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Regulation of intracellular pH in proximal tubules of avian long-looped mammalian-type nephrons

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Brokl, Olga H., Christina L. Martinez, Apichai Shuprisha, Diane E. Abbott, and William H. Dantzler. Regulation of intracellular pH in proximal tubules of avian long-looped mammalian-type nephrons. *Am. J. Physiol. 274 (Regulatory Integrative Comp. Physiol. 43):* R1526–R1535, 1998.—In nonperfused proximal tubules isolated from chicken long-looped mammalian-type nephrons, intracellular pH (pH_i), measured with the pH-sensitive fluorescent dye 2',7'-bis(2-carboxyethyl)-5(6)-carboxyfluorescein, was ~ 7.3 under control conditions (HEPES-buffered medium with pH 7.4 at 37°C) and was reduced to ~ 7.0 in response to NH_4Cl pulse. The rate of recovery of pH_i from this level to the resting level was 1) significantly reduced by the removal of Na^+ from the bath, 2) significantly increased by the removal of Cl^- from the bath, 3) unchanged by the removal of both Na^+ and Cl^- from the bath, 4) significantly reduced by the addition of either ethylisopropylamiloride or DIDS to the bath, 5) significantly increased by a high bath K^+ concentration, and 6) unchanged by the addition of Ba^{2+} to the bath. These data suggest that both Na^+ -coupled and Cl^- -coupled basolateral acid-base fluxes are involved in determining the rate of recovery of pH_i after acidification. The most likely ones to be important in regulating pH_i are a Na^+/H^+ exchanger and a Na^+ -coupled $\text{Cl}^-/\text{HCO}_3^-$ exchanger. In birds, long-looped mammalian-type nephrons resemble short-looped transitional nephrons but differ markedly from superficial loopless reptilian-type nephrons.

chickens; ammonium chloride pulse; intracellular acidification; intrinsic buffering capacity; sodium-coupled basolateral acid-base fluxes; chloride-coupled basolateral acid-base fluxes

THE ORGANIZATION OF THE avian kidney is highly complex. It involves two major nephron populations with a gradation between them (6, 8, 25). The most superficial cortical nephrons (often called reptilian-type nephrons) (12) are small, lack loops of Henle, and empty at right angles into collecting ducts (6, 25, 26). The deepest medullary nephrons (often called mammalian-type nephrons) (12) are large and have complex convoluted proximal tubules and long loops of Henle lying parallel to collecting ducts (6, 25). The gradation between the extremes of these nephron types consists of transitional nephrons (sometimes referred to as "short-looped mammalian-type" nephrons) (6). These nephrons have relatively straight proximal tubules and short-looped intermediate segments that do not lie parallel to collecting ducts (6, 25). In our initial work with isolated avian proximal tubules (7, 13), we worked only with transitional nephrons because these were the only nephrons from which we could tease proximal tubules without the aid of enzymatic agents for *in vitro* studies. More recently, however, we have been able to tease proximal tubules, first from superficial loopless reptilian-type

nephrons (20), and, in the current study, from long-looped mammalian-type nephrons.

The proximal tubules of avian nephrons, like those of nephrons from other tetrapod vertebrates, must help deal with systemic acid or base loads while maintaining their own acid-base homeostasis. Some *in vivo* micro-puncture studies on superficial loopless reptilian-type avian nephrons (18, 19) and *in vitro* microperfusion studies on short-looped transitional avian nephrons (7) indicate that along the proximal tubule there is little acidification of luminal fluid, that net bicarbonate reabsorption proceeds at the same rate as net fluid reabsorption, and that net fluid reabsorption is not dependent on bicarbonate reabsorption. In a series of studies, we have begun to examine the regulation of intracellular pH (pH_i) under various conditions in proximal tubules from all three populations of nephrons. We began with proximal tubules from the short-looped transitional nephrons (13), continued with proximal tubules from superficial loopless reptilian-type nephrons (20), and, in the present study, have examined proximal tubules from long-looped mammalian-type nephrons. These studies were all performed in nonperfused tubules with totally collapsed lumens, and regulation of pH_i was only considered with regard to the basolateral membrane (13, 20). The results of the previous studies as well as the present study indicate that 1) resting pH_i is ~ 7.3 – 7.4 in both transitional short-looped nephrons (13) and long-looped mammalian-type nephrons (present study), whereas it is ~ 7.1 – 7.2 in superficial loopless reptilian-type nephrons (20); 2) acidification in response to NH_4Cl pulse (21) is qualitatively similar in all three nephron types (13, 20; present study); and 3) the rate of recovery of pH_i from acidification and maintenance of resting pH_i are apparently dependent to a varying extent on commonly suggested basolateral acid-base transporters (primarily a Na^+/H^+ exchanger and a Na^+ -dependent $\text{Cl}^-/\text{HCO}_3^-$ exchanger) in both transitional short-looped nephrons (13) and long-looped mammalian-type nephrons (present study), whereas they are apparently dependent on some other type of Na^+ -dependent basolateral acid-base transporter in loopless reptilian-type nephrons (20).

METHODS

Preparation of isolated renal tubules. Female White Leghorn chickens, 1–3 mo old, were decapitated. Their kidneys were flushed *in situ* via the aorta with chilled (4°C) avian Ringer solution, quickly removed, and placed in the same solution (see below for composition) on ice. Tubules were dissected from thin, vertical slices of kidney without the aid of enzymatic agents as described previously (7). Normally, we used only one tubule segment from each bird (total of 48) in these

experiments. The dissections were performed in chilled, oxygenated medium in a dissection dish maintained on ice. As pointed out previously (6, 7, 12), avian nephrons vary from very small, simple, superficial cortical nephrons without loops of Henle to large, deep medullary nephrons with long loops of Henle. Thus the nephrons can be readily identified in fresh tissue by their size and form as well as their position within the kidney (6). Proximal segments (~500 μm in length) were dissected from the deepest mammalian-type nephrons with long loops of Henle extending into the medullary region and from transitional nephrons with short loops of Henle located wholly in the deep cortical region. The proximal tubules from the long-looped nephrons are always highly convoluted, whereas those from the short-looped transitional nephrons are relatively straight (6). In addition, the proximal tubules of the long-looped nephrons are substantially longer than those of the short-looped transitional nephrons (6). In our initial work (7, 13), we used only proximal tubules from short-looped transitional nephrons because they were the only proximal segments that we were able to tease from avian tissue without the aid of enzymatic agents in lengths sufficient for microperfusion. However, we have now learned to tease out proximal tubules from loopless reptilian-type nephrons (20) and from long-looped nephrons to use in the nonperfused state for measurements of pH_i . Each nephron type is teased free of all surrounding tubule segments and cells. The lumens of proximal tubules from long-looped nephrons, like the lumens of proximal tubules from loopless (20) and short-looped nephrons (12), collapsed rapidly so that no fluid was detectable in them. For a few experiments, we also teased out proximal tubules from transitional short-looped nephrons. Dissections were performed in chilled medium (4°C), but all experiments were performed at 37°C.

