression in mammalian cells. Two days posttransfection, cells were loaded with BCECF-AM as described in NBC3 transport assay. Coverslips were mounted in a
fluorescence cuvette and allowed to equilibrate in Ringer buffer containing 1 mM amiloride bubbled with oxygen to ensure bicarbonate-free conditions. Cells were then perfused consecutively for 200 s with Ringer buffer without sodium bicarbonate containing varying concentrations of NH₄Cl. Intracellular \( \text{NH}_4^+ \) concentration (\( \text{NH}_4^+_{\text{c}} \)) was calculated from the Henderson-Hasselbalch equation, and the intrinsic buffering capacity (\( \beta_{\text{in}} \)) was then calculated as \( \Delta[\text{NH}_4^+] / \Delta \text{pH} \) (28). The total buffering capacity of the system (\( \beta_{\text{total}} \)) was then determined as \( \beta_{\text{total}} = \beta_{\text{in}} + \beta_{\text{CO2}} \), where \( \beta_{\text{CO2}} = 2.3 \times \text{[HCO}_3^-] \) (25).

Total proton flux was calculated as \( \text{J}_\text{p} = \Delta[\text{NH}_4^+] / \Delta \text{pH} \) (25).

**Antibody preparation.** A synthetic peptide comprised of the COOH-terminal 18 amino acids of human NBC3 was synthesized yielding peptide NH₂-ISFEDEPRKKKYDAETSL-COOH. The peptide was coupled to keyhole limpet hemocyanin and subsequently injected into two rabbits numbered SN 338-1 and -2. Serum from each rabbit was monitored on immunoblots containing NBC3 protein until a maximal immune response was observed. Rabbits were killed, exsanguinated, and sera-designated SN 338-1 and -2 were isolated. Only the SN 338-1 has a high anti-NBC3 titer. Peptide synthesis and antisera production were completed by SynPep (Dublin, CA).

**Immunodetection.** Transfected cells were washed in PBS buffer (in mM: 140 NaCl, 3 KCl, 6.5 Na₂HPO₄, 1.5 KH₂PO₄, pH 7.5), and lysates of the whole tissue culture cells from each dish were prepared by the addition of 0.5 ml SDS-PAGE sample buffer containing complete miniprotease inhibitor cocktail (Roche). Total protein content was measured by using the Bradford protein assay (5).

Samples (5 μg) were resolved by SDS-PAGE on 8% acrylamide gels (18). Proteins were transferred to PVDF membranes (35). Membranes were blocked by incubation for 1 h in TBST-M buffer [0.1% (vol/vol) Tween-20, 137 mM NaCl, 20 mM Tris, pH 7.5 (TBST) containing 5% (wt/vol) nonfat dry milk] and then incubated overnight in 10 ml TBST-M, containing 1:5,000 diluted affinity purified rabbit anti-NBC3 antibody or 1:3,000 diluted sheep anti-CAIL antibody, and then incubated with TBST-M containing 1:3,000 diluted donkey anti-rabbit IgG (Santa Cruz Biotechnology) or donkey anti-sheep IgG (Santa Cruz Biotechnology) conjugated to horseradish peroxidase. After a final wash with TBST buffer (3 times), blots were visualized by using ECL reagent, and a Kodak Image Station 440CF.

**Cell surface processing assay.** The fraction of protein processed to the cell surface was quantified as described (9), using the membrane-impermeant biotinylating reagent sulfo NHS-SS-biotin.

**Confocal microscopy.** Cells grown on poly-L-lysine-coated, 18-mm diameter coverslips were transiently transfected, as described above. The coverslips were transferred to 35-mm petri dishes. Cells were fixed for 30 min in 2% (wt/vol) paraformaldehyde in PBS containing calcium (PBSc; in mM: 1 CaCl₂, 140 NaCl, 3 KCl, 6.5 Na₂HPO₄, 1.5 KH₂PO₄, pH 7.5). After two washes with PBSc, the cells were incubated for 25 min in permeabilization buffer [in mM: 300 sucrose, 50 NaCl, 3 MgCl₂, 20 HEPES, pH 7.4, and 0.5% (vol/vol) Triton X-100]. Coverslips were washed three times with PBSc and blocked for 25 min in 10% serum in PBSc. Coverslips were incubated with a 1:50 dilution of sheep anti-CAII antibody (Serotec) in PBSc and 4% calf serum. Coverslips were washed three times with PBSc and 4% calf serum and then incubated for 45 min in a dark chamber with a 1:100 dilution of biotinylated anti-rabbit IgG antibody (Santa Cruz Biotechnology) in PBSc and 4% serum. After three washes with PBSc and 4% serum, the coverslips were incubated for 45 min in a dark chamber with a 1:100 dilution of streptavidin fluorescein conjugate (Amersham). Images were collected by using a Zeiss LSM 510 laser scanning confocal microscope mounted on an Axiovert 100M controller with a ×63 (NA1.4) lens.

