



Published in final edited form as:

*Invest Ophthalmol Vis Sci.* 2009 April ; 50(4): 1791–1800. doi:10.1167/iovs.08-2487.

## Studies on bicarbonate transporters and carbonic anhydrase in porcine non-pigmented ciliary epithelium

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### Abstract

**Purpose**—Bicarbonate transport plays a role in aqueous humor (AH) secretion. Here, we examined bicarbonate transport mechanisms and carbonic anhydrase (CA) in porcine non-pigmented ciliary epithelium (NPE).

**Methods**—Cytoplasmic pH ( $\text{pH}_i$ ) was measured in cultured porcine NPE loaded with BCECF. Anion exchanger (AE), sodium bicarbonate cotransporter (NBC) and CA were examined by RT-PCR and immunolocalization. AH secretion was measured in the intact porcine eye using a fluorescein dilution technique.

**Results**—Anion exchanger AE2, CAII and CAIV were abundant in the NPE layer. In cultured NPE superfused with a  $\text{CO}_2/\text{HCO}_3^-$  free HEPES buffer, exposure to a  $\text{CO}_2/\text{HCO}_3^-$ -containing buffer caused a rapid acidification followed by a gradual  $\text{pH}_i$  increase. Subsequent removal of  $\text{CO}_2/\text{HCO}_3^-$  with HEPES buffer caused rapid alkalization followed by gradual  $\text{pH}_i$  decrease. The rate of gradual alkalization after addition of  $\text{HCO}_3^-/\text{CO}_2$  was inhibited by sodium-free conditions, DIDS, CA inhibitors acetazolamide and methazolamide but not by Na-H exchange inhibitor dimethylamiloride or low chloride buffer. The phase of gradual acidification after removal of  $\text{HCO}_3^-/\text{CO}_2$  was inhibited by DIDS, acetazolamide, methazolamide and by low chloride buffer. DIDS reduced baseline  $\text{pH}_i$ . In the intact eye, DIDS and acetazolamide reduced AH secretion by 25% and 44% respectively.

**Conclusion**—The results suggest the NPE uses a  $\text{Na}^+/\text{HCO}_3^-$  cotransporter to import bicarbonate and a  $\text{Cl}^-/\text{HCO}_3^-$  exchanger to export bicarbonate. CA influences the rate of bicarbonate transport. AE2, CAII and CAIV are enriched in the NPE layer of the ciliary body and their coordinated function may contribute to AH secretion by effecting bicarbonate transport into the eye.

## Keywords

aqueous humor; AE2; bicarbonate; carbonic anhydrase; ciliary epithelium

## INTRODUCTION

Aqueous humor (AH) is formed by active transport of solutes followed by osmotic flux of water across the double layer ciliary epithelium<sup>1, 2</sup>. This process requires coordinated action of several different ion transport mechanisms and ion channels<sup>3, 4</sup>. The ciliary epithelium bilayer consists of two types of cells joined by gap junctions in an apex to apex orientation<sup>5</sup>. The inner layer of pigmented ciliary epithelium (PE) faces the stromal blood supply and the outer nonpigmented ciliary epithelium (NPE) faces the AH in the posterior chamber of the eye. Solute transport across the bilayer gives rise to osmotic water movement, resulting in AH formation. The ability of carbonic anhydrase (CA) inhibitors to reduce the rate of AH formation points to an important role for CA<sup>6-9</sup>.

Mammalian CA is a family of enzymes consisting of at least 10 members, some localized in the cytoplasm and some membrane-associated<sup>10, 11</sup>. All members catalyze the reversible interconversion of CO<sub>2</sub> and HCO<sub>3</sub><sup>-</sup><sup>12, 13</sup>. In human erythrocytes it has been shown that the speed of the reaction is increased 23,000 fold by CA<sup>14</sup>. By increasing the availability of HCO<sub>3</sub><sup>-</sup>, CA can have significant effect on bicarbonate and proton transporter mechanisms. Thus sodium-hydrogen exchanger NHE1 function is influenced by CA inhibitors<sup>15, 16</sup> as is anion exchanger (AE)-mediated bicarbonate and chloride transport<sup>17-19</sup>. Here we show enrichment of AE2, CAII and CAIV in the porcine ciliary NPE layer. For consistency we also used the porcine eye to examine aqueous secretion and as a source of NPE cells for primary culture. In cultured porcine NPE, studies were conducted to determine effects of DIDS and acetazolamide on cytoplasmic pH responses to the HCO<sub>3</sub><sup>-</sup> addition or removal. We also provide evidence for a reduced AH formation rate in arterially perfused intact eyes treated with DIDS and acetazolamide, inhibitors of bicarbonate transporters and CA respectively.

## MATERIALS AND METHODS

### Perfused eye preparation

Fresh pig eyes were obtained from a local abattoir and perfused based on a similar method as described earlier<sup>20, 21</sup>. The eye was placed in a circulating warming jacket maintained at 37° C and covered with an insulated plastic cup. The ophthalmic artery was cannulated and the eye perfused with Krebs' solution at 37°C, that contained (mM) NaCl, 118; KCl, 4.0; MgSO<sub>4</sub>, 1.2; CaCl<sub>2</sub>, 2.0; NaHCO<sub>3</sub>, 25; KH<sub>2</sub>PO<sub>4</sub>, 1.2; glucose, 10; reduced glutathione, 1.0; ascorbate 0.05 and allopurinol, 1.8, at pH 7.4. The solution was bubbled with O<sub>2</sub> containing 5% CO<sub>2</sub>. Allopurinol, a xanthine oxidase inhibitor, was added to the perfusate to suppress oxidative damage and reperfusion injury. Using a peristaltic pump (Watson Marlow, 505S) perfusion flow was increased stepwise to 1.5 ml.min<sup>-1</sup>.

The anterior chamber was cannulated with two 23G needles connected to silicon rubber tubing filled with 1.04 ml of artificial AH containing (in mM): NaCl, 110; KCl, 3; CaCl<sub>2</sub>, 1.4; MgCl<sub>2</sub>, 0.5; KH<sub>2</sub>PO<sub>4</sub>, 0.9; NaHCO<sub>3</sub>, 30; glucose, 6; ascorbic acid, 3 sodium and fluorescein, 0.0186 at pH 7.6 and equilibrated with 95% O<sub>2</sub> - 5% CO<sub>2</sub>. The artificial AH formed a loop which circulated via a pump at 0.2 ml.min<sup>-1</sup> from the anterior chamber to a spectrophotometer cuvette (Pharmacia Biotech, Spectronic 2000) then back to the anterior chamber. Absorbance was recorded every 5 min at 490 nm. The two needles were kept wide apart to optimize fluid mixing. A third 23G needle in the anterior chamber was connected to a water manometer to measure intraocular pressure.

The AH formation rate was estimated from the rate of fluorescein dilution (decrease in absorbance) that occurred due to continuous secretion of AH into the eye. After a settling in period, the plot of  $\log_e$  [absorbance] vs time (min) was a straight line whose slope is the rate constant,  $K_{out}$  ( $\text{min}^{-1}$ ) of aqueous flow. Test agents were added to the arterial perfusate (i.e., to the stromal side) at a fixed concentration (100 $\mu\text{M}$  for DIDS and 500 $\mu\text{M}$  for acetazolamide).  $K_{out}$  determined 30 min before the addition of DIDS or acetazolamide to the arterial perfusate was considered as the control value for comparison with  $K_{out}$  measured in the presence of the test compound.

### Isolation and culture of porcine NPE

Porcine non-pigmented ciliary epithelium (NPE) was established in primary culture using a modification of our previous technique<sup>22</sup>. Porcine eyes were dissected to obtain the entire ring of NPE remained attached to the vitreous, leaving the pigmented cell (PE) layer attached to the ciliary body. The NPE ring was separated from the vitreous using fine scissors then cut into 1–2 mm pieces. NPE from 5–7 eyes was pooled and transferred to a 90 mm petri dish containing 15 ml of 0.015% collagenase A and 500 U/ml of hyaluronidase (Sigma St. Louis, MO, USA) in a collagenase buffer containing (in mM): NaCl 66.7, KCl 13.4, HEPES 3.8,  $\text{CaCl}_2$  4.8, pH 7.4. The petri dish was placed for 5–7 min on a rotary shaker in a 37°C incubator then removed from the shaker and the collagenase and hyaluronidase neutralized by adding excess (7ml) of a 1:1 mixture of newborn calf serum (NCS) and fetal bovine serum (FBS). NPE cells were dispersed by gentle trituration using a round-tipped pasteur pipette and pelleted by centrifugation at 2,000 rpm (670g) for 10 min at 4°C. The pellet was dispersed and the cells incubated without changing the medium for 3–4 days in a small volume of Dulbecco's modified Eagle's medium (DMEM) (Sigma, St Louis, MO) containing 10% FBS and 100 IU/ml gentamycin at 37°C in 5%  $\text{CO}_2$ /95% air. Thereafter the medium was them changed every alternate day. The cells grow to confluence in 7–10 days. At confluence the cells were trypsinized and seeded at a density of  $2 \times 10^4$  -  $5 \times 10^4$  cells. $\text{cm}^2$  for subsequent passages. In the studies reported here, only the 4<sup>th</sup> passage cells prior to confluence were used.

### Immunolocalization

Immunolocalization studies were conducted using fresh pig eyes. The cornea was removed then 4–6 mm of the anterior sclera was carefully peeled from the choroid all around the globe using a pair of curved scissors. The corneal remnant at the limbus was used as the handle to facilitate this dissection. The whole iris-ciliary body along with the lens and anterior vitreous was then removed from the posterior part of the globe. The tissue was placed in a petri dish (corneal face down) containing ice-cold Ringer solution comprising (mM) NaCl 113, KCl 4.6,  $\text{NaHCO}_3$  21.0,  $\text{MgSO}_4$  0.6, D-glucose 7.5, glutathione (reduced form) 1.0,  $\text{Na}_2\text{HPO}_4$  1.0, HEPES 10.0, and  $\text{CaCl}_2$  1.4, pH adjusted to 7.4. and the lens removed by cutting the zonules. Vitreous was carefully trimmed away then the iris was dissected away to leave the ciliary body which was fixed in formalin and used to prepare 5–7 $\mu\text{m}$  paraffin sections. Cultured NPE cells also were used for immunolocalization. The cells, which were grown on specially designed chamber slides (Nalge Nunc, Lab-Tek II Chamber Slides), were washed with PBS containing 1.0mM  $\text{MgCl}_2$  and 0.1mM  $\text{CaCl}_2$  then fixed in acetone for 2 min at room temperature. To probe for the cytoplasmic protein CAII, the cells were permeabilized before fixation using 0.1% Triton X-100 in PBS.

