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Effect of sodium concentration and mucosal pH on apical uptake of acetate and butyrate, and barrier function of the isolated bovine ruminal epithelium

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ABSTRACT

This study was conducted to investigate the role of Na^+ on ruminal short-chain fatty acid (SCFA) absorption and barrier function when isolated ruminal epithelium was exposed to high and low pH ex vivo. Nine Holstein steer calves (322 ± 50.9 kg of body weight) consuming 7.05 ± 1.5 kg dry matter of a total mixed ration were euthanized and ruminal tissue was collected from the caudal-dorsal blind sac. Tissues were mounted between 2 halves of Ussing chambers (3.14 cm^2) and exposed to buffers that contained low (10 m*M*) or high (140 m*M*) Na^+ with low (6.2) or high (7.4) mucosal pH. The same buffer solutions were used on the serosal side except that pH was maintained at 7.4. Buffers used to evaluate SCFA uptake contained bicarbonate to determine total uptake or excluded bicarbonate and included nitrate to determine noninhibitable uptake. Bicarbonate-dependent uptake was calculated as the difference between the total and noninhibitable uptake. Acetate (25 m*M*) and butyrate (25 m*M*) were spiked with 2-^{3}H-acetate and 1-^{14}C-butyrate, respectively, and were then added to the mucosal side, incubated for 1 min, and tissues were analyzed to evaluate rates of SCFA uptake. Tissue conductance (G_t) and the mucosal-to-serosal flux of 1-^{3}H-mannitol were used to assess barrier function. There were no Na^+ × pH interactions for butyrate or acetate uptake. Decreasing mucosal pH from 7.4 to 6.2 increased total acetate and butyrate uptake, and bicarbonate-dependent acetate uptake. Flux of 1-^{3}H-mannitol was not affected by treatment. However, high Na^+ concentration reduced G_t and prevented an increase in G_t from flux period 1 to flux period 2. The results of this study indicate that although providing more Na^+ to the ruminal epithelium does not affect SCFA uptake or mannitol flux, it may help stabilize tissue integrity.

Key words: sodium, rumen, barrier function

INTRODUCTION

Sodium (Na^+) is the most abundant cation in ruminal digesta and is likely the most regulated ion transported across the reticulo-ruminal epithelium (Bennink et al., 1978; Tucker et al., 1988). Luminal Na^+ supply originates from feed and water sources; however, recycling of Na^+ back to the rumen via sodium bicarbonate in saliva is a major contributor to ruminal Na^+ supply (Bailey and Balch, 1961). It has been estimated that lactating dairy cows produce 170 to 310 L (Bowman et al., 2003) of saliva daily with Na^+ concentrations ranging from 140 to 165 m*M* (Silanikove and Tadmor, 1989), of which less than 10% reaches the duodenum (Khorasani et al., 1997; Leonhard-Marek et al., 2010). This implies that most of the Na^+ entering the reticulo-rumen is absorbed by the reticulo-rumen, omasum, and abomasum (Leonhard-Marek et al., 2010).

Sodium disappearance from the reticulo-rumen is facilitated by electroneutral Na^+ transport along with electrogenic absorption pathways (Leonhard-Marek et al., 2010). Electroneutral absorption is mediated by the Na^+/H^+ exchanger (**NHE**) that absorbs Na^+ in exchange for H^+, driven by the Na^+ gradient created by the basolateral Na^+/K^+ ATPase (Leonhard-Marek et al., 2010). It is suggested that this pathway is indirectly stimulated by the absorption of fermentation end products, particularly short-chain fatty acids (**SCFA;** Sehested et al., 1996, 1999). Short-chain fatty acids in the protonated state can diffuse across the apical membrane and upon appearance in the cytosol will dissociate and induce intracellular acidification upregulating apical NHE (Schweigel et al., 2005; Etschmann et al., 2009). Absorption of SCFA is considered a desirable outcome as they provide ruminants with metabolizable energy (Bergman, 1990) and contribute to the stabilization of ruminal pH (Penner et al., 2009). However, when the rate of acid production exceeds the rate of acid removal, SCFA accumulate resulting in a simultaneous increase in ruminal osmolality and acidification of ruminal digesta, thus impairing absorption of SCFA (Penner et al., 2010; Meissner et al., 2017). Low ruminal pH may increase the proportion of SCFA in the

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undissociated state (**HSCFA**), facilitating uptake into the ruminal epithelium via lipophilic diffusion (Gäbel et al., 1991). Following absorption, undissociated SCFA will rapidly deprotonate due to the intracellular pH ($\mathbf{pH_i}$) of 7.4. Hence apical Na^+/H^+ exchangers and an adequate luminal supply of Na^+ are required to extrude protons back into the lumen in exchange for Na^+ helping to stabilize pH_i and maintain cellular functions and integrity (Gäbel et al., 2002).

