JCI The Journal of Clinical Investigation

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J Clin Invest. 1985;75(6):2056-2064. https://doi.org/10.1172/JCI111925.

Research Article

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Cyclic Adenosine Monophosphate-stimulated Bicarbonate Secretion in Rabbit Cortical Collecting Tubules

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Abstract

We studied the effects of cyclic AMP (cAMP) on HCO_3^- transport by rabbit cortical collecting tubules perfused in vitro. Net HCO_3^- secretion was observed in tubules from $NaHCO_3$-loaded rabbits. 8-Bromo-cAMP–stimulated net HCO_3^- secretion, whereas secretion fell with time in control tubules. Both isoproterenol and vasopressin (ADH) are known to stimulate adenylate cyclase in this epithelium; however, only isoproterenol stimulated net HCO_3^- secretion.

The mechanism of cAMP-stimulated HCO_3^- secretion was examined. If both HCO_3^- and H^+ secretion were to occur simultaneously in tubules exhibiting net HCO_3^- secretion, cAMP might increase the net HCO_3^- secretory rate by inhibiting H^+ secretion, by stimulating HCO_3^- secretion, or both. These possibilities were examined using basolateral addition of the disulfonic stilbene (4,4′-diisothiocyanostilbene-2,2′-disulfonate (DIDS). In acidifying tubules from NH_4Cl-loaded rabbits, DIDS eliminated HCO_3^- reabsorption, a result consistent with known effects of DIDS as an inhibitor of H^+ secretion. In contrast, cAMP left acidification (H^+ secretion) intact. DIDS applied to HCO_3^- secretory tubules failed to increase the HCO_3^- secretory rate, indicating minimal H^+ secretion in HCO_3^--secreting tubules. Thus, inhibition of H^+ secretion by cAMP could not account for the cAMP-induced stimulation of net HCO_3^- secretion.

cAMP-stimulated HCO_3^- secretion was reversibly eliminated by 0 Cl perfusate, whereas luminal DIDS had no effect. Bath amiloride (1 mM) failed to eliminate cAMP-stimulated HCO_3^- secretion when bath $[Na^+]$ was 145 mM or 5 mM. cAMP depolarized the transepithelial voltage. The collected fluid $[HCO_3^-]$ after cAMP could be accounted for by electrical driving forces, suggesting that cAMP stimulates passive HCO_3^- secretion. However, cAMP did not alter HCO_3^- permeability measured under conditions expected to inhibit transcellular HCO_3^- movement (0 Cl^- solutions and bath DIDS). This measured HCO_3^- permeability was not high enough to account, by passive diffusion, for the HCO_3^- fluxes observed in Cl^--containing solutions.

We conclude the following: 1) cAMP increased net HCO_3^- secretion by stimulating HCO_3^- secretion and not by inhibiting H^+ secretion; 2) this HCO_3^- secretion may have occurred by Cl-HCO_3^- exchange; 3) Na^+-H^+ exchange appeared not to play a role in basolateral H^+ extrusion under these conditions; and 4) the stimulation of HCO_3^- secretion by isoproterenol, but not ADH, suggests the existence of separate cell cAMP pools or cellular heterogeneity in this cAMP response.

Introduction

Net HCO_3^- secretion has been described in cortical collecting tubules dissected from alkali-loaded rats and rabbits (1–4), but little is known about the hormonal control of this process. Although initial studies by Knepper et al. suggested that chronic mineralocorticoid administration stimulates HCO_3^- secretion by the cortical collecting tubule (5), subsequent investigation has shown that this stimulation is secondary to the accompanying metabolic alkalosis rather than a direct hormonal affect on HCO_3^- transport (6). In contrast, cyclic nucleotides directly stimulate HCO_3^- secretion in a number of epithelia including salivary gland (7), small intestine (8), and choroid plexus (9). The present studies on rabbit cortical collecting tubules have focused on cyclic AMP (cAMP) and two hormones known to stimulate adenylate cyclase in this nephron segment, namely, vasopressin (ADH)[1] and isoproterenol (10). Our experiments demonstrate that cAMP stimulated HCO_3^- secretion and address the mechanism of this stimulation.

Methods

General microperfusion methods. In vitro microperfusion of cortical collecting tubules was performed as previously described (11). In studies examining HCO_3^- secretion, tubules were dissected from rabbits given normal rabbit chow ad lib. and drinking water consisting of 0.075 M $NaHCO_3$ and 0.1 M glucose for 3–14 d before study. In studies examining acidification (HCO_3^- reabsorption), tubules were dissected from rabbits starved for 3 d and given a drinking solution of 0.075 M NH_4Cl and 0.1 M glucose for 3–7 d before study. Female New Zealand white rabbits from either of these two groups were killed by decapitation, the left kidney was quickly removed, 1–2-mm coronal slices were cut, and dissection was performed at 4°C. Tubules were transferred to a thermostatically controlled chamber of 1 ml vol and were connected to perfusing and holding pipettes. Continuous bath exchange at ≥0.6 ml/min was begun, and the temperature of the chamber was warmed to 38°C. After a 60-min equilibration period, collections were begun.

Collected fluid spontaneously filled a calibrated volume pipette (CVP) under water-equilibrated mineral oil. CVP volumes ranged from 23–33 nl. In all periods, 3–4 collections for HCO_3^- concentration (total

A portion of this work was presented at the 17th Annual Meeting of the American Society of Nephrology, Washington, DC, and has appeared in abstract form. 1985. *Kidney Int.* 27:288.

Received for publication 10 December 1984 and in revised form 18 February 1985.

J. Clin. Invest.

0021-9738/85/06/2056/09 $1.00
Volume 75, June 1985, 2056–2064

1. *Abbreviations used in this paper:* ADH, vasopressin; CVP, calibrated volume pipette; DIDS, 4,4′-diisothiocyanostilbene-2,2′-disulfonate; J_v, net volume flux; tCO_2, total CO_2; TMA, tetramethylammonium; V_T, transepithelial voltage.

CO_2 [tCO_2], see below) were bracketed by 2–3 collections for net volume flux (J_v). At the end of each experiment, the perfusate was calibrated as follows. The perfusion pipette was advanced into a second pipette ("calibrating pipette") containing Sylgard (Dow Corning Corp., Midland, MI) backed by water-equilibrated mineral oil. Perfusate was then forced between the Sylgard and oil. The CVP was filled at 38°C and analyzed for either [^{3}H]inulin counts or HCO_3^- concentration, each of which was measured in triplicate at least.