Tissue sections or cultured NPE cells prior to confluence were incubated at room temperature for 90 min in 10% goat serum in PBS (blocking buffer). Primary antibodies directed against either AE2 (10 $\mu\text{g/ml}$ ), CAII (10 $\mu\text{g/ml}$ ) or CAIV (20 $\mu\text{g/ml}$ ) were added for 24h at 4°C. Control specimens received only the blocking buffer. The specimens were washed with PBS and incubated 24h at 4°C with fluorescent secondary antibody (Alexa fluor 488 or 546 conjugated to either goat anti rabbit or anti mouse IgG, 1:200 dilution). The specimens were examined

using a Zeiss microscope (Axiovert 200M) and photographed using a Hamamatsu digital camera (C4742-95).

Some samples were prepared for confocal microscopic study. In these cases, nuclear counterstaining with TO-PRO-3 iodide (Invitrogen, Carlsbad, CA) was used to visualize the nucleus and the images were captured using a Zeiss LSM 510 meta-NLO confocal microscope (Carl Zeiss Inc, Thornwood, NY). Fluorescence excitation was achieved using 488, 543 and 633nm laser excitation wavelengths for FITC, rhodamine and TO-PRO-3 iodide respectively.

## RT-PCR

**Design of oligonucleotide probes for porcine AE1, AE2, AE3 and kNBC1**—There is currently no published sequence for the porcine ortholog of AE1. The bovine AE1 sequence (GenBank Acc# NM181036) was used to probe for a related sequence in the partially sequenced porcine genome (NCBI). This query resulted in several highly conserved hits located in proximity on chromosome 12 (genomic locales: 98698–98874 bp; 101907–102086 bp; 102522–102711 bp; 105983–106259 bp). Nucleotide sequences in these locales were 83–87% identical to the open reading frame of human AE1, but less than 80% (60–80%) identical to human AE2 and AE3, suggesting that the mined sequence is that of porcine AE1 (pAE1). The oligonucleotide probes used were sense: 5'-GTGACATCACAGACGCCTTGA-3' and antisense: 5'-CTCTGGTTTGCTGACGATCA-3'. The porcine ortholog of AE2 has been fully sequenced (GenBank Acc# AF120099), and the oligonucleotide probes used were sense: 5'-AGGAGATCTTCGCCTTCCTC-3' and antisense: 5'-AGCATCCAGGCATTTCATCT-3'. There is currently no published sequence for pAE3, and a sequence similar to hAE3 could not be found in the porcine genome. Nucleotide sequences conserved between the human, mouse, rabbit, rat and monkey AE3 cDNA sequences were used to design oligonucleotide probes for pAE3 (sense: 5'-AAGACCTTGGCTGTGAGCAG-3' and antisense: 5'-GCTGCTCCAAGAAAGGCAC-3'). The porcine ortholog of kNBC1 has been cloned (NM001030533), and the oligonucleotide probes used were sense: 5'-TCTTTTGCCTCTTTGCTGGT-3' and antisense: 5'-GCTTGAACCTCACTTGGCACA-3'.

**RNA isolation and RT-PCR**—Total RNA from porcine renal cortex, cardiac muscle, native NPE and primary cultures of NPE at passage #4 was isolated using RNA-Bee (Tel-Test, Inc., Friendswood, TX). Porcine kidney was used as a positive control for AE1 and AE2, whereas porcine myocardial tissue served as a control for AE3. One microgram of total RNA was reverse transcribed using the QuantiTect Reverse Transcription kit according to the manufacturer's protocol (Qiagen, Valencia, CA). This protocol includes a step to remove genomic DNA. Four microliters of cDNA was used in the PCR reaction. The PCR components were assembled followed by a single denaturing step for 2 min at 94C. This was followed by 35 cycles of: 94C for 30 sec, 55C for 30 sec and 72C for 2 min. A final elongation step of 7 min (72C) was included after the last cycle. PCR products were separated on 1% agarose gels and visualized with ethidium bromide. Amplified products were purified with the QIAquick gel extraction kit (Qiagen), and sequences were confirmed with an Applied Biosystems 3730xl DNA analyzer at the University of Arizona sequencing facility. The partially cloned pAE1 and pAE3 sequences were 87% and 92% identical to the hAE1 and hAE3 sequences, respectively. The cloned pAE2 sequence was identical to the previously published pAE2 sequence.

## Measurement of cytoplasmic pH

NPE cells grown to pre-confluence on 35mm plastic dishes (Corning) were loaded for 10 min with the pH-sensitive dye BCECF-AM (5.0 $\mu$ M) as described earlier<sup>23</sup>, placed in a temperature controlled perfusion micro-incubator (PDMI-2, Harvard Biosciences, Holliston, MA) on the stage of an upright epifluorescence microscope (Nikon Eclipse 80i, Japan) and superfused with a HEPES buffer containing (in mM): NaCl, 137; KCl, 4.5; D-glucose, 6.0; MgCl<sub>2</sub>, 1.0;

CaCl<sub>2</sub>, 1.5 and HEPES, 10.0, adjusted with NaOH to pH 7.35. The flow rate was 3.0 ml.min<sup>-1</sup>. Cytoplasmic pH (pH<sub>i</sub>) was recorded using an Incyt imaging system (Intracellular Imaging Inc., Cincinnati OH) with an emission wavelength of 535nm and alternating excitation wavelengths of 488 nm and 460nm. pH<sub>i</sub> was calculated from the fluorescence intensity ratio I<sub>488</sub>/I<sub>460</sub>.

The cells were first superfused with the HEPES buffer for 5 min to obtain a stable pH<sub>i</sub> baseline then the superfusate was switched to bicarbonate/CO<sub>2</sub> buffer containing (in mM): NaCl, 117; KCl, 4.5; NaHCO<sub>3</sub>, 20; D-glucose, 6.0; MgCl<sub>2</sub>, 1.0; CaCl<sub>2</sub>, 1.5 and HEPES, 10.0 adjusted to pH 7.35 and equilibrated by gassing with 5% CO<sub>2</sub> and 95% air. In some experiments either a sodium-free or a low chloride buffer was used. The sodium-free bicarbonate /CO<sub>2</sub> solution contained (in mM): choline chloride, 117; choline bicarbonate, 20; KCl, 4.5; D-glucose, 6.0; MgCl<sub>2</sub>, 1.0 and CaCl<sub>2</sub>, 1.5 and HEPES 10. The low chloride buffer contained (in mM): sodium gluconate, 117; potassium gluconate, 4.5; NaHCO<sub>3</sub>, 20; glucose, 6.0; MgCl<sub>2</sub>, 1.0 and CaCl<sub>2</sub>, 2.5; HEPES, 10. To prepare sodium-free/ bicarbonate-free or low chloride/bicarbonate-free buffers, the bicarbonate was omitted and replaced with an equimolar amount of either sodium gluconate or choline chloride, respectively.

## Reagents

Mouse anti-human carbonic anhydrase IV monoclonal antibody was obtained from R & D Systems Inc. MN. Rabbit anti CAII polyclonal antibody was purchased from Santa Cruz Biotechnology, Inc, CA. Rabbit anti AE2 polyclonal antibody was obtained from Alpha Diagnostics International, TX. Secondary antibodies used to probe the bound primary antibodies were Alexa Fluor 488 goat anti-rabbit IgG and Alexa Fluor 546 goat anti-mouse IgG (Invitrogen Carlsbad, CA). Acetazolamide, methazolamide, 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid (DIDS) and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich, St Louis, MO, USA. 2',7'-bis(2-carboxyl)-5(6)-carboxyfluorescein-acetoxyethyl ester (BCECF) was purchased from Invitrogen. All other chemicals were purchased from Sigma-Aldrich. Stock solutions of test compounds were prepared in DMSO before addition to the cell superfusate or to the whole eye perfusate. Control solutions received DMSO only.

## Statistical analysis

A two-sample 't' test was used to analyze unpaired data and paired 't' test was used to compare paired samples. One way analysis of variance (ANOVA) followed by Bonferroni's post hoc multiple comparison tests was used to compare differences for more than two groups of data. A probability (P) value of <0.05 was considered significant.

## RESULTS

### The influence of DIDS and acetazolamide on aqueous humor formation

The rate of AH formation was measured in the intact, arterially perfused eye. Under control conditions the rate constant for AH formation ( $K_{out} \cdot \text{min}^{-1} \times 10^{-4}$ ) was  $28.0 \pm 2.0$  (n=5). To obtain the rate constant for AH formation under drug-treated condition, the anion transport inhibitor DIDS or the carbonic anhydrase inhibitor acetazolamide was added to the arterial perfusate. This represents stromal application. DIDS was added at a final concentration of either 10 or 100  $\mu\text{M}$  and acetazolamide was added at a final concentration of 500  $\mu\text{M}$ . At a concentration of 100  $\mu\text{M}$ , DIDS significantly reduced the rate constant for AH to  $20.0 \pm 2.0$  (n=5) (Fig 1A). The carbonic anhydrase inhibitor acetazolamide (500  $\mu\text{M}$ ) produced more pronounced reduction of the rate constant for AH formation to  $15.0 \pm 2.0$  (n=6) (Fig 1B).

## Expression and localization of chloride-bicarbonate exchanger (AE2) and carbonic anhydrase in porcine NPE

Since DIDS and acetazolamide reduce the rate of AH formation, experiments were conducted to examine chloride-bicarbonate exchanger and carbonic anhydrase expression in ciliary epithelium. Immunolocalization and RT-PCR studies were carried out to examine chloride-bicarbonate exchangers (AEs) in the ciliary epithelium bilayer and cultured NPE. AE2 was detected in the NPE cell layer where it was enriched at the basolateral membrane (Fig 2A). AE2 immunoreactivity was also present in cultured NPE (Fig 2B). AE1 and AE3 was not detected (result not shown). RT-PCR studies show presence of AE2 mRNA in both the native and cultured porcine NPE. AE1 or AE3 mRNA was not found (Fig 3). Carbonic anhydrase IV (CAIV) was localized to the surface of the NPE (Fig 4) but was not detectable in the PE layer. However, we acknowledge that the presence of heavy pigmentation in the PE cells might have masked the detection of fluorescence in these cells. In contrast, carbonic anhydrase II (CAII) distribution in the ciliary body was widespread as revealed by laser confocal microscopy (Fig 5A). CAII was abundant in cytoplasm of the NPE. CAIV and CAII immunoreactivity also was detected in the cultured NPE by laser confocal (Fig. 4B) and epifluorescence microscopy (Fig. 5B) respectively.

