Remodeling the cellular profile of collecting ducts by chronic carbonic anhydrase inhibition

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and absent from the inner stripe (IS) and the inner medullary collecting duct (IMCD). A-IC gradually disappear from the proximal third and are virtually absent in the terminal two-thirds of the IMCD (13, 28). However, both A-IC and B-IC are found in the IMCD of newborn rats. They progressively disappear from the deep IMCD during the first 3 wk after birth (28), but IC gradually increase in number in other collecting duct regions, including the cortex during postnatal development.

Little information is available concerning the mechanism by which different cell types arise in the renal collecting duct system. In the developing kidney, growth factors and hormones as well as changes in the pH, osmolarity, and variations in the extracellular electrolyte composition (45) play important roles in the differentiation of IC and PC. Because both A-IC and B-IC are present in the fetal rat kidney, these subtypes of IC seem to proliferate as a result of programmed differentiation during development (28). Furthermore, the cause of the selective depletion of IC from the tip of the papilla during postnatal development remains poorly understood (31), although Kim et al. (25) showed that IC appear to be deleted from the medullary collecting duct by two distinct mechanisms: type B-IC undergo apoptosis and subsequent phagocytosis by neighboring PC, whereas type A-IC are eliminated by extrusion into the tubule lumen.

We have previously shown that IC are greatly depleted and replaced by PC in medullary collecting ducts of CAII-deficient mice (8). This suggests a potential role for CA in regulating cell-type diversity in collecting ducts. In this study, we examined the effect of pharmacological inhibition of CA activity on rat kidney collecting ducts. By using antibodies against cell-type-specific proteins, we show that a significant remodeling of the cellular profile of this tubule segment occurs in adult rats after 2 wk of acetazolamide treatment via osmotic pumps.

METHODS

Animals. Adult Sprague-Dawley rats were maintained on a standard diet and had free access to water. They were implanted with Alzet osmotic pumps and treated with acetazolamide (15 mg·kg⁻¹·day⁻¹), a CA inhibitor, for up to 2 wk. The animals were anesthetized with Nembutal (Abbott Laboratories, North Chicago, IL, 40 mg/kg body weight ip), and the osmotic pumps were implanted beneath the skin at Laboratories, North Chicago, IL, 40 mg/kg body weight ip), wk. The animals were anesthetized with Nembutal (Abbott after 7 and 14 days, respectively

Systemic and urinary biological parameters. In an initial set of experiments, six animals were implanted with osmotic pumps and treated for up to 2 wk (acetazolamide-treated rats, n = 6; control rats, n = 4). After 7 and 14 days, they were put into metabolic cages for 48 h to monitor weight, plasma creatinine, bicarbonate and chloride levels, and urinary creatinine, sodium, potassium, and pH.

Table 1. Plasma parameters and weight of control and acetazolamide-treated animals after 7 and 14 days, respectively

