Intracellular pH Regulation in Rabbit Renal Medullary Collecting Duct Cells

Role of Chloride-Bicarbonate Exchange

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Abstract

The renal medullary collecting duct (MCD) secretes protons into its lumen and HCO_3^- into its basolateral space. Basolateral HCO_3^- transport is thought to occur via Cl/HCO_3^- exchange. To further characterize this Cl/HCO_3^- exchange process, intracellular pH (pH_i) regulation was monitored in freshly prepared rabbit outer MCD cells. Cells were separated by protease digestion and purified by Ficoll gradient centrifugation. pH_i was estimated fluorometrically using the entrapped intracytoplasmic pH indicator, 6-carboxyfluorescein. Cells were preincubated in bicarbonate-containing solutions and then abruptly diluted into bicarbonate-free media. The MCD cell pH_i response to abrupt removal of $\text{CO}_2/\text{HCO}_3^-$ included an initial alkalization due to rapid CO_2 efflux, followed by an acidification due to HCO_3^- efflux and a gradual recovery to the resting pH_i of 7.24 ± 0.06 partly due to the action of a plasma membrane H^+ -ATPase. The initial alkalization required a $\text{CO}_2/\text{HCO}_3^-$ gradient and did not occur in the presence of acetazolamide. The acidification phase required intracellular HCO_3^- and extracellular Cl , which was consistent with a Cl/HCO_3^- exchange. MCD HCO_3^- efflux exhibited saturable kinetics for extracellular Cl , with a Michaelis constant (K_m) of 29.9 ± 7.7 mM. HCO_3^- efflux also exhibited preference for halides over NO_3^- , SCN^- , and gluconate, and striking sensitivity to disulfonic stilbene and acetazolamide inhibition, with an apparent K_i of 5×10^{-7} M for DIDS. The final pH_i recovery required intracellular ATP, which indicated that Cl/HCO_3^- and H^+ -ATPase activities are present in the same cells in these suspensions. The results provide direct evidence for MCD Cl/HCO_3^- exchange and describe some of the properties of this transport process.

Introduction

The mammalian medullary collecting duct (MCD)¹ is the final site of urinary acidification. Acid secretion is known to occur in this segment in a sodium-independent fashion against a steep electrochemical gradient (1). Protons are thought to be transported from the cytoplasm to the lumen via a plasma membrane

H^+ -ATPase (2, 3), while the alkali equivalents generated by acid secretion are converted to HCO_3^- by intracellular carbonic anhydrase (4). The resulting HCO_3^- is thought to exit the basolateral membrane in exchange for extracellular Cl via a Cl/HCO_3^- exchanger (5, 6). Evidence has been obtained in studies using the isolated perfused MCD that favors a role for basolateral Cl/HCO_3^- exchange in bicarbonate reabsorption in this segment. In this preparation, substitution of the impermeant anion, gluconate, for Cl on the basolateral side increased intracellular pH (7) and abolished bicarbonate reabsorption (5). Also, addition of the anion exchange inhibitor, SITS, to the basolateral solution inhibited bicarbonate reabsorption (5).

To further characterize the properties of Cl/HCO_3^- exchange in the MCD, we monitored intracellular pH (pH_i) regulation in suspensions of cells isolated from rabbit outer MCD using a fluorescent intracellular pH probe (3). In these experiments cells were preloaded with HCO_3^- and abruptly diluted into HCO_3^- -free solutions with on-line measurement of pH_i .

