A FUNCTIONAL AND MOLECULAR STUDY OF MECHANISMS CONTRIBUTING TO THE REMOVAL OF PROTONS FROM THE RUMEN

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Content

Chapter 1: Literature Review

The importance of the rumen as a fermentational chamber in ruminants has been known for decades. Symbiotic microorganisms break down fibrous plant components, which represent an indigestible feed source in other mammals. The short chain fatty acids (SCFA) produced represent an exploitable energy source for the host animal. However, the purpose of the rumen exceeds its mere function as a fermentation chamber. The hollow organ is lined by the ruminal epithelium which has a transport capacity for numerous minerals and metabolic compounds so that the ruminal absorption contributes to energy and protein metabolism as well as to ion balance and mineral homeostasis. The maintenance of optimal fermentation and absorption capacities is crucial, particularly during challenging situations, such as increased nutrient demand or a changed feed composition. The aim of this literature review is 1.) to provide an overview of the function and importance of the rumen and its epithelium, 2.) to present the current state of knowledge on pH-buffering of the ruminal fluid and its implications in ruminal acidosis, 3.) to give an introduction to ruminal nitrogen transport and its effect on nitrogen homeostasis with potential consequences for ruminal buffering, and 4.) to discuss current models for the transport of organic and inorganic cations and anions across the ruminal epithelium. The review thus highlights the central role of the ruminal epithelium in nutrient supply and overall animal health, providing the theoretical background and a short outline for this thesis.

The present thesis is based on three peer-reviewed manuscripts:

1.) Hille, K.T., Hetz, S.K., Rosendahl, J., Braun, H.-S., Pieper, R., and Stumpff, F. (2016). Determination of Henry's constant, the dissociation constant, and the buffer capacity of the bicarbonate system in ruminal fluid. Journal of Dairy Science *99*, 369–385.

2.) Rosendahl, J., Braun, H.S., Schrapers, K.T., Martens, H., and Stumpff, F. (2016). Evidence for the functional involvement of members of the TRP channel family in the uptake of Na^+ and NH_4^+ by the ruminal epithelium. Pflügers Archiv-European Journal of Physiology *468*, 1333–1352.

3.) Schrapers, K.T., Sponder, G., Liebe, F., Liebe, H., and Stumpff, F. (2018). The bovine TRPV3 as a pathway for the uptake of Na^+, Ca^{2+}, and NH_4^+. PLOS ONE *13*, e0193519.

The Rumen

Different Fermentation Strategies of Herbivores

Herbivorous animals benefit from the fact that plants represent a diverse and easily obtainable feed source. The composition of plant material changes throughout the year; the amount of non-digestible fiber, like hemicelluloses, celluloses or lignin increases when plants mature. The enzymes of mammals are incapable of breaking down fibrous components due to the β-1,4 glycosidic bonds between the linked glucose molecules. However, several classes of microorganisms, such as protozoa, archaea, bacteria and fungi are capable to produce an enzyme called cellulase, which breaks down fibrous components via hydrolyzation. Fibers are converted to CO_2, methane, and SCFA; mainly acetate, propionate, and butyrate.

In the course of evolution, herbivores have developed specialized fermentation chambers in their gastrointestinal tract to host these symbiotic microorganisms. SCFA produced by the host animals microbiota can be absorbed via the intestinal epithelium and thus represent obtainable energy sources (Bergman, 1990). In certain herbivores different evolutionary strategies emerged with distinct sections of the intestine being used as fermentative chambers, i.e. a specialized foregut, caecum or colon. The development of intestinal fermentation chambers by herbivores drastically increased their ability to utilize nutrients from plant material.

The Rumen and its Role for Nutrient Digestibility

The forestomach of ruminants is a highly specialized compartment of the gastrointestinal tract, the largest part of which is called the rumen. As mentioned, this large hollow organ serves as a fermentation chamber for the digestion of plant material and it optimizes the utilization of nutrients from plant-based diets with low protein and high fiber content. The symbiotic microbial population produces high quality protein, as well as energy rich SCFA from sources unavailable for mammalian digestive enzymes. In addition, the rumen epithelium accounts for significant amounts of the absorption of both calcium and magnesium and it plays a central role in nitrogen homeostasis and overall energy supply (Loeffler and Gäbel, 2013). Ruminants can adapt to a largely divergent of feed composition, mainly mediated by changes in the ruminal fermentation pattern and changes in the ruminal epithelium. There is reason to believe that the activity of ruminal transport mechanisms as

such as enzymes, carriers, ion channels and epithelial barrier function can be regulated to optimize absorption mechanisms (Aschenbach et al., 2011; Baldwin and Connor, 2017; Dieho et al., 2017; Kim et al., 2016; Zhao et al., 2017). A proper function of the rumen is essential for the supply with nutrients and thus the animal's health.

Fermentation in the foregut offers high benefits for an animal's nutrition by improving digestibility of nitrogen and energy (Annison, 1956; Bach et al., 2005; Bergman, 1990). The main benefit of foregut fermentation is that the entire nutritional content of the microbial population itself, such as protein, minerals, vitamins and energy pass on into the following gastro-intestinal tract (GIT), where these nutrients can be fully reclaimed. In contrast, hindgut fermenters such as humans, horses or pigs excrete the microbials produced with the feces (Bergman, 1990). In animals receiving a low protein diet, nitrogen utilization is increased: low value plant protein is converted to high value microbial protein. Furthermore, non-protein nitrogen (NPN) like urea or ammonia, which represent unavailable nitrogen sources for the mammalian digestive tract, can be utilized by the ruminant since ruminal microorganisms can incorporate these nitrogen compounds to build up microbial protein which is absorbed later in the small intestines (Harmeyer and Martens, 1980; Kennedy and Milligan, 1980; Reynolds and Kristensen, 2008). Foregut fermenters are thus an evolutionarily successful group, and can accordingly be found in almost every habitat on earth.

Grazing ruminants naturally feed on low protein diets, but in modern farming systems, cattle are offered high protein diets with the intention of improving growth and milk yield. However, with increasing amounts of nitrogen offered in feed, the utilization of nitrogen decreases and high amounts are released into the environment via urine and feces (Kohn et al., 2005; Pearson and Smith, 1943).

The Forestomaches of Ruminants – Advantages and Benefits

The foregut is separated into three distinct sections: reticulum, rumen and omasum. The rumen is the largest fermentation chambers. During rumination, content from the rumen is regurgitated via the reticulum to be re-chewed again. Sufficiently degraded ingesta leave the rumen and enter the omasum, where large amounts of bicarbonate, ions and water are absorbed (Edrise and Smith, 1979; Martens and Gabel, 1988; Schultheiss and Martens, 1999). Finally, the ingesta enter the abomasum, which is comparable to the acid producing stomach of monogastric species. The function and anatomy of the lower intestine are comparable between ruminants and monogastrics (Loeffler and Gäbel, 2013).

In order to demonstrate the importance of ruminal fermentation, some numbers are summarized for cattle – the target species chosen in the present thesis. The rumen of mature cattle contains about 100 l of ruminal fluid (Johnson and Combs, 1991). A cow is able to ingest up to 20 kg of dry matter per day, from which the microbial population can form up to 100 mol SCFA (Allen, 1997; Bergman, 1990). These SCFA are mainly absorbed in the rumen and constitute an important energy precursor which covers up to 75% of the cow's total energy requirement by metabolization of SCFA.

To optimize ruminal fermentation, a cow produces between 220 and 250 l of saliva per day (Cassida and Stokes, 1986; Maekawa et al., 2002) containing about 130 mM $NaHCO_3$ (Storm et al., 2013) in order to maintain the ruminal pH at a level above 6, which is required to preserve optimal fermentation conditions (Aschenbach et al., 2011). The supply of nitrogen for the growth of microbial population is regulated by rumino-hepatic recycling, ensuring a sufficient supply of nitrogen for microbial growth and preventing a toxic accumulation of nitrogen in the rumen (Harmeyer and Martens, 1980; Kennedy and Milligan, 1980; Reynolds and Kristensen, 2008).

Rumen Development and Histology

How does the Rumen Develop?

After birth, a calf receives milk containing high amounts of easily digestible lactose, fat and protein. The rumen of the newborn calf is rudimentary and anatomically arranged so that the milk passes directly to the abomasum. Accordingly, the GIT of calves is functionally comparable to monogastric species with an acid producing stomach followed by the small and large intestine.

Naturally, calves receive milk from their dam until the age of about 10 months (Reinhardt and Reinhardt, 1981). During this period, they gradually start to ingest plants, which triggers the development of the rumen (Steele et al., 2016). In modern farming systems, calves are fed increasing amounts of specially adapted starter concentrates and weaned at the age of about three to five months, after which their diet changes rapidly from liquid milk or milk substitute to solid, plant-based feed (Myers et al., 1999).

At weaning, the underdeveloped rumen may account only for about 30% of the volume capacity of the whole GIT, but most of the microbial population is already present (Steele et al., 2016). The ruminal microbiome is primed at birth provided that the calf has contact to its dam. The microbial population of the colostrum, skin, feces or saliva is transferred and stored in the underdeveloped rumen. Until weaning, the community develops and contains a variety of protozoa, bacteria, fungi and archaea. In order to cope with the changed feed composition and nutrient demand after weaning, the rumen enlarges to about 70% of the volume capacity of the whole GIT (Warner et al., 1956). This development is triggered by fermentation processes of symbiotic microbes, mainly related to the production of butyrate (Sakata and Tamate, 1978). The ruminal microbiota are then able to break down fibrous feed compounds, producing SCFA in addition to methane or CO_2. Additionally, essential vitamins such as vitamin B and vitamin K are synthetized by the microbial metabolism. Until the age of about two years, the rumen enlarges to its final size and develops its full function.

The Histology of the Ruminal Epithelium

The rumen possesses large papillae to increase its surface which are visible macroscopically. The papillae size depends on the feeding regime and can reach a length between 1 and 5 mm as reported for sheep (Coyle et al., 2016; Gäbel et al., 1987). In contrast to other intestinal epithelia, the rumen is covered with a stratified squamous epithelium which can be separated into four distinct layers: the lowest cell layer is called stratum basale (~10µm). It is followed by the stratum spinosum (~ 25µm), which constitutes the metabolically active part of the epithelium. Thus, ketone bodies are produced here from SCFA. Both of these layers are covered by the stratum granulosum (~ 25µm), which shows a high abundance of tight junction proteins like claudin 1, 4, 7 and occludin (Greco et al., 2018; Stumpff et al., 2011) as well as other connecting proteins such as desmosomes to strengthen the epithelium against mechanical forces during rumen contraction (Graham and Simmons, 2005). The upmost layer consists of keratinocytes that lack a cell nucleus and metabolic activity called stratum corneum (~ 25µm), which protects the epithelium from mechanical damage (Steele et al., 2016). This cell layer is continuously desquamated as the lower cells are pushed to the top. The histology of ruminal epithelium resembles that of human skin although lacking serous glands and surface lipids in line with different physiological functions of both epithelia (Blaydon and Kelsell, 2014).

The whole epithelium represents a functional syncytium with gap junction proteins like connexin 43 (Graham and Simmons, 2005). In models, this syncytium is seen as a functional entity with an apical and basolateral membrane.

Rumen Function and Performance Associated Challenges

Different challenges have emerged for cattle and sheep in the present modern farming systems, which are outlined with special regard to dairy cows. Domestication of cattle started about 10 000 years ago, when animals grazed on fibrous plants with a low content of protein (Diamond, 2002). As mentioned before, the rumen evolved as a fermentation chamber to achieve sufficient energy and protein supply from low-nutrient feed. During the last few decades livestock production has been revolutionized due to the introduction of professional farming systems. Within less than 60 years the mean annual milk yield in Germany increased from 3395 kg per cow in 1960 to 8563 kg in 2016 (Statista, 2018), which corresponds to an increase by +150%. In order to achieve this enormous increment, selective breeding strategies were combined with an adaptation of animal feed composition. High yielding cattle receive diets containing high amounts of easily digestible starch from grain or maize with large implications for microbial fermentation (Lana et al., 1998). Hay is usually silaged to increase digestibility, ruminal turnover and hence feed intake. Additionally, feed is supplemented with protein from soy or sugar rich sources like molasses and fruit pulps. Although nutrient supply is enhanced, these changes in the diet pose a challenge to ruminal microbiota, epithelial homeostasis, and animal health (Ceciliani et al., 2017; Fleischer et al., 2001).