Transepithelial voltage (V_T) was measured by connecting a Ringer-agarose bridge to the perfusion pipette and to a calomel electrode. A similar bridge in the bath, also connected to a calomel electrode, completed the circuit. V_T (mV) was measured with an electrometer (Keithley Instruments, Inc., Cleveland, OH) and continuously recorded on a strip-chart recorder. Voltages at the perfusion pipette were reported referenced to the bath as ground.

All tubules were perfused at or near 1 nl/mm tubule length per min. The mean paired difference between all control period and all experimental period perfusion rates was 0.015±0.03 (SD) nl/mm tubule length per min.

Solutions. The composition of perfusing and bathing solutions is shown in Table I. Solutions A, C, and E were used as perfusates, and solutions B, D, and F as baths. The combination A and B was used in most protocols; solutions C and D were used to determine passive HCO_3^- permeability; solution E was used for luminal Cl removal; and solution F was used for some of the bath amiloride studies. All solutions were gassed with 95% O_2/5% CO_2. Tetramethylammonium (TMA) was generated from TMA-OH and CO_2. [^{3}H]Inulin (New England Nuclear, Boston, MA), exhaustively dialyzed to remove fragments < 3,000 mol wt, was added to perfusates as a volume marker. Preliminary experiments showed that tubule viability was somewhat higher in albumin-containing baths as opposed to Ringers alone, and that the responses to hormones and inhibitors were qualitatively the same with or without albumin. Therefore, bovine serum albumin (BSA) (CRG-7; Armour Pharmaceutical Co., Tarrytown, NY) was used in all baths except the 0 Cl bath (solution D).

Microcalorimetry. In all of the present studies except those examining HCO_3^- permeability, bicarbonate concentrations of perfused and collected fluid were operationally defined as the tCO_2, measured by microcalorimetry (Picapnotherm; World Precision Instruments, Inc., New Haven, CT) with the realization that this approximation systematically overestimates [HCO_3^-] by the amount of dissolved CO_2, which is 1.2 mM at a constant P_{CO_2} of 40 mmHg. With regard to tCO_2 measurements, we were able to consistently exceed the manufacturer's stated sensitivity by modifying the LiOH crystal as follows. Relatively large anhydrous LiOH crystals (#44102, −4±14 mesh; Alfa Products, Morton Thiokol Inc., Danvers, MA) were stored in desiccating Drierite (W. A. Hammond Drierite Co., Xenia, OH). Just before use, crystals were heated to 200°C for 10 min, and then hand-carved under a dissecting microscope with a razor blade and forceps into a thin wafer 1 mm square and ~⅓–½ mm thick. One surface of this wafer was chiseled with forceps so that it was concave, and the crystal was mounted with this concave surface toward the thermistor bead. This method consistently yielded signals of ≥25 integrator units/pmol tCO_2, and water blanks of ≤10 integrator units/nl of sample.

Table I. Composition of Perfusing and Bathing Solutions

Solution	A	B	C	D	E	F
Na^+	145	145	145	145	145	5
K^+	5	5	5	5	5	5
Cl^-	115	115	0	0	0	115
HCO_3^-	25	25	5	25	25	25
Ca^{++}	1.8	1.8	1.8	1.8	1.8	1.8
Mg^{++}	1.0	1.0	1.0	1.0	1.0	1.0
PO_4^-	2.3	2.3	0	2.3	2.3	2.3
SO_4^-	1.0	1.0	1.0	1.0	1.0	1.0
Acetate	10	10	0	0	10	10
Glucose	8	8	13	8	8	8
Alanine	5	5	5	5	5	5
Gluconate	0	0	135	115	115	0
TMA	0	0	0	0	0	140
BSA	0	1	0	0	0	1

Concentrations in millimolars except BSA which is in grams per deciliters.

Calculations. Net volume flux was calculated from the equation $J_v = (V_i - V_0)/L$. V_0 is the collection rate (nanoliters per minute) measured directly; V_i is the perfusion rate (nanoliters per minute) calculated from $V_i = V_0$ (cpm_0/cpm_i), where cpm_0 and cpm_i are perfused and collected fluid ^{3}H counts per minute per nanoliter; and L is tubule length measured by eyepiece micrometer. Net HCO_3^- flux, $J_{HCO_3^-}$ (picomoles per millimeter per minute), was calculated from the following equation: $J_{HCO_3^-} = [(V_i)(tCO_2)_{in} - (V_0)(tCO_2)_{out}]/L$, where $(tCO_2)_{in}$ and $(tCO_2)_{out}$ are perfused and collected fluid tCO_2 concentrations (mM), and V_i, V_0, and L have their aforestated meanings. Since determinations of tCO_2 were alternated with J_v measurements, the V_i term for each $J_{HCO_3^-}$ was calculated from the measured V_0 and the average J_v for that experimental period. By convention, negative values for $J_{HCO_3^-}$ signify net HCO_3^- secretion and positive values net reabsorption. Tubules were excluded if the J_v exceeded ±0.1 nl/mm per min in the absence of ADH or cAMP, i.e., if there was a significant inulin leak.

The V_T required to account for the collected fluid [HCO_3^-] by passive diffusion along electrical gradients was calculated from the Nernst equation (12):

$$E_{HCO_3^-}(\text{mV}) = \frac{-RT}{F} \ln \frac{[HCO_3^-]_{bath}}{[HCO_3^-]_{out}},$$

where R is the gas constant, T is degrees Kelvin, F is the Faraday constant, and $[HCO_3^-]_{bath}$ and $[HCO_3^-]_{out}$ are bath and collected fluid tCO_2 concentrations (mM), respectively, measured by microcalorimetry.

The apparent HCO_3^- permeability was calculated from the increase in luminal [HCO_3^-] above a 5-mM perfusate value using the Goldman-Hodgkin-Katz equation (12):

$$P_{HCO_3} = \frac{J_{HCO_3}}{(2\pi r)(\xi)\left\{\dfrac{C_b - \dfrac{C_i^l + C_0^l}{2}\, e^{-\xi}}{1 - e^{-\xi}}\right\}},$$

where $\xi = FV_T/RT$, r is tubule radius (assumed = 10 μm), J_{HCO_3} is net HCO_3^- flux, C_b is bath HCO_3^- concentration, C_i^l is perfusate HCO_3^- concentration, and C_0^l is collected fluid HCO_3^- concentration, all determined by microcalorimetry; V_T is corrected for liquid junction potentials; and F, R, and T are as described above. Because these experiments involved measuring collected fluid tCO_2 concentrations of around 5–8 mM, the 1.2 mM contribution of dissolved CO_2 would contribute proportionately more to the estimation of true [HCO_3^-] than in experiments with collected fluid [HCO_3^-] of 25–40 mM. Therefore, in these HCO_3^- permeability studies, 1.2 mM was subtracted from all tCO_2 concentrations to more closely estimate true [HCO_3^-].

