Bicarbonate-dependent and bicarbonate-independent mechanisms contribute to nondiffusive uptake of acetate in the ruminal epithelium of sheep

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Aschenbach JR, Bilk S, Tadesse G, Stumpff F, Gäbel G. Bicarbonate-dependent and bicarbonate-independent mechanisms contribute to nondiffusive uptake of acetate in the ruminal epithelium of sheep. Am J Physiol Gastrointest Liver Physiol 296: G1098–G1107, 2009. First published March 5, 2009; doi:10.1152/ajpgi.90442.2008.—The present study investigated the significance of apical transport proteins for ruminal acetate absorption and their interaction with different anions. In anion competition experiments in the washed reticularum, chloride disappearance rate (initial concentration, 28 mM) was inhibited by the presence of a short-chain fatty acid mixture (15 or 30 mM of each acetate, propionate, and butyrate). Disappearance rates of acetate and propionate, but not butyrate (initial concentration, 25 mM each) were diminished by 40 or 80 mM chloride. In isolated ovine ruminal epithelium mounted in Ussing chambers, an increase in chloride concentration from 4.5 to 90 mM led to a decrease of apical acetate uptake at a concentration of 0.5 mM. Mucosal nitrate inhibited acetate uptake most potently whereas sulfate had no effect. Decreasing pH effects on acetate absorption originate mainly from modulation of apical acetate transport and the copresence of other anions (Cl−, HCO3−, NO3−) constitute only a small fraction in the HSCFA-SCFA− acid-base equilibrium, e.g., only 1% at pH 6.8. Consequently, the effective luminal concentration driving apical uptake of HSCFA may be as little as ~1 mM. Although this might not be a severe restriction for a very lipophilic acid like butyric acid, diffusive absorption should be much more difficult for acetic acid. Lipid bilayer permeability decreases from butyric to acetic acid by a factor of ~14 (42). Furthermore, butyrate but not acetate is intensely metabolized inside the ruminal epithelial cells (14, 28), establishing concentration gradients that drive passive diffusion primarily of butyrate. Last but not least, it is also difficult to explain why a decrease in luminal pH (i.e., an increase in the proportion of luminal HSCFA) never stimulated SCFA absorption to the extent predicted for lipophilic diffusion (11, 12).

Problems could be resolved if one assumed significant participation of anion-transporting proteins in SCFA absorption. Based on the strong coupling between SCFA absorption and bicarbonate secretion demonstrated in vivo (13), this could likely be via an SCFA−/HCO3− exchanger in the apical membrane (13, 26, 38). A suggestion has been that this SCFA−/HCO3− exchanger could be identical to the ruminal Cl−/HCO3− exchanger (26). Since intraruminal chloride concentrations are usually very low (e.g., 16 mM; Ref. 19), SCFA− would be in a good position to compete for transport via such a common exchanger. Alternatively, apical monocarboxylate cotransport via monocarboxylate transporter (MCT) has been proposed as an acetate import pathway in the goat rumen (22, 23). MCT are usually very low (e.g., 16 mM; Ref. 19), SCFA− would be in a good position to compete for transport via such a common exchanger. Alternatively, apical monocarboxylate transporter (MCT) has been proposed as an acetate import pathway in the goat rumen (22, 23). MCT are primarily proton/monocarboxylate cotransporters (4, 35) but may also operate in a HCO3−-dependent mode (17).

The present study proceeded from the assumption that the paradigm of almost exclusively passive permeation of SCFA is most questionable for acetate, which is the quantitatively most important but least lipophilic SCFA. Therefore, this study was intended to provide conclusive support for a main role of apical transport proteins in ruminal acetate absorption. In a combined approach in vivo and in vitro, several interactions between acetate transport and the copresence of other anions (Cl−, HCO3−, NO3−) were uncovered. From this we can extract not only convincing evidence for an SCFA−/HCO3− exchanger; for...
the first time, we demonstrate bicarbonate-independent uptake of acetate anions across the apical membrane of ruminal epithelial cells. Neither uptake component is identical to MCT.

MATERIAL AND METHODS

In Vivo Studies (Washed and Temporarily Isolated Reticulorumen)

Animals. Six Merino wethers aged 2–4 yr, with permanent ruminal fistulae in the dorsal ruminal sac, were used. The sheep were fed 200 g of oats per 50 kg body wt per day. Meadow hay and water were offered ad libitum.

Determination of SCFA and chloride disappearance. The washed and temporarily isolated reticulorumen technique was used as described in detail by Kramer et al. (26). In brief, reticulorunal content was removed through the fistula, stored in a closed container at 37–40°C, and replaced after the experiment. The reticulum was rinsed clear with buffer solution. A saliva collector was placed in the distal part of the esophagus, and suctioned saliva was bypassed into the omasum via a balloon catheter. This balloon catheter also avoided outflow of experimental solution to distal parts of the gastrointestinal tract. For determination of disappearance rates, 2.5 liters of buffer solution was sampled before and after the experiment. All solutions were kept at 37°C until analysis.