## Cytoplasmic pH response of cultured NPE to $\text{HCO}_3^-/\text{CO}_2$ addition

Figure 6 shows a typical cytoplasmic pH ( $\text{pH}_i$ ) response of cultured NPE when the bathing medium was switched for 5 min from bicarbonate-free HEPES-buffered solution to a  $\text{HCO}_3^-/\text{CO}_2$ -buffered solution. Baseline  $\text{pH}_i$  in  $\text{HCO}_3^-/\text{CO}_2$ -free HEPES buffer was  $7.25 \pm 0.13$ . When the superfusate was switched to  $\text{HCO}_3^-/\text{CO}_2$  buffer there was a rapid fall in  $\text{pH}_i$  ( $0.4 \pm 0.02$  units,  $n=10$ ) followed by gradual recovery towards baseline. Subsequent replacement of  $\text{HCO}_3^-/\text{CO}_2$  medium with HEPES-buffered solution caused  $\text{pH}_i$  to rise sharply ( $0.4 \pm 0.04$  units,  $n=10$ ) then gradually recover towards the baseline. The  $\text{pH}_i$  response was examined under several different conditions: in sodium-free buffer, in low chloride buffer, in the presence of DMA, in the presence of DIDS and in the presence of acetazolamide or methazolamide.

The addition of  $\text{HCO}_3^-/\text{CO}_2$  acidifies the cells due to rapid diffusion of  $\text{CO}_2$  into the cells, its carbonic anhydrase-mediated conversion to  $\text{H}_2\text{CO}_3$  and subsequent dissociation of  $\text{H}_2\text{CO}_3$  to  $\text{HCO}_3^-$  and  $\text{H}^+$ . After this, we suggest the gradual alkalization towards baseline occurs as the result of bicarbonate entry. DIDS at a concentration of  $100 \mu\text{M}$  prevented the gradual alkalization (Fig 7 & Fig 8). Sodium-free buffer also abolished the gradual alkalization and on replacement of external sodium, the rate of  $\text{pH}_i$  recovery returned to the normal (Fig 8). However, the rate of gradual alkalization was not significantly different in low chloride buffer or in the presence of  $100 \mu\text{M}$  DMA (Fig 8). The findings are consistent with the notion that the gradual increase of  $\text{pH}_i$  is due to bicarbonate entry via a sodium-dependent anion transporter. Both acetazolamide ( $500 \mu\text{M}$ ) and methazolamide ( $100$  &  $500 \mu\text{M}$ ) significantly reduced the rate of gradual alkalization (Fig 8).

The removal of external  $\text{HCO}_3^-/\text{CO}_2$  causes  $\text{pH}_i$  to increase sharply due to the rapid exit of  $\text{CO}_2$ . After this, we suggest, the gradual acidification toward baseline was due to chloride-sensitive  $\text{HCO}_3^-$  efflux. The evidence is as follows. DIDS significantly inhibited the gradual acidification (Fig 9). The rate of gradual acidification also was reduced in low chloride buffer (Fig 9). The rate of gradual acidification measured in the presence of the sodium-hydrogen exchange inhibitor, DMA ( $100 \mu\text{M}$ ), was not significantly different from the control rate (Fig 9). The findings are consistent with the gradual reduction of  $\text{pH}_i$  due to bicarbonate exit via a sodium-independent anion exchanger. The carbonic anhydrase inhibitors acetazolamide ( $500 \mu\text{M}$ ) and methazolamide ( $100$  &  $500 \mu\text{M}$ ) both completely inhibited the gradual acidification (Fig 9).

### Effect of DIDS and acetazolamide on baseline cytoplasmic pH

Since DIDS blocked and acetazolamide reduced the rate of cytoplasmic pH recovery in bicarbonate-containing buffer and since both the drugs reduced AH formation in isolated intact eye preparations, we studied the effect of these drugs on the baseline cytoplasmic pH of cultured NPE. DIDS (100 $\mu$ M) caused a significant progressive reduction of baseline cytoplasmic pH (Fig 10). After 20 min, the time point at which measurement of AH formation was started in the intact eye experiments, DIDS had lowered pH by ~0.6 pH units. In cells exposed to acetazolamide (500 $\mu$ M) pH<sub>i</sub> constantly was slightly lower than control pH<sub>i</sub> but at any one time point the difference was not significant (Fig 10). In control cells there is a slight and gradual drift in cytoplasmic pH, which may be due to dye bleaching.

## DISCUSSION

Three lines of evidence point to expression of the AE2 chloride-bicarbonate exchanger in porcine NPE; RT-PCR detection of mRNA, protein immunolocalization, and the observation of chloride-sensitive pH responses. Consistent with a previous report from the human ciliary body<sup>24</sup>, RT-PCR detected neither AE1 nor AE3. The ability of the ciliary epithelium bilayer to form aqueous humor is determined by the location of transport proteins and immunolocalization studies revealed expression of AE2 in the NPE layer. AE2 appeared most dense at the NPE basolateral surface, but it was not strictly limited to the cell border. The apparent cytoplasmic signal could stem from nonspecific antibody binding or the intracellular presence of AE2 trafficking vesicles. AE2 expression is consistent with earlier functional evidence for an electroneutral sodium-independent and DIDS-sensitive Cl<sup>-</sup>/HCO<sub>3</sub><sup>-</sup> exchange mechanism in native rabbit NPE<sup>25</sup>. Together with AE2, the porcine NPE layer also displayed abundant CAII, which appeared in the cytoplasm, and CAIV which was localized to the membrane. To our knowledge, this is the first report on localization of AE2 in native porcine ciliary epithelium but the presence of carbonic anhydrase has been demonstrated earlier in rabbit, monkey and human ciliary body using histochemical methods<sup>26–28</sup>. In human NPE, carbonic anhydrase was reported at the basal and lateral membranes as well as in the cytoplasm<sup>28</sup>. As well as CAII, other carbonic anhydrases reported in human ciliary body include membrane bound isoforms CAIX and CAXII<sup>29</sup>. Western blot analysis and functional studies using a membrane impermeable carbonic anhydrase inhibitor pointed to the presence of the membrane bound CAIV in rabbit NPE<sup>30</sup>. Matsui and coworkers suggested that CAIV could be linked to chloride/bicarbonate exchanger function in the NPE<sup>9</sup>.

Using cultured porcine NPE we were able to examine anion transport indirectly by measuring pH<sub>i</sub> responses. Although there are likely to be significant differences between native and cultured cells, porcine NPE in primary culture was found to maintain expression of AE2, CAII and CAIV and NBC as judged by RT-PCR and immunolocalization. Earlier we have shown similar patterns of nitric oxide synthase (NOS 1, 2 and 3 isoforms), Na,K-ATPase (alpha 1, 2 and 3 isoforms)<sup>22</sup>, and NHE (NHE1, 3 and 4 isoforms), aquaporin (AQP-1 and AQP-4) and connexins (Cx50 and Cx43) (unpublished observation, 2007) in cultured and native porcine NPE.

In cells superfused with a CO<sub>2</sub>/HCO<sub>3</sub><sup>-</sup> free HEPES buffer, exposure to a CO<sub>2</sub>/HCO<sub>3</sub><sup>-</sup> containing buffer caused a rapid acidification followed by a gradual pH<sub>i</sub> increase. Subsequent replacement of CO<sub>2</sub>/HCO<sub>3</sub><sup>-</sup> with HEPES buffer caused rapid alkalization followed by gradual pH<sub>i</sub> decrease. A similar response to CO<sub>2</sub>/HCO<sub>3</sub><sup>-</sup> addition and removal has been reported by Wolosin and coworkers in native rabbit NPE cells<sup>25, 31</sup> who suggested the initial rapid pH<sub>i</sub> changes likely resulted from rapid entry or exit of CO<sub>2</sub>. In the present study we focused on the subsequent slower pH<sub>i</sub> changes that are dependent, at least in part, on bicarbonate transport.

A gradual  $\text{pH}_i$  increase back toward baseline was observed following the rapid acidification caused by addition of  $\text{CO}_2/\text{HCO}_3^-$ . Alkalinizing systems reported in the ciliary epithelium include  $\text{Na}^+-\text{HCO}_3^-$  cotransporter (NBC)<sup>32</sup>, Na-H exchanger (NHE)<sup>33</sup> and vacuolar H-ATPase (V-ATPase)<sup>34, 35</sup>. Here, the rate of alkalization was completely inhibited by Na-free solutions and DIDS, but not significantly altered in cells exposed to low chloride solution or to the NHE inhibitor DMA. The results are consistent with a  $\text{Na}^+-\text{HCO}_3^-$  cotransporter that enables external bicarbonate to enter the cell. This notion fits with an earlier proposal that a  $\text{Na}^+-\text{HCO}_3^-$  cotransporter and  $\text{Cl}^-/\text{HCO}_3^-$  exchanger are the two principal determinants of NPE cytoplasmic pH<sup>25</sup>. The lack of sensitivity to DMA suggests NHE-mediated proton export does not contribute to the observed alkalization response. This apparently contrasts with our previous finding that porcine native and cultured NPE express abundant quantities of NHE1, NHE3 and NHE4 and that DMA significantly inhibits  $\text{pH}_i$  recovery in this cell after acidification by a 20 mM ammonium chloride pulse (Shahidullah, et al. IOVS 2007;48:ARVO E-Abstract). The explanation may lie in the fact that the smaller degree of acidification caused by exposure to  $\text{CO}_2/\text{HCO}_3^-$  (~0.4 pH unit below baseline) compared to 20mM ammonium chloride pulse (>1 pH unit below baseline) is not sufficient to cause activation of NHE. It is well known that NHE activity is regulated primarily by  $\text{pH}_i$  and increases markedly in response to intracellular acidosis<sup>36</sup>. Such NHE activation is thought to occur through the interaction of  $\text{H}^+$  with an allosteric modifier site within the transport domain<sup>37, 38</sup>. The ability of DIDS to completely inhibit the NPE alkalization response following acidification by  $\text{CO}_2/\text{HCO}_3^-$  addition argues against the contribution of NHE or H-ATPase to the  $\text{pH}_i$  rise observed in the present studies.