Reagents. Arginine ADH, 8-bromo-cAMP, isoproterenol, Na, K, and Ca gluconate, TMA-Cl, and TMA-OH were purchased from Sigma Chemical Co., (St. Louis, MO). Amiloride was a kind gift of the Merck Sharp & Dohme Div., West Point, PA. 4,4'-Diisothiocyanostilbene-2,2'-disulfonate (DIDS) was purchased from Pierce Chemical Co. (Rockford, IL).

Acid-base parameters. In most rabbits, urine pH and whole blood pH (drawn from an ear vein into a heparinized syringe) were measured at 38°C under oil by pH electrode. Blood was collected at decapitation, allowed to clot, centrifuged, and serum [HCO_3^-] measured by microcalorimetry.

Statistics. The values from three to four collections per experimental period were averaged to yield a single value for that tubule. Thus, n refers to number of tubules. The t test was used for paired and unpaired comparisons as appropriate. Differences were considered significant if $P < 0.05$. Mean values are reported as ±SE unless otherwise indicated.

Results

Systemic acid-base parameters. The results of urine and blood pH and serum [HCO_3^-] measurements on HCO_3^--loaded and NH_4Cl-loaded, starved rabbits are shown in Table II. There were highly significant differences in all three parameters between the two groups.

cAMP, ADH, and isoproterenol effects on net HCO_3^- secretion. In this group of experiments, net HCO_3^- secretory rates were measured in tubules harvested from HCO_3^--loaded rabbits; perfusate was solution A, and bath was solution B. Fig. 1 shows the results of time control experiments. The HCO_3^- secretory rate fell with time (paired Δ = +3.84±1.09 pmol/mm per min, $P < 0.02$). The V_T (−8.3±2.4 mV) did not significantly change with time (paired Δ = −1.5±1.6 mV).

In contrast to these spontaneous time-dependent changes, 8-bromo-cAMP (0.1 mM) added to the bath (Fig. 2) stimulated net HCO_3^- secretion (paired Δ = −4.53±1.29 pmol/mm per min, $P < 0.025$ by paired analysis, $P < 0.001$ by unpaired analysis to time controls). Associated with this change in HCO_3^- flux, cAMP depolarized V_T from −21.8±4.7 to +12.8±5.7 mV (paired Δ = +34.6±7.9 mV, $P < 0.02$). Perfusion rates in control and experimental periods were not significantly different (paired Δ = 0.046±0.019 nl/mm length per min).

Both ADH and isoproterenol stimulate adenylate cyclase in the rabbit cortical collecting tubule (10). Therefore, we examined the effects of these two hormones on HCO_3^- secretion. Fig. 3 shows the effects of 100 μU/ml bath ADH on HCO_3^- secretion. $J_{HCO_3^-}$ fell with ADH (paired Δ = +4.29±1.09 pmol/mm per min), a change that is not significantly different from that observed in time-control experiments (Fig. 1). ADH depolarized V_T from −24.8±6.7 to −16.8±3.2 mV (paired Δ = +8.0±6.4 mV, NS).

In contrast, as shown in Fig. 4 addition of isoproterenol (10^{-6} M) to the bath resulted in a stimulation of HCO_3^- secretion in eight of nine tubules (paired Δ = −1.89±0.89 pmol/mm per min, $P = 0.07$). When compared with time controls, the change in HCO_3^- secretion induced by isoproterenol was highly significant ($P < 0.005$). Perfusion rates in the two periods were not significantly different (paired Δ = 0.003±0.008 nl/mm length per min). Isoproterenol, like cAMP, depolarized V_T from −24.8±5.6 to −3.8±5.0 mV (paired Δ = +21.2±4.2 mV, $P < 0.001$).

Table II. Systemic Acid-Base Parameters

	Urine pH	Blood pH	Serum [HCO_3^-]
HCO_3^--loaded, fed (n)	8.22±0.04 (55)	7.56±0.01 (54)	24.7±0.6 (38)
NH_4Cl-loaded, starved (n)	5.16±0.45* (8)	7.35±0.04* (9)	12.6±2.2* (7)

* Significantly different from HCO_3^--loaded group at $P < 0.0001$.

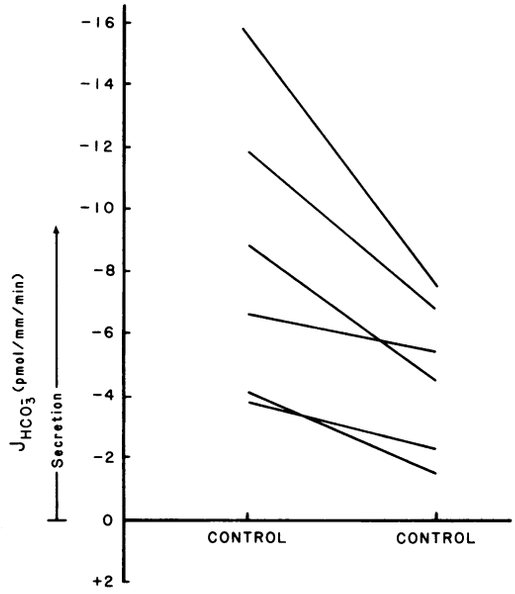

Figure 1. Rate of HCO_3^- secretion in cortical collecting tubules dissected from HCO_3^--loaded rabbits as a function of time perfused in vitro. The two periods were separated by 30 min. Perfusate and bath contained 25 mM HCO_3^-. Mean bicarbonate fluxes for the two periods are −8.48±1.92 and −4.65±0.98 pmol/mm per min.

Neither the change in V_T produced by cAMP nor that produced by isoproterenol showed a significant correlation with the baseline values of V_T (r = −0.69 and −0.50, respectively; NS). Likewise, the changes in HCO_3^- secretion produced by cAMP and isoproterenol did not correlate with the changes

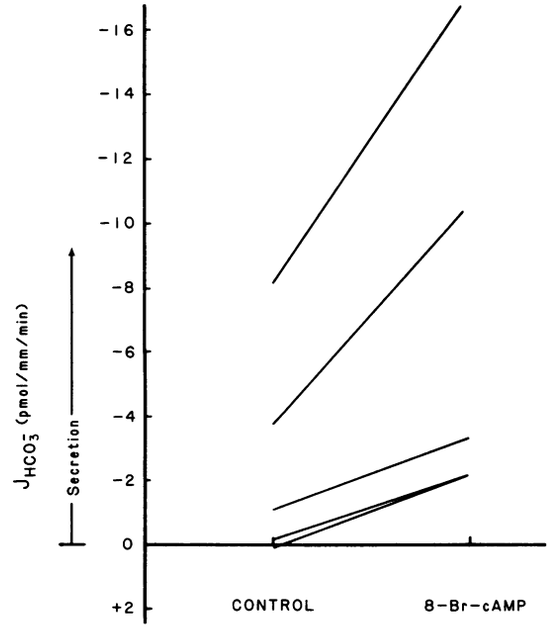

Figure 2. Effect of 8-bromo-cAMP (8-Br-cAMP) (0.1 mM) added to the bath on HCO_3^- secretion. Conditions are as in Fig. 1. Mean HCO_3^- secretory rates for the control and 8-Br-cAMP periods are −2.49±1.58 and −6.96±2.88 pmol/mm per min, respectively. The increase is significantly different from time controls in Fig. 1 ($P < 0.001$).