Ringer composition. The components of the avian Ringer solutions used in these studies are shown in Table 1. All solutions were buffered with HEPES. *Solution 1* is the basic solution established previously for flushing the kidneys and for dissection of avian renal tubules (7). As noted in our previous work (7, 13), although the osmolality of this solution with the extra sucrose (Table 1) is substantially above the

normal plasma osmolality (23), we found it to be the best solution for dissecting these tubules *in vitro*. There was no apparent change in cell volume of tubules maintained in this solution. *Solution 2*, which was identical to *solution 1* except for the removal of the extra sucrose (Table 1), was used for the initial incubation period with the pH-sensitive fluorescent dye (see below). The sucrose was removed to avoid possible interference with the dye uptake or calibration. *Solution 3* was the standard solution used for the control pH_i measurements and for studies involving the addition of DIDS and ethylisopropylamiloride (EIPA). This solution was identical to *solution 2* except that all extra organic substrates (except glucose) were replaced with NaCl to prevent any possible effect of these substrates on pH_i . *Solutions 4-10* involved modifications in *solution 3* designed to examine the effects of Na^+ , Cl^- , K^+ , and Ba^{2+} on pH_i (see RESULTS). The pH of each solution was adjusted to 7.4 with 1 N NaOH, 1 N KOH, or Tris-base, as appropriate. When 20 mM NH_4Cl was present in the medium, the concentration of NaCl was reduced by an equimolar amount to maintain the osmolality and ionic strength approximately constant. The osmolality of all solutions except *solution 1* was ~290 mosmol/kgH₂O (Table 1) and was checked regularly with a vapor pressure osmometer. The solutions were continuously bubbled with 100% O₂ and were assumed to be nominally HCO_3^- free in the absence of tissue.

Measurement of pH_i in single renal tubules. We used the pH-sensitive fluorescent dye 2',7'-bis(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF) to measure pH_i in a manner similar to that described by others and used previously by us (13, 14, 16, 20). For these measurements, we used a dual-wavelength spectrofluorimeter built around an Olympus IMT-2 inverted epifluorescence microscope. A 100-W mercury arc lamp was used as an excitation source, and specific excitation wavelengths for measurement of pH using BCECF were selected by a filter wheel mounted to the shaft of a high-speed motor. This filter wheel was composed of hemispherical filters centered at 445 (isobestic wavelength) and 495 nm. As the wheel spun, sequential excitation wavelengths were transmitted. The selected excitation light was directed to the sample by a matched dichroic mirror. To prevent

Table 1. Avian Ringer solutions

	Solutions									
	1	2	3	4	5	6	7	8	9	10
NaCl	100	100	120				50	120	115	45
NMDG-Cl				120						
Na-gluconate					120					
NMDG-gluconate						120				
HEPES	25	25	25	25	25	25	25	25	25	25
Sucrose	100									
K ₂ HPO ₄	3	3	3	3	3	3	3			
KH ₂ PO ₄					5	5				
KCl	5	5	5	5			75	5	5	75
MgSO ₄	1	1	1	1	1	1	1			
MgCl ₂								1	1	1
CaCl ₂	1.2	1.2	2.7	2.7			2.7	2.7	2.7	2.7
Ca(OH) ₂					2.7	2.7				
BaCl ₂								5	10	10
D-Glucose	8	8	8	8	8	8	8	8	8	8
L-Proline	5	5								
Hemicalcium lactate	1.5	1.5								
Na-pyruvate	10	10								
Glutathione	3	3								
Adenosine	5	5								
Osmolality	~380	~290	~290	~290	~290	~290	~290	~290	~290	~290

Values for compounds given in mM; values for osmolality given in mosmol/kgH₂O. NMDG, *N*-methyl-D-glucamine.

photodamage to the dye-loaded cells from the excitation light, a neutral density filter (ND 2, Oriel) was placed in front of the illumination site. The emitted fluorescent light passed through the dichroic mirror and a wavelength-specific emission filter (530 nm for BCECF). The fluorescent light emitted from a selected region of the sample was collected by a Hamamatsu HC120-03 photomultiplier tube operated in photon-counting mode. The dark current of the photomultiplier tube was zeroed out. Collection of fluorescent light was synchronized to the wheel rotation and excitation filter position. Synchronization, speed selection, and data collection were controlled by a microcomputer running custom software. The integrated average of 30 measurements per second was collected at 1-s intervals.

Individual tubules were held with Cell-Tak in an appropriate bathing chamber on the stage of the microscope and incubated in *solution 2* with the acetoxymethyl ester (AM) form of BCECF (6 μM) (first dissolved in dimethyl sulfoxide) for 45 min at 25°C. The AM form of BCECF readily enters the cells, where the ester is cleaved by nonspecific esterases yielding the impermeant, fluorescent form of the dye. After the loading period, the bath was replaced with dye-free *solution 3*. The tubule and bathing chamber were rinsed several times with this dye-free solution to remove remaining extracellular dye before beginning an experiment. The Cell-Tak held the tubule in place so that we could continue to visualize the same tubule area after repeated changes in the bathing solution. For collection of fluorescent light, the microscope was equipped with a Zeiss 63 \times Neofluor oil-immersion objective (1.25 numerical aperture). Wavelength-specific fluorescence was collected as described above over a 15- to 32- μm diameter area of nonperfused tubule. The ratio of fluorescence at 495/445 nm was then used as a measurement of pH_i to eliminate influence of changes in dye content or cell shape. Calibration of the pH sensitivity of intracellular BCECF was performed for each tubule at the end of each experiment. This involved monitoring the 495/445 nm ratio at various values of pH_i by incubating the tubule in a solution with high K^+ containing the ionophore 13 μM nigericin (which exchanges K^+ for H^+ and sets pH_i to approximate extracellular pH) (24). The calibration curve was linear between pH 6.5 and 8.0. Autofluorescence was insignificant compared with the fluorescence from BCECF and was taken into account by the calibration procedure.

Exposure to NH_4Cl pulse. To alter pH_i , we exposed single tubules for 60–90 s to 20 mM NH_4Cl in the bathing medium, as in our previous studies (13, 14, 20, 21). NH_3 diffuses across cell membranes much more readily than NH_4^+ (9). Therefore, NH_3 rapidly enters the tubule cells and combines with free intracellular H^+ to form NH_4^+ and alkalize the cell interior. In principle, pH_i should increase until the intracellular NH_3 concentration ($[\text{NH}_3]_i$) is equal to the extracellular NH_3 concentration. NH_4^+ enters the cells more slowly than NH_3 , leading to a gradual decrease in pH_i over the exposure period. When NH_4Cl is then removed from the bathing medium, free NH_3 diffuses rapidly from the cells, leaving behind free H^+ and producing rapid acidification of the cell interior. The rate of recovery from acid pH_i to control resting pH_i then gives a measure of the regulation of pH_i by acid/base fluxes.

Determination of rate of pH_i change, intrinsic buffering capacity, NH_3 flux across the basolateral membrane, and permeability of basolateral membrane to NH_3 during NH_4Cl pulse experiments. We measured the rate of change of pH_i (dpH_i/dt) and calculated the total buffering capacity (β_i), NH_3 flux across the basolateral membrane (J_{NH_3}), and the permeability of the basolateral membrane to NH_3 (P_{NH_3}) during the initial alkalization (addition of NH_4Cl to the bath) and

acidification (removal of NH_4Cl from the bath). These calculations were performed as in our previous studies (13, 14, 20) and are described briefly below.

dpH_i/dt was measured directly. The intrinsic buffering capacity (β_i , mM H^+/pH U) was then calculated from the following equation

$$\beta_i = \Delta[\text{H}^+]_i/\Delta\text{pH}_i \quad (1)$$

In *Eq. 1*, $\Delta[\text{H}^+]_i$ is the change in the amount of H^+ in the cells by virtue of the NH_3 loading or removal and ΔpH_i is the change in pH_i .

