

## Research Article

# The Vacuolar-Type H<sup>+</sup>-ATPase in Ovine Rumen Epithelium is Regulated by Metabolic Signals

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In this study, the effect of metabolic inhibition (MI) by glucose substitution with 2-deoxyglucose (2-DOG) and/or application of antimycin A on ovine rumen epithelial cells (REC) vacuolar-type H<sup>+</sup>-ATPase (vH<sup>+</sup>-ATPase) activity was investigated. Using fluorescent spectroscopy, basal pHi of REC was measured to be 7.3 ± 0.1 in HCO<sub>3</sub><sup>-</sup>-free, glucose-containing NaCl medium. MI induced a strong pHi reduction (-0.44 ± 0.04 pH units) with a more pronounced effect of 2-DOG compared to antimycin A (-0.30 ± 0.03 versus -0.21 ± 0.03 pH units). Treatment with foliomycin, a specific vH<sup>+</sup>-ATPase inhibitor, decreased REC pHi by 0.21 ± 0.05 pH units. After MI induction, this effect was nearly abolished (-0.03 ± 0.02 pH units). In addition, membrane-associated localization of vH<sup>+</sup>-ATPase B subunit disappeared. Metabolic control of vH<sup>+</sup>-ATPase involving regulation of its assembly state by elements of the glycolytic pathway could provide a means to adapt REC ATP consumption according to energy availability.

## 1. Introduction

Resulting from its considerable role in the absorption of nutrients, mainly of short chain fatty acids (SCFAs) and of electrolytes [1–3], the rumen epithelium ranks among the tissues with high metabolic rates [4, 5]. A main proportion of the rumen ATP utilization is related to activity of a Na<sup>+</sup>/K<sup>+</sup>-ATPase that has been shown to be expressed at high levels [6–8] in the cell membrane of rumen epithelial cells (REC) [9, 10]. In addition, functional vacuolar-type H<sup>+</sup> pumps (vH<sup>+</sup>-ATPase) are existent in REC [10, 11]. The vH<sup>+</sup>-ATPase is well known as being present in intracellular membrane components such as endosomes, lysosomes, clathrin-coated vesicles, and the Golgi complex [12–15]. The pump-mediated acidification of such cell compartments is required for a variety of processes, including transcytosis of receptor-ligand complexes and other molecules, for example, NH<sub>3</sub>/NH<sub>4</sub><sup>+</sup>, coupled transport of neurotransmitters and protein breakdown [16, 17]. In addition, a link between electrogenic H<sup>+</sup> secretion by vH<sup>+</sup>-ATPases localized on the

cell membrane and ion transport and/or the regulation of cytosolic pH has been found in osteoclasts [18], macrophages [19], and various epithelia, for example, frog and toad skin, mammalian renal collecting duct, endolymphatic sac of the inner ear, and epididymis [20–25].

The existence of the vH<sup>+</sup>-ATPase as an active transport mechanism in addition to the Na<sup>+</sup>/K<sup>+</sup>-ATPase implies a special functional role of the protein in the rumen. We have shown that the pump plays a considerable role in REC pHi regulation being responsible for about 30% of total H<sup>+</sup> release [11]. Moreover, indirect evidence for the involvement of vH<sup>+</sup>-ATPase in ruminal transport processes comes from experiments showing that mucosal nitrate, known to inhibit vH<sup>+</sup>-ATPase activity [20], reduced propionate and Cl<sup>-</sup> absorption markedly [26, 27]. Folioimycin, a specific vH<sup>+</sup>-ATPase blocker [28], has been found to inhibit the uptake of Mg<sup>2+</sup> into REC [29].

In our previous study [10], a variable subcellular distribution of vH<sup>+</sup>-ATPase in cell membranes and/or cytosolic pools of the more lumenally oriented cell layers

(stratum spinosum, stratum granulosum) of the rumen epithelium has been observed. We speculate that this flexible location could reflect reversible recycling of ruminal  $vH^+$ -ATPase between the plasma membrane and a pool of cytoplasmic vesicles and/or dissociation of  $V_1$  catalytic complex from membrane-bound  $V_0$  domains. In various epithelia and other cell types, such mechanisms are known to be involved in the regulation  $vH^+$ -ATPase activity [12–15, 30–32]. Regulatory factors in ruminal  $vH^+$ -ATPase recycling are unknown but for yeasts [33–36] and renal epithelia [37]; metabolic control has been demonstrated. Physiological signals that modulate  $vH^+$ -localization and activity include  $pH_i$ ,  $HCO_3^-$ ,  $pCO_2$ , and glucose [14, 15, 18, 37, 38], all related to cell metabolism.

The present study was designed to investigate a possible modulation of ruminal  $vH^+$ -ATPase activity by substrate/energy availability. To do this, we used fluorescent spectroscopic  $pH_i$  measurements to study the effects of glucose removal and/or reduction of the cellular ATP concentration ([ATP]) on  $vH^+$ -ATPase functional activity. In addition, Western blot and immunocytochemistry are used to analyze if changes of  $vH^+$ -ATPase expression and localization play a role in adaptation of the pump activity.

## 2. Material and Methods

**2.1. Materials.** Medium 199, trypsin, glutamine, antibiotics (gentamycin, nystatin, kanamycin, penicillin-streptomycin), fetal calf serum (FCS), and Dulbecco's phosphate-buffered saline (DPBS) were purchased from PAN Biotech (Aidenbach, Germany). HyQTase was obtained from Thermo Fisher Scientific (Bonn, Germany). BCECF-AM and pluronic acid were from Molecular Probes Inc. (Eugene, OR). Foliomycin, amiloride, antimycin A, and 2-deoxyglucose (2-DOG) were from Sigma Aldrich (Munich, Germany). All chemicals for Western blot analysis were purchased from Carl Roth (Karlsruhe, Germany).

**2.2. Antibodies.** The monoclonal mouse antibodies used in this study were specific for 60-kDa subunit of the yeast  $vH^+$ -ATPase (13D11-B2, Molecular Probes) and the  $\alpha$  subunit of the sheep  $Na^+/K^+$ -ATPase (M7-PB-E9, Affinity Bioreagents). Both antibodies have been shown to detect the sheep proteins specifically [10, 11]. Relevant secondary antibodies conjugated to Alexa fluor 488 (Invitrogen) were used for immunocytochemistry. For Western blotting, a horseradish-peroxidase (HRP)-conjugated antibody (ECL Anti-mouse IgG) obtained from Amersham Bioscience was used.

**2.3. Tissue Preparation and Cell Culture.** The ruminal tissues were obtained from a local slaughter house. Samples were excised from the forestomachs of sheep within 10 minutes of slaughter. Two pieces of rumen tissue, each about 100 cm<sup>2</sup>, were taken from the Atrium ruminis, washed at least three times in ice-cold phosphate-buffered saline (PBS) containing penicillin-streptomycin, and then transported to the laboratory in the same solution. There, rumen

papillae were removed by scissors and washed three times in antibiotic-containing PBS and ones in antibiotic-free PBS. Then, primary cultures of ruminal epithelial cells (REC) were prepared as described by Galfi et al. [39]. Briefly, REC were isolated by fractional trypsination and those cell fractions containing mostly cells of the strata spinosum and basale were grown in Medium 199 containing 15% FCS, 1.36 mM glutamine, 20 mM HEPES, and antibiotics (50 mg/L gentamycin, 100 mg/L kanamycin,  $2.4 \times 10^5$  U/L nystatin) in an atmosphere of humidified air-5% CO<sub>2</sub> at 38°C. From day 2 of culture, the medium was nystatin-free and contains 10% FCS only. The experiments were performed 5-6 days after seeding.