<table>
<thead>
<tr>
<th></th>
<th>Control (n = 4)</th>
<th>AC (n = 6)</th>
<th>Control (n = 4)</th>
<th>AC (n = 6)</th>
<th>Control (n = 4)</th>
<th>AC (n = 6)</th>
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<tr>
<td>Weight, g</td>
<td>325 ± 6</td>
<td>330 ± 3</td>
<td>356 ± 7</td>
<td>354 ± 6</td>
<td>380 ± 12</td>
<td>374 ± 7</td>
</tr>
<tr>
<td>Bicarbonate, mmol/l</td>
<td>19 ± 1</td>
<td>16 ± 1</td>
<td>18 ± 1</td>
<td>18 ± 1</td>
<td>19 ± 1</td>
<td>17 ± 1</td>
</tr>
<tr>
<td>Chloride, mmol/l</td>
<td>108 ± 2</td>
<td>112 ± 2</td>
<td>111 ± 2</td>
<td>110 ± 1</td>
<td>109 ± 1</td>
<td>113 ± 1</td>
</tr>
<tr>
<td>Creatinine clearance, ml·min⁻¹·100g⁻¹</td>
<td>0.82 ± 0.05</td>
<td>0.76 ± 0.04</td>
<td>0.69 ± 0.05</td>
<td>0.69 ± 0.05</td>
<td>0.59 ± 0.02</td>
<td>0.65 ± 0.05</td>
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Values are means ± SE. AC, acetazolamide.
affinity-purified rabbit polyclonal antibody IC270 raised against glutathione S-transferase-NHE-RF fusion protein amino-acids 270–358; this antibody has been characterized previously (21) and was kindly provided by Dr. Vijaya Ramesh, Massachusetts General Hospital, Boston, MA; 4) a rabbit polyclonal antibody raised against CAII from red blood cells (kindly provided by Dr. W. Sly, Wash. Univ., St. Louis, MO) used at a 1:100 dilution; 5) a rabbit polyclonal antibody raised against the second external loop of AQP2 at a 1:100 dilution (22); and 6) a rabbit polyclonal affinity-purified antibody raised against the COOH-terminal decapeptide of AQP4 at a dilution of 1:25 (49).

Secondary antibodies used were either goat anti-rabbit immunoglobulin (IgG) coupled to FITC (Kirkegaard & Perry, Gaithersburg, MD), goat anti-rabbit IgG coupled to indocarbocyanine (CY3, Jackson ImmunoResearch, West-Grove, PA), and donkey anti-chicken IgG coupled to FITC or CY3 (Jackson ImmunoResearch, West-Grove, PA). Some 4-μm sections were double stained to confirm the identity of positive and negative cell types that were detected with single staining and for the purpose of morphological measurements on the cells of interest. Both primary antibodies were applied at the same time by using the appropriate final dilutions. In a second step, both secondary antibodies were also applied simultaneously.

Some sections were double stained with anti-NHE-RF and anti-AE1 antibodies. Because both antibodies were raised in rabbit, an amplification procedure was used to allow staining of sections with two primary antibodies raised in the same species. Briefly, the first affinity-purified antibody, anti-NHE-RF, was applied at a dilution of 1:10, a concentration that was too low to be detectable by conventional application.

<table>
<thead>
<tr>
<th>Day 0</th>
<th>Day 7</th>
<th>Day 14</th>
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<tbody>
<tr>
<td></td>
<td>Control</td>
<td>AC</td>
</tr>
<tr>
<td>24-h Urinary volume, ml/day</td>
<td>13 ± 2</td>
<td>15 ± 2</td>
</tr>
<tr>
<td>Urinary potassium, mmol/day</td>
<td>4.8 ± 0.3</td>
<td>5 ± 0.6</td>
</tr>
<tr>
<td>Urinary Na, mmol/day</td>
<td>1.6 ± 0.3</td>
<td>2 ± 0.1</td>
</tr>
<tr>
<td>Urinary pH</td>
<td>8.4 ± 0.2</td>
<td>8.5 ± 0.1</td>
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Values are means ± SE. *P < 0.05 vs. control group.

Fig. 1. Double staining of the 31-kDa subunit of vacuolar H^+-ATPase (green) and anion exchanger AE1 (red) in a 4-μm section of control (A) and acetazolamide-treated kidney cortex (B). Arrows indicate B-intercalated cells (IC) stained with anti-H^+-ATPase antibodies, diffusely or on the basolateral membrane (some B-IC also have bipolar H^+-ATPase staining). A-IC are stained on their apical membrane with anti-AE1 antibodies, respectively. The number of B-IC decreased significantly in cortical collecting ducts of acetazolamide-treated animals. Bar = 10 μm.
of a secondary fluorescent antibody in these sections, as determined in preliminary experiments. The dilute anti-NHE-RF antibody was detected by using a tyramide amplification kit (NEN Life Science Products) with tyramide-FITC as a fluorescent reagent, according to the manufacturer's instructions. The sections were then incubated conventionally with anti-AE1 and secondary goat-anti-rabbit CY3 as described above. No cross reactivity between the two sets of reagents was detectable under these conditions.