In vivo studies have shown that when cattle are exposed to an acute bout of ruminal acidosis, SCFA absorption rates decline and saliva production increases (Schwaiger et al., 2013). The etiology of this response is not well characterized but it has been theorized that it might be a compensatory mechanism of inadequate Na^+ availability in the rumen (Mooney, 2006). In addition, cattle experiencing heat stress have reduced feed intake, reduced rumination, and increased salivary loss from excessive drooling which may result in depleted ruminal Na^+ reserves and electrolyte imbalances (Dijkstra et al., 2012; Burhans et al., 2022). Theoretically, limitations in ruminal Na^+ availability may limit removal of intracellular protons after SCFA uptake, thus challenging pH_i and the ability to regulate barrier function. The strong coupling between SCFA absorption and active Na^+ transport has been demonstrated (Diernaes et al., 1994; Leonhard-Marek et al., 2010); however, there is limited understanding on how Na^+ supply affects SCFA transport mechanisms. Sehested et al. (1996, 1999) evaluated the relationship between Na^+ and butyrate by measuring butyrate flux at Na^+ concentrations of 5, 10, 30, and 140 m*M* at pH 7.3. They observed that net butyrate flux was unaffected by Na^+ concentration; however, mucosal-to-serosal butyrate flux was stimulated when Na^+ concentration was increased from 5 to 30 m*M*, with no further stimulation at 140 m*M*. Although the mechanisms of SCFA transport have been well characterized, the plausible consequences of a Na^+ deficiency on apical uptake of SCFA have not been elucidated.

Therefore, we hypothesized that increasing ruminal Na^+ concentration during reduced pH may indirectly stimulate apical SCFA uptake thus improving barrier function in isolated ruminal epithelia. The objectives of this study were to investigate whether Na^+ availability limits apical uptake of SCFA during reduced luminal pH and the corresponding effect on the paracellular movement of mannitol across the isolated ruminal epithelia.

MATERIALS AND METHODS

This study was conducted between March 2021 and May 2021 at the University of Saskatchewan in the Livestock Research Building (Saskatoon, SK, Canada). All procedures were preapproved by the University of Saskatchewan's Animal Research Ethics Board and adhered to the guidelines for humane animal use by the Canadian Council of Animal Care (Ottawa, Ontario, Canada).

Animal Feeding Regimen and Housing

Nine weaned Holstein steer calves that were approximately 7 to 9 mo of age were sourced from a single calf grower, but originated from 2 commercial dairy farms, and used in this study. Before euthanasia, calves weighed 322 ± 50.9 kg (mean ± SD) and were consuming 7.05 ± 1.5 kg (mean ± SD) DM of TMR. The number of calves and resulting experimental replication used was based on the ability to detect differences using data from previously published studies in our laboratory (Penner et al., 2010; Wilson et al., 2012; Schurmann et al., 2014). Upon arrival, calves were fed an all-hay diet ad libitum (84.0% DM, 10.6% CP, 59.8% aNDFom and 8.9% ash) for 2 weeks, group housed in an indoor pen bedded with straw, and had free access to water. After 2 weeks of environmental adaptation, all calves were transitioned onto a TMR formulated using the Nutritional Dynamic System (Reggio Emilia, Italy) until euthanasia. The TMR contained (DM basis) 55.4% barley silage, 35.8% pellet (barley grain, canola oil, urea, monensin, vitamins and minerals) and 8.8% chopped straw. The diet contained: 49.7% DM, 11.8% CP, 29.3% starch, 3.9% ether extract providing 17.5 Mcal/d ME, and 643.7 g/d MP and monensin at 22 mg/kg. Calves were fed the TMR for at least 25 d before euthanasia and the TMR was offered ad libitum by ensuring refusals equated to 10% of the weight originally offered. Calves were moved into individual 5-m^2 pens with rubber mats and straw bedding 6 d before euthanasia. The amount of feed provided (0700 h) and refused (0630 h) were recorded daily for each calf while individually housed. Body weight was measured at 0600 h, 6 d before slaughter and on the day of slaughter.

Rumen Tissue and Ruminal Fluid Collection

Calves were slaughtered by captive-bolt stunning, pithing, and exsanguination at 1100 h (4 h after feeding). A single calf was handled on each day to facilitate ex vivo measurements in Ussing chambers. Immediately after confirming death, the abdominal cavity was opened and samples of ruminal digesta and ruminal tissue were collected. Ruminal digesta was mixed before collection of a subsample, which was then strained through 2 layers of cheesecloth. The pH of the strained ruminal fluid was then immediately measured using a

Table 1. Buffer compositions for washing and transport of ruminal epithelial tissue, and ex vivo Ussing chamber experiments

	Transport and barrier function buffer[2]		Bicarbonate-free with nitrate buffer[3]		Bicarbonate-containing buffer[4]	
Substance,[1] m*M*	Low Na^+	High Na^+	Low Na^+	High Na^+	Low Na^+	High Na^+
$CaCl_2$	1	1	0	0	0	0
Ca-gluconate	0	0	1	1	1	1
$MgCl_2$	1.25	1.25	0	0	0	0
Mg-gluconate			1.25	1.25	1.25	1.25
KCl	5.5	5.5	0	0	0	0
K-gluconate	86	0	95.6	5.5	116.1	5.5
NaH_2PO_4	0.6	0.6	0.6	0.6	0.6	0.6
Na_2HPO_4	2.4	2.4	2.4	2.4	2.4	2.4
Na-gluconate	0	86	4.6	94.6	0	110.6
L-Glutamine	1	1	1	1	1	1
HEPES-free acid	10	10	10	10	10	10
$NaHCO_3$	0	24	0	0	4.6	24
$KHCO_3$	24	0	0	0	19.4	0
Na-lactate	4.6	4.6	0	0	0	0
Na-acetate	0	10	0	0	0	0
K-acetate	10	0	0	0	0	0
Na-propionate	0	10	0	0	0	0
K-propionate	10	0	0	0	0	0
Butyric acid	10	10	0	0	0	0
Acetazoleamide	0	0	0.1	0.1	0	0
Glucose	10	10	10	10	10	10
Mannitol	0	0	22	22	22	22
Na-nitrate	0	0	0	40	0	0
K-nitrate	0	0	40	0	0	0
Osmolarity, mOsmol/L	286 ± 4.4	289 ± 1.9	301 ± 3.5	306 ± 1.7	296 ± 0.9	304 ± 1.0

[1]All buffers, with the exception of the buffer used for washing and transport of tissues, contained 60 mg/L penicillin G, 100 mg/L kanamycin, and 50 mg/L flurocytosine.

