

## Altered Na<sup>+</sup>/K<sup>+</sup>-ATPase expression plays a role in rumen epithelium adaptation in sheep fed hay *ad libitum* or a mixed hay/concentrate diet

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**ABSTRACT:** In this study we investigated rumen papillae morphology and the localization and expression of the Na<sup>+</sup>/K<sup>+</sup>-ATPase in eight sheep fed hay *ad libitum* (h) or hay *ad libitum* plus additional concentrate (h/c). Four sheep were provided with the *ad libitum* h-diet for the complete three-week experimental period. The second group of four sheep received the h-diet for only one week and was fed the mixed hay/concentrate (h/c) diet for another two weeks. The amount of concentrate supplement was stepwise increased from 150 to 1000 g/day and given in two meals. Following slaughter rumen papillae from the atrium ruminis (AR), the rumen ventralis (RV) and the ventral blind sac (BSV) were fixed and examined for morphological changes and Na<sup>+</sup>/K<sup>+</sup>-ATPase localization by morphometric methods and immunohistochemistry. Ruminal epithelial cells (REC) originating from the strata basale to granulosa were also isolated. Cellular Na<sup>+</sup>/K<sup>+</sup>-ATPase expression (mRNA and protein) and differentiation state were determined by RT-PCR, Western blot, and flow cytometry. Compared with data from h-fed sheep, morphometric analysis revealed an increased length and width of rumen papillae in h/c-fed sheep, resulting in a marked 41% and 62% increase in rumen papillae surface in AR and RV, respectively. The rumen mucosa of h/c-fed sheep was characterized by a predominant stratum corneum (42 ± 0.7 µm vs. 28 ± 0.5 µm), but the thickness of the metabolically active cell layers remained unchanged. REC suspensions from sheep fed the h/c diet generally contained more cells (7.30 ± 0.83 vs. 3.49 ± 0.52 × 10<sup>7</sup>/ml; *P* < 0.001) and an increased proportion of REC positive for basal cytokeratin and for the differentiation marker cytokeratin 10 (*P* < 0.05). Cellular (cell membrane) and epithelial (stratum basale to stratum granulosum) Na<sup>+</sup>/K<sup>+</sup>-ATPase localization was similar between rumen regions and was not changed by concentrate feeding. After two weeks on the h/c-diet, a 96% increase in the absolute number of Na<sup>+</sup>/K<sup>+</sup>-ATPase-positive REC (6.56 ± 0.84 vs. 3.35 ± 0.51 × 10<sup>7</sup>/ml; *P* = 0.003) and a 61% elevation (*P* = 0.043) in Na<sup>+</sup>/K<sup>+</sup>-ATPase protein expression in REC from the upper third of the suprabasal cell layers were found. Moreover, a two-fold (*P* = 0.001) elevation in cell membrane surface area accompanied by a reduction (1.19 × 10<sup>-7</sup> ± 1.72 × 10<sup>-9</sup> arbitrary units (AU)/cm<sup>2</sup> vs. 1.73 × 10<sup>-7</sup> ± 8.16 × 10<sup>-9</sup> AU/cm<sup>2</sup> in the h-group; *P* < 0.001) in specific Na<sup>+</sup>/K<sup>+</sup>-ATPase fluorescence per cm<sup>2</sup> of cell membrane surface area was observed after h/c-feeding. Na<sup>+</sup>/K<sup>+</sup>-ATPase α subunit mRNA expression was also reduced (*P* < 0.0001) from 0.154 ± 0.013 to 0.057 ± 0.004 pg per pg S18 mRNA control in the h/c-compared with the h-group. Thus, the h/c-diet led to a rapid increase in REC number and total cell membrane surface area in metabolically active and resorptive cell layers and was accompanied by a reduction in Na<sup>+</sup>/K<sup>+</sup>-ATPase mRNA expression and abundance per cell membrane surface area.

**Keywords:** sheep; rumen epithelial cells; transport protein; ruminal mucosa morphology; metabolizable energy

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**List of abbreviations:** AR = atrium ruminis, AU = arbitrary units, BSV = ventral blind sac; h = diet hay *ad libitum* diet, h/c = diet hay *ad libitum* plus additional concentrate, ME = metabolizable energy, PBS = phosphate-buffered saline, REC = rumen epithelial cells, RV = rumen ventralis, SCFA = short chain fatty acids, SC = stratum corneum, SB = stratum basale, SG = stratum granulosum

In ruminants, the forestomach, particularly the rumen, is responsible for a larger part of nutrient and electrolyte absorption. About 65% to 85% of the short-chain fatty acids (SCFA) produced by intraruminal carbohydrate fermentation are absorbed across the rumen epithelium which is also known to be the main site of  $\text{Na}^+$  and  $\text{Mg}^{2+}$  uptake (Dobson, 1959; Pfeiffer and Rahman, 1974; Remond et al., 1995). The transport capacity of the rumen epithelium for these substrates is known to increase markedly with higher levels of metabolizable energy (ME) intake and feeding of high-concentrate rations (Gaebel et al., 1987; Sehested et al., 1997; Uppal et al., 2003; Shen et al., 2004; Etschmann et al., 2009). Higher transport rates are thought to result from well-described morphological transformations (Dirksen et al., 1984) leading to a size increase in the rumen epithelium papillae and consequently to an enlargement of the available absorptive surface area (Liebich et al., 1987; Shen et al., 2004). In addition, early functional adaptation processes involving the altered activity and/or expression of cellular transport proteins have been described (Storeheier et al., 2003; Uppal et al., 2003; Shen et al., 2004; Etschmann et al., 2009). In accordance with a specific role for active transport proteins in rumen epithelial adaptation, a significant enhancement of  $\text{Na}^+/\text{K}^+$ -ATPase activity by concentrate feeding has been found (Kristensen et al., 1995; McLeod and Baldwin, 2000). The  $\text{Na}^+/\text{K}^+$ -ATPase is a key transport element required for the establishment of electrochemical gradients driving cellular transport and substrate flow across epithelia (Zouzoulas et al., 2005). The  $\text{Na}^+$ -motive force generated by the  $\text{Na}^+/\text{K}^+$ -ATPase is essential for the absorption of sodium (Ferreira et al., 1966; Harrison et al., 1975) and is indirectly related to SCFA transport via the  $\text{Na}^+/\text{H}^+$  exchanger (NHE) (Sehested et al., 1996). In addition, the enzyme is involved in basic processes such as cell maintenance and proliferation (Burke et al., 1991). In the rumen epithelium, a  $\text{Na}^+/\text{K}^+$ -ATPase is expressed at extremely high levels (Kristensen et al., 1995; Hansen 1998; Graham and Simmons, 2005; Albrecht et al., 2008). Its ac-

tivity accounts for ~22–25% of rumen  $\text{O}_2$  and, consequently, ATP utilization (Kelly et al., 1993).