Chloride concentration was measured with ion-selective electrodes (NOVA 12, NOVA Biomedical) and chloride concentration by atomic absorption spectrophotometry (AAS 30, Carl Zeiss, Jena, Germany). SCFA concentration was determined by capillary gas chromatography after centrifugation (2,000 g, 5 min) and deproteinization (30 μl formic acid/500 μl sample, followed by centrifugation at 2,000 g for 5 min), using 4-methylvaleric acid as internal standard (Shimadzu GC15, Shimadzu, Japan; capillary column: length 15 m; diameter 0.248 mm; mobile phase: DB-FFAP, i.e., polyethylene glycol modified with nitroterephallic acid; flame ionization detector; Ref. 45). Osmolarity was determined by freezing point depression (Knauer Osmometer, Berlin, Germany). For the calculation of disappearance rates, the actual volume of buffer solution at the time of sampling was calculated from the measured chloride concentration, by using the equation described by Care et al. (8).

Buffer solutions used in vivo. Solution used for washing the reticulorumen contained (in mM) 100 NaCl, 20 NaHCO3, and 10 propionic acid. Standard buffer solution used to measure chloride disappearance rate in the absence of single SCFA (Fig. 1) contained (in mM) 20 NaCl, 25 NaHCO3, 30 acetic acid, 30 propionic acid, 30 butyric acid, 60 NaOH, 15 KOH, 5 K2HPO4, 2 CaCl2, 2 MgCl2, and 20 mannitol. A lower SCFA concentration of 15 mM or omission of butyrate at concentrations of 15 mM (A) or 30 mM each (B). To achieve acetate-, propionate-, or butyrate-free conditions, the respective acid was replaced by gluconate. Initial pH was 6.0 and initial chloride concentration was 28 mM in both setups. Data represent means with SE of n = 5 (A) or n = 6 (B) independent observations. * Data in the same graph are different if they do not share a common letter (P < 0.05). Asterisks indicate differences from the equivalent column in A (**P < 0.05; ***P < 0.01).

In Vitro Studies (Ussing Chamber Experiments)

Animals and preparation of epithelia. Adult (2–5 yr old) sheep (Ovis aries, Merino breed) were fed 100 g concentrate per day and had ad libitum access to good-quality meadow hay (first cut) and water for at least 14 days before the experiments. Sheep were stunned by captive-bolt pistol and killed by exsanguination. After exenteration, a ~300 cm2 piece of the ventral ruminal sac was cut off the ruminal wall and repeatedly rinsed with buffer solution (for composition of buffer solution, see Buffer solutions used for acetate and lactate uptake). Epithelial pieces were prepared and mounted in Ussing chambers as described by Gäbel et al. (15) and incubated under short-circuit conditions.

Uptake measurements. The uptake technique for ruminal epithelia has been described in detail previously (1). Briefly, varying concentrations of acetate spiked with D-[U-14C]acetate (final activity: 4 kBq/ml; Moravec Biochemicals, Brea, CA) or 0.5 mM L-lactate spiked with L-[1-14C]lactate (final activity: 5 kBq/ml; American Radiolabeled Chemicals, St. Louis, MO) were added to the mucosal side for 1 min. After three washings, the mucosal area previously exposed to the uptake buffer was lysed with 4 ml ice-cold NaOH (100 mM) for 3 min in a lysing device. Cornified aggregates were removed from the lystate by centrifugation (3,000 g, 4°C, 15 min). Radioactivity of lysates was measured in duplicate by scintillation counting (Wallac 1409 LSC, Berthold Technologies, Bad Wildbad, Germany), by use of Aquasafe 300 Plus (Zinsser Analytic, Maidencourt, UK) as scintillation fluid. Uptake was corrected for lystate protein content, which was determined in triplicate by the method of Smith et al. (40).
Buffer solutions used for acetate and L-lactate uptake. The standard buffer recipe for use in vitro contained (in mM) 80 NaCl, 5.5 KCl, 1 CaCl₂, 1.25 MgCl₂, 0.6 NaH₂PO₄, 2.4 Na₂HPO₄, 1 L-glutamine, 24 NaHCO₃, 10 HEPES, 10 glucose, 50 mannitol, 25 mg/l colistin methanesulfonate, and 100 mg/l cefuroxime. To obtain a solution with 4.5 mM chloride (Fig. 3), NaCl and KCl were replaced by Na gluconate and K gluconate. In completely chloride-free solutions (Figs. 4–9), CaCl₂ and MgCl₂ were replaced by Ca gluconate and Mg gluconate, additionally. The standard recipe with or without chloride produced HCO₃⁻/H₂CO₃-containing buffers at pH 7.4. A decrease to pH 6.1 was achieved by replacing 20 mM gluconic acid for 20 mM mannitol. Bicarbonate-free solutions were prepared by exchange of 24 mM Na gluconate for 24 mM NaHCO₃. This maneuver decreased buffer pH, which required addition of 5 mM choline for readjustment to pH 7.4 or 2 mM gluconic acid for adjustment to pH 6.1. Both additions were osmotically compensated by equimolar omission of mannitol.