[2]Low Na^+ concentration buffer to assess mannitol flux was also used to wash and transport the tissue.

[3]Used to determine bicarbonate-independent nitrate-insensitive acetate and butyrate uptake.

[4]Used to determine total acetate and butyrate uptake.

portable pH meter (Accumet AP110, Fischer Scientific, Ottawa, ON, Canada). The pH meter was calibrated daily using standard pH solutions 4 and 7. Thereafter, a subsample of strained ruminal fluid was preserved with 25% (wt/v) metaphosphoric acid, placed on ice, and stored at −20°C until analysis. Ruminal fluid was analyzed for SCFA concentration using gas chromatography (Agilent Technologies Inc., Santa Clara, CA) as described by Khorasani et al. (1996). Additionally, 300 cm^2 of ruminal epithelial tissue was collected from the caudal-dorsal blind sac. The caudal-dorsal blind sac was selected because of the homogeneous papillae size and density to minimize variation. Tissue was washed until clean with preheated (38.5°C) buffer (Table 1) that was adjusted to a pH of 7.4. The buffer was continuously gassed with carbogen (95% O_2 and 5% CO_2). Thereafter, the submucosal and muscular layers were carefully stripped from the epithelium (Aschenbach et al., 2002). The ruminal epithelial tissue was placed in fresh buffer, gassed with carbogen, and transported to the laboratory.

Within 30 min of harvesting, ruminal epithelial tissue was cut into strips which were then mounted in a total of 24 Ussing chambers, each with an exposed surface area of 3.14 cm^2. Incubation conditions were designed to test the main effects of Na^+ concentration, mucosal buffer pH, and their interaction on barrier function of the ruminal epithelia and on the pathways for acetate and butyrate uptake. As buffer solutions differed among treatments (Table 1), chambers were allocated to buffer solutions at the start of each day and tissues were randomly assigned to a chamber with 2 technical replicates for each treatment. After mounting tissues, 30 min were allotted for equilibration of electrophysiology. All tissues were incubated under short-circuit current ($\mathbf{I_{sc}}$) conditions using a computer-controlled voltage-clamp device (Dipl.-Ing. K. Mussler, Scientific Instruments, Aachen, Germany) as previously described by (Aschenbach and Gäbel, 2000). Briefly, each half of the Ussing chamber (mucosal and serosal) were connected using paired Argenthal reference electrodes (Mettler Toledo, Urdorf, Switzerland) bathed in 3 *M* KCl, via agar bridges (3% agar melted in 3M KCL) to measure the transepithelial potential difference. Then with the use of an automatic voltage-clamp device an equal and opposite magnitude of current was passed from Ag-Cl

electrodes connected to each half of the chamber by salt bridges to eliminate passive ion transport via an electrical gradient. Ohm's law was used to determine tissue conductance ($\mathbf{G_t}$) by measuring the impulse-induced change in voltage difference following the application of short bipolar current impulses (100 μA) every 6 s to yield a measured change in the voltage.

Buffer Solutions for Incubation of Ruminal Epithelia

Tissues were exposed to buffers to evaluate the effect of Na^+ concentration, pH, and their interaction in a 2 × 2 factorial treatment arrangement (Figure 1). As such, buffers contained either a high (140 m*M*) or low (10 m*M*) Na^+ concentration achieved by substituting Na^+ and K^+ (Table 1). The same buffer solutions were used for the mucosal and serosal sides to minimize differences in ion gradients across the tissue. The Na^+ concentrations chosen were based on suitability for ex vivo culture and to provide a large magnitude of difference between treatments while keeping other molecule concentrations equal between the serosal and mucosal buffers (K, Cl, Mg, Ca, gluconate, mannitol). Others have used similar Na^+ concentrations in buffer solutions to test their hypotheses. For example, Etschmann et al. (2009) and Schurmann et al. (2014) used Na^+ concentrations of 80 to 95 m*M* to evaluate ruminal Na^+ transport during dietary adaptation. Additionally, Kramer et al. (1996) used the isolated and washed reticulo-rumen technique to determine SCFA disappearance in buffer solutions that contained either 0 or 100 m*M* of Na^+. For pH, buffers used for incubation on the mucosal side were adjusted to a pH of either 7.4 ± 0.03 or 6.2 ± 0.03 using potassium hydroxide or gluconic acid. The chosen pH values were used to result in near maximal rates of acetate and butyrate uptake rather than excessively challenge tissues with low pH thereby increasing tissue permeability. Gaebel et al. (1989) reported that reducing pH below 5.5 in Ussing chambers reduced mucosal-to-serosal Na^+ and Cl^- flux along with inducing cellular alterations that they suggested impaired active transport by inhibiting the NHE. However, Gäbel et al. (1991) reported that reducing pH from 7.3 to 6.5 stimulated Na^+ transport. Therefore, the chosen high pH (7.4) was equivalent to serosal pH commonly used in Ussing chamber studies and a reduction in pH to 6.2 was used as the low pH value. Buffer pH was adjusted and confirmed using a pH meter (Accumet AP110, Fischer Scientific, Ottawa, ON, Canada) that was calibrated on each day using standard pH buffer solutions (pH 4, 7, and 10). All buffers were maintained at 38.5°C using circulating water jackets and were mixed by gas lift. Buffers that contained bicarbonate were gassed with 95% O_2/5% CO_2 and bicarbonate-free buffers (described below) were gassed with 100% O_2. Buffer chemicals and antibiotics were purchased from Sigma-Aldrich Co. (St. Louis, MO).

