Acidic pH and short-chain fatty acids activate Na⁺ transport but differentially modulate expression of Na⁺/H⁺ exchanger isoforms 1, 2, and 3 in omasal epithelium

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ABSTRACT

Low sodium content in feed and large amounts of salivary sodium secretion are essential requirements to efficient sodium reabsorption in the dairy cow. It is already known that Na⁺/H⁺ exchange (NHE) of the ruminal epithelium plays a key role in Na⁺ absorption, and its function is influenced by the presence of short-chain fatty acids (SCFA) and mucosal pH. By contrast, the functional role and regulation of NHE in omasal epithelium have not been completely understood. In the present study, we used model studies in small ruminants (sheep and goats) to investigate NHE-mediated Na⁺ transport and the effects of pH and SCFA on NHE activity in omasal epithelium and on the expression of NHE isoform in omasal epithelial cells. Conventional Ussing chamber technique, primary cell culture, quantitative PCR, and Western blot were used. In native omasal epithelium of sheep, the Na⁺ transport was electroneutral, and it was inhibited by the specific NHE3 inhibitor 3-[2-(3-guanidino-2-methyl-3-oxo-propenyl)-5-methyl-phenyl]-N-isopropylidene-2-methyl-acrylamide dihydrochloride, which decreased mucosal-to-serosal, serosal-to-mucosal, and net flux rates of Na⁺ by 80% each. The application of low mucosal pH (6.4 or 5.8) in the presence of SCFA activated the Na⁺ transport across omasal epithelium of sheep compared with that at pH 7.4. In cultured omasal epithelial cells of goats, mRNA and protein of NHE1, NHE2, and NHE3 were detected. The application of SCFA increased NHE1 mRNA and protein expression, which was most prominent when the culture medium pH decreased from 7.4 to 6.8. At variance, the mRNA and protein expression of NHE2 and NHE3 were decreased with low pH and SCFA, which was contrary to the published data from ruminal epithelial studies. In conclusion, this paper shows that (1) NHE1, NHE2, and NHE3 are expressed in omasal

epithelium; (2) NHE3 mediates the major portion of transepithelial Na⁺ transport in omasal epithelium; and (3) SCFA and acidic pH acutely activate Na⁺ transport but suppress the expression of NHE2 and NHE3 in the longer term. By contrast, the expression of NHE1 is increased by SCFA and acidic pH, indicating a prominent role for NHE1 in the regulation of intracellular pH of omasal epithelium. Our results suggest a regulatable Na⁺ absorption in ruminal and omasal epithelium. It is of benefit for intracellular pH homeostasis and highly relevant to dairy cows fed on high-concentrate diets.

Key words: Na⁺/H⁺ exchanger, mRNA and protein, short-chain fatty acids and pH, omasal epithelial cell

INTRODUCTION

In the ruminant forestomach (especially its reticulo-rumen compartment), the ingesta are broken down by resident anaerobic microbes through fermentation. The major fermentation products are short-chain fatty acids (SCFA, p*K*_a ~4.8, e.g., acetic, propionic, and butyric acid). A currently emerging area with crucial importance for mammalian health is determining the manner in which such metabolites and nutrients derived from commensal bacteria regulate host growth, function, and homeostasis (Brestoff and Artis, 2013), including forestomach epithelial functions and morphology (Penner et al., 2011; Martens et al., 2012). Previous studies have shown that gene expression in ruminal epithelium is altered significantly by high-concentrate intake (Taniguchi et al., 2010; Bondzio et al., 2011; Penner et al., 2011), subacute ruminal acidosis (Steele et al., 2011a), as well as ruminal acidic pH and SCFA (Yang et al., 2012; Yan et al., 2014). However, little is known about how these conditions affect epithelial function and homeostasis in the omasum, the compartment located between the reticulorumen and the abomasum. The omasum contributes significantly to the absorption of SCFA, water (von Engelhardt and Haufler 1975; Holtenius and Björnhag, 1989), Na⁺ (Martens and Gäbel, 1988), Mg²⁺ (Edriss and Smith, 1986), and HCO₃⁻

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(Wegeler, 2008) from the ruminal outflow, as well as to the secretion of Cl^- from the cytoplasm to the omasal lumen. As such, the omasum plays an important role in the electrolyte homeostasis of ruminants.

The role of the omasum in electrolyte homeostasis appears especially important with regard to Na^+ homeostasis of high-yielding dairy cows. Assuming that daily salivary secretion increases to ~ 250 L/d during lactation (Maekawa et al., 2002; Aschenbach et al., 2011) with a salivary sodium concentration of up to ~ 140 mM (Grace, 1988), dairy cows may direct up to 35 mol/d of salivary Na^+ into the forestomachs, which is more than their whole body content of Na^+ . This implies that an effective and rapid reabsorption of Na^+ in the proximal parts of the gastrointestinal tract is needed to prevent Na^+ depletion. To support the physiological needs, dairy cows invert a net disappearance of Na^+ from the portal-drained viscera into a net Na^+ appearance within the first weeks of lactation (Reynolds et al., 1991).

It is well known that the absorption of Na^+ is coupled with the absorption of SCFA in rumen (Gäbel et al., 1991b; Schurmann et al., 2014) and omasum (Ali et al., 2006). The absorption of SCFA in the forestomach epithelium occurs via 2 mechanisms: the undissociated form is absorbed via lipophilic diffusion (Gäbel et al., 2002; Ali et al., 2006; Aschenbach et al., 2011), whereas the dissociated form (SCFA^-) is absorbed via $\text{SCFA}^-/\text{HCO}_3^-$ exchange (Gäbel et al., 1991a; Aschenbach et al., 2009, 2011). Both mechanisms cause an increase of proton concentration in the epithelial cytoplasm and a decrease of intracellular pH (pH_i ; Müller et al., 2000; Aschenbach et al., 2011). The Na^+/H^+ exchangers (NHE) facilitate the exchange of one extracellular Na^+ for one intracellular H^+ . The NHE contributes not only to the maintenance of pH_i homeostasis (Müller et al., 2000), cell volume regulation, and fluid balance, but also to the transcellular absorption of NaCl and, in omasum, NaHCO_3 (Martens et al., 1991; Zachos et al., 2005). The mRNA of *NHE1*, *NHE2*, and *NHE3* has already been demonstrated in the ruminal epithelium of sheep (Schweigel et al., 2005) and goat (Yang et al., 2012; Yan et al., 2014). Ruminal NHE proteins have been detected in different epithelial strata of ovine (Rabbani et al., 2011) and bovine rumen (Graham et al., 2007; Rabbani et al., 2011). The functional importance of NHE for ruminal Na^+ transport has been demonstrated by the application of high concentrations of amiloride (1 mM mucosal side), a relatively unspecific inhibitor of NHE, which reduced mucosal-to-serosal flux rate of Na^+ by 70% (Martens and Gäbel 1988). In omasal epithelium, electroneutral Na^+ transport has also been suggested to occur via NHE (Martens and Gäbel, 1988; Ali et al., 2006), but the involved isoforms have not yet been

identified. Hence, the aim of the current study was to identify Na^+ transport by NHE3 and the modulation of NHE1, NHE2, and NHE3 by the luminal factors SCFA and pH, with established small-ruminant models. To address the first objective, we studied the effects of the NHE3-specific blocker {3-[2-(3-guanidino-2-methyl-3-oxo-propenyl)-5-methyl-phenyl]-*N*-isopropylidene-2-methyl-acrylamide dihydrochloride; **S3226**} and the effects of acidic pH and SCFA on NHE-mediated Na^+ transport in native omasal epithelium of sheep by the conventional Ussing chamber technique. To address the second objective, the effects of acidic pH and SCFA on *NHE1*, *NHE2*, and *NHE3* mRNA and protein expression were studied in primary culture of omasal epithelial cells of goats by quantitative PCR (qPCR) and Western blot.

MATERIALS AND METHODS

Ethical Approval

All experiments on sheep were performed according to German laws for the protection of animals and were approved by the local authorities, the Landesamt für Gesundheit und Soziales, Berlin (Permit: T0064/99).

Experimental Design

Two experiments were designed. The Ussing chamber technique was used to study the responses of Na^+ transport in native omasal epithelium of sheep to acute acidic pH and SCFA treatment. Primary cell culture of omasal epithelial cells of goats was performed to determine the long-term effects of SCFA and acidic pH on mRNA and protein levels of NHE isoforms 1, 2, and 3.

