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Evidence for NHE3-mediated Na transport in sheep and bovine forestomach

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Rabbani I, Siegling-Vlitakis C, Noci B, Martens H. Evidence for NHE3-mediated Na transport in sheep and bovine forestomach. *Am J Physiol Regul Integr Comp Physiol* 301: R313–R319, 2011. First published May 25, 2011; doi:10.1152/ajpregu.00580.2010.—Na absorption across the cornified, multilayered, and squamous rumen epithelium is mediated by electrogenic amiloride-insensitive transport and by electroneutral Na transport. High concentrations of amiloride (>100 μM) inhibit Na transport, indicating Na^+/H^+ exchange (NHE) activity. The underlying NHE isoform for transepithelial Na absorption was characterized by mucosal application of the specific inhibitor HOE642 for NHE1 and S3226 for NHE3 in Ussing chamber studies with isolated epithelia from bovine and sheep forestomach. S3226 (1 μM; NHE3 inhibitor) abolished electroneutral Na transport under control conditions and also the short-chain fatty acid-induced increase of Na transport via NHE. However, HOE642 (30 μM; NHE1 inhibitor) did not change Na transport rates. NHE3 was immunohistochemically localized in membranes of the upper layers toward the lumen. Expression of NHE1 and NHE3 has been previously demonstrated by RT-PCR, and earlier experiments with isolated rumen epithelial cells have shown the activity of both NHE1 and NHE3. Obviously, both isoforms are involved in the regulation of intracellular pH, pH_i. However, transepithelial Na transport is only mediated by apical uptake via NHE3 in connection with extrusion of Na by the basolaterally located Na-K-ATPase. The missing involvement of NHE1 in transepithelial Na transport suggests that the proposed "job sharing" in epithelia between these two isoforms probably also applies to forestomach epithelia: NHE3 for transepithelial transport and NHE1 for, among others, pH_i and volume regulation.

rumen; Na^+/H^+ exchange; NHE inhibitors

SODIUM IS THE MAJOR CATION in isotonic saliva of ruminants (2), with 1.2–1.5 mol Na being daily secreted by an adult sheep (8), which is flowing into the rumen together with 0.1–0.2 mol dietary Na. Some 50% of salivary Na and a corresponding amount of dietary Na is absorbed from the rumen against a concentration and electrical gradient (blood-side positive), indicating an active transport mechanism (8). Early in vitro studies with isolated rumen epithelium demonstrated a net Na absorption (J_{net} Na) in the mucosal-serosal direction under Ussing chamber conditions, confirming the assumption of active Na transport (39). Furthermore, J_{net} Na is larger than the short circuit current (I_{sc}), indicating electroneutral Na transport (39). This in vitro observation (J_{net} Na > I_{sc}) has been confirmed in all following studies with rumen from sheep (11), goats (5), cattle (5, 38), or deer (40).

Further characterization of Na transport has revealed that, in the absence of permeable anions, J_{net} Na equals I_{sc} (5). I_{sc} and, hence, electrogenic Na transport, is abolished by serosal ouabain (17), is insensitive to mucosal amiloride (28), is modulated by divalent cations (22), and is mediated by a potential sensitive cation conductance in the apical membrane (20, 25).

Electroneutral Na transport is generally larger than the I_{sc} and depends on the presence of CO_2/HCO_3^- and Cl^- in the buffer solution (5, 13, 28). Because Cl^- is also actively absorbed (37, 39), coupled with Na (27), Chien and Stevens (5) have proposed a NaCl cotransport or alternatively parallel-working Na^+/H^+ (NHE) and Cl^-/HCO_3^- exchangers. The proposed model of Na absorption via NHE has been corroborated by the stimulation of electroneutral Na transport by short-chain fatty acids (SCFA) (7, 15, 38), by its inhibition through mucosal amiloride (1 mM) (28, 38), and indirectly, with its inhibition by acetazolamide (10, 38). Obviously, two uptake mechanisms of Na (electroneutral and electrogenic) exists in the luminal membrane of the rumen epithelium, and transepithelial transport of Na is accomplished by extrusion of Na via the Na-K-ATPase in deeper cell layers of the epithelium (16, 19, 26, 34).

Na^+/H^+ exchange was first described by Murer et al. (30), and currently, 10 isoforms (NHE1–10) have been described (1, 9, 21). NHE1 is ubiquitously expressed and, among others, is involved in the regulation of cell volume and pH (18). In epithelia, NHE1 is located in the basolateral membrane (6, 33) and is considered as a "housekeeper." NHE3 is present in the apical membrane of many epithelia, such as the small and large intestine, gall bladder, and various parts of the kidney tubule (9).

The localization of NHE3 in the apical membrane and NHE1 in the basolateral membrane of polarized cells has led, in epithelia, to the generally accepted model of "job sharing" between these two NHE isoforms: NHE3 mediates transepithelial electroneutral Na transport (9), and NHE1 is involved in the regulation of pH and homeostasis of cell volume (33).

In a recent study, Graham et al. (16) have demonstrated the expression of mRNA of NHE1–3 and NHE8 by RT-PCR in bovine rumen epithelium. In contrast to the general assumption, Graham et al. showed with immunostaining that NHE1 is apically localized in the stratum granulosum of the multilayered squamous rumen epithelium. Graham et al. (16) also discuss the interaction of NHE1 with SCFA transport, as has been proposed by Gäbel and Sehested (14): the uptake of SCFA across the luminal membrane by nonionic diffusion, the release of protons, and the activation of NHE. Furthermore, the stimulation of NHE1 should lead to the generation of "local-

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ized extracellular acidity to promote nonionic diffusional uptake of SCFA" (16). As a consequence, the SCFA-dependent increase of transepithelial Na transport (15, 38) should be mediated by NHE1 in the luminal membrane, in contrast to the general model of the functions of NHE3 and NHE1 in epithelia (see above) and our previous findings concerning the inhibition of Na transport via NHE by amiloride (28).