Measurement of Epithelial Barrier Function

Tissue conductance and the mucosal-to-serosal flux of 1-^{3}H-mannitol ($\mathbf{J_{MS\text{-}mannitol}}$; Perkin-Elmer Inc. Boston, MA) were used to assess tissue barrier function in 8 of the 24 Ussing chambers (Schweigel et al., 2005). After 30 min to allow for stabilization of electrophysiology, ^{3}H-mannitol (111 kBq/15 mL) was added to the mucosal column and isotope was allowed to equilibrate for 45 min before the first sample collection (Sehested et al., 1996). Thereafter, 2 45-min flux periods were used to measure $J_{MS\text{-}mannitol}$. Hot samples (taken from the same side as radioactivity addition; 100 μL) were collected at the beginning of the first flux period and at the end of the second flux period. The samples were then placed in a vial and 400 μL of fresh buffer was added along with 5 mL of scintillation cocktail (Ultima Gold; Perkin-Elmer, Waltham, MA). Samples were allowed to sit for 12 h before reading the ionized radiation by scintillation counting (Tricarb 2910, Perkin-Elmer, Waltham, MA). Buffer samples were collected from the serosal side (500 μL) at the beginning and end of each flux period, 5 mL of scintillation cocktail was added, and samples were analyzed by scintillation counting. After collecting the serosal samples, 500 μL of fresh buffer solution was added to the serosal side to maintain volume and hydrostatic pressure. Subsequently, the $J_{MS\text{-}mannitol}$ was calculated as described Gäbel et al. (1991). The average G_t and I_{sc} for each flux period were summarized and used for statistical analysis.

Determination of Acetate and Butyrate Uptake

Acetate and butyrate uptake were evaluated in 16 of the 24 Ussing chamber using a 2 × 2 factorial arrangement to determine the effect of Na^+ concentration, pH, and their interaction on total uptake, bicarbonate-dependent, and bicarbonate-independent nitrate-insensitive pathways (Aschenbach et al., 2009; Penner et al., 2009). The uptake of acetate and butyrate was determined as previously described by Aschenbach et al. (2002). Briefly, tissues were incubated for 30 min in buffers that contained bicarbonate (for determination of total uptake) or that were free of bicarbonate and contained nitrate (for determination of bicarbonate-dependent nitrate-insensitive uptake; Table 1). These

