Mechanism of Basolateral Membrane
H⁺/OH⁻/HCO₃⁻ Transport in the Rat Proximal Convoluted Tubule

A Sodium-coupled Electrogenic Process

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ABSTRACT In order to examine the mechanism of basolateral membrane H⁺/OH⁻/HCO₃⁻ transport, a method was developed for the measurement of cell pH in the in vivo doubly microperfused rat proximal convoluted tubule. A pH-sensitive fluorescein derivative, (2',7')-bis(carboxyethyl)-(5,6)-carboxyfluorescein, was loaded into cells and relative changes in fluorescence at two excitation wavelengths were followed. Calibration was accomplished using nigericin with high extracellular potassium concentrations. When luminal and peritubular fluids were pH 7.32, cell pH was 7.14 ± 0.01. Decreasing peritubular pH from 7.32 to 6.63 caused cell pH to decrease from 7.16 ± 0.02 to 6.90 ± 0.03. This effect occurred at an initial rate of 2.4 ± 0.3 pH units/min, and was inhibited by 0.5 mM SITS. Lowering the peritubular sodium concentration from 147 to 25 meq/liter caused cell pH to decrease from 7.20 ± 0.03 to 6.99 ± 0.01. The effect of peritubular sodium concentration on cell pH was inhibited by 0.5 mM SITS, but was unaffected by 1 mM amiloride. In addition, when peritubular pH was decreased in the total absence of luminal and peritubular sodium, the rate of cell acidification was 0.2 ± 0.1 pH units/min, a >90% decrease from that in the presence of sodium. Cell depolarization achieved by increasing the peritubular potassium concentration caused cell pH to increase, an effect that was blocked by peritubular barium or luminal and peritubular sodium removal. Lowering the peritubular chloride concentration from 128 to 0 meq/liter did not affect cell pH. These results suggest the existence of an electrogenic, sodium-coupled H⁺/OH⁻/HCO₃⁻ transport mechanism on the basolateral membrane of the rat proximal convoluted tubule.

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INTRODUCTION

The mammalian proximal convoluted tubule (PCT) absorbs 80% of filtered bicarbonate. This occurs by transcellular proton secretion. Protons are secreted from the cell across the apical membrane at least partially by an Na/H antiporter, and possibly by a sodium-independent mechanism. The mechanism by which protons (or their equivalent) enter the cell across the basolateral membrane remains unknown. Previous studies from our laboratory (2, 4) have demonstrated that the rate of transcellular proton secretion is very sensitive to peritubular pH and have suggested that this interaction occurs on the basolateral membrane.

In order to examine the mechanism of basolateral membrane H⁺/OH⁻/HCO₃⁻ transport in the mammalian PCT, it is necessary to measure cell pH in these small cells (~8 μm diam). While investigators have succeeded recently in impaling cells with pH-sensitive microelectrodes (40, 47), such studies are technically difficult, and the electrodes may damage the cells. Thomas et al. (45) have developed a technique for nontraumatically loading pH-sensitive fluorescein derivatives into small cells, thus allowing the measurement of cell pH.

The purposes of the present studies were to develop a technique for the measurement of cell pH and to examine the mechanism of basolateral membrane proton transport in the in vivo doubly microperfused rat PCT. Cells were loaded using the method of Thomas et al. (45), with a fluorescein derivative developed by Tsien and co-workers (34, 37), (2',7')-bis(carboxyethyl)-(5,6)-carboxyfluorescein (BCECF). The mechanism of basolateral membrane proton transport was examined using a specially designed peritubular capillary perfusion pipette, which allowed rapid changes in peritubular fluid. The results demonstrate that proton transport across the basolateral membrane occurs by an electrogenic, sodium-coupled H⁺/OH⁻/HCO₃⁻ transporter similar to that previously found in the salamander proximal tubule (11).

METHODS

Experiments were performed using male Wistar rats (Charles River Breeding Laboratories, Wilmington, MA) weighing 208–257 g. The rats were prepared for microperfusion as previously described (2, 3). Briefly, rats were anesthetized with an intraperitoneal injection of Inactin (100–120 mg/kg) and placed on a heated table that maintained body temperature at 37°C. The right femoral artery was catheterized for monitoring blood pressure and obtaining blood samples. The left kidney was exposed using a flank incision and immobilized in a Lucite cup. The ureter was cannulated (PE-50) to ensure the free drainage of urine. Throughout the experiment, rats were infused intravenously with a bicarbonate Ringer’s solution (mM: 105 NaCl, 25 NaHCO₃, 4 Na₂HPO₄, 5 KCl, 1 MgSO₄, 1.8 CaCl₂) at 1.6 ml/h. The proximal tubular transit time was measured after the injection of 0.02 ml of 10% lissamine green dye intravenously, and only kidneys in which the transit time was <12 s were accepted for study. At the completion of surgery, a blood sample was obtained for determination of pH and P₄CO₂ (model 165 blood gas analyzer, Corning Glass Works, Medfield, MA). The rats had normal systemic acid-base parameters: pH 7.44 ± 0.01; P₄CO₂, 37.8 ± 1.1 mmHg; [HCO₃⁻], 24.4 ± 0.6 mM.

All experiments were paired, comparing two peritubular solutions, a control and an experimental solution. Pipettes were placed using a Leitz dissecting microscope (Leitz Wetzlar, Rockleigh, NJ). The lumen of a PCT was perfused at 40 nl/min using a thermally
insulated microperfusion pump (Wolfgang Hampel, Berlin, Federal Republic of Germany) with a 6–8-μm-tip pipette, as previously described [2, 3].

Peritubular capillaries were perfused with a 10–13-μm-tip pipette designed to allow rapid changes between two perfusion fluids. Two pieces of pulled polyethylene tubing (PE-10) inserted into the pipette carried the two perfusion solutions to the pipette tip. Fluid was pushed out of the pipette tip by hydraulic pressure, which was switched from one series of PE tubing to the other by Hamilton valves (HV and HVP valves, Hamilton Co., Reno, NV). Simultaneously with switching the perfusion pressure from one series of inner tubing to the other, an outlet valve was briefly opened in the back of the peritubular capillary pipette holder, which caused rapid flow from the tubing and mixing at the tip. This method allowed rapid fluid changes in the peritubular capillary perfusate.

