Inhibition of Cortical Collecting Tubule Chloride Transport by Organic Acids

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Abstract

Cl self-exchange by the rabbit cortical collecting tubule (CCT) occurs via an apical anion exchanger in series with a basolateral Cl conductance. We studied the effects of organic acids on CCT Cl self-exchange. We found no evidence for transport of acid anions by the self-exchange system. Rather, Cl self-exchange was inhibited by a variety of organic acids. The degree of inhibition correlated with the chloroform/water partition coefficient and was enhanced by lowering pH, indicating inhibition by the lipid-soluble, protonated species. Inhibition by the representative acid iso-butyrate was dose-dependent and showed sidedness (basolateral > apical). Iso-butyrate also reversibly reduced transepithelial conductance without altering K permeability, suggesting inhibition of the principal cell basolateral Cl conductance. Because small organic compounds with similar lipid solubilities but no carboxyl group had no effect, both the carboxyl group and the lipid-solubility of organic acids appear to be important. The results are consistent with blockade of chloride channels by organic acids.

Introduction

The transcellular transfer of Cl across the cortical collecting tubule (CCT)¹ occurs through a population of intercalated cells specialized for HCO₃ secretion. These cells, called β intercalated cells (1), have an anion exchanger on their apical (luminal) membrane and a Cl conductive pathway (channel) on the basolateral membrane. Although this transepithelial anion exchange process secretes HCO₃, the system operates more often in a Cl self exchange mode. In the absence of exogenous CO₂ and HCO₃, transcellular Cl transport occurs exclusively by self-exchange (2, 3).

In order to understand this transepithelial anion exchange system more completely, we investigated the effects of organic anions on Cl transport. We anticipated two general outcomes. First, organic anions could have been transported as substrates as is the case for some organic anions transported on the red cell band 3 exchanger (4). Precedence for transepithelial organic anion transport can be found in the proximal renal tu-

bule, in which organic acids absorbed via a luminal membrane Na (lactate) cotransporter (5) exit the basolateral membrane via a conductive pathway (6). Additional examples of epithelial organic acid transport can be found in the gallbladder (7) and small intestine (8). Because these transport systems involve anion exchangers, we reasoned that there may be similarities with the CCT anion transport system.

Second, we recognized that organic acids might inhibit transepithelial anion exchange. Organic acids have been shown to inhibit membrane conductive pathways (9–12) and might not substitute on all anion exchangers. We had a special a priori interest in the effect of two organic anions on CCT Cl transport: lactate, by virtue of its being perhaps the most abundant organic anion, and formate, an anion which stimulates Cl transport in the proximal tubule (13) and which may have a major role in volume absorption (14, 15).

The results of our experiments provide no evidence for significant transport of organic acids; rather they demonstrate one mechanism whereby organic acids can inhibit Cl transport systems.

Methods

General microperfusion methods. Segments of rabbit cortical collecting tubule were perfused in vitro by techniques previously described (9, 16). Briefly, New Zealand white rabbits weighing 1.5–2.5 kg were given free access to water and standard rabbit chow. After decapitation, the left kidney was quickly removed and cut into 1–2-mm thick coronal slices. Cortical collecting tubules were dissected at 0–4°C, transferred to a Lucite chamber thermally regulated at 37±0.5°C, and mounted in glass pipettes at both ends. The perfusion pipette served as a voltage-sensing electrode for transepithelial voltage (V_T) measurements and also as a pathway for injected current for transepithelial conductance (G_T) measurements (9). Perfusate and control bath solutions contained: 105 mM NaCl, 25 mM Na-gluconate, 5 mM Na-Hepes, 5 mM H-Hepes, 5 mM KCl, 2.3 mM Na₂HPO₄, 1.0 mM MgCl₂, 3.3 mM CaCl₂, and 8.3 mM dextrose. In experiments in which the effects of various organic acids on lumen-to-bath tracer fluxes were examined, the concentration of Na-gluconate was reduced by the concentration of these organic acids used. When Cl was removed from the bath solution, gluconate was substituted. The concentration of calcium salts was appropriately adjusted to keep the ionized Ca concentration at 1.0 mM in every solution according to the gluconate concentration used (17).

The pH of perfusate and bath solutions was 7.37–7.42 after gassing with 100% O₂. For bath solutions of pH 7.0, the ratio of Na-Hepes and H-Hepes was adjusted appropriately. All perfusates contained 50 μ M amiloride in order to eliminate active Na and K transport and minimize the electrical driving force for Cl diffusion (2). All bath solutions (except in experiments involving ⁸⁶Rb flux measurements) contained 0.1 mM 8-bromo-cAMP to avoid the time-dependent decrement of Cl transport (2).

Tracer flux measurements. Three kinds of tracer flux measurements were performed using ³⁶Cl, ⁸⁶Rb, and [¹⁴C]iso-butyrate. All perfusates contained exhaustively dialyzed methoxy-[³H]inulin as a volume marker. Net volume reabsorption (J_v , nl mm⁻¹ min⁻¹) was calculated as previously described (18). Experiments in which J_v were more than ±0.1 nl mm⁻¹ min⁻¹ were discarded.