Study on Na^+ Transport

Animals. For Ussing chamber experiments, the omasal epithelial tissues were obtained from adult sheep (*Ovis aries*, $n = 78$) of both sexes. The sheep had free access to a commercially available lick stone (containing Na, Mg, Ca, K, P, SO_4^{2-} , Fe, Cu, Mn, Zn, and so on for mineral supplement) and tap water, and were fed on hay ad libitum at least 3 wk before the experiment.

Isolation and Incubation of Omasal Epithelium. The experiments were essentially performed as described previously (Ali et al., 2006). The sheep for experiments were stunned by captive bolt and killed by exsanguination. The forestomach, including the abomasums, was taken out of the abdominal cavity within 2 to 3 min after killing. The omasum was separated from the reticulum and the abomasum. Six to eight

large leaves were removed from the wall of the omasum and cleaned by buffer solution 1, which was composed of (in mM) 115 NaCl, 25 NaHCO_3 , 1.0 NaH_2PO_4 , 2.0 Na_2HPO_4 , 5 KCl, 8 glucose, 1.0 CaCl_2 , 1.0 MgCl_2 , and 2.5 glutamine. The solution had a pH of 7.4 and a temperature of 38.0°C , and the osmolality was adjusted to 280 mM by mannitol. It was gassed with 95% O_2 –5% CO_2 . The collected tissues, bathed in buffer solution 1, were taken to the laboratory within 30 min. One piece of the epithelium (3×3 cm) was mounted between the 2 halves of an Ussing chamber to give an exposed area of 3.14 cm^2 . The mounted tissue was bathed on each side with 16 mL of serosal (i.e., blood side) buffer solution (buffer 1) by using a gas-lift system driven by a mixture of O_2 – CO_2 (95%–5%) at 38°C . After a 20- to 30-min equilibration period, the mounted tissues with a conductance not more than $6.0\text{ mS}\cdot\text{cm}^{-2}$ and short-circuit current (I_{sc}) not less than $1.0\text{ }\mu\text{eq}\cdot\text{cm}^{-2}\cdot\text{h}^{-1}$ were paired to determine the Na^+ flux rates. For each set of experiments, 6 pairs of epithelia (when 12 chambers were used) or 12 pairs (when 24 chambers were used) were available at the same time and allocated to the experimental groups. In all Ussing chamber experiments, the buffer solution 1 was used as the serosal buffer. The buffer solution on the mucosal side (buffer 2) contained (in mM) 55 NaCl, 25 NaHCO_3 , 1.0 NaH_2PO_4 , 2.0 Na_2HPO_4 , 10 KCl, 8 glucose, 25 Na-acetate, 10 Na-propionate, 5 Na-butyrate, 2.5 glutamine, 1.0 CaCl_2 , 1.0 MgCl_2 , and 15 mannitol. Where applicable, the pH of the mucosal solution was acidified by tris (hydroxymethyl) aminomethane (Tris)-HCl.

Electrical Measurements. Electrical measurements were continuously obtained from a computer-controlled voltage-clamp device (Mussler, Aachen, Germany). Modified tips filled with KCl-agar were positioned ~ 3 mm from each surface of the tissue and connected to Ag-AgCl electrodes for measurement of the transepithelial potential difference. Similar tips were inserted ~ 2 cm from the surface of the tissue for the application of clamp current. The tissues were incubated under short-circuit conditions, that is, with transepithelial potential difference clamped to zero. Under these conditions, the clamp current completely neutralizes the electrogenic charge transfer (I_{sc}) by the epithelium; the height of the clamp current can thus be used to determine I_{sc} . Transepithelial conductance was calculated by measuring the displacements in transepithelial potential difference, caused by the application of a bipolar pulse of $100\text{ }\mu\text{A}$ and 1 s duration.

Measurements and Calculation of Na^+ Flux Rates. To the hot (i.e., labeled) side of the epithelium, $^{22}\text{Na}^+$ (70 kBq) was added. After a 10-min equilibration period, the first hot sample (0.1 mL) was taken

from the hot side in each chamber. After 30 min, cold samples (P_0 to P_3 , 1 mL) were taken from the contralateral cold side every 30 min. Together with P_3 , a second hot sample was taken from the hot side. The sample volume of cold samples was replaced by the corresponding buffer. The radioactivity of samples was measured by a β -counter (LKB; Wallac-PerkinElmer, Überlingen, Germany). To calculate mucosal-to-serosal flux rates ($J_{\text{ms}}\text{Na}$) and serosal-to-mucosal flux rates ($J_{\text{sm}}\text{Na}$) of Na^+ , the increase in radioactivity on the cold side was calibrated to the mean of the specific activity of the 2 hot samples. The net Na^+ flux rate ($J_{\text{net}}\text{Na}$) was calculated as $J_{\text{net}}\text{Na} = J_{\text{ms}}\text{Na} - J_{\text{sm}}\text{Na}$. If not stated otherwise, flux-rate data represent mean values of 3 consecutive flux periods.

Setups of Ussing Chamber Experiments

Na^+ Transport. Omasal tissues (8) were collected from 2 sheep and mounted in Ussing chambers with buffer solution 2 adjusted to pH 6.4 on the mucosal side. After the electrical parameters were stable, Na^+ fluxes were measured in epithelial pairs.

Influence of NHE3 Inhibitor. Two sets of experiments were performed with omasal and ruminal epithelium, respectively. In the first set of experiments, a total of 6 pairs of omasal tissues from 2 sheep were randomly allocated into a S3226 group and a control group (3 pairs each). The highly selective blocker of NHE3, S3226 ($1\text{ }\mu\text{M}$), was added to the mucosal side (buffer solution 2, pH 6.4) of the S3226 group 30 min before the first flux period. In the second experimental set, 6 pairs of ruminal epithelium from 2 sheep were randomly allocated into an S3226 group and a control group (3 pairs each). The method of S3226 treatment was the same as that in the first set of inhibitory experiments.

Effect of Luminal pH. Tissues were randomly allocated into 3 groups with mucosal buffer 2 adjusted to pH 7.4, 6.4, and 5.8 (7 or 9 pairs each). Flux rate data of this experimental series represent mean values of 4 consecutive flux periods.

Omasal Epithelial Morphology and Cell Culture in Goats

The experiments on goats were approved by the Animal Care and Use Committee of Nanjing Agricultural University. The experiments were approved under the act "The State Science and Technology Commission of P. R. China, 1988."

Collection of Omasal Samples from Goats. The goats for experiments were stunned by captive bolt and

killed by exsanguination. The omasum was separated from reticulorumen, abomasum, and connective and adipose tissue. Leaves for the observation of omasal epithelial morphology were collected and fixed in 4% formaldehyde solution. For cell culture, omasal laminae were isolated from the underlying muscular sheets. The epithelia were sunk into ice-cold PBS (pH 7.4) and transferred to the cell culture laboratory.

Morphological Observations on Omasal Epithelium. After fixation for 24 h in 4% formaldehyde solution, the samples of omasal laminae were rinsed in water, dehydrated in graded concentrations of ethanol (30, 50, 70, 90, 95, and 100%), cleared in dimethyl benzene, and then saturated with and embedded in paraffin. For light microscopic observations (Olympus microscope DX-53, Shinjuku-ku, Tokyo, Japan), cross sections (5 μ m in thickness) were cut perpendicularly to the long axis and stained with hematoxylin and eosin.

Cell Culture. To obtain individual epithelial cells, the omasal epithelium was repeatedly digested with 0.25% trypsin in D-Hanks' balanced salt solution with antibiotics until individual epithelial cells appeared in the digestion solution. Cells were collected and washed with culture medium and antibiotics, and their viability (>98%) was confirmed with trypan blue staining. Cell density was adjusted to 1×10^6 cells/mL in high-glucose (4,500 mg/L) Dulbecco's modified Eagle's medium (GIBCO BRL, Grand Island, NY) with 10% fetal bovine serum (FBS, Sijiqing, Hangzhou, China), 2 mM L-glutamine (Sunshine, Nanjing, China), 100 U/mL of penicillin, and 100 mg/mL of streptomycin. The cells were then seeded into 100-cm² plastic tissue culture flasks (Corning, New York, NY) and incubated at 37°C in an incubator (5% CO₂) for 24 h. Thereafter, the cells were treated with a medium of pH 7.4 or 6.8 in the absence or presence of 20 mM SCFA in the medium. After an incubation period of 24 h, the cells were harvested by scraping and transferred to -80°C for storage until they were analyzed for mRNA level of NHE. The 20 mM of SCFA treatment included sodium acetate, sodium propionate, and sodium butyrate at concentrations of 12, 5, and 3 mM, respectively (Merck, Darmstadt, Germany). The data for the cell culture are representative of the independent experiments performed for the cultured omasal epithelial cells that were collected from each of the 4 goats.