A gradual acidification back towards baseline was observed after the rapid  $\text{pH}_i$  rise on removal of extracellular  $\text{CO}_2/\text{HCO}_3^-$  and return to HEPES buffer. The rate of gradual acidification was inhibited by both DIDS and low chloride solution. The results are consistent with a chloride-bicarbonate exchanger, such as AE2, that enables bicarbonate to exit the cell in exchange for external chloride entry. Removal of extracellular chloride results in the reversal of the exchanger due to favorable gradient for AE2-mediated chloride efflux in the presence of ample extracellular bicarbonate. The gradual acidification could be abolished by carbonic anhydrase inhibitors, either acetazolamide or methazolamide. The sodium-hydrogen exchange inhibitor DMA (100  $\mu\text{M}$ ) had no effect on the gradual acidification. The results are consistent with findings in other tissues where AE2-mediated bicarbonate export effects recovery from alkaline loading<sup>39, 40</sup>. The sensitivity of the gradual acidification to acetazolamide or methazolamide can be explained if bicarbonate transport is rate-limited by availability of cytoplasmic  $\text{HCO}_3^-$  and can be inhibited by build up of extracellular  $\text{HCO}_3^-$ . On this basis, cytosolic CAII might provide the “push” for bicarbonate transport by making  $\text{HCO}_3^-$  available to the cytoplasmic face of AE2, and CAIV-catalyzed conversion of  $\text{HCO}_3^-$  to  $\text{CO}_2$  in the extracellular unstirred layer might provide the “pull” by diminishing the concentration of  $\text{HCO}_3^-$  at the extracellular AE2 face. Such a “push-pull” mechanism, resulting from CA activity on both sides of the NPE basolateral membrane, could accelerate AE2-mediated bicarbonate transport into the eye. The pH alkalization response described above, which we propose is mediated by bicarbonate uptake via  $\text{Na}^+-\text{HCO}_3^-$  cotransport, was only partially inhibited by carbonic anhydrase inhibitors acetazolamide or methazolamide. This may reflect the greater availability of  $\text{HCO}_3^-$  in the extracellular solution compared to cytoplasm.

The fact that the gradual alkalization after cytoplasmic acidification was abolished by DIDS and sodium-free solution but was insensitive to extracellular chloride removal indicates that the alkalization is due to NBC-mediated bicarbonate entry into the cells. Since AE2 in the NPE cells localized mostly on the basolateral surface, and because AE2 enables bicarbonate to exit the cell in exchange for external chloride entry, it is tempting to speculate that the NBC might be localized on the apical membrane of the NPE cells. Apical localization of NBC has



been reported in other secretory tissues, such as in human parotid and sublingual duct<sup>41</sup> and in rat pancreatic duct<sup>42</sup> epithelium.

The ability of acetazolamide and DIDS to abolish  $\text{HCO}_3^-$  export by cultured porcine NPE is interesting in light of the ability DIDS and acetazolamide to reduce AH secretion in the intact porcine eye. The findings are consistent with a contribution of bicarbonate transport to AH formation by the porcine eye. Acetazolamide reduced the rate of aqueous humor secretion by 40% and DIDS inhibited AH secretion by 25%. DIDS previously has been reported to reduce aqueous formation in the bovine eye<sup>20</sup>. It is interesting to note that DIDS significantly reduced basal cytoplasmic pH in cultured NPE, lowering  $\text{pH}_i$  by ~0.6 pH units after 20 min. In the perfused intact eye experiments, a 20 min interval was allowed to establish drug effect prior to measuring the effect of DIDS on AH formation rate. It should also be noted that DIDS is not a selective inhibitor of  $\text{Cl}^-/\text{HCO}_3^-$  exchangers such as AE2. It also inhibits  $\text{Na}^+/\text{HCO}_3^-$  cotransport<sup>24, 43</sup>, and chloride channels<sup>44</sup>. The carbonic anhydrase inhibitor, acetazolamide had little effect on basal cytoplasmic pH in the cultured NPE, even though it had a robust effect in reducing AH formation rate in the perfused intact eye. One explanation for this apparent discrepancy might be that *in vitro* and with continuous  $\text{CO}_2$  bubbling of the bathing medium there might be sufficient hydration of  $\text{CO}_2$  in the absence of the catalysis by the carbonic anhydrase. Since  $\text{CO}_2$  is a membrane permeable, such noncatalytic hydration would be equally effective in the bathing medium as well as in the cytoplasm of cultured NPE. Perhaps this could help maintain the normal  $\text{HCO}_3^-$  transport and acid-base balance in the cultured cells. In the intact eye preparation or *in vivo* situation, aqueous humor  $\text{CO}_2$  equilibration might be less efficient and hence sufficient hydration of  $\text{CO}_2$  would be more dependent on the carbonic anhydrase. Consequently, inhibition of carbonic anhydrase would cause insufficient hydration of  $\text{CO}_2$  and hence insufficient production of  $\text{HCO}_3^-$  inside the cells for transport into the aqueous by basolateral AE2. On this basis, inhibition of AE2 by DIDS would inhibit transport of bicarbonate into the aqueous in exchange for  $\text{Cl}^-$ . Inhibition of carbonic anhydrase would cause depletion of  $\text{HCO}_3^-$  required for transport by the AE2 and so have a similar effect; less bicarbonate transport to the aqueous and hence reduction in the rate of AH formation. Reduction of  $\text{pH}_i$ , observed in response to DIDS in the present experiments, may also slow down the overall metabolic rate in the NPE, in a way that leads to inhibition of AH formation.

In contrast to the minimal effect of acetazolamide on  $\text{pH}_i$  in porcine NPE in the present study, a 0.2 pH units reduction of  $\text{pH}_i$  was detected in rabbit transformed NPE<sup>30</sup>. This may be due to a difference in the contribution of bicarbonate transport to the formation of AH in different species. For example, it had been shown previously that bicarbonate depletion in the bathing medium of rabbit ciliary body preparation completely reversed the electrical polarity<sup>45</sup>, whereas the same maneuver only inhibited the short circuit current ( $I_{sc}$ ) by approximately 30% in the ox<sup>46</sup> and ~90 in the pig<sup>47</sup>. According to these results, the importance of  $\text{HCO}_3^-$  transport in the porcine ciliary body falls between that of rabbit and ox.

In summary, our results suggest that porcine NPE uses a  $\text{Na}^+/\text{HCO}_3^-$  cotransporter to import bicarbonate and a  $\text{Cl}^-/\text{HCO}_3^-$  exchanger to export bicarbonate. Carbonic anhydrase influences the rate of bicarbonate transport. The  $\text{Cl}^-/\text{HCO}_3^-$  exchanger AE2 and carbonic anhydrases CAII and CAIV are enriched in the NPE layer of the ciliary body and their coordinated function may contribute to aqueous humor secretion by effecting bicarbonate transport into the eye.