NHE1 is inhibited by amiloride in the micromolar range (IC_{50}, 10.7 μM) (35), which is without an effect on J_{ms} Na (mucosal to serosal sodium flux) in studies of isolated sheep rumen epithelium (28). Higher concentrations (1 mM) significantly reduce J_{ms} and J_{net} Na in sheep (28) and bovine rumen epithelium (38). Furthermore, the recovery of pH_i in ruminal epithelial cells (REC) after an acid load by butyrate is significantly reduced by amiloride, by S3226 (inhibitor of NHE3), and by HOE642 or HOE694 (inhibitor of NHE1) (29, 36).

These data clearly indicate the physiological presence of NHE1 and NHE3 in rumen epithelium (29, 36), and the corresponding RT-PCR products with a high-sequence homology to human NHE3 and NHE1 have been detected (36); however, the specific functional relevance of the transepithelial Na transport and/or regulation of pH_i cannot be deduced unequivocally from our current knowledge. Therefore, we have decided to study Na transport in the forestomach epithelia of cattle and sheep with the aid of the more specific NHE blockers HOE642 (NHE1) and S3226 (NHE3). Most of the studies were performed with tissues from sheep because ruminal Na transport has been primarily studied in this species, and consequently, the knowledge about electrogenic and electroneutral Na transport is well established in this tissue (14–15, 25, 28–29, 36). Experiments with bovine epithelia were included from a comparative point of view. The inhibitors were applied at the mucosal side because the electroneutral absorption of Na should be primarily characterized.

MATERIAL AND METHODS

Animals. Three sheep with an age between 6 and 9 mo and a body weight of 30–40 kg were fed hay ad libitum for at least 3 wk before the experiment. The sheep had free access to tap water and a mixture of minerals (lick stone for sheep). Holstein bull calves were fed colostrum for 3 or 4 days postpartum and were then placed into individual pens in a climate-controlled room. From the 4th day, the calves were fed milk replacer (Denkamilk, Fa. Denkavit, Warendorf, Germany), according the recommendations of the producer. In addition to the milk replacer, chopped hay and a starter diet for calves (ForFarmers, Lochem, The Netherlands) were offered from the 2nd wk. Tap water was available ad libitum. Dry matter intake (hay and starter diet) increased from 100 to 200 g/day during the 2nd wk to 3 kg/day at the 10th wk, when the calves were killed with a mean body weight of 112 kg. The weight of the reticulorumen (organ plus content) was 14.5 kg or some 13% of the body weight; this structure was considered as a functional forestomach. Sheep and bull calves were housed and treated in accordance with the regulations of experimental animals of the county Berlin and the Faculty of Veterinary Medicine, according the law of animal welfare (§4 of the German Law of Animal Welfare, license no. T0064/99 of the Landesamt für Gesundheit, Berlin).

Tissue collection and preparation. The preparation and incubation of the forestomach epithelium have been described in detail by Martens et al. (28). Briefly, after the stunning by a captive bolt and exsanguinations of sheep/calves at a local slaughterhouse, the reticulorumen, together with the omasum, was removed from the abdominal cavity within 2–3 min. A 250-cm^2 piece of rumen wall was taken from the ventral sac, repeatedly cleaned in a buffer solution, and stripped from the muscle layer. The tissues were transported to the laboratory in a buffer (in mmol/l: 115 NaCl, 25 $NaHCO_3$, 0.4 NaH_2PO_4, 2.4 Na_2HPO_4, 5 KCl, 5 glucose, 1.2 $CaCl_2$, 1.2 $MgCl_2$; pH 7.4 at 38.0°C, 280 mosmol/kg) solution, which was gassed with 95% O_2-5% CO_2. Pieces of the epithelium (3 × 3 cm) were mounted between the two halves of an Ussing chamber to give an exposed area of 3.14 cm^2. The mounted tissues were bathed on each side with 16-ml buffer solution by using a gas lift system and were gassed with 95% O_2-5% CO_2 at 38°C, pH 7,4. The SCFA buffer contained (in mmol/l): 20 NaCl, 1 $CaCl_2$, 1 $MgCl_2$, 25 $NaHCO_3$, 1 KH_2PO_4, 2 K_2HPO_4, 48 Na-acetate, 24 Na-propionate, 8 Na-butyrate, 10 glucose, and 20 Na-gluconate. In SCFA-free buffer solutions, SCFA were replaced by Na-gluconate. Table 1 shows the application of the buffer solutions. Mucosal pH was adjusted to 6.4 in the presence of carbogen with 1 M HCl and controlled before and during the experiment; serosal pH was 7.4 during the whole experimental period. The osmolarity in all cases was adjusted to 290 ± 10 mosmol/l with mannitol after adjusting the pH. For both calves and sheep, all tissues were divided into four groups: *1*) control (without inhibitors) *2*) 1 mM amiloride, *3*) 30 μM NHE1 inhibitor (HOE 642), and *4*) 1 μM NHE3 inhibitor (S3226). Amiloride was purchased from Sigma-Aldrich (Munich, Germany), while the inhibitors of NHE1 (HOE642) and NHE3 (S3226) were kindly supplied by Dr. J. Pünter (Sanofi-Aventis, Frankfurt, Germany). ^{22}Na was purchased from Hartmann Analytik (Braunschweig, Germany). All reagents used were of analytic grade.

Electrical measurement. 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 (PD_t). Similar types of tips were inserted ~2 cm from the surface of the tissue for the application of current (I_{sc}). The tissues were incubated under short-circuit conditions. Transepithelial conductance (G_t) was calculated by measuring the displacements in the potential difference (ΔPD), caused by the application of a bipolar pulse of 100 μA, 1-s duration, and any changes in its amplitude. Since the inhibitors were applied only on the mucosal side, for the interpretation of I_{sc}, only m-s tissues were used.