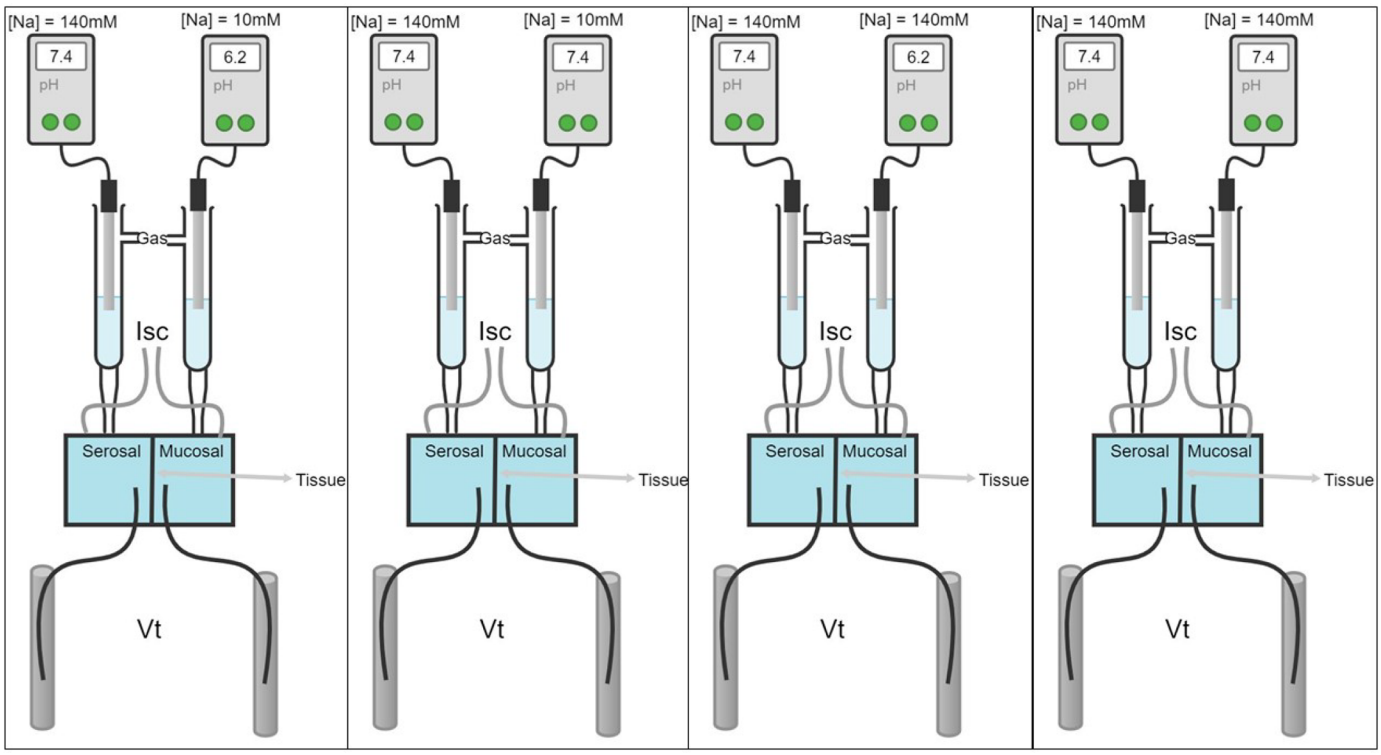

Figure 1. Diagrammatic representation of Ussing chambers and the treatment structure used to test the effect of low versus high Na^+ (10 vs. 140 m*M*) and low and high mucosal pH (6.2 vs. 7.4), and their interaction. Isc = short circuit current; Vt = transepithelial voltage.

buffer solutions did not contain SCFA but rather contained glucose as an energy source. Thereafter, a solution containing acetate and butyrate was added to the mucosal side to achieve final concentrations of 25 m*M* for each SCFA (total of 50 m*M*). The SCFA solutions were spiked with [2-^{3}H]-acetate (100 kBq ^{3}H-acetate/15 mL; Perkin-Elmer Inc. Woodbridge, ON, Canada) and [1-^{14}C]-butyrate (74 kBq ^{14}C-butyrate/15 mL; Moravek Biochemicals Inc., Brea, CA). This concentration (50 m*M*) was chosen because previous work showed that tissues mounted in Ussing chambers exposed to SCFA closer to physiological concentrations of 100 m*M* (60 m*M* acetate, 30 m*M* propionate and 10 m*M* butyrate; Meissner et al., 2017) or greater than 30 m*M* butyrate (Greco et al., 2018) had negative effects on tissue integrity, potentially impairing SCFA uptake. Propionate uptake was not included in this experiment because propionate is intermediate to acetate and butyrate in terms of chain length, lipophilicity, and extent of metabolism by the rumen epithelium (butyrate > propionate > acetate; Aschenbach et al., 2011). Therefore, this experiment along with others (Penner et al., 2009; Schurmann et al., 2014) just evaluated the transport mechanisms of acetate and butyrate, making the inference that propionate uptake is likely in between that of acetate and butyrate. Tissues used ex vivo are much more sensitive and cannot be exposed to similar in vivo conditions without affecting tissue viability. Epithelia were subsequently allowed a 1-min incubation before the buffer was removed, and epithelia were washed 3 times with ice-cold buffer (18 s/wash) to halt protein-mediated transport and remove radioactivity that was not bound to the tissue. Epithelia were then dismounted and transferred to a pre-cooled lysing device with an exposed surface area of 2.84 cm^2. Epithelia were lysed using 4 mL of ice-cold Solvable (Perkin-Elmer, Waltham, MA) for 3 min. The lysate was removed and centrifuged at 3,000 × *g* at 4°C for 15 min to remove cornified epithelial cells from the lysate. Radioactivity of lysates were assessed in duplicate by adding 5 mL of liquid scintillation fluid (Ultima Gold; Perkin-Elmer, Waltham, MA) to 500 μL of sample and using scintillation counting (Tricarb 2910, Perkin-Elmer, Waltham, MA). Protein content of the lysate was measured in triplicate according to the method of Smith et al. (1985). Rates of uptake were calculated as described by Aschenbach et al. (2002) and values were corrected for lysate protein content. The G_t and I_{sc} values obtained during uptake measurements were not used for statistical analysis because buffer solutions did not contain SCFA and, therefore, were not representative of treatment conditions.

Table 2. Effects of ex vivo buffers differing in Na^+ (10 vs. 140 m*M*) and mucosal pH (6.2 vs. 7.4) on the mucosal-to-serosal flux of mannitol ($J_{MS\text{-}mannitol}$), short-circuit current (I_{sc}), and tissue conductance (G_t) in isolated ruminal epithelia obtained from Holstein calves

	High Na^+		Low Na^+			*P*-value		
Variable	High pH	Low pH	High pH	Low pH	SEM[1]	Na^+	pH	Na^+ × pH
$J_{MS\text{-}mannitol}$,[2,3] mmol/(cm^2 × h)	72.88	84.38	75.12	69.69	8.729	0.37	0.66	0.23
I_{sc},[4] mEq/(cm^2 × h)	0.96	0.37	0.09	−0.57	0.120	<0.001	<0.001	0.74
G_t,[2] mS/cm^2	4.86	5.18	8.81	6.98	0.678	<0.001	0.20	0.071

[1]SEM for the interaction is reported.

[2]$J_{MS\text{-}mannitol}$ and Gt were greater in flux period 2 than flux period 1 ($P < 0.001$).

[3]There was a flux × Na interaction, with high Na^+ having lower G_t values between flux 1 and 2 ($P = 0.03$). All values were different from each other ($P < 0.05$).

[4]I_{sc} values are different ($P < 0.05$) from zero.

