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Research Article

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Cyclic Adenosine Monophosphate–stimulated Anion Transport in Rabbit Cortical Collecting Duct

Kinetics, Stoichiometry, and Conductive Pathways

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Abstract

Cyclic AMP stimulates HCO_3 secretion and Cl self-exchange in rabbit cortical collecting tubule. We found that varying peritubular [Cl] changed the Cl self-exchange rate with saturation kinetics (K_m, 3–4 mM). HCO_3 secretion also showed saturation kinetics as a function of mean luminal [Cl] (K_m, 4–11 mM). Both Cl self-exchange and Cl-HCO_3 exchange thus appear to be carrier-mediated. Addition/removal of basolateral HCO_3 qualitatively changed Cl and HCO_3 transport as expected for Cl-HCO_3 exchange, but quantitatively changed Cl absorption more than HCO_3 secretion. The diffusive Cl permeability and the transepithelial conductance in the presence of HCO_3/CO_2 and cAMP were higher than in their absence suggesting that HCO_3/CO_2 and cAMP together increase a conductive Cl pathway parallel to a 1:1 Cl-HCO_3 exchanger. Thus, cAMP not only stimulates the overall process of anion exchange (probably by increasing an electroneutral exchanger and/or a series Cl conductance), but also stimulates a Cl conductance parallel to the exchange process.

Introduction

Both the in vitro turtle urinary bladder and the rabbit cortical collecting tubule secrete HCO_3, particularly when the animal has been alkali-loaded. In the absence of cyclic adenosine monophosphate (cAMP), it appears that this HCO_3 secretion occurs in both epithelia via 1:1 electroneutral Cl-HCO_3 exchange (1–4). Addition of basolateral HCO_3 thus induces HCO_3 secretion and Cl reabsorption of equal and opposite magnitude (1–3). Evidence obtained in the cortical collecting tubule suggests that: (*a*) the responsible anion exchanger is located at the apical membrane (lowering basolateral Cl concentration acidifies the majority of intercalated cells, presumably by favoring apical Cl entry into and exchange-mediated HCO_3 exit out of the cell) (4), and (*b*) Cl brought into the cell by this exchanger probably exits via a basolateral Cl conductance (5). The overall process of Cl-HCO_3 exchange thus must be considered in terms of such a "double-membrane" model (6).

In the turtle bladder, cAMP or phosphodiesterase inhibitors change the nature of HCO_3 secretion. These agents increase the transepithelial conductance and induce electrogenic HCO_3 secretion, presumably via an apical HCO_3 conductive pathway (6, 7). Because apical application of the Cl channel blocker anthracene-9-carboxylic acid reduces the effect of cAMP on electrogenic HCO_3 secretion, and because cAMP reduces net Cl reabsorption, it has been suggested that the putative cAMP-induced apical anion conductance in turtle bladder is poorly selective, allowing for either: (*a*) electrogenic HCO_3 secretion, or (*b*) recycling of Cl (brought into the cell by an apical Cl-HCO_3 exchanger) across the apical membrane (6).

We have recently reported cAMP effects on HCO_3 and Cl transport by the rabbit cortical collecting tubule (8–10). These results differed somewhat from those in the turtle bladder in suggesting a predominance of electroneutral exchange pathways in the cortical collecting tubule. In the presence of HCO_3/CO_2, we found that cAMP does stimulate net HCO_3 secretion, but because luminal Cl removal inhibits this HCO_3 secretion, it seems likely that Cl-HCO_3 exchange is involved (8). In the absence of exogenous HCO_3/CO_2, Cl transport by the cortical collecting tubule occurs largely via Cl self-exchange, and this self-exchange process is also stimulated by cAMP (9). A substantial number of other similarities between HCO_3 secretion and Cl self-exchange have led us to suggest that the two are alternate modes of operation of a single anion exchange process, and that stimulation of this exchange process accounts for the observed effects of cAMP on cortical collecting tubule anion transport (8–10).

Because of the apparent differences in cAMP action with respect to electrogenic anion transport in the turtle bladder as opposed to the cortical collecting tubule, we have extended our previous tubule studies. The present experiments address the following three questions. Do cAMP-stimulated Cl self-exchange and HCO_3 secretion exhibit saturation-type kinetics as a function of Cl concentration, as would be expected for carrier-mediated processes? Is the stoichiometry of Cl reabsorption and HCO_3 secretion 1:1 in the presence of cAMP? Is there evidence for a cAMP-induced anion conductance in the cortical collecting tubule?

Methods

General microperfusion methods. Cortical collecting tubules were dissected from New Zealand white rabbits and perfused as previously described (8–10). All rabbit drinking water consisted of 0.075 M $NaHCO_3$ and 0.1 M glucose for 3–14 d before study. All perfusates contained 50 μM amiloride. The perfusion rate was 1–4 nl/min in experiments in which fluxes were calculated from measurements on collected fluid, and >20 nl/min in experiments in which fluxes were calculated from bath collections or in which transepithelial conductance was measured. The bath flow was continuous, single-pass, and at a fixed rate of either 0.6 or 1.2 ml/min for each experiment.

Transepithelial voltage was measured using a circuit previously described (7–9) with the following exception. When bath Cl concentration needed to be maintained at absolute minimal levels (Fig. 3, Table III) the single-pass bath outflow line emptied into a separate, 1-ml constant-

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volume reservoir into which was inserted the bath (ground) calomel electrode. The bath outflow line thus served as the bath electrical bridge. In experiments in which bath or perfusate Cl concentrations were varied, the respective bridges were 150 mM Na gluconate in 3% agarose. Liquid-junction potentials of these bridges with solutions of varying Cl concentrations were measured directly in the perfusion apparatus and the measured transepithelial voltages were corrected accordingly. For example, the junction voltage between a 150-mM Na gluconate bridge and a 150-mM NaCl solution was 11.0 mV.

Solutions. The composition of perfusing and bathing solutions is shown in Table I. The "standard HCO_3" and "0 Cl HCO_3" solutions were equilibrated with 94% O_2/6% CO_2 at 37°C to pH 7.4–7.5. All Hepes solutions were equilibrated with 100% O_2 and had a pH of 7.4–7.5. When gluconate was present in bath solutions, total Ca^{2+} concentration was adjusted so that the uncomplexed, "free" Ca^{2+} concentration was maintained constant at about 2 mM (11).

The "simple Cl" solutions were used as perfusates. These perfusates were equilibrated with either 100% O_2 or 94% O_2/6% CO_2 depending upon experimental conditions as follows. When the corresponding bath solution was a HCO_3-containing one, these perfusates were bubbled with 94% O_2/6% CO_2 and had a pH of ~6.7. When the corresponding bath solution was CO_2-free and Hepes-buffered, the simple Cl perfusates were equilibrated with 100% O_2 and had a pH of ~7.0. All perfusates contained 50 μM amiloride. For cable analysis (see below) perfusates also contained 4 mM $BaNO_3$.