SCFA – the Main Fermentation Product

Short chain fatty acids account for 75% of the energy supply in cattle (Bergman, 1990) and thus SCFA production, absorption, and metabolization represent a key element in animal nutrition.

Ruminal microbiota break down complex carbohydrates to $SCFA^-$ + H^+, with a daily production of about 100 moles, yielding a ratio of about 70:20:10 for acetate, propionate, and butyrate, respectively (Bergman, 1990). Almost all SCFA are absorbed by the rumen and the omasum. The fate of the three main SCFA was investigated as early as the 1940s and 1950s by the group around Phillipson (Danielli et al., 1945; Masson and Phillipson, 1951; Phillipson

and McAnally, 1942). Most of the acetate and propionate are absorbed by the ruminal epithelium and transported to the liver. Acetate is metabolized to Acetyl-CoA in the liver or by extrahepatic tissues (e.g. the adipose tissue) where it is converted for lipogenesis. Conversely, the majority of propionate is used as a precursor for gluconeogenesis via transformation to succinyl-CoA and oxaloacetate by the liver. Given the fact that sugars in the feed are mostly fermented to SCFA in the rumen, this is one of the most important metabolic pathways in ruminants, especially during milk production. Studies measuring net glucose absorption in the blood of portal drained viscera (PDV) offered negative values indicating that the intestines consume all glucose absorbed (Archibeque et al., 2006). Dairy cows produce about 3 kg glucose per day in early lactation; and propionate serves as main precursor (>60%) of the carbohydrate compound (Aschenbach et al., 2010; Huntington et al., 2006) for gluconeogenesis in the liver, highlighting the importance of the absorption of SCFA produced by the rumen. Butyrate, the third most abundant SCFA in the rumen, is mainly metabolized by the ruminal epithelium. It accounts for 90% of the energy expenditure of the rumen (Rémond et al., 1995). Additionally, a minor part of butyrate passes on into the blood stream and is subsequently used for ketogenesis in the liver; only traces of butyrate were detectable in the general blood circulatory system (Black et al., 1961).

Ruminal Buffering

If adequate buffering fails, the ruminal pH will drop. This has severe consequences on numerous fermentation parameters, absorption and epithelial integrity, and it often occurs as a consequence of modern livestock feeding strategies.

During the fermentation of carbohydrates, a fast production of $SCFA^-$ occurs correlated to large amounts of protons released into the ruminal fluid. The equilibrium constant pK of $SCFA^-$/HSCFA is ~ 4.8, which is much too low to ensure an optimal environment for ruminal microbiota. To stabilize the pH at levels > 6, the produced acids have to be buffered by HCO_3^- which is mainly provided either by saliva or secreted by the ruminal epithelium (Aschenbach et al., 2011; Counotte et al., 1979; Kohn and Dunlap, 1998).

In the past, it was suggested that buffering through CO_2/HCO_3^- might be negligible because typically, the ruminal pH is below the pK of CO_2/HCO_3^- (Counotte et al., 1979). The misapprehension here was that the rumen was treated as a closed buffer system with constant amounts of acids and bases. However, bicarbonate reacts with protons and disintegrates to water and CO_2, which then escape via the ructus, resulting in a continuous in- and efflux of

buffer molecules. Hence, as is the case with plasma, the rumen needs to be considered an open buffer system in which the amount of acid is not constant but determined by the partial pressure of CO_2 (pCO_2) (Hille et al., 2016; Kohn and Dunlap, 1998).

The buffer capacity represents a quantifiable parameter of ruminal fluid reflecting the resilience of the fluid to cope with acidotic challenges. Somewhat curiously perhaps, no attempts have been made so far to determine this parameter. The Henderson-Hasselbalch equation (equation 1) is the underlying equation:

[1] $$\mathrm{pH} = \mathrm{pK}_{\mathrm{H_2CO_3}} + \log_{10}\left(\frac{[\mathrm{HCO_3^-}]}{\alpha \cdot \mathrm{pCO_2}}\right) = \mathrm{pK}_{\text{acetic acid}} + \log_{10}\left(\frac{[\text{acetate}^-]}{[\text{acetic acid}]}\right)$$

In principle the pH can be calculated using the constants pK (equilibrium constant for CO_2/HCO_3^-), α (the solubility of CO_2), and the partial pressure of CO_2 (pCO_2) in the solution. However, the few studies that exist on the chemistry of ruminal buffering (Kohn and Dunlap, 1998; Turner and Hodgetts, 1955) have been criticized since they utilized constants for aqueous solutions (van Slyke, 1922). The first manuscript contributing to the present thesis aimed to measure α and pK for the CO_2/HCO_3^- system in ruminal fluid under physiological conditions and to determine the contribution of different buffer systems to the total buffer capacity of ruminal fluid (Hille et al., 2016).

The contribution of bicarbonate to ruminal buffering and its concentration can be calculated once the constants needed for the Henderson-Hasselbalch equation are known. This is also essential for determining the driving forces of ruminal epithelial transport processes involving HCO_3^- and the neutralization of protons emerging from SCFA production.

Feed Induced Ruminal Acidosis

In order to provide sufficient energy for cattle with high production rates of milk or high growth rates, rapidly fermentable carbohydrate feed sources like maize or wheat are fed. During fast fermentation, the acid produced in the rumen needs to be buffered to ensure pH values optimal for ruminal fermentation processes and ruminal epithelial integrity.

When low fiber diets are fed, rumination and chewing decrease. This lowers salivary flow, resulting in a reduction of the inflow of buffering $NaHCO_3$ and phosphate contained in the saliva of the ruminant (Humer et al., 2018; Zebeli et al., 2012). As a result, ruminal pH drops which causes a disorder called subacute ruminal acidosis (SARA). Several problems are associated with SARA, including an impaired barrier function of the ruminal epithelium and a

shift in the population of ruminal microbiota towards species that produce lactate instead of SCFA. In addition, a high acid load inhibits the fermentation processes with a negative impact on nutrient utilization (Humer et al., 2018; Kleen et al., 2003; Plaizier et al., 2008).

Different adaptation mechanisms to concentrate rich feed were observed over the last few years, thus improving the understanding of the animal's regulation mechanism when facing acidotic challenges. After feeding a high concentrate feed in order to experimentally induce SARA, the absorption rate of SCFA and lactate increased for the whole rumen in dairy cows (Qumar et al., 2016). Moreover, the expression of acid base transporters in ruminal epithelium was shown to be regulated in sheep facing an increased epithelial acid load (Kuzinski et al., 2012; Mirzaei-Alamouti et al., 2016; Penner et al., 2011; Yang et al., 2012)

Despite the well-known associated health problems, the diagnosis of SARA remains difficult. Determining the pH of ruminal fluid is still technically demanding and as emerges from our study (Hille et al., 2016), the applied methods to sample ruminal fluid (ruminocentesis and aspirating ruminal fluid via oro-ruminal tube) may distort pH measurements. In the last decades, specialized pH-measuring probes were developed to measure pH in the rumen over a course of several hours or even weeks. Data measured by these sensors displayed significant daily fluctuations of ruminal pH levels (Duffield et al., 2004; Neubauer et al., 2018). The expensive sensors are mainly used for scientific purposes and their data output does yield sufficiently reliable information, as the measurement accuracy is low and there is no scientific consent over an adequate pH threshold for acidosis.

On top of the issues with sampling and the measurement technique, the ruminal pH merely depicts the number of free protons and has no explanatory power concerning the buffer capacity of the ruminal fluid and its resilience towards a further acid load. To determine these valuable parameters, a precise determination of the constants pK and α of the HCO_3^-/CO_2 buffer systems are needed (see equation [1).

Nitrogen Homeostasis and Rumino-Hepatic Recirculation

Both energy and protein need to be supplied in a sufficient amount and an available form, in order to ensure a proper nutrition of the animals (Raubenheimer and Simpson, 1998). Nitrogen supply and availability are limiting factors towards the animal's growth rate and milk production.

Optimal Nitrogen Utilization at Low Protein Diets

At low protein intake, the utilization efficiency of nitrogen is strongly increased in ruminants when compared to other herbivores. Low value plant protein is deaminated by microbial organisms and the resulting ammonia can be incorporated into high grade microbial protein with essential amino acids. Furthermore, non-protein nitrogen sources like urea can be used for microbial protein synthesis. In the latter parts of the GIT, which resembles that of monogastric animals, this microbial protein is digested and absorbed. Conversely, the protein formed by fermentational processes in the hindgut are lost with the feces. As a result of forestomach fermentation, the rumen microbiota can thus improve nitrogen utilization and produce valuable protein while producing SCFA from indigestible fiber.

Rumino-hepatic Recirculation Ensures Optimal Nitrogen Supply for Ruminal Microbiota

As a product of the microbial metabolism, ammonia is released into the ruminal fluid. The optimal concentration of ammonia to ensure proper fermentation processes and optimal microbial protein production ranges from 3.5 to 6 mM (Satter et al., 2002; Slyter et al., 1979). These levels can be manifolds higher shortly after feeding, with ammonia concentrations reaching values of up to 20 mM (Thompson et al., 1981) although feeding an experimental diet supplemented with 5% urea to sheep can increase ruminal ammonia concentration to 40 mM within one hour (Broderick and Wallace, 1988). Ammonia is absorbed via the ruminal epithelium and transported to the liver. Traditionally this has been seen as a passive process in order to keep the ruminal ammonia concentration below a toxic level. Depending on the feed source absorption can account for up to 60% of the ingested nitrogen and reach daily amounts of up to 23 moles of ammonia (Delgado-Elorduy et al., 2002a, 2002b). Upon its arrival at the hepatic tissue, the toxic ammonia needs to be converted to a non-toxic molecule before entering the body circulation.

Experiments using different infusion rates of ammonium acetate into a mesenteric vein revealed the ability of the liver to maintain constant ammonia blood levels of 0.04 mM in the circulatory system for infusion rates of up to 15.5 mM/min (Symonds et al., 1981). This corresponds to a detoxification of 22 moles of ammonia per day which is in accordance with the daily absorption of ammonia described in the study mentioned above. Inside the hepatocytes, 2 moles of ammonia are converted to 1 mole of urea and water while consuming 4 moles of ATP. If the remaining ammonia level in blood is still too high, hepatocytes localized downstream can convert glutamate and ammonia to glutamine which is utilized for

protein synthesis, energy supply or acid base regulation in the kidney (Taylor and Curthoys, 2004). However, particularly in animals on low protein diets, large amounts of urea are not excreted via urine but recirculated to the rumen, thus greatly improving nitrogen utilization (Abdoun et al., 2010; Lu et al., 2014a; Reynolds, 1992; Reynolds and Kristensen, 2008). Ruminal secretion of urea is mediated by the urea transporter B (UT-B) which is expressed in all layers of the ruminal epithelium except the stratum corneum (Coyle et al., 2016; Stewart et al., 2005). This process is known as urea nitrogen salvaging, up to about 75% of the synthetized urea is secreted to the rumen (Theurer et al., 2002). In the rumen, urea is converted by microbial ureases to NH_3 accessible for microbial nitrogen metabolism (Pearson and Smith, 1943). It has been estimated that about 50% of the nitrogen in microbial proteins is synthetized from ammonia in the ruminal fluid (Hristov and Broderick, 1994). Via the microbiota found in the rumen, the toxic metabolite ammonia can thus be incorporated to high value microbial protein accessible for protein digestion in the posterior GIT.

