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Involvement of the putative anion transporter 1 (SLC26A6) in permeation of short chain fatty acids and their metabolites across the basolateral membrane of ovine ruminal epithelium

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DEDICATION

To my family especially my mother for her continuous support with endless love
To soul of my father

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ABBREVIATIONS

ac	**Acetate**
AE2	**Anion exchanger 2**
ANOVA	**Analysis of variance**
ATP	**Adenosine triphosphate**
ATPase	**Adenylpyrophosphatase**
BLMV	**basolateral membrane vesicles**
bu	**Butyrate**
C°	**Celsius centigrade**
Ca	**Calcium**
cAMP	**Cyclic adenosine monophosphate**
CFEX	**Cl^- / formate exchanger**
CHC	**α-cyano-4-hydroxycinnamic acid**
Cl	**Chloride**
cm^2	**Square centimetres**
CO_2	**Carbon dioxide**
Cy 3	**Carbocyanin 3**
DAPI	**4',6-Diamidino-2-phenylindol**
DIDS	**4,4′-diisothiocyano-2,2′-stilbenedisulfonic acid**
DMI	**Dry matter intake**
DNA	**Desoxyribonucleic acid**
DRA	**Downregulated in adenoma**
EIPA	**Ethylisopropyl amiloride**
Fig	**Figure**
g	**Gram**
GIT	**Gastrointestinal tract**
G_t	**Tissue conductance**
h	**Hour**
H^+	**Proton**
H_2CO_3	**Carbonic acid**
H_2O	**Water**
HCO_3^-	**Bicarbonate ion**
HEPES	**4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid**

HSCFA	**Undissociated short-chain fatty acids**
I_{sc}	**Short-circuit current**
J_{ms}	**Unidirectional ion transport in mucosal to serosal direction**
J_{sm}	**Unidirectional ion transport in serosal to mucosal direction**
K	**Potassium**
Kg	**Kilogram**
l	**Liter**
Log	**Logarithm**
MCT	**Monocarboxylate**
Mg	**Magnesium**
min	**Minute**
ml	**Millilitre**
mM	**Millimolar**
mRNA	**Messenger RNA**
mV	**Millivolt**
µmol	**Micromol**
N	**Number of experimental animals**
n	**Number of tissues/epithelia**
Na	**Sodium**
NHE	**Na^+/H^+-exchanger**
NO_3^-	**Nitrate ion**
P	**p value, probability**
PAT1	**Putative anion transporter 1**
PBS	**Phosphate Buffered Saline**
pCMB	**p-chloromercuribenzoate**
pCMBA	**p-chloromercuribenzoic acid**
pCO_2	**Partial pressure of CO_2**
PD	**Potential difference**
PD_t	**Transepithelial potential difference (mV)**
pH	**Potentia hydrogenii**
pH_i	**Intracellular pH**
pHMB	**p-hydroxymercuribenzoic acid**
pK-valu	**The negative base-10 logarithm of the acid dissociation constant of a solution**
RNA	**Ribonucleic acid**

R_t	Transepithelial resistance
RT-PCR	Reverse transcriptase polymerase chain reaction
sat-1	Sulphate anion transporter-1
$SCFA^-$	Anion of short-chain fatty acids
SCFA	Short-chain fatty acids
SEM	Standard error of the mean
SITS	4-acetamido-4-isothiocyanostilbene-2,2-disulfonate
SO_4^{2-}	Sulphate ion

1 Introduction

Ruminants consume plant material containing complex carbohydrates. Consequently, they depend mainly on the fermentation capability of the micro-organisms which habitat their forestomach. The end products of the microbial fermentation of carbohydrates are short-chain fatty acids (SCFA; principally acetic acid, propionic acid and n-butyric acid) (BERGMAN. 1990; KRISTENSEN et al. 2000). SCFA serve as the essential source of energy for the ruminant (75-80% of animal`s maintenance energy requirements) (GÄBEL et al. 2002). Moreover, SCFA are necessary for the synthesis of milk fat and lactose (ALUWONG et al. 2010). Most of the SCFA produced in the rumen are absorbed directly across the ruminal wall (PENNER et al. 2009c; ASCHENBACH et al. 2011). Due to the importance of SCFA for the welfare of ruminants, the mechanisms behind their absorption are under scientific focus.

The ruminal epithelium is a stratified squamous epithelium consisting of the *stratum basale*, the *stratum spinosum*, the *stratum granulosum*, and the *stratum corneum* (STEVEN and MARSHALL 1970; GRAHAM and SIMMONS 2005). Regarding its transport properties, ruminal epithelium can be simplified as a functional unit with an apical membrane on the lumen side and a basolateral membrane on the blood side (GÄBEL et al. 2002). Concerning the permeation of SCFA across this tissue, it has been shown previously that it occurs mainly via transcellular pathways, and that paracellular permeation plays a minor role (STEVENS and STETTLER 1966b; SEHESTED et al. 1999b, RACKWITZ et al. 2012). The transcellular permeation includes the apical uptake of SCFA, their intraepithelial degradation and the extrusion of SCFA and/or their metabolites to the serosal side. Mechanisms suggested to be involved in the apical uptake of SCFA into the ruminal epithelium are 1) lipophilic diffusion of undissociated (protonated) SCFA (HSCFA) (WALTER and GUTKNECHT 1986; RECHKEMMER et al. 1995; GÄBEL et al. 2002); 2) proton-coupled transport of SCFA anions ($SCFA^-$) via monocarboxylate transporter (MCT) 4 (KIRAT et al. 2007); 3) uptake of $SCFA^-$ in exchange for bicarbonate (BILK. 2008; ASCHENBACH et al. 2009). The molecular correlate for the latter could be the anion exchanger proteins anion exchanger 2 (AE2), putative anion transporter 1 (PAT1) or down-regulated in adenoma (DRA). These proteins have been found at mRNA level in cultured ruminal epithelial cells as well as in intact ruminal epithelium (BILK et al. 2005). But, so far there is no functional evidence for the involvement of these proteins in SCFA transport across ruminal epithelium. Inside the ruminal epithelium, SCFA mainly

occur in the dissociated form due to higher intracellular pH values (MÜLLER et al. 2000). SCFA (primarily butyrate) are partially metabolized intracellularly to ketone bodies (D-3-hydroxybutyric acid and acetoacetic acid) and lactic acid (REMOND et al. 1995). These substances are even less lipophilic in comparison to the undissociated form of SCFA (LEO et al. 1971). Thus, SCFA and their metabolites essentially require protein mediated mechanisms for their extrusion across the basolateral membrane of the ruminal epithelium.

One of the mechanisms suggested to be involved in the extrusion of $SCFA^-$ across the basolateral membrane of the ruminal epithelium is the monocarboxylate transporter 1 (MCT1). MCT1 has been localized immunohistochemically in the basolateral membrane of ovine ruminal epithelium (MÜLLER et al. 2002). This finding was supported by KIRAT et al. (2006) in caprine, and by GRAHAM et al. (2007) in the bovine ruminal epithelium. Functionally, MCT1 operates as a proton-coupled transporter for monocarboxylates and SCFA in several tissues including caprine and ovine rumen epithelium (MÜLLER et al. 2002; KIRAT et al. 2006). However, studies on colon epithelium suggested that MCT1 could also act as anion exchanger (REYNOLDS et al. 1993; TYAGI et al. 2002; DUDEJA and RAMASWAMY 2006). This suggestion can be transferred to ruminal epithelium, since DENGLER et al. (2015) found bicarbonate dependent transport mechanism at the basolateral side that turned out to be sensitive to MCT1 inhibitors. However, in these experiments, the inhibition of MCT1 abolished only half of bicarbonate dependent SCFA. This suggests the involvement of further anion exchanger(s) in the basolateral extrusion of SCFA. Promising candidates underlie this exchange could be the above mentioned proteins PAT1 and DRA. They belong to the SLC26 gene family (PAT1: SLC26A6; DRA: SLC26A3) and facilitate not only the exchange of chloride and bicarbonate but also that of larger organic anions like oxalate (ALPER and SHARMA 2013). Especially PAT1 which has been reported to facilitate the transport of organic acids as well as inorganic anions (JIANG et al. 2002; CHERNOVA et al. 2005; SHCHEYNIKOV et al. 2006; NOZAWA et al. 2004; ALVAREZ et al. 2004) should be taken in focus. Moreover, PAT1 has been detected in other parts of the digestive tract such as duodenum and proximal colon (WANG et al. 2002; ALPER and SHARMA 2013).Therefore, the present study was designed to pursue the question whether PAT1 and/or DRA could be involved in the efflux of SCFA or their metabolites across the basolateral membrane of the ruminal epithelium.

2 Literature Review

2.1 Importance of short-chain fatty acid production for ruminants

Ruminants consume complex carbohydrates such as cellulose, hemicellulose and pectin which can't be digested by the enzymes secreted by the glandular epithelium of the digestive tract of ruminants (BERGMAN. 1990). Consequently, the micro-organisms living inside the ruminant's digestive tract have a symbiotic relationship with the host animal (BERGMAN. 1990; KRISTENSEN et al. 2000; ALUWONG et al. 2010). Anatomically, ruminants have unique fermentation and mixing vats, particularly the rumen and reticulum (reticulorumen).They provides an ideal environment for the microbial growth and fermentation of the carbohydrates. The rumen particularly assort as the main fermentation vat by providing a large space for fermentation and a variety of microbes (BERGMAN. 1990; ALUWONG et al. 2010). The main products of the microbial fermentation of the polysaccharides are short-chain fatty acids (SCFA), principally acetic acid, propionic acid and n-butyric acid (BOGGS et al. 1959; STEVENS and STETTLER 1966; BERGMAN. 1990; DIERNAES et al. 1994; KRISTENSEN et al. 1996). Moreover, ammonia, carbon dioxide and methane are also produced during the fermentation process (FAHEY and BERGER 1988; GÄBEL et al. 2001). SCFA are considered as an essential source of energy for ruminants (75%-80% of their maintenance energy requirements) (BERGMAN. 1990; GÄBEL et al. 2002). Acetate alone provides 50% of the caloric energy contained in the three SCFA, while each of propionate and butyrate provide 25% of the energy (BERGMAN. 1975; BERGMAN.1990). Propionate is the main exogenous precursor for the hepatic gluconeogenesis to provide circulatory glucose in ruminants (BAIRD et al. 1980; HUNTINGTON et al. 1981). Moreover, propionate metabolism produces L-lactate which is also considered as a precursor for hepatic gluconeogenesis (HUNTINGTON et al. 1981). Therefore, propionate represents the main source for the synthesis of the milk lactose and availability of the circulatory glucose (ALUWONG et al. 2010). On the other hand, circulatory acetate is considered as the main source for fatty acid synthesis and lipogenesis in adipose tissue and muscles as well as the synthesis of milk fat (BERGMAN. 1975). Beside the nutritive and energetic importance of the SCFA production, they play an endocrine regulatory role for insulin secretion (MANNS et al. 1967; BASSETT. 1975; WEEKES. 1991). Furthermore, HARADA and KATO (1983) reported that SCFA have influenced the exocrine secretion of the pancreas. With regards to rumen, SCFA, at least

butyrate, enhance the epithelial development and differentiation (TAMATE et al. 1962; SAKATA and YAJIMA 1978). Therefore, effective production and absorption of the SCFA from the forestomach is crucial for the health and welfare of ruminants.

There are a lot of discussions concerning the quantitative production of SCFA in the rumen. The quantitative production of SCFA in the reticulorumen depends on many factors such as feeding time, quantity and quality of the food (MARTIN et al. 2001; KRISTENSEN et al. 1996). Concerning the feed quality, study by SUTTON et al. (2003) in dairy cattle using infusion technique reported that the difference in the total SCFA production between normal (7.8 concentrates, 5.1 hay) and low-roughage diet (11.5 concentrates, 1.2 hay) is ranged between 79.8 and 90.0 mol/d, respectively.

The level of the feed intake also influences the production of the SCFA. Study by MARTIN et al. (2001) in sheep using isotopic tracing technique reported that the difference between low 45% *ad libitum* consumption and high 90% *ad libitum* consumption intake is between 8.8 and 14.8 mol/d, respectively. ASCHENBACH et al. (2011) pointed out that the isotopic tracing technique has limitation in the sense that the technique is susceptible to carbon sequestration by ruminal microbes and microbial carbon inter-conversion of SCFA as a source of error. Study by LONCKE et al. (2009) using meta-analysis of the published data to measure the net portal appearance of the SCFA suggested that 1g / (d·kg BW) increase in rumen fermentable OM intake induced 5.93 mmol/(d·kg BW) increase in SCFA production. However, ASCHENBACH et al. (2011) reported that portal appearance studies systematically lead to underestimation of the produced acids due to the differences between the rate of acid production and acid removal, as well as SCFA metabolism by the pre-gastric epithelia.

2.2 Apical uptake of short-chain fatty acids from the rumen

Earlier study by STEVENS and STETTLER (1966b) demonstrated that the increase of the electrical potential gradient had no influence on the absorption of SCFA. Therefore, it can be concluded that the permeation of SCFA across the ruminal epithelium occurs mainly transcellularly and the paracellular pathway plays a minor role. Consequently, the transcellular route has been considered as the main pathway for SCFA absorption across the ruminal epithelium.

The forestomach of the ruminants plays the main role in the microbial fermentation of feed and the production of SCFA, as well as the major role in the absorption of these products. It has been demonstrated that most of the SCFA produced in the rumen are absorbed across the epithelium directly into the blood (BARCROFT et al. 1944; MARSHALL and PHILLIPSON. 1945). Reviewing the previous findings, ASCHENBACH et al. (2011) pointed out that 50 to 85% of the SCFA produced in the rumen is directly absorbed across the reticulorumen wall. While the remaining SCFA passes to the following parts of the digestive tract (VON ENGELHARDT and HAUFFE 1975; PETERS et al. 1990). Due to the vital importance of the SCFA absorption across ruminal epithelium, a lot of work has been done on the transport of these substrates.

The rumen epithelium is classified as stratified squamous epithelium containing leaf like papillae which increase the absorptive service area (STEVEN and MARSHALL 1970). Histologically, the rumen epithelium consists of four layers: the *stratum corneum*, the *stratum granulosum*, the *stratum spinosum* and the *stratum basale* (STEVEN and MARSHALL 1970). Regarding absorption processes, the epithelium is simplified as a functional unit with an apical (lumen)-facing barrier, and a basolateral (blood)-facing membrane (MARTENS and GÄBEL 1988; STEVEN and MARSHALL 1970). Thus, the transcellular route has to permeate two main barriers, the uptake across the apical membrane and the extrusion across the basolateral membrane.

The apical uptake of SCFA is the first step to deliver the metabolic energy for the animal. Moreover, the apical absorption of SCFA provides substrates for the intracellular metabolisms and, hence, provides energy for the rumen epithelium. In addition, the intracellular metabolism of SCFA creates a concentration gradient which enhances the apical absorption of SCFA (STEVENS and STETTLER 1966a). ASCHENBACH et al.

(2011) pointed out that the apical absorption of SCFA is determined mainly by the rumen epithelium itself, but it is influenced also by microbial dynamics, energy input and the physiochemical factors.

Several mechanisms have been suggested to be involved in the apical uptake of SCFA and some of them have been confirmed. Generally, SCFA exist in the ruminal lumen in two forms: 1) the protonated or undissociated form (HSCFA), 2) the ionized or dissociated form ($SCFA^-$). HSCFA are lipid soluble and can directly permeate the epithelial membrane (LEO et al. 1971; WALTER and GUTKNECHT 1986). In contrast, the dissociated form of SCFA is not lipophilic. Thus, it needs specific protein mediated transport mechanisms to be introduced into the epithelial cells. Therefore, the apical ruminal uptake of SCFA could occur either by passive diffusion of HSCFA or by a secondary active transport of $SCFA^-$ (SEHESTED et al. 1996b).

2.2.1 Apical uptake of undissociated SCFA from the rumen

An early study by WALTER et al. (1982) reported that weak acids such as SCFA in aqueous solution exist in both the undissociated form (HSCFA) and the dissociated form ($SCFA^-$). HSCFA can permeate the lipid bi-layer of biological membranes by diffusion because they are lipid soluble (WALTER et al 1982; WALTER and GUTKNECHT 1986). The high production rate of SCFA inside the lumen creates a concentration gradient between the lumen and blood which could reach a ratio of 20:1 – 100:1 (ANNISON et al. 1957; REYNOLDS and HUNTINGTON 1988). This chemical gradient between the lumen and blood is the only driving force for the permeation of HSCFA.

The diffusion of the HSCFA across the apical membrane simultaneously eliminates protons from the lumen which in term regulates the intraruminal pH (RECHKEMMER et al. 1994). Several studies confirmed the role of lipophilic diffusion of HSCFA in the apical uptake of SCFA (BUGAUT. 1987; LOPEZ et al. 2003; GRAHAM et al. 2007). Based on the different chain length of SCFA, they show different lipophilicity which in tern leads to different efficiency in their permeability. According to WALTER and GUTKNECHT (1986), the permeability of propionic acid is 5.1 times higher than that of acetic acid, and butyric acid is 2.1 times more permeable than propionic acid. This finding is in qualitative agreement with other in vivo studies in sheep and cattle which revealed that the clearance

rate of SCFA was in order of acetic acid < propionic acid < butyric acid (WEIGAND et al, 1972a; THORLACIUS and LODGE 1973; GÄBEL et al. 1991; DIJKSTRA et al. 1993; SEHESTED et al. 1999 a).

In aqueous solution, the undissociated form (HSCFA) and dissociated form ($SCFA^-$) of SCFA are in equilibrium (WALTER et al. 1982; LEHNINGER et al. 1998). This balance depends on the pK value of the short-chain fatty acid and the pH of the solution according to the Henderson-Hasselbalch equation **[**pH = pK + log (SCFA- / HSCFA)**],** (LEHNINGER et al. 1998). The ruminal pH ranges form 5.5 to 7.0 (MARTENS. 2005; BILK et al. 2008) and the pK value of the short-chain fatty acids is ~4.8 (LEHNINGER et al. 1998). Therefore, lowering the intraruminal pH should increase the concentration of the HSCFA and, as a consequence, will lead to an increased absorption rate. Previous studies showed a correlation between ruminal pH and the absorption rate of SCFA in sheep and cattle (STEVENS and STETTLER 1966a; DIJKSTRA et al. 1993; KRAMER et al. 1996; SEHESTED et al. 1999b).

On the other hand, STEVENS and STETTLER (1967) reported that even under elimination of the trans-epithelial electrochemical gradient a net transport of SCFA has occurred. Moreover, when considering the effect of the pH on HSCFA concentration, according to the Henderson-Hasselbalch equation, the low pH should increase the concentration of HSCFA and hence stimulate the absorption rate of SCFA (GABEL et al. 2002). However, several studies reported that the low pH induced stimulation of SCFA was not similar as that predicted by Henderson-Hasselbalch equation (DIJKSTRA et al. 1993; KRAMER et al. 1996; SEHESTED et al. 1999). Therefore, there should be other mechanisms for apical uptake beside the lipophilic diffusion of SCFA. Furthermore, because of the lipophilicity of the SCFA which reported by DIJKSTRA et al. (1993) is that propionic acid had a clearance rate just 1.6 times more than acetic acid, and butyric acid had a clearance rate 1.2 times more than propionic acid. Pending on that, if SCFA are absorbed only by lipophilic diffusion of HSCFA, the difference in the absorption rate between the three SCFA should be noticeable. However, previous studies confirmed that the differences in the absorption rate of SCFA according to the lipophilicity were smaller than predicted (GÄBEL et al. 1991; SEHESTED et al. 1999b). In summary, the apical uptake of SCFA doesn’t occur only by the lipophilic diffusion of HSFA and other mechanisms should be involved.

2.2.2 Apical uptake of dissociated fatty acids from the rumen

As mentioned in section 2.2.1, lipophilic diffusion of HSCFA can't be the essential way for the permeation of SCFA across ruminal epithelium. Therefore, other mechanisms should participate effectively in the apical uptake of $SCFA^-$.

Numerous studies have been conducted to determine alternative mechanisms of SCFA uptake across the apical membrane of the ruminal epithelium. One mechanism suggested to participate in the apical uptake of SCFA is the exchange of $SCFA^-$ for bicarbonate (HCO_3^-) (GÄBEL et al. 1991). The authors observed a relationship between ruminal bicarbonate secretion and luminal disappearance of SCFA in isolated and washed reticulo-rumen of sheep. Later, in vitro and in vivo studies on sheep ruminal epithelium by KRAMER et al. (1996) reported that an increase of the mucosal chloride concentration as a competitive substrate for SCFA leads to a significant drop in the mucosal-serosal flux of propionate (in vitro), SCFA net absorption (in vivo). This competition between chloride and SCFA provides a hint for a participation of anion exchangers in this process owing to the fact that chloride has been used as an exchanger substrate for anion exchange in several studies (KNAUF et al. 2001; JIANG et al. 2002; KO et al. 2002; WANG et al. 2002; XIE et al. 2002). Furthermore, the authors applied apically 4,4′-diisothiocyano-2,2′-stilbenedisulfonic acid (DIDS) which is considered as unspecific anion transport blocker (KNICKELBEIN et al. 1985; ALPER et al. 2002; JIANG et al. 2002; IKUMA et al. 2003). Also, the authors applied nitrate (NO_3^-) which has been reported as a competitive inhibitor for anion exchange (MEIER et al. 1985; SEIFTER and ARONSON 1984). Both inhibitors markedly reduced the apical uptake of propionate which supports the existence of anion exchanger in the apical side of the ruminal epithelium.