Microcalorimetry. Bicarbonate concentrations of perfused and collected fluid were measured using microcalorimetry as previously described (8). Microcalorimetry measures total CO_2, which includes HCO_3, dissolved CO_2 (~1.2 mM at P_{CO_2} of 40 mmHg), and H_2CO_3 (negligible under the present conditions). When perfused fluids are nominally HCO_3 free, dissolved CO_2 may represent a significant fraction of total CO_2. In our experiments we wished to measure only HCO_3 addition by the tubule to the HCO_3-free simple Cl perfusates. To achieve this, these perfusates were equilibrated with the same gas mixture as that of the corresponding bathing solution, and the total CO_2 concentration of the perfusates was then measured in a separate calibrating step (8). With this method, the total CO_2 of perfused fluid bubbled with 94% CO_2/6% CO_2 was 1.02±.25 mM (SD, n = 38). Any increment of collected fluid total CO_2 concentration over this value when the bath solution is equilibrated with 94% O_2/6% CO_2 thus represents true HCO_3 addition to the fluid by the tubule.

Chloride concentrations. Chloride concentrations on bulk solutions were measured by chloridometer (Buchler-Cotlove, Laboratory Glass and Instruments, New York) with sample dilution and titration rates adapted for extremely low Cl concentrations where appropriate as described by Fischer et al. (12).

For measurement of Cl concentrations in nanoliter samples, two different, overlapping methods were used. For Cl concentrations ≥ 12 mM, ^{36}Cl was used because of the ease and accuracy of isotope methods. ^{36}Cl (Amersham Corp., Arlington Heights, IL) was added to the perfusate as the Na salt at a concentration of 12–40 μCi/ml. Because of the low specific activity of ^{36}Cl, the cold NaCl concentration was adjusted downward as needed. Collection pipette volumes of 40 nl yielded total ^{36}Cl counts per minute at least 10-fold background. In perfusates containing 12 mM Cl, all of the Cl in the solution, both isotopic and cold, derived from the isotope stock solution. In three such perfusates we measured the total Cl concentration by microtitration; these were within 5% of the values calculated from scintillation counts and the listed specific activity.

At Cl concentrations < 12 mM we could not use ^{36}Cl because of insufficient counts in collected fluid. We also found that standard microtitration gave poorly reproducible end-points on 20-nl samples containing Cl concentrations < 5 mM (<100 pmol). We therefore constructed a miniature double-barreled silver metal Cl electrode (13). This was inserted down the lumen of an oversized collection pipette directly into the collected fluid. As shown in Fig. 1 *a*, a Teflon-coated silver wire (A-M Systems, Everett, WA), 0.003 in. in diameter was threaded down a glass capillary tube. This tube, in turn, was glued to a larger diameter glass tube which, when filled with 1 M KNO_3 in 4% agar, served as the reference electrode. Fig. 1 *b* shows the calibration at 37°C of a representative electrode in pure KCl solutions of varying ionic strength (orthodox method), and in 150-mM constant-ionic strength mixed KCl-K gluconate solutions (unorthodox method) such as those used in the present experiments. The responses were slightly non-Nernstian at Cl concentrations < 1.0 mM. The average slope of 18 electrodes calibrated by the unorthodox method in bulk solutions was 53.9±1.4 mV over the 1–10 mM range. In practice, a decreased slope after multiple uses could be restored to normal by carefully cutting the tip of the silver wire to expose fresh

Table I. Composition of Bathing (Basolateral) and Perfusing (Luminal) Solutions

	Baths				Perfusates			
Solution	0 Cl Hepes	2 Cl Hepes	10 Cl Hepes	40 Cl Hepes	Standard Hepes	Standard HCO_3	0 Cl HCO_3	Simple Cl
NaCl	0	2	10	40	130	115	0	A
$NaHCO_3$	0	0	0	0	0	25	25	0
KCl	0	0	0	0	5	5	0	0
Na_2HPO_4	2.3	2.3	2.3	2.3	2.3	2.3	2.3	0
$CaCl_2$	0	0	0	0	2	2	0	0
Na-gluconate	114	112	105	80	0	0	100	B
K-gluconate	5	5	5	5	0	0	5	5
Ca (gluconate)$_2$	10	10	9	7.5	0	0	10	0
Na-Hepes	5	5	5	5	5	0	0	0
H^+-Hepes	5	5	5	5	5	0	0	0
D-Glucose	8	8	8	8	8	8	8	13
For simple Cl perfusates the values of A and B were:								
A	0	2	5	12	45	100	145	
B	145	143	140	133	100	45	0	

All concentrations in millimolars. When $BaNO_3$ was added to perfusates, the Na_2HPO_4 concentration was reduced to 1 mM.

