Basolateral Na-H Exchange in the Rabbit Cortical Collecting Tubule

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ABSTRACT We used the intracellular absorbance spectrum of the dye 4′,5′-dimethyl-5- (and -6-) carboxyfluorescein (Me_2CF) to measure intracellular pH (pH_i) in the isolated, perfused cortical collecting tubule (CCT) of the rabbit nephron. The incident spot of light was generally 10 μm in diameter, large enough to illuminate from two to six cells. No attempt was made to distinguish principal from intercalated cells. All experiments were carried out in HCO_3^--free Ringer to minimize HCO_3^- transport. When cells were acid-loaded by briefly exposing them to Ringer containing NH_4^+ and then withdrawing the NH_4^+, pH_i spontaneously recovered from the acid load. The pH_i recovery was best fit by the sum of two exponentials. When the acid loading was performed in the absence of Na^+, the more rapid of the two phases of pH_i recovery was absent. The remaining slow phase never returned pH_i to normal and was sometimes absent. Returning Na^+ to the lumen had only a slight effect on the pH_i recovery. However, when Na^+ was returned to the basolateral (i.e., blood-side) solution, pH_i recovered rapidly and completely. The apparent K_m for basolateral Na^+ was 27.3 ± 4.5 mM. The basolateral Na-dependent pH_i recovery was reversibly inhibited by amiloride. We conclude that the mechanism responsible for the rapid phase of pH_i recovery is an Na-H exchanger confined primarily, if not exclusively, to the basolateral membrane of the CCT.

INTRODUCTION

The cortical collecting tubule (CCT) of the rabbit plays a major role in the transport of Na^+, K^+, and HCO_3^- by the kidney. Experiments on the isolated, perfused CCT have shown that this segment reabsorbs Na^+ and secretes K^+ (Grantham et al., 1970). If the donor animal is fed a normal or acid diet, the CCT reabsorbs HCO_3^- (McKinney and Burg, 1977, 1978*a*; Koeppen and Helman, 1982), whereas if the animal is fed an alkaline diet, HCO_3^- is secreted (McKinney and Burg, 1977, 1978*b*; Lombard et al., 1983). Moreover, there is evidence that transport function may be segregated by cell type. The more

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numerous principal cells (Kaissling and Kriz, 1979) appear to be involved in Na^+ and K^+ transport (Wade et al., 1979; O'Neil and Hayhurst, 1984), whereas the intercalated cells, which are rich in carbonic anhydrase (Dobyan et al., 1982), may be responsible for HCO_3^- transport. Recent evidence on the rabbit CCT (Schwartz and Al-Awqati, 1985) and its analogue, the turtle bladder (Stetson and Steinmetz, 1985), suggests that HCO_3^- reabsorption and secretion may be mediated by different subpopulations of these carbonic anhydrase–rich cells.

There is still uncertainty about the mechanisms of H^+ and HCO_3^- transport in each of these cell types. Although the principal cells are not thought to be involved in the transepithelial transport of H^+ or HCO_3^-, they presumably have a requirement for the regulation of their internal pH that is similar to that of other cells (see Roos and Boron, 1981). This could be accomplished, as in most vertebrate cells, by an Na-H exchanger (Boron, 1983). Considering the likely transmembrane gradients for H^+ and Na^+, one would predict that such an Na-H exchanger would probably be at the basolateral, rather than the luminal, membrane (Boron, 1983). By analogy to the turtle bladder (see reviews by Steinmetz and Andersen, 1982; Al-Awqati, 1978), it is thought that the cells responsible for HCO_3^- reabsorption (presumably the intercalated cells or a subpopulation thereof) possess an electrogenic H^+ pump that extrudes H^+ across the luminal membrane. In addition, it is thought that HCO_3^- exits the cell across the basolateral membrane via a Cl-HCO_3 exchanger. By analogy with the turtle bladder (Cohen, 1980), the cells that secrete HCO_3^- may have a Cl-HCO_3 exchanger at the luminal membrane, and an unspecified H^+-extruding mechanism at the basolateral membrane. The luminal Cl-HCO_3 exchanger hypothesis is consistent with the observation that alkali secretion by the CCT is influenced by the concentration of luminal Cl^- (Laski et al., 1983). The observation that transepithelial HCO_3^- secretion is not inhibited by the removal of Na^+ (Schuster, 1985; Star et al., 1985) suggests that the basolateral H^+-extruding mechanism is not an Na-H exchanger.

One approach for determining the identity and location of H^+ and HCO_3^- transport systems in the CCT is to investigate the mechanisms by which intracellular pH (pH_i) is regulated. Such a study could also identify mechanisms of potential importance for the transepithelial transport of HCO_3^-. In the present study, we have examined pH_i regulation in the isolated, perfused CCT. pH_i was calculated from the intracellular absorbance spectrum of the pH-sensitive dye 4′,5′-dimethyl-5- (and -6-) carboxyfluorescein (Me_2CF), an approach previously tested in the salamander proximal tubule (Chaillet and Boron, 1985). Because the incident light was a spot 10 μm in diameter, focused on a group of two to six random cells, our pH_i measurements presumably reflect a weighted average of the pH_i values of both principal and intercalated cells. No attempt was made to distinguish these cell types. We found that the recovery of pH_i from an intracellular acid load usually occurred in two distinct phases. One was independent of Na^+ and relatively slow. The other required basolateral Na^+, was inhibited by amiloride, and was substantially faster. Inasmuch as this rapid component is present in the absence of HCO_3^-, it is probably mediated by a basolateral Na-H exchanger.

A portion of this work has been published in abstract form (Chaillet and Boron, 1984).

METHODS

General

The chamber and the optical system were the same as those described in the preceding paper (Chaillet and Boron, 1985), except that the diameter of the beam of light focused on the tubule was usually 10 μm. Absorbance spectra of the intracellular dye were recorded approximately once every 2 s by computer.

TABLE I

Compositions of Solutions

Component	(1) Standard HEPES	(2) NH_4^+ HEPES	(3) pH 6.8 HEPES	(4) 0-Na, HEPES	(5) 0-Na, NH_4^+ HEPES	(6) 0-Na, high-K^+ HEPES
Na^+	146.4	126.4	141.6	0	0	0
K^+	5.0	5.0	5.0	5.0	5.0	105.0
NH_4^+	0	20.0	0	0	20.0	0
$NMDG^+$	0	0	0	146.4	126.4	46.4
Mg^{2+}	1.2	1.2	1.2	1.2	1.2	1.2
Ca^{2+}	1.0	1.0	1.0	1.0	1.0	1.0
meq(+)	155.8	155.8	151.0	155.8	155.8	155.8
Cl^-	122.0	122.0	128.0	122.0	122.0	122.0
$H_2PO_4^-$	0.4	0.4	1.0	0.4	0.4	0.4
$HPO_4^=$	1.6	1.6	1.0	1.6	1.6	1.6
$Acetate^-$	10.0	10.0	10.0	10.0	10.0	10.0
$HEPES^-$	17.8	17.8	7.6	17.8	17.8	17.8
$SO_4^=$	1.2	1.2	1.2	1.2	1.2	1.2
meq(−)	155.8	155.8	151.0	155.8	155.8	155.8
Glucose	5.5	5.5	5.5	5.5	5.5	5.5
Alanine	5.0	5.0	5.0	5.0	5.0	5.0
HEPES	14.4	14.4	24.6	14.4	14.4	14.4
pH	7.4	7.4	6.8	7.4	7.4	7.4

The concentrations of all components are in millimolar.

