# Basolateral Membrane $Na^+$-independent $Cl^-/HCO_3^-$ Exchange in the Inner Stripe of the Rabbit Outer Medullary Collecting Tubule

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ABSTRACT The inner stripe of the outer medullary collecting tubule is a major distal nephron segment in urinary acidification. To examine the mechanism of basolateral membrane $H^+/OH^-/HCO_3^-$ transport in this segment, cell pH was measured microfluorometrically in the inner stripe of the rabbit outer medullary collecting tubule perfused in vitro using the pH-sensitive fluorescent dye, (2',7')-bis(carboxyethyl)-(5,6)-carboxyfluorescein. Decreasing peritubular pH from 7.4 to 6.8 (changing $[HCO_3^-]$ from 25 to 5 mM) caused a cell acidification of 0.25 ± 0.02 pH units, while a similar luminal change resulted in a smaller cell acidification of only 0.04 ± 0.01 pH units. Total replacement of peritubular $Cl^-$ with gluconate caused cell pH to increase by 0.18 ± 0.04 pH units, an effect inhibited by 100 μM peritubular DIDS and independent of $Na^+$. Direct coupling between $Cl^-$ and base was suggested by the continued presence of peritubular $Cl^-$ removal-induced cell alkalinization under the condition of a cell voltage clamp ($K^+$-valinomycin). In addition, 90% of basolateral membrane $H^+/OH^-/HCO_3^-$ permeability was inhibited by complete removal of luminal and peritubular $Cl^-$. Peritubular $Cl^-$-induced cell pH changes were inhibited two-thirds by removal of exogenous $CO_2/HCO_3^-$ from the system. The apparent $K_m$ for peritubular $Cl^-$ determined in the presence of 25 mM luminal and peritubular $[HCO_3^-]$ was 113.5 ± 14.8 mM. These results demonstrate that the basolateral membrane of the inner stripe of the outer medullary collecting tubule possesses a stilbene-sensitive $Cl^-/HCO_3^-$ exchanger which mediates 90% of basolateral membrane $H^+/OH^-/HCO_3^-$ permeability and may be regulated by physiologic $Cl^-$ concentrations.

## INTRODUCTION

The inner stripe of the outer medullary collecting tubule ($OM_iCT$) is a high capacity distal segment for proton secretion [$HCO_3^-$ absorption] (Lombard et al., 1983; Atkins and Burg, 1985) and is thought to be a key segment in the final acidification of tubular fluid by the kidney. There is no evidence for $HCO_3^-$ secretion (Lombard

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TABLE I

*Compositions of Solutions*

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Na | 145 | 145 | 145 | 25 | 25 | 145 | 0 | 0 | 135 | 135 | 135 | 135 | 145 | 145 | 145 |
| K | 5 | 5 | 5 | 125 | 125 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 |
| $Mg^{2+}$ | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| $Ca^{++}$ | 1.6 | 1.6 | 7.0 | 1.6 | 7.0 | 7.0 | 1.6 | 7.0 | 7.0 | 2.0 | 7.0 | 1.6 | 6.8 | 5.8 | 4.3 |
| Choline | | | | | | | 25 | 25 | | | | | | | |
| *N*-methyl-D-glucosamine | | | | | | | 120 | 120 | | | | | | | |
| $Cl^-$ | 123.2 | 143.2 | 0 | 123.2 | 0 | 0 | 123.2 | 0 | 0 | 104 | 0 | 103.2 | 10 | 20 | 40 |
| $HCO_3^-$ | 25 | 5 | 25 | 25 | 25 | 5 | 25 | 25 | 0 | 0 | 25 | 25 | 25 | 25 | 25 |
| $HPO_4^-$ | 2.5 | 2.5 | 2.5 | 2.5 | 2.5 | 2.5 | 2.5 | 2.5 | 2.5 | 2.5 | 2.5 | 2.5 | 2.5 | 2.5 | 2.5 |
| $SO_4^-$ | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| Gluconate$^-$ | | | 134 | | 134 | 154 | | 134 | 139 | 25 | 114 | 0 | 123.6 | 111.6 | 88.6 |
| Glucose | 8.3 | 8.3 | 8.3 | 8.3 | 8.3 | 8.3 | 8.3 | 8.3 | 8.3 | 8.3 | 8.3 | 8.3 | 8.3 | 8.3 | 8.3 |
| Alanine | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 |
| HEPES | | | | | | | | | 25 | 25 | 25 | 25 | | | |

All units are millimolar.

et al., 1983; Atkins and Burg, 1985) or active $Na^+$ transport in this segment (Stokes, 1982).

Proton secretion across the apical membrane is thought to be effected by a $H^+$-ATPase (Gluck and Al-Awqati, 1984; Stone et al., 1984; Zeidel et al., 1986*a*; Silva et al., 1987; Brown et al., 1988). Current evidence suggests that the alkali equivalents generated within the cell by apical membrane proton secretion exit the cell across the basolateral membrane in exchange for $Cl^-$. This is based on studies that found that transepithelial $HCO_3^-$ absorption was dependent on $Cl^-$ and was abolished by peritubular addition of 4-acetamido-4′-isothiocyano-2,2′disulfonic stilbene (SITS) (Stone et al., 1983), an inhibitor of the red blood cell $Cl^-/HCO_3^-$ exchanger. Additional evidence suggesting the existence of basolateral membrane $Cl^-/HCO_3^-$ exchange is the labeling of the basolateral membrane of $OM_iCT$ cells by monoclonal and polyclonal antibodies raised against both the cytoplasmic and membrane domains of the erythrocyte band 3 anion exchange protein (Schuster et al., 1986; Wagner et al., 1987; Verlander et al., 1988).

The purpose of this study was to examine whether a functional $Cl^-/HCO_3^-$ exchanger was present on the basolateral membrane of the rabbit $OM_iCT$ using the measurement of cell pH ($pH_i$). $pH_i$ was measured using the pH-sensitive dye, (2′,7′)-bis-(carboxyethyl)-(5,6)-carboxyfluorescein (BCECF). The results demonstrate that the basolateral membrane possesses a stilbene-sensitive $Na^+$-independent $Cl^-/HCO_3^-$ exchanger which is responsible for 90% of the basolateral membrane $H^+/OH^-/HCO_3^-$ permeability. Under physiologic conditions, this transporter is regulated by changes in peritubular $Cl^-$ concentration within the physiologic range.

## METHODS

The technique of in vitro microperfusion of isolated rabbit $OM_iCT$ was used as previously described (Hays et al., 1986). Briefly, female New Zealand White rabbits weighing 1.5–2.0 kg were maintained on standard laboratory chow and tap water ad lib. Animals were decapitated and the left kidney was rapidly removed, decapsulated, and sliced into 1-mm coronal slices. Slices were placed in an oxygenated bathing solution at 4°C (pH 7.4, solution 1, Table I). $OM_iCT$ segments were identified and dissected free as previously described (Hays et al., 1986). To avoid the outer stripe, perfused segments were dissected from the inner half of the inner stripe. Tubules were transferred into a bath chamber with a volume of ~90 μl, constructed of black lucite to minimize light reflection. The peritubular fluid was continuously exchanged at ~10 ml/min by hydrostatic pressure. With this setup, a complete fluid exchange occurs within 1 s. Tubular lumens were perfused at flow rates of 50–100 nl/min. Bath pH was monitored continuously by placing a commercial flexible pH electrode into the bath (MI-5089; Microelectrodes, Inc., Londonberry, NH). Bath solutions were prewarmed at 37°C, continuously equilibrated with 95% $O_2$/5% $CO_2$, and passed to the bath chamber through $CO_2$-impermeable tubing (Clarkson Controls and Equipment Co., Detroit, MI). Bath temperature of 37 ± 0.3°C was maintained by a specially designed water-jacketed glass coiled tubing placed in line just before the bath chamber.