J_{NH_3} ($\text{nmol}\cdot\text{cm}^{-2}\cdot\text{s}^{-1}$) was calculated from the following equation

$$J_{\text{NH}_3} = (\text{dpH}_i/\text{dt} \times \Delta[\text{NH}_3]_i)/(\Delta\text{pH}_i \times S/V) \quad (2)$$

In *Eq. 2*, the surface area (S) and tubular volume (V) are calculated from the measured tubule length and diameter, and dpH_i/dt and ΔpH_i are as defined above. $\Delta[\text{NH}_3]_i$, the change in $[\text{NH}_3]_i$, equals the total amount of NH_3 that has moved across the basolateral membrane. Assuming, as noted above, that intracellular NH_4^+ is formed from NH_3 that has entered the cell and is reduced through the movement of NH_3 from the cell, we can calculate $\Delta[\text{NH}_3]_i$ from the change in the intracellular NH_3 and NH_4^+ concentrations at maximum pH_i after NH_4Cl addition and at resting pH_i before NH_4Cl removal. In this calculation, the relative intracellular concentrations of NH_3 and NH_4^+ are determined from the pH_i with the Henderson-Hasselbalch equation on the assumption that the pK'_a for the reaction $\text{NH}_3 + \text{H}^+ \leftrightarrow \text{NH}_4^+$ equals 9.0 at 37°C (9). Finally, P_{NH_3} (cm/s) was calculated from the following relationship

$$P_{\text{NH}_3} = J_{\text{NH}_3}/\Delta[\text{NH}_3]_i \quad (3)$$

in which the terms have been defined above.

Protocol for experiments. A single tubule from a single bird was used for each experiment. The total experimental period after loading with dye was 30 min, a time found to be appropriate to maintain sufficient dye in the tubule so that the counts remained high enough for accurate measurements. The standard protocol involved three experimental manipulations accompanied by three control manipulations. For example, this usually involved the following sequence: 1) a measurement of control resting pH_i , 2) a control NH_4Cl pulse with return to control resting pH_i , 3) one or two NH_4Cl pulses with an experimental manipulation of the bathing medium during the recovery to control resting pH_i , 4) a control NH_4Cl pulse with return to control resting pH_i , 5) one or two NH_4Cl pulses with an experimental manipulation of the bathing medium (the number of experimental manipulations depended on whether one or two were performed at *step 3*, and 6) a final control NH_4Cl pulse with return to control resting pH_i . If, at any point, pH_i failed to return to control (within ± 0.05 pH units as determined by computer averaging of the control tracings) or if the tubule shifted so that focus was not maintained at the same position (a very uncommon occurrence), the experiment was terminated. The control values before and after each experimental manipulation were averaged together by computer for comparison with the experimental value for that tubule only. In experiments in which effects on resting pH_i only were determined, control resting pH_i was measured before and after each experimental change in the bath. Again, the control resting pH_i values before and after each experimental manipulation (1–2 min of stable tracing) were averaged together by computer for comparison with the experimental value (also averaged by

Table 2. Resting pH_i and response of pH_i to NH_4Cl pulse in proximal tubules from chicken long-looped mammalian-type nephrons

Status	pH_i
Resting (control tubules)	7.29 ± 0.02 (39)
Resting before NH_4Cl addition	7.29 ± 0.03 (39) ^{a,b,c}
Maximum after NH_4Cl addition	7.84 ± 0.06 (39) ^{a,d,e}
Resting before NH_4Cl removal	7.66 ± 0.04 (39) ^{b,d,f}
Minimum after NH_4Cl removal	7.00 ± 0.03 (39) ^{c,e,f}

Values are means \pm SE; numbers in parentheses indicate number of tubules. Differences between intracellular pH (pH_i) values in response to NH_4Cl pulse were analyzed by paired analysis, with each tubule serving as its own control. Values with same letter superscript are significantly different ($P < 0.05$) by paired analysis.

computer for a period of 1–2 min of stable tracing) for that tubule only. If resting pH_i did not return to control (within ± 0.05 pH units) after an experimental manipulation, the experiment was terminated and that experimental manipulation discarded. Also, if the calibration curve at the end of each experiment was not linear over the appropriate range, the experiment was discarded. Although there was some noise in the tracings (see RESULTS), this did not reflect an actual change in the signal and was averaged out by the computer. The computer averaging and the comparison of experimental values with their own controls in the same tubules permitted accurate differentiation of pH values.

Chemicals. Nigericin and DIDS were purchased from Sigma. BCECF and EIPA were purchased from Molecular Probes. All other chemicals were purchased from standard sources and were of the highest purity available.

Statistics. Values are summarized as means \pm SE (n = number of tubules with each tubule from a different animal). Significant differences between values were determined with Student's t -test for paired or unpaired data, as appropriate. Linear regression analyses were performed for calibrations as required. In all analyses, differences were considered statistically significant when $P < 0.05$.

RESULTS

Control measurements of pH_i . We first measured the resting pH_i in isolated proximal tubules from chicken

long-looped mammalian-type nephrons before studying changes in pH_i . As summarized in Table 2, the resting pH_i was ~ 7.3 .

Response of pH_i to exposure to NH_4Cl pulse. The effects of an NH_4Cl pulse on pH_i are summarized for all individual tubules studied in Table 2. The results are also shown for individual chicken proximal tubules in Figs. 1–5. The expected pattern was observed, with both the maximum pH_i in the presence of NH_4Cl (~ 7.8) and the minimum pH_i after removal of NH_4Cl (~ 7.0) being significantly different from the resting pH_i (Table 2).

Rate of pH_i change, intrinsic buffering capacity, NH_3 flux across the basolateral membrane, and permeability of basolateral membrane to NH_3 during NH_4Cl pulse experiments. The measurements of dpH_i/dt and the results of the calculations of β_i , J_{NH_3} , and P_{NH_3} are shown in Table 3. These values are very similar for both the alkalization and acidification phases. For comparison, the values obtained on proximal tubules from chicken loopless reptilian-type and short-looped transitional nephrons and snake and rabbit nephrons in our previous studies (13, 14, 20) are also shown in Table 3 (see DISCUSSION).

Effects of Na^+ , Cl^- , or Na^+ and Cl^- removal on rate of recovery from acid pH_i to control resting pH_i . We examined the rate of recovery of pH_i (dpH_i/dt) from the acid value following removal of NH_4Cl to the control resting value in these renal proximal tubules from chicken long-looped mammalian-type nephrons. The average control value for this recovery rate is given in Table 4, in which it is compared with the values for proximal tubules from chicken loopless reptilian-type and short-looped transitional nephrons and snake and rabbit nephrons obtained in our previous studies (13, 14, 20) (see DISCUSSION). The control recovery is also shown for individual tubules in Figs. 1–5.

