Bicarbonate Transport Mechanisms in the *Ambystoma* Kidney Proximal Tubule: Transepithelial Potential Measurements

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Received August 17, 1990

Modes of bicarbonate entry from tubule lumen to cell were examined in isolated *Ambystoma* proximal tubules, using determinations of transepithelial potential differences (ΔVj). (1) Upon removal of luminal substrate, tubules first equilibrated in bilateral (lumen and bath) 94.72 mM Cl– and 10 mM HCO3– yielded a change in Vj between the experimental and control circumstances of +1.8 mV (ΔVj). (2) The identical experiment conducted under the condition of symmetrical 4.72 mM Cl– produced a ΔVj of +7.6 mV. This reduction of luminal and bath Cl– generates an amplification of ΔVj by a factor of 4.4 and reflects a substantial increase in the paracellular Cl– shunt resistance. Ensuing experiments were conducted in bilateral nominally Cl–-free solutions and in the absence of luminal substrate. The experimental protocols are divided into several situations where HCO3– is removed from the lumen, bath, or lumen and bath; the HCO3– removal sequences are repeated in the presence of luminal SITS and then after SITS washout. 0.5 mM SITS (4-acetoamido-4-isothiocyanostilbene-2,2’-disulfonate) was applied exclusively to the luminal perfusate. (1) Removal of luminal HCO3– in the absence of SITS produces a ΔVj of −1.9 mV whereas, in the presence of SITS, the ΔVj measures −1.3 mV. Subsequent removal of luminal HCO3– in the presence of bath HCO3– (in the presence of luminal SITS) yields a ΔVj of −1.0 mV. All of these measurements reflect a decrease in HCO3– current across the basolateral membrane Na+ (HCO3–) co-transporter; the role of a possible Cl–/Anion– antiport cannot be assessed. (2) Removal of bath HCO3– in the absence of SITS yields a ΔVj of +1.5 mV whereas, in the presence of SITS, the ΔVj value measures +1.2 mV. Subsequent removal of bath HCO3– in the absence of luminal HCO3– (in the presence of SITS) yields a ΔVj of +0.8 mV. These experiments are consistent with an increase in HCO3– current across the basolateral Na+ (HCO3–) co-transporter, do not rule out the possibility of an apical HCO3– conductance pathway, and diminish the likelihood of an apical Cl–/HCO3– antiport system.

INTRODUCTION

The role of pH regulation and HCO3– transport cannot be underestimated since control of intracellular pH is essential to cell growth, differentiation, endocrine processes, and metabolism. The process of intracellular acidification may be mediated by the efflux of base (i.e., HCO3– ions or related equivalent) across the cell membrane. If this mechanism carries a net charge across the cell membrane, then the system is termed a “rheogenic HCO3– transport process.” Rheogenic intracellular acidification may occur via the Na+ (HCO3–) co-transporter or a conductive HCO3– pathway. In both transport models, HCO3– efflux from the intracellular milieu promotes intracellu-

Abbreviations: ΔVj: change in Vj PVP: polyvinylpyrrolidone SITS: 4-acetoamido-4-isothiocyanostilbene-2,2’-disulfonate Vj: transepithelial potential differences

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lar acidification. The following section presents documentation of rheogenic HCO$_3^-$ pathways within isolated tubule, single cell, and membrane vesicle preparations; in vivo micropuncture data are only described for rat renal HCO$_3^-$ transport mechanisms, as the majority of such studies have employed the microperfused rat kidney preparation.

**The Na$^+$ (HCO$_3^-$)$_n$ Co-Transporter**

The Na$^+$ (HCO$_3^-$)$_n$ symporter system functions independently of Cl$^-$ movement and is inhibited by disulfonic stilbene derivatives; the stoichiometry for this mechanism as HCO$_3^-$ equivalent per Na$^+$ equivalent exceeds unity, ~3, and appears to be highly species-specific. The rheogenic Na$^+$ (HCO$_3^-$)$_n$ transporter has been first identified in the basolateral membrane of *Ambystoma* proximal tubule cells [1] and has also been found in individual cultured monkey kidney cells [2] and bovine corneal endothelium [3]. Electrophysiological studies in *Ambystoma* proximal tubule basolateral membrane [1] demonstrate outward movement of HCO$_3^-$, Na$^+$, and negative charge, which require a stoichiometry of at least 2 HCO$_3^-$ for 1 Na$^+$, whereas, in *Necturus* proximal tubule, a ratio of 3 HCO$_3^-$ to 1 Na$^+$ is necessary [4]. Experiments in mammalian proximal tubule epithelia provide further evidence for the existence of a rheogenic Na$^+$ (HCO$_3^-$)$_n$ co-transport system. Investigations in isolated perfused rabbit proximal tubules delineate a SITS-sensitive Na$^+$ (HCO$_3^-$)$_n$ symporter located within the basolateral membrane using electrophysiological measurements [5,6,7], using a pH-sensitive fluorometric dye [8] or basolateral membrane vesicles [9,10]. The rabbit basolateral membrane vesicle experiments by Akiba et al. are consistent with a co-transport stoichiometry of 2 HCO$_3^-$:1 Na$^+$ [9]. Using fluorescent dyes in *in vivo* rat proximal tubule preparations, a Na$^+$ (HCO$_3^-$)$_n$ symporter is found which is inhibited by disulfonic stilbenes [11,12], and an electrophysiological study suggests a stoichiometry of 3 HCO$_3^-$:1 Na$^+$ [13]. Rat renal cortex membrane vesicle data, using a $^{22}$Na$^+$ tracer technique, are congruent with *in vivo* findings [14]. Recent evidence has firmly established the stoichiometry of the Na$^+$ (HCO$_3^-$)$_n$ symporter in the mammalian proximal tubule as 3 HCO$_3^-$:1 Na$^+$ [15].

**The Conductive HCO$_3^-$ Pathway**

Demonstration of a conductive HCO$_3^-$ pathway requires several lines of evidence: (1) a rapid depolarization across a given membrane in the presence of low HCO$_3^-$ bathing solutions, (2) a concomitant decrease in intracellular pH, (3) requirement that the effect be independent of Na$^+$ and that any altered K$^+$ conductance subsequent to a change in intracellular pH be excluded (e.g., the application of Ba$^{++}$ can be used to block K$^+$ channels), and (4) a persistent effect in the presence of disulfonic stilbenes. If the experimental data fulfill these criteria, it is likely that such an intracellular acidification mechanism exists within a given cell. Some epithelia may exhibit two modes of rheogenic HCO$_3^-$ transport: the conductive HCO$_3^-$ pathway and the Na$^+$ (HCO$_3^-$)$_n$ co-transporter as shown in *Necturus* proximal tubule [4,16]. Evidence for a conductive HCO$_3^-$ pathway within *Necturus* proximal tubule is suggested by experiments of Matsumura et al., who found basolateral membrane depolarizations mediated by HCO$_3^-$ efflux, independent of K$^+$ conductance changes and uninhibitable with 4-acetoamido-4-isothiocyanostilbene-2,2'-disulfonate (SITS) [16]. Data from *in vitro* mammalian proximal tubules, however, are less well defined. The possibility of a conductive HCO$_3^-$ pathway in the rabbit proximal tubule basolateral membrane was
initially proposed by Sasaki and Berry, who found that Ba\(^{++}\) blockade of the acetazolamide-sensitive component of proximal absorption produced a 41 percent decrease in net CO\(_2\) absorption and symmetrical deletion of Cl\(^-\) decreased net CO\(_2\) absorption by 30 percent [17]. These microcalorimetric data are, however, merely suggestive of HCO\(_3^-\) efflux mediated by a conductive pathway. Furthermore, these investigations are refuted by direct electrophysiological measurements in which the basolateral membrane depolarization observed under the condition of low bath HCO\(_3^-\) was obliterated with Ba\(^{++}\) application and, therefore, appears to be a function of K\(^+\) conductance [18]. Likewise, the rapid, spiking basolateral membrane depolarization observed within in vivo rat proximal tubules in response to low extracellular HCO\(_3^-\) [19,20] appears to be largely due to the rheogenic Na\(^+\)(HCO\(_3^-\))\(_n\) co-transport system [11,12,13]. Nonetheless, experiments measuring basolateral membrane potential changes in the symmetrical absence of HCO\(_3^-\) demonstrate a persistent basolateral membrane depolarization which is not inhibited by SITS or Ba\(^{++}\) and remains in the absence of luminal Na\(^+\) [21]. It is possible that a conductive HCO\(_3^-\) pathway manifests itself only under conditions of HCO\(_3^-\) deletion or operates, silently, in parallel with the Na\(^+\) (HCO\(_3^-\))\(_n\) co-transporter. Rat renal membrane vesicle studies have shown the presence of a H\(^+\)/OH\(^-\) conductance in basolateral membrane vesicles [22].

