

Cytoplasmic pH Responses to Carbonic Anhydrase Inhibitors in Cultured Rabbit Nonpigmented Ciliary Epithelium

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Abstract. Carbonic anhydrase (CA) inhibitors lower the rate of aqueous humor (AH) secretion into the eye. Different CA isozymes might play different roles in the response. Here we have studied the effects of carbonic anhydrase inhibitors on cytoplasmic pH (pH_i) regulation, using a dextran-bound CA inhibitor (DBI) to selectively inhibit membrane-associated CA in a cell line derived from rabbit NPE. pH_i was measured using the fluorescent dye BCECF and the pH_i responses to the cell permeable CA inhibitor acetazolamide (ACTZ) and DBI were compared. ACTZ markedly inhibited the rapid pH_i changes elicited by bicarbonate/ CO_2 removal and readjustment but DBI was ineffective in this respect, consistent with the inability of DBI to enter the cell and inhibit cytoplasmic CA isozymes. Added alone, ACTZ and DBI caused a similar reduction (0.2 pH units) of baseline pH_i . We considered whether CA-IV might facilitate H^+ extrusion via Na-H exchange. The Na-H exchanger inhibitor amiloride (1 mM) reduced pH_i 0.52 ± 0.10 pH units. In the presence of DBI, the magnitude of pH_i reduction caused by amiloride was significantly ($P < 0.05$) reduced to 0.26 ± 0.09 pH units. ACTZ similarly reduced the magnitude of the pH_i reduction. DBI also reduced by ~40% the rate of pH_i recovery in cells acidified by an ammonium chloride (20 mM) prepulse; a reduction in pH_i recovery rate was also caused by ACTZ and amiloride. DBI failed to alter the pH_i alkalization response caused by elevating external potassium concentration, a response insensitive to amiloride but sensitive to ACTZ. These observations are consistent with a reduction in Na-H exchanger activity in the presence of

DBI or ACTZ. We suggest that the CA-IV isozyme might catalyze rapid equilibration of H^+ and HCO_3^- with CO_2 in the unstirred layer outside the plasma membrane, preventing local accumulation of H^+ which competes with sodium for the same external Na-H exchanger binding site. Inhibition of CA-IV could produce pH_i changes that might alter the function of other ion transporters and channels in the NPE.

Key words: pH — Carbonic anhydrase — Ciliary epithelium — Acetazolamide

Introduction

Carbonic anhydrase catalyzes the reversible hydration of CO_2 , producing carbonic acid which subsequently decomposes to HCO_3^- and H_3O^+ . There are multiple CA isozymes with the cytoplasmic isozyme CA-II generally being the most predominant (Sly & Hu, 1995). One CA isozyme, CA-IV, is a plasma membrane protein and there is considerable evidence that it exerts an influence on membrane transport mechanisms which shift protons or HCO_3^- . CA-IV has been proposed to facilitate recycling of extracellular H^+ and HCO_3^- at the outer surface of the plasma membrane by catalyzing the generation of CO_2 which diffuses back into the cell (Sly & Hu, 1995). In renal proximal tubule, for example, CA-IV has a key role in the absorption of bicarbonate from the lumen (Lucci et al. 1983).

CA inhibitors lower the rate of aqueous humor secretion into the eye and are widely used to reduce intraocular pressure (IOP) in the treatment of glaucoma (Maren, 1984). Aqueous humor formation is driven osmotically by solute transport across the ciliary epithelium bilayer comprising the nonpigmented epithelium (NPE) on the aqueous side and the pigmented epithelium (PE) on the blood side (Davson, 1990). It has been established that CA inhibitors reduce not only fluid forma-

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tion but also cause marked inhibition in the net rate of bicarbonate and sodium movement from blood to aqueous (Maren, 1984). However, it is not known whether CA-IV inhibition might contribute to these CA inhibitor responses. There have been relatively few physiologic studies of CA-IV in ciliary epithelium. In an elegant study with the isolated rabbit ciliary epithelium bilayer, a preparation which retains cell polarity, Matsui et al. (1996) have suggested that the NPE cell layer has a membrane-associated CA but it was not detected in PE. Inhibition of the membrane-associated carbonic anhydrase at the NPE side of the bilayer caused a marked increase in short circuit current across the tissue. Using cultured rabbit NPE, we determined ~20% of total CA activity is associated with the plasma membrane. (Wu, Delamere & Pierce, 1997). In the same study, the plasma membrane-associated CA was found to be resistant to SDS, a characteristic associated specifically with the CA-IV isozyme and Western blot confirmed the presence of CA-IV immunoreactive polypeptide. In addition, CA inhibitors were found to cause small changes in cytoplasmic pH (pH_i). The present study was conducted to determine whether inhibition of membrane-associated carbonic anhydrase changes pH_i responses to maneuvers which acidify or alkalinize the cell. We synthesized a dextran-bound CA inhibitor (DBI) and compared cellular pH responses to this and acetazolamide, a cell permeable CA inhibitor. We provide evidence suggesting the CA-IV isozyme could have a role in maintaining cytoplasmic acid-base balance in the cultured NPE. Since the study was conducted using an NPE cell line which is not well polarized into apical and basolateral domains, we were not able to study how inhibition of membrane-associated carbonic anhydrase might alter vectorial transport.