We hypothesize that feed-induced changes in  $\text{Na}^+/\text{K}^+$ -ATPase expression and/or activity are a major component of the functional adaptation of the rumen epithelium to a high-energy diet. Therefore, using isolated rumen epithelial cells (REC), we have analyzed the mRNA and protein expression of ruminal  $\text{Na}^+/\text{K}^+$ -ATPase in sheep fed hay *ad libitum* only or a mixed hay/concentrate diet for two weeks. In addition, the cellular and epithelial localization of the  $\text{Na}^+/\text{K}^+$ -ATPase and the feed-induced morphological alterations in the epithelium were monitored in three rumen regions (AR, RV, BSV) by immunohistochemistry and morphometric analysis.

## MATERIAL AND METHODS

### Materials

Trypsin, glutamine, antibiotics (penicillin-streptomycin), fetal calf serum (FCS) and Dulbecco's phosphate-buffered saline (DPBS) were purchased from PAN Biotech (Aidenbach, Germany). All chemicals for Western blot analysis were purchased from Carl Roth (Karlsruhe, Germany).

### Antibodies

The monoclonal mouse antibody used in this study is specific for the  $\alpha$  subunit of sheep  $\text{Na}^+/\text{K}^+$ -ATPase (M7-PB-E9, Affinity Bioreagents) and has been shown to detect the sheep protein specifically (Albrecht et al., 2008). Mouse monoclonal anti-cytokeratin 10 [DE-K10] and anti-basal cell cytokeratin [RCK103] antibodies were obtained from abcam (Cambridge, UK). Relevant secondary antibodies conjugated to Alexa Fluor 488 (Invitrogen) or FITC (Abcam, Cambridge, UK) were used for flow cytometry and immunohistochemistry, respectively. For Western analysis of  $\text{Na}^+/\text{K}^+$ -ATPase expression, we employed a horseradish peroxidase (HRP)-

conjugated antibody (ECL<sup>TM</sup> sheep Anti-mouse) obtained from Amersham Bioscience.

### Animals and experimental design

Eight six month-old male castrated lambs were fed meadow hay *ad libitum* for one week. Thereafter, the animals were divided into two groups of four sheep. The control group received meadow hay *ad libitum* for another two weeks (h), and the second group was fed with a mixed meadow hay *ad libitum*/concentrate (h/c) diet over the same time period. The amount of the concentrate was stepwise increased from 150–1000 g/day and was given in two meals at 7 am and 2 pm. The chemical composition of the concentrate is shown in Table 1. Water was available *ad libitum*.

### Sample preparation

**Tissue preparation.** Samples were excised from the rumen of sheep within 10 min of slaughter. Tissue pieces of at least 100 cm<sup>2</sup> each were taken from three regions, viz., (1) the AR, (2) the RV and (3) the BSV, and washed three to five times in ice-cold divalent-free phosphate-buffered saline (PBS) containing penicillin-streptomycin. Then, after one wash in the same solution without antibiotics tissue pieces (1 cm<sup>2</sup> surface) from all three areas were

Table 1. Metabolizable energy content (in MJ/kg dry matter, DM) and chemical composition of the concentrate mixture\* fed to those sheep receiving the mixed hay/concentrate diet for 14 days

Metabolizable energy (MJ/kg DM)	10.2
Nutrient composition (% DM)	
Crude protein	16
Crude fat	3.2
Crude fiber	9.5
Crude ash	9.5
Minerals (% DM)	
Calcium	1.5
Phosphorus	0.5
Sodium	0.4

\*Concentrate mixture: 15% sugar beet slices, 15% oat, 15% wheat bran, 12% wheat gluten food meal, 10% barley malt germs, 10% rape expeller, 8% corn, 6% sugar beet molasses, 4.5% soybean solvent extracted oil meal, 3.2% CaCO<sub>3</sub>, 0.8% NaCl

fixed for morphometric and immunohistochemical studies. The remaining tissue was transferred to fresh ice-cold divalent-free PBS with penicillin-streptomycin, transported to the laboratory, and stored for one hour at –4 °C before the preparation of rumen epithelial cells (REC).

**Isolation of REC.** Rumen papillae were removed by scissors and then washed three times in divalent-free PBS with antibiotics and once in antibiotic-free PBS without Ca/Mg. Thereafter, REC were prepared by fractional trypsinization as described by Galfi et al. (1980). The composition of the cell fractions was evaluated by light microscopy, and fractions one and two (mostly consisting of cells from the SC) were discarded. In order to investigate the existence of functionally different cell types in the rumen epithelium, three groups consisting of (1) fractions 3 to 5 = G1, (2) fractions 6 to 8 = G2, and (3) fractions 9 and 10 = G3 were collected. Some REC from each group were used to extract total protein or RNA. The samples were stored at –80 °C until analysis by Western blot and quantitative reverse transcription polymerase chain reaction (qRT-PCR). Some REC were fixed with methanol for flow-cytometric analysis of Na<sup>+</sup>/K<sup>+</sup>-ATPase, basal cell cytokeratin and cytokeratin 10 abundance.

### Light microscopy and morphometry of rumen papillae

Samples from AR, RV, and BSV were fixed in a 4% neutral formaldehyde solution. After rinsing with water, samples were dehydrated in a graded series of ethanol (30%, 50%, 70%, 90%, and absolute ethanol), cleared with benzene and saturated with and embedded in paraffin. Sections of 5 µm thickness were taken from 30 papillae and stained with haematoxylin/eosin. The length and width of the papillae were determined by the computer-operated *Image C* picture analysis system (Imtronic GmbH, Berlin, Germany) and the IMES analysis program, using a colour video camera (SONY 3 CCD, Sony Electronics Inc., Tokyo, Japan) and a light microscope (Axiolab, Carl Zeiss Jena, Germany). The number of papillae per cm<sup>2</sup> mucosa was estimated using a video camera equipped with a picture analysis system. The total surface of papillae per cm<sup>2</sup> mucosa was determined as length × width × 2, multiplied by the number of papillae/cm<sup>2</sup> (Hofmann and Schnorr, 1982). In addition, the widths of the ruminal mucosa and of the SC were measured.

## Immunohistochemistry

Rumen papillae were fixed in 4% paraformaldehyde in PBS overnight, washed in PBS, frozen in liquid nitrogen, and cryosectioned on a Leica CM3050 S (Leica, Bensheim, Germany). Sections (6 µm) were washed three times with PBS, permeabilized by incubation for 20 min in PBS containing 0.1% Triton X-100, and blocked with 10% rabbit serum in PBS + Triton X-100 for 15 min (all at room temperature). Subsequently, sections were incubated overnight at 4 °C with primary antibody (diluted 1 : 50 with 2% serum in PBS + Triton X-100) in a humidity chamber. After being washed three times in PBS, sections were incubated for 45 min at room temperature in the dark with an appropriate secondary antibody labelled with Alexa Fluor 488 (Molecular Probes, Eugene, OR), diluted 1 : 500 in PBS + Triton X-100. Nuclei were usually counterstained with 1 µg/ml propidium iodide in PBS. Sections were covered with MobiGLOW mounting medium (MoBiTec, Göttingen, Germany) and appropriate cover-slips. Sections incubated with goat serum in PBS + Triton X-100 instead of primary antibody were used as negative controls and showed no unspecific binding of secondary antibodies in rumen epithelial cells.