Nitrogen Utilization Decreased at Performance Oriented Diets

To increase milk yield and growth in modern farming systems, cattle are offered high protein diets with up to 20% protein content (Krause et al., 2002; Slyter et al., 1979). The high nitrogen influx elevates ruminal NH_3 concentration, which needs to be hepatically detoxified to urea to ensure optimal conditions for ruminal microbiota. In growing goats, very low levels of dietary protein (7%) resulted in a low urea concentration of 0.8 mM in the plasma. In contrast, a supply with 14% crude protein feed increased the plasma urea level up to 5.8 mM (Muscher et al., 2010). When the urea concentration in blood increases, more urea is secreted into the rumen via UT-B, driven by the elevated concentration gradient (Stewart et al., 2005). However, high levels of ruminal ammonia may inhibit the ruminal UT-B (Lu et al., 2014b), so that while the absolute amounts of urea entering the rumen increase with high dietary protein intakes, the relative proportion of urea recycled to the rumen decreases. Simultaneously, and both in absolute and relative terms, the amount of nitrogen loss via the kidneys increases. The fate of dietary nitrogen is summarized in Figure 1.

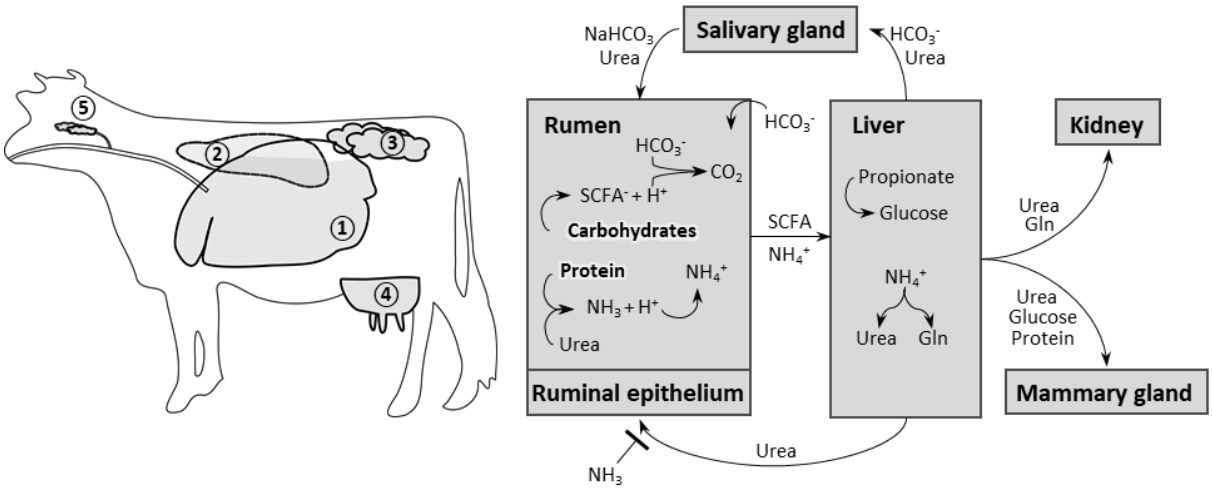

Figure 1: The fate of dietary nitrogen: feed compounds enter the rumen ① where protein and carbohydrates are degraded by microbial fermentation. Ammonia leaves the rumen and is detoxified to urea or glutamine in the liver ②. Urea is excreted via kidney ③ or the mammary gland ④. Also, urea can be re-circulated and is secreted to saliva in salivary glands ⑤ or the rumen epithelium. The secretion of urea by the ruminal epithelium is inhibited by increased ruminal NH_3 concentrations. For further details see text.

The fine tuning of nitrogen requirements and excretion is one of the main challenges for cattle nutrition. Urea and protein rich feed like soy are supplemented to the diet in order to ensure high milk and protein yields in dairy cows and fast growth rates in beef cattle, respectively. The apparent digestibility determined as the difference between dietary input and fecal output was found to be at 65% in pregnant non-lactating cows, but urinary nitrogen output accounted for 55% of dietary input. Therefore the nitrogen retention was rather low in in this study: only 12% of the dietary nitrogen input was not excreted (Plaizier et al., 2000). Most of nitrogen is excreted via urine in the form of urea which correlates linearly with nitrogen supply in feed. Additionally NH_3 and NH_4^+ are excreted through the kidney to balance the acid base status (Weiner and Verlander, 2017).

The low retention rates imply nitrogen emission to the environment. The urea in manure or slurry is converted to ammonia, nitrate and nitrous oxides (NOx) which increase nitrification of the ground water. Livestock farming contributes to 75% and cattle to about 50 % of all nitrogen emissions in Northern Europe (Koerkamp et al., 1998). Ammonia and nitrogenous compounds are relevant climate gases and it has been suggested that they contribute more to global warming than CO_2 (Schneider, 1990). Given that ammonia that leaves the fermentative

parts of the gut and thus escapes conversion to microbial protein, the mechanism of ammonia absorption in cattle is crucial for minimizing environmental pollution and improving protein efficiency in animal production.

Recirculation of Ammonia – a Waste of Energy?

The detoxification of up to 22 moles of NH_3 per day is very energy demanding. Beside the stabilized NH_3 concentration in the rumen, the rumino-hepatic recirculation may have additional beneficial effects on ruminal homeostasis: if ammonia is absorbed in the form of NH_4^+, each mole of ammonium shuttles one mole of protons from the ruminal fluid to the blood side (Aschenbach et al., 2011).

Ammonia is a weak base with an equilibrium between NH_3 and NH_4^+ at its pK of 9.11. For a long time, it was assumed that ammonia leaves the rumen via lipid diffusion in the form of NH_3, but its chemical and biophysical properties contradict this statement: at the physiological ruminal pH of below 6.4, less than 0.1% of ammonia is present in the form of NH_3 compared to more than 99.9% of protonated NH_4^+. Moreover, NH_3 is a highly polar molecule which would impair its ability to diffuse through the lipid bilayer of cell membranes (Weiner and Verlander, 2011). Furthermore, numerous studies have established that NH_4^+ is transported through the isolated ruminal epithelium inducing current characteristics which are comparable to currents of K^+ transporting proteins (Abdoun et al., 2005; Bödeker and Kemkowski, 1996; Rosendahl et al., 2016).

The similarity between the transport mechanisms of potassium and ammonium may be explained by their remarkably similar biophysical properties. Both cations display almost the same ionic radius, mobility rate, and hydration energy (Weiner and Verlander, 2011), lowering the ability of channels to distinguish between the cations. When NH_4^+ enters the cell, it dissociates within the slightly alkaline cytosolic milieu and protons are released. Pending on the form in which ammonia crosses the basolateral membrane (NH_3 or NH_4^+), an intracellular acidification is to be expected. Using isolated ruminal epithelium, a decrease of intracellular pH was shown when NH_4^+ was applied to the mucosal side (Lu et al., 2014a; Rosendahl et al., 2016), supporting the hypothesis that the protonated NH_4^+ is absorbed by the ruminal epithelium.

Upon ruminal absorption of NH_4^+, the rumino-hepatic circulation ensures both, nitrogen homeostasis and pH regulation of the ruminal fluid. Yet the molecular identity of NH_4^+ transporting protein in the rumen remains unclear. The second and third manuscripts

presented in this thesis aimed to elucidate the protein mediating NH_4^+ transport (Rosendahl et al., 2016; Schrapers et al., 2018).

Buffering Agents for the Ruminal pH

As described above, microbial fermentation processes in the rumen produce about 100 moles of $SCFA^-$ daily which is associated with a production of 100 moles of protons. The pH of ruminal fluid needs to be maintained above levels with negative impact on fermentation patterns and epithelial barrier function (Greco et al., 2018; Khafipour et al., 2009; Plaizier et al., 2017). Different threshold values have been suggested for healthy fermentation, but the pH most commonly used as a threshold for ruminal acidosis is 5.8 (Humer et al., 2018; Kleen et al., 2003). SCFA have a pK of 4.8 which means that the ratio of HSCFA: $SCFA^- + H^+$ equals one at a pH of 4.8. At a physiological ruminal pH of 5.8, the majority (95%) of the SCFA are present in the dissociated form ($SCFA^- + H^+$) and these protons need to be buffered. Buffering compounds are supplied from saliva and the ruminal epithelium as described in the following section.

Saliva and its Buffering Compounds

The saliva contributes to ruminal buffering in a major way: lactating dairy cows produce about 250 liters of saliva on a daily basis. It has been suggested that fibrous compounds increase the rumination time which is associated with a higher production of saliva (Allen, 1997; Silanikove and Tadmor, 1989). The importance of saliva for ruminal buffering presents itself when one considers the composition of saliva. Based on the chemical analysis of parotid saliva of sheep, the following composition was found: 117 mM $NaHCO_3$, 26 mM Na_2HPO_4, 8 mM NaCl, 8 mM KCl, 0.2 mM $CaCl_2$ and 0.3 mM $MgCl_2$ at a pH of 8.2 (McDougall, 1948). Similar concentrations of ions were determined in consecutive studies (Silanikove and Tadmor, 1989; Storm et al., 2013). The concentration of urea in saliva was estimated to be 3 mM (Storm et al., 2013), which contributes to buffering as discussed. Considering all the protons which theoretically can be bound to all of the buffering agents found in saliva, one liter has the capacity to neutralize 149 mmoles protons. Based on a 250 l daily production the saliva can account for about 40 mole protons bound given that all buffer agents are saturated with protons (Table 1).

Table 1: Theoretical contribution of different buffer agents from saliva to ruminal buffering

Buffer	Possible protons bound	Maximum buffer capacity	
		[mmole H^+/l]	[moles H^+/250l]
$NaHCO_3$	$HCO_3^- + H^+$	117	29.25
$NaHPO_4^-$	$HPO_4^{2-} + H^+$	26	6.5
urea	$CH_4N_2O \rightarrow 2\ NH_3 + 2H^+$	6	1.5

Considering a daily production of 100 moles of $SCFA^- + H^+$, saliva thus contributes considerably to ruminal buffering, although other buffering mechanisms are required in order to maintain ruminal pH. These mechanisms include the secretion of HCO_3^- and urea by the ruminal epithelium, and the absorption of protons – e.g. in the form of HSCFA or in the form of ammonium (NH_4^+).

Absorption Mechanisms expressed by the Ruminal Epithelium

Absorption of SCFA by the Ruminal Epithelium

Classically, it was suggested that the uncharged HSCFA diffuse through the lipid cell membrane driven by the chemical gradient. However as early as the 1940s and 1950s, cracks began to emerge in this picture. Thus, when a Na-acetate solution is placed in the rumen, the acetate ion is absorbed with Na^+ as a counterion (Phillipson and McAnally, 1942).

The most common model for the absorption of SCFA is depicted below (Figure 2) and has been repeatedly reviewed (Aschenbach et al., 2011; Stumpff, 2018). There are different electroneutral and electrogenic transport mechanisms involved in the absorption of SCFA. Certain quantities of SCFA enter the cell vial lipid diffusion in the form of HSCFA. Since intracellular pH is higher than the pH of SCFA, 99% of intracellular HSCFA dissociates to $SCFA^-$ and H^+ acidifying the cytosol. These protons can be released from the cell by an apical sodium-proton exchanger (NHE3) (Rabbani et al., 2011). Sodium is extruded through the basolateral membrane via the Na^+-K^+-ATPase, with potassium leaving the cell via basolateral K^+-channels to maintain intracellular K^+ homeostasis. Two main apical anion exchangers are involved in the absorption of $SCFA^-$ in exchange for HCO_3^-: DRA (down regulated in adenoma) and PAT1 (putative anion transporter) (Bilk et al., 2005; Gäbel and Aschenbach,

2006). Additionally, uptake of SCFA was shown to be mediated by MCT4 (monocarboxylate transporter 4), involving the co-transport of $SCFA^-$ and H^+ (Aschenbach et al., 2009). All these mechanisms acidify the cytosol.

At the basolateral membrane, SCFA and Cl^- can leave the cell via AE2 (anion exchanger 2) using an antiport with HCO_3^-. This transport system results in a net transport of SCFA from the apical to the basolateral side and a net transport of HCO_3^- in the opposite direction providing HCO_3^- as a buffer agent for ruminal pH maintenance.

Additionally SCFA and other anions can pass the basolateral membrane via a maxi-anion channel, which displays a remarkably high conductance for anions (350pS for Cl^- and 142pS for $acetate^-$ (Stumpff et al., 2009)) stabilizing intracellular osmolarity (Georgi et al., 2014; Stumpff et al., 2009). Efflux of $SCFA^-$ via this mechanism is driven by the potential generated by the basolateral Na^+/K^+-ATPase and mediates a net absorption of Na^+ and $SCFA^-$, with protons recirculated into the rumen via NHE3. This mechanism explains why protons frequently accumulate in the rumen causing ruminal acidosis when secretion of buffers with saliva is insufficient.