ASCHENBACH et al. (2009) pointed out the importance of bicarbonate-independent mechanisms in the apical uptake of $SCFA^-$. In their study, the presence of SCFA mixture in a bicarbonate-free buffer solution at the apical side of ruminal epithelium inhibited chloride disappearance rate from the reticuloumen. Vice versa the presence of chloride reduced acetate and propionate disappearance in washed reticulorumen in sheep. Moreover, the authors showed that the stimulatory effect of decreased pH values on the apical uptake of acetate was slight in the case of the bicarbonate absence. Hence, ASCHENBACH et al.

(2011) reported that the enhanced SCFA uptake at low ruminal pH could be attributed to the bicarbonate gradient across the apical membrane and not for the pH values.

In other gastrointestinal epithelial parts, notable anion exchanger proteins have been detected. The down regulated in adenoma (DRA, SLC26A3) and putative anion exchanger 1 (PAT1, SLC26A6) have been detected at the apical side of the small intestine and colon of human aid mice (HOGLUND et al. 1996a; WANG et al. 2002). Other studies also postulated that MCT1 could work as anion exchanger in the other part of the digestive tract such as rat colon (REYNOLDS et al. 1993; DUDEJA and RAMASWAMY 2006)

With regards to ruminal epithelial cells, BILK et al. (2005) found DRA, PAT1 and anion exchanger 2 (AE2) at mRNA level in both cultured ruminal epithelial cells and intact ruminal epithelium. This study assumed that DRA and PAT1 are responsible for the direct secretion of bicarbonate into the lumen and that ruminal $SCFA^-$ are used as a substrate for exchange with bicarbonate via DRA and PAT1. Furthermore, members of the monocarboxylate transporter family (MCT, SLC16H) have been localized immunohistochemically in the ruminal epithelium. KIRAT et al. (2007) located MCT4 in the lumen oriented cell layer of the ruminal epithelium and assumed participation of MCT4 in the apical acetate transport. However, ASCHENBACH et al. (2009) contradicted this assumption and indicated that neither bicarbonate dependent nor the bicarbonate independent apical uptake of acetate is mediated by apical MCTs.

2.3 Intraepithelial metabolism of short-chain fatty acids

After their apical uptake, SCFA are metabolized within the ruminal epithelium to some extent (MASSON and PHILLIPSON 1951; WEIGAND et al, 1975; BERGMAN. 1990; KRISTENESN et al. 1998; KRISTENESN and HARMON 2004). The extent of this intraepithelial breakdown of the SCFA is discussed controversially, in particular for acetate and butyrate. Previous studies have pointed out that the ruminal epithelium metabolizes 30, 50 and 90% of the absorbed acetate, propionate and butyrate, respectively (BERGMAN. 1990; BRITTON and KREHBEIL 1993). However, a study by KRISTENSEN and HARMON (2004) suggested that this rate of intra- epithelial catabolism of SCFA would produce more ATP than needed. Moreover, an *in vitro* study by SEHESTED et al. (1999b) showed that catabolism of acetate inside ruminal epithelium is negligible. Using a portal drainage technique in sheep, KRISTENSEN et al. (2000a) showed that a small portion of

acetate and propionate are catabolised inside the epithelium. The controversy was in the extent of the catabolism of acetate and propionate. Nevertheless, all studies concerning intraepithelial metabolism of SCFA reported a high level of catabolism of n-butyrate inside the epithelial cells (BERGMAN. 1990; BRITTON and KREHBEIL 1993; KRISTENSEN and HARMON 2004). Furthermore, an *in vivo* study using the washed reticulorumen technique supported the suggestion that only n-butyrate is broken down to a higher extent that may reach 95% (KRISTENSEN and HARMON 2004).

The explanation for the different catabolic rates of the SCFA could be the activation process inside the ruminal epithelium. For the initiation of intraepithelial metabolism of SCFA, the first step is the formation and activation of two acyl-CoA syntheses (SCAIFE and TICHIVANGANA 1980). According to the kinetic properties, the activation of the acyl-CoA occurs in the order of acetate ≤ propionate ≤ n-butyrate (SCAIFE and TICHIVANGANA 1980). After this activation, acetate and butyrate are degraded aerobically to CO_2 and water or an-aerobically to acetoacetate and D-3-hydroxybutyrate (BERGMAN. 1990; KRISTENSEN et al 2000b; GÄBEL et al 2002). Concerning propionate, the final product of the an-aerobic intraepithelial catabolism is lactic acid (WEIGAND et al. 1975). The activation of the catabolic process is affected by n-butyrate as reported by HARMON et al. (1991) which revealed that the presence of n-butyrate could depress the activation of acetate and propionate, but the presence of acetate and propionate had no influence on the activation of n-butyrate.

Intraepithelial catabolism of the SCFA has obvious advantages because it improves the apical uptake SCFA by maintaining the concentration gradient between the rumen and the cytosol (GÄBEL et al. 2002). This view is supported by GÄBEL et al. (2001) who reported that the hardly metabolizable iso-butyrate is much less transported across ovine ruminal epithelium than the well metabolizable n-butyrate. Furthermore, the CO_2 produced by intraepithelial metabolism can be converted to HCO_3^- which serves as counter anion for apical $SCFA^-$ uptake via anion exchange (see section 2.2.2) (ASARI et al. 1989; AMASAKI et al. 1991; SEHESTED et al. 1999; GÄBEL et al. 2002).

Also, the intraepithelial catabolism of the SCFA is the main source of energy for the epithelial cells instead of glucose (BALDWIN and JESSE 1992; BRITTON and KREHBIEL 1993; GÄBEL et al. 2002). Furthermore, the ketone bodies produced from intraepithelial catabolism of the SCFA bypass the liver and serve as an energy supply for the extra-hepatic tissue such as the heart muscle, kidney, mucosal cells of the intestine, mammary

gland and also the brain (ROBINSON and WILLIAMSON 1980; HEITMANN et al. 1987.; LEHNINGER et al. 1998; GÄBEL et al. 2002; MORRIS. 2005). Some studies indicated that the excessive production of n-butyrate had a harmful effect locally in the rumen and also systematically (OWENS et al. 1998; REMESY et al. 1995; GÄBEL et al. 2002). Therefore, the degradation of n-butyrate to D-3-hydroxybutyrate could prevent the local and systemic side effects of n-butyrate (GÄBEL et al. 2002). On the other hand, the ketone bodies produced are less lipophilic than the SCFA and can hardly cross the basolateral membrane by lipophilic diffusion (LEO et al. 1971; GÄBEL et al. 2002). Thus, the accumulation of ketone bodies inside the ruminal epithelial cells would affect intraepithelial pH and osmolality resulting in acidification and swelling of the cell (GÄBEL et al. 2002) if these substrate would not be sufficiently extruded to the blood.

2.4 Mechanisms for the basolateral discharge of the short-chain fatty acids

As described before, the apical uptake of SCFA and their intraepithelial metabolism result in high intraepithelial concentration of $SCFA^-$ and ketone bodies. Ketone bodies and lactate should be efficiently extruded from the ruminal epithelial cells to prevent a lethal drop in intracellular pH and counteract osmotic load of the cytosol. However, D-3-hydroxybutyric acid, acetoacetic acid, and lactic acid are even less lipophilic than SCFA in their undissociated form (LEO et al. 1971). Therefore, they cannot cross the membrane well by free diffusion. Additionally, D-3-hydroxybutyric acid, acetoacetic acid, and lactic acid are almost completely dissociated at physiological intraepithelial pH (~7.4) (MÜLLER et al. 2000), implying that membrane permeation by free diffusion is very low. Consequently, specific transport mechanism for rapid export of ketone bodies and lactate are required in the basolateral membrane of ruminal epithelial cells. Although, lipophilic diffusion across basolateral membrane of the rumen could not completely excluded from the process, but it should play a minor role due to two factors: (1) the concentration gradient of SCFA between cytosol and blood is low compared to the concentration gradient of SCFA between ruminal lumen and epithelial cells (DENGLER et al. 2014) and (2) the concentration of the intracellular HSCFA is low because of the slightly alkaline intracellular pH (MÜLLER et al. 2000). However, there are few studies focusing on the transport of SCFA across the basolateral membrane of the ruminal epithelium.

Nevertheless, some hints can be derived from studies on basolateral membrane functions in other digestive tract tissues of different species.

2.4.1 Basolateral extrusion of short-chain fatty acids in other gastrointestinal tract epithelia

Generally, in monogastric herbivores, omnivorous and ruminants, SCFA are produced by microbial fermentation of the polysaccharides in the colon and the cecum (BUGAUT. 1987). Likewise, acetate, butyrate and propionate are the main end products of the microbial fermentation of dietary fibres in human colon (CUMMINGS. 1981; CLAUSEN and MORTENSEN 1995). MCNEIL (1984) reported that 5-10 % of the energy requirements of humans are provided by absorption of SCFA from the large intestine. Also, 40 % of energy requirement of rabbits and ponies is contributed to the cecal SCFA (GLINSKY et al. 1976). Moreover, SCFA are considered as a major energy supplement and nutrient for the large intestinal epithelial cells (ROEDIGER. 1982; CUMMINGS et al. 1981). Butyrate in particular, promotes the growth and proliferation of the colon mucosa and induces cell differentiation (MORTENSEN et al. 1991; TREEM et al. 1994). It also, regulates fluid and electrolyte Two mechanisms are suggested to contribute in the SCFA transport in the large intestine: passive diffusion of the protonated form of SCFA and/or carrier mediated SCFA/HCO_3^- exchange for the ionized form of SCFA (SELLIN et al. 1993; CHARNEY et al. 1998; RAJENDRAN and BINDER 2000; VON ENGELHARDT et al. 1993). A functional study by CHARNEY et al. (1998) suggested that SCFA are mainly transported across the rat colon by non-ionic diffusion. Furthermore, study by SELLIN et al. (1993) on rabbit supported the non-ionic diffusion of SCFA across colon epithelium. In contrast, a study regarding the membrane permeability in the caecum and proximal colon of guinea pig by BUSCHE et al. (2002) suggested that the protein mediated transport of SCFA across the basolateral side plays a minor role in contrast to the apical side. The authors pointed out that the apical membrane in proximal colon and caecum considers more effective barriers for non ionic diffusion of protonated SCFA than the basolateral membrane. This finding was approved functionally by VON ENGELHARDT et al. (1997). In their study, the removal of bicarbonate had more influence on the mucosal to serosal flux of propionate than the serosal to mucosal flux across guinea pig distal colon. This finding suggested that the anion exchange mechanism is more involved in the apical uptake of the propionate than in the basolateral extrusion of propionate in the colon epithelium.

Nevertheless, several studies have underlined the role of anion exchange mechanism at the basolateral membrane of proximal and distal colonic epithelial cells. REYNOLDS et al. (1993) detected an anion exchanger in basolateral membrane vesicles (BLMV) of the distal colon of rat. Where the acidic pH enhanced butyrate uptake by BLMV more than the alkaline one. In this study, increasing the outward gradient of bicarbonate enhanced butyrate uptake by BLMV. In addition, the uptake of butyrate was inhibited when the anion exchange inhibitor DIDS was applied. The authors also concluded that the type of the carrier mediated the anion exchange mechanism on the basolateral side differs from the one present on the apical side in colonic epithelium cells.

In another study by TYAGI et al. (2002) using the same approach in human proximal colon, basolateral transport of butyrate was enhanced in the presence of an outwardly directed HCO_3^- gradient. While the butyrate flux was inhibited by use of the anion exchange inhibitor niflumic acid but not by DIDS or 4-acetamido-4′-isothiocyanostilbene-22′-disulfonate (SITS). A molecular study by RAJENDRAN and BINDER (2000) characterized and identified several anion exchangers in the colonic epithelium. Based on their finding, the authors suggested that SCFA are transported by anion exchanger 1 (AE1) in the apical side and anion exchanger 2 (AE2) in the basolateral side. KIRAT et al. (2006a) located MCT1 in the basolateral membrane of the bovine caecum, proximal colon and distal colon using immunohistochemistry and confocal laser microscopy. The authors suggested that MCT1 is the main route for SCFA efflux across the basolateral membrane of bovine large intestine and also regulates the intracellular pH. Furthermore, monocarboxylate transporter 1 (MCT 1) has been located in basolateral membrane of small intestine and large intestine of different species and suggested to act as a proton-SCFA symporter (KIRAT et al. 2009). In pectin-fed rats, MCT1 has been detected immunohistochemically in the basolateral membrane of stomach, small intestine and large intestine (KIRAT et al. 2009). The authors have also detected MCT1 in the basolateral membrane of caecum and colon of the control rats. Moreover, MCT1 has been detected immunohistochemically in the small intestine and large intestine of the dog (SHIMOYAMA et al. 2007). In contrast, MCT1 has been detected in the apical side of human colonic epithelial cells (RITZHAUPT et al. 1998a).

In summary, SCFA produced in other regions of the GI-tract by microbial fermentation of carbohydrate, mainly in the large intestine, could efflux basolaterally either by passive diffusion of their protonated form or mediated by transport proteins for their ionic form. These proteins could either be anion exchanger proteins such AE2 or proton coupled transporters such as MCT1.

2.4.2 Basolateral extrusion of short-chain fatty acids in ruminal epithelium

The basolateral extrusion of SCFA across the ruminal epithelium is still under discussion. A functional study by SEHESTED et al. (1999b) revealed that the transport of butyrate and its metabolites across the basolateral membrane of the ruminal epithelium occurs partially by passive diffusion, however an anion-exchange for bicarbonate is also involved.

Up to now, MCT1 is the only transporting protein that has been detected and proved to be involved in the basolateral extrusion of SCFA in ruminal epithelium. In addition, a channel in the basolateral side of the rumen epithelium has been proposed to transport SCFA (STUMPFF et al. 2009). MCT1 is a member of the monocarboxylate transporter family (SLC6 family) which has 14 members (POOLE and HALESTRAP 1993). In an early study on lactate and pyruvate transport into human erythrocytes, it was recognized that the transport was mediated by a carrier later named monocarboxylate transporter (MCT) (DEUTICKE. 1982). The different characteristics of monocarboxylate transporter observed after extensive characterization in erythrocytes, cardiac myocytes and hepatocytes led to the proposal that a family of various MCTs might exist (POOLE and HALESTRAP 1993). MCT1 has been detected for the first time in the rat and rabbit erythrocytes (POOLE and HALESTRAP 1993). MCT2 has been identified by GARCIA et al. (1995) in the rat liver. MCT3 has been detected in chick retinal pigment epithelium (GROLLMAN et al. 2000). MCT4, MCT5, MCT6 and MCT7 have been identified in a study for the MCT-like sequences from the human cDNA libraries by PRICE et al. (1998). MCT8 gene has been isolated by positional cloning in a study regulation and chromosomal basis of X chromosome inactivation (LAFRENIERE et al. 1994). MCT9, MCT11, MCT12, MCT13 and MCT14 have been detected in the analysis of the human genomic expressed sequence tag (EST) databases (HALESTRAP and PRICE 1999, HALESTRAP and MEREDITH 2004).

MCT1, as reported by HALESTRAP and MEREDITH (2004) is the most widely expressed member of the monocarboxylate transporter family. With regards to gastrointestinal tract, MCT1 has been detected by molecular biology and immunohistochemistry in several tissues. MCT1 is expressed in the reticulum, rumen, omasum, abomasums, caecum, proximal colon, distal colon and small intestine of goats (KIRAT et al. 2006b). Concerning localization, MCT1 has been detected in the basolateral membrane of the rumen epithelium in sheep and goat and that of rumen and caecum epithelia in cattle (MÜLLER et

al. 2002; KIRAT et al. 2006b; KIRAT and KOTO 2006; GRAHAM et al. 2007). In contrast, MCT1 has been detected in the apical side of colon epithelium in human and pig (RITZHAUPT et al. 1998a; CUFF et al. 2000; GILL et al. 2005).

Regarding substrate specificity, MCT1 transports mainly monocarboxylates (especially lactate) (HALESTRAP et al. 1999). Lactate is considered to be the main end product of the glycolysis and the source of energy in several cells in the body such as erythrocytes, white muscle cells, tumour cells, liver cells, kidney cells and neurons (POOLE and HALESTRAP 1993). MCT1 has been reported as H^+/lactate co-transporter that can also transport monocarboxylic acids with chain lengths of C2-C5 (POOLE and HALESTRAP 1993; WANG et al. 1996). For a proper cell surface expression and function, MCT1 needs another cell surface glycoprotein called CD147 (KIRK et al. 2000, GRAHAM et al. 2007).

MCT1 inhibitors can be divided into three types. First, large molecular or aromatic monocarboxylates such as 2-oxo-4-methylpentanoate, phenylpyruvate and derivatives of the α-cyanocinnamic acid as α-cyano-4-hydroxycinnamic acid (CHC).These inhibitors act as competitive inhibitors (POOLE and HALESTRAP 1993; GARCIA et al. 1995; HALESTRAP and MEREDITH 2004). Second, various amphiphilic substances with divergent structure, which include bioflavonoids such quercetin and phloretin. These inhibitors work as a potent inhibitors (POOLE and HALESTRAP 1993; HALESTRAP and MEREDITH 2004). Third, stilbene derivatives such as 4,4’-diisothiocyanostilbene-2,2’-disulphonate (DIDS) and 4,4'-2,2'-disulfonate Dibenzamidostilben (DBDS). These inhibitors produce a reversible inhibition in the short-term incubation. On the other hand, prolonged incubation with thiol reagent p-chloromercuribenzene sulphonate (pCMBS), produce irreversible inhibition (POOLE and HALESTRAP 1993; HALESTRAP and MEREDITH 2004)

MÜLLER et al. (2002) conducted experiments in cultures of ovine ruminal epithelial cells concerning the effects of monocarboxylates uptake on intracellular pH (pH_i). The study revealed that the addition of DIDS as MCT1 inhibitor blocked the pH_i recovery after the extrecelluar buffer was uploaded with D-3-hydroxybutyrate, acetoacetate and lactate. Furthermore, the authors detected MCT1 immunohistochemically in the *stratum basale* of the ruminal epithelium, and characterized MCT1 as a H^+ and monocarboxylic acids co-transporter. Consequently, extrusion of monocarboxylates by MCT1 prevents the cell swelling because monocarboxylates are osmotically active. Additionally, MTC1 prevents inhibition of intracellular metabolism which would be caused by the drop of intracellular pH and accumulation of metabolites. In addition to MCT1, MCT4 has been detected by KOHO

et al. (2005) in the rumen epithelium of free-ranging and captive reindeer using immunoblotting technique. They revealed that the expression of MCT1 and MCT4 in the rumen epithelium was higher than that in the epithelium of small intestine which could reflect their functional importance. The expression of MCT4 in the forestomach epithelium was confirmed later by KIRAT et al. (2007), where the transport protein was immunohistochemically located in the *stratum corneum and startum*. Moreover, KIRAT et al. (2006 a) detected the expression of MCT1 protein in the rumen and reticulum epithelia of sheep, using reverse transcriptase polymerase chain reaction (RT-PCR) techniques and Western blot analysis. The authors found higher expression of MCT1 protein in the reticulum and rumen epithelia compared to other parts of the GIT, and attributed this to the high SCFA production in these organs. Further, the authors confirmed their findings using immunohistochemical technique. Later, KIRAT et al. (2006 b) discovered that MCT1 is expressed mainly in the basolateral membrane of the rumen epithelium, particularly in the *stratum basale* and *stratum spinosum* thus supprting the previous findings of MÜLLER et al. (2002). The authors also conducted a functional study in the caprine rumen using the MCT1 inhibitors, p-chloromercuribenzoate (pCMB) and p-chloromercuribenzoic acid (pCMBA) which reduced the efflux of the acetate and butyrate. Therefore, the authors suggested that MCT1 plays a role in the extrusion of SCFA across the basolateral membrane. KIRAT et al. (2006a) demonstrated that pharmacological inhibition of the basolateral MCT1 could diminish transepithelial transfer of the hardly metabolizable acetate. However, the incubation buffer contained also butyrate which is metabolized within the epithelium to a great extent. Therefore, the inhibitory effects found in the studies of KIRAT et al. (2006b) might be due to indirect effects resulting from a diminished efflux of metabolites of n-butyrate. Moreover, GRAHAM et al. (2007) detected MCT1 and MCT2 at mRNA level in isolated bovine ruminal epithelium cells. Thereafter, an immunohistochemical study detected only MCT1 in the *stratum basale* of the rumen epithelium. A Study by STUMPFF et al. (2009) using patch-clamp technique pointed out to the presence of an anion channel in cultured ruminal epithelial cells. The authors proved that the channel is responsible for the conductance of Cl^-, NO_3^-, HCO_3^- and acetate. Therefore, they suggested that this channel could play a role in the extrusion of the SCFA across the basolateral membrane of the rumen epithelium. A review by LEONHARD-MAREK et al. (2010) almost ruled out the passive diffusion of SCFA across basolateral membrane of the rumen epithelium due to the intracellular dissociation of HSCFA. Furthermore, the authors suggested that SCFA metabolites are extruded through proton coupled transport process by MCT1, while the ionized form of SCFA are extruded via

electrical channel coupled with Na^+, which is mainly influenced by Na^+/K^+- ATPase pump. Recent functional studies by DENGLER et al. (2014) found a bicarbonate dependent transport mechanism at the basolateral side of ruminal epithelium which turned out to be sensitive to MCT1 inhibitors. The study also found a correlation between basolateral HCO_3^- concentration and the extrusion of SCFA. The authors also inhibited the efflux of SCFA by use of the anion exchanger inhibitor nitrate in the basolateral buffer solution. Furthermore, basolateral application of p-hydroxymercuribenzoic acid (pHMB) as MCT1 inhibitor in the presence of HCO_3^- inhibited the serosal to mucosal flux of acetate and also caused a partial inhibition of the butyrate flux. The pHMB sensitive HCO_3^- dependent transport was lower than the nitrate sensitive part. This indicates the existence of other anion exchanger(s) participating in the basolateral extrusion of SCFA from the ruminal epithelium.