Immunofluorescence was detected using a Nikon Microphot SA fluorescence microscope (Nikon Instruments Europe B.V., Netherlands) and an image analysis system equipped with CELL<sup>^</sup>F image analysis software and a CC-12 high resolution colour camera (OSIS, Münster, Germany).

## Detection of the Na<sup>+</sup>/K<sup>+</sup>-ATPase mRNA transcript by qRT-PCR

Total RNA was isolated by a standard procedure (Total RNA isolation kit from Macherey & Nagel, Düren, Germany following the manufacturer's protocol). The concentration and quality of the extracted RNA were measured by using a NanoDrop ND-1000 Spectrophotometer (Peqlab Biotechnology GmbH, Erlangen, Germany). The ratios of absorbance at 260 and 280 nm of all preparations were approximately 2.0. The integrity of RNA was checked by denaturing agarose gel electrophoresis and ethidium bromide staining. The iScript<sup>™</sup> cDNA Synthesis Kit (Bio-Rad Laboratories GmbH, Munich, Germany) was used to synthesize cDNA from 100 ng total RNA from

each sample according to the manufacturer's instructions. A negative control, without reverse transcriptase, was processed for each sample to detect possible contamination with genomic DNA or environmental DNA.

The abundance of mRNA for the ribosomal protein S18 (S18) and for the Na<sup>+</sup>/K<sup>+</sup>-ATPase α subunit was quantified by qRT-PCR using the iCycler and the iQ-SYBR green supermix (Bio-Rad Laboratories GmbH, Munich, Germany) as described previously (Loehrke et al., 2005; Ulbrich et al., 2006). Briefly, 1 µl aliquots of each RT reaction (1/20 of total) were primed, in each 10 µl PCR, with gene-specific oligonucleotides (final concentration of 0.2 µM) for S18 (gi 74268022: 5'-CTT AAA CAG ACA GAA GGA CGT GAA-3' and 5'-CCA CAC ATT ATT TCT TCT TGG ACA-3'; transcript size: 218 bp) and Na<sup>+</sup>/K<sup>+</sup>-ATPase α (gi 115305283: 5'-GAG ATT ACC CCC TTC CTG ATA TTT-3' and 5'-TGG ATC ATA CCA ATC TGT CCA TAG-3'; transcript size: 227 bp). The primers were designed to span a corresponding intron and to anneal at 60 °C to published cDNA and gene sequences. PCR was performed over 40 cycles for 180 s at 94 °C and 10 s at 94 °C, followed by 30 s at 60 °C and 225 s at 70 °C. The specificity of amplification was determined by melting curve analysis and agarose gel electrophoresis in comparison with an oligonucleotide molecular mass ladder to confirm that the calculated molecular mass of the cDNA corresponded to the produced cDNA. The cDNA structure was checked by sequencing. Each cDNA was quantified in duplicate; the average value of each sample value minus the corresponding negative control value was used to calculate the cDNA product corresponding to the abundance of mRNA. The amount of Na<sup>+</sup>/K<sup>+</sup>-ATPase α subunit mRNA was normalized against the housekeeping gene S18.

## Western blot analysis

For Western blots, total protein from freshly isolated and washed REC was extracted using the M-PER Mammalian Protein Extraction Reagent (Pierce, Bonn, Germany), complemented with Halt<sup>™</sup> protease inhibitor cocktail (Pierce, Bonn, Germany). Protein concentrations were determined by means of the Bradford assay (Bio-RAD, Munich, Germany). Protein samples (20 µg) were separated by SDS (12.5%)-polyacrylamide gel electrophoresis and subsequently transferred to polyvinylidene flu-



oride (PVDF) membranes (GE Healthcare, Munich, Germany). After transfer, membranes were blocked with 3% non-fat dry milk in PBS (pH 7.5) containing 0.05% Tween 20 (PBS-T) for 2 h and washed in PBS-T (1 × 10 min and 2 × 5 min). Thereafter, membranes were incubated at 4 °C with the primary antibody (anti-Na<sup>+</sup>/K<sup>+</sup>-ATPase: 1 : 1000 dilution) overnight, washed three times (1 × 15 min, 2 × 5 min) with PBS-T, and incubated for 1 h with HRP-conjugated secondary anti-mouse IgG (1 : 10 000 dilution) antibody. Then, after three washes (1 × 10 min and 2 × 5 min) in PBS-T, membranes were developed with the ECL Western Blotting Substrate (Pierce). For size comparison, the Precision Plus Protein WesternC Standard (161-0376, Bio-RAD) and the Precision Protein StrepTactin-HRP Conjugate (161-0381, Bio-RAD) were used. Density quantification was performed by ImageJ 1.41 software (National Institutes of Health).

### Flow cytometry

Methanol-fixed REC were incubated overnight at 4 °C with anti-Na<sup>+</sup>/K<sup>+</sup>-ATPase antibody (10 µg/ml) dissolved in 10mM PBS with 0.2% bovine serum albumin and 1mM EDTA, pH 7.3. After warming to room temperature, cells were washed twice in PBS-EDTA and incubated for 1 h in a 200-fold dilution (4 µg/ml) of Alexa-Fluor 488-conjugated anti-mouse-IgG(ab')<sub>2</sub> (Molecular Probes, Eugene, OR). The anti-Na<sup>+</sup>/K<sup>+</sup>-ATPase antibody was omitted from control incubations. After a further two washes in PBS-EDTA, quantitative analysis of cellular fluorescence was carried out by flow cytometry to analyze the cells simultaneously, with regard to size, granularity, and ATPase abundance (portion of protein-expressing cells and relative fluorescence intensity per single cell). Cytokeratin 10 and basal cell cytokeratin abundance was determined in the same way, but the incubation with the specific antibodies (7.5 µg/ml) was performed for two hours only, and secondary FITC-conjugated anti-mouse-IgG (4 µg/ml) antibodies were employed.

Flow-cytometric analysis was performed as described previously (Schweigel et al., 2006). Briefly, an argon-laser-equipped flow cytometer (Coulter-XL, Beckmann, Krefeld, Germany) was used to record emissions of multiple fluorescence (green, orange, red) excited at 488 nm (counting 5000 cells). Particle size was calibrated using standard beads (Coulter). Cells of interest were identified (1) by

establishing a histogram on the basis of cell size and granularity, (2) by establishing a fluorescence histogram, and (3) by projecting the fluorescence onto the size-granularity histogram. Subsequently, the cells were gated, and the portion of fluorescent cells and their fluorescence intensity were automatically computed.