RNA Isolation and Quantitative Real-Time PCR

Total RNA was isolated from cultured cells of goats with acid guanidinium thiocyanate-phenol-chloroform (Chomczynski and Sacchi, 2006). The RNA concentration and purity were assessed as described by Yang et

al. (2014). The RNA concentration was quantified by measuring absorbance at 260 and 280 nm using a Bio-photometer (Eppendorf, Hamburg, Germany). The absorption ratio (260/280 nm) of all samples was between 1.8 and 2.0. Aliquots of RNA samples were subjected to electrophoresis through a 1.4% agarose-formaldehyde gel to verify integrity. Concentration of RNA was adjusted to 1 μ g/ μ L based on optical density, and the RNA was stored at -80°C (Yang et al., 2012). By using MMLV-reverse transcriptase, cDNA was synthesized according to the manufacturer's instructions (Fermentas, Burlington, Canada). Relative mRNA expression was analyzed by quantitative real-time PCR (qPCR) by using the MyiQ2 2-color real-time PCR detection system (Bio-Rad Laboratories Inc., Hercules, CA). Prior to target gene quantification, 3 common ovine housekeeping genes (*GAPDH*, 18S, and β -actin) were tested (Steele et al., 2011b). Gene *GAPDH* exhibited a lower standard deviation (0.40) and variance (0.16) compared with the other 2 housekeeping genes (0.58 and 0.31 for 18S, 1.60 and 2.55 for β -actin) and was, therefore, used as the single internal control to normalize the qPCR results. Real-time PCR was performed in a total volume of 20 μ L containing $1 \times$ iQ SYBR Green Supermix (Bio-Rad Laboratories Inc.), a mixture of forward and reverse primers (500 nM each), 1 ng of cDNA template, and sterile water. An initial cycle at 95°C for 30 s was used to denature the cDNA. This was followed by 45 PCR cycles consisting of a denaturation step at 95°C for 5 s and primer annealing and extension at 54°C for 30 s (NHE1 and NHE2) or 60°C for 30 s (NHE3).

The PCR primers used in this experiment (Table 1) were chosen from previous publications. The individual NHE isoforms are highly conserved among different species, for example, mammalian NHE1 homologs have 93 to 96% amino acid identity (Fliegel and Frolich, 1993). Therefore, primers of sheep NHE1 and NHE3 described by Etschmann et al. (2006) were used in the present experiment. The latter authors had conducted sensitivity, specificity, and efficiency of quantitative real-time PCR assays by using 10-fold cDNA dilution series and subsequent sequence analysis of the resulting PCR products. For NHE2, the primer set of bovine NHE2 described by Graham et al. (2007) was used. In our preliminary experiments, the resulting qPCR product of NHE2 was sequenced (GenBank: KT 223566) by Shanghai Invitrogen Biological (Shanghai, China). This sequence was compared with goat gene sequences (GenBank: XM_005686441.1) by using the Blast program and showed a 99% sequence identity. Furthermore, the sizes of NHE1, NHE2, and NHE3 products were confirmed by agarose gel electrophoresis. The sizes of de-

Table 1. Oligonucleotide primers for reference and target genes expressed in goat omasal epithelial cells in primary culture

Gene	Primer sequence ¹	Reference	Size (bp)
<i>GAPDH</i>	F: 5-TTGTCTCCTGCGACTTCA-3 R: 5-CCACCACCCTGTTACTGTT-3	HM043737.1	135
<i>NHE1</i>	F: 5-CCTCTACAGCTACATGGCCTAC-3 R: 5-GGGAGATGTTGGCTTCCA-3	Etschmann et al., 2006	113
<i>NHE2</i>	F: 5-TTGGAGAGTCCCTGCTGAAC-3 R: 5-GGCCGTGATGTAGGACAAAT-3	Graham et al., 2007	257
<i>NHE3</i>	F: 5-AGCTACGTGGCCGAGGG-3 R: 5-AGACAGAGGCCTCCACGGT-3	Etschmann et al., 2006	121

¹F = forward; R = reverse.

tected bands were consistent with their expected sizes by previous reports (Table 2; *NHE1*: 113 bp, *NHE2*: 257 bp, and *NHE3*: 121 bp).

Before qPCR were performed, the amplification efficiencies of all primers were calculated by using a standard dilution series. For each qPCR sample, a melting curve analysis was performed. Gene expression was normalized to *GAPDH* mRNA ($\Delta\text{Ct} = \text{Ct Target} - \text{Ct GAPDH}$). The relative expression values, compared with the control group, were calculated by using the $2^{-\Delta\Delta\text{Ct}}$ method, as described by Livak and Schmittgen (2001). All samples were analyzed in triplicate.

Western Blot Analysis

Protein was extracted from cultured omasal epithelial cells by using RIPA lysis buffer (PR11101, Yuan-PingHao Bio, Beijing, China) according to the manufacturer's recommendations. Protein concentration was determined by the bicinchoninic acid method (PT0102, Beyotime Institute of Biotechnology, Shanghai, China), using bovine serum albumin as a standard. The protein samples were solubilized in $5 \times$ SDS sample buffer (40 mM Tris-HCl, 4 mM EDTA, 10% SDS, 40% glycerol, 0.04% bromophenol blue, and 8% β -mercaptoethanol) and boiled for 5 min before SDS-PAGE in 10% separating gels. Proteins were then electro-transferred to

0.45 μM polyvinylidene fluoride membranes (Millipore Corporation, Bedford, MA) by semi-dry electrophoretic transfer (Bio-Rad Laboratories Inc.) and blocked with 5% milk powder in Tris-buffered saline with 0.1% Tween 20 for 2 h at room temperature. Membranes were probed with primary antibodies overnight at 4°C, washed 5 times with Tris-buffered saline containing 0.1% Tween 20, and then incubated for 1 h at room temperature with an appropriate horseradish peroxidase-labeled secondary antibody (Bioworld Technology, St. Louis Park, MN, Cat No: BS13278). The membranes were washed 5 more times, and signals were detected by enhanced chemiluminescence (Biouniquer Technology Co. LTD., Nanjing, China). The antibody against *GAPDH* was purchased from Bioworld Technology Inc. (Cat No: MB001). Rabbit polyclonal antibodies for *NHE1* and *NHE3* were purchased from Millipore Ltd. (Cat No: AB3081 and AB3085, Temecula, CA), and *NHE2* (H-172) was purchased from Santa Cruz Biotechnology Inc. (Cat No: sc-30167, Santa Cruz, CA). These commercial antibodies were used previously to verify the expression of *NHE1* and *NHE3* proteins in the ovine rumen and kidney (Rabbani et al., 2011), of *NHE1* in rat brain (Castañeda-Corral et al., 2012), and *NHE2* in rat small intestine (Uray et al., 2011). In our preliminary experiments, the detected band sizes of *NHE1*, *NHE2*, and *NHE3* were 90 kDa in ruminal and omasal epithelium of goats. The sizes of detected bands of *NHE1*, *NHE2*, and *NHE3* were consistent to the sizes predicted by the manufacturers of the antibodies.

Table 2. Sodium ion transport across omasal epithelium of sheep¹

Item	Value
$J_{\text{ms}}\text{Na}$ ($\mu\text{mol}\cdot\text{cm}^{-2}\cdot\text{h}^{-1}$)	11.06 ± 2.13
$J_{\text{sn}}\text{Na}$ ($\mu\text{mol}\cdot\text{cm}^{-2}\cdot\text{h}^{-1}$)	6.32 ± 1.73
$J_{\text{net}}\text{Na}$ ($\mu\text{mol}\cdot\text{cm}^{-2}\cdot\text{h}^{-1}$)	4.74 ± 1.77
I_{sc} ($\mu\text{eq}\cdot\text{cm}^{-2}\cdot\text{h}^{-1}$)	1.47 ± 0.24
G_t ($\text{mS}\cdot\text{cm}^{-2}$)	1.50 ± 0.19
N/n	2/8

¹Values are mean $\pm$ SD. $J_{\text{ms}}\text{Na}$ = mucosal-to-serosal flux rates of Na^+ ; $J_{\text{sn}}\text{Na}$ = serosal-to-mucosal flux rates of Na^+ ; $J_{\text{net}}\text{Na}$ = net Na^+ flux rate; I_{sc} = short-circuit current; G_t = transepithelial conductance; N = number of sheep; n = number of tissues.

Statistical Analyses

Data are expressed as the means $\pm$ standard deviations. Differences were considered significant when $P < 0.05$. Data involving more than 2 groups were assessed by one-way ANOVA and Student-Newman-Keuls post hoc analysis after testing for homogeneity of variance. Independent *t*-test (2-tailed test) was used in comparisons between 2 groups. The general linear model

with relevant interactions was used to determine the significance of the effects of SCFA and pH on NHE1, NHE2, and NHE3 expression. All statistical analyses were performed by using SPSS (SPSS version 13.0.1 for Windows; SPSS Inc., Chicago, IL) software packages.