In these tubules with completely collapsed lumens, recovery should take place through ion fluxes across the basolateral membrane. A number of basolateral

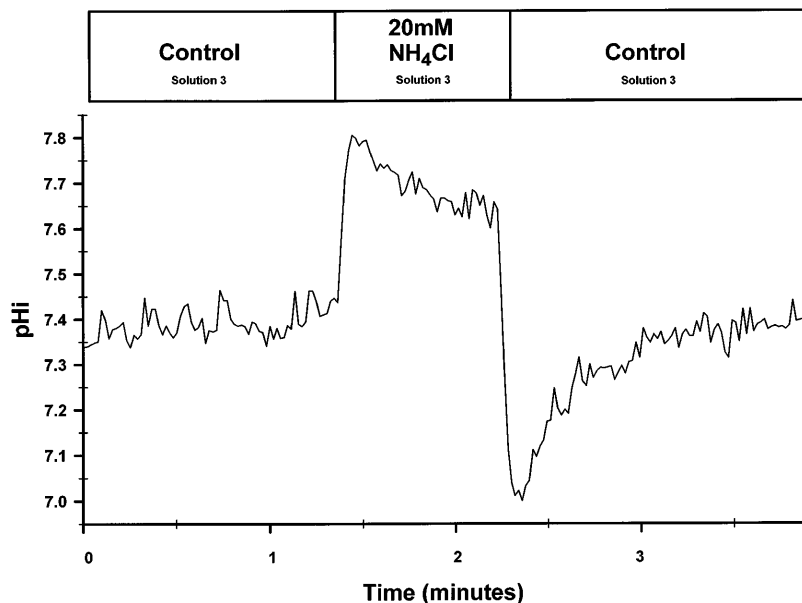


Fig. 1. Intracellular pH (pH_i) in single proximal tubule from chicken long-looped mammalian-type nephron during various modifications in bathing medium. Boxes above tracing indicate when medium is control Ringer (control) (solution 3, Table 1) or control Ringer (solution 3, Table 1) with 20 mM NaCl replaced by 20 mM NH_4Cl .

Table 3. dpH_i/dt , β_i , J_{NH_3} , and P_{NH_3}

Tubule	dpH_i/dt , pH U/s $\times 10^{-2}$	β_i , mM H ⁺ /pH U	J_{NH_3} , nmol \cdot cm ⁻² \cdot s ⁻¹	P_{NH_3} , cm/s $\times 10^{-3}$
<i>During change of pH_i from control resting to maximum value after NH_4Cl addition</i>				
Chicken long-looped proximal tubules	15.5 \pm 1.19 (40) ^a	23.96 \pm 2.52 (35)	1.77 \pm 0.17 (38) ^{b,c}	3.63 \pm 0.34 (38) ^{e,f,g}
Chicken short-looped proximal tubules	14.6 \pm 0.73 (59)	20.36 \pm 1.61 (48)	2.43 \pm 0.20 (59) ^b	4.95 \pm 0.41 (59) ^e
Chicken loopless proximal tubules	13.6 \pm 1.19 (32)	28.11 \pm 3.95 (32)	1.85 \pm 0.16 (32)	3.77 \pm 0.33 (32)
Snake distal-proximal tubules	8.3 \pm 0.50 (25) ^a	22.03 \pm 4.83 (27)	1.27 \pm 0.16 (25) ^c	6.43 \pm 0.82 (25) ^f
Rabbit proximal S2 tubules	11.5 \pm 1.80 (6)	51.06 \pm 13.25 (6)	4.04 \pm 0.78 (6) ^d	8.83 \pm 1.70 (6) ^g
<i>During change of pH_i from resting before NH_4Cl removal to minimum value after NH_4Cl removal</i>				
Chicken long-looped proximal tubules	14.7 \pm 0.98 (42) ^{h,i,j}	25.31 \pm 3.15 (37) ^k	2.07 \pm 0.27 (37) ^{l,m}	3.87 \pm 0.43 (36) ⁿ
Chicken short-looped proximal tubules	11.4 \pm 0.69 (38) ^h	25.37 \pm 2.08 (48)	3.52 \pm 0.37 (38) ^l	7.19 \pm 0.76 (38) ⁿ
Chicken loopless proximal tubules	13.1 \pm 0.90 (32)	26.99 \pm 2.88 (32)	1.88 \pm 0.15 (32)	3.83 \pm 0.32 (32)
Snake distal-proximal tubules	5.5 \pm 0.40 (25) ⁱ	17.72 \pm 2.60 (27)	0.80 \pm 0.10 (25) ^m	4.07 \pm 0.50 (25)
Rabbit proximal S2 tubules	6.8 \pm 1.30 (6) ^j	54.09 \pm 11.63 (6) ^k	2.77 \pm 0.86 (6)	6.05 \pm 1.87 (6)

Values are means \pm SE; numbers in parentheses indicate number of tubules. Data on chicken loopless reptilian-type nephrons are from Ref. 19, data on chicken short-looped transitional nephrons are from Ref. 12, and data on snake and rabbit tubules are from Ref. 13. dpH_i/dt , rate of pH_i change; β_i , intrinsic buffering capacity; J_{NH_3} , NH_3 flux across basolateral membrane; P_{NH_3} , permeability of basolateral membrane to NH_3 . Values for chicken loopless, chicken short-looped, snake, or rabbit tubules that differ significantly ($P < 0.05$) from corresponding values for chicken long-looped tubules are shown by matching letter superscripts.

Na^+ -coupled acid or base transporters have been identified in various segments of amphibian or mammalian proximal renal tubules (10). These include 1) Na^+/H^+ exchange that can be inhibited by amiloride or amiloride derivatives (4, 10), 2) Na^+ -coupled Cl^-/HCO_3^- exchange moving HCO_3^- into the cells (3, 10, 11) that can be inhibited by DIDS and other disulfonic stilbene compounds (10), and 3) electrogenic $Na^+-HCO_3^-CO_3^{2-}$ cotransport moving HCO_3^- out of the cells (1, 5, 10, 11, 27) that can also be inhibited by DIDS (5, 10). Basolateral Na^+ -independent Cl^-/HCO_3^- exchange moving HCO_3^- out of the cells that can be inhibited by DIDS has also been described (2, 3, 22). Because nothing was known about the regulation of pH_i in proximal tubules from these long-looped mammalian-type avian nephrons, we examined the effects of removing Na^+ , Cl^- , or both Na^+ and Cl^- from the bathing medium on the rate of recovery (dpH_i/dt) from acid pH_i to control resting pH_i . The results are summarized in Table 5 and shown for individual tubules in Figs. 2 and 3.

When all the Na^+ alone was removed from the bathing medium (solution 4, Table 1), the rate of recovery was significantly depressed by $\sim 60\%$ (Table 5). This inhibitory effect is also apparent in the response of the individual tubule shown in Figs. 2 and 3.

Table 4. Control values for dpH_i/dt from acid pH_i to control resting pH_i

Tubule Segment	dpH_i/dt , pH U/s $\times 10^{-3}$
Chicken long-looped proximal tubule	5.94 \pm 0.53 (39)
Chicken short-looped proximal tubule	5.67 \pm 0.40 (48)
Chicken loopless proximal tubule	4.98 \pm 0.38 (32)
Snake distal-proximal tubule	2.52 \pm 0.37 (21)
Rabbit proximal S2 tubule	8.77 \pm 1.68 (3)

Values are means \pm SE; numbers in parentheses indicate number of tubules. Data on chicken loopless reptilian-type nephrons are from Ref. 19, data on chicken short-looped transitional nephrons are from Ref. 12, and data on snake and rabbit tubules are from Ref. 13. Value for snake tubules is significantly different ($P < 0.05$) from values for chicken and rabbit tubules.