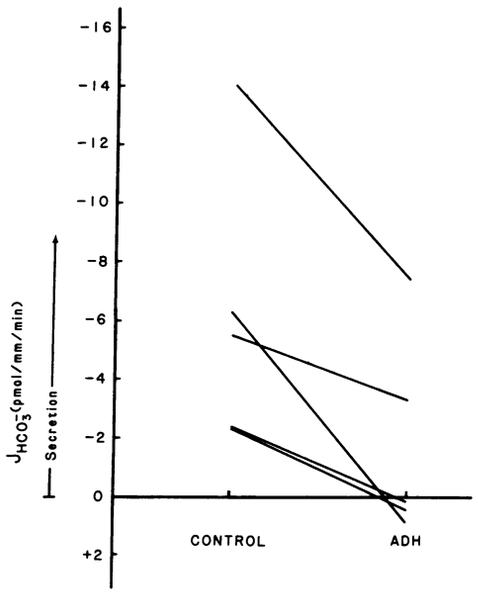

Figure 3. Effect of bath ADH (100 μU/ml) on HCO_3^- secretion. Conditions as per Fig. 1. Mean HCO_3^- secretory rates for the control and ADH periods are −6.10±2.13 and −1.81±1.57 pmol/mm per min, respectively. The change with ADH is not significantly different from the time controls of Fig. 1.

in V_T produced by these agents (r = −0.24 and 0.44, respectively; NS). However, both the increase in HCO_3^- secretion produced by cAMP and that produced by isoproterenol were significantly correlated with the respective baseline HCO_3^- secretory rates (r = 0.98, $P < 0.01$ for cAMP; r = 0.80, $P < 0.01$ for isoproterenol).

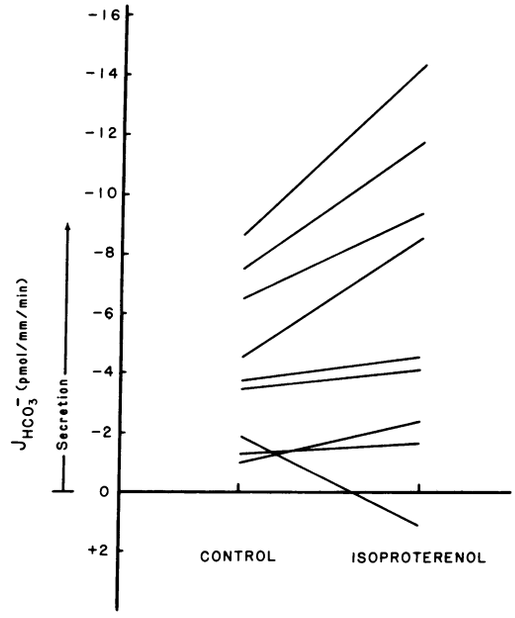

Figure 4. Effect of bath isoproterenol (10^{-6} M) on HCO_3^- secretion. Conditions as per Fig. 1. Mean HCO_3^- secretory rates for the control and isoproterenol periods are −4.28±0.91 and −6.18±1.71 pmol/mm per min, respectively. The increase in HCO_3^- secretion is significantly different from time controls in Fig. 1 ($P < 0.001$).

Stimulation of HCO_3^- secretion vs. inhibition of H^+ secretion. In HCO_3^--secreting tubules, it is possible that both HCO_3^- secretion and H^+ secretion may occur simultaneously, with flux rates for HCO_3^- secretion exceeding those of H^+ secretion (excluding differential leak components). If this were the case, cAMP could stimulate *net* HCO_3^- secretion by inhibiting H^+ secretion, by stimulating HCO_3^- secretion, or both. In the following experiments, we addressed these possibilities, using basolateral addition of the disulfonic stilbene DIDS. The perfusate and bath solutions were the same as in the previous studies.

The effect of basolateral DIDS on H^+ secretion was examined in tubules dissected from NH_4Cl-loaded, starved rabbits (Fig. 5). These tubules exhibited baseline net reabsorption of HCO_3^- that was reduced to zero after addition of bath DIDS; i.e., H^+ secretion was completely inhibited (paired Δ = −3.70±1.16 pmol/mm per min, $P < 0.05$; post-DIDS = −1.69±1.92 SD pmol/mm per min, NS from zero). V_T did not significantly change with DIDS (paired Δ = −1.0±1.9 mV, NS). These results are consistent with the known effects of basolateral disulfonic stilbenes on acidification in the turtle bladder (13), medullary collecting tubule (14), and cortical collecting tubule (15), and demonstrate that bath DIDS was a potent inhibitor of H^+ secretion in our system. In time controls from acid-loaded, starved rabbits (n = 4, not shown), the HCO_3 reabsorptive rate did not change (1.94±1.32 period 1, 1.45±1.63 period 2, Δ = NS). As shown in Fig. 5, cAMP addition to acidifying tubules produced a small fall in net HCO_3^- reabsorption (paired Δ = −1.24±0.42 pmol/mm per min, $P < 0.05$), but, unlike DIDS, left a sizeable rate of acidification intact (post-cAMP = 2.40±0.88 SD pmol/mm per min, $P < 0.01$ compared with zero).