Materials and Methods

CHEMICALS

Amiloride and acetazolamide (ACTZ) were purchased from Sigma (St. Louis, MO). BCECF-AM was purchased from Molecular Probes (Eugene, OR). Water insoluble compounds were dissolved in a minimum volume of dimethyl sulfoxide (DMSO) or methanol (<0.1% final concentration). Equal amounts of DMSO or methanol were added to control solutions.

CELL CULTURE

The experiments were conducted using a cell line derived from SV 40 virus-transformed rabbit nonpigmented ciliary epithelium (NPE). This cell line, kindly provided by Dr. M. Coca-Prados (Yale University), has been used previously for a number of ion transport studies (Delamere et al. 1993; Dong, Delamere & Coca-Prados, 1994). Monolayers of cells were grown on 35-mm diameter petri dishes at 37°C in Dulbecco's modified Eagle's medium (DMEM) (GIBCO, Gaithersburg, MD) supplemented with 10% fetal bovine serum, penicillin (100 U/ml) and

streptomycin (100 µg/ml) under a humidified atmosphere of 5% CO₂ and 95% air. The medium was changed every two days. It took 3–5 days for cells to reach confluence after each split.

MEASUREMENT OF INTRACELLULAR pH BY SPECTROFLUOROMETRY

The fluorescent pH sensitive dye, 2',7'-biscarboxyethyl-5(6)-carboxyfluorescein acetoxymethyl ester (BCECF-AM) was used to measure intracellular pH in monolayers of cultured rabbit cells which were continuously superfused (2.5 ml/min) with artificial aqueous humor (AAH) containing (in mmoles/l) NaCl: 117.0, KCl: 4.5, HEPES: 10.0, NaHCO₃: 20.0, glucose: 6.0, MgCl₂: 1.0, CaCl₂: 1.5, at pH 7.3. The AAH was continuously bubbled with 5% CO₂ and 95% air. Bicarbonate-free solutions, made by omitting NaHCO₃ and substituting an equimolar amount of NaCl, were bubbled with nitrogen. Sodium-free solutions were made substituting choline chloride and choline bicarbonate for sodium chloride and sodium bicarbonate respectively. Potassium-rich AAH was made by substituting an equimolar amount of potassium chloride for sodium chloride. The superfusate pH was monitored by a pH electrode (Fisher, Pittsburgh, PA). Prior to each experiment, the cells were incubated 1 hr in AAH containing BCECF-AM (1.5 µM in AAH) then washed three times with AAH before the petri dish was placed on the stage of a fluorescence microscope (Zeiss, Thornwood, NY) equipped with an Attofluor fluorescence intensity quantification system. A water jacket was used to maintain temperature in the dish at 37°C.

Fluorescence intensity of BCECF was measured at an emission wavelength of 520 nm using alternating excitation wavelengths of 460 nm and 488 nm. The fluorescence intensity ratio (I_{488}/I_{460}) as a function of intracellular pH was calibrated at the end of each experiment by superfusing the cells with a range of potassium-rich buffer solutions which contained 10 µM nigericin (Sigma Chemical, St. Louis, MO). Nigericin mediates membrane K⁺-H⁺ exchange and in combination with a potassium-rich buffer serves to equilibrate intracellular pH with extracellular pH (Thomas et al. 1979). The potassium-rich solution contained 110 mM KCl, 20 mM NaCl and 20 mM of a buffer selected to control pH. The buffer 2-(N-morpholino) ethanesulfonic acid (MES, $pK_a = 6.1$) was used to set pH in the range 6.0 to 6.5; piperazine-N,N'-bis (2-ethanesulfonic acid) (PIPES, $pK_a = 6.8$) was used to set pH at 7.0; N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES, $pK_a = 7.5$) was used to set pH at 7.4; N-tris(hydroxymethyl) methyl-3-aminopropanesulfonic acid (TAPS, $pK_a = 8.4$) was used to set pH at 8.0 (Bassnett, 1990).

SYNTHESIS OF A DEXTRAN-BOUND CARBONIC ANHYDRASE INHIBITOR

A dextran-bound carbonic anhydrase inhibitor (DBI), composed of an aminothiadiazole sulfonamide which is covalently bound to a dextran, was synthesized by a modification of the method described by Tinker, Coulson & Weiner (1981). Aminoethyl dextran was linked to 2-succinylamido-1,3,4-thiadiazole-5-sulfonamide using 1-(3-dimethylaminopropyl)-3-ethyl-carbodiimide as the coupling reagent. After synthesis, the DBI was dialyzed exhaustively against deionized water using a 3500 Dalson cutoff membrane (Spectrum Medical Industries, Houston, TX). The average MW of the dextran was 9400. At a concentration of 0.2 mg/ml, DBI causes 96 ± 4% inhibition of bovine CA-II which was not significantly different from the inhibition of 100 ± 7% caused by 0.2 mg/ml (1 mM) acetazolamide (Wu et al., 1997).