Slides were mounted in a 2:1 mixture of Vectashield (Vector Laboratories, Burlingame, CA) mounting medium and 1.5 M Tris solution (pH 8.9). Some sections were examined with a Nikon Eclipse 800 epifluorescence photomicroscope (Nikon Instruments, Garden City, NY) and photographed using Kodak TMAX 400 black and white film push-processed to 1600 ASA. Other images were acquired digitally from the Nikon 800 by using a Hamamatsu Orca charge-coupled device (CCD) camera or from a Nikon FXA photomicroscope with an Optronics 3-bit CCD color camera. They were stored on an Apple Macintosh Power PC 8500 and analyzed by using IPLab Spectrum version 3.1a image capture and analysis software (Signal Analytics, Vienna, VA). Image montages were arranged by using Adobe Photoshop 4.0, and hardcopies were produced by using an Epson Stylus 600 ink jet printer.

Quantification in the cortex by using \( \text{H}^{+}\)-ATPase and AE1 antibodies. The quantitative studies were performed on one randomly chosen section from each animal for each set of incubations. Tubules that were sectioned perpendicularly to the basement membrane were used for the quantification. The number of IC was counted in the cortex of kidney sections double stained for \( \text{H}^{+}\)-ATPase and AE1 (acetazolamide treated \( n = 6 \), control \( n = 6 \)). A total of 50 pictures were digitally acquired (28 from the control group and 22 from the acetazolamide-treated group). Double-stained cells (apical \( \text{H}^{+}\)-ATPase and basolateral AE1) were counted as A-IC. Cells with staining for \( \text{H}^{+}\)-ATPase but no AE1 were counted as B-IC. Nonstained cells were identified as PC. The percentages of A-IC and B-IC were calculated from the number of A-IC and B-IC relative to the total number of IC in each tubule.

Quantification in the inner stripe of the outer medulla by using \( \text{H}^{+}\)-ATPase, AQP2, and AQP4 antibodies. The number of stained IC and PC in the inner stripe was counted by using the \( \text{H}^{+}\)-ATPase and AQP2 antibodies, respectively, as cell-specific markers. Digital images, showing IS sections, were counted for each group of animals (1-wk acetazolamide treated \( n = 2, 6 \) pictures; control \( n = 3, 9 \) pictures; 2-wk acetazolamide treated \( n = 6; 12 \) pictures; control \( n = 6, 11 \) pictures). A total of 38 digital images were examined. Collecting ducts in the IS were distributed regularly throughout each image, and the total number of cells were counted and expressed per total area of each photograph. The percentages of IC and PC were then estimated from the number of positive cells relative to the total number of cells in each tubule stained with \( \text{H}^{+}\)-ATPase and AQP2 antibodies, respectively. The number of IC was also counted on 23 black

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**Fig. 2.** Effect of acetazolamide administration on the percentage of IC and principal cells (PC; A) and on the percentage of A-IC and B-IC (B) in the cortical collecting duct (CCD). Cell types were identified by using a double staining for AE1 and \( \text{H}^{+}\)-ATPase (means \( \pm \) SE). *\( P < 0.01 \) vs. control.

**Fig. 3.** Identification of B-type intercalated cells was confirmed by performing a double staining to detect sodium/hydrogen exchanger regulatory factor (NHE-RF; green) and AE1 (red). NHE-RF-positive B-cells were frequently seen in control collecting ducts (A, arrows). Acetazolamide-treated collecting ducts (B) showed a striking decrease in the number of NHE-RF-positive cells, whereas AE1-positive A-IC were abundant. The arrowhead indicates an AE1-positive red blood cell in A. The proximal tubule brush border shows a variable amount of NHE-RF staining in both controls and acetazolamide-treated rats. Bar = 15 \( \mu \)m.
and white prints from the IS at a final magnification of ×98. Apical and basolateral membrane length of IC and PC was measured by using IPLab Spectrum software on digitized images. The apical length was measured in IC and PC stained for H\textsuperscript{1}-\textsuperscript{ATPase} and AQP2, respectively, and the basolateral length was measured in IC and PC stained with AE1 and AQP4, respectively, to highlight the membrane domain of interest. A total of 341 cells were counted (1-wk acetazolamide-treated rats, n = 3; controls for 1-wk rats, n = 2; 2-wk acetazolamide-treated rats, n = 6; controls for 2-wk rats, n = 6).

