Carbonic Anhydrase II and Alveolar Fluid Reabsorption during Hypercapnia

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Carbonic anhydrase II (CAII) plays an important role in carbon dioxide metabolism and intracellular pH regulation. In this study, we provide evidence that CAII is expressed in both type I (AECI) and type II (AECII) alveolar epithelial cells by RT-PCR and Western blotting in freshly isolated rat cells. These results were further confirmed by double immunostaining with CAII antibodies and AECI- or AECII-specific markers in freshly isolated alveolar epithelial cells and rat lung tissues. Inhibition of CAII by acetazolamide or methazolamide delayed the decrease in the intracellular pH observed during hypercapnia in cultured AECI, AECII, and AECI-like cells. In an isolated-perfused rat lung model, alveolar fluid reabsorption significantly decreased during high CO₂ exposure, which was not prevented by carbonic anhydrase inhibition. Thus, we provide evidence that CAII is expressed in rat alveolar epithelial cells and does not regulate lung alveolar fluid reabsorption during hypercapnia.

Keywords: carbonic anhydrase II; rat alveolar epithelial cells; intracellular pH; alveolar fluid clearance; hypercapnia

Carbonic anhydrase (CA) can accelerate the hydration and dehydration of CO₂ with a 500,000- to 1,000,000-fold increase over the uncatalyzed rate at 37°C; it is ubiquitously expressed and found in most tissues (1). CA was first discovered in human erythrocytes in 1932 by Meldrum and Roughton, and more than 15 isoenzymes of carbonic anhydrases have since been reported (2, 3). Among the carbonic anhydrase isoenzymes, CAII has the highest catalytic efficiency (10⁶ reactions/s) and is the isoenzyme that has been studied the most (4). Using an *in vitro* lung model and the CAII inhibitor, acetazolamide, Crandall and O'Brasky first demonstrated the role that CAII plays in CO₂ elimination by the lungs (5). CAII-deficient mice have been reported to develop respiratory acidosis as a consequence of CO₂ retention in the lungs (6).

The alveolar epithelium is composed of type I and type II epithelial cells (AECI and AECII), where AECI cover approximately 95% of the alveolar surface area and play an important role in fluid clearance (7–10). AECII cover less than 5% of alveolar area and contribute to fluid transport and surfactant protein secretion (11). Fleming and coworkers reported that CAII is expressed in AECII (12); however, there are no reports studying whether CAII is expressed in AECI.

It has been proposed that CO₂ elimination by the lungs may involve the activation of apical H⁺ channels (13). Also, intra-

CLINICAL RELEVANCE

This is the first report showing that carbonic anhydrase (CA) protein is expressed in type I epithelial cells and in which this activity was measured. Inhibition of CA activity in the alveolar epithelium does not have an effect on alveolar fluid reabsorption.

cellular acidification of AECII by CO₂ triggers Na⁺-dependent transports (14). Alveolar fluid reabsorption (AFR) is mainly driven by vectorial Na⁺ transport via apical Na⁺ channels and the Na,K-ATPase, which is located in the basolateral membrane of the alveolar epithelium (15–22). We have recently observed that increased pCO₂ decreases AFR in rats by inhibiting Na,K-ATPase function (23).

In this study, we set out to determine whether rat AECI, AECII, and lung tissues express CAII and whether carbonic anhydrase plays a role in alveolar fluid reabsorption in normal or hypercapnic conditions using an isolated-perfused rat lung model. We found that carbonic anhydrase II is expressed in both alveolar epithelial type I and type II cells, and although we confirmed the previous report that alveolar fluid reabsorption is inhibited by hypercapnia (23), these effects are not modified by carbonic anhydrase activity.

MATERIALS AND METHODS

All animals used in this study were provided with food and water *ad libitum*. Animals were handled according to National Institutes of Health guidelines and Institutional Animal Care and Use Committee-approved experimental protocols.