Methods

Isolation of MCD cells. The technique for preparing fresh suspensions of MCD cells has been previously described (3). Briefly, male New Zealand white rabbits were killed by cervical dislocation and exsanguinated via the carotid artery. All enzyme treatments and centrifugations were carried out in Joklik's minimal essential medium containing 10% fetal bovine serum. The kidneys were perfused free of blood and treated with 0.2% collagenase. The inner stripe of the outer medulla was excised from each kidney and finely minced. The tissue was incubated for 1 h in 0.2% collagenase at 37°C under 95% $\text{O}_2/5\%$ CO_2 , and the resulting digest centrifuged to harvest the suspended tubule segments. The tubules were incubated repeatedly in 0.25% trypsin solution at room temperature under 95% $\text{O}_2/5\%$ CO_2 for 20 min. At the end of each incubation the undigested tubules were pelleted and returned to the trypsin, and the suspended cells were harvested and pooled on ice. The resulting cell suspension containing all medullary cell types was layered over 60 ml continuous Ficoll gradients (2.5–43%) and centrifuged for 45 min at 2,300 g. The gradients were harvested in 4-ml fractions, and the cells in each fraction were washed free of Ficoll in a 10-fold excess of Joklik's medium and centrifuged at 300 g for 10 min. MCD cells were found in the upper two fractions (3). The MCD cells prepared in this manner resembled closely MCD principal cells *in situ* by both light and electron microscopy, and no intercalated cells were found. As discussed below and in a previous paper, it is likely that the principal cells in this segment participate in acidification *in vivo* (3, 6). The MCD fraction was purified for both carbonic anhydrase and ADH-stimulated adenylate cyclase, which are two enzymes known to be present at high activity in MCD (8, 9). In addition, these cells regulate pH_i and partly transport protons via a plasma membrane proton-ATPase (3).

Measurement of intracellular pH. The technique of measurement of pH_i of MCD cells has been described previously (3, 10). The medium used for all experiments was a nominally bicarbonate-free Ringer's, which was composed of NaCl (130 mM), KCl (5 mM), Tris-Hepes (10 mM, pH 7.40), MgSO_4 (0.4 mM), CaCl_2 (2 mM), and Na_2HPO_4 (2 mM). In solutions containing bicarbonate, 25 mM NaHCO_3 was substituted iso-

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1. *Abbreviations used in this paper:* ADH, antidiuretic hormone; MCD, medullary collecting duct; MES, 2-[N-morpholine]-ethanesulfonic acid; pH_i , intracellular pH.

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tonically for NaCl. Bicarbonate-containing solutions were gassed with 5% CO₂/95% O₂; PCO₂ and HCO₃⁻ in these solutions, measured using a Corning blood gas analyzer, were 40 mmHg and 25 mM, respectively. The pH of all solutions was checked immediately before use and adjusted as needed to pH 7.40 with small amounts of 1 M HCl or 1 M NaOH. Cells were resuspended in nonbicarbonate Ringer's and incubated for 15–20 min in 10 μ M 6-carboxyfluorescein diacetate at 25°C and an extracellular pH titrated to 6.50 by addition of small amounts of 330 mM 2-[N-morpholino]ethanesulfonic acid (MES). The diacetate form rapidly enters the cells, where it is converted to the optically active 6-carboxyfluorescein by intracellular esterases (10). Once loaded with 6-carboxyfluorescein, cells were washed thoroughly with nonbicarbonate Ringer's pH 7.40 to remove MES and extracellular dye. The fluorescence of dye-loaded cells was monitored using an Aminco-Bowman fluorescence spectrophotometer equipped with a temperature-controlled water jacket and a mechanical stirrer. Excitation wavelengths were 492 and 450 nm and emission wavelength was 530 nm. One-million cells were diluted into 3.5 ml of nonbicarbonate Ringer's and stirred in the fluorimeter at 37°C. At the end of each run the cells were pelleted and the fluorescence of the supernatant assayed directly. By subtracting this extracellular signal from the total signal, the signal generated by the intracellular dye could be calculated.

Standard curves relating pH_i and fluorescence excitation ratio were obtained daily (3) by placing the cells in medium containing 137 mM KCl and adding the K/H ionophore, nigericin. Addition of small amounts of concentrated MES to the medium produced incremental acidification, thus generating the curve. The fluorescence excitation ratios obtained from each curve were compared with these standard curves to estimate pH_i.