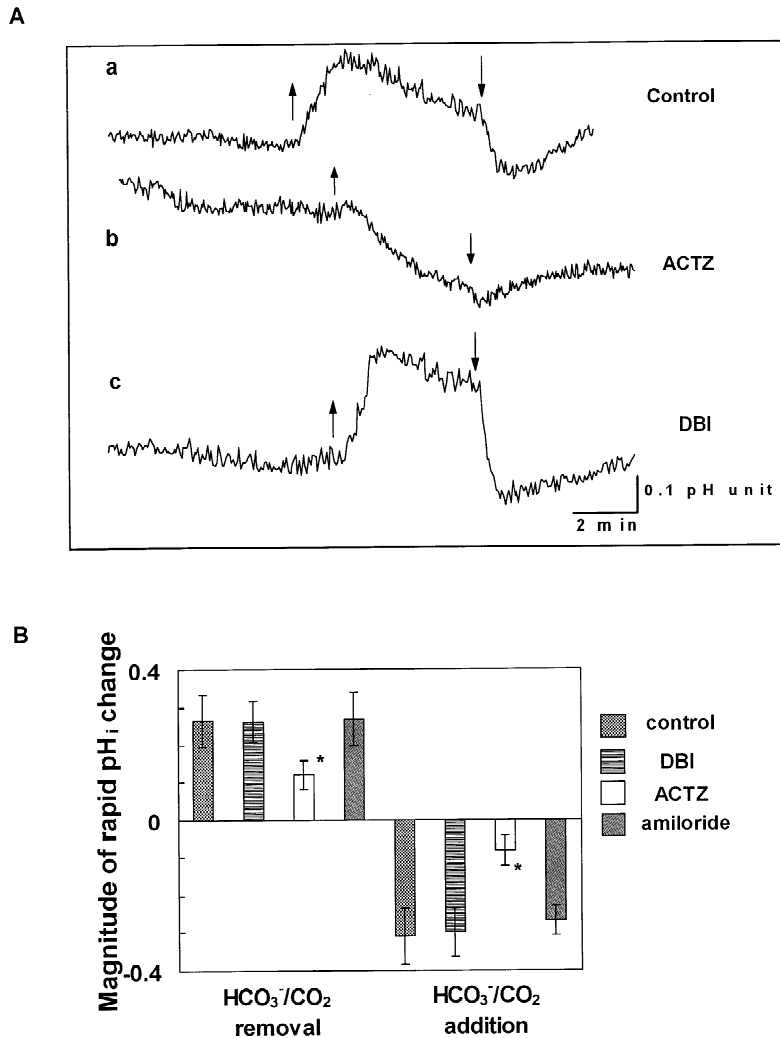


Fig. 1. Cytoplasmic pH responses to bicarbonate/CO₂ removal and readdition. Cultured cells were first superfused with control solution. The upper panel (A) shows the records from typical experiments; pH_i is shown plotted against time. After the pH_i had stabilized for at least 5 min, the superfusate was switched to bicarbonate/CO₂-free AAH for ~5 min (↑) then returned to control (bicarbonate/CO₂-containing) solution (↓). Record (a) was obtained in control cells (no CA inhibitor). Records (b) and (c) were obtained from cells exposed to 0.2 mg/ml ACTZ and 0.2 mg/ml DBI respectively. In each experiment the magnitude of the rapid pH_i changes in response to bicarbonate/CO₂ removal and readdition was recorded. The lower panel (B) shows the means of results from 5–10 different experiments. The steady state pH_i value prior to bicarbonate (CO₂) removal was 7.14 ± 0.10 (*n* = 10) in control cells, 6.96 ± 0.12 (*n* = 8) in ACTZ-treated cells, 6.94 ± 0.11 (*n* = 7) in DBI-treated cells and 6.75 ± 0.11 (*n* = 5) in amiloride-treated cells. The vertical bars indicate SD. Significant difference from control (*P* < 0.05) is indicated by *.

DATA ANALYSIS

In each BCECF experiment, fluorescence data were collected from ~50 cells in the monolayer and the signal was averaged. *n* values signify the number of separate and independent experiments. Unless otherwise noted, values are reported as mean ± SD and statistical analysis was conducted using Student's *t* test. Values of *P* < 0.05 were considered to indicate significant differences.