**GST-fusion protein purification.** GST constructs (NBC3Ct, CAB1, CAB2, or S1132A) were expressed in E. coli BL21 Codon Plus and purified as previously described (32). A synthetic peptide from NBC3Ct corresponds to amino acids 1127–1214 of human NBC3.

**In vitro phosphorylation by PKA.** GST or GST-NBC3Ct were treated with PKA in either of two ways: 1) with [γ-32P]ATP to measure incorporation of phosphate by scintillation counting and 2) with nonradioactive ATP for analysis by mass spectrometry and microtiter plate binding assays. GST or GST-NBC3Ct (0.1 nmol) was mixed with 2,500 units of PKA catalytic subunit (or equivalent volume of water) in PKA reaction buffer (200 μM ATP, 10 mM MgCl₂, 50 mM Tris-HCl, pH 7.5) or PKA reaction buffer supplemented with 500 μCi/μmol [γ-32P]ATP. Samples were then incubated at 30°C for 10 min. Nonradioactive samples were characterized by matrix-assisted laser desorption/ionization-time of flight, using a Voyager De-Pro mass spectrometer from Applied Biosystems (Foster City, CA). Radioactive samples were precipitated with TCA/DOC (5% TCA containing 0.3% deoxycholate), centrifuged at 14,000 rpm for 5 min, and washed twice with saturated TCA/DOC solution. Samples were then resuspended in 0.5 M NaOH, and radioactivity was counted by a Beckman LS 6500 scintillation system (Fullerton, CA).

**Microtiter plate binding assay.** The microtiter plate CAB assay is modified from an assay previously reported (39). Purified CAB (200 ng/well) was immobilized onto 96-well microtiter plates by 30 min of incubation at room temperature in ELISA buffer (150 mM NaCl, 100 mM Na₂HPO₄, pH 6.0) containing 1.25 mg/ml 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-p-toluene sulinate. Plates were washed (3 ×5 min) with PBS and blocked at room temperature for 1.5 h with PBS containing 2% BSA. Plates were washed twice with antibody buffer [100 mM NaCl, 5 mM EDTA, 0.25% (wt/vol) gelatin, 0.05% (vol/vol) Triton X-100, 50 mM Tris-HCl, pH 7.5] and then once with antibody buffer containing 1 mM DTT. Plates were incubated with varied concentrations of GST or GST-NBC3Ct with or without PKA phosphorylation, overnight at room temperature. For pH titration experiments, antibody buffer was prepared containing 25 mM MES and 25 mM MOPS in place of 50 mM Tris-HCl, and the buffer pH was adjusted appropriately. The following day, plates were washed with antibody buffer (3 × 5 min) and then incubated with rabbit polyclonal anti-GST (1:5,000 dilution) in antibody buffer at room temperature for 2 h. Plates were washed and incubated with donkey anti-rabbit IgG (1:5,000 dilution) in antibody buffer at room temperature for 2 h. Plates were washed a final time then developed with orthophenylenediamine dihydrochloride substrate with absorbance at 450 nm, read by using a Labsystem Multiskan MCC ELISA microplate reader (MTX Lab System, McLean, VA) when sufficient color had developed. To estimate the affinity of the association between CAB and NBC3Ct, double reciprocal plots of NBC3Ct vs. %binding were prepared. The \( K_d \) for the interaction was estimated from the negative inverse of the \( x \)-intercept.

**Sequence and statistical analysis.** The amino acid sequence of the NBC3 COOH-terminal tail was analyzed for the presence of consensus PKA phosphorylation sites using Peptools software. Statistical analysis was performed by using Excel Software (Microsoft). Groups were compared with one-way ANOVA, followed by Tukey’s test with \( P < 0.05 \) considered significant. For other experiments, paired \( t \)-test was used with \( P < 0.05 \) considered significant.

**RESULTS**

**NBC3 transport activity and interaction with CAII.** To measure NBC3 HCO₃⁻ transport activity, HEK-293 cells were transiently transfected with NBC3, and the recovery rate from an acid load was induced by 40 mM NH₄Cl prepulse was assessed (Fig. 1A). Although the initial characterization of human NBC3 (23) suggested that the protein is amiloride sensitive, a subsequent report (confirmed here) showed that NBC3 is not amiloride sensitive (22). Thus all NBC3 transport assays were performed in the presence of 5 μM EIPA to block activity of the endogenous NHE. The average rate of recovery
from acid load was $0.080 \pm 0.007$ pH/min (2.1 mM/min) for NBC3 transfected cells and $0.037 \pm 0.004$ pH/min (0.97 mM/min) for vector-alone transfected cells. The background recovery rate of the sham cells may be attributable to the endogenous NBC3 expression that has been reported in HEK-293 cells (22). Background activity was subtracted from all measurements of NBC3 pH recovery activity.