**2.4. Solutions for  $pH_i$  Measurements.** Control experiments were performed in  $HCO_3^-$ -free, HEPES-buffered measuring solution (in mM): 125 NaCl, 5 KCl, 1 CaCl<sub>2</sub>, 2 MgCl<sub>2</sub>, 5 glucose, 10 HEPES, pH 7.1. In experiments designed to reduce the energy metabolism of REC (metabolic inhibition): (1) glucose was removed from the medium and antimycin A (5  $\mu$ M), a known inhibitor of Complex III of the mitochondrial respiratory chain, was added; (2) glucose was replaced with 20 mM 2-DOG to inhibit the glycolytic pathway; (3) antimycin A (5  $\mu$ M) and 2-DOG (20 mM) were added with concomitant glucose removal. The osmolarity of all solutions was adjusted to 280 mOsmol/kg using D-mannit. All experiments were performed in the nominal absence of CO<sub>2</sub>/HCO<sub>3</sub><sup>-</sup>, to suppress Na<sup>+</sup>-HCO<sub>3</sub><sup>-</sup> symporter related  $pH_i$  regulation [40] and to enable comparability with results of our previous studies [10, 11]. Amiloride (250  $\mu$ M) and foliomycin (2  $\mu$ M), known as specific inhibitors of Na<sup>+</sup>/H<sup>+</sup> exchanger (NHE) and  $vH^+$ -ATPase, respectively, were used to differentiate Na<sup>+</sup>- and pump dependent H<sup>+</sup>-secretion.

**2.5. Measurement of  $pH_i$  by Spectrofluorometry.** For the determination of  $pH_i$ , cells were loaded with 1  $\mu$ M BCECF-AM for 30 minutes and subsequently washed twice in DPBS. REC were incubated for a further 30 minutes to allow complete de-esterification and washed twice before measurement of fluorescence. Intracellular pH was detected by measuring the fluorescence of the probe-loaded REC in a spectrofluorometer (LS-50 B, Perkin-Elmer) equipped with a fast-filter accessory that allowed fluorescence to be measured at 20-ms intervals with excitation for BCECF at 440 and 480 nm and emission at 515 nm. All measurements were made at 37°C in a 3-ml cuvette containing 2 ml cell suspension (10% cytocrit) under stirring. BCECF signals were calibrated to pH by placing the cells in medium containing 135 mM KCl and the ionophore nigericin (10  $\mu$ M) to equilibrate intra- and extracellular [H<sup>+</sup>]. The procedure was repeated for various pH values between 6.0 and 8.0. For data evaluation, 10-s data sets were each averaged at the beginning of the measurement and then after 50, 100, 150, 200, 250, 300, 350, 400, 450, 500, and 550 s. The final  $pH_i$  was determined as the mean  $pH_i$  of the last 10 s of the measurement. Thus, for the calculation of any given  $pH_i$  value, 500 data points were used.

**2.6. Immunocytochemistry.** REC ( $2 \times 10^6$  cells/ml) were grown on sterile glass cover slips (Neolab, Germany) for 24 to 48 hours. Thereafter, a metabolic inhibition (20 minutes) was performed as described above. After being rinsed twice with PBS, REC were fixed in methanol (10 minutes at  $-20^\circ\text{C}$ ). If not otherwise stated, all the following steps were carried out at room temperature. After two PBS washes, cells were permeabilized in 0.25% Triton X-100 for  $\text{vH}^+$ -ATPase or in  $100 \mu\text{M}$  digitonin for  $\text{Na}^+/\text{K}^+$ -ATPase for 10 minutes and again rinsed three times with PBS. Nonspecific binding of IgG was suppressed by incubation of specimens with 7% goat serum in PBS for 20 minutes. Subsequently, cells were rinsed with PBS (three times for 5 minutes) and then incubated overnight at  $4^\circ\text{C}$  with the primary anti- $\text{vH}^+$ -ATPase antibody ( $13 \mu\text{g}/\text{mL}$ ) or the primary anti- $\text{Na}^+/\text{K}^+$ -ATPase antibody ( $15 \mu\text{g}/\text{mL}$ ) solved in PBS with 1% BSA; PBS-BSA. After being rinsed three times with PBS, cells were incubated for 2 hours with the secondary, Alexa Fluor-488-labeled goat anti-mouse  $\text{IgG}_1(\gamma 1)$  antibody (1 : 200 in PBS-BSA). After three changes of PBS (5 minutes each), nuclei were counterstained with 300 nM of 4,6-diamidino-2-phenylindole (DAPI) in S-buffer (containing: 75 mM KCl, 3 mM  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 1 mM EGTA, 0.2 mM dithiothreitol, 10 mM imidazol,  $1 \mu\text{g}/\text{mL}$  aprotinin, 0.1 mM phenylmethane sulfonyl-fluoride). Cover slips were then mounted with  $30 \mu\text{L}$  mounting medium (Dianova, Hamburg, Germany). Digital images were acquired by using a fluorescence microscope Olympus IX50 (Hamburg, Germany) and MetaMorph version 7.5.2.0 and AutoDeblur version 1.4.1 software (Visitron Systems GmbH, Puchheim, Germany).

**2.7. Western Blot Analysis.** For Western blots, total protein from washed REC was extracted by use of the M-PER Mammalian Protein Extraction Reagent (Pierce), complemented with Halt protease inhibitor cocktail (Pierce). The protein concentration was determined by using the Bradford assay (Bio-Rad, Munich, Germany). Protein samples ( $10\text{--}115 \mu\text{g}$ ) were separated by SDS (12.5%)-polyacrylamide gel electrophoresis and subsequently transferred to polyvinylidene fluoride (PVDF) membrane (GE Healthcare). 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 hours and washed two times for 5 minutes in PBS-T. Thereafter, membranes were incubated at  $4^\circ\text{C}$  with the primary antibodies (anti- $\text{vH}^+$ -ATPase: 1 : 5,000 dilution; anti  $\text{Na}^+/\text{K}^+$ -ATPase: 1 : 1,000 dilution) overnight, washed three times ( $1 \times 15$  minutes,  $2 \times 5$  minutes) with PBS-T, and incubated for 1 hour with HRP-conjugated secondary antibodies (1 : 10,000 dilution). Then, after three washings ( $1 \times 10$  minutes and  $2 \times 5$  minutes) in PBS-T, membranes were developed with ECL Western Blotting Substrate (Pierce).

**2.8. Rumen Fluid Analysis.** Rumen fluid was taken from the perforated rumen immediately after slaughtering. Its pH was measured directly after sampling with a glass electrode (N 1042A, pH meter CG 841, Schott, Mainz, Germany). Then, the rumen fluid was strained through 4 layers of gauze and prepared for SCFA analysis. First, a mixture of 5 mL rumen

fluid and 2 mL iso-capronic acid (internal standard) was centrifuged at  $3000 \times g$  at  $4^\circ\text{C}$  for 20 minutes. The filtered ( $0.22 \mu\text{m}$  pore size) supernatant was used to measure the SCFA concentration by gas chromatography (Shimadzu GC-14A, Shimadzu Corporation, Kyoto, Japan) on a capillary column (Free Fatty Acid Phase,  $25 \text{ m} \times 0.25 \text{ mm}$ , Machery-Nagel GmbH & Co. KG, Düren, Germany) according to the method of Geissler et al. [41].