If HCO_3^--secreting tubules from $NaHCO_3$-loaded rabbits have a significant degree of H^+ secretion occurring simultaneously with HCO_3^- secretion, complete inhibition of H^+ secretion by bath DIDS would be expected to increase the net

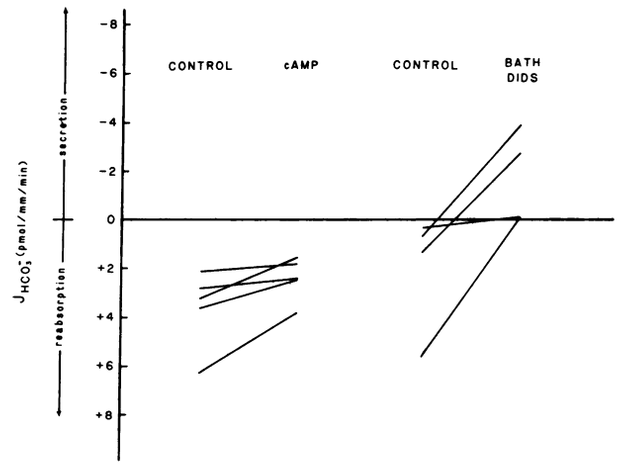

Figure 5. Effect of 8-bromo-cAMP (cAMP) (0.1 mM) (*left*) and bath DIDS (0.1 mM) (*right*) on HCO_3^- reabsorption in tubules from NH_4Cl-loaded, starved rabbits. Mean HCO_3^- fluxes for control and experimental periods are the following: (*left*) 3.63±0.72 and 2.40±0.39 pmol/mm per min, respectively, and (*right*) 2.02±1.25 and −1.69±0.96 pmol/mm per min, respectively. The post-DIDS values are not significantly different from zero; the post-cAMP values are significantly different from zero.

HCO_3^- secretory rate. As shown in Fig. 6, addition of bath DIDS to HCO_3^--secreting tubules had no significant effect on net HCO_3^- secretion (paired $\Delta = +1.62\pm0.71$ pmol/mm per min, NS to time controls), indicating minimal H^+ secretion in these tubules. Together, the results shown in Figs. 5 and 6 make it extremely unlikely that cAMP stimulates HCO_3^- secretion (Fig. 2) by inhibiting H^+ secretion.

Possible Cl^--HCO_3^- exchange. Because cAMP increased net HCO_3^- secretion and made the lumen voltage more positive, cAMP-induced electrogenic HCO_3^- secretion appeared unlikely. Therefore, we considered the possibility of an electroneutral process, i.e., Cl^--HCO_3^- exchange. Fig. 7 shows the effects of replacing luminal Cl with gluconate (solution E) on cAMP-stimulated HCO_3^- secretion. It is evident that Cl removal reversibly eliminated HCO_3^- secretion, consistent with Cl^--HCO_3^- exchange (paired $\Delta = +13.11\pm3.12$ pmol/mm per min, $P < 0.02$). Although luminal Cl^- removal tended to make V_T more lumen-negative, this change was not statistically significant (Cl present: -2.0 ± 11.0 mV; 0 Cl: -27.2 ± 12.2 mV; paired $\Delta = -25.2\pm11.3$ mV, NS).

Results using the Cl^--HCO_3^- exchange inhibitor DIDS (0.1 mM) in Cl-containing (solution A) perfusate are shown in Fig. 8. First, it should be noted that the control HCO_3^- secretory rate in the presence of luminal DIDS (-6.65 ± 2.22 pmol/mm per min) was not significantly different from control period values pooled from other groups. Therefore, luminal DIDS did not inhibit non-cAMP-stimulated HCO_3^- secretion. Moreover, cAMP still stimulated HCO_3^- secretion in the presence of luminal DIDS (paired $\Delta = -2.63\pm1.61$ pmol/mm per min, $P < 0.01$ to time controls, NS to cAMP group).

Role of basolateral Na^+-H^+ exchange in HCO_3^- secretion. Net cell-to-lumen HCO_3^- secretion must involve disposal of a H^+ across the basolateral membrane or movement of HCO_3^- into the cell across this membrane. Proton extrusion might result from primary active transport (H^+ ATPase) or secondary active transport, e.g., Na^+-H^+ exchange. The latter exchanger has been proposed to exist at the basolateral membrane of rabbit cortical collecting tubules (16). Therefore, we attempted to inhibit cAMP-stimulated HCO_3^- secretion by pharmacologic inhibition of the Na^+-H^+ exchanger. Tubules from HCO_3^--loaded rabbits were perfused with solution A containing 5×10^{-4} M amiloride, and bathed in either solution B (145 mM Na) or solution F (5 mM Na). In experiments with bath solution B (145 mM Na), 8-bromo-cAMP (0.1 mM) was added to the bath after V_T had stabilized. cAMP increased the lumen positivity in each tubule (pre-cAMP: $+15.8\pm7.5$ mV; post-cAMP: $+30.2\pm7.1$ mV, $\Delta = +14.4\pm2.0$, $P < 0.001$, $n = 5$). After V_T had again stabilized, control collections for $J_{HCO_3^-}$

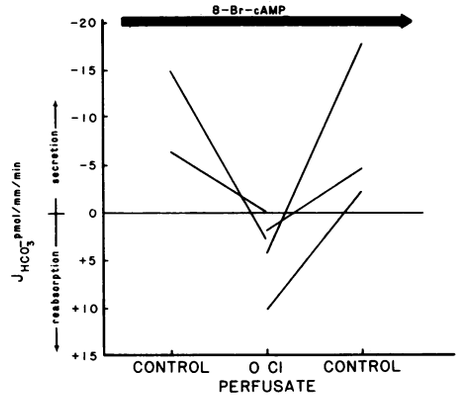

Figure 7. Effect of Cl removal (gluconate substitution) from the lumen on cAMP-stimulated HCO_3^- secretion. Mean control and 0 Cl HCO_3^- secretory rates are -9.19 ± 3.00 and $+3.88\pm1.72$ pmol/mm per min, respectively. The fall in HCO_3^- secretory rate is significant ($P < 0.02$).

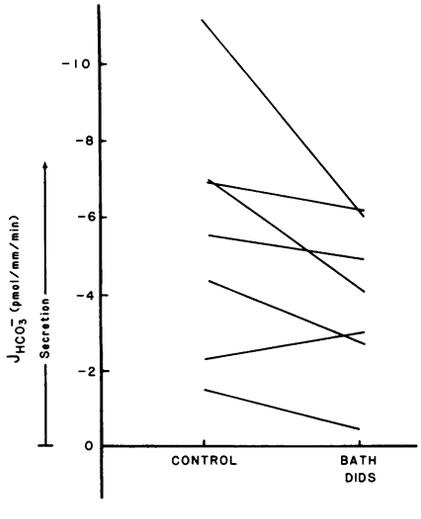

Figure 6. Effect of bath DIDS (0.1 mM) on HCO_3^- secretion in tubules dissected from HCO_3^--loaded rabbits. Mean HCO_3^- secretory rates for control and bath DIDS periods are -5.42 ± 1.21 and -3.80 ± 0.75 pmol/mm per min, respectively. The fall in HCO_3^- secretion is not significantly different from that of time controls shown in Fig. 1.

