Regulation of Rabbit Medullary Collecting Duct Cell pH by Basolateral Na^+/H^+ and Cl^-/Base Exchange

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Abstract

The collecting duct of the inner stripe outer medulla (OMCDi) is a major site of distal nephron acidification. Using the pH sensitive fluorescent dye 2'-7'-bis(carboxyethyl)-5,6,-carboxy-fluorescein (BCECF) and quantitative spectrofluorometry to measure intracellular pH in isolated perfused OMCDi, we have characterized basolateral transport processes responsible for regulation of intracellular pH. Experiments suggesting the existence of basolateral Cl^-/base exchange were performed. In HCO_3^- containing buffers, bath Cl^- replacement resulted in reversible alkalinization of the OMCDi from 7.22±0.05 to 7.57±0.12. Similarly 0.1 mM bath 4',4'-diisothiocyanostilbene-2,2'-disulphonic acid (DIDS) alkalinized the OMCDi from 7.14±0.09 to 7.34±0.09 and blocked further alkalinization by bath Cl^- removal (Δ = + 0.02 pH units). The concentration dependence kinetics of Cl^-/base exchange revealed a $K_{1/2}$ of 10 mM for external Cl^- with a V_{max} of 0.50 pH U/min. Experiments suggesting the existence of basolateral Na^+/H^+ exchange were also performed. Replacement of bath Na^+ by tetramethylammonium resulted in reversible cell acidification (7.14±0.09 to 6.85±0.1). Tubules that were acidified by a brief exposure to NH_4Cl displayed recovery of cell pH back to baseline at a rate that was highly dependent on bath Na^+ concentration. Half maximal recovery rate was achieved at 7 mM bath Na^+ and V_{max} was 0.605 pH U/min. The Na^+-dependent rate of cell pH recovery after acidification was blocked by 0.2 mM bath amiloride. These results suggest that intracellular pH in the OMCDi is regulated by parallel basolateral Na^+/H^+ exchange and Cl^-/base exchange.

Introduction

Distal nephron acidification of the urine is critical to maintain net acid-base balance. It is in this portion of the nephron that H^+ secretion titrates nonbicarbonate buffers, resulting in the renal regeneration of HCO_3^- (1, 2). The outer medullary collecting duct-inner stripe (OMCDi)[1] has been identified as a major site of distal acidification (3, 4). In isolated perfused OMCDi, acidification, as measured by HCO_3^- absorption rates, correlates well with the simultaneously measured lumen positive transepithelial voltage (4). Both voltage and HCO_3^- absorption are unaffected by complete removal of peritubular or luminal Na, distinguishing the major mechanism of HCO_3^- reabsorption in this segment from that of more proximal nephron segments. In contrast, both voltage and HCO_3^- absorption are markedly decreased by peritubular Cl^- removal or the peritubular addition of disulfonic stilbene derivatives (such as 4',4'-diisothiocyanostilbene-2,2'-disulfonic acid [DIDS] or 4-acetamido-4' isothiocyanato stilbene 2,2' disulfonic acid [SITS]) (5), which are known inhibitors of band 3-mediated Cl^-/HCO_3^- exchange in the red blood cell (6). Based on experimental results obtained in the turtle bladder and the isolated perfused OMCDi, a model for HCO_3^- absorption by this segment has been developed (7, 8) in which intracellular water is decomposed into protons and hydroxyl ions. An apical membrane electrogenic H^+ translocating ATPase pumps intracellular protons into the urine, titrating luminal buffers. The remaining intracellular base exits across the basolateral membrane in exchange for Cl^- via an electroneutral anion exchanger.

Recently, immunohistochemical studies have provided independent evidence for this model and have further characterized the OMCDi cell. Using antibodies against the red blood cell anion exchange protein (anti-band 3 antibodies) several investigators (9–11) have demonstrated the presence of basolateral staining of the OMCDi. Brown et al. (9) have shown apical staining of the OMCDi using anti-H^+ATPase antibodies. Nonetheless direct functional confirmation for the presence of basolateral Cl^-/base exchange in the OMCDi is lacking.

The apparent lack of an effect of ouabain or Na^+ removal on net proton secretion by the OMCDi is intriguing. The existence of basolateral Na^+/K^+ ATPase in the OMCDi has been demonstrated by immunohistochemical techniques (12). Furthermore, ouabain sensitive ATPase activity is markedly induced in this segment by hypokalemia (13). The current model for the OMCDi however, does not include a functional role for this basolateral Na/K ATPase. Several studies have suggested the ubiquitous presence of Na^+/H^+ exchange in mammalian cells (14, 15), and an important role for this exchanger in both the maintenance of cell pH and cell volume regulation (14–16). The existence of Na^+/H^+ exchange in the OMCDi would require the presence of a counterbalancing Na^+ extrusion mechanism. This Na^+ entry step thus would provide a functional role for the Na/K ATPase in this segment.

The present studies were therefore designed to examine directly whether basolateral Na/H and Cl^-/base exchange exist in the OMCDi. Using the in vitro microperfused OMCDi, we

Presented in part at the 1988 Annual Meeting of the American Federation for Clinical Research, Washington, DC, and at the 1988 Annual Meeting of the American Society of Nephrology, San Antonio, TX.

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Received for publication 9 December 1988 and in revised form 17 March 1989.

1. *Abbreviations used in this paper:* BCECF, 2',7'-bis-(carboxyethyl)-5,6-carboxy-fluorescein; DIDS, 4',4'-diisothiocyanostilbene-2,2'-disulphonic acid; NMDG, *N*-methyl-D-glucamine; OMCDi, outer medullary collecting duct, inner stripe; TMA, tetramethylammonium.

J. Clin. Invest.

0021-9738/89/09/0996/09 $2.00
Volume 84, September 1989, 996–1004

measured intracellular pH with the fluorescent dye, 2',7'-bis-(carboxyethyl)-5,6-carboxyfluorescein (BCECF). The regulation of OMCDi cell pH was studied under conditions designed to stimulate specifically or inhibit specifically, either basolateral Cl^-/base exchange or Na^+/H^+ exchange.