## Acknowledgments

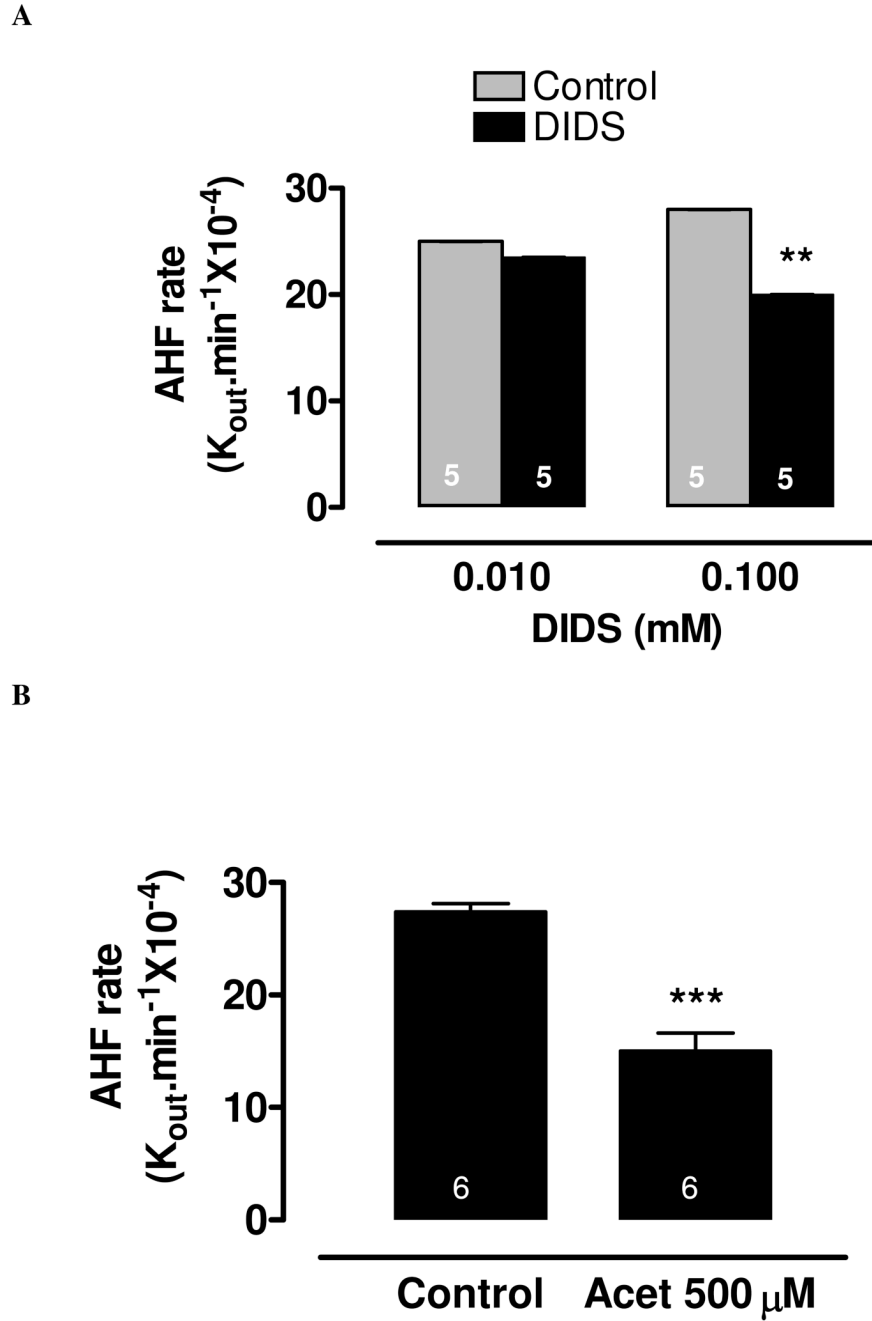
Funding: NIH grant number EY006915

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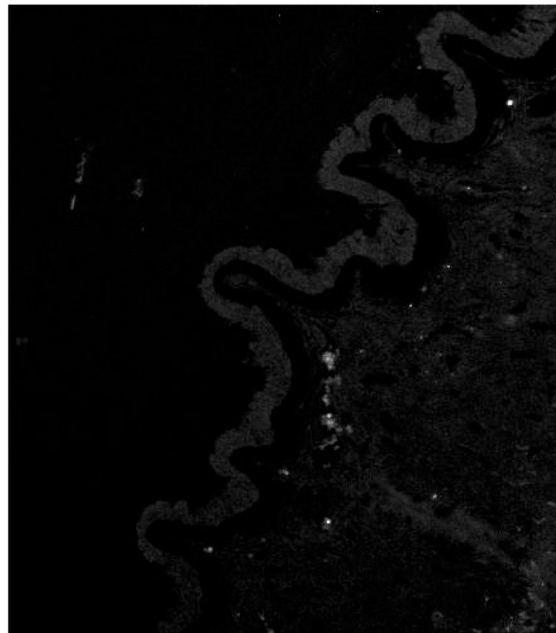
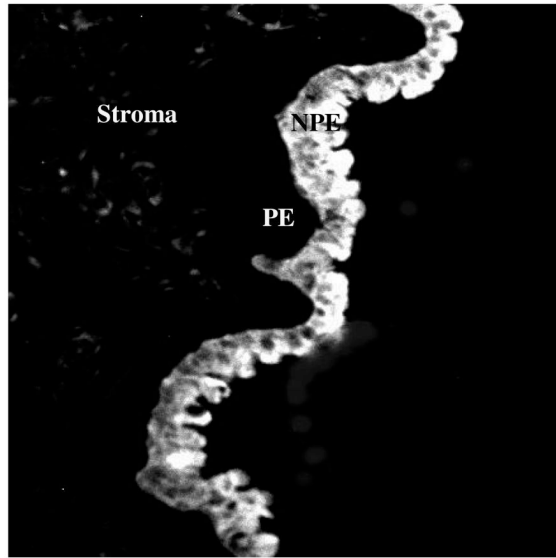
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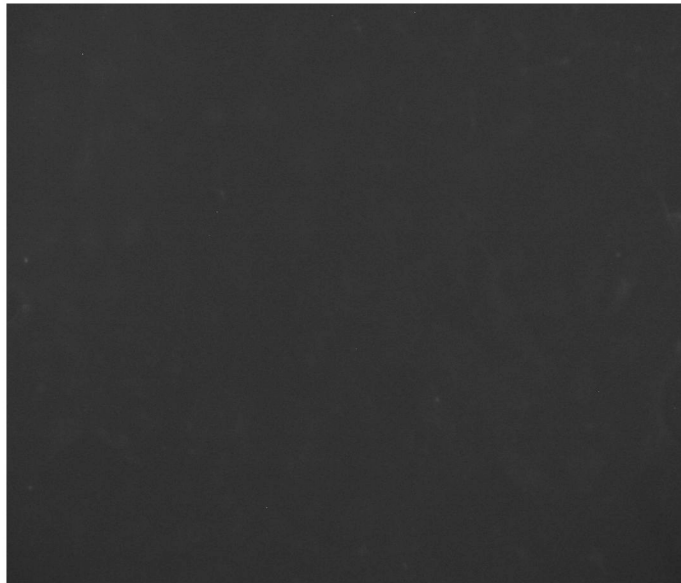
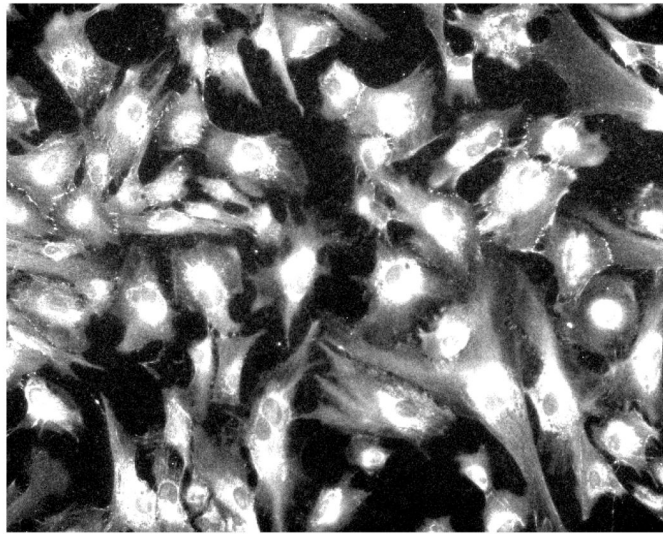
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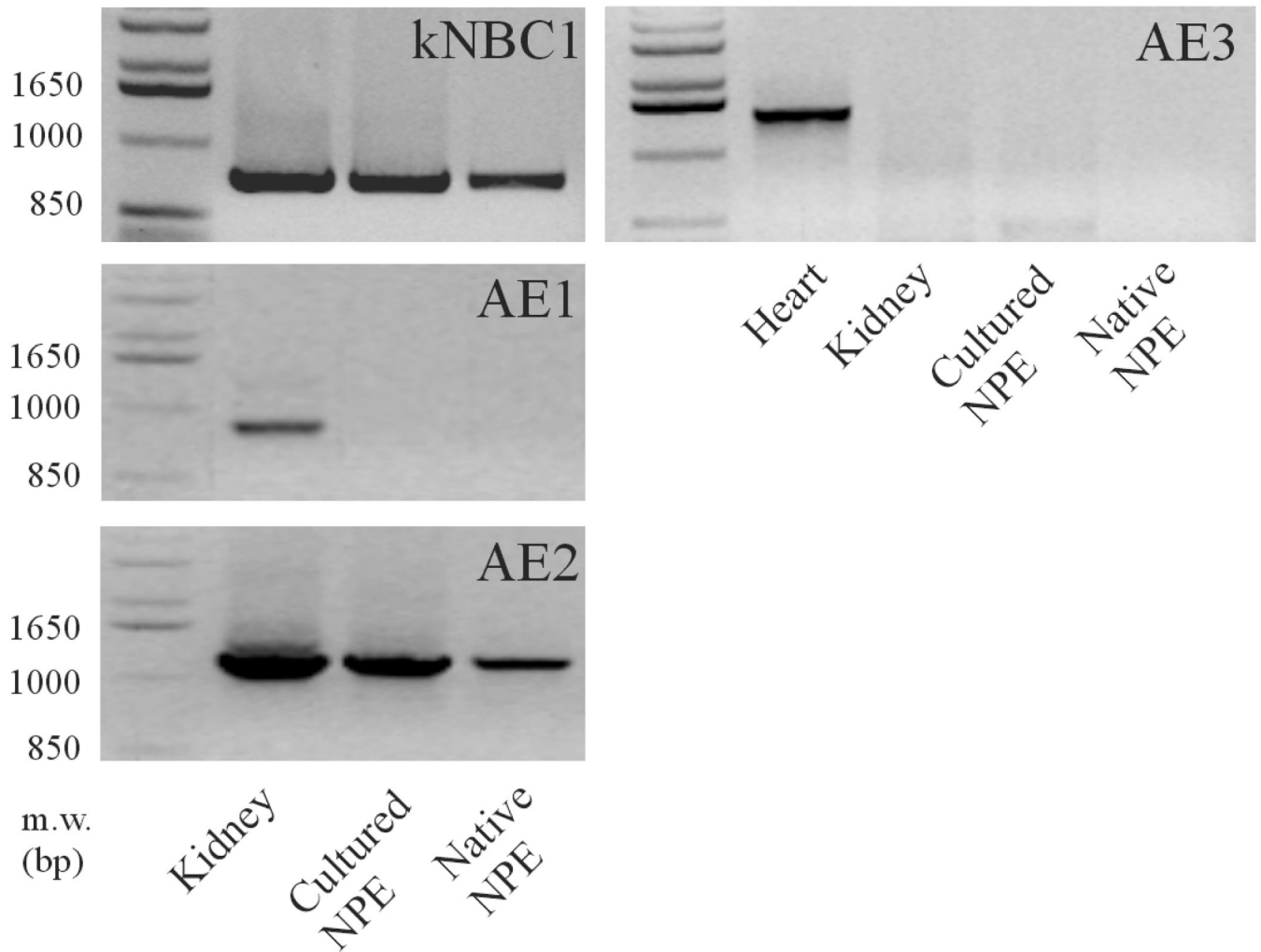
**Figure 1.** The effect of DIDS (10μM & 100μM) (A) and acetazolamide (500μM) (B) on AH formation rate measured in the porcine isolated perfused whole eye preparation. AH formation was measured by a fluorescein dilution technique. The results are expressed as a rate constant (K<sub>out</sub>.min<sup>-1</sup> X 10<sup>-4</sup>), and are shown as mean±SEM of 5 independent experiments for each condition. The rate measured during the first 30 min prior to drug addition was taken as the control value. After the addition of drug, a 20 min period was allowed to establish the drug effect, then the rate was measured over the subsequent 60 min. Significant differences from control were indicated by \*\**P*<0.01 (panel A) and \*\*\**P*<0.001 (panel B).