However, further transporters are expressed in the basolateral membrane mediating the export of protons (MCT1: co-transport of $SCFA^-$ and H^+ and NHE1: Na^+-H^+-exchanger (Dengler et al., 2014; Graham et al., 2007)) or the import of HCO_3^- (NBC: Na^+-$2HCO_3^-$ cotransporter) in order to maintain intracellular pH.

The transport mechanisms for the absorption of H^+ and $SCFA^-$ and the excretion of HCO_3^- as a buffer agent for ruminal fluid need to be tightly regulated. There are reasons to believe that ruminal tissue has high capacities to adapt to different challenges regarding acid load or shifts in the fermentational pattern by changing the expression of the transport proteins presented above (Dengler et al., 2015; Mirzaei-Alamouti et al., 2016; Penner et al., 2011). When adaptational mechanisms break down, ruminal acidosis may threaten the health of the animal (Kleen et al., 2003; Zebeli et al., 2012).

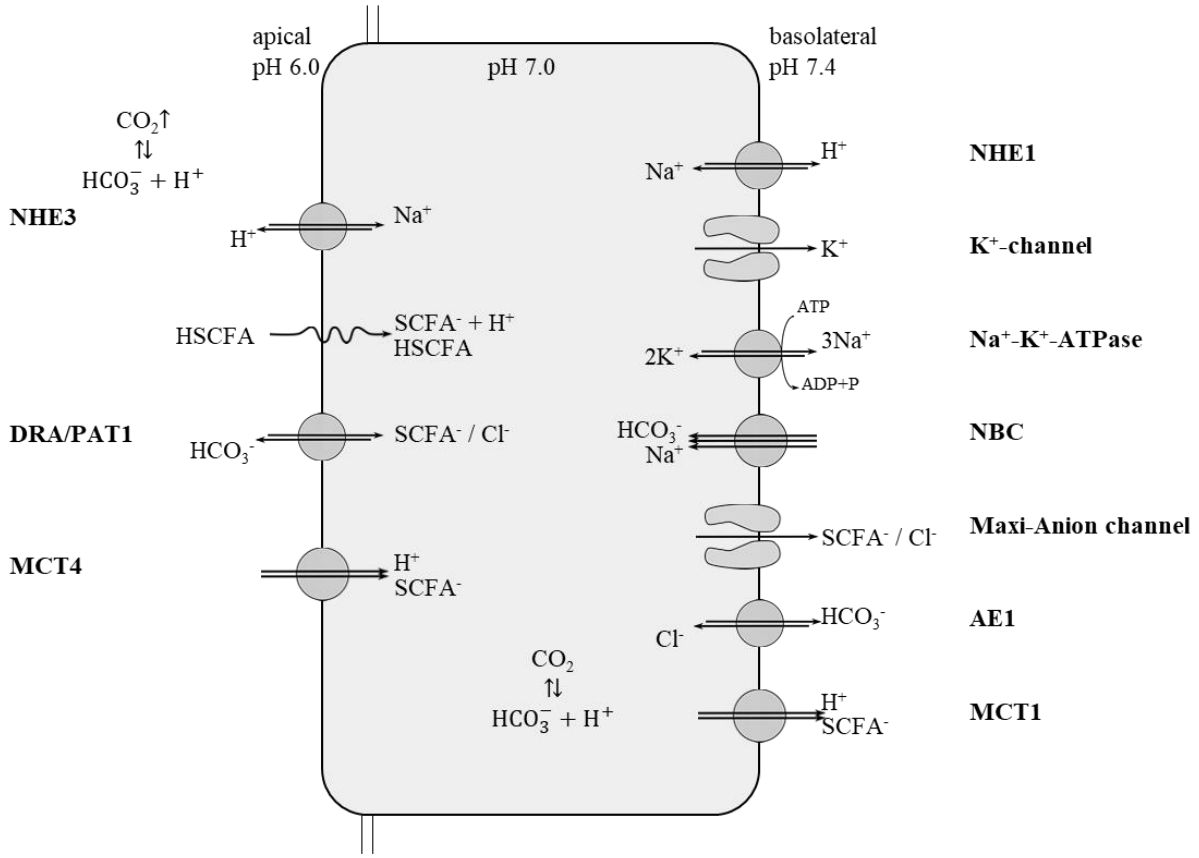

Figure 2: Transport mechanisms for the absorption of SCFA across the ruminal epithelium: HSCFA can enter the functional syncytium by lipid diffusion. Apically the transporters DRA, PAT1 and MCT4 are involved in the channel mediated uptake of $SCFA^-$. At the basolateral side $SCFA^-$ are extruded via MCT1, AE1 or maxi-anion channels. Different exchangers contribute to pH regulation (NHE1 and 3, NBC) energized by the Na^+-gradient generated by a basolateral Na^+-K^+-ATPase.

Absorption of Sodium by the Ruminal Epithelium

For each liter of saliva, about 170 mmoles sodium are secreted as a counter cation for bicarbonate and phosphate resulting in a total daily secretion of 43 moles of sodium (McDougall, 1948; Silanikove and Tadmor, 1989). This rather large amount needs to be re-absorbed to ensure sufficient sodium supply for salivary production.

The absorption of up to 80% of the Na^+ from ruminal fluid was observed in vivo (Dobson, 1959). A large fraction of this sodium is absorbed by NHE3, which exchanges sodium ions for protons on the apical cell membrane. Additionally an electrogenic transport of sodium was confirmed in the isolated ruminal epithelium (Ferreira et al., 1966; Martens and Gabel, 1988), but its molecular basis has long been elusive. The epithelia Na^+ channel ENaC – the most

obvious candidate channel which is expressed by the intestine, kidneys, and the mammary glands – is not functionally expressed by the rumen since the application of amiloride did not influence transepithelial currents (Leonhard-Marek et al., 2010).

The first significant hint concerning the channels involved was found when the divalent cations Ca^{2+} and Mg^{2+} were removed from the mucosal solution. This resulted in an increase of Na^+ currents (Schultheiss and Martens, 1999) across the omasum of sheep with similar effects for other monovalent cations such as K^+, Li^+, Rb^+ or Cs^+. Consecutive studies demonstrated similar results for isolated ruminal epithelium and isolated ruminal epithelial cells (Leonhard-Marek, 2002; Leonhard-Marek et al., 2005). The authors concluded involvement of non-selective cation channels (NSCC), which were described earlier in the gastrointestinal tract of amphibians, avians and mammals (see Leonhard-Marek, 2002).

Absorption of Ammonium by the Ruminal Epithelium

Since the putative channel was proposed to mediate the transport of various cations, it might be a candidate for the absorption of NH_4^+. As described above, the rumen epithelium of cattle can absorb up to 20 moles of ammonia per day. There are two possible pathways for the absorption of ammonia: an electroneutral transport of NH_3 or an electrogenic transport of NH_4^+. The absorption of NH_3 via Rhesus proteins or aquaporins as shown for renal epithelium (Weiner and Verlander, 2011) would result in an electroneutral process with an alkalization of the cytosol. Conversely the absorption of NH_4^+ should lead to a depolarization of the epithelium and an acidification of the cytosol. The absorption of NH_4^+ was demonstrated using isolated ruminal epithelium where the mucosal application of NH_4Cl induced an increased current reflecting the absorption of NH_4^+ (Bödeker and Kemkowski, 1996). The authors suggested a transport mechanism similar to K^+ absorption because both currents were partially blocked by quinidine. The NH_4^+ induced current was further increased after the removal of Ca^{2+} and Mg^{2+} (Rosendahl et al., 2016), suggesting identity with the divalent-sensitive NSCC described above (Leonhard-Marek et al., 2005).

A recent study of the sheep rumen investigated the effect of NH_4^+ application on the intracellular pH of isolated ruminal tissue. It confirmed the effect of NH_4^+ induced currents as well as tissue conductance of NH_4^+ and demonstrated a concentration dependent intracellular depolarization of the apical membrane with an acidification after the application of NH_4^+. This effect was enhanced when the mucosal pH was lowered from pH 7.4 to pH 6.4 (Lu et al., 2014a). These results indicate that ammonia is absorbed in the form of protonated NH_4^+ with

subsequent release of protons, leading to an increase of the electroneutral absorption of Na^+ in exchange with H^+ via NHE3 (Abdoun et al., 2005). A subsequent study on bovine ruminal tissue confirmed the depolarizing and acidifying effects of NH_4^+ (Rosendahl et al., 2016). Investigations using isolated ruminal epithelial cells support the hypothesis that NSCC are involved in the apical cation absorption when investigated with the whole cell patch clamp technique (Rosendahl et al., 2016).

Summarizing the state of research on NSCC, different studies reveal evidences for the expression of NSCC with a conductance for monovalent cations like Na^+, K^+ or Cs^+ (for summary see Figure 3). However, the molecular basis of this NSCC remains unclear. Building on a previous thesis suggesting that the NSCC is a member of the TRP family of ion channels (Rosendahl, 2014), the present thesis aimed to investigate one candidate protein, the TRPV3, in detail and to compare its properties with the functionally verified NSCC in the intact ruminal epithelium.

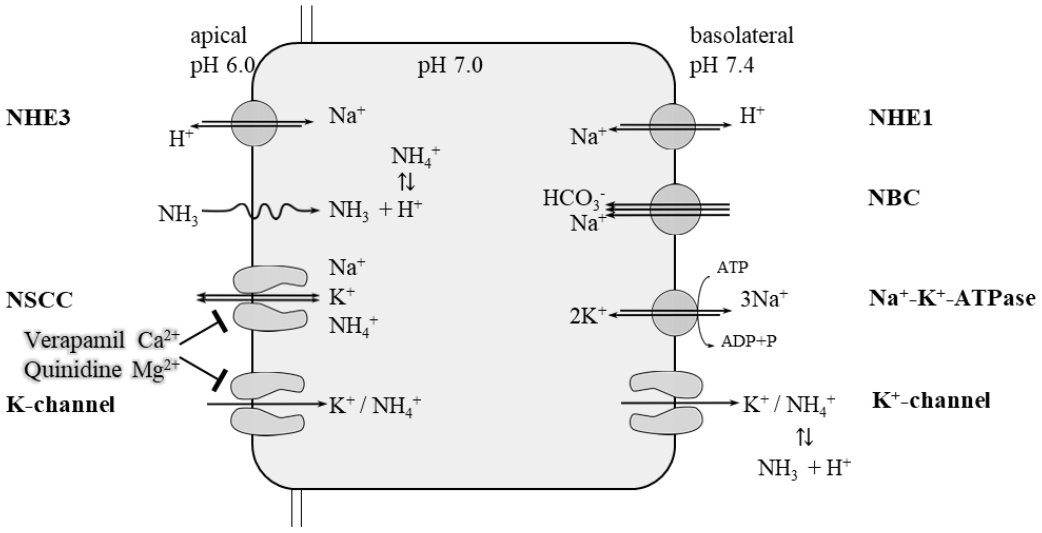

Figure 3: current model of ruminal cation absorption: apical absorption is mediated via electroneutral exchange (NHE3) and electrogenic channel-mediated transport (K^+-channels and NSCC). Electrogenic transport can partially be inhibited by the presence of divalent cations or quinidine and verapamil. Basolateral transport involves NHE1, NBC and K-channels as well as the Na^+-K^+-ATPase.

Chapter 2: Objective and Outline of the Thesis

The present thesis attempted to fill two important gaps in the knowledge about pH homeostasis in the rumen:

a) What are the relevant constants for the CO_2/HCO_3^- buffering system in the rumen?
b) Which channels mediate the efflux of protons from the rumen with NH_4^+?

Sufficient buffering of acids is necessary to maintain the nutrient supply and epithelial integrity in ruminats, with a special focus on livestock cattle kept under a performance-oriented feeding system. Since ruminal acidosis poses a high risk for animal health, understanding the individual components of the ruminal buffering system is essential. In the first part of the study we focused on the main buffer systems CO_2 and SCFA and determined the dissociation constants (pK) and α, the solubility of CO_2 in ruminal fluid at physiological conditions. For this purpose we used a newly developed titration technique and a modification of the classical Astrup technique with ruminal fluid obtained from ruminally fistulated cows. Not surprisingly perhaps, the constants α and pK were similar to those determined for human blood (**Paper 1** (Hille et al., 2016)).