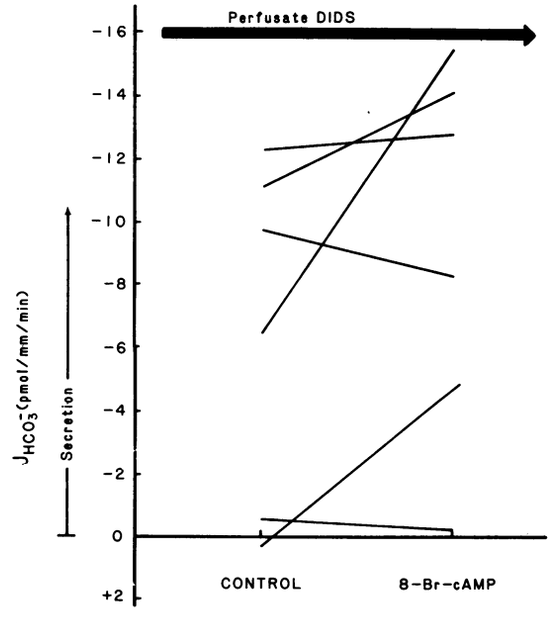

Figure 8. Control HCO_3^- secretion and response to 8-bromo-cAMP (0.1 mM) in tubules perfused with DIDS (0.1 mM) in the perfusate from the start of perfusion. Mean control and post-cAMP HCO_3^- secretion rates are -6.65 ± 2.22 and -9.29 ± 2.44 pmol/mm per min, respectively. The rise in HCO_3^- secretory rate is not significantly different from that seen with cAMP alone (Fig. 2).

were made. When bath Na = 5 mM (solution F), bath cAMP was present from the start of perfusion. After control collections were made, 1 mM amiloride was added to each bath, and HCO_3^- flux was remeasured. The combination of 5 mM Na and 1 mM amiloride should inhibit Na^+-H^+ exchange by ~90% (17). The results are shown in Fig. 9. Bath amiloride failed to alter $J_{HCO_3^-}$ in normal bath (paired $\Delta = +1.13 \pm 1.96$, $n = 5$, NS) or in bath with 5 mM Na (paired $\Delta = +0.36 \pm 1.76$, $n = 4$, NS).

Active vs. passive cAMP-stimulated HCO_3^- secretion. Because both isoproterenol and cAMP depolarized V_T to a more lumen-positive value, it is possible that the increase in collected fluid [HCO_3^-] in response to these agents resulted simply from passive diffusion of HCO_3^- down its electrical gradient. Indeed, as shown in Table III, comparing the collected fluid [HCO_3^-] after cAMP or isoproterenol with the V_T (measured at the perfusion end of the pipette), using the Nernst equation, shows that these HCO_3^- concentrations can be accounted for by the electrical driving forces. Although it is possible that slower perfusion rates would have allowed collected fluid [HCO_3^-] to rise to values higher than those dictated by passive distribution, such slow perfusion rates are technically difficult.

Instead, we addressed this issue by measuring what we have operationally defined as the passive HCO_3^- permeability. In these experiments, tubules were dissected from HCO_3^--loaded rabbits. The perfusate contained 0 Cl and 5 mM HCO_3^- (solution C), and the bath 0 Cl and 25 mM HCO_3 (solution D) plus 0.1 mM DIDS. This combination of solutions should inhibit active HCO_3^- reabsorption (DIDS) and Cl^--HCO_3^- exchange (0 Cl perfusate), while providing a chemical HCO_3^- gradient (20 mM) for passive diffusion from the bath into the lumen. By measuring the rise in collected fluid [HCO_3^-] over perfused values and knowing V_T, a permeability coefficient for HCO_3^- can be calculated.[2] Table IV shows the results of these experiments. The control period passive HCO_3^- permeability coefficient was $2.89 \pm 0.82 \times 10^{-6}$ cm/s. After 8-bromo-cAMP (0.1 mM) addition to the bath, $P_{HCO_3^-}$ was $1.88 \pm 1.11 \times 10^{-6}$ cm/s (paired $\Delta = -0.97 \pm 0.54 \times 10^{-6}$ cm/s, NS).

Discussion

These results show that cAMP and isoproterenol stimulate net HCO_3^- secretion in rabbit cortical collecting tubules. In contrast, HCO_3^- secretion falls spontaneously with time or with ADH addition. The reason(s) for the spontaneous decline with time are unclear at present. Two possibilities seem likely, however. First, the prevailing in vivo ionic milieu may somehow regulate HCO_3^- transport. If this is so, then the "signals" that regulated transport in vivo may have been lost in vitro when P_{CO_2}, [HCO_3], [Cl], and pH of the bathing solutions were "clamped" at "normal" values. Second, the fall with time may be owing

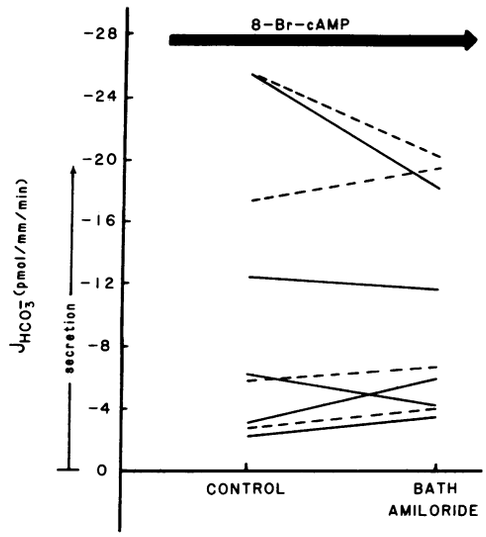

Figure 9. Effect of bath amiloride (10^{-3} M) on cAMP-stimulated HCO_3^- secretion in bath containing 145 mM Na (—) or 5 mM Na (- - -) (TMA substitution). Perfusate contained amiloride 5×10^{-4} M from the start of perfusion. Mean HCO_3^- secretory rates for the control and bath amiloride periods are the following: -9.89 ± 4.30 and -8.76 ± 2.63 pmol/mm per min, respectively, in the [Na] = 145 mM group; and -12.87 ± 5.26 and -12.51 ± 4.16 pmol/mm per min, respectively, in the [Na] = 5 mM group. None of the changes are significant.

to the absence in vitro of regulatory hormones that were present in vivo (i.e., catecholamines). In the same way that hydraulic conductivity of cortical collecting tubules falls in vitro as the effects of in vivo ADH reverse, so might an in vivo catecholamine effect on HCO_3^- transport decline in vitro. These two possibilities will require further study.