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1. *Abbreviations used in this paper:* CCT, cortical collecting tubule; CVT, calibrated volume pipette; GABA, γ -amino butyric acid; G_T , transepithelial conductance; J_v , net volume reabsorption; MAPTAM, 1,2-bis-5-methyl-amino-phenoxyethane-*N,N,N'*-tetraacetoxymethyl acetate.

^{86}Rb flux measurements were performed and expressed as the rate coefficient (K_{Rb} , nm s^{-1}) as previously described (19). In these experiments tubules were perfused at slow rates ($3\text{--}7\text{ nl min}^{-1}$). Lumen-to-bath ^{86}Rb fluxes were determined from the difference in ^{86}Rb counts between the perfusate and the fluids collected in a calibrated volume pipette (CVP).

^{36}Cl flux measurements were performed by both CVP and bath collection methods. In the case of bath collection, tubules were perfused at rapid rates ($> 20\text{ nl min}^{-1}$). Lumen-to-bath ^{36}Cl fluxes were determined from ^{36}Cl counts in timed collections (5 min) of bath fluid and the Cl rate coefficient (K_{Cl} , nm s^{-1}) was calculated using the following expression: $K_{\text{Cl}} = C_b/C_o^*At$ where C_b is the counts appearing in the bath over time t , C_o^* is the concentration of tracer in the perfused fluid, and A the area of the tubule calculated from the inner diameter. Both ends of the tubule were tightly sealed with unpolymerized Sylgard (Dow Corning Corp., Midland, MI) to avoid leakage of the perfusate into the bath. Experiments in which $> 1\%$ of perfused $[^3\text{H}]\text{inulin}$ counts appeared in the collected bath fluid were discarded. As a further check on the accuracy of the bath vs. the CVP collection methods, we compared the K_{Cl} values in tubules where Cl-Cl and Cl- HCO_3 exchange had been eliminated by removing Cl and HCO_3 from the bath (2, 3). The low value of K_{Cl} obtained under these conditions increases the probability of uncovering a technical artifact. K_{Cl} values were $26.4 \pm 2.3\text{ nm s}^{-1}$ ($n = 11$) by the CVP collection method and $30.7 \pm 2.6\text{ nm s}^{-1}$ ($n = 19$) by bath collection method (NS, unpaired t test). We conclude that either method will provide an accurate estimate of the lumen-to-bath tracer flux.

In many experiments the data are expressed in terms of Cl self-exchange. We have previously shown (2) that, under the present experimental conditions, the Cl self-exchange rate is the difference between K_{Cl} in the presence and absence of bath Cl. This method was used in the present protocols: the last experimental period was one in which bath Cl was removed (gluconate replacement) and K_{Cl} measured. This K_{Cl} value was then subtracted from other K_{Cl} values in the same experiment to obtain Cl self-exchange rates.

For $[^{14}\text{C}]\text{iso-butyrate}$ flux measurements, tubules were perfused at rapid rates ($> 20\text{ nl min}^{-1}$) and both CVP and bath collections were made. CVP collection was used to compare the counts of $[^{14}\text{C}]\text{iso-butyrate}$ between the perfusate and the collected fluid. The rate coefficient for $[^{14}\text{C}]\text{iso-butyrate}$ was obtained by the bath collection method.

Transepithelial conductance measurements. The transepithelial conductance (G_T) was measured by techniques previously described (9). Constant-current square-wave pulses ($40\text{--}75\text{ nA}$, 100 ms duration) were injected into the tubule lumen via the perfusion pipette. An electrical bridge was balanced for the particular perfusate used, thus allowing voltage deflections at the perfusion pipette to be nulled in the absence of a tubule. Voltage deflections at the distal end of the tubule were measured by a Ag-AgCl electrode placed in a microelectrode filled with 3 M KCl in agar. The G_T (mS cm^{-2}) was calculated using cable analysis as previously described from our laboratory (9).

Partition coefficients. Partition coefficients in chloroform/aqueous phase systems (K_p^{chloro}) were determined for nonionic forms of several organic acids, and in olive oil/aqueous and ether/aqueous for iso-butyrate using a modified radiotracer method previously described (20). In general, 1 ml of aqueous solution, pH 1.5 containing $0.03\text{--}0.07\text{ }\mu\text{Ci}$ of ^{14}C -labeled organic acids was mechanically shaken with 3 ml of non-aqueous phase solution for 1 h at room temperature. After centrifugation for 15 min at $1,500\text{ g}$, samples were carefully taken from both phases and counted in 3a70 cocktail (Research Products Corp., Mt. Prospect, IL). ^{14}C counts per minute were converted to disintegrations per minute using measured counting efficiencies in water and the nonaqueous phase liquid. Partition coefficients (K_p) for undissociated forms of organic acids were calculated according to formulae previously described (20); the calculations shown below use the example of the chloroform/aqueous partition coefficient: $K_p^{\text{chloro}} = r(1 + 10^\alpha)$, where $r = ^{14}\text{C}(\text{dpm})/\text{ml}$ in chloroform phase/ $^{14}\text{C}(\text{dpm})/\text{ml}$ in aqueous phase, and $\alpha = \text{pH}(\text{aqueous phase}) - \text{pKa}$. pKa is the dissociation constant for the organic acid being examined.