**Experimental Hypothesis**

In *Ambystoma* isolated perfused proximal tubules, under the conditions of zero substrate and symmetrical Cl\(^-\) deletion, a transepithelial potential difference of approximately \(-2.0\) mV is still measurable. Possible ionic mechanisms for this potential difference include apical Na\(^+\) channels which permit residual Na\(^+\) entry along with minimal functioning of Na\(^+\)-substrate symport or a reversal of the basolateral Na\(^+\) (HCO\(_3^-\))\(_n\) co-transporter. This study examines the role of rheogenic HCO\(_3^-\) transport mechanisms using transepithelial potential recordings in the microperfused, in vitro *Ambystoma* proximal tubule through serial lumen, bath, and lumen plus bath HCO\(_3^-\) deletions in the presence and absence of luminal SITS (4-acetamido-4-isothiocyanostilbene-2,2'-disulphonate).

**MATERIALS AND METHODS**

**Animals**

Female neotenic tiger salamanders (*Ambystoma tigrinum*) were procured from New Mexico and Arizona by Charles Sullivan (Nashville, TN). Salamanders ranging between 55.0 and 98.0 grams were selected for kidney dissection. Anesthesia was induced by immersion in 0.2 percent tricaine methanesulfonate (titrated to a pH of 7.5 with NaHCO\(_3\)). The kidneys were dissected by standard technique [23] and then quickly immersed in a bathing solution of standard substrate Ringer (solution a, Table 1). Each kidney was divided into five transverse sections; those segments reserved for future dissection were maintained in solution a (bubbled with 98.5 percent O\(_2\) and 1.5 percent CO\(_2\), refer to Table 1) on a bed of ice. Tubules were initially dissected under 50 × magnification; final trimming and dilation of tubule orifices required 200 × magnification. The tubule was severed from the glomerulus at a distance of approximately 300 μm. Proximal tubule lengths ranged between 1,400–2,400 μm (mean = 1,871 ± 365 μm; n = 19).
TABLE 1
Composition of Solutions: Standard Ringer and 0-Substrate Ringer Solutions

|       | a     | b     | c     | d     |
|-------|-------|-------|-------|-------|
| Na⁺   | 100.95| 100.90| 100.95| 103.40|
| K⁺    | 2.50  | 2.50  | 2.50  | 2.50  |
| Mg²⁺  | 1.00  | 1.00  | 1.00  | 1.00  |
| Ca²⁺  | 1.80  | 1.80  | 1.80  | 1.80  |
| Lys⁺  | 0.22  | —     | 0.22  | 0.22  |
| mM (+) | 106.47| 106.20| 106.47| 108.92|
| mEq (+)| 109.27| 109.00| 109.27| 111.72|
| Cl⁻   | 94.72 | 98.10 | 94.72 | 94.72 |
| HCO₃⁻ | 10.00 | 10.00 | 10.00 | 10.00 |
| H₂PO₄⁻| 0.10  | 0.10  | 0.10  | 0.10  |
| HP0₄⁻ | 0.40  | 0.40  | 0.40  | 0.40  |
| Glu⁻  | 0.05  | —     | 0.05  | 0.05  |
| Lac⁻  | 3.60  | —     | 3.60  | 3.60  |
| X⁻    | —     | —     | —     | 2.45  |
| mM (-) | 108.87| 108.60| 108.87| 111.32|
| mEq (-)| 109.27| 109.00| 109.27| 111.72|
| Glucose| 2.22  | —     | 2.22  | 2.22  |
| Gln   | 0.52  | —     | 0.52  | 0.52  |
| Ala   | 0.55  | —     | 0.55  | 0.55  |
| PVP (15 g/l)| —   | —     | +     | —     |
| Dye   | —     | —     | —     | +     |

Solutions are in mM unless indicated otherwise. Solution a, standard substrate Ringer. Solution b, zero-substrate Ringer. Solution c, PVP standard substrate Ringer used for proximal tubule dissections. Solution d, dye standard substrate Ringer. All solutions were equilibrated with 98.5 percent O₂ and 1.5 percent CO₂ and had a pH of 7.5. Solution c contained 15 g/l PVP (polyvinylpyrrolidone), GAF Corporation Plasdone C, average molecular weight 40,000 daltons. Solution d included 1 g/l (0.1 percent) Hercules green dye No. 1, which contains an additional 2.45 mM Na⁺ as compared to Solution a. X⁻ refers to the anion fraction of the dye in Solution d.

Perfusion of Proximal Tubules

The standard technique of Ambystoma proximal tubule perfusion is fully described by Sackin and Boulpaep [23]; an outline of this method is provided below. The perfusion apparatus was equipped with six gravity-feed reservoirs for rapidly altering luminal perfusates. The bath chamber also included two gravity-feed reservoirs; the bathing solution could be replaced in less than five seconds, as determined empirically with timing of dye clearance. Each solution was delivered through CO₂-impermeable Saran tubing (Clarkson Equipment and Controls, Detroit, MI). The tubule was clearly visualized in the bathing chamber with a Zeiss inverted microscope and then gently attached to the holding pipette with suction (this force was immediately released upon tubule attachment and returned to atmospheric pressure), and a perfusion pipette was gradually introduced into the tubule lumen. This maneuver effectively forms a seal that is mechanically and electrically tight. A similar concentric pipette arrangement was connected to the distal end of the tubule (Fig. 1). Luminal perfusate is collected by the innermost pipette on the left-hand side of Fig. 1; this collection pipette is connected to a suction source. All perfusion rates were maintained at greater than 20 nl/minute to prevent significant alteration of fluid composition as a result of reabsorption along the tubule length.