In addition to the determination of these buffer constants, the concept of base excess, which is well known from blood acid base balance, was modified according to conditions in the rumen. Ruminal fluid was equilibrated with 5% and 100% CO_2 at 37°C and the amount of acid or base needed to adjust the pH to 6.2 and 5.8 was measured. The base excess of ruminal fluid obtained from hay fed cows was relatively high, confirming a high rate of HCO_3^- secretion in relation to the SCFA produced. The ruminal base excess could provide information about the resilience of the buffer system towards an increase in the acid load. The main buffer substance in the rumen, which maintains pH despite a high production of $SCFA^+ + H^+$, is bicarbonate, which is secreted from saliva as well as by the ruminal epithelium. In addition, ammonia released from rumino-hepatic recirculation of urea is protonated at a physiological ruminal pH, contributing to the neutralization of H^+.

While the transport mechanisms for $SCFA^-$, H^+ and HCO_3^- have been under investigation for decades (Aschenbach et al., 2009; Bilk et al., 2005; Bugaut, 1987; Georgi et al., 2014; Masson and Phillipson, 1951), the molecular basis for the efflux route of NH_4^+ and many other cations remained unknown. There are numerous studies *in vivo* or *in vitro* which

demonstrate remarkable absorption capacities of the rumen for both sodium and ammonium (Abdoun et al., 2005, 2006; Ferreira et al., 1972). Recent studies in our lab supported the hypothesis that NH_4^+ was mainly absorbed in the cationic form via transport proteins rather than via non-ionic diffusion of NH_3 (Abdoun et al., 2005; Lu et al., 2014a). Numerous observations support the notion that a non-selective cation channel mediates this transport. For clarification as to which channel or channels may contribute to cation transport in the rumen, studies were conducted using electrophysiological measurement techniques (whole cell patch clamp, micro-electrode, and Ussing chamber) as well as quantitative PCR (**Paper 2** (Rosendahl et al., 2016)).

Using isolated bovine ruminal epithelial cells, the conductance to NH_4^+, Na^+ and K^+ was shown to be sensitive to the removal of divalent cations, an observation consistent with previous studies of intact ovine ruminal epithelium (Leonhard-Marek, 2002; Leonhard-Marek et al., 2005). Further experiments on bovine ruminal tissue were performed using ion selective microelectrodes and Ussing chamber technique. The acidification of the intracellular milieu was shown after the application of NH_4^+, confirming previous results in the ovine tissue (Lu et al., 2014a). The transepithelial currents were partially blocked by verapamil, clearly indicating a transcellular transport pathway for NH_4^+. In experiments on ruminal tissue, addition of bumetanide, an inhibitor of the basolateral NKCC (Na^+-K^+-$2Cl^-$ co-transporter) expressed by many other epithelia (Xu et al., 1994), did not affect the transepithelial currents. This argues against chloride secretion as a contributor to the transepithelial currents measured across the ruminal epithelium in Ussing chamber experiments.

Based on the known characteristics of NSCCs, various terpenoids were tested for their ability to induce currents across the epithelium. Some of the terpenoids are known fragrances which activate TRP channels in the olfactory and gustatory system. The transient receptor potential (TRP) channel family is expressed in various tissues including the skin, central nervous system and intestine (Holzer, 2011a; Nilius and Voets, 2005). Effects on currents were clearly seen for a variety of compounds including menthol and thymol, suggesting the involvement of TRPV3 and TRPA1 on the absorption of monovalent cations, since TRPM8 was not expressed (Holzer, 2011b; Patel et al., 2007; Rosendahl et al., 2016). The presence of these genes was confirmed using quantitative PCR. Since some TRP channels are known to transport calcium, a further experiment was conducted to investigate the effects of menthol and thymol on net calcium transport of the ruminal epithelium. We were able to show a dose

dependent effect on calcium absorption after the application of menthol and thymol, respectively.

The previous paper demonstrated that menthol and thymol induce currents which might be mediated by the activation of TRPA1, TRPV3 or / and TRPM8. Since TRPM8 was not expressed (Rosendahl et al., 2016), the involvement of the bovine TRPV3 (bTRPV3) on cation transport was chosen for further investigations. The bTRPV3 was transiently expressed in HEK-293 cells and conductance for different cations was investigated using both the single channel and whole cell patch clamp technique. The responsiveness of TRPV3 expressing cells on specific agonists was tested using whole cell patch clamp technique. Additionally, the conductance of TRPV3 for calcium was tested using intracellular calcium imaging in the presence of agonists (**Paper 3** (Schrapers et al., 2018)).

These investigations revealed that the properties of bTRPV3 match the characteristics previously described for the NSCC in ruminal tissue and cells. Whole cell patch clamp measurements confirmed the monovalent cation conductance for K^+ and Na^+ and its sensitivity to the presence of divalent cations. Additionally, a conductance for NH_4^+ was demonstrated for bTRPV3, which has not been investigated before for any member of the TRP channel family. Furthermore, Na^+ and K^+ currents were activated by the application of known TRPV3 agonists (menthol, carvacrol, thymol and 2-APB). Using the single channel patch clamp technique, the conductance was determined for different cations, revealing decreasing permeabilities in the order of $NH_4^+ > Na^+ > Mg^{2+} > Ca^{2+} > NMDG^+$. A time dependent increase in the open probability of bTRPV3 channel was measured after patch excision. In these experiments it could be shown that the single channel conductance for Na^+ and NH_4^+ did not change with time after excision, so that in our experiments pore dilation as previously proposed for TRPV3 (Chung et al., 2005) did not play a role. Using intracellular calcium imaging, the application of menthol was shown to increase the intracellular calcium concentration.

Summarizing the results, the current thesis demonstrates that the bovine representative of TRPV3 facilitates both the transport of monovalent cations such as NH_4^+ or Na^+ and the divalent cation Ca^{2+}. These findings are in accordance with the characteristics of the putative NSCC previously described for native ruminal cells. It should be added that the data of this thesis additionally support a certain conductance for Mg^{2+}. These results to not rule out a contribution of other potential TRP channels on cation absorption by the ruminal epithelium. Since this is the first time that a role for TRP channels in the transport of ammonium as an

important product of protein catabolism has been shown, implications for the function of this poorly understood family of ion channels in other tissues arise (Nilius and Bíró, 2013).

Chapter 3: Determination of Henry's Constant, the Dissociation Constant, and the Buffer Capacity of the Bicarbonate System in Ruminal Fluid

The manuscript

Hille, K.T., Hetz, S.K., Rosendahl, J., Braun, H.-S., Pieper, R., and Stumpff, F. (2016). Determination of Henry's constant, the dissociation constant, and the buffer capacity of the bicarbonate system in ruminal fluid. Journal of Dairy Science *99*, 369–385.

was published in the Journal of Dairy Science. https://doi.org/10.3168/jds.2015-9486

Contribution	Detailed Description	Contributor
Conceptualization	Design and Aim of the Study	Stumpff, Schrapers (nee Hille)
Data Curation	Data Management	Schrapers, Stumpff, Hetz
Formal Analysis	Data Analysis	Schrapers, Stumpff
Investigation	Sample Acquisition and Performing of Experiments	Schrapers, Hetz, Braun, Rosendahl
Methodology	Adaptation of Titration and Astrup Technique	Stumpff, Schrapers, Hetz
Resources	Providing Materials and Instruments	Hetz, Pieper, Stumpff
Software	Programming	Stumpff
Visualization	Data Presentation	Schrapers, Stumpff
Writing – Original Draft Preparation	Preparation of the Manuscript	Schrapers, Stumpff
Writing – Review & Editing	Finalizing of the Manuscript	Stumpff, Schrapers, Hetz, Braun, Rosendahl, Pieper

Chapter 4: Evidence for the Functional Involvement of Members of the TRP Channel Family in the Uptake of Na^+ and NH_4^+ by the Ruminal Epithelium.

The manuscript

Rosendahl, J., Braun, H.S., Schrapers, K.T., Martens, H., and Stumpff, F. (2016). Evidence for the functional involvement of members of the TRP channel family in the uptake of Na^+ and NH_4^+ by the ruminal epithelium. Pflügers Archiv-European Journal of Physiology *468*, 1333–1352.

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https://doi.org/10.1007/s00424-016-1835-4

Contribution	Detailed Description	Contributor
Conceptualization	Design and Aim of the Study	Stumpff, Rosendahl, Martens
Data Curation	Data Management	Rosendahl, Stumpff, Braun, Schrapers
Formal Analysis	Data Analysis	Rosendahl, Braun, Stumpff, Schrapers
Investigation	Sample Acquisition and Performing of Experiments	Molecular Biology: Braun Ussing Chamber: Schrapers Microelectrode and Patch Clamp: Rosendahl
Methodology	Adaptation of Microelectrode	Stumpff, Rosendahl
Resources	Providing Materials and Instruments	Stumpff
Software	Programming: Patch Clamp Analysis	Stumpff
Visualization	Data Presentation	Rosendahl, Stumpff
Writing – Original Draft Preparation	Preparation of the Manuscript	Stumpff, Rosendahl, Braun, Schrapers
Writing – Review & Editing	Finalizing of the Manuscript	Stumpff, Rosendahl, Braun, Schrapers, Martens

Chapter 5: The Bovine TRPV3 as a Pathway for the Uptake of Na^+, Ca^{2+}, and NH_4^+

The manuscript

Schrapers K.T., Sponder G., Liebe F., Liebe H., Stumpff F. (2018) The bovine TRPV3 as a pathway for the uptake of Na^+, Ca^{2+}, and NH_4^+. PlosOne, 13, e0193519.

was published in PlosOne. https://doi.org/10.1371/journal.pone.0193519

Contribution	Detailed description	Contributor
Conceptualization	Design and Aim of the Study	Stumpff, Schrapers, Sponder
Data Curation	Data Management	Stumpff, Schrapers, Liebe F, Sponder
Formal Analysis	Data Analysis	Schrapers, Liebe F & H, Sponder, Stumpff
Investigation	Sample Acquisition and Performing of Experiments	Patch clamp: Schrapers, Liebe F $[Ca^{2+}]_i$: Liebe F, Liebe H, Schrapers Molecular biology: Sponder
Methodology	Design of the TRPV3 Construct	Sponder
Resources	Providing Materials and Instruments	Stumpff, Sponder
Software	Programming (Patch Clamp and $[Ca^{2+}]_i$ Analysis)	Stumpff, Liebe H
Visualization	Data Presentation	Stumpff, Schrapers, Liebe F & H, Sponder
Writing – Original Draft Preparation	Preparation of the Manuscript	Stumpff, Schrapers, Liebe F, Sponder
Writing – Review & Editing	Finalizing of the Manuscript	Stumpff, Schrapers, Liebe F & H, Sponder

Chapter 6: General Discussion

The first part of this study aimed at determining the buffer capacity of ruminal fluid at physiological conditions (**Paper 1** (Hille et al., 2016)). It was clearly shown that the CO_2/HCO_3^- buffer constants pK and α (solubility of CO_2) do not deviate significantly from the values known for human blood (Andersen, 1963; Hille et al., 2016). Given the very different composition of the two solutions, this result is not trivial. However, at the same time, it must be stressed that at total osmolarity of <400 mosmol/kg, the major interactions of CO_2 will be with the aqueous phase. The findings of this study further show that ruminal buffer capacity and pH can be adequately described by the contribution of $SCFA^-$/HSCFA and CO_2/HCO_3^- buffer systems. Phosphate buffer may contribute in a minor way, but it does not account for significant amounts.