Biological Preparation

CCTs were obtained from pathogen-free, female New Zealand white rabbits (Dutchland, Inc., Reston, VA), weighing 2–5 lbs. Single tubules were isolated in cold HEPES Ringer (solution 1, below), titrated to pH 7.40 at 4°C. After transfer to the chamber, the tubule was perfused at 37°C by the method of Burg et al. (1966). The length of the exposed tubule between the perfusion pipettes was ~200 μm. The tubules were acclimatized at 37°C for ~1 h, part of which time was used for loading the cells with dye.

Solutions

The compositions of the Ringer solutions are given in Table I. The solutions were buffered with HEPES to the appropriate pH. For the determination of the intracellular dye calibration spectra, nigericin (Calbiochem-Behring Corp., La Jolla, CA) was added to

solution 6 from a stock solution (10 mM in ethanol) to a final nigericin concentration of 10 μM. The colorless dye precursor 4′,5′-dimethyl-5- (and -6-) carboxyfluorescein diacetate (Me_2CFAc_2) was obtained from Molecular Probes, Inc., Junction City, OR. For loading cells with dye, Me_2CFAc_2 was added to solution 3 from a stock solution (100 mM in dimethyl sulfoxide) to a final concentration of 100 μM. *N*-Methyl-D-glucamine (NMDG) and HEPES were obtained from Sigma Chemical Co., St. Louis, MO. Ringer solutions with an Na^+ concentration between 0 and 145 mM were made by mixing solutions 1 and 4. Amiloride hydrochloride (a gift of Merck, Sharpe & Dohme, West Point, PA) was sometimes added to solutions 1 and 4 and those containing intermediate concentrations of Na^+.

RESULTS

Intracellular pH Calibration of the Dye

In the preceding paper (Chaillet and Boron, 1985), on the salamander proximal tubule, we demonstrated that steady state values of pH_i calculated from absorbance spectra Me_2CF are within ~0.1 pH unit of those obtained with pH-sensitive microelectrodes. The two techniques also agree closely during rapid pH_i transients. Because the absorbance spectrum of the intracellular dye in these salamander experiments differed from that obtained in vitro, we performed an intracellular dye calibration. pH_i was nominally clamped to extracellular pH (pH_o) by exposing the tubule to a solution containing the K-H exchanger nigericin, and having a K^+ activity chosen to match the intracellular K^+ activity. Intracellular dye absorbance spectra were then obtained over a range of pH_o values. In the present experiments, we performed a similar intracellular dye calibration on the rabbit CCT. Inasmuch as there are no published data on the K^+ activity of the rabbit CCT, we assumed a value of 80 mM (a concentration of ~105 mM, assuming an intracellular K^+ activity coefficient of 0.75). This represents the same fraction of total osmolality as measured in the salamander proximal tubule (Sackin and Boulpaep, 1981), and is within the range of measured K^+ levels in mammalian tubules in general (see Boron and Sackin, 1983). Furthermore, we have shown that in the salamander (Chaillet and Boron, 1985), modest changes in $[K^+]_o$ have little effect on the intracellular calibration spectra.

Fig. 1*A* illustrates an experiment on the CCT in which we varied pH_o from 5.89 to 8.15 in the presence of 10 μM nigericin and 105 mM K^+ (solution 6). The ordinate is the ratio of the peak absorbance (510 nm) to the absorbance at the in vitro isosbestic wavelength (470 nm). Representative spectra, obtained after $A(510)/A(470)$ reached a steady value, are plotted in Fig. 1*B*, scaled to an absorbance of unity at 470 nm. The $A(510)/A(470)$ ratios from the spectra of Fig. 1*B* are plotted as a function of pH in Fig. 1*C*. The curve drawn through the points is the result of a nonlinear least-squares fit to a standard pH titration curve, having a pK_a of 7.36 ± 0.01 at 37°C. In the salamander proximal tubule, the in vivo pK_a at 23°C is 7.35 ± 0.03, shifted to a value 0.44 greater than the in vitro pK_a (Chaillet and Boron, 1985). Although we have no in vitro data at 37°C, it is likely that a similar pK_a shift occurs in the rabbit CCT.

The above calibration procedure is subject to the same uncertainties as outlined in the preceding work on salamander proximal tubules (Chaillet and Boron, 1985). Because these uncertainties are of a quantitative nature, however, they are unlikely to affect the general conclusions of the present study.

The measured value of pH_i in these experiments is necessarily a mean value for all the cells illuminated by the incident light (see Discussion). Our spot of

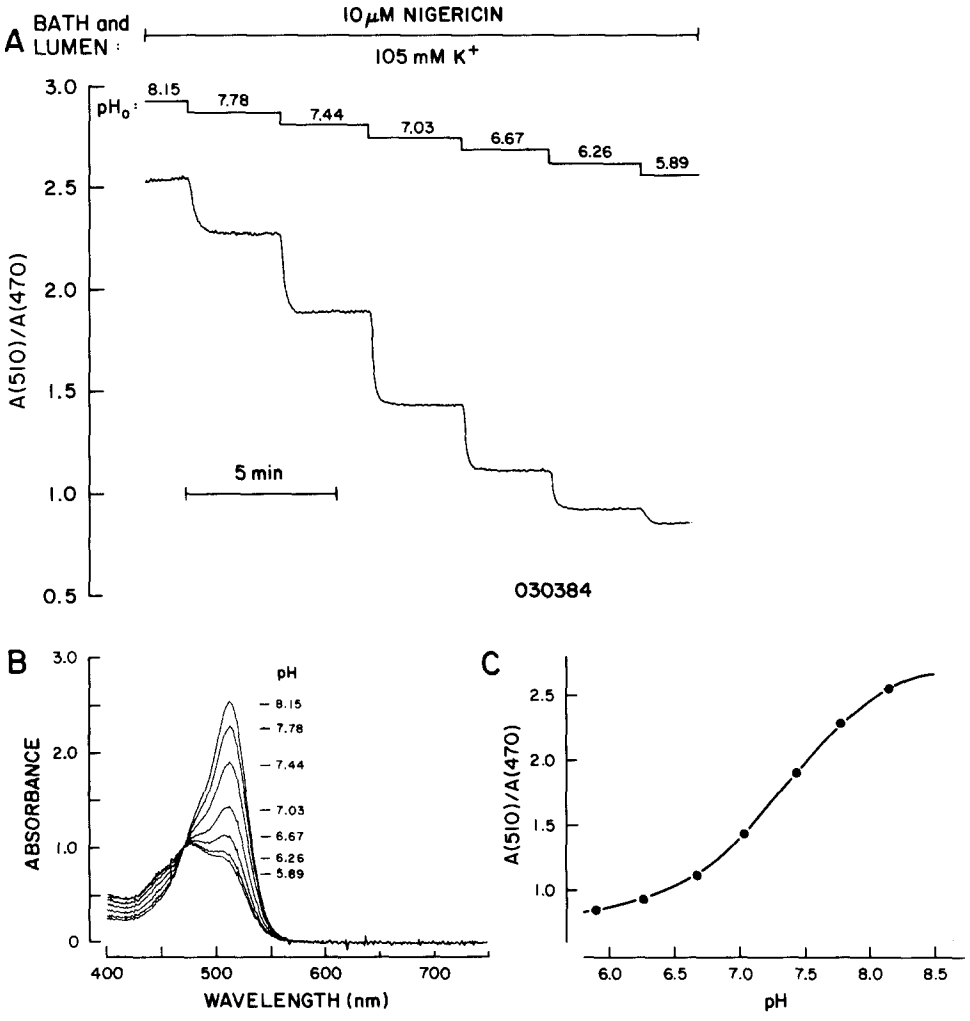

FIGURE 1. Calibration of intracellular dye. (*A*) Time course of $A(510)/A(470)$ while pH_i was varied. See text for details. The initial $A(510)/A(470)$ at pH 8.15 (solution 6) was 2.53. (*B*) Absorbance spectra obtained during periods of stable $A(510)/A(470)$ in the experiment of *A*. (*C*) In vivo dye calibration curve. The points are derived from the spectra of *B*. The curve drawn through the points is a nonlinear least-squares fit to the data. The pK_a is 7.36 ± 0.01, and the upper and lower asymptotes are 2.84 ± 0.01 and 0.78 ± 0.01, respectively.

light was sufficiently small (i.e., 10 μm) that, in some experiments, probably only cells of the majority type (i.e., principal cells) were illuminated. Only in this fortuitous case would the measured pH_i pertain to a single cell type. In most experiments, cells of the minority type (i.e., intercalated cells) were probably also illuminated, so that the measured pH_i would pertain to both cell types.