RESULTS

Na⁺ Transport in Sheep Omasum

The isolated native omasal epithelium of sheep exhibited a net transport of Na⁺ from the mucosal to the serosal side (Table 2). The $J_{ms}Na$ ($11.06 \pm 2.13 \mu\text{eq}\cdot\text{cm}^{-2}\cdot\text{h}^{-1}$) was significantly higher than $J_{sm}Na$ ($6.32 \pm 1.73 \mu\text{eq}\cdot\text{cm}^{-2}\cdot\text{h}^{-1}$). The resulting $J_{net}Na$ ($4.74 \pm 1.77 \mu\text{eq}\cdot\text{cm}^{-2}\cdot\text{h}^{-1}$) was significantly larger than I_{sc} ($1.47 \pm 0.24 \mu\text{eq}\cdot\text{cm}^{-2}\cdot\text{h}^{-1}$), indicating that most of the Na⁺ transport is electroneutral.

Effect of NHE3 Inhibitor on Na⁺ Transport in Sheep Omasum

A specific NHE3 inhibitor (S3226, 1 μM) was used to characterize NHE3-mediated Na⁺ transport in both omasal and ruminal epithelium (Tables 3 and 4). In comparison with the control, the presence of S3226 in the luminal buffer solution caused a reduction of the omasal $J_{ms}Na$, $J_{sm}Na$, and $J_{net}Na$ by 80.0%, 80.7%, and 79.5%, respectively ($P < 0.05$). Similarly, in ruminal epithelium, S3226 inhibited $J_{ms}Na$ by 69.3% and $J_{net}Na$ by 80.3% ($P < 0.05$), but $J_{sm}Na$ did not change significantly ($P > 0.05$). The $J_{net}Na$ was significantly higher than the I_{sc} (Tables 3 and 4) under all experimental conditions in both tissues.

Effect of Luminal pH and SCFA on Na⁺ Transport in Sheep Omasum

All the data presented in the previous paragraphs were obtained by using a 40-mM SCFA-containing solution with pH 6.4 on the mucosal side. These obtained results support the assumption of electroneutral Na⁺ transport via NHE, which should be responsive to mucosal pH (Laghmani et al., 1997). In the following experiments, we therefore tested the effect of increasing or decreasing mucosal pH on Na⁺ flux rates by using the same buffer 2 with 40 mM SCFA on the mucosal side. An increase of mucosal pH from 6.4 to 7.4 led to a significant decrease of $J_{ms}Na$ and $J_{net}Na$ by 39.1% and 19.7%, respectively ($P < 0.05$; Table 5). Vice versa, when mucosal pH was decreased from 6.4 to 5.8, $J_{ms}Na$, $J_{sm}Na$, and $J_{net}Na$ increased by 55.0%, 70.9%, and 37.9% ($P < 0.05$; Table 5), respectively, compared

Table 3. Effect of S3226¹ (1 μM) on Na⁺ transport across omasal epithelium in hay-fed sheep²

Item	Control	S3226
$J_{ms}Na$ ($\mu\text{mol}\cdot\text{cm}^{-2}\cdot\text{h}^{-1}$)	14.77 ± 4.64	$2.95 \pm 0.46^*$
$J_{sm}Na$ ($\mu\text{mol}\cdot\text{cm}^{-2}\cdot\text{h}^{-1}$)	6.02 ± 1.83	$1.16 \pm 0.43^*$
$J_{net}Na$ ($\mu\text{mol}\cdot\text{cm}^{-2}\cdot\text{h}^{-1}$)	8.75 ± 3.54	$1.79 \pm 0.28^*$
I_{sc} ($\mu\text{eq}\cdot\text{cm}^{-2}\cdot\text{h}^{-1}$)	1.36 ± 0.16	1.10 ± 0.26
G_t ($\text{mS}\cdot\text{cm}^{-2}$)	1.40 ± 0.06	1.61 ± 0.32
N/n	2/6	2/6

¹S3226 = 3-[2-(3-guanidino-2-methyl-3-oxo-propenyl)-5-methyl-phenyl]-N-isopropylidene-2-methyl-acrylamide dihydrochloride.

²Values are mean $\pm$ SD. $J_{ms}Na$ = mucosal-to-serosal flux rates of Na⁺; $J_{sm}Na$ = serosal-to-mucosal flux rates of Na⁺; $J_{net}Na$ = net Na⁺ flux rate; I_{sc} = short-circuit current; G_t = transepithelial conductance; N = number of sheep; n = number of tissues.

*Different from respective value of control: $P < 0.05$.

with those at pH 6.4. Changes of mucosal pH from pH 6.4 to 7.4 or 5.8 did not alter I_{sc} (Table 5). These data indicate that mucosal acidification affects the electroneutral Na⁺ transport via NHE. An activation of apical (i.e., mucosal) Na⁺ uptake upon an extracellular acidification can only be explained by a concurrent increase in intracellular proton concentration, either via proton uptake with undissociated SCFA or via HCO_3^- loss, mediated by $\text{SCFA}^-/\text{HCO}_3^-$ exchange (see Introduction).

Omasal Epithelial Morphology in Goats

The mucosal surface of goat omasum is covered by stratified squamous epithelium (Figure 1). Four strata of cell layers composed the epithelium: stratum corneum, stratum granulosum (**SG**), stratum spinosum, and stratum basale (**SB**). The lamina propria beneath the epithelium contains connective tissue, capillary network, and the smooth muscles.

Table 4. Effect of S3226¹ (1 μM) on Na⁺ transport across ruminal epithelium in hay-fed sheep²

Item	Control	S3226
$J_{ms}Na$ ($\mu\text{mol}\cdot\text{cm}^{-2}\cdot\text{h}^{-1}$)	10.56 ± 2.26	$3.24 \pm 0.43^*$
$J_{sm}Na$ ($\mu\text{mol}\cdot\text{cm}^{-2}\cdot\text{h}^{-1}$)	1.73 ± 0.35	1.50 ± 0.15
$J_{net}Na$ ($\mu\text{mol}\cdot\text{cm}^{-2}\cdot\text{h}^{-1}$)	8.83 ± 2.45	$1.74 \pm 0.36^*$
I_{sc} ($\mu\text{eq}\cdot\text{cm}^{-2}\cdot\text{h}^{-1}$)	1.23 ± 0.24	$0.69 \pm 0.08^*$
G_t ($\text{mS}\cdot\text{cm}^{-2}$)	2.79 ± 0.32	2.95 ± 0.26
N/n	2/6	2/6

¹S3226 = 3-[2-(3-guanidino-2-methyl-3-oxo-propenyl)-5-methyl-phenyl]-N-isopropylidene-2-methyl-acrylamide dihydrochloride.

²Values are mean $\pm$ SD. $J_{ms}Na$ = mucosal-to-serosal flux rates of Na⁺; $J_{sm}Na$ = serosal-to-mucosal flux rates of Na⁺; $J_{net}Na$ = net Na⁺ flux rate; I_{sc} = short-circuit current; G_t = transepithelial conductance; N = number of sheep; n = number of tissues.

*Different from respective value of control: $P < 0.05$.

Figure 1. Morphology of native omasal epithelium of goat (hematoxylin-eosin staining, 1:400). SC = stratum corneum; SG = stratum granulosum; SS = stratum spinosum; SB = stratum basale; ML = smooth muscle; CT = connective tissue with blood capillaries. Scale bar = 20 μm .

Expression and Modulation of NHE Isoforms 1, 2, and 3 in Goat Omasum

To identify the expression and transcriptional modulation of NHE3 and other NHE isoforms by SCFA and low pH, first, the qPCR technique was used to quantify the gene expression. The mRNA of NHE isoforms 1, 2, and 3 were detected in cultured omasal epithelial cells (Figure 2). Changes of SCFA and pH in the culture medium affected the mRNA expression of the NHE isoforms differently. A change of culture medium pH from 7.4 to 6.8 did not affect the NHE1 mRNA level significantly (Figure 2A). However, the addition of 20 mM of SCFA to medium at pH 7.4 or 6.8 led to significant increases of NHE1 mRNA levels by 39.6 and 49.5%, respectively ($P < 0.05$), compared with that at pH 7.4

or 6.8 without SCFA. The greatest NHE1 mRNA level was observed when incubation medium with SCFA was adjusted to pH 6.8 ($P < 0.05$; Figure 2A), indicating that the stimulating effect of SCFA on NHE1 mRNA expression was potentiated by low pH conditions. The latter was statistically supported by a significant $\text{pH} \times \text{SCFA}$ interaction ($P < 0.01$; Table 6).