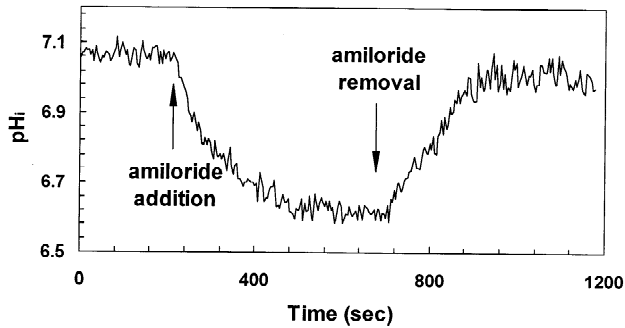
Results

THE INFLUENCE OF Ca INHIBITORS ON THE pH_i RESPONSE TO BICARBONATE REMOVAL

Cultured monolayers of NPE cells were pretreated with BCECF-AM then superfused with artificial aqueous humor (AAH). After a stable baseline cytoplasmic pH (pH_i) was obtained, the superfusate was switched to bicarbonate/CO₂-free AAH. In control cells (absence of CA inhibitor) this caused an immediate pH_i increase fol-

lowed by a more gradual decline (Fig. 1). After 5–10 min in bicarbonate-free medium, pH_i stabilized at 6.89 ± 0.10, a reduction of 0.24 ± 0.10 pH units from the starting pH_i of 7.13 ± 0.11 measured in the same cells (mean ± SD; *n* = 14). The pH_i response reversed when bicarbonate was returned to the superfusate. Based on studies in other tissues (Zeidel, Patricio & Seifter, 1986; Burkhardt, Sato & Frömter, 1984), the rapid component of the pH_i change observed upon external bicarbonate removal or addition is generally dependent upon carbonic anhydrase. In keeping with this scheme, the magnitude of the rapid pH_i increase was significantly smaller when external bicarbonate was removed in the presence of 0.2 mg/ml ACTZ. The rapid pH_i decrease caused by bicarbonate readdition was also inhibited by ACTZ. In contrast, 0.2 mg/ml DBI caused no significant change in the magnitude of the rapid pH_i responses to bicarbonate/CO₂ removal and readdition. For comparison, 1.0 mM amiloride also caused no significant change in the magnitude of the pH_i responses. These findings suggest that

A



B

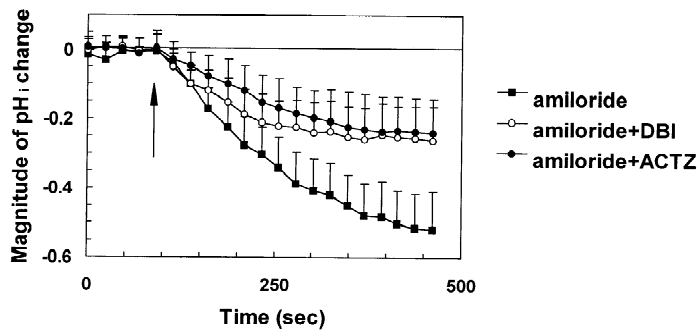


Fig. 2. The influence of amiloride on pH_i . The upper panel (A) shows a typical response. Cells were first superfused with control solution. After the pH_i had stabilized for at least 5 min, the superfusate was switched (\uparrow) to contain 1.0 mM amiloride for ~ 8 min then amiloride was removed (\downarrow). In two different sets of experiments, the cells were first superfused ~ 5 min with solution containing 0.2 mg/ml DBI or 0.2 mg/ml ACTZ then the superfusate was switched (arrow) to contain 1 mM amiloride in the continued presence of DBI or ACTZ. The magnitude of the pH_i change is shown plotted against time. Each set of data represents the mean \pm SD of results from 6–15 experiments. Baseline pH_i was 7.09 ± 0.08 in control (no CA inhibitor) cells, 6.93 ± 0.10 in the DBI group prior to amiloride treatment and 6.91 ± 0.11 in the ACTZ group prior to amiloride treatment.

although cytosolic CA inhibition alters the rapid pH_i responses to external bicarbonate changes, selective inhibition of membrane-associated CA is ineffective in this respect. The inability of DBI to alter the rapid pH_i response caused by bicarbonate removal is consistent with the inability of DBI to cross the plasma membrane, a characteristic confirmed earlier in studies with red blood cells (Wu et al., 1997).

THE INFLUENCE OF Ca INHIBITORS ON RESTING pH_i

Added alone, DBI reduced pH_i by 0.22 ± 0.06 pH units (mean \pm SD; $n = 10$). A pH_i decrease of 0.21 ± 0.05 (mean \pm SD; $n = 13$) was observed in cells that received ACTZ, which inhibits both cytosolic and membrane-associated CA. This pattern of responses, which has been described in detail by Wu et al. (1997), is consistent with a contribution of CA-IV to cytoplasmic pH regulation. As a control experiment to address the possibility that simple addition of dextran might cause cytosolic pH changes, we confirmed that the addition of dextran alone did not pH_i ; 5 min after the addition of 0.2 mg/ml dextran, pH_i was 7.10 ± 0.07 which was not significantly different from the starting pH_i of 7.11 ± 0.06 (mean \pm SD; $n = 4$).