In summary, there are four pathways for the extrusion of SCFA and/or their metabolites across the basolateral membrane of the ruminal epithelium (Fig. 1). These include: (1) passive diffusion of HSCFA which appears to play a minor role in view of excessive dissociation of HSCFA due to the alkaline intracellular pH, (2) the symport with H^+ via MCT1, (3) antiport with HCO_3^- (or other anions) which is at least mediated by MCT1, and (4) the permeation of $SCFA^-$ via an anion channel.

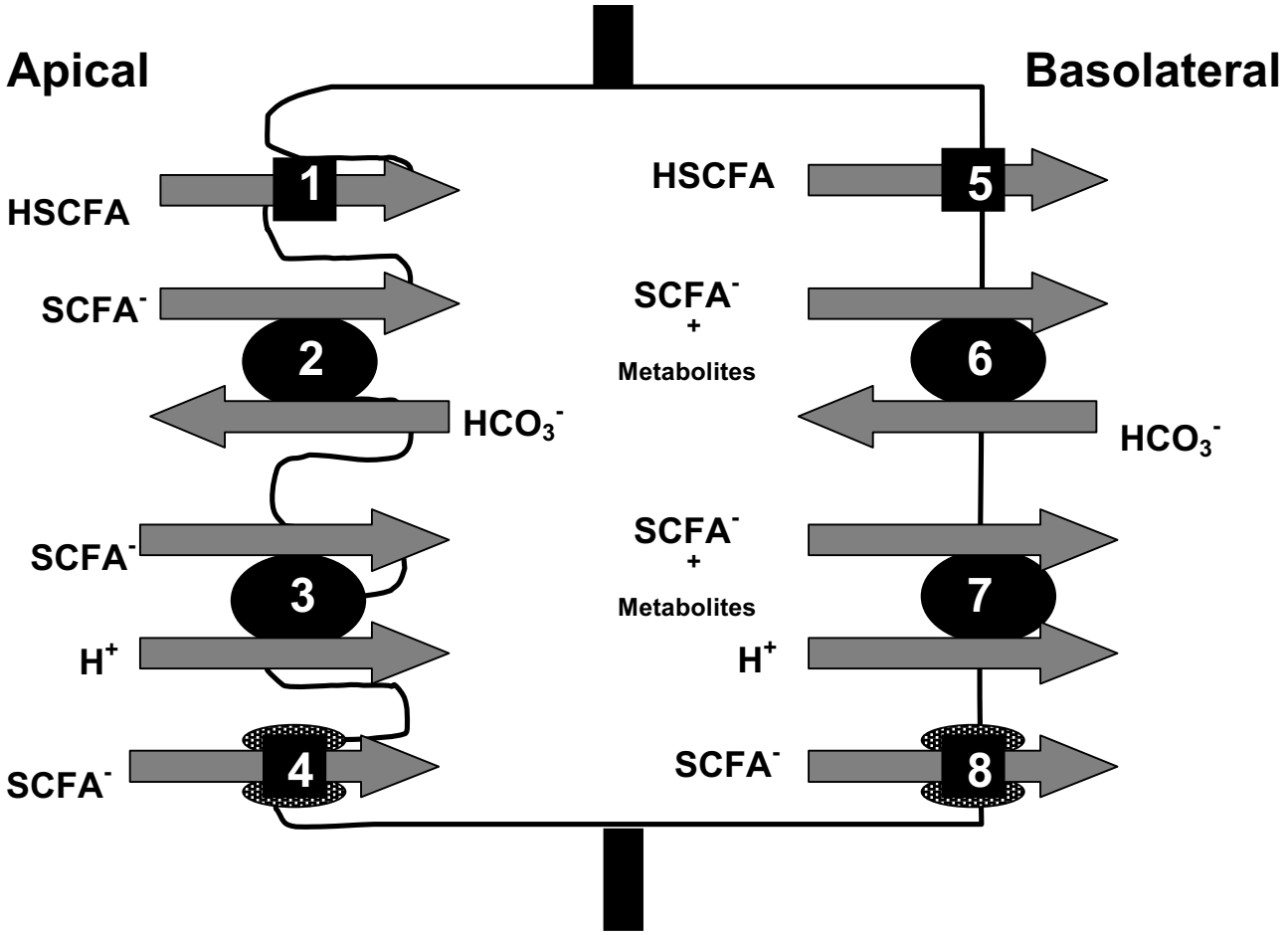

Figure 1: Tentative model for the transport of SCFA across the ruminal epithelium.

Apical side:

(1) Apical uptake of HSCFA by lipophilic diffusion (STEVENS and STETTLER 1966b; SEHESTED et al. 1999b)

(2) Exchange of $SCFA^-$ for HCO_3^- (GÄBEL et al 1991a; KRAMER et al. 1996:; ASCHENBACH et al. 2009)

(3) Co-transport of $SCFA^-$ with H^+ which has been suggested to function through MCT4 (KIRAT et al. 2007)

(4) Apical transport of acetate via an anion channel (RACKWITZ et al. 2012)

Basolateral side:

(5) Lipophilic diffusion of HSCFA (DANIELLI et al. 1945)

(6) Basolateral extrusion of $SCFA^-$ and/or their metabolite in exchange for HCO_3^- using MCT1 (DENGLER et al. 2014)

(7) Basolateral extrusion of $SCFA^-$ and their metabolites together with the H^+ via MCT1 (KIRAT et al. 2006b; GRAHAM et al 2007)

(8) Permeation of $SCFA^-$ via an anion channel (STUMPFF et al. 2009)

2.4.3 Further candidate proteins for extrusion of $SCFA^-$ in exchange for HCO_3^-

DENGLER et al. (2014) found a bicarbonate dependent transport mechanism which turned out to be sensitive to MCT1 inhibitors at the basolateral side of rumen epithelium. However, in these experiments the inhibition of MCT1 abolished bicarbonate dependent transport only by half. This suggests the involvement of further anion exchanger(s) in the basolateral extrusion of SCFA. BILK et al. (2005) detected anion exchanger 2 (AE2), downregulated in adenoma (DRA), and putative anion transporter (PAT1) at mRNA level in ovine cultured ruminal epithelial cells and intact ruminal epithelium. Therefore, these proteins are promising candidates for anion exchange. However, there is no functional evidence so far for the involvement of these proteins in SCFA transport across the ruminal epithelium.

The following parts of literature review will concern with the above mentioned proteins as possible candidates for the basolateral extrusion of SCFA and/or their metabolites in the ruminal epithelial cells.

2.4.3.1 Putative Anion transporter 1 (PAT1 = SLC26A6)

The putative anion transporter 1 (PAT1) is a member of the distinct anion transporter family SLC6 (EVERETT and GREEN 1999). PAT1 has been identified and mapped in human by LOHI et al. (2000). The authors suggested that PAT1 participates in the anion exchange in the pancreatic duct and kidney. Thereafter, PAT1 has been detected in a wide range of tissues. PAT1 is expressed in the brush border membrane of proximal tubular cells of the kidney (KNAUF et al. 2001; WANG et al. 2002), the tubulo-vesicular membranes of the stomach, the apical membrane of the duodenum and colon (PETROVIC et al. 2002; WANG et al. 2002), the apical membrane of the pancreatic duct (GREELEY et al. 2001; KO et al. 2002; ISHIGURO et al. 2007), and the plasma membrane of the ventricular myocytes (ALVAREZ et al. 2004). In most of the studies, PAT1 was localized apically. Furthermore, it has been demonstrated in intact ruminal epithelial cells at mRNA level (BILK et al. 2005).

With regards to the transport properties, human and murine PAT1 were shown to mediate electrically silent Cl^-/HCO_3^- and Cl^-/HO^- exchange (KO et al. 2002). In contrast, PAT1 was shown to mediate electrogenic Cl^-/ oxalate exchange in the mouse oocytes (CHERNOVA et al. 2005). A study concerning Cl^-/HCO_3^- stoichiometry of the transporter, proposed that PAT1 mediates 2 Cl^- for 1 HCO_3^- (SHCHEYNIKOV et al. 2006). A study by KNAUF et al. (2001) revealed that PAT1 is the main candidate for the apical Cl^- / formate exchange in the proximal tubules of the murine kidney. Therefore, PAT1 was known in the mouse as Cl^- / formate exchanger (CFEX) or (Slc26ac). Moreover, PAT1 functions as a Cl^- / oxalate, Cl^- / formate, Cl^- / HCO_3^-, Cl^- / HO^-, Cl^- / SO_4^{2-}, SO_4^{2-} / oxalate and oxalate / formate exchanger in oocytes expressing PAT1(JIANG et al. 2002; KO et al. 2002; WANG et al. 2002; XIE et al. 2002).

Concerning the gastro-intestinal tract epithelium, WANG et al. (2002) reported that PAT1 is located in the mouse duodenum particularly in the membrane of the villus brush border. The authors pointed out that PAT1 is the main protein mediating the apical Cl^- / HCO_3^- exchange in the duodenum in order to protect the epithelium against the harmful effects of the acid. Thereafter, WANG et al. (2005) confirmed the importance of PAT1 in Cl^- / HCO_3^- exchange by using SlC26a6-knockout mice which showed a significant decrease in Cl^- / HCO_3^- exchange activity in the duodenum. On the other hand, concerning the role of PAT1 in SCFA transport, NOZAWA et al. (2004) described PAT1 as a candidate of SCFA transporting proteins in the small intestine. The authors detected an uptake of acetate, propionate and butyrate in PAT1-transfected HEK-293 cells. Moreover, a functional study on the distal ileum of mice by FREEL et al. (2006) proved that PAT1 mediates the efflux of oxalate in the ileum. The same result was achieved in the duodenum by JIANG et al. (2006). Results from knockout mice experiments by TUO et al. (2006) suggested that PAT1 activity for the secretion of HCO_3^- is activated by prostaglandin E2 (PGE2) through Ca^{2+}-dependent pathway and not by cyclic adenosine monophosphate (cAMP)-dependent pathway. SIMPSON et al. (2007) confirmed that PAT1 is the main candidate for Cl^- / HCO_3^- and SO_4^{2-} / HCO_3^- exchanges in the apical membrane of murine duodenal villus epithelium. Moreover, the authors revealed that PAT1 regulates the intra-cellular pH (pHi) in the murine duodenal epithelium in combination with Na+ / H+ exchanger 1 (NHE1). On the other hand, WALKER et al. (2010) suggested that all previous studies for the role of PAT1 as a mediator for Cl^- / HCO_3^- exchange had ignored the interference of glucose transporters which could inhibit the activity of the PAT1 in the villus epithelium of duodenal mucosa. Since membrane depolarization or cell acidification during glucose transport may

obscure PAT1 activity. Concerning ruminant forestomach, BILK et al. (2005) suggested a role for PAT1 in the transport of SCFA across the ruminal epithelium.

PAT1 has been reported in several studies as a DIDS sensitive transporter (JIANG et al. 2002; WANG et al. 2002; PETROVIC et al. 2002; FREEL et al. 2006). Furthermore, transport of oxalate by human PAT1 has been inhibited by niflumic acid and tenidap (CHERNOVA et al. 2005).

2.4.3.2 Down-regulated in adenoma (DRA = SLC26A3)

Down-regulated in adenoma (DRA) is also a member of the SLC26 family which is distributed widely in the body of animal and human (EVERETT and GREEN 1999; KEREM et al. 1989). The DRA gene has been isolated and identified for the first time by SCHWEINFEST et al. (1993) at mRNA level in the mucosa of the human colon. It is called DRA because its expression was decreased in case of adenomas and adenocarcinomas of the colon (SCHWEINFEST et al. 1993). Thereafter, DRA has been located in the columnar cells of the colon mucosal epithelium in the mouse (BYEON et al. 1996). A study by JACOB et al. (2002) revealed that DRA is localized apically in the brush border of the colon and duodenum in human, rat and rabbit. DRA has also been expressed in other membranes such as the apical domain of the pancreatic duct cells (GREELEY et al. 2001), apical membrane of the trachea epithelium (WHEAT et al. 2000), and in the epithelia of the eccrine sweat glands and seminal vesicles (HAILA et al. 2000).

Concerning the properties of the transporter, a study by KO et al. (2002) in oocytes and HEK-293 cells postulated that DRA is an electrogenic transporter that exchanges two Cl^- for one HCO_3^-. In contrast, MELVIN et al. (1999) in their study on mice colonic epithelium have showed that DRA is an electroneutral Cl^- / HCO_3^- exchanger. This finding was confirmed by LAMPRECHT et al. (2005) in DRA-transfected HEK-293 cells. Furthermore, DRA is considered mainly as Cl^- / HCO_3^- exchanger which also mediates SO_4^{2-} / HCO_3^- exchange (SILBERG et al. 1995; BYEON et al. 1998; MELVIN et al. 1999; KO et al. 2002; WHITTAMORE et al. 2013). DRA has also been reported to transport hydroxide and nitrate. A study by MOSELEY et al. (1999) in Xenopus oocytes pointed out that DRA mediates Cl^- / OH^- exchange. This finding was supported by ALREFAI et al. (2001) study on Caco-2 cell line as a model for human intestine, which indicated DRA ability to mediate SO_4^{2-} / OH^- exchange. Furthermore, STERLING et al. (2002) reported Cl^- / NO_3^- activity for DRA in transfected HEK 293 cells. Concerning the possible role of DRA in SCFA

transepithelial transport, VIDYASAGAR et al. (2004) suggested that DRA could contribute in the SCFA transport across the epithelium of rat distal colon.

With respect to the inhibitor sensitivity of DRA, it has been described as DIDS sensitive (SILBERG et al. 1995; ALREFAI et al. 2001; JACOB et al. 2002; WHITTAMORE et al. 2013).

2.4.3.3 Anion exchanger 2 (AE2 = SLC4A2)

Anion exchanger (AE2) or the Na^+-independent Cl^- / HCO_3^- exchanger belongs to the SLC4 gene family which mediates electroneutral exchange of monovalent anions. The main substrates are Cl^- and HCO_3^- (ALPER et al. 2002; ROMERO et al. 2004; ROMERO et al. 2013). It has been initially cloned by DEMUTH et al. (1986) from human kidney and lymphoma cells. AE2 has three isomers that differ mainly in their cytoplasmic N terminal but they have the same transport characteristics to exchange extracellular Cl^- by an intracellular HCO_3^- (ROMERO et al. 2013). Among the AEs, AE2 is the most widely distributed one; it is expressed in gastric parietal cells (STUART-TILLEY et al. 1994), renal collecting duct epithelial cells (ALPER et al. 1997; STUART-TILLEY et al. 1998), choroid-plexus epithelial cells (ALPER et al. 1994) and colonic enterocytes (ALPER et al. 1999). In these studies, it has been mentioned that AE2 is expressed mostly in the basolateral membrane of the studied cells.

AE2 has been proposed to regulate mainly Cl^- / HCO_3^- exchange. However, a study by STUART-TILLEY et al. (1994) showed that AE2 plays a role in the secretion of H^+ in the gastric parietal cells by exporting HCO_3^- into the blood so as to balance H^+ pumped into the lumen of gastric gland. Furthermore, ALPER et al. (1994) suggested that AE2 could participate in the transepithelial flux of Na^+ and Cl^- across the choroid-plexus epithelial cells. It has also been suggested that AE2 could play a role in the regulation of intracellular pH (JIANG et al. 1994). A study by STEWART et al. (2002) in Xenopus oocytes using $^{36}Cl^-$ efflux technique confirms that AE2 has an influence on both intracellular and extracelluler pH. Moreover, ROMERO et al. (2013) postulated that AE2 could also regulate the cell volume by taking up Cl^-.

Concerning AE inhibitors, it has been reported that all AEs are functionally blocked by stilbene derivatives like SITS and DIDS (BARZILAY et al. 1979; CABANTCHIK and GREGER 1992; ROMERO 2004). However, GARCIA et al. (1997) noticed that DIDS did not provide full blocking features for AE2.

2.5 Literature implications for this study

The previous sections indicated the possible existence of protein mediated transport mechanisms for the extrusion of SCFA and their metabolites across the basolateral membrane of the rumen epithelium. Up to date, the proton-coupled MCT1 transporter is the only transport protein which has been reported to be expressed in the basolateral membrane of the rumen epithelium. In addition to that, there are some hints that MCT1 could function as a SCFA/HCO_3^- exchanger in the rumen epithelium. Nevertheless, the incomplete inhibition of SCFA transport across the basolateral membrane by MCT1 blockers suggests the existence of other transport mechanism(s). Expected possible candidates for such pathway are the anion exchangers PAT1 and DRA which have been detected at mRNA level in cultured ruminal epithelial cells as well as in intact rumen epithelium. Therefore, the aim of this study is to investigate the role of anion exchanger proteins in the basolateral extrusion of SCFA and/or their metabolites in ovine ruminal epithelium. The following questions are addressed by using Ussing chamber technique and immunohistochemistry:

1. Is there any functional involvement of PAT1 or DRA in the basolateral efflux of SCFA and/or their metabolites across the basolateral membrane of ovine ruminal epithelial cells?
2. Are these proteins located in the basolateral membrane of ovine ruminal epithelial cells?

3 Materials and Methods

3.1 Animals

Adult Merino breed sheep (1 to 6 years old) were used in the experiments. The sheep were obtained from Oberholz teaching and experimental farm affiliated to the Faculty of Veterinary Medicine – Leipzig. Sheep were kept at least for two weeks prior to the experiments in the stable of the institute of veterinary physiology, University of Leipzig. During which sheep were fed on hay ad libitum, and had free access to clean fresh water. Slaughtering of the animals was carried out in the Institute of Anatomy, University of Leipzig. Where, Sheep were stunned by captive bolt pistol and killed by exsanguination. Thereafter, the abdominal cavity was opened and the ventral ruminal sac (approximately 300 cm^2) was removed. The experiments were performed according to the German legislation on the protection of animals and were reported to the State Directorate - Leipzig under the file number T 88/13.

3.2 Ussing chamber studies

Ussing chamber technique is an *in vitro* technique that developed by Hans Ussing (USSING and ZERHAN. 1951). Later on the technique was adapted to fit with forestomach epithelial tissues (FERREIRA et al. 1966). Since then, the technique has been used to measure the flux of numerous molecules across the rumen epithelium, such as: chloride (GÄBEL et al. 1991b), sodium (LODEMANN and MARTENS 2006), magnesium (LEONARD-MAREK et al. 1998), urea (ABDOUN et al. 2010) and SCFA (ASCHENBACH et al. 2009; DENGLER et al. 2014).

3.2.1 Buffer solutions

For cleaning, preparation and transport of the epithelium, basic buffer was used (mM: 115 NaCl, 5.5 KCl, 1.25 $CaCl_2$,1.25 $MgCl_2$, 0.6 NaH_2PO_4, 2.4 Na_2HPO_4, 1 L-glutamine, 10 glucose, 10 HEPES-free acid and 13 mannitol; gassed with oxygen). For all experiments, gluconate containing buffer (gluconate buffer) was applied to the hot side (mM: 110 Na-gluconate, 5.5 K-gluconate, 2 Ca-gluconate, 2 Mg-gluconate, 1.5 NaH_2PO_4, 1.5 Na_2HPO_4, 1 L-glutamine, 10 glucose, 10 HEPES-free acid, 10 Na-acetate or 10 Na-butyrate, gassed with oxygen). Different types of buffers were used on the cold side according to the type of

the experiment. The composition of these buffer solutions is stated in the respective sections. The pH of the buffer solutions was adjusted to 7.4 by using HCl, gluconic acid or NaOH. Ethoxyzolamide was used to suppress carbonic anhydrase except in the buffer solutions containing $HCO3^-$. Mannitol was used to adjust the osmolarity of the buffer solutions to 288 ± 5 mOsmol. All chemicals were obtained from Sigma Aldrich/Fluka, Carl Roth Germany, VWR Germany or Merck Schuchardt OHG Germany. The gas was obtained from Linde Gas Germany.

3.2.2 Preparation of ruminal epithelium

After dissection of the ventral ruminal sac, the contents of the sac was emptied and the tissue was rinsed for several times with warm (38 °C) O_2-gassed basic buffer solution until the solution remained clear. Thereafter, the serosa and the muscle layers were manually removed (stripped) from mucosa. This action was performed because the submucosa is composed of collagen and cellular elements such as fibroblasts which; 1) acts as a structural diffusion barrier for isotope molecules, 2) reduces the short circuit current conditions that will be applied to the Ussing chamber apparatus, and 3) alters the unidirectional movement of molecules (BINDER and RAWLINS 1973; CLARKE. 2009). Moreover, the removal of mucosal layer reduces the effect of intrinsic neuromuscular system (CLARKE. 2009). The term ´´epithelium`` is used throughout this study for the tissue obtained from this stripping procedure. The epithelium was then transferred to laboratory in warmed (38 °C) O_2-gassed basic buffer solution. In the laboratory, the epithelium was cut into squares of 4 X 4 cm and mounted in Ussing chambers as described by GÄBEL et al. (1991).