Materials. Collagenase was obtained from Worthington Biochemical Corp., Freehold, NJ; Joklik's minimal essential medium was obtained from Gibco, Grand Island, NY; Ficoll was obtained from Pharmacia Fine Chemicals, Piscataway, NJ; and 6-carboxyfluorescein diacetate was obtained from Molecular Probes, Junction City, OR. 4-acetamido-4'-isothiocyanostilbene-2,2'-disulfonate (SITS) and 4,4'-diisothiocyanostilbene-2,2'-disulfonate (DIDS) were obtained from Pierce Chemical Co., Rockford, IL. Both SITS and DIDS were kept frozen, in the dark, and dissolved into stock solutions immediately before use and kept in the dark. Furosemide was a gift of Hoescht-Roussel Pharmaceuticals, Somerville, NJ. Bumetanide was a gift of Hoffmann-LaRoche Pharmaceuticals, Nutley, NJ. All other reagents were obtained from Sigma Chemical Co., St. Louis, MO and were of analytical grade.

Statistics. In all bar graphs a single "n" represents the mean of duplicate or triplicate determinations of pH_i time course on a given cell preparation. Unless specified experimental results are given for the mean $\pm$ SEM for at least three separate cell preparations. Protocols for which representative experiments are presented were also performed on at least three different cell preparations.

Results

To determine the effect on pH_i of bicarbonate efflux from cells, carboxyfluorescein-loaded MCD cells were resuspended in bicarbonate-containing Ringer's for at least 20 min and then diluted abruptly into a cuvette containing nonbicarbonate Ringer's in the fluorimeter. Fig. 1 shows a typical pH_i time course obtained in this manner. The pH_i response observed when CO₂/HCO₃⁻ preloaded cells were diluted into nonbicarbonate Ringer's can be divided into three phases: an initial alkalinization to pH_i = 7.45 $\pm$ 0.03 (*n* = 22), completed before the fluorescence measurements began, an acidification to pH_i = 6.93 $\pm$ 0.03 (*n* = 22), occurring over the first 4 min, and a final recovery of pH_i to a resting level of 7.24 $\pm$ 0.06 (*n* = 3). Because the stirrer on the Aminco-Bowman fluorimeter could not rapidly mix the cells, readings of fluorescence were delayed until 30 s after the cells

Figure 1. Effect of HCO₃⁻ gradient on pH_i. 6-Carboxyfluorescein-loaded MCD cells were preincubated in HCO₃⁻-containing Ringer's as described in Methods and added to HCO₃⁻-free Ringer's (closed circles) or to HCO₃⁻-containing Ringer's (open circles).

The time course of pH_i was followed as described in the text. These time courses are representative of identical protocols performed on at least three different MCD cell preparations.

were added. Thus, the time course of the initial alkalinization of the cells could not be observed directly. When MCD cells were incubated in HCO₃⁻-containing Ringer's and diluted into the same medium (open circles, Fig. 1), pH_i remained stable at 7.20 $\pm$ 0.03 (*n* = 3). The almost instantaneous alkalinization from a pH_i of 7.20–7.45 can be attributed to more rapid efflux from the cells of CO₂ than HCO₃⁻. In numerous studies of intracellular pH, this more rapid permeance of CO₂ than HCO₃⁻ has allowed investigators to alkalinize or acidify the intracellular space (11). Direct measurements showing the high permeability of biological and artificial membranes to CO₂ (12) support the idea that the initial alkalinization was due to CO₂ efflux.

Since the acidification phase shown in Fig. 1 was likely to be caused by bicarbonate efflux down its chemical gradient, this phase was examined in further detail. In Fig. 2, the curve labeled "HCO₃_{in}, Cl_{out}" represents a typical time course of the acidification response. To allow quantitative comparison of experimental groups, the first three minutes of this time course were fitted to the following linear equation form: pH_i = *k*(*t*) + pH_{i(t=0)}, where *k* is the fitted constant that describes the initial rate of acidification, *t* is the time after the cells reached maximum pH_i, and pH_{i(t=0)} was the maximal intracellular pH. *r* values from these linear fits averaged 0.96. Fitting pH_i time courses to a linear function relating pH_i to time requires no assumptions regarding the mechanism of pH_i response, but merely provides a straightforward means of quantitatively comparing experimental groups (3, 13, 14).