The protocol for most of the studies involved equilibration of the tubule for 3–5 min with a control solution in the peritubular capillary and a control solution (the same control solution unless otherwise stated) in the lumen. After measurements were made, the peritubular fluid was changed to the experimental solution and measurements were made for 1–2 min. Then the peritubular capillary solution was changed back to the control solution for 1–2 min, during which measurements were made (recovery period).

Control studies were performed to examine whether changes in peritubular capillary perfusion fluid would affect luminal fluid composition. [3H]Inulin was placed in the peritubular capillary perfusion fluid, and fluid was collected from the lumen by micro-puncture. From the 3H counts in the collected fluid, it was calculated that 0.5 ± 0.1 nl/min of peritubular fluid appeared in the lumen. This is <2% of the luminal perfusion rate and thus should have minimal effects on luminal composition.

The perfusion solutions are listed in Table I. 4-Acetamido-4′-isothiocyanostilbene-2,2′-disulfonate (SITS) was obtained from International Chemical and Nuclear (Cleveland, OH). Amiloride was obtained from Merck, Sharp & Dohme (Rahway, NJ). In all studies, the peritubular capillary perfusion pipette contained a control and an experimental solution. Luminal perfusion pipettes were filled with the control solution (unless otherwise stated) containing 0.025% FD + C green dye no. 3 and 40 μg/ml (5 × 10⁻⁵ M) of the acetoxymethyl derivative of BCECF (BCECF-AM) (Research Development Corp., Toronto, Canada). This compound does not fluoresce and is lipid soluble. It rapidly diffuses into cells where cytoplasmic esterases cleave off the acetoxymethyl groups, forming the fluorescent BCECF, which has four negative charges and leaves the cell slowly. Cells were usually loaded until sufficient visible fluorescence was achieved (minutes). In preliminary studies, the half-time for loss of BCECF from the cell was found to be 10–12 min, which was greater than that of other fluorescein derivatives at 37°C in the rat PCT.

In preliminary studies, it was demonstrated that tubules perfused with BCECF-AM had normal rates of volume (2.0 ± 0.3 nl/mm-min) and bicarbonate transport (150 ± 26 peq/mm-min) (2–4). Thus, the dye itself is not cytotoxic. The dye can, however, cause photodynamic damage to the cell. Indeed, if prolonged light exposure occurred, cells swelled and cell pH decreased. When this was noted, data were rejected. To avoid this problem, minimal light exposure was used.

Cell pH Measurement

After placement of the pipettes, the dissecting microscope was moved out of position, and a Leitz epifluorescence microscope (MPV compact system, Leitz Wetzlar) was moved into position. In general, fluorescence intensity was greatest in the cells of the loop the perfusion pipette was in and was smaller in subsequent loops. Lumens, capillaries, and surrounding structures did not fluoresce above background fluorescence. Fluorescence was measured in the loop containing the perfusion pipette, but always away from the site
TABLE I

*Perfusion Solutions (mM)*

|          | 25 Bicarbonate | 5 Bicarbonate | Low-Na Choline | Low-Na TMA | 0 Na, 25 bicarbonate | 0 Na, 5 bicarbonate | 5 K | 50 K | 0 Na, 5 K | 0 Na, 50 K | 0 Cl | 128 Cl | 50 Na, 25 bicarbonate |
|----------|----------------|---------------|----------------|------------|-----------------------|----------------------|-----|------|----------|------------|------|--------|-----------------------|
| Na⁺      | 147            | 147           | 25             | 25         | 102                   | 102                  |     |      | 147      | 147        | 147  | 50     | 147                   |
| K⁺       | 5              | 5             | 5              | 5          | 5                     | 5                    | 50  | 50   | 5        | 5          | 5    | 5      | 5                     |
| Ca²⁺     | 1.8            | 1.8           | 1.8            | 1.8        | 1.8                   | 1.8                  | 1.8 | 1.8  | 1.8      | 1.8        | 1.4  | 1.4    | 1.4                   |
| Mg²⁺     | 1              | 1             | 1              | 1          | 1                     | 1                    | 1   | 1    | 1        | 1          | 1    | 1      | 1                     |
| Choline²⁺|                |               | 122            |            | 147                   | 147                  | 45  | 147  | 102      |            |      |        | 97                     |
| TMA⁺     |                |               |                |            | 122                   |                      |     |       |          |            |      |        |                       |
| Ba²⁺     |                |               |                |            |                       | ±2                   | ±2  |       |          |            |      |        |                       |
| Cl⁻      | 128.6          | 148.6         | 128.6          | 128.6      | 128.6                 | 128.6                | 128.6 |      | 127.8    | 128.6      |      |        |                       |
| HCO₃⁻    | 25             | 5             | 25             | 25         | 25                    | 25                   | 25  | 25   | 25       | 25         | 25   | 25     |                       |
| HPO₄⁻    | 1              | 1             | 1              | 1          | 1                     | 1                    | 1   | 1    | 1        | 1          | 1    | 1      |                       |
| SO₄⁻     | 1              | 1             | 1              | 1          | 1                     | 1                    | 1   | 1    | 1        | 1          | 1    | 1      |                       |
| Gluconate|                |               |                |            |                       | 137.5                |      |       |          |            |      |        |                       |
| Glucose  | 5              | 5             | 5              | 5          | 5                     | 5                    | 5   | 5    | 5        | 5          | 5    | 5      | 5                     |
| Alanine  | 5              | 5             | 5              | 5          | 5                     | 5                    | 5   | 5    | 5        | 5          | 5    | 5      | 5                     |
| Urea     | 5              | 5             | 5              | 5          | 5                     | 5                    | 5   | 5    | 5        | 5          | 5    | 5      | 5                     |
| Bubbled  |                |               |                |            |                       |                      |     |       |          |            |      |        |                       |
| Percent O₂/percent CO₂ | 93/7         | 93/7         | 93/7           | 93/7       | 93/7                  | 93/7                 | 93/7 | 93/7 | 93/7     | 93/7        | 93/7 | 93/7    | 93/7                  |
of tubular puncture and never through the glass pipettes. This was done by use of an adjustable measuring diaphragm. Measurements were made at a magnification of 100. Although the size of the measured area varied, it was usually ~60–80 μm square. The measured loop was always well within the capillary perfusion area. Background fluorescence was measured in a tubule that did not contain the dye, but was within the area of capillary perfusion. Background varied only slightly from tubule to tubule. The signal-to-background ratio varied from ~10 to 60 at 500 nm excitation, and from 5 to 20 at 450 nm excitation (see below).