Quantification in the IMCD by using antibodies against the 31-kDa subunit of the H\textsuperscript{+}-ATPase. The number of IC in the IMCD was counted on sections of the inner medulla stained for the 31-kDa subunit of H\textsuperscript{+}-ATPase. Two black and white prints taken with a Nikon 800 microscope (final magnification ×98) were counted for each animal (2-wk acetazolamide treated, n = 9, controls n = 9). The first set of pictures was taken from the proximal region of the inner medulla and included the initial 1 mm that extended from the beginning of the inner medulla toward the tip of the papilla. The second set of pictures was taken more distally and included an additional 1 mm. Together, both pictures accounted for about one-third of the total length of the inner medulla (i.e., the region where IC are located in the IMCD). Thirty-six prints were examined overall. Data were expressed as the total number of IC per unit area for the first and second set of pictures separately, and for both sets of images combined.

Electron microscopy. For electron microscopy, some kidneys were fixed in 2.5% glutaraldehyde containing 0.1-M cacodylate buffer, pH 7.4. Tissues were chopped into smaller pieces (about 1 mm\textsuperscript{3}) and immersed in the same fixative, as described previously (8). They were then postfixed for 1 h in 2% osmium tetroxide, stained en bloc with uranyl acetate, dehydrated in graded ethanol, and embedded in Epon (Electron Microscopy Sciences, Ft. Washington, PA). Thin sections were stained with uranyl acetate and lead citrate prior to examination with a Philips model CM10 electron microscope (Philips, Mahwah, NJ).

Western blotting. Rats were perfused through the left ventricle with PBS (pH 7.4) as described above. A kidney was removed, cut into smaller pieces with a razor blade, transfected into ice-cold homogenization buffer, and weighed. Kidney samples were homogenized in 10 ml of homogenization buffer (250 mM sucrose, 1 mM EDTA, 18 mM HEPES-Tris, pH 7.4) per gram of tissue in the presence of Complete, a cocktail of protease inhibitors (Boehringer Mannheim, Germany). Tissue was homogenized by using 20 strokes with a glass potter (model C-925, Thomas) equipped with a tight-fitting Teflon pestle. Homogenates were centrifuged for 10 min (1,000 g, 4°C). The supernatant (S1) was either aspirated or further centrifuged (30 min, 10,000 g, 4°C). The resulting pellet (P2) was kept on ice for SDS-PAGE, and the supernatant S2 was centrifuged for 1 h (100,000 g, 4°C). Protein concentration was measured after solubilization of the membranes in 0.1% SDS with the Pierce-bicinchoninic acid protein assay reagent (Pierce, Rockford, IL) by using albumin as standard (44). All pellets and supernatants were diluted 3:1 in 4× Laemmli (reducing) sample buffer (Boston BioProducts, Ashland, MA) and boiled for 5 min. Samples were loaded at 10 μg protein/lane onto SDS-polyacrylamide (12%) minigels (Bio-Rad, Richmond, CA) and separated by using the Laemmli method (29). Proteins were transferred onto Immobilon-P transfer membrane (Millipore Bedford, MA) in a Bio-Rad (Richmond, CA) semidry transfer cell. Membranes were blocked in buffer (5% nonfat dry milk in 15 mM NaCl, 5 mM Tris·HCl, 0.3% Tween 20, pH 7.0) for 1 h at 20°C. Membranes were then incubated with the primary antibodies (H\textsuperscript{+}-ATPase, CAII, and AQP2) diluted 1:500, 1:1000, and 1:1,000 respectively. Goat anti-chicken or anti-rabbit HRP-
conjugated secondary antibodies were diluted 1:12,000 and 1:10,000 respectively. Washes between and after incubations were repeated four times. Detection of antibody binding was performed with the enhanced chemiluminescence method (Amersham Life Sciences, Buckinghamshire, UK) by using Kodak X-Omat blue XB-1 film. Films were scanned and quantitatively analyzed by using NIH Image 1.62 software.