Flux studies. Unidirectional mucosal (m) to serosal (s) and serosal (s) to mucosal (m) fluxes (J_{ms}, J_{sm}) of Na were determined on paired tissues from the same rumen under short-circuit conditions. The tissue conductance (G_t) of each tissue in a pair did not differ by more than 25%. Net transepithelial fluxes were calculated as the difference between the two oppositely directed unidirectional fluxes ($J_{net} = J_{ms} - J_{sm}$). Usually, the flux rates were studied in 12 pairs of tissues from the same rumen simultaneously. When the electrical parameters have been stabilized (generally 30–40 min after mounting), the mucosal buffer was changed (SCFA and pH 6.4), and the tissues were short-circuited. Then, the inhibitors were added on the mucosal side of

Table 1. *A general description for the experimental animals and buffer solution used in the study showing pH and the application of SCFA*

Species	Buffer Composition		*N/n*
	Mucosal	Serosal	
Calf	+SCFA 80 mM, pH 6.4	−SCFA, pH 7.4	3/35
Sheep	+SCFA 80 mM, pH 6.4	−SCFA, pH 7.4	3/32
Sheep	−SCFA, pH 6.4	−SCFA, pH 7.4	3/34

All animals and used buffer compositions were divided into four groups: *1*) control *2*) 1 mM amiloride, *3*) 30 μM NHE1 inhibitor (HOE 642), and *4*) 1 μM NHE3 inhibitor (S3226). The inhibitors were only added to the mucosal side. The results are presented in Tables 2-4. SCFA, short-chain fatty acids. *N* is number of animals; *n* is number of paired tissues.

both J_{ms} and J_{sm} tissues, whereas one group was always kept as control (without inhibitor). ^{22}Na (80 kBq) was added to the mucosal side of one tissue of each pair and to the serosal side of the other ("hot side"). Samples (1 ml) were taken from the unlabeled ("cold") side after an equilibration period of half an hour at 30-min intervals for three flux periods. The flux rates were calculated for each flux period, and the mean was taken as the transport rate. Each sample was replaced by an equal volume of corresponding buffer solution. Samples (100 μl) from the labeled bathing solution ("hot side") were taken before the first and after the last flux period for the calculation of the specific radioactivity. ^{22}Na was assayed in scintillation liquid (Rotiszint, Roth-Karlsruhe, Germany) by using a β-counter (LKB Wallace-Perkin-Elmer; Überlingen, Germany).

Immunochemistry. Rumen tissue of sheep was washed and stripped as described above, and the epithelium was fixed in 4% neutral buffered formaldehyde. Rumen slices were dehydrated and embedded in paraffin. Tissue sections (3 μm thick) were mounted on SuperFrost Plus adhesive glass slides (Menzel-Gläser, Braunschweig, Germany), deparaffinized in Roti-Histol (T6640, Carl Roth, Karlsruhe, Germany), and rehydrated in isopropanol and graded ethanol.

Endogenous peroxidase activity was blocked by 30-min incubation in 0.3% H_2O_2 in methanol. The slides were washed, and nonspecific binding sites were blocked with 2.5% blocking horse serum:RTU (ready to use) for 20 min (ImmPRESS universal antibody kit VC-MC-7500, Vector Laboratories, Burlingame, CA). The sections were incubated overnight with the primary antibody, namely, polyclonal rabbit anti-NHE3 (AB3085; Chemicon International, Temecula, CA), at a dilution of 1:100 in 5% horse serum in PBS at 4°C and washed for 10 min in PBS. The appropriate ImmPRESS anti-mouse/rabbit Ig:HRP reagent (ImmPRESS universal antibody kit VC-MC-7500; Vector Laboratories) was added to the sections and incubated for 30 min at room temperature. Slides were again rinsed, and immunostaining was developed with the peroxidase substrate HistoGreen (E109, Linaris, Wertheim, Germany) for 2–5 min under visual control (microscope). After the slides had been washed for 2–5 min in PBS and for 30 s in distilled water, they were counterstained with Mayer's Hemalum solution (T865; Carl Roth, Karlsruhe, Germany), followed by washes in cold running water (5 min). Subsequently, sections were dehydrated (3 × 30 s) with ethanol and (2 × 30 s) in xylene before being mounted with HistoPerm (E6001, Linaris, Wertheim, Germany). Immunocytochemistry was carried out on rumen tissue obtained from three animals.

For negative controls, tissues were probed with the respective preimmune serum instead of immune serum or purified antibodies (Fig. 2*B*). Rat kidney (Fig. 2*C*) was used as a positive tissue control (4), and the heart as a negative tissue control (Fig. 2*D*). All controls were treated with the same procedure as that described above.

Statistics. All evaluations were carried out by using Sigma Stat (3.0.1) and Sigma Plot program version 8.0 for Windows (SPSS, Chicago, IL). Results are given as means ± SD. Comparison between the groups was carried out in the form of Student's *t*-test (Tables 2–4) or paired *t*-test (Fig. 1). *P* values of <0.05 were considered significant. *N* refers to the number of experimental animals, whereas *n* refers to the number of paired tissues.