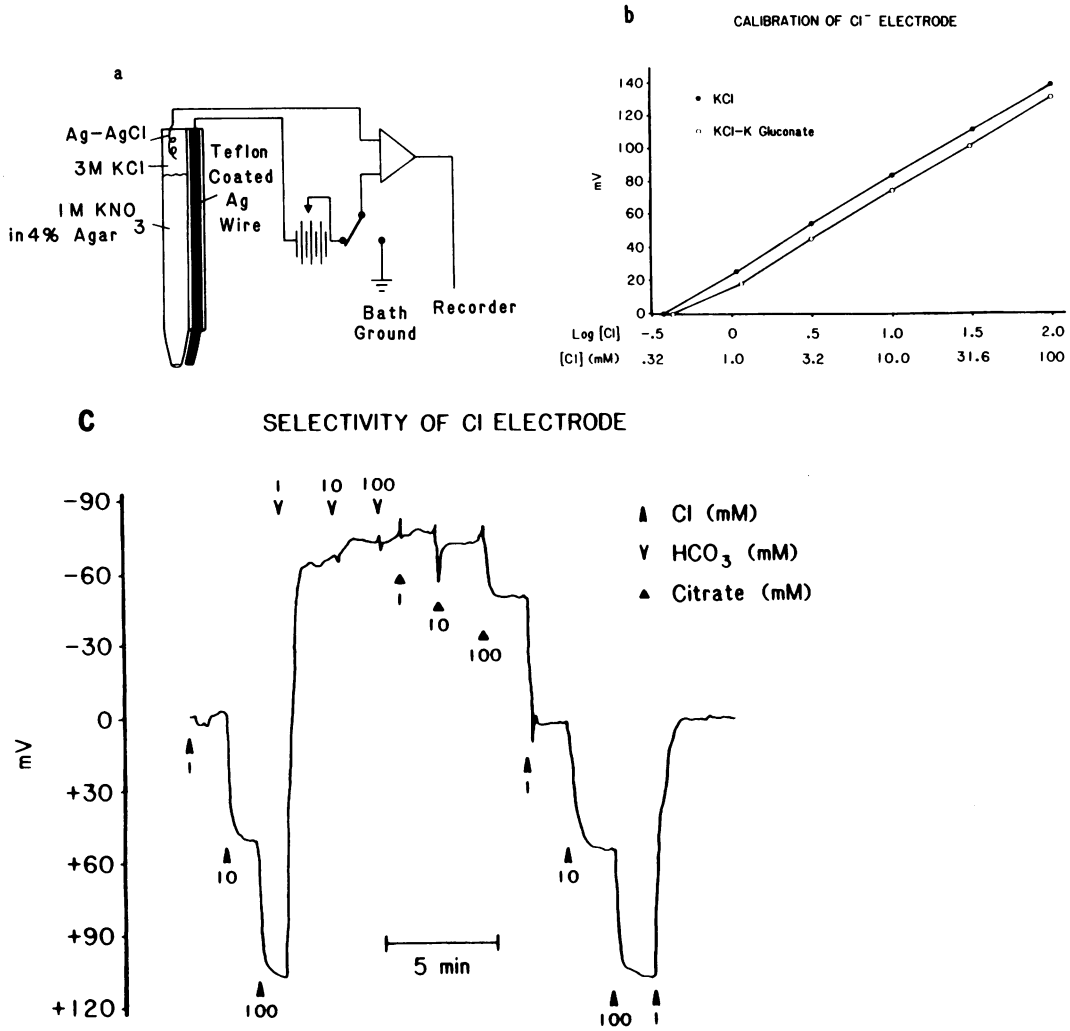

Figure 1. Miniature Cl-selective electrode. (*a*) Double-barrel design allows direct measurement of Cl concentration (activity). (*b*) Typical calibration at 37°C in bulk solution. See Methods for details. (*c*) Response to 1, 10, or 100 mM solutions of KCl, $KHCO_3$, or K citrate. At the indicated points, the electrode was placed into the respective bulk solutions and the resulting voltage recorded. The silver metal is >100 times as sensitive to Cl as to the other anions.

metal. Fig. 1 *c* shows that Ag metal is highly selective for Cl over the anions HCO_3 or citrate. During an experiment, the electrode was calibrated in bulk solution at 37°C, was then inserted into the collected fluid to measure the collected fluid Cl concentration, and was then recalibrated in bulk solution. The electrode voltage was recorded on a strip-chart recorder. From continuous recordings even small amounts of electrode drift could be detected; when drift did occur, it appeared to be linear, allowing reasonably accurate interpolation of sample Cl concentrations. Perfusate Cl concentrations were measured using the electrode and perfusate calibration methods previously described (8). In experiments using a perfusate Cl of 12 mM, both ^{36}Cl and the electrode were used; the results agreed within 15%.

Flux measurements. All perfusates for flux measurements contained exhaustively dialyzed methoxy-[^{3}H]inulin as a volume marker. HCO_3 fluxes were measured by previously-described methods (8). When perfusates are nominally HCO_3-free and the bath contains 25 mM HCO_3, net HCO_3 flux approximates the unidirectional bath-to-lumen flux.

Lumen-to-bath Cl fluxes were measured in one of two ways. At slow perfusion rates, fluxes were calculated from the difference between perfused and collected fluid ^{36}Cl counts, as previously described (9, 10). At rapid perfusion rates (>20 nl/min), Cl fluxes were determined using timed bath collections; unidirectional lumen-to-bath ^{36}Cl fluxes were calculated according to Burg and Green (14). By either method, experiments with significant [^{3}H]inulin leaks (>1%) were discarded.

Conductance measurements. The transepithelial conductance, G_T[1] (millisiemans per square centimeter) was determined using standard cable analysis as developed by Helman (15) and as used in our laboratory (16). Constant-current injections of 10–70 nA and 100 ms duration were injected into the tubule lumen via the perfusion pipette. An electrical bridge allowed voltage deflections at the perfusion pipette to be nulled for a given perfusate in the absence of a tubule. A sample-and-hold device sent the resulting voltage deflections at each end of the tubule (measured at 100 ms) to a strip-chart recorder. Voltage deflections at the collection end were measured using a Ag-Ag Cl electrode. To ensure maximum sensitivity and reproducibility of G_T measurements, the following steps were taken: (*a*) Extreme care was taken to electrically seal both ends of the tubule with a 50:50 mixture of unpolymerized Sylgard and Hysol

1. *Abbreviations used in this paper:* DIDS, 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid; GHK, Goldman-Hodgkin-Katz equation; G_T, transepithelial conductance; IBMX, isobutylmethylxanthine; *I*eq, equivalent current; V_T, transepithelial voltage.

200 fluid (200 centi Stokes viscosity, Dexter Manufacturing, Olean, NY). At the perfusion end, this usually required careful imposition of slight positive pressure behind the Sylgard-Hysol mixture in the holding pipette so as to seal the tubule tightly around the perfusion pipette. (*b*) Because slight temperature variations unbalance the electrical bridge and change voltage deflections at the perfusion pipette, the bath was water-jacketed to ±0.5°C. During an experiment, bath temperature was continuously monitored on a third recorder channel (1 cm deflection/°C), and G_T measurements from the tracing were made only at constant temperatures. (*c*) Only lumen-positive current injection was used so as to maximize voltage-dependent Ba^{2+} blockade of K channels (17). (*d*) Adequate current was injected so that the voltage deflections at the collection pipette were ≥10 mV (10 mV = 1 in. pen deflection).

In some experiments, an equivalent current, *I*eq ($\mu A/cm^2$) was calculated from G_T and the spontaneous transepithelial voltage (V) measured at the perfusion pipette. In order for *I*eq to be valid, the relationship of injected current to voltage deflection must be linear over the range of spontaneous transepithelial voltages observed. With amiloride and barium in the perfusate, we found that this relationship was linear over a range of 0 to +100 mV.

Reagents and statistics. 8-bromo-cAMP was purchased from Sigma Chemical Co. (St. Louis, MO).

Results are expressed as mean±SE. Data were analyzed by paired or nonpaired *t* test as appropriate.