Reagents and isotopes. 8-Bromo-cyclic adenosine 3'5'-monophosphate (8-Br-cAMP), formate, L(+)-lactate, *n*-valerate, *n*-butyrate, propionate, caprylate, benzoate, γ -amino butyric acid (GABA), and naloxone were purchased from Sigma Chemical Co. (St. Louis, MO). Acetate and iso-butyrate were purchased from Fisher Scientific Co. (Fair Lawn, NJ). 1,2-bis-5-methyl-aminophenoxyethane-*N,N,N'*-tetraacetoxymethyl acetate (MAPTAM) was purchased from Calbiochem-Behring (La Jolla, CA). Amiloride was a gift of the Merck, Sharp and Dohme Division (West Point, PA). Methoxy- $[^3\text{H}]\text{inulin}$, ^{36}Cl , ^{86}Rb , $[^{14}\text{C}]\text{iso-butyrate}$, $[^{14}\text{C}]\text{acetate}$, $[^{14}\text{C}]\text{formate}$, and $[^{14}\text{C}]\text{lactate}$ were purchased from New England Nuclear (Boston, MA).

Statistics. Statistical analyses were performed by paired and unpaired t test or ANOVA as appropriate. If ANOVA showed a significant difference between groups, the studentized range test was used to examine for differences between individual groups. Results are expressed as means $\pm$ SE.

Results

Effect of bath iso-butyrate on K_{Cl} . We first examined the effect of bath iso-butyrate on the ^{36}Cl lumen-to-bath tracer rate coefficient (K_{Cl}). As shown in Fig. 1, 25 mM bath iso-butyrate reversibly reduced K_{Cl} from 141.0 ± 17.0 to $33.4 \pm 7.3\text{ nm s}^{-1}$ ($\Delta K_{\text{Cl}} = -107.6 \pm 14.7\text{ nm s}^{-1}$, $P < 0.001$). Given the limited temporal resolution of flux studies, the onset of action appeared to occur in less than 15 min and recovery occurred within 30 min . Table I shows the dose-response of the bath iso-butyrate effect on K_{Cl} . Four concentrations of iso-butyrate ($1, 2.5, 10,$ and 25 mM) were sequentially examined in this order in each experiment. At a concentration of 25 mM , iso-butyrate produced a reduction of K_{Cl} not different from the value obtained with a Cl-free bath solution. Because K_{Cl} values in the Cl-free period most likely reflect paracellular Cl diffusion (2, 9), the differences between K_{Cl} values in each period and that of the Cl-free period are reasonable estimates of Cl self-exchange. The fractional inhibition of Cl self exchange by each dose of iso-butyrate was calculated in this fashion and the results plotted in Fig. 2 A. Fig. 2 B demonstrates a double reciprocal plot of the data in Fig. 2 A. The IC_{50} from this double reciprocal plot was 2.3 mM . From this value the dose-response curve in Fig. 2 A was drawn.

Rank order inhibitory potency of organic acids on Cl self-exchange. We compared the effect of iso-butyrate on Cl self-exchange with the effects of eight other organic acids: formate, acetate, L(+)-lactate, *n*-butyrate, propionate, *n*-valerate, caprylate, and benzoate. These organic acids were examined sequentially and in random order in two separate groups of

Figure 1. Effect of bath iso-butyrate on lumen-to-bath ^{36}Cl tracer rate coefficient. Mean decrement was $107.6 \pm 14.7\text{ nm s}^{-1}$.

Table I. Dose Response of Bath Iso-Butyrate Effect on the Cl Efflux Rate Coefficient (K_{Cl})

	Control*	Iso-butyrate concentration				0 Cl*
		1 mM	2.5 mM	10 mM	25 mM	
K_{Cl} , nm s ⁻¹ (n = 5)	132.8 ±8.5	100.7 ±8.8	84.2 ±7.5	44.9 ±3.9	31.3 ±3.5	29.4 ±4.7

* Iso-butyrate concentration = 0 mM.

experiments; their concentrations were all 10 mM (bath side). All experiments included a control period as well as iso-butyrate and Cl-free periods. Fractional inhibition of Cl self-exchange for each organic acid was calculated by subtracting the K_{Cl} in the 0 Cl period from the values obtained in the previous periods. As shown in Table II, all but formate showed significant inhibitory effects on Cl self-exchange. Caprylate was the most potent of the organic acids examined. Among the other aliphatic compounds, iso-butyrate, propionate, *n*-butyrate, and *n*-valerate demonstrated similar potencies.

pH influence on iso-butyrate inhibition of Cl self-exchange. To determine whether the pH of iso-butyrate-containing bath solutions might influence the ability of iso-butyrate to inhibit Cl self-exchange, we compared the inhibitory effect of 3 mM iso-butyrate bath solutions at pH 7.0 and at pH 7.4. Based on a pKa of 4.85 (21), the calculated concentrations of (undissociated) iso-butyric acid in these two solutions were 22.1 μ M at pH 7.0 and 9.0 μ M at pH 7.4. We previously showed (3) that varying the pH of HEPES-buffered bath solutions over the range of 7.0 to 7.4 itself had no effect on K_{Cl} . Therefore, any difference in the inhibitory effects of 3 mM iso-butyrate on Cl self-exchange as a function of bath pH could be reasonably attributed to a difference in the concentration of (undissociated) iso-butyric acid. The experiments were conducted in four periods, the first and last being the control and the 0 Cl bath periods, respectively. The sequence of iso-butyrate expo-

Figure 2. (A) Fractional inhibition of Cl self-exchange by iso-butyrate. Cl self-exchange values were calculated by subtracting the value of K_{Cl} in a Cl-free bath from those obtained at each iso-butyrate concentration. (B) Double reciprocal plot of the data in A. IC_{50} of 2.3 mM was used to generate the curve in A. $n = 5$.

