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Carbonic Anhydrase II Binds to and Enhances Activity of the Na^+/H^+ Exchanger*

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We examined the ability of carbonic anhydrase II to bind to and affect the transport efficiency of the NHE1 isoform of the mammalian Na^+/H^+ exchanger. The C-terminal region of NHE1 was expressed in *Escherichia coli* fused with an N-terminal glutathionine *S*-transferase or with a C-terminal polyhistidine tag. Using a microtiter plate binding assay we showed that the C-terminal region of NHE1 binds carbonic anhydrase II (CAII) and binding was stimulated by low pH and blocked by antibodies against the C-terminal of NHE1. The binding to NHE1 was confirmed by demonstrating protein-protein interaction using affinity blotting with CAII and immobilized NHE1 fusion proteins. CAII coimmunoprecipitated with NHE1 from CHO cells suggesting the proteins form a complex *in vivo*. In cells expressing CAII and NHE1, the H^+ transport rate was almost 2-fold greater than in cells expressing NHE1 alone. The CAII inhibitor acetazolamide significantly decreased the H^+ transport rate of NHE1 and transfection with a dominant negative CAII inhibited NHE1 activity. Phosphorylation of the C-terminal of NHE1 greatly increased the binding of CAII. Our study suggests that NHE1 transport efficiency is influenced by CAII, likely through a direct interaction at the C-terminal region. Regulation of NHE1 activity by phosphorylation could involve modulation of CAII binding.

The Na^+/H^+ exchanger (NHE)[1] is a ubiquitously expressed integral membrane glycoprotein that functions to exchange one intracellular proton for one extracellular sodium, thereby protecting cells from intracellular acidification (1). Several known isoforms of the Na^+/H^+ exchanger have been designated NHE1–NHE7. NHE1 was the first isoform cloned (2) and is ubiquitously expressed in the plasma membrane of mammalian cells, with the other isoforms having more restricted tissue distributions (3). In mammals, NHE1 plays a key role in regulation of cell pH, cell volume, and cell proliferation (4). It is also critically involved in the damage that occurs to the myocardium with ischemia and reperfusion (5).

The Na^+/H^+ exchanger (NHE1 isoform) consists of two structural and functional domains, a 500 amino acid N-terminal membrane domain that is responsible for ion transport, and a C-terminal cytoplasmic domain of ~300 amino acids that regulates activity of the membrane domain (1). The large cytoplasmic domain is involved in protein-protein interactions with a number of proteins including calcineurin homologous protein (6), calmodulin (7), and heat shock protein (8). In addition the Na^+/H^+ exchanger is subject to regulation by phosphorylation that stimulates transport activity (9).

Carbonic anhydrases catalyze the hydration of CO_2 to produce HCO_3^- and H^+. The predominant cytoplasmic isozyme is carbonic anhydrase II (CAII) (10). CAII deficiency is associated with osteopetrosis, renal tubular acidosis, and cerebral calcification (11). In the red blood cell, CA activity is required for efficient Cl^-/HCO_3^- exchange by the anion exchanger AE1 (12). Recently it was discovered (13) that CAII binds directly to AE1. The binding site was located in acidic residues (^{887}DADD) of the C-terminal cytosolic region of AE1 (14). The binding site for AE1 was localized to the basic N-terminal of CAII (15).

Since the activity of CAII can result in proton production, an association of CAII with the Na^+/H^+ exchanger could facilitate proton removal. Several reports have supported the notion that the Na^+/H^+ exchanger is in some way associated with CA and AE. It was demonstrated earlier that the CA inhibitor acetazolamide could result in a reduction of Na^+/H^+ exchanger activity (16). The Na^+/H^+ exchanger and the AE both localize to the same protruding lamellipodium regions of some cell types (17). Also, the AE has long been shown to be linked to the cytoskeleton (18), and the Na^+/H^+ exchanger has also recently been shown to be linked to the cytoskeleton (19). It is also interesting to note that the presence of NHE1 has been shown to be essential for the regulation or functional expression of HCO_3^--dependent and -independent transporters in neurons (20), suggesting that AE and CA interact with and may in some way be regulated by the Na^+/H^+ exchanger. In this report we examine the hypothesis that NHE1 binds to CAII. We demonstrate a direct interaction of CAII with the Na^+/H^+ exchanger and give evidence of effects on activity. The results support the hypothesis that Na^+/H^+ exchanger activity is linked to bicarbonate-based pH regulation possibly through carbonic anhydrase activity.

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[1] The abbreviations used are: NHE, Na^+/H^+ exchanger; CAII, carbonic anhydrase II; NTA, nitrilotriacetic acid; MEM, minimum essential medium; HA, hemagglutinin; RIPA, radioimmune precipitation assay buffer; TBS, Tris-buffered saline; PBS, phosphate-buffered saline; Ab, antibody; DSP, dithiobis(succinimidylpropionate).

EXPERIMENTAL PROCEDURES

Materials—Restriction enzymes, *E. coli* BL21-SI, pDest 17, and related GATEWAY™ cloning items were from Invitrogen. pGEX-3X, glutathione-Sepharose 4B and protein A-Sepharose CL-4B were from Amersham Biosciences. Glutathione, CAII protein (from rabbit), nigericin, phenylenediamine, and acetazolamide were from Sigma. Rabbit anti-human CAII polyclonal antibody was from Abcam Ltd, (Cambridge, UK), and rabbit anti-hemagglutinin (HA) and Protein G-PLUS agarose were from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit anti-His tag antibody was purchased from BioWorld, Dublin, OH. Conjugated antibodies were from Jackson ImmunoResearch (Mississauga, Ont.). Ni-NTA-agarose resin was from Qiagen (Valencia, CA). α-Casein (dephosphorylated) was from Sigma and casein kinase II (human recombinant) was from Cederlane laboratories (Hornby, Ont.). DSP (dithiobis(succinimidylpropionate)) was purchased from Pierce.

Construction and Purification of Na^+/H^+ Exchanger Fusion Proteins—The C-terminal 178 amino acid sequence of the rabbit cardiac Na^+/H^+ exchanger was expressed as a fusion protein with GST (GST178) using the plasmid pGEX-3X as described previously (21). The *E. coli* TOPP2 strain was induced with 1 mM isopropylthiol- β-D-galactoside. GST178 was purified via glutathione-Sepharose 4B affinity chromatography as described earlier (21). The C-terminal 182 amino acids of the rabbit Na^+/H^+ exchanger (NHE1) were expressed as a fusion protein with a C-terminal histidine tag (His182) using the plasmid pDest 17 and the Gateway™ Cloning System. The *E. coli* strain BL21-SI strain was induced with 0.3 M NaCl for 3 h. His182 protein was purified via Ni-NTA affinity chromatography as described by the manufacturer (Qiagen).