RESULTS

Effect of NHE inhibitors on Na transport across rumen epithelium of calves (with SCFA). Transport rates of Na, J_{ms}, J_{sm}, and J_{net}, and the electrophysiological parameters I_{sc} and G_t of isolated calf rumen epithelium are summarized in Table 2. Under control conditions, J_{net} Na was larger than I_{sc}, confirming all previous observations. Mucosal amiloride (1.0 mM) caused a numerical decrease of J_{ms} and J_{net} Na of 30–40%, indicating an inhibition of NHE. The NHE3 blocker S3226 (1.0 μM) significantly reduced J_{ms} Na from 3.11 ± 0.31 to 1.63 ± 0.22 μEq·cm^{-2}·h^{-1} and, hence, J_{net} from 2.34 ± 0.33 to 1.12 ± 0.28 μEq·cm^{-2}·h^{-1}. The short circuit current (I_{sc}) and J_{net} Na were almost identical in the presence of mucosal S3226 (1.07 ± 0.05 vs. 1.12 ± 0.28 μEq·cm^{-2}·h^{-1}), indicating inhibition of electroneutral Na transport via NHE3. However, the NHE1 inhibitor HOE642 [30 μM; IC_{50} 0.08 μM; (34)] failed to cause any significant inhibition of Na transport. It is important to mention here that the tissue conductance (G_t) among various groups did not change significantly (Table 2) and should be considered as a control parameter of tissue integrity during the time course of the experiment. The I_{sc} of bovine rumen epithelium was not changed by the inhibitors (Table 2).

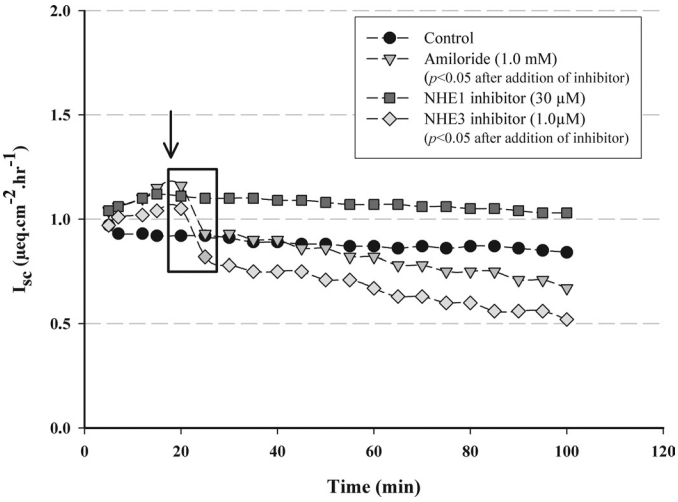

Fig. 1. Effect of Na^+/H^+ exchange (NHE) inhibitors on I_{sc} of sheep rumen epithelium (without short chain fatty acids, SCFA). The arrow indicates the mucosal addition of amiloride (1 mM), HOE642 (30 μM), or S3226 (1 μM).

Effect of NHE inhibitors on Na transport across rumen epithelium of sheep. An identical design was chosen for corresponding studies with sheep rumen epithelium. We sought to determine the isoforms of NHE responsible for both SCFA-independent (Table 3) and SCFA-stimulated (Table 4) electroneutral Na transport (7, 15, 38). The NHE inhibitors applied on the mucosal side showed a similar response compared with the experiments with calves. In the absence of SCFA, amiloride, and the NHE1-specific inhibitor, HOE642 did not significantly change J_{ms} and J_{net} Na. Again, the NHE3 specific inhibitor, S3226, caused a significant decrease of J_{ms} Na from 3.14 ± 0.76 to 1.70 ± 0.43 μEq·cm^{-2}·h^{-1}. Despite a substantial, but nonsignificant, inhibition of J_{net} Na, I_{sc} was almost identical to J_{net} Na (0.74 vs. 0.87 μEq·cm^{-2}·h^{-1}) in the presence of S3226 (Table 3).

The presence of SCFA caused approximately an 80 to 90% increase of J_{ms} and J_{net} Na and again amiloride or S3226 but not HOE642 caused a significant decrease of these flux rates, indicating that the stimulation of Na transport by SCFA represented the activation of NHE3. The magnitude of this inhibition was larger for S3226 than amiloride (Table 4).

Notably, both in the presence and in the absence of SCFA, I_{sc}, which represents electrogenic Na transport, was significantly reduced after the addition of S3226 (Tables 3 and 4) and by amiloride (not significant). However, this effect on I_{sc} exhibited some delay, and a new steady state of I_{sc} was

Table 2. *Effects of mucosal addition of amiloride, HOE642, or S3226 on unidirectional Na flux rates of bovine rumen epithelium (with mucosal SCFA)*

	J_{ms} Na	J_{sm} Na	J_{net} Na	I_{sc}	G_t	
	$\mu Eq \cdot cm^{-2} \cdot hr^{-1}$				mS/cm^2	*N/n*
Control	3.11 ± 0.31[a]	0.77 ± 0.12	2.34 ± 0.33[a,1]	0.98 ± 0.11[2]	1.34 ± 0.28	3/9
Amiloride, 1 mM	2.11 ± 0.42	0.80 ± 0.35	1.31 ± 0.48[b]	0.95 ± 0.09	2.55 ± 0.68	3/9
HOE642, 30 μM	3.04 ± 0.51	0.81 ± 0.43	2.23 ± 0.84	1.05 ± 0.04	1.79 ± 0.18	3/8
S3226, 1 μM	1.63 ± 0.22[b]	0.51 ± 0.14	1.12 ± 0.28[b]	1.07 ± 0.05	1.26 ± 0.19	3/9

Values are expressed as means ± SD. Values in the same column bearing different superscripts and numerals in the same row bearing different values are significantly different ($P < 0.05$).

obtained after some 5–10 min (Fig. 1). Mucosal addition of HOE642 did not produce any effect on I_{sc}.

Immunolocalization of NHE3. Immunocytochemistry demonstrated various intensities of staining in the rumen epithelium of the various animals. In two of three epithelia, the strongest intensity appeared in the stratum granulosum (luminal cells of ovine rumen); the intensity decreased from the stratum spinosum through to the stratum basale without any staining in the stratum corneum (Fig. 2*A*). One epithelium showed evenly distributed staining in the stratum granulosum, stratum spinosum, and stratum basale.