Table II. Chloroform/Water Partition Coefficients (K_p^{chloro}) and Fractional Inhibition of Cl Self-exchange by Organic Acids

Organic acid	Fractional inhibition of Cl self-exchange %	n	K_p^{chloro}	pKa
Formate	6.3±3.0	5	7.9×10^{-3} *	3.75
Acetate	30.0±4.9	5	2.5×10^{-2} 3.2×10^{-2} *	4.76
L(+)-Lactate	38.1±7.5	5	4.0×10^{-3} *	3.86
<i>n</i> -Valerate	51.6±5.9	5	7.9×10^{-1}	4.84
<i>n</i> -Butyrate	64.6±6.5	5	5.0×10^{-1}	4.83
Propionate	68.7±4.5	5	1.6×10^{-1}	4.87
Iso-butyrate	72.7±4.0	10	6.3×10^{-1} 6.3×10^{-1} *	4.85
Benzoate	89.5±1.4	5	3.2	4.21
Caprylate	93.8±2.7	5	25.1	4.90

* Measured values. Other K_p^{chloro} values taken from ref. 20.

n = number of tubules. pKa values from reference 5. All concentrations = 10 mM, pH 7.4.

sure at the different pH values was randomized. As shown in Table III, iso-butyrate at a pH of 6.98 was more effective at inhibiting K_{Cl} than at 7.37. The mean paired difference in the K_{Cl} values for these solutions was 27.0 ± 6.6 nm s⁻¹ ($P < 0.02$). Fig. 3 shows the fractional inhibition of Cl self-exchange as a function of the calculated (undissociated) iso-butyric acid concentration (μ M) in these experiments. The solid line depicts the dose-response relationship predicted from the data of Table I and Fig. 2 A, expressed in terms of (undissociated) iso-butyric acid concentration rather than butyrate. The fractional inhibition of Cl self-exchange in the pH-shift experiments was not significantly different from the predicted values. These results indicate that iso-butyrate's ability to inhibit Cl self-exchange is influenced by the pH in a manner consistent with inhibition by the undissociated form.

Partition coefficients, pKa, and fractional inhibition of Cl self-exchange. Having demonstrated that the concentration of (undissociated) iso-butyric acid is important for inhibition of Cl self-exchange, we next examined the relationship between the partition coefficients of undissociated forms of organic acids and their abilities to inhibit Cl self-exchange.

Chloroform/water partition coefficients for many undisso-

Table III. Bath Iso-Butyrate Effect on Cl Self-exchange as a Function of pH

	Control	IB 3 mM	IB 3 mM	0 Cl
	pH 7.36 ±0.08	pH 7.37 ±0.14	pH 6.98 ±0.16	pH 7.42 ±0.15
K_{Cl} , nm s ⁻¹ (n = 5)	183.4 ±13.6	111.2 ±17.3	84.2 ±14.7	28.4 ±4.9
Fractional inhibition of Cl self-exchange (%)		48.3 ±8.3	65.7 ±6.9	
P		<0.02		

K_{Cl} , lumen-to-bath Cl rate coefficient; IB, iso-butyrate. P value compares the IB periods.

Figure 3. Influence of pH on iso-butyrate inhibition of Cl self-exchange. The curve is drawn from the data presented in Fig. 2 but displayed as a function of (undissociated) iso-butyric acid rather than the ionized form. The two points are from the data in Table III and are not different from the values predicted from the curve.

ciated organic acids and bases (K_p^{chloro}) have been previously determined by Jackson et al. (20). Using the methods they described, we determined partition coefficient values for several organic acids whose values were not available (Table II). Fig. 4 shows that there is a good correlation between fractional inhibition of Cl self-exchange by organic acids and their log K_p^{chloro} ($r = 0.90$, $P < 0.002$). These results suggest that the lipid solubility of organic acids determines their potency in inhibiting Cl self-exchange.

Table II also shows the pKa of the organic acids examined. Unlike the situation with chloroform-water partition coefficients, the correlation between the pKa and the fractional inhibition of Cl self-exchange is poor ($r = 0.549$, NS).