In the experiments shown in Fig. 6, HCO₃⁻-containing and HCO₃⁻-free buffers were prepared Cl⁻ free as described above. Additionally, 15 mM choline gluconate were exchanged for 30 mM mannitol to raise gluconate concentration >120 mM. This gluconate could then be exchanged equimolarly by 60 mM NO₃⁻ and up to 60 mM acetate in the mucosal uptake buffer to measure concentration kinetics of acetate uptake. In the experiment shown in Fig. 7, a Cl⁻- and HCO₃⁻-free solution was used with 0 mM K⁺ and 13 mM Na⁺, which was achieved by equimolar replacements of K⁺ and Na⁺ ions by choline.

All buffers were adjusted to an osmolality of 285 mosmol/kg by use of mannitol, whereas choline and gluconic acid served to adjust pH. Bicarbonate-containing solutions were gased with carbogen (5% CO₂–95% O₂), bicarbonate-free solution with 100% O₂ at 37°C. Washing and transportation of epithelia after slaughter were performed with the serosal incubation buffer used in the respective experiments (HCO₃⁻ containing in all experiments except Fig. 7).

Nitrate and sulfate additions to the mucosal side were performed 2 min before addition of radiolabeled acetate. Control epithelia were sham treated with gluconate. DIDS (200 mM stock solution in DMSO), ethoxyzolamide (50 mM stock solution in DMSO), p-chloromercuribenzoic sulfonic acid (pCMBS; 400 mM stock solution in distilled water), p-hydroxymercuribenzoate sodium (pHMB; 400 mM stock solution in 0.1 M NaOH), and phloretin (20 mM stock solution in ethanol) were added 2 min before measurement of acetate uptake in the experiments shown in Figs. 3, 5, and 7–9. Control epithelia received an equivalent amount of vehicle. In the experiments shown in Figs. 4 and 6, ethoxyzolamide was directly dissolved in the HCO₃⁻-free, HEPES-buffered solutions.

Chemicals. Where no manufacturer is specified in the text, chemicals were obtained from Merck (Darmstadt, Germany), Roth (Karlsruhe, Germany), or Sigma-Aldrich (Deisenhofen, Germany).

Ethical Approval

Experiments were in accordance with the German legislation on the protection of animals. The in vivo studies were preapproved by the Regierungspräsidium Leipzig under TVV-No. 13/97. The in vitro studies were communicated to the Regierungspräsidium Leipzig under AZ 24-9162.11-01-T58/04.

Fig. 2. Influence of increasing chloride concentrations on the disappearance rates of acetate (ac; A) propionate (prop; B), and butyrate (but; C) from the washed reticulorumen. Intraruminal solutions contained a mixture of 25 mM each acetate, propionate, and butyrate. Initial pH and initial chloride concentration were 6.0 and 28 mM, respectively. Data represent means with SE (n = 4). a,bMeans within the same graph are different if they do not share a common letter (P < 0.05).

Fig. 3. Influence of chloride and DIDS on the apical uptake of 0.5 mM acetate (U_ac) at 2 different pH values. Concentrations of mucosal chloride ([Cl⁻]_m; 4.5 vs. 90 mM) and DIDS (0 vs. 0.2 mM) were varied in a mucosal, bicarbonate-containing solution buffered to either pH 7.4 (A) or pH 6.1 (B). mgprot·min⁻¹·μmol⁻¹. Milligrams of protein. Data represent means with SE of n = 9 epithelia from 5 sheep. a,bValues within the same graph are different if they do not share a common letter (P < 0.05).
Statistics

Results are shown as arithmetic means with SE. Differences between two means were tested by using Student’s t-test paired or unpaired as appropriate (normal distribution) or Mann-Whitney rank sum test (no normal distribution). Repeated-measures (RM) ANOVA was applied to compare multiple means. If RM ANOVA indicated a significant difference between means, subsequent Student-Newman-Keuls test was done to determine which of the means differed from each other. Nonlinear regression of acetate uptake ($U_{ac}$) with acetate concentrations was estimated by the Marquard-Levenberg algorithm with the following equation:

$$U_{ac} = U_{ac-max} \left( \frac{[acetate]}{K_{0.5}} \right)$$

where $U_{ac-max}$ is the maximal acetate uptake at saturating concentration, [acetate] is acetate concentration, and $K_{0.5}$ is the acetate concentration at 0.5 $U_{ac-max}$. Statistical tests and nonlinear regression fitting were performed by using the statistical software Jandel SigmaStat 3.5 (SPSS, Chicago, IL). Results were regarded as significantly different when $P < 0.05$.