In contrast to NHE1, acidic pH ($P < 0.01$) or SCFA ($P < 0.01$) displayed inhibitory effects on mRNA of NHE2 and NHE3 (Table 6). When SCFA were present in the culture medium, a change of pH from 7.4 to 6.8 caused significant decreases of mRNA ($P < 0.05$; Figures 2B and 2C) by about 20% for NHE2 and NHE3. The supplementation of medium with SCFA (20 mM) induced an even stronger reduction of mRNA level by about 80 to 90% for NHE2 and NHE3 ($P < 0.05$) compared with the respective values without SCFA. Given this very dominant effect of SCFA, the effect of pH was reduced to numerical difference in the presence of 20 mM SCFA, evidenced by a significant $\text{pH} \times \text{SCFA}$ interaction ($P < 0.01$) for NHE2 and a similar trend ($P = 0.12$) for NHE3 (Table 6).

The expression of NHE1, NHE2, and NHE3 in cultured omasal epithelial cells was also verified by Western blot. The changes in NHE1, NHE2, and NHE3 protein expression qualitatively followed the changes in mRNA expression (Figure 3). The only exception was that the decrease in NHE2 and NHE3 protein induced by the presence of 20 mM SCFA was more subtle compared with the decrease in their mRNA expression (compare Figure 2B and 2C with Figure 3B and 3C).

DISCUSSION

It is well known that the omasum plays a central role in the absorption of fluid and electrolytes and, as such, affects electrolyte homeostasis in ruminants (Leonhard-Marek et al., 2010). One key electrolyte is Na^+ , whose absorption is influenced by the presence of SCFA (Ali

Table 5. Effect of mucosal pH on Na^+ transport across omasal epithelium in hay-fed sheep¹

Item	pH		
	7.4	6.4	5.8
$J_{\text{ms}}\text{Na}$ ($\mu\text{mol}\cdot\text{cm}^{-2}\cdot\text{h}^{-1}$)	4.31 ± 1.27^a	6.96 ± 2.12^b	10.79 ± 1.93^c
$J_{\text{sm}}\text{Na}$ ($\mu\text{mol}\cdot\text{cm}^{-2}\cdot\text{h}^{-1}$)	1.62 ± 0.35^a	3.61 ± 1.00^b	6.17 ± 0.53^c
$J_{\text{net}}\text{Na}$ ($\mu\text{mol}\cdot\text{cm}^{-2}\cdot\text{h}^{-1}$)	2.69 ± 1.20^a	3.35 ± 1.59^b	4.62 ± 1.16^c
I_{sc} ($\mu\text{eq}\cdot\text{cm}^{-2}\cdot\text{h}^{-1}$)	1.29 ± 0.36	1.31 ± 0.35	1.27 ± 0.23
G_t ($\text{mS}\cdot\text{cm}^{-2}$)	2.02 ± 0.37	1.93 ± 0.34	1.97 ± 0.48
N/n	3/14	3/14	3/18

^{a-c}Values within a column differ if they do not share a common superscript, $P < 0.05$.

¹Values are mean $\pm$ SD. $J_{\text{ms}}\text{Na}$ = mucosal-to-serosal flux rates of Na^+ ; $J_{\text{sm}}\text{Na}$ = serosal-to-mucosal flux rates of Na^+ ; $J_{\text{net}}\text{Na}$ = net Na^+ flux rate; I_{sc} = short-circuit current; G_t = transepithelial conductance; N = number of sheep; n = number of tissues.

Figure 2. Effects of pH and short-chain fatty acids (SCFA) on *NHE1* (A), *NHE2* (B), and *NHE3* (C) mRNA expression of goat omasal epithelial cells in primary culture ($n = 4$). The primer for *NHE1* and *NHE3* was used as described by Etschmann et al. (2006). The primer for *NHE2* was used as described by Graham et al. (2007). The levels of gene expression were quantified with real-time PCR in comparison with *GAPDH*. ^{a-c}Values (means $\pm$ SD) within one graph differ if they do not share a common letter: $P < 0.05$. Main effects and interactions are presented in Table 6.

et al., 2006). The current study, by using 2 established small-ruminant models (sheep and goat) that have been used for functional studies in forestomach epithelia (Martens and Gäbel, 1988; Ali et al., 2006; Yang et al., 2012; Yan et al., 2014), identified the NHE isoform responsible for bulk Na^+ absorption and then the effects of pH and SCFA on this NHE activity in native omasal epithelium, as well as on the expression of the 3 NHE isoforms in cultured omasal epithelial cells.

NHE3 Mediates Na^+ Transport in Sheep Omasum

With the mucosal presence of 40 mM of SCFA at pH 6.4, all tested $J_{\text{net}}\text{Na}$ were significantly greater than concurrent I_{SC} (Tables 2, 3, and 4), which resembles early findings in the ruminal epithelium of cattle and goats (Stevens, 1964; Chien and Stevens, 1972) and more recent observations in the omasal epithelium of sheep (Ali et al., 2006). It indicates that the major portion of Na^+ absorption in omasum of goats and cattle, as well as in rumen of sheep, does not contribute to net charge transfer across the epithelium and is hence electroneutral. This is in line with earlier studies demonstrating that Na^+ transport in the forestomach involves both electrogenic and electroneutral mechanisms with a predominance of electroneutral pathways (Martens et al., 1991). Further experiments were conducted in the present study to identify the NHE isoform that mediates the major portion of the electroneutral Na^+ transport in omasum and, for comparison, in rumen. With mucosal application of the NHE3-specific inhibitor S3226 (1 μM), $J_{\text{ms}}\text{Na}$ and $J_{\text{net}}\text{Na}$ were decreased in the omasal epithelium by 80% each. Similarly to omasum, $J_{\text{ms}}\text{Na}$ and $J_{\text{net}}\text{Na}$ were decreased by 69% and 80%, respectively, in the ruminal epithelium. The latter confirmed the results of a previous study on rumen epithelium (Rabani et al., 2011) in which the application of S3226 at mucosal side also decreased $J_{\text{ms}}\text{Na}$ and $J_{\text{net}}\text{Na}$ in sheep (by 46% and 57%, respectively) and calves (by 48% and 52%, respectively). Because S3226 has been used to elucidate NHE3-mediated Na^+ flux in a variety of other epithelial cell types (Schwark et al., 1998; Krishnan et al., 2003), these findings endorse the fact that NHE3 mediates bulk Na^+ transport in the mucosal-to-serosal direction not only in the ruminal but also in the omasal epithelium. Because S3226 was applied in the mucosal solution, this implies an apical localization of NHE3 in the omasal epithelium.

We have included some morphological investigations in this study to demonstrate that the morphology of goat omasal epithelium is similar to published results for cattle, buffalo, and sheep (Stumpff et al., 2011). The omasal epithelium of goat, sheep, and cattle contains 4 strata of cell layers. The cells in stratum corneum are

Table 6. Significance (*P*-value) of effects of short-chain fatty acids (SCFA) and pH on mRNA and protein expression of NHE1, NHE2, and NHE3 in goat omasal epithelial cells in primary culture¹

Item	mRNA			Protein		
	<i>NHE1</i>	<i>NHE2</i>	<i>NHE3</i>	NHE1	NHE2	NHE3
pH	0.05	<0.01	<0.05	<0.01	<0.01	0.08
SCFA	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
pH × SCFA	0.29	<0.05	0.22	<0.01	<0.01	0.12

¹Data sets for the stated significances are provided in Figure 2 (mRNA) and Figure 3 (protein). The general linear model with relevant interactions was used to determine the significance of the effects of SCFA and pH on mRNA or protein levels of NHE1, NHE2, and NHE3.

keratinized, devoid of nuclei. These cells form an outer mechanical barrier. Just beneath this layer, the costaining of cell borders for claudin-1, claudin-4, and occludin have been observed (Stumpff et al., 2011) in the SG of omasal and ruminal epithelium of sheep. The tight junction proteins have also been shown by electron microscopy in the SG of the bovine rumen (Henrikson and Stacy, 1971; Graham and Simmons, 2005). Therefore, Graham and Simmons (2005) have concluded that the SG cells constitute the apical diffusion barrier. They have further shown that SG cells are interlinked by connexin 43 with each other and with the (basolateral) cells in the stratum spinosum and SB to form a functional syncytium.