To determine the role of extracellular Cl and intracellular HCO₃⁻ on the acidification response, the substitution experiments shown in Fig. 2 were performed. When the impermeant anion, gluconate, was substituted for Cl in the extracellular medium (HCO₃_{in}, Gluc_{out}), the acidification response was markedly in-

Figure 2. Cl/HCO₃⁻ exchange. 6-Carboxyfluorescein-loaded MCD cells were preincubated in HCO₃⁻-containing Ringer's (HCO₃_{in}) or in HCO₃⁻-free Ringer's titrated to pH 8.30 with KOH (HCO₃-free). At time zero, cells were added to HCO₃⁻-free Ringer's containing 135 mM Cl⁻ (Cl_{out})

or to an identical solution with gluconate salts replacing Cl (GLUC_{out}). These time courses are representative of identical protocols performed on three different MCD cell preparations.

hibited. When MCD cells were alkalinized (by preincubation in nonbicarbonate Ringer's titrated to pH 8.40 with 1 N KOH) and then added to the usual Cl-containing nonbicarbonate Ringer's (HCO_3 -free, Cl_{out} , final extracellular pH = 7.40), the acidification response was again markedly inhibited. This result indicates that HCO_3 in the presence of similar transmembrane pH gradients was a far better "substrate" for the acidification response than OH^- . The dependence of the acidification response on intracellular HCO_3 and extracellular Cl indicates that this process occurs via a Cl/HCO_3 exchange.

The next experiments were designed to determine whether MCD cell Cl/HCO_3 exchange is carrier-mediated and to describe some of the properties of this transport process. Fig. 3 shows the effect of varying extracellular Cl concentration on the rate of the acidification response. MCD cells were loaded with CO_2/HCO_3 as described above and added to solutions containing mixtures of 130 mM NaCl and 130 mM sodium gluconate. The acidification rates were determined and plotted against the extracellular Cl concentration (Fig. 3, left). Note that the acidification rate reached a plateau as the Cl concentration rose, thus indicating a saturable process. A Lineweaver-Burk plot (Fig. 3, right) permitted calculation of the apparent Michaelis constant (K_m) and maximum velocity (V_{max}) for Cl of Cl/HCO_3 exchange. The values obtained from identical experiments on three separate MCD cell preparations were apparent $K_m = 29.9 \pm 7.7$ (SE) mM, and $V_{\text{max}} = 0.00314 \pm 0.00067$ pH U/s. Correlation coefficients averaged 0.98. In these experiments, intracellular Cl concentration was not measured. Although the level of intracellular Cl will influence the response of Cl/HCO_3 exchange to varying extracellular Cl concentrations, it is valid to compare different extracellular Cl concentrations if intracellular Cl is initially the same for each extracellular Cl level. Since the cells for each run on a given day were taken from a single suspension that was preincubating in CO_2/HCO_3 , it is likely that the intracellular Cl concentration was identical for each extracellular Cl concentration at the start of each rate determination.

To determine the anion selectivity of MCD Cl/HCO_3 exchange, the rate of the acidification response was measured in the presence of different extracellular anions. Since use of concentrations far in excess of the apparent K_m for Cl might have obscured differences between anions, all experiments were car-

Figure 3. Kinetics of Cl/HCO_3 exchange. 6-Carboxyfluorescein-preloaded MCD cells were preincubated with HCO_3 -containing Ringer's and added to HCO_3 -free Ringer's containing varying concentrations of Cl. Gluconate salts were used to replace Cl salts isotonicity. (Left) Acidification rates were obtained as described in text and are plotted on the ordinate. Extracellular Cl concentration is plotted on the abscissa. (Right) Double reciprocal plot of data from left panel. The apparent K_m for Cl in this MCD cell preparation was 16.9 mM. Each data point is the mean of duplicate or triplicate rate determinations at the [Cl] given.