**2.9. Light Microscopy and Morphometry of Rumen Papillae.** Samples of the rumen wall ( $1 \text{ cm}^2$  surface) were obtained from identical sites of the atrium ruminis and fixed in 4% neutral formaldehyde solution for morphometric investigations. After rinsing with water, the rumen wall tissues were dehydrated in a graded series of ethanol (30%, 50%, 70%, 90%, and absolute ethanol), cleared with benzene, saturated with and embedded in paraffin. At each sampling, sections of  $5 \mu\text{m}$  thickness were made of 30 papillae and stained with haematoxylin/eosin. The length and width of papillae were determined by the computer-operated *Image C* picture analysis system (Intronic GmbH, Berlin, Germany) and the IMES analysis program, by using a color video camera (SONY 3 CCD) and a light microscope (Axiolab, Carl Zeiss Jena, Germany). The number of papillae per  $\text{cm}^2$  mucosa was estimated by using a video camera equipped with a picture analysis system. According to Hofmann and Schnorr [42], the total surface of papillae per  $\text{cm}^2$  mucosa was determined as length  $\times$  width  $\times 2$ , multiplied by the number of papillae/ $\text{cm}^2$ .

**2.10. Statistical Analysis.** If not otherwise stated, data are presented as means  $\pm$  standard error (SE). Significance was determined by Student's *t*-test or the paired *t*-test as appropriate.  $P < .05$  was considered to be significant. All statistical calculations were performed by using SigmaStat (Jandel Scientific).

### 3. Results

**3.1. Basal Intracellular pH of REC and Effects of Glucose Removal, 2-DOG, and Antimycin A on  $\text{pH}_i$ .** BCECF-loaded REC were suspended in either HEPES-buffered Na-medium (control) or HEPES-buffered, glucose-free Na-medium with 2-DOG and/or antimycin A, and  $\text{pH}_i$  was measured over a 10-min period. The appropriate concentrations of 2-DOG (20 mM) and antimycin A ( $5 \mu\text{M}$ ) were chosen from dose-response experiments (Figure 1). Characteristic original traces showing the time course of the REC  $\text{pH}_i$  for all four conditions are shown in Figure 2(a). Data on the  $\text{pH}_i$  reduction induced by either antimycin A or 2-DOG alone or by a combination of both metabolic inhibitors are summarized in Figure 2(b). The initial and final  $\text{pH}_i$  of control REC incubated in HEPES-buffered NaCl-medium were  $7.2 \pm 0.1$  and  $7.3 \pm 0.1$ , respectively. The presence of antimycin A and/or 2-DOG in the media led to a reduction of  $\text{pH}_i$  (Figures 2(a) and 2(b)). In REC exposed to antimycin A, 2-DOG or antimycin A and 2-DOG, the  $\text{pH}_i$  measured at the beginning of the measuring period was reduced

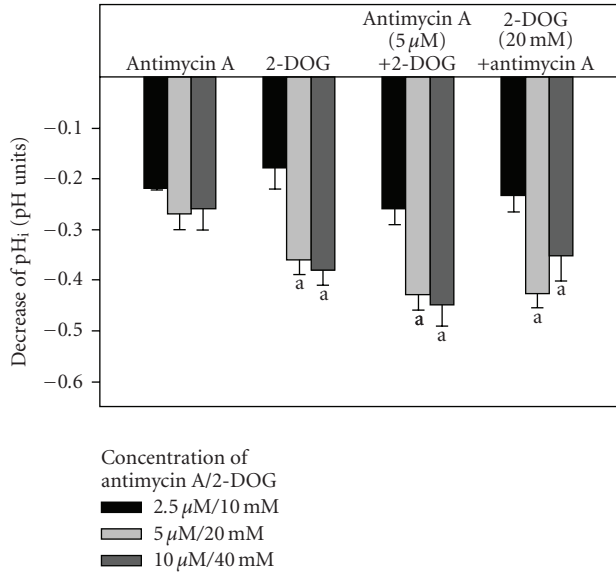
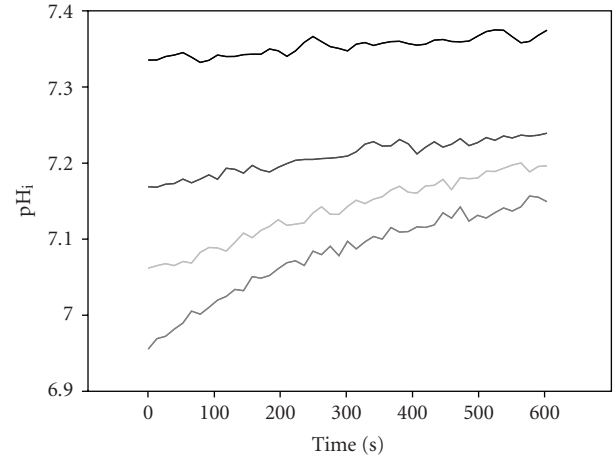


FIGURE 1: Results of dose-response experiments with antimycin A and/or 2-DOG. The  $pH_i$  of ruminal epithelial cells was measured in either HEPES-buffered  $HCO_3^-$ -free Na-medium containing 5 mM glucose (control) or in glucose-free media with blockers as indicated. The mean  $pH_i$  reduction from the  $pH_i$  measured in control medium with glucose is given. Values are means  $\pm$  SE from 3 single measurements;  $^aP < .05$  versus the lowest dosage used.

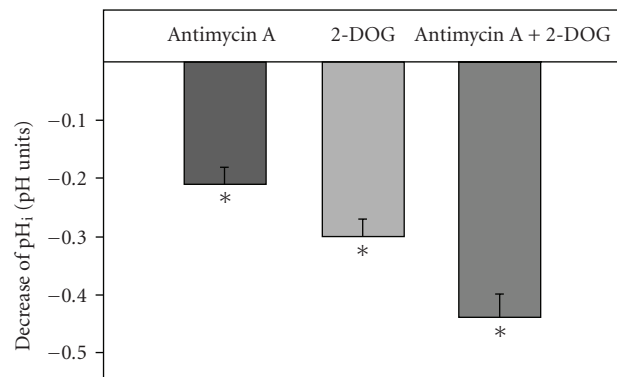
by  $0.21 \pm 0.03$ ,  $0.35 \pm 0.04$ , and  $0.44 \pm 0.04$  pH units, respectively. With antimycin A or both inhibitors in the medium, this  $pH_i$  reduction was stable in most cases. However, if 2-DOG only was present, the effect was slightly diminished and the observed  $pH_i$  decrease amounted to  $0.30 \pm 0.03$  pH units at the end of the measuring period.