The molecular experiments of the present study show that mRNA and protein of NHE1, NHE2, and NHE3 are expressed in the omasal epithelium. Previous immunohistochemical studies on rumen have localized these 3 NHE isoforms in all strata of the functional syncytium. It has been demonstrated that expression of NHE1 (bovine rumen, Graham et al., 2007) and NHE3 (ovine rumen, Rabbani et al., 2011) increased from the SB toward the SG, whereas NHE2 is predominantly present in the SB (bovine rumen, Graham et al., 2007). The proposed apical localization of NHE1 and basolateral localization of NHE2 in bovine rumen (Graham et al., 2007) appears rather strange because this is opposite to their localization in the intestine. In the intestine NHE2, together with NHE3, typically resides in the apical membrane, whereas NHE1 is present at the basolateral membrane (Hoogerwerf et al., 1996). Nevertheless, even when assuming that NHE1 and NHE3 express simultaneously in the apical membrane of omasal epithelium, our study is consistent with the conclusion that NHE3 mediates bulk Na^+ transport in the mucosal-to-serosal direction (He and Yun, 2010), at least when sheep are fed on a hay-based diet.

Acidic pH and SCFA Activate Na^+ Transport

Acidity and SCFA exert roles on Na^+/H^+ exchange in many cell types and tissues. For example, acidity

activated NHE1 in renal cells (Odunewu and Fliegel, 2013), metabolic acidosis increased Na^+/H^+ exchange in rat intestine (Lucioni et al., 2002), and SCFA modulated intestinal Na^+ and water absorption (Binder and Mehta, 1989; Krishnan et al., 2003). The effect of luminal SCFA on net Na^+ absorption has been attributed to increased Na^+/H^+ exchange in proximal (Zaharia et al., 2001) and distal colon (Binder and Mehta, 1989), as well as in bovine and ovine rumen (Sehested et al., 1996; Uppal et al., 2003). The present study shows that the efficacy of SCFA to increase omasal Na^+ absorption is pH dependent. An acidification of the mucosal solution from pH 7.4 to pH 6.4 or 5.8 caused significant increases of J_{msNa} , J_{smNa} , and J_{netNa} . Such stimulation of Na^+ transport by acidic pH and SCFA has already been observed in the epithelium of omasum (Ali et al., 2006) and rumen (Abdoun and Martens, 2000). It is noteworthy that this relationship is bilateral in the omasal epithelium (Ali et al., 2006). An inhibition of apical NHE by using amiloride does not only inhibit apical Na^+ but also SCFA absorption in the omasum (Ali et al., 2006). But, the latter is in contrast to the rumen of sheep, in which amiloride is ineffective on SCFA absorption (Kramer et al., 1996). A bidirectional coupling of Na^+ and SCFA absorption in omasum via NHE can be explained by the intracellular acidification, caused by undissociated SCFA influx and intracellular dissociation into SCFA^- and H^+ , the latter activating Na^+ absorption via NHE. At the same time, the back-flow of H^+ into the lumen via NHE facilitates further absorption of SCFA by increasing the proportion of the extracellular undissociated SCFA for lipophilic influx. The mutual interactions between the absorption of Na^+ and SCFA transport via NHE are of physiological significance when considering that salivary Na^+ secretion and ruminal production of SCFA mostly increase in parallel as, for example, in postpartum dairy cows. This interaction ensures that Na^+ secreted with saliva is reabsorbed together with SCFA to restore electrolyte homeostasis and absorb energy substances. Furthermore, the H^+ recycling into the omasal lumen via NHE could help to ensure a maximal absorption of SCFA

Figure 3. Effects of pH and short-chain fatty acids (SCFA) on protein abundances of NHE1 (A), NHE2 (B) and NHE3 (C) of goat omasal epithelial cells in primary culture ($n = 4$). The NHE1, NHE2, and NHE3 proteins were detected by antibody, and the protein abundances were analyzed by Western blot in comparison with GAPDH. The quantitative data are shown in bar charts and representative blots. ^{a-c}Values (means $\pm$ SD) differ if they do not share a common letter: $P < 0.05$. Main effects and interactions are presented in Table 6.

and HCO_3^- in this forestomach compartment. It is of importance to prevent an outflow of significant amounts of SCFA into the abomasum. The latter would inhibit the motility (Svendsen, 1969) and emptying rate of the abomasum (Bolton et al., 1976). In addition, an outflow of HCO_3^- together with SCFA into the abomasum could favor gas accumulation and cause abomasal displacement—a disease quite commonly observed in postpartum dairy cows.

The acute activation of Na^+/H^+ exchange by low intracellular pH has been proposed to occur through the interaction of H^+ with an allosteric modifier site (pH_i sensor) within the transporter domain of NHE (Wakabayashi et al., 1994; Donowitz et al., 2013). The intracellular pH-dependent activation of NHE has been confirmed in mesangial cells (Boyarsky et al., 1990) and in rat ventricular myocytes (Wakabayashi et al., 1994). In addition to the pH_i sensor mechanism, the mechanism of phosphorylation of NHE may also play a role in the interplay between SCFA and Na^+ transport. The C-terminal cytoplasmic domain of NHE3 contains multiple putative phosphorylation sites that are probable targets of protein kinases of signal transduction pathways (He and Yun, 2010).

Acidic pH and SCFA Differentially Regulate NHE1, NHE2, and NHE3

To investigate the long-term regulation of NHE by acidic pH and SCFA, primary cultures of omasal epithelial cells were treated by acidic pH and SCFA individually or jointly in the present study. The mRNA and protein levels of NHE1 increased significantly in the medium containing SCFA, especially with low pH. This agrees with observations in primary cultures of ruminal epithelial cells, in which both the individual and joint application of acidic pH and SCFA increased NHE1 mRNA expression significantly (Yang et al., 2012). Similarly, Igarashi et al. (1992) reported that metabolic acidosis stimulated the mRNA expression of NHE1 in the porcine renal epithelial cell line LLC-PK1. In contrast to NHE1, however, in the present study the mRNA and protein levels of NHE2 and NHE3 decreased significantly in primary cultures of omasal epithelial cells treated by acidic pH and SCFA. This partly resembles the results obtained previously in inner medullary collecting duct cells, in which an acid loading has caused an increased level of NHE1 mRNA but a decreased level of mRNA of NHE2 (Soleimani et al., 1994). However, it differs markedly from results of many previous studies. In primary cultures of ruminal epithelial cells, the treatment of acidic pH and SCFA caused increase of mRNA expression of NHE3 along with NHE1, whereas NHE2 was unaffected (Yang et

al., 2012). Chronic acid exposure also enhanced NHE3 expression in OKP (a clonal opossum kidney cell line, Amemiya et al., 1995) and rat renal epithelial cells (Ambuhl et al., 1996). In the intestine, acid loading typically increased both apical NHE2 and NHE3 expression (Lucioni et al., 2002) but did not affect basolateral NHE1 expression (Lucioni et al., 2002). In brief, the responses of NHE isoform 1, 2, and 3 to environmental pH and SCFA appear tissue and isoform specific, with responses of omasal NHE distinct from those of ruminal and intestinal NHE.

The NHE isoforms have been characterized by their diversity of functions and distributions in cell types and tissues. In general, NHE1 is responsible for pH_i recovery after an acid load (Müller et al., 2000; Ford et al., 2002), whereas NHE2 has been found to mediate Na^+ absorption in rat distal colon (Krishnan et al., 2003) and has a role in the repair of epithelial damage in mouse intestine (Xue et al., 2011). The NHE3 is thought to mediate the bulk of Na^+ and water absorption by the intestine, especially the colon (He and Yun, 2010). The differential responses of expression of NHE isoforms to acidic pH and SCFA in the present study may, therefore, suggest that NHE3 plays a primary role in Na^+ transport across the omasal epithelium. The NHE1, however, exerts a primary role in pH_i regulation, when the omasal cells are facing the challenges of increased levels of SCFA and protons.

CONCLUSIONS

Omasum is one of the important organs in maintaining the electrolyte and water homeostasis of ruminant animals. The present study, together with prior experiments, provides new insights into the molecular identity, relevance, and regulation of omasal NHE. First, we demonstrated the presence of NHE1, NHE2, and NHE3 in omasal epithelium on the mRNA and protein level. Second, NHE3 was identified as the main Na^+ transport pathway found on the luminal side, which is not only responsible for bulk Na^+ absorption across the epithelium, but also mediates some serosal-to-mucosal back flux of Na^+ . Third, the microbial fermentation products SCFA and their released protons acutely activate Na^+ transport but suppress mRNA and protein levels of NHE2 and NHE3 in the longer term regulation, leading to downregulation of NHE2 and NHE3 protein. Concurrently, the mRNA and protein levels of NHE1 have been increased. If NHE1 was localized in the basolateral membrane (as is the case in intestine and many other tissues), it would mean that omasal epithelial cells adapt to an increased acid load by diverting proton extrusion from the apical membrane

(via NHE3) to the basolateral membrane (via NHE1). This may be a suitable mechanism for maintenance of systemic Na^+ concentration and pH_i homeostasis, when dairy cows fed on high-concentrate diets.