In the second part of this study we demonstrated the involvement of TRP channels in cation absorption of ruminal tissue (**Paper 2** (Rosendahl et al., 2016)). Previous studies suggested that ammonia released from the rumino-hepatic recirculation of urea may be absorbed in the form of NH_4^+ resulting in a net absorption of protons. This contributes to maintaining ruminal pH despite the production of acids from fermentation processes. It was therefore a further goal of this study to determine the molecular identity of the transporting protein for NH_4^+. Different specific agonists applied to ruminal tissue were able to activate TRP channels. The functional data suggest the involvement of TRPA1, TRPV3 and/or TRPM8 (Holzer, 2011b; Nilius and Szallasi, 2014). On the level of mRNA, the expression of TRPM8 was not detected excluding TRPM8 from candidate genes conducting NH_4^+. Furthermore, the specific TRPM8 agonist icillin had no effect on the currents across the isolated ruminal epithelium (Rosendahl et al., 2016). The expression of non-selective channels TRPA1, TRPV3 and TRPV4 was shown, but no expression of TRPV1 or TRPV2 was detected. Moreover, the expression of the magnesium transporter genes TRPM6 and TRPM7 was demonstrated. Conversely we confirm that any expression of the TRPV5 and TRPV6 is discrete (Wilkens et al., 2009). These were the first data offering an insight into the molecular basis of non-selective cation transport in rumen tissue and isolated ruminal cells. Furthermore, the contribution of TRP channels to calcium absorption across ruminal tissue was demonstrated by inducing increased calcium

absorption across ruminal tissue in the presence of TRP agonists, revealing a novel transport pathway for ruminal calcium absorption.

The third part of this study aimed to investigate the characteristics of the candidate protein bTRPV3 with special regard to its ammonium and calcium permeability (**Paper 3** (Schrapers et al., 2018)). Whole cell and single channel patch clamp experiments with HEK-293 cells overexpressing bTRPV3 demonstrate a conductance for NH_4^+, and displayed an inhibition of monovalent currents in the presence of divalent cations. In experiments on the single channel level, the large conductance of bTRPV3 made it possible to clearly distinguish between endogenous cation channels expressed by the HEK-293 cells and the TRPV3 channels in the overexpressing system.

For the first time, the molecular identity of a NH_4^+ transporting channel expressed by ruminal tissue was unveiled. This contributes to the hypothesis that the rumino-hepatic recirculation of nitrogen may contribute to ruminal pH homeostasis because the absorption of NH_4^+ results in an absorption of H^+ in contrast to the proposed diffusive uptake of NH_3. In addition to the transport of monovalent cations, the bTRPV3 displays a conductance for calcium, which was measured directly using whole cell and single channel patch clamp measurements, and indirectly via intracellular calcium imaging. The application of TRPV3 agonists increased both monovalent currents and intracellular calcium levels. Since the classic calcium transporting proteins TRPV5 and TRPV6 are not expressed by the rumen, the TRPV3 emerges as a prime candidate protein for ruminal calcium absorption.

The implications of these findings for the transport physiology of the rumen and animal health are discussed in the following section.

Buffering of Ruminal Fluid

As summarized above, the buffer constants for the CO_2/HCO_3^- buffer system known from blood were confirmed for ruminal fluid. The main difference between both systems is the high partial pressure of carbon dioxide (pCO_2) in ruminal fluid. Hence, the contribution of different buffer systems on ruminal pH can be calculated as follows. From the Henderson-Hasselbalch equation [1, equation [2 can be derived, where A^- represents the acid and HA the base, respectively. For the CO_2/HCO_3- buffer system, A^- equals the concentration of HCO_3^- and HA

represents the concentration of H_2CO_3 calculated as the product of α the solubility of CO_2 and its partial pressure pCO_2.

[2] $$A^- = \frac{(A^- + HA) \cdot 10^{pH-pK}}{1+10^{pH-pK}}$$

This equation allows the calculation of the concentrations of HSCFA, $SCFA^-$ and HCO_3^- for different pH levels and different pCO_2 levels as presented in Table 2. The chosen pCO_2 of 35 and 65 kPa represent the limit values reported for ruminal fluid (Kölling, 1974; McArthur and Miltimore, 1961).

Table 2: Concentration of protonated and unprotonated SCFA and ammonia (in mmol/l) at different pH values and the corresponding HCO_3^- concentration at different levels of pCO_2.

	pH = 6.2	pH = 5.8	pH = 5.4
HSCFA	3.829	9.091	20.076
$SCFA^-$	96.171	90.909	79.924
NH_4^+	5.993	5.997	5.999
NH_3	0.007	0.003	0.001
HCO_3^- at pCO_2=35 kPa	10.076	4.011	1.597
HCO_3^- at pCO_2=65 kPa	18.712	7.450	2.966

HSCFA + $SCFA^-$ = 100mM, NH_3 + NH_4^+ = 6 mM

At the pH level of 6.2, between 80 and 90% of the bicarbonate entering the rumen neutralizes protons and is converted to CO_2 and water. The proportion of protonated HSCFA is very low, supporting models of protein mediated transport (Aschenbach et al., 2009; Stumpff et al., 2009). Almost all of the NH_3 entering the rumen is protonated and less than 10µM NH_3 are present in the ruminal fluid.

When ruminal pH decreases to the acidotic value of 5.4, the proportions of the components changed massively. These low pH values are commonly measured in beef cattle or in dairy cows that are offered a ration with a high level of concentrate feed. The amount of HSCFA increases by a factor of 5, strongly promoting the diffusive uptake of HSCFA. This results in a simultaneous increase of the acid load which has to be extruded from the ruminal epithelial cells. Notably, intracellular protons need to be buffered to maintain cell viability and cell function. Furthermore, the amount of HCO_3^- decreased to values of 1.5 to 3 mM, implying that HCO_3^- entering the rumen with saliva or through the ruminal wall is almost entirely converted to CO_2, so that an almost equimolar amount of protons will be neutralized for each mole of HCO_3^- entering the rumen. This also means that since residual bicarbonate in the

ruminal fluid is minimal, further acid production from fermentation will result in steep changes in the ruminal pH.

Evaluation of Ruminal Acidosis – a Proposal

As summarized in the introduction, up to 100 moles of SCFA are produced by microbial fermentation of carbohydrates. Carbohydrates like starch are easily fermentable resulting in a rapid elevation of the acid load, which increases the risk of subacute ruminal acidosis (SARA) (Enemark, 2008; Humer et al., 2018). The impact of the feed composition on ruminal pH with special attention on epithelial integrity and animal health has been discussed for a long time (Humer et al., 2018; Kleen et al., 2003; Zebeli et al., 2012). In these studies, different measurement techniques were applied to investigate the effect of feed composition on SCFA production and acid load. More recent approaches used direct measurements of the ruminal pH with sensors that are placed inside the rumen and record daily changes of the ruminal pH (Dohme et al., 2008; Gasteiner et al., 2012). The main advantage of these sensors is that continuous profiles of the ruminal pH can be obtained as measured directly in the rumen. Unfortunately, the accuracy of commercially available sensors is rather low, since once inside the rumen, recalibration is not possible. Furthermore, the location of the sensor is questionable: the sensor ought to be located in the reticulum, which displays remarkably higher pH values than the rumen, but rumination movements may transport the sensor to the rumen, resulting in a sharp decline of measured pH (Schrapers et al., 2016).

Classical sampling techniques to measure ruminal pH use aspiration through an oro-ruminal tube placed into the rumen or the reticulum. The disadvantage of this method is a high risk of contamination of the ruminal fluid with alkaline saliva and the location of sampling cannot be determined sufficiently. Ruminocentesis is another aspiration technique, where a cannula is punctured through the abdominal wall into the ventral ruminal sack to sample ruminal fluid. This procedure is highly controversial with a high risk for postoperative problems, leading to reduced feed intake and pain (Enemark, 2008; Kleen et al., 2003). Both sampling techniques are not able to display the circadian fluctuation of the pH and merely represent a momentary status. Additionally, both sampling techniques depend on the aspiration of ruminal fluid. The negative pressure used to obtain the sample directly influences the pCO_2 within it. Reducing the pCO_2 from 60 to 30 kPa will result in a shift of pH from 5.8 to about 6.1 and thus strongly distort the result and its explanatory power (Hille et al., 2016). Especially at the

physiologically relevant pH levels above pH 5.5, the contribution of CO_2/HCO_3^- buffer capacity is increased, meaning that the escape of CO_2 from the sample will influence the pH values measured. Somewhat surprisingly perhaps, this relative basic reason for the great variability of pH in clinical studies of ruminal acidosis has not been discussed in the literature.

To evaluate the challenge of a further acid load and its impact on acid-base balance in ruminal fluid, a new evaluation method was proposed similar to the base excess measurements performed in blood (Hille et al., 2016). Since pCO_2 is set to a fixed level, this technique allows the evaluation of the acid-base balance and the resilience of the ruminal fluid to further acid loads. Remarkable differences in base excess were observed for cows fed a hay-based diet compared to a performance-oriented diet with high amounts of concentrate feed demonstrating the depletion of ruminal buffer capacity in cows offered the second diet. In comparison to measurements using intraruminal sensors, a clear disadvantage of this approach is the need for samples of ruminal fluid. However, the limited reliability of ruminal sensors limits their clinical usefulness. Accordingly, most studies continue to rely on analyses of ruminal fluid. In such studies and in analogy to what has long been standard practice in analyzing systemic acid-base status, the determination of base excess in addition to simply measuring the ruminal pH as the exclusive parameter to test for SARA appears mandatory.

Subsequent studies have investigated the role of CO_2 in the ruminal fluid and its properties as an acid. It was suggested that the amount of dissolved CO_2 (dCO_2) (as measured via a CO_2 sensor) determines the pH level (Laporte-Uribe, 2016). Based on his measurements, the author suggested that the amount of dCO_2 exceeds the amount of CO_2 calculated from $pCO_2 \cdot \alpha$ (partial pressure of CO_2 and solubility), and concluded that ruminal fluid does not behave like an "ideal" liquid with a set value of α. However, one would expect different pCO_2 levels in ruminal fluid *in vivo*, caused by different fermentation patterns in different layers (Tafaj et al., 2004), with values clearly distinct from the pCO_2 in the gaseous phase above the ruminal fluid. Additionally, it must be pointed out that *in vivo*, the ruminal fluid is not in equilibrium, caused by feed intake and fermentational processes, resulting in variable pH and pCO_2 values depending on time and place measured in the rumen (Tafaj et al., 2004). The local variability of pCO_2 is almost certainly the major reason why in the study of Laporte-Uribe, dCO_2 could not be predicted from pCO_2 as measured in the gas phase above the ruminal mat. Conversely, and since the value of α is almost identical in ruminal fluid (Hille et al., 2016), Ringer solution (van Slyke, 1922), and plasma, it is not expected that changes in α can account for the large variations of dCO_2 found in the rumen *in vivo* as suggested (Laporte-Uribe, 2016).

The importance of CO_2 on the rumen epithelial health and integrity was demonstrated in a recent study (Rackwitz and Gäbel, 2018). The authors investigated the effect of SCFA and low pH values on the conductance and short circuit current in the presence and absence of CO_2. They concluded that at pH values of 5.5 and 5.0 the amount of HSCFA entering the epithelial cells increases considerably, resulting in an intracellular acidification with a subsequent increase in conductance, which indicates an impaired ruminal barrier function. This effect was diminished in the presence of CO_2 in the buffer solutions. CO_2 can permeate the epithelial membrane and dissociate into HCO_3^- and H^+, which will acidify the cytosolic space, but also increase its buffer capacity and thus, the resilience of the epithelium towards addition of further acid. Furthermore, the accumulation of HCO_3^- may lead to an increase in the absorption of $SCFA^-$ in exchange for HCO_3^- via DRA or PAT1 (see Figure 2). This can eventually buffer the apical microclimate and thus reduce the driving force for the diffusive absorption of HSCFA. It was concluded that CO_2 has a protective role in counteracting the SCFA induced epithelial damage at low pH values.

The review and the original research article summarized above proposed the physically dissolved CO_2, dCO_2 as a parameter which should be evaluated to assess the severity of ruminal acidosis. The concept of dCO2 and the determination of ruminal base excess (introduced in Hille et al., 2016) both target the buffer capacity of the CO_2/HCO_3^- system and its contribution to pH buffering in ruminal fluid and the epithelium. Further investigations are needed to evaluate the application potential on the diagnosis of SARA and its severity.