DISCUSSION

The data of the present study are in agreement with findings in many previous studies of Na transport in rumen epithelium: J_{net} Na in the mucosal-serosal direction, with $J_{net} > I_{sc}$ (11, 15, 28, 36, 38–39), and stimulation of J_{net} Na by SCFA (7, 15, 38). Furthermore, the inhibition of Na transport by amiloride and S3226 supports the early conclusion that electroneutral Na transport is mediated via NHE (5, 28). The pronounced or missing effects of various inhibitors of NHE give further insight into the isoform of NHE that is probably involved.

HOE642 as a specific inhibitor for NHE1 was ineffective in all experiments. By contrast, S3226 as a specific inhibitor of NHE3 significantly reduced J_{ms} Na and abolished electroneutral Na transport, because the remaining J_{net} Na was not significantly different from I_{sc}, despite some variations. The used concentrations of the inhibitors (30 μM HOE642 and 1 μM S3226) were far above the IC_{50} for the respective NHE isoform, according to the studies of Schwark et al. (35), but much lower than the concentrations that probably caused the unspecific effects on other NHE isoforms (see Table 2 in Ref. 35). In the study of Schwark et al. (35), a variety of cells were used for testing the specificity of the inhibitors, and it was shown that the specificity was independent of the cell system. Our results are in general agreement with this observation.

Amiloride (1 mM) was simply used as a demonstration of the effects known from previous experiments with rumen epithelium and served as a further control within the framework of this study (28, 38). The NHE3 in bovine rumen epithelium seems to be less sensitive to amiloride, with Diernaes et al. (7) observing no effect of amiloride (1 mM) on Na transport. In the present study, amiloride reduced J_{ms} Na in calf rumen (Table 2) by only 20% (not significant), and a similar (%), but significant, decrease of J_{ms} Na was noticed by Sehested et al. (38) in studies with bovine rumen.

SCFA stimulated Na transport (Table 4) (7, 14–15, 38), and the increase was inhibited by amiloride or S3226, further supporting the conclusion that electroneutral Na transport is mediated via NHE and via the isoform NHE3. A similar approach for identifying the isoform NHE3 was applied in a study of Na transport via Na^+/H^+ exchanger in rat nephron (41).

This observation is in contrast to the discussion of Graham et al. (16), who have demonstrated, by RT-PCR, the presence of NHE1−3 and NHE8 in bovine rumen and who conclude, from immunostaining, that NHE1 is "predominantly localized to the stratum granulosum with a progressive decrease toward stratum basale". Consequently, the authors discuss this isoform with regard to the well-known interaction between the nonionic diffusional uptake of SCFA, the intracellular release of protons, the activation of NHE1, and Na influx.

The application of the specific inhibitor HOE642 in the present study does not support the conclusions of Graham et al. (16), and the demonstrated immunostaining of NHE3 predominantly in the luminal layer of epithelium is in agreement with the effect of inhibitors. Special attention has been paid to the avoidance of unspecific immunostaining. No staining has been observed in the absence of the primary antibody and the detection of NHE3 in the kidney, and its absence in heart as negative controls supports the conclusion that NHE3 staining is specific.

Table 3. *Effects of mucosal addition of amiloride, HOE642, or S3226 on unidirectional Na flux rates of sheep rumen epithelium (without SCFA)*

	J_{ms} Na	J_{sm} Na	J_{net} Na	I_{sc}	G_t	
	$\mu Eq \cdot cm^{-2} \cdot h^{-1}$				mS/cm^2	*N/n*
Control	3.14 ± 0.76[a]	1.12 ± 0.27[a]	2.02 ± 0.85	1.07 ± 0.22[a]	3.56 ± 0.15	3/8
Amiloride, 1 mM	2.24 ± 0.51	1.09 ± 0.24	1.15 ± 0.59	0.98 ± 0.15	4.49 ± 0.47	3/8
HOE642, 30 μM	2.90 ± 0.57	1.13 ± 0.17	1.77 ± 0.60	1.07 ± 0.15	3.72 ± 0.51	3/9
S3226, 1 μM	1.70 ± 0.43[b]	0.83 ± 0.26[b]	0.87 ± 0.52	0.74 ± 0.14[b]	3.51 ± 0.40	3/9

Values are expressed as means ± SD. Values in the same column bearing different superscripts are significantly different ($P < 0.05$) to the control.

Table 4. *Effects of mucosal addition of amiloride, HOE642, or S3226, on unidirectional Na flux rates of sheep rumen epithelium (with mucosal SCFA)*

Group	J_{ms} Na	J_{sm} Na	J_{net} Na	I_{sc}	G_t	*N/n*
	$\mu Eq \cdot cm^{-2} \cdot h^{-1}$				mS/cm^2	
Control	5.71 ± 1.31[a]	1.77 ± 0.49	3.94 ± 1.42[a]	1.16 ± 0.42[a]	4.04 ± 0.45	3/9
Amiloride, 1 mM	2.39 ± 0.45[b]	1.45 ± 0.30	0.94 ± 0.54[b]	0.85 ± 0.18	4.69 ± 1.18	3/7
HOE642, 30 μM	5.35 ± 1.10	1.66 ± 0.31	3.69 ± 1.16	1.18 ± 0.22	5.02 ± 1.14	3/8
S3226, 1 μM	2.22 ± 0.67[c]	1.55 ± 0.30	0.67 ± 0.22[c]	0.59 ± 0.23[b]	4.62 ± 1.04	3/8

Values are expressed as mean ± SD. Values in the same column bearing different superscripts are significantly different ($P < 0.05$) from the control.

The possible reasons for the discrepancy with the results of Graham et al. (16) are, however, not clear. It could be that NHE1 is present in the stratum granulosum but does not significantly contribute to transepithelial Na transport. NHE1 has been demonstrated in the stratum corneum of the epidermis (32) and is essential for the acidification of surface pH and the function of epidermal barrier. However, in contrast to the slightly acid pH (6.2–6.4) of the epidermis (3), the surface of the rumen epithelium exhibits an alkaline pH (7.4–7.5), which is modified by the luminal Cl concentration, supporting the assumption that the surface pH of this epithelium is mainly influenced by Cl^-/HCO_3^--exchange (23).