Calculations and Statistical Analysis

The uptakes of acetate and butyrate are reported as total uptake, bicarbonate-dependent uptake, and bicarbonate-independent, nitrate-insensitive uptake as previously described (Aschenbach et al., 2009; Penner et al., 2009). Briefly, bicarbonate-dependent uptake was calculated as the difference between SCFA uptake in bicarbonate-containing buffer solutions (i.e., total uptake) and SCFA uptake in bicarbonate-free nitrate-containing buffer solutions (i.e., bicarbonate-independent, nitrate-insensitive). The bicarbonate-dependent uptake for butyrate was not calculated as uptake measured in the bicarbonate-free nitrate-containing buffers (i.e., bicarbonate-independent nitrate-insensitive) was numerically equal to or greater than the uptake measured in the bicarbonate-containing buffers (i.e., total uptake).

The $J_{MS\text{-}mannitol}$, G_t, I_{sc}, and the uptake of acetate and butyrate were analyzed as a randomized complete block design with a 2 × 2 factorial treatment design using the MIXED procedure of SAS (version 9.4; SAS Institute). The model for the $J_{MS\text{-}mannitol}$ data and corresponding G_t and I_{sc} included the fixed effect of in vitro treatment (Na^+ concentration and pH) and the 2-way interaction with the random effect of calf. A repeated measures statement was used with in vitro measurement period (2 45-min flux periods) as the repeated measure and tissue within calf included as the subject. Compound symmetry covariance structure was used as this matrix yielded the lowest Akaike's and Bayesian information criterion and was the most appropriate. Uptake of acetate and butyrate included the same fixed effects of Na^+ concentration, pH, and their interaction, with calf as the subject. When the *F*-test was significant, the TUKEY mean separation test was used to determine whether means differed. All data were tested for normality using PROC UNIVARIATE to evaluate the distribution of the presented histogram. Differences between treatments were considered significant when $P < 0.050$. Data were reported as means ± SEM. The studentized residuals for G_t of tissues used in flux measurements depicted increasing residual variance with increasing predicted mean. The following data transformations were tested: log, square root, fifth root and second power; however, the tested transformations did not improve the residual distribution and, therefore, the untransformed data are presented. The 3-way interaction of flux × Na^+ × pH was not statistically significant ($P > 0.050$) for $J_{MS\text{-}mannitol}$, G_t, or I_{sc} and is not presented in tables. Electrophysiology during uptake measurements were not reported because tissues were not exposed to normal physiological conditions (without SCFA), thus making interpretation of the results misleading.

RESULTS

The mean ruminal SCFA concentration was 98.7 m*M* (mean ± 23.11) and the molar proportions of individual SCFA were (mol/100 mol): acetate, 59.1 ± 5.52 propionate, 21.8 ± 4.60; isobutyrate 0.8 ± 0.17; butyrate, 13.7 ± 2.07; isovalerate, 2.3 ± 0.42; valerate 1.7 ± 0.35; and caproate, 0.7 ± 0.13. Mean ruminal pH at the time of euthanasia was 6.61 ± 0.42.

Epithelial Barrier Function

We observed no interaction between Na^+ concentration and pH for $J_{MS\text{-}mannitol}$ (Table 2). However, $J_{MS\text{-}mannitol}$ was greater in flux period 2 than flux period 1 [94.7 vs. 56.6 mmol/(cm^2 × h); $P < 0.001$, data not shown]. Tissues exposed to a high Na^+ concentration had greater I_{sc} compared with those exposed to low Na^+ concentration ($P < 0.001$). Additionally, low pH reduced Isc [−0.10 μEq/(cm^2 × h); $P < 0.001$]. A flux period × Na^+ concentration interaction was detected for G_t, with tissues exposed to a high Na^+ concentration having a lower G_t value and a smaller change in G_t from flux period 1 to flux period 2 than low Na^+ ($P = 0.030$, Figure 2).

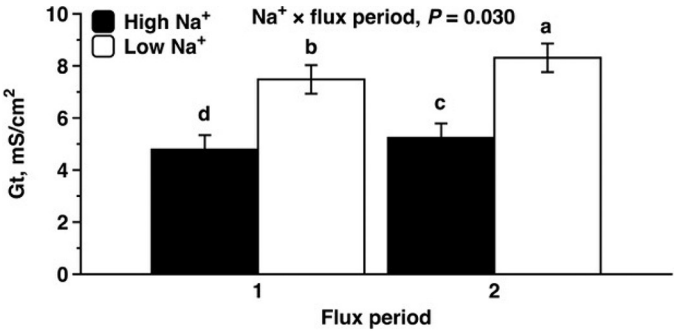

Figure 2. The interaction between Na^+ (10 vs. 140 m*M*) and flux period (2 consecutive 45-min periods) on tissue conductance (G_t) of isolated ruminal epithelium from Holstein steers. Error bars represent the SEM.

Acetate and Butyrate Uptake

Apical acetate and butyrate uptakes were not affected by Na^+ concentration or the Na^+ × pH interaction (Table 3). Total and bicarbonate-dependent uptakes of acetate ($P \leq 0.012$) were stimulated when mucosal pH was reduced from 7.4 to 6.2, but bicarbonate-independent, nitrate-insensitive acetate uptake only tended to increase when mucosal pH was reduced to 6.2 ($P = 0.095$). Similarly, total butyrate ($P < 0.001$) uptake was greater at pH 6.2. We observed no pH effect on bicarbonate-independent, nitrate-insensitive butyrate uptake ($P = 0.62$).