Prior to initiating each experiment, tubular integrity was assessed by luminal
perfusion with standard substrate Ringer solution with 0.1 percent Hercules green dye No. 1 (H. Kohnstamm, New York); either standard dye substrate Ringer solution (i.e., solution \( d \) or solution \( c' \), Tables 1 and 2) was employed, depending upon the experimental protocol requirements. As reported previously, individual cellular staining is

![Diagram of in vitro perfusion apparatus](image)

**FIG. 1.** Illustration of *in vitro* perfusion apparatus as described within the Materials and Methods section. Adapted and modified from [23], with permission from the authors.

| TABLE 2 Composition of Solutions: Low Cl\(^-\) Standard HCO\(_3\)^- Ringer and 0-Substrate Standard HCO\(_3\)^- Ringer Solutions |
|---|---|---|
| | \( a' \) | \( b' \) | \( c' \) |
| Na\(^+\) | 100.95 | 100.90 | 103.40 |
| K\(^+\) | 2.50 | 2.50 | 2.50 |
| Mg\(^{++}\) | 1.00 | 1.00 | 1.00 |
| Ca\(^{++}\) | 1.80 | 1.80 | 1.80 |
| Lys\(^+\) | 0.22 | — | 0.22 |
| mM (+) | 106.47 | 106.20 | 108.92 |
| mEq (+) | 109.27 | 109.00 | 111.72 |
| Cl\(^-\) | 4.72 | 4.50 | 4.72 |
| HCO\(_3\)^- | 10.00 | 10.00 | 10.00 |
| H\(_3\)PO\(_4\)^- | 0.10 | 0.10 | 0.10 |
| HPO\(_4\)^{2-} | 0.40 | 0.40 | 0.40 |
| Glu\(^-\) | 0.05 | — | 0.05 |
| Lac\(^-\) | 3.60 | — | 3.60 |
| Cyclamate\(^-\) | 90.00 | 93.60 | 90.00 |
| X\(^-\) | — | — | 2.45 |
| mM (-) | 108.87 | 108.60 | 111.32 |
| mEq (-) | 109.27 | 109.00 | 111.72 |
| Glucose | 2.22 | — | 2.22 |
| Gln | 0.52 | — | 0.52 |
| Ala | 0.55 | — | 0.55 |
| Dye | — | — | + |

Solutions are in mM unless indicated otherwise. *Solution a’,* low Cl\(^-\) standard HCO\(_3\)^- substrate Ringer. *Solution b’,* low Cl\(^-\) zero substrate standard HCO\(_3\)^- Ringer. *Solution c’,* low Cl\(^-\) dye standard HCO\(_3\)^- substrate Ringer. All solutions were equilibrated with 98.5 percent O\(_2\) and 1.5 percent CO\(_2\) and titrated to a pH of 7.5 with 0.5 M NaOH and 0.5 M cyclamic acid. *Solution c’* included 1 g/l (0.1 percent) Hercules green dye No. 1. X\(^-\) refers to the anion dye fraction in *Solution c’*.
TABLE 3
Composition of Solutions: Low Cl\textsuperscript{−} Standard HEPES and 0-Substrate HEPES Solutions

|          | \(d'\) | \(e'\) |
|----------|--------|--------|
| Na\textsuperscript{+} | 97.65  | 97.60  |
| K\textsuperscript{+}  | 2.50   | 2.50   |
| Mg\textsuperscript{2+} | 1.00   | 1.00   |
| Ca\textsuperscript{2+} | 1.80   | 1.80   |
| Lys\textsuperscript{+} | 0.22   | —      |
| mM (+) | 103.17 | 102.90 |
| mEq (+) | 105.97 | 105.70 |
| Cl\textsuperscript{−} | 4.72   | 4.50   |
| HCO\textsubscript{3}− | —      | —      |
| H\textsubscript{2}PO\textsubscript{4}− | 0.10   | 0.10   |
| HPO\textsubscript{4}2− | 0.40   | 0.40   |
| HEPES\textsuperscript{−} | 6.70   | 6.70   |
| Glu\textsuperscript{−} | 0.05   | —      |
| Lac\textsuperscript{−} | 3.60   | —      |
| Cyclamate\textsuperscript{−} | 90.00  | 93.60  |
| mM (−) | 105.57 | 105.30 |
| mEq (−) | 105.97 | 105.70 |
| HEPES (neutral) | 6.70 | 6.70 |
| Glucose | 2.22 | — |
| Gln | 0.52 | — |
| Ala | 0.55 | — |

Solutions are in mM unless indicated otherwise. Solution \(d'\), low Cl\textsuperscript{−} standard HEPES. Solution \(e'\), low Cl\textsuperscript{−} zero substrate HEPES. All solutions were equilibrated with 100 percent O\textsubscript{2} and titrated to a pH of 7.5 with 0.5 M NaOH and 0.5 M cyclamic acid. HEPES was purchased from Sigma Chemical Co. (St. Louis, MO).

0.275 g/l (0.5 mM) SITS (4-acetoamido-4-isothiocyanostilbene-2,2'-disulfonate) was added to solutions in Tables 2 and 3 according to experimental protocol. SITS was obtained from International Chemical and Nuclear (Cleveland, OH).

associated with tubular damage; therefore, such tubules were excluded from all studies [23].

Composition of Solutions

The composition of solutions used in both the standard substrate Ringer and the low Cl\textsuperscript{−} experiments are fully described in Table 1, Table 2, and Table 3 [3]. Solution c (Table 1) was used for fine tube dissections as the addition of polyvinylpyrrolidone (PVP) substantially decreases electrostatic interactions between the dissecting forceps and isolated proximal tubule. For microperfusion, no colloids or albumin were added to the bath. Solutions were prepared fresh daily, titrated to a pH of 7.5, and equilibrated with appropriate gases at least 30 minutes before experimentation. All trials were conducted in an average ambient temperature of 22 ± 1 degrees Celsius.

Measurement of Transepithelial Potential Difference

Determination of the transepithelial potential difference (\(V_3\)) is schematically represented in Fig. 1. A complete electrical circuit was achieved by a 3 percent agar standard substrate Ringer solution bridge, which was immersed into the luminal perfusate solution. Either solution a or solution a' was used for the agar bridges, depending upon whether the trial was a physiological saline or low Cl\textsuperscript{−} experimental
sequence; refer to Tables 1 and 2. The opposite end of the agar bridge contacted an AgAgCl half-cell holder filled with the experimentally appropriate standard substrate Ringer solution, as described above. A half-cell symmetrical to the perfusion half-cell was placed in the bathing solution and connected to ground. Transepithelial potential differences (the voltage potential difference between lumen and bath) were determined with high-impedance electrometers (W-P Instruments, New Haven, CT, Model 750) and recorded on a chart recorder (Rikadenki, Model B-161).

Theoretically, in the situation of symmetrical solutions (i.e., solution a, as perfusate, bath, agar bridges, and half-cell solution), liquid junction potentials should be nonexistent. With different solutions, however, measurable liquid junction potentials can be anticipated. For this reason, all junction potentials were measured directly against an ideal flowing 3M KCl reference electrode, and appropriate corrections were only included in recorded data for circumstances where measurable junction potentials were obtained [24]. Measurable liquid junction potentials ranging from −0.3 to −2.6 mV were only detected in all cases where standard HEPES solution (solution d', refer to Table 3) served as the bathing solution. Therefore, in these circumstances, the recorded junction potential corresponding to each tubule experiment was subtracted from the actual recording. Such corrections were not necessary in the other experimental situations.

Experimental Protocols

Prior to initiating the extensive transepithelial potential determinations in low Cl⁻ media, five tubules were first equilibrated in bilateral (bath and lumen) 94.72 mM Cl⁻ with a symmetrical HCO₃⁻ source and exposed to sequential switches from luminal standard substrate Ringer (solution a, Table 1) to luminal 0-substrate standard Ringer (solution b, Table 1) while maintaining a constant standard substrate Ringer bath (solution a, Table 1). Measurements were not made until a steady state was achieved, usually within one to two minutes.