Effect of naloxone, *n*-butanol, and GABA on K_{Cl} . It is conceivable that the undissociated forms of organic acids inhibit K_{Cl} by interacting with the cell membrane as uncharged molecules by creating a more disordered lipid domain. To determine whether the inhibitory effect of these compounds on Cl self-exchange is specific for organic acids, we first examined the effect of naloxone on K_{Cl} . Naloxone is an organic base, but its K_p^{chloro} is reasonably close to that of iso-butyrate (about 2.0×10^{-1}) (20). As shown in Table IV, 1 mM naloxone had no effect on K_{Cl} . It should be noted that, based on the naloxone pKa of 6.93, the calculated concentration of the undissociated form of naloxone in these experiments is 662 μM . In contrast, the calculated undissociated concentration of iso-butyric acid is 27 μM at 10 mM total iso-butyrate (pH 7.4). Viewed from

Figure 4. Fractional inhibition of Cl self-exchange as a function of the chloroform:water partition coefficient (K_p^{chloro} , log scale). Squares represent K_p^{chloro} values determined by Jackson et al. (20), circles represent values determined by us.

Table IV. Effect of Naloxone, *n*-Butanol, GABA, MAPTAM, and Cyanide on K_{Cl}

Agent	<i>n</i>	Control	Experimental	Mean paired difference	<i>P</i>
		<i>K</i> _{Cl} , nm s ⁻¹			
Naloxone 1 mM	5	208.6	215.2	6.6	NS
		±52.2	±57.3	±17.0	
<i>n</i> -Butanol 1 mM	7	296.4	300.4	3.9	NS
		±34.4	±32.4	±9.6	
GABA 10 mM	5	185.2	187.0	1.8	NS
		±49.0	±47.3	±5.5	
MAPTAM 0.1 mM	4	179.7	185.7	6.1	NS
		±26.9	±28.4	±8.4	
Cyanide 1 mM	5	376.2	299.9	-76.4	<0.01
		±55.1	±46.0	±15.0	

Agents were added to bath solution.

this perspective of lipid solubility, it is clear that no effect on K_{Cl} is produced by naloxone at a concentration 25 times higher than that concentration of iso-butyric acid causing a 70–80% inhibition of self-exchange.

Table IV also shows the effect of the alcohol of butyric acid, *n*-butanol, on K_{Cl} . 1 mM *n*-butanol had no effect on K_{Cl} . We estimated K_p^{chloro} for *n*-butanol as follows. We used published values of $K_p^{\text{olive oil}}$ and K_p^{ether} for *n*-butanol (22). We assumed that the ratio of partition coefficients determined in two different oil/water systems is the same for all organic compounds. That is, $K_p^{\text{chloro}}/K_p^{\text{ether}}$ for iso-butyrate should equal $K_p^{\text{chloro}}/K_p^{\text{ether}}$ for *n*-butanol. Therefore, we measured K_p^{chloro} and K_p^{ether} for iso-butyrate and used the published value for K_p^{ether} of *n*-butanol to derive a K_p^{chloro} for *n*-butanol of 6.3×10^{-1} . An identical value was obtained using the ratio $K_p^{\text{chloro}}/K_p^{\text{olive oil}}$. This value of K_p^{chloro} for *n*-butanol is identical to that for iso-butyrate and similar to that for *n*-butyrate (5.0×10^{-1} , Table II). Thus, at the same extracellular concentrations, *n*-butanol and iso-butyric acid would produce the same concentration in the membrane. Nonetheless, 1,000 μM of *n*-butanol had no effect on K_{Cl} , whereas 27 μM of the equally lipid-soluble *n*-butyric acid inhibited K_{Cl} by 65% (Table II). We interpret these results with naloxone and *n*-butanol as indicating that the effect of the organic acids is not related solely to their lipid solubility, but is also dependent on the presence of the carboxyl group in the organic acid.

We also considered that these organic acids may be interacting with a regulatory site on or near the Cl transport pathway. The structure of these compounds is close to that of GABA, well known for its stimulatory effect on Cl channels in central nervous system neurons (23). We reasoned that butyric acid, or one of the family of organic acids shown to inhibit Cl self-exchange, might be interacting with a GABA receptor. As shown in Table IV, 10 mM GABA had no effect on K_{Cl} . This result makes it unlikely that there is a GABA receptor regulating this Cl transport pathway. Parenthetically, it is of interest to note that the addition of an amino group on the molecule eliminates the inhibitory effect of the parent organic acid. It is possible that the most important factor in this inactivation is the greatly lower lipid solubility of GABA compared to butyric acid.

Effect of MAPTAM and cyanide on K_{Cl} . We examined two possible mechanisms of action of organic acids that might involve alterations in intracellular environment. One possibility is that intracellular ionized Ca^{2+} was reduced. The reduction could be owing to the well-known effect of these compounds to bind Ca^{2+} (24), or their stimulation of mitochondrial Ca^{2+} uptake (25). To test this possibility, we added MAPTAM, a calcium chelator derived from EGTA (26) to the bath. If we assume that, at equilibrium, (a) the intracellular concentrations of MAPTAM and iso-butyrate are 0.1 and 25 mM, respectively, and (b) the ratio of MAPTAM/iso-butyrate affinity constants for calcium is 10,000:1 (24, 26), then MAPTAM would be predicted to chelate significantly more intracellular calcium than iso-butyrate. As shown in Table IV, 100 μ M MAPTAM (dissolved in dimethyl sulfoxide) had no effect on K_{Cl} . Therefore, it is unlikely that the effect of organic acids on Cl self exchange is mediated by a reduction in intracellular levels of ionized calcium.