**3.2. Effect of Foliomycin on the  $pH_i$  of REC before and after Metabolic Inhibition with Glucose Substitution by 2-DOG and Antimycin A Application.** Next, we investigated whether a decreased  $vH^+$ -ATPase activity was responsible for the  $pH_i$  reducing effect of metabolic inhibition (MI). Therefore, BCECF-loaded REC were suspended in foliomycin ( $2 \mu$ M)-containing HEPES-buffered Na-medium or glucose-free, HEPES-buffered Na-medium with antimycin A ( $5 \mu$ M), 2-DOG (20 mM), and foliomycin ( $2 \mu$ M). Then, the  $pH_i$  was measured continuously over a 10-min period. Control measurements were performed with cells handled in the same way, but without foliomycin present in the solutions. In comparison to the  $pH_i$  of control cells measured in HEPES-buffered Na-medium, foliomycin-treated REC showed a decreased  $pH_i$  (Figures 3(a) and 3(b)). The mean  $pH_i$  reduction observed in foliomycin-treated REC amounted to  $0.21 \pm 0.05$  pH units (Figure 3(b)). As shown in Figures 3(a) and 3(B), the foliomycin effect was nearly abolished when REC were incubated in antimycin A- and 2-DOG-containing glucose-free media. Under such conditions, the  $pH_i$  difference between control (HEPES-buffered, glucose-free Na-medium with antimycin A and 2-DOG) and foliomycin-treated REC amounted to  $0.03 \pm 0.02$  pH units only (Figure



— Control  
— Antimycin A  
— 2-DOG  
— Antimycin A + 2-DOG

(a)



(b)

FIGURE 2: Effect of glucose replacement by 2-DOG and/or antimycin A application on  $pH_i$  of ruminal epithelial cells. Measurements were made in either HEPES-buffered  $HCO_3^-$ -free Na-medium containing 5 mM glucose (control) or in glucose-free media with blockers as indicated. (a): The figure shows original traces from one experiment. (b): To summarize the inhibitor effects, the mean  $pH_i$  reduction from the  $pH_i$  measured in control medium with glucose and without metabolic inhibitors has been calculated and is shown for each condition. Inhibitor concentrations are  $5 \mu$ M and 20 mM for antimycin A and 2-DOG, respectively. Values are means  $\pm$  SE from 8 to 9 single measurements;  $*P < .05$  versus control.

3(b)). These results point to a fast deactivation of  $vH^+$ -ATPase activity after MI with antimycin A and 2-DOG. Regulatory mechanisms responsible for such adaptive response could be reversible disassembly of the  $V_0$  and  $V_1$  subunits of the pump [30, 33] and/or a translocation between the cell membrane and intracellular compartments [43].

Thus, in a next step we tested whether MI affect the distribution of  $vH^+$ -ATPase and performed an immunofluorescence study using an antibody directed against the 60-kDa subunit of the yeast  $vH^+$ -ATPase which has been shown to detect  $vH^+$ -ATPase in ovine rumen epithelium [10].

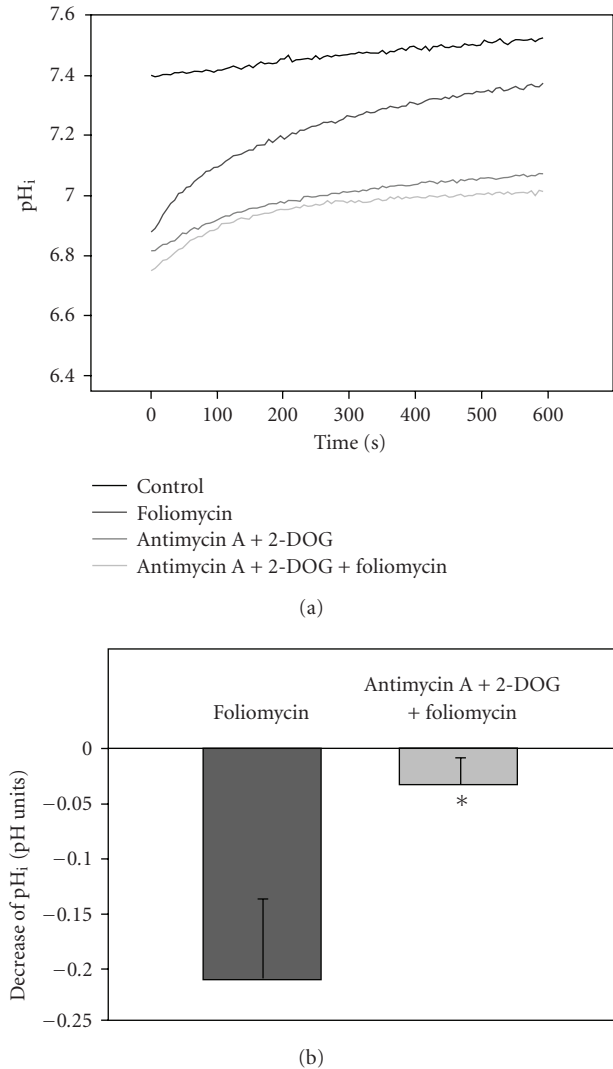


FIGURE 3: Effect of the  $vH^+$ -ATPase inhibitor foliomyacin before and after metabolic inhibition (MI) induced by glucose replacement with 2-DOG and application of antimycin A on  $pH_i$  of ruminal epithelial cells (REC). Foliomyacin was added to REC suspended in either HEPES-buffered  $HCO_3^-$ -free Na-medium containing 5 mM glucose (control;  $n = 6$ ) or in glucose-free media with 2-DOG and antimycin A ( $n = 7$ ). A: The figure shows original traces from one experiment. B: To summarize the foliomyacin effects, the mean  $pH_i$  reduction from the  $pH_i$  measured in respective control medium without inhibitor has been calculated and is shown for both conditions. Values are means  $\pm$  SE; \* $P < .05$  versus control.

As a control, the  $Na^+/K^+$ -ATPase which is constantly found in the cell membrane of REC [10] has been investigated using an  $\alpha$  subunit-specific anti- $Na^+/K^+$ -ATPase antibody.

**3.3. Vacuolar  $H^+$ -ATPase Localization before and after Metabolic Inhibition.** As expected, the  $\alpha$  subunit-specific anti- $Na^+/K^+$ -ATPase antibody identified the protein in the cytoplasmic membrane of REC and its localization was not changed by a 20-min incubation in glucose-free, antimycin A- and/or 2-DOG-containing media (Figure 4(A), a–c).

In comparison with the  $Na^+/K^+$ -ATPase, the  $vH^+$ -ATPase distribution showed a much higher variability and dependence on the substrate availability. Examples of characteristic staining patterns are given in figure 4(B). When incubated in glucose-containing standard medium, REC  $vH^+$ -ATPase appeared in the cell membrane and/or perimembrane area as well as in the cytoplasm of REC (Figure 4(B), a). The cytosolic  $vH^+$ -ATPase staining is clearly defined and either distributed throughout the cytoplasm or concentrated around the nucleus. After glucose deprivation and application of 2-DOG (Figure 4(B), b), no membrane staining can be found and typically the  $vH^+$ -ATPase staining is clustered in the perinuclear area of the cells. Compared to control conditions, glucose deprivation and combined application of 2-DOG and antimycin A (Figure 4(B), c) induced appearance of diffuse, cytosolic  $vH^+$ -ATPase staining.