Cell Culture and Transfections—A Chinese hamster ovary cell line (AP1 cells) that was previously selected to lack endogenous NHE activity (22) was grown in a humidified atmosphere of 5% CO_2 and 95% air in α-MEM medium supplemented with 10% (v/v) fetal bovine serum, 25 mM HEPES, penicillin (100 units/ml), and streptomycin (100 μg/ml), pH 7.4 at 37 °C. Stable transfections were made and selected by the calcium phosphate technique essentially as described earlier (23). The plasmid pYN4+ contains the HA-tagged NHE1 isoform of the human Na^+/H^+ exchanger (23), and the plasmid pJRC36 encodes human CAII (24). Both were behind the constitutively active cytomegalovirus (CMV) promoter and were used to stably transfect AP1 cells as described earlier (23). Where indicated, transient transfections were used to introduce plasmids as described earlier (24). For dominant negative experiments an inactive mutant of CAII was used, which possessed the V143Y mutation (24, 25).

Measurement of Intracellular pH—NHE activity was measured fluorometrically using 2′,7 -bis(2-carboxyethyl)-5 (6) carboxyfluorescein-AM (BCECF-AM) essentially as described previously (26, 27). pH regulation by the Na^+/H^+ exchanger was examined in (un- or mock-transfected) AP1 cells, AP1/pYN4+ stably transfected cells and AP1/pYN4+/pJRC36 stably transfected cells. Cells were grown on glass coverslips and the acetoxymethyl ester of BCECF-AM was used to measure pH_i. Cells on the coverslips were incubated with BCECF-AM for 18 min at 37 °C and placed into a holder device and inserted into a fluorescence cuvette at room temperature. The cuvette was supplied with 5 mM HEPES buffer bubbled with 100% O_2 (pH 7.4 ± 0.5) with a constant flow of 3.5 ml/min and shifted into a buffer containing 25 mM HCO_3^- buffer whenever appropriate. HCO_3^- solutions were bubbled with 5% CO_2, 95% air to create dissolved CO_2 where appropriate as described earlier (27). The intracellular pH was measured using the dual excitation single emission ratio technique with a PTI spectrofluorophotometer. Excitation wavelengths were 440 and 490 nm, and the emission wavelength was 520 nm. Acetazolmide (100 μM) was added into the buffer when indicated. A calibration curve for intracellular dye was generated using the high K^+-nigericin technique for each coverslip (24). The ΔpH/min was calculated using Sigma plot software. The use of CO_2/HCO_3^- to acidify cells for measurement of Na^+/H^+ exchanger activity has been used earlier (28, 29).

Western Blot of Na^+/H^+ Exchanger and Carbonic Anhydrase—AP1 cells transfected with pYN4+ or both pYN4+ and pJRC36 were grown in 60-mm Petri dishes. They were treated with RIPA lysate buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 0.25% sodium deoxycholate, and a proteinase inhibitor mixture) (8) to extract cellular proteins. Total cell extracts were fractionated by 12% SDS-PAGE and transferred to nitrocellulose as described earlier (8). Mouse monoclonal anti-HA tag antibody (1:2000) and rabbit anti-human CAII antibody (1:50,000) were used to check for the expression of transfected proteins.

To examine CAII binding to the Na^+/H^+ exchanger immobilized on nitrocellulose, GST, GST178, and His182 proteins were separated on 12% SDS-PAGE and then transferred to nitrocellulose membranes (8). Nitrocellulose membranes were blocked with 10% (w/v) skim milk powder in TBS (20 mM Tris, pH 7.4, 137 mM NaCl) for 5 h at 4 °C. They were then incubated with 10 μg of CAII with 1% (w/v) skim milk powder in TBS and rocked gently overnight at 4 °C. Membranes were washed with TBS for 4 × 15 min at room temperature. The nitrocellulose was then incubated with rabbit anti-CAII antibody (1:50,000) in TBS with 1% skim milk powder for 2 h at room temperature followed by washing for another hour with TBS. Further amplification was achieved by a subsequent incubation with goat anti-rabbit-horseradish peroxidase antibodies. Reactive bands were visualized by the Amersham Biosciences Enhanced Chemiluminescence system.

Co-immunoprecipition of NHE1 and CAII—All steps were performed at 4 °C unless otherwise noted. AP1 cells, AP1/pYN4+, and AP1/pYN4+/pJRC36-transfected cells were washed with phosphate-buffered saline (PBS, 150 mM NaCl, 5 mM sodium phosphate, pH 7.4) and frozen in 2 ml of RIPA buffer in the absence of detergent (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 80 mM NaF, 5 mM EDTA, 1 mM EGTA, 1 mM sodium orthovanadate, and proteinase inhibitor mixture) by placing cells on dry ice. Cells were defrosted and removed from the flask followed by sonication for 15 s. The lysate was centrifuged (35,000 × *g* for 1 h), and the pellet containing the Na^+/H^+ exchanger was resuspended and sonicated for 15 s in 2 ml of RIPA buffer with detergent (1% Nonidet P-40, 0.5% deoxychlolate). After centrifugation at 10,000 × *g* for 30 min, the supernatant was collected for immunoprecipitation. The supernatant, containing Na^+/H^+ exchanger, was rocked overnight with 7.5 μl of rabbit anti-HA tag polyclonal antibody. Protein A-Sepharose was added, and the sample was incubated for a further 2 h. The resin was washed with RIPA buffer, and bound protein was solubilized with SDS-PAGE sample buffer. Proteins were transferred to nitrocellulose after SDS-PAGE and probed with anti-CAII antibody. For some experiments to obtain a more quantitative co-immunoprecipitation of CAII and the Na^+/H^+ exchanger a cross-linking reagent was used. DSP was added to cells at a final concentration of 2 mM for 30 min at room temperature. The reaction was terminated by addition of Tris, pH 7.5, to a final concentration of 10 mM. Cells were then washed with phosphate-buffered saline, and the immunoprecipitation was continued as described above.