To minimize motion, the distal end of tubule was sucked gently into a collection pipette. In addition, the average length of the tubule exposed to the bath fluid was limited to ~250–500 μm. The tubules were loaded with the acetoxymethyl derivative of BCECF (BCECF-AM, Molecular Probes, Eugene, OR), 10 μM, from the bath. The loading solution was similar to solution 1 (Table I) except that it was titrated to pH 7.20 by HCl addition to aid in tubule

loading. Loading was continued until signal to background fluorescence at the 450 nm excitation wavelength was ≥ 20:1, usually requiring 10–15 min. Tubules were then washed with solution 1, (Table I) at pH 7.4 for a minimum of 10 min followed by the control solution of each experiment for at least an additional 5–10 min.

Luminal and peritubular solutions used in this set of studies are listed in Table I. Added calcium was increased in $Cl^-$-free solutions to maintain ionized [$Ca^{++}$] similar in all solutions (Alpern and Chambers, 1987). $CO_2/HCO_3^-$-free solutions were bubbled with 100% oxygen passed through a 3 N KOH trap. With these precautions, bath total $CO_2$ is undetectable (Krapf et al., 1987*a*). 4,4′-diisothiocyano-stilbene-2,2′-disulfonate (DIDS), nigericin, valinomycin, and all solution salts were purchased from Sigma Chemical Co. (St. Louis, MO).

### *Cell pH Measurement*

BCECF has peak excitation at 504 nm that is pH sensitive and an isosbestic point at which fluorescence excitation is independent of pH at 436 nm; peak emission is at 526 nm (Rink et al., 1982; Moolenaar et al., 1983; Alpern, 1985). Epifluorescence was measured in these studies alternately at 500 and 450 nm excitation with fluorescent emission measured at 530 nm as previously described (Alpern, 1985). The ratio of fluorescence with excitation at 500 and 450 nm is independent of dye concentration and optical pathway, and is an index of $pH_i$.

Fluorescent emission was measured with an inverted epifluorescent microscope (Nikon Diaphot, Nikon Inc., Garden City, NY) attached to a dual excitation microspectrofluorimeter which allows rapid alternation between two excitation wavelengths (SPEX CM-1; Spex Industries, Edison, NJ). Fluorescence was measured using a 20× objective, on an area of the tubule ~150 μm in length and including the entire width of the tubule. Generally, the measured segment started 50–100 μm from the perfusion pipette. No attempts were made to measure fluorescence from single cells, the implications of which are addressed in the Discussion. Background fluorescence at each of the excitation wavelengths was measured on the tubule, before loading with BCECF, and the results were subtracted from the measured fluorescence during the experiment. A fluorescent ratio was then calculated as the ratio of fluorescence with 500 nm excitation divided by that with 450 nm excitation. The initial rate of change in the fluorescence excitation ratio was defined by the slope of a line drawn tanget to the initial deflection [$d(F500/F450)/dt$].

### *Buffer Capacity*

The buffer capacity was determined using the technique of rapid $CO_2$ addition as described by Roos and Boron (1981). Tubules were perfused at pH 7.4 in $Cl^-$-free, $CO_2/HCO_3^-$-free, HEPES-buffered solutions (solution 9, Table I). Luminal and peritubular solutions then were rapidly changed to similar solutions containing 40 mmHg $P_{CO_2}$ and 25 mM $HCO_3^-$ (solution 11, Table I). In an additional set of studies, buffer capacity was measured in the presence of 2 mM cyanide to prevent contributions from active transport mechanisms to the measured buffer capacity. This measurement in the presence of cyanide was felt to be a more accurate estimate of the cell's true buffer capacity.

After $CO_2/HCO_3^-$ addition, cells initially acidify because of $CO_2$ entry and then show a slow alkalinization that is due to $HCO_3^-$ entry into the cell and $pH_i$ defense. The buffer capacity was calculated from the initial acidification. To correct for the late alkalinization, the initial $pH_i$ change was calculated by extrapolating back to the time of the fluid exchange, as described by Roos and Boron (1981). Since one $HCO_3^-$ is formed for each $H^+$ released, the amount of acid added to the cell is given by $\Delta[HCO_3^-]_i$, the intracellular [$HCO_3^-$] at the peak of the cell acidification. The non-$CO_2/HCO_3^-$ buffer capacity, $\beta_{non\text{-}CO_2/HCO_3}$ (mmol·liter$^{-1}$·pH

unit$^{-1}$), is given by the formula:

$$\beta_{\text{non-CO}_2/\text{HCO3}} = [HCO_3^-]_i/\Delta pH_i \quad (1)$$

where $\Delta pH_i$ is the measured $pH_i$ change. $[HCO_3^-]_i$ is calculated from the peak values of $pH_i$ and $PCO_2$:

$$[HCO_3^-]_i = \alpha \cdot PCO_2 \cdot 10^{(pH_i - pK)} \quad (2)$$

where $\alpha$ is the solubility of $CO_2$ in water, and a pK of 6.1 was used.

In studies performed in the absence of $CO_2/HCO_3^-$, the total buffer capacity ($\beta_T$) equals $\beta_{\text{non-CO}_2/\text{HCO3}}$. In studies performed in the presence of $CO_2/HCO_3^-$, the total buffer capacity of the cell is the sum of both the non-$CO_2/HCO_3^-$ buffer capacity plus the $CO_2/HCO_3^-$ buffer

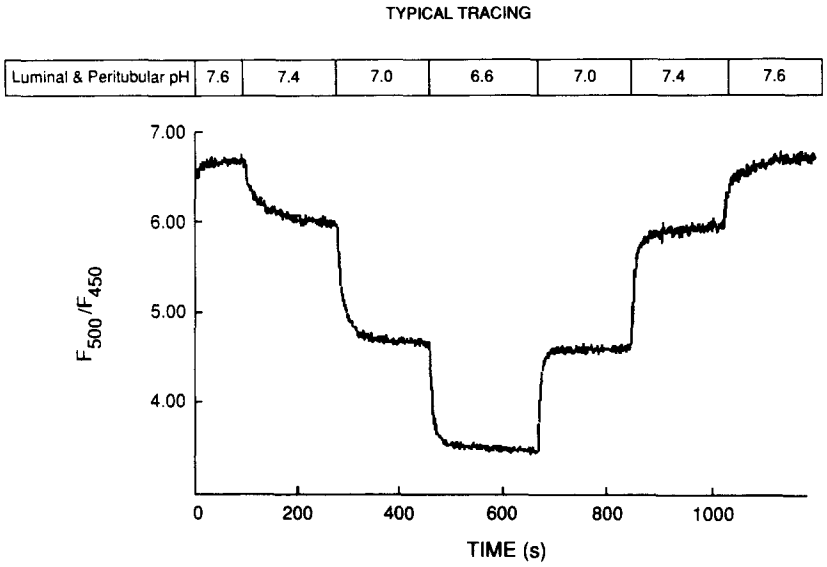


FIGURE 1. Intracellular dye calibration: typical study. The ratio of fluorescence with 500 and 450 nm fluorescence is plotted on the *y* axis.

capacity. The $CO_2/HCO_3^-$ buffer capacity of the cell, $\beta_{CO_2}$, was calculated from the formula (Roos and Boron, 1981):

$$\beta_{CO_2} = 2.3[HCO_3^-]_i \quad (3)$$

and was individually calculated for each tubule.