3.2.3 Incubation

Twenty four Ussing chambers are available in the laboratory of the Institute of veterinary Physiology, University of Leipzig. A chamber consists of two equal halves (Fig. 2); between them the epithelium has to be mounted. Thus, dividing the chamber into two equal compartments, one which represents the blood side (serosal or basolateral side) and the other represents lumen side (mucosal or apical side). The rumen epithelium was mounted in the chamber such that the tissue matches the serosal and mucosal side appropriately. Small holes were cut in the tissue to fit over pins attached to the chamber, in order to secure the tissue in the centre of the two halves of the chamber. The area exposed to the buffer solutions was 3.14 cm^2. Silicon rings were placed on both sides of

the epithelium to minimize edge damage. Each side of the Ussing chamber is connected to a reservoir that contains a buffer solution with specific component to meet essential requirements and condition of the experiment. Furthermore, the buffer is heated by a water jacket surrounding the buffer solution, which maintains the temperature of the buffer and tissue at physiological temperature (38 °C). The buffer solution is gassed with either carbogen (95% O2, 5% CO2) or oxygen, pending on the aim of the measurement. Under bicarbonate-free conditions, oxygen gassing is used in order to prevent exogenous production of bicarbonate and maintaining pH at the appropriate level (CLARKE. 2009). Gassing also allows mixing of the buffer solution (LI et al. 2004). Carbogen gas helps to overcome the lack of hemoglobin that would otherwise be supplied by blood in vivo (CLARKE. 2009).

3.2.4 Electrophysiological parameters

Electrophysiological data were recorded using a computer-controlled measuring and computing unit (Voltage-clamp system, Engineering Office for Measurement and Data Technology, Mußler, Aachen, Germany). The transepithelial potential difference (PD_t) was measured using two AgCl half-cells and a millivoltmeter connected to the mucosal and serosal side of the chambers by agar-gel bridges filled with KCl-agar. Inside the chamber, the ends of the voltage bridges are located close to the tissue on both the serosal and mucosal side. The self-potential and the inherent resistance of the system were determined before the experiment and used as a correction factor for the data obtained during the experiments. The open circuit mode was used for the calibration and preparation of the technique. While, all experiments were carried out under short-circuit conditions, where the transepithelial potential difference (PD_t) was clamped to 0 mV. Under such condition (short-circuit condition) there is no electrogenic driving force available for passive and paracellular transport. The transepithelial conductance (G_t) was determined by a computer- controlled device connected to the chambers via pairs of agar-gel NaCl electrodes. The tissue conductance (G_t) was corrected for the conductance of the buffer solution, measured before mounting the epithelia. PD_t and short circuit current (I_{sc}), were recorded throughout the experiments in 6 seconds intervals.

Open-circuit

In this technique the potential difference remained unaffected (unchanged). To calculate the tissue conductance (G_t) and the short circuit current (I_{sc}), currents of 100 µA/second, in

alternating direction were applied across to the tissue. The tissue conductance (G_t) was determined by changes of the PD_t according to Ohm's law ($R = \Delta PD_t / \Delta I$).

Short circuit current (I_{sc})

This technique is a particular form of the voltage-clamp technique. In which, the transepithelial potential difference (PD_t) is clamped to 0 mV through application of an external current. The current equals net charge transfer because it has the same value but is oppositely directed. Under these conditions, the short-circuit current (I_{sc}) is equivalent to the sum of all electrogenic ions movement across the epithelial tissue. LI et al. (2004) described I_{sc} as the sum of all anion and cation flow during the time that PD_t is short-circuited.

The voltage-clamp technique enables the measurement of the electrical parameters (PD_t, G_t and I_{sc}), where G_t is the reciprocal value of the tissue resistance (R_t). The potential difference (PD_t) was directly measured, the tissue resistance (R_t) or the tissue conductance (G_t) were calculated according to Ohm's law. Therefore, a current of known strength and duration should be applied across to the epithelium tissue. The calculation based on the change in PD_t caused by this current. By the help of this technique, and through the application of an external current, the transepithelial PD_t can be clamped to a certain value, so that the electrophysiological conditions for the ions transport could be changed.

3.3 Experimental procedure

All experiments were conducted under short-circuit conditions. The experiment was started after an equilibration period with the respective puffer solutions for about 30 minutes. Only the epithelial tissues with a transepithelial conductance (G_t) of not more than 5 mS/cm^2 were used in the experiments. G_t above this value indicates the damage of the ruminal tissue and the respective epithelia were excluded from the experiment (CLARKE. 2009). In the current experiments, epithelial tissues with approximately similar G_t values were paired in an experimental unit, where up to 25% difference in the electrophysiological values between the two epithelial tissues in one pair was accepted.

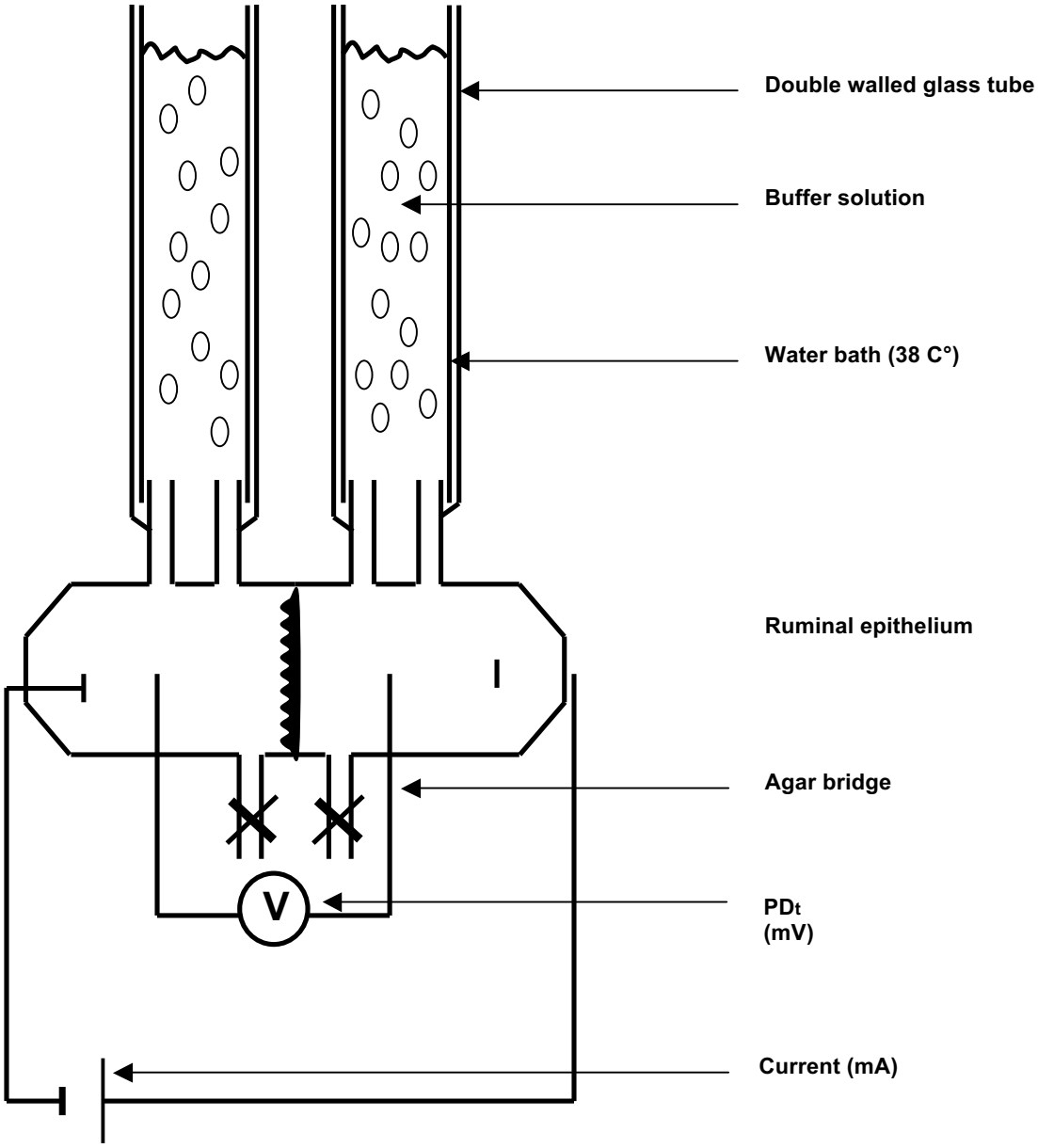

Fig 2: Ussing chamber model

The description of the model is described in section 3.2.3.

3.3.1 Determination of the unidirectional SCFA flux rate

To measure the unidirectional flux rates of acetate (ac) or butyrate (bu), the respective substrate labelled with the radioactive isotope ^{14}C was added to mucosal (mucosal to serosal flux; J_{ms}) or serosal buffer solution (serosal to mucosal flux; J_{sm}). After the equilibration period (30 minute), the labelled substrate was added to the respective side (hot side). Radioactivity was measured in a liquid scintillation counter (Liquid Scintillation Analyser Tri Carb 2818 TR, Perkin Elmer Inc., USA). Radioactivity was measured in decays per minute (dpm) after adding scintillator to the samples (Aquasafe 300; Fa. Zinsser Analytic, Frankfurt, Germany). In one tissue, the flux measurement is always unidirectional. Samples of 800 µl were taken from the cold side every 30 min. The volume of the cold sample was replaced by the same amount of the respective buffer solution. Meanwhile, 100 µl samples were taken from the hot side at the beginning and the end of the experiment, where their averaged dpm were used to calculate the specific activity used on the hot side. Fluxes were calculated by using simple ratio equation as follows:

$$J^{S}_{unidir} = \frac{\frac{V_{g(n)}}{V_c} * dpm_n - \left(\frac{V_g}{V_c} * dpm_{(n-1)} - dpm_{(n-1)} \right)}{\frac{dpm_h}{S} * \Delta t * A}$$

J^{S}_{unidir}	=	Unidirectional flux rate of the substance	(µmol·cm^{-2}·h^{-1})
$V_{g\,(n)}$	=	Total volume of the buffer on cold side	(15 ml)
V_C	=	Volume of the cold sample	(0.8 ml)
dpm_n	=	Activity in the cold sample at time point	
dpm_h	=	Activity in the hot sample	
		(Average of the two samples taken at the beginning and the end of the experiment)	
S	=	Volume of the hot sample	(0.1 ml)
Δt	=	Duration of the flux period	(0.5 h)
A	=	Exposed epithelial area	(3.14 cm^2)

Regarding the appearance of the labelled ac or bu in the unlabelled side, the calculation includes both original ac or bu and their metabolites.

3.4 Experimental setups

3.4.1 Sensitivity of J_{sm}^{ac} to inhibitors

3.4.1.1 Effect of nitrate and pHMB on J_{sm}^{ac}

The study conducted by DENGLER et al. (2014) suggested that there is more than one anion exchange mechanism involved in the extrusion of SCFA or their metabolites across the basolateral membrane of the ruminal epithelium. In this experimental setup, we switched the direction of the flux to J_{sm} (Normally *in vivo* the flux is J_{ms}) so that we can manipulate the transport properties of the basolateral membrane which is necessary to understand the mechanism(s) of SCFA transport. This experimental setup examined the role of anion exchanger proteins and MCT1 in the transport of SCFA. In order to examine the role of anion exchangers in the permeation of acetate across the basolateral membrane of ruminal epithelium, J_{sm} of acetate was measured for two flux periods of 30 min each, in the presence or absence of anion exchanger inhibitors and MCT1 inhibitors.

Gluconate buffer was applied on the serosal side (hot side) where Cl^- was omitted. While, chloride containing buffer was used on the mucosal side (cold side) (mM: 110 NaCl, 5.5 KCl, 2 $CaCl_2$, 2 $MgCl_2$, 1.5 NaH_2PO_4, 1.5 Na_2HPO_4, 1 L-glutamine, 10 glucose and 10 HEPES-free acid). So that chloride wouldn't act as competitor for SCFA permeation, but was available as a counter-anion for anion exchange. After allowing the epithelia to equilibrate for 30 min, ^{14}C labelled acetate was added on the serosal side (hot side) (final activity in the incubation solution: 3.3 kBq / ml). Thereafter, NO_3^- (47 mM) was applied on the serosal side as non-specific inhibitor of anion exchange by adding 2 ml of 400mM stock solution (added to 15 ml buffer solution). The added NO_3^- slightly changed the osmolality on the serosal side. To correct for this alteration in the osmolality, same amount of Na-gluconate was added on the mucosal side and led to the same change in the osmolality. Na-gluconate was also added in the same amount and final concentration on both sides of the control group.

For the other experimental group, p-hydroxymercuribenzoic acid (pHMB) as MCT1-inhibitor) was added (1.6 mM) on the serosal side. Control group was left without adding any inhibitor to differentiate between MCT1 and anion exchange mediated transport. Epithelia were incubated with either NO_3^- or MCT1-inhibitors alone for one hour. Thereafter, for the second incubation period, a combinatory inhibitory effect was measured by adding MCT1-inhibitor to epithelia pre-incubated with NO_3^- and vice versa for another hour (Table 1). Samples were taken from the mucosal side (cold side) at 30 minutes

intervals, and from the serosal side (hot side) at the beginning and the end of the experiment for the measurement of the J_{sm}^{ac}.

Table 1: The contents of the serosal buffer during first and second flux period

First flux period **(0-60 min)**	Na-gluconate	Na-gluconate	NO_3^-	−	pHMB	pHMB	NO_3^-
Second flux period **(90-150 min)**	−	NO_3^-	Na-gluconate	pHMB	−	NO_3^-	pHMB

3.4.1.2 Effect of DIDS, NO_3^- and pHMB on J_{sm}^{ac}

The influence of DIDS on the anion exchange proteins and MCT1 has been described previously (see sections 2.4.1 and 2.4.3). Therefore, we investigated the effect of DIDS on the basolateral extrusion of acetate. To achieve this, the J_{sm} of ac were measured again during two fluxes periods. In this experiment, the same buffers solutions as in the previous experiment were used. After addition of ^{14}C labelled ac to the serosal side, either DIDS (1 mM) as an inhibitor of anion exchangers or NO_3^- (50 mM) as non-specific inhibitor of anion transport or pHMB as MCT1 inhibitor were added. Each inhibitor was applied individually, and the J_{sm} ac was measured for one hour for the first flux period. Thereafter, the inhibitors were combined by adding each inhibitor to epithelia pre-incubated with another inhibitor during the first flux period. Sodium gluconate was applied on the mucosal side in case of NO_3^- addition on the serosal side to balance differences in osmolarity due to addition of NO_3^-. Samples were taken from both sides during two hours and the J_{sm} ac was determined as describe above.

3.4.2 Effect of the basolateral replacement of the anions on the extrusion of SCFA

3.4.2.1 Effect of Cl^- and NO_3^- on J_{ms} of acetate and butyrate

As mentioned before (see section 2.2.2) both anions (Cl^- and NO_3^-) have been used to compete with SCFA for the transport across the ruminal epithelium in the same direction. Based on that, this experiment was designed to investigate whether the presence of either Cl^- or NO_3^- in the basolateral buffer solution could act as counter anions for exchange with SCFA and thus enhance $J_{ms}^{ac,\ bu}$. To achieve this, 24 chambers were divided into three groups. Gluconate containing buffer was applied on the mucosal side (hot side) of all experimental groups, and the serosal side of the control group. Whereas in the other two groups, 50 mM of sodium gluconate on the serosal side was substituted by the same amount of either NaCl or $NaNO_3$. All buffer solutions were gassed with oxygen. Thereafter, labelled ac or bu was added to the mucosal side (hot side), and $J_{ms}^{ac\ or\ bu}$ was measured for one hour (first flux period (0 – 60 min). Then, the buffer solutions of the serosal sides were drained and replaced by gluconate buffer in all experimental groups. After 30 min of equilibration, $J_{ms}^{ac\ or\ bu}$ was measured for another hour (second flux period (90 – 150 min)). The same setup was conducted in other chambers but we added 50 mM of sodium gluconate in the serosal side in the first hour and then replaced by buffer containing either 50 mM of NaCl or $NaNO_3$. The results were presented as the difference between first and second flux period (ΔJ_{ms}) = ($J_{ms}^{90-150\ min} - J_{ms}^{0-60\ min}$). The results were calculated for each group and compared to the ΔJ_{ms} of the control group.

3.4.2.2 Effect of SO_4^{2-} on J_{ms} of acetate and butyrate

Based on the results obtained from the effect of Cl^- and NO_3^- as substrate for exchange with SCFA, we planned to characterize the properties of the putative candidate transporting protein. The affinity of sulphate to be used as substrate by PAT1 has been described in previous chapter (see section 2.4.3). Accordingly, the same protocol as in the previous experiment was conducted to functionally characterize PAT1. Gluconate containing buffer was added to the mucosal side (hot side) of all experimental groups, and the serosal side of the control group. While, 50 mM of sodium gluconate on the serosal side was replaced by the same amount of Na_2SO_4 in the treatment group. Osmolality of the buffer solutions was adjusted to 288 ± 5 mOsmol. The flux rate was measured for one

hour {first flux period (0 – 60 min)} after adding labelled ac and bu in the mucosal side. In the second flux period, the buffer solutions on the serosal side were replaced by gluconate containing buffer solution and the flux rate was measured for another hour {second flux period (90 – 150 min)}. The results were presented as a reduction of J_{ms} (ΔJ_{ms}) ($J_{ms}^{90-150\ min} - J_{ms}^{0-60\ min}$) by anion replacement in the sulphate group compared to the reduction occurred in the control group.

3.4.3 Effect of different anions available in the serosal solution on J_{ms} of acetate and butyrate

Based on the results obtained from the previous experiments, we planned to compare the affinity of different anions to be used as a substrate by PAT1, without changing the buffer on the serosal side. To achieve this, the epithelia were divided into four experimental groups. All groups were incubated with gluconate containing buffer solution on the mucosal side (hot side). The serosal side (cold side), received the same gluconate buffer solution for the control group. Whereas, 50 mM of Na-gluconate in the serosal side buffer was replaced by 50 mM of either Cl^-, NO_3^- or SO_4^{2-}, respectively in the treatment groups. The buffers in all groups were gassed with O_2 (Table 2). Labelled acetate or butyrate was applied on the mucosal side after the equilibration period. Thereafter, samples were taken every 30 min from both sides for three hours, and J_{ms} of acetate or butyrate was calculated as stated above.

3.5 Immunohistochemistry

3.5.1 Preparation of the samples

The immunohistochemical studies were performed on frozen sections of ruminal epithelium. After killing of the sheep, tissue pieces from the ventral ruminal sac were removed and washed in phosphate-buffered saline (PBS; 0.1 M phosphate, 0.88% NaCl). Then, they were cut into 0.5 x 0.5 cm pieces, transferred to a PBS / NaN_3 buffer solution containing 30% sucrose and stored overnight at 4 °C. Sucrose was used to prevent crystal formation during freezing (TOKUYASU and SINGER 1976). Thereafter, tissue samples were embedded in Tissue Tek O.C.T. Compound (Fa. Sakura via Fa. Vogel GmbH & Co KG SA 4583) overnight at -20 °C. The preparation of cryostat sections was performed by cutting the tissue cubes on a freezing microtome (CM1850, Leica, Germany). The thickness of sections was 10 µm. The sections were placed on poly-L-lysine coated slides. Then, the slides were stored at -80 °C until further processing.

3.5.2 Fixation and staining of the samples

Cryostat sections were fixed using HOPE® (Hepes-glutamic acid buffer mediated Organic solvent Protection Effect). In particular, sections were incubated with HOPE I solution for 5 min, then with HOPEII solution for 5 min. Thereafter, sections were washed and incubated for 60 min in PBS / NaN_3-HS-TritonX100 solution (96% PBS / NaN_3, 4% horse serum (HS), 0.1% TritonX100) in a humid chamber at room temperature. The detergent (Triton X-100) (93443, Sigma, Germany) was used to increase the permeability of the cell membrane to allow binding between the antibody and intracellularly localized antigens. Horse serum was used to saturate non-specific antibody binding and to minimize non-specific background of the immunohistochemical staining. After preparation, sections were washed three-times for 10 min each with PBS solution. Then the sections were incubated with primary antibodies overnight at room temperature. Rabbit-anti-DRA antibody (ABIN 404755, antibodies-online, Aachen, Germany) and rabbit-anti-PAT1 antibody (ABIN 971716, antibodies-online, Aachen, Germany) were used in a dilution of 1:500 in PBS / NaN_3-HS-TritonX100 solution. Secondary antibody was anti-rabbit IgG raised in donkey and conjugated to Cyanine dyes 3 (Cy3) in dilution of 1:500. Sections were washed again three times with PBS for 10 min. To differentiate between epithelium and subepithelial layers, nuclei were stained using 4', 6-diamidino-2-phenylindole (DAPI). After a further

washing with PBS for 10 min, sections were covered in a solution of PBS / NaN_3 and 80% glycerol. Subsequently, the preparations were stored protected from light at 4 °C, in order to avoid fading of the fluorescence intensity of the fluorochrome, until analysis.

Negative controls (without primary antibodies) were performed to demonstrate the specificity of the immunohistochemical staining. Additionally, specificity of the primary antibodies was further confirmed by incubating the primary antibodies prior to staining for 24 hours with the following blocking peptides in a dilution of 1:250 in PBS / NaN_3-HS-TritonX100 solution: in case of DRA ABIN 983932 (Antibodies-online, Aachen, Germany), and in case of PAT1 ABIN 983935, (Antibodies-online, Aachen, Germany). After the incubation of the sections with the antibody/peptide solution, the cryostat sections were washed 3 times for 10 minutes each in PBS. Then the preparations were incubated in secondary antibody diluted in a PBS / NaN_3-HS-TritonX100 solution for 2.5 hours protected from light in a humid chamber.