A further observation supports the conclusion that transepithelial Na transport is mediated by NHE3 and not by NHE1. Schweigel et al. (36) have shown that an increase of the luminal osmotic pressure causes a significant inhibition of J_{ms} and J_{net} Na and, after a detailed discussion, conclude that this inhibition of J_{ms} and J_{net} Na is caused by an effect of osmotic pressure on NHE3 activity. By contrast, hyperosmolarity causes a rapid alkalinization in isolated rumen epithelial cells, indicating an increased NHE1 exchange activity (36). The assumption of NHE3-mediated electroneutral transport is further supported by the observation that cAMP inhibits Na transport across the rumen epithelium (12, 42). As is well

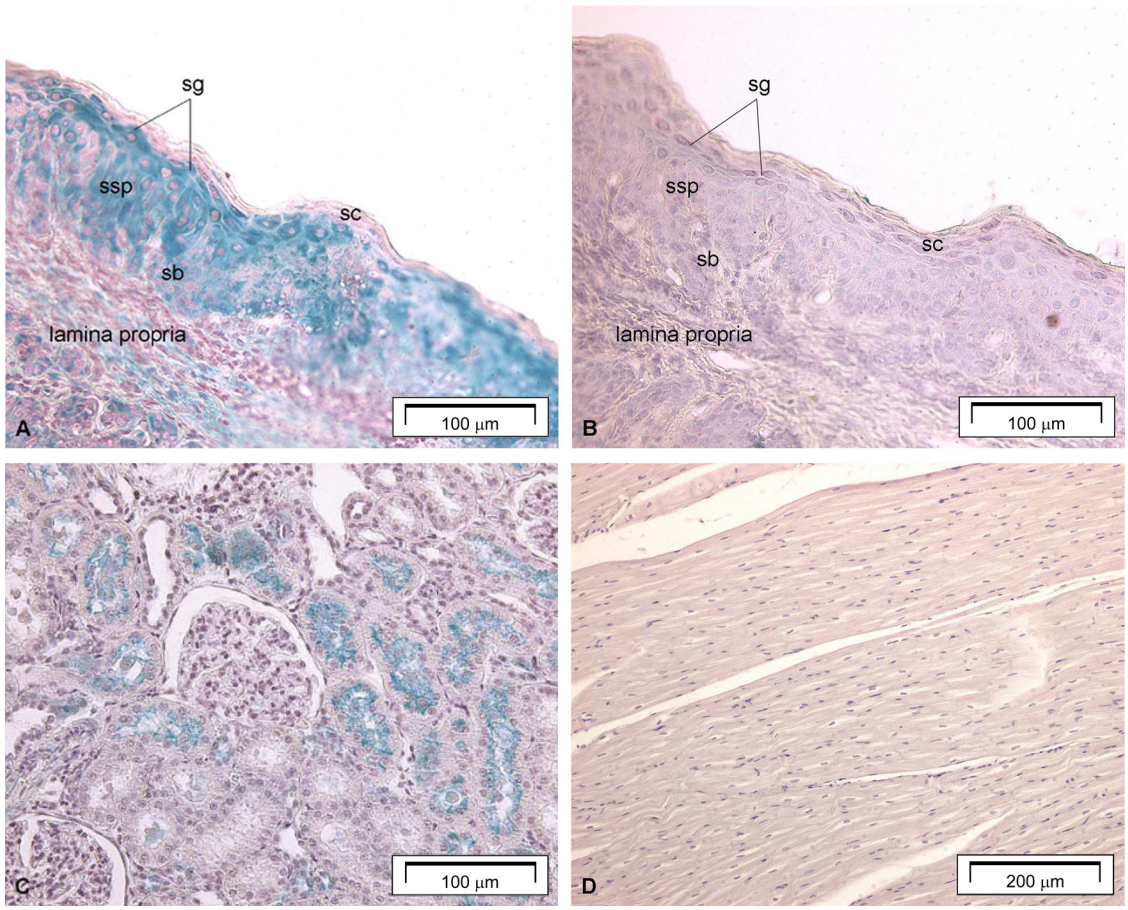

Fig. 2. Immunolocalization of NHE3. *A*: ovine rumen epithelium (blue): Immunostaining for NHE3 is most intense at the lumen facing membrane in cells of the stratum granulosum (sg) with a decrease in intensity across the stratum spinosum (ssp) and stratum basale (sb). Note the negative staining in the stratum corneum (sc). *B*: negative control with the respective preimmune serum (diluted 1:1,500) and counterstained with Mayer's Hemalum solution. *C*: positive tissue control: rat kidney (4). *D*: negative tissue control: ovine heart.

established, cAMP inhibits NHE3 via the activation of PKA and not NHE1 (31).

On the other hand, NHE1 activity has been demonstrated in studies of the regulation of pH_i in isolated REC (29, 36). In these studies, HOE642 and S3226 (36) or HOE694 (29) significantly reduces the recovery of pH_i after an acid load by butyrate. Interestingly, the effects of HOE642 and S3226 are almost additive, indicating the activity of both isoforms in REC (36).

Graham et al. (16) have further demonstrated the presence of mRNA of NHE3 and NHE1 in bovine rumen; this is in agreement with our findings in sheep rumen (36). However, immunostaining by Graham et al. (16) with regard to NHE has only been performed with antibodies against NHE1 and NHE2, but not against NHE3. The reasons for the restriction to NHE1 and NHE2 are not known, and the authors do not further mention NHE3, despite detection of its mRNA.