Microtiter Plate Binding Assay—Purified CAII (0.2 μg/well) was immobilized onto 96-well microtiter plates by overnight incubation in buffer containing 1.25 mg/ml of 1-cyclohexyl-3-(2-morpholinoethy) carbodiimide metho-*p*-toluene sulfonate in 150 mM NaCl, 100 mM sodium phosphate, pH 6.0 at 4 °C. Plates were then washed extensively with PBST (150 mM NaCl, 5 mM sodium phosphate, 0.1% Triton X-100, pH 7.5) and blocked for 1 h at 37 °C in PBS with 0.5% bovine serum albumin. The bound CAII was shown to be active by an esterase assay (13). Plates were washed with PBST and incubated for 1 h with 0–100 nM GST178 or His182 in Ab buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 5 mM EDTA, 0.1% $C_{12}E_8$, 0.25% gelatin) at 37 °C. Purified GST was used as a control. Plates were washed with PBST and incubated with rabbit anti-GST (1:4000) antibody or with anti-His tag antibody (1:2000) for 1 h at 37 °C. Plates were further washed, and goat anti-rabbit horse radish peroxidase (1:4000) antibody was added for 1 h at 37 °C. Red color was developed by 0.1% *o*-phenylenediamine in substrate buffer (50 mM citric acid, 5 mM sodium phosphate, pH 5.0, 0.09% H_2O_2). The reaction was terminated with 50 μl of 3 M H_2SO_4 per well, and absorbance of microtiter plates was read at A_{450}. For some experiments the pH or incubation time of Ab buffer was varied as indicated in the figure legends. In other experiments an antibody against the C-terminal 178 amino acids of the Na^+/H^+ exchanger (described earlier, Ref. 8) was premixed with His182 in the Ab buffer at the indicated concentrations. This was then immediately incubated with immobilized CAII.

In Vitro Phosphorylation of Proteins—In some experiments cell extracts from rabbit ventricular muscle were used to phosphorylate the His182 fusion protein. Cell extracts and *in vitro* phosphorylation of the His182 fusion protein were as described earlier (9). In some cases primary cultures of isolated myocyte cells were grown overnight in serum-free medium (unstimulated), as opposed to serum-containing medium (stimulated) to reduce the activity of NHE1-directed protein kinases as described earlier (9). After phosphorylation (or mock phosphorylation of controls) by cell extracts the Na^+/H^+ exchanger fusion protein (His182) was removed from the cell extracts using Ni-NTA-linked agarose. Phosphorylated and non-phosphorylated His182 proteins were used to examine CAII binding to the Na^+/H^+ exchanger immobilized on nitrocellulose as described above. To confirm that equal amounts of phosphorylated and non-phosphorylated protein were present, nitrocellulose transfers were examined by Ponceau S staining.

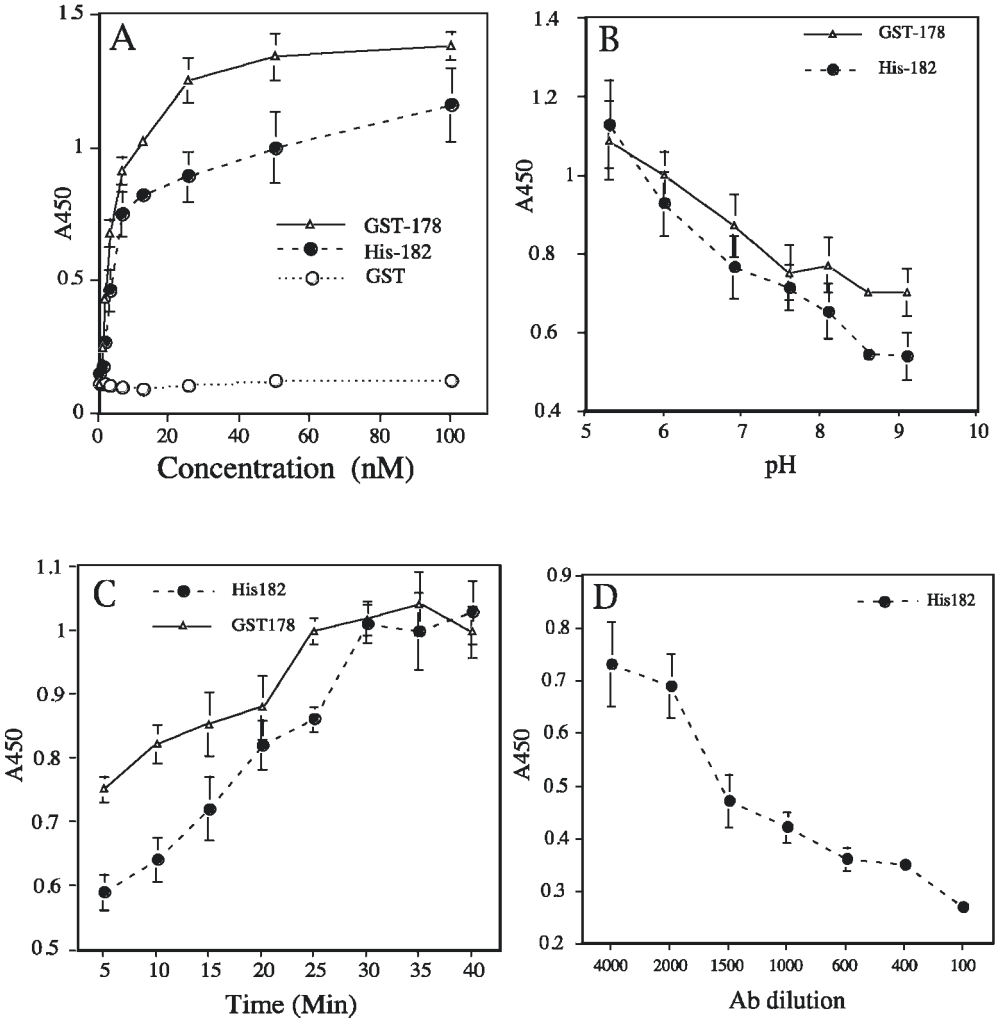

FIG. 1. **Solid phase microtiter plate binding assay for Na^+/H^+ exchanger and CAII interactions.** CAII was immobilized to microtiter plates as described under "Experimental Procedures." The fusion protein concentration was 20 nM (*B–D*). *A*, increasing concentrations of GST178, His182, or GST proteins were added to CAII immobilized to microtiter plates. Bound proteins were detected with anti-GST antibody or anti-His tag antibody. *B*, effect of varying the pH of interaction medium on interaction of NHE1 fusion proteins with immobilized CAII (0.2 μg/well). *C*, effect of varying the time of incubation between CAII and NHEI fusion proteins. *D*, inhibition of CAII and NHE1 interaction by addition of varying amounts of anti-NHE1 antibody. Results are mean ± S.E. of at least four experiments.

Some *in vitro* phosphorylation experiments contained ^{32}P to confirm *in vitro* phosphorylation of the protein. In these experiments the final ATP concentration was 250 μM. In experiments without ^{32}P labeling the final ATP concentration was 1 mM.