Thereafter, low Cl⁻ solution trials were started in an effort to disclose small transepithelial potential differences (V2) in the presence and absence of luminal and basolateral HCO₃⁻ (i.e., a low Cl⁻ state effectively increases paracellular resistance, as this pathway represents the major route for chloride current in the "leaky" proximal tubule membranes of Ambystoma). Experiments were completed on 14 acceptable proximal tubules with an average testing duration of 4.5 ± 0.6 hours. Schematically, the experimental sequence can be divided into three portions: pre-SITS trials, SITS trials, and post-SITS (control) trials.

All perfusion experiments were completed between August and November 1986.

RESULTS

Effect of Organic Substrate Removal on Transepithelial Potential at Physiological Concentrations of Cl⁻

Initially, transepithelial potential recordings from five Ambystoma proximal tubules were examined in the presence and absence of luminal substrate. The luminal perfusate was either standard substrate HCO₃⁻ Ringer (solution a) or 0-substrate standard HCO₃⁻ Ringer (solution b), whereas the bathing solution was consistently standard substrate HCO₃⁻ Ringer (solution a) in both trials (refer to Table 1 for
TABLE 4
Direct Measurements of Transepithelial Potential Differences (V3): Low Cl⁻ Experimental Protocol

| Trial No. | Lumen                   | Bath                   | n    | $\bar{x} \pm$ SD (in mV) |
|-----------|-------------------------|------------------------|------|--------------------------|
| I         | Standard Ringer (Solution a') | Standard Ringer (Solution a') | 56   | $-10.2 \pm 4.1$ mV       |
| II        | 0-Substrate Ringer (Solution b') | Standard Ringer (Solution a') | 84   | $-2.2 \pm 1.2$ mV        |
| III       | 0-Substrate HEPES (Solution e') | Standard Ringer (Solution a') | 84   | $-3.4 \pm 2.0$ mV        |
| IV        | 0-Substrate HEPES (Solution e') | Standard HEPES (Solution d') | 42   | $-2.0 \pm 1.4$ mV        |
| V         | 0-Substrate HEPES* (Solution e'* ) | Standard Ringer (Solution a') | 42   | $-2.9 \pm 2.0$ mV        |
| VI        | 0-Substrate Ringer* (Solution b'* ) | Standard Ringer (Solution a') | 42   | $-1.5 \pm 1.6$ mV        |
| VII       | 0-Substrate HEPES* (Solution e'* ) | Standard HEPES (Solution d') | 28   | $-1.6 \pm 2.2$ mV        |
| VIII      | 0-Substrate Ringer* (Solution b'* ) | Standard HEPES (Solution d') | 56   | $-0.7 \pm 1.3$ mV        |
| IX        | 0-Substrate HEPES (Solution e') | Standard HEPES (Solution d') | 28   | $-1.7 \pm 1.6$ mV        |
| X         | 0-Substrate HEPES (Solution e') | Standard Ringer (Solution a') | 56   | $-2.7 \pm 2.0$ mV        |
| XI        | 0-Substrate Ringer (Solution b') | Standard Ringer (Solution a') | 42   | $-1.8 \pm 1.7$ mV        |
| XII       | Standard Ringer (Solution a') | Standard Ringer (Solution a') | 28   | $-5.8 \pm 3.1$ mV        |

Table 4 was derived from experiments completed on 14 proximal tubules (August to November 1986). Solution compositions and letter designations are described fully in Tables 2 and 3. $n =$ Number of direct observations. SD = Standard deviation. $\bar{x} =$ Average measurement. $t$-values for the independent data presented in this chart can be found in Table 6. Refer to Legend 1 for experimental key. * = those solutions containing 0.5 mM SITS (refer to Table 3).

solution compositions). Each solution contained physiological concentrations of chloride (Table 1).

In the presence of substrate, the lumen demonstrates a potential difference of $-2.3 \pm 0.5$ mV ($n = 12$) with respect to the bath. This finding is expected, as the major absorption of sodium occurs along the proximal tubule and is co-transported with glucose and various amino acids. If the [Na plus substrate] influx represents the only source of current, one anticipates a progression toward zero potential with the removal of luminal substrate; however, this result is not the case. Experimentally, removal of luminal substrate leaves a small, yet persistent, transepithelial potential difference of $-0.5 \pm 0.3$ mV ($n = 11$). The effect of luminal substrate removal is statistically significant ($p < 0.001$ with df = 21; unpaired Students' $t$-test). Likewise, the difference in potential $\Delta V_3$ between the condition of luminal standard substrate Ringer and 0-substrate standard Ringer (solution a to b) and vice versa (solution b to a) with a constant standard substrate Ringer bath (solution a), shows an average $\Delta V_3$...
**Table 5**
Comparisons for Independent Data Derived from Table 4; Transepithelial Potential Differences (V₃):
Low Cl⁻ Experimental Protocol

| Comparison (control versus experimental) | [df] | t-value | p       |
|------------------------------------------|------|---------|---------|
| Pre-SITS Trials                          |      |         |         |
| I versus II                              | 138  | 5.3     | <0.001  |
| II versus III                            | 166  | 5.1     | <0.001  |
| III versus IV                            | 124  | 3.9     | <0.001  |
| SITS Trials                              |      |         |         |
| VI versus V                              | 96   | 3.9     | <0.01   |
| V versus VII                             | 68   | 2.9     | <0.01   |
| VIII versus VII                          | 82   | 3.0     | <0.01   |
| VI versus VIII                           | 110  | 2.8     | <0.01   |
| Post-SITS Trials                         |      |         |         |
| IX versus X                              | 82   | 2.4     | <0.02   |
| X versus XI                              | 96   | 2.4     | <0.02   |
| XI versus XII                            | 68   | 7.0     | <0.001  |
| Pre-versus Post-SITS Trials              |      |         |         |
| IX versus IV                             | 68   | 0.8     | <0.4    |
| X versus III                             | 138  | 2.0     | <0.05   |
| XI versus II                             | 124  | 1.4     | <0.2    |
| XII versus I                             | 82   | 5.4     | <0.001  |
| Effect of SITS Addition (Control)        |      |         |         |
| II versus VI                             | 138  | 2.8     | <0.1    |
| III versus V                             | 124  | 1.2     | <0.3    |
| IV versus VII                            | 68   | 1.1     | <0.3    |

Table 5 represents analysis of data presented in Table 4, using comparisons for independent data with calculated t-values using the unpaired Students' t-test. df = degrees of freedom. t = t-value. p = probability. Refer to Legend 1 and Table 4 for appropriate key.

of +1.8 ± 0.2 mV (n = 16) and a significant statistical difference (p < 0.001, df = 15; paired Students' t-test).