Materials

DNase I, rat immunoglobulin (Ig)G, acetazolamide, and methazolamide were from Sigma-Aldrich (St. Louis, MO). Dextran 40 was from Amersham Biosciences (Piscataway, NJ). Nylon meshes (160, 37, 20 µm) were from Tetko (Elmsford, NY). Porcine pancreas elastase was from Worthington Biochemical (Lakewood, NJ). Bovine serum albumin (BSA), normal goat serum, goat anti-mouse IgG, Texas Red-conjugated goat anti-mouse IgG, and fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG were from Jackson ImmunoResearch Laboratories (West Grove, PA). Rabbit antibody against CAII was from Research Diagnostics, Inc. (Flanders, NJ). Monoclonal antibody against surfactant protein D (SP-D) was from Cell Sciences (Canton, MA). Monoclonal antibody against surfactant protein B (SP-B) was from Neomarkers (Fremont, CA). Monoclonal anti-rat leukocyte common antigen antibody was from Accurate (Westbury, NY). Monoclonal antibody (E11) against type I alveolar epithelial cell α protein (T1α) was a gift from Dr. Antoinette Wetterwald (University of Berne, Berne, Switzerland) and Dr. Mary C. Williams (Boston University, Boston, MA). CELLlection Pan Mouse IgG kit, sheep anti-rat IgG magnetic beads, and goat anti-mouse IgG beads were from Dynal Biotech (Lake Success, NY). RPMI 1640 Medium containing 25 mM HEPES buffer was from Irvine Scientific Inc. (Santa Ana, CA).

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Cell Isolation and Cell Culture

AECI were isolated following the previous described methods (10). Briefly, lungs from ~280-g male Sprague-Dawley rats were perfused via the pulmonary artery with RPMI 1640 medium containing 25 mM Hepes (solution I) at 37°C. Lungs were lavaged with solution II (PBS [pH 7.4] containing 0.06 mg/ml EGTA, 0.06 mg/ml penicillin G, and 0.1 mg/ml streptomycin sulfate) at 37°C and then were instilled with 9 ml solution I with 10% dextran (MW 4000; Sigma-Aldrich) containing 4.5 U/ml elastase at 37°C for a total of 40 minutes. After elastase digestion, the lung tissue was dissected in 20% fetal bovine serum (FBS) and 2 mg/ml DNase in solution I and minced by chopping. The lung fragments were gently agitated for 5 minutes and filtered through 160- μ m and 37- μ m nylon mesh once. The resultant cell suspension was centrifuged and resuspended in solution I followed by panning on rat IgG-coated bacteriological plates for 30 minutes at 37°C. The supernatant cells were centrifuged, resuspended in solution I including 1% FBS and 50 μ g DNase/ml, and incubated with 20 μ g/ml of rat IgG and anti-rat leukocyte common antigen monoclonal antibody ascites at 4°C for 30 minutes with gentle rotation. Cells were then washed three times with solution I with 1% FBS (Solution III) to remove unbound antibodies, and incubated with mouse anti-rat T1 α antibody (20 μ g/ml) for 1 hour at 4°C with gentle rotation. After incubation with anti-rat T1 α antibody, the unbound antibodies were removed by washing with solution III three to four times. The AECI cells were then isolated and released using Collection anti-mouse IgG kit based on the instruction of the manufacturer. The viability (~95%) and purity (~90%) of the final AECI cells were checked by immunocytochemistry labeling (24, 25). Fresh isolated AECI were cultured in fibronectin-coated (Costar, Cambridge, MA) plates in Dulbecco's modified Eagle's medium (DMEM) with 4.5 g/L glucose, 4.5 g/L L-glutamine, 4.5 g/L sodium pyruvate, 20% FBS, and 50 μ g/ml gentamicin.

Type II alveolar epithelial cells were isolated as previously reported protocols (7, 10, 24) with the following modifications. During the panning step, the cells were incubated twice with rat IgG-coated plates at 37°C in 10 ml RPMI 1640 medium with 25 mM Hepes (30 min per incubation). The cells from the supernatant were centrifuged for 8 minutes at 250 $\times$ g and resuspended with solution III at a concentration of approximately 10 to 20 $\times$ 10⁶ cells/ml. Rat IgG, anti-rat leukocyte common antigen monoclonal antibody ascites, and mouse anti-rat T1 α antibody were incubated with cells at 4°C for 1 hour with gentle rotation. The cells were washed three times with Solution III, then magnetic beads coated with secondary antibodies, sheep anti-rat IgG, and goat anti-mouse (DynaL Biotech) were incubated with the cells at 4°C for 20 minutes with gentle rotation. A magnet was applied to negatively remove macrophages, leukocytes and type I cells. The resultant cells contained less than 0.5% type I cells by immunofluorescence and over 98% type II cells by the Papanicolaou staining (25). Yields were approximately 15 $\times$ 10⁶/rat. Freshly isolated AECII were cultured in a complete medium (DMEM with 20 mM Hepes, 10% FBS, 2 mmol/L L-glutamine, 40 μ g/ml gentamicin, 100 U/ml penicillin, and 100 μ g/ml streptomycin). Cell culture medium was changed every other day. AECII cultured on plastic for 5 days were called AECI-like cells.