### *Dye Calibration*

Fluorescence excitation ratios were calibrated intracellularly using the method of Thomas et al. (1979). Tubules were bathed and perfused with well-buffered solutions (25 mM HEPES, 60 mM phosphate, and appropriate $[HCO_3^-]$) of varying pH containing 7 $\mu$M nigericin (a K/H antiporter) and 120 mM $K^+$. The tubules were loaded with BCECF before exposure to nigericin, and then were bathed and perfused with the above solutions at different pH values. Fig 1 shows a typical calibration tracing. These studies established a linear relationship

between the fluorescence excitation ratios and $pH_i$ values from pH 6.6 to 7.6 with an $r$ value of 0.999 in 12 tubules. The mean and standard errors for the fluorescent ratios at each of the pHs were: pH 7.6, 7.86 ± 0.13; pH 7.4, 6.98 ± 0.08; pH 7.0, 5.24 ± 0.06; and pH 6.6, 3.89 ± 0.08. Because of the small amount of variability between tubules, a calibration generated in 12 tubules was used to convert $F500/F450$ fluorescent ratios to pH units in all experimental studies. Rates of change of the fluorescent ratio were converted to rates of change of cell pH ($dpH_i/dt$) by dividing by the slope of the calibration curve [$d(F500/F450)/dpH_i$].

### *Calculation of Proton Fluxes*

The proton fluxes ($J_H$, $pmol \cdot mm^{-1} \cdot min^{-1}$) induced by the maneuvers in the different protocols were calculated using the formula:

$$J_H = dpH_i/dt \cdot V/mm \cdot \beta_T \quad (4)$$

where $V/mm$ is the cellular volume of the tubules per millimeter of length. For an outer

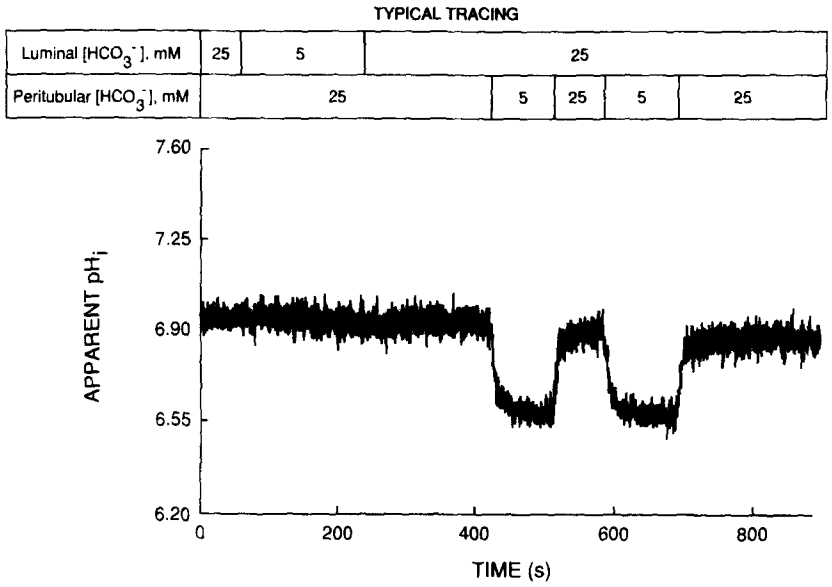


FIGURE 2. Effect of ambient pH on $pH_i$: typical study. See text for explanation.

tubular diameter of 39 μm and an inner diameter of 29 μm, $V/mm$ is $5.34 \times 10^{-10}$ liter/mm.

### *Statistics*

Results are reported as means ± standard error. The data were analyzed using the two-tailed Students $t$ test for paired data.

## RESULTS

### *Effect of Ambient pH on Cell pH*

When tubules were loaded with BCECF as described, the dye appeared evenly distributed in all cells. In preliminary studies, we attempted to load tubules from the

lumen (Weiner and Hamm, 1988), or with varying bath concentrations of BCECF-AM (2–15 $\mu$M), but in no case could we achieve apparent selective loading of individual cells. In tubules bathed and perfused with a control solution containing 25 mM $HCO_3^-$ (pH 7.4, solution 1, Table I), $pH_i$ was 7.03 ± 0.03 ($n = 44$).

The first set of studies was designed to determine the relative potency of the apical and basolateral membrane transporters in controlling $pH_i$. Tubules were initially bathed and perfused with a control solution containing 25 mM $HCO_3^-$ (pH 7.4, solution 1, Table I). During the experimental period luminal or peritubular perfusate was changed to a solution containing 5 mM $HCO_3^-$ (pH 6.8, solution 2, Table I). Fig 2 shows a typical tracing. Decreasing luminal pH and [$HCO_3^-$] caused a small but detectable $pH_i$ decrease which was reversible. When peritubular pH was then decreased, the decline in $pH_i$ was more marked. In 10 paired tubules, a change in peritubular [$HCO_3^-$] from 25 to 5 mM resulted in a significant cell acidification from 6.87 ± 0.05 to 6.59 ± 0.04 ($P < 0.001$) and a return to 6.81 ± 0.04 ($P < 0.001$) during the recovery period. A similar luminal change in these same tubules resulted in a smaller acidification from 6.94 ± 0.06 to 6.90 ± 0.06 ($P < 0.05$), which was also reversible with $pH_i$ returning to 6.94 ± 0.06 upon return to the control luminal fluid ($P < 0.05$).

The mean $pH_i$ change was 0.25 ± 0.02 pH units for a peritubular change compared with only 0.04 ± 0.01 pH units after a luminal change ($P < 0.001$). These experiments demonstrate that as in the proximal tubule (Alpern and Chambers, 1986; Krapf et al., 1987*b*), basolateral membrane transporters appear to have a greater effect on $pH_i$ than apical membrane transporters in the $OM_iCT$.

### *Effect of Peritubular $Cl^-$ Removal on Cell pH*

The next set of studies was designed to examine whether $Cl^-$ interacts with the basolateral membrane $H^+/OH^-/HCO_3^-$ pathway. Tubules were bathed and perfused with solutions containing 25 mM $HCO_3^-$ and 123.2 mM $Cl^-$ (pH 7.4, solution 1, Table I). During the experimental period, $Cl^-$ was removed from the peritubular solution and replaced with gluconate (pH 7.4, solution 3, Table I). In each tubule this experimental maneuver was performed first in the absence and then in the presence of 100 $\mu$M peritubular DIDS, an anion exchange inhibitor. Shown in Fig. 3 is a typical tracing. Peritubular $Cl^-$ removal resulted in a rapid cell alkalinization that was reversible. Subsequent addition of 100 $\mu$M peritubular DIDS in the presence of peritubular $Cl^-$ resulted in a slow alkalinization of the cell. Then, in the presence of DIDS, the effect of peritubular $Cl^-$ removal on $pH_i$ was inhibited. In six paired tubules, peritubular $Cl^-$ removal alkalinized cells from 7.14 ± 0.08 to 7.29 ± 0.09 ($P < 0.005$), and readdition caused $pH_i$ to return to 7.08 ± 0.06 ($P < 0.01$). Addition of 100 $\mu$M peritubular DIDS significantly alkalinized cells from 7.09 ± 0.06 to 7.15 ± 0.05 ($P < 0.05$), and inhibited the cell alkalinization that occurred in response to peritubular $Cl^-$ removal (control 7.15 ± 0.05; experimental 7.21 ± 0.07, $P$ = NS; recovery 7.18 ± 0.06, $P$ = NS). These experiments demonstrate the existence of a stilbene-sensitive basolateral membrane pathway for the movement of $H^+/OH^-/HCO_3^-$ that is modulated by peritubular $Cl^-$. The alkalinization that occurs when peritubular DIDS is added suggests that the transporter normally operates to extrude base from the cell.

*Effect of Peritubular $Cl^-$ Removal in the Presence of a Voltage Clamp*

The above results are consistent with a basolateral member $Cl^-$/base$^-$ exchange process, but are also consistent with a $Cl^-$ conductance functioning in parallel with a voltage-sensitive, $Cl^-$-independent $H^+/OH^-/HCO_3^-$ pathway. To address this, two sets of studies were performed. The first set of studies was designed to examine the effect of peritubular $Cl^-$ removal on $pH_i$ in the presence of a cell voltage clamp. In the presence of a voltage clamp, peritubular $Cl^-$ removal should not result in cell alkalinization by parallel $Cl^-$ and $H^+/OH^-/HCO_3^-$ conductances. In contrast, voltage clamp will not prevent the effect of pertibular $Cl^-$ removal on a $Cl^-$/base exchange process.