Results

Stability of HCO_3 secretion in 0 Cl bath with cAMP. We have previously reported that net HCO_3 secretion falls spontaneously with time in vitro (8). We have also noted a similar time-dependent decline in Cl self-exchange (9). In the case of Cl transport, we reported that the presence of cAMP in the bath from the onset of perfusion prevented the decline, suggesting that withdrawal of a cAMP-stimulating hormone that had been present in vivo accounted for the in vitro decline (9). We thus began the present studies by examining whether cAMP also prevents the time-dependent decline of HCO_3 secretion in vitro. Because many of the subsequent experiments were done with bath Cl = 0 mM, we also needed to determine that HCO_3 secretion rates were stable in the absence of bath Cl. Therefore, tubules were bathed in the 0 Cl HCO_3 solution. As shown in Table II, the presence of cAMP from the onset of perfusion maintained HCO_3 secretion constant. Therefore, it seems likely that, as with Cl self-exchange, absence of a cAMP-stimulatory hormone in vitro caused the previously observed spontaneous in vitro decline in HCO_3 secretion.

Table II. Cyclic AMP Prevents Time-dependent Changes in HCO_3 Secretion

Period	$J^{net}_{HCO_3}$
	pmol/mm per min
1	15.5±3.6
2	15.9±4.4
Paired change	0.4±0.8

Periods 1 and 2 were separated by 1 h. $n = 4$. The perfusate was standard HCO_3 containing 50 μM amiloride and the bath 0 Cl HCO_3. 0.1 mM 8-Bromo-cAMP was present from the start of perfusion. The paired change is not significant.

Kinetics of cAMP-stimulated Cl self-exchange. We next examined the rate of cAMP-stimulated Cl self-exchange as a function of basolateral Cl concentration. The purpose was twofold: saturation-type kinetics would lend support to the idea that this large component of lumen-to-bath ^{36}Cl flux is carrier-mediated, and for practical reasons, the data would indicate how rigorously Cl must be eliminated from the bath in order to stop lumen-to-bath Cl tracer flux from occurring via self-exchange.

Six tubules were perfused with standard Hepes containing ^{36}Cl. The bathing solutions were varied randomly between 0 Cl, 2 Cl, 10 Cl, 40 Cl, or standard Hepes (see Table I). Cyclic AMP was present throughout. With each different bath, lumen-to-bath ^{36}Cl fluxes were measured using timed bath collections. Samples for scintillation counting were bracketed by collections for total Cl concentration by chloridometer. Because of the relatively slow bath exchange rates, bath [Cl] did not reach 0 mM even in the 0 Cl Hepes solution (see legend, Fig. 2 for measured bath Cl concentrations). In previous studies in which a bath [Cl] of 0 mM was achieved, we found a (presumably diffusional) Cl permeability coefficient of 2.2×10^{-6} cm/s (8). Therefore, in the present experiments we calculated a predicted "diffusional" J^{lb}_{Cl} for each experimental period from the permeability coefficient, the measured electrical driving force (V_T), and the Goldman-Hodgkin-Katz (GHK) equation (18). This predicted "diffusional" flux was then subtracted from the total measured J^{lb}_{Cl} at each bath Cl concentration to yield a mediated J^{lb}_{Cl}. The results are shown in Fig. 2. The Michaelis-Menten kinetic constants (K_m) were calculated from Lineweaver-Burk and Eadie-Hofstee plots, and also by the nonparametric Cornish-Bowden method (19). Depending upon the method used, the apparent epithelial K_m for bath Cl was 2.8–4.2 mM, and the maximum velocity (V_{max}) 75.2–86.5 pmol/mm per min. These results indicate that physiological basolateral Cl concentrations are saturating with respect to cAMP-stimulated Cl self-exchange, and that Cl self-exchange is probably carrier mediated.

Kinetics of cAMP-stimulated Cl-HCO_3 exchange. We next examined the Cl-dependence of HCO_3 secretion in the presence of cAMP. The general strategy was similar to that of the previous section, except that the exchanged anion species and the sidedness of the Cl effect were different. Six different groups of tubules (5–11 tubules per group) were each perfused with one of the simple Cl perfusates shown in Table I (nominal Cl concentrations of 2, 5, 12, 45, 100, and 145 mM). The bath solution

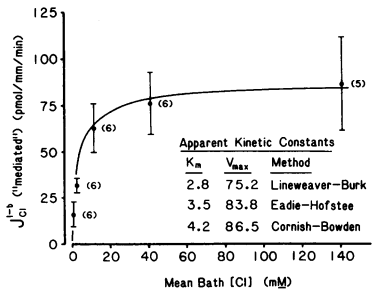

Figure 2. Dependence of cAMP-stimulated Cl self-exchange on bath (basolateral) Cl concentration. Bath solutions (in random order) were: 0 Cl, 2 Cl, 10 Cl, 40 Cl, and standard Hepes. Cl concentrations actually measured in the perfusion chamber effluent were 0.7±0.4, 2.7±0.2, 11.6±1.0, 40.8±0.7, 140.5±0.6 in millimolars. Number of flux data points available at each concentration from six tubules is shown (paired protocol). Apparent epithelial kinetic constants were calculated by three different methods from the mean flux values (see text); the line is that predicted from the Cornish-Bowden–derived kinetic constants (19).

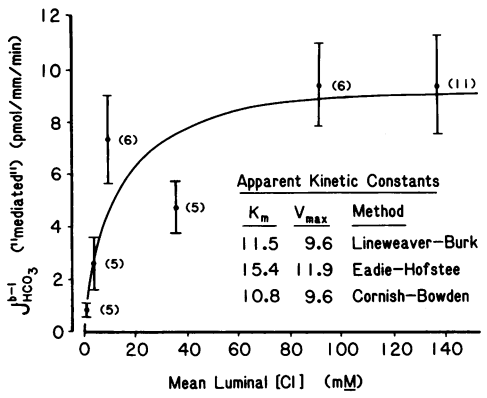

Figure 3. Dependence of cAMP-stimulated HCO_3 secretion on luminal Cl concentration. Measured (arithmetic) mean luminal Cl concentrations were 1.0±0.1, 4.2±0.2, 9.8±0.5, 36.8±1.2, 91.7±1.2, 137.9±0.9 in millimolars. The number of tubules contributing to each data point is shown (unpaired protocol). Apparent epithelial kinetic constants and the derived line were determined as in Fig. 2.

was 0 Cl HCO_3 and contained cAMP. Perfusate and collected fluid HCO_3 concentration were determined by microcalorimetry, and Cl concentrations by Cl electrode and/or ^{36}Cl (see Methods). Omitting Cl from the basolateral bathing solution served two purposes: Cl self-exchange is eliminated (see above), so that the lumen-to-bath ^{36}Cl flux represents Cl-HCO_3 exchange plus Cl diffusion (9), and the rate of proton secretion is negligible (12, 20).