In other experiments (not shown), NBC3 activity was measured by using the same assay as above except using Na$^+$-free Ringers buffer (Na$^+$ replaced by choline) containing 1 mM amiloride. After maximum acidification was achieved, the rate of pH$_i$ recovery was measured for 1 min as $0.021 \pm 0.003$ pH/min ($n = 3$). Three minutes after maximum acidification, perfusion solution was switched to Na$^+$-containing Ringer buffer, containing 1 mM amiloride. The recovery rate then accelerated to 0.113 \pm 0.008 pH/min. Peak acidification was pH 6.64 \pm 0.04. We conclude that NBC3-transfected cells express a pH recovery activity that is Na$^+$-dependent and amiloride insensitive, consistent with NBC3 (22). The low level of pH recovery in the absence of Na$^+$ underscores the absence of Na$^+$-independent pH recovery activity.

NBC3 contains two CAB motifs in its cytoplasmic COOH terminus (31). Functional assays were performed to assess the effects of CAB on NBC3 transport activity (Figs. 1 and 2). To examine whether CAB affects NBC3 HCO$_3^-$ transport activity, we assayed NBC3 activity in the absence and presence of the functionally inactive CAII V143Y mutant. CAII is endogenously expressed in HEK-293 cells (Fig. 2A) at sufficient levels so that overexpression has previously been shown to have no effect on HCO$_3^-$ transport activity (32). However, overexpression of the V143Y mutant will displace wild-type CAII from its cytoplasmic binding sites. The V143Y point mutation reduces CAII catalytic activity to 1/3,000th of wild-type activity yet preserves wild-type structure (8) so that the mutant is able to compete with wild-type protein for interactions with physiological binding partners (32). Transfection of HEK-293 cells with the V143Y CAII mutant increased total CAII expression \sim 20-fold as assessed by densitometry compared with untransfected cells, which indicates that the amount of mutant CAII greatly exceeds wild-type in transfected cells (Fig. 2A). Anti-NBC3 antibody SN 338-1 recognized a band with molecular weight consistent with NBC3 in NBC3-transfected HEK-293 cells, but not in untransfected cells (Fig. 2A). This indicates the specificity of antibody SN 338-1 to recognize NBC3. The doublet band likely arises from differential glycosylation of NBC3, but was not further characterized.

Cotransfection of CAII V143Y and NBC3 had no effect on NBC3 expression level (Fig. 2B). However, cotransfection with the V143Y mutant reduced NBC3 transport activity by $31 \pm 3\%$ (Figs. 1 and 2C). The reduction of NBC3 activity in the presence of V143Y CAII suggests that displacement of wild-type CAII from a binding site on NBC3 reduces NBC3 transport activity. Localization of wild-type CAII to a binding site on NBC3 is thus required for full transport activity.

Rates of change of pH$_i$ are highly dependent on buffer capacity of the cell. Intrinsic buffer capacity ($\beta_i$) was measured for HEK-293 cells transfected with NBC3 or cotransfected with NBC3 and CAII. In the pH region in which measurements
of NBC3 transport rate were made, $\beta_i$ was 11.2 ± 4.5 mM/pH at pH 6.82 ± 0.08 and 13.3 ± 1.3 mM/pH at pH 6.84 ± 0.06 for cells transfected with NBC3 alone and NBC3/CAII cotransfected cells, respectively. $\beta_i$ measurements for NBC3 and NBC3/CAII are representative of all experiments performed here, because NBC3 transfected cells are likely to have the same $\beta_i$ as cells transfected with NBC3 mutants. Similarly, cells are likely to have the same $\beta_i$ whether transfected with wild-type or point mutant CAII. We conclude that there is no significant difference in $\beta_i$ for the HEK-293 cells under the various transfections states studied in these experiments. At pH 6.83 the buffer capacity resulting from bicarbonate ($\beta_{\text{CO}_2}$) is 14.1 mM/pH; $\beta_{\text{total}}$ is thus estimated as 26.3 mM/pH. To compare rates of pH$_i$ recovery mediated by NBC3, it is essential that the pH$_i$ recovery be measured from the same pH value. In the experiments described above, the minimum pH$_i$ in experiments to measure NBC3 activity was 6.67 ± 0.09 and 6.59 ± 0.06 for NBC3 and NBC3/V143Y CAII cotransfections, respectively. There was no statistically significant difference between these values.