Figure 2A



**Figure 2B**

**Figure 2.** Immunolocalization of  $\text{Cl}^-/\text{HCO}_3^-$  exchanger AE2 in the porcine ciliary body where it appears on the NPE basolateral membrane (A). PE, pigmented epithelium; NPE, nonpigmented epithelium (Original Magnification 200x). AE2 also was detected in cultured NPE (B) (4<sup>th</sup> passage cells are shown). The negative controls, in which the primary antibody was replaced by PBS, show no staining. (Magnification 200x)

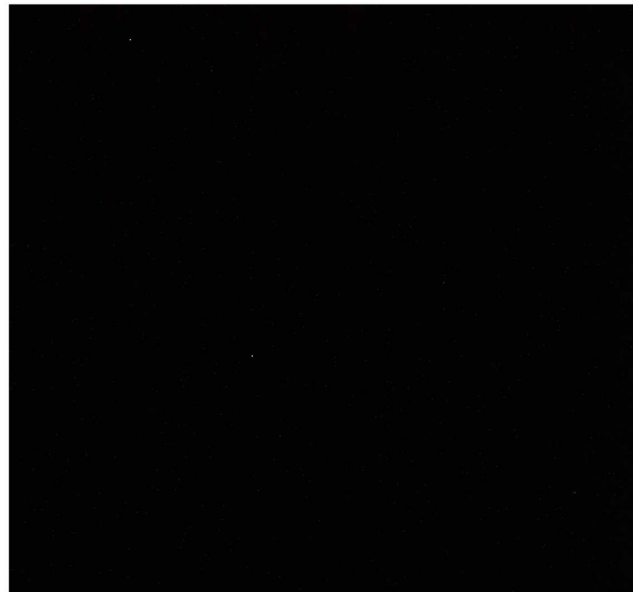
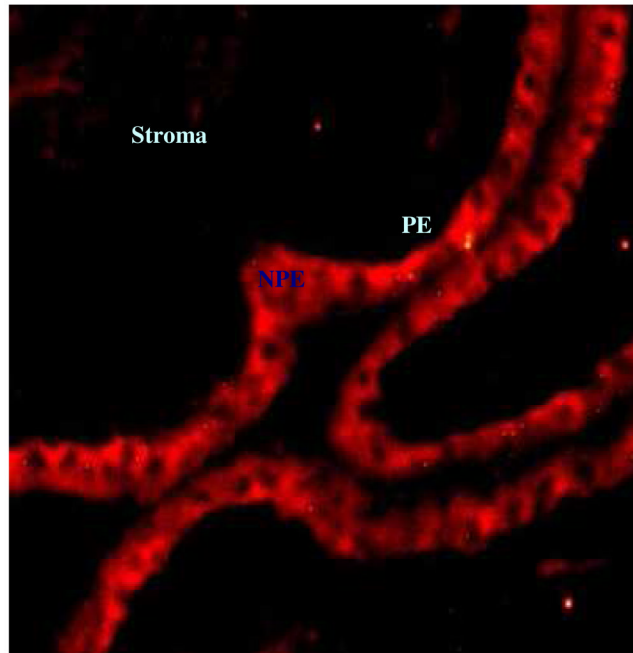


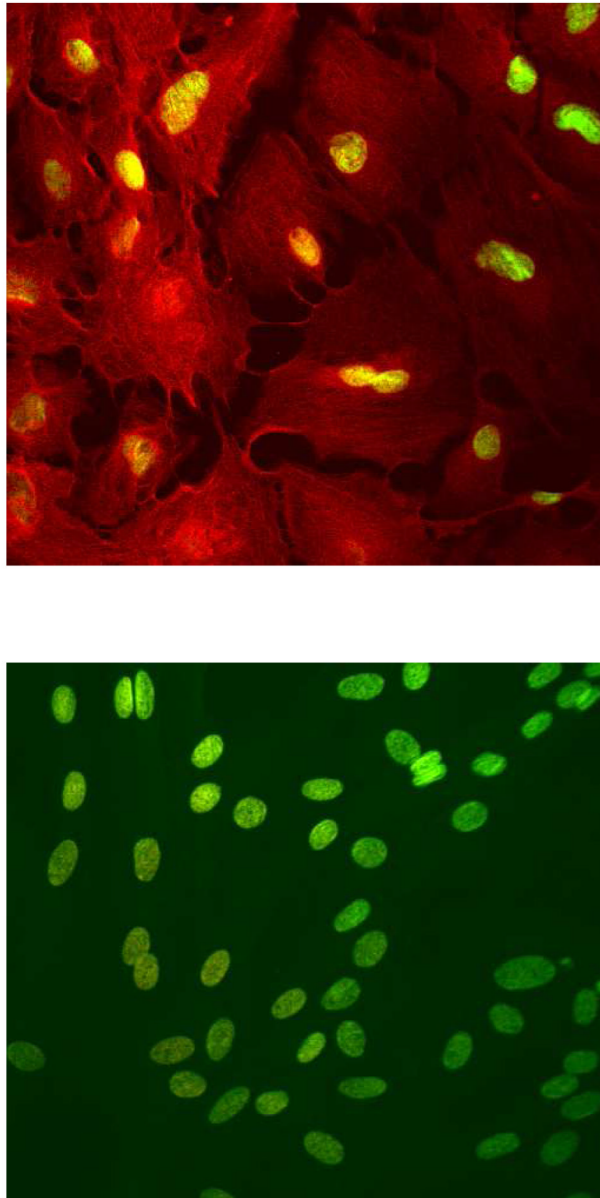
**Figure 3.**

RT-PCR for AE1, AE2, AE3 and kNBC1 in native and primary cultures of porcine non-pigmented epithelium (NPE). Porcine renal cortex (kidney) and porcine cardiac muscle (heart) served as positive controls for AE1, AE2, kNBC1 and AE3, respectively. Amplified products were separated on 1% agarose gels and visualized with ethidium bromide. The resulting amplified cDNA products were gel purified and their sequences were confirmed with DNA sequencing.



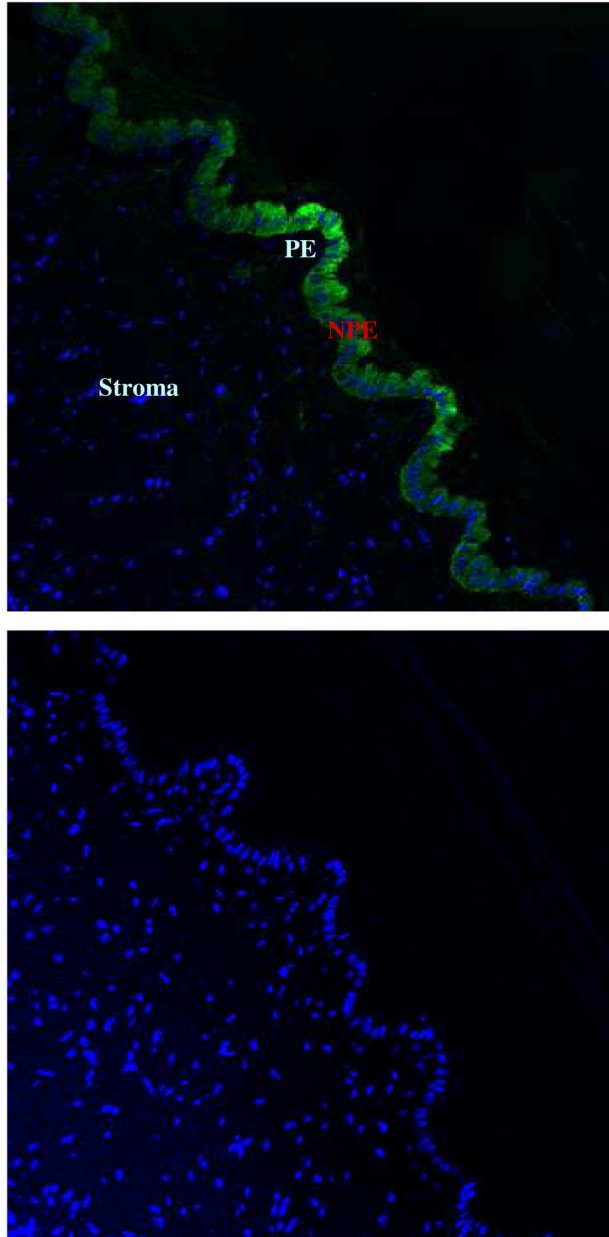
Figure 4A

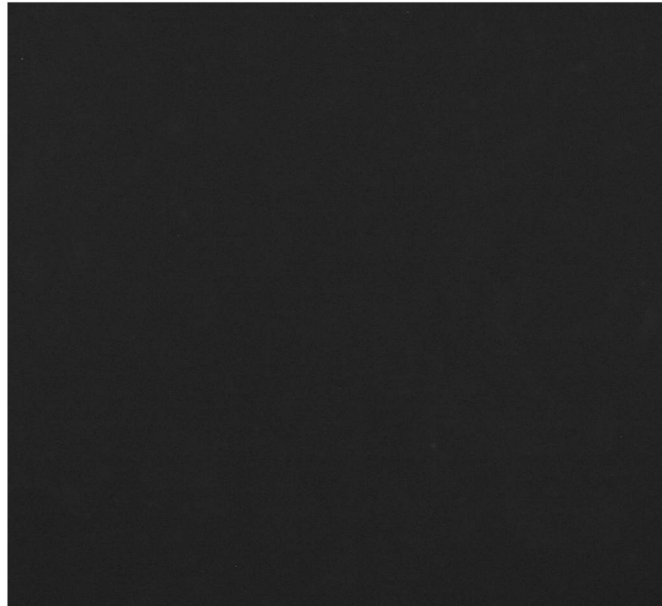
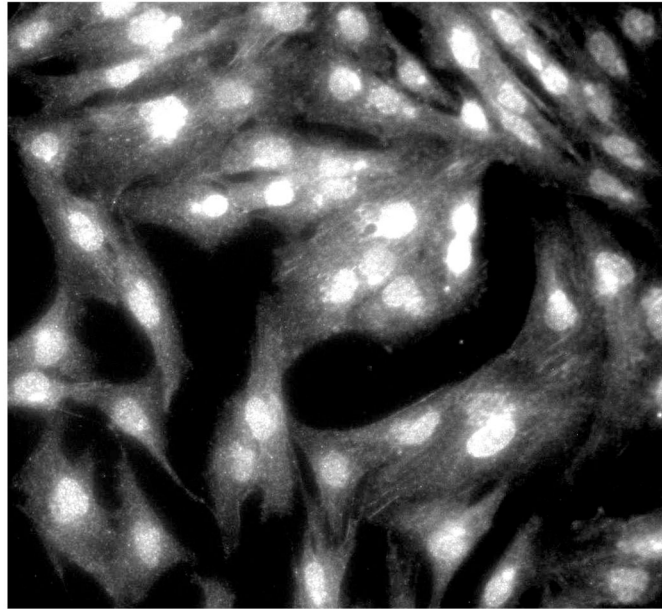