### Statistical analysis

If not otherwise stated, data are presented as means ± standard error (SE). Significance was determined by Student's *t*-test or the paired *t*-test as appropriate. *P* < 0.05 was considered to be significant. All statistical calculations were performed using SigmaStat (Jandel Scientific).

## RESULTS AND DISCUSSION

### Animals

Before starting the two-week experimental period of differential feeding, sheep from both groups had a mean body weight of 43 ± 2 kg, and no change (0.7 ± 0.9 kg) occurred in the h-group. At the end of the experiment, the mean body weight of h/c-fed sheep was significantly higher when compared with that of the h-fed sheep and showed an increase of 3.0 ± 0.5 kg. As the hay intake per sheep and day was the same in both dietary groups (about 1 kg), this gain resulted from the extra energy supplied via the concentrate supplementation.

### Morphology of ruminal mucosa

In agreement with results of other authors (Shen et al., 2004), the length, width, and, thus, surface of the rumen papillae was in the order of AR > RV > BSV in both feeding groups (Table 2). These results were related to the higher post-feeding concentrations of SCFA and the lower rumen fluid pH values observed in the AR compared with the lower parts (RV, BSV) of the rumen (Martin et al., 1999). Concentrate feeding induced a significant reduction in rumen papillae density. However, morphometric analysis of single papillae from all three regions of the rumen (Table 2) revealed an increased length and width in h/c- compared with h-fed sheep. The observed changes were most prominent in the

Table 2. Morphometric parameters of rumen papillae in the atrium ruminis, rumen ventralis, and ventral blind sac (mean  $\pm$  SE;  $n = 4$  per diet)

	Atrium ruminis		
	h diet	h/c diet	<i>P</i> -value
Length (mm)	3.39 $\pm$ 0.21	4.64 $\pm$ 0.18	0.001
Width (mm)	1.85 $\pm$ 0.07	2.19 $\pm$ 0.07	0.008
Density ( $n/cm^2$ )	58 $\pm$ 1	51 $\pm$ 1	0.001
Surface ( $mm^2/cm^2$ )	736 $\pm$ 65	1038 $\pm$ 43	0.003
	Rumen ventralis		
	h diet	h/c diet	<i>P</i> -value
Length (mm)	2.08 $\pm$ 0.20	2.93 $\pm$ 0.13	0.005
Width (mm)	1.58 $\pm$ 0.08	1.96 $\pm$ 0.07	0.006
Density ( $n/cm^2$ )	62 $\pm$ 1	58 $\pm$ 1	0.018
Surface ( $mm^2/cm^2$ )	413 $\pm$ 56	671 $\pm$ 53	0.026
	Ventral blind sac		
	h diet	h/c diet	<i>P</i> -value
Length (mm)	1.97 $\pm$ 0.15	2.42 $\pm$ 0.13	0.045
Width (mm)	1.49 $\pm$ 0.03	1.65 $\pm$ 0.04	0.010
Density ( $n/cm^2$ )	63 $\pm$ 0	58 $\pm$ 1	$\leq 0.001$
Surface ( $mm^2/cm^2$ )	371 $\pm$ 35	460 $\pm$ 26	0.069

AR and RV resulting in a marked 41% and 62% increase, respectively, in papillae surface in these rumen regions of h/c-fed sheep. A 57% to 95% elevation in rumen papillae size was also found in concentrate-fed sheep (Goodlad, 1981), goats (Shen et al., 2004) and bulls (Shen et al., 2005). Together with other factors, such as lower intraruminal pH values (Gaebel et al., 1987; Brossard et al., 2004) and increased blood flow (Dobson, 1984; Thorlacius, 1972), this strongly supported the passive absorption of non-dissociated SCFA by passive diffusion (Bugaut, 1987; Gaebel et al., 1987; Sehested et al., 2000).

Importantly, with regard to the rumen mucosa (h-group: 142  $\pm$  2  $\mu$ m; h/c-group: 161  $\pm$  4  $\mu$ m), the higher epithelial surface area of h/c-fed sheep resulted from an increased thickness of the SC only (Table 3). Its width increased from 28  $\pm$  0.5  $\mu$ m in h-fed sheep to 42  $\pm$  0.7  $\mu$ m in h/c-fed sheep, and no inter-region differences were observed. Thus, the SC accounted for 19% and 26% of the rumen mucosa in h- and h/c-fed sheep, respectively (Table 3). In accordance with our results, Gaebel et al. (1987) found an increased number of cell layers (12–18 layers) forming the SC in sheep fed 64% or 90% concentrate compared with hay-fed sheep (2–3 layers). These changes also demonstrated the

growing importance of the protective function of the rumen epithelium with respect to concentrate feeding, a function achieved by a higher degree of differentiation and keratinization. One major factor responsible for the initiation of these processes was demonstrated to be the SCFA butyrate (Galfi et al., 1983). In our study, the ruminal butyrate concentration, as measured by gas chromatography, amounted to 6.8  $\pm$  0.4 mmol/l in h-fed sheep and increased to 17.3  $\pm$  1.2 mmol/l ( $P < 0.001$ ) in h/c-fed sheep. Butyrate is known to act as a reversible mitosis inhibitor, thereby initiating the process of REC differentiation (Galfi et al., 1981). It also induces distinct changes such as the appearance of refractive granules, an increased number of filamentous bundles, cell membrane thickening, nucleus degeneration, the formation of keratohyalin-like granules and the accumulation of the enzyme alkaline phosphatase in the cell membrane (Galfi et al., 1982, 1983, 1991) characteristic of the enhanced keratinization of REC. The thickness of the metabolically active cell layers (stratum basale to stratum granulosum) was not different between the feeding groups (Table 3). However, in accordance with a higher degree of differentiation, more cells positive for cytokeratin 10 (1.35  $\pm$  0.45 vs. 0.43  $\pm$  0.14  $\times 10^7$ /ml;  $P < 0.05$ ) were found in REC from

Table 3. Effect of diet on the thickness of rumen mucosa and on the width of various cell layers forming this mucosa (mean  $\pm$  SE;  $n = 4$  per diet)