RESULTS

In Vivo Studies

Influence of SCFA on chloride disappearance. Absorption of chloride from the rumen is thought to occur predominantly via Cl$^-$/HCO$_3^-$ exchange (31). Results of previous in vitro studies suggested that the same transport protein might be used for SCFA/HCO$_3^-$ exchange (26). If this was indeed a relevant pathway in vivo, one would expect mutual competition between SCFA and chloride absorption. Therefore, we investigated the influence of all three SCFA on chloride disappearance rate while holding pH (initially 6.0) and chloride concentration (initially 28 mM) in the physiological range. Figure 1 demonstrates the chloride disappearance in the presence of an SCFA mixture containing either 15 mM (Fig. 1A) or 30 mM (Fig. 1B) of each acetate, propionate, and butyrate. The doubling of luminal SCFA concentration from 45 mM (15 mM each; Fig. 1A) to 90 mM (30 mM each; Fig. 1B) reduced chloride disappearance rate by approximately half ($P < 0.05$; compare controls in Fig. 1, A vs. B). To elucidate whether the decrease in chloride disappearance rate was caused by one specific SCFA, we omitted each SCFA separately from the buffer solution. At both SCFA concentrations tested, omission of either acetate or propionate had a large stimulatory effect on chloride disappearance rate whereas butyrate omission showed smaller effects ($P < 0.05$; Fig. 1).

Fig. 4. Effect of mucosal pH and HCO$_3^-$ availability on $U_a$ at a mucosal pH (pH$_m$) of 7.4 or 6.1. Mucosal acetate concentration was either 0.5 mM (A) or 10 mM (B) in HCO$_3^-$-containing or HCO$_3^-$-free (HEPES-buffered) solution. HEPES-buffered solutions contained 50 nM ethoxyzolamide. The serosal pH was always adjusted to 7.4. In HCO$_3^-$-containing solution, the latter implicated a HCO$_3^-$ gradient from the serosal ($[\text{HCO}_3^-]_s$) to the mucosal side ($[\text{HCO}_3^-]_m$) of 22.5:1.1 mM at mucosal pH 6.1. Data represent means with SE; HCO$_3^-$-buffered solution: $n = 10$ epithelia of 5 sheep, HEPES-buffer solution: $n = 11$ epithelia of 6 sheep. $a$, $b$, $c$Columns within the same graph are different if they do not share a common letter ($P < 0.05$).

Fig. 5. $U_{ac}$ at mucosal acetate concentration of 0.5 mM (A and B) or 10 mM acetate (C and D) in HCO$_3^-$-containing or HCO$_3^-$-free (HEPES-buffered) solution. Serosal and mucosal pH were adjusted to 7.4 and 6.1, respectively, resulting in a serosal-to-mucosal HCO$_3^-$ gradient of 22.5:1.1 mM in HCO$_3^-$-containing solution. In A and C, all solutions contained 50 nM ethoxyzolamide. NO$_3^-$ was added at the indicated concentration to the mucosal side ($[\text{NO}_3^-]_m$). Data represent means with SE of $n = 11$ epithelia of 11 sheep. $a$, $b$, $c$, $d$, $e$Columns within the same graph are different if they do not share a common letter ($P < 0.05$).
mucosal acetate concentration (5–60 mM) in HCO₃⁻ no effect of SCFA chain length (i.e., lipophilicity) on basal propionate, and butyrate in the absence of chloride, indicating disappearance rates were not significantly different for acetate, propionate, and butyrate (Fig. 2). A first finding was that and butyrate were measured in the presence of 0, 40, and 80 bidirectional, the disappearance rates of acetate, propionate, and butyrate were fitted with the Marquard-Levenberg algorithm to the Michaelis-Menten equation. Data represent means with SE of n = 10 epithelia from 21 animals were used.

\[ U_{ac} = \frac{k_{max} [HCO_3^-]}{K_m + [HCO_3^-]} \]

\[ \Delta U_{ac} = \frac{k_{max} [HCO_3^-]}{K_m + [HCO_3^-]} - \frac{k_{max} [HCO_3^-]}{K_m + [HCO_3^-]_{m}} \]

\[ P_{ac} = \frac{K_m}{K_m + [HCO_3^-]_{m}} \]

\[ P_{ac} = \frac{K_m}{K_m + [HCO_3^-]_{m}} \]

Values at the same acetate concentration are different if they do not share a common letter (P < 0.05).