Ruminal acidosis remains a clinical problem which is mainly induced by feed compositions that are not appropriate for ruminants. The high amounts of easily digestible carbohydrates pose a severe challenge for ruminal buffer capacity and epithelial transport mechanisms. A deeper understanding of the impact of SARA not just on microbial populations but also epithelial transport mechanisms and epithelial integrity is needed to prevent harmful conditions for the animal (Aschenbach et al., 2011; Greco et al., 2018; Lu et al., 2014a; Plaizier et al., 2017).

Ammonia Absorption and its Contribution to Nitrogen and pH Homeostasis

Various studies of the ruminal epithelium and isolated ruminal cells have revealed that the absorption of ammonia from ruminal fluid occurs in the form of NH_4^+ (Abdoun et al., 2005; Bödeker and Kemkowski, 1996; Rosendahl et al., 2016), resulting in a net acid absorption

which alleviates the acid load of ruminal fluid. This finding clearly highlights the positive effect of rumino-hepatic circulation. The absorption of ammonia by the epithelium limits the amount of ammonia in the ruminal fluid and thus ensures non-toxic levels to optimize microbial fermentation. The hepatic conversion to urea with subsequent re-secretion results in a net removal of protons from the rumen but also warrants sufficient nitrogen supply for microbial protein synthesis. The rumino-hepatic circulation hence optimizes the homeostasis of nitrogen. The concomitant absorption of protons represents a further benefit for ruminal pH homeostasis.

Usually cattle have slightly alkalotic blood pH values, which is caused by the high amounts of K^+ and Na^+ absorbed from their feed. In the feedstuff, these "strong" cations are associated with organic anions (e.g. aconitate$^-$) balancing the charge, so that the feed and the ruminal fluid are electrically neutral. These organic anions are fermented to $SCFA^-$, which are absorbed and metabolized to HCO_3^-, again conserving the charge. This explains the metabolic alkalosis of the ruminant. The protons absorbed together with NH_4^+ are partially released after entering the blood stream and can thus reduce systemic base excess.

In this context, it is interesting to note that in mammals, the glutamine synthetase in hepatocytes is activated when plasma pH levels decrease. Acidosis thus increases the conversion of glutamate and NH_4^+ to glutamine, which can be used for energy and protein metabolism (Taylor and Curthoys, 2004). Whether or not this effect is of relevance in cattle has yet to be shown, but it is intriguing to speculate that a shift in the absorption of ammonia away from NH_3 towards NH_4^+ may lead to a shift away from the production of the waste product urea and towards more production of glutamine, an important precursor of many non-essential amino acids.

The studies presented above demonstrate the contribution of the bTRPV3 channel to the absorption of NH_4^+, which was the first molecular identification of a NH_4^+ transporting protein in the rumen epithelium (Schrapers et al., 2018). Further channels expressed in rumen like TRPA1 and TRPV4 may also contribute to NH_4^+ absorption. Since a number of TRP channels can be specifically modulated by applying various monoterpenes, further research is needed to understand the role of TRP channels in nitrogen metabolism with further implications on the absorption of ammonium by the GIT of monogastric species. Additionally, our findings may have implications for the skin, which like the rumen, is a squamous keratinized epithelium and which was shown to express TRPV3 (Xu et al., 2006). This new field of research offers diverse possibilities for the regulation of nitrogen retention

and absorption with possible effects on nitrogen homeostasis, excretion, and environmental pollution.

TRP Channels – an Unknown Player Amongst Ruminal Transport Mechanisms

Investigating the molecular basis of the functionally described non-selective cation channels (NSCC) (Leonhard-Marek et al., 2010), studies on isolated ruminal epithelium uncovered the involvement of TRP channels on cation absorption (Rosendahl et al., 2016; Schrapers et al., 2018). TRP channels are found ubiquitously in diverse species including the model species nematode (*Caenorhabditis elegans*), fruit fly (*Drosophila melanogaster*) and mouse (*Mus musculus*).

The 28 different TRP channels found in mammals are divided into six subfamilies: TRPA (Ankyrin), TRPC (Canonical), TRPM (Melastatin), TRPML (Mucolipin), and TRPV (Vanilloid), TRPP (Polycystin). All TRP proteins are composed of six transmembrane domains and residues protruding into the intracellular matrix which are responsible for their distinct activation mechanisms. Four TRP proteins assemble to form a channel around a pore which serves as a selectivity filter for different cations (Nilius and Voets, 2005; Owsianik et al., 2006). Usually, four identical TRP proteins form a pore region, but some members of the TRP protein family can form heteromeres with intermediate characteristics (Cheng et al., 2012).

TRP channels were first described in sensory systems. Some are associated with temperature sensing and possess distinct activation thresholds; e.g. TRPV2 is activated at severe heat (> 52°C), TRPV1 at intermediate heat (>43°C), TRPV3 at moderate warmth (30-39°C) and TRPA1 is activated at noxious cold (<17°C) (Venkatachalam and Montell, 2007). TRPV1 is a classical pain sensor which is activated by heat, yet it also reacts to perturbances of the pH level (acetic or alkaline) and to capsaicin, the active ingredient of chilies (reviewed in Nilius and Szallasi, 2014). Furthermore, some TRP channels are activated by alkaloids or terpenes like menthol, citral or vanillin (Nilius and Appendino, 2013).

While recent research on TRP channels mainly focuses on their contribution to sensation and pain, TRP channels have been found to be expressed in numerous other tissues including keratinocytes, the central nervous system, the kidneys, the reproductive system and the GIT with numerous different functions (Holzer, 2011a; Vriens et al., 2009). Certain members of the TRP family are mainly involved in the homeostasis of calcium and magnesium: thus

TRPV5 and TRPV6 mediate the absorption of calcium by the intestine and in the kidney (den Dekker et al., 2003; Nilius et al., 2000; Peng et al., 1999) while TRPM6 and TRPM7 are Mg^{2+} selective channels (Nadler et al., 2001; Schweigel et al., 2008).

In the present studies, the involvement of TRP channels on cation absorption in the rumen was demonstrated on a molecular as well as a functional level (Rosendahl et al., 2016; Schrapers et al., 2018). The bovine TRPV3 was shown to contribute to the absorption of NH_4^+, Na^+, Ca^{2+} and possibly Mg^{2+}.

Calcium Absorption in the Rumen

Calcium is a key element in animal nutrition. Apart from its structural importance in the skeleton, it is an important second messenger in practically every cell of the body, mediating diverse responses that range from muscle contraction to apoptosis (Kimura et al., 2006; Trevisi and Minuti, 2018). Especially after parturition, large amounts of calcium need to be mobilized in cows for colostrum and milk production. Decreased blood calcium levels are directly associated with milking periods (Littledike and Goff, 1987). In principle large amounts of Ca^{2+} can be mobilized from the bone under the influence of parathyroid hormone (PTH). However, due to the metabolic alkalosis of the ruminant, this mechanism is suppressed, making cows highly susceptible to hypocalcemia during early lactation. The amount of calcium absorbed by the rumen mainly depends on the supply of calcium from the feed offered as shown by Khorasani and coworkers (1997). Cows which were fed a low calcium diet (115g/day) showed a net secretion into the rumen of -0.9 g/day. In the same study, the supply of a high calcium diet with 231 g/day resulted in a calcium net absorption of 49.8 g/day. The absorption from the small and large intestine was not influenced by the amount of dietary calcium ranging from 24.6 to 36.8g/day (Khorasani et al., 1997).

Calcium absorption across the rumen was shown to occur in a transcellular and a paracellular manner (Schröder et al., 2015; Wilkens et al., 2016). In Ussing chamber experiments using isolated ruminal tissue, the presence or absence of other anions and cations in the buffer had a high impact on the transport of calcium (Schröder et al., 1997). In the presence of SCFA, the absorption of calcium was increased compared to other anions (e.g. $gluconate^-$) present in the mucosal buffer (Leonhard-Marek et al., 2007). The authors suggested an elevated transport of calcium via a Ca^{2+}-H^+- exchanger. An alternative explanation is the activation of TRP channels by acidification or cell swelling (Chokshi et al., 2012; Vriens et al., 2004).

Basolaterally, calcium is either actively exported by a Ca^{2+}-ATPase PMCA1 (Schröder et al., 2015) or passively in exchange for sodium.

Hypocalcemia in High Yielding Dairy Cows

Under optimal feeding conditions, the calcium intake from feed and the absorption in the GIT is sufficient. Calcium homeostasis is mainly challenged directly after calving. In non-dairy cows, the calcium required for fetal development is roughly identical to the amount required for the production of colostrum and milk after parturition with the amounts required increasing only gradually thereafter (Ramberg Jr et al., 1984). Conversely, high yielding dairy cows require a rapid increase in calcium immediately after parturition, which exceeds the amount of calcium available in blood. Only 3 to 4 g of calcium are available in the blood of a cow with a healthy serum calcium level of 2.25 mM (Goff, 2008). Yet, a daily supply of more than 40g of calcium is needed to maintain homeostasis in order to produce 40 liters of milk with a calcium content of about 1.1 g/l (Lindmark-Månsson et al., 2003). The tremendous gap between the required amount of calcium and freely available calcium in the blood needs to be filled for the maintenance of calcium homeostasis. Calcium can be mobilized both from the bone and increased absorption from the GIT, regulated by parathyroid hormone and by 1.25-dihydroxy vitamin D3 (Horst et al., 1994; Weiss et al., 2015).

If the shortage cannot be compensated, the serum calcium level decreases and subclinical or clinical hypocalcemia may occur. When serum calcium concentration drops below the critical level of 1.4 mM, the threshold for clinical hypocalcemia or milk fever is reached (DeGaris and Lean, 2008). The reduced calcium level impairs neuro-muscular transmission and induces parturient paresis, which is usually fatal (DeGaris and Lean, 2008). When serum calcium concentration drops below 2 mM, subclinical hypocalcemia occurs with a strong impact on the animals health: subclinical hypocalcemia is associated with an impaired immune function (Martín-Tereso and Martens, 2014), diseases associated with muscle weakness like replaced abomasum, mastitis, metritis, retained placenta (Goff, 2008; Riond, 2001), and a reduced reproductive performance (Caixeta et al., 2017). In mild cases, the low level of serum calcium is usually compensated for within 3 to 5 days after parturition; the release of PTH induces the mobilization of calcium from the bone (Martín-Tereso and Verstegen, 2011; Suzuki et al., 2008) and calcium homeostasis is adapted to the calcium demand for milk production.

There are different strategies to prevent hypocalcemia. Feeding low level calcium diets *ante partum* supports the mobilization of calcium from bone tissue decreasing the time for calcium

mobilization after calving. The feeding strategy of a dietary cation anion difference (DCAD) *ante partum* induced metabolic acidosis, which activates the release of PTH and thus helps to release calcium from the bone and reduces the severity of calcium shortage (Martín-Tereso and Martens, 2014; Wilkens et al., 2016).

After calving, calcium supplementation is used in many farms to decrease the risk of hypocalcemia. Despite different prevention strategies, the risk of subclinical hypocalcemia remains very high. Recent studies suggested an probability of 40 to 50 % for multiparous cows in German and US farms, respectively (Reinhardt et al., 2011; Venjakob et al., 2017). In the latter study hypocalcemia was associated with the high risk of secondary diseases, even in farms using the above-mentioned prevention strategies. Understanding the mechanism of calcium absorption by the ruminal epithelium is required to gain an understanding of factors that might mitigate the gap between calcium demand and supply after parturition.

Involvement of TRP Channels in Ruminal Calcium Absorption

The classical calcium transporters are TRPV5 and TRPV6 and are mostly expressed in kidney- and gut tissue, respectively (Suzuki et al., 2008). Unexpectedly, previous studies, including our own study, have failed to demonstrate the expression of these channels by the rumen (Rosendahl et al., 2016; Wilkens et al., 2009). Our study on the native isolated ruminal epithelium confirmed an increased calcium absorption when the known TRP agonists menthol and thymol were applied to the mucosal side. In order to elucidate the involvement of TPRV3 in calcium absorption, we overexpressed bTRPV3 and examined its calcium conductance (Schrapers et al., 2018). We could demonstrate that TRPV3 displays a conductance of ~ 35 pS for calcium. Moreover, intracellular calcium imaging showed an increased intracellular calcium level when TRPV3 was activated with menthol. Applying magnesium to the bath solution initially decreased the intracellular calcium concentration, but it recovered to former levels. These findings imply an interaction between magnesium and calcium ions, possibly as summarized in Figure 5.