In some experiments we examined the binding of CAII to phosphorylated and unphosphorylated casein. To phosphorylate casein (4 μg) was treated with casein kinase 2 enzyme in a reaction consisting of 100 mM Tris-HCl, pH 8.0, 20 mM $MgCl_2$, 50 mM KCl, 100 mM NaCl, 2.5 mM EGTA, 0.2 mM EDTA, 3 mM ATP (or 1 μl of [γ-^{32}P]ATP) and 1 μl of kinase (500 units/μl) for 30 min at 30 °C as described by others (30). Some *in vitro* phosphorylation experiments contained ^{32}P to confirm *in vitro* phosphorylation of the protein. Phosphorylated and unphosphorylated casein was then used to examine CAII binding while immobilized on nitrocellulose as described above for the Na^+/H^+ exchanger.

RESULTS

Protein Production and Purification—To study the C-terminal region of the Na^+/H^+ exchanger it was produced as two independent fusion proteins. The fusion proteins contained amino acids 635–816 or 639–816, respectively, of the rabbit NHE1 protein with histidine and GST tags respectively. The identities of the induced proteins were confirmed using an antibody generated against the C-terminal region of Na^+/H^+ exchanger (not shown) (8). The proteins were purified using the standard procedures with either glutathione-Sepharose or Ni-NTA affinity chromatography.

Na^+/H^+ Exchanger Binding to CAII—A solid phase binding assay was used to examine the interaction between the C terminus of the Na^+/H^+ exchanger (GST178, His182) and CAII. CAII was immobilized on microtiter plates and possessed enzymatic activity indicating it had retained a native conformation (13). The binding curves of GST178, His182 and GST to immobilized CAII (Fig. 2*A*) showed that the amount of GST178 and His182 binding increased with increasing concentrations and saturated at higher levels. Under the identical conditions, only a low background binding to GST was observed, suggesting the binding was caused by the Na^+/H^+ exchanger part of the molecules. There was no indication of cooperatively from the shape of the curve. We tested the effect of varying the pH of the incubation medium on the interaction between CAII and NHE1. The results (Fig. 1*B*) showed that in acidic medium the interaction between NHE1 and CAII was increased. A time course (Fig. 1*C*) of the interaction between NHE1 and CAII showed that the association was time-dependent reaching saturation in 30 min. To confirm that the interaction was specifically due to the association between the C terminus of NHE1 and CAII we used an antibody generated against an independently made, different fusion protein of NHE1 directed toward the C-terminal 178 amino acids (8). The results (Fig. 2*D*)

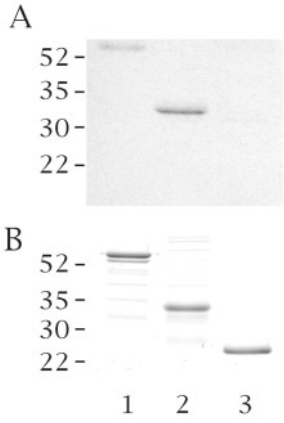

FIG. 2. **Affinity blotting assay of CAII with Na^+/H^+ exchanger fusion proteins.** Fusion proteins were separated with SDS-PAGE and transferred to nitrocellulose. *Lanes 1–3* are 10 μg of the proteins GST178, His182, and GST proteins. *A*, nitrocellulose transfer of proteins probed with CAII. *B*, Coommassie Blue stain of corresponding proteins in SDS-PAGE. Results are typical of three experiments.

showed that this antibody blocked the association of CAII with NHE1.

To confirm the results of the solid phase assay we used an affinity blotting technique. Equal amounts (10 μg) of purified GST178, His182, or GST were run on SDS-PAGE, transferred to nitrocellulose membranes, and probed with CAII. The results (Fig. 2) showed that both the His-tagged and the GST-tagged Na^+/H^+ exchanger C-terminal proteins (GST178 and His182) bound CAII. Purified GST alone did not bind CAII.

In Vivo Interactions of CAII and NHE1—To determine if the Na^+/H^+ exchanger can interact with CAII *in vivo* we made stable cell lines of AP1 cells expressing the Na^+/H^+ exchanger (pYN4+) alone or cells expressing the Na^+/H^+ exchanger plus CAII (pYN4+/pJRC36). Fig. 3 illustrates the analysis of the cell lines. Fig. 3*A* demonstrates the presence of the Na^+/H^+ exchanger protein in cells lines transfected with HA-tagged Na^+/H^+ exchanger protein. We usually found a larger form of the NHE1 protein of ~105–110 kDa plus a smaller form about 90–95 kDa in size. This result is commonly found with the smaller isoform representing unglycosylated or partially glycosylated protein (26, 31). Fig. 3, *B* and *C*, demonstrate the presence of CAII and Na^+/H^+ exchanger, respectively, in cells stably co-transfected with pYN4+/pJRC36 plasmids. To examine if an interaction between CAII and the Na^+/H^+ exchanger occurs *in vivo*, co-transfected actively growing cells were used for immunoprecipitation with anti-HA antibody. Fig. 3*D* illustrates the results of immunoblotting of the immunoprecipitates. *Lanes 1* and *2* contained CAII immunoreactive protein of the same size as purified CAII protein (*lane 4*). *Lane 3* contained immunoprecipitates of untransfected AP1 cells and did not show any CAII immunoreactive species. The results show that a complex of CAII and NHE1 can be isolated from co-transfected cells. In this experiment we did not use a cross-linker to secure the CAII to the Na^+/H^+ exchanger, and therefore the results could be described qualitatively only.

Physiologic Effects of CAII on $Na^+/\dot{V}AH^+$ Exchanger Activity—To determine if CAII binding influences the activity of the Na^+/H^+ exchanger we examined pH regulation in AP1, AP1/pYN4+, and AP1/pYN4+/pJRC36 cells. Fig. 4*A* illustrates examples of the effects obtained during transient induction of acid load by shifting cells from 0_2-gassed nominally CO_2-free medium to CO_2/HCO_3^--containing medium as described by others (27–29). AP1 cells, lacking the Na^+/H^+ exchanger, showed only very small amounts of alkalization (recovery) after acid load. Stably transfected AP1/pYN4+ cells showed a much