**3.4. Effect of Amiloride on the  $pH_i$  of REC before and after Metabolic Inhibition with Glucose Substitution by 2-DOG and Antimycin A Application.** As in our previous investigation with ovine and bovine REC [10, 11], a more or less expressed compensatory  $pH_i$  increase was seen after application of foliomyacin. In  $Na^+$ -containing media, an NHE is known to be responsible for this effect [40] as application of the NHE inhibitor amiloride reduced REC  $pH_i$  by 65% [11]. Therefore, we repeated the above described experiments to investigate the NHE activity before and after MI with 2-DOG and antimycin A. Experiments were performed as described for the first series, however, in addition to foliomyacin (2  $\mu$ M), the NHE inhibitor amiloride (250  $\mu$ M) was used alone or in combination with the  $vH^+$ -ATPase blocker.

Surprisingly, the results differ from that seen before. MI induced a  $pH_i$  decrease of  $0.10 \pm 0.03$  pH units only (Figure 5), which corresponds to a 75% reduction of the effect compared with the results of the first experiment. In addition, the mean inhibitory effect of foliomyacin was strongly diminished ( $-0.05 \pm 0.03$  pH units; Figure 5). Interestingly, there was a coupling between MI and the foliomyacin effect. Figure 5 shows that REC responding to substrate deprivation and application of metabolic inhibitors were also foliomyacin-sensitive. In such cells  $pH_i$  was reduced by  $0.14 \pm 0.03$  or  $0.10 \pm 0.05$  pH units after MI induction or exposure to foliomyacin. In contrast, REC that did not respond to MI were foliomyacin-insensitive (Figure 5).

In Figure 6, the inhibitory effects of foliomyacin and/or amiloride before and after MI are summarized. Only results from measurements with REC responding to metabolic inhibition are included and for comparison data from the first series of experiments are also shown. Figure 6 shows that compared to series 1 (a) the foliomyacin effect was strongly reduced ( $-0.10 \pm 0.02$  pH units versus  $-0.27$  pH units) and (b) the residual foliomyacin effect ( $-0.08 \pm 0.03$  pH units) was not influenced by MI. As in series 1, foliomyacin application led to a compensatory  $pH_i$  increase, an effect that was reduced after MI. A strong amiloride-sensitive component was observed. At the beginning and end of the measurement, the  $pH_i$  of amiloride-treated REC was reduced by  $0.83 \pm 0.05$  and  $0.89 \pm 0.06$  pH-units, respectively.

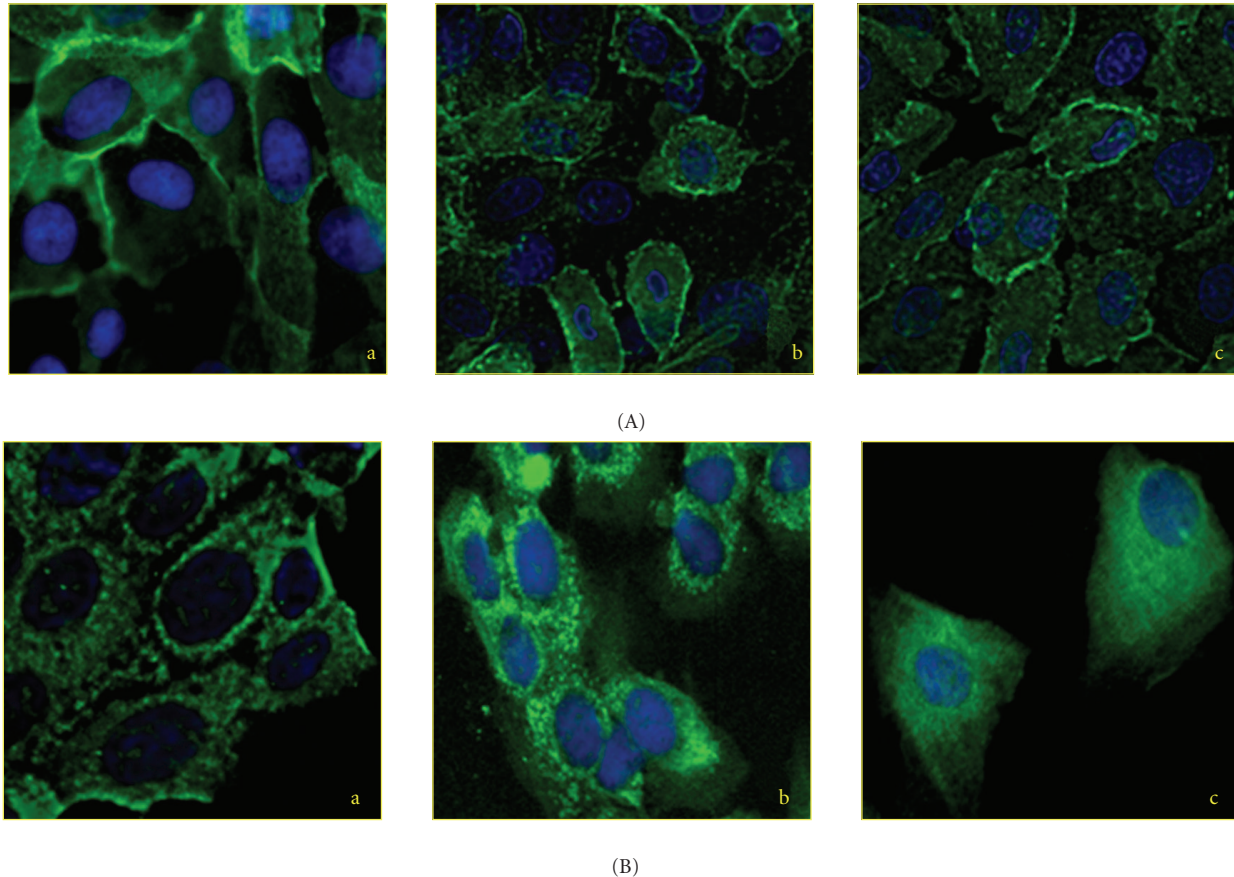


FIGURE 4: Immunolocalization of Na<sup>+</sup>/K<sup>+</sup>-ATPase α-subunit (A, green) and of vH<sup>+</sup>-ATPase B subunit (B, green) in ovine REC. Monoclonal mouse antibodies against sheep Na<sup>+</sup>/K<sup>+</sup>-ATPase α-subunit or yeast vH<sup>+</sup>-ATPase 60 kDa subunit, and an Alexa fluor 488 conjugated secondary antibody were used. The nuclei are counterstained with DAPI (blue). Compared to control conditions (Ba) glucose substitution with 2-DOG (Bb) and complete MI (glucose substitution with 2-DOG and antimycin A application; Bc) induced loss of membrane staining and appearance of diffuse, cytosolic staining of vH<sup>+</sup>-ATPase B subunits. In contrast, the Na<sup>+</sup>/K<sup>+</sup>-ATPase was always localized to the cell membrane and not influenced by metabolic inhibition (A a–c).

The foliomyacin- and amiloride-sensitive components were additive under control conditions and the observed pHi decrease amounted to  $0.93 \pm 0.05$  at the start and to  $0.95 \pm 0.08$  at the end of the measuring period. MI led to a reduction of the amiloride-sensitive, NHE-related component. At the end of the measurement the pHi decrease was only  $0.69 \pm 0.06$  pH units.