RT-PCR

Total RNAs from purified rat lung alveolar epithelial cells were isolated using RNeasy Mini kit (Qiagen, Valencia, CA) and incubated with DNase I enzyme for 20 minutes at room temperature. First-strand cDNA synthesis was performed using Superscript first-strand synthesis system (Invitrogen, Carlsbad, CA). The primers for rat carbonic anhydrase II were 5'-AAGAGCAACGGACCAGAGAA-3' (forward) and 5'-GGCAGGTCCAATCTTCAAAA-3' (reverse); the primers for glyceraldehyde-3-phosphate dehydrogenase (GAPDH; as a positive control) were 5'-ACCACAGTCCATGCCATCAC-3' (forward) and 5'-TCCACCACCCTGTTGCTGTA-3' (reverse). The conditions for PCR amplification were: 10 mM of Tris-HCl (pH 9.0 at 25°C), 50 mM of KCl, 0.1% Triton X-100, 1.8 mM of MgCl₂, 0.16 mM of dNTP mix, 1.6 μ M of each primer, and 1 unit of DNA *Taq* polymerase, with 20 ng of cDNA in a final reaction volume of 25 μ l. Cycling conditions were 94°C for 2 minutes, 55°C for 40 seconds, 72°C for 2 minutes, two cycles; 94°C for 30 seconds, 55°C for 30 seconds, 72°C for 1.5 minutes, 35 cycles; and 72°C for 8 minutes. Agarose-gel (1.5%) electrophoresis and ethidium bromide staining were used to visualize PCR bands.

Western Blotting

Purified AECI and AECII were lysed by adding lysis buffer (20 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β -glycerolphosphate, 1 mM Na₃VO₄, 1 μ g/ml leupeptin, 1 mM PMSF) and centrifuged at 14,000 $\times$ g, 4°C, for 5 minutes to eliminate the insoluble materials. Protein was quantified by Bradford assay (Bio-Rad, Hercules, CA) and equal amounts of protein were resolved by 10% polyacrylamide gel. Thereafter, proteins were transferred onto nitrocellulose membranes (Optitran; Schleicher and Schuell, Keene, NH) using a semi-dry transfer apparatus (Bio-Rad). Incubation with specific antibodies was performed overnight at 4°C. Blots were developed with an enhanced chemiluminescence detection kit (ECL⁺; Amersham, Buckinghamshire, UK) used as recommended by the manufacturer.

Immunocytochemistry and Immunohistochemistry

Purified rat AECI and AECII were cytospun to a coverslip, washed once with PBS buffer, and immediately fixed in 4% paraformaldehyde at 4°C overnight. Immunocytochemistry was performed as described before (7). Antigen retrieval in 0.5 M Tris-HCl with 5% Urea, pH 9.5 was used before blocking in PBS with 10% normal goat serum, 0.1% BSA, 0.3% TX-100. A 1-hour incubation with primary antibody, rabbit against carbonic anhydrase II, was performed after washing with PBS including 0.1% BSA and 0.3% TX-100 (buffer A) once, followed by washing with buffer A three times. Thereafter, mouse anti-rat T1 α (AECI-specific marker) or mouse anti-SP-B antibodies (AECII specific marker) was added to label AECI or AECII. The secondary antibodies are FITC-conjugated goat anti rabbit IgG and Texas Red-labeled goat anti-mouse IgG for polyclonal and monoclonal antibodies, respectively. An anti-fade mounting media (Innovex Biosciences, Richmond, CA)

Figure 1. Characterization of carbonic anhydrase II (CAII) in rat alveolar epithelial cells. (A) RT-PCR using mRNA obtained from rat alveolar type I (AECI) and type II (AECII) cells. Primers used for amplification are listed in MATERIALS AND METHODS. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used to verify quality of cDNA and reaction without enzyme was used as the negative control. Kidney was used as a positive control. (B) Western blots of cell lysates from rat AECI and AECII cells were performed with antibodies against CAII, the specific AECI marker T1 α , and the specific AECII marker surfactant protein D (SP-D). Actin was used as a loading control. MW, molecular weight marker.