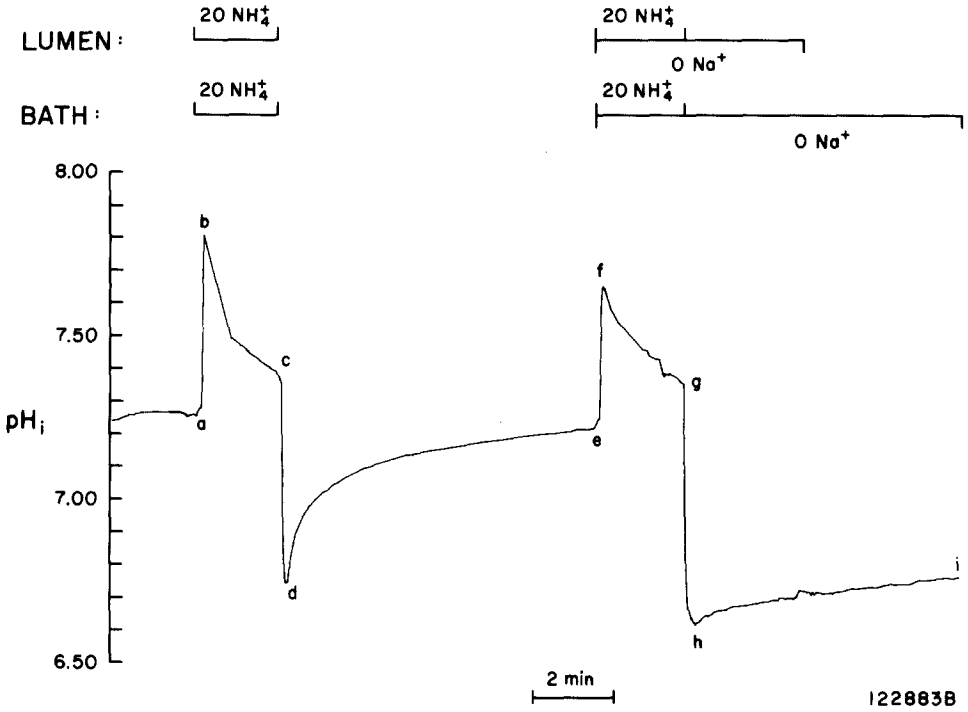

FIGURE 2. pH_i recovery from an NH_4^+-induced acid load in the presence and absence of Na^+. The pH_i recovery (*de*) from the first NH_4^+-induced acid load had a rapid and a slower phase. The time constants of this double-exponential pH_i recovery were 16.3 ± 1.4 and 187.3 ± 10.4 s, respectively. In the absence of Na^+, the pH_i recovery (*hi*) from the acid load lacked the rapid phase. The remaining slow phase had a time constant of 231.0 ± 16.8 s. At the end of the experiment, intracellular dye calibration spectra were obtained. The resultant in vivo pH titration curve was then used to compute the ordinate scale.

In a total of 38 CCTs, the average initial absorbance at 470 nm was 0.113 ± 0.006. Assuming an average total path length of 15 μm through the tubule cells, and an extinction coefficient of 1.9×10^4 M^{-1} cm^{-1}, this corresponds to an average intracellular dye concentration of 4.0 ± 0.2 mM. This is within the range of 2–4 mM for the intracellular dye concentration in the previous study on the salamander proximal tubule.

Recovery of pH_i from an Acid Load

Fig. 2 illustrates an experiment in which cells of a CCT were twice acid-loaded by a 2-min exposure to 20 mM NH_4^+ (Boron and De Weer, 1976). The rapid rise

of pH_i during *ab* was due to the influx of the weak-base NH_3. The subsequent slow decline of pH_i (*bc*) was caused by the slower, passive influx of the weak-acid NH_4^+, possibly augmented by Cl-OH exchange or by NH_4^+ uptake mediated by the Na pump. Upon removal of the external NH_4^+, pH_i rapidly fell (*cd*) to a value ~0.7 below the initial value (compare *a* and *d*). In the presence of extracellular Na^+, pH_i spontaneously recovered (*de*) from this acid load, as a result of one or more pH_i-regulatory mechanisms. The time course of this pH_i recovery is fitted by the sum of two exponentials, a rapid phase with a time constant of 16.3 ± 1.4 s and a slow phase with a time constant of 187.3 ± 10.4 s. The tubule cells were then acid-loaded for a second time (*e–h*), but in the total absence of extracellular Na^+ (replaced by $NMDG^+$, solutions 5 and 4). This Na^+ removal blocked the rapid-phase pH_i recovery, leaving only a slow phase (*hi*) with a time constant of 231.0 ± 16.8 s. The similarity in slow-phase time constants for segments *de* and *hi* suggests, but does not prove, that the slow phases in the presence (*de*) and absence (*hi*) of Na^+ may be mediated by the same Na-independent mechanism.

We performed a total of nine experiments in which the pH_i recovery in the absence of Na^+ was allowed to continue until pH_i reached a stable value. Fig. 3*A* illustrates one of three experiments in which the slow-phase pH_i recovery was virtually absent. Fig. 3*B* is one example of the remaining six experiments, in which the slow-phase pH_i recovery was present. The measured pH_i clearly stabilized at a level far below the initial value. In these six experiments, the average pH_i recovery amounted to 14.6% of the initial acid load.

Calculation of Intracellular Buffering Power

Experiments of the type shown in Fig. 3 can also be used to determine intracellular buffering power (see Boron, 1977). By definition, the intracellular buffering power is the magnitude of the intracellular acid load (i.e., the amount of strong acid added to the cell, given in millimolar) divided by the resultant fall of pH_i. The magnitude of the acid load is the calculated $[NH_4^+]_i$ at the time just before removal of external NH_4^+. Upon removal of the external NH_4^+ and NH_3, this internal NH_4^+ dissociates into NH_3 (which passively leaves the cell) and H^+. The latter is trapped inside and constitutes the intracellular acid load. The pH_i decrease is simply the fall of pH_i that occurs when the external NH_4^+ is removed. The accuracy with which we can measure the pH_i decrease is limited by two factors, both of which relate to the low values to which pH_i falls the after removal of the external NH_4^+. First, such low pH_i values are far from the apparent pK of the dye, limiting the dye's sensitivity. Second, the effects of nigericin calibration errors can be expected to be greatest at extreme pH_i values (Chaillet and Boron, 1985).

An accurate determination of intracellular buffering power also requires the blockade of all pH-related ion transport systems. This requirement was approximately met in a total of 14 experiments, the 9 discussed in conjunction with Fig. 3, and 5 others. In all cases, the Na-H exchanger was blocked by Na^+ removal, and potential HCO_3^- transporters were inhibited by the nominal removal of HCO_3^-. Although the slow-phase mechanism of Fig. 3*B* was not blocked, the resultant pH_i changes were so slow as to negligibly affect the calculation. The result was a mean intracellular buffering power of 24.7 ± 1.7 mM. This value

includes the buffering power of all non-CO_2 or intrinsic intracellular buffers, and could be contaminated to some extent by HEPES buffer, which may have entered the cells.