On the other hand, when all the Cl^- alone was removed from the bathing medium (solution 5, Table 1), the rate of recovery increased by over 60% (Table 5). This stimulatory effect is also shown for an individual tubule in Fig. 3. When both Na^+ and Cl^- were removed from the bathing medium (solution 6, Table 1), the rate of recovery was essentially unchanged from the control level (Table 5). This lack of change is also illustrated by the response of a single tubule (Fig. 2). It appears that the removal of both Na^+ and Cl^- simultaneously canceled out the effect of removing either ion alone on the rate of recovery.

Effects of EIPA and DIDS on rate of recovery from acid pH_i to control resting pH_i . In view of the inhibitory effect of Na^+ removal on the rate of recovery of pH_i , we examined the effect of EIPA, an amiloride analog that is a particularly potent inhibitor of Na^+/H^+ exchange, on the rate of recovery in the presence of Na^+ (standard control solution 3, Table 1) in these tubules. As summa-

Table 5. Effect of treatments on dpH_i/dt from acid pH_i to control resting pH_i in proximal tubules from chicken long-looped mammalian-type nephrons

Treatment	dpH_i/dt , pH U/s $\times 10^{-3}$	
	Before treatment	During treatment
0 Na^+	6.24 \pm 0.85 (14) ^a	2.47 \pm 0.42 (14) ^a
0 Cl^-	4.68 \pm 0.52 (23) ^b	7.64 \pm 1.10 (23) ^b
0 Na^+ , 0 Cl^-	5.37 \pm 0.64 (13)	5.05 \pm 0.41 (13)
EIPA (1.0 mM)	7.30 \pm 0.96 (12) ^c	4.77 \pm 0.69 (12) ^c
DIDS (0.25 mM)	7.24 \pm 1.03 (12) ^d	4.34 \pm 0.77 (12) ^d
High K^+ (75 mM)	7.14 \pm 1.13 (10) ^e	9.32 \pm 1.54 (10) ^e
Ba^{2+} (5 mM)	4.08 \pm 0.84 (9)	3.88 \pm 0.88 (9)

Values are means \pm SE for all tubules studied; numbers in parentheses indicate number of tubules. Although mean values for all tubules are shown for the sake of presentation, effect of each treatment on dpH_i/dt was evaluated statistically by paired analysis with each tubule serving as its own control. Values for treatments that differ significantly ($P < 0.05$) by paired analysis from corresponding controls are shown by matching letter superscripts. EIPA, ethylisopropylamiloride.

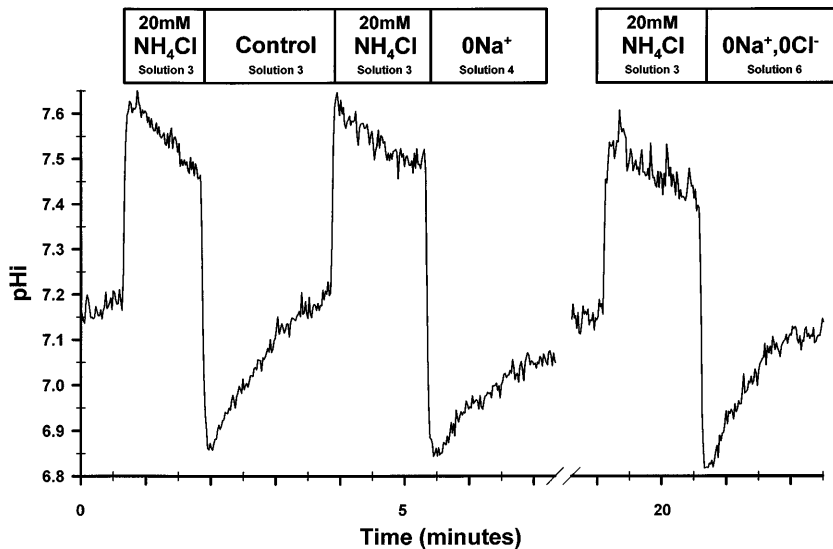


Fig. 2. pH_i in single proximal tubule from chicken long-looped mammalian-type nephron during various modifications in bathing medium. Designations in boxes above tracing indicate same changes as in Fig. 1, with the addition of Na^+ -free Ringer (0 mM Na^+) (solution 4, Table 1) and Na^+ - and Cl^- -free Ringer (0 mM Na^+ , Cl^-) (solution 6, Table 1).

alized in Table 5 and shown for an individual tubule in Fig. 4, 1 mM EIPA in the bathing medium reduced the rate of recovery by $\sim 35\%$. In the present study, we used 1 mM EIPA because we wished to be certain that there was an effect in view of the fact that even this concentration of EIPA had no effect on loopless reptilian-type nephrons (20). However, a few experiments with 100 μM EIPA gave a similar depression in rate of recovery (reduction of $2.39 \pm 0.50 \times 10^{-3}$ pH U/s; $P < 0.05$ for paired analysis; $n = 4$).

We also examined the effects of DIDS on the rate of recovery of proximal tubules from these nephrons in the presence of Na^+ and the nominal absence of HCO_3^- (standard HEPES-buffered control solution 3, Table 1). As summarized for all tubules in Table 5 and shown for an individual tubule in Fig. 4, 0.25 mM DIDS in the bathing medium significantly reduced the rate of recovery by $\sim 40\%$. We used this concentration of DIDS for comparison with our previous work on short-looped reptilian-type nephrons (20). However, in a few additional experiments with 100 μM DIDS, we obtained

almost the same depression of recovery rate (depression of $2.88 \pm 0.67 \times 10^{-3}$ pH U/s; $P < 0.05$ by paired analysis; $n = 4$).

Effects of high K^+ concentration and Ba^{2+} on rate of recovery from acid pH_i to control resting pH_i . As noted above, at least one basolateral mechanism that might be involved in regulation of pH_i , the Na^+ - HCO_3^- - CO_3^{2-} cotransporter that moves HCO_3^- out of the cells, is electrogenic. If this process were functioning during recovery of pH_i , it would tend to reduce the rate of recovery. However, because the process is electrogenic, its function would be influenced by changes in the basolateral membrane potential. A reduction in membrane potential should reduce its function and thus reduce its tendency to delay recovery of pH_i . To examine this possibility, we undertook maneuvers designed to reduce the basolateral membrane potential. Although we did not measure membrane potential directly in these studies, we assumed that a high concentration of K^+ in the bathing medium or the addition of Ba^{2+} to the