Statistics. Data were analyzed by using IPLab Spectrum 3.1a running on a Power Macintosh 8500. Values are means ± SE, and significance levels were calculated by using the two-tailed Student’s t-test for unpaired samples using Statview software version 4.5 1.1 (Abacus Concepts, Berkeley, CA).

RESULTS

Systemic and urinary biological parameters. The blood and urine parameters of control and acetazolamide-treated animals after 7 and 14 days are summarized in Tables 1 and 2, respectively. No difference in plasma bicarbonate was observed after 7 and 14 days between the acetazolamide-treated and the control animals. No change in renal function was observed. Acetazolamide induced a significant but transient increase in diuresis and in natriuresis (day 7, P < 0.05). Urinary pH did not change significantly compared with controls.

A-IC and B-IC in the cortical collecting duct. The 31-kDa subunit of the vacuolar H⁺-ATPase was distributed in IC, either on the apical membrane, on the basolateral membrane, or on cytoplasmic vesicles. IC exhibiting apical staining for H⁺-ATPase associated with a basolateral staining for AE1 were identified as A-IC. IC exhibiting positive staining for H⁺-ATPase and no staining for AE1 were considered to be B-IC (Fig. 1). The percentage of IC and PC relative to the total number of cells was similar in all groups (Fig. 2A). In control rats, the percentages of A-IC and B-IC were 64 ± 4 and 36 ± 4%, respectively, which agrees with previously published data (Fig. 2B) (12). However, in acetazolamide-treated animals, the percentage of A-IC increased significantly and that of B-IC decreased significantly (82 ± 2 and 18 ± 2%, P < 0.01 vs. control) (Fig. 2B).

To confirm that the H⁺-ATPase-positive but AE1-negative IC identified in our quantitative study were
indeed B cells, we examined the qualitative pattern of expression of NHE-RF in control and acetazolamide-treated rat kidneys. We have previously reported that NHE-RF, a PDZ-domain protein originally identified as a regulatory factor for the sodium/hydrogen exchanger NHE3 (54) is specifically expressed in B-IC but not A-IC in cortical collecting ducts (10). As shown in Fig. 3A, NHE-RF positive IC were always AE1 negative as previously described and were readily detectable in control collecting ducts. However, in collecting ducts from acetazolamide-treated rats, NHE-RF-positive cells were much less abundant. In the example illustrated in Fig. 3B (from an animal that had a dramatic loss of B-IC based on data from the quantitative study described above), they were virtually undetectable. In contrast, AE1-positive IC were numerous in cortical collecting ducts from this rat.

IC in the inner stripe of the outer medulla. In the inner stripe of control rats, the percentages of IC and PC in the collecting duct were 37 ± 3 and 63 ± 3%, respectively (Fig. 4A). In 2-wk acetazolamide-treated animals (Fig. 4B), the percentage of IC increased significantly (48 ± 2%, P < 0.05 vs. control group) and the percentage of PC decreased significantly (52 ± 2%, P < 0.05 vs. control group), whereas the total number of cells remained unchanged (Fig. 5).