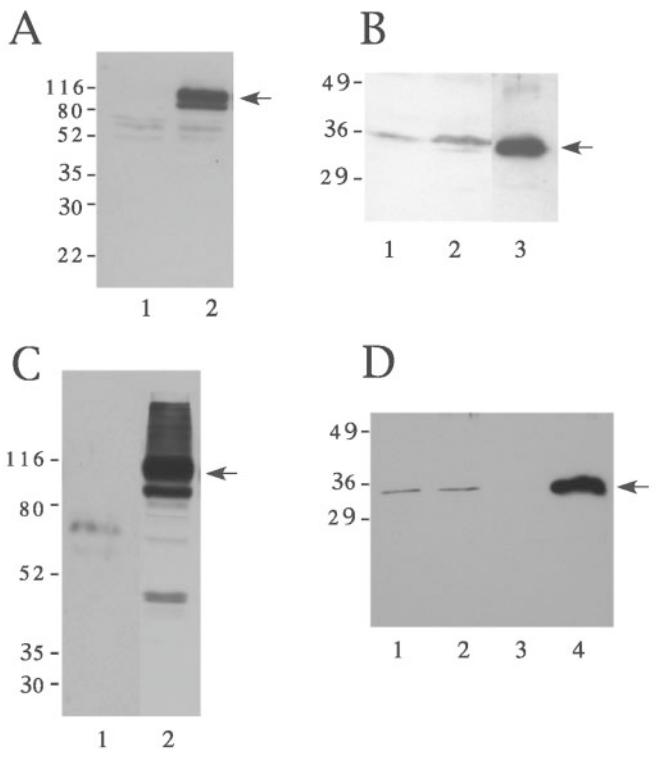

FIG. 3. **Immunoblot of NHE1 and CAII in cell lines stably transfected with Na^+/H^+ exchanger and CAII.** *A*, immunoblot with anti-HA antibody (directed against the HA tag of the Na^+/H^+ exchanger). *Lane 1*, mock-transfected AP1 cells. *Lane 2*, AP1 cells transfected with the pYN4+ plasmid containing HA-tagged Na^+/H^+ exchanger (*NHE1*). *Arrow* indicates the position of the 105–110 kDa NHE1 immunoreactive band. *B*, immunoblot probed with anti-CAII antibody. *Lane 1*, mock-transfected AP1 cells. *Lane 2*, AP1 cells transfected with the pYN4+ plasmid containing HA-tagged Na^+/H^+ exchanger (NHE1) and pJRC36 containing CAII. *Lane 3*, positive control, 1 μg of purified CAII protein. *Arrow* indicates the position of the CAII immunoreactive bands. *C*, immunoblot probed with anti-HA antibody. *Lane 1*, mock-transfected AP1 cells. *Lane 2*, AP1 cells transfected with the pYN4+ plasmid containing HA-tagged Na^+/H^+ exchanger (*NHE1*) and pJRC36 containing CAII. *Arrow* indicates the position of the major 105–110-kDa NHE1 immunoreactive band. *D*, immunoblot probed with anti-CAII antibody of immunoprecipitates. The immunoprecipitation was with anti-HA antibody. *Lane 1*, AP1 cells transfected with the pYN4+ plasmid (alone) containing the Na^+/H^+ exchanger. *Lane 2*, AP1 cells transfected with pYN4+ and pJRC36 plasmids containing the Na^+/H^+ exchanger and CAII respectively. *Lane 3*, mock-transfected AP1 cells. *Lane 4*, positive control, 1 of μg purified CAII protein. *Arrow* indicates the position of the CAII immunoreactive bands.

greater recovery (Fig. 4*A*), indicating that the Na^+/H^+ exchanger extruded H^+s and alkalinized the cells. The results shown were typical of several independently made cell lines. Stable transfectants of AP1/pYN4+/pJRC36 cells also alkalinized after acid load; however, their recovery was faster than AP1/pYN4+ cells, indicating that cotransfection of CAII with NHE1 stimulated H^+ transport by the Na^+/H^+ exchanger (Fig. 4*A*). This effect was reduced by treatment with acetazolamide. Fig. 4*B* summarizes these results. The amiloride analogue 5-(*N*,*N*-hexamethylene)-amiloride inhibited the recovery indicating that it was due to the Na^+/H^+ exchanger (not shown). Fig. 4*B* also shows that treatment with the CA inhibitor acetazolamide can affect Na^+/H^+ exchanger activity of (AP1/pYN4+). The H^+ transport rate of AP1/NHE1 cells was reduced 37 ± 7.8% (n = 5) when cells were treated with 100 μM acetazolamide (Fig. 4*B*). Acetazolamide also reduced the transport rate of AP1/NHE1/CAII cells by 74% (n = 5). To confirm that a direct interaction between CAII and NHE1 was necessary for stimulation of NHE1 activity, we transiently trans-