We have previously estimated the nonexchange-mediated, presumed diffusional HCO_3 permeability of cortical collecting tubules in the presence of cAMP at 1.9×10^{-6} cm/s (8). As with the Cl self-exchange experiments in the previous section, we therefore took the total $J^{bl}_{HCO_3}$ values at each luminal [Cl] and subtracted a predicted diffusional component using this HCO_3 permeability, the measured electrical and chemical driving forces, and the GHK equation. The results are shown in Fig. 3. Although the data demonstrate variability because of the unpaired experimental design, the calculated mediated $J^{bl}_{HCO_3}$ values fit reasonably well with saturation-type kinetics as a function of mean luminal [Cl]. Depending upon the reciprocal method used, the apparent epithelial K_m of HCO_3 secretion for luminal [Cl] was 10.8–15.4 mM, with a V_{max} of 9.6–11.9 pmol/mm per min.

Several studies have suggested that our previous estimate of HCO_3 permeability (8) yielded an upper-limit value, and that the HCO_3 permeability of the cortical collecting tubule may be lower (21, 22). We therefore calculated kinetic parameters assuming a P_{HCO_3} of zero. In this case, the range of K_ms for luminal Cl by the three reciprocal methods is 3.4 to 4.9 mM, and the range of V_{max} values is 10.8 to 12.5 pmol/mm per min. It thus seems likely that the true K_m for luminal Cl is between 3.4 and 15.4 mM. The kinetics suggest a carrier-mediated process. These results also indicate that rates of HCO_3 secretion typically seen after cAMP (8) can be entirely accounted for by a Cl-dependent process.

Stoichiometry of HCO_3 secretion vs. Cl absorption. If HCO_3 secretion in the presence of cAMP occurs by 1:1 Cl-HCO_3 exchange, one might expect that perturbations in the rate of HCO_3 secretion, induced by adding or removing HCO_3 from the basolateral solution, would produce changes in Cl reabsorption of equal magnitude (1–3). To test this hypothesis, we added or removed bath HCO_3 (Hepes replacement) and measured the

Table III. Changes in $J_{HCO_3}{}^{b-l}$ and $J_{Cl}{}^{l-b}$ Upon Adding or Removing Bath HCO_3 (Hepes Replacement) in the Presence of cAMP

	Mean luminal [Cl]	HCO_3/CO_2bath			Hepes bath			Paired changes			
		V_T	$J_{HCO_3}{}^{b-l}$	$J_{Cl}{}^{l-b}$	V_T	$J_{HCO_3}{}^{b-l}$	$J_{Cl}{}^{l-b}$	V_T	$J_{HCO_3}{}^{b-l}$	$J_{Cl}{}^{l-b}$	ΔFlux
	mM	*mV*			*mV*			*mV*			
	90.8	20	8.7	16.8	14	1.4	15.7	−6	−7.3	−1.1	+6.2
	92.4	22	13.8	31.9	11	7.9	18.5	−11	−5.9	−13.4	−7.5
	88.9	56	19.9	40.1	15	1.5	14.1	−41	−18.4	−26.0	−7.6
	33.9	47	7.8	23.0	14	−0.6	10.0	−33	−8.5	−13.0	−4.5
	9.8	85	12.0	18.7	29	0.9	7.0	−56	−11.1	−11.7	−0.6
	36.7	28	5.5	12.9	5	0.7	7.1	−23	−4.8	−5.8	−1.0
	30.7	32	9.5	18.4	12	−1.5	4.4	−20	−11.1	−14.0	−2.9
	10.3	23	17.4	23.0	10	5.1	4.9	−13	−12.3	−18.0	−5.7
	41.5	21	9.9	17.2	14	5.6	11.6	−7	−4.3	−5.6	−1.3
	42.6	45	4.2	18.9	18	1.5	7.3	−27	−2.7	−11.6	−8.9
	144.4	24	10.7	19.4	13	2.1	1.1	−11	−8.6	−18.3	−9.7
	142.7	58	10.4	29.2	15	1.2	9.1	−43	−9.2	−20.0	−10.8
	144.1	40	10.2	19.7	17	1.5	9.9	−23	−8.6	−9.8	−1.2
	11.4	40	6.7	11.4	10	0.0	2.9	−30	−6.9	−8.5	−1.6
Mean		38.6	10.5	21.5	14.1	2.0	8.8	−24.6	−8.5	−12.6	−4.1
SE		5.0	1.1	2.1	1.4	.8	1.3	4.0	±1.1	1.7	±1.2
P								<0.001	<0.001	<0.001	<0.005

ΔFlux, change in $J_{Cl}{}^{l-b}$ minus change in $J_{HCO_3}{}^{b-l}$. Flux units are picomoles per millimeter per minute. Bath was 0 Cl HCO_3 or 0 Cl Hepes in random order. Perfusates were 12, 45, 100, and 145 mM Cl simple Cl solutions (see Table I) containing 50 μM amiloride. 0.1 mM 8-bromo-cAMP was present in the bath throughout.

effect on J_{Cl}^{lb} and $J_{HCO_3}^{bl}$. The bath solution was 0 Cl HCO_3 or 0 Cl Hepes. Actual bath [Cl] was measured at 48.7±16.8 μM, a concentration that would be expected to support a ^{36}Cl self-exchange rate of <1.0 pmol/mm per min (Fig. 2). The rate of proton secretion should also be negligible with this bath Cl concentration (20).

The results are displayed in Table III. Two points should be noted. First, in the absence of bath HCO_3 the transepithelial voltage was 24.6±4.0 mV less positive than in the presence of bath HCO_3. With amiloride and barium in the lumen, and in the absence of bath Cl, it is unlikely that changes in Na^+, K^+, or H^+ transport account for these voltage changes. Rather, as will be developed below, it appears likely that this lumen-positive voltage represents a Cl diffusion voltage that is sensitive to the presence of HCO_3/CO_2.

Second, bath HCO_3 removal lowered both HCO_3 secretion and Cl reabsorption, but the change in the Cl flux exceeded the change in HCO_3 flux (Δ flux term, Table III). If the Cl-HCO_3 exchange stoichiometry were 1:1, the two fluxes should have changed by comparable amounts. The decrease in the lumen-positive voltage by 25 mV upon removing bath HCO_3 would tend to lower diffusive HCO_3 secretion and increase diffusive Cl reabsorption. That is, upon removing bath HCO_3 the voltage effect on diffusive fluxes would tend to make total HCO_3 secretion fall more than total Cl reabsorption. The opposite was observed. This observation suggested one of two possibilities; the stoichiometry of Cl-HCO_3 exchange in the presence of cAMP is not 1:1; or in the presence of HCO_3/CO_2 there is a separate pathway for transepithelial ^{36}Cl transfer that is parallel to a 1:1 Cl-HCO_3 exchanger.