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REFERENCES

- Abdoun, K., and H. Martens. 2000. Luminal pH-dependent effects of ammonia on Na transport across the ruminal epithelium of sheep *in vitro*. *Proc. Soc. Nutr. Physiol.* 9:127.
- Ali, O., Z. Shen, U. Tietjen, and H. Martens. 2006. Transport of acetate and sodium in sheep omasum: Mutual, but asymmetric interactions. *J. Comp. Physiol. B* 176:477–487.
- Ambuhl, P. M., M. Amemiya, M. Danczkay, M. Lotscher, B. Kaissling, O. W. Moe, P. A. Preisig, and R. J. Alpern. 1996. Chronic metabolic acidosis increases NHE3 protein abundance in rat kidney. *Am. J. Physiol.* 271:F917–F925.
- Amemiya, M., Y. Yamaji, A. Cano, O. W. Moe, and R. J. Alpern. 1995. Acid incubation increases NHE-3 mRNA abundance in OKP cells. *Am. J. Physiol.* 269:C126–C133.
- Aschenbach, J. R., S. Bilk, G. Tadesse, F. Stumpff, and G. Gäbel. 2009. Bicarbonate-dependent and bicarbonate-independent mechanisms contribute to non diffusive apical uptake of acetate in the ruminal epithelium of sheep. *Am. J. Physiol. Gastrointest. Liver Physiol.* 296:G1098–G1107.
- Aschenbach, J. R., G. B. Penner, F. Stumpff, and G. Gäbel. 2011. Ruminant Nutrition Symposium: Role of fermentation acid absorption in the regulation of ruminal pH. *J. Anim. Sci.* 89:1092–1107.
- Binder, H. J., and P. Mehta. 1989. Short-chain fatty acids stimulate active sodium and chloride absorption *in vitro* in the rat distal colon. *Gastroenterology* 96:989–996.
- Bolton, J. R., A. M. Merritt, G. M. Carlson, and W. J. Donawick. 1976. Normal abomasal electromyography and emptying in sheep and the effects of intra-abomasal volatile fatty acid infusion. *Am. J. Vet. Res.* 37:1387–1392.
- Bondzio, A., C. Gabler, B. Badewien-Rentzsch, P. Schulze, H. Martens, and R. Einspanier. 2011. Identification of differentially expressed proteins in ruminal epithelium in response to a concentrate supplemented diet. *Am. J. Physiol. Gastrointest. Liver Physiol.* 301:G260–G268.
- Boyarsky, G., M. B. Ganz, E. J. Cragoe Jr., and W. F. Boron. 1990. Intracellular-pH dependence of Na-H exchange and acid loading in quiescent and arginine vasopressin-activated mesangial cells. *Proc. Natl. Acad. Sci. USA* 87:5921–5924.
- Brestoff, J. R., and D. Artis. 2013. Commensal bacteria at the interface of host metabolism and the immune system. *Nat. Immunol.* 14:676–684.
- Castañeda-Corral, G., H. I. Rocha-González, C. I. Araiza-Saldaña, G. C. Vidal-Cantú, J. Miguel Jiménez-Andrade, J. Murbartíán, and V. Granados-Soto. 2012. Blockade of peripheral and spinal Na^+/H^+ exchanger increases formalin-induced long-lasting mechanical allodynia and hyperalgesia in rats. *Brain Res.* 1475:19–30.
- Chien, W. J., and C. E. Stevens. 1972. Coupled active transport of Na and Cl across forestomach epithelium. *Am. J. Physiol.* 223:997–1003.
- Chomczynski, P., and N. Sacchi. 2006. The single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction: Twenty-something years on. *Nat. Protoc.* 1:581–585.