Figure 4. Effects of anion substitution on acidification rate. 6-Carboxyfluorescein-preloaded MCD cells were preincubated with HCO_3 -containing Ringer's and added to solutions of varying anion content. Each solution was made up of 70 mM gluconate salts (Na^+ and K^+) and 65 mM Na anion. Results are expressed as the percentage of the rate of acidification observed using an extracellular solution containing 65 mM NaCl on the same MCD preparation. Results represent the mean and standard error of duplicate determinations on three different MCD cell preparations.

ried out with 65 mM extracellular sodium gluconate and 65 mM extracellular sodium anion. Fig. 4 shows the results of duplicate determinations performed on three different MCD cell preparations. We obtained the ranking $\text{Cl} > \text{Br} > \text{I} > \text{SCN} = \text{NO}_3 > \text{gluconate}$. These results demonstrate specificity of MCD Cl/HCO_3 exchange for halides.

The effects of inhibitors of anion transport on MCD Cl/HCO_3 exchange were also examined. Fig. 5 shows, in a representative experiment, the effect of the stilbenes, SITS and DIDS, on the acidification response. These anion exchange inhibitors markedly inhibited Cl/HCO_3 exchange when present in the extracellular medium at 10^{-4} M. Fig. 6 shows the relative rates of the acidification response in the presence of various anion transport inhibitors at 10^{-4} M concentration. SITS and DIDS diminished the rate of the acidification response by over 80%. These molecules act as potent inhibitors of the red cell anion exchanger, band 3 (15). Phloretin, a less potent inhibitor of band 3 (15) reduced the acidification rate by 50%. Furosemide, which inhibits $\text{Na}/\text{K}/\text{Cl}$ co-transport in the thick ascending limb (16), and acts as a weak band 3 inhibitor (17), also partially inhibited MCD cell Cl/HCO_3 exchange. Interestingly, bumetanide, another potent inhibitor of $\text{Na}/\text{K}/\text{Cl}$ co-transport (18), showed only slight inhibition of the acidification response. Probenecid, which inhibits organic anion transport in the proximal tubule (19), exhibited slight inhibition of MCD Cl/HCO_3 exchange. The carbonic anhydrase inhibitor, acetazolamide, was a potent inhibitor of Cl/HCO_3 exchange. As with the other inhibitors shown in this table, cells were not pretreated with acetazolamide, and were only exposed to it when added to the HCO_3 -free medium. To assess the sensitivity of MCD Cl/HCO_3 exchange to stilbene inhibition, the acidification rate was measured in the presence of varying concentrations of DIDS. As shown in Fig. 7, half-maximal inhibition of acidification occurred at a DIDS concen-

Figure 5. Effects of stilbene inhibitors on Cl/HCO_3 exchange. 6-Carboxyfluorescein-loaded MCD cells were preincubated with HCO_3 -containing Ringer's and added to HCO_3 -free Ringer's. Where indicated, 0.1 mM SITS or DIDS were present in the extracellular medium at the start of the time course.

Figure 6. Effects of inhibitors on Cl/HCO_3 exchange. Protocol was as described in Fig. 5. Each inhibitor was present in the HCO_3 -free Ringer's at 10^{-4} M. MCD cells were not pretreated with inhibitor before time courses began. Results are expressed as percentage of acidification rate observed with the same MCD cell preparation in the absence of inhibitor. Results represent the mean and standard error of duplicate or triplicate time courses performed with each inhibitor on three different MCD cell preparations. CONT, control; ACTZ, acetazolamide; PHL, phloretin; FUR, furosemide; BUM, bumetanide; and PROB, probenecid.

tration of $\sim 5 \times 10^{-7}$ M, a level comparable with that exhibited by red cell anion exchange (15).

Since MCD cells recover from acid loading by extruding protons via a plasma membrane H^+ -ATPase (3), we examined the mechanism of pH_i recovery after the acidification response characterized above. In Fig. 8, untreated MCD cells (control) and MCD cells poisoned with potassium cyanide in the absence of extracellular glucose (KCN) were preloaded with CO_2/HCO_3 and added to nonbicarbonate Ringer's. Although the initial al-

Figure 7. Effect of varying [DIDS] on rate of Cl/HCO_3 exchange. Protocol was as in Fig. 5. DIDS concentration was varied and the percentage inhibition determined as in Fig. 6 and plotted as a function of [DIDS].