We also considered the possibility that organic acids might inhibit Cl self exchange by altering cellular metabolism, for example by undergoing intramitochondrial β -oxidation to acetyl-CoA (27). To determine whether Cl self-exchange is linked to metabolism, we tested the effect of cyanide on K_{Cl} . Cyanide blocks the reduction of oxygen catalyzed by cytochrome aa₃ in mitochondria (28). Electrolyte transport dependent on ATP hydrolysis would be inhibited by cyanide. Table IV shows that 1 mM NaCN reduced K_{Cl} from 376.2 ± 55.1 to 299.9 ± 46.0 nm/s ($\Delta K_{Cl} = -76.4 \pm 15.0$ nm/s, $P < 0.01$). This result implies that a small fraction of Cl self-exchange is dependent on intact mitochondrial function. However, the fact that K_{Cl} is reduced by both cyanide and by organic acids makes it unlikely that stimulation of cellular metabolism plays a major role in the inhibition of Cl self-exchange by organic acids.

Sidedness of iso-butyrate effect on K_{Cl} . The results described above were obtained in experiments that examined the effects of organic acids on Cl self-exchange from the bath (basolateral) side. As previously described (1), it appears that transcellular Cl movement in the rabbit CCT intercalated cell occurs via an apical electroneutral entry step in series with a basolateral conductive pathway. Because of this polarity of Cl transport components, we examined the effects of organic acids on Cl self-exchange from the luminal side and compared lumen and bath effects on Cl self-exchange using 25 mM iso-butyrate. As shown in Fig. 5, using rapid perfusion rates and bath collections to measure K_{Cl} , 25 mM bath iso-butyrate reduced Cl self-exchange by 85%, whereas 25 mM lumen iso-butyrate reduced Cl self exchange by only 22% ($P < 0.001$). One possible reason for this difference in potency might be that the iso-butyrate concentration decayed axially along the tubule length owing to efflux into the bath. To examine this possibility, we measured counts of [¹⁴C]iso-butyrate in perfusates and in collected fluid at rapid perfusion rates; they were $20,931 \pm 103$ and $20,778 \pm 106$ cpm, respectively. These results indicate that axial changes in lumen iso-butyrate concentration cannot account for the sidedness of the compound's effect on K_{Cl} . The preferential inhibition from the basolateral side strongly suggests, but does not prove, an effect on the basolateral membrane.

We also calculated the lumen-to-bath rate coefficient for [¹⁴C]iso-butyrate (measured simultaneously by bath and CVP collections) using the calculated iso-butyrate concentration of

Figure 5. Fractional inhibition of Cl self-exchange by 25 mM iso-butyrate from bath and from lumen. Results were obtained in a paired, randomized fashion. $P < 0.001$. $n = 6$.

24.92 mM. The butyrate rate coefficient was 41.8 ± 3.4 nm/s ($n = 5$).

Effect of bath iso-butyrate on transepithelial conductance and Rb flux. We next tested the hypothesis that organic acids have their effect on Cl self-exchange by some action on a conductive pathway. We have previously modeled Cl transport as occurring via the β -intercalated cell (1). As modeled, this cell has no apical ion conductive pathways (29, 30), and thus changes in the basolateral Cl conductance are "invisible" to transepithelial conductance determinations. The principal cell also contains a Cl conductance at the basolateral membrane (31) and, in contrast to the β -intercalated cell, changes in this Cl conductance can be determined by transepithelial conductance measurements because the apical membrane of this cell type is conductive (29, 32). In Hepes-buffered solutions, and in the presence of lumen amiloride, G_T represents two conductances in series in principal cells: an apical K conductance and basolateral K and Cl conductances (31). An agent lowering basolateral membrane conductance could do so by lowering either the basolateral K and/or Cl conductances. Although G_T measurements alone cannot distinguish between these possibilities, it is possible to assess relative effects of an agent on these two ion pathways by comparing its effects on G_T and the lumen-to-bath ⁸⁶Rb rate coefficient (K_{Rb}). Maneuvers that reduce G_T but have no effect on K_{Rb} most likely are reducing the basolateral Cl conductance. We have used a similar construct to deduce the effect of Cl channel blockers on the principal and intercalated cell (9).

The effect of 25 mM bath iso-butyrate on G_T is displayed in Fig. 6. Iso-butyrate reversibly reduced G_T from 9.3 ± 1.7 to 6.5 ± 1.3 mS cm⁻² ($P < 0.005$). Recovery G_T was 9.1 ± 1.6 mS cm⁻². These changes in G_T were maximal within 1 min, were constant during the exposure, and were reversed within 5 min of washout. In contrast, Fig. 7 shows that 25 mM bath iso-butyrate did not reduce but, in fact, increased K_{Rb} from 70.2 ± 11.8 to 80.5 ± 10.1 nm/s ($\Delta K_{Rb} = 10.3 \pm 2.2$ nm/s, $P < 0.001$). Although we are unable to explain this increase, this result makes it unlikely that iso-butyrate reduced K conductances. Rather, 25 mM bath iso-butyrate probably reduced the principal cell basolateral Cl conductance. By analogy, it seems likely that organic acids reduce Cl tracer flux through β -type intercalated cells by a similar inhibition of the basolateral Cl conductance in that cell.