	Atrium ruminis		<i>P</i> -value
	h diet	h/c diet	
Mucosa ( $\mu\text{m}$ )	147.25 $\pm$ 4.48	174.50 $\pm$ 7.98	0.025
SC ( $\mu\text{m}$ )	27.75 $\pm$ 1.25	43.50 $\pm$ 1.71	< 0.001
SB-SG	119.50 $\pm$ 4.21	131.00 $\pm$ 6.72	0.197
% SC	18.87 $\pm$ 0.85	24.97 $\pm$ 0.70	0.001
(SB-SG)/SC	4.33 $\pm$ 0.22	3.01 $\pm$ 0.11	0.002
Rumen ventralis			
Mucosa ( $\mu\text{m}$ )	140.75 $\pm$ 2.56	160.25 $\pm$ 5.79	0.022
SC ( $\mu\text{m}$ )	29.25 $\pm$ 0.85	42.50 $\pm$ 3.23	0.007
SB-SG	111.50 $\pm$ 1.85	117.75 $\pm$ 2.96	0.123
SC (%)	20.77 $\pm$ 0.33	26.43 $\pm$ 1.10	0.003
(SB-SG)/SC	3.82 $\pm$ 0.08	2.80 $\pm$ 0.15	< 0.001
Ventral blind sac			
Mucosa ( $\mu\text{m}$ )	137.25 $\pm$ 4.91	149.50 $\pm$ 3.97 <sup>a</sup>	0.100
SC ( $\mu\text{m}$ )	27.50 $\pm$ 0.65	41.00 $\pm$ 0.91	< 0.001
SB-SG	109.75 $\pm$ 4.61	108.50 $\pm$ 3.07 <sup>a</sup>	0.829
SC (%)	20.09 $\pm$ 0.65	27.43 $\pm$ 0.15 <sup>a</sup>	0.029
(SB-SG)/SC	3.99 $\pm$ 0.15	2.65 $\pm$ 0.02 <sup>a</sup>	0.029

SC = stratum corneum, SB-SG = stratum basale to stratum granulosum

<sup>a</sup>atrium ruminis vs. ventral blind sac,  $P < 0.05$

h/c-fed sheep. Cytokeratin 10 has been shown to be a differentiation marker appearing in cells from the upper stratum spinosum to stratum granulosum (Eckert et al., 1997).

As shown in Table 3, inter-region differences were found between AR and BSV of h/c-fed sheep only. The mucosa width of the BSV was decreased, resulting from the reduced thickness (21%) of the stratum basale to stratum granulosum cell layers in the BSV compared with the AR region.

Compared with controls, REC suspensions from sheep fed the h/c diet generally contained more cells ( $7.30 \pm 0.83$  vs.  $3.49 \pm 0.52 \times 10^7/\text{ml}$ ;  $P < 0.001$ ), showed an elevated protein content ( $891 \pm 76$  vs.  $287 \pm 56 \mu\text{g}/\text{ml}$ ;  $P < 0.001$ ) in the germinative and absorptive REC populations (G2, G3) and a decreased RNA-to-protein ratio ( $0.13 \pm 0.01$  vs.  $0.50 \pm 0.05 \mu\text{g}/\text{ml}$ ;  $P < 0.005$ ). This indicates cellular hyperplasia, viz., a higher capacity to synthesize protein and to proliferate. Because of this, differentiated cells enter the upper epithelial layers faster (Eckert et al., 1997).

### Localization and protein expression of the $\text{Na}^+/\text{K}^+$ -ATPase in rumen epithelium

With the exception of the SC, the  $\text{Na}^+/\text{K}^+$ -ATPase was present in all REC and was strictly localized to the cell membrane (Figure 1). This was in accordance with results from studies with bovine rumen epithelium (Graham and Simmons, 2005; Albrecht et al., 2008). Cellular and epithelial  $\text{Na}^+/\text{K}^+$ -ATPase localization was similar between rumen regions and was not altered by concentrate feeding. As only cornified cells express no  $\text{Na}^+/\text{K}^+$ -ATPase protein, the pump can be used as a marker to identify cells from the metabolically active cell layers (Figure 1). Using flow cytometry and a  $\alpha_1$ -subunit-specific anti-sheep  $\text{Na}^+/\text{K}^+$ -ATPase antibody, the protein was found in  $95 \pm 1\%$  and  $89 \pm 3\%$  of REC isolated from h or h/c epithelia. However, as mentioned above, REC suspensions from h/c-fed sheep contained many more cells than that from h-fed sheep resulting in a 96% increase in the absolute number