Analysis

The excitation spectra of BCECF in solutions of varying pH are shown in Fig. 1. It can be seen that BCECF has a peak excitation at 504 nm that is pH sensitive, and an isosbestic point where fluorescence excitation is independent of pH at 436 nm. Peak emission is at 526 nm.

![Figure 1](image)

**Figure 1.** Excitation spectra of 2 μM BCECF in solutions of 50 mM HEPES, 50 mM Tris, titrated to the indicated pH values. Emission was measured at 530 nm. Note that an isosbestic point exists where fluorescence is independent of pH at 436 nm. Below this wavelength, the lines cross such that there is more fluorescence in more acid solutions. Spectra were measured with an SLM 8000 spectrofluorimeter (SLM Instruments, Urbana, IL), and were corrected for wavelength-dependent variations in lamp intensity.

For these studies, fluorescence was measured alternately with epifluorescence at 500 and 450 nm excitation and with 530 nm emission (interference filters, Corion Corp., Holliston, MA). Fluorescence was always measured with 500 nm excitation, followed by 450 nm excitation, followed again by 500 nm excitation. All results were corrected by subtracting background. The fluorescence excitation ratio \( \left( \frac{F_{500}}{F_{450}} \right) \) was calculated as the mean of the two 500-nm excitation measurements divided by the 450-nm excitation measurement. The use of the fluorescence excitation ratio provides a measurement that is unaffected by changes in the dye concentration.

In order to define the relation between the fluorescence excitation ratio and cell pH using the epifluorescent microscope, calibration studies were performed in two ways.
First, the dye was calibrated in vitro by preparing well-buffered solutions of various pH's, placing them under oil, and measuring the fluorescence excitation ratio. The results are shown in Fig. 2 (open circles) for a potassium phosphate buffer. The results were not affected by replacing potassium with sodium or by adding a bicarbonate-CO$_2$ buffer.

To determine whether the dye behaved similarly in the cell, calibration studies were performed using the method of Thomas et al. (45). Tubules were perfused with well-buffered solutions (25 mM HEPES, 60 mM phosphate, and appropriate bicarbonate concentrations) containing 10 μg/cc nigericin (a K/H antiporter) and 120 meq/liter potassium (estimated to approximate cell potassium activity [16, 19, 46]) at various pH's.

![Figure 2. Dye calibration by epifluorescent microscopy. The results of the in vitro (10 μM BCECF; open circles) and in vivo (solid circles) calibrations are shown. The pK's of the dye are shifted upward by ~0.6 pH units intracellularly.](image)

In this setting, cell pH is predicted to equal extracellular pH. Cells were loaded with BCECF before exposure to nigericin and were then perfused with the above solutions in the lumen and capillary or in the capillary alone. Both methods gave similar results. Over

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1 Because of the likelihood that the activity coefficient for potassium is lower intracellularly than extracellularly, a slightly lower extracellular potassium concentration (120 meq/liter) was used than that found for the intracellular potassium concentration by the microprobe (140 meq/liter) (46). Microelectrode studies have found intracellular potassium activities of 66 (16) and 82 meq/liter (19). Using an activity coefficient of 0.73, which Edelman et al. (19) measured for potassium in a Ringer's bicarbonate solution, the extracellular potassium activity in our solutions would be 88 meq/liter. In addition, because of the high concentrations of dibasic phosphate in our solutions, the activity coefficient would be expected to be somewhat lower than 0.73, and thus the potassium activities would be somewhat lower than 88 meq/liter. Errors in the estimation of cell potassium activity will have only a small effect on cell pH. A 20% error will lead to a 0.08 pH unit error. In addition, if a significant potassium conductance persists after nigericin application, intracellular potassium activity will move toward extracellular potassium activity.
~5–10 min, cell pH approached a steady value, which was used as the result. This method has been validated in careful studies by Chaillet (17), where pH-sensitive changes in absorbance spectra were measured. Nevertheless, these calibrations provide only an estimate of cell pH. In addition, as will be discussed later, this method of calibration yielded cell pH values similar to those obtained with pH-sensitive microelectrodes (40, 47).

The results of this calibration (solid circles, Fig. 2) demonstrate that the effect of cell pH on the fluorescence excitation ratio intracellularly is shifted from the results of the solution calibration (open circles, Fig. 2). The apparent shift in the pK of the dye intracellularly is similar to that reported by Chaillet (17) for absorbance of dimethyl-(5,6)-carboxyfluorescein in the salamander proximal tubule. All results of pH measurements will subsequently be presented using the intracellular nigericin calibration.

In some of the studies, an attempt was made to measure an initial rate of cell pH change. When cell pH changed slowly, the fluorescence excitation ratio changed linearly with time, and the initial slope was used as the initial rate (method 1). When cell pH changed rapidly, it was not possible to follow both excitation wavelengths quickly enough. Preliminary studies showed that fluorescence with 450 nm excitation was unaffected by cell pH.2 Fluorescence was therefore followed with 500 nm excitation on a chart recorder (model 689M, Hewlett-Packard Co., Palo Alto, CA) as the peritubular fluid was changed. The slope of a line drawn tangent to the initial deflection [d(F_{500})/dt] defined the initial rate of change in 500 nm fluorescence. Measurement of fluorescence with 450 nm excitation just before and after the peritubular capillary change demonstrated a small and insignificant rate of change in 450 nm fluorescence over this time period. The rate of change in the fluorescence excitation ratio [d(F_{500}/F_{450})/dt] could then be calculated using the formula:

\[ \frac{d(F_{500}/F_{450})}{dt} = \frac{[d(F_{500})/dt]}{F_{450}}, \]  

where \( F_{450} \) represents the calculated 450-nm excitation fluorescence corrected for background at the time of the peritubular capillary fluid change (interpolated from the measurements before and after the fluid change) (method 2).