3.5.3 Evaluation

The evaluation of the staining and expression of the proteins was performed using an inverted fluorescence microscope (IX50, Olympus GmbH, Japan) (Japan Cell ^ F 2.6, Olympus Soft Imaging Solutions GmbH). This epifluorescence microscope was equipped with a digital camera and a computer-based image analysis system (F-view; Olympus GmbH). Cy3 labelled antibodies were detected with a wavelength of 530-560 nm (Emission filter HQ5450/30x). DAPI has a wavelength from 330 to 385 nm (Excitation filter BP330-385). For every preparation, three fluorescence images were taken. For all fluorescence images, the magnification used was 200-fold and the exposure time was 200 ms.

3.6 Statistical analysis

Evaluation and display of data was carried out using SigmaPlot 11.0 software (Systat Software Inc., USA). Results are given as means ± SEM (standard error of mean). N represents the number of animals used, and n represents the number of epithelia per treatment. The data of each treatment (n) were pooled for each animal (N) for the statistical analysis. To determine significant differences between two groups, paired student's *t*-test (normal distribution) or Mann-Whitney rank Test (no normal distribution) were used. One way ANOVA with subsequent Holm-Sidak-Test was conducted to compare many groups. Results are assigned as significantly different at $P < 0.05$.

4 Results

4.1 Inhibitor sensitivity

4.1.1 Effect of nitrate and pHMB on J_{sm}^{ac}

In this experiment, J_{sm}^{ac} was initially measured in the presence or absence of either NO_3^- or pHMB in the serosal solution. Then, NO_3^- was added to the serosal solution of chambers pre-incubated with pHMB and vice versa. Thereafter, J_{sm}^{ac} was measured for a second flux period. In the first flux period, the presence of NO_3^- (50 mM) reduced J_{sm}^{ac} significantly by 57 ± 9% compared to control (Fig. 3). Likewise, presence of pHMB (1.6 mM) reduced J_{sm}^{ac} significantly by 51 ± 5% compared to control (Fig. 3).

With regards to the second flux period, the control group showed a slight, but not significant reduction in J_{sm}^{ac} compared to the first flux period (-20 ± 4% of first flux period; Fig. 4). When NO_3^- was added alone in the second flux period, a significant reduction in the J_{sm}^{ac} was detected (-60 ± 3% of first flux period). When NO_3^- applied to epithelia pre-incubated with pHMB, an additional decrease of J_{sm}^{ac} was observed (-66 ± 2% of first flux period) as shown in Fig. 4. Subsequently we tested the effect of pHMB on the epithelia pre-incubated with NO_3^- (Fig. 5). In epithelia pre-incubated with NO_3^-, the addition of pHMB reduced J_{sm}^{ac} by -39 ± 10% of first flux period.

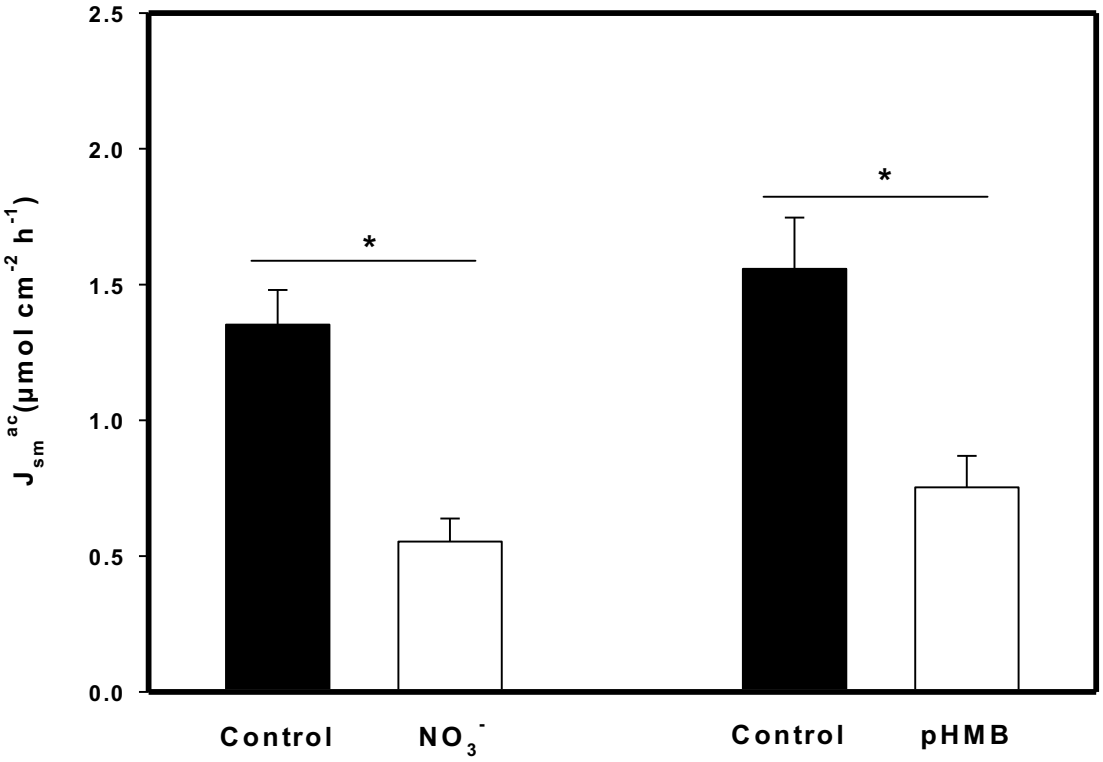

Fig 3: Effect of NO_3^- and pHMB on J_{sm}^{ac}

Epithelia were incubated in the presence or absence of either 50 mM NO_3^- or 1.6 mM pHMB in the serosal solution for one hour. Asterisk indicates a significant difference between the values after adding the inhibitors ($p < 0.05$; paired t-test). Data represent means ± SEM; statistics was made on basis of N = 5 (n = 10) for each treatment.

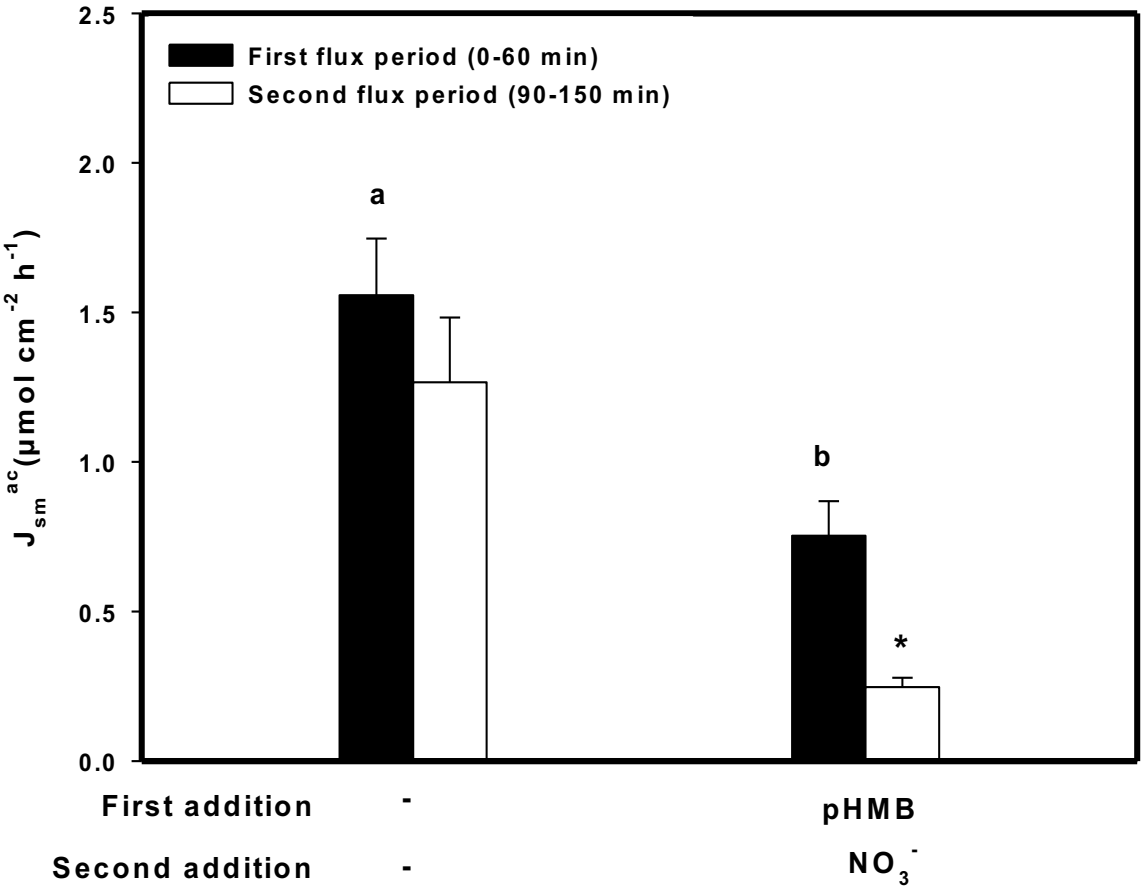

Fig 4: Inhibitory effect of NO_3^- and pHMB applied in combination on J_{sm}^{ac}

J_{sm}^{ac} was first measured for one hour in the presence or absence of 1.6 mM pHMB (first row of X-axes labels; black bars). Thereafter, nitrate (second row of X-axes labels; white bars) was added to the serosal side preincubated with pHMB. J_{sm}^{ac} was measured for another hour (second flux period 90-150 min). Different letters mark significant differences between the values of the first flux period ($p < 0.05$; paired t-test); Asterisk indicates significant differences between the fluxes of the first and second flux period ($p < 0.05$; paired t-test). Data represent means ± SEM; statistic was made on basis of N = 5 (n = 10) for each treatment.

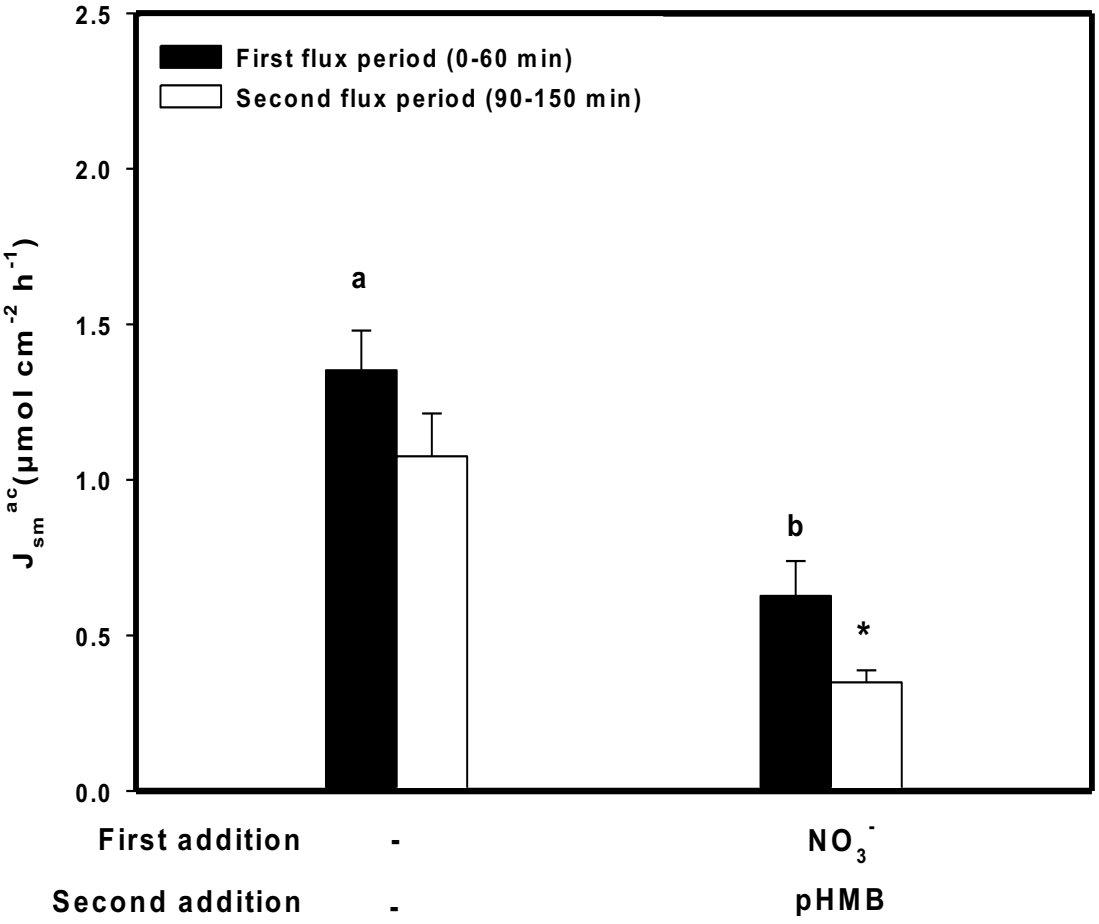

Fig 5: Inhibitory effect of pHMB and NO_3^- applied in combination on J_{sm}^{ac}

J_{sm}^{ac} was first measured for one hour in the presence or absence of 50 mM NO_3^- (first row of X-axes labels; black bars). Thereafter, pHMB (second row of X-axes labels; white bars) was added to the serosal side preincubated with NO_3^-. J_{sm}^{ac} was measured for another hour (second flux period 90-150 min). Different letters mark significant differences between the values of the first flux period ($p < 0.05$; paired t-test); Asterisk indicates significant differences between the fluxes of the first and second flux period ($p < 0.05$; paired t-test). Data represent means ± SEM; statistic was made on basis of N = 5 (n = 10) for each treatment.

4.1.2 Effect of DIDS, pHMB and NO_3^- on J_{sm}^{ac}

In these experiments, the effects of DIDS alone or in a combination with pHMB and NO_3^- on J_{sm}^{ac} were investigated. The same approach as in the previous experiment was conducted. Addition of 1 mM of DIDS alone had no effect on J_{sm}^{ac} in comparison to control group (Fig. 6).

Moreover, DIDS had no additional effect on J_{sm}^{ac} in epithelia pre-incubated with NO_3^-. Also, when DIDS was applied to epithelia pre-incubated with pHMB, there was no additional reduction of J_{sm}^{ac} (Fig. 6). However, when NO_3^- or pHMB was applied to epithelia pre-incubated with DIDS, a significant reduction in J_{sm}^{ac} was observed (Fig. 6).

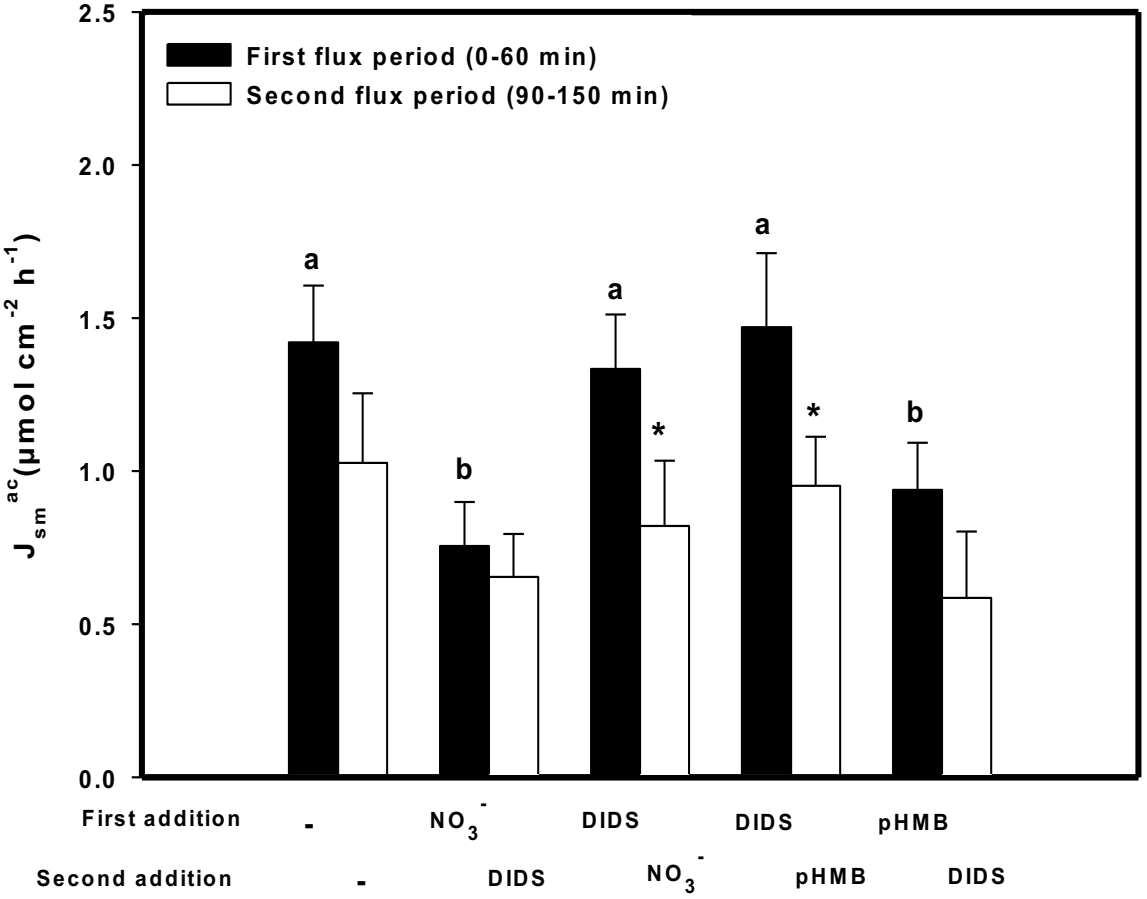

Fig 6: Inhibitory effect of DIDS, pHMB and NO_3^- applied in combination on J_{sm}^{ac}

J_{sm}^{ac} was first measured for one hour in the presence or absence of 1 mM DIDS, 50 mM NO_3^- or 1.6 mM pHMB (first row of X-axes labels; black bars). After that, a second inhibitor (second row of X-axes labels; white bars) was added to the serosal side. J_{sm}^{ac} was measured for another hour (second flux period 90-150 min). Different letters mark significant differences between the values of the first flux period ($p < 0.05$; One way repeated measures ANOVA + subsequent Holm-Sidak-Test); Asterisk indicate significant differences between the fluxes of the first and second flux period ($p < 0.05$; paired t-test). Data represent means ± SEM; statistics was made on basis of N = 5 (n = 10) for each treatment.

4.2 Effect of Cl^- and NO_3^- on J_{ms} of acetate and butyrate

In the former experiments, we studied J_{sm}^{ac} assuming that the basolateral exchange operates also in the uptake mode. Under physiological conditions, however, the exchange has to export the SCFA out of the cell. Moreover, the basolateral exchange might also be responsible for the extrusion of metabolites from the butyrate breakdown.

To investigate the influence of different anions in the serosal solution (potential partners for exchange) on the basolateral extrusion of acetate and butyrate (or its metabolites), J_{ms} of radiactivelly labelled acetate or butyrate was measured during two flux periods. Due to radioactive labelling the transport of both the original compound and of the metabolites are detected. Measuring flux of radiolabelled acetate mostly indicates the transport of intact acetate, while determining butyrate fluxes mostly represents the transport of its metabolites (see section 2.3).

In the first flux period (0 – 60 min), J_{ms} of acetate and butyrate was measured by using buffer solution containing 50 mM chloride or nitrate on the serosal side (or gluconate buffer as a control) (see section 3.2.1). After one hour of flux detection, serosal buffer solution in all groups was exchanged for gluconate buffer (see section 3.2.1), and J_{ms} was measured for another hour (90 – 150 min). Moreover, in another experimental group we added first gluconate buffer on the serosal side for one hour and then replaced it by a buffer solution containing 50 mM chloride or nitrate.

To evaluate the effect of the anions replaced, the difference between first and second flux period (ΔJ_{ms}) was calculated ($J_{ms}^{90-150\ min} - J_{ms}^{0-60\ min}$). Negative ΔJ_{ms} indicate that the omission of the anion lead to a diminution of mucosal to serosal flux.

When serosal buffer containing Cl^- was replaced by a chloride free solution, ΔJ_{ms}^{ac} was significantly greater than the time dependent ΔJ_{ms} observed under control conditions (Fig. 7). Likewise, replacement of chloride free buffer on the serosal side by a Cl^- containing buffer enhanced J_{ms} of acetate. On the other hand, replacement of NO_3^- by gluconate also diminished mucosal to serosal flux. Consequently ΔJ_{ms}^{ac} was significantly greater in this group than time dependent change in the control group. Unexpectedly, replacement of NO_3^- free buffer in the serosal side by a NO_3^- containing buffer reduced J_{ms} of acetate (Fig. 7).

Concerning butyrate, ΔJ_{ms}^{bu} was significantly greater when serosal buffer containing Cl^- was replaced by a chloride free solution than the time dependent ΔJ_{ms} observed under control conditions (Fig. 8). Also, Cl^- has enhanced J_{ms}^{bu} when chloride free buffer on the serosal side was replaced by Cl^- containing buffer. Replacement of NO_3^- by gluconate also

diminished butyrate mucosal to serosal flux (Fig. 8). Consequently, ΔJ_{ms}^{bu} was significantly greater in this group than time dependent change in the control group. In contrast to that but similar to the effect observed when determining acetate fluxes (Fig. 7), replacement of NO_3^- free buffer in the serosal side by NO_3^- containing buffer solution did not significantly influence J_{ms} of butyrate (Fig. 8).