As shown in Figs. 6 and 7A, IC from 2-wk acetazolamide-treated animals appeared bulkier, protruded markedly into the lumen, and showed a significant increase in their apical membrane length compared with control rats (20.9 ± 0.5 vs. 14.6 ± 0.4 μm, P < 0.05). In addition, a significant increase in the length of the IC basolateral membrane was observed in acetazolamide-treated animals after 1 wk (23.6 ± 0.9 vs. 21.4 ± 0.5 μm, P < 0.05) and 2 wk (25.8 ± 0.4 vs. 23.7 ± 0.5, P < 0.05) of treatment (Fig. 7B).

IC in the IMCD. In the inner medulla, the number of IC was lower in rats treated with acetazolamide for 2 wk compared with control animals (Fig. 8). This reduction was marginally significant in the proximal region that included the first 1 mm of the inner medulla (113 ± 22 vs. 155 ± 22 IC/mm², P = 0.09). However, a clearly significant reduction was observed in the second 1 mm located more distally (11 ± 4 vs. 40 ± 11 IC/mm², P < 0.05) (Fig. 9).

Electron microscopy. By conventional thin-section electron microscopy, IC and PC could be clearly distinguished in all regions of the collecting duct. In control rats, IC had morphological features similar to those that have been previously described, with numerous mitochondria, a large number of intracellular vesicles, and apical microvilli (Fig. 10A). In 2-wk acetazolamide-treated animals, IC were increased in size, bulkier, and protruded markedly in the lumen (Fig. 10B). The number of intracellular vesicles seemed to be decreased in these cells although this was not evaluated quantitatively. Apical microvilli were more developed in acetazolamide-treated rats.

Western blotting. S3, P3, and P2 fractions were obtained from kidney inner stripe (corresponding to the cytosolic, microsomal, and plasma membrane fractions of the cells, respectively). An S1 fraction (total homogenate) was obtained from whole papilla. Western blotting with the anti H⁺-ATPase antibody showed a single band in all samples. As shown in Figs. 11 and 12, this band was more intense in the membrane and cytosolic fractions of acetazolamide-treated rats compared with control rats. Quantitative studies showed a significant increase in the amount of protein detected in the lanes corresponding to the cytosolic fraction of 2-wk acetazolamide-treated rats (P < 0.05) and a difference which did not reach statistical significance in the membrane fraction. No significant difference was observed between control and acetazolamide-treated rats when samples were probed with antibodies against CAII and AQP2 (data not shown).

DISCUSSION

We have previously shown that IC are absent from medullary collecting ducts of CAII-deficient mice (8),
which suggests a potential role for CA in determining the cellular composition of this segment of the urinary tubule. Our present study shows that chronic (2-wk) CA inhibition by acetazolamide induces a marked remodeling of collecting ducts in all regions of the kidney. In the cortex of acetazolamide-treated animals there was a significant decrease in the number of B-IC and a corresponding increase in the percentage of A-IC compared with control rats. In some extreme cases, B-IC were rarely found in the cortical collecting duct (see Fig. 1B). However, the ratio of total IC to PC was no different in acetazolamide-treated rats and control rats, suggesting that CA inhibition favors the acid-secreting IC phenotype to the detriment of bicarbonate-secreting B cells. In the inner stripe of the outer medulla, acetazolamide induced a significant increase in the number of IC and a decrease in the number of PC. The size of IC was also increased. These changes were associated with a higher level of expression of the H\(^+\)-ATPase in cytosolic fractions measured by Western blotting, although the amount associated with membrane fractions was not increased significantly. A previous study on the effect of acetazolamide on collecting duct morphology was performed by Lonnerholm et al. (30). By using CA as a marker of IC, their major conclusion was that IC from acetazolamide-treated rats appeared bulkier than in control rats. Our study supports this finding in the inner stripe collecting duct.