Comparisons within the same tubule were made using the paired \( t \) test. Group comparisons not within the same tubule were made using the unpaired \( t \) test. In no series were all the tubules from the same rat. Calibration data were fit using linear regression. Results are reported as means ± SEM.

RESULTS

Effect of Peritubular pH on Cell pH

In the first set of studies, the effect of changes in peritubular pH on cell pH was examined (Fig. 3). In these studies, capillaries were perfused with a solution containing 25 meq/liter bicarbonate (pH 7.32; perfusate, 25 bicarbonate; Table I) in the initial control period and in the recovery period. During the experimental period, capillaries were perfused with a solution containing 5 meq/liter 2When fluorescence was measured with 450 nm excitation, the dye behaved as if at an isosbestic point (where fluorescence is independent of pH). This is due to the fact that the mercury arc lamp used for these experiments has a large intensity peak at 436 nm, the true isosbestic point of BCECF. Thus, even with a 450-nm interference filter (bandwidth, 8 nm), the dominant exciting wavelength appears to be near 436 nm. In addition, there may be a shift of the isosbestic point within the cell.
bicarbonate (pH 6.63; perfusate, 5 bicarbonate; Table I). As in most of the studies to follow, lumens were perfused throughout the experiment with the solution used in the initial control and recovery periods. Cell pH was 7.16 ± 0.02 in the initial control period, decreased to 6.90 ± 0.03 in the experimental period, and returned to 7.10 ± 0.02 in the recovery period (P < 0.001, control vs. experimental; P < 0.001, experimental vs. recovery). These experiments demonstrate the existence in the basolateral membrane of a pathway for movement of H⁺/OH⁻/HCO₃⁻.

Effect of Peritubular Sodium Concentration on Cell pH

The next set of studies was designed to examine whether sodium interacts with the basolateral membrane H⁺/OH⁻/HCO₃⁻ pathway. For these studies, the control perfusate was perfusate 25 bicarbonate (sodium concentration, 147 meq/liter), and the experimental perfusates contained 25 meq/liter sodium. Sodium was replaced by either choline or tetramethylammonium (TMA) (low-Na choline or low-Na TMA; Table I). The results are shown in Fig. 4. In the choline tubules, pH was 7.20 ± 0.03 in the control period, decreased to 6.99 ± 0.01 in the experimental period, and recovered to 7.19 ± 0.03 in the recovery period (P < 0.005, control vs. experimental; P < 0.005, experimental vs. recovery). To ensure that the observed cellular acidification was due to sodium removal rather than to choline addition, a similar set of studies was performed with TMA substitution. In these tubules, cell pH was 7.16 ± 0.02 in the control period, 7.00 ± 0.02 in the experimental period, and 7.22 ± 0.06 in the recovery period (P < 0.001, control vs. experimental; P < 0.05, experimental vs. recovery). Thus, lowering the peritubular sodium concentration leads to cell acidification.
Rate of Cell pH Change in Response to a Change in Peritubular pH

The effect of peritubular sodium concentration on cell pH could be due to a number of different mechanisms (see Discussion). To further explore the mechanism of this interaction, the rate of change in cell pH in response to altered peritubular pH was examined in the total absence of sodium and compared with the rate of change in the presence of sodium. Fig. 3 shows the effect of peritubular acidification on cell pH in the presence of sodium. As described in the Methods (method 2), it was possible to estimate an initial rate for the change in cell pH when peritubular pH was changed. A typical experiment is shown in Fig. 5, which demonstrates that fluorescence with 450 nm excitation is independent of cell pH, whereas that measured with 500 nm excitation is markedly pH dependent. Similar studies performed on six tubules gave an initial rate of cell pH change of 2.4 ± 0.3 pH units/min.

In order to repeat these studies in the absence of sodium, both lumen and peritubular capillary were perfused with a sodium-free solution (0 Na, 25 bicarbonate; Table 1) in the control period. Cell pH in this setting was 7.48 ± 0.03, a value more alkaline than in the presence of sodium (P < 0.001). Thus, removal of luminal and peritubular sodium leads to alkalinization of the cell. The implications of this finding will be discussed below.

When the peritubular capillary perfusate was then changed to a more acid but still sodium-free solution (0 Na, 5 bicarbonate; Table 1), cell pH decreased, but
the rate of change was extremely slow. Fig. 6 shows a plot of the time course of the cell pH change. From the initial slopes of these lines (method 1, Methods section), the initial rate of cell pH change was 0.2 ± 0.1 pH units/min (P < 0.001 vs. rate in the presence of sodium). Thus, the removal of sodium from luminal and peritubular fluids decreased the apparent permeability of the basolateral membrane to H⁺/OH⁻/HCO₃⁻ by >90%.

Effect of Cell Depolarization on Cell pH

To examine whether the H⁺/OH⁻/HCO₃⁻ exit step was rheogenic, the effect of cell depolarization on cell pH was examined. For these studies, the control

**Figure 5.** A typical experiment demonstrating the effect of rapid changes in peritubular pH on fluorescence with 450 and 500 nm excitation. The bars at the top of figure indicate peritubular pH and excitation wavelength (λex). Between peritubular pH bars, blood comes into the field from surrounding capillaries and partially quenches the fluorescence. When the exciting wavelength is 500 nm, changes in peritubular pH lead to rapid changes in fluorescence, the initial rate of which is shown by the straight lines. When the excitation wavelength is 450 nm, fluorescence is unaffected by changes in peritubular and cell pH (isosbestic point).
FIGURE 6. Time course of change in cell pH when peritubular pH is changed from 7.32 to 6.63 (time = 0). Both luminal and peritubular fluids contain no sodium. The ordinate shows the fluorescence excitation ratio on the left and the calculated cell pH on the right.