Methods

Microperfusion. Female New Zealand white rabbits were decapitated. The left kidney was removed, sliced into sagittal sections 1-mm thick and placed into a chilled 475-mosM dissection medium. Single OMCDi were dissected as previously described (3). For these studies the distal most part of the OMCDi nearest the first branch point at the inner medullae were used. The OMCDi was then transferred to a low volume laminar flow perfusion chamber (17) on an inverted microscope. Tubules were cannulated and perfused with an isotonic Hepes-buffered (pH = 7.40), Na-free, Cl^--containing solution (see Solutions). The initial bath solution was an isotonic NaCl, HCO_3^-/CO_2 buffer. The bath was preheated through a 38°C water-jacketed line and bath temperature was continuously maintained at 37°C by maintaining flow rate > 1 ml/min and monitoring bath temperature as previously described (3). Bath flow rate was controlled using a perfusion pump (Sage Instruments, Inc., Cleveland, OH). Flow was initially set at 1.17 ml/min and subsequently increased to 2.5–3.5 ml/min during data collection to ensure rapid (< 0.2 s) washout of the perfusion chamber. Bath solutions were exchanged by means of a low volume four-way distribution valve (Hamilton Co., Reno, NV).

Measurement of cell pH. Perfused tubules were loaded for 10 min from the bath with 2 μM of the acetoxymethylester derivative of BCECF (BCECF-AM). The loaded tubule was then alternately excited at 24 Hz with 495 and 440 nm light by an optical chopper and two monochromators (Deltascan; Photon Technology Inc., New Brunswick, NJ) coupled to the microperfusion chamber on an inverted microscope stage (Diaphot; Nikon, Inc., Instrument Div., Garden City, NY). The excitation source was directed to the stage using an 500 nm dichroic mirror and a 63× (Neofluor; Carl Zeiss, Inc., Thornwood, NJ) objective lens as the condenser. Emitted fluorescent light then passed through a 520–560-nm filter (Nikon). The area of the tubule from which emitted light intensity was quantitated was 40 μm wide by 15 μm long. Fluorescence was recorded in counts per second by a photomultiplier tube (Hamamatsu Photonics, K.K., Toyooka Vill., Japan) linked to a personal computer. The ratio of fluorescent intensity using 495 vs. 440 nm excitation was measured in real time. As has been reported by several investigators, this ratio initially increased with time and then gradually fell over the next 20 min (18, 19). After this ratio stabilized the experiment was started.

At the end of each experiment the fluorescent ratio was calibrated to cell pH by addition of nigericin (10 μM) and 105 mM KCl-Hepes buffers of four different pH values (6.34, 6.82, 7.31, and 7.77). From the mean 495/440 ratio obtained at each respective pH, a regression line of ratio-versus cell pH was plotted. This line was used to replot the real-time ratio data as cell pH vs. time for that specific tubule. The mean relationship between 495/440 ratio and pH for these studies was 1.95±0.258 ratio units per pH unit (Figs. 1 and 2). OMCDi autofluorescence, determined before loading with BCECF, was < 5% of the total fluorescent intensity after loading with BCECF and so was discounted.

Protocols

Resting cell pH. The 495/440 emission ratio was take in either HCO_3^- containing (buffer 1) or HCO_3^--free (buffer 4) media before any experimental maneuvers were performed.

Chloride removal. Perfusate was Na and HCO_3^- free but contained Cl^- (buffer 5). The bath solutions were changed from 120 mM Cl^- (buffer 1; see below) to an identical buffer in which all Cl^- was replaced with 120 mM gluconate containing bath. After a new steady state ratio was achieved (90 s later) the solution was changed back to the chloride-containing solution. Since gluconate complexes with calcium, the calcium content in gluconate containing solutions was increased appropriately to maintain extracellular Ca^+ activity constant (buffer 2).

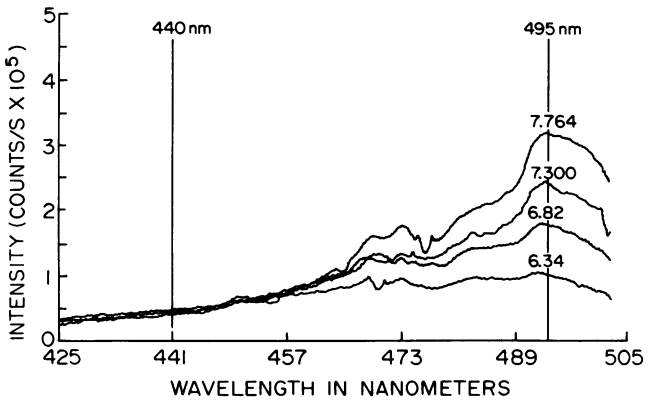

Figure 1. Excitation spectra of intracellular BCECF in OMCDi. OMCDi were loaded with 2 μM BCECF-AM for 10 min at 37°C. Flourescent emission intensity between 520–560 nm was recorded. The excitation wavelengths were scanned from 425–505 nm. Spectra were obtained while intracellular pH was clamped using four different Hepes buffered, high K^+/nigericin baths set at pH 6.34, 6.82, 7.30, and 7.764 (37°C). It can be seen that emission intensity using 440 nm excitation was pH insensitive, while emission using 495 excitation was highly dependent on pH.

Peritubular DIDS. Tubules were perfused in buffer 1 until a stable baseline was achieved. After 300 s of baseline recording the bath was changed to 0.1 mM DIDS added to buffer 1. After a new steady state was achieved, the bath was changed to buffer 2 to examine the effects of Cl^- removal in DIDS-treated tubules.

Dependence of recovery from cell alkalinization on peritubular Cl^- concentration. Tubules were bathed in buffer 1 and then alkalinized by changing to buffer 3, which was identical to the Cl^--free containing buffer 2 except 25 mM of gluconate were replaced with HCO_3^-, making this buffer's HCO_3^- content 50 mM. This maneuver allowed reproducible and repeatable realkalinization of the OMCDi after several Cl^- exposures.