**Influence of chloride on SCFA disappearance.** To investigate whether the interaction between chloride and SCFA is bidirectional, the disappearance rates of acetate, propionate, and butyrate were measured in the presence of 0, 40, and 80 mM chloride. The solution contained 25 mM each acetate, propionate, and butyrate (Fig. 2). A first finding was that decrease of mucosal pH from 7.4 to 6.1 approximately doubled apical acetate uptake at low pH. The previous experiments demonstrated that low mucosal pH stimulates acetate uptake by low mucosal pH was reproducible in HCO₃⁻-containing solution at two different acetate concentrations, 0.5 and 10 mM (P < 0.05). In HCO₃⁻-containing solution, this could be attributable to changes in the Henderson-Hasselbalch equilibria of either SCFA/HSCFA or HCO₃⁻/H₂CO₃ or both. To elucidate the contribution of increased bicarbonate dependency of pH effect. The previous experiment had shown a stimulation of acetate uptake by low mucosal pH (Fig. 3, A vs. B). Figure 4 demonstrates that the increase of acetate uptake by low mucosal pH was reproducible in HCO₃⁻-containing solution at two different acetate concentrations, 0.5 and 10 mM (P < 0.05). In HCO₃⁻-containing solution, this could be attributable to changes in the Henderson-Hasselbalch equilibria of either SCFA/HSCFA or HCO₃⁻/H₂CO₃ or both. To elucidate the contribution of increased bicarbonate availability of HSCFA (i.e., lipophilic diffusion) to increased acetate uptake, HCO₃⁻ was omitted from the solution. The HCO₃⁻-free, HEPES-buffered solution additionally contained ethoxyzolamide to block conversion of metabolically generated CO₂ to HCO₃⁻ by carbonic anhydrase. In this HCO₃⁻-free setup, a decrease of mucosal pH from 7.4 to 6.1 induced no significant (Fig. 4A) or only small increases in acetate uptake (P < 0.05; Fig. 4B). The latter indicated that an increased proportion of protonated acetate hardly contributed to the overall increase of acetate uptake at low pH.

**Cell metabolism as HCO₃⁻ source/effect of nitrate.** The previous experiments demonstrated that low mucosal pH stimulates acetate uptake by low mucosal pH was reproducible in HCO₃⁻-containing solution at two different acetate concentrations, 0.5 and 10 mM (P < 0.05). In HCO₃⁻-containing solution, this could be attributable to changes in the Henderson-Hasselbalch equilibria of either SCFA/HSCFA or HCO₃⁻/H₂CO₃ or both. To elucidate the contribution of increased bicarbonate availability of HSCFA (i.e., lipophilic diffusion) to increased acetate uptake, HCO₃⁻ was omitted from the solution. The HCO₃⁻-free, HEPES-buffered solution additionally contained ethoxyzolamide to block conversion of metabolically generated CO₂ to HCO₃⁻ by carbonic anhydrase. In this HCO₃⁻-free setup, a decrease of mucosal pH from 7.4 to 6.1 induced no significant (Fig. 4A) or only small increases in acetate uptake (P < 0.05; Fig. 4B). The latter indicated that an increased proportion of protonated acetate hardly contributed to the overall increase of acetate uptake at low pH.

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of 0.5 mM acetate, uptake in HCO₃⁻ buffer was not different from the uptake in HEPES buffer (Fig. 5B). Consequently, uptake at 0.5 mM acetate was not suppressed in HEPES buffer as long as metabolism-derived CO₂ could be converted to HCO₃⁻. By contrast, conversion of metabolism-derived CO₂ was not sufficient to drive acetate uptake at 10 mM acetate. HCO₃⁻ buffer was essential for maximum transport rates at this elevated concentration. It was irrelevant whether the HCO₃⁻ buffer contained ethoxyzolamide or not (compare first columns in Fig. 5, C and D), indicating that influx of extracellular HCO₃⁻ already allowed for maximum transport and did not require support by carbonic anhydrase.

A second intention of this experimental series was to test the effect of NO₃⁻ on acetate uptake. NO₃⁻ is a known inhibitor of ruminal chloride (44) and propionate absorption (26). Mucosal addition of 10 mM NO₃⁻ decreased apical uptake of acetate in all setups to a larger extent than switching from HCO₃⁻ buffer to HEPES buffer (P < 0.05; Fig. 5, A–D). Moreover, the uptake remaining in the presence of NO₃⁻ was not (Fig. 5, A, B, and D) or only marginally different (P < 0.05; Fig. 5C) in HCO₃⁻ and HEPES buffer. Together, this indicated that nitrate inhibits the HCO₃⁻-dependent plus an HCO₃⁻-independent portion of acetate uptake.

Uptake at increasing acetate concentrations. Concentration dependence of acetate uptake was investigated initially in a lower concentration range (20 μM–10 mM). However, there was no obvious saturation (data not shown). We then investigated uptake at mucosal acetate concentrations between 5 and
60 mM (Fig. 6A). Uptake increased with increasing acetate concentration in HCO\textsubscript{3}\textsuperscript{-}-containing buffer. In HEPES-buffered solution containing 50 \textmu M ethoxyzolamide, apical acetate uptake was significantly reduced at all acetate concentrations (P < 0.05; Fig. 6A). Addition of 60 mM NO\textsubscript{3} to the HEPES-buffered solution reduced apical uptake further at any investigated acetate concentration (P < 0.05) but 60 mM (P = 0.09; Fig. 6A).