**Effect of PKA on NBC3 transport activity.** In the proximal tubule, PKA-dependent phosphorylation of NBC1 reduces transport activity and HCO$_3^-$ reabsorption (29). Recently, an important phosphorylation site, Ser$^{982}$, has been identified that shifts the NBC1a transport stoichiometry from 3:1 to 2:1 (HCO$_3^-$/Na$^+$) in proximal tubule cells (12). It is interesting that analysis of the NBC3Ct sequence with Peptools software revealed the presence of a consensus PKA phosphorylation site (S1132) in NBC3Ct. Notably this site was identified as a low stringency site and does not form a strong consensus site. The presence of a potential PKA phosphorylation site, however weak, suggested the possibility that PKA could phosphorylate NBC3Ct, reducing activity through displacement of CAII or by other means. We therefore characterized the effect of PKA on NBC3 transport activity. HEK-293 cells, transiently transfected with NBC3, were incubated with the membrane-permeant PKA agonist, 8-bromo-adenosine (8-Br-cAMP; 100 μM). The compound reduced pH$_i$ recovery rate by 38 ± 7%, compared with the rate of pH$_i$ recovery after an initial acid load without treatment (Figs. 1C and 2C). As a control, the rate of pH$_i$ recovery was measured after two successive acid loads, without 8-Br-cAMP treatment. The rates of pH$_i$ recovery were indistinguishable ($\Delta$PH/min = 0.072 ± 0.001 (acidotic pH$_i$ = 6.70 ± 0.05) and 0.071 ± 0.003 (acidotic pH$_i$ = 6.71 ± 0.03) for recovery from the first and second pulse, respectively). Treatment with 8-Br-cAMP in the presence of CAII V143Y reduced pH$_i$ recovery by 69 ± 12% compared with untreated NBC3 transfected cells (Fig. 2C). The additive nature of the CAII V143Y and 8-Br-cAMP effects suggests that cAMP does not mediate its effects on NBC3 transport activity through modulation of CAII/NBC3 interaction.

To verify the results with 8-Br-cAMP, experiments were also performed with the adrenergic agonist forskolin (5 μM). Cells treated with forskolin for 10 min before recovery from acid load had pH$_i$ recovery rate 44% ± 3% lower than the rate before forskolin treatment (Fig. 2C). This indicates that mobilization of cAMP at physiological levels inhibits NBC3 to an extent similar to 100 μM 8-Br-cAMP. To examine whether the inhibitory effects of cAMP on NBC3 are mediated through PKA, NBC3-transfected cells were preincubated with the PKA inhibitor, H89 (10 μM) for 5 min and then for a further 10 min with H89 and forskolin (5 μM). Treatment with H89 reduced the inhibitory effect of forskolin on NBC3 activity to 10 ± 3% (Fig. 2C), which was not significantly different from untreated NBC3. There was no difference in the level of acidification between the groups of transfected cells in these experiments. Average minimum pH$_i$ was 6.67 ± 0.09, 6.69 ± 0.11, 6.61 ± 0.04, 6.69 ± 0.01, and 6.74 ± 0.02 for NBC3 alone, NBC3/8-Br-cAMP, NBC3/V143Y CAII, NBC3/forskolin, and NBC3/H89/forskolin, respectively. We conclude that physiological levels of cAMP inhibit NBC3 activity and that PKA is responsible for the inhibition.

**CAII physically interacts with NBC3Ct in a pH-dependent manner.** We then examined the possibility that the functional interaction between CAII and NBC3 may be mediated by a direct physical interaction between these two proteins. A microtiter-binding assay was performed in which CAII was im-

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**Fig. 2.** Expression of NBC3 and CAII constructs in HEK-293 cells and summary of NBC3 activity. **A:** expression of CAII V143Y relative to endogenous wild-type CAII expression was measured in lysates from HEK-293 cells either sham-transfected or expressing NBC3 or NBC3 and CAII V143Y used for transport experiments and probed on immunoblots for CAII as indicated (bottom). **B:** expression of NBC3 and NBC3 CAB1 and CAB2 mutants measured in lysates used for transport experiments and probed on immunoblots using the SN 338-2 antibody. The major immunoreactive band shown migrated between the 233-kDa myosin marker and the 135-kDa β-galactosidase marker and had a relative molecular mass of ~150 kDa. **C:** average pH$_i$ recovery rate is expressed as a percentage of wild-type NBC3 recovery. Forskolin, 8-Br-cAMP, and H89 were used at 5 μM, 100 and 10 μM, respectively. Error bars represent standard error (n = 3–4 cells). *Statistical difference P < 0.05 relative to wild-type NBC3 (unpaired, 2-tailed t-test).
mobilized to the bottom of a microtiter dish well. Plates were then washed, overlaid with GST fusion proteins, and the amount of bound protein quantified by using an anti-GST antibody and a tertiary development system (39). We performed the assay using GST alone and GST.NBC3Ct [NBC3Ct corresponds to amino acids 1127–1214 of human NBC3] at concentrations from 0–200 nM. We observed saturation binding kinetics for GST.NBC3Ct with a $K_d$ value of 101 nM (Fig. 3A). The V143Y mutant also bound GST.NBC3Ct, but with a $K_d$ value of 227 nM, which is still well within the range of normal protein-protein interaction affinities (data not shown). The CAII/NBC3Ct interaction was verified by dot-blot assay with purified NBC3Ct bound to a nylon membrane, overlaid with a cellular extract from HEK-293 cells, and developed with an anti-CAII antibody (data not shown). As shown in Fig. 3B, the NBC3Ct/CAII interaction is pH-dependent, with greater binding at acid pH, which is consistent with a role in pH-dependent regulation of NBC3 activity. Pretreatment of the GST.NBC3Ct fusion protein with PKA had no significant effect on NBC3Ct/CAB over the pH 5–8 range (Fig. 3B). For example, at pH 7.25, binding of CAII was 17.2 ± 3.6 and 13.6 ± 2.5% of maximum binding for GST.NBC3Ct without and with PKA treatment, respectively, which was statistically indistinguishable. We conclude that PKA does not alter CAII/NBC3 binding kinetics by phosphorylation of NBC3Ct.