DISCUSSION

In the present study, we observed no effect of increasing Na^+ concentration or interactions between Na^+ concentration and buffer pH on acetate or butyrate uptake. Past research has focused on flux-based measurements and reported a positive correlation between the mucosal-to-serosal SCFA and Na^+ flux (Gäbel et al., 1991; Sehested et al., 1999; Schurmann et al., 2014). Reasoning for the consistently observed coupling of SCFA and Na^+ flux is proposed to be mediated through increased passive diffusion of undissociated SCFA, thus requiring upregulation of NHE1 or basolateral NHE2 and NHE3 to regulate pH_i (Sehested et al., 1996; Gäbel et al., 2002; Yang et al., 2012). Given that apical uptake is likely to represent the rate-limiting component for SCFA transport (Penner et al., 2009; Stumpff, 2018), differences might be more apparent when assessing uptake rather than flux. However, we did not observe an effect of Na^+ concentration or an interaction between Na^+ concentration and mucosal pH on acetate or butyrate uptake. Results of our study suggest that Na^+ supply does not appear to affect SCFA uptake, at least when pH is reduced to 6.2. In support of our results, Kramer et al. (1996) used the isolated and washed reticulo-rumen technique in sheep and demonstrated no effect of increasing Na^+ concentrations from 0 m*M* to 100 m*M* on SCFA disappearance from the reticulo-rumen. Furthermore, those authors used theophylline (an inhibitor of NHE) and reported a reduction in Na^+ absorption without any effect on SCFA disappearance. Sehested et al. (1996) reported increased $J_{MS\text{-}butyrate}$ at pH 7.3 when Na^+ concentration was increased from 5 to 30 m*M*, although we observed no further increases in $J_{MS\text{-}butyrate}$ when Na^+ was increased from 30 to 140 m*M*. The latter studies suggest a stimulatory effect of SCFA on Na^+ absorption; however, the results of the present support the findings of Sehested et al. (1996)

Table 3. Effects of ex vivo buffers differing in Na^+ concentrations (10 vs. 140 m*M*) and mucosal pH (6.2 vs. 7.4) on apical uptake of ^{3}H-acetate and ^{14}C-butyrate in isolated ruminal epithelia obtained from Holstein calves

	High Na^+		Low Na^+			*P*-value		
Variable	High pH	Low pH	High pH	Low pH	SEM[1]	Na^+	pH	Na^+ × pH
Acetate uptake, nmol × mg_{prot} × min^{-1}								
Total[2]	54.5	69.3	55.0	80.7	8.18	0.25	<0.001	0.29
Bicarbonate-dependent[3]	13.0	23.4	11.2	30.1	8.30	0.65	0.012	0.44
Bicarbonate-independent, nitrate-insensitive[4]	41.5	45.9	43.7	50.7	4.76	0.29	0.095	0.70
Butyrate uptake,[5] nmol × mg_{prot} × min^{-1}								
Total[2]	54.3	81.6	51.1	88.0	8.24	0.80	<0.001	0.47
Bicarbonate-independent, nitrate-insensitive[4]	68.9	67.9	61.0	67.8	7.31	0.50	0.62	0.51

[1]SEM for the interaction is reported.

[2]Total acetate and butyrate uptakes represent total uptake of ^{3}H-acetate and ^{14}C-butyrate in the presence of bicarbonate.

[3]Bicarbonate-dependent acetate uptake represents the uptake of ^{3}H-acetate requiring bicarbonate, calculated as the difference between total and bicarbonate-independent uptake.

[4]Bicarbonate-independent, nitrate-insensitive acetate and butyrate uptakes represent uptakes of ^{3}H-acetate and ^{14}C-butyrate in the absence of bicarbonate and in the presence of nitrate. This uptake represents the noninhibitable uptake.

[5]Bicarbonate-dependent uptake for butyrate was not reported because bicarbonate-independent nitrate-insensitive uptake was greater than total uptake.

suggesting that luminal Na^+ concentration does not limit apical uptake of SCFA but could possibly mediate transport processes on the basolateral membrane.

Fractional absorption rates among the 3 major SCFA are comparable at physiological ruminal pH; however, as pH declines butyrate absorption increases up to approximately 2 times that of acetate (Aschenbach et al., 2011; Stumpff, 2018). We observed that reducing mucosal pH from 7.4 to 6.2 increased bicarbonate-dependent and total uptake of acetate and the total uptake of butyrate with no response for the noninhibitable uptake of acetate and butyrate (i.e., bicarbonate-independent, nitrate-insensitive). The stimulatory effect of reduced pH on anion exchange transport is a well-documented response ex vivo (Kramer et al., 1996; Aschenbach et al., 2009), and is likely a result of low mucosal pH increasing the HCO_3^- gradient between the cytosol and the lumen driving anion exchange (Aschenbach et al., 2011). The increase in SCFA uptake at a pH of 6.2 versus 7.4 observed in the present study would be expected to impose a greater challenge to pH_i without inducing severe acidotic conditions that may damage the isolated epithelia and compromise SCFA transport. As such, severe reductions in mucosal pH have been reported to eliminate the stimulatory effect of low pH on SCFA absorption and can compromise cellular homeostasis and elicit compensatory mechanisms that reduce SCFA transport (Penner et al., 2009; Wilson et al., 2012; Meissner et al., 2017). Gaebel et al. (1989) demonstrated that reducing pH to 5.5 in Ussing chambers reduced mucosal-to-serosal Na^+ and Cl^- flux and caused cellular alterations. However, Gäbel et al. (1991) reported that reducing pH from 7.3 to 6.5 stimulated Na^+ transport. Hence, in the present study the low pH value of 6.2 was chosen to promote SCFA and Na^+ absorption and the greater acetate and butyrate uptake confirm the model was sufficient in increasing SCFA transport rates. It can be argued that a pH of 6.2 may have limited the amount of protonated SCFA (Aschenbach et al., 2011) reducing the requirement for upregulation of NHE to expel protons back into the lumen, thereby minimizing the potential effects of Na^+ concentration. However, decreasing luminal pH from 7.4 to 6.2 theoretically would increase the proportion of undissociated acetate and butyrate by a factor of 15. Therefore, the absent effect of reducing pH on stimulating passive diffusion challenges the dogma that low pH drives the permeation of HSCFA into the epithelia requiring NHE to maintain pH_i but rather, that apical $SCFA^-/HCO_3^-$ exchange and basolateral Na^+/HCO_3^- cotransport work simultaneously for maintenance of pH_i. In agreement with Aschenbach et al. (2009), the present study provides support for the notion that low pH modulates apical uptake of SCFA via transporter-mediated mechanisms to a greater extent than passive diffusion without a further stimulatory effect of Na^+ concentration.