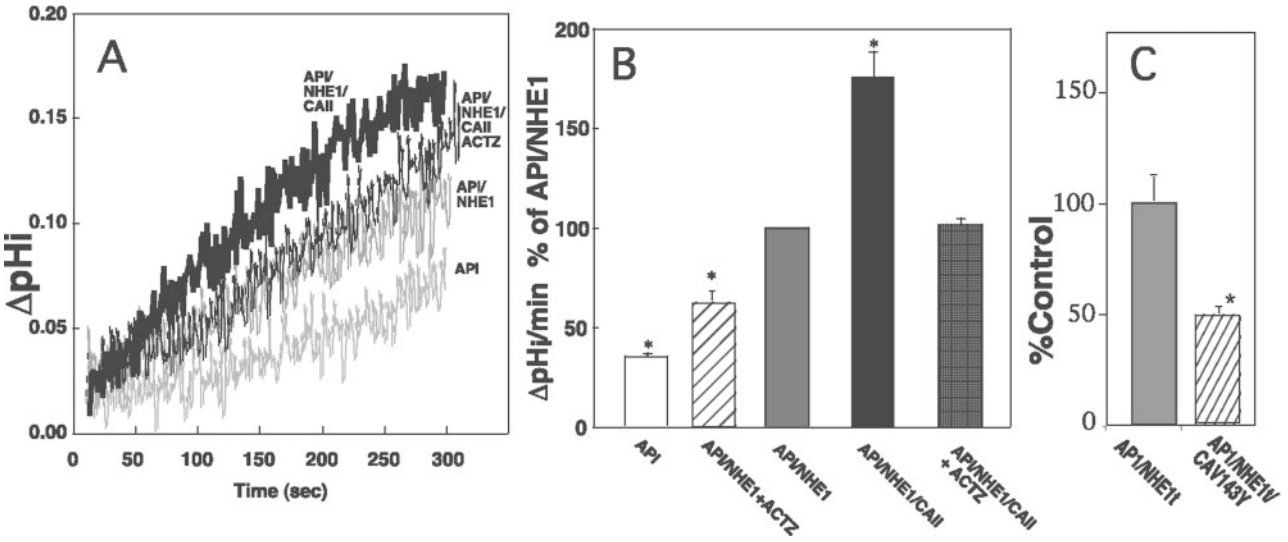

FIG. 4. **Effects of CAII expression and acetazolamide on H^+ transport rate of AP1 cells in the presence of bicarbonate.** Cell lines were initially bathed in O_2 bubbled HEPES-containing buffer (pH 7.4 ± 0.5) and then shifted to CO_2/HCO_3^--containing buffer to create a bicarbonate-dependent acid load. The rate of recovery from an acid load was quantified as described earlier (27). *A*, examples of effects on intracellular pH. Representative effects on rate of recovery from an acid load induced by CO_2/HCO_3^- are illustrated. *AP1* refers to AP1 cells that do not have an endogenous Na^+/H^+ exchanger. *AP1/NHE1* refers to AP1 cells stably transfected with the plasmid pYN4+ that expresses the Na^+/H^+ exchanger (NHE1). AP1/pYN4+/CAII are AP1 cells stably transfected with NHE1 and the plasmid pJRC36 that expresses CAII. ACTZ indicates that the appropriate cells were treated with 100 μM acetazolamide. *B*, summary of H^+ transport rates of Na^+/H^+ exchanger and Na^+/H^+ exchanger and CAII-transfected cells. Results are mean ± S.E. of at least seven experiments. Abbreviations are as in *A*. *Asterisk* indicates statistically significant difference from AP1/NHE1 cells at $p < 0{,}05$. *C*, effect of dominant negative CAII with a V143Y mutation on Na^+/H^+ exchanger activity of AP1 cells. AP1 cells stably transfected with NHE1 were transiently transfected with either empty vector (*AP1/NHE1*) or CAII with a V143Y mutation (*AP1/NHE1/CAV143Y*). The activity of NHE1 was measured as described in the legend for Fig. 4. Results are mean ± S.E. of four experiments.

fected the dominant negative CAII mutant with a V143Y mutation into AP1/pYN4+ cells. We found that this resulted in approximately a 50% decrease in NHE1 activity (Fig. 4*C*, $n = 4$) likely due to displacement of endogenous CAII from its binding site on NHE1. Transfection with equivalent amount of vector without CAII had no effect on NHE1 activity (not shown).

Effects of Phosphorylation on CAII Binding to NHE1—Because phosphorylation of NHE1 has been shown to be stimulatory to activity (9) we examined if phosphorylation could influence the binding of CAII to the C-terminal of NHE1. Fig. 5*A* (*lane 1*) confirms that cell extracts from rabbit ventricles phosphorylate the His182 fusion protein. As a control, we also phosphorylated commercially obtained casein, using casein kinase II (*lane 3*). We then examined the effect of phosphorylation of CAII binding. Fig. 5*B* compares the binding of CAII to equal amounts of phosphorylated (*lane 1*) and non-phosphorylated (*lane 2*) His182 protein and phosphorylated (*lane 3*) and non-phosphorylated (*lane 4*) casein. Phosphorylated NHE1 C-terminal protein bound much larger amounts of CAII than unphosphorylated protein. The effect was seen in over seven independent experiments with the His182 protein. Phosphorylation of the NHE1 protein also caused a slight mobility shift in the protein typical of proteins with added phosphate moieties. Neither phosphorylated or non-phosphorylated casein bound CAII. To compare the effect of different amounts of phosphorylation activity from cells we examined the effects of isolated myocyte extracts treated or untreated with serum as described under "Experimental Procedures." Fig. 5*C* shows that unstimulated extracts phosphorylated the His182 protein to a lesser degree than stimulated extracts. The same extracts were used to treat the His182 protein and then the binding of CAII was examined. The results are shown in Fig. 5*D*. Extracts from active, stimulated cells that caused a higher degree of phosphorylation (*lanes 2* and *4*, *stimulated*), resulted in greater binding of the CAII protein to His182 than cells that caused a lesser degree of phosphorylation (*lanes 1* and *3*). The amount of increase in CAII binding by increased levels of phosphorylation was between 45 and 60% in three different experiments. To examine the effect of phosphorylation *in vivo* on the binding of CAII to the Na^+/H^+ exchanger we used a cross-linking reagent, DSP, to make the linkage between the two proteins more stable and more quantitative during the immunoprecipitation process. DSP contains a thiol-cleavable linkage, and the samples were incubated in SDS-PAGE sample buffer containing β-mercaptoethanol prior to electrophoresis. The results are shown in Fig. 5*E*. *Lanes 2* and *3* illustrate immunoprecipitated CAII from cells transfected with Na^+/H^+ exchanger and CAII. The amount immunopreciptated from cells in the presence of serum (*lane 2*) was always greater than that in the absence of serum (*lane 3*). *Lanes 4* and *5* illustrate a similar experiment but with cells transfected with only the Na^+/H^+ exchanger. More CAII immunoprecipited in the presence of serum (*lane 4*) than in its absence (*lane 5*). In addition the amount of CAII immunoprecipitated in these cells in the presence of serum (*lane 4*) was reduced by about 40%, compared with cells transfected with additional CAII (*lane 2*). In the absence of serum there was no difference in the amount immunoprecipitated from cells transfected with or without exogenous CAII, and this amount was always small, about 25–35% of the amount of CAII immunoprecipitated from serum-stimulated cells. Reprobing the immunoblot with anti-HA antibody demonstrated that the equivalent amount of Na^+/H^+ exchanger was present in *lanes 2–5* (not shown).

DISCUSSION

The Na^+/H^+ exchanger is an essential part of pH homeostasis in mammalian cells. Regulation of the Na^+/H^+ exchanger has been the subject of many investigations but is still not well understood at the molecular level. The C-terminal, hydrophilic domain of Na^+/H^+ exchanger regulates the activity of the membrane domain that transports the Na^+ and H^+ ions (4, 32).