To investigate further the ionic current source(s) for this persistent luminal potential difference after substrate deletion, Cl⁻ was symmetrically removed from the lumen and bath. The *Ambystoma* proximal tubule epithelium behaves as a "leaky" membrane, where Cl⁻ represents the primary carrier of paracellular current. Therefore, symmetrical deletion of apical and basolateral Cl⁻ will substantially increase transepithelial resistance and thereby amplify V₃. Indeed, evidence for the anticipated amplification of transepithelial potential recordings is shown in Table 4, where symmetrical removal of Cl⁻ yields a potential difference of −10.2 ± 4.1 mV (n = 56) for tubules exposed to luminal and bath low Cl⁻ standard substrate Ringer (solution a', refer to Table 2). This value is statistically different from the identical experiment under physiological Cl⁻ conditions (p < 0.001, df = 66; unpaired Students' t-test) and demonstrates an increase in V₃ by a factor of 4.4. Likewise, luminal substrate removal (solution b, Table 2) in the low Cl⁻ condition produces a depolarization of the lumen to −2.2 ± 1.2 mV (n = 84, refer to Table 4). This value is statistically different from the parallel experiment under physiological Cl⁻ conditions (p < 0.001, df = 93; unpaired Students' t-test) and also reveals an increase in V₃ by a factor of 4.4.

In the presence or absence of luminal substrate, the deletion of Cl⁻ increases V₃ by
the same multiplication factor of 4.4. Among these comparisons, Cl\(^-\) is the only modified ion concentration; hence this 4.4-fold amplification of \(V_3\) must be attributed to Cl\(^-\). Symmetrical removal of Cl\(^-\) reflects a parallel increase in the paracellular resistance \(R_3\) which, therefore, must be extremely Cl\(^-\)-selective (see [25] for a complete discussion of equivalent electrical circuit analysis). Clearly, this procedure is a valid maneuver for further investigation of small ionic current source(s) independent of luminal substrate transport mechanisms.

**Low Cl\(^-\)** **Experimental Protocol**

The following series of trials examine the apical and basolateral membrane effects of HCO\(_3\)^\(-\) removal under low Cl\(^-\) conditions. These measured effects may or may not be directly attributed to HCO\(_3\)^\(-\). The low Cl\(^-\) trials are schematically arranged into three categories: pre-SITS, SITS, and post-SITS sequences \((n = 14\) tubules\). Legend 1 illustrates the transepithelial potential comparisons completed for each experimental situation \((\Delta V_3\) measurements\). Each measurement in the pre-SITS and SITS comparisons is presented as a removal of luminal substrate, luminal HCO\(_3\)^\(-\), or bath HCO\(_3\)^\(-\). In Legend 1, the control situation is noted on the far left-hand side of the table, whereas the experimental condition is listed on the right-hand side. Experimentally, however, this situation was not the case: sometimes the control preceded the experimental circumstance and vice versa. In those situations where HCO\(_3\)^\(-\) was removed first from the lumen and then from the bath, a time gap must exist between the comparisons listed in Legend 1. Obviously, symmetrical deletion of HCO\(_3\)^\(-\) was not completed in a single step because isolated effects of one-sided HCO\(_3\)^\(-\) deletions could not otherwise be explored.

Potentially, HCO\(_3\)^\(-\) exchangers on the apical or basolateral membrane surfaces may be blocked with pharmacological agents. For the SITS trials, SITS \((4\)-acetoamido-\(4\)-isothiocyanostilbene-\(2,2\)'-disulfonate\) was applied exclusively to the lumen; therefore, transepithelial measurements only reflect changes due to apical anion exchange mechanisms. Control comparisons with and without the addition of luminal SITS will also be discussed. As SITS may have damaging effects, pre- and post-SITS trials are sequenced in a mirror-image fashion; hence the pre-SITS trials monitor the reversibility of SITS effects in washout trials, check tube viability after approximately 4.5 hours of *in vitro* perfusion, and demonstrate any long-term intracellular composition changes. Essentially, these comparisons check for internal consistency.

Tables 4, 5, and 6 and Legend 1 present the data of the following section on results. Tables 4 and 5 give actual \(V_3\) measurements and Table 6 the \(\Delta V_3\) measurement comparisons. Legend 1 may be referred to as a guide for understanding the comparisons among experimental maneuvers, using roman numeral designations.

**Effect of Substrate Deletion: Low Cl\(^-\)** **Experiments**

A complete description of the equivalent electrical circuit model is presented in [25]. Various current-producing (rheogenic) transport systems have been identified in proximal tubule epithelia. These mechanisms may be symporters which carry one or more ions with an uncharged substrate or with other ion(s). Alternatively, antiport systems may exist, which transport one ionic species into the cell and another ionic species out of the cell. In this situation, an imbalance between the number of ions carried by the antiport must exist in order to produce a net current across a given membrane barrier. In the *Ambystoma* model, current from primary or secondary
Comparison (control versus experimental) | Experimental Effects
---|---
I versus II | *Pre-SITS Trials* Luminal substrate removal
II versus III | Luminal HCO$_3^-$ removal
III versus IV | Bath HCO$_3^-$ removal
VII versus VI | *SITS Trials* Luminal HCO$_3^-$ removal
V versus V | Bath HCO$_3^-$ removal
VIII versus VII | Luminal HCO$_3^-$ removal
VI versus VIII | Bath HCO$_3^-$ removal
IX versus X | *Post-SITS Trials* Bath HCO$_3^-$ addition
X versus XI | Luminal HCO$_3^-$ addition
XI versus XII | Luminal substrate addition

**Effect of SITS Addition (Control)**

|   |   |
|---|---|
| IX versus IV | 0-Substrate HEPES (lumen); Substrate HEPES (bath) 0-Substrate HCO$_3^-$ Ringer (lumen); Substrate HCO$_3^-$ Ringer (bath) |
| X versus III | (bath) |
| XI versus II | Substrate HCO$_3^-$ Ringer (lumen and bath) |
| XII versus I | |

**Legend 1**

Legend 1, serves as a guide for understanding coding used in further data analysis for all low Cl$^-$ experiments (Table 6).

Note: Legend 1 illustrates the transepithelial potential comparisons completed for each experimental situation. Each measurement in the pre-SITS and SITS comparisons is presented as a removal of luminal substrate, luminal HCO$_3^-$, or bath HCO$_3^-$ . The control situation is noted on the far left-hand side of the table, whereas the experimental condition is listed to the right of the control.

Experimentally, this was not the case: sometimes the control preceded the experimental circumstance and vice versa (refer to Table 4). In those situations where HCO$_3^-$ was removed first from the lumen and then from the bath, an interlude must exist between the comparisons listed in this table. Obviously, symmetrical deletion of HCO$_3^-$ was not completed in a single step because isolated effects of one-sided HCO$_3^-$ deletions could not, otherwise, be explored.

The pre- and post-SITS trials are written as mirror-image comparisons; therefore, all perturbations of HCO$_3^-$ and substrate are designated as additions rather than as removals.

active sources across the basolateral membrane is designated $i_{a}^{b}$, whereas similar current across the apical membrane is denoted as $i_{a}^{a}$. Either current may be negative or positive.