After alkalinization had stabilized, the bath was changed to solutions of increasing Cl^- concentration with 25 mM HCO_3^-. The solutions of varying Cl^- concentrations were made up by mixing varying

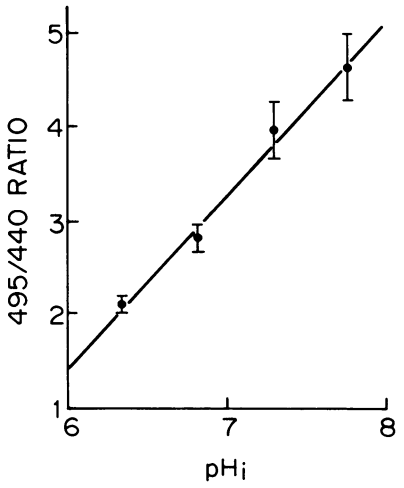

Figure 2. Relationship between OMCDi cell pH and emission ratio. Ratio of BCECF loaded OMCDi emission intensity, alternating excitation between 495 and 440 nm (24 Hz). The data represent the mean ratios obtained in 56 separate tubules yielding a mean slope of 1.95±0.26 ratio unit/pH unit. Each tubule was calibrated at the end of an experiment and the calibration slope for that particular tubule used to translate the ratio values for that tubule into pH.

proportions of buffer 1 and buffer 2. The Cl^- concentrations tested were 0, 1.8, 9, 18, 33, and 90 mM Cl^-. For data analysis, initial rate of cell acidification ($\Delta pH/\Delta time$) for 0 Cl^-/25 mM HCO_3^- was determined, and subtracted from the rates obtained for higher Cl^- concentrations. This allowed us to assess the rate of change in cell pH due exclusively to Cl^- concentration. After this initial rate was determined we realkalinized the OMCDi followed by exposure to the next highest Cl^- concentration tested. The rates of correction in an individual tubule were normalized to the mean correction rate at 90 mM Cl^- for the entire group of tubules. The mean correction rate at each Cl^- concentration tested was then obtained and fit to a nonlinear regression curve (Graphpad; ISI Software, Philadelphia, PA).

Effect of Na^+ removal. NaCl containing Hepes-buffered, HCO_3^- free (buffer 4) bath was used initially to establish a baseline ratio. The bath was then switched to a Na^+-free, Tetramethylammonium (TMA) substituted (buffer 5), solution for 5–7 min. A pH reading was recorded at 5 min after sodium Na^+ removal. The bath was then switched back to buffer 4.

Effect of Na^+ removal and Na^+ concentration on recovery from cell acidification. In the first protocol tubules were equilibrated initially in HCO_3^- free NaCl buffer (buffer 4). The bath was then changed to buffer 5 (TMACl, Hepes buffered) to which 20 mM NH_4Cl had been added. After 120 s in this solution the bath was changed to buffer 5 without NH_4Cl. This resulted in an abrupt and reproducible acidification of the OMCDi. The OMCDi was then exposed to bath solutions of increasing Na^+ concentration. Specifically 5, 25, 50, and 125 mM Na^+ containing solutions were made up by mixing appropriate amounts of buffer 4 and 5. After an initial recovery rate was established, the buffer was changed back to buffer 5 in between each Na^+ containing solution.

For data analysis, the 0 Na rate of recovery was subtracted from the rate of recovery in each Na containing solution. The rate of recovery at any given Na concentration was then normalized to the group mean rate of recovery at 125 mM Na^+. The mean rates of recovery were plotted vs. Na^+ concentration and fit to a curve by nonlinear regression analysis.

In a separate protocol, rates of recovery from acidification (NH_4Cl^- washout) were compared for the same tubule in the presence and absence of Na^+. For this protocol *N*-methyl-D-glucamine (NMDG) was used as the Na^+ substitute instead of TMA. In this way nonspecific effects of TMA were excluded. In the first half of the protocol, the tubule was equilibrated in buffer 4, and then exposed to 20 mM NH_4Cl in buffer 4 for 120 s. The NH_4Cl was washed away resulting in an abrupt acidification. The initial rate of increase in cell pH was determined by the slope of the increase in ratio over the first 15–25 s after the nadir was reached. After the cell pH returned to baseline, a second 20 mM NH_4Cl pulse was performed in NMDDG-Cl solution (buffer 6). The initial rate (~ 15–25 s) of cell pH recovery in the absence of Na^+ was measured. Cell pH was allowed to reach a steady state. In some cases NaCl was then added back to ensure that readdition of Na^+ increased the rate of recovery.

Blockade of Na^+-dependent recovery of pH by amiloride. Tubules were equilibrated in buffer 4 and then exposed to an NH_4Cl pulse and washout using Na^+-free solutions (buffer 5) as above. A baseline rate of recovery was established, and then the bath was changed to 0.2 mM amiloride in buffer 5. In the presence of 0.2 mM amiloride 50 mM Na^+ was added to the bath. After 3–4 min, the bath was changed back to an amiloride-free and Na^+-free solution. Finally 50 mM Na^+ (no amiloride) was added back to the bath and a rate of change in cell pH was established.

Solutions

The initial bath contained: Na, 135 mM; Cl^-, 120 mM; K, 5 mM; HCO_3^-, 25 mM; Ca^{2+}, 2.4 mM; PO_4, 2.4 mM; SO_4, 1 mM; Mg^{2+}, 1 mM; alanine, 5 mM; glucose, 8 mM; and was acetate free (buffer 1) (Table I). HCO_3^- free solutions were buffered with 30 mM Hepes. For Na-free solutions, Na was replaced with either TMA or NMDG. For Na^+-free/HCO_3^- free solutions, pH was titrated to 7.37 using either a 2-M stock solution of TMA hydroxide or NMDG. For Cl^- free solutions, Cl^- was replaced with gluconate. For acidification using NH_4Cl pulse-and-washout 20 mM of either NaCl, NMDGCl, or TMACl were replaced with 20 mM NH_4Cl. In those experiments where the effect of Na concentration was examined, the desired Na concentration was achieved by mixing the appropriate volume of Na free and sodium containing buffers. Similarly, the effect of Cl^- concentration was examined by mixing the appropriate proportions of Cl^- free and Cl^- containing buffers. The osmolality of all solutions was 280–300 mosM. HCO_3/CO_2-buffered solutions were bubbled with 95% 02/5% CO_2 before use and the pH of all solutions was corrected to within 0.03 pH U of 7.37 before experimentation.