Figure 2. CAII is expressed in purified rat AECI and AECII cells. Immunocytochemistry: the purified AECI and AECII were cytospun to a coverslip, and immediately fixed in 4% paraformaldehyde at 4°C overnight. The slides were examined using a Nikon Eclipse E800 fluorescence microscope, and the images were processed by MetaMorph software. Representative images of the specific AECI marker T1α and CAII double labeling (first row), the specific AECII marker surfactant protein B (SP-B) and CAII double labeling (second row), and negative controls (third row) are shown. Magnification: $\times 400$. BF, bright field.

was used to fix the coverslip to a slide. The slides were examined using a Nikon Eclipse E800 fluorescence microscope (Nikon Instruments Inc., Melville, NY), and the images were processed by MetaMorph software. Negative control of both AECI and AECII were performed following the same procedure but without primary antibody.

Rat lung tissues for immunohistochemistry were perfused with ice-cold PBS to remove the blood and then fixed in 4% paraformaldehyde overnight. Lungs were embedded in paraffin and 4- μ m lung tissue sections were cut and sections were placed on glass slides. Slides were deparaffinized in xylene for 5 minutes (three times) and then rehydrated in 100%, 95%, 70% ethanol and PBS. Immunohistochemistry was performed as described before (7). Negative controls were performed following the same procedure but without primary antibody.

Intracellular pH Measurement

Purified rat AECI, AECII, and AECI-like cells were cultured on a 40-mm circular glass coverslip in a 60-mm cultural plate, 1×10^6 cells per plate. They were preincubated with 1.5 μ M 2',7'-bis-(carboxyethyl)-

5,6-carboxyfluorescein (BCECF/AM) for 30 minutes at 37°C. After incubation, cover slips were placed in an environmental chamber, maintained at 37°C and continuously perfused with equilibrated media. BCECF fluorescence in the chamber was monitored continuously through the desired excitation wavelength (500 and 440 nm) with an emission wavelength of 520 nm. Signals were processed by MetaFluor Software (Molecular Devices Corp. Downingtown, PA). Intracellular pH (pH_i) measurements were conducted with a multi-mode inverted Microscopy (Nikon TE2000; Nikon Instruments). Inhibition of CAII was conducted by pre-incubation with 100 μ M acetazolamide or 100 μ M methazolamide 30 minutes. The solutions with normal P_{CO_2} (~ 40 mm Hg) and high P_{CO_2} (~ 80 mm Hg) were not HCO_3^- -free and were prepared with DMEM-Ham's F-12 medium-Tris base (3:1:0.5) containing 10% FBS, with 2 mmol/L L-glutamine, 40 μ g/ml gentamicin, 100 U/ml penicillin, and 100 μ g/ml streptomycin. The medium for normal P_{CO_2} was placed overnight in a cell-culture incubator with 5% CO_2 ; the medium for high P_{CO_2} was incubated overnight in a humidified chamber (C-174 Chamber, Biospherix, Ltd., Redfield, NY) with 20% CO_2 .

Figure 3. CAII is expressed in the rat lung alveolar epithelium. Immunohistochemistry: paraffin-embedded tissue sections (4 μ m) were dewaxed with xylene and rehydrated. The slides were examined using a Nikon Eclipse E800 fluorescence microscope, and the images were processed by MetaMorph software. Representative images of the specific AECI marker T1α and CAII double labeling (first row), the specific AECII marker SP-B and CAII double labeling (second row), and negative controls (third row) are shown. Magnification: first and second rows, $\times 1,000$; third row, $\times 400$. BF, bright field.