Comparison I versus II (refer to Table 5) examines the effect of luminal substrate deletion in the presence of apical and basolateral HCO$_3^-$ sources. The major defined source of positively charged apical membrane current arises from the Na$^+$-substrate co-transporter; therefore, removal of luminal substrate will substantially decrease Na$^+$ current (i.e., the positive $i_{a}^{a}$ current) across the apical membrane. Sodium-dependent
TABLE 6
Paired Comparison (Delta Values) for Data Derived from Table 4 Using Correlated Data; Transepithelial Potential Differences (V3); Low Cl− Experimental Protocol

| Comparison (control versus experimental) | Effect Examined | n   | $\bar{x} \pm SD$ | df   | t-value | p     |
|-----------------------------------------|-----------------|-----|-----------------|------|---------|-------|
| **Pre-SITS Trials**                     |                 |     |                 |      |         |       |
| I versus II Substrate removal (L)        | 98              | +7.6 $\pm$ 2.8 mV | 97  | 26.2    | <0.001 |
| II versus III HCO$_3^ −$ removal (L)     | 70              | −1.9 $\pm$ 1.3 mV | 69  | 12.7    | <0.001 |
| III versus IV HCO$_3^ −$ removal (B)     | 84              | +1.5 $\pm$ 1.0 mV | 83  | 15.0    | <0.001 |
| **SITS Trials**                         |                 |     |                 |      |         |       |
| VI versus V HCO$_3^ −$ removal (L)       | 42              | −1.3 $\pm$ 0.8 mV | 41  | 13.0    | <0.001 |
| V versus VII HCO$_3^ −$ removal (B)      | 14              | +1.2 $\pm$ 0.8 mV | 13  | 6.0     | <0.001 |
| VIII versus VII HCO$_3^ −$ removal (L)   | 42              | −1.0 $\pm$ 0.7 mV | 41  | 10.0    | <0.001 |
| VI versus VIII HCO$_3^ −$ removal (B)    | 70              | +0.8 $\pm$ 0.3 mV | 69  | 16.0    | <0.001 |
| **Post-SITS Trials**                    |                 |     |                 |      |         |       |
| IX versus X HCO$_3^ −$ addition (B)      | 56              | −0.9 $\pm$ 0.8 mV | 55  | 9.0     | <0.001 |
| X versus XI HCO$_3^ −$ addition (L)      | 42              | +1.2 $\pm$ 1.0 mV | 41  | 8.0     | <0.001 |
| XI versus XII Substrate addition (L)     | 42              | −4.0 $\pm$ 1.5 mV | 41  | 16.7    | <0.001 |

Table 6 represents analysis of data presented in Table 4 using correlated data (delta values) with calculated t-values (applying the paired Student’s t-test). n = the number of comparisons for each set. $\bar{x}$ = average delta value. df = degrees of freedom. t = t-value. p = probability. “L” and “B” refer to luminal and bath perfusates, respectively. Refer to Legend 1 and Table 4 for appropriate keys.

Electrogenic transporters along the basolateral membrane will be secondarily affected by a decrease in the positive $i^p_3$, namely the $3\text{Na}^+/2\text{K}^+$ antiport [24] and $\text{Na}^+(\text{HCO}_3^−)_n$ [1] symport systems. Likewise, Na$^+$ extrusion across the basolateral membrane will also decrease (i.e., the positive $i^p_1$). Using the equivalent circuit model equation for $V_3$ [25], if the positive $i^p_1$ and the positive $i^p_3$ decrease, the net change in $V_3$ or $\Delta V_3$ ought to be a positive value. Experimentally, the results concur with the predicted theoretical model, yielding a $\Delta V_3$ value of $+7.6 \pm 2.8$ mV (refer to Table 6).

Effect of Luminal HCO$_3^ −$ Removal in the Absence of Luminal SITS

In the absence of luminal SITS, removal of HCO$_3^ −$ from the apical side with a constant HCO$_3^ −$ bath is compared in trials II versus III (refer to Table 6). Deleting HCO$_3^ −$ from the lumen produces a CO$_2$ gradient favoring intracellular CO$_2$ exit across the apical membrane, thereby decreasing intracellular CO$_2$, H$_2$CO$_3$, H$^+$, and HCO$_3^ −$ (see Fig. 2). This hypothesis, of course, assumes CO$_2$ and H$_2$CO$_3$ diffuse more rapidly across the apical membrane than HCO$_3^ −$ movement through other transport processes. Before removal of luminal HCO$_3^ −$, rheogenic HCO$_3^ −$ transport across the basolateral membrane is directed such that negative charges are extruded from cell to bath. With a decrease in intracellular HCO$_3^ −$, bicarbonate current across the basolateral membrane via the electrogenic symporter, $\text{Na}^+(\text{HCO}_3^−)_n$ and thus the negative $i^p_1$, is diminished and may actually reverse. Hence, the direct effect of luminal HCO$_3^ −$ removal is a decrease in the negative $i^p_1$ and an expected negative $\Delta V_3$ measurement. The experimental result is congruent with this expectation, producing a $\Delta V_3$ of $−1.9 \pm 1.3$ mV (Table 6). Theoretically, this finding is a direct manifestation of a diminished $i^p_1$ current; therefore, one expects the resulting $\Delta V_3$ tracing to reach a steady-state condition quickly.

Experimentally, a stable equilibrium state is attained in approximately 30 seconds.
Effect of Luminal HCO₃⁻ Removal in the Presence of Luminal SITS

Trial VI versus V is analogous to II versus III except for the addition of 0.5 mM luminal SITS. The argument for generating a negative $\Delta V_3$ is the same as in the previous case; however, one must also consider that potential apical HCO₃⁻ or equivalent anion exchangers are blocked in this experiment. Applying the equivalent circuit model, the negative $i'_0$ should decrease during luminal HCO₃⁻ removal and, if present, any negative $i'_2$ could be blocked by luminal SITS. As predicted, the experimental $\Delta V_3$ is negative ($-1.3 \pm 0.8$ mV, refer to Table 6). Tracings from this trial are similar to comparison II versus III with a stable equilibrium state attained in approximately 30 seconds.

A somewhat similar perturbation of HCO₃⁻ transport mechanisms is achieved with SITS trial VIII versus VII. In this case, HCO₃⁻ is removed from the lumen in the absence of HCO₃⁻ in the bath. Before HCO₃⁻ is deleted from the lumen, intracellular HCO₃⁻ readily exits the cell across the basolateral membrane down a favorable HCO₃⁻ electrochemical gradient. This bicarbonate exit should yield levels of intracellular HCO₃⁻, H₂CO₃, and CO₂ which are lower than control. With the omission of apical HCO₃⁻, a decrease in the negative $i'_0$ current is anticipated, due to the direct effect of diminished outward HCO₃⁻ current; however, the resulting negative $\Delta V_3$ recording may be transient since intracellular HCO₃⁻, CO₂, and H₂CO₂ are not being replenished by an external source in this circumstance. An additional explanation for such a transient effect may be attributed to a change in K⁺ conductance. Indeed, apical exit of CO₂ and H₂CO₃ at a rate exceeding apical HCO₃⁻ would raise cellular pH and increase potassium channel conductance [26]. Since the number of K⁺ channels is greatest along the basolateral membrane [24], the basolateral membrane potential would hyperpolarize, yielding an eventual negative $\Delta V_3$. Experimentally, the direct effect attributable to diminished basolateral HCO₃⁻ current is confirmed by a $\Delta V_3$ of $-1.0 \pm 0.7$ mV with a steady state being attained in 30 seconds. The tracing does not
show an eventual decline of the $\Delta V_3$ over the course of 2.5 minutes, however; therefore, the sustained hyperpolarization may be an indirect manifestation of intracellular alkalinization.