Table I. Solutions

	1	2	3	4	5	6
Na^+	135	135	135	125	—	—
Cl^-	120	—	—	110	110	110
TMA^+	—	—	—	—	125	—
$NMDG^+$	—	—	—	—	—	125
Gluconate	—	120	95	—	—	—
HCO_3^-	25	25	50	—	—	—
$Hepes^-$	—	—	—	15	15	15
H^+Hepes	—	—	—	15	15	15
Ca^{2+}	2.4	7.0	7.0	2.4	2.4	2.4

(All values given in millimolars.) In addition each solution also contained K^+ 5.0, PO_4 2.4, Mg^{2+} 1.0, $SO_4^=$ 1.0, alanine 5, and glucose 8 mM and were titrated to pH 7.37.

Reagents

DIDS (Pierce Chemical Co., Rockford, IL) was dissolved into distilled water as a 10^{-2} M stock and frozen at 20°C. Amiloride (Sigma Chemical Co.) was made the day of use as a 10–2-M stock in TMACl, Hepes-buffered solution. BCECF-AM (Molecular Probes, Junction City, OR) 50 μg dissolved fresh each day in 20 μl DMSO then pipetted into control buffer to achieve a concentration of 2 μM. All other chemicals and reagents were purchased from Sigma Chemical Co. (St. Louis, MO).

Statistics and calculations

All results are presented as the transformation of the 495/440 ratio to cell pH as discussed above. Fit slopes for the calibration were performed by linear regression. Results are presented as mean±SE. Within a single group comparison between two periods was by paired Student's *t* test. When comparisons between multiple groups was performed, one-way ANOVA was used. For ion concentration dependence studies examining the rate of change of cell pH, the initial rate over the first 20 s was used. The curve drawn through these data points was plotted using nonlinear regression analysis (Graphpad).

Results

Resting cell pH in the OMCDi. Resting cell pHi measurements were taken in a total of 36 tubules. In pH 7.37 HCO_3^-/CO_2-buffered solutions, resting pHi was 7.15±0.04 (n = 14). In pH 7.37 Hepes-buffered (HCO_3^-/CO_2 free) solutions resting cell pHi was 7.19±0.05 (n = 22, $P > 0.25$ vs. HCO_3^-/CO_2 solutions).

OMCDi cell alkalinization by peritubular Cl^- removal. In nine experiments, peritubular Cl^- was completely replaced with gluconate while maintaining the peritubular pH (7.37), CO_2 (40), and HCO_3^- (25 mM) constant. Cl^- removal reversibly alkalinized the OMCDi cell by 0.39±0.09 pH units from a

resting pHi of 7.22±0.06 to 7.57±0.12 ($P < 0.025$) with recovery to 7.23±0.07 after return to control Cl^--containing solutions (see Fig. 3). The alkalinization was rapid (0.50±0.07 pH U/min). Plateau values determined 90 s after a change in bath chloride concentration.

OMCDi cell alkalinization by peritubular DIDS. 0.1 mM DIDS added to the bath resulted in reproducible intracellular alkalinization with a mean rate of 0.07±0.02 pH U/min from a mean resting pHi of 7.14±0.09 to 7.34±0.09 after DIDS ($n = 3$, $P < 0.05$) (Fig. 4). After treatment with DIDS, replacement of peritubular Cl^- with gluconate failed to significantly alkalinize the OMCDi +DIDS/+Cl^- = 7.29±0.10 to post-DIDS/−Cl^- = 7.31±0.10. This is in contrast to the effect of Cl^- replacement in the absence of DIDS: ΔpHi −DIDS/±Cl^- = 0.36±0.06 vs. ΔpHi +DIDS/±Cl^- = 0.02±0.46; $P < 0.025$.

Dependence on [Cl^-] of OMCDi pHi recovery rate after cell alkalinization. The effect of increasing Cl^- concentrations on cell pH recovery from high HCO_3^- and Cl^- removal was examined. The results from a typical experiment are shown in Fig. 5. The mean rates of recovery for seven tubules are plotted in Fig. 6. These results demonstrate a half-maximal rate of recovery is achieved by roughly 10.0±4.1 mM peritubular Cl^-, yielding a maximal rate of pHi recovery from this maneuver of 0.50±0.06 pH U per minute.

OMCDi acidification by peritubular Na^+ removal. To examine the potential role of Na^+/H^+ exchange in regulation of cell pH in the OMCDi peritubular Na^+ was replaced on a molar basis by TMA^+. These experiments were performed in Hepes-buffered solutions (HCO_3^-/CO_2 free) to minimize the contribution of HCO_3^- linked transport processes to cell pH regulation. In three separate experiments (Fig. 7), the pHi in the OMCDi was reversibly acidified by Na^+ removal. Intracellular pH fell from 7.14±0.09 to 6.85±0.10 after Na^+ removal ($P < 0.025$). Return to Na^+-containing bath resulted in partial recovery to 6.96±0.09.

We further documented that this process was independent from Cl^-/base exchange (DIDS inhibitable) by showing replacement of Na by TMA resulted in cell acidification even in tubules previously treated with DIDS. In DIDS-treated tubules resting pH was 7.48±0.26 and fell to 7.11±0.23 after complete replacement of Na^+ with TMA ($P < 0.05$). Upon return to control Na^+ containing conditions, cell pH alkalinized to pH 7.24±0.28.

Inhibition of cell pH recovery from an acid load by peritubular Na^+ removal. Two different experimental protocols were performed. In the initial series of experiments tubules were exposed to a NaCl free, NH_4Cl pulse (20 mM) (NaCl replaced by TMACl). After the NH_4Cl was washed out, the initial rate of pHi recovery was determined and then the tubule was exposed to progressively increasing concentrations of Na (5, 25, 50, and 125 mM NaCl). The tubule was returned to a Na free solution after each exposure to NaCl containing solutions, to provide a clear demarcation between the change in recovery rate seen at each different Na concentration (Fig. 8). In any given tubule, the rate of cell pH recovery for each Na concentration was normalized to the mean of the maximal rates for this experimental group ($n = 6$).

These results were fit by nonlinear regression analysis to a rectangular hyperbolic curve (Fig. 9). It can be seen from these results that there is a steep increase in the rate of recovery from an acid load as peritubular NaCl concentrations were increased from 5 to 25 mM. Above 25 mM bath Na the rate of recovery increased at a much slower rate. The apparent V_{max} for the Na-dependent process is 0.605±.02 pH U/min with a K_m for Na of 6.93±1.2 mM.