Isolated Perfused Lung Model

The isolated perfused lung model was described previously (9, 26). Briefly, rats were anesthetized with Nembutal (50 mg/kg), and the lungs were removed *en bloc* after a 10-minute ventilation with 100% O₂. The pulmonary artery and left atrial appendage were cannulated and perfused with a buffered physiologic solution (135.5 mM Na⁺, 119.1 mM Cl⁻, 25 mM HCO₃⁻, 4.1 mM K⁺, 2.8 mM Mg²⁺, 2.5 mM Ca²⁺, 0.8 mM SO₄²⁻, 8.3 mM glucose) including 3% BSA. Trace amount of FITC-labeled albumin was added to the perfusate to monitor protein leakage from the vascular space into the alveolar space. The recirculating volume of the perfusion system with the constant pressure was 90 ml. Pulmonary circulation and inflow and outflow pressures were set at 12 and 0 cm H₂O, respectively, and vascular pressures were recorded every 10 seconds with a multichannel recorder (Cyber Sense Inc., Nicholasville, KY). The lungs were immersed in a "pleural" bath (100 ml) filled with the same BSA solution. The entire system was maintained at 37°C in a water bath. Perfusate pH was maintained at 7.40 by bubbling with a gas mixture of 95% O₂/5% CO₂. The lungs were then instilled via the tracheal cannula in two sequential phases leaving a total of 5 ml volume of the BSA solution containing 0.1 mg/ml EBD-albumin, 0.02 µCi/ml of ²²Na⁺, and 0.12 µCi/ml of ³H-mannitol in the rat airspaces. Samples were taken from the instillate, perfusate, and bath solutions after an equilibration time of 10 minutes from the instillation and 60 minutes later as reported previously; to ensure a homogenous sampling of the instillate, a volume of 2 ml was aspirated and reintroduced into the airspaces three times before removing each sample (27, 28). All samples were centrifuged at 3,000 × *g* for 10 minutes. Absorbance analysis of the supernatant or EBD albumin was performed at 620 nm in a Hitachi model U2000 spectrometer (Hitachi, San Jose, CA). Analysis of FITC-albumin (excitation 487 nm and emission 520 nm) was performed in a Barnstead Turner Quantech fluorometer (Barnstead Turner, Dubuque, IA). Scintillation counts (for ²²Na⁺ and ³H-mannitol) were measured in a Beckman β counter (model LS 6500; Beckman Instruments Inc., Fullerton, CA).

Statistical Analysis

Data are expressed as mean ± SEM. Data were compared using ANOVA adjusted for multiple comparisons with the Dunnett test. When comparisons were performed between two groups of values significance was evaluated by Student's *t* test. A *P* value less than 0.05 was considered significant.

RESULTS

CAII Is Expressed in AECl and AECII

As shown in Figure 1A, CAII is expressed in both AECl and AECII cells at mRNA level. Western blotting with an antibody against CAII (Figure 1B) confirmed expression of CAII protein in both rat AECl and AECII. T1α and SP-D were used to confirm the AECl and AECII cell purity, respectively. Actin was used for normalization of protein loading. CAII expression in rat AECl and AECII was confirmed by immunocytochemistry and immunohistochemistry in purified rat AECl, AECII, and paraffin-embedded rat lung tissues (Figures 2 and 3).

Acetazolamide and Methazolamide Delayed the Intracellular pH Decrease Caused by High CO₂

Intracellular pH (pH_i) decreased in AECl, AECII, and AECl-like cells when Pco₂ increased from approximately 40 mm Hg to approximately 80 mm Hg (Figure 4). Pre-incubation with 100 µM acetazolamide or 100 µM methazolamide for 30 minutes delayed the decrease in pH_i. This effect was possibly due to inhibition of cytosolic carbonic anhydrases, suggesting that carbonic anhydrase activity is present in the cytosol of AECl and AECII.

Inhibition of Carbonic Anhydrase Activity by Acetazolamide or Methazolamide Does Not Affect Alveolar Fluid Reabsorption during Hypercapnia

As shown in Figure 5A, alveolar fluid reabsorption significantly decreased when Pco₂ increased from approximately 40 mm Hg

Figure 4. Carbonic anhydrase regulates the decrease in intracellular pH due to high CO₂. Intracellular pH (pH_i) was measured in the presence or absence of acetazolamide or methazolamide in (A) rat AECl cells, (B) rat AECII cells, and (C) rat AECl-like cells exposed to a Pco₂ of 80 mm Hg. Graph represents mean ± SEM (*n* = 3). **P* < 0.05; ***P* < 0.01; &*P* < 0.05; &&*P* < 0.01.

to approximately 60 mm Hg. Addition of 100 µM acetazolamide or 100 µM methazolamide in the vascular space did not affect the impaired alveolar fluid reabsorption caused by increased CO₂. FITC-labeled albumin concentration was not altered for the six different conditions (Figure 5B), suggesting no significant changes in alveolar epithelium permeability. Flow values remained constant with treatments suggesting no effects of the CA inhibitors in the vasculature (Table 1).