The present data suggest that acetazolamide treatment triggers a series of events that would tend to increase net proton secretion in both cortical and OMCDs. Morphological alterations in IC similar to those described here occur after induction of acute and chronic metabolic and respiratory acidosis in rats (32). In the inner medulla, however, acetazolamide treat-

Fig. 8. Double staining using H\(^+\)-ATPase (green) and AE1 antibodies (red) on control (A) and acetazolamide (B) sections of the inner medulla (IM). The number of IC in the distal part of the inner medulla/papilla decreased markedly in the acetazolamide-treated rats. Bar = 250 μm.

Fig. 9. Effect of acetazolamide on the number of IC in the inner medullary collecting ducts (IMCD) (means ± SE). Proximal region (Prox) includes the first 1 mm of the inner medulla. Distal region (Dist) includes the second 1 mm located more distally. Total, both regions pooled together. *P < 0.05 vs. control group.
ment reduced the number of IC. We have previously reported a similar, but more pronounced, depletion of inner medullary IC in CAII-null mice (8). However, CA inhibition for 2 wk did not result in a loss of IC from other collecting duct segments. On the contrary, IC were more numerous and more “developed” in the inner stripe of acetazolamide-treated rats. Although it is possible that more prolonged treatment with acetazolamide might induce a more widespread loss of IC in medullary collecting ducts, it is also possible that a lack of (or inhibition of) CA activity has different effects on collecting duct cells during postnatal renal development and in adult animals.

It has been known for many decades that the collecting duct undergoes morphological adaptation to acid-base disturbances (32, 53). Metabolic acidosis induces changes consistent with increased activity of A-IC and decreased activity of B-IC (6, 7, 39, 50), whereas the opposite changes are seen in systemic alkalosis (6, 7, 39, 51). However, in most previous studies, the population of A-IC vs. B-IC has appeared to remain constant, implying that the observed changes occurred in populations of cells with a fixed A or B phenotype. Chronic metabolic acidosis or alkalosis in late pregnancy or during initial lactation in rats led to a decrease and an increase, respectively, in the percentage of B-IC in the pups as defined by the location of plasma membrane “studs” (37), an ultrastructural marker of the vacuolar H⁺-ATPase (11). After ammonium chloride loading, a significant increase in apical H⁺-ATPase staining and apical membrane area in AE1-positive IC was observed in addition to a reduction in the number of IC with basolateral H⁺-ATPase staining (50). Morphological modifications of A-IC and B-IC have also been observed in the kidneys of K⁺-depleted rats (38, 43, 46). Although in our study plasma potassium data were not available, urinary potassium was similar in both groups, suggesting that potassium balance was similar in acetazolamide-treated rats and in control animals.

Interpretation of some previous studies is confounded, however, by the existence of AE1-negative cells that express apical H⁺-ATPase, as well as some cells expressing intermediate patterns of H⁺-ATPase staining (5, 7, 26). In the absence of double-staining for both AE1 and H⁺-ATPase, these cells would be identified as A cells based simply on morphological criteria or H⁺-ATPase staining alone. Furthermore, A-IC can have very low levels of AE1 staining under some conditions (39). Our conclusion obtained from AE1- and H⁺-ATPase-stained sections, i.e., that B cells are reduced in number in acetazolamide-treated rats, was, therefore, reevaluated in sections stained for NHE-RF, a recently identified marker of B-IC (10). Qualitative examination of the sections revealed a marked reduction in the number of cells stained for NHE-RF in the cortical collecting ducts of acetazolamide-treated animals, consistent with a loss of B-IC and supporting our data using AE1 and H⁺-ATPase staining patterns to distinguish A-IC and B-IC.