The cells within the ruminal epithelium operate as a syncytium to absorb SCFA transcellularly while providing a selectively permeable barrier to prevent paracellular translocation of bacteria and antigens (Aschenbach et al., 2019). Previous ex vivo experiments have focused on the strong coupling between Na^+ and SCFA transport (Gäbel et al., 1991; Sehested et al., 1996, 1999) and mRNA expression of transport proteins (Schweigel et al., 2005; Schurmann et al., 2014) with minimal experimentation on how Na^+ concentration may affect ruminal barrier function. In the present study, Na^+ concentration had no effect on the $J_{MS\text{-}mannitol}$ under reduced pH conditions further supporting the notion that Na^+ may not affect permeability under iso-osmotic conditions. Interestingly, a flux period by Na^+ interaction was detected, with elevated Na^+ reducing G_t and minimizing the magnitude of increase in G_t from flux period 1 to flux period 2 and tending to reduce the change in I_{sc} from flux period 1 to flux period 2. Increasing G_t over time is a normal observation in Ussing chambers (Penner et al., 2010; Wilson et al., 2012) and is likely a response of decreasing tissue viability over time. These findings support the hypothesis that Na^+ may help stabilize tissue integrity in the short term with possible long-term effects on barrier function. The outcome of higher I_{sc} and elevated Na^+ concentration implies a net movement of cations from the mucosal-to-serosal bathing solutions, which could indicate increased cation absorption or increased anion (HCO_3^-) secretion. However, in the present study it is difficult to decipher whether cation absorption or anion secretion is the leading cause resulting in the observed difference in I_{sc}. Moreover, it should be recognized that the ex vivo tissue culture system does not prevent accumulation of metabolites in the buffer solutions resulting in differing conditions than occurs in vivo due to blood flow.

The stabilization of G_t could be related to improved stabilization of pH_i associated with proton neutralization via basolateral Na^+/HCO_3^- symport (Aschenbach et al., 2009) or proton extrusion via NHE isoforms and MCT1 (Aschenbach et al., 2011). Under normal in vivo conditions, arterial HCO_3^- (21–27 mEq/L; Tucker et al., 1988; Sanchez et al., 1994) and Na^+ (140–160 mEq/L; Tucker et al., 1988; West et al., 1992) concentrations are tightly regulated and may compensate for reduced ruminal Na^+ concentrations to provide adequate substrates (Na^+ and HCO_3^-) for SCFA absorption and maintenance of pH_i. As increasing the Na^+ concentration reduced G_t within both flux period and minimized the change in G_t between flux period 1 and flux period 2, our data partially support the notion that Na^+ plays

a role in maintaining tissue integrity likely through regulating pH_i. The lack of difference for $J_{MS\text{-}mannitol}$ in response to Na^+ concentration could be that the allotted time (2 45 min flux periods) was insufficient for pH to elicit an effect on mannitol permeation, given that high Na^+ reduced G_t over time. Additionally, Penner et al. (2010) demonstrated that during a mucosal acid challenge (from pH 6.1 to 5.2) we observed no change in G_t and $J_{MS\text{-}mannitol}$ within 60 min after an acidic challenge but showed an increase in G_t and $J_{MS\text{-}mannitol}$ during the recovery period after removal of the acidic challenge (120 to 180 min following the acidic insult). They concluded that the delayed onset of paracellular movement was a result of cell swelling, masking the opening of the paracellular space due to conformational changes in tight junction proteins and cellular damage (Gaebel et al., 1989). Although the pH values in the latter study were different than the ones tested in the present study, it is plausible that the effects of low pH could be more prominent following resolution of low pH conditions and with extended durations of measurement (Penner et al., 2010). Regardless, the lacking effect of Na^+ on the $J_{MS\text{-}mannitol}$ suggests that given an adequate supply of luminal or arterial substrates (Na^+, HCO_3^-) the ruminal epithelium may be capable of regulating SCFA absorption to maintain barrier function.

CONCLUSIONS

In summary, Na^+ supply does not appear to limit apical SCFA transport mechanisms measured ex vivo resulting in no additional protective effect against the permeation of mannitol; however, increasing Na^+ concentration may help to maintain tissue integrity over time. The results presented lead to the interpretation that the rumen epithelium is a dynamic tissue with the ability to extract available substrates from mucosal or serosal supply to drive SCFA absorption while maintaining barrier function, but these mechanisms do not appear to be contingent on luminally available Na^+.

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