FIGURE 7. Effect of cell depolarization on cell pH. Capillaries were perfused with solutions containing either 5 or 50 meq/liter potassium. In the figure on the left, experiments were performed in the absence of barium; in the figure on the right, experiments were performed in the presence of 2 mM barium. The ordinate shows the fluorescence excitation ratio on the left and the calculated cell pH on the right.
and thus depolarizes the cell and blocks any effects of peritubular potassium concentration on cell potential difference (PD) (9, 12). The previous studies (changing the peritubular potassium concentration from 5 to 50 meq/liter) were repeated with 2 mM barium added to both control and experimental perfusates. Cell pH was 7.40 ± 0.06 in the control period, unchanged at 7.37 ± 0.07 in the experimental period, and again unchanged at 7.36 ± 0.08 in the recovery period (all changes were not significant [NS]) (right panel, Fig. 7). The cell alkalization observed in the control and recovery periods is consistent with the effect of barium-induced cell depolarization on a rheogenic H⁺/OH⁻/HCO₃⁻ pathway. In addition, the failure of changes in potassium concentration to affect cell pH in the presence of barium demonstrates a dependence on an intact potassium conductance, and is inconsistent with a direct interaction between potassium and the H⁺/OH⁻/HCO₃⁻ pathway.

To examine whether cell depolarization was affecting cell pH by an effect on a sodium-dependent transport mechanism, the effect of increasing peritubular potassium concentration in the absence of sodium was studied. In these experiments, sodium was totally replaced by choline in luminal and peritubular perfusates (perfusates 0 Na, 5 K and 0 Na, 50 K; Table I). Cell pH was 7.46 ± 0.03 in the control period (capillary [K⁺], 5 meq/liter), unchanged at 7.45 ± 0.03 in the experimental period (capillary [K⁺], 50 meq/liter), and 7.45 ± 0.02 in the recovery period (all changes NS) (Fig. 8). Thus, the effect of cell PD on cell pH was abolished in the absence of sodium.

Effect of Changes in Peritubular Chloride Concentration on Cell pH

To examine whether chloride interacted with the basolateral membrane H⁺/ OH⁻/HCO₃⁻ transporter, the effect of total chloride removal from the peritubular capillaries was studied. In these experiments, chloride was replaced with gluconate (perfusate 0 Cl, Table I). 6.25 mM calcium was added to this perfusate to maintain a normal ionized calcium concentration, which was measured and found to be 1.09 mM. The control perfusate for these studies (128 Cl; Table I) was similar to the standard control solution, but was designed to have the same ionized calcium concentration as in the experimental perfusate. Cell pH was 7.07 ± 0.02 in the control period, 7.08 ± 0.03 in the experimental period, and 7.10 ± 0.02 in the recovery period (P < 0.025, control vs. experimental; NS, experimental vs. recovery) (Fig. 9). Although the 0.01 pH unit increase from control to experimental was significant, the change was small and was due to a slight drift in cell pH as cell pH continued to rise further in the recovery period. Thus, the peritubular chloride concentration does not affect cell pH.

Effects of Inhibitors on the Basolateral Membrane H⁺/OH⁻/HCO₃⁻ Transport Mechanism

In order to further examine the mechanism of interaction between sodium and the H⁺/OH⁻/HCO₃⁻ transporter, the effects of SITS and amiloride were examined. In the first set of studies, capillaries were perfused with perfusate 25 bicarbonate during the control and recovery periods, and with perfusate 5 bicarbonate in the experimental period (Table I). In the absence of SITS, results
were similar to the results shown in Fig. 3. Cell pH decreased from $7.14 \pm 0.02$ in the control period to $6.92 \pm 0.03$ in the experimental period, and returned to $7.12 \pm 0.03$ in the recovery period ($P < 0.001$, control vs. experimental; $P < 0.001$, experimental vs. recovery; $n = 5$). Fig. 10 shows the results of similar studies performed in the presence of 0.5 mM SITS. SITS was added to the
perfusates 25 and 5 bicarbonate, and tubules were exposed to SITS for at least 5 min before any measurements. Cell pH was 7.52 ± 0.03 in the control period, 7.52 ± 0.03 in the experimental period, and 7.54 ± 0.02 in the recovery period (all changes NS). Thus, SITS blocked the peritubular membrane H⁺/OH⁻/HCO₃⁻ permeability and, as expected for such an effect, alkalized the cell.

If the basolateral membrane H⁺/OH⁻/HCO₃⁻ transporter is coupled to sodium, it might be predicted that SITS would also inhibit the effect of peritubular sodium concentration on cell pH. Capillaries were perfused with perfusate 25 bicarbonate in the control and recovery periods, and with perfusate low-Na choline in the experimental period (Table I). In the absence of SITS, results were similar to the results of Fig. 4. Cell pH decreased from 7.11 ± 0.06 in the control period to 6.96 ± 0.05 in the experimental period, and returned to 7.13 ± 0.07 in the recovery period (P < 0.025, control vs. experimental; P < 0.025, experimental vs. recovery; n = 3). When the experiments were repeated with 0.5 mM SITS added to both peritubular perfusates (5 min exposure before measurements), cell pH was 7.43 ± 0.02 in the control period, 7.42 ± 0.03 in the experimental period, and 7.41 ± 0.02 in the recovery period (all changes NS, Fig. 11). Thus, SITS inhibits the effects of peritubular sodium and peritubular pH on cell pH.

To further examine the possibility that the basolateral membrane transport mechanism was an electroneutral Na/H antiporter, as described in the apical membrane and other tissues, the effect of peritubular amiloride on the sodium-dependent cell acidification was examined. Because at least part of the amiloride-
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NS NS

pH 7.43±0.02 7.42±0.03 7.41±0.02

PERITUBULAR [Na⁺], meq/liter

147 25 147

FIGURE 11. Effect of peritubular sodium on cell pH in the presence of 0.5 mM SITS. Capillaries were perfused with SITS-containing solutions (0.5 mM) with either 147 or 25 meq/liter sodium. The ordinate shows the fluorescence excitation ratio on the left and the calculated cell pH on the right.