Figure 8. Effect of ATP depletion on pH_i recovery. 6-Carboxyfluorescein-preloaded MCD cells were preincubated in HCO_3 -containing Ringer's and added to HCO_3 -free Ringer's. KCN: Cells were pretreated with 2 mM KCN; 2 mM

KCN was also present in the HCO_3 -free Ringer's. We have previously shown (3) that cells treated with KCN in the absence of glucose are rapidly depleted of ATP. These time courses are representative of identical protocols performed on three different MCD cell preparations.

kalinization and acidification responses were similar in the two protocols, the cells poisoned with KCN did not restore pH_i to the usual resting level of 7.20 but instead continued to acidify. As described previously, MCD cells treated with KCN in the absence of glucose are rapidly depleted of $>95\%$ of cellular ATP (3, 20). Thus, Fig. 8 indicates that recovery from CO_2/HCO_3 -induced acidification is ATP-dependent and provides evidence that Cl/HCO_3 exchange occurred in the same cell as ATP-dependent proton extrusion.

Since the bicarbonate-induced acidification response appeared to be more rapid when ATP-dependent proton extrusion was inhibited (see Fig. 8), it seemed likely that our measurements of acidification rates in unpoisoned cells reflected the rate of bicarbonate-induced acidification minus the rate of H^+ -ATPase-mediated proton extrusion. Since the opposing effects on pH_i of these two transport mechanisms might have altered the apparent relationship between external Cl concentration and HCO_3 efflux, we repeated the Cl kinetics experiments in cells exposed to KCN in the absence of glucose. When H^+ -ATPase was inhibited by ATP depletion, saturability was again observed. The apparent V_{max} in H^+ -ATPase-inhibited cells was 0.00356 ± 0.000163 pH U/s ($n = 2$ cell preparations), and the apparent K_m was 14.8 ± 3.5 mM, with correlation coefficients for the fitted Lineweaver-Burk plots averaging 0.98.

Discussion

Plasma membrane Cl/HCO_3 exchange is known to participate in a wide variety of epithelial transport functions. Net Cl transport is mediated in part via Cl/HCO_3 exchange in mouse thick ascending limb (20), amphibian gall bladder (21), and intestinal epithelia (22). Volume regulation in response to hypertonic stimuli has recently been shown to be mediated by Cl/HCO_3 exchange coupled to Na/H exchange in mouse thick ascending limb (23) and amphibian gall bladder (24). In epithelial cells specialized to secrete luminal acid, basolateral Cl/HCO_3 exchange functions to restore intracellular pH by secreting into the basolateral space alkali equivalents generated by the luminal secretion of acid (25). Such acid-secreting epithelia include gastric mucosa (26), amphibian bladder (27), and MCD (5). Evidence favoring basolateral Cl/HCO_3 exchange in the isolated, perfused mammalian medullary collecting duct includes the cellular alkalization and inhibition of lumen-to-bath HCO_3 transport, which accompanies removal of basolateral Cl (5, 7). Furthermore, addition of SITS to basolateral solutions inhibits HCO_3 reabsorption (5). The ability to monitor pH_i in MCD cell suspensions provides a straightforward means of measuring and characterizing Cl/HCO_3 exchange in an epithelial tissue specialized to secrete acid.

There is direct histochemical and electrophysiological evidence that inner stripe MCD principal cells participate in acidification *in vivo*. Thus these cells stain strongly for carbonic anhydrase *in situ* (28). In addition, isolated perfused inner stripe collecting ducts reabsorb bicarbonate (4, 5) and have a single cell population by electrophysiologic criteria (6). Since principal cells make up the vast majority of cells in this segment (29), principal cells must be responsible for acid secretion *in vivo*. The cells used in these experiments express morphologic and enzymatic characteristics of principal cells of the inner stripe of the outer MCD (3). Their viability has been confirmed by measurement of O_2 consumption, ATP levels, and the demonstration of pH_i regulation (3). In the cortical collecting duct and the turtle bladder, acid secretion is thought to occur only via the intercalated cells (7, 25). Because of the striking morphologic differences between principal cells in the inner stripe and in the cortex (29), it is impossible to assign functions to inner stripe collecting duct cell types based on analogy to these other tissues.