Tubules were initially bathed and perfused with a solution containing 123.2 mM $Cl^-$ and 5 mM $K^+$ (pH 7.4, solution 1, Table I). The voltage clamp was produced by

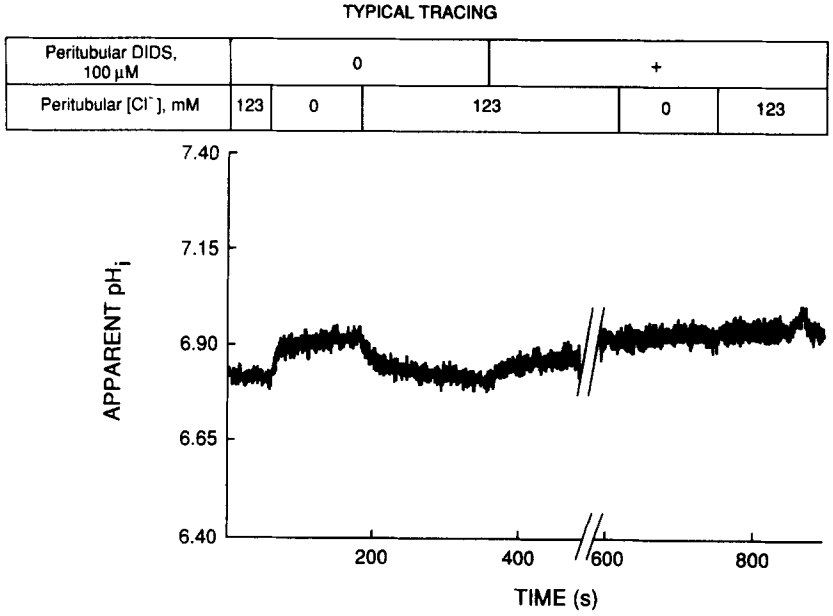


FIGURE 3. Effect of peritubular $Cl^-$ on $pH_i$: typical study. See text for explanation.

bathing and perfusing the tubules with a solution containing 123.2 mM $Cl^-$ and 125 mM $K^+$ (pH 7.4, solution 4, Table I), with 5 $\mu$M valinomycin added to the peritubular solution. During the experimental period, peritubular $Cl^-$ was replaced by gluconate, once again with 5 $\mu$M valinomycin added (pH 7.4, solution 5, Table I). Fig. 4 shows a typical tracing. When tubules were exposed to the voltage-clamping solutions, a slow alkalinization of the cells occurred. Subsequent replacement of $Cl^-$ by gluconate resulted in a rapid and reversible cell alkalinization. In six paired tubules, a slow significant alkalinization of $pH_i$ was found in all tubules with application of the voltage-clamping solutions ($7.17 \pm 0.06$ to $7.27 \pm 0.08$, $P < 0.05$). Cells then alkalinized from $7.27 \pm 0.08$ to $7.74 \pm 0.14$ ($P < 0.001$) when $Cl^-$ was replaced by

gluconate, and then returned to 7.41 ± 0.08 ($P < 0.005$) with readdition of peritubular $Cl^-$. The alkalinization of the cell upon peritubular $Cl^-$ removal in the presence of a voltage clamp suggests the direct coupling of $Cl^-$ and $H^+/OH^-/HCO_3^-$. Unfortunately, interpretation of the above studies relies on knowledge that the cell voltage was indeed clamped and unaffected by peritubular [$Cl^-$] changes. Because we did not measure cell voltage, an additional set of experiments was performed to rule out parallel conductances.

### *$Cl^-$ Dependence of Basolateral Membrane $H^+/OH^-/HCO_3^-$ Permeability*

If the basolateral membrane contains parallel $Cl^-$ and $H^+/OH^-/HCO_3^-$ conductances, total luminal and peritubular $Cl^-$ removal should not affect the $H^+/OH^-/$

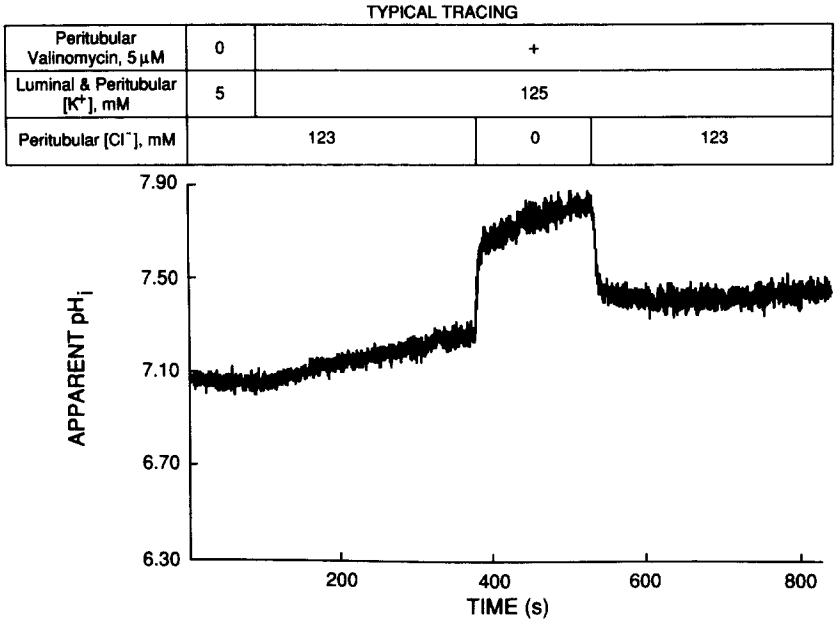


FIGURE 4. Effect of peritubular $Cl^-$ removal in the presence of voltage clamp. See text for explanation.

$HCO_3^-$ permeability of this membrane. On the other hand, any contribution of a $Cl^-/base^-$ exchanger to this permeability would be inhibited by $Cl^-$ removal. To estimate $H^+/OH^-/HCO_3^-$ permeability, the effect on $pH_i$ of lowering peritubular [$HCO_3^-$] from 25 to 5 mM was examined in the presence and complete absence of luminal and peritubular $Cl^-$ (pH 7.4, solutions 1 and 3; pH 6.8, solutions 2 and 6, Table I). Fig. 5 shows a typical tracing. Tubules were first perfused in the absence of luminal and peritubular $Cl^-$. In this setting, lowering [$HCO_3^-$] from 25 to 5 mM resulted in a slow acidification of the cell which was reversible. Luminal and peritubular $Cl^-$ addition resulted in a rapid cell acidification, as observed above with peritubular $Cl^-$ addition. Lowering peritubular [$HCO_3^-$] from 25 to 5 mM in the pres-

ence of luminal and peritubular $Cl^-$ then resulted in a rapid and reversible cell acidification as seen in the previous experiments described above.

In 10 paired tubules, a reduction of peritubular [$HCO_3^-$] from 25 to 5 mM in the absence of luminal and peritubular $Cl^-$, acidified the cells from 7.19 ± 0.08 to 7.05 ± 0.07 ($P < 0.002$). Upon return to 25 mM peritubular $HCO_3^-$, $pH_i$ rose to 7.09 ± 0.08 ($P < 0.05$). $Cl^-$ addition to both the luminal and peritubular fluid caused cells to acidify from 7.09 ± 0.08 to 6.84 ± 0.05 ($P < 0.001$). In the presence of $Cl^-$, decreasing peritubular [$HCO_3^-$] from 25 to 5 mM caused $pH_i$ to acidify from 6.84 ± 0.05 to 6.61 ± 0.04 ($P < 0.001$), an effect that was fully reversible upon return to the control 25 mM $HCO_3^-$ solution (6.83 ± 0.05 [$P < 0.001$]).