- Donowitz, M., C. Ming Tse, and D. Fuster. 2013. SLC9/NHE gene family, a plasma membrane and organellar family of Na^+/H^+ exchangers. *Mol. Aspects Med.* 34:236–251.
- Edriss, B. M., and R. H. Smith. 1986. Exchanges of magnesium and phosphorus at different sites in the ruminant stomach. *Arch. Tierernähr.* 36:1019–1027.
- Etschmann, B., K. S. Heipertz, A. von der Schulenburg, and M. Schweigel. 2006. A vH^+ -ATPase is present in cultured sheep ruminal epithelial cells. *Am. J. Physiol. Gastrointest. Liver Physiol.* 291:G1171–G1179.
- Fliegel, L., and O. Frolich. 1993. The Na^+/H^+ exchanger: An update on structure, regulation and cardiac physiology. *Biochem. J.* 296:273–285.
- Ford, P., V. Rivarola, A. Kierbel, O. Chara, M. Blot-Chabaud, N. Farman, M. Parisi, and C. Capurro. 2002. Differential role of Na^+/H^+ exchange isoforms NHE-1 and NHE-2 in a rat cortical collecting duct cell line. *J. Membr. Biol.* 190:117–125.
- Gäbel, G., J. R. Aschenbach, and F. Müller. 2002. Transfer of energy substrates across the ruminal epithelium: Implications and limitations. *Anim. Health Res. Rev.* 3:15–30.
- Gäbel, G., M. Bestmann, and H. Martens. 1991a. Influences of diet, short-chain fatty acids, lactate and chloride on bicarbonate movement across the reticulorumen wall of sheep. *Zentralbl. Veterinarmed. A* 38:523–529.
- Gäbel, G., S. Vogler, and H. Martens. 1991b. Short-chain fatty acids and CO_2 as regulators of Na^+ and Cl^- absorption in isolated sheep rumen mucosa. *J. Comp. Physiol. B* 161:419–426.
- Grace, N. D. 1988. Effect of varying sodium and potassium intakes on sodium, potassium, and magnesium contents of the ruminoreticulum and apparent absorption of magnesium in non-lactating dairy cattle. *N. Z. J. Agric. Res.* 31:401–407.
- Graham, C., I. Gatherer, I. Haslam, M. Glanville, and N. L. Simmons. 2007. Expression and localization of monocarboxylate transporters and sodium/proton exchangers in bovine rumen epithelium. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 292:R997–R1007.
- Graham, C., and N. L. Simmons. 2005. Functional organization of the bovine rumen epithelium. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 288:R173–R181.
- He, P., and C. C. Yun. 2010. Mechanisms of the regulation of the intestinal Na^+/H^+ exchanger NHE3. *J. Biomed. Biotechnol.* 2010:238080.
- Henrikson, R. C., and B. D. Stacy. 1971. The barrier to diffusion across ruminal epithelium: A study by electron microscopy using horseradish peroxidase, lanthanum and ferritin. *J. Ultrastruct. Res.* 34:72–82.
- Holtenius, K., and G. Björnag. 1989. The significance of water absorption and fibre digestion in the omasum of sheep, goats and cattle. *Comp. Biochem. Physiol. A Comp. Physiol.* 94:105–109.
- Hoogerwerf, W. A., S. C. Tsao, O. Devuyt, S. A. Levine, C. H. Yun, J. W. Yip, M. E. Cohen, P. D. Wilson, A. J. Lazenby, C. M. Tse, and M. Donowitz. 1996. NHE2 and NHE3 are human and rabbit intestinal brush-border proteins. *Am. J. Physiol.* 270:G29–G41.
- Igarashi, P., M. I. Freed, M. B. Gan, and R. F. Reilly. 1992. Effects of chronic metabolic acidosis on Na^+/H^+ exchangers in LLC-PK1 renal epithelial cells. *Am. J. Physiol.* 263:F83–F88.
- Kramer, T., T. Michelberger, H. Gürtler, and G. Gäbel. 1996. Absorption of short chain fatty acids across ruminal epithelium of sheep. *J. Comp. Physiol. B* 166:262–269.
- Krishnan, S., V. M. Rajendran, and H. J. Binder. 2003. Apical NHE isoforms differentially regulate butyrate-stimulated Na absorption in rat distal colon. *Am. J. Physiol. Cell Physiol.* 285:C1246–C1254.
- Laghmani, K., P. Borensztein, P. Ambühl, M. Froissart, M. Bichara, O. W. Moe, R. J. Alpern, and M. Paillard. 1997. Chronic metabolic acidosis enhances NHE-3 protein abundance and transport activity in the rat thick ascending limb by increasing NHE-3 mRNA. *J. Clin. Invest.* 99:24–30.
- Leonhard-Marek, S., F. Stumpff, and H. Martens. 2010. Transport of cations and anions across forestomach epithelia: Conclusions from in vitro studies. *Animal* 4:1037–1056.
- Livak, K. J., and T. D. Schmittgen. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)). *Methods* 25:402–408.
- Lucioni, A., C. Womack, M. W. Musch, F. L. Rocha, C. Bookstein, and E. B. Chang. 2002. Metabolic acidosis in rats increases intestinal NHE2 and NHE3 expression and function. *Am. J. Physiol. Gastrointest. Liver Physiol.* 283:G51–G56.
- Maekawa, M., K. A. Beauchemin, and D. A. Christensen. 2002. Effect of concentrate level and feeding management on chewing activities, saliva production, and ruminal pH of lactating dairy cows. *J. Dairy Sci.* 85:1165–1175.
- Martens, H., and G. Gäbel. 1988. Transport of Na and Cl across the epithelium of ruminant forestomachs: Rumen and omasum. *Comp. Biochem. Physiol. A Comp. Physiol.* 90:569–575.
- Martens, H., G. Gäbel, and B. Strozky. 1991. Mechanism of electrically silent Na and Cl transport across the rumen epithelium of sheep. *Exp. Physiol.* 76:103–114.
- Martens, H., I. Rabbani, Z. Shen, F. Stumpff, and D. Deiner. 2012. Changes in rumen absorption processes during transition. *Anim. Feed Sci. Technol.* 172:95–102.
- Müller, F., J. R. Aschenbach, and G. Gäbel. 2000. Role of Na^+/H^+ exchange and HCO_3^- transport in pH recovery from intracellular acid load in cultured epithelial cells of sheep rumen. *J. Comp. Physiol. B* 170:337–343.
- Odunewu, A., and L. Fliegel. 2013. Acidosis-mediated regulation of the NHE1 isoform of the Na^+/H^+ exchanger in renal cells. *Am. J. Physiol. Renal Physiol.* 305:F370–F381.
- Penner, G. B., M. A. Steele, J. R. Aschenbach, and B. W. McBride. 2011. Ruminant Nutrition Symposium: Molecular adaptation of ruminal epithelia to highly fermentable diets. *J. Anim. Sci.* 89:1108–1119.
- Rabbani, I., C. Siegling-Vlitakis, B. Noci, and H. Martens. 2011. Evidence for NHE3-mediated Na transport in sheep and bovine forestomach. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 301:R313–R319.
- Reynolds, C. K., G. B. Huntington, H. F. Tyrrell, and P. J. Reynolds. 1991. Net absorption of macrominerals by portal-drained viscera of lactating Holstein Cows and beef steers. *J. Dairy Sci.* 74:450–459.
- Schurmann, B. L., M. E. Walpole, P. Górka, J. C. Ching, M. E. Loewen, and G. B. Penner. 2014. Short-term adaptation of the ruminal epithelium involves abrupt changes in sodium and short-chain fatty acid transport. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 307:R802–R816.
- Schwark, J. R., H. W. Jansen, H. J. Lang, W. Krick, G. Burckhardt, and M. Hropot. 1998. S3226, a novel inhibitor of Na^+/H^+ exchanger subtype 3 in various cell types. *Pflügers Arch.* 436:797–800.
- Schweigel, M., M. Freyer, S. Leclercq, B. Etschmann, U. Lodemann, A. Bottcher, and H. Martens. 2005. Luminal hyperosmolarity decreases Na transport and impairs barrier function of sheep rumen epithelium. *J. Comp. Physiol. B* 175:575–591.
- Sehested, J., L. Diernaes, P. D. Moller, and E. Skadhauge. 1996. Transport of sodium across the isolated bovine rumen epithelium: Interaction with short-chain fatty acids, chloride and bicarbonate. *Exp. Physiol.* 81:79–94.
- Soleimani, M., G. Singh, G. L. Bizal, S. R. Gullans, and J. A. McAteer. 1994. Na^+/H^+ exchanger isoforms NHE-2 and NHE-1 in inner medullary collecting duct cells. Expression, functional localization, and differential regulation. *J. Biol. Chem.* 269:27973–27978.
- Steele, M. A., J. Croom, M. Kahler, O. AlZahal, S. E. Hook, K. Plautz, and B. W. McBride. 2011a. Bovine rumen epithelium undergoes rapid structural adaptations during grain-induced subacute ruminal acidosis. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 300:R1515–R1523.
- Steele, M. A., G. Vandervoort, O. AlZahal, S. E. Hook, J. C. Matthews, and B. W. McBride. 2011b. Rumen epithelial adaptation to high-grain diets involves the coordinated regulation of genes involved in cholesterol homeostasis. *Physiol. Genomics* 43:308–316.
- Stevens, C. E. 1964. Transport of sodium and chloride by the isolated rumen epithelium. *Am. J. Physiol.* 206:1099–1105.

- Stumpff, F., M. I. Georgi, L. Mundhenk, I. Rabbani, M. Fromm, H. Martens, and D. Günzel. 2011. Sheep rumen and omasum primary cultures and source epithelia: Barrier function aligns with expression of tight junction proteins. *J. Exp. Biol.* 214:2871–2882.
- Svendsen, P. 1969. Etiology and pathogenesis of abomasal displacement of cattle. *Nord. Vet. Med.* 21(Suppl. 1):1–60.
- Taniguchi, M., G. B. Penner, K. A. Beauchemin, M. Oba, and L. L. Guan. 2010. Comparative analysis of gene expression profiles in ruminal tissue from Holstein dairy cows fed high or low concentrate diets. *Comp. Biochem. Physiol. Part D Genomics Proteomics* 5:274–279.
- Uppal, S. K., K. Wolf, S. S. Khahra, and H. Martens. 2003. Modulation of Na^+ transport across isolated rumen epithelium by short-chain fatty acids in hay- and concentrate-fed sheep. *J. Anim. Physiol. Anim. Nutr. (Berl.)* 87:380–388.
- Uray, K. S., S. K. Shah, R. S. Radhakrishnan, F. Jimenez, P. A. Walker, R. H. Stewart, G. A. Laine, and C. S. Cox Jr. 2011. Sodium hydrogen exchanger as a mediator of hydrostatic edema-induced intestinal contractile dysfunction. *Surgery* 149:114–125.
- von Engelhardt, W., and R. Hauffe. 1975. Funktionen des Blättermagens bei kleinen Hauswiederkäuern. IV: Resorption und Sekretion von Elektrolyten. *Zbl. Vet. Med. A* 22:363–375.
- Wakabayashi, S., B. Bertrand, M. Shigekawa, P. Fafournoux, and J. Pouyssegur. 1994. Growth factor activation and " H^+ -sensing" of the Na^+/H^+ exchanger isoform 1 (NHE1). *J. Biol. Chem.* 269:5583–5588.
- Wegeler, C. 2008. Transport von HCO_3^- am isolierten Psalterepithel des Schafes –Charakterisierung und Einfluss der Fütterung/Fütterung. Thesis. Free Univ. Berlin, Germany.
- Xue, L., E. Aihara, T. C. Wang, and M. H. Montrose. 2011. Trefoil factor 2 requires Na/H exchanger 2 activity to enhance mouse gastric epithelial repair. *J. Biol. Chem.* 286:38375–38382.
- Yan, L., B. Zhang, and Z. Shen. 2014. Dietary modulation of the expression of genes involved in short-chain fatty acid absorption in the rumen epithelium is related to short-chain fatty acid concentration and pH in the rumen of goats. *J. Dairy Sci.* 97:5668–5675.
- Yang, W., Z. Shen, and H. Martens. 2012. An energy-rich diet enhances expression of Na^+/H^+ exchanger isoform 1 and 3 messenger RNA in rumen epithelium of goat. *J. Anim. Sci.* 90:307–317.
- Yang, W., H. Sheng, J. W. Thompson, S. Zhao, L. Wang, P. Miao, X. Liu, M. A. Moseley, and W. Paschen. 2014. Small ubiquitin-like modifier 3-modified proteome regulated by brain ischemia in novel small ubiquitin-like modifier transgenic mice: Putative protective proteins/pathways. *Stroke* 45:1115–1122.
- Zachos, N. C., M. Tse, and M. Donowitz. 2005. Molecular physiology of intestinal Na^+/H^+ exchange. *Annu. Rev. Physiol.* 67:411–443.
- Zaharia, V., M. Varzescu, I. Djavadi, E. Newman, R. W. Egnor, J. Alexander-Chacko, and A. N. Charney. 2001. Effects of short chain fatty acids on colonic Na^+ absorption and enzyme activity. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* 128:335–347.