Irrespective of the presence or absence of SCFA, amiloride, or S3226 (significantly) reduces I_{sc} (Tables 3 and 4, Fig. 1), suggesting that electrogenic Na transport is also inhibited by blocking the PD-dependent cation conductance of the apical membrane (20, 22, 25). However, the change in I_{sc} occurs with some delay after the mucosal addition of amiloride or S3226. The effect on I_{sc} might thus indirectly be caused by the inhibition of NHE3, the decrease of intracellular pH possibly causing changes of ion conductance and hence of the flow and direction of ions. This conclusion of indirect effects is supported by the observation that amiloride in the micromolar range, which inhibits the classical epithelial Na channel, is without effect on NHE and I_{sc} in sheep rumen (28). Notably, in the presence of mucosal amiloride or S3226, J_{net} and I_{sc} are not significantly different, but J_{net} is numerically higher (Table 4). This small gap probably represents J_{net} K flux in the serosal-mucosal direction (24).

Significance and Perspectives

The interaction between SCFA and NHE3 demonstrated here is of significant physiological importance under in vivo conditions because the apical uptake of SCFA during feeding causes intracellular release of protons. Acidification of the cytosol by proton release would be cytotoxic without the observed coordination of proton extrusion via NHE. Apical NHE3 activity is, therefore, the first line of defense against epithelial acid load by SCFA; the challenge of enhanced fermentation and production of SCFA after feeding concentrated nutrients causes a mitigating increase of NHE activity (40).

Further, the findings of the current study support the conclusion that transepithelial electroneutral Na transport in rumen is mediated by NHE3 and not by NHE1. Na is taken up across the apical membrane via NHE3, and transepithelial Na transport is accomplished by extrusion via the basolaterally located Na-K-ATPase (19). This supposition of Na uptake via NHE3 is in agreement with our previous finding that low concentrations of amiloride that are known to inhibit NHE1 do not change Na transport (28) and with the observation that cAMP inhibits Na transport (12, 42). NHE1 activity is present in ruminal epithelial cells and is involved in the regulation of pH_i (29, 38) but probably does not contribute to transepithelial Na transport. Hence, classical "job sharing" in epithelia between NHE3 (transepithelial Na transport) and NHE1 (regulation of pH_i) probably also occurs in the rumen epithelium. This is in line with the presence of NHE1 as shown by Graham et al. (16) but contradicts their conclusions.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

REFERENCES

1. **Alexander RT, Grinstein S.** Na^+/H^+ exchangers and the regulation of volume. *Acta Physiol (Oxf)* 187: 159–167, 2006.
2. **Bailey CB, Balch CC.** Saliva secretion and its relation to feeding in cattle. 1. The composition and rate of secretion of parotid saliva in a small steer. *Br J Nutr* 15: 371–382, 1961.
3. **Behne MJ, Meyer JW, Hanson KM, Barry NP, Murata S, Crumrine D, Clegg RW, Gratton E, Holleran WM, Elias PM, Mauro TM.** NHE1 regulates the stratum corneum permeability barrier homeostasis. Microenvironment acidification assessed with fluorescence lifetime imaging. *J Biol Chem* 277: 47399–47406, 2002.
4. **Biemesderfer D, Rutherford PA, Nagy T, Pizzonia JH, Abu-Alfa AK, Aronson PS.** Monoclonal antibodies for high-resolution localization of NHE3 in adult and neonatal rat kidney. *Am J Physiol Renal Physiol* 273: F289–F299, 1997.
5. **Chien WJ, Stevens CE.** Coupled active transport of Na and Cl across forestomach epithelium. *Am J Physiol* 223: 997–1003, 1972.
6. **Coupaye-Gerard B, Bookstein C, Duncan P, Chen XY, Smith PR, Musch M, Ernst SA, Chang EB, Kleyman TR.** Biosynthesis and cell surface delivery of the NHE1 isoform of Na^+/H^+ exchanger in A6 cells. *Am J Physiol Cell Physiol* 271: C1639–C1645, 1996.
7. **Diernaes L, Sehested J, Moller PD, Skadhauge E.** Sodium and chloride transport across the rumen epithelium of cattle in vitro: effect of short-chain fatty acids and amiloride. *Exp Physiol* 79: 755–762, 1994.
8. **Dobson A.** Active transport through the epithelium of the reticulo-rumen sac. *J Physiol* 146: 235–251, 1959.
9. **Donowitz M, Li X.** Regulatory binding partners and complexes of NHE3. *Physiol Rev* 87: 825–872, 2007.
10. **Emanovic D, Harrison FA, Keynes RD, Rankin JC.** The effect of acetazolamide on ion transport across isolated sheep rumen epithelium. *J Physiol* 254: 803–812, 1976.
11. **Ferreira HG, Harrison FA, Keynes RD, Zurich L.** Ion transport across an isolated preparation of sheep rumen epithelium. *J Physiol* 222: 77–93, 1972.
12. **Gäbel G, Butter H, Martens H.** Regulatory role of cAMP in transport of Na^+, Cl^-, and short-chain fatty acids across sheep ruminal epithelium. *Exp Physiol* 84: 333–345, 1999.
13. **Gäbel G, Martens H.** Transport of Na^+ and Cl^- across the forestomach epithelium: mechanism and interactions with short-chain fatty acids. In: *Physiological Aspects of Digestion and Metabolism in Ruminants,* edited by T. Tsuda, Y. Sasaki, R. Kawashima; San Diego, CA: Academic, 1991, p. 129–151.
14. **Gäbel G, Sehested J.** SCFA transport in the forestomach of ruminants. *Comp Biochem Physiol A Physiol* 118: 367–374, 1997.
15. **Gäbel G, Vogler S, Martens H.** 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, 1991.
16. **Graham C, Gatherar I, Haslam I, Glanville M, Simmons NL.** Expression and localization of monocarboxylate transporters and sodium/proton