The initial rate of cell acidification ($dpH_i/dt$) induced by lowering bath [$HCO_3^-$]

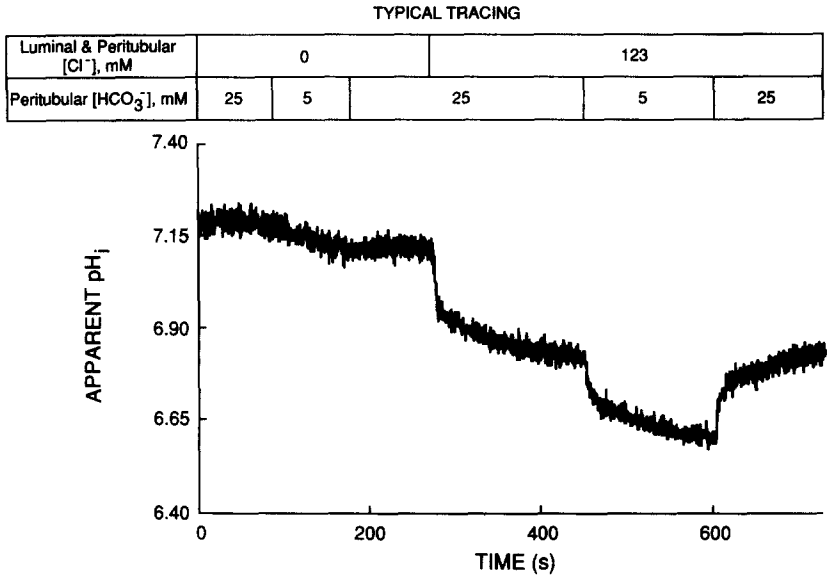


FIGURE 5. Measurement of basolateral $HCO_3^-$ permeability in the absence and presence of ambient $Cl^-$: typical study. See text for explanation.

was inhibited by 89% in the complete absence of luminal and peritubular $Cl^-$, 0.11 ± 0.02 pH units/min vs. 0.98 ± 0.14 pH units/min ($P < 0.001$). These initial rates of acidification demonstrate $Cl^-$ dependence of ~90% of basolateral membrane $H^+/OH^-/HCO_3^-$ movement, and further suggest the existence of $Cl^-/base^-$ exchange.

### *$Na^+$ Dependence of Basolateral Membrane $Cl^-/Base^-$ Exchange*

In the proximal tubule most of apparent $Cl^-/base^-$ exchange has been found to be $Na^+$ dependent, and attributed to a $Na^+$ $(HCO_3^-)_2/Cl^-$ exchanger (Guggino et al., 1983; Alpern and Chambers, 1987; Sasaki and Yoshiyama, 1988). The next set of studies was designed to examine whether $Na^+$ is required for $Cl^-/base^-$ exchange in

this segment. Tubules initially were bathed and perfused with 25 mM $HCO_3^-$ and 145 mM $Na^+$ (pH 7.4, solution 1, Table I). Peritubular and luminal $Na^+$ were then replaced by *N*-methyl-D-glucosamine and choline (pH 7.4, solution 7, Table I). At varying intervals, peritubular $Cl^-$ was replaced by gluconate either in the presence or absence of $Na^+$ (pH 7.4, solutions 3 and 8, Table I). Fig. 6 shows a typical tracing. In the presence of $Na^+$, replacement of peritubular $Cl^-$ by gluconate resulted in a rapid and reversible cell alkalinization as above. After peritubular and luminal $Na^+$ replacement, $pH_i$ decreased. In the absence of luminal and peritubular $Na^+$, replacement of peritubular $Cl^-$ still resulted in a rapid and reversible cell alkalinization, a finding that was consistent in six tubules. Return to the control 145 mM $Na^+$ luminal and peritubular solutions resulted in cell alkalinization. These data demon-

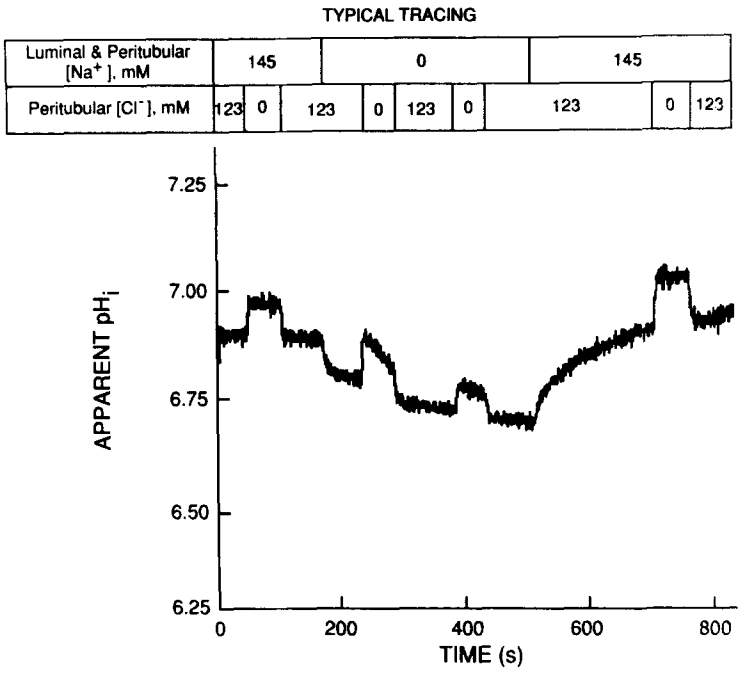


FIGURE 6. Effect of peritubular $Cl^-$ removal in the presence and absence of ambient $Na^+$ on $pH_i$: typical study. See text for explanation.

strate two important findings. First, the results demonstrate a $Na^+$-dependent transport process involved in $pH_i$ regulation, likely a $Na^+$-$H^+$ antiporter present on the basolateral membrane as suggested in preliminary studies by Breyer and Jacobson (1988). Second, $Cl^-$/base$^-$ exchange on the basolateral membrane occurs in the absence of luminal and peritubular $Na^+$, and thus most of $Cl^-$/base$^-$ exchange is not dependent on $Na^+$.

### *$CO_2$ Dependence of $Cl^-$/Base$^-$ Exchange*

The purpose of the next set of studies was to examine whether the transporter was a $Cl^-/HCO_3^-$ exchanger or a $Cl^-/OH^-$ exchanger (equivalent to an HCl cotrans-

porter). Tubules were initially bathed and perfused with $Cl^-$-free and $CO_2/HCO_3^-$-free solutions that were HEPES buffered to pH 7.4 (solution 9, Table I). During the experimental period, peritubular $Cl^-$ was added in the absence of $CO_2/HCO_3^-$ (pH 7.4, solution 10, Table I). This maneuver was then repeated in the presence of HEPES-buffered solutions containing 40 mmHg $CO_2$ and 25 mM $HCO_3^-$ (pH 7.4, solutions 11 and 12, Table I). Shown in Fig. 7 is a typical tracing. In the absence of exogenous $CO_2/HCO_3^-$ peritubular $Cl^-$ addition resulted in a rapid and reversible cell acidification. After peritubular and luminal addition of $CO_2/HCO_3^-$, a rapid cell acidification occurred, followed by a slow alkalinization. Under these conditions peritubular $Cl^-$ addition resulted in a rapid reversible cell acidification. In seven tubules, perfused and bathed in the absence of $Cl^-$ and $CO_2/HCO_3^-$, $pH_i$ was