The HCO\textsubscript{3}\textsuperscript{-}-dependent part of acetate uptake was calculated as the difference of acetate uptakes in HCO\textsubscript{3}\textsuperscript{-} buffer and HEPES buffer. It could be fitted to a Michaelis-Menten equation with an apparent \( K_m \) = 54.1 \pm 17.5 mM and a maximal uptake \( U_{\text{ac-max}} = 22.5 \pm 4.3 \text{ nmol mg}^{-1} \text{ min}^{-1} \) (Fig. 6B). The HCO\textsubscript{3}\textsuperscript{-}-independent part of acetate uptake was calculated as the difference of acetate uptakes in HEPES buffer without and with NO\textsubscript{3}. It could be fitted to a Michaelis-Menten equation with an apparent \( K_m \) = 11.8 \pm 4.8 mM and a maximal uptake \( U_{\text{ac-max}} = 7.7 \pm 1.0 \text{ nmol mg}^{-1} \text{ min}^{-1} \) (Fig. 6B). \( K_m \) and \( U_{\text{ac-max}} \) were both larger for HCO\textsubscript{3}\textsuperscript{-}-dependent uptake compared with HCO\textsubscript{3}\textsuperscript{-}-independent uptake; \( P < 0.05 \) and \( P < 0.01 \), respectively.

Effect of mucosal SO\textsubscript{4}\textsuperscript{2-} on acetate uptake. Having discovered interference of Cl\textsuperscript{-} or NO\textsubscript{3} with acetate uptake, we aimed in the next set of experiments to test the effect of the divalent anion SO\textsubscript{4}\textsuperscript{2-}. However, uptake of acetate was not different in the absence of SO\textsubscript{4}\textsuperscript{2-} (0.80 \pm 0.20 nmol mg\textsuperscript{-1} min\textsuperscript{-1}; \( n = 6 \)) compared with the mucosal presence of 5 mM SO\textsubscript{4}\textsuperscript{2-} (0.90 \pm 0.34 nmol mg\textsuperscript{-1} min\textsuperscript{-1}; \( n = 6 \)).

Effect of inhibitors for MCT on acetate and \( l \)-lactate uptake. Previous studies had led to the conclusion that MCT might be involved in apical uptake of acetate in the ruminal epithelium of goats (22, 23). To clarify the role of MCT in apical acetate uptake in our model, two different classes of MCT inhibitors, 400 \mu M pCMBS and 20 \mu M phloretin (35), were applied to the mucosal solution prior to measurement of acetate uptake. Under HCO\textsubscript{3}\textsuperscript{-}-free conditions and an acetate concentration of 10 mM, however, none of these inhibitors decreased uptake of acetate (Fig. 7). To verify that MCT does not contribute to apical acetate uptake, acetate concentration was decreased to 0.5 mM and comparative measurements were performed in both HCO\textsubscript{3}\textsuperscript{-}-containing and HCO\textsubscript{3}\textsuperscript{-}-free buffer solutions. Additionally, a chloride-free variant of the mercurial MCT inhibitors, pHMB, was used to avoid any interference by residual free chloride. As observed before (Fig. 5), the omission of HCO\textsubscript{3}\textsuperscript{-} significantly reduced acetate uptake (compare Fig. 8, A and B). However, neither 800 \mu M pHMB nor 100 \mu M phloretin had an effect on acetate uptake, either in HCO\textsubscript{3}\textsuperscript{-}-containing or in HCO\textsubscript{3}\textsuperscript{-}-free buffer (Fig. 8). This finally disproved involvement of apical MCT in acetate transport and could indicate either complete absence of apical MCT or negligible transport rates. To differentiate between the latter two possibilities, comparative measurements were performed with the MCT model substrate, \( l \)-lactate (18, 35). At a concentration of 0.5 mM, uptake of \( l \)-lactate was an order of magnitude smaller than that of acetate (P < 0.01; compare scaling in Figs. 8 and 9). However, \( l \)-lactate uptake was reduced by both 800 \mu M pHMB and 100 \mu M phloretin (P < 0.05; Fig. 9), suggesting that MCT is present apically but operates at low transport rates.

**DISCUSSION**

Despite several decades of research, we are currently only starting to realize that absorption of SCFA from the rumen has functional complexity. There is no uniform mechanism for SCFA absorption. Lipophilic diffusion has long been regarded as such a uniform mechanism (2, 7, 16). However, lipophilic diffusion strongly depends on the proportion of undissociated HSCFA and chain length. The proportion of HSCFA is approximately similar for acetate, propionate, and butyrate at any given pH because of a similar pK value of 4.8 (9). However, chain lengths differ, which makes butyrate ~14 times more permeant than acetate (42). Since butyrate and acetate had similar absorption rates during the present in vivo studies (Fig. 2), the assumption that butyrate was absorbed predominantly by lipophilic diffusion would logically imply that lipophilic absorption of acetate should not be larger than ~1/14 (i.e., 7%) of total acetate absorption. The expected contribution of lipophilic acetate absorption during the in vivo studies would decrease further when taking into account the intensive intracellular metabolism of butyrate (14, 28) and would increase when assuming efficient basolateral exit pathways for acetate (22, 41). To exclude these additional influences, we attempted the in vitro approach to assess transport pathways for acetate at the level of the apical membrane.