**Effect of Bath HCO$_3^-$ Removal in the Absence of Luminal SITS**

In the absence of apical HCO$_3^-$ and SITS, basolateral HCO$_3^-$ deletion is studied in comparison III versus IV (refer to Table 6). Before excluding HCO$_3^-$ from the bath, the cell is in a physiologically altered state where both HCO$_3^-$ and CO$_2$ enter the cell across the basolateral membrane, following their electrochemical gradient, and principally CO$_2$ exits across the apical membrane. This process results in an increased intracellular pH. Subsequent removal of HCO$_3^-$ from the bath has the direct effect of restoring HCO$_3^-$ exit across the basolateral membrane. With a large increase in the negative $i_1^i$ current, the $\Delta V_3$ measurement should become positive; however, the effect is probably quickly washed out. Comparison III versus IV shows the predicted $+1.5 \pm 1.0$ mV value (Table 6); however, the tracing is stable with a steady-state value achieved in 1.5 minutes. Finally, in condition IV, where the HCO$_3^-$ buffer system is removed symmetrically, the $\Delta V_3$ measurement is not significantly different from condition II, in which HCO$_3^-$ is available in both luminal and bath perfusates (refer to Table 4).

**Effect of Bath HCO$_3^-$ Removal in the Presence of Luminal SITS**

Trial V versus VII is similar to comparison III versus IV except for the addition of luminal SITS. As argued above, the HCO$_3^-$ bath deletion results in an increase in the negative $i_1^i$ with an expected positive $\Delta V_3$ measurement. The experimental $\Delta V_3$ is $+1.2 \pm 0.8$ mV (refer to Table 6). In this trial, any existing SITS-sensitive anion exchangers are blocked at the apical membrane. Unfortunately, experiments V and VII were not contiguous recordings; hence, the possible transient qualities of the electrical effects cannot be assessed.

In the last comparison (VI versus VIII), HCO$_3^-$ is removed from the bath in the presence of apical SITS and HCO$_3^-$ . Under these conditions, the HCO$_3^-$ gradient favors CO$_2$ and HCO$_3^-$ exit across the basolateral membrane, which would result in a net efflux of negative charge; i.e., the negative $i_1^i$ increases, whereas any negative $i_2^i$, if present, is blocked by SITS. The anticipated $\Delta V_3$ measurement should be positive, as confirmed by an experimental $\Delta V_3$ of $+0.8 \pm 0.3$ mV (refer to Table 6); the steady-state value is achieved in 30 seconds and remains constant.

**SITS Effects**

Under the conditions of HCO$_3^-$ present in the lumen and bath, in the lumen only, or in the bath only, the addition of luminal SITS depolarizes the epithelium by about $+1.0$ mV. These differences are statistically different from the identical pre-SITS conditions ($p < 0.001$ and df = 13 for each comparison). Due to these electrophysiologic effects of SITS in control situations, it is impossible to apply analysis of variance tests to compare identical HCO$_3^-$ deletions in the pre-SITS and SITS trials.

When SITS is present in the bath, depolarization of $V_3$ should be due to a direct action on the Na$^+$(HCO$_3^-$)$_a$ symporter. SITS in the lumen could have a depolarizing effect if it blocked putative Cl$^-$-HCO$_3^-$ exchangers on the luminal membrane, thereby preventing apical escape of HCO$_3^-$ and decreasing intracellular H$^-$ concentration [1]. As previously discussed, this circumstance favors basolateral HCO$_3^-$ exit; i.e., the
negative i‡ increases, whereas intracellular alkalization activates K⁺ channels hyper-polarizing the basolateral membrane. A preponderance of the former effect would explain the positive ΔV₃.

**Pre-SITS Versus Post-SITS Trials**

The intent of mirror-image comparisons between pre- and post-SITS trials is to determine whether or not the effects of bath and luminal HCO₃⁻ additions as well as luminal substrate addition are recovered after SITS has been washed out. The magnitude of the measurements is not critical, as SITS is recognized to have metabolic side effects with a high receptor binding affinity. Furthermore, the mirror-image experimental results in the pre- and post-SITS circumstances will probably not be identical, as the average in vitro perfusion duration was on the order of 4.5 hours and this interval may produce irreversible metabolic changes.

Indeed, the tubules remain responsive in SITS washout comparisons. Since the pre- and post-SITS trials are mirror-image comparisons, the ΔV₃ measurements in the post-SITS trials are electrically oriented in the opposite direction from the pre-SITS trials, as expected. This result indicates that the tubules remain viable after washout of SITS exposure for a lengthy experimental duration (refer to Table 5 for independent data). Not surprisingly, however, in each experimental setting, the post-SITS ΔV₃ recordings are smaller in absolute magnitude and statistically different from their pre-SITS counterparts (p ≤ 0.01 and df = 13 for each comparison). Furthermore, due to the accumulated effects of prolonged in vitro perfusion, analysis of variance tests cannot be applied to compare ΔV₃ measurements in the appropriate pre-SITS versus SITS experiments.

**DISCUSSION**

**The Role of Rheogenic HCO₃⁻ Transport as a Potential Source of Experimentally Measured ΔV₃ Recordings**

The low Cl⁻ experiments examine the effects of lumen, bath, and lumen plus bath HCO₃⁻ removal in the presence or absence of luminal SITS. The objective of these protocols is to explore the possible role of an apical, SITS-sensitive, rheogenic HCO₃⁻ (or equivalent anion) transporter. As substrates (notably lactate [27]) are removed from the lumen and Cl⁻ is symmetrically reduced from both cell membrane surfaces, any measured ΔV₃ recording may reflect primary HCO₃⁻ transport events at the apical membrane or secondary events caused by the basolateral Na⁺(HCO₃⁻)ₙ symporter. The basolateral Na-K-ATPase current (+i‡) ought to remain constant, as neither cation is manipulated in these tests. As previously discussed, pH-dependent changes in K⁺ channel conductance may contribute to the observed ΔV₃.

Figure 3 presents two theoretical models for rheogenic HCO₃⁻ transport. These include a channel for HCO₃⁻ and an electrogenic Na⁺(HCO₃⁻)ₙ co-transporter. Four modes of possible Na⁺(HCO₃⁻)ₙ transport are depicted in Fig. 3; these are thermodynamically indistinguishable variants.

**Physiological Cl⁻ Concentrations**

In the bilateral presence of physiological concentrations of Cl⁻ and substrate, the absolute V₃ value measures −2.3 ± 0.5 mV. This value is less than reported by others
in *Ambystoma* proximal tubules, ranging from $-2.9$ mV to $-5$ mV, using tubule lengths ranging from 500 to 1,000 $\mu$m [1,24,27,28,29]. As the range of tubule lengths in the present experiments varied from 1,400 to 2,400 $\mu$m, the probability for tubule damage increased throughout all dissection and perfusion manipulations. Such damage, not revealed by discrete cellular staining during the Hercules dye maneuver, may lower the transepithelial potential difference. It is commonly observed that luminal substrate deletion depolarizes the proximal tubule epithelium toward zero [29]. Under physiological circumstances, luminal substrate omission produces a mean $V_3$ recording of $-0.5 \pm 0.3$ mV.