The bicarbonate preloading protocol used in these experiments was designed to allow exchange of extracellular Cl for intracellular HCO_3^- , the direction of transport expected to occur *in vivo*. The initial alkalization that we observed in these studies was due to rapid efflux of CO_2 from the cells. The known high permeability of CO_2 in biological membranes favors this view (12). Further evidence that the rise in pH_i was due to CO_2 efflux, includes the lack of alkalization in the absence of CO_2/HCO_3^- gradients (Fig. 1) or in the absence of functional carbonic anhydrase.

Although our methods do not provide a direct measure of the intracellular HCO_3^- concentration, a calculated value of 15.1 mM could be expected at a PCO_2 of 40 mmHg and a pH_i of 7.20, which were the conditions present when the cells were suspended in CO_2/HCO_3^- Ringer's medium (see Fig. 1). After the cells were added to a bicarbonate-free Ringer's, a HCO_3^- gradient was established across the cell membrane. This gradient could be dissipated by efflux of HCO_3^- anion, or by conversion of HCO_3^- to H_2CO_3 and CO_2 , with rapid efflux of CO_2 from the cells. Since the latter mechanism involves removal of a proton and HCO_3^- from the cell as CO_2 and H_2O , it cannot account for the striking acidification that we observed in these cells. Thus, the acidification and dissipation of the bicarbonate gradient could only have been caused by efflux of bicarbonate from the cells. This bicarbonate efflux was markedly inhibited when the impermeant anion, gluconate, was substituted for Cl in the extracellular medium (Fig. 2). This observation parallels the findings in turtle bladder (27) and perfused rabbit MCD (5), in which basolateral Cl was necessary for net acid transport, and is in agreement with the recent demonstration that removal of basolateral Cl alkalinizes pH_i (7). The finding that intracellular alkalinity alone, in the absence of bicarbonate, could not duplicate the acidification response, indicates that the bicarbonate efflux mechanism operates far more efficiently with HCO_3^- than with OH.

The demonstration that MCD Cl/ HCO_3^- exchange exhibits saturability with respect to extracellular Cl (Fig. 3) provides evidence that this exchange is carrier mediated. The apparent K_m s for Cl that were calculated in this study are similar to values obtained for the red blood cell anion exchanger, which range from 33 to 65 mM, depending on the transport function being studied (15, 30). In recent studies of the effect of basolateral Cl on turtle bladder acidification, Fischer et al. reported an apparent

K_m for Cl in the micromolar range (27). The differences between their results and ours are not readily explained, but may be due to tissue and species differences and the fact that in their studies, transepithelial acid transport was measured using short circuit current, while in our study the cell pH was measured directly.

The Cl saturability that we observed in this study could reflect the kinetics of binding of Cl to a Cl/ HCO_3^- exchange protein or the effects of raising extracellular Cl concentration on MCD cell membrane potential via a saturable Cl channel. Alterations of membrane potential via a Cl conductance could only affect HCO_3^- efflux if HCO_3^- exit occurs via an electrogenic conductance. Recently, Koeppen has measured directly the membrane potential of rabbit inner stripe MCD cells using intracellular electrodes and the *in vitro* perfusion technique (6). His experiments showed that abrupt changes in extracellular Cl, but not in HCO_3^- , provoke rapid alterations in membrane potential (6), which indicates that the MCD cell possesses electrogenic Cl but not HCO_3^- conductance (6). In this context, our finding of saturability to Cl likely reflects the interaction of Cl with a Cl/ HCO_3^- exchanger.