**3.5. Western Analysis.** Using Western analysis, we investigated the expression of vH<sup>+</sup>-ATPase B subunit and Na<sup>+</sup>/K<sup>+</sup>-ATPase α subunit protein expression in REC protein extracts from sheep slaughtered during the first or second series of experiments. Characteristic examples of immunoblots are given in Figure 7. Only with protein samples from series one, a clear 60-kDa band representing the vH<sup>+</sup>-ATPase B subunit was seen (Figure 7, lane 1). However, with protein extracts obtained during the second experimental series, the 60-kDa band was very weak or completely absent (Figure 7, lane 2-3). In contrast, a distinct 110-kDa band was constantly detected showing the presence of Na<sup>+</sup>/K<sup>+</sup>-ATPase α subunit (Figure 7, lane 4–6) in all the samples.

**3.6. Ruminal Fluid Analysis and Morphometry.** We speculate that varying feeding conditions of sheep could be responsible for the different results seen in both series. To get some information on prior slaughter nutrition, we started rumen fluid analysis (SCFA concentration, pH) and morphometric measurements of the rumen papillae at the end of the second series of experiments. Figure 8 shows measured rumen fluid pH values and the accompanying SCFA concentrations determined from 22 sheep. As expected, an inverse relationship ( $y = -57.7x + 462.6$ ;  $r^2 = 0.61$ ) between ruminal fluid pH and the amount of SCFA was observed (Figure 8). The mean pH value amounted to  $7.1 \pm 0.1$  with minimum and maximum values ranging from 6.4 to 7.9. The mean concentration of SCFA was  $51.6 \pm 5.7$  mM/l and minimum to maximum levels ranged from 11.7 to 115.7 mM/l. For comparison, data from a preliminary feeding experiment with sheep fed hay ad libitum are also given in Figure 8. Although morphometric data showed no significant differences in rumen papillae surface from sheep with ruminal fluid pH values  $<6.9$  ( $n = 6$ ) or  $>6.9$  ( $n = 16$ ), there was a tendency to higher surfaces ( $872.0 \pm 47.8$  mm<sup>2</sup>/cm<sup>2</sup>) in the

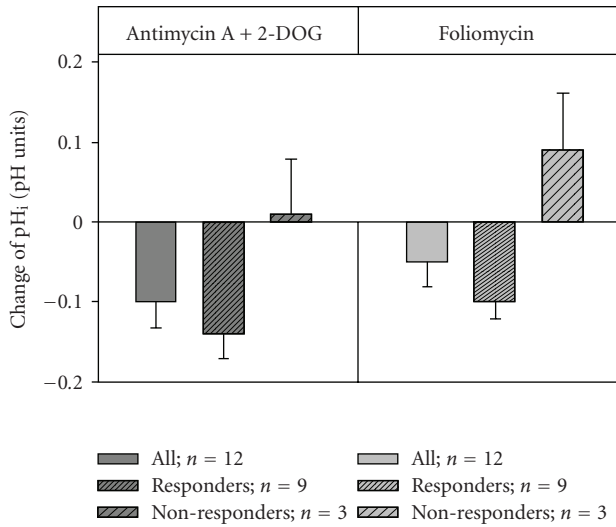


FIGURE 5: Effect of metabolic inhibition (MI) and of the  $vH^+$ -ATPase inhibitor foliomycin in the second series of experiments. Experiments were performed as described in Figures 2 and 3. Note the coupling between MI and foliomycin sensitivity. Values are means  $\pm$  SE;  $n$  = number of single experiments = number of animals.

low-pH group when compared to the high-pH group ( $797.7 \pm 27.1 \text{ mm}^2/\text{cm}^2$ ).

#### 4. Discussion

To date, the functional role and the regulation of the recently described ruminal  $vH^+$ -ATPase [10, 11] is not well understood. Based on our own work [10] and that of other investigators [15, 37, 44] showing metabolic regulation of the pump, we assume that the availability of substrates and of energy are main regulatory factors to adapt ruminal  $vH^+$ -ATPase activity. To prove this hypothesis, we here investigated the effect of MI induced by glucose removal and application of inhibitors of the glycolytic pathway (2-DOG) and/or of mitochondrial ATP production (antimycin A) on  $vH^+$ -ATPase activity.

**4.1. Basal  $pH_i$  of REC.** The  $pH_i$  ( $7.2$  to  $7.3 \pm 0.1$ ) of REC suspended in control solution (HEPES-buffered,  $\text{HCO}_3^-$ -free  $\text{Na}^+$ -media with  $5 \text{ mM}$  glucose) was in the range of  $7.1$  to  $7.5$  reported for REC [11, 40, 45] and other cell types [44, 46–48] under  $\text{HCO}_3^-$ -free conditions.

**4.2. Effect of Metabolic Inhibition on  $pH_i$  of REC.** Although oxidative metabolism of SCFA, mainly of butyrate and propionate, is the main energy source for metabolically matured REC [2, 49–51], glucose has also been shown to be used at a basal rate in vivo and in vitro [5, 51–54]. The metabolism of SCFA has been shown to be supported by glucose [55] and particularly it is known to exert a positive effect on ruminal butyrate metabolism [49]. Addition of glucose to butyrate in the incubation medium decreases the

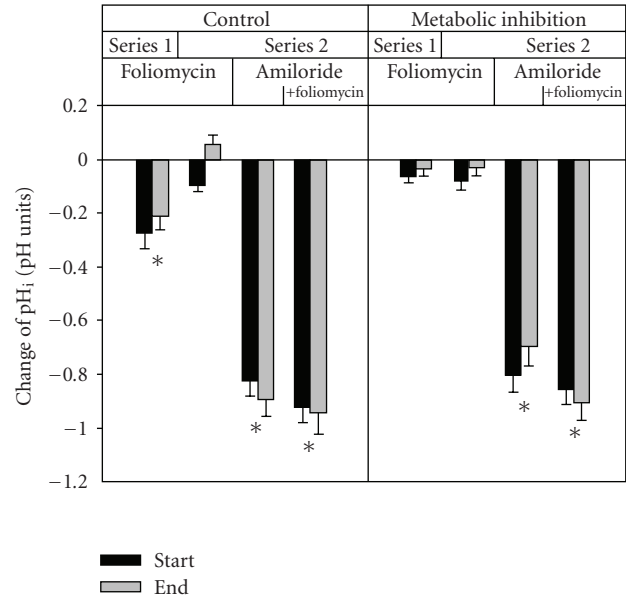


FIGURE 6: Effect of  $vH^+$ -ATPase and  $\text{Na}^+/\text{H}^+$  exchanger inhibitors on REC  $pH_i$  before and after metabolic inhibition. Experiments were performed as described in Figure 3, however, in addition to foliomycin ( $2 \mu\text{M}$ ), amiloride ( $250 \mu\text{M}$ ) was used alone or in combination with the  $vH^+$ -ATPase blocker. For comparison, the foliomycin effects observed in series 1 of our experiments are also shown. Values are means  $\pm$  SE; \*  $P < .05$ .

rate of butyrate oxidation to  $\text{CO}_2$  and increases the rate of ketogenesis [49]. Besides glutamine, glucose is the main metabolic fuel in cultured REC and known to be primarily metabolized through glycolysis [53]. In this study, inhibition of the glycolytic pathway by substitution of glucose with 2-DOG induced a significant reduction of  $pH_i$  ( $-0.35 \pm 0.04$  pH units) in REC showing that their ability to regulate  $pH_i$ , at least partly, depends on this metabolic pathway. Like glucose, its analogue 2-DOG is transported into the cells and is phosphorylated into 2-DOG-6-phosphate. However, then it is not further metabolized and accumulates inside the cells causing energy deprivation. Even in the absence of glucose, antimycin A application reduced the REC  $pH_i$  to a lesser extent ( $-0.21 \pm 0.03$  pH units) than 2-DOG showing that an intact glycolytic pathway is more important than mitochondrial ATP production for  $\text{H}^+$  secretion under our experimental conditions. This is in accordance with our data showing that the combination of glucose substitution with 2-DOG and antimycin A led to a stable  $pH_i$  decrease by  $0.44 \pm 0.04$  pH units.