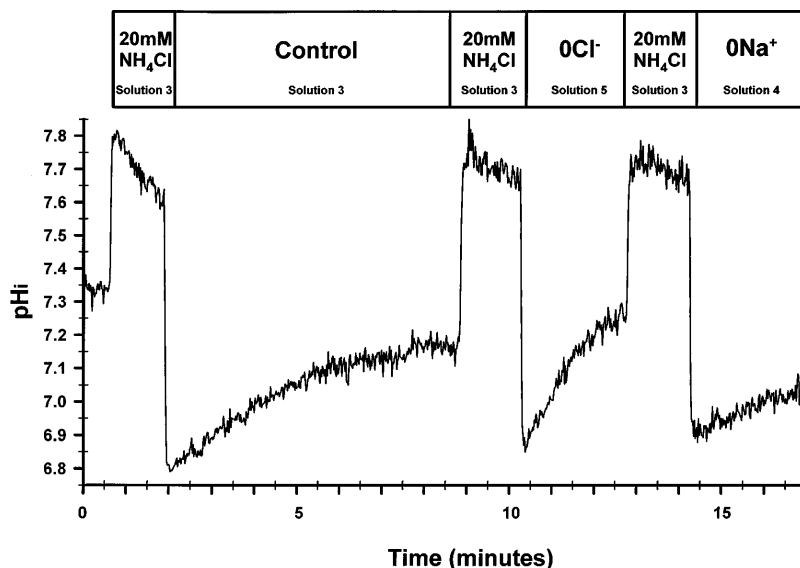
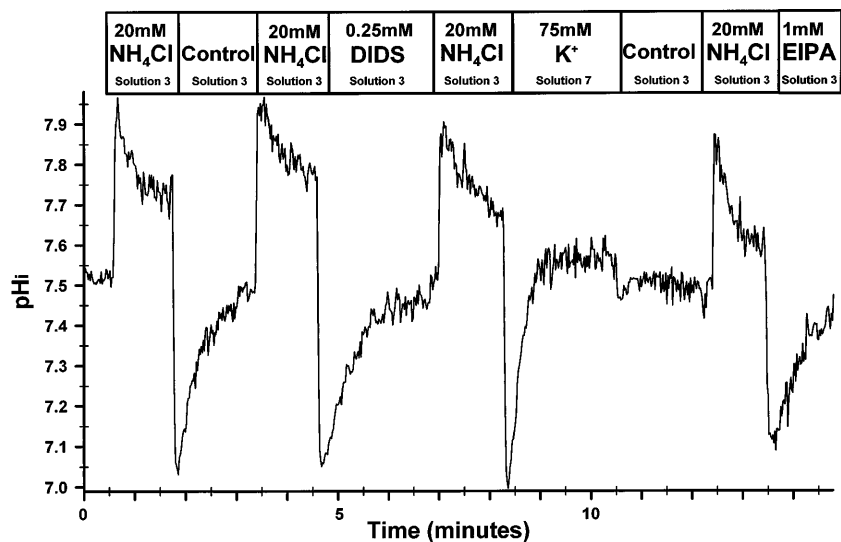


Fig. 3. pH_i in single proximal tubule from chicken long-looped mammalian-type nephron during various modifications in bathing medium. Designations in boxes above tracing indicate same changes as in Figs. 1 and 2, with the addition of Cl^- -free Ringer (0 mM Cl^-) (solution 5, Table 1).

Fig. 4. pH_i in single proximal tubule from chicken long-looped mammalian-type nephron during various modifications in bathing medium. Designations in boxes above tracing indicate same changes as in Fig. 1, with the addition of 1 mM ethylisopropylamiloride (EIPA) and 0.25 mM DIDS (*solution 3*, Table 1) and high K^+ (75 mM K^+) (*solution 7*, Table 1).



bathing medium would reduce the membrane potential, as they do in renal tubules from other species (14). Therefore, we examined the effects of increasing the K^+ concentration in the bathing medium to 75 mM (*solution 7*, Table 1) and of adding Ba^{2+} (5 mM) (*solution 8*, Table 1) to the bathing medium on the rate of recovery of pH_i . The results are shown in Table 5 and Figs. 4 and 5. As would be expected if the mechanism suggested above played a role in pH_i recovery, increasing the K^+ concentration to 75 mM produced a significant increase in the rate of recovery (Table 5; Fig. 4). However, in contrast to this expectation, the addition of 5 mM Ba^{2+} to the bathing medium had no effect on the rate of recovery (Table 5, Fig. 5). Because of the lack of effect of 5 mM Ba^{2+} on the rate of recovery, we also explored the effects of 10 mM Ba^{2+} (*solution 9*, Table 1) and of 10 mM Ba^{2+} plus 75 mM K^+ (*solution 10*, Table 1) on the rate of recovery in four experiments. The addition of 10 mM Ba^{2+} had no effect (Fig. 5), and the combination of 10 mM Ba^{2+} and 75 mM K^+ produced exactly the stimulatory effect on the rate of recovery as 75 mM K^+ alone (Fig. 5; statistical comparison of effects: $0.70 < P < 0.80$).

In our previous study on proximal tubules from transitional short-looped nephrons (13), we had not examined the effects of high K^+ or Ba^{2+} (and thus the apparent effects of changing basolateral membrane potential) on the rate of recovery of pH_i . Therefore, in the present study, we also examined the effects of these treatments on the rate of recovery of pH_i in proximal tubules from transitional short-looped nephrons. The results are shown in Table 6. As in the proximal tubules from long-looped nephrons, increasing the K^+ concentration to 75 mM produced a significant increase in the rate of recovery whereas 5 mM Ba^{2+} had no effect (Table 6).

Effects of removal of Na^+ , Cl^- , or both Na^+ and Cl^- , of high K^+ concentration, and of the addition of EIPA, DIDS, and Ba^{2+} on resting pH_i . In view of the effects of some of these treatments on the rate of recovery of pH_i , we also examined the effects of all of them on the resting pH_i . The results are summarized in Table 7. Removal of Na^+ produced a highly significant (~ 0.3 pH units) decrease in pH_i . Removal of both Na^+ and Cl^- produced a smaller (~ 0.14 pH unit) but still statisti-

Fig. 5. pH_i in single proximal tubule from chicken long-looped mammalian-type nephron during various modifications in bathing medium. Designations in boxes above tracing indicate same changes as in Fig. 1, with the addition of Ba^{2+} (5 mM Ba^{2+} or 10 mM Ba^{2+}) (*solutions 8 and 9*, Table 1) and Ba^{2+} plus high K^+ (10 mM Ba^{2+} , 75 mM K^+) (*solution 10*, Table 1).