The in vitro part of the present study clearly showed that a decrease in luminal pH from 7.4 to 6.1 (i.e., an increase in undissociated acids by a factor of 19) led to only barely measurable increases of apical acetate uptake in HEPES-buffered solution (Fig. 4). Earlier studies suggested that such low or missing pH effects on SCFA absorption are attributable to apical Na\textsuperscript{+}/H\textsuperscript{+} exchange (NHE) enriching protons near the ruminal wall. The NHE would generate a high proportion of HSCFA at the epithelial surface largely independent of bulk pH (11, 12). Indeed, apical NHE is very active in ruminal epithelia and its inhibition by amiloride reduces the absorptive net flux of Na\textsuperscript{+} by ~70% (31). However, amiloride has negligible effects on the ruminal permeation of propionate (26) and butyrate (38), which contradicts an NHE-driven transport of HSCFA. The present study now demonstrates that a pH effect on apical acetate uptake is only to a minor part due to the increased availability of protons. In contrast, it is largely dependent on the presence of HCO\textsubscript{3} (Fig. 4). This is congruent with predominant participation of an acetate/HCO\textsubscript{3} exchange in acetate absorption. At mucosal pH 6.1, HCO\textsubscript{3} concentration amounts to 1.1 mM, thereby generating an HCO\textsubscript{3} gradient from the cell interior to the mucosal solution as a driving force for acetate/HCO\textsubscript{3} exchange. The proteins underlying this transport process could be identical to those responsible for Cl\textsuperscript{-}/HCO\textsubscript{3} exchange (26) as suggested by the interaction between chloride and acetate transport observed in vivo and in vitro (Figs. 1–3).

The experimental setup shown in Fig. 5 demonstrated that cellular HCO\textsubscript{3} for apical acetate/HCO\textsubscript{3} exchange is replenished mainly from extracellular sources. The source can be either HCO\textsubscript{3} import from the serosal side (likely via the basolateral Na\textsuperscript{+}/HCO\textsubscript{3} cotransporter described previously; Ref. 20) or extracellular CO\textsubscript{2} that is converted by cellular carbonic anhydrase to HCO\textsubscript{3} after entering the cell (29). Of these possibilities, HCO\textsubscript{3} import alone was sufficient to continuously drive the exchanger at an acetate concentration of 10
mM in the present study (Fig. 5C). It seems plausible to assume that HCO$_3^-$ generation from extracellular CO$_2$ may support the HCO$_3^-$ supply for the exchanger in vivo since CO$_2$ constitutes up to 70% of the gas phase of the rumen (3). However, CO$_2$ generation by epithelial cell metabolism was only sufficient to drive acetate uptake at the very low concentration of 0.5 mM in the present study (Fig. 5B) but not at a concentration of 10 mM acetate (Fig. 5D). Thus metabolic CO$_2$ seems quantitatively irrelevant for acetate/HCO$_3^-$ exchange when considering that intraruminal acetate concentrations often exceed 60 mM in vivo (27, 32).

The observation that absorption of SCFA was linked to ruminal appearance of HCO$_3^-$ originates back to the year 1951 (33). For a long time, it was explained with a model of purely lipophilic diffusion of protonated acetate. Intraruminal carbonic acid was suggested to provide protons to make acetate diffusible, leaving HCO$_3^-$ behind in the rumen (2). Gäbel et al. (13) later introduced the idea that a direct exchange of SCFA-- for HCO$_3^-$ might be a better explanation for SCFA/HCO$_3^-$ coupling. One main argument against SCFA--/HCO$_3^-$ exchange was that SCFA absorption does not show saturation with increasing acetate concentration (7, 30). The present study demonstrates that this is partly attributable to a very high $K_m$ value of acetate/HCO$_3^-$ exchange (Fig. 6). A $K_m$ of 54 mM largely prevents saturation of acetate absorption in the physiological range of intraruminal acetate concentrations. With low experimental resolution, this may easily be interpreted as quasi-linear increases of acetate absorption with increasing acetate concentrations.

In addition to SCFA--/HCO$_3^-$ exchange, the present study provided, for the first time, functional proof of HCO$_3^-$-independent apical uptake of acetate (Fig. 5 and 6). HCO$_3^-$-independent uptake was sensitive to inhibition by nitrate and also had a $K_m$ in the millimolar range (12 mM).