The cAMP stimulation of HCO_3^- secretion in tubules dissected from $NaHCO_3$-loaded rabbits appeared not to result from inhibition of H^+ secretion, as judged from experiments using basolateral DIDS as an inhibitor of H^+ secretion. We have assumed that, as in the turtle bladder (13), basolateral DIDS inhibits H^+ secretion but does not affect HCO_3^- secretion, and our own data suggest that this assumption holds true for the collecting tubule. In tubules exhibiting baseline net H^+ secretion (NH_4Cl-loaded rabbits), addition of basolateral DIDS completely abolished acidification, whereas cAMP left intact a sizeable rate of acidification. Moreover, when basolateral DIDS was added to HCO_3^--secreting tubules from $NaHCO_3$-loaded rabbits, it failed to reverse the time-dependent fall in HCO_3^- secretion, indicating that there was a minimal H^+ secretory flux in HCO_3^--secreting tubules. If complete inhibition of acidification by DIDS failed to stimulate net HCO_3^- secretion in tubules from $NaHCO_3$-loaded rabbits (Fig. 6), then cAMP could not have stimulated net HCO_3^- secretion (Fig. 2) solely by inhibiting H^+ secretion in these tubules. Therefore, cAMP must have stimulated HCO_3^- secretion directly. This conclusion is also supported by the significant correlation between baseline HCO_3^- secretory rates and the increments in HCO_3^- secretion induced by cAMP or isoproterenol. That is, the more an individual tubule was poised toward HCO_3^- secretion and away from H^+ secretion, the more responsive it was to cAMP.

Further experiments have addressed possible cellular mechanisms of cAMP-stimulated HCO_3^- secretion, i.e., HCO_3^- movement across the apical membrane and H^+ extrusion

2. There are several possible mechanisms for HCO_3^- appearance in the lumen other than passive diffusion: (*a*) CO_2 could diffuse into the lumen and become hydrated to carbonic acid, which could dissociate to H^+ and HCO_3^-; (*b*) HCO_3^- reaching the lumen by diffusion could be titrated by H^+ from luminal buffers; and (*c*) carbonic acid could diffuse into the lumen and dissociate into H^+ and HCO_3^-. As discussed by Alpern et al. (18), in the absence of luminal carbonic anhydrase and non-HCO_3^- buffers, conditions that pertain to the present experiments, the contribution of these mechanisms to luminal HCO_3^- appearance is neglibible.

Table III. Equilibrium Values of HCO_3 Versus V_T

	Control			Experimental		
	V_T	E_{HCO_3}	$V_T - E_{HCO_3}$	V_T	E_{HCO_3}	$V_T - E_{HCO_3}$
cAMP (n = 5)	−21.8±4.8	+1.9±0.8	−19.8±5.2*	+12.8±5.7	+6.3±3.2	+6.2±4.4
Isoproterenol (n = 9)	−24.8±5.6	+1.7±0.9	−26.5±5.3*	−3.8±5.0	+3.5±1.6	−7.3±5.3

All values in millivolts. * $V_T - E_{HCO_3}$ value significantly different from zero.

across the basolateral membrane. Because the cAMP effect is associated with a change in the V_T toward a more lumen-positive value, cAMP-stimulated electrogenic HCO_3^- secretion appears unlikely. Therefore, it is possible that HCO_3^- secretion due to cAMP occurs via an electroneutral process, i.e., Cl^--HCO_3^- exchange. This possibility is supported, though certainly not proved, by our experiments in which cAMP-stimulated HCO_3^- secretion was completely inhibited by luminal Cl^- removal. Seemingly against this mechanism are the negative results with luminal DIDS. It should be recalled, however, that Husted et al. (13) found no effect of apical disulfonic stilbenes on apparent Cl^--HCO_3^- exchange in the turtle bladder, as opposed to unequivocal effects of stilbenes on basolateral Cl^--HCO_3^- exchange (19). It is not clear if our negative results indicate inaccessibility of DIDS to a DIDS-sensitive site, Cl^--HCO_3^- exchange mediated by a DIDS-insensitive protein, or absence of Cl^--HCO_3^- exchange at the apical membrane. Further experiments will be required to prove that cAMP-stimulated HCO_3^- secretion occurs via apical Cl^--HCO_3^- exchange. At the basolateral membrane, H^+ extrusion (or HCO_3^- uptake) must occur if cell-to-lumen HCO_3^- movement is to result in net HCO_3^- secretion. Proton extrusion across this membrane could conceivably occur via a H^+-ATPase or via Na^+-H^+ exchange. Because Na^+-H^+ exchange has been proposed to exist in the cortical collecting tubule (16), we tested for such a mechanism using pharmacologic inhibition of Na^+-H^+ by bath amiloride. Regardless of whether bath [Na^+] was 145 mM or 5 mM, 1 mM amiloride had no effect on cAMP-stimulated HCO_3^- secretion. Because the combination of 5 mM Na^+ and 1 mM amiloride inhibits Na^+-H^+ exchange in proximal tubule brush-border membrane vesicles by >90% (17), the present negative results suggest that basolateral Na^+-H^+ exchange did not play a role in H^+ disposal behind cAMP-stimulated HCO_3^- secretion.

cAMP and isoproterenol both depolarized the V_T toward a more lumen-positive value, a change favoring passive HCO_3^- accumulation in the lumen down electrical gradients. We cannot be certain from the present data whether cAMP-stimulated HCO_3^- secretion represented active transport, passive transport, or some combination of the two. When cAMP- or isoproterenol-treated tubules are considered as a group (Table III), the collected fluid HCO_3^- concentration can be entirely accounted for by the electrochemical driving forces; i.e., HCO_3^- secretion could be passive. On the other hand, several lines of evidence argue against purely passive HCO_3^- transport. First, ADH, like cAMP, tended to depolarize V_T, but ADH was associated with a fall in HCO_3^- secretion. Second, there was no correlation between the magnitude of the change in V_T and that of HCO_3^- secretion produced by cAMP or isoproterenol. Thus, changes in V_T are not necessarily accompanied by changes in HCO_3^- flux, which would be expected for passive diffusion. Others have also found V_T and HCO_3^- flux to be poorly correlated (1). Third, under conditions that we would expect to inhibit transcellular HCO_3^- transport (0 Cl^- solutions plus bath DIDS), cAMP produced no significant change in the HCO_3^- permeability coefficient (Table IV). If we assume that cAMP effects on paracellular HCO_3^- permeability are the same in the presence and absence of Cl^- and bath DIDS, then the HCO_3^- permeability measured in 0 Cl^- solutions and with bath DIDS can be compared with that permeability that would be theoretically required to yield, via passive diffusion, the cAMP- and isoproterenol-stimulated HCO_3^- fluxes measured in the presence of Cl^- and without bath DIDS. Such calculations (using the Goldman-Hodgkin-Katz equation) show that a HCO_3^- permeability of 19.1 × 10^{-6} cm/s would be required to account, by diffusion, for the observed HCO_3^- secretory flux in Cl^--bathed cAMP-treated tubules, and of 37.7 × 10^{-6} cm/s in the isoproterenol-treated tubules. Both of these values are significantly different from the measured post-cAMP HCO_3^- permeability of 1.88±2.48 (SD) × 10^{-6} cm/s. The major drawback of this approach is that we do not know whether possible cAMP effects on paracellular HCO_3^- permeability would or would not be expressed in 0 Cl^- solutions and in the presence of bath DIDS. Further experiments involving measurements of cellular and paracellular HCO_3^- conductance in the presence of cAMP will be required to answer this question. Even if