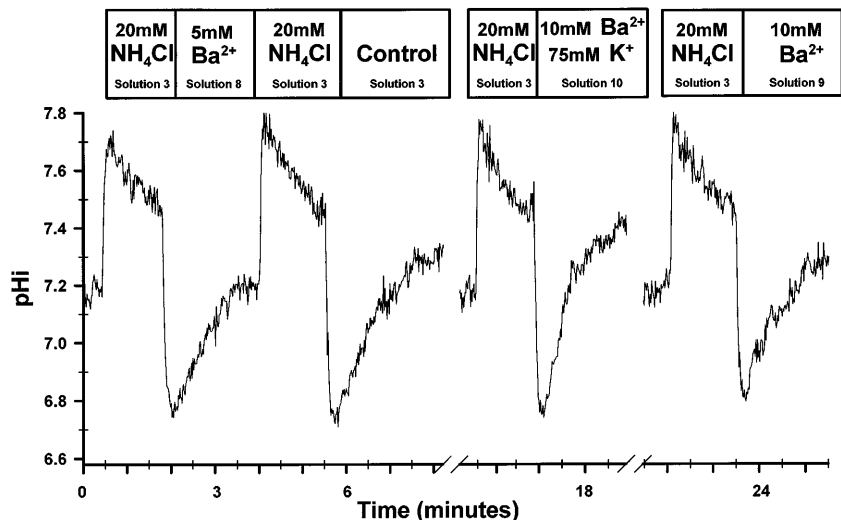


Table 6. *Effects of high K⁺ and Ba²⁺ on dpH_i/dt from acid pH_i to control resting pH_i in proximal tubules from chicken transitional short-looped nephrons*

Treatment	dpH _i /dt, pH U/s × 10 ⁻³	
	Before treatment	During treatment
High K ⁺ (75 mM)	3.62 ± 0.54 (9) ^a	6.17 ± 0.03 (9) ^a
Ba ²⁺ (5 mM)	3.62 ± 0.54 (9)	3.61 ± 0.97 (9)

Values are means ± SE for all tubules studied; numbers in parentheses indicate number of tubules. Although mean values for all tubules are shown for the sake of presentation, effect of each treatment on dpH_i/dt was evaluated statistically by paired analysis, with each tubule serving as its own control. Values for treatments that differ significantly ($P < 0.05$) by paired analysis from corresponding controls are shown by matching letter superscripts.

cally significant decrease in pH_i, whereas removal of Cl⁻ alone had no effect. Increasing the K⁺ concentration to 75 mM, which should have markedly reduced the basolateral membrane potential, produced a significant increase in resting pH_i. However, the addition of 5 mM Ba²⁺ to the bathing medium, which should also have reduced the basolateral membrane potential, had no effect on resting pH_i. Neither EIPA nor DIDS had any effect on resting pH_i.

DISCUSSION

The present study indicates that regulation of pH_i in nonperfused proximal tubules of chicken long-looped mammalian-type nephrons is essentially the same as that in nonperfused proximal tubules of chicken short-looped transitional nephrons (13) (also called short-looped mammalian-type nephrons) (6), whereas it is substantially different from that in nonperfused proximal tubules of chicken loopless reptilian-type nephrons studied under the same conditions (20). The resting pH_i (~7.30) in proximal tubules from long-looped mammalian-type nephrons in HEPES-buffered medium was essentially the same as the resting pH_i in proximal tubules from transitional short-looped nephrons in HEPES-buffered medium measured in our previous

Table 7. *Effects of treatments on resting pH_i in proximal tubules from chicken long-looped mammalian-type nephrons*

Treatment	Resting pH _i	
	Control	After change
0 Na ⁺	7.24 ± 0.04 (16) ^a	6.91 ± 0.05 (16) ^a
0 Cl ⁻	7.23 ± 0.03 (24)	7.22 ± 0.03 (24)
0 Na ⁺ , 0 Cl ⁻	7.25 ± 0.04 (17) ^b	7.11 ± 0.04 (17) ^b
EIPA (1 mM)	7.36 ± 0.03 (12)	7.40 ± 0.04 (12)
DIDS (0.25 mM)	7.35 ± 0.03 (12)	7.32 ± 0.04 (12)
High K ⁺ (75 mM)	7.34 ± 0.04 (10) ^c	7.52 ± 0.01 (10) ^c
Ba ²⁺ (5 mM)	7.18 ± 0.06 (7)	7.19 ± 0.05 (7)

Values are means ± SE for all tubules studied; numbers in parentheses indicate number of tubules. Although mean values for all tubules are shown for the sake of presentation, effect of each treatment on resting pH_i was evaluated statistically by paired analysis, with each tubule serving as its own control. Values for changes that differ significantly ($P < 0.05$) by paired analysis from corresponding controls are shown by matching letter superscripts.

study (13). However, it was significantly higher than the resting pH_i (~7.20 or less) in proximal tubules from loopless reptilian-type nephrons also examined previously under these same conditions (20). It was also significantly higher than the resting pH_i in snake and rabbit proximal tubules in HEPES-buffered media, measured by others and by us with the same pH-sensitive fluorescent dye (BCECF) technique (9, 14, 16).

The general pattern of the response of pH_i to an NH₄Cl pulse was similar in proximal tubules from these chicken long-looped mammalian-type nephrons, chicken short-looped transitional nephrons (13), chicken loopless reptilian-type nephrons (20), snake nephrons, and rabbit nephrons (14). There were, however, some minor quantitative differences in the information derived from these responses between proximal tubules from different chicken nephron populations and proximal tubules from snake and rabbit nephrons, as shown in Table 3. As a general pattern, proximal tubules from all three types of chicken nephrons tended to have a greater dpH_i/dt than snake proximal tubules and a lower β_i than rabbit proximal tubules (13, 14, 20) (Table 3).

With regard to regulation of pH_i, the observations on the factors influencing recovery from the acid value following the NH₄Cl pulse to the normal, slightly alkaline value are particularly revealing. The control rate of recovery was about the same in proximal tubules from all three types of chicken nephrons (Table 4). This rate in chicken proximal tubules was not significantly different from that in rabbit proximal tubules, but was more than twice that in snake proximal tubules (Table 4). The more rapid rate of recovery of pH_i in the chicken and rabbit tubules than in the snake tubules may reflect primarily the temperature differences between the studies. Snake tubules were studied at 25°C (14), whereas chicken and rabbit tubules were studied at 37°C (13, 14, 20).

In proximal tubules from chicken long-looped mammalian-type nephrons, as in proximal tubules from chicken short-looped transitional nephrons (13), both the removal of Na⁺ from the bath and the addition of EIPA or amiloride to the bath in the presence of Na⁺ depressed the rate of recovery of pH_i, strongly suggesting that Na⁺/H⁺ exchange at the basolateral membrane plays a role in this recovery process (Fig. 6, *transporter 1*). In contrast, in proximal tubules from chicken loopless reptilian-type nephrons (20) and from snake nephrons (14), removal of Na⁺ from the bath depressed the rate of recovery but even 1 mM EIPA or amiloride had no effect on the rate of recovery. Therefore, a basolateral amiloride-inhibitable Na⁺/H⁺ exchanger appears very likely to exist in proximal tubules of avian long-looped mammalian-type nephrons, avian short-looped transitional nephrons (13), amphibian nephrons (4), and mammalian nephrons (10), but not in the proximal tubules of avian loopless reptilian-type nephrons (20) and reptilian nephrons (14).