In a separate group of experiments two sequential NH_4Cl pulses and washouts were performed: first with, and then without peritubular Na^+. Complete NaCl replacement was achieved using NMDG-Cl in this protocol. The results of a typical experiment are shown in Fig. 10. The nadir pH after

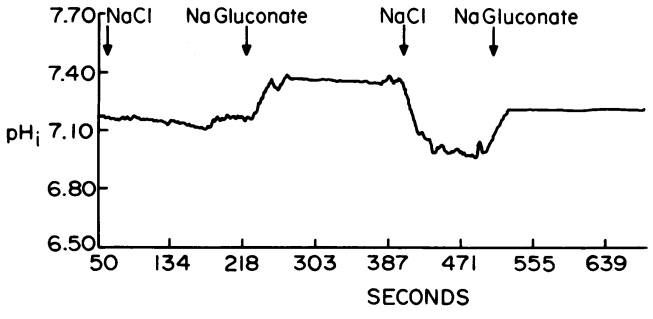

Figure 3. Effect of bath Cl^- replacement on cell pH. Studies were performed in the presence of a HCO_3^-/CO_2 buffer. Cl^- was replaced with gluconate.

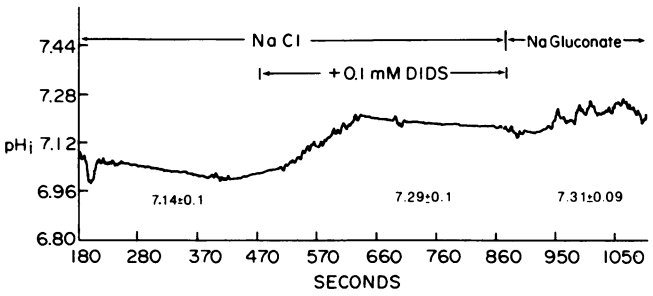

Figure 4. Effect of bath DIDS on cell pH. In the last part of this tracing bath Cl^- was removed after DIDS pretreatment. In contrast to the results depicted in Fig. 3, no significant change in cell pH was observed.

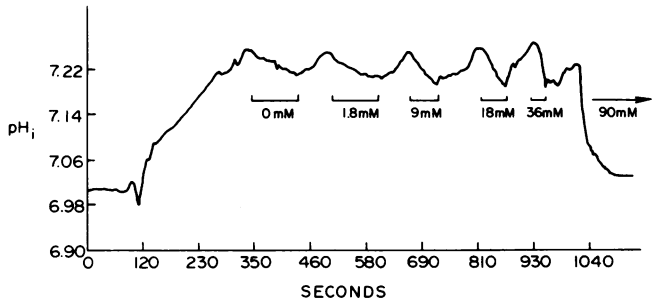

Figure 5. Representative tracing of protocol measuring the Cl^- concentration dependence of the basolateral Cl^-/base exchanger. OMCDi were perfused in the setting of HCO_3^-/CO_2. At 120 s the bath was changed to one that was Cl^- free, pH 7.70, and 50 mM HCO_3^-. After a steady state alkaline pH was achieved, the 0 Cl^-, high HCO_3^- buffer was sequentially alternated with a series of 25 mM HCO_3^- (pH 7.40) buffers, having progressively higher chloride concentrations (0, 1.8, 9, 18, 36, and 90 mM). After a reliable slope was determined the bath was changed back to the high HCO_3^- buffer.

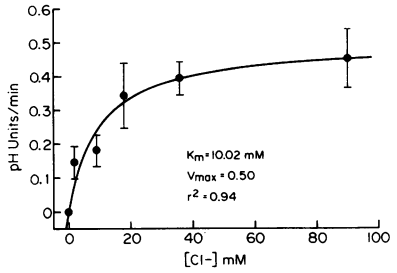

Figure 6. Saturation kinetics of basolateral Cl^-/base exchange in OMCDi. Mean rates of change in cell pH at varying basolateral Cl^- concentrations are plotted. These values were taken from seven experiments, done according to the protocol depicted in Fig. 5. Results were fit to a rectangular hyperbola as discussed in Methods.

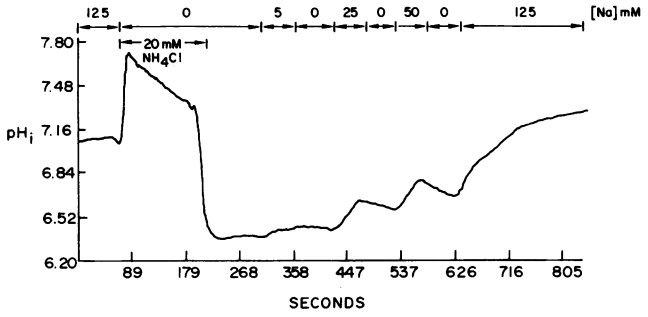

Figure 8. Recovery of cell pH after an acid load is dependent on bath Na^+ concentration. After equilibration in HCO_3^- free, NaCl bath, OMCDi's were exposed to 20 mM NH_4Cl in Na free bath for 120 seconds. Washout of NH_4Cl, with Na-free bath resulted in a brisk acidification. The initial recovery of cell pH in the absence of bath Na^+ was determined, then the Na^+-free bath was alternated with baths of progressively increasing Na^+ concentration (5, 10, 25, 50, and 125 mM). Rates of recovery in the first 20 s were determined.

NH_4Cl washout was the same in the presence and absence of peritubular NaCl (+Na: 6.71±0.11 vs. −Na: 6.68±0.18, P = NS). After the pHi nadir was reached the initial rate of recovery (first 20 s) was determined. In each case the initial rate of recovery of cell pH was greater in the presence of peritubular NaCl than in its absence (+Na 0.96±0.25; −Na 0.23±0.05, $P < 0.01$). These effects of Na removal were reversible as can be seen in Fig. 10 where readdition of peritubular NaCl to the acid loaded tubule, resulted in a markedly enhanced recovery of OMCDi cell pH.

Amiloride blockade of Na^+-dependent OMCDi cell pH recovery from an acid load. In four experiments the Na^+ dependent recovery from cell acidification (NH_4Cl washout) was blocked by 0.2 mM bath amiloride (Fig. 11). The rate of cell pH recovery with 50 mM peritubular Na was 0.036±0.144 pH U/min in the presence of amiloride was vs. 0.22±0.05 pH U/min after amiloride was washed out ($P < 0.01$). These results show that amiloride blocks Na-dependent pH recovery from an acid load, and this blockade is reversible.