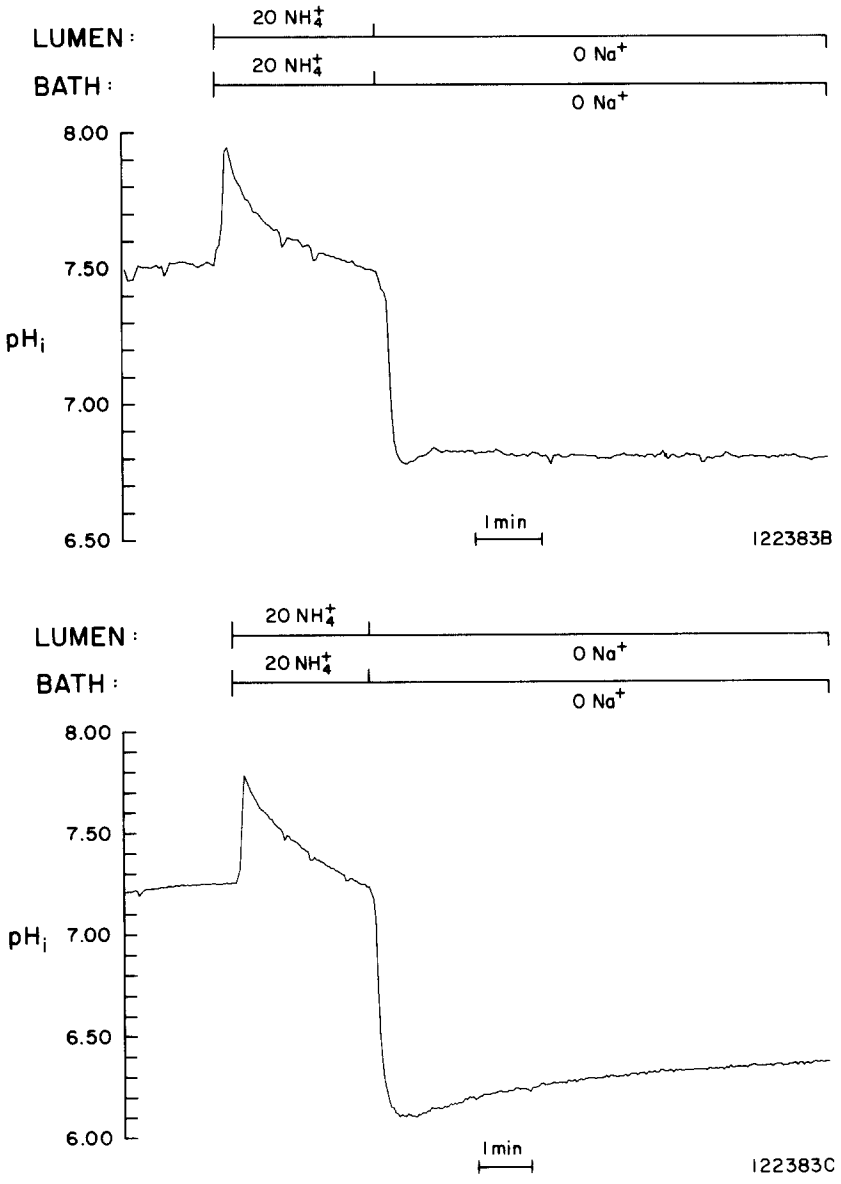

FIGURE 3. Two examples of pH_i recovery in the absence of Na^+. In both *A* and *B*, the cells were acid-loaded with a brief pulse of 20 mM NH_4^+ to the lumen and bath in the absence of Na^+ (solution 5). Removal of NH_4^+, in the continued absence of Na^+ (solution 4), caused a rapid acidification in both examples. In *A*, there was no recovery of pH_i. In *B*, pH_i partially recovered, with a time constant of 124 s.

The Na^+ Dependence of the Rapid-Phase Mechanism

Fig. 2 demonstrated that the rapid-phase pH_i recovery mechanism is blocked by removing Na^+ from both the lumen and bath. To determine the sidedness of this Na^+ dependence, we performed the experiment of Fig. 4. In the absence of Na^+, the pH_i recovery (*ab*) from the NH_4^+-induced acid load was very slow. This suggests that the slow-phase mechanism is relatively inactive. The addition of 145 mM Na^+ to only the lumen produced a very slow pH_i recovery (*bc*). The subsequent removal of luminal Na^+ caused a slow pH_i decline (*cd*). When 145 mM Na^+ was then added to the bath alone, pH_i recovered rapidly and completely (*de*). The maximal rate of pH_i recovery in *de* (0.0182 pH/s) was 13-fold greater

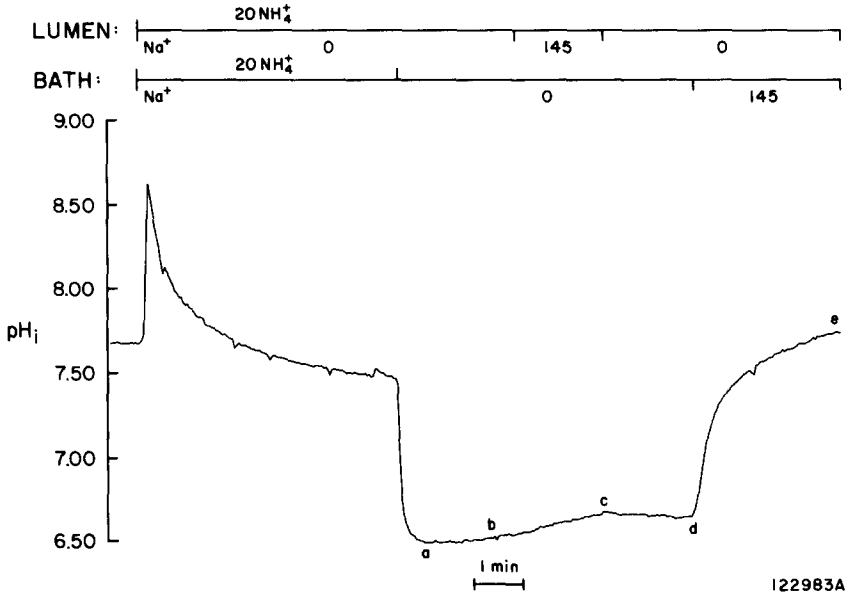

FIGURE 4. Dependence of the pH_i recovery on luminal vs. basolateral Na^+. The cells were acid-loaded by briefly pulsing with 20 mM NH_4^+ (solution 5). See text for details.

than that in *bc* (0.0014 pH/s). In a total of five similar experiments, the factor was 13.7 ± 4.7. Thus, the Na-dependent, rapid-phase mechanism is confined primarily, if not exclusively, to the basolateral membrane.

In order to quantify the basolateral Na^+ dependence of the rapid-phase pH_i recovery, we performed a series of experiments similar to the one of Fig. 5. After the cells were acid-loaded in the absence of luminal and basolateral Na^+, the slow-phase mechanism caused a partial pH_i recovery (*ab*), followed by a slow and modest decline of pH_i to a stable value (*bc*). Then, in the absence of any slow-phase contribution to pH_i recovery, we determined the effect of three concentrations of basolateral Na^+ on the rate of rapid-phase pH_i recovery. When 4.4 mM Na^+ was added, the pH_i recovery was approximately linear, with a rate

of 0.0011 pH/s (*cd*). This Na-induced recovery was halted upon removal of basolateral Na^+, and pH_i slowly declined (*de*). The addition of 29 mM Na^+ (*ef*) resulted in a substantially higher initial rate of pH_i recovery, 0.0063 pH/s. The subsequent removal of Na^+ caused a pH_i decline (*fg*). This acidification is consistent with a persistent acid load in the absence of the Na-dependent acid extrusion mechanism or, more likely, a reversal of the Na-dependent mechanism. Indeed, we observed that amiloride, an inhibitor of Na-H exchange, slowed this pH_i decline (not shown). Finally, the addition of 145 mM Na^+ (solution 1) resulted in a rapid, complete recovery of pH_i (*gh*). Segment *gh* was well fitted by a single exponential, having a time constant of 41.2 s.