**NBC3 recruits CAII to the plasma membrane.** We further investigated the CAII/NBC3 physical interaction in transiently transfected HEK-293 cells. CAII was overexpressed by transient transfection and cells were then treated with an anti-CAII antibody. Confocal immunofluorescence indicated a predominant cytoplasmic localization for CAII (Fig. 4A). In contrast, cotransfection of CAII with NBC3 recruited CAII to the plasma membrane consistent with association with NBC3 (Fig. 4B). Cotransfection of CAII with a deletion mutant of NBC3 lacking the COOH-terminal domain restored the diffuse cytoplasmic localization of CAII (Fig. 4C). At the exposure levels used in these experiments, endogenous CAII (expressed at levels 20-fold lower than the level induced by transfection with CAII cDNA) was not visible (data not shown). These experiments demonstrate that NBC3 expression recruits CAII to the plasma membrane and that the NBC3 COOH-terminal domain is required to mediate this interaction.

In Fig. 4B, it is surprising that although there is intense membrane staining, cytosolic CAII staining is weak, despite the fact that the cells are transfected with CAII. We suspect that the fixation methods used in the immunocytochemistry experiments result in some loss of cytosolic contents. It stands to reason that free cytosolic components will be more labile than membrane proteins fixed in a bilayer or proteins bound to membrane proteins. Thus the lower-than-expected cytosolic staining may reflect loss of free cytosolic CAII during slide processing. It may also reflect the result that localized CAII at the membrane will stain much more intensely than diffuse cytosolic CAII.

**PKA does not phosphorylate NBC3Ct in vitro.** NBC3 interacts with the membrane scaffolding protein NHE regulatory factor (NHERF) via a PDZ binding motif at the extreme COOH terminus of NBC3 (22). NHERF, in turn, interacts with the PKA-associated protein AKAP ezrin, which binds the regulatory domain of PKA (42). The NBC3Ct-NHERF interaction suggests that NBC3Ct may be a substrate for PKA, especially in light of our finding that 8-BrCAMP treatment reduces NBC3 transport activity. We therefore investigated the possibility that PKA phosphorylates NBC3Ct. GST.NBC3Ct was treated in vitro with the catalytic subunit of PKA. PKA-mediated phosphorylation of NBC3Ct was first assessed by mass spectrometry. Addition of phosphate adds 80 Da to protein mass, yet no 80-Da shifted peak could be detected when PKA-treated NBC3Ct was analyzed (data not shown). To verify this finding, NBC3Ct was PKA-treated in the presence of [γ-32P]ATP. Again, no phosphorylation of NBC3Ct was detected (data not shown). We conclude that the effect of PKA on NBC3 activity in transiently transfected HEK-293 cells does not involve phosphorylation of the NBC3 COOH-terminal domain.

**Identification of the site of CAII interaction on NBC3Ct.** Site-directed mutagenesis of the putative CAB motif of AE1 has shown that the minimum binding requirements for CAII are a hydrophobic residue (usually leucine) followed by at least two acidic residues within the next four amino acids (38).
COOH terminus of AE1 contains four putative motifs, yet only the most membrane-proximal motif binds CAII (38). The COOH-terminal domain of NBC3 contains two consensus CAB motifs (CAB1 and CAB2) (Fig. 5A). In a previous report, limited proteolysis revealed two proteolytically sensitive regions in NBC3Ct (Fig. 5A), which were interpreted to be conformationally open potential protein binding sites (20). The most membrane-proximal protease-sensitive region is very close to CAB1. To examine the role of CAB1 and CAB2, we replaced the acidic residues within the putative CAB motifs with the corresponding amide residue (mutation to LNNLM and LQNNN for CAB1 and CAB2) (Fig. 5A), to neutralize charge in the CAB site with the simplest change possible. The pHᵢ recovery rates of the NBC3-CAB1 and CAB2 mutants were measured in transiently transfected HEK-293 cells using the NH₄Cl prepulse method of acid loading. The NBC3-CAB1 mutant had a transport rate that was 29 ± 22% of wild type, whereas the CAB2 mutant recovery rate was not statistically different from wild type (Fig. 5B). The dramatic effect of the CAB1 mutations is not explained by a failure of the protein to be processed appropriately to the cell surface, because assays of cell surface processing revealed that the fraction of total cellular NBC3 expressed at the plasma membrane was 48, 67, and 53% (n = 2) for wild-type NBC3, NBC3-CAB1, and NBC3-CAB2, respectively. The difference in rate of change of pHᵢ mediated by the CAB1 mutants also did not result from differences in the starting pHᵢ in transport assays; minimum pHᵢ values were 6.62 ± 0.03 and 6.55 ± 0.06 for the CAB1 and CAB2 mutants, respectively, which represents no statistically significant difference.