FIGURE 12. Effect of peritubular sodium on cell pH in the presence and absence of 1 mM amiloride. Capillaries were perfused with solutions containing either 50 or 0 meq/liter sodium. Lumens were perfused with a solution containing 147 meq/liter sodium. In the figure on the left, experiments were performed in the absence of amiloride; in the figure on the right, experiments were performed in the presence of 1 mM amiloride. The ordinate shows the fluorescence excitation ratio on the left and the calculated cell pH on the right.
induced inhibition of the Na/H antiporter is due to competition with sodium (24, 29), these studies were performed with relatively low peritubular sodium concentrations. Capillaries were perfused in the control and recovery periods with perfusate 50 Na, 25 bicarbonate, and in the experimental period with perfusate 0 Na, 25 bicarbonate (Table I). Throughout the experiments, lumens were perfused with perfusate 25 bicarbonate. Thus, these studies examined the effect of lowering the peritubular sodium concentration from 50 to 0 meq/liter while the lumen contained 147 meq/liter sodium.

In control studies (left panel, Fig. 12), cell pH was 7.14 ± 0.02 in the control period, decreased to 6.96 ± 0.01 in the experimental period, and returned to 7.13 ± 0.02 in the recovery period (P < 0.001, control vs. experimental; P < 0.001, experimental vs. recovery). Next, these experiments were repeated in the presence of 1 mM amiloride added to both peritubular capillary perfusates (right panel, Fig. 12). Cell pH was 7.09 ± 0.03 in the control period, decreased to 6.94 ± 0.02 in the experimental period, and returned to 7.10 ± 0.03 in the recovery period (P < 0.001, control vs. experimental; P < 0.001, experimental vs. recovery). The similar changes in cell pH observed in the absence and presence of amiloride suggest that the effect of peritubular sodium concentration on cell pH is not mediated by an electroneutral Na/H antiporter.

In order to further examine this question, the rate constants for the change in cell pH in response to lowering the peritubular sodium concentration from 50 to 0 meq/liter were compared (method 2, Methods section). In the absence of amiloride, the cell acidified at a rate of 1.4 ± 0.2 pH units/min, while in the presence of amiloride, the cell acidified at a rate of 1.3 ± 0.2 pH units/min (NS). Thus, amiloride does not inhibit the effect of the peritubular sodium concentration on cell pH.

DISCUSSION

Methods

In these studies, a method was developed for the measurement of cell pH in the in vivo doubly microperfused rat PCT using an intracellularly trapped fluorescein derivative whose excitation spectrum is exquisitely pH sensitive. Using the method of Thomas et al. (45), tubules were perfused with the acetoxymethyl derivative of BCECF, BCECF-AM. This compound does not fluoresce, is lipid soluble, and easily diffuses into cells. In cells, cytoplasmic esterases cleave off the acetoxymethyl groups, forming the negatively charged, fluorescent compound BCECF, which leaves the cell very slowly.

Thomas et al. have shown in Ehrlich ascites cells that 6-carboxyfluorescein (6-CF), loaded intracellularly in this manner, is confined to the cytoplasm and does not significantly enter mitochondria (an alkaline compartment). While similar studies have not been performed for BCECF in the proximal tubule, it is reasonable to conclude that since there are more negative charges on BCECF than 6-CF, this molecule will also not enter mitochondria. The dye may enter acid intracellular vesicles, but should not selectively accumulate in these compartments. In support of this, when proximal tubules were perfused with fluo-
rescein-bound dextran, the probe was endocytosed and appeared as fluorescent
granules in the apical cytoplasm (42, and unpublished observation). When cells
were loaded with BCECF, the dye appeared as a smooth ribbon (at magnifications
of 100 and 250), which suggests more even distribution. In summary, while some
BCECF may enter mitochondria or acidic intracellular vesicles, the majority
remains in the cytoplasm.

In order to estimate the value of cell pH, it was necessary to calibrate BCECF
intracellularly. For this purpose, the method of Thomas et al. (45) was used.
Tubules were perfused with nigericin (a K/H antiporter) and a potassium
concentration that approximated cell potassium activity (16, 19, 46), at various
external pHs. This method causes cell pH to approximate external pH. Chaillet
(17) has performed extensive studies in the salamander proximal tubule, mea-
suring pH-sensitive shifts in the absorbance spectrum of dimethyl-6-carboxyfluor-
rescein, which demonstrated that this calibration method gives cell pH values
similar to those obtained with a pH microelectrode. Indeed, the value that we
obtained for cell pH (see below) is similar to that found with pH-sensitive
microelectrodes in the rat PCT perfused in vivo (47) and in the rabbit proximal
straight tubule perfused in vitro (40). The calibration studies demonstrated that
the pK of the dye is shifted upward by 0.6 pH units intracellularly. This finding
is possibly due to protein binding and is similar to that of Chaillet (17) for
absorbance of dimethyl-6-carboxyfluorescein in the salamander proximal tubule.

Cell pH

The cell pH in these studies was 7.14 ± 0.01 (n = 32) when tubules were perfused
symmetrically (lumen and capillary) with the control perfusate (25 bicarbonate;
Table I). As stated above, this value is similar to the cell pH values of 7.17 and
7.10 obtained using pH-sensitive microelectrodes in the rat PCT and rabbit
proximal straight tubules, respectively (40, 47). This cell pH is lower than that
obtained using DMO in tubular suspensions (10, 30, 44), a difference that is
probably due to the fact that DMO measurements are disproportionately
weighted by alkaline compartments such as mitochondria (1). With a cell PD of
−70 mV (13), a cell pH of 7.14 implies that protons can passively enter the cell
but must be actively extruded.5

Effect of Peritubular pH on Cell pH

When the pH of the peritubular capillary fluid was decreased from 7.32 to 6.63,
cell pH decreased by 0.26 pH units from 7.16 to 6.90. The change in cell pH
occurred rapidly at an initial rate of 2.4 pH units/min.