DISCUSSION

CAII is ubiquitously expressed in mammalian cells, where it catalyzes CO₂ hydration and regulates pH_i (5, 29). In the lungs, CAII plays an active role in the CO₂ elimination through the alveolar epithelium. However, it is unknown whether it is expressed in type I alveolar epithelial cells and whether it has a

Figure 5. Carbonic anhydrase inhibition did not prevent the decrease on alveolar fluid reabsorption (AFR) during exposure to high CO₂ in the isolated-perfused rat lung model. (A) Isolated rat lungs were perfused for 1 hour with 40 (CT, control) or 60 (HC, hypercapnia) mm Hg CO₂ in the presence or absence of 100 μ M acetazolamide or 100 μ M methazolamide and AFR was measured as described in MATERIALS AND METHODS. (B) Albumin flux from the pulmonary circulation into the alveolar space was determined from the fraction of fluorescein isothiocyanate (FITC)-labeled albumin placed in the perfusate that appeared in the alveolar instillate during the experimental protocol. Graph represents mean $\pm$ SEM ($n = 3$). *** $P < 0.001$.

role in fluid transport, an important function of the alveolar epithelium. In this study, we show that carbonic anhydrase activity does not play a role in alveolar fluid reabsorption in normal or hypercapnic conditions.

The alveolar epithelium is composed of AECl and AEcII. AECl, with typical thin cytoplasmic extensions, cover over 95% of the alveolar epithelium surface area and contribute approx-

imately 60% of lung basal liquid clearance. Our data show that CAII is expressed in freshly isolated rat AECl, AEcII, and AECl-like cells (AEcII cultured for 5–7 d) at mRNA and protein level (see Figures 1–3), in agreement with the data of Fleming and colleagues (12), who reported CAII expression in rat AEcII cells. Inhibition of carbonic anhydrase activity by acetazolamide or methazolamide in AECl, AEcII, and AECl-like cells delayed the decrease in pH_i under hypercapnic conditions, suggesting that carbonic anhydrase activity is present in these cells.

To study the role of CAII in alveolar fluid reabsorption in basal and hypercapnic conditions, an isolated-perfused lung model was used in the presence or absence of acetazolamide. Acetazolamide is lipophilic and can easily diffuse into the cytosol of the cells, inhibiting cytosolic carbonic anhydrases (30). To confirm our findings we also used the carbonic anhydrase inhibitor methazolamide, which diffuses better into tissues (31). We found that both inhibitors did not affect the basal AFR, nor did they prevent the hypercapnia-mediated decrease of AFR. Thus, we reason that the hydration of CO₂ by carbonic anhydrases (32) is not a limiting step in AFR in our isolated-perfused lung model.

We found no changes in the fluxes of the perfusates in the isolated fluid lung model (see Table 1), which excludes some of the reported systemic effects, independent of carbonic anhydrase inhibition by acetazolamide. Acetazolamide has been reported to attenuate hypoxic pulmonary vasoconstriction by modulating Ca²⁺ responses independently of carbonic anhydrase inhibition (33, 34). Our data contrast with previous reports in which acetazolamide increased subretinal fluid absorption in rabbits, but these effects may have been due to its systemic effects involving the adrenergic pathway (30).

In summary, we provide first evidence that CAII is expressed in rat type I alveolar epithelial cells at mRNA and protein level. Double labeling with CAII and type I alveolar epithelial cell marker (T1 α) further confirms that CAII is expressed in rat type I alveolar epithelial cells and lung tissues. Inhibition of carbonic anhydrase activity by acetazolamide or methazolamide delayed hypercapnia-mediated pH_i decrease, which suggests that there is carbonic anhydrase activity in alveolar epithelial cells, although it does not appear to play a role in lung alveolar fluid reabsorption during hypercapnia.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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TABLE 1. FLOWS DID NOT CHANGE WITH THE DIFFERENT EXPERIMENTAL PROTOCOLS

	Flux (ml/min)	
	Average	SEM
CT		
CT	11.6	0.2
HC	10.8	0.4
100 μ M Acetazolamide		
CT	11	0.6
HC	10.3	0.3
100 μ M Methazolamide		
CT	10.7	0.7
HC	10	0.6

Definition of abbreviations: CT, control; HC, hypercapnia.

The pulmonary circulation perfusate flow was measured in all the conditions tested.

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