4.2.1 Effect of SO_4^{2-} on J_{ms} of acetate and butyrate

Sulphate ion (SO_4^{2-}) has been reported to have an affinity to PAT1 mediated transport in several previous studies (see section 2.4.3). Therefore, we conducted the same experiments as described above using buffer solution containing 50 mM SO_4^{2-} (section 4.2). ΔJ_{ms}^{ac} was significantly greater when serosal buffer containing SO_4^{2-} was replaced by a sulphate free solution than the time dependent ΔJ_{ms} observed under control conditions (Fig. 9). Regarding butyrate, replacement of SO_4^{2-} by gluconate also diminished mucosal to serosal butyrate flux. The ΔJ_{ms}^{bu} was greater in this group than the time dependent change in the control group, but did not reach the significant level (Fig. 10).

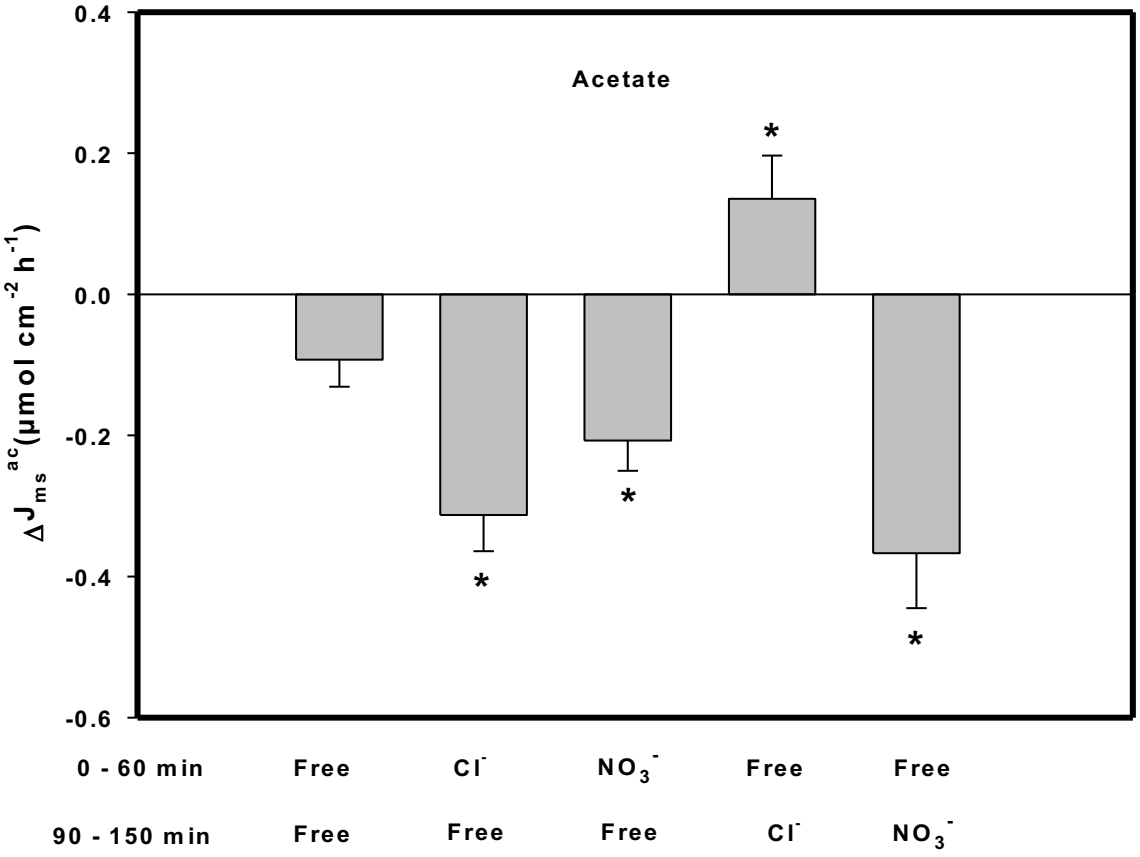

Fig 7: Effect of anion replacement on Δ J_{ms}^{ac}

Mucosal to serosal acetate flux (J_{ms}^{ac}) was measured for one hour (0 – 60 min) in epithelia incubated with either 50 mM of Cl^-, NO_3^- or gluconate buffer on the serosal side and served as control (first row of X-axes labels). Then, all buffers on the serosal side were replaced by the buffer stated in the second row of X-axes labels, and J_{ms}^{ac} was measured for another hour (90 – 150 min). The reduction of J_{ms} was quantified by calculating the difference between the second and the first flux period ($\Delta J_{ms} = J_{ms}^{90\text{- }150\text{ min}} - J_{ms}^{0 - 60\text{ min}}$). Asterisk mark significant differences between control and experimental groups ($p < 0.05$; paired t-test). Data represent means ± SEM; on the basis of N = 8 for each treatment (n = 16).

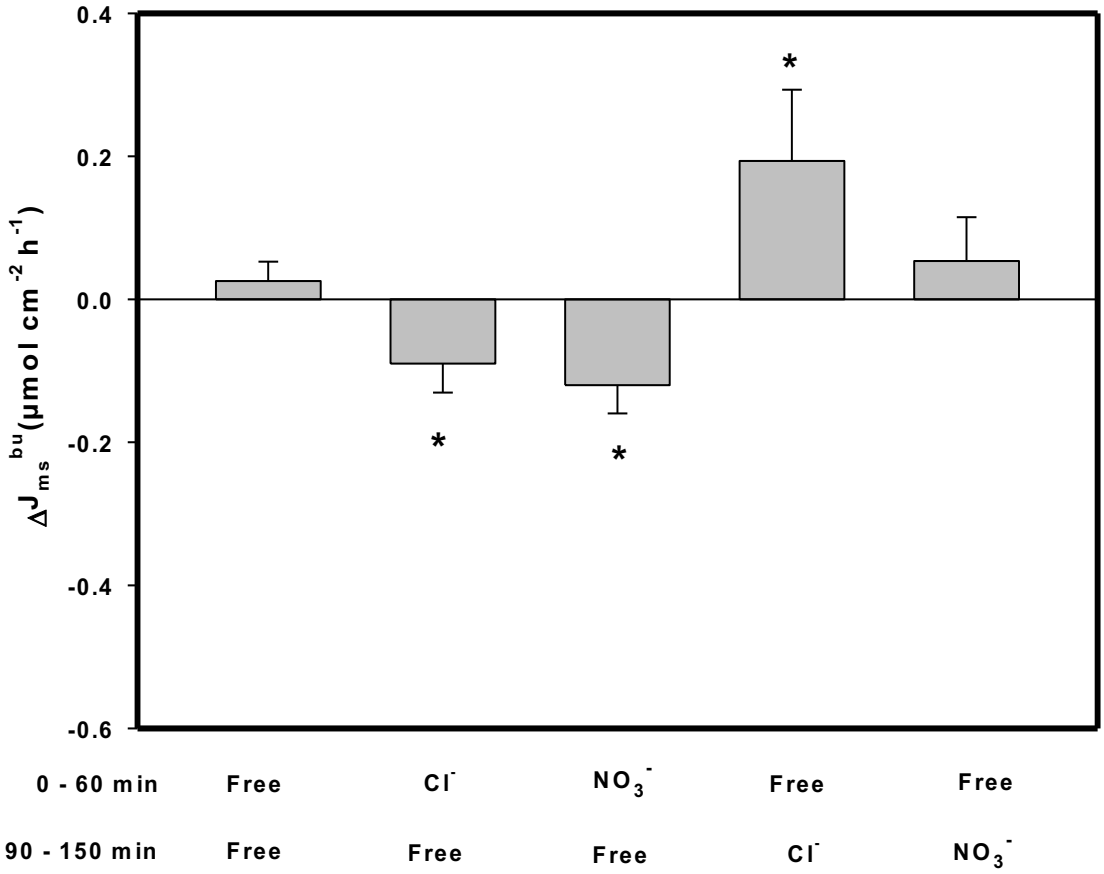

Fig 8: Effect of anion replacement on Δ J_{ms}^{bu}

Mucosal to serosal butyrate flux (J_{ms}^{bu}) was measured for one hour (0 – 60 min) in epithelia incubated with 50 mM of Cl^-, NO_3^- or gluconate buffer on the serosal side and served as control (first row of X-axes labels). Then, all buffers on the serosal side were replaced by the buffer stated in the second row of X-axes labels and J_{ms}^{bu} was measured for another hour (90 – 150 min). The reduction of J_{ms} was quantified by calculating the difference between the second and the first flux period ($\Delta J_{ms} = J_{ms}^{90\text{- }150\text{ min}} - J_{ms}^{0\text{ – }60\text{ min}}$). Asterisk mark significant differences between control and experimental groups ($p < 0.05$; paired t-test). Data represent means ± SEM; on the basis of N = 8 for each treatment (n = 16).

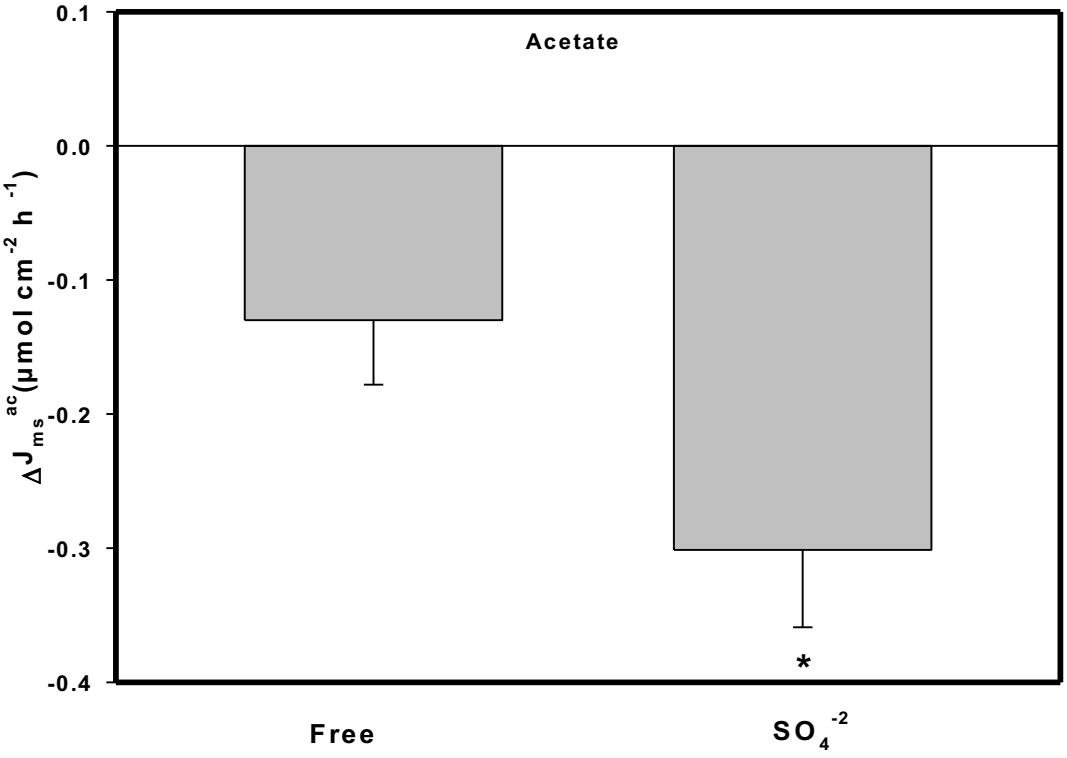

Fig 9: Effect of sulphate replacement on Δ J_{ms}^{ac}

J_{ms}^{ac} was measured for one hour (0 – 60 min) in epithelia incubated with 50 mM SO_4^{2-} buffer on the serosal side. Gluconate buffer served as control. Then, all buffers on the serosal side were replaced by gluconate containing buffer (buffer A), and J_{ms}^{ac} was measured for another hour (90 – 150 min). The reduction of J_{ms} ($\Delta J_{ms} = J_{ms}^{90\text{- }150\text{ min}} - J_{ms}^{0\text{ – }60\text{ min}}$) by anion replacement was compared to the control group. Asterisks mark a significant deference to the control group ($p < 0.05$; paired t-test). Data represent means ± SEM; on the basis of N = 8 for each treatment (n = 16).

Fig 10: Effect of sulphate replacement on Δ J_{ms}^{bu}

J_{ms}^{bu} were measured for one hour (0 – 60 min) in epithelia incubated with 50 mM SO_4^{2-} buffer on the serosal side. While, gluconate buffer was served as control. Then, all buffers on the serosal side were replaced by gluconate containing buffer (buffer A), and J_{ms}^{bu} was measured for another hour (90 – 150 min). The reduction of J_{ms} ($\Delta J_{ms} = J_{ms}^{90-150\ min} - J_{ms}^{0-60\ min}$) by anion replacement was compared to the control group. There was no significant deference between the control group and SO_4^{2-} ($p < 0.05$; paired t-test). Data represent means ± SEM; on the basis of N = 8 for each treatment (n = 16).

4.3 Effect of Cl^-, NO_3^- or SO_4^{2-} when present in the serosal solution for 150 min

In an additional experimental series, we compared the effects of prolonged epithelial serosal exposure to Cl^-, NO_3^- or SO_4^{2-} on mucosal to serosal flux of acetate and butyrate. J_{ms} was measured during 150 min.

In the first 30 min, J_{ms}^{ac} under control conditions amounted to 1.14 ± 0.10 µmol cm^{-2} h^{-1} (Fig. 11). During the same flux period, the presence of sulphate in the serosal buffer efficiently enhanced J_{ms}^{ac} to 1.63 ± 0.11 µmol cm^{-2} h^{-1}. The stimulation persisted all the time of observation. The presence of Cl^- in the serosal buffer solution enhanced J_{ms}^{ac} in the first 30 min (1.37 ± 0.10 µmol cm^{-2} h^{-1}) but the enhancement did reach significant level only from 60 min on (Fig. 11). Throughout the whole time observed, J_{ms}^{ac} of epithelia incubated in NO_3^- containing buffer solution did not differ from J_{ms}^{ac} of the epithelial incubated under control conditions.

J_{ms}^{bu} showed slight higher values in all experimental groups compared to the values of the J_{ms}^{ac}. Under control condition, J_{ms}^{bu} in the first 30 min was 1.25 ± 0.04 µmol cm^{-2} h^{-1} (Fig. 12). Presence of sulphate in the serosal buffer efficiently enhanced J_{ms}^{bu} in the respective time (1.85 ± 0.06 µmol cm^{-2} h^{-1}). The stimulatory effect persisted during 150 min. Presence of Cl^- in the serosal buffer only slightly but not significantly altered J_{ms}^{bu} after 30 min (Fig. 12). NO_3^- exerted not a definite effect.

In summary: Like in the experiments with short term incubation, both SO_4^{2-} and Cl^- enhanced J_{ms}^{ac}. NO_3^- showed again unexpected result by not influencing J_{ms}^{ac} Concerning butyrate, SO_4^{2-} did enhance the J_{ms}^{bu} significantly during all time points and was the most efficient anion in compare to the other anions and the control group. Cl^- in the serosal buffer solution enhanced J_{ms}^{bu} slightly but this effect did not reach the significant level as in the short term incubation experiments.

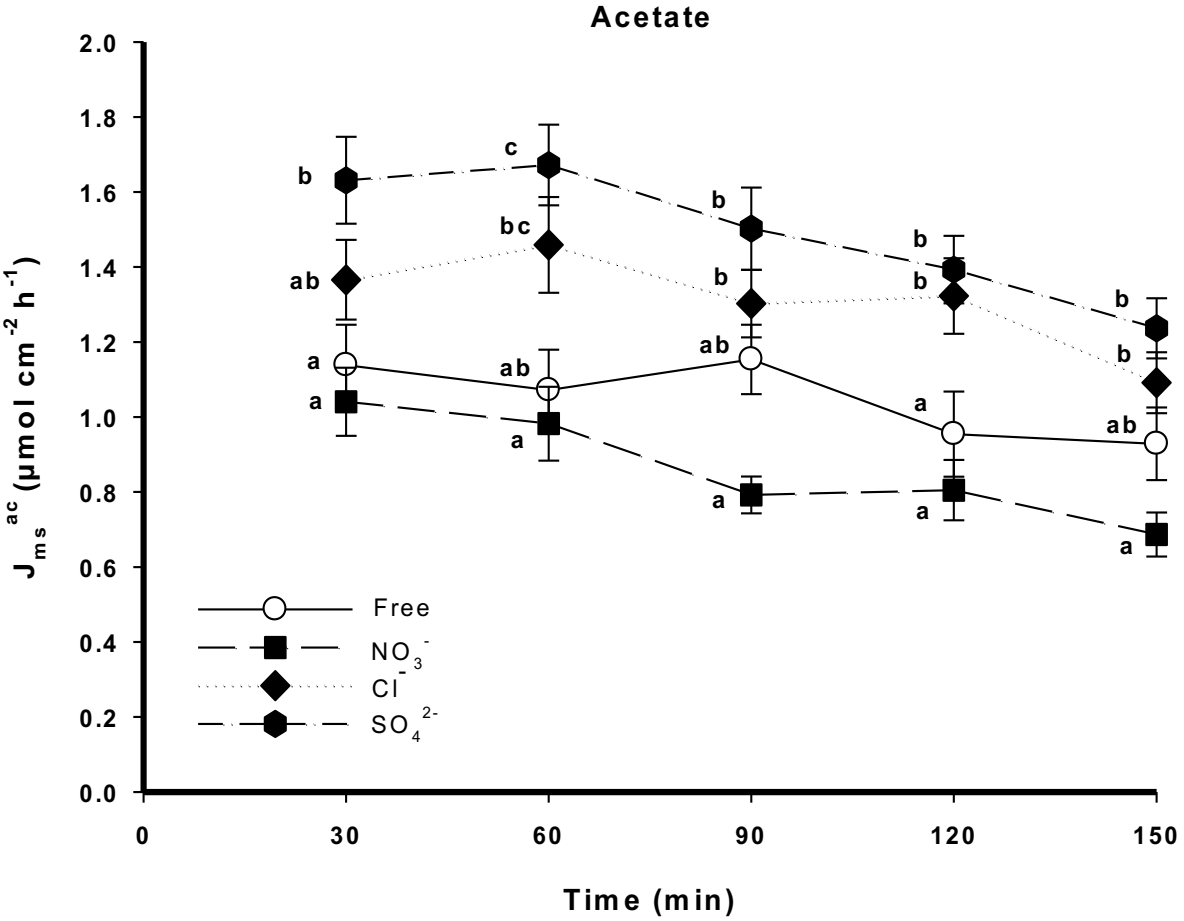

Fig 11: Effects of long term incubation by NO_3^-, Cl^- or SO_4^{2-} buffer solutions on the serosal side on J_{ms}^{ac}

Epithelia were incubated in gluconate containing buffer on the mucosal side, and buffer solutions containing 50 mM of either NO_3^-, Cl^-, SO_4^{2-} or gluconate (control) on the serosal side, respectively for 150 min. Different letters mark significant differences between the J_{ms}^{ac} of respective time point in the X-axes ($p < 0.05$; One way repeated measures ANOVA + subsequent Holm-Sidak-Test); data represent means ± SEM on the basis of N = 6 (n = 12) for each treatment.

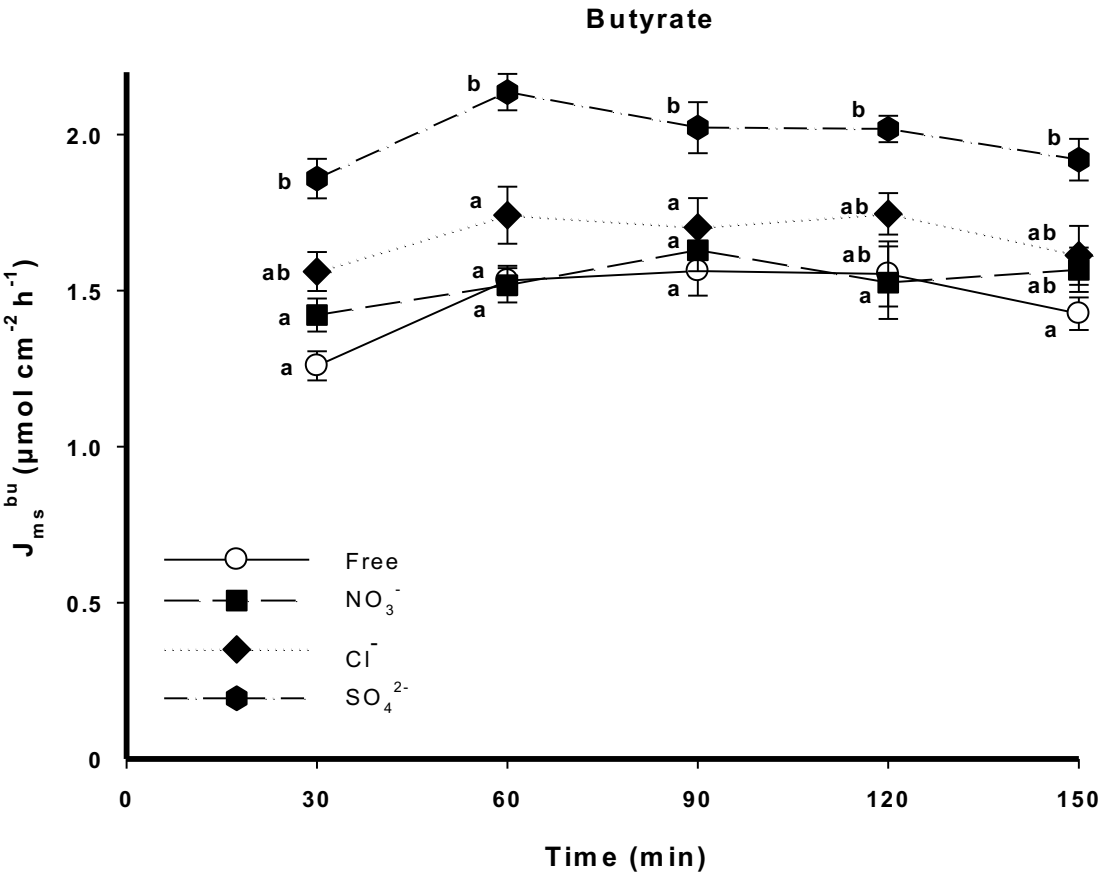

Fig 12: Effects of long term incubation with NO_3^-, Cl^- or SO_4^{2-} buffer solutions on the serosal side on J_{ms}^{bu}

Epithelia were incubated in gluconate containing buffer on the mucosal side, and buffer solutions containing 50 mM of either NO_3^-, Cl^-, SO_4^{2-} or gluconate (control) on the serosal side, respectively for 150 min. Different letters mark significant differences between the J_{ms}^{bu} of respective time point in the X-axes ($p < 0.05$; One way repeated measures ANOVA + subsequent Holm-Sidak-Test); data represent means ± SEM on the basis of N = 6 (n = 12) for each treatment.