**4.3. Effect of Foliomycin on the  $pH_i$  of REC before and after Glucose Removal and Metabolic Inhibition with Antimycin A and/or 2-DOG.** In yeast [34, 35, 56], various mammary cell types [18, 37, 46], and turtle urinary bladder [57], a coupling between  $vH^+$ -ATPase activity and cellular energetic processes has been demonstrated. To evaluate the role of metabolic regulation on ruminal  $vH^+$ -ATPase activity, we investigated the effect of its specific inhibitor foliomycin before and

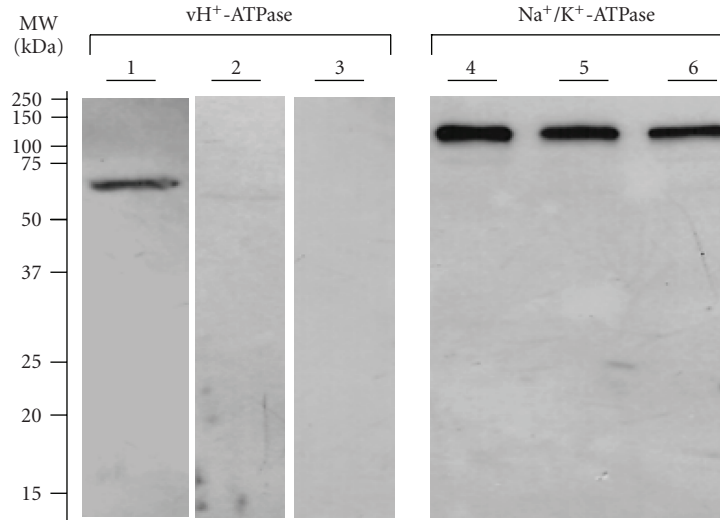


FIGURE 7: Immunoblot of the  $vH^+$ -ATPase subunit B ( $\approx 60$  kDa) and the  $Na^+/K^+$ -ATPase  $\alpha$  subunit ( $\approx 110$  kDa) in ovine REC. Protein extracts were prepared from REC isolated during series 1 (lane 1 and 4) or series 2 (lane 2, 3, 5 and 6) of the experiments. For the  $Na^+/K^+$ -ATPase  $\alpha$  subunit, a band at the expected size of approximately 110 kDa was always detected (lane 4–6). In contrast, a clear 60 kDa-band for  $H^+$ -ATPase subunit B protein was found with protein extracts from series 1 only (lane 1). With protein extracts from REC with low metabolic activity, staining was very weak (lane 2) or completely absent (lane 3).

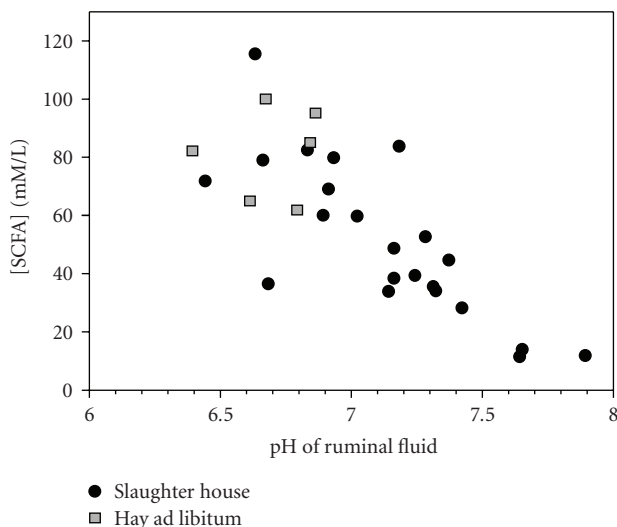


FIGURE 8: Ruminal fluid concentrations of short chain fatty acids and accompanying pH values. Ruminal fluid were obtained from sheep ( $n = 22$ ) slaughtered during the second experimental period. For comparison, data from sheep fed hay ad libitum are also given. Note the high proportion of samples showing high pH values and compared to hay fed sheep low SCFA concentrations indicative of low feed intake.

after MI with 2-DOG and antimycin A in glucose-free media. Under control conditions, the foliomyacin-induced  $pH_i$  decrease amounted to  $0.21 \pm 0.05$  pH units at the end of the measurements, which is in agreement with results ( $-0.18 \pm 0.07$  pH units) seen in a previous study with sheep REC [11]. Very similar effects of  $vH^+$ -ATPase inhibition with bafilomycin  $A_1$  or foliomyacin ( $-0.16$  to  $-0.26$  pH

units) have been reported in studies with cells of the human eccrine sweat duct [58], cultured rabbit non-pigmented ciliary epithelium [59] and alveolar macrophages [19]. Thus, a remarkable part of the REC  $H^+$  secretion resulted from  $vH^+$ -ATPase activity.

The  $vH^+$ -ATPase-related  $pH_i$  component ( $0.21 \pm 0.05$  pH units) was nearly abolished ( $0.03 \pm 0.02$  pH units) after substitution of glucose by the glycolytic inhibitor 2-DOG and treatment with the mitochondrial electron transport inhibitor antimycin A. This result clearly shows that the  $pH_i$  reduction observed after MI mainly results from deactivation of REC  $vH^+$ -ATPase. As 2-DOG exerts stronger effects on REC  $pH_i$  than treatment with antimycin A, it can be assumed that ruminal  $vH^+$ -ATPase activity mainly depends on an intact glycolytic pathway. Glucose deprivation and/or inhibition of glycolysis has also been shown to reduce or prevent  $vH^+$ -ATPase activity in cells of the medullary collecting duct [44, 60], the porcine kidney proximal tubule cell line LLC-PK1 [15, 37], and yeast cells [35, 56]. In addition, glucose-induced activation of  $vH^+$ -ATPase activity has been shown [18, 37]. A coupling between the ATP-generating glycolytic pathway and  $vH^+$ -ATPase-mediated  $H^+$  secretion is supported by the findings that iodoacetate, an inhibitor of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), reduced  $vH^+$ -ATPase activity [46] and that the enzyme coimmunoprecipitates with the pump [35]. Also, the rate limiting enzyme of glycolysis, phosphofructokinase-1 interacts with  $vH^+$ -ATPase, a subunit which has been suggested to play a role crucial for proton translocation [61]. A direct physical interaction between the  $vH^+$ -ATPase E, B and a subunits, and aldolase, an enzyme responsible for cleavage of fructose-6-phosphate in the glycolytic pathway, has been demonstrated [34, 35]. With glucose present, this interaction increased dramatically leading to the suggestion



that aldolase acts as a glucose sensor and regulates  $vH^+$ -ATPase assembly, expression, and activity via direct physical association and by providing ATP for  $H^+$  extrusion from cytosol [35, 36].