exchangers in bovine rumen epithelium. *Am J Physiol Regul Integr Comp Physiol* 292: R997–R1007, 2007.
17. **Harrison FA, Keynes RD, Rankin JC, Zurich L.** The effect of ouabain on ion transport across isolated sheep rumen epithelium. *J Physiol* 249: 669–677, 1975.
18. **Kapus A, Grinstein S, Wasan S, Kandasamy R, Orlowski J.** Functional characterization of three isoforms of the Na^+/H^+ exchanger stably expressed in Chinese hamster ovary cells. ATP dependence, osmotic sensitivity, and role in cell proliferation. *J Biol Chem* 269: 23544–23552, 1994.
19. **Kristensen NB, Hansen O, Clausen T.** Measurement of the total concentration of functional Na^+, K^+-pumps in rumen epithelium. *Acta Physiol Scand* 155: 67–76, 1995.
20. **Lang I, Martens H.** Na transport in sheep rumen is modulated by voltage-dependent cation conductance in apical membrane. *Am J Physiol Gastrointest Liver Physiol* 277: G609–G618, 1999.
21. **Lee SH, Kim T, Park ES, Yang S, Jeong D, Choi Y, Rho J.** NHE10, a novel osteoclast-specific member of the Na^+/H^+ exchanger family, regulates osteoclast differentiation and survival. *Biochem Biophys Res Commun* 369: 320–326, 2008.
22. **Leonhard-Marek S.** Divalent cations reduce the electrogenic transport of monovalent cations across rumen epithelium. *J Comp Physiol [B]* 172: 635–641, 2002.
23. **Leonhard-Marek S, Breves G, Busche R.** Effect of chloride on pH microclimate and electrogenic Na^+ absorption across the rumen epithelium of goat and sheep. *Am J Physiol Gastrointest Liver Physiol* 291: G246–G252, 2006.
24. **Leonhard-Marek S, Martens H.** Effects of potassium on magnesium transport across rumen epithelium. *Am J Physiol Gastrointest Liver Physiol* 271: G1034–G1038, 1996.
25. **Leonhard-Marek S, Stumpff F, Brinkmann I, Breves G, Martens H.** Basolateral Mg^{2+}/Na^+ exchange regulates apical nonselective cation channel in sheep rumen epithelium via cytosolic Mg^{2+}. *Am J Physiol Gastrointest Liver Physiol* 288: G630–G645, 2005.
26. **Marshall AB, Steven DH.** Branching cells in the rumen epithelium. *J Physiol* 204: 13P–14P, 1969.
27. **Martens H, Blume I.** Studies on the absorption of sodium and chloride from the rumen of sheep. *Comp Biochem Physiol A* 86: 653–656, 1987.
28. **Martens H, Gabel G, Strozyk B.** Mechanism of electrically silent Na and Cl transport across the rumen epithelium of sheep. *Exp Physiol* 76: 103–114, 1991.
29. **Müller F, Aschenbach JR, Gabel G.** Role of Na^+/H^+ exchange and HCO_3^- transport in pHi recovery from intracellular acid load in cultured epithelial cells of sheep rumen. *J Comp Physiol [B]* 170: 337–343, 2000.
30. **Murer H, Hopfer U, Kinne R.** Sodium/proton antiport in brush-border-membrane vesicles isolated from rat small intestine and kidney. *J Am Soc Nephrol* 9: 143–150, 1976.
31. **Orlowski J, Grinstein S.** Diversity of the mammalian sodium/proton exchanger SLC9 gene family. *Pflügers Arch* 447: 549–565, 2004.
32. **Sarangarajan R, Shumaker H, Soleimani M, Le Poole C, Boissy RE.** Molecular and functional characterization of sodium–hydrogen exchanger in skin as well as cultured keratinocytes and melanocytes. *Biochim Biophys Acta* 1511: 181–192, 2001.
33. **Schelling JR, Abu Jawdeh BG** Regulation of cell survival by Na^+/H^+ exchanger-1. *Am J Physiol Renal Physiol* 295: F625–F632, 2008.
34. **Schnorr B.** Histochemical, electron microscopic and biochemical studies on ATPases in the forestomach epithelium of goats. *Z Zellforsch Mikrosk Anat* 114: 365–389, 1971.
35. **Schwark JR, Jansen HW, Lang HJ, Krick W, Burckhardt G, Hropot M.** S3226, a novel inhibitor of Na^+/H^+ exchanger subtype 3 in various cell types. *Pflügers Arch* 436: 797–800, 1998.
36. **Schweigel M, Freyer M, Leclercq S, Etschmann B, Lodemann U, Bottcher A, Martens H.** Luminal hyperosmolarity decreases Na transport and impairs barrier function of sheep rumen epithelium. *J Comp Physiol [B]* 175: 575–591, 2005.
37. **Scott D.** Absorption of chloride from the rumen of the sheep. *Res Vet Sci* 11: 291–293, 1970.
38. **Sehested J, Diernaes L, Moller PD, Skadhauge E.** Transport of sodium across the isolated bovine rumen epithelium: interaction with short-chain fatty acids, chloride and bicarbonate. *Exp Physiol* 81: 79–94, 1996.
39. **Stevens CE.** Transport of sodium and chloride by the isolated rumen epithelium. *Am J Physiol* 206: 1099–1105, 1964.
40. **Storeheier PV, Sehested J, Diernaes L, Sundset MA, Mathiesen SD.** Effects of seasonal changes in food quality and food intake on the transport of sodium and butyrate across ruminal epithelium of reindeer. *J Comp Physiol [B]* 173: 391–399, 2003.
41. **Vallon V, Schwark JR, Richter K, Hropot M.** Role of Na^+/H^+ exchanger NHE3 in nephron function: micropuncture studies with S3226, an inhibitor of NHE3. *Am J Physiol Renal Physiol* 278: F375–F379, 2000.
42. **Wolffram S, Frischknecht R, Scharrer E.** Influence of theophylline on the electrical potential difference and ion fluxes (Na, Cl, K) across the isolated rumen epithelium of sheep. *Zentralbl Veterinarmed A* 36: 755–762, 1989.