CAB mutations were recapitulated into NBC3 COOH-terminal domain GST fusion proteins GST.NBC3CtCAB1 and GST.NBC3CtCAB2, and the effect of CAII/NBC3 physical interaction was assessed. Microtiter dish CAII-binding assays revealed that GST.NBC3CtCAB1 mutant bound 7 ± 2% of the amount of CAII bound by wild-type NBC3, whereas GST.NBC3CtCAB2 binding was not statistically different from wild-type binding (Fig. 5C). We conclude that CAB1 motif mediates the CAII/NBC3 interaction.

**DISCUSSION**

Mechanisms to regulate the activity of membrane transport proteins are limited, because much of the protein is buried in the lipid bilayer, inaccessible to direct interaction with modulators. Only one surface of plasma membrane transporters is accessible to the cytosol, compounding the problem. Membrane transport can be chronically regulated by changes of expression level or acutely regulated by insertion of previously translated vesicle-localized transporters into the membrane, as occurs for Glut4 (34) and renal H⁺-ATPase. How else can membrane transport be regulated acutely? Renal HCO₃⁺ transport is acutely inhibited by cAMP-coupled agonists (2, 16). In this report, we examined the acute regulation of the NBC3 Na⁺/HCO₃⁻ cotransporter by cAMP and by the cytosolic enzyme CAII which catalyzes the reaction HCO₃⁻ + H⁺ ⇌ CO₂ + H₂O to produce the substrate for transport by NBC3 or in reverse mode to consume HCO₃⁻. CAB has large effects on the Cl⁻/HCO₃⁻ exchange activity of AE family members (30, 32). The presence of a consensus PKA site beside a consensus CAB site in NBC3 (Fig. 5) led to the following potential
Fig. 5. Identification of the CAII binding site within NBC3Ct. A: amino acid sequence of NBC3Ct (residues 1127-1214) with potential and utilized cleavage sites for trypsin indicated by single and double lines under the bar, respectively (20). Putative carboxylic anhydrase binding (CAB) motifs, CAB1 and CAB2, are indicated above the bar. The vertical arrow represents the potential PKA phosphorylation site identified by Peptools software. B: rate of pH recovery from acid loads was measured for HEK-293 cells transfected with wild-type NBC3 (WT) or NBC3-CAB1 (CAB1 was mutated to LNLNM) and NBC3-CAB2 mutants (CAB2 was mutated to LQNNN). C: binding of wild-type NBC3 and NBC3-CAB1 and CAB2 mutants to CAII, measured by using microtiter plate assay. Error bars represent SE (n = 3). *Statistical difference P < 0.05 relative to wild-type NBC3 (unpaired, 2-tailed t-test).

regulatory model for NBC3. cAMP-coupled agonists activate PKA, which in turn phosphorylates the NBC3 COOH terminus, displacing CAII from its binding site and thereby decreasing transport activity. Here we explored this regulatory mechanism and found that although parts were accurate, the model on the whole did not hold together.

We explored the interaction between CAII and NBC3. Consistent with what was found for Cl⁻/HCO₃⁻ exchangers, a single CAB site in the cytosolic COOH terminus was sufficient to stabilize the NBC3/CAII interaction. Microtiter plate binding assays revealed that the CAII/NBC3 binding had sufficient affinity (101 nM) to be physiologically relevant. Mutation of two aspartic acid residues (D1135 and D1136) in the CAB1 consensus CAB site of the NBC3 COOH terminus was sufficient to reduce CAII interaction with the COOH-terminal domain to only 7% of wild-type levels. The presence of only this single CAB site on NBC3 is supported by immunofluorescence data, which showed that, whereas wild-type NBC3 induced CAII to localize to the plasma membrane, cells transfected with an NBC3 deletion mutant lacking its COOH terminus previously shown to localize to intracellular membranes (20) showed a broad cytosolic CAII distribution, as found for cells not transfected with NBC3. Because the CAII/NBC3 interaction occurred in vitro, we conclude that the interaction is direct and does not require accessory factors.