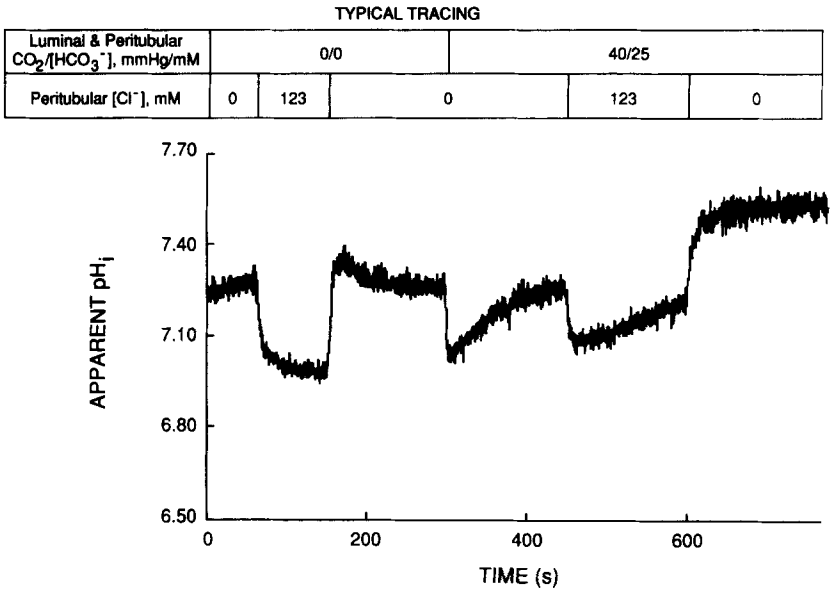


FIGURE 7. Effect of peritubular $Cl^-$ addition in the absence and presence of $CO_2/HCO_3^-$: typical study. See text for explanation.

6.99 ± 0.13. After peritubular $Cl^-$ addition, cells acidified to 6.81 ± 0.12 ($P < 0.002$) and returned to 6.99 ± 0.14 ($P < 0.001$) when peritubular $Cl^-$ was once again removed. In the presence of $CO_2/HCO_3^-$, peritubular $Cl^-$ addition caused $pH_i$ to decrease from 6.99 ± 0.12 to 6.84 ± 0.10 ($P < 0.002$), and subsequent peritubular $Cl^-$ removal, caused $pH_i$ to increase from 6.89 ± 0.11 to 7.10 ± 0.14 ($P < 0.002$).

Transporter activity was assessed from the average of the $J_H$ (Eq. 4) obtained upon $Cl^-$ addition and removal. The $dpH_i/dt$ in the absence of exogenous $CO_2/HCO_3^-$ was 1.86 ± 0.26 pH units, and in its presence was 2.20 ± 0.38 pH units/min. To calculate $J_H$ from $dpH_i/dt$, buffer capacities under these two conditions were calculated. The non-$CO_2/HCO_3^-$ buffer capacity was determined from the effect of

sudden $CO_2/HCO_3^-$ addition in the above studies (see Methods), and found to be 33.4 ± 7.0 mmol·liter$^{-1}$·pH unit$^{-1}$ ($pH_i$ changed from 7.00 to 6.78). To obtain a better estimate of cell buffer capacity without contribution from active transport processes and processes secondarily coupled to active transport, buffer capacity was measured with isohydric $CO_2/HCO_3^-$ addition in the presence of 2 mM cyanide. In these studies, the non-$CO_2/HCO_3^-$ buffer capacity was 18.5 ± 1.1 mmol·liter$^{-1}$·pH unit$^{-1}$ ($pH_i$ changed from 6.88 to 6.64). This value was used in subsequent calculations. Total buffer capacity in the presence of $CO_2/HCO_3^-$ was 44.8 ± 5.7 mmol·liter$^{-1}$·pH unit$^{-1}$ (Eq. 3). Using these buffer capacities, the $J_H$ induced by peritubular $Cl^-$ addition in the presence of exogenous $CO_2/HCO_3^-$ (53.7 ± 14.6 pm·mm$^{-1}$·min$^{-1}$) was inhibited 66% in the absence of exogenous $CO_2/HCO_3^-$ (18.4 ± 3.6 pm·mm$^{-1}$·min$^{-1}$; $P < 0.05$).

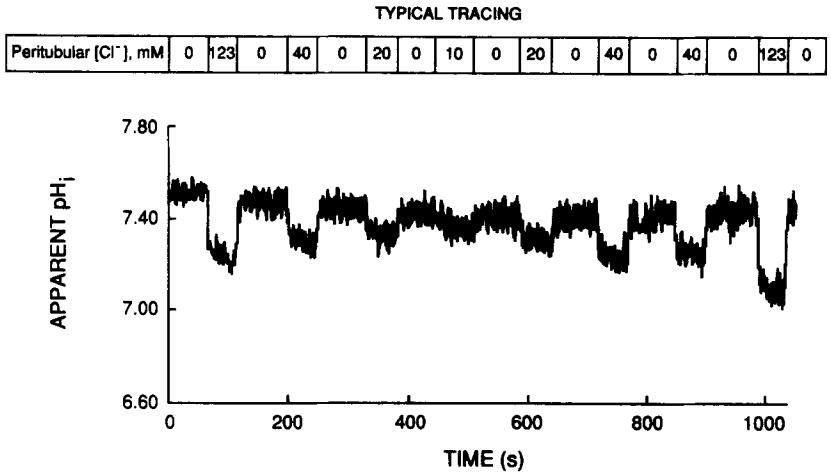


FIGURE 8. Effect of varying peritubular $Cl^-$ concentration additions on cell $pH_i$: typical study. See text for explanation.

These results suggest that the majority of $Cl^-$-coupled transport is mediated by a $CO_2/HCO_3^-$-dependent mechanism. The component remaining in the absence of $CO_2/HCO_3^-$ could represent $Cl^-/OH^-$ exchange, or could be due to a $Cl^-/HCO_3^-$ exchanger using metabolically produced $CO_2/HCO_3^-$. In previous studies, we addressed this problem by inhibiting metabolic $CO_2$ production with cyanide (Krapf et al., 1987*a*). When a similar maneuver was used in the present studies, cyanide not only completely inhibited the response to peritubular $Cl^-$ addition in the absence of exogenous $CO_2/HCO_3^-$, but also inhibited the response in the presence of exogenous $CO_2/HCO_3^-$ (where metabolic processes are not required for $CO_2/HCO_3^-$ availability). Because these results suggested a nonspecific effect of cyanide on the transporter (see Discussion), it was not possible to use the cyanide experiments to exclude $Cl^-/OH^-$ exchange. These studies, however, demonstrate that at least two-thirds of $Cl^-$/base$^-$ exchange requires $CO_2/HCO_3^-$, and most likely represents a $Cl^-/HCO_3^-$ exchanger.

*Apparent $K_m$ for $Cl^-$ of the $Cl^-/HCO_3^-$ Exchanger*

In the last set of studies, the apparent $K_m$ for peritubular $Cl^-$ was determined in the presence of 25 mM luminal and peritubular [$HCO_3^-$]. Tubules were initially bathed and perfused with $Cl^-$-free solutions, and peritubular additions of 10, 20, 40, and 123.2 mM $Cl^-$ (pH 7.4, solutions 3, 13, 14, 15, and 1, respectively, Table I) were examined. The order of the $Cl^-$ additions was varied from tubule to tubule. The tracing shown in Fig. 8 is typical. When 123.2 mM peritubular $Cl^-$ was added, cells rapidly and reversibly acidified. As peritubular [$Cl^-$] additions were reduced to 40, 20, and 10 mM, cell acidification occurred to a lesser degree and at a slower rate.