**Figure 4B**

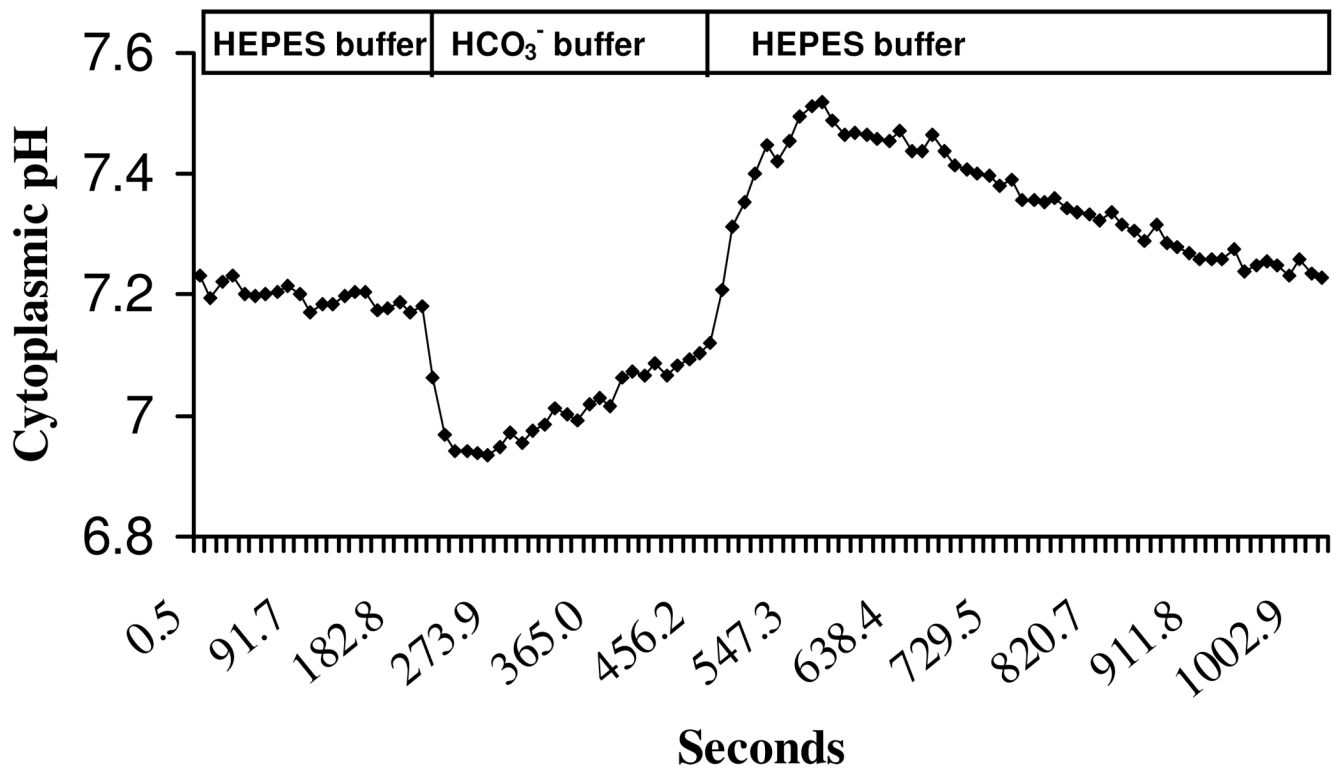
**Figure 4.** Immunolocalization of carbonic anhydrase IV in the porcine ciliary body where it appears on the NPE membrane (A). PE, pigmented epithelium; NPE, nonpigmented epithelium (Original magnification 200x). Carbonic anhydrase IV also was detected in cultured NPE by laser confocal microscopy (B) (4<sup>th</sup> passage cells are shown). The negative controls, in which the primary antibody was replaced by PBS, show no staining (Original magnification 400x).

Figure 5A



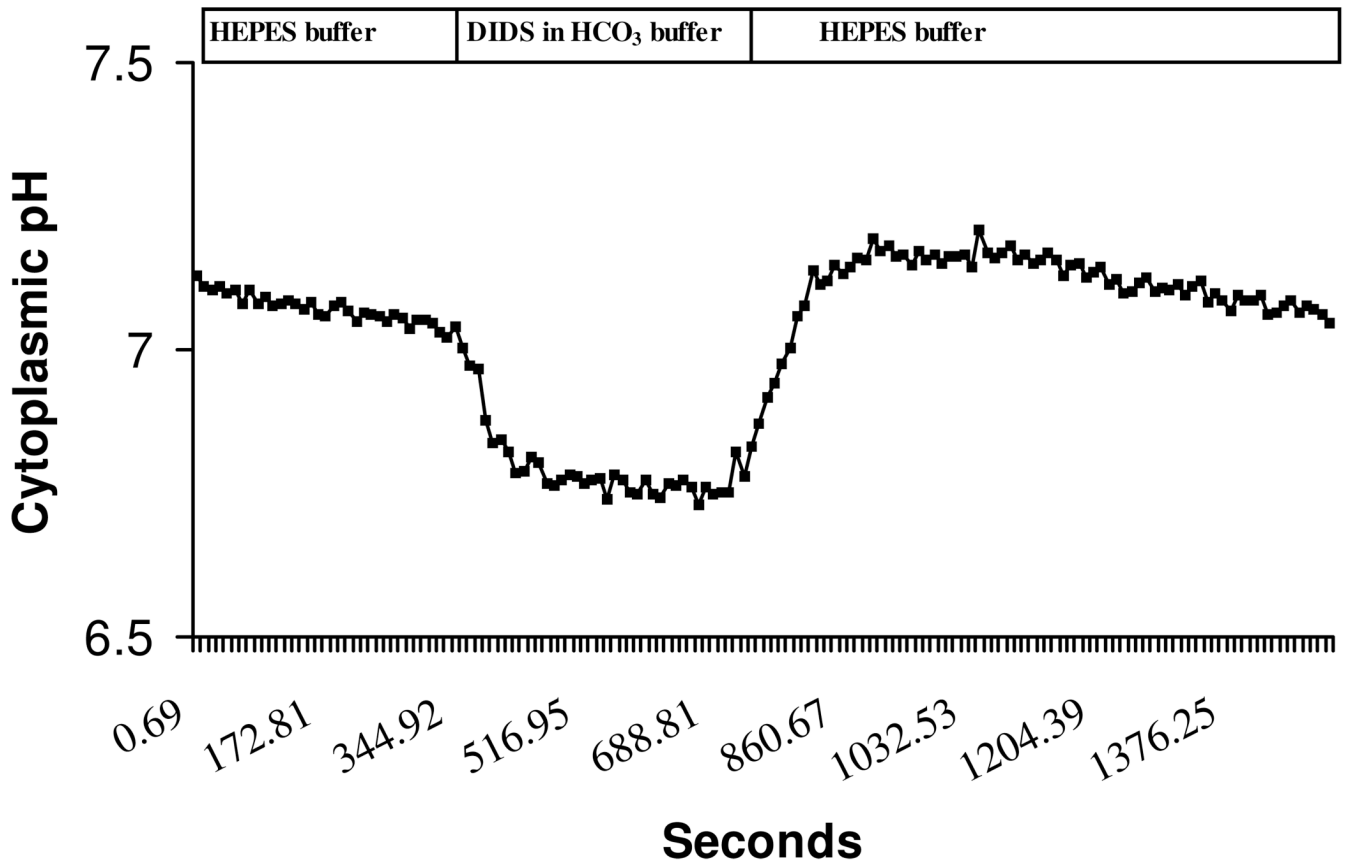
**Figure 5B**

**Figure 5.** Immunolocalization of carbonic anhydrase II in the porcine ciliary body by laser confocal microscopy, where it appears within the cytoplasm of the NPE (A). PE, pigmented epithelium; NPE, nonpigmented epithelium (Original magnification 200x). Carbonic anhydrase II also was detected in cultured NPE (B) (4<sup>th</sup> passage cells are shown). The negative controls, in which the primary antibody was replaced by PBS, show no staining (Original magnification 200x).



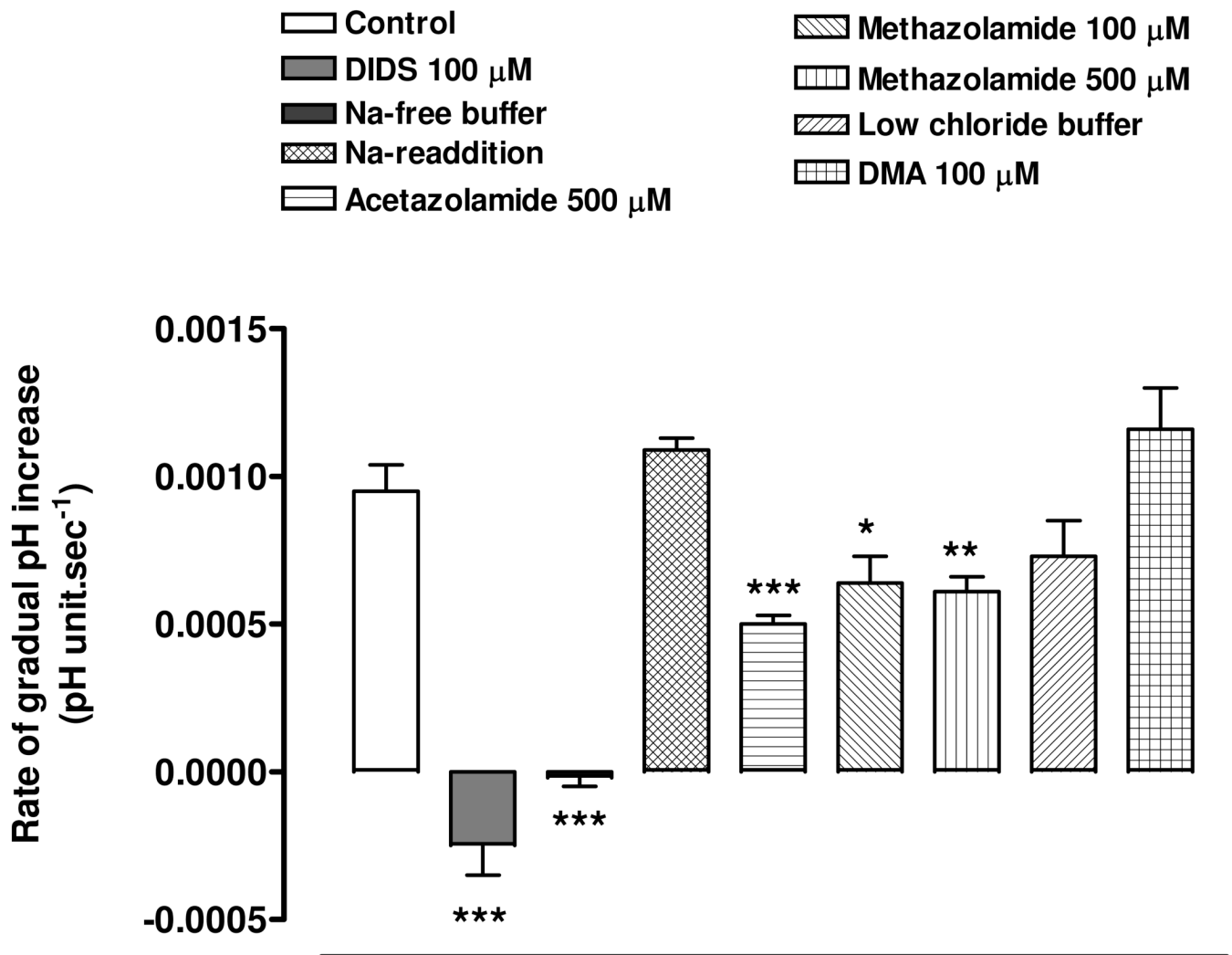
**Figure 6.**

A typical cytoplasmic pH ( $\text{pH}_i$ ) response of BCECF-loaded porcine non-pigmented ciliary epithelial cells to the addition and subsequent removal of  $\text{HCO}_3^-/\text{CO}_2$  from the bathing solution. The cells first were superfused with bicarbonate-free HEPES buffered Krebs' solution for 5 min to obtain a stable baseline. The superfusate was then switched to  $\text{HCO}_3^-/\text{CO}_2$  buffer. This caused a rapid fall in  $\text{pH}_i$ , which gradually recovered towards baseline. Subsequent removal of  $\text{HCO}_3^-/\text{CO}_2$  and replacement with HEPES buffer caused a rapid  $\text{pH}_i$  rise followed by a gradual recovery towards baseline.