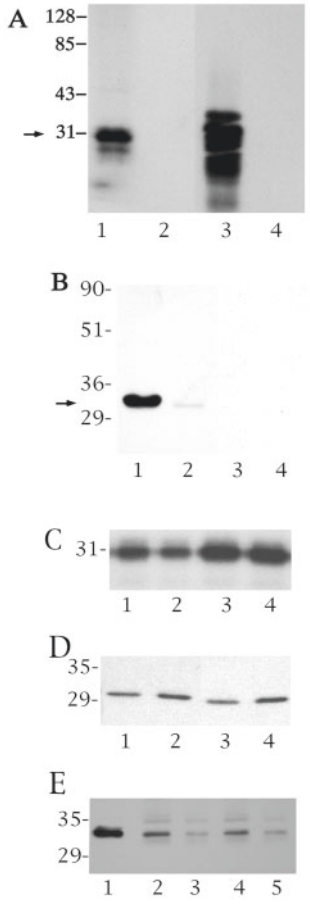

FIG. 5. **Effect of phosphorylation on CAII binding to the Na^+/H^+ exchanger.** *A–D*, purified His182 protein was incubated with ATP and a cell lysate prepared from rabbit ventricular myocardium. *A*, autoradiogram of His182 fusion protein and casein phosphorylated in the presence of [γ-^{32}P]ATP. *Arrow* denotes the phosphorylated His182 protein. The His182 fusion protein containing the C-terminal 182 amino acids of the Na^+/H^+ exchanger was phosphorylated *in vitro* with rabbit ventricular cell extracts, and casein was phosphorylated with casein kinase II as described under "Experimental Procedures." *Lane 1*, His182 protein phosphorylated with cell extracts. *Lane 2*, His182 protein treated with the presence of phosphorylating buffer but in the absence of cell extracts. *Lane 3*, casein treated with casein kinase II in a phosphorylating buffer. *Lane 4*, casein treated with phosphorylating buffer in the absence of kinase. *B*, affinity blotting assay of phosphorylated and non-phosphorylated proteins. Equal amounts of His182 and casein proteins were separated by SDS-PAGE and transferred to nitrocellulose as described under "Experimental Procedures." Nitrocellulose transfer of proteins was probed with CAII protein as described in the legend to Fig. 2. Lanes are as described for Fig. 5. The *arrow* indicates location of CAII binding to His182. *C*, autoradiogram of phosphorylation of the His182 protein by unstimulated (*lanes 1* and *2*) and stimulated (*lanes 3* and *4*) rat cardiomyocyte extracts prepared as described under "Experimental Procedures." *D*, affinity blotting assay of phosphorylated His182 fusion protein treated with unstimulated (*lanes 1* and *3*) and stimulated (*lanes 2* and *4*) rat cardiomyocyte extracts. Equal amounts of His182 proteins were separated by SDS-PAGE and transferred to nitrocellulose as described under "Experimental Procedures." Nitrocellulose transfer of proteins was probed with CAII protein as described in the legend to Fig. 2. Results are typical of three independent experiments. *E*, immunoblot of immunoprecipitates probed with anti-CAII antibody. The immunoprecipitation was with anti-HA antibody. Samples were treated with DSP prior to immunoprecipitation and were finally heated in sample buffer containing β-mercaptoethanol as described under "Experimental Procedures." *Lane 1*, positive control, 1 μg of purified CAII protein. *Lanes 2* and *3*, immunoprecipitate from cells transfected with pYN4+ and pJRC36 plasmids. *Lanes 4* and *5*, immunoprecipitate from cells transfected with pYN4+. *Lanes 2* and *4*, immunoprecipitate from cells grown in the presence of serum. *Lanes 3* and *5*, immunopreciptate from cells grown in the absence of serum for 16 h.

The cytoplasmic, C-terminal of the exchanger is over 300 amino acids in length and can be divided into four distinct subdomains that are involved in regulation. These include an ATP-dependent regulation, phosphorylation region, and binding regions for calcineurin homologous protein and calmodulin (1). It is still unclear if there are other proteins involved in the interactions of the C-terminal of the Na^+/H^+ exchanger.

Recently, it was found that CAII could interact with the C-terminal of the anion exchanger by binding with an acidic amino acid cluster (^{887}DADD) (13, 14). Removal of the DADD sequence resulted in a loss of CAII binding (14). The binding may allow the formation of a metabolon between AEI and CAII that functions to channel the products of the carbonic anhydrase reaction to the anion exchanger (25, 33). Several observations suggested that CAII might also associate with the Na^+/H^+ exchanger. First, there is a general structural and functional similarity between the anion exchanger and Na^+/H^+ exchanger, with both proteins having large interior cytoplasmic domains with internal acidic amino acids. Second, several reports have suggested that the Na^+/H^+ exchanger is associated with CA and AEs (16, 20, 34). Third, since CA catalyzes the hydration of CO_2 to produce a proton and bicarbonate, it might also co-localize with NHE1 to improve efficiency of proton removal, similar to CAII and the AE. It is of note that the C-terminal 178 amino acids of NHE1 contain 12 aspartate and 17 glutamate residues that could be involved in forming a binding site for CAII.

Our study demonstrated that the C-terminal 178 amino acids of NHE1 can bind CAII in microtiter plate binding assays and in affinity blotting assays with immobilized Na^+/H^+ exchanger (Figs. 1 and 2). It was clear that the Na^+/H^+ exchanger part of these fusion proteins was responsible for the binding since GST alone did not bind to CAII. CAII was found co-imunoprecipitating with the Na^+/H^+ exchanger from cells either transfected or not transfected with CAII (Fig. 3*D*). However the AP1 cells we used in this study possessed endogenous CAII (Fig. 3*B*, *lane 1*). Overall our results clearly indicate an interaction between CAII and the Na^+/H^+ exchanger both *in vivo* and *in vitro*.

To examine the effects of CAII binding on Na^+/H^+ exchanger activity *in vivo*, pH regulation of transfected cells was measured. Our results showed that cells transfected with both Na^+/H^+ exchanger and CAII have a higher H^+ transport rate compared with cells transfected with Na^+/H^+ exchanger alone. The H^+ transport rate in cotransfected cells increased 76%, which suggests that CAII could stimulate Na^+/H^+ exchanger activity. In addition, the CAII inhibitor acetazolamide significantly decreased the H^+ transport rates by the Na^+/H^+ exchanger. This result further demonstrated that CAII activity influences activity of the Na^+/H^+ exchanger. It was notable that transfection with a dominant negative inactive carbonic anhydrase mutant resulted in a decrease in activity of the Na^+/H^+ exchanger protein (Fig. 4*C*). Overexpression of a dominant negative CAII would cause displacement of endogenous CAII from its binding site on NHE1 by the inactive CAII mutant. Therefore we interpret the inhibitory effect of V143Y CAII on the Na^+/H^+ exchanger activity as an indication that direct binding by an active CAII protein is necessary for stimulatory activity.