Discussion

The present results demonstrate that organic acids inhibit Cl transport in the rabbit CCT. More potent when applied to the

Figure 6. Effect of 25 mM bath iso-butyrate on trans-epithelial conductance (G_T). Mean paired difference 2.7 ± 0.4 mS/cm².

basolateral rather than the apical membrane, the inhibition does not appear to be related to changes in cell metabolism, cytosolic calcium concentration, or the pKa of the organic acids. Rather, the inhibition correlates well with the chloroform/water partition coefficients of the acids and with the concentration of the protonated form. Thus, the inhibition is probably mediated by the undissociated, lipid-soluble form of the acids. Conductance measurements and measurements of Rb flux are consistent with inhibition of a basolateral Cl conductance on principal cells.

As recently reviewed (1), the majority of transcellular Cl transport by the rabbit CCT is mediated by the β -type intercalated cell. This cell contains an apical membrane anion exchanger. Chloride is brought into the cell electroneutrally in exchange for a secreted HCO_3^- . In an alternative (and more frequent) mode of operation, one Cl is exchanged for another, a process termed self-exchange that effects no net flux and performs no work. Cl tracer brought into the cell at the apical membrane crosses the basolateral membrane via a Cl channel (9). If exogenous HCO_3^- and CO_2 are omitted from the perfusion and bathing solutions, the exchange process is constrained to function in a Cl self-exchange mode. The Cl self-exchange mode represents a convenient and sensitive way to study the combined processes of apical anion exchange in series with basolateral conductive Cl exit.

Transport of Iso-butyrate. Because organic anions can be transported on anion exchangers in various epithelia (5–8, 13, 14), we postulated that the same might hold true in CCT. Such was not the case. We will consider first the data bearing on whether iso-butyrate is transported out of the lumen via the β -intercalated cell apical anion exchanger. From previous ex-

Figure 7. Effect of 25 mM bath iso-butyrate on the lumen-to-bath ^{86}Rb rate coefficient. Mean paired increase was 10.3 ± 2.2 nm s⁻¹.

periments (2, 9) and the 0 bath Cl experiments in Table I, we know that the paracellular lumen-to-bath Cl (diffusive) flux (expressed as a rate coefficient) is ~ 30 nm s⁻¹. The mobility of the iso-butyrate anion is 0.43 that of Cl (33). Therefore, assuming the tight junction does not select organic over inorganic anions, one would predict a paracellular (diffusional) iso-butyrate anion permeation of $\sim 0.43 \times 30 = 13$ nm s⁻¹. We measured the total lumen-to-bath iso-butyrate anion rate coefficient as 41.8 ± 3.4 nm s⁻¹. Subtracting the predicted diffusive component of 13 nm s⁻¹, we are left with a predicted cellular rate coefficient of ~ 29 nm s⁻¹. This low value stands in contrast to the typical Cl rate coefficient of 100–400 nm s⁻¹ (2, 3, 9, and Tables I, III, and IV, and Fig. 1), suggesting that very little transport of iso-butyrate as the anion takes place via the exchanger.

If this “cellular” butyrate rate coefficient is recalculated using the concentration of iso-butyric acid (70 μM) instead of the total iso-butyrate concentration, the rate coefficient is 10.2 ± 1.2 μM s⁻¹.² This permeability coefficient is indistinguishable from the butanol permeability in CCT of 12.2 ± 0.5 μM s⁻¹ reported by Schafer and Andreoli (34). Thus, assuming that iso-butyrate and iso-butyric acid permeation occur via both ionic and nonionic diffusion, and assuming that butanol permeability is a reasonable approximation of (undissociated) butyric acid permeability, then all of the predicted “cellular” iso-butyrate flux of 28.6 nm s⁻¹ can be theoretically accommodated by absorption as the undissociated form. Carrier-mediated transport of iso-butyrate anion by the anion transport pathway must therefore be close to zero.

Mechanism of inhibition. The mechanism(s) involved in the inhibition of Cl transport by organic acids cannot be deduced completely from the present experiments, but there are some clues. The inhibition is not owing to competition by the organic anions for Cl transport as is the case for the monocarboxylate transporter of the proximal tubule (5, 6, 35): in the proximal tubule, organic acids are themselves transported, their inhibitory potency on lactate transport is inversely related to size, and their effect is strongly related to the charge density of the carboxyl group. In contrast, organic acids in the CCT are not themselves transported, their inhibitory potency is more directly related to size, and their effect is mediated by the undissociated moiety.