The inhibitory effect of Na⁺ removal on the rate of recovery in proximal tubules from chicken long-looped

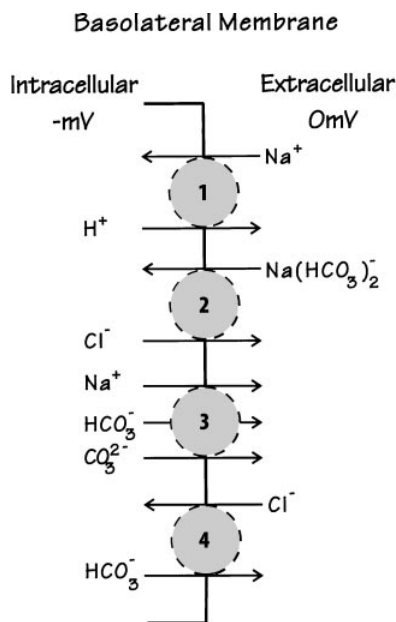


Fig. 6. Diagram illustrating some basolateral acid or base transporters for regulating pH_i as described previously for mammalian proximal tubules (see text).

nephrons could reflect the presence of a DIDS-inhibitable basolateral Na^+ -coupled $\text{Cl}^-/\text{HCO}_3^-$ exchanger moving HCO_3^- into the cells, as described for mammalian tubules (Fig. 6, *transporter 2*) (2, 3, 10, 11). Some HCO_3^- (mostly generated by the tubule cells) must be present even in nominally HCO_3^- -free solutions, such as those used in the present experiments and our other experiments on avian nephrons (13), and Na^+ -dependent HCO_3^- transporters apparently function under these circumstances in mammalian nephrons (10, 15). The observation in the present study that the addition of DIDS to the bath in the presence of Na^+ inhibited the rate of recovery of pH_i to about the same extent as Na^+ removal suggests that a Na^+ -coupled $\text{Cl}^-/\text{HCO}_3^-$ exchanger might play a significant role in regulating pH_i in chicken long-looped mammalian-type nephrons. This possibility is also strongly supported by the observation that removal of Cl^- from the bathing medium increased the rate of recovery of pH_i , a result anticipated if this transporter was present and functioning because HCO_3^- movement into the cells would be enhanced. Similar data support the presence of such a basolateral transporter in chicken short-looped transitional nephrons (13). However, the lack of effect of Cl^- removal on recovery of pH_i in proximal tubules from chicken loopless reptilian-type nephrons suggests that this transporter is not a likely mechanism for regulating pH_i in those nephrons (20).

A basolateral DIDS-sensitive, electrogenic Na^+ - HCO_3^- - CO_3^{2-} cotransporter that moves HCO_3^- out of the proximal tubule cells (Fig. 6, *transporter 3*) has been reported for amphibians and mammals and plays an important role in transepithelial HCO_3^- reabsorption in these other vertebrate classes (1, 5, 10, 17, 27). However, the evidence for such a transporter in avian proximal tubules is conflicting in this and our previous

studies (13, 20). Its presence is supported by the effects of Na^+ replacement and a high K^+ concentration on the rate of recovery of pH_i in all three avian nephron types. However, the lack of effect of Ba^{2+} in all nephron types appears to militate against such a transporter. This observation does not completely rule out the presence of such a transporter because there might be no Ba^{2+} -sensitive K^+ channels in this membrane and Ba^{2+} might not have any effect on membrane potential. This appears to be likely in view of the observation in this study and our preceding one on loopless nephrons (20) that a high K^+ concentration enhanced recovery of pH_i to the same extent whether Ba^{2+} was present or not. Because of the extreme fragility of these isolated avian nephrons, we have not attempted to measure directly the membrane potential with microelectrodes as we have in other species (14).

Finally, however, the Na^+ - HCO_3^- - CO_3^{2-} cotransporter (Fig. 6, *transporter 3*) is inhibitable by DIDS in other species (5, 10), and such inhibition should enhance the recovery of pH_i after acidification. In the present study and in our previous studies (13, 20), DIDS reduced the rate of recovery of pH_i in proximal tubules from all three nephron types. In the case of the long-looped mammalian-type nephrons and the short-looped transitional nephrons, these data suggest that *transporter 3* (Fig. 6), if present and if sensitive to DIDS, is less important in the recovery process (and, presumably, in the maintenance of resting pH_i) than the Na^+ -coupled $\text{Cl}^-/\text{HCO}_3^-$ exchanger (Fig. 6, *transporter 2*).

The stimulation of the rate of recovery of pH_i by Cl^- removal could reflect depressed extrusion of HCO_3^- from the cells (or actual uptake of HCO_3^-) via a basolateral Na^+ -independent $\text{Cl}^-/\text{HCO}_3^-$ exchanger (Fig. 6, *transporter 4*) in proximal tubules from chicken long-looped mammalian-type nephrons in the present study and in proximal tubules from chicken short-looped transitional nephrons in our earlier study (13). Such a renal transporter has been described in other vertebrate species (2, 3, 22). DIDS should inhibit this transporter (2, 3, 22), and such inhibition should lead to an increase in the rate of recovery of pH_i after acidification. However, DIDS depressed the rate of recovery in both long-looped mammalian-type nephrons and short-looped transitional nephrons (13). This observation suggests that, if both this Na^+ -independent exchanger and the Na^+ -coupled exchanger discussed above (Fig. 6, *transporter 2*) are present and inhibited by DIDS, the Na^+ -coupled exchanger is more important than the Na^+ -independent exchanger in the process of pH_i recovery after acidification. There is no evidence for a basolateral Na^+ -independent $\text{Cl}^-/\text{HCO}_3^-$ exchanger in the loopless reptilian-type nephrons (20).

In both the chicken long-looped mammalian-type nephrons in the present study and the chicken short-looped transitional nephrons in the previous study (13), the rate of recovery of pH_i after acidification proceeded at the control rate even in the absence of both Na^+ and Cl^- . We can only speculate on the manner in which this occurs. If there is a Na^+ -independent $\text{Cl}^-/\text{HCO}_3^-$ exchanger in the basolateral membrane, HCO_3^- might

enter the cells via reversal of this exchanger even with a very low concentration of HCO_3^- in the bath. At the same time, acid might leave the cells by some pathway independent of Na^+ and Cl^- . As an example, it might leave as NH_4^+ , probably through K^+ channels. The loss of acid and uptake of base by Na^+ - and Cl^- -independent pathways might result in essentially the same rate of recovery of pH_i as in the presence of Na^+ and Cl^- .

Perspectives

The data from this and our previous studies (13, 20) indicate that basolateral regulation of pH_i is essentially the same in isolated nonperfused proximal tubules from chicken long-looped mammalian-type nephrons and short-looped transitional nephrons (often referred to as "short-looped mammalian-type nephrons" because of the presence of the loop segment) (6), whereas it is distinctly different in isolated nonperfused proximal tubules from chicken loopless reptilian-type nephrons. In short- and long-looped mammalian-type nephrons, only a Na^+/H^+ exchanger and possibly a Na^+ -dependent Cl/HCO_3^- exchanger (Fig. 6, *transporters 1* and *2*) seem likely to be important in the normal regulation of pH_i .

In the loopless reptilian-type nephrons, not one of the four mechanisms for basolateral acid or base transfer discussed above (Fig. 6) appears to play a role in regulating pH_i (20). Instead, some totally different Na^+ -dependent mechanism for moving HCO_3^- into the cells across the basolateral membrane may be present (20). However, determining with certainty the existence of such a basolateral HCO_3^- transporter requires more detailed study of the dependence of pH_i regulation on the availability of extracellular HCO_3^- in reptilian-type nephrons. Finally, at least in terms of basolateral acid-base transporters, these avian reptilian-type nephrons may actually resemble true reptilian nephrons more closely than they do either short- or long-looped mammalian-type avian nephrons (13, 14, 20). Much more clearly remains to be learned about these apparent differences between nephron populations in the avian kidney and how they may be related to maintenance of both pH_i and overall acid-base balance.

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