Molecular candidates for HCO$_3^-$-dependent and HCO$_3^-$-independent SCFA transport are numerous and could be members of the SLC4A, SLC16A, SLC21A, SLC22A, and SLC26A families. So far, systematic screening has been performed only for the family of monocarboxylate transporters (SLC16A), which translocate monocarboxylic acids in either HCO$_3^-$-independent (4, 35) or HCO$_3^-$-dependent modes (17). Molecular screening has identified MCT1 in ovine (35) and caprine rumen (22), MCT1 (SLC16A1) and MCT4 (SLC16A3) in reindeer rumen (25), as well as MCT1, MCT2 (SLC16A7), and MCT4 in bovine rumen (16, 23). Of these, MCT1 protein has a proven role in SCFA absorption but is located at the basolateral pole in ovine ruminal epithelial cells (35). This would leave a possibility for apical SCFA uptake to MCT4 (23) because MCT2 protein was either not detectable in immunohistochemical studies (25) or not detectable apically (16), at least in reindeer and cattle. Proceeding from MCT4 as the likely apical isoform and from a very poor affinity of MCT4 to acetate (10), it was not surprising to find no involvement of apical MCT in acetate absorption in the present study (Figs. 7 and 8). However, we clearly showed that MCT is present at the apical side mediating permeation of lactic acid albeit at low rates. The low activity of apical MCT, in turn, is in agreement with previous studies in vivo, showing that lactate is absorbed at only one-tenth the rate of SCFA from the forestomach content of sheep (43). The poor absorption of lactate has health consequences; lactate accumulation in the rumen due to excessive bacterial production and insufficient epithelial absorption is considered the main pathogenetic principle in the development of acute ruminal acidosis, a sometimes fatal disease after carbohydrate overload (36).

At variance to our findings, a previous report in goats proposed an involvement of apical MCT in ruminal acetate transport (22) that was later attributed to isofrom 4 by the same group (23). These previous reports may not necessarily conflict with our study. Goats also appear to be more tolerant to intraruminal lactate than sheep (34) and may have different expression levels of MCT both at the apical and at the basolateral side. What finally matters for the functional interpretation of the present study is that neither the HCO$_3^-$-independent nor the HCO$_3^-$-dependent component of acetate uptake were mediated by apical MCT. Thus, especially, the discovered HCO$_3^-$-independent uptake of acetate constitutes a completely novel entity in the rumen.

Although the functional significance of members of the SLC16A family awaits further evaluation, future screening should increasingly focus on members of the SLC4A, SLC21A, SLC22A, and SLC26A families of anion transporters. So far, the downregulated in adenoma (DRA, SLC26A3) and the putative anion transporter 1 (PAT1, SLC26A6) have been identified in ovine rumen (6), where PAT1 has a reported capacity to transport SCFA (37). However, there could be several other candidates. For example, OAT2 (SLC22A7) and OAT7 (SLC22A9) have recently been identified as hepatic uptake carriers for propionate and butyrate, respectively (21, 39).

In comparing the results of the present study to those of previous investigations, it becomes clear that the ruminal epithelium has evolved a so-far-unrecognized diversity of transport pathways for SCFA. For example, there appear to be different proteins for the HCO$_3^-$-dependent transport of propionate and acetate across the apical membrane. Propionate/ HCO$_3^-$ exchange in ovine rumen has been shown to be sensitive to inhibition by low concentration of DIDS (100 μM; Ref. 26). By contrast, 200 μM DIDS did not reduce acetate uptake under four different experimental conditions in the present study (Fig. 2). Secondly, propionate transport was depressed more by the copresence of chloride than acetate transport in the in vivo part of this study (Fig. 1). If one assumed that lower lipophilicity of acetate needs to be compensated by a higher acetate/HCO$_3^-$ exchange, one would have expected larger chloride interaction on acetate transport compared with propionate transport. Thus it appears that several transport proteins with differing SFCAs, DIDS, and chloride affinities are involved in apical uptake of individual SCFA.

Specifically for acetate, the present study leads to the conclusion that protein-mediated transport pathways play a major role in its absorption. Acetate is absorbed via both HCO$_3^-$-dependent and HCO$_3^-$-independent mechanisms. The HCO$_3^-$-dependent mechanism can be stimulated by an HCO$_3^-$ gradient. At the gradient chosen in the present study (22.5 mM serosal vs. 1.1 mM mucosal), the HCO$_3^-$-dependent mechanism had a lower acetate affinity than the HCO$_3^-$-independent mechanism but a three times larger transport capacity. This high transport capacity is a good precaution for a dominance of acetate/ HCO$_3^-$ exchange at physiological intraruminal acetate concentrations. Although the bicarbonate gradient is mostly inverted in vivo because of the high intraruminal bicarbonate concen-
tration (3, 24), previous studies showed a high degree of coupling between SCFA absorption and $\text{HCO}_3^-$ secretion (13), suggesting that the high acetate gradient occurring in vivo is an equally effective driving force for the exchanger. The competition between chloride and acetate/proprionate disappearance observed in the present study further extends this conclusion. The acknowledgment of transport proteins as main mechanisms of acetate absorption implies the possibility to adjust transport capacity via transcriptional and/or posttranscriptional regulation. Therefore, molecular identification of the involved transport proteins will be vital to understand the mechanisms of diet adaptation in ruminants in the future.

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