Another characteristic of mediated transport is ion specificity. The anion ranking, $Cl^- > Br^- > I^-$ (Fig. 4) is similar to that seen in anion exchange in human red blood cells (30, 31) and in microvillus membrane vesicles prepared from Necturus proximal tubule (32), and may reflect size selectivity of the exchanger. Because NO_3^- and SCN^- were far less effective than the halides in stimulating HCO_3^- efflux, we suggest that MCD Cl/ HCO_3^- exchange is electroneutral. If, as is true in many biological membranes (32–35), SCN^- and NO_3^- exhibit a higher passive permeance than Cl, the presence of these two more permeant anions at high extracellular concentrations might be expected to hyperpolarize the cells and stimulate electrogenic HCO_3^- efflux. Thus, the inability of these anions to allow an acidification response much higher than that of the impermeant gluconate suggests that MCD Cl/ HCO_3^- exchange is not electrogenic, but that occurs via a direct exchange process. However, since the basolateral membrane of MCD cells perfused *in vitro* exhibits a Cl conductance (6), it is not certain that either SCN^- or NO_3^- permeate the MCD membrane more rapidly than Cl. The evidence stating that MCD basolateral HCO_3^- flux is electrically silent (6) leads us to favor electroneutral Cl/ HCO_3^- exchange as the mechanism of HCO_3^- exit from this cell.

The inhibitor sensitivities of MCD Cl/ HCO_3^- exchange were examined to allow comparison of anion exchange in this tissue with that of other systems. The stilbenes, SITS and DIDS, are relatively specific and potent inhibitors of red blood cell anion exchange (15). Sensitivity to these inhibitors has been demonstrated for turtle bladder (25) and MCD bicarbonate transport (5), in Necturus gall bladder (21) and proximal tubule (32), and also in mammalian proximal tubule (36) and thick ascending limb (20). In all of these studies, stilbene concentrations of 10^{-4} M or higher were used. MCD Cl/ HCO_3^- exchange was highly sensitive to these stilbenes (Figs. 5 and 6); the apparent K_i for DIDS was $\sim 5 \times 10^{-7}$ M, which is similar to the sensitivity of red blood cell anion exchange to this inhibitor (1.2×10^{-6} M, 15). The relative insensitivity of MCD Cl/ HCO_3^- exchange to furosemide, bumetanide, and probenecid distinguishes this transport process from Na/K/Cl co-transport of the thick ascending limb, and from the organic anion transport of the proximal tubule. The finding that furosemide is a more effective inhibitor of Cl/ HCO_3^- exchange than bumetanide is similar to

results obtained for *Necturus* microvillus vesicle anion exchange (37).

The apparent inhibition of anion exchange by acetazolamide may have been caused by direct interaction of this carbonic anhydrase inhibitor with the Cl/HCO_3 exchanger. Preincubation of MCD cells with acetazolamide prevented initial alkalization due to CO_2 efflux, and prevented the subsequent acidification (due to HCO_3 efflux). In the inhibitor protocols (Fig. 6), acetazolamide was present only when the HCO_3 -preloaded cells were added to the HCO_3 -free Ringer's; in these protocols, normal alkalization was observed. Once the cell had alkalized (due to CO_2 efflux), inhibition of carbonic anhydrase was expected to diminish the protonation of intracellular HCO_3 to H_2CO_3 and provide more HCO_3 for efflux and acidification of the cell interior. Thus, carbonic anhydrase inhibition was expected to increase rather than decrease the acidification rate. Direct interaction of carbonic anhydrase inhibitors with anion transport processes has been reported in human red blood cell and in mammalian proximal tubule (36). Further studies using carbonic anhydrase inhibitors that cannot enter the cell will be necessary to determine whether these inhibitors interact directly with MCD Cl/HCO_3 exchange.

The recovery of MCD cells from intracellular acidification induced by exposure to mineral acids and acetate salts, and by withdrawal of ammonium salts, is mediated in part via a plasma membrane H^+ -ATPase (3). As shown in Fig. 8, recovery of these cells from an acid load induced by HCO_3 efflux was also critically dependent on cellular ATP. Taken together, these results indicate that both Cl/HCO_3 exchange and H^+ -ATPase activity are present in the MCD cell.

In summary, the MCD cell pH_i response to abrupt removal of CO_2/HCO_3 includes an initial alkalization due to CO_2 efflux, followed by an acidification (due to HCO_3 efflux) via Cl/HCO_3 exchange, with a gradual recovery to the resting pH_i of 7.20 partly due to the action of a plasma membrane H^+ -ATPase. MCD Cl/HCO_3 exchange requires intracellular HCO_3 and extracellular Cl , and is a saturable process that exhibits anion specificity and sensitivity to stilbene and acetazolamide inhibition.

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