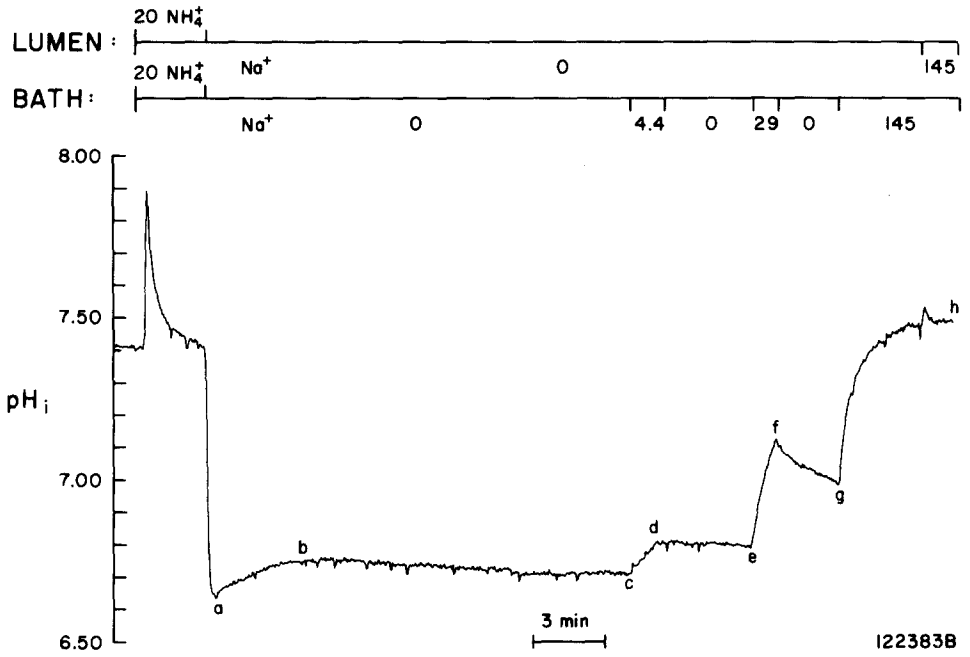

FIGURE 5. Basolateral Na^+ dependence of the rapid phase of pH_i recovery. After an NH_4^+-induced acid load, pH_i partially recovered in the absence of lumen and bath Na^+ (*ab*), and then declined slightly (*bc*). Basolateral Na^+ was periodically raised to 4.4 (*cd*), 29 (*ef*), and 145 mM (*gh*), interspersed with periods of Na^+ removal.

Because the rates of pH_i recovery are clearly pH_i dependent (note the exponential shape of the pH_i recovery in segment *gh* in Fig. 5), we compared pH_i recovery rates for the three Na^+ concentrations at the same pH_i. The aforementioned pH_i recovery rates in 4.4 and 29 mM Na^+ were obtained at the same pH_i, ~6.85. In order to estimate the recovery rate in 145 mM Na^+ at this same pH_i, we fitted the *gh* pH_i recovery to a single exponential and extrapolated this fitted exponential curve down to pH 6.85. At this pH_i, the extrapolated curve had a slope of 0.0163 pH/s. We performed a series of similar experiments at basolateral Na^+ concentrations of 4.4, 15, 29, and 145 mM Na^+. In each case, the pH_i recovery rates at these concentrations were normalized to the rate at 145 mM

Na^+, at the same pH_i. This analysis assumes that $[Na^+]_i$ is the same at different basolateral Na^+ concentrations. Because the cells were exposed to Na-free solutions for such a long time before the addition of basolateral Na^+, it is likely that $[Na^+]_i$ was uniformly low in all cases. However, the kinetics at a low $[Na^+]_i$ are not necessarily the same as at a more physiological value. These data are presented in Fig. 6. The curve is the result of a nonlinear least-squares curve fit to the Michaelis-Menten equation. The apparent K_m for basolateral Na^+ was 27.3 ± 4.5 mM. These results are consistent with the hypothesis that the rapid-phase pH_i recovery is caused by a basolateral Na-H exchanger.

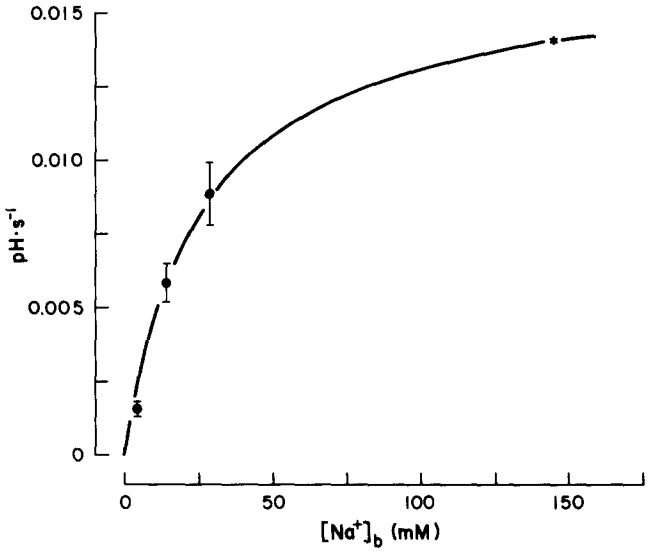

FIGURE 6. Basolateral Na^+ dependence of the rapid-phase pH_i recovery. The data are derived from the experiment of Figs. 5 and 16 similar experiments. For each experiment, the initial pH_i recovery rates in 4.4 (n = 6), 15 (n = 10), or 29 (n = 7) mM Na^+ were normalized to the recovery rate at the same pH_i in 145 mM Na^+. The mean initial recovery rate in 145 mM Na^+ was 0.014 pH/s. The normalized recovery rates are plotted vs. $[Na^+]_b$, with the vertical lines indicating standard errors. The curve drawn through the points is the result of a nonlinear least-squares fit to a variant of the Michaelis-Menten equation (Boron, 1985) that forces the curve to pass through the mean initial recovery rate in 145 mM Na^+ (indicated by the asterisk). V_{max} was 0.0167 ± 0.0028 pH/s, and K_m was 27.2 ± 4.5 mM.

Amiloride Sensitivity of the Rapid-Phase pH_i Recovery Mechanism

In order to further test the hypothesis that the rapid-phase pH_i recovery is due to a basolateral Na-H exchanger, we examined the sensitivity of the rapid-phase mechanism to amiloride, a competitive inhibitor of Na-H exchange (Kinsella and Aronson, 1981). Fig. 7*A* illustrates an experiment in which cells were acid-loaded in the absence of Na^+. When the NH_4^+ was removed, 50 μM amiloride was simultaneously added to the bath. In the absence of Na^+, pH_i did not recover (*ab*). Upon the addition of 15 mM Na^+ to the bath, the recovery of pH_i was approximately linear, with a rate of 0.0015 pH/s (*bc*). The subsequent simulta-