4.4 Immunohistochemistry

The immunohistochemical study was conducted on frozen sections from the ventral ruminal sac of sheep. The staining was performed to detect the anion exchanger proteins PAT1 and DRA.

Concerning PAT1, the fluorescence was detected in the *stratum basale* of ovine ruminal epithelium. The signal corresponded predominantly to the basal cell boundaries in all tissues analyzed (Fig. 13). A weaker fluorescence signal was also detected within the cytoplasm (Fig. 13). The fluorescent signal was absent in slides incubated only with secondary antibody or when the primary antibody was pre-incubated with blocking peptide (Fig. 14). Regarding the expression of DRA protein, in three sections obtained from three different sheep, no fluorescent signal for DRA could be detected.

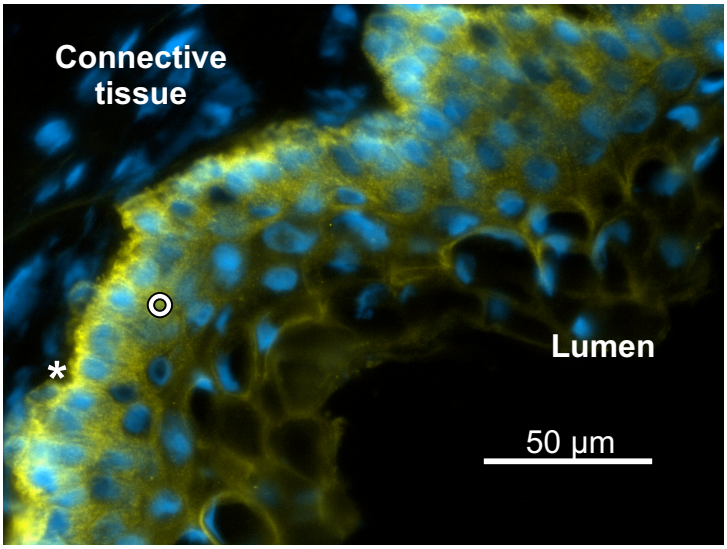

Fig 13: Immunohistochemical staining using primary antibody for PAT1 in a dilution of 1:500.

Cryostat sections were obtained from the epithelium of rumen ventral sac and stained against putative anion transporter 1 (PAT1) (in yellow). Cellular nuclei are labelled with DAPI (in blue). Asterisk indicates the strong fluorescent signal for PAT1 in the basal cell boundaries. The circle indicates a weak fluorescent signal within the cytoplasm. The orientation of the epithelia is shown by indicating the lumen (apical) and the connective tissues.

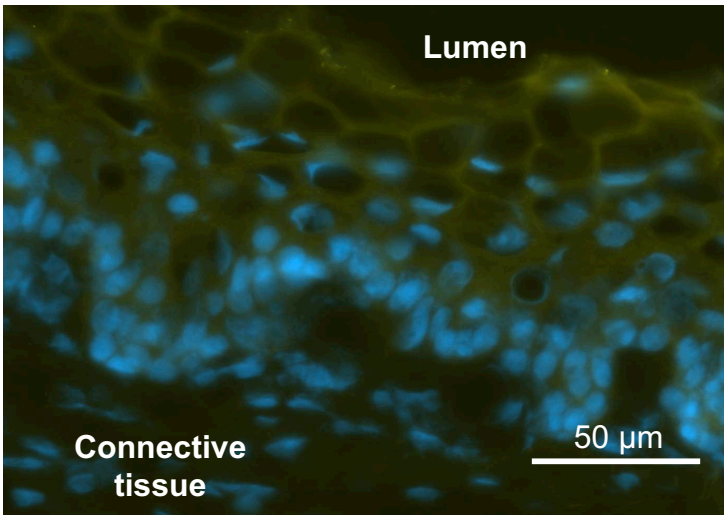

Fig 14: Immunohistochemical staining using primary antibody for PAT1 pre-incubated with specific peptide.

No specific staining for PAT1 could be seen in the section. The cellular nuclei were additionally labelled with DAPI (in blue). The scale bar corresponds to 50 µM.

5 Discussion

The involvement of bicarbonate dependent mechanisms in the efflux of SCFA and/or their metabolites across the basolateral membrane of ruminal epithelium was recently shown by DENGLER et al. (2014). The mechanisms revealed in that study can be most likely considered as anion exchangers. The present study supports this conclusion and provides clue that one of the mechanisms behind this anion exchange is PAT1.

After uptake into the ruminal epithelial cells, SCFA are metabolized inside the cells to different extents. While intraepithelial metabolism of acetate is negligible (KRISTENSEN et al. 2000a), butyrate is almost completely broken down to ketone bodies (KRISTENSEN et al. 2000b). Consequently, both SCFA and ketone bodies would accumulate inside the epithelium if protein mediated mechanisms for the extrusion of these substrates to the basolateral side didn't exist. This would harm the epithelial cells through alteration of the intracellular pH and increasing the osmotic load of the cytosol.

DENGLER et al. (2014) showed that the efflux rate of acetate and butyrate (metabolites) was higher across the basolateral side than across the apical side. The authors concluded that the basolateral efflux of SCFA and/or their metabolites is more efficient than the one in the apical side. One of the mechanisms to release SCFA and ketone bodies basolaterally is the proton coupled transport via MCT1 (MÜLLER et al. 2000; KIRAT et al. 2006b; GRAHAM et al. 2007). In addition, the permeation via an anion channel permeable to SCFA is suggested (STUMPFF et al. 2009; GEORGI et al. 2013). Ussing-chamber studies by DENGLER et al. (2014) revealed a significant role of HCO_3^- in the basolateral extrusion of SCFA across ruminal epithelium, thus indicating to the involvement of a bicarbonate dependent anion exchange.

Regarding the molecular identity of the transport mechanisms, MCT1 is the only transport protein for SCFA that has definitely been detected so far on the basolateral membrane of the ruminal epithelium immunohistochemically (mostly in *Stratum basale*) (MÜLLER et al. 2002; KIRAT et al. 2006a; KIRAT et al. 2006b). The functional role of MCT1 in the extrusion of the SCFA across basolateral membrane of the ruminal epithelium has been confirmed in the study of KIRAT et al. (2006). Regarding the transport properties, MCT1 was first shown to functionally act as proton-coupled transporter for monocarboxylates and SCFA in several tissues including caprine and ovine rumen (MÜLLER et al. 2002; KIRAT et al. 2006). However, studies on colon epithelium revealed that MCT1 could also act as anion exchanger (REYNOLDS et al. 1993; TYAGI et al. 2002). This could also be the case

in ruminal epithelium, since DENGLER et al. (2014) found that bicarbonate dependent transport mechanism is sensitive to MCT1 inhibitors at the basolateral side of the epithelium. However, the inhibition of MCT1 abolished bicarbonate dependent transport only by half in these experiments. This suggests the involvement of further anion exchanger(s).

BILK et al. (2005) have detected the anion exchangers PAT1, DRA and AE2 in addition to MCT1 at mRNA level in cultured ruminal epithelial cells as well as in the intact ruminal epithelium. The authors suggested that these proteins could be involved in the permeation of SCFA across the ruminal epithelium. Several studies detected anion exchange mechanisms at the apical side of the ruminal epithelium (GÄBEL et al 1991a; KRAMER et al. 1996; ASCHENBACH et al. 2009), but none of these studies identified the involved transport proteins. Hence, it is unclear so far if the anion exchangers detected at mRNA level are responsible for apical SCFA uptake only or could also be involved in the basolateral extrusion of these substrates.

In the present study we figured out that beside MCT1, there are other transport proteins participating in the basolateral extrusion of SCFA and/or their metabolites in the ovine ruminal epithelium. In the following sections we will discuss our findings according to the series of the experiments which led to the final conclusions.

In the current study we used acetate as representative for un-metabolized SCFA. It has been reported that only a negligible portion of acetate is catabolised inside the epithelium (KRISTENSEN et al. 2000a). Therefore, determining fluxes of radiolabelled acetate indicates that acetate can enter the cells and then appear in its original form, i.e. not metabolized, on the other side of the epithelium. Determining fluxes of radiolabelled butyrate however, includes detection of both un-metabolized butyrate and butyrate metabolites. In the following, acetate is discussed first.

5.1 Ussing chamber experiments

5.1.1 Effect of Cl^- and NO_3^- on J_{ms} of acetate

Functional existence of an anion exchanger protein mediating SCFA transport in the basolateral membrane of the ruminal epithelium was detected by ion replacement studies. We tested the effect of various anions present on the serosal side on the mucosal to serosal flux of acetate (Fig. 7). We hypothesized that the presence of respective anions should enhance the basolateral extrusion of acetate if the anion exchanger is accepting the ions presented in the experimental setup.

Such an anion replacement approach was used before to detect the involvement of anion exchange mechanism in the apical uptake of the SCFA (KRAMER et al. 1996; ASCHENBACH et al. 2009). These studies used Cl^- as a competitive anion for SCFA transport via anion exchanger substrate with HCO_3^-. Beside Cl^-, NO_3^- was used previously to detect an anion exchange mechanisms in the apical side of ruminal epithelium (ASCHENBACH et al. 2009). DENGLER et al. (2014) also used NO_3^- to detect such mechanism in the basolateral side of the ruminal epithelium.

In the current study, the transport of acetate from the mucosal to the serosal side was decreased when Cl^- containing buffer solution on the serosal side was switched for Cl^- free solution (Fig. 7). Likewise, switching Cl^- free solution for Cl^- containing buffer solution on the serosal side has enhanced acetate transport.

Concerning NO_3^-, switching NO_3^- containing buffer for NO_3^- free buffer in the serosal solution decreased the transport of mucosal to serosal flux of acetate (Fig. 7). Unexpectedly, switching NO_3^- free buffer for NO_3^- containing buffer in the serosal solution decreased acetate transport. Long term incubation with NO_3^- did not influence mucosal to serosal flux of acetate compared to the values of the control group (Fig. 11). Up to now, the inconsistent influence of NO_3^- on acetate flux from mucosal to serosal side cannot be explained.

In contrast, Cl^- was consistently able to enhance the basolateral transport of acetate in the ruminal epithelium suggesting involvement of anion exchange proteins accepting chloride in the basolateral extrusion of acetate

5.1.2 Effect of nitrate and pHMB on J_{sm} of acetate

To further investigate the participation of anion exchanger proteins, we tested the possible inhibitory effects on the serosal to mucosal flux of acetate. The serosal to mucosal pathway is quantitatively insignificant under physiological conditions, but we used this artificial setup to characterize the functional properties of transport proteins assuming that the proteins are able to transport substrates in both directions when the respective gradients are present. This setup (i.e. determining serosal to mucosal flux of SCFA) has also been used before in flux studies of SCFA across the ruminal epithelium (SEHESTED et al. 1999b; DENGLER et al. 2014).

First NO_3^- was applied on the serosal side. When determining serosal to mucosal flux of acetate, NO_3^- was initially assumed to act as a competitive inhibitor competing with acetate for the binding side of the anion exchange. NO_3^- has been reported previously as inhibitor for anion exchange in the kidney (SEIFTER and ARONSON 1984) and in ruminal epithelium cells (WÜRMLI et al. 1987). It has been reported to inhibit the apical uptake of SCFA and affirm the existence of anion exchange mechanism in the ovine ruminal epithelial cells (KRAMER et al. 1996; ASCHENBACH et al. 2009).

In the present experiments, NO_3^- significantly inhibited J_{sm}^{ac} when added on the serosal side (Fig. 3). This finding further supports the assumption that anion exchangers are involved in the permeation of acetate across the basolateral membrane of ruminal epithelium. The study conducted by DENGLER et al. (2014) indicated that the transport of acetate across basolateral membrane of the ruminal epithelium is mediated mainly by $SCFA^-$/ HCO_3^- and linked to MCT1. Therefore, beside NO_3^-, we also used pHMB as a specific inhibitor for MCT1 (POOLE and HALESTRAP 1993; HALESTRAP and MEREDITH 2004) to compare the effect of the combination of both pHMP and NO_3^- to that of the inhibitors when used separately. In the current experiments, pHMB significantly reduced J_{sm}^{ac} (Fig. 3), suggesting an involvement of MCT1 in the transport of acetate across ovine ruminal epithelium.

However, when a combination of both pHMB and NO_3^- was applied on the serosal side, an additional inhibitory effect on J_{sm}^{ac} was observed. The additional inhibition of J_{sm}^{ac} occurred by pHMB in epithelia pre-incubated with NO_3^- was 39% (Fig. 5). Whereas, the additional inhibition of J_{sm}^{ac} occurred by NO_3^- in the epithelia pre-incubated with pHMB was 66 % (Fig. 4). The additional effects of both inhibitors applied in combination gives a clue that

anion exchanger(s) other than MCT1 are also involved in the basolateral extrusion of acetate in ovine ruminal epithelium.

5.1.3 Effect of DIDS, pHMB or NO_3^- on J_{sm} of acetate

To elucidate the participation of transport proteins other than MCT1 in the basolateral extrusion of acetate we applied DIDS, pHMB or NO_3^- in combination.

DIDS has been reported to inhibit the anion exchanger proteins PAT1 & DRA, and has been used to characterize functional properties of those proteins (JIANG et al. 2002; WANG et al. 2002; PETROVIC et al. 2002; FREEL et al. 2006; SILBERG et al. 1995; ALREFAI et al. 2001; JACOB et al. 2002; WHITTAMORE et al. 2013). Moreover, DIDS is also known as an inhibitor of MCT1 in plasma membrane (POOLE and HALESTRAP 1993; HALESTRAP and MEREDITH 2004).

Therefore, we investigated the effect of DIDS on J_{sm}^{ac} and compared its effect with the inhibitory effects caused by NO_3^- and pHMB. Since DIDS has been reported to inhibit both MCT1 and anion exchangers, we expected a remarkable decline of J_{sm}^{ac} in epithelia incubated with DIDS. However, DIDS didn't show any inhibitory effect on J_{sm}^{ac} when applied alone or when added to epithelia pre-incubated with NO_3^- or pHMB (Fig. 6).

To explain this controversy, it is worth to mention that the effect of DIDS is not as uniform as reported for other inhibitors like ethylisopropyl amiloride (EIPA) which is used for inhibition of NHE (YU et al. 1993; KUWAHARA et al. 1994). Nevertheless, our findings are in agreement with that of DENGLER et al. (2014) who reported an unaffected J_{ms}^{ac} by serosal DIDS application despite the proved MCT1 involvement in acetate permeation. Similar to our findings and that of DENGLER et al. (2014), a previous study by HADIIAGAPIOU et al. (2000) also showed that MCT1 is insensitive to 1 mM DIDS. MCT1 insensitivity to DIDS has also been observed in a study on butyrate transport in human colon (TYAGI et al. 2002). The authors attributed these differences in DIDS sensitivity of SCFA transport to the species variations.

However, the lacking DIDS effect is in contrast to the findings of STUMPFF et al. (2009) in cultured ruminal epithelial cells. They revealed the presence of an anion channel sensitive to DIDS and pHMB. The authors indicated that the channel plays a role in the transport of $SCFA^-$ across the basolateral membrane of ruminal epithelium. The lacking effect of DIDS in the current study may lead to the conclusion that DIDS sensitive anion channel is not involved in the basolateral acetate permeation at least under herein applied conditions.

Concerning the identity of transporters responsible for the exchange of SCFA for chloride in addition to MCT1, some studies point to the SLC26A gene family, particularly PAT1 and DRA. PAT1 has been reported in several studies to mediate Cl^- transport across certain membranes in exchange with a wide variety of substrates such HCO_3^-, HO^-, SO_4^{2-}, oxalate and formate (KNAUF et al. 2001; JIANG et al. 2002; KO et al. 2002; WANG et al. 2002; XIE et al. 2002; CHERNOVA 2004; SHCHEYNIKOV et al. 2006). DRA has been described mainly as Cl^- / HCO_3^- exchanger (MELVIN et al. 1999; KO et al. 2002; LAMPRECHT et al. 2005). DRA has also been reported to facilitate the transport of SO_4^{2-}, NO_3^-, OH^- and SCFA (MELVIN et al. 1999; ALREFAI et al. 2001; STERLING et al. 2002; VIDYASAGAR et al. 2004; WHITTAMORE et al. 2013).

5.1.4 Effect of SO_4^{2-} on J_{ms} of acetate

The assumption that PAT1 and/or DRA are involved in the permeation of SCFA is further supported by the results obtained in the experiments using SO_4^{2-}. SO_4^{2-} is a hydrophilic anion that cannot cross the lipid-bilayer of cell membranes passively. Several studies have been conducted to identify the proteins responsible for SO_4^{2-} transport (MARKOVICH. 2001; MARKOVICH and ARONSON 2007). The SLC26 gene family which includes both PAT1 and DRA has been identified as sulphate permease gene family which transports sulphate by anion exchange (MARKOVICH. 2001). However, there is no study so far indicating that MCT1 could transport or use SO_4^{2-} as a substrate. A review by HALESTRAP. (2012) showed that MCT1 facilitates the co-transport of several monocarboxylate anions with H^+, and in some cases in exchange with another monocarboxylate but not with SO_4^{2-}.

Based on the available information, we expected that the presence of SO_4^{2-} in the serosal buffer solution would enhance the basolateral extrusion of acetate if PAT1 or DRA are involved. The same approach as in the previous experiments was conducted to investigate the influence of SO_4^{2-} on the basolateral extrusion of acetate. The obtained results showed that the transport of acetate was significantly increased when the serosal buffer solution contained SO_4^{2-} (Fig. 9). Sulphate sensitivity observed in the present experiment strongly suggests the involvement of PAT1 and/or DRA in the efflux of acetate. MARKOVICH. (2001) suggested the link between PAT1 and SO_4^{2-} transport. The authors attributed that to the chemical structure of PAT1 which shares 59% amino acid identity with rat sulphate anion transporter-1 (sat-1). Furthermore, a functional study on *Xenopus* oocytes by JIANG

et al. (2002) indicated that PAT1 has a high tendency to mediate SO_4^{2-} transport. Moreover, PAT1 has been described as SCFA transporter in small intestine (NOZAWA et al. 2004).

Concerning DRA, it has also been reported to share a significant homology with sat-1 (MARKOVICH. 2001). Moreover, a study by ALREFAI et al. (2001) on Caco-2 cells indicated that DRA is able to mediate SO_4^{2-} / OH^- exchange. Whereas, other studies have reported that DRA mediates SO_4^{2-} / HCO_3^- exchange (SILBERG et al. 1995; BYEON et al. 1998; MELVIN et al. 1999; KO et al. 2002; WHITTAMORE et al. 2013). However, since both PAT1 and DRA are linked to SO_4^{2-} transport, we could not figure out which of these proteins are involved in basolateral extrusion of acetate in ruminal epithelium.

5.1.5 Comparison between different anions as exchange substrate for the basolateral extrusion of acetate

Up to this point, the obtained results support the participation of anion exchange mechanism for the basolateral extrusion of acetate by PAT1 and/or DRA. In this stage, the effect of different anions on J_{ms} of acetate was evaluated and compared. Buffers containing either Cl^-, NO_3^- or SO_4^{2-} were incubated on the serosal side for 150 min without replacing to be used as exchange substrate. This study also demonstrated a stimulatory effect of Cl^- and SO_4^{2-}, with SO_4^{2-} being the most effective substrate which support our finding in the previous experiment for the involvement of PAT1 and/or DRA for basolateral extrusion of acetate (Fig. 11).

However, NO_3^- didn't show any influence on the J_{ms} of acetate (Fig. 11). Thus, the effect of NO_3^- in this experiment was different from those experiments in which it showed an inhibitory effect on the serosal to mucosal transport of acetate (Fig. 3).