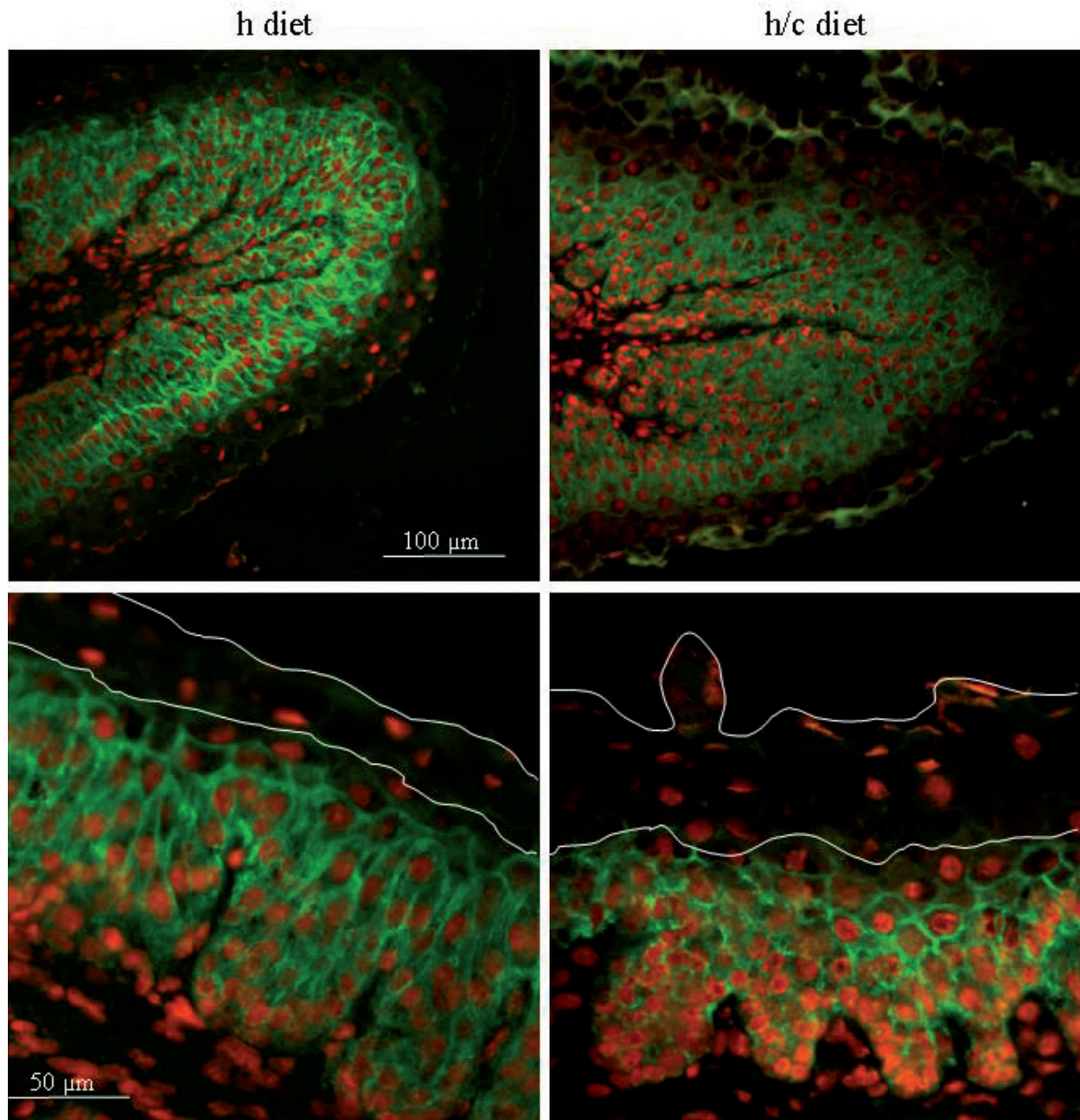


Figure 1. Immunolocalization of the  $\text{Na}^+/\text{K}^+$ -ATPase  $\alpha$  subunit (green) in the atrium ruminis of sheep fed hay *ad libitum* (h diet) or hay *ad libitum* + concentrate (h/c diet) for 14 days. Cells were co-stained with propidium iodide (red) to label nuclei. With the exception of the stratum corneum (SC), the  $\text{Na}^+/\text{K}^+$ -ATPase exists in the cell membrane of all REC and thus, can be used to differentiate between cornified and metabolically active cells. Note the predominant SC (area enclosed by white lines) in h/c fed sheep

of  $\text{Na}^+/\text{K}^+$ -ATPase-positive REC ( $6.56 \pm 0.84$  vs.  $3.35 \pm 0.51 \times 10^7/\text{ml}$ ;  $P = 0.003$ ) after concentrate feeding (Figure 2A).

Next, we investigated the expression of the  $\text{Na}^+/\text{K}^+$ -ATPase protein by Western blotting (Figure 2B). Typical Western blots of whole cell protein extracts derived from G1 to G3 REC fractions from

h- or h/c-fed sheep are presented in Figure 2C. As in bovine rumen tissue (Graham and Simmons, 2005), a major band at about 110–120 kDa together with a smaller one at about 97 kDa represented the  $\alpha$ -subunits of the ovine  $\text{Na}^+/\text{K}^+$ -ATPase protein. Both bands were stronger in G1 of h/c-fed sheep. Indeed, downstream quantification of the 97-kDa



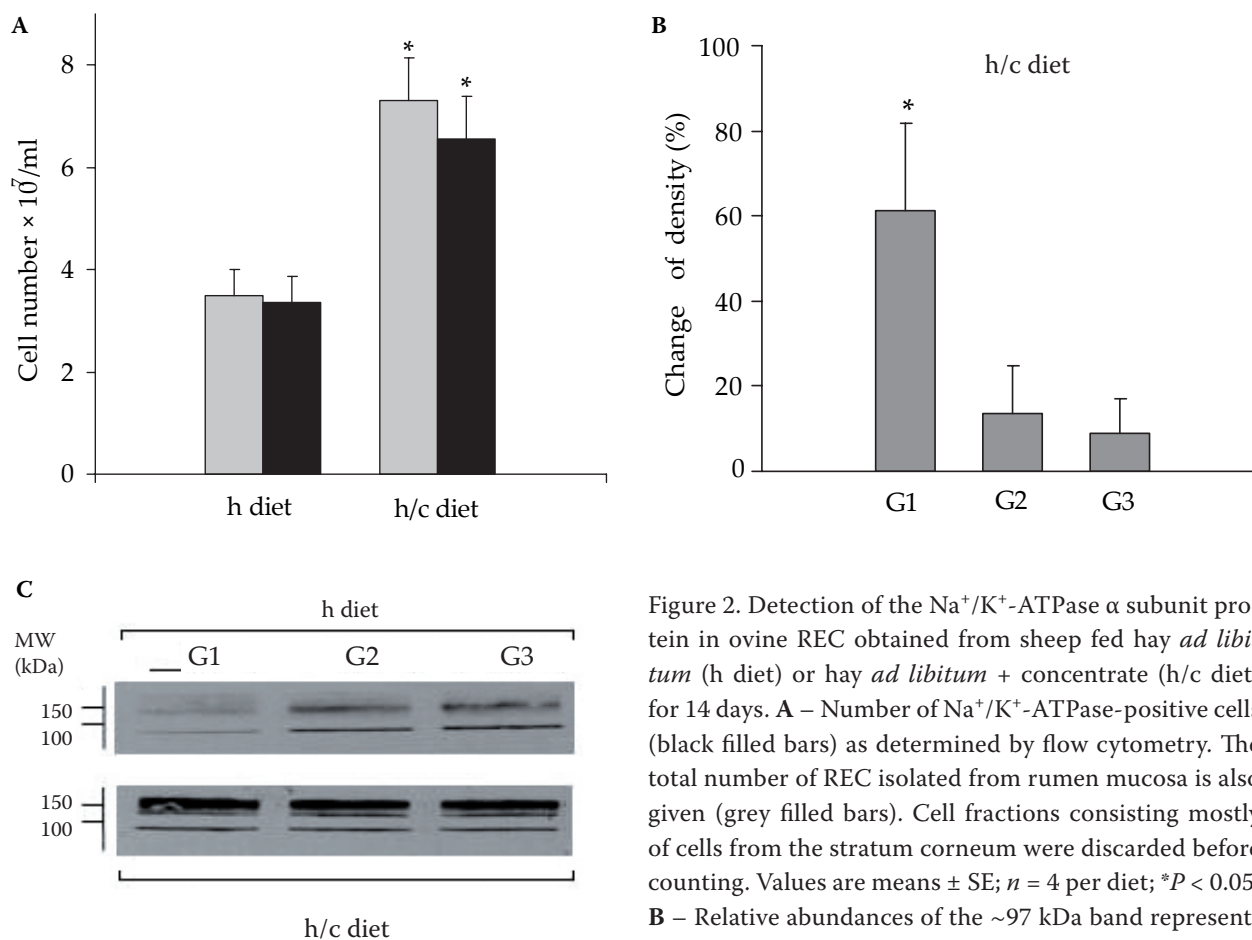


Figure 2. Detection of the Na<sup>+</sup>/K<sup>+</sup>-ATPase α subunit protein in ovine REC obtained from sheep fed hay *ad libitum* (h diet) or hay *ad libitum* + concentrate (h/c diet) for 14 days. **A** – Number of Na<sup>+</sup>/K<sup>+</sup>-ATPase-positive cells (black filled bars) as determined by flow cytometry. The total number of REC isolated from rumen mucosa is also given (grey filled bars). Cell fractions consisting mostly of cells from the stratum corneum were discarded before counting. Values are means ± SE; *n* = 4 per diet; \**P* < 0.05. **B** – Relative abundances of the ~97 kDa band representing the α subunit protein in ovine REC isolated from

sheep fed the h- or h/c-diet. Densitometric analysis was performed using ImageJ freeware. The percentage increase in Na<sup>+</sup>/K<sup>+</sup>-ATPase α subunit protein abundance in REC from h/c-fed sheep in relation to its abundance in REC from h-fed sheep is shown. **C** – Representative example of a Na<sup>+</sup>/K<sup>+</sup>-ATPase α subunit Western blot, used for the ImageJ analysis. The anti-Na<sup>+</sup>/K<sup>+</sup>-ATPase α subunit antibody detected bands at the expected sizes of ~110–120 and ~97 kDa, respectively. Values are means ± SE; *n* = 4 per diet; \**P* < 0.05