It is not surprising that different parts of the collecting duct exhibit different morphological responses to acetazolamide treatment. Rabbit outer and inner medullary collecting ducts vary in susceptibility to CA inhibitors and in their response to stimulation with CO₂ (35, 36). It is likely that the concentration of acetazolamide in the inner medulla is higher than in the outer medullary or cortical collecting ducts, and more complete CA inhibition in the inner medulla could lead to the observed decrease in the number of IC in this region. How might CA inhibition affect the differentiated phenotype of an epithelial cell? Whereas acetazolamide treatment results in a transient systemic acidosis of variable duration (23, 33, 34) and

Fig. 10. Electron micrographs showing morphological features of intercalated cells from control (A) and acetazolamide (B) collecting ducts in the IS. Control IC are smaller, with fewer apical microvilli and numerous apical vesicles. Acetazolamide induces a large amplification of the apical surface, which has many microvilli. There are few vesicles in the cytoplasm. The cell protrudes into the tubule lumen. Bar = 1 μm.
could, therefore, mimic the effects of metabolic acidosis on IC, it also induces an acute increase in intracellular pH (pHi) in IC (40), whereas the pH i of PCs does not change. The effect of chronic acetazolamide treatment on pHi in IC is not known, but pH i changes can affect many cellular processes, including regulation of acid-base transporter trafficking and insertion (4, 19), as well as gene expression (17, 42, 48).

In mammals, the acute response to CA inhibition is an increase in the excretion of bicarbonate, sodium and potassium, an increase in urinary flow, and titratable acid. However, the loss of bicarbonate and sodium is self-limited on continued administration of the drug, probably because the initial acidosis resulting from bicarbonate loss activates bicarbonate reabsorption by CA-independent mechanisms. In our study, major morphological modifications of A-IC were observed after 2 weeks of acetazolamide treatment despite the fact that no significant systemic acidosis was observed compared with controls. Therefore, the persistence of an “activated” form of the A-IC phenotype in the cortex and outer medulla does not seem to require the continued stimuli of systemic CO2, plasma bicarbonate, or pH imbalance. Although our present study did not screen for an initial transient acidosis at early time points after CA inhibition, as shown in previous reports (23, 33, 34), our data suggest that 1) a possible transient initial systemic acidosis induced by acetazolamide triggered a series of events that leads to the development of “activated” A-IC, and “inhibition” of the B-IC phenotype, which persisted after 2 wk; 2) the continued inhibition of CA at the cellular level was itself responsible for the establishment of the “activated” A-IC phenotype, perhaps by altering pH i regulation and modifying gene expression (see above); and 3) the systemic acid/base parameters providing feedback to IC are either too small to measure accurately after renal compensation has occurred, or are oscillating and were missed by our sampling procedure. It has been shown that acute acid/base disturbances can alter IC characteristics after just a few hours (39, 41), but for how long these changes can be maintained after the initial stimulus was not examined. Indeed, acidosis, when occurring either in animals or in humans after acetazolamide treatment, peaks usually after 1.5 to 5 days (23). It seems paradoxical that A-IC developed morphological characteristics compatible with a high rate of proton secretion (31, 32) under conditions in which one of the key components of their acidification mechanism, CAII, was inhibited. However, this could reflect a compensatory upregulation of one component of the proton secretory machinery in response to the complete or partial inactivation of another component, i.e., CAII. This would at least partially explain the apparently normal acid/base status of the acetazolamide-treated animals, as well as in most acetazolamide-treated patients (24).

Whether the reduction in the number of B-IC observed in the cortex of acetazolamide-treated rats could be attributed to an interconversion of B-IC into A-IC or was associated with a selective depletion of B-IC to-
gether with an increased proliferation of A-IC remains uncertain. In vitro, IC have been reported to switch polarity, with bicarbonate-secreting cells transforming into proton-secreting cells under some culture conditions (3, 47). Furthermore, there are several older studies in the literature addressing the issue of phenotype switching (i.e., conversion between principal and IC) in collecting duct epithelial cells, dating back to the previous century (see Ref. 53 for a review of this literature). So far, however, no clear evidence in favor of this process has been obtained by using the new generation of cell-specific antibodies and probes. The fact that IC and PC could have a common cellular origin has also been proposed by Fejes-Toth et al. (18). Our present data clearly show that the cellular profile of collecting ducts can be modulated in adult animals by CA inhibition. This result sets the stage for future studies aimed at identifying the mechanism(s) by which this process takes place.

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