We addressed the issue of stoichiometry in another way. As previously discussed, we have reported that the Cl permeability in the absence of HCO_3 secretion, Cl self-exchange, and cAMP (conditions that should allow only Cl diffusion) is 2.24±.66 $\times 10^{-6}$ cm/s (9). This reference value is shown as the left-hand bar in Fig. 4. In the present studies, we perfused a group of 10 tubules with 145 mM Cl simple Cl perfusate; the bath was 0 Cl HCO_3 with cAMP. $J_{HCO_3}^{bl}$ and J_{Cl}^{lb} were measured simultaneously. If we set two conditions: the passive "diffusive" HCO_3 permeability is 1.9×10^{-6} cm/s (8); and Cl-HCO_3 exchange is 1:1, then we can calculate the diffusive Cl permeability that would be necessary to account for the observed nonmediated J_{Cl}^{lb} under the present conditions. The results are shown in Table IV. A P_{Cl} of $7.79 \pm 1.19 \times 10^{-6}$ cm/s would be required. This is significantly greater than our previous result in the absence of HCO_3 or cAMP. If we set $P_{HCO_3} = 0$, then a P_{Cl} of $6.60 \pm 1.06 \times 10^{-6}$ cm/s is required (Table IV). As shown in Fig. 4, this P_{Cl} is still significantly greater than that we previously measured in the absence of HCO_3 and cAMP. Our data are consistent with cAMP and HCO_3 inducing or increasing a Cl pathway separate from 1:1 Cl-HCO_3 exchange.

Table IV. Estimated Passive Cl Permeabilities If Cl-HCO_3 Exchange is 1:1

P_{HCO_3}	J_{Cl}^{lb} (Total)	$J_{HCO_3}^{bl}$ (Mediated)	J_{Cl}^{lb} (Residual)	Required P_{Cl}
10^{-6} cm/s				10^{-6} cm/s
1.9	28.4±2.2	7.54±0.74	20.9±2.18	7.79±1.19
0.00	28.4±2.2	10.3±0.79	18.1±2.18	6.60±1.06

Perfusate was 145 mM Cl simple Cl containing 50 μM amiloride. Bath was 0 Cl HCO_3 with 0.1 mM 8-bromo-cAMP. Mean transepithelial Cl gradient was 139.7±1.1 mM, mean transepithelial voltage was 29.0±4.3 mV. If Cl-HCO_3 stoichiometry is 1:1, then J_{Cl}^{lb} (total) $- J_{HCO_3}^{bl}$ (mediated) = J_{Cl}^{lb} (residual). From J_{Cl}^{lb} (residual) and the measured electrochemical driving forces, P_{Cl} was calculated using the GHK equation. P_{HCO_3} value of 1.9×10^{-6} cm/s is from reference 8. Fluxes are in picomoles per millimeter per minute. $n = 10$.

Effect of cAMP on G_T and equivalent current in the presence of HCO_3/CO_2. We next directly addressed the hypothesis that cAMP increases a conductive Cl pathway in the presence of HCO_3/CO_2. It should be noted that we previously found no effect of cAMP on transepithelial conductance in the absence of HCO_3/CO_2 (16). Tubules were perfused with amiloride and Ba^{2+} (4 mM) to block principal cell conductances (23, 24). Both perfusate and bath were symmetrical standard HCO_3 solutions. After allowing endogenous cAMP effects to decline in vitro for 90 min, we measured G_T and V_T. Then cAMP was added and the measurements were repeated when a steady state had been achieved. As shown on the left in Fig. 5, in the presence of Cl in the perfusate and bath, cAMP increased G_T from 1.66±0.29 to 2.49±0.39 mS/cm² (paired Δ = +0.84±0.24, $P < 0.02$). In contrast, when a separate set of tubules was perfused and bathed in 0 Cl HCO_3 solutions, cAMP failed to change G_T (2.41±0.56 to 2.40±0.53, paired Δ = −0.01±0.04 mS/cm², NS). These data are consistent with a cAMP-induced increase in Cl conductance. Because HCO_3 was present symmetrically in both cases, the data are not consistent with an effect of cAMP on a HCO_3 conductance.

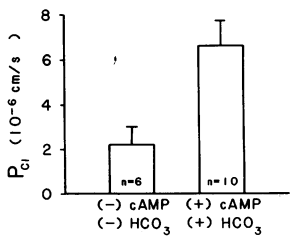

Figure 4. Cl permeabilities in the absence (*left*) and presence (*right*) of cAMP and HCO_3. Values on the left are from reference 9 and were measured directly in the absence of Cl self-exchange and HCO_3 secretion. Values on the right are calculated from the present data as per Table IV, assuming a Cl-HCO_3 exchange stoichiometry of 1:1 and a P_{HCO_3} of zero ($P < 0.005$).

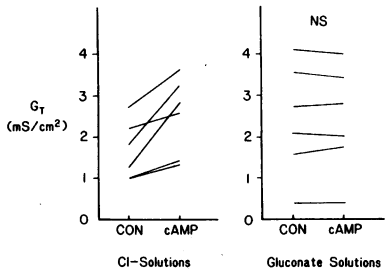

Figure 5. Effect of 0.1 mM 8-bromo-cAMP on transepithelial conductance, G_T (mS/cm²), in the presence of luminal $BaNO_3$ (4 mM) and amiloride (50 μM). In the left panel ($P < 0.02$), perfusion and bathing solutions were standard HCO_3 solutions (Cl = 124 mM). In the right panel, all Cl in both perfusing and bathing solutions was replaced by gluconate (0 Cl HCO_3 solutions). CON, control.

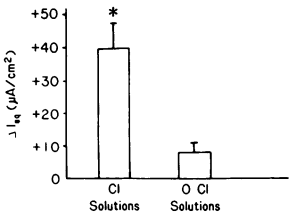

Figure 6. Change in equivalent short-circuit current, *I*eq ($\mu A/cm^2$), produced by 0.1 mM 8-bromo-cAMP. Values are calculated from the corresponding tubules in Fig. 4. Positive current values represent flow of cations into the lumen or anions from the lumen. * Change in *I*eq significantly different from zero. Note that no cAMP-induced negative equivalent current flow (i.e., electrogenic HCO_3 secretion) is seen in Cl-free solutions.

From the open-circuit transepithelial voltage and the G_T, we calculated the effect of cAMP on the equivalent current, *I*eq ($\mu A/cm^2$) under these two conditions. As shown in Fig. 6, in Cl-containing solutions cAMP increased the flow of positive equivalent current into the lumen from 2.44±3.25 to 42.0±9.29 $\mu A/cm^2$ (paired Δ = +39.5±7.8, $P < 0.01$). In contrast, in Cl-free solutions (0 Cl HCO_3) cAMP did not significantly change *I*eq (6.32±3.7 to 13.9±4.7 $\mu A/cm^2$, paired Δ = +7.6±3.2, NS compared with zero). It should be noted that we did not observe a flow of negative equivalent current into the lumen which would have suggested electrogenic HCO_3 secretion. This agrees with our previous results in which, under similar 0 Cl conditions, cAMP failed to increase HCO_3 secretion (8).