THE INFLUENCE OF Ca INHIBITORS ON THE pH_i RESPONSE TO AMILORIDE

The ability of DBI to reduce pH_i could be explained if CA-IV in some way facilitates proton extrusion via the Na-H exchanger. To examine the Na-H exchanger, cultured NPE cells were exposed to amiloride (Fig. 2). At a concentration of 1 mM, amiloride reduced pH_i by 0.52 ± 0.10 pH units (mean \pm SD; $n = 15$), bringing pH_i to a new set point of 6.56 ± 0.10 . A similar pH_i change was observed in cells exposed to $100 \mu\text{M}$ dimethylamiloride (*data not shown*). Removal of external sodium also reduced pH_i , causing a reduction of 0.65 ± 0.07 pH units (mean \pm SD; $n = 5$). In comparison, lowering the external chloride concentration to 5 mM increased pH_i by 0.23 ± 0.09 pH units to a set point of 7.30 ± 0.10 (mean \pm SD; $n = 5$).

In the presence of DBI, amiloride caused a significantly smaller pH_i reduction (Fig. 2), changing pH_i by only 0.26 ± 0.09 (mean \pm SD; $n = 8$). Thus selective inhibition of CA-IV reduces the amiloride-sensitive pH_i component. General CA inhibition had a similar effect. In cells exposed to ACTZ, amiloride caused a pH_i reduction of 0.24 ± 0.09 (mean \pm SD; $n = 6$). Since both DBI and ACTZ reduced pH_i prior to amiloride addition, the final pH_i set point measured 5 min after amiloride

addition was 6.66 ± 0.12 and 6.65 ± 0.11 in DBI- and ACTZ-treated cells respectively. This similarity between the pH_i set point in the presence of amiloride alone, amiloride plus DBI or amiloride plus ACTZ suggests that the effects of CA inhibition and Na-H inhibition on pH_i are not additive. Instead, the results are consistent with a partial inhibition of Na-H exchanger activity when membrane-associated CA-IV is inhibited.

THE INFLUENCE OF Ca INHIBITORS ON THE pH_i RESPONSE TO AMMONIUM CHLORIDE REMOVAL

Cultured NPE cells were exposed to 20 mM external ammonium chloride. After 3–4 min, the ammonium chloride was removed, causing a rapid cytoplasmic acidification followed by a slower pH_i recovery (pH_i increase). The rate of pH_i recovery is dependent in part on the rate of outwardly directed proton transport. Amiloride slowed pH_i recovery (Fig. 3), a response consistent with a contribution of Na-H exchanger-mediated outward proton transport to the pH_i increase. To test the idea that CA-IV inhibition slows the Na-H exchanger, the rate of pH_i recovery from an ammonium chloride prepulse was measured in the presence of DBI. DBI also slowed pH_i recovery. A similar reduction in the pH_i recovery rate was observed in the presence of ACTZ. The rate of pH_i recovery was 0.30 ± 0.12 pH units/min (mean \pm SD; $n = 12$) in control cells but was 0.15 ± 0.07 and 0.15 ± 0.06 pH units/min in the presence of DBI and amiloride respectively. For comparison, the rate of pH_i recovery was 0.15 ± 0.05 pH units/min in the presence of ACTZ. In each case, the pH_i recovery rate in treated cells was significantly ($P < 0.05$) lower than the rate measured in control cells. Although carbonic anhydrase inhibitors and amiloride both reduced the pH_i recovery rate, no additional rate decrease was observed when the two agents were applied simultaneously. When cells were exposed to amiloride and DBI added together or amiloride and ACTZ added together, the pH_i recovery rate was 0.19 ± 0.07 and 0.19 ± 0.08 pH units/min respectively which was no slower than the recovery rate seen with amiloride alone.

Following an ammonium chloride prepulse, a sizable component of pH_i recovery persists in the presence of amiloride, suggesting the contribution of mechanisms in addition to Na-H exchange. In sodium-free medium, the rate of pH_i recovery was reduced to 0.05 ± 0.02 pH units/min (mean \pm SE; $n = 4$) which was significantly ($P < 0.05$) less than the rate observed in the presence of amiloride. This could possibly be explained by the presence of a sodium-dependent bicarbonate uptake mechanism. This notion is supported by the detection of a reduced pH_i recovery rate (0.16 ± 0.02 pH units/min; mean \pm SE; $n = 6$) in bicarbonate/ CO_2 -free medium as compared to the recovery rate in control medium.