Interaction between CAII and NBC3 is highly significant to NBC3 activity. Displacement of wild-type CAII from its binding site on the NBC3 COOH terminus was accomplished by expression of functionally inactive V143Y mutant CAII at levels 20-fold higher than wild-type CAII. V143Y CAII expression reduced NBC3 HCO₃⁻ transport activity by 31 ± 3%, in the absence of effects on NBC3 expression level or cell surface processing. More profound was the effect of mutation of the CAB site (CAB1) on NBC3 activity; NBC3 activity was inhibited by 71%. The significance of the result was underscored by the lack of effect on transport activity of mutation of the second consensus CAB site CAB2, which did not bind CAII. The larger effect of CA binding site mutation than V143Y CAII on NBC3 activity was also found for AE1 (32).

This likely reflects the fact that V143Y CAII was expressed at only 20-fold higher levels than wild-type CAII, so that ~5% of NBC3 molecules would be occupied by wild-type CAII. Also, V143Y CAII retains 1/3,000th of its catalytic activity. CAII, with turnover of 10⁶ s⁻¹ still retains a turnover rate that is significant relative to the turnover rate for NBC3, which is likely 10⁶-10⁷ s⁻¹.

Interaction between CAII and NBC3 increases the rate of pH recovery mediated by NBC3 in transfected cells. NBC3 is an electroneutral cotransporter that does not transport ions that can be detected with fluorescent dyes, which eliminates electrophysiological and fluorescent techniques for direct measurement of ion flux, respectively. Instead, we used the pH sensitive dye, BCECF, to determine changes in pHᵢ, which in turn was an indirect measurement of HCO₃⁻ flux because of the equilibrium reaction HCO₃⁻ + H⁺ ⇌ CO₂ + H₂O. Concentration gradients are often thought not to exist within the cytosol, because the rate of diffusion within free solution is much greater than across the plasma membrane. However, Vaughan-Jones and colleagues (36, 44) have established that H⁺ movement in biological systems is much slower than in nonphysiological solvents, because of fixed buffer molecules, viscosity, and other factors. How does CAII/NBC3 interaction facilitate NBC3 HCO₃⁻ transport? The acceleration of transport rate we observed with CAII requires direct binding by NBC3, because the expression of dominant-negative V143Y CAII and mutation of the NBC3 CAB1 site both reduced transport rate in the presence of endogenous wild-type CAII expression. Because NBC3 transport is driven by the Na⁺ and HCO₃⁻ gradients present at the surfaces of NBC3, localization of CAII to the COOH terminus of NBC3 will maximize the rate of HCO₃⁻ conversion to CO₂ at the site in which NBC3 brings HCO₃⁻ into the cell. This minimizes the local (HCO₃⁻) and maximizes the transmembrane (HCO₃⁻) gradient across each individual NBC3 molecule, maximizing transport rate.

In this report, we did not examine the effect of V143Y CAII on the efficiency of processing of NBC3 to the cell surface. If V143Y CAII inhibited NBC3 cell surface processing, we would observe a decrease in transport activity, which we
found. Although we did not measure the level of cell surface expression of NBC3 in the presence and absence of V143Y CAII, we believe that this is unnecessary for the following reasons. First, we measured the effect of V143Y CAII on the level of cell surface expression for AE1 (32) and NBC1 (1). In neither case with transporters related to NBC3 did we see an effect of V143Y CAII on surface processing efficiency. Second, we determined the cell surface processing of NBC3 vs. the NBC3 CAB1 and CAB2 mutants. Because the mutants were as well processed as wild-type NBC3, we were able to conclude that the impairment of NBC3 CAB1 function resulted from impaired transport rate, not inefficient cell surface processing. The NBC3 CAB1 data support the role of CAII interaction in activation of NBC3, consistent with the V143Y/NBC3 data. Thus we cannot absolutely rule out the possibility the V143Y CAII acts through effects on NBC3 cell surface processing. However, the balance of data is consistent with a model in which interaction of CAII with the NBC3 COOH-terminal region activates NBC3 transport function.

We found that treatment of NBC3-expressing HEK-293 cells with the membrane-permeant cAMP-derivative 8-Br-cAMP and the adrenergic agonist forskolin inhibited NBC3-mediated pH recovery rate by 38 ± 7 and 44 ± 3%, respectively, representing a strong, acute regulation of transport activity. Transport inhibition resulted from cAMP stimulation of PKA, because the PKA inhibitor H89 blocked the cAMP effect. Presence of a consensus PKA phosphorylation site adjacent to CAB1 suggested that PKA might mediate NBC3 inhibition by displacement of CAB. However, three lines of evidence showed that this was not the case. In vitro treatment of a GST fusion protein of the NBC3 COOH terminus with PKA did not result in any phosphorylation of the protein, as measured by mass spectrometry and radioactive phosphate incorporation. Second, treatment with PKA did not affect the in vitro interaction between CAII and NBC3Ct. Finally, the effects of V143Y CAII and 8-Br-cAMP were additive, suggesting that they act through independent pathways.