Discussion

The OMCDi is a major site of urinary acidification. Acidification in this segment is driven by an apical cell membrane electrogenic H^+ translocating ATPase (7, 9, 20). Base exit, along the basolateral cell membrane, maintains intracellular pH, balancing apical membrane H^+ transported into the urine (5, 7, 21). The present studies address the mechanism by which intracellular base, exits across the basolateral cell membrane. In addition, these studies examine Na^+-dependent mechanisms by which intracellular pH in the OMCDi is regulated.

These studies provide direct functional evidence for the existance of a Cl^- dependent antiluminal base exit mechanism. Removal of peritubular Cl^- results in reversible cell alkalinization. The observation that DIDS also produces cell alkalinization argues that base accumulation due to ongoing H^+ secretion is a major mechanism for cell alkalinization consequent to peritubular Cl^- removal (in addition to possible reversal of the Cl^-/HCO_3^- exchanger). Exposure of the OMCDi to peritubular DIDS resulted in gradual cell alkalinization and prevented further alkalinization when Cl^- was subsequently removed from the peritubular environment. These results suggest that DIDS affects the same pathway for base exit as affected by Cl^- removal. The recent demonstration of band 3-like immunoreactivity on the basolateral membrane in the OMCDi (10, 11) is consonant with the existence of a DIDS sensitive, Cl^-/base exchanger at this site. Previous functional studies demonstrate that net acidification in the OMCDi is blocked by either peritubular application of the disulfonic stilbene derivative SITS, or the removal of peritubular Cl^- (5, 7). While inhibition of acidification by peritubular Cl^- removal could be due to direct inhibitory effects of Cl^- depletion on the H^+ ATPase (22, 23), this effect is not compatible with the cell alkalinization produced by Cl^- removal or the inhibition of urinary acidification by peritubular SITS. Recent studies in the isolated perfused OMCDi with 6-carboxyfluorescein-

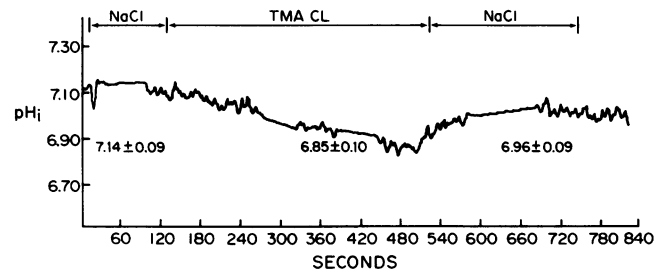

Figure 7. Effect of basolateral Na removal on cell pH. OMCDi were bathed in Hepes-buffered HCO_3^-/CO_2 free solutions. Na was replaced with TMA keeping bath pH constant.

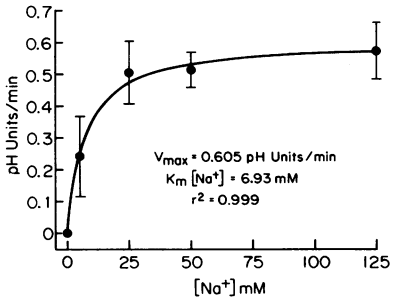

Figure 9. Saturation kinetics of basolateral Na^+/H^+ exchange in OMCDi. Mean rates of change in cell at varying bath Na^+ concentrations are plotted. These values were determined from 6 experiments done according to the protocol depicted in Fig. 8. Results were fit to a rectangular hyperbola according as discussed in Methods.

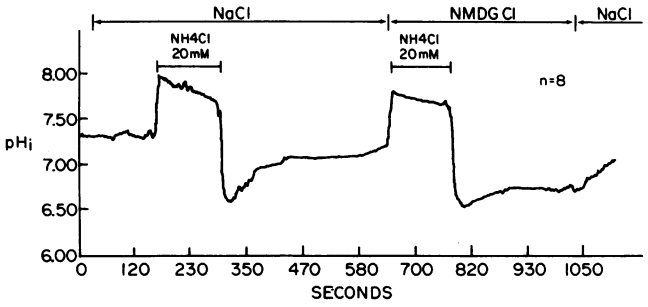

Figure 10. *N*-methyl-D-glucamine replacement of Na^+ also reveals Na^+ dependence of cell pH recovery after an acid load. In these studies bath Na^+ was replaced with *N*-methyl-D-glucamine to rule out an independent cation effect of TMA. Cell pH recovery from NH_4Cl washout was first measured in the presence and then the absence of Na^+ in the same tubules.

loaded tubules also document cell alkalinization after application of peritular DIDS or bath chloride removal (24). It is conceivable that inhibition of net acidification in this setting is a direct consequence of cell alkalinization (25).

In addition to providing evidence for Cl^--dependent base exchange in the basolateral membrane, we characterized the kinetic parameters of this exchanger. We examined the dependence of cell pH recovery (after alkalinization) on external Cl^- concentration. Half-maximal Cl^- dependent recovery rates ($K_{1/2}$) occurred at a peritubular Cl^- concentration of ~ 10 mM with a V_{max} of 0.50 pH U/min. Similar studies on suspensions of rabbit OMCDi cells yielded a $K_{1/2}$ of roughly 30 mM and a V_{max} of 0.118 pH U/min (26). There are several potential reasons for these modest discrepancies. In these earlier studies, the cells were introduced into a CO_2/HCO_3^- free medium with varying concentrations of ambient Cl^-. The present studies were performed in the presence of CO_2/HCO_3^- throughout. The effects of these methodologic differences are difficult to predict. It is also conceivable that the measured time points for pH recovery rate in the previous studies do not reflect true initial rates, hence accounting for the modest discrepancy in $K_{1/2}$ and the lower V_{max}. In the present studies we examined the drop in pH for the first 20 s after chloride replacement, in the previous study the earliest time point examined was 30 s.