This rapid change in cell pH demonstrates a high permeability to H+ /OH− /
HCO3− on the basolateral membrane, and is consistent with previous studies,
which have suggested a marked sensitivity of proximal tubular proton secretory
rate to peritubular pH (2, 4). Changes in peritubular pH lead to rapid changes
in cell pH, which will then secondarily alter the electrochemical driving forces
5 Using a cell pH of 7.14, a peritubular pH of 7.32, and a cell PD of −70 mV (13), the
electrochemical driving force for proton influx is:

\[ 61.5 \times (-\Delta pH) - PD = 61.5 \times (-0.18) + 70 = 59 \text{ mV}. \]
across the apical membrane transport mechanisms (Na/H antiporter and H⁺-ATPase) and change their rate. In addition, Aronson et al. (5) have demonstrated that cell pH can allosterically regulate the rate of the Na/H antiporter, and Schwartz and Al-Awqati (42) and Cannon et al. (15) have suggested that changes in cell pH can lead to exocytotic fusion with the apical membrane of vesicles containing proton pumps. Thus, through all of these mechanisms, the rate of acidification is exquisitely sensitive to peritubular pH and to secondary changes in cell pH (2, 4).

The fact that the cell pH change was only a fraction of the change in peritubular pH is due to the above-described apical membrane transport mechanisms, which serve not only to regulate the rate of acidification, but also to protect cell pH (38), and is possibly also due to the fact that the basolateral membrane transporter is sodium-coupled. This lesser change in cell pH than in peritubular pH agrees with the results of Struyvenberg et al. (44) in proximal tubule suspensions.

**Effects of Cell PD on Cell pH**

When cells were depolarized by increasing the peritubular potassium concentration from 5 to 50 meq/liter (9, 12), cell pH increased from 7.12 to 7.30. Electrophysiological studies have demonstrated that the addition of barium to the peritubular fluid inhibits the potassium conductance, which causes the cell to depolarize and prevents changes in peritubular potassium concentration from affecting cell PD (9, 12). When barium was added to the peritubular fluid, cell pH increased to 7.40, and was subsequently unaffected by changes in peritubular potassium concentration.

These studies demonstrate that potassium does not interact directly with the H⁺/OH⁻/HCO₃⁻ transporter, but rather that changes in cell PD can drive H⁺ into or out of the cell. The present studies do not address whether the PD-sensitive H⁺/OH⁻/HCO₃⁻ pathway is on the apical or basolateral membrane. However, electrophysiologic studies have identified a large H⁺/OH⁻/HCO₃⁻ conductance on the basolateral membrane, but not on the apical membrane of the rat and rabbit PCT (7, 13). Thus, the effect of cell PD is probably on the flux across a basolateral membrane H⁺/OH⁻/HCO₃⁻ transporter. The presence of an electrogenic H⁺/OH⁻/HCO₃⁻ transporter on the basolateral membrane is consistent with studies that have demonstrated inhibition of proximal tubular proton secretion secondary to barium-induced cell depolarization (39).

**Sodium Dependence of Basolateral Membrane Transporter**

When the sodium concentration in the peritubular capillary was lowered from 147 to 25 meq/liter, cell pH decreased by 0.2 pH units. This cellular acidification could have occurred by a number of different mechanisms: (a) parallel Na⁺ and H⁺ conductances; (b) an Na⁺/Ca²⁺ antiporter (21, 31, 33), with increased cell Ca²⁺ concentration increasing basolateral membrane H⁺ influx or decreasing apical membrane H⁺ efflux (32); or (c) a directly coupled Na⁺/H⁺ antiporter or Na⁺/HCO₃⁻ symporter, either electroneutral (1:1) (18, 23, 28, 35, 41) or electrogenic (1:>1) (11).

To further examine these possibilities, the effect of total sodium removal (luminal and peritubular) on basolateral membrane H⁺/OH⁻/HCO₃⁻ apparent
permeability was examined. When peritubular pH was decreased from 7.32 to 6.63, cell pH decreased at an initial rate of 2.4 pH units/min in the presence of sodium, and 0.2 pH units/min in the absence of sodium. This >90% inhibition is not compatible with parallel conductances (mechanism 1), as a simple H⁺/OH⁻/HCO₃⁻ conductance pathway should not be inhibited by sodium removal. In addition, electrophysiological studies have failed to find a basolateral membrane sodium conductance (7, 12). This effect of sodium removal also is not consistent with a calcium interaction (mechanism 2). When sodium is removed from the peritubular side alone, or the luminal and peritubular sides, cell calcium would be expected to rise. However, when sodium is removed from the peritubular side alone, there is an increased rate of basolateral membrane H⁺ influx, and when sodium is removed from the luminal and basolateral sides, there is a decreased rate. The results of all these studies are consistent with mechanism 3, an Na⁺/HCO₃⁻-coupled mechanism (either electroneutral or electrogenic). This mechanism is also supported by the observation that 0.5 mM SITS inhibited the effects of both peritubular pH and sodium on cell pH.

Are Sodium-dependent and PD-dependent Transporters the Same?

To examine whether the PD effect was on a sodium-dependent transporter or on a parallel pathway, the effect of cell depolarization was examined in the absence of sodium. Siebens and Boron (43) have found in the salamander proximal tubule that increasing the peritubular potassium concentration from 5 to 50 meq/liter in the absence of sodium leads to cell depolarization. When peritubular potassium was increased in the absence of sodium in the present studies, cell pH was unaffected. Thus, these studies suggest that the PD-sensitive, basolateral membrane H⁺/OH⁺/HCO₃⁻ transporter is sodium dependent. In addition, the failure of amiloride to inhibit the effect of sodium on cell pH provides evidence against a basolateral membrane electroneutral Na/H antiporter (24, 29).

The finding of an electrogenic NaHCO₃ cotransporter agrees with electrophysiological studies in the Ambystoma and Necturus proximal tubule, and in the rat and rabbit PCT, which have all found that lowering the peritubular sodium concentration depolarizes the cell (7, 11, 12, 33). If lowering the peritubular sodium concentration acidified the cell by a nonelectrogenic mechanism parallel to the H⁺/OH⁺/HCO₃⁻ conductance, then the more acid cell would have hyperpolarized. In addition, if a significant sodium conductance existed, the cells should have hyperpolarized. Biagi (7) actually found that lowering the peritubular sodium concentration caused a spike depolarization in the rabbit PCT, which suggests a sodium-coupled electrogenic process.