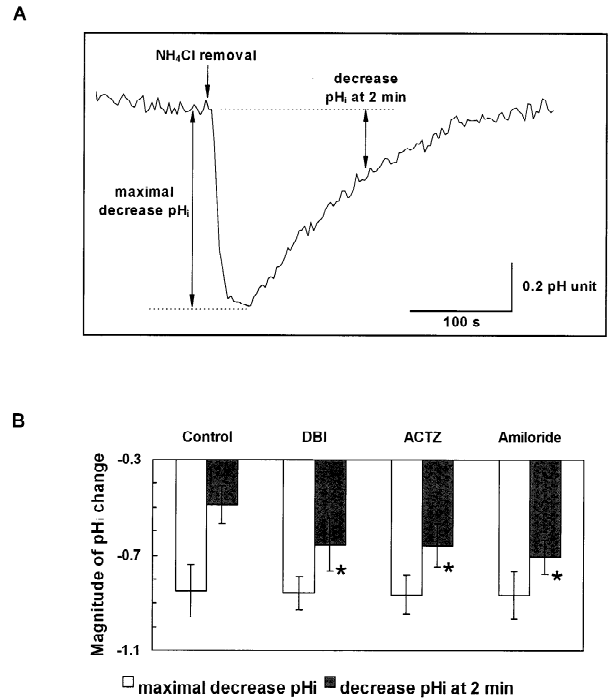


Fig. 3. The influence of carbonic anhydrase inhibitors on pH_i recovery after an ammonium chloride prepulse. Cells were exposed to 20 mM ammonium chloride for 3–4 min then superfused with control (no NH_4Cl) AAH. pH_i was measured continuously. A typical response is shown in panel A. In some cells, the maneuver was carried out in the presence of 0.2 mg/ml DBI or 0.2 mg/ml ACTZ or 1.0 mM amiloride added 5 min prior to the ammonium chloride. Using the reference point of the pH_i value at the start of pH_i recovery, we calculated the magnitude of the maximal pH_i decrease after ammonium chloride removal (open bars) and the magnitude of the pH_i decrease remaining after 2 min of recovery (shaded bars); the results are shown in Panel B. The data represent the mean \pm SD of 4–12 experiments. *indicates a significant difference from control ($P < 0.05$).

THE INFLUENCE OF Ca INHIBITORS ON THE pH_i RESPONSE TO POTASSIUM-RICH MEDIUM

Increasing external potassium causes a rapid pH_i increase (Fig. 4). In a previous study, this response was found to be partially suppressed by the H^+ -ATPase inhibitor bafilomycin A₁ (Wu & Delamere, 1998). In that same study, the response was found to be insensitive to amiloride, suggesting that the pH_i increase is not dependent on Na-H exchanger activity. Based on the thinking that CA-IV inhibition could partially inhibit Na-H exchanger activity, we predicted that DBI would not change the pH_i response to increasing external potassium. This was the case; increasing external potassium to 81.5 mM increased pH_i by 0.51 ± 0.12 ($n = 9$) and 0.53 ± 0.13 ($n = 23$) pH units in the presence and absence of DBI respectively (mean \pm SD). In contrast, the pH_i increase of 0.36 ± 0.14 ($n = 7$) observed when 81.5 mM potassium was added in the presence of ACTZ was significantly lower ($P <$

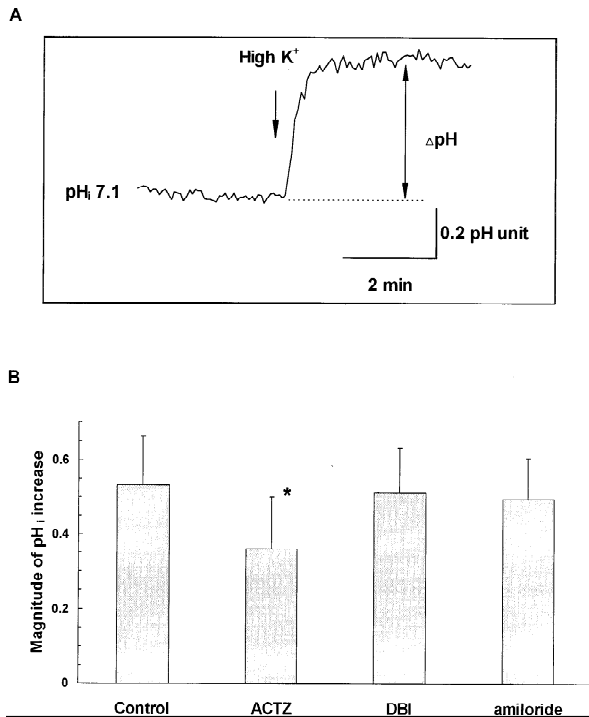


Fig. 4. The pH_i response to an increase of external potassium concentration. Cells were first superfused with control AAH then the potassium concentration in the superfusate was increased to 81.5 mM. pH_i was monitored continuously. A typical response is shown in panel A where the first vertical arrow indicates potassium addition. In some sets of cells, 0.2 mg/ml DBI or 0.2 mg/ml ACTZ or 1.0 mM amiloride was added \sim 5 min prior to the increase of external potassium. The magnitude of the pH_i increase caused by 81.5 mM external potassium is shown in panel B. The steady state pH_i prior to potassium addition was 7.15 ± 0.14 in control cells, 7.01 ± 0.11 in DBI-treated cells, 6.98 ± 0.08 in ACTZ-treated cells and 6.67 ± 0.08 in amiloride-treated cells. The data represent the mean \pm SD of results from 5–23 experiments. Significant difference from control ($P < 0.05$) is indicated by *.