Effects of HCO_3/CO_2 on G_T and equivalent current in the presence of cAMP. Because the stoichiometry and Cl permeability studies above suggested that the diffusional Cl permeability might be regulated by HCO_3/CO_2, we tested the hypothesis that, in the presence of cAMP, HCO_3/CO_2 induces or increases a Cl conductance. First, we perfused tubules with 145 mM Cl simple Cl perfusate; the bath was first 0 Cl Hepes and was then changed to 0 Cl HCO_3. Each bath contained cAMP. These experimental conditions thus are analogous to those in Table III in which HCO_3/CO_2 changed J^{lb}_{Cl} disproportionately. G_T and V_T were measured, and *I*eq was calculated with each bath. *I*eq is usually calculated using symmetrical solutions, referred to as an equivalent short-circuit current, and has significance in terms of active transport. In Table V, the equivalent current was calculated under nonsymmetrical conditions (lumen > bath Cl gradient and bath > lumen Hepes or HCO_3 gradient). In the presence of luminal amiloride and barium and in the absence of bath Cl, it is unlikely that electrogenic transport of Na^+, K^+, or H^+, respectively, are contributing to *I*eq. Given that the Cl permeability and gradient are larger than those for HCO_3, it seems likely that *I*eq under these conditions is dominated by passive Cl diffusion. The results are shown in Table V. Bath HCO_3/CO_2 addition produced an abrupt increase in the transepithelial voltage which peaked at ~5 min. The calculated increase in equivalent current flow at this peak response (expressed as a flux) was 23.6±9.7 pmol/mm per min. The transepithelial voltage then declined gradually such that 30 min after HCO_3/CO_2 addition it was not significantly different from control values. The transepithelial conductance, however, continued to increase during 30 min. The calculated equivalent current at 30 min thus had declined from the peak value and was only 11.2±5.8 pmol/mm per min above pre-HCO_3/CO_2 values (P = 0.06 vs. control). This increment above control is not statistically different from the HCO_3/CO_2-induced change in J^{lb}_{Cl} which was unaccounted for by Cl-HCO_3 exchange in Table III (4.1±1.2 pmol/mm per min). The numerically somewhat larger increment in *I*eq could be due to the transient nature of the response to HCO_3/CO_2: *I*eq was last measured at 30 min, whereas Cl fluxes were averaged over 90 min. These results suggest that HCO_3/CO_2, in the presence of cAMP, induces a conductance parallel to a 1:1 Cl-HCO_3 exchanger, which can account for the nonexchange-mediated lumen-to-bath Cl flux seen in Table III.

If HCO_3/CO_2 induces or increases a transepithelial Cl conductive pathway in the presence of cAMP, then HCO_3/CO_2 should have no effect on G_T in the absence of Cl. As shown in Table VI, under such conditions HCO_3/CO_2 did not change G_T.

In the symmetrical absence of Cl, HCO_3 addition also failed to significantly change V_T (paired Δ = +0.6±1.4 mV, NS). Therefore, it is clear that induction by HCO_3/CO_2 of a large lumen-positive V_T (Table III) requires luminal Cl. It seems unlikely that electrogenic H^+ secretion plays a role in this V_T change because H^+ secretion by the medullary collecting duct is independent of luminal Cl (20). Rather, the most likely explanation is that the HCO_3-induced lumen-positive V_T observed in the presence of lumen Cl represents a Cl diffusion voltage.

Discussion

The present studies demonstrate two important new features about cAMP-stimulated Cl and HCO_3 transport in rabbit cortical collecting tubules. First, both cAMP-stimulated Cl self-exchange and Cl-dependent HCO_3 secretion exhibit saturation-type kinetics with respect to basolateral and luminal Cl concentration, respectively. Second, cAMP and HCO_3/CO_2 appear to act syn-

Table V. Change in Calculated Current Induced by Addition of Bath HCO_3/CO_2 in the Presence of cAMP

	Transepithelial voltage			Transepithelial conductance			Equivalent current		
	Control	5 min	30 min	Control	5 min	30 min	Control	5 min	30 min
	mV	*mV*	*mV*	*mS/cm²*	*mS/cm²*	*mS/cm²*	*μA/cm²*	*μA/cm²*	*μA/cm²*
	36.3±3.1	57.8±7.9	38.8±6.3	1.14±.21	1.46±.29	1.68±.27	42.9±10.2	91.3±26.6	65.8±14.6
Paired Δ vs. control		+21.5±6.7	+2.4±6.3		+0.32±0.10	+0.53±0.23		+48.4±19.9*	+22.9±11.9*
P		<0.02	NS		<0.05	<0.05		<0.05	=0.06

* 48.4±19.9 $\mu A/cm^2$ = 23.6±9.7 pmol/mm per min and 22.9±11.9 $\mu A/cm^2$ = 11.2±5.8 pmol/mm per min. n = 6. Perfusate was 145 mM Cl simple Cl and contained 4 mM $BaNO_3$ in addition to 50 μM amiloride. The control bath solution was 0 Cl Hepes; the bath at 5 and 30 min was 0 Cl HCO_3. 0.1 mM 8-bromo-cAMP was present throughout.

Table VI. Effect of HCO_3/CO_2 on Transepithelial Conductance in the Absence of Cl

Bath and perfusate	G_T
	mS/cm²
0 Cl Hepes	1.88±0.46
0 Cl HCO_3	1.76±0.38
Paired change	−0.12±0.10

0.1 mM 8-bromo-cAMP was present throughout. $n = 5$. The mean paired change is not significant. Perfusates contained 4 mM $BaNO_3$ and 50 μm amiloride.

ergistically to increase or induce a Cl conductive pathway parallel to the Cl-HCO_3 exchange system.

Our previous experiments demonstrated that cAMP stimulates Cl-dependent HCO_3 secretion and Cl self-exchange (8–10). Using paired analysis of the stoichiometry of Cl reabsorption and HCO_3 secretion in the present study, we found that the addition of basolateral HCO_3 caused a larger increase in (unidirectional) Cl reabsorption than in (unidirectional) HCO_3 secretion. It was thus necessary to postulate a HCO_3-dependent increase in Cl permeability parallel to a 1:1 Cl-HCO_3 exchanger in order to accommodate the observed flux data in the presence of cAMP (Tables III and IV). This permeability appears to be conductive; the size of the HCO_3/CO_2/cAMP-induced conductance change appears adequate to account for the observed discrepancy in the stoichiometry (Table III vs. Table V).