The kinetics of this transporter were determined from the rate of change in $pH_i$ ($dpH_i/dt$) in response to $Cl^-$ addition. When more than one measurement was made with the same $Cl^-$ concentration on a tubule, these were averaged to provide a result for that tubule. The initial acidification rate ($dpH_i/dt$) in eight tubules with addition of 10 mM $Cl^-$ was 0.36 ± 0.06 pH units/min; 20 mM, 0.67 ± 0.10 pH units/min; 40 mM, 1.30 ± 0.27 pH units/min; and 123.2 mM, 2.48 ± 0.62 pH units/min. Fig. 9 shows a Lineweaver-Burk plot of the data, with the drawn line fit

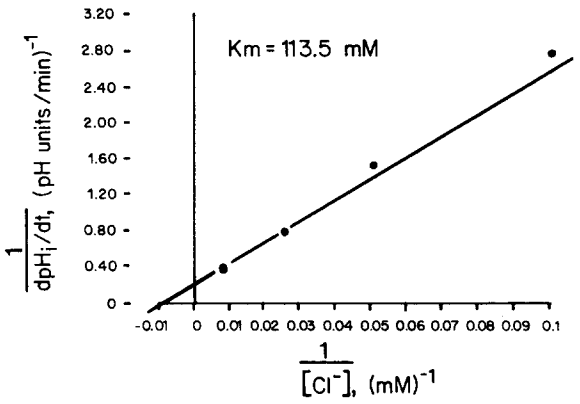


FIGURE 9. Acidification rate as a function of peritubular [$Cl^-$]: Lineweaver-Burk transformation.

by the weighted linear regression method of Wilkinson (1961). Using this fit, the apparent $K_m$ for $Cl^-$ was 113.5 ± 14.8 mM and the $V_{max}$ was 4.8 ± 0.4 pH units/min. Both Hanes Wolf and Eadie Hofstee fits yielded similar values ($K_m$ 128.2 mM; $V_{max}$ 5.1 pH units/min). These units demonstrate an apparent $K_m$ for $Cl^-$ in the range of interstitial [$Cl^-$].

## DISCUSSION

In the present studies we measured $pH_i$ using the pH-sensitive intracellularly trapped fluorescent dye, BCECF, in the $OM_iCT$ perfused in vitro. As previously described, a ratio of fluorescence with 500 and 450 nm excitation was obtained, which was consistent and was a sensitive index of $pH_i$. Using the nigericin calibration technique, a $pH_i$ of 7.03 ± 0.03 was found under control conditions. Unfortunately, there are presently no measurements of $pH_i$ using microelectrodes with which this value can be compared.

In these studies $H^+/HCO_3^-$ transport mechanisms were studied in the inner stripe of the outer medulla. To avoid contamination with outer stripe, all tubules were dissected from the inner half of the inner stripe. In the rat, the inner stripe of the outer medullary collecting tubule clearly contains two cell types: an intercalated cell, similar to that felt to mediate $H^+/HCO_3^-$ transport in the outer stripe and cortical collecting tubule; and a second cell which has been referred to as a principal cell (Madsen and Tisher, 1986). In the rabbit outer medullary collecting tubule, however, intercalated cells (defined ultrastructurally and with antibodies against carbonic anhydrase II) decrease in frequency along its length, constituting only 10% of the cells in the outer half of the inner stripe and rarely being found in the inner half of the inner stripe (Madsen et al., 1989). While the remaining cell type appears similar to a principal cell, it is unlikely to be a principal cell in that this segment does not actively transport $Na^+$ and $K^+$, characteristics associated with principal cells (Stokes, 1982).

Recently, Ridderstrale et al. (1988) have classified cells of the $OM_iCT$ as inner stripe cells. Although these investigators found ultrastructural heterogeneity between cells of the inner stripe, with regard to the number of subapical vesicles, number of mitochondria, and density of rod-shaped apical intramembranous particles, it was felt that the results were more consistent with a variable pattern of one cell type rather than two distinct cell types. All cells of the inner region of the inner stripe stained positive for carbonic anhydrase and contained Na-K ATPase localized to the basolateral membrane (Ridderstrale et al., 1988). Schuster et al. (1986) found that 43% of cells in the rabbit inner stripe were positive for band 3 and a mitochondrial marker. Based on the results of Madsen et al. (1989) and Ridderstrale et al. (1988), these cells cannot be intercalated cells, and most likely represent one part of the spectrum of inner stripe cells. In addition, electrophysiologic studies of the rabbit inner stripe have identified only one cell type, a cell with electrical properties very different from that of principal cells (i.e., no significant apical membrane conductances) (Koeppen, 1985, 1987).

On the basis of these results, we feel that the inner stripe of the outer medullary collecting tubule is composed mostly of "inner stripe cells" (Ridderstrale et al., 1988), with a few intercalated cells in the outer part of the $OM_iCT$ that are not present in the inner part of the $OM_iCT$. Since this segment secretes $H^+$, and does not actively transport $Na^+$ or $K^+$, we presume that this cell type mediates $H^+$ transport. In our studies, tubules were dissected from the inner half of the inner stripe. If there are two cell types in this segment, our measurements are an average of these two cell types. In that cells mediating $H^+$ transport may have higher $H^+/OH^-/HCO_3^-$ transport rates, the observed $pH_i$ changes may be weighted by these cells.

### *Relative Effects of Basolateral and Apical Membrane Transporters on $pH_i$*

In previous studies in the proximal tubule, we found that changes in peritubular pH had a greater effect on $pH_i$ than similar changes in luminal pH (Alpern and Chambers, 1986; Krapf et al., 1987*b*). In fact, dominance of $pH_i$ by the basolateral membrane transporters was so striking in the proximal tubule that it was necessary to inhibit the basolateral membrane transporters in order to study apical membrane transporter effects on $pH_i$. Therefore, the first study that we performed was to

examine the relative effects of luminal vs. peritubular fluid pH changes on $pH_i$. The present results were similar to those in the proximal tubule. Peritubular acidification lowered $pH_i$ to an extent six times greater than that seen with a similar acidification of the luminal fluid.

*Basolateral Membrane Stilbene-sensitive $Cl^-/HCO_3^-$ Exchange*

In the next series of studies, peritubular $Cl^-$ removal was found to cause a cell alkalinization that was reversible and completely blocked by 100 $\mu$M DIDS. While these studies suggested the presence of $Cl^-/HCO_3^-$ exchange, they were also compatible with a $Cl^-$ conductance in parallel with a voltage-sensitive $H^+/OH^-/HCO_3^-$ pathway, with one of the two pathways inhibitable by DIDS. Indeed, Koeppen (1985) has shown a significant basolateral membrane $Cl^-$ conductance in this segment. The presence of direct coupling between $Cl^-$ and base was suggested by two findings: (*a*) changes in peritubular $[Cl^-]$ caused similar changes in $pH_i$ in the presence of a voltage clamp; and (*b*) 90% of basolateral membrane $H^+/OH^-/HCO_3^-$ permeability was dependent on the presence of peritubular $Cl^-$. In agreement with these results, Koeppen (1985) found no evidence for a $H^+/OH^-/HCO_3^-$ conductance in these cells.

In the proximal tubule, Guggino et al. (1983), Alpern and Chambers (1987), and Sasaki and Yoshiyama (1988) described a $Na^+$-dependent $Cl^-/HCO_3^-$ exchanger which may run as a $Na^+$ $(HCO_3^-)_2/Cl^-$ exchanger. To examine whether the apparent $Cl^-/HCO_3^-$ exchange seen in the present studies was due to such a transporter, we examined the effect of luminal and peritubular $Na^+$ removal on the $Cl^-/HCO_3^-$ exchanger. While in the absence of luminal and peritubular $Na^+$, peritubular $Cl^-$ addition and removal continued to affect $pH_i$, there was a tendency toward a smaller $\Delta pH_i$ in the absence of $Na^+$ (17% inhibition). While this effect may have indicated some $Na^+$ dependence of $Cl^-/HCO_3^-$ exchange, these studies were complicated by the fact that luminal and peritubular $Na^+$ removal also caused cell acidification. Therefore it is also possible that $pH_i$ modulated the $Cl^-/HCO_3^-$ exchanger. Indeed, Paradiso et al. (1986) have previously shown that $pH_i$ is an important regulator of $Cl^-/HCO_3^-$ exchange in gastric gland cells, with decreases in $pH_i$ lowering transporter activity. In any case, most of the $Cl^-$-induced change in $pH_i$ persisted in the absence of luminal and peritubular $Na^+$, indicating that most of $Cl^-/HCO_3^-$ exchange activity is independent of $Na^+$.