Up to now, the unaffected J_{ms} of acetate by serosal application of NO_3^- cannot definitely be explained. However, NO_3^- has been used previously as an inhibitor for anion exchange in the apical and basolateral sides of the ruminal epithelium (ASCHENBACH et al. 2009; DENGLER et al. 2014). The proved inhibitory effect of NO_3^- on the serosal to the mucosal flux of acetate (Fig. 3) in combination with the unaffected mucosal to serosal flux (Figs. 11 and 12), suggests that NO_3^- does not function as anionic substrate binding to the transporting site but might inhibit anion exchange mechanisms by other effects.

5.2 Immunohistochemistry

The functional findings in the present study pointed to the involvement of PAT1 and/or DRA in the basolateral extrusion of SCFA and/or their metabolites across ovine ruminal epithelial. This assumption is supported by immunohistochemical staining at least for PAT1.

In our study, the use of PAT1 antibody revealed fluorescence which was detectable predominantly in the basal cell layers (*stratum basale*) of ruminal epithelium and restricted to the cell membranes (Fig. 13). Immunohistochmical findings are in harmony with the functional results which suggested the role of PAT1 in the basolateral extrusion of the SCFA and/or their metabolites in ruminal epithelium. Previous studies have detected PAT1 in the other part of gastrointestinal tract such stomach, duodenum and colon (PETROVIC et al. 2002; WANG et al. 2002). The apical localization of the PAT1 in these studies was predominant. WANG et al. (2002) pointed out that the expression pattern of PAT1 in the gastrointestinal tract is completely opposite that for DRA. Whereas DRA is predominantly expressed in the large intestine, the expression levels of PAT1 are very high in small intestine and stomach but are low in the colon. Concerning DRA, we did not detect fluorescence in any layer of the ruminal epithelium. This was astonishing since BILK et al. (2005) has detected both PAT1 and DRA on mRNA level in cultured ruminal epithelial cells as well as in intact ruminal epithelium. To definitely exclude expression of DRA protein in the forestomach, further studies are required using additional ovine-specific DRA antibodies.

5.3 Comparison between basolateral extrusion of butyrate and acetate

Although the results discussed so far indicated to the involvement of at least two transporters in the basolateral permeation of acetate, it has to be questioned whether the anion exchanger also permits the extrusion of SCFA metabolites.

Acetate has been reported to be slightly catabolised inside the ruminal epithelial cells (BERGMAN. 1990; BRITTON and KREHBEIL 1993; KRISTENSEN et al. 2000a), while butyrate might be broken down up to 90-95% (BERGMAN. 1990; BRITTON and KREHBEIL 1993; KRISTENSEN and HARMON 2004).

Thus measuring the transepthelial transport of radioactively labelled butyrate, will give hint on how its metabolites are permeating the epithelial cells membranes. The main metabolites of butyrate catabolism are acetoacetate, D-3-hydroxybutyrate and lactic acid (WEIGAND et al. 1975; KRISTENSEN et al. 2000b; GÄBEL et al. 2002). According to the structure of these metabolites they can hardly be extruded via lipophilic diffusion (LEO et al.1971). Thus, they need a protein mediated transport mechanism.

Similar to acetate, the transport of butyrate (metabolites) from the mucosal to the serosal side was decreased when Cl^- containing buffer solution on the serosal side was switched to Cl^- free solution (Fig. 8). Also, switching NO_3^- containing buffer to NO_3^- free buffer on the serosal side has decreased the transport of mucosal to serosal flux of butyrate (metabolites) (Fig. 8). Moreover, SO_4^{2-} as a transporting substrate for PAT1 or DRA has increased the transport of butyrate (metabolites) when the serosal buffer solution contained SO_4^{2-} (Fig. 10). Also, in the long term incubation series, presence of sulphate in the serosal buffer efficiently enhanced J_{ms}^{bu} and the stimulation persisted all the time of observation (Fig. 12) These results indicate that butyrate and/or its metabolites may also be extruded across the basolateral membrane of ovine ruminal epithelium via anion exchange.

Regarding the molecular structure of the transporter responsible for extrusion of butyrate and its metabolites, it might also at least partly be the MCT1 discussed above. The role of MCT1 (and PAT1 and DRA) in the transport of butyrate and its metabolites might have been further elucidated by measuring J_{sm} fluxes.

However, we did not use butyrate in studies on J_{sm} since the butyrate metabolites seem to prefer the mucosal to serosal pathway much more than the other direction due to the presence and effectiveness of MCT1 located in the basolateral membrane (DENGLER et al. 2014). Consequently large part of butyrate taken up basolaterally is probably be recycled as metabolites into the serosal solution and does not appear in the mucosal solution. KRISTENSEN et al. (1999b) found that the amount of butyrate and/or its metabolites reaching the mucosal side was 38% of the total amount of the butyrate transported when studying J_{sm}, however 95% of the transported butyrate and/or its metabolites appear on the serosal side when studying J_{ms}.

Thus at this moment, we cannot definitely outline whether the transporter mediating efflux of butyrate metabolites is only MCT1 and/or a combined action of MCT1, PAT1 and DRA.

Nevertheless, the studies shown in Figs. 11 and 12 underline that the protein mediated transport of butyrate seems to play a similar quantitative role than in the case of acetate since the effect of different anions applied in the basolateral side was similar in acetate

and butyrate. In contrast to our findings, the study by DENGLER et al. (2014) detected a difference in change of J_{ms} between acetate and butyrate using different amount of HCO_3^- in the serosal side or different values of pH and pCO_2. The authors further pointed out that pHMB as specific inhibitor of MCT1 decreased the J_{ms} of acetate but did not inhibit J_{ms} of butyrate.

5.4 Conclusions

Due to the alkaline cytosolic pH, SCFA are present inside the cells mainly as anions which can hardly cross basolateral side by lipophilic passive diffusion. Moreover, a high portion of SCFA is metabolized intracellularly to D-3-hydroxybutyric acid, acetoacetic acid and lactic acid. On the other side, both intact SCFA and their metabolites need to be extruded efficiently across the basolateral side to avoid cell damage. Before this study, MCT1 was the only transport protein that has been intensively functionally characterized to be involved in SCFA transport, and localized in the basolateral membrane of the ovine ruminal epithelium.

The results obtained in the current study indicate that:

- Besides MCT1 there are other transport proteins participating in the basolateral extrusion of the SCFA and/or their metabolites in the ovine ruminal epithelium. The abundance of transport proteins allows the rumen epithelium to acclimate to the amount of SCFA produced in the rumen and taken up from the cytosol into the blood.

- This transport protein(s) can be characterized as anion exchanger accepting chloride and thus are constantly driven by the high chloride concentration in the blood.

- It may be assumed that there are more mechanisms than PAT1, DRA and MCT1 yet to be addressed for basolateral extrusion of SCFA. Further researches are needed to be conducted on both sides of the ruminal epithelium to discover these mechanisms.

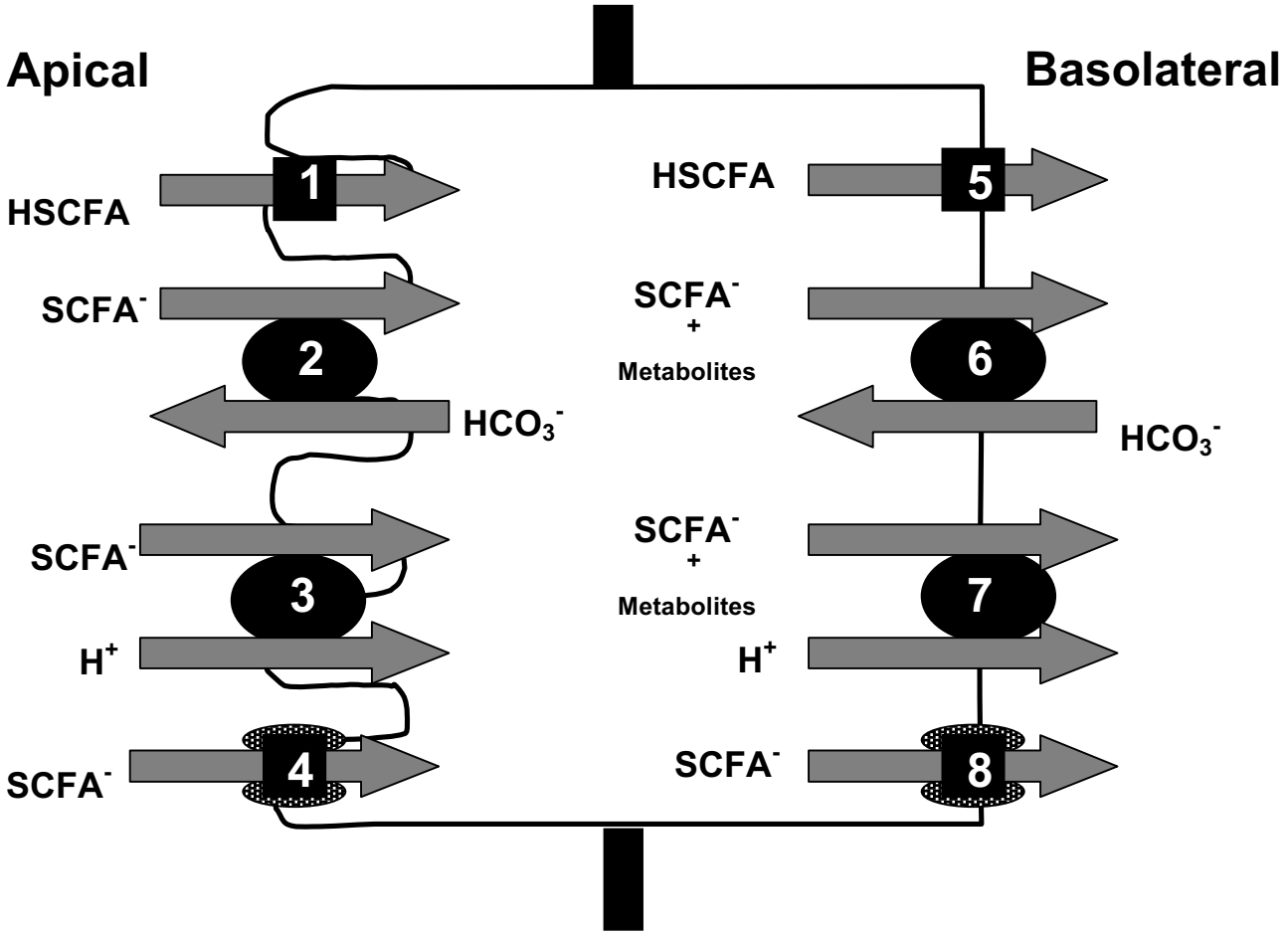

Figure 15: Tentative model for the transport of SCFA across the ruminal epithelium.

Apical side:

(1) Apical uptake of HSCFA by lipophilic passive diffusion (STEVENS and STETTLER 1966b; SEHESTED et al. 1999b)

(2) Exchange of $SCFA^-$ for HCO_3^- (GÄBEL et al. 1991a; KRAMER et al. 1996:; ASCHENBACH et al. 2009)

(3) Co-transport of $SCFA^-$ with H^+ which has been suggested to function through MCT4 (KIRAT et al. 2007)

(4) Apical transport of acetate via an anion channel (RACKWITZ et al. 2012)

Basolateral side:

(5) Lipophilic passive diffusion of HSCFA (DANIELLI et al. 1945)

(6) In exchange for HCO_3^- using MCT1 (DENGLER et al. 2014) or PAT1 or DRA (present study)

(7) H^+ coupled transport MCT1 (KIRAT et al. 2006b; GRAHAM et al. 2007)

(8) Permeation of $SCFA^-$ via an anion channel (STUMPFF et al. 2009)

SUMMARY

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Involvement of the putative anion transporter 1 (SLC26A6) in permeation of short chain fatty acids and their metabolites across the basolateral membrane of ovine ruminal epithelium

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Keywords: Basolateral extrusion of short chain fatty acids, rumen epithelium, Anion exchanger

Introduction: Microbial fermentation of carbohydrates in forestomach of ruminants produces large amounts of short-chain fatty acids (SCFA, mainly acetic acid, propionic acid, and n-butyric acid). The majority of these substrates is taken up directly across the ruminal wall. After luminal uptake into the epithelial cells, SCFA mainly occur in the dissociated form due to the intracellular pH of ~7.4. Moreover, a big portion of SCFA is metabolised within the cytosol. Main end products of epithelial SCFA metabolism are ketone bodies (D-3-hydroxybutyric acid and acetoacetic acid) and lactic acid. Both intact SCFA and ketone bodies and lactate need to be efficiently extruded from the ruminal epithelial cells to prevent a lethal drop of intracellular pH and counteract osmotic load of the cytosol. All these substances are less lipophilic in comparison to the undissociated form of SCFA. Thus, dissociated SCFA ($SCFA^-$) and their metabolites need protein mediated mechanisms for the extrusion across the basolateral side of ruminal epithelium. One mechanism suggested to be involved in the extrusion of $SCFA^-$ across basolateral membrane of the ruminal epithelium is the monocarboxylate transporter 1 (MCT1). Functionally, MCT1 was first assumed to operate as proton-coupled transporter for monocarboxylates including SCFA. Nonetheless, a recent study found a bicarbonate dependent anion exchange mechanism which turned out to be sensitive to MCT1 inhibitors at the basolateral side of the ruminal epithelium pointing to the ability of MCT1 to act as an anion exchanger. However, in these experiments the inhibition of MCT1 abolished bicarbonate dependent transport only by half. This suggests the involvement of further anion exchanger(s) in the transport of SCFA across the basolateral membrane of ruminal epithelium. Promising candidates to underlie this exchange are the putative anion

exchanger 1 (PAT1) and a transport protein designated „down-regulated in adenoma“ (DRA).

Materials and Methods: Sheep rumen epithelium was mounted in Ussing chambers under short-circuit conditions. Radioactively labelled acetate (ac) was added to the serosal side. Serosal to mucosal flux of ac (J_{sm}^{ac}) was measured with or without anion exchange inhibitors (50 mM NO_3^- or 1 mM DIDS) or the MCT1 inhibitor p-hydroxy mercuribenzoic acid (pHMB; 1.5 mM) in the serosal buffer solution. The inhibitors were added alone or in combination with each other. Furthermore, mucosal to serosal flux of radioactivelly labelled ac or butyrate (bu) ($J_{ms}^{ac,\ bu}$) was measured in the presence or absence of SO_4^{2-}, Cl^- or NO_3^- (50 mM respectively) as exchange substrate in the serosal buffer solution. Immunohistochemical staining was conducted to locate PAT1 and DRA by use of commercially available antibodies.

Results: NO_3^- and pHMB significantly reduced J_{sm}^{ac} by 57 % and 51 %, respectively. When pHMB was applied after pre-incubation with NO_3^- an additional inhibition of J_{sm}^{ac} was observed. Vice versa, NO_3^- further inhibited J_{sm}^{ac} when epithelia were pre-incubated with pHMB before. DIDS had no inhibitory effect on SCFA flux. Serosal presence of SO_4^{2-} or Cl^- enhanced J_{ms}^{ac} significantly. Regarding bu, Cl^- or SO_4^{2-} also enhanced J_{ms}^{bu} significantly. The different anions available in the serosal buffer solution numerically enhanced J_{ms} in the order of $SO_4^{2-} > Cl^-$ for both ac and bu, which corresponds to the known affinity sequence of PAT1 and DRA. Immunohistochemistry revealed localization of PAT 1 in the *stratum basale*, whereas DRA was not detectable using this method.

Conclusions: Basically, this study supports the suggestion that MCT1 works as an anion exchanger in ruminal epithelium. In addition, it clearly shows that there is at least one further anion exchanger involved in the basolateral extrusion of SCFA and their metabolites. The functional and immunohistochemical findings suggest that PAT1 holds a significant role in this respect.

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ZUSAMMENFASSUNG

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Beteiligung des putativen Anionentransporters 1 (SLC26A6) an der Permeation von kurzkettigen Fettsäuren und deren Metaboliten über die basolaterale Membran des ovinen Pansenepithels

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Schlüsselwörter: Ausschleusung der kurzkettigen Fettsäuren, Pansenepithel, Anionenaustauscher

Einleitung: Die mikrobielle Fermentation von Kohlenhydraten im Vormagen der Wiederkäuer erzeugt große Mengen an kurzkettigen Fettsäuren (SCFA, hauptsächlich Azetat, Propionat und n-Butyrat). Der Großteil dieser Substrate wird direkt über die Pansenwand aufgenommen. Nach der luminalen Aufnahme in die Epithelzellen liegen SCFA im Cytosol der Epithelzellen, aufgrund des intrazellulären pH-Wertes von ~ 7,4, hauptsächlich in ihrer dissoziierten Form ($SCFA^-$) vor. Außerdem wird ein großer Teil der aufgenommenen SCFA im Zellinneren metabolisiert. Die Hauptmetabolite des intraepithelialen SCFA-Stoffwechsels sind Ketonkörper (D-3-Hydroxybuttersäure und Azetoazetat) und Laktat. Sowohl intakte SCFA als auch Ketonkörper und Laktat müssen effizient aus den Pansenepithelzellen ausgeschleust werden, um einerseits ein Absinken des intrazellulären pH-Wertes zu verhindern und andererseits der osmotischen Belastung des Cytosols entgegenzuwirken. Da die genannten Substanzen wenig lipophil sind und deshalb die Zellmembran kaum durch Diffusion überwinden können, werden für ihre Ausschleusung proteinvermittelte Mechanismen benötigt. Ein Mechanismus, der an der Ausschleusung von $SCFA^-$ über die basolaterale Membran des Pansenepithels bereits nachgewiesen werden konnte, ist der Monocarboxylattransporter 1 (MCT1). Funktionell wurde der MCT1 zunächst als protonengekoppelter Transporter für Monocarboxylate einschließlich der SCFA betrachtet. Eine kürzlich veröffentlichte Studie zeigte jedoch, dass an der basolateralen Seite des Pansenepithels ein Bikarbonat-abhängiger Anion-Austauschmechanismus vorhanden ist, der eine Sensitivität gegenüber MCT1-Inhibitoren ausweist. Dies deutet darauf hin, dass der MCT1 auch als Anionenaustauscher arbeiten könnte. In den erwähnten Versuchen wurde der Bikarbonat-abhängige Transport durch MCT1-Hemmstoffe jedoch nur etwa auf die Hälfte reduziert. Dies deutet auf die

Beteiligung weiterer Anionenaustauscher beim Transport von SCFA über die basolaterale Membran des Pansenepithels hin. Mögliche Transportproteine, die diesen Austausch vermitteln könnten, sind der putative Anionentransporter 1 (PAT1) und ein als „down-regulated in adenoma“ (DRA) bezeichneter Transporter.

Material und Methoden: Pansenepithel von Schafen wurde in Ussing-Kammern eingespannt und unter Kurzschlussbedingungen inkubiert. Radioaktiv markiertes Azetat (ac) wurde der serosalen Pufferlösung zugegeben und der Azetatflux von serosal nach mukosal (J_{sm}^{ac}) in An- und Abwesenheit von Anionenaustausch-Inhibitoren (50 mM NO_3^- oder 1 mM DIDS) oder des MCT1-Hemmstoffes p-Hydroxymercuribenzoesäure (pHMB; 1,5 mM) in der serosalen Pufferlösung gemessen. Die Inhibitoren wurden allein oder in Kombination miteinander eingesetzt. Weiterhin wurde der Flux von radioaktiv markiertem Azetat oder Butyrat von mukosal nach serosal ($J_{ms}^{ac,bu}$) gemessen. Dies geschah in An- oder Abwesenheit von SO_4^{2-}, Cl^- oder NO_3^- (jeweils 50 mM) als Austauschersubstrat in der serosalen Pufferlösung. Zur Lokalisierung von PAT1 und DRA im Pansenepithel wurden immunhistochemische Färbungen mit kommerziell erhältlichen Antikörpern durchgeführt.

Ergebnisse: NO_3^- und pHMB reduzierten J_{sm}^{ac} um 57% bzw. 51%. Wenn pHMB nach Vorinkubation mit NO_3^- angewendet wurde, konnte eine zusätzliche Hemmung des J_{sm}^{ac} beobachtet werden. Genauso konnte eine zusätzliche Hemmung des J_{sm}^{ac} durch NO_3^- beobachtet werden, wenn die Epithelien vorher mit pHMB inkubiert waren. DIDS hatte keinen hemmenden Effekt auf den J_{sm}^{ac}. Die Anwesenheit von SO_4^{2-} oder Cl^- in der serosalen Pufferlösung erhöhte signifikant den J_{ms}^{ac} und. J_{ms}^{bu}. Die Höhe der Zunahme des J_{ms} war bei den verschiedenen Anionen in der serosalen Pufferlösung unterschiedlich. Sowohl für Azetat als auch Butyrat konnte ein stärkerer Einfluss von SO_4^{2-} als von Cl^- beobachtet werden, was den in der Literatur beschriebenen Affinitätssequenzen von PAT1 und DRA entspricht. Immunhistochemisch wurde die Lokalisation des PAT 1 im Stratum basale des Pansenepithels nachgewiesen. Der DRA konnte durch diese Methode nicht nachgewiesen werden.

Schlussfolgerung: Die vorliegende Studie unterstützt die Hypothese, dass der MCT1 im Pansenepithel als Anionenaustauscher fungiert. Zudem konnte gezeigt werden, dass mindestens ein weiterer Anionenaustauscher an der basolateralen Ausschleusung von SCFA und deren Metaboliten beteiligt ist. Die funktionellen und immunhistochemischen Ergebnisse deuten darauf hin, dass PAT1 in dieser Hinsicht eine bedeutende Rolle spielt.

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