0.05). For comparison, amiloride caused no significant change in the pH_i response to increased external potassium (Fig. 4).

Discussion

Since CA inhibitors are a highly effective means of reducing aqueous humor formation, there has been considerable interest in the role of CA in the ciliary epithelium. There are several CA isozymes with distinct properties (Sly & Hu, 1995) and this raises the prospects for possible therapeutic uses of isozyme-selective CA inhibitors. Matsui et al. (1996) have suggested that a functional membrane-associated CA is localized on the NPE but not the underlying PE. These investigators suggested that membrane-associated carbonic anhydrase is linked to the function of a chloride/bicarbonate exchanger at the basolateral side of the NPE. The NPE expresses multiple

Na,K-ATPase isoforms (Ghosh et al. 1990), is rich in mitochondria and has a highly invaginated basolateral surface. Active transport of solute across the NPE basolateral membrane is thought to drive fluid (aqueous humor) secretion into the eye (Davson, 1990). In a biochemical study using cultured rabbit NPE, we measured a significant component of membrane-associated CA which we identified as the CA-IV isozyme (Wu et al., 1997). Here we present evidence suggesting that under certain conditions, selective inhibition of outward-facing CA-IV is sufficient to change pH_i responses in the cultured NPE.

As in other cells, the NPE has a high activity of cytosolic CA and only \sim 20% of the overall CA activity appears to be associated with the plasma membrane (Wu et al., 1997). The action of cytosolic CA is evident from the ability of ACTZ to inhibit the rabbit pH_i increase caused by removal of external bicarbonate. A similar action of ACTZ has been reported by Zeidel et al. (1986) in rabbit kidney collecting duct. The likely interpretation of this response is that on bicarbonate/ CO_2 removal, cytosolic CA facilitates rapid generation then exit of CO_2 produced from cellular HCO_3^- and H^+ . This could account for the observed rapid pH_i increase that is prevented by the cell permeable CA inhibitor ACTZ. On bicarbonate/ CO_2 replenishment, rapid CO_2 entry followed by CA-catalyzed production of cytoplasmic H^+ and HCO_3^- is the likely cause of the observed rapid pH_i reduction which also was inhibited by ACTZ. A similar rapid pH_i decrease in response to bicarbonate addition has been reported in native rabbit NPE by Wolosin et al. (1993). The ability of ACTZ to block rapid pH_i responses to HCO_3^- addition/removal fits with our earlier proposal that ACTZ penetrates into the cytoplasm (Wu et al., 1997). Importantly, DBI did not alter the magnitude of the rapid pH_i responses to bicarbonate/ CO_2 removal or addition suggesting that inhibition of outward-facing CA-IV does not contribute to the observed ACTZ effects. This fits with the proposal, based on studies in renal tubule, that CA-IV serves primarily to catalyze the reversible CO_2 hydration reaction extracellularly in the unstirred layers adjacent to the plasma membrane (Sly & Hu, 1995; Lucci, et al., 1983).

We reported earlier that selective CA-IV inhibition with DBI causes a change in resting pH_i (Wu et al., 1997). Here we report that DBI also slows the rate of pH_i recovery in cells acidified by an ammonium chloride prepulse. These responses to DBI suggest CA-IV could play a role in the maintenance of cytoplasmic acid-base balance in the NPE. It is noteworthy that the pH_i change elicited by ACTZ is no greater than that caused by DBI. We considered the mechanism by which CA-IV inhibition could reduce pH_i . If inhibition of CA-IV were to lead to a reduction in the rate of outward proton transport this could explain the observed cytoplasmic acidifica-

tion. In different tissues it has been suggested that CA might facilitate ion transport mediated by the Na-H exchanger (Knickelbein, Aronson & Schron, 1985; Dagher, Egnor & Charney, 1993; Helbig et al., 1989). In studies here with cultured rabbit NPE, partial inhibition of the Na-H exchanger when CA-IV is inhibited could explain why the magnitude of the pH_i response to amiloride was reduced in the presence of either DBI or ACTZ. Importantly, the effects of DBI and amiloride were not additive. Inhibition of CA-IV by DBI reduced the size of the amiloride-inhibitable pH_i component and it is noteworthy that overall CA inhibition by ACTZ produced an identical response.

The notion that CA-IV facilitates Na-H exchanger activity fits with the observed effects of DBI on pH_i recovery following cytoplasmic acidification caused by an ammonium chloride prepulse. Following this ammonium chloride maneuver, it is established that proton export via the Na-H exchanger contributes to the rate of pH_i recovery and indeed the rate of recovery was reduced by 48% in the presence of amiloride. The pH_i recovery rate was also reduced by 38% in the presence of DBI and 38% in the presence of ACTZ and these responses are consistent with reduced Na-H exchanger activity in cells where CA-IV activity is inhibited. This is also consistent with the finding that the effects of amiloride and either carbonic anhydrase inhibitor were not additive. It should be noted that proton export via H^+ -ATPase also appears to contribute to pH_i recovery (Wu & Delamere, 1998).