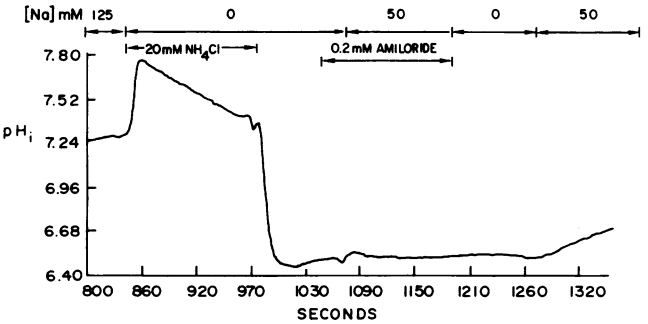

Figure 11. Bath amiloride blocks Na^+ dependent recovery of cell pH. OMCDi were exposed to NH_4Cl washout in Na^+ free bath. 200 μM amiloride was then added to the bath flowed by 50 mM Na. The amiloride and Na^+ were then washout out and the tubule was re-exposed to 50 mM Na in the absence of amiloride. The rate of cell pH recovery in the presence and absence of amiloride were compared.

Alternatively the averaged rate of correction for a large sample of cells, harvested in a different manner than dissection of single tubules, might respond in a different fashion than the small sample of cells from which our rate was determined. This is especially true if all cells in the cell suspension are not transporting at equivalent rates.

Studies of the red cell band 3 anion exchanger that have examined the Cl^- affinity of the external anion binding site, suggest a $K_{1/2}$ of roughly 3 mM (26). However these studies also demonstrate that this binding affinity is highly dependent on the experimental conditions especially regarding the internal Cl^- concentration, as well as temperature and external pH (27, 28). Nevertheless, in other studies examining Cl^-/HCO_3^- exchange in nucleated mammalian cells, similar $K_{1/2}$ values for external Cl^- concentration were obtained. In pulmonary alveolar cells Nord et al. (29) found an external Cl^- $K_{1/2}$ of 11 mM. In MDCK cells an external Cl^- $K_{1/2}$ was obtained of 6 mM (30) and in Vero cells the external $K_{1/2}$ for Cl^- was 7 mM (31). These values are in close agreement with those obtained in the present studies. Of course the $K_{1/2}$ obtained in the present studies pertains only to the effect of Cl^- on pH recovery rates from cell alkalinization and cannot be extrapolated to predict the basolateral Cl^- concentration dependency of net acidification in the OMCDi.

The present studies did not address whether Cl^-/base exchange was Na^+ dependent. In invertebrates and some mammalian cells, a Na^+-dependent, Cl^-/HCO_3^- exchanger has been found to play a role in the defense of cell pH from an acid load (15). The inwardly directed Na^+ gradient provides the energy for HCO_3^- entry in exchange for Cl^-. This exchanger has an optimal efficacy when intracellular pH is acidic (15, 32). This process is inhibited by DIDS but not blocked by amiloride. In contrast, Na^+ independent Cl^-/HCO_3^- exchange exhibits an pH optimum in the alkaline range (15, 32). Thus Na^+-dependent Cl^-/HCO_3^- exchange is more effective in defense of cell pH against acid loads, while Cl^-/HCO_3^- exchange is more effective in defense of cell pH against alkaline loads.

Although we did not show directly that the Cl^-/base exchanger in the OMCDi was Na^+ independent, these studies did demonstrate the Na^+-dependent regulation of intracellular pH in the OMCDi is separate from the DIDS sensitive Cl^--dependent regulatory step. Thus while DIDS failed to block cell acidification after Na^+ removal, DIDS completely blocked cell alkalinization due to chloride removal. In addition, Na^+ dependent recovery of cell pH from acidification occurred in the absence of extracellular HCO_3^-/CO_2. Thus regulation of cell pH after acidification could not have resulted from Na^+ dependent HCO_3^-/Cl^- exchange since this would require extracellular HCO_3^- to support Na^+/HCO_3^- symport into the cell. Further studies will have to be performed to characterize the ionic requirements of the Cl^-/base exchange mechanism.

In contrast to Cl^-/HCO_3^- exchange, Na^+/H^+ exchange is highly efficient in acid intracellular conditions (15, 33). We specifically examined if the OMCDi possess a Na^+/H^+ exchanger as a defense mechanism against cell acidification. We observed that the rate of cell pH recovery after NH_4Cl washout, was highly dependent on basolateral Na^+ concentration and could be blocked by amiloride. The Na^+ concentration dependence of pH recovery was saturable with a half-maximal recovery rate ($K_{1/2}$) estimated to be achieved at roughly 7 mM peritubular Na^+. $K_{1/2}$ values for external Na concentration ranging from 3 to 50 mM have been obtained in numerous cell

types examining Na^+/H^+ exchange (34). The $K_{1/2}$ for Na^+ in the present studies is in good agreement with the $K_{1/2}$ of of 6.3 mM and 13.3 mM for Na^+/H^+ exchange obtained in brush border membrane vesicles (35, 36), but is somewhat lower than that determined in the rabbit cortical collecting tubule (37) or the late S_3 segment of the rabbit proximal tubule, ~ 28 mM (38). However the present data are consistent with the general observation that the $K_{1/2}$ of Na^+/H^+ exchange is achieved at external Na^+ concentrations well below physiologic concentrations of Na^+.

Studies in the isolated perfused rabbit cortical collecting duct have also suggested the existence of basolateral Na^+/H^+ exchange in this segment (37). Additionally, numerous studies demonstrate that Na^+/H^+ exchange exists in cultured inner medullary collecting duct cells (33, 39). However, work performed on fresh suspensions of cells from the OMCDi failed to demonstrate Na^+/H^+ exchange (40). In those studies suspensions of OMCDi cells were separated from rabbit kidney by density gradient centrifugation. Neither replacement of Na^+ with K^+, 0.5 mM ouabain, or 0.5 mM amiloride inhibited pHi recovery rate after an acetate load. Intracellular pH recovery rates in those studies were ~ 0.177 pH U/min under in the presence of Na^+. It should be noted that this rate is markedly lower than the rate of recovery of cell pHi after an acid load in the present studies (0.177 vs. 0.61 to 0.93 pH U/min, depending on the protocol used). The recovery rates in those earlier studies (40), are comparable to those obtained in the present studies in the absence of Na^+ (0.23 pH U/min). Thus it is conceivable that the basal Na^+/H^+ exchange activity was inhibited in the former studies by some step in the isolation procedure.