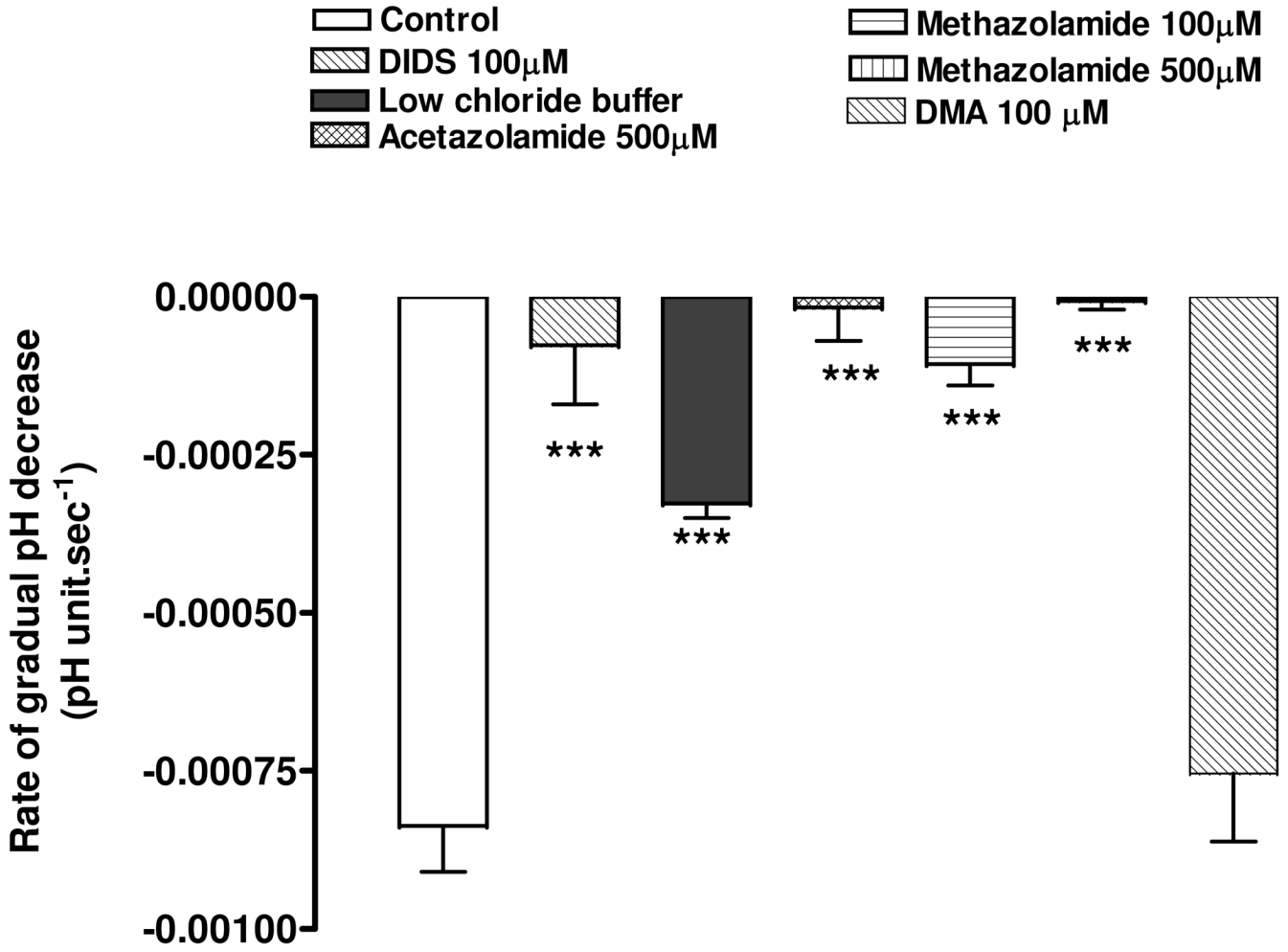
**Figure 7.**

A typical cytoplasmic pH ( $pH_i$ ) response of BCECF-loaded NPE to the addition and subsequent removal of  $HCO_3^-/CO_2$  from the bathing solution in the presence of DIDS ( $100 \mu M$ ). The cells first were superfused with bicarbonate-free HEPES buffered Krebs' solution for 5 min to obtain a stable baseline, then the superfusate was then switched to  $HCO_3^-/CO_2$  buffer for 5 min.



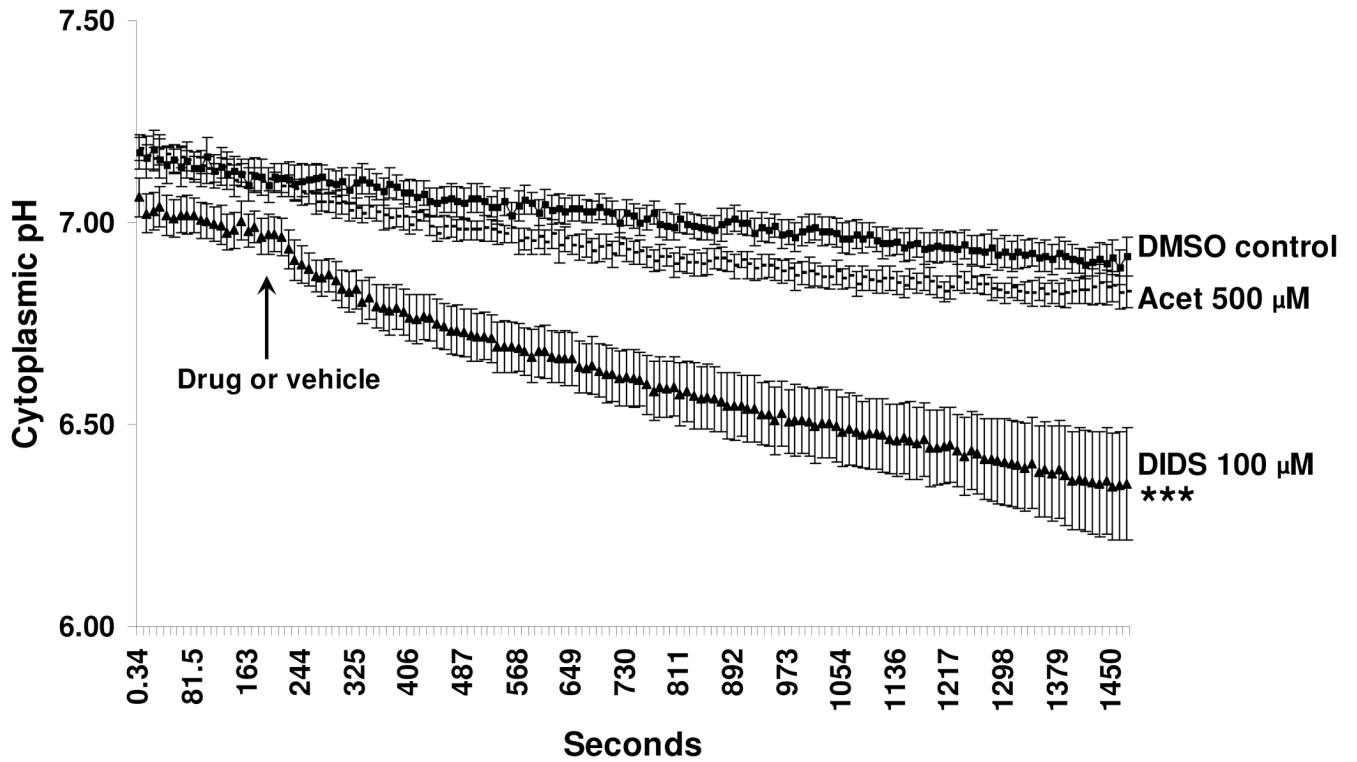
**Figure 8.**

The effect of DIDS (100  $\mu\text{M}$ ), sodium-free buffer, low chloride buffer, CA inhibitors acetazolamide (500  $\mu\text{M}$ ) and methazolamide (100 and 500 $\mu\text{M}$ ) and sodium-hydrogen exchange inhibitor DMA (100  $\mu\text{M}$ ) on the rate of gradual alkalization towards baseline following the rapid  $\text{pH}_i$  fall caused by the addition of  $\text{HCO}_3^-/\text{CO}_2$  to the bathing medium. The results are the mean $\pm$ SEM of data from 5 or 10 independent experiments. A significant difference from control is indicated by \* $P$ <0.05, \*\* $P$ <0.01 or \*\*\* $P$ <0.001.



**Figure 9.** The effect of DIDS (100 µM), low chloride buffer, CA inhibitors acetazolamide (500 µM) and methazolamide (100 and 500µM) and sodium-hydrogen exchange inhibitor DMA (100 µM) on the rate of gradual acidification towards baseline following the rapid pH<sub>i</sub> rise caused by the removal of HCO<sub>3</sub><sup>-</sup>/CO<sub>2</sub> and replacement with HEPES buffer. The results are the mean±SEM of data from 6 or 10 independent experiments. A significant difference from control is indicated by \*\*\**P* < 0.0001.





**Figure 10.**

The effect of DIDS and acetazolamide on baseline cytoplasmic pH of porcine cultured NPE. BCECF-loaded cells were first superfused with bicarbonate-containing buffer for 3 min to establish baseline cytoplasmic pH. At this point DIDS 100 μ, acetazolamide 500 μM or vehicle DMSO 0.1% was introduced (arrow) and data collection was continued for a further 20 min. The results are shown as mean±SEM of 7–10 independent experiments. At the final time point,  $pH_i$  in DIDS-treated cells showed a significant difference from control \*\*\* $P < 0.001$ .