Because organic acids tend to lower intracellular pH by virtue of their low pKa and by their ability to enter the cell by non-ionic diffusion, one might postulate that these agents alter Cl transport via a cell pH effect. In this regard, it is important to recognize that the final cell pH in the presence of continuous exposure to external organic acids is a function of the balance between the rate of the cell’s proton-extruding mechanisms and the extrusion mechanisms for the associated anion. In most cells, the steady state cell pH is usually more acidic in the presence of an organic acid (36). It is therefore conceivable that a lowering of cell pH may have caused the inhibition of Cl transport reported here. We have not yet directly measured changes in cell pH in CCT under these conditions. However, we have found in separate studies that addition of exogenous CO_2 , a maneuver that consistently lowers cell pH in other cells (36), actually stimulates CCT Cl self-exchange (3; and unpub-

2. This rate coefficient is calculated assuming an iso-butyric acid concentration of 70 μM which obtains at pH 7.4 and an iso-butyrate concentration of 25 mM.

lished observations). Thus, it seems unlikely that a lowering of cell pH by organic acids is the sole mechanism by which these agents inhibit Cl transport.

Another possible mechanism of inhibition by organic acids, especially butyrate, is via alteration of nuclear function. Of particular relevance to the CCT, butyrate has been shown to partially inhibit the aldosterone-induced increase in Na transport by the toad urinary bladder (37). Such an effect is thought to involve acetylation of histones and alteration of differentiation (38, 39). These effects require hours to days to develop, whereas the reduction of CCT Cl transport occurs in minutes or seconds. Therefore, it seems unlikely that these mechanisms play a role in altering Cl transport in the present experiments.

Interaction with membrane lipids. The effects of lipid soluble compounds on membrane transport processes have been the subject of considerable investigation. For purposes of this discussion, we will divide their effects into three general categories: (a) interaction with, and disruption of the lipid bilayer, (b) alteration of membrane surface charge, and (c) specific interaction of a portion of the inhibitor molecule with a charged site in the transport protein in combination with an interaction of the hydrophobic region of the inhibitor molecule with the lipid domain.

The first of these three mechanisms is best exemplified by the anesthetics. Anesthetics are thought to produce their effects on ionic currents in nerve membranes by disrupting the lipid organization (40, 41). Although the specific nature of this interaction is probably complex, the general effect has been attributed to changes in membrane fluidity and/or the membrane dipole potential (42–44). This class of interaction is characteristic of many kinds of lipid soluble substances such as alkanes, alcohols, unsaturated hydrocarbons, undissociated organic acids, and nonpolar compounds (11, 41, 42, 45). Of relevance to the present data, studies of organic compounds in nerve membranes demonstrate that the IC_{50} of the undissociated acid was approximately the same as the IC_{50} of the corresponding alcohol (42). This mechanism is thus not a likely explanation for our results since *n*-butanol in substantially higher concentrations than the undissociated butyric acid had no effect on Cl transport (Table IV).

With regard to the second mechanism, alteration in surface charge has been postulated by Caffier et al. (10) to explain the reduction in Cl conductance and increase in cation conductance produced by octanoate in frog skeletal muscle. They postulated that this effect was owing to an increase in surface charge resulting from the insertion of the non-polar tail of the organic acid into the lipid domain. Although we are not certain whether or not that explanation obtains for frog muscle, it seems unlikely in the CCT. If surface charge were the major factor, titration of the charges by lowering the pH should have mitigated the inhibitory effect, rather than enhance it as was observed (Table III).

The last putative mechanism to be considered is that a charged part of the inhibitor molecule interacts with a charged site on the transport protein, and that a hydrophobic part of the inhibitor molecule interacts with the lipid domain of the membrane. This particular hypothesis appears to have the most merit in explaining our CCT data. Our data indicate that a wide variety of organic acids can interact specifically with Cl conductive pathways in these epithelial cells. There appear to be two requirements: a carboxyl group and a hydrophobic

domain. These organic acids thus behave in a fashion similar to a large number of organic acids derived from anthracene and diphenylamines (9, 12) whose potency has been attributed to the presence both of a carboxyl group and of a hydrophobic region.

The site of action of these organic acids on Cl self-exchange cannot be deduced with certainty. However, we suspect that the majority of the inhibition is occurring at the basolateral Cl channel rather than the apical exchanger for the following reasons: (a) the effect is more pronounced from the basolateral side than the apical side (Fig. 5); (b) the reduction of the trans-epithelial conductance (Figs. 6 and 7) is probably an effect on a Cl conductive pathway (albeit on another cell type); (c) the effect of these organic acids on the CCT is the same as the effect of other agents known to inhibit Cl conductive pathways in other cells (9, 12); and (d) there is a precedent for organic acid inhibition of Cl channels in muscle cells (10). The clear conclusion in this regard must await additional experiments. We cannot exclude the possibility that these agents affect the electrically silent anion exchanger and note that other inhibitors of Cl channels can inhibit electrically silent pathways (12, 46).

The physiologic importance of the inhibition of Cl conductive pathways by organic acids is probably minimal. We note that lactate, the acid most likely to be available in millimolar concentrations, has only a small effect on Cl transport (Table II). The others tested are less likely to be present in such concentrations. The inhibition described herein, then, may prove most valuable to our understanding of the interaction of Cl channel inhibitors with the Cl channel. Additionally, the discovery that butyrate can inhibit Cl conductances suggests that the mechanism of longterm (days) butyrate effects on cell function may need reexamination. Although histone acetylation, etc., clearly occurs (38, 39), it is possible that some of the previously described effects on intact cells result from a primary blockade of anion channels.

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