The marked stimulation by Na^+ of pHi recovery after an acid load, reversible blockade by amiloride, and insensitivity of this process to DIDS, provides several lines of evidence favoring the existence a basolateral Na^+/H^+ exchanger in the OMCDi. Indeed, given the ubiquitous nature of Na^+/H^+ exchange (14, 15, 35), it would be surprising to find a normal cell lacking this exchange process. Nevertheless, since the OMCDi does not participate in net Na^+ absorption, the role of the Na^+/H^+ exchanger in this segment remains a matter of speculation. Given that the OMCDi is located in a hypertonic portion of the medulla and is subject to rapid osmotic shifts dependent on the water balance of the animal, we would speculate that one role of the Na^+/H^+ exchanger is to participate in volume regulation. Parallel activation of Cl^-/base exchange and Na^+/H^+ exchange would allow net NaCl influx or efflux as has been described for other cells (41, 42). Other roles for the Na^+/H^+ exchanger in regulation of net proton secretion or signal transduction may also be important (15, 43).

At present, we cannot be certain if the two basolateral exchange mechanisms, Na^+/H^+ exchange and Cl^-/base exchange, reside in the same cell or separately expressed in neighboring cells. Studies suggest cellular heterogeneity in the collecting duct in several mammalian species. There are, however, significant differences between the degree of heterogeneity in cortical versus medullary segments (9, 10, 44, 45) as well as species differences (rat shows much more distinctive cellular heterogeneity than the rabbit [12, 21]). In the rabbit cortical collecting duct there is good evidence for three types of cells (21, 44, 46): The principal cell appears to be primarily involved in water, Na^+ and K^+ transport; the alpha type intercalated cell that appears to mediate unidirectional H^+ secretion; and the β-intercalated cell mediating HCO_3^- secretion. In contrast the heterogeneity of the rabbit OMCDi is much less striking (12, 21).

Functionally, OMCDi specializes in acidification and does not participate in active, net Na^+, or K^+ transport, or show evidence for HCO_3^- secretion (3, 47, 48). Ridderstrale et al. examined carbonic anhydrase histochemistry, Na/K ATPase distribution, and distribution of rod-shaped intramembranous particles, and were unable to make any qualitative distinctions between cell types in the rabbit OMCDi (12). Furthermore, the electrophysiological characteristics of the rabbit OMCDi cells display a monotonous homogeneity when examined by microelectrode impalements, in contrast to the two cell types observed in the outer stripe of the OMCD (8). These observations argue for the existence of one cell type in the OMCDi.

In contrast, Lefurgey and Tisher reported roughly 20% of the rabbit OMCDi cells appeared to be intercalated cells by light, and electron microscopy (44). Schuster et al. (10) found 50% of cells in the OMCDi stained for the mitochondrial marker succinate dehydrogenase and basolateral band 3, while Burnatowska-Hledin and Spielman (49) found only 17% of cells to stain similarly. These latter results quantitatively agree with those of Satlin and Schwartz (50). In summary it may be, as suggested by Ridderstrale et al., that the distinctions between the cell types in the rabbit OMCDi are quantitative ones, possibly due to technique sensitivity and relative density of various transporters etc., but not true qualitative differences between cell types.

We would speculate that all cells in this segment are likely to have parallel basolateral Na^+/H^+ exchange and Cl^-/base exchange. In the present studies we could not discriminate between cell types because all cells loaded equally with BCECF. In each experiment we took measurements from roughly 30 cells (using a slit of 15 μm long by 40 μm wide and assuming a cell length of 8 μm and that light was gathered from the whole circumference of the tubule). In every experiment we were able to demonstrate the respective effects of Na^+ replacement or Cl^- removal. The fact that Cl^- removal resulted in an apparent steady state alkalinization without recovery does not argue against the existence of a parallel basolateral Na^+/H^+ exchanger in the same cell, since as mentioned, the functional pH optimums for these transporters are at opposite ends of the spectrum for cell pH. Thus Na^+/H^+ exchange activity, predictably, would be inhibited in the alkaline range. The same is true for Cl^-/base exchange in the acid range (15, 32, 33).

If all cells participate in H^+ secretion in the OMCDi then one might predict that Na^+ removal, and cell acidification should have significant effects on net H^+ secretory rates. However an effect of Na^+ removal on acidification rates in OMCDi has not as yet been observed (4). This apparent inconsistency however presumes that acidifying the cell, would measurably increase acidification rates. There is good evidence this may not be the case. Alkalinizing the cell may have much more impressive effects on H^+ secretion rates than acidifying the cell. Cohen and Steinmetz (51) found in turtle bladder that maneuvers which lowered cell pH below the resting value of 7.28, resulted in less of an increase in acidification rates than the inhibition obtained when cell pH was increased to a comparable extent. This resulted in a hyperbolic relationship be-

tween acidification rates and cell pH such that in as cell pH fell below the resting level the increase in acidification tended to flatten out. From these results one might predict that a maneuver which acidifies the OMCDi cell might not cause a brisk increase in acidification. We would also point out that the previous studies (4) in which HCO_3^- absorption in the OMCDi was measured after Na^+ replacement were not performed in a paired fashion. These studies were designed to determine whether Na^+ removal abolished OMCDi acidification, so modest increases in HCO_3^- absorptive rates might have been missed. Thus we do not believe there is any functional evidence against the existence of basolateral Na^+/H^+ exchange in all cells of the OMCDi. In contrast histologic studies demonstrate heterogeneity of basolateral band 3 staining. As discussed above, however, one cannot be certain from these studies that antigen density is heterogeneous as opposed to true functional heterogeneity. Only by measuring single cell pH in the isolated perfused OMCDi can this issue be addressed adequately.

In conclusion these studies demonstrate the presence of basolateral Na^+/H^+ exchange and Cl^-/base exchange in the OMCDi. As might be predicted the Na^+-dependent exchange process was blocked by amiloride and the Cl^- dependent exchange process was blocked by DIDS. The $K_{1/2}$ of these exchange processes for external Na^+ and Cl^-, respectively, is well below the physiologic concentrations of these ions in blood. Thus pH gradient from blood-to-cell, or cell Na^+ and Cl^- concentrations may provide a more significant stimulus to these exchange process than do blood Na^+ and Cl^- concentration. It remains to be determined if these exchangers are present in all, or only some cells in the OMCDi.

Acknowledgments

The authors would like to thank Ms. Deidrienne Fredin and Melinda Mosely for their skillful technical assistance.

Dr. Breyer is the recipient of a Veterans Administration Career development award. This study was supported by National Institutes of Health grant AM-37097.

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