There is evidence that a bafilomycin A_7 -sensitive H^+ -ATPase might also contribute to the pH_i recovery after an ammonium chloride prepulse (Wu & Delamere, 1998). We considered the possibility that CA-IV inhibition could reduce H^+ -ATPase-mediated outward proton transport. H^+ -ATPase function is linked to carbonic anhydrase in kidney proximal tubule where both mechanisms act together to reabsorb bicarbonate (Alpern, 1990). However, such interdependence between H^+ -ATPase and CA-IV seems unlikely in the NPE since DBI failed to change the magnitude of the pH_i increase caused by an increase of external potassium concentration yet this depolarization-induced pH_i increase can be almost completely attributed to H^+ -ATPase stimulation and is not detectably inhibited by either SITS, amiloride or external chloride removal (Wu & Delamere, 1998). It is noteworthy that the depolarization-induced pH_i increase is not altered by amiloride suggesting Na-H exchanger mediated proton export does not participate. Such an absence of a Na-H exchange component fits well with the lack of a detectable DBI effect since we suggest CA-IV inhibition elicits pH_i responses through a mechanism involving reduction of Na-H exchanger activity. The ability of ACTZ to partially inhibit the depolarization-induced alkalization could originate from CA-II inhibition which limits the rate of generation of cytoplas-

mic H^+ from CO_2 to replenish cytoplasmic protons pumped outward by the stimulated H^+ -ATPase. It is noteworthy that aqueous humor secretion can be reduced both by ACTZ (Maren, 1984) and bafilomycin A_7 , a specific inhibitor of vesicular H^+ -ATPase (Wax et al., 1997).

Wolosin et al. (1991) have suggested that the NPE could have two types of bicarbonate transport mechanisms, one sodium-dependent and one sodium-independent. A sodium-dependent bicarbonate uptake process could explain why, after an ammonium chloride prepulse, there is a sodium-sensitive component of pH_i recovery that apparently persists in the presence of amiloride. A chloride-bicarbonate exchange mechanism which shifts bicarbonate outward could explain the pH_i increase which occurs when the external chloride concentration is lowered. Matsui et al. (1996) have proposed that membrane-associated carbonic anhydrase could influence the chloride/bicarbonate exchange mechanism by preventing accumulation of a locally high bicarbonate concentration at the outward face of the NPE basolateral membrane. It is possible that the Na-H exchanger and chloride/bicarbonate exchanger shift protons and bicarbonate ions outward in parallel and that CA-IV facilitates the transport of both ions. This idea has yet to be tested.

In summary, we suggest that in cultured rabbit NPE CA-IV facilitates the outward transport of protons via the Na-H exchanger. By acting to catalyze rapid equilibration of H^+ and HCO_3^- with CO_2 in the unstirred layer outside the plasma membrane, CA-IV could prevent the build up of H^+ concentration at the extracellular side of the Na-H exchanger. Inhibition of CA-IV with DBI or ACTZ could lower pH in the unstirred layer and since H^+ and Na^+ compete for the same extracellular binding site (Aronson, 1985) this could inhibit the activity of the Na-H exchanger. In the PE cell layer, Na-H exchange might have a specialized role, importing sodium from the plasma which subsequently passes to the NPE where Na,K-ATPase shifts it across the basolateral membrane (Helbig et al., 1989). However, the NPE is not thought to absorb sodium from the aqueous humor. In the NPE it is more likely that the Na-H exchange mechanism has the main purpose of extruding protons to maintain cytoplasmic pH in this mitochondria-rich cell. Acting together, the Na-H exchanger and CA-IV might serve to regulate cytoplasmic acid-base balance in the NPE. Cytoplasmic pH regulation could be particularly important in NPE where there is considerable transepithelial solute and water movement and even small mismatches in bicarbonate entry vs. exit could cause large pH_i swings. Brubaker has calculated that the amount of fluid passing through the NPE cell from blood to aqueous is equivalent to a turnover in cell volume every 3 min (Brubaker, 1984). If carbonic anhydrase inhibition were to cause

intracellular acidification of the ciliary epithelium in vivo, this could alter many biochemical processes. Importantly, cytoplasmic acidification is known to cause marked inhibition of Na,K-ATPase activity and cause a marked inhibition of active sodium-potassium transport (Eaton, Hamilton & Johnson, 1984; Greiber, O'Neill & Mitch, 1995). Active sodium-potassium transport provides the driving force for aqueous humor secretion (Davson, 1990) and indeed Maren (1984) has suggested that blood-to-aqueous sodium transport is inhibited in animals given systemic carbonic anhydrase inhibitors. The present study highlights the ability of a selective CA-IV inhibitor to produce pH_i responses that are equivalent to those elicited by ACTZ. This raises the possibility that CA-IV inhibition, perhaps by causing changes of pH_i , could possibly account for some of the physiological effects which cause a reduced rate of aqueous humor formation in humans and animals treated with ACTZ.

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