**Low Cl⁻ Experiments**

The effect of symmetrical Cl⁻ removal produces a consistent 4.4 amplification of all $V_3$ recordings. In the situation of symmetrical low Cl⁻ bilateral standard HCO₃⁻ Ringer, Sackin and Boulpaep [30] observed a $V_3$ measurement of $-19.0 \pm 2.5$ mV, whereas we noted a $-10.2 \pm 4.1$ mV recording in the same circumstance. This discrepancy may reflect the greater tubule lengths of our tubules. Removing luminal substrates predictably diminishes the source of apical current, the positive $i_2^+$, largely due to Na⁺/substrate co-transport. Such mechanisms are well described in the rat [31,32,33] and amphibian proximal tubule [29]. The experimental results concur with expected findings (refer to Table 6). In the absence of luminal substrate and symmetrical Cl⁻ deletion, what is the source of the observed $-2.2 \pm 1.2$ mV transepithelial measurement? Candidates include apical Na⁺ channels which permit residual Na⁺ entry along with minimal functioning of Na⁺/substrate symport and possibly Na⁺(HCO₃⁻)$_n$ symport, which is reversed such that HCO₃⁻ is transported from the basolateral membrane into the cell. It should be recognized that if a basolateral Na⁺(HCO₃⁻)$_2$ transporter exists with a reversal potential close to the basolateral membrane potential, it may contribute a positive or negative current across that membrane ($i_0$). It is also possible that any incoming apical HCO₃⁻ current is offset by
such a reversible basolateral \( \text{Na}^+ (\text{HCO}_3^-) \) transporter. To address this problem, serial lumen, bath, and lumen plus bath \( \text{HCO}_3^- \) deletions were examined with and without the addition of luminal SITS.

**Removal of Luminal \( \text{HCO}_3^- \)**

Deletion of luminal \( \text{HCO}_3^- \) alone (comparison II versus III) essentially measures the decrease in \( \text{HCO}_3^- \) current (the negative \( i_D^p \)) across the basolateral membrane via the \( \text{Na}^+ (\text{HCO}_3^-) \) transporter. Possible apical \( \text{Cl}^- - \text{HCO}_3^- \) antiport would also be blocked with the omission of luminal \( \text{Cl}^- \) and \( \text{HCO}_3^- \); these effects, of course, are not measurable in \( V_3 \) recordings, as \( \text{Cl}^- - \text{HCO}_3^- \) antiport represents an electroneutral process. The presence of luminal SITS and absence of luminal \( \text{HCO}_3^- \) (comparison VI versus V) is essentially the same as comparison II versus III, with the addition that possible rheogenic luminal \( \text{HCO}_3^- \) transporters and \( \text{Cl}^- - \text{Anion}^- \) or \( \text{Cl}^- - \text{HCO}_3^- \) pathways are inhibited. In both comparisons, the steady state is attained within 30 seconds; presumably, both tracings reflect the rapid decrease in \( \text{HCO}_3^- \) current across the basolateral membrane. The role of possible apical \( \text{Cl}^- - \text{Anion}^- \) antiport cannot be assessed, due to the multiple effects of SITS addition; however, it is unlikely that this mechanism plays a significant role when \( \text{Cl}^- \) is deleted on both sides.

**Removal of Bath \( \text{HCO}_3^- \)**

The removal of luminal and bath \( \text{HCO}_3^- \) should only reflect a decrease in basolateral \( \text{HCO}_3^- \) current across the \( \text{Na}^+ (\text{HCO}_3^-) \) co-transporter and produce some steady-state intracellular alkalinization (comparison VIII versus VII). The measured \( \Delta V_3 \) value exhibits a hyperpolarization either as a result of increased \( \text{K}^+ \) channel conductance or of a dampened negative \( (i_D^p) \) current.

Experiment III versus IV, where bicarbonate is deleted from the bath in the absence of luminal \( \text{HCO}_3^- \) is the basolateral equivalent of comparison VIII versus VII. The effect is consistent with increased negative \( \text{HCO}_3^- \) current \( i_D^p \) across the basolateral membrane but not with any increased \( \text{K}^+ \) channel conductance following intracellular alkalinization. Potential effects of an apical \( \text{Cl}^- - \text{HCO}_3^- \) or \( \text{HCO}_3^- \) conductance pathway are inhibited in this trial. The same comparison, with the addition of luminal SITS (V versus VII), likewise demonstrates a predictable increase in the negative \( i_D^p \) current across the basolateral membrane. Potential effects of apical \( \text{Cl}^- - \text{HCO}_3^- \) or \( \text{Cl}^- - \text{Anion}^- \) exchange and \( \text{HCO}_3^- \) conductance are blocked in this experimental sequence.

The last experimental comparison (VI versus VIII) is the only trial which does not solely monitor rheogenic basolateral \( \text{HCO}_3^- \) current, because the basolateral bicarbonate removal is performed in the presence of \( \text{HCO}_3^- \) in the lumen. If a rheogenic apical \( \text{HCO}_3^- \) pathway existed, removal of bath \( \text{HCO}_3^- \) in the presence of luminal SITS and \( \text{HCO}_3^- \) should produce an increase in \( \text{HCO}_3^- \) current across both membranes (i.e., an increase in the negative \( i_D^p \) and the negative \( i_D^i \)), which would result in a net positive \( \Delta V_3 \) value greater than that in comparison V versus VII (\( \text{HCO}_3^- \) bath deletion in the presence of luminal SITS and absence of luminal \( \text{HCO}_3^- \)). A larger positive \( \Delta V_3 \) was not found. These trials cannot, however, be quantitatively compared for reasons delineated earlier. The role of a possible apical SITS-sensitive exchanger seems unlikely in these trials, which were performed in low \( \text{Cl}^- \) solutions.
CONCLUSION: ELECTRICAL ROLE OF HCO$_3^-$

As hypothesized earlier, the $-2.2$ mV $V_3$ value observed in the absence of luminal substrate and the presence of symmetrical HCO$_3^-$ cannot be directly attributed to an apical HCO$_3^-$ and/or basolateral Na$^$(HCO$_3^-$)$_n$ rheogenic current source because the net effect of these processes would produce a negative $i_t^i$ and negative $i_t^2$, yielding a positive net $V_3$ measurement. Experimentally, this result is not the case. It should be noted that transepithelial voltage measurements are an indirect technique for monitoring membrane current as a function of the basolateral Na$^$(HCO$_3^-$)$_n$ symport.

A luminal HCO$_3^-$ conductance pathway is unlikely, since decreasing luminal pH from 7.5 to 6.8 by decreasing luminal HCO$_3^-$ has no significant effect on apical membrane polarization in Ambystoma [1]. Furthermore, existence of an apical Na$^$(HCO$_3^-$)$_n$ co-transporter is not consistent with the finding that removal of luminal Na$^+$ in the presence of an apical HCO$_3^-$ source has minimal effects [1]. An apical electroneutral Cl$^-$/HCO$_3^-$ antiport has recently been disproven. Removal of luminal HCO$_3^-$ produces a decrease in intracellular Cl$^-$ activity of $1.0 \pm 0.3$ mM, which is incompatible with a Cl$^-$/HCO$_3^-$ model [34]. Finally, the addition to a HCO$_3^-$-free lumen of the disulfonic stilbene derivative DIDS increases intracellular Cl$^-$ activity by $1.7 \pm 0.6$ mM; these observations discount the likelihood of an apical Cl$^-$-Anion$^-$ antiport [34].

In conclusion, this study indicates: (1) the $-2.2$ mV $V_3$ observed under the conditions of symmetrical HCO$_3^-$ and low Cl$^-$ solutions and in the absence of luminal substrate is not due to apical or basolateral rheogenic bicarbonate pathways, and (2) changes in HCO$_3^-$ basolateral current are measurable at the transepithelial level during serial bath and/or luminal HCO$_3^-$ deletions.

ACKNOWLEDGEMENTS

This work was supported by grants DK-13844 and DK-17433 from the National Institute of Diabetes and Digestive and Kidney Diseases.

We are grateful to Subrata Tripathi, M.D., for his peerless instruction in proximal tubule perfusion technique.

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