REC conversion of glucose to pyruvate and  $CO_2$  is rather small in proportion to conversion to lactate [53] and thus, an increased lactate production is indicative of higher rates of glycolysis [62]. Interestingly, it was found, that at  $pH_e = 7.4$ , REC converted 3-fold more D-glucose than n-butyrate and produced about two-fold more lactate than at  $pH_e = 6.2$  [62]. Since glycolysis generates protons, its activation at higher  $pH_e$  will stimulate  $vH^+$ -ATPase-mediated  $H^+$  efflux and thereby, will help to maintain SCFA absorption under such conditions. Provision of extracellular protons is critical for diffusion-mediated uptake of SCFA in their undissociated form [3, 50].

In this study, deactivation of  $vH^+$ -ATPase activity induced by metabolic inhibition has been shown to be a fast process appearing within a few minutes. A general mechanism responsible for such response to energy deprivation could be reversible disassembly of the catalytic  $V_O$  and the proton-translocating  $V_1$  domains of the pump [30, 33, 56]. In yeast cells, glucose deprivation induced a disassembly of 75% of the assembled  $vH^+$ -ATPase complexes in as little as 5 minutes [63]. In accordance with our previous study with bovine rumen epithelium [10], ovine REC  $vH^+$ -ATPase B subunit was found in close vicinity of the cell membrane as well as in the cytosolic compartment under control conditions (glucose-containing NaCl-medium). However, while  $Na^+/K^+$ -ATPase used as control protein was always membrane-bound, we here showed for the first time that the B subunit associated with the REC  $vH^+$ -ATPase  $V_1$  domain shows diffuse cytosolic distribution after MI. The data provide first evidence that the reduction of  $vH^+$ -ATPase-mediated transmembrane proton efflux observed after substrate and energy deprivation of REC could result from a higher proportion of disassembled  $V_1$  and  $V_O$  sectors. Since  $vH^+$ -ATPases are major cellular proteins that can consume significant amounts of total cellular ATP, their graduated disassembly could help to conserve energy under such conditions. However, with the method used in this study, we were not able to exclude endocytosis of  $vH^+$ -ATPase-bearing vesicles from the cell membrane into the cytosolic compartment. Endo- and exocytotic translocations have been demonstrated in epithelia of kidney, pancreas, and placenta [13–15, 64] as an additional mechanism for regulating  $vH^+$ -ATPase activity.

**4.4. A High NHE Activity Was Observed in REC with Low Glycolytic and  $vH^+$ -ATPase Activity.** REC belong to cells that have a specific requirement for high levels of proton transport and possess  $Na^+/H$  exchangers of subtype 1 to 3 [40, 45, 65] and monocarboxylate transporter 1 (MCT1) [66, 67] in addition to the  $vH^+$ -ATPase. The partial recovery of  $pH_i$  from the acidosis induced by MI or foliomycin application may result from compensatory activation of one or both of these  $H^+$ -secreting transport proteins. The NHE has been shown to be most important for REC  $pH_i$ -

regulation under  $HCO_3^-$ -free conditions responsible for about 70% of proton secretion and the remaining proton secretion was related to  $vH^+$ -ATPase [11]. Therefore, our second series of experiments was originally designed to investigate the role of NHE before and after MI.

Unexpectedly however, the effect of MI was decreased by 75% in our secondary series of experiments and this was accompanied by a strongly reduced (–81%) foliomycin effect. These results point to an impairment of the glycolytic pathway in those REC and corroborate with tight coupling between glycolysis and/or components of the glycolytic pathway and  $vH^+$ -ATPase activity. Because REC used in this study were isolated from rumen tissue obtained from a local slaughter house, we can only speculate on the reasons for the very different metabolic properties of the cells. However, the latter has been shown to depend on the level of metabolizable energy (ME) intake and on the type of nutrition [51, 68]. Ruminal fluid analysis showed a high proportion of samples (16 out of 22) showing pH values  $>6.9$  and [SCFA]  $<60$  mM/l (Figure 8) indicating decreased ME intake [51]. In a preliminary feeding experiment with sheep fed hay ad libitum (Figure 8) lower ruminal pH values ( $6.7 \pm 0.2$ ) and higher [SCFA] ( $81.7 \pm 15.5$  mM/l) were observed than in this study ( $7.1 \pm 0.1$ ;  $51.6 \pm 5.7$  mM/l).

A tendency for a reduced capacity to oxidize glucose in REC isolated from low-intake sheep had been observed [51]. Different feeding conditions are also known to induce changes of the chemical composition of the ruminal fluid. As shown by Kauffold et al. [68], such changes are most strongly expressed between rations consisting of fresh green feed or maize silage and such consisting of concentrate or dried green feeds. The former feeding conditions led to a fast decrease of metabolic activity and protein synthesis in the germinative layers of the rumen epithelium characterized by low  $O_2$ -consumption and REC nucleus diameter [68].

It seems therefore possible that adaptation to low energy availability that could include reduced proton production from glycolysis [69, 70] gives an explanation for the low or absent  $vH^+$ -ATPase activity. This hypothesis is supported by our finding that the expression of  $vH^+$ -ATPase B subunit protein was drastically reduced or absent in protein extracts from REC used in the second series of experiments. The B subunit is essentially involved in the regulation of normal trafficking, assembly, and activity of  $vH^+$ -ATPase [36, 71].

Interestingly, REC identified to have low metabolic and  $vH^+$ -ATPase activity were characterized by a very high amiloride-sensitive component of  $pH_i$  reflecting NHE activity. The amiloride-induced reduction of  $pH_i$  ( $-0.89 \pm 0.06$  pH-units) was as high as that from butyrate-stimulated REC ( $-1.00 \pm 0.25$  pH units) [72]. An increased activity of NHE has also been shown to occur during early stages of apoptosis in response to growth factor withdrawal [73].

Elevation of NHE activity may compensate for the loss of  $vH^+$ -ATPase-related  $H^+$  extrusion thereby improving  $pH_i$  homeostasis in energy-deprived REC. However, after induction of MI, NHE activity decreases slowly resulting in a reduction of the amiloride effect to 0.69 pH units.

An explanation is a reduced ability to maintain low intracellular  $[Na^+]$  by  $Na^+/K^+$ -ATPase leading to slow dissipation of the transmembrane  $Na^+$  gradient [74]. Inhibition of REC  $Na^+/K^+$ -ATPase has been shown to reduce  $pH_i$  by lowering NHE activity [45].

In conclusion, our results demonstrate metabolic regulation of ruminal  $vH^+$ -ATPase activity. A fast reduction of  $vH^+$ -ATPase mediated proton extrusion occurs few minutes after initiation of MI by glucose substitution with 2-DOG and application of antimycin A. An intact glycolytic pathway seems to be more important for  $vH^+$ -ATPase activity regulation than mitochondrial ATP production. The very fast response possibly results from disassembly of the  $V_1$  and  $V_O$  domains of the pump. Prolonged energy deficiency may result in a higher level of disassembled, inactive  $vH^+$ -ATPase complexes but also led to a reduced expression of the essential B subunit of the pump. This may provide a means to control ruminal epithelial ATP consumption in dependence of substrate and energy availability.

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