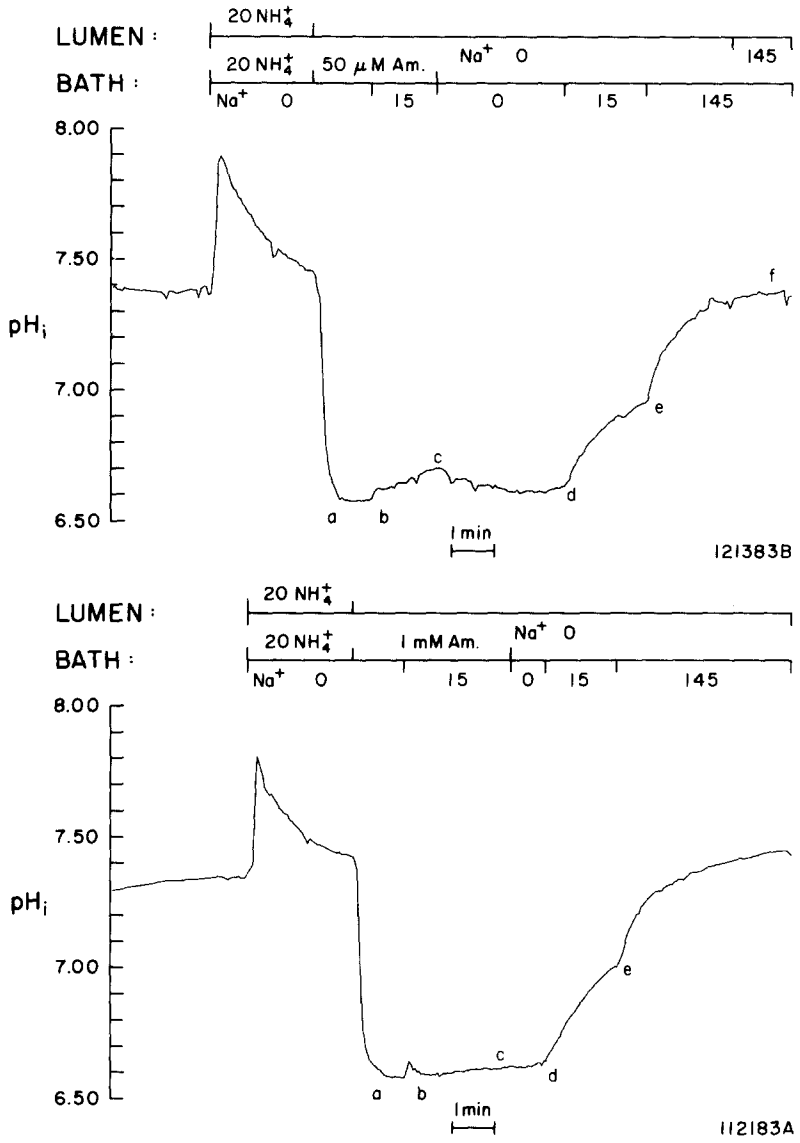

FIGURE 7. Effect of basolateral amiloride on the rapid-phase pH_i recovery. (*A*) After a brief exposure to 20 mM NH_4^+ in the absence of Na^+ (solution 5), 50 μM amiloride was added to the bath at the same time that NH_4^+ was removed (solution 4). The addition of 15 mM Na^+ to the bath, in the continued presence of 50 μM amiloride, caused pH_i to recover at a rate of 0.0015 pH/s (*bc*). The addition of 15 mM Na^+ in the absence of amiloride caused pH_i to recover with an initial rate of 0.0065 pH/s (*de*). (*B*) In an experiment similar to that of *A*, but on a different tubule, the effect of 1 mM amiloride was tested. The addition of 15 mM Na^+ to the bath in the presence of amiloride caused only a negligible pH_i recovery (*bc*), whereas the addition of 15 mM Na^+ to the bath in the absence of amiloride caused pH_i to recover with an initial rate of 0.0061 pH/s.

neous removal of Na^+ and amiloride caused pH_i to slowly decline and level off (*cd*). The addition of 15 mM bath Na^+, now in the absence of amiloride, caused pH_i to recover more rapidly (*de*), with an initial rate of 0.0065 pH/s. The difference in the pH_i recovery rates between *bc* and *de* corresponds to an inhibition by amiloride of 77%. In a total of three similar experiments, 50 μM amiloride inhibited the pH_i recovery in 15 mM Na^+ by 62 ± 7%. The experiment of Fig. 7*B* is similar to that of Fig. 7*A*, except that 1 mM amiloride was present in the bath. Whereas in Fig. 7*A* there was still a partial pH_i recovery in the presence of 50 μM amiloride, 1 mM amiloride completely inhibited pH_i recovery in 15 mM Na^+ (compare *bc* and *de* in Fig. 7*B*). Taken together, the Na^+ dependence and the amiloride sensitivity indicate that the rapid-phase mechanism is a basolateral Na-H exchanger.

DISCUSSION

Relative Contributions of Principal vs. Intercalated Cells to Measured pH_i Values

Inasmuch as the CCT has been shown to be composed of about two-thirds principal cells and one-third intercalated cells (Kaissling and Kriz, 1979), in most cases the pH_i values we measured for the CCT reflect an average pH_i for both cell types. Their relative contributions to the measured pH_i depend in a complex way on at least five parameters, each of which may have different values in the two cell types.

(*a*) pH_i determines not only the shape of the absorbance spectrum, but its sensitivity to pH changes. Thus, all else being equal, cells with pH_i values nearer the pK of the dye tend to make greater contributions to the mean pH_i.

(*b*) The position of the cells in the light path also influences the degree to which they contribute to the average pH_i. For cells in series with one another, it is easily shown that the absorbances are additive, and that the average pH_i is weighted in favor of the cell with the higher dye absorbance (which is affected both by pH_i and dye concentration). For cells in parallel with one another, it can be shown that the average absorbance is a weighted mean[1] of the individual absorbances, and that the average pH_i is weighted in favor of the cell with the lower dye absorbance.

(*c*) The spectral properties of Me_2CF in vitro are different from those of Me_2CF incorporated into salamander proximal tubule cells (Chaillet and Boron, 1985). In particular, the intracellular dye has a relatively flattened absorbance spectrum that is red-shifted by ~5 nm, as well as an elevated pK_a'. Should small differences exist between the spectral properties of the dye in different cell types of the rabbit CCT, the average pH_i could be biased toward one of them.

(*d*) The projected area and thickness that the cells present to the light path also affect the contribution of their pH_i values to the average pH_i. Regardless of

[1] For n cells that are parallel with one another in the light path, each with an absorbance A_i, and each illuminated by a fraction f_i of the total incident light, the average absorbance A is given by:

$$A = -\log \sum_{i=1}^{n} [f_i \cdot 10^{-A_i}].$$

whether the cells are in parallel or in series, other factors being equal, the average pH_i is weighted toward the cell for which the projected area in the light path is greater. If cells have different thicknesses, however, the average pH_i will be biased toward the cell with the greater thickness if the cells are in series, and toward the cell with the lesser thickness if the cells are in parallel (see *b* above).

(*e*) The effect of dye concentration on average pH_i can be predicted from *b* above. For cells in series, all other things being equal, the average pH_i is weighted toward the cell having the greater dye concentration, whereas for cells in parallel, the opposite is true. There are no data on the relative intracellular concentrations of fluorescein derivatives for the cell types of the CCT. However, one would expect $[Me_2CF]_i$ to depend on at least three factors: (*i*) the rate at which the dye precursor (an ester derivative of the dye) enters the cell during the period of dye loading, (*ii*) the rate at which the intracellular dye is formed as the precursor is hydrolyzed spontaneously and by native intracellular esterases, and (*iii*) the rate at which the dye leaks from the cell. In their experiments on Ehrlich ascites tumor cells, J. A. Thomas et al. (1979) found that the appearance of intracellular fluorescein derivatives depends critically upon extracellular pH in a way that suggests that it is the protonated, neutral form of the esterified dye precursor that enters the cell. We have noted a similar trend in our experiments. Regarding the rate of hydrolysis of Me_2CFAc_2 by intracellular esterases, it is interesting to note that carbonic anhydrase II, which is present at high levels in the intercalated cells of the CCT, has weak esterase activity (Hopkinson et al., 1974). However, there are no data that address the issue of whether carbonic anhydrase II significantly increases the esterase activity of intercalated cells, or whether there is a difference in relative esterase activities of principal and intercalated cells. Finally, as far as factor *iii* is concerned, we have noted that one of the most important determinants of $[Me_2CF]_i$ in our experiments is the rate at which the dye leaks from the cells. It should be noted that experiments can be divided into two parts, the period of dye loading, and the period during which pH_i is actually measured. During the loading period, $[Me_2CF]_i$ is determined by the interaction of factors *i–iii*. The subsequent time course of $[Me_2CF]_i$ depends on the $[Me_2CF]_i$ obtaining at the end of the loading period, and on the dye leakage rate. The actual importance of factors *i–iii* for determining $[Me_2CF]_i$ in either the CCT as a whole or in the individual cell types is not known.