Similar observations on cAMP/HCO_3/CO_2-dependent anion conductances have been made in the turtle bladder. Although Husted et al. found that Cl reabsorptive fluxes and HCO_3 secretory fluxes changed by equal amounts upon adding serosal HCO_3 (2), examination of the data of Leslie et al. (1) shows that HCO_3 addition altered Cl reabsorption significantly more than HCO_3 secretion. This agrees with our results in the presence of cAMP (Table III). In the presence of a phosphodiesterase inhibitor (isobutylmethylxanthine, IBMX), Ehrenspeck found that HCO_3 addition significantly increased the mean transepithelial conductance of turtle bladders from 0.35 to 0.61 mS/cm² (25). Similarly, Satake et al. found that addition of cAMP plus IBMX to turtle bladders from alkali-loaded animals increased the conductance by 0.26±0.03 mS/cm² (7). Because the bathing solutions in the latter study were Cl free, the conductance change observed by Satake et al. appears to represent a HCO_3 conductance. Subsequent studies by Stetson et al. have suggested, however, that the anion conductance stimulated by cAMP in the turtle bladder might be poorly selective for HCO_3 over Cl (6). In this regard, cAMP has been found to increase the serosal-to-mucosal Cl flux and therefore decrease net Cl reabsorption in two separate studies in turtle bladder (6, 26). If cAMP were to increase an apical Cl conductance as proposed by Stetson et al. (6), this should represent a route other than the Cl-HCO_3 exchanger by which ^{36}Cl could traverse the epithelium from mucosa to serosa, i.e., one would expect that the absorptive Cl tracer flux would also have increased with cAMP. Although the trend in their study was in this direction, the change was not statistically significant.

The physical location of the cAMP/HCO_3/CO_2-induced conductance is unknown. Cyclic AMP has been reported to alter tight-junction conductances, but these effects have been in "leaky epithelia" such as proximal tubule (27) and goldfish intestine (28). We recently provided evidence that the paracellular pathway of the rabbit cortical collecting tubule is nonselective for anions over cations (29). These experiments were done in the absence of HCO_3/CO_2 and cAMP. We thus cannot exclude the possibility that the paracellular Cl permeability is higher in the presence of HCO_3/CO_2 and cAMP. Our present data do not support the notion, however, that the epithelium becomes "Cl-selective" in the presence of HCO_3/CO_2 and cAMP. We can compare the measured G_T value of 2.49 mS/cm² in the presence of cAMP and symmetrical standard HCO_3 solutions to the calculated partial ionic conductance of the epithelium to Cl (using a P_{Cl} of 7.8×10^{-6} cm/s [Table IV], $V_T = +23$ mV, mean [Cl] = 124 mM, and a modified GHK equation [21]). The calculated Cl partial ionic conductance, 1.42 mS/cm² is still only 57% the total transepithelial conductance.

Another alternative is that cAMP changes the apical cell membrane Cl conductance. There is good evidence that cAMP causes the induction or appearance of previously quiescent Cl conductances in the apical membrane of both Cl-secreting (30) and -absorbing (31) epithelia. The apparent role in the first case is to stimulate net Cl transport, whereas in the second the Cl conductance inhibits Cl reabsorption. The available electrophysiological data on cortical collecting tubule cells have argued against any apical membrane Cl conductances (5, 23, 24). Again, however, the studies were not done in the presence of cAMP. If cAMP were to induce an intercalated cell apical membrane Cl conductance, it might do so in either HCO_3-secreting cells, H^+-secreting cells, or both. As long as there is some route for basolateral Cl exit in each cell type, either event would increase the transepithelial lumen-to-bath ^{36}Cl flux, and would be seen as a shunt Cl permeability relative to the apical Cl-HCO_3 exchanger. Our data thus are compatible with either or both of these possibilities. It will require additional study to determine the exact physical location of the conductance changes we have observed.

Our kinetic data add to the growing body of evidence that HCO_3 secretion is mediated by a Cl-HCO_3 exchanger (3, 4) and that this exchanger can function in a Cl self-exchange mode (9, 10). It is important to note that the K_m and V_{max} values derived here for the two processes represent lumped transepithelial kinetic parameters and cannot be considered to be constants in the true enzyme sense. Assuming the exchanger is located on the apical membrane (4), the kinetic constants include a contribution by the series basolateral membrane. Cyclic AMP could stimulate the apical exchanger directly, it could increase the series basolateral Cl conductance, or both. Our data do not allow us to differentiate these possibilities. Despite these limitations, the kinetic values are of interest for several reasons.

First, we found a good Michaelis-Menten fit of Cl self-exchange to bath Cl concentration (Fig. 2). This is somewhat surprising, given previous data on the trans dependence of Cl flux in frog skin (32). Nonetheless, the overall basolateral K_m of 2.8–4.2 mM makes it unlikely that small changes from normal in blood Cl concentration per se, such as those seen in metabolic alkalosis (33), could regulate HCO_3 secretion by altering the ratio of Cl self-exchange to Cl-HCO_3 exchange. Moreover, the V_{max} values for the two processes indicate that, under maximal stimulation, the exchanger probably turns over in the self-exchange mode at ~7–10-fold the rate of the Cl-HCO_3 mode.

Second, half-maximal rates of HCO_3 secretion were seen at luminal Cl concentrations of between 4 and 11 mM. This K_m range is similar to the turtle bladder value of roughly 4.2 mM, which one can calculate from the data of Leslie et al. (1). Similarly, the data of Garcia-Austt et al. (34) in cortical collecting tubules from desoxycorticosterone-treated rabbits yield, from unidirectional HCO_3 secretory rates measured at two different luminal Cl concentrations, an estimated luminal K_m for Cl of ~1.7 mM. Our luminal K_m is not close, however, to the value of 0.13 mM found by Fischer et al. for the turtle bladder basolateral Cl-HCO_3 exchanger (12). The K_m for Cl of the basolateral Cl-HCO_3 exchanger in cortical collecting tubules is not known. If it is found to be significantly lower than the luminal K_m, it would suggest that the two exchangers are different. Because HCO_3 secretion/Cl self-exchange and HCO_3 reabsorption are differentially sensitive to disulfonic stilbenes (8, 9, 20, 35), and because the apical and basolateral membranes of intercalated cells show differential immunohistochemical evidence of band 3 protein (36, 37), it is possible that the exchangers are, indeed, different.

Finally, the luminal K_m for Cl of 4–11 mM is important in considering whole kidney physiology. This relatively high-affinity system could function to effectively lower lumen Cl concentration and/or to secrete HCO_3 in states of Cl-depletion alkalosis. Indeed, recent models of Cl-depletion alkalosis suggest that the collecting duct plays the most important role in excreting HCO_3 upon Cl replacement (33). A significantly higher K_m for Cl would be incompatible with such a regulatory function.

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