In more recent work, Biagi and Brown (8) have examined the time course of inhibition of basolateral membrane conductances by SITS. Both the spike depolarization in response to lowering the peritubular bicarbonate concentration and the spike depolarization in response to lowering the peritubular sodium concentration were inhibited within 1 min by SITS (Biagi, B., personal communication). Inhibition of the steady state depolarization in response to lowering the sodium concentration, the secondary increase in the potassium transference number, and the secondary cell hyperpolarization, however, did not occur until
~4-6 min after the application of SITS. The reason for the delay in these secondary effects is presently not clear, but the simultaneous inhibition of the bicarbonate- and sodium-dependent spikes is consistent with a cotransport process.

**Chloride Dependence of Basolateral Membrane Transporter**

In order to examine whether chloride interacted with the basolateral membrane transporter, the effect of chloride removal from the peritubular fluid was examined. When the peritubular chloride concentration was decreased from 128 to 0 meq/liter, cell pH was unaffected. These results imply that the presently described transporter does not interact with chloride.

Recently, Grassl et al. (22) have found a Cl⁻/HCO₃⁻ antiporter in basolateral membrane vesicles from rabbits. In agreement with this, Nakhoul and Boron (36) have found a basolateral membrane Cl⁻/HCO₃⁻ exchanger in the rabbit proximal straight tubule. The present studies find no evidence for a Cl⁻/HCO₃⁻ exchanger in the basolateral membrane. However, Fisher et al. (20) have found that the Cl⁻/HCO₃⁻ exchanger on the basolateral membrane of the turtle bladder has a \( K_m \) for chloride of 0.13 mM. While we have measured our perfusates and found that the chloride concentration is <0.05 meq/liter, it is unlikely that the interstitial chloride concentration is this low. We therefore cannot eliminate the possibility of a high-affinity Cl⁻/HCO₃⁻ exchanger. The absence of an effect of chloride concentration in the peritubular space, however, agrees with the results of transport studies in the rabbit PCT, which have found that total luminal and peritubular chloride replacement does not affect the rate of acidification (14, 39).

**General Comments**

The present studies demonstrate that a sodium-coupled, chloride-independent, electrogenic H⁺/OH⁻/HCO₃⁻ transport mechanism is present in the basolateral cell membrane of the PCT. The fact that this transporter is electrogenic suggests that the HCO₃⁻:Na⁺ stoichiometry is >1.

This transporter is very similar to the SITS-sensitive, electrogenic Na(HCO₃)ₙ (\( n > 1 \)) transporter recently found by Boron and Boulaep (11) in the basolateral membrane of the salamander proximal tubule. Jentsch and co-workers (25, 26) have also recently described such a transporter in cultured bovine corneal epithelium. In the present study, we have not addressed whether the basolateral membrane transporter actually transports HCO₃⁻ or H⁺/OH⁻ (i.e., requires CO₂), because of the difficulty in removing CO₂ from the tubule in vivo.

The apical membrane acidification mechanism is at least partially an electro-neutral Na/H antiporter (18, 23, 28, 35, 41). In the present studies, when sodium was removed completely from luminal and peritubular fluids (presumably stopping all sodium-dependent transport processes), cell pH became alkaline relative to control. In this setting, cell pH is far above electrochemical equilibrium, sodium-dependent apical and basolateral membrane transport mechanisms are inoperative, and it might be predicted that basal metabolism would generate
acid, which would acidify the cell. The cell alkalinity observed in this setting thus suggests the existence of a sodium-independent mechanism for proton efflux from the cell, possibly an apical membrane proton translocating ATPase, as has been suggested (6, 18, 23, 27, 42).

The basolateral membrane transporter must transport bicarbonate or its equivalent from the cell into the peritubular space. The process can occur passively, as there is an ∼59-mV electrochemical driving force favoring it. Each sodium ion has a 123-mV driving force favoring sodium entry into the cell, which if coupled to bicarbonate transport in a 1:1 or 1:2 ratio would promote bicarbonate influx rather than efflux. However, with a stoichiometry of 1 sodium to 2 bicarbonate ions, the estimated driving forces are such that the transporter is only 5 mV away from functioning in the bicarbonate efflux direction. Thus, small errors in the actual cell pH or PD would allow a 1:2 stoichiometry. Future studies will be required to resolve the exact stoichiometry, but the electrogenicity implies that there are at least two bicarbonate ions for each sodium ion.

An Na⁺(HCO₃⁻)₂ symporter might have advantages for protection of cell pH. In the presence of a large, simple H⁺/OH⁻/HCO₃⁻ permeability, the equilibrium cell pH would be 6.18, and it would be difficult to maintain a cell pH of 7.14. If the only mechanism for basolateral membrane proton influx were coupled to sodium, the driving force for proton influx would be smaller, and it would be simpler to defend cell pH.

In summary, the present studies demonstrate that fluorescent probes provide a sensitive method for measurement of cell pH in vivo. Using this method, we have shown that >90% of proton influx (bicarbonate efflux) across the basolateral membrane occurs by an electrogenic, sodium-coupled H⁺/OH⁻/HCO₃⁻ transporter. Thus far, this transporter has only been described in some epithelia that effect vectorial transport of sodium bicarbonate (e.g., proximal renal tubule, corneal epithelium [11, 25, 26]). Whether this transport mechanism is present in other sodium bicarbonate-transporting epithelia (e.g., intestine, pancreas, biliary tract, choroid plexus) deserves further study.

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Using a cell sodium concentration (Naₙ) of 20 meq/liter (46), a peritubular sodium concentration (Naₚ) of 147 meq/liter, and a cell PD of −70 mV (13), the electrochemical driving force for sodium influx is:

\[61.5 \log(\text{Na}_p/\text{Na}_n) - \text{PD} = (61.5 \times 0.87) + 70 = 123 \text{ mV}.

Assuming a simple H⁺/OH⁻/HCO₃⁻ pathway, a cell PD of −70 mV (13), and a peritubular pH (pHₚ) of 7.32, the equilibrium cell pH (pHₑ) would be:

\[\text{pH}_e = \frac{\text{PD} + (61.5 \times \text{pH}_p)}{61.5} = 6.18.

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