In order to examine whether the exchanger transported $HCO_3^-$ or $H^+/OH^-$, the ability of the transporter to run in the absence of exogenous $CO_2/HCO_3^-$ was examined. Removal of exogenous $CO_2/HCO_3^-$ inhibited the effect of $Cl^-$ addition on $dpH_i/dt$ by 67%. This suggested that at least this fraction was mediated by $Cl^-/HCO_3^-$ exchange. The component remaining in the absence of exogenous $CO_2/HCO_3^-$ could represent $Cl^-/OH^-$ exchange, but may also represent $Cl^-/HCO_3^-$ exchange with metabolic generation of $HCO_3^-$. In previous studies in the proximal tubule, we found that one-third of $Na^+/3HCO_3^-$ transporter activity remained in the absence of exogenous $CO_2/HCO_3^-$, and that this component was eliminated by inhibited of metabolic $CO_2$ production by 2 mM cyanide (Krapf et al., 1987*a*). This maneuver was attempted in these tubules, and indeed inhibited most of the remain-

ing effect of $Cl^-$ on $pH_i$. Unfortunately, these studies were difficult to interpret because cyanide also inhibited the effect of $Cl^-$ on $pH_i$ in the presence of exogenous $CO_2/HCO_3^-$. No such effect was seen in the studies on the proximal tubule (Krapf et al., 1987*a*). This effect of cyanide may represent a general toxicity toward the epithelium, or may represent an ATP dependence of the $Cl^-/HCO_3^-$ exchanger.

In summary, these studies demonstrate a $Na^+$-independent, $Cl^-/HCO_3^-$ exchanger which may also use $OH^-$ as a substrate. While our data do not specify a stoichiometric ratio, this transporter has generally been found to be electroneutral implying a 1:1 stoichiometry. The absence of a rapid effect of peritubular $[HCO_3^-]$ on cell voltage in this segment (Koeppen, 1985) suggests electroneutrality and thus a 1:1 stoichiometry. In our studies cell depolarization by valinomycin plus high extracellular $[K^+]$ consistently caused cell alkalinization, which could be interpreted as indicating an electrogenic pathway. However, this observation can be explained either by cell depolarization causing an increase in cell $[Cl^-]$, which then drives $HCO_3^-$ into the cell across the $Cl^-/HCO_3^-$ exchanger, or by an effect of cell depolarization on the apical membrane $H^+$ pump.

The finding of a basolateral membrane $Na^+$-independent $Cl^-/HCO_3^-$ exchanger agrees with the labeling of this membrane with antibodies against band 3 protein (Schuster et al., 1986; Wagner et al., 1987; Verlander et al., 1988). In addition, our results agree with the results of Schwartz et al. (1985), and the preliminary results of Breyer and Jacobson (1988), who have found basolateral membrane $Cl^-/HCO_3^-$ exchange using cell pH measurements in the $OM_iCT$, and of Zeidel et al. (1986*b*), who found $Cl^-/HCO_3^-$ exchange in suspensions of $OM_iCT$ tubules. $Cl^-/HCO_3^-$ exchange has been found in several different cell types including the red blood cell (Gunn et al., 1973; Wieth and Bruhm, 1985), nerve and muscle tissue (Roos and Boron, 1981; Wieth and Bruhm, 1985), small and large intestine (Schultz, 1979; Fondocaro, 1986), gallbladder (Reuss and Costantin, 1984), and neutrophil (Simchowitz and Roos, 1985), as well as in proton-secreting epithelia such as the gastric mucosa (Rehm, 1967; Muallem et al., 1985, 1988; Paradiso et al., 1986, 1987), and turtle bladder (Ehrenspeck and Brodsky, 1976; Cohen et al., 1978; Fischer et al., 1983).

### *Physiologic Role of the $Cl^-/HCO_3^-$ Exchanger*

The $Cl^-/HCO_3^-$ exchanger demonstrated in these studies is believed to mediate base exit across the basolateral membrane, effecting transepithelial $HCO_3^-$ absorption. This conclusion is based on a number of observations. First, 90% of basolateral membrane $H^+/OH^-/HCO_3^-$ permeability is dependent on $Cl^-$ and most likely represents this transport mechanism. Second, Stone et al (1983) observed that $Cl^-$ removal from luminal and bath fluids inhibited transepithelial $H^+$ secretion in the $OM_iCT$. While this effect may be due to a $Cl^-$ dependence of the apical membrane $H^+$-ATPase (Kaunitz et al., 1985), it can also be explained by a $Cl^-$-coupled transporter on the basolateral membrane. Third, SITS, an inhibitor of this transport mechanism, inhibits acidification in this segment when applied from the peritubular side (Stone et al., 1983). Lastly, in the present studies DIDS applied to the bath caused a cell alkalinization consistent with this transporter running in the $HCO_3^-$

efflux direction. Based on the electrophysiologic data of Koeppen (1985), it is believed that $Cl^-$, which enters the cell in exchange for $HCO_3^-$, exits across a basolateral membrane $Cl^-$ conductance.

*Kinetics*

An important physiologic question is whether changes in the $Cl^-$ concentration of the medullary interstitium can regulate the rate of transepithelial $H^+$ secretion in the medullary collecting duct. Since volume contraction is known to increase medullary interstitial [$Cl^-$] (Atherton et al., 1971), this could provide a mechanism by which volume contraction would stimulate renal acidification.

To address whether interstitial $Cl^-$ concentration could regulate the $Cl^-/HCO_3^-$ exchanger, an apparent $K_m$ was determined for this transporter. As shown in Fig. 9, the $K_m$ for $Cl^-$ was 113.5 mM, implying that $Cl^-$ concentrations within the physiologic range are able to regulate $Cl^-/HCO_3^-$ exchanger rate, and secondarily regulate transepithelial acidification rate. The high $K_m$ for $Cl^-$ found in these studies differs from those observed by other investigators. In outer medullary collecting duct cells in suspension, Zeidel et al. (1986*b*) found a $K_m$ of 29.9 mM for the $Cl^-/HCO_3^-$ exchanger. In the studies of Zeidel et al. (1986*b*), the effect of $Cl^-$ on transporter rate was examined in the absence of extracellular $HCO_3^-$. Studies from the red cell have shown that $HCO_3^-$ competes with $Cl^-$ at a single site on the band 3 $Cl^-/HCO_3^-$ exchanger (Gunn et al., 1973; Dalmark, 1976; Wieth, 1979). Thus, an apparent $K_m$ for $Cl^-$ measured in the presence of $HCO_3^-$ (as ours was measured) would be expected to be higher than one measured in the absence of $HCO_3^-$. The marked difference between our results and those obtained by Fischer et al. (1983) in the turtle bladder ($K_m$ = 0.13 mM) may be explained by differences in species.

In any case, the conditions under which the present $K_m$ for $Cl^-$ was measured are physiologic and show that under physiologic conditions, peritubular $Cl^-$ can regulate $Cl^-/HCO_3^-$exchange rate. If the competitive model for $Cl^-$ and $HCO_3^-$ on this transporter is true, in metabolic alkalosis where extracellular fluid volume is an important regulator of renal acidification, the apparent $K_m$ for $Cl^-$ could be shifted even higher. Thus, these studies suggest that interstitial $Cl^-$ concentration could be an important regulator of acidification rate and provide a possible mechanism by which extracellular fluid volume status could regulate acidification.

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