signal with ImageJ software established that the pixel density of G1 was increased by 61 ± 21% in the h/c-group compared with the h-group (*P* = 0.043; Figure 2B). No difference in the signal intensity was found between the G2 and G3 REC fractions from the differently fed sheep. Thus, compared with the h-group, more Na<sup>+</sup>/K<sup>+</sup>-ATPase protein was found in the first REC fractions (three to five = G1) from h/c sheep. G1 is known to contain more cells from the upper third of the suprabasal cell layers, a rumen epithelial region in which NHE subtype 1 shows its highest expression level (Graham et al., 2007). Concentrate feeding promotes the NHE-related Na<sup>+</sup> uptake, which results from an elevated rate of diffusional SCFA uptake accompanied by an increased cellular acid load (Jessop, 2000; Uppal et al., 2003; Shen et al., 2004). To maintain the trans-

membrane Na<sup>+</sup> gradient and normal intracellular [Na<sup>+</sup>], excess Na<sup>+</sup> has to be removed by the Na<sup>+</sup>/K<sup>+</sup>-ATPase (Jessop, 2000; Albrecht et al., 2008); this might explain its higher abundance in the upper cell layers of h/c-fed compared with h-fed sheep as found in the present study. This interpretation of our data is in agreement with the early work of Henrikson (1971). Using immunohistochemical techniques, Henrikson found an enrichment of Na<sup>+</sup>/K<sup>+</sup>-ATPase in the middle layers of sheep rumen epithelium. In addition, functional data from a study with lambs fed a 75% forage or concentrate diet at about 2 × ME (McLeod and Baldwin, 2000), respectively, show a 36% increase in specific Na<sup>+</sup>/K<sup>+</sup>-ATPase activity with the latter treatment.

Using flow-cytometric analysis, we were able to quantify not only the number of Na<sup>+</sup>/K<sup>+</sup>-ATPase-

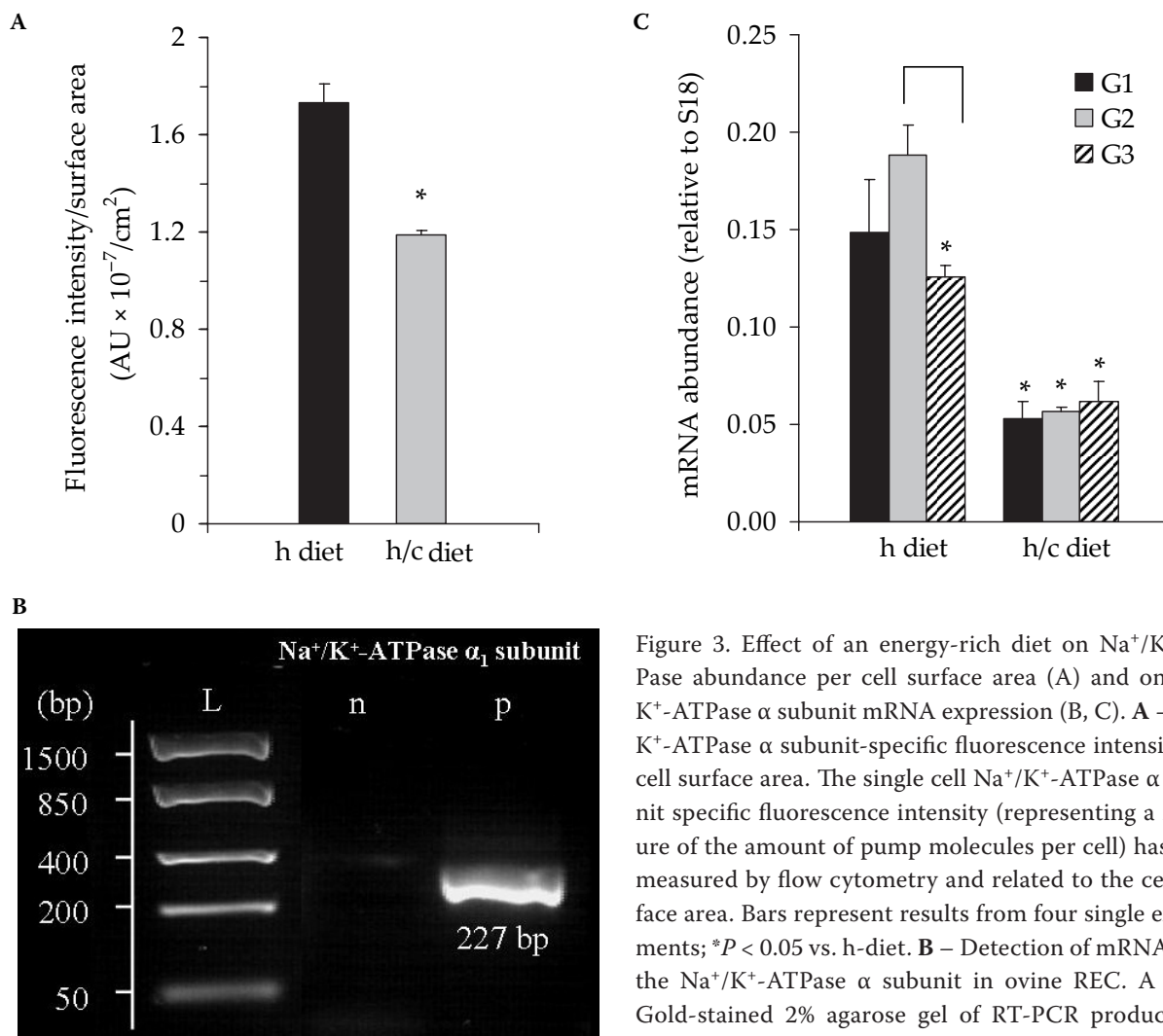


Figure 3. Effect of an energy-rich diet on Na<sup>+</sup>/K<sup>+</sup>-ATPase abundance per cell surface area (A) and on Na<sup>+</sup>/K<sup>+</sup>-ATPase α subunit mRNA expression (B, C). **A** – Na<sup>+</sup>/K<sup>+</sup>-ATPase α subunit-specific fluorescence intensity per cell surface area. The single cell Na<sup>+</sup>/K<sup>+</sup>-ATPase α subunit specific fluorescence intensity (representing a measure of the amount of pump molecules per cell) has been measured by flow cytometry and related to the cell surface area. Bars represent results from four single experiments; \**P* < 0.05 vs. h-diet. **B** – Detection of mRNA from the Na<sup>+</sup>/K<sup>+</sup>-ATPase α subunit in ovine REC. A SYBR Gold-stained 2% agarose gel of RT-PCR products for DNA size ladder (bp, lane L), negative control (lane n, without reverse transcriptase), and Na<sup>+</sup>/K<sup>+</sup>-ATPase α subunit (lane p) are shown. The product obtained with the Na<sup>+</sup>/K<sup>+</sup>-ATPase α subunit-specific primer has the expected size of 227 bp. **C** – Expression of Na<sup>+</sup>/K<sup>+</sup>-ATPase α subunit mRNA in group 1 (G1) to group 3 (G3) REC fractions of sheep fed the hay or the hay/concentrate diet for 14 days. Values have been normalized to the S18 signal and are shown as means ± SE; *n* = 4 per diet; \**P* < 0.05