Table IV. Passive HCO_3^- Permeability Coefficients

	Perfused [HCO_3^-]	Collected [HCO_3^-]	J_{HCO_3}	V_T	P_{HCO_3}
	mM	*mM*	*pmol/mm per min*	*mV*	*10^{-6} cm/s*
Control (n = 5)	5.1±0.3	7.8±0.5	−2.40±0.69	+6.2±0.4	2.86±0.82
8-bromo-cAMP (0.1 mM) (n = 5)	5.1±0.3	6.9±0.7	−1.67±1.00	+9.6±1.0	1.88±1.11
Mean paired change, cAMP vs. control	0.0±0	−0.9±0.4	+0.73±0.48	+3.4±1.1	−0.97±0.54
P	NS	NS	NS	<0.05	NS

cAMP stimulates HCO_3^- secretion via only passive mechanisms, the present experiments, performed under Na-transporting conditions, suggest that this effect is large and potentially physiologically important.

We can only speculate on the mechanism of the depolarization of V_T produced by cAMP. At the apical membrane, cAMP could decrease Na^+ conductance or increase K^+ conductance. Because cAMP appears to have no effect on Na^+ transport (20, 21) and has been reported to inhibit K^+ secretion (21), these two mechanisms appear unlikely. cAMP could increase electrogenic H^+ secretion. In the absence of a change in transepithelial H^+ conductance, depolarization of V_T due to a H^+ current would dictate an increase in H^+ secretory flux. If this mechanism pertained, one might have expected to see an increase in HCO_3^- reabsorption upon addition of cAMP to acidifying tubules. This was not observed (Fig. 5), though admittedly an accompanying cAMP-stimulated electroneutral HCO_3^- secretion could have obliterated the signal. Measurements of equivalent reverse short circuit current before and after cAMP should help address this possibility. At the basolateral membrane, large conductances for K^+ and Cl^- have been described (22, 23). Either a decrease in K^+ conductance or an increase in Cl^- conductance at this membrane would move the basolateral membrane voltage away from E_K and toward E_{Cl} (i.e., less negative). In the absence of changes in apical membrane voltage, such a change would depolarize V_T. Because the basolateral Cl conductance appears to be highly variable (23), and because cAMP increases Cl conductances in a number of epithelia (24–27), it is possible that cAMP increases basolateral Cl conductance in this epithelium. Such an effect might enhance apical Cl^--HCO_3^- exchange by favoring conductive cell Cl exit at the basolateral membrane. This possibility must also be directly examined. Such a scheme does not exclude direct cAMP effects on a Cl^--HCO_3^- exchanger.

The present results can be compared with those in the turtle bladder, in which cAMP has been reported to inhibit H^+ secretion (28), stimulate H^+ secretion (29), increase passive HCO_3^- permeability (30), and stimulate electrogenic HCO_3^- secretion (31). Although we cannot rule out a small inhibition of H^+ secretion in the cortical collecting tubule by cAMP, such an effect by itself cannot account for the changes in HCO_3^- secretion. cAMP certainly does not stimulate H^+ secretion alone, nor does it appear to stimulate electrogenic HCO_3^- secretion. As previously discussed, we cannot rule out an effect of cAMP on passive HCO_3^- permeability in Cl^--containing solutions.

A very important result of our studies is that isoproterenol, but not ADH, mimicked the cAMP effect on HCO_3^- secretion. Both isoproterenol and ADH stimulate adenylate cyclase in this epithelium (10). However, whereas ADH stimulates osmotic water flow (32), isoproterenol does not (33); and whereas isoproterenol inhibits K^+ secretion, ADH does not (21). In addition, Tago et al. have recently reported that isoproterenol and cAMP stimulate lumen-to-bath ^{36}Cl movement, whereas ADH does not (34). All of these studies strongly suggest the existence of either separate pools of hormone-sensitive adenylate cyclase/cAMP in the same collecting tubule cell and/or cellular heterogeneity in the distribution of hormone-linked adenylate cyclase. The latter possibility seems particularly likely, given the existence of at least two cell types (principal and intercalated) in this nephron segment (35). Because intercalated cells contain abundant carbonic anhydrase (36), it seems likely that isoproterenol-stimulated HCO_3^- secretion, and perhaps Cl reabsorption by Cl^--HCO_3^- exchange (33, 34), are accomplished by intercalated cells. Proof of this hypothesis will require combined morphological and functional studies.

Finally, the present results are relevant to the pathophysiology of volume-contraction, Cl-depleted metabolic alkalosis. It has been known for some time that Cl^- is critical to the correction of volume-contraction metabolic alkalosis (37). Volume contraction clearly stimulates renal efferent nerve activity (38), and cortical collecting tubules appear to receive innervation and to possess β-adrenergic receptors (39, 40). Therefore, cortical collecting tubules under volume-contracted conditions in vivo might be expected to correspond to our in vitro tubules treated with isoproterenol. In vivo studies have shown that, under volume-contracted conditions, provision of Cl alone without concomitant volume expansion increases urinary Cl concentration and allows correction of metabolic alkalosis (41). Although this phenomenon has previously been attributed to alterations of plasma renin and aldosterone levels (41), the present studies (Fig. 7) suggest that a rise above very low levels in luminal $[Cl^-]$ could allow the β-adrenergic–stimulated cortical collecting tubule to secrete HCO_3^-, and thus contribute to correction of metabolic alkalosis. That renal nerves may play a role in this process is further suggested by the rapidity with which Cl^- administration (and volume expansion) can repair metabolic alkalosis despite glomerular filtration rate being held constant (42). A reduction in adrenergic-stimulated collecting tubule HCO_3^- secretion may also conceivably play a role in the lower urine P_{CO_2} concentrations observed in acutely volume-expanded animals (43).

Acknowledgments

I thank Drs. John Stokes, Michael Welsh, and Russell Husted for numerous fruitful discussions and for careful critique of the manuscript, and Ms. Syd Harned for expert secretarial assistance.

This work was supported, in part, by grants from the American Heart Association (Iowa Affiliate), the Veterans Administration, National Institutes of Health grants 1-K08-AM01343-01 and AM 25231, and Program Project grant HL 14388.

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