These data need to be interpreted carefully. Because the phosphorylation experiment was performed in vitro, it is possible that some cytosolic factor, like the PKA scaffolding proteins ezrin (42) and NHERF (22), may be required for phosphorylation of NBC3. Indeed, NHERF has already been shown to interact with NBC3 (22). Second, the definitive experiment would be to assay the effect of 8-Br-cAMP on the NBC3-CAB1 mutant, which cannot bind CAII. If cAMP acts by PKA-mediated displacement of CAII, then 8-Br-cAMP would have no effect on the CAB1 mutant. Conversely, if cAMP acts independently of CAII, it would still inhibit transport activity. Unfortunately this experiment could not be reliably performed, because the activity of the CAB1 mutant is already too low to be able to observe further reductions of activity. If the NBC3 COOH-terminal domain is not required for regulation by PKA, then it suggests that the NH2-terminal cytoplasmic domain is the site of action of PKA. Consistent with this possibility, analysis of the human NBC3 sequence reveals that, whereas the COOH-terminal domain has a single consensus PKA phosphorylation site (S1132, Fig. 5A), the NH2-terminal cytoplasmic domain has six, at amino acids 182, 225, 242, 260, 276, and 301. It is also possible that PKA does not act directly on NBC3. PKA may phosphorylate some protein, which is able to regulate NBC3 transport activity.

One other report (22) has examined the effect of cAMP on NBC3 transport activity. In that study, cAMP was mobilized by forskolin treatment of NBC3-transfected HEK-293 cells, the same used in the present report. The results of that paper are difficult to compare directly to the present study. In the previous report, it was found that expression of the CFTR Cl- channel did not affect NBC3 activity. However, treatment of NBC3/CFTR cotransfected cells with forskolin inhibited the rate of NBC3-mediated HCO3- transport by 67 ± 11%, compared with forskolin-treated cells that expressed NBC3 alone. This article did not report on the effect of forskolin on NBC3 in the absence of CFTR. The report also differed in methodology. In the present report, cells were incubated with forskolin or 8-Br-cAMP for 10–15 min before measuring the effect of the agent on NBC3 activity; in the previous paper (22), forskolin was added just before (estimated as 2 min) measurement of pH recovery rate. We found a lower degree of NBC3 inhibition by 8-Br-cAMP (38 ± 7%) and forskolin (44 ± 3%), which was independent of CFTR expression, because HEK-293 cells do not endogenously express CFTR (41). By combining the two data sets, it appears that cAMP mobilization inhibits NBC3 transport activity and that this inhibition is potentiated in the presence of CFTR. Prolonged adrenergic stimulation results in NBC3 inhibition even in the absence of CFTR.

The present report does conflict with the earlier report (22) in the effect of the PKA inhibitor H89. Here, H89 fully blocked the inhibitory effect of forskolin on NBC3. In contrast, the earlier report found that H89 had a slight additional inhibitory effect on forskolin-treated NBC3 but blocked the inhibitory effect of CFTR on NBC3 activity. The basis for the difference in results is not clear. However, it may be related to methodology. In the present report, effects of compounds were internally controlled. That is, NBC3 transport rate was measured for NBC3 alone and again after treatment of the same set of cells. Summarized results presented in Fig. 2 are the average of these internally controlled data. The difference may also relate to the length of time of incubation with H89. We incubated with H89 for 5 min before treatment with forskolin for a total of 15–20 min of H89 treatment. In the previous report, H89 and forskolin were coadministered, which may have reduced the efficacy of H89.

Although our data do not support modulation of CAB as the mechanism by which cAMP regulates transport, our data do suggest that during extreme acidosis, pH may regulate transport activity, in part through increasing CAB to NBC3. Binding between CAII and NBC3 was pH dependent with an acidic maximum and reduced binding at alkaline pH, similar to what was previously found for the interaction of the AE1 Cl-/HCO3- exchanger with CAII (39). The increased interaction of CAII at acid pH would help to activate NBC3 during recovery from acid loads, a physiological function of the transporter.

We examined the regulation of the human NBC3 Na+/HCO3- cotransporter by CAII, cAMP, and pH. CAII binds amino acids D1135-D1136 in the COOH-terminal cytoplasmic tail of NBC3. Inhibition of the NBC3/CAII interaction, either by expression of functionally inactive CAII or mutation of the CAB site on NBC3, reduced NBC3 activity, consistent with a model in which CAII interaction activates NBC3 transport activity. Because the CAII/NBC3 interaction increases at acid pH, the interaction contributes to pH-dependent regulation of
NBC3 activity. Activation of the cAMP-coupled signaling pathway with 8-BrcAMP or forskolin reduced NBC3 transport rate. However, we could find no evidence for phosphorylation of the NBC3 COOH-terminal tail by PKA. Similarly, the additivity of the 8-BrcAMP and V143Y CAIL effects led us to conclude that cAMP inhibits NBC3 activity through a mechanism independent of CAIL.

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