Even though the measured pH_i is a complex average of the pH_i values of the different cell types of the CCT, changes in this measured pH_i can still provide valuable information about individual acid/base transport systems. This is exemplified by the experiment of Fig. 5, in which kinetic details of Na-H exchange could be assessed even though the CCT possesses a second mechanism for pH_i recovery.

The Two Phases of pH_i Recovery from an Intracellular Acid Load

A major conclusion of this study is that there are at least two mechanisms by which the pH_i of the CCT can increase after an acute intracellular acid load. The first, or slow-phase, mechanism is independent of Na^+, and causes only a partial recovery of the apparent pH_i. Furthermore, the slow-phase mechanism

was virtually absent in about one-third of the experiments in which we carefully searched for it. This slow-phase pH_i recovery could simply have been due to the passive transport of H^+ or a buffer such as HEPES, or to the electrogenic H^+ pump that is believed to be present in this nephron segment. Inasmuch as there are no specific inhibitors of the H^+ pump, and the nonspecific inhibitors would be expected to cause pH_i changes for other reasons, we made no attempt to further characterize the slow-phase pH_i recovery mechanism. We conclude that the second, or rapid-phase, mechanism for pH_i recovery from an acid load is probably a basolateral Na-H exchanger (see below).

It is possible that the slow-phase and/or the rapid-phase mechanisms are present in both principal and intercalated cells. This hypothesis requires that the slow-phase mechanism be absent in about one-third of the experiments, and that it not be capable of accomplishing a complete pH_i recovery in the absence of Na^+. An alternative hypothesis is that the two phases of pH_i recovery have their origins in the two cell types, the intercalated cells (or a subpopulation thereof) and the principal cells. The intercalated cell, which is analogous to the "dark" or "mitochondria-rich" cell of the turtle bladder, constitutes about one-third of the total cell number in the CCT (Kaissling and Kriz, 1979), and is believed to possess an H^+ pump. Our data obtained in Na-free Ringer also can be accounted for if (*a*) the slow-phase mechanism is confined to the intercalated cells (or a subpopulation), and (*b*) the rapid-phase mechanism is present in at least the principal cells, and possibly in the intercalated cells as well. In experiments in which the illuminating spot of light fell only on principal cells, the measured pH_i would not recover at all from an acid load, inasmuch as the Na-H exchanger would be blocked by Na^+ removal. In experiments in which the spot fell on both intercalated and principal cells, the apparent pH_i would only partially recover. That is, the pH_i of the minority, intercalated cells might recover fully or partially, whereas the pH_i of the majority, principal cells would once again fail to recover. As a result, the mean pH_i of the illuminated cells would only partially recover. This hypothesis can be tested only by studying pH_i transients in identified intercalated and principal cells.

Characteristics of the Basolateral Na-H Exchanger

A second conclusion of this study is that the rapid-phase mechanism of pH_i recovery is due to a basolateral Na-H exchanger. Although other Na-dependent, pH_i-regulating mechanisms have been described (R. C. Thomas, 1977; Boron et al., 1981; Boron and Russell, 1983; Boron and Boulpaep, 1983*b*), these are all HCO_3^- dependent. Inasmuch as our experiments were performed in the nominal absence of HCO_3^-, it is unlikely that the rapid-phase pH_i recovery mechanism could be mediated by one of these aforementioned HCO_3^- transporters. Furthermore, the amiloride sensitivity of this mechanism strongly suggests that this Na-dependent mechanism is an Na-H exchanger. Our apparent K_m for external Na^+, 27 mM, is somewhat larger than the values of 6.3 (Kinsella and Aronson, 1981) and 13.3 mM (Warnock et al., 1982) obtained for Na-H exchangers of brush-border membrane vesicles of the rabbit renal cortex. However, our value is somewhat lower than the value of 42 mM (at pH_o 7.5) for the Na-H exchanger

of the MDCK kidney cell line (Rindler and Saier, 1981). Our observed inhibition of the Na-H exchanger with 50 μM amiloride in the presence of 15 mM Na^+ (i.e., 62%) is also consistent with the apparent inhibition constants found in membrane vesicles (Kinsella and Aronson, 1981). The Na-H exchanger described in the present study is stimulated by intracellular acid loads (i.e., low values of pH_i). If we assume the intracellular buffering power to be reasonably independent of pH_i, then the exponential time course of the pH_i recovery implies that the Na-H exchange rate is high at low pH_i values, and falls linearly toward zero as pH_i approaches a "threshold" value of ~7.5. This pH_i dependence is shared with the HCO_3^--dependent pH_i regulator of invertebrates as well as the Na-H exchanger of vertebrate cells (see Roos and Boron, 1981; Boron, 1983). The only other report of an Na-H exchanger in a native renal cell concerns the salamander proximal tubule, which was studied with pH- and Na-sensitive microelectrodes (Boron and Boulpaep, 1983*a*). This preparation is noteworthy in that it possesses both luminal and basolateral Na-H exchangers.

Significance of the Basolateral Na-H Exchanger

POSSIBLE ROLE IN TRANSEPITHELIAL HCO_3^- SECRETION Our data suggest that most, and possibly all, cells of the CCT possess a basolateral Na-H exchanger. Such an exchanger could participate in two activities: alkali secretion and pH_i regulation. The luminal step of alkali secretion would presumably involve the efflux of HCO_3^-, possibly in exchange for Cl^-, and would acidify the cell interior. If an Na-H exchanger were present in the basolateral membrane of the same cell, this intracellular acid load would stimulate the Na-H exchanger to extrude acid across the basolateral membrane, and thereby complete the process of transepithelial HCO_3^- secretion. However, the recent observations by Schuster (1985) and Star et al. (1985), that Na^+ is not required for HCO_3^- secretion, indicate that Na-H exchange does not play an important role in this process. This suggests that the cells responsible for HCO_3^- secretion have at most a small degree of basolateral Na-H exchange activity.

ROLE IN pH_i REGULATION All cells of the CCT must deal with the problem of pH_i regulation. The intercalated cells, for which there may be two subpopulations (Schwartz and Al-Awqati, 1985; Stetson and Steinmetz, 1985), could in principle accomplish this pH_i regulation by means of the same H^+ pump that presumably is responsible for HCO_3^- reabsorption and secretion. Our data are consistent with this hypothesis, as well as the variant in which intercalated cell pH_i regulation is augmented by a basolateral Na-H exchanger. We propose that pH_i regulation for the principal cells is probably accomplished by the basolateral Na-H exchanger identified in the present study. This hypothesis is supported by the fact that we observed the basolateral Na-H exchanger in all our experiments. The confinement of the Na-H exchanger to the basolateral membrane might be expected (Boron, 1983), inasmuch as the in vivo lumen-to-cell gradients for Na^+ and H^+ could be unfavorable for acid extrusion by Na-H exchange. Similar basolateral Na-H exchangers might also be anticipated in other renal tubule cells not involved in acid secretion.