Phosphorylation of the Na^+/H^+ exchanger has been shown to stimulate activity in the heart and other tissues. Phosphorylation has also been localized to the C-terminal 178 amino acids of the protein (9) similar to the region that we found binds CAII. We found that phosphorylation of the Na^+/H^+ exchanger greatly increased CAII binding (Fig. 5). The effect was specific to CAII since phosphorylation of casein did not result in CAII

binding. The effect of phosphorylation occurred *in vitro*, and we also found that serum treatment of cells increases the amount of CAII that co-immunoprecipitated with the Na^+/H^+ exchanger (Fig. 5*E*). We have earlier shown that such serum stimulation causes phosphorylation of the Na^+/H^+ exchanger *in vivo* (9). Since phosphorylation has been shown to stimulate the activity of the Na^+/H^+ exchanger, and since we found that expression of CAII protein stimulated Na^+/H^+ exchanger activity, this leaves open the possibility that the mechanism by which phosphorylation stimulates the activity is through increased CAII binding. However at present, this only remains a theory and further experiments are necessary to explore this possibility. It is interesting to note that the pH dependence of interaction of CAII with the Na^+/H^+ exchanger (Fig. 1*B*) is consistent with the known activity profile of NHE1, which is activated by decreases in intracellular pH.

In summary, our results show that CAII can bind to the C-terminal of the Na^+/H^+ exchanger *in vitro* and *in vivo*. The interaction can influence pH regulation of Na^+/H^+ exchanger in mammalian cells. Where CAII binds on the C-terminal of Na^+/H^+ exchanger and how CAII interacts with Na^+/H^+ exchanger remains undefined. Further experiments are necessary to define the binding site of CAII. Our results support the earlier suggestions that CAII, the AE, and Na^+/H^+ exchanger activity may be linked together in a functional complex or metabolon involved in intracellular bicarbonate and pH regulation (20, 34). Future experiments will further explore this possibility and the regulatory role that phosphorylation plays in protein-protein interactions that modulate Na^+/H^+ exchanger activity.

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REFERENCES

1. Fliegel, L. (2001) *Basic Res. Cardiol.* **96,** 301–305
2. Sardet, C., Franchi, A., and Pouysségur, J. (1989) *Cell* **56,** 271–280
3. Counillon, L., and Pouyssegur, J. (2000) *J. Biol. Chem.* **275,** 1–4
4. Wiebe, C. A., DiBattista, E. R., and Fliegel, L. (2001) *Biochem. J.* **357,** 1–10
5. Avkiran, M. (2001) *Basic Res. Cardiol.* **96,** 306–311
6. Lin, X., and Barber, D. L. (1996) *J. Biol. Chem.* **93,** 12631–12636
7. Wakabayashi, S., Ikeda, T., Iwamoto, T., Pouyssegur, J., and Shigekawa, M. (1997) *Biochemistry* **36,** 12854–12861
8. Silva, N. L. C. L., Haworth, R. S., Singh, D., and Fliegel, L. (1995) *Biochemistry* **34,** 10412–10420
9. Moor, A. N., and Fliegel, L. (1999) *J. Biol. Chem.* **274,** 22985–22992
10. Sly, W. S., and Hu, P. Y. (1995) *Annu. Rev. Biochem.* **64,** 375–601
11. Sly, W. S., Whyte, M. P., Sundaram, V., Tashian, R. E., Hewett-Emmett, D., Guibaud, P., Vainsel, M., Baluarte, H. J., Grushkin, A., Al-Mosawi, M., Sakati, N., and Ohlsson, A. (1985) *N. Eng. J. Med.* **313,** 139–145
12. Cousin, J. L., Motais, R., and Sola, F. (1975) *J. Physiol. (London)* **253,** 385–399
13. Vince, J. W., and Reithmeier, R. A. F. (1998) *J. Biol. Chem.* **273,** 28430–28437
14. Vince, J. W., and Reithmeier, R. A. F. (2000) *Biochemistry* **39,** 5527–5533
15. Vince, J. W., Carlsson, U., and Reithmeier, R. A. (2000) *Biochemistry* **39,** 13344–13349
16. Wu, Q., Pierce, W. M., Jr., and Delamere, N. A. (1998) *J. Membr. Biol.* **162,** 31–38
17. Klein, M., Seeger, S. B., Alper, S. L., and Schwab, A. (2000) *J. Gen. Physiol.* **115,** 599–607
18. Davis, L. H., Otto, E., and Bennett, V. (1991) *J. Biol. Chem.* **266,** 11163–11169
19. Denker, S. P., Huang, D. C., Orlowski, J., Furthmayr, H., and Barber, D. L. (2000) *Mol. Cell* **8,** 1425–1436
20. Yao, H., Ma, E., Gu, X.-Q., and Haddad, G. G. (1999) *J. Clin. Invest.* **104,** 637–645
21. Wang, H., Silva, N. L. C. L., Lucchesi, P. A., Haworth, R., Wang, K., Michalak, M., Pelech, S., and Fliegel, L. (1997) *Biochemistry* **36,** 9151–9158
22. Rotin, D., Steele-Norwood, D., Grinstein, S., and Tannock, I. (1989) *Cancer Res.* **49,** 205–211
23. Murtazina, BR., Booth, B. J., Bullis, B. L., Singh, D. N., and Fliegel, L. (2001) *Eur. J. Biochem.* **268,** 1–13
24. Sterling, D., and Casey, J. R. (1999) *Biochem. J.* **344,** 221–229
25. Sterling, D., Reithmeier, R. A. F., and Casey, J. R. (2001) *J. Biol. Chem.* **276,** 47886–47894
26. Wang, H., Singh, D., and Fliegel, L. (1998) *Arch. Biochem. Biophys.* **358,** 116–124
27. Tang, X.-B., Kovacs, M., Sterling, D., and Casey, J. R. (1999) *J. Biol. Chem.* **274,** 3557–3564
28. Perez, N. G., Alvarez, B. V., Camilion de Hurtado, M. C., and Cingolani, H. E. (1995) *Circ. Res.* **77,** 1192–1200
29. Murray, G. M. M. J., Trumble, W., and Magnuson, B. A. (2001) *BioTechniques* **30,** 1036–1041
30. Landesman-Bollag, E., Romieu-Mourez, R., Song, D. H., Sonenshein, G. E., Cardiff, R. D., and Seldin, D. C. (2001) *Oncogene* **20,** 3247–3257
31. Noel, J., Roux, D., and Pouyssegur, J. (1996) *J. Cell Sci.* **109,** 929–939
32. Fliegel, L. (1999) *J. Thromb. Thrombolysis* **8,** 9–14
33. Reithmeier, RA. (2001) *Blood Cells Mol. Dis.* **27,** 85–89
34. Mason, M. J., Smith, J. D., Garcia-Soto, J. J., and Grinstein. S. (1989) *Am. J. Physiol.* **256,** C428–C433

Carbonic Anhydrase II Binds to and Enhances Activity of the Na^+/H^+ Exchanger

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