positive REC, but also the Na<sup>+</sup>/K<sup>+</sup>-ATPase-specific fluorescence intensity per single cell as a measure of the cellular amount of Na<sup>+</sup>/K<sup>+</sup>-pumps. This value was  $74 \pm 11$  AU in control cells from h-fed sheep and dropped to  $54 \pm 8$  AU after concentrate feeding. In other cell systems cell density has been found to be an important signal determining the Na<sup>+</sup>/K<sup>+</sup>-ATPase number per cell. Elevated numbers of pumps have been observed in subconfluent compared to confluent cultures (Burke et al., 1991). Considering the diameter and total number of REC isolated from G1 to G3 fractions, we calculated a two-fold elevation of the cell membrane surface area ( $335.0 \pm 41.6$  cm<sup>2</sup>) in h/c-fed sheep compared with controls ( $156.6 \pm 26.1$  cm<sup>2</sup>; *P* = 0.001). However, as shown in Figure 3A, the spe-

cific Na<sup>+</sup>/K<sup>+</sup>-ATPase fluorescence per cm<sup>2</sup> of cell membrane surface area was reduced from  $1.73 \times 10^{-7} \pm 8.16 \times 10^{-9}$  AU/cm<sup>2</sup> in the h-group to  $1.19 \times 10^{-7} \pm 1.72 \times 10^{-9}$  AU/cm<sup>2</sup> (*P* < 0.001) in the h/c-group. Our data are in accordance with the reduction of Na<sup>+</sup>/K<sup>+</sup>-ATPase-binding sites (–15%, Kristensen et al., 1995) and of net Na<sup>+</sup> transport per surface area (Gaebel et al., 1987) seen after concentrate feeding. They also explain reported results showing lower portal-drained viscera O<sub>2</sub> consumption in heifers fed a 75% concentrate compared with heifers fed a 75% forage diet (Reynolds et al., 1991). Thus, a reduction in Na<sup>+</sup>/K<sup>+</sup>-ATPase expression per surface area can be assumed to be the main energy-saving mechanism of single REC from h/c-fed sheep. In agreement with this

hypothesis, the total  $O_2$  consumption of isolated REC *in vitro* was not affected by changes in the dietary forage-to-concentrate ratio (McLeod and Baldwin, 2000).

### $Na^+/K^+$ -ATPase $\alpha$ subunit mRNA expression

As shown in Figure 3B, PCR revealed the presence of the  $Na^+/K^+$ -ATPase  $\alpha$  subunit in REC. The product obtained corresponded to the calculated base number (227 bp) of the sequence produced by the primers. This result was confirmed by sequencing of the PCR products, yielding an identity of 98% homology. The levels of  $Na^+/K^+$ -ATPase  $\alpha$  subunit mRNA were  $0.154 \pm 0.013$  and  $0.057 \pm 0.004$  pg per pg S18 mRNA control in the h- and h/c-REC fractions, respectively, and were thus significantly ( $P < 0.0001$ ) reduced in the latter. In our recent study with cows, the mean amount of  $Na^+/K^+$ -ATPase  $\alpha$  subunit mRNA was determined to be  $0.090 \pm 0.002$  pg per pg of S18 mRNA, which is in good agreement with the results of the present study of sheep. Figure 3C presents the relative concentration of  $Na^+/K^+$ -ATPase  $\alpha$  subunit mRNA for G1 ( $0.148 \pm 0.028$  vs.  $0.053 \pm 0.008$  pg per pg S18 mRNA), G2 ( $0.188 \pm 0.016$  vs.  $0.057 \pm 0.003$  pg per pg S18 mRNA), and G3 ( $0.126 \pm 0.006$  vs.  $0.062 \pm 0.012$  pg per pg S18 mRNA) fractions of REC obtained from h-fed and h/c-fed sheep, respectively. The strong  $62 \pm 4\%$  reduction in  $Na^+/K^+$ -ATPase  $\alpha$  subunit mRNA levels observed after consumption of the high energy diet corresponded to the reduced  $Na^+/K^+$ -ATPase  $\alpha$  subunit protein abundance per cell and  $cm^2$  surface area. Penner et al. (2009) found only a non-significant 18% and 29% decrease in  $Na^+/K^+$ -ATPase and *NHE1* gene expression, respectively, in cows fed high (64%) versus low (8%) concentrate. However, in contrast to our study, they used complete ruminal tissue samples including mucosal and submucosal layers for their experiments. The expression profile of cells originating from the submucosal connective tissue thus probably differs from that of epithelial cells. As concentrate feeding leads to a massive proliferation of this part of rumen papillae (Liebich et al., 1987), a higher proportion of cells from connective tissue may be responsible for expression biases. For example, in our study, the reduction in  $Na^+/K^+$ -ATPase  $\alpha$  subunit expression was lower ( $-51\%$ ) in G3 fractions of h/c-fed sheep containing an increased number of cells positive for basal cytokeratins ( $4.3 \times 10^7/ml$ )

compared with the G1 fractions ( $-64\%$ ;  $1.2 \times 10^7/ml$ ) from these animals. Thus, we suspect that chronic adaptation of the expression of the  $Na^+/K^+$ -ATPase  $\alpha$  subunit to changes in the diet energy level involves transcriptional control.

### CONCLUSION

Feeding a mixed hay/concentrate diet to sheep leads not only to an enlargement of the rumen papillae surface area but also to a marked increase in the number of cells in the basal and parabasal layers of the epithelium resulting in a two-fold elevation in the cell membrane surface area and a higher number of  $Na^+/K^+$ -ATPase-positive REC. Compared with hay-fed sheep, more  $Na^+/K^+$ -ATPase protein is found in REC from the upper third of the suprabasal cell layers, a rumen epithelial region in which NHE-dependent  $Na^+$  absorption is highest. Functional adaptation of rumen epithelial cells to high-concentrate feeding also includes a lowering of  $Na^+/K^+$ -ATPase mRNA expression and a decreased amount of  $Na^+/K^+$ -ATPase protein per cell membrane surface area. The reduced abundance of this major energy-consuming process will make more energy available for other cellular functions such as protein synthesis and proliferation (Remond et al., 1995). Additional research is needed to further probe the differences between the cell types of the multilayered rumen epithelium to better understand functional adaptation processes.

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