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REFERENCES

Al-Awqati, Q. 1978. H^+ transport in urinary epithelia. *Am. J. Physiol.* 235:F77–F88.

Boron, W. F. 1977. Intracellular pH transients in giant barnacle muscle fibers. *Am. J. Physiol.* 233:C61–C73.

Boron, W. F. 1983. Transport of H^+ and of ionic weak acids and bases. *J. Membr. Biol.* 72:1–16.

Boron, W. F. 1985. Intracellular pH-regulating mechanism of the squid axon. Relation between the external Na^+ and HCO_3^- dependences. *J. Gen. Physiol.* 85:325–345.

Boron, W. F., and E. L. Boulpaep. 1983*a*. Intracellular pH regulation in the renal proximal tubule of the salamander. Na-H exchange. *J. Gen. Physiol.* 81:29–52.

Boron, W. F., and E. L. Boulpaep. 1983*b*. Intracellular pH regulation in the renal proximal tubule of the salamander. Basolateral HCO_3^- transport. *J. Gen. Physiol.* 81:53–94.

Boron, W. F., and P. De Weer. 1976. Intracellular pH transients in squid giant axons caused by CO_2, NH_3, and metabolic inhibitors. *J. Gen. Physiol.* 67:91–112.

Boron, W. F., W. C. McCormick, and A. Roos. 1981. pH regulation in barnacle muscle fibers: dependence on extracellular sodium and bicarbonate. *Am. J. Physiol.* 240:C80–C89.

Boron, W. F., and J. M. Russell. 1983. Stoichiometry and ion dependencies of the intracellular-pH-regulating mechanism in squid giant axons. *J. Gen. Physiol.* 81:373–399.

Boron, W. F., and H. Sackin. 1983. Measurement of intracellular ionic composition and activities in renal tubules. *Annu. Rev. Physiol.* 45:483–496.

Burg, M., J. Grantham, M. Abramow, and J. Orloff. 1966. Preparation and study of fragments of single rabbit nephrons. *Am. J. Physiol.* 210:1293–1298.

Chaillet, J. R., and W. F. Boron. 1984. Basolateral Na-H exchange in the rabbit cortical collecting tubule. *Fed. Proc.* 43:1089. (Abstr.)

Chaillet, J. R., and W. F. Boron. 1985. Intracellular calibration of a pH-sensitive dye in isolated, perfused salamander proximal tubules. *J. Gen. Physiol.* 86:765–794.

Cohen, L. 1980. HCO_3-Cl exchange transport in the adaptive response to alkalosis by turtle bladder. *Am. J. Physiol.* 239:F167–F174.

Dobyan, D. C., L. S. Magill, P. A. Friedman, S. C. Hebert, and R. E. Bulger. 1982. Carbonic anhydrase histochemistry in rabbit and mouse kidneys. *Anat. Rec.* 204:185–197.

Grantham, J. J., M. B. Burg, and J. Orloff. 1970. The nature of transtubular Na and K transport in isolated rabbit renal collecting tubules. *J. Clin. Invest.* 49:1815–1826.

Hopkinson, D. A., J. S. Coppock, M. F. Muhlemann, and Y. H. Edwards. 1974. The detection and differentiation of the products of the human carbonic anhydrase loci, CA_I and CA_{II}, using fluorogenic substrates. *Ann. Hum. Genet.* 38:155–162.

Kaissling, B., and W. Kriz. 1979. Structural analysis of the rabbit kidney. *Adv. Anat. Embryol. Cell Biol.* 56:1–123.

Kinsella, J. L., and P. S. Aronson. 1981. Amiloride inhibition of the Na^+-H^+ exchanger in renal microvillus membrane vesicles. *Am. J. Physiol.* 241:F374–F379.

Koeppen, B. M., and S. I. Helman. 1982. Acidification of luminal fluid by the rabbit cortical collecting tubule perfused in vitro. *Am. J. Physiol.* 242:F521–F531.

Laski, M. E., D. G. Warnock, and F. C. Rector, Jr. 1983. Effects of chloride gradients on total CO_2 flux in the rabbit cortical collecting tubule. *Am. J. Physiol.* 244:F112–F121.

Lombard, W. E., J. P. Kokko, and H. R. Jacobson. 1983. Bicarbonate transport in cortical and outer medullary collecting tubules. *Am. J. Physiol.* 244:F289–F296.

McKinney, T. D., and M. B. Burg. 1977. Bicarbonate transport by rabbit cortical collecting tubules: effect of acid and alkali loads in vivo on transport in vitro. *J. Clin. Invest.* 60:766–768.

McKinney, T. D., and M. B. Burg. 1978*a*. Bicarbonate absorption by rabbit cortical collecting tubules in vitro. *Am. J. Physiol.* 234:F141–F145.

McKinney, T. D., and M. B. Burg. 1978*b*. Bicarbonate secretion by rabbit cortical collecting tubules in vitro. *J. Clin. Invest.* 61:1421–1427.

O'Neil, R. G., and R. A. Hayhurst. 1984. Functional differentiation of cell types of perfused rabbit cortical collecting duct (CCD). *Fed. Proc.* 43:302. (Abstr.)

Rindler, M. J., and M. H. Saier, Jr. 1981. Evidence for Na^+/H^+ antiport in cultured dog kidney cells (MDCK). *J. Biol. Chem.* 256:10820–10825.

Roos, A., and W. F. Boron. 1981. Intracellular pH. *Physiol. Rev.* 61:296–434.

Sackin, H., and E. L. Boulpaep. 1981. Isolated perfused salamander proximal tubule. II. Monovalent ion replacement and rheogenic transport. *Am. J. Physiol.* 241:F540–F555.

Schuster, V. L. 1985. Cyclic-AMP-stimulated bicarbonate secretion (BiS) in rabbit cortical collecting tubules (CCT). *Kidney Int.* 27:288. (Abstr.)

Schwartz, G. J., and Q. Al-Awqati. 1985. Two functionally distinct types of mitochondria-rich (MR) cells in cortical collecting tubule (CCT) as determined by changes in cell pH (pH_i) in individually identified cells. *Kidney Int.* 27:288. (Abstr.)

Star, R., M. Knepper, and M. Burg. 1985. Bicarbonate secretion by rabbit cortical collecting duct: role of chloride/bicarbonate exchange. *Kidney Int.* 27:289. (Abstr.)

Steinmetz, P. R., and O. S. Andersen. 1982. Electrogenic proton transport in epithelial membranes. *J. Membr. Biol.* 65:155–174.

Stetson, D. L., and P. R. Steinmetz. 1985. A subpopulation of carbonic anhydrase-rich (CA) cells in turtle bladder: possible role in urinary HCO_3^- secretion. *Kidney Int.* 27:289. (Abstr.)

Thomas, J. A., R. N. Buchsbaum, A. Zimniak, and E. Racker. 1979. Intracellular pH measurements in Ehrlich ascites tumor cells utilizing spectroscopic probes generated in situ. *Biochemistry.* 81:2210–2218.

Thomas, R. C. 1977. The role of bicarbonate, chloride and sodium ions in the regulation of intracellular pH in small neurones. *J. Physiol. (Lond.).* 273:317–338.

Wade, J. B., R. G. O'Neil, J. L. Pryor, and E. L. Boulpaep. 1979. Modulation of cell membrane area in renal collecting tubules by corticosteroid hormones. *J. Cell Biol.* 81:439–445.

Warnock, D. G., W. W. Reenstra, and V. J. Yee. 1982. Na^+/H^+ antiporter of brush border vesicles: studies with acridine orange uptake. *Am. J. Physiol.* 242:F733–F739.