Already in the first studies on mineral absorption, Care and coworkers showed that there was a negative correlation between magnesium absorption and luminal calcium concentration (Care et al., 1984).

Involvement of TRP Channels in Ruminal Magnesium Absorption

Magnesium is an essential mineral. In cattle, magnesium homeostasis depends primarily on efficient absorption by the rumen (Martens et al., 1976). One of the present studies (Rosendahl et al., 2016) confirmed the expression of the classical magnesium transporting TRP channel TRPM7 in ruminal epithelium as shown previously (Schweigel et al., 2008). Additionally, the expression of the magnesium transporting channel TRPM6 was demonstrated for the first time in ruminal tissue. However, additional TRP channels may be involved in the ruminal absorption of magnesium. Single channel patch clamp measurements of bTRPV3 revealed a conductance for magnesium as depicted below (Figure 4). These preliminary measurements clearly need further confirmation but are interesting indeed since unlike TRPM6 and TRPM7, TRPV3 can be activated by various agonists which might enhance magnesium absorption by the ruminal epithelium.

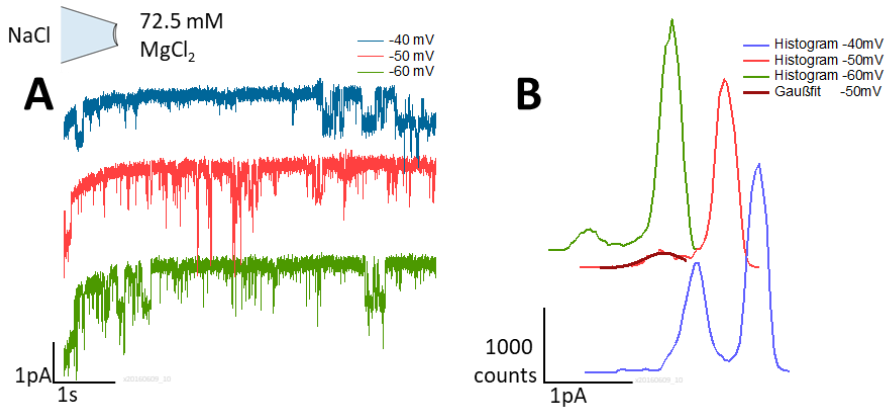

Figure 4: single channel recordings of bTRP3 with NaCl in the pipette solution and 72.5 mM $MgCl_2$ in bath solution. Single channel opening events were visible at negative potentials (-60mV to -40mV) (A). Single channel conductance was estimated using histograms resulting a conductance to magnesium of ~ 35 pS (B).

Involvement of TRP Channels in Cation Absorption – Concluding Remarks

The presented studies clearly displayed the involvement of bTRPV3 on ruminal absorption of monovalent cations such as NH_4^+, Na^+, K^+, Ca^{2+} and possibly also Mg^{2+} (Rosendahl et al., 2016; Schrapers et al., 2018). These were the first studies to determine the molecular identity of a channel mediating the absorption of Ca^{2+} and NH_4^+ in ruminal epithelium with multiple

implications for our understanding of ruminal function and posing and questions for future research. The results are summarized in Figure 5.

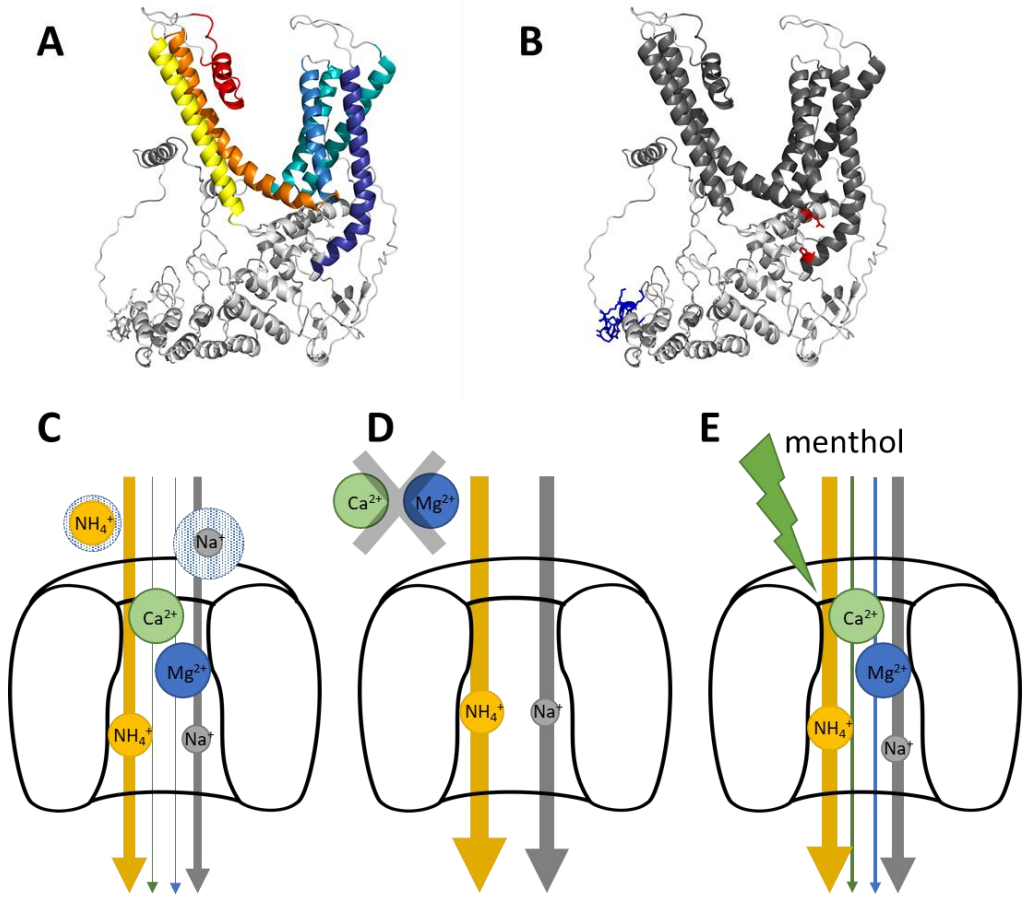

Figure 5: model of the structure and function of bTRPV3: A) cartoon of bTRPV3 with its six membrane spanning helices (coloured) and the pore region (red). Intracellular portions of the proteins are depicted in grey. Four of these subunits form one channel mediating the transport of cations. B) The APB-2-binding side (red) and calmodulin binding side (blue) are highlighted. 3-D models were assigned from the bTRPV3 sequence XM_015458625.1 and visualized by RaptorX (Källberg et al., 2012). Panels C, D and E attempt to visualize the transport characteristics of a homomer of four bTRPV3 subunits using a simplified model. C) In the presence of divalent cations, both Na^+ and NH_4^+ are transported accompanied by a smaller amount of Ca^{2+} and Mg^{2+}. The conductance is heavily influenced both by the energy required to strip the hydration shell (blue circle) and by interaction between monovalent and divalent cations in the pore. D) In the absence of divalent cations, the transport of monovalent cations increases since the larger ions Ca^{2+} and Mg^{2+} do not interfere with the channel pore. E) The activation of TRPV3 by agonists like menthol very rapidly increased the whole cell

conductance of bTRPV3. Since the pipette solution was heavily buffered, this suggests direct effects on the open probability and/or conductance of pre-existing channels. The arrow strength corresponds to the conductance of cations, the size of ion circle depicts the size and mobility of the different cations. For A-E) channels are depicted with extracellular side on top and intracellular side on the bottom

Potential Interactions Between Ammonia and Calcium Absorption

Due to a plant based feed rich in organic anions bound to strong cations such as Na^+ and K^+, herbivores tend towards metabolic alkalosis. As mentioned, this impairs the availability of calcium negatively since an alkalotic blood pH decreases the secretion of PTH and prevents the mobilization of calcium from the bone needed for milk synthesis. If a significant amount of ammonia is absorbed in the form of protonated NH_4^+, it will result in a net transport of 1 mole of H^+ per mole ammonia resulting in a slight acidification of blood with implications on PTH release.

Recent studies on growing goats suggest a further interaction between nitrogen and calcium homeostasis. The impact of reduced nitrogen and calcium was assessed regarding calcium absorption, blood calcium level, and bone formation (Elfers et al., 2015, 2016). Reducing calcium in feed did not impact the calcium level in the blood, because it was compensated by increased bone resorption and intestinal calcium absorption triggered by the expected regulation mechanism of PTH and calcitriol levels. When nitrogen supply was reduced, the result significantly changed: blood calcium level decreased, calcium absorption from the jejunum decreased and the calcitriol level decreased in parallel. The bone resorption marker osteocalcin was strongly increased but none of those mechanisms could restore the blood calcium levels. The authors concluded that there is a negative impact of a reduced nitrogen supply on calcium homeostasis. It is intriguing to speculate that the link between both absorption mechanisms could possibly be represented by the TRP channels which mediate both the absorption of NH_4^+ and calcium. Until now, the regulation of TRPV3 expression levels in the ruminal epithelium has not been investigated, but high levels of ruminal ammonium might induce the expression of TRP channels not only in the rumen, but possibly also in other parts of the gut. This hypothesis very clearly merits further investigation, but studies along these lines may contribute to a better understanding of the interactions of these regulatory mechanisms.

Furthermore, studies summarized in this thesis (Rosendahl et al., 2016; Schrapers et al., 2018) show that TRPV3 can be activated by various chemical compounds including menthol, thymol, carvacrol and others. These essential oils are found in herbs that are partially present in the feed of grazing sheep and cattle. These compounds can be added to performance oriented TMR (total mixed ratio) and may increase the absorption of calcium, magnesium and nitrogen. In addition, the above mentioned essential oils can be added to feed in order to alter ruminal fermentation (Oh et al., 2017). Accordingly, the addition of specific essential oils to performance oriented diets might improve mineral homeostasis and nitrogen utilization at the same time.

The Current Cation Absorption Model in the Ruminal Epithelium

This study provides novel insights into the transport proteins involved in the absorption of cations by the ruminal epithelium. Based on a study of the effects of essential oils on native isolated ruminal epithelium (Rosendahl, 2014), clear evidence for the capacity of the bovine representative of TRPV3 to transport NH_4^+ is shown (Schrapers et al., 2018). This is the first time that a member of the TRP channel family has been implicated in the transport of ammonium as an important product of amino acid metabolism. It should be stressed, however, that alternative pathways may contribute to NH_3/ NH_4^+ absorption. This includes other TRP channels, in particular TRPA1 and TRPV4, which have been demonstrated on the level of mRNA in ruminal epithelium (Rosendahl et al., 2016), but also rhesus proteins or aquaporins, which mediate ammonia transport in the renal epithelium (Weiner and Verlander, 2017).

In addition, bTRPV3 emerges from this study as a prime candidate for the uptake of Ca^{2+} not just in overexpressing cells (Schrapers et al., 2018), but also in by the intact isolated ruminal epithelium (Rosendahl et al., 2016). Given that this pathway has long remained obscure (Wilkens et al., 2009), this finding has important implications for understanding calcium absorption in ruminants. The non-selective nature of the channel also facilitates the absorption of Na^+, K^+ and possibly even Mg^{2+} (Schrapers et al., 2018). The regulatory mechanisms behind the expression level and regulation of the activity of TRPV3 are still awaiting investigation.

Furthermore, this study presents clear evidence for the involvement of bTRPV3 in the response of native ruminal epithelium to essential oils such as menthol or thymol. Consecutive studies should evaluate a possible positive impact of the feeding of essential oils on the homeostasis of calcium and nitrogen.

Summary

A functional and molecular study of mechanisms contributing to the removal of protons from the rumen.

The rumen of cattle and sheep is a large fermentation chamber where symbiotic microbiota break down otherwise indigestible fibrous components of feed to short chain fatty acids (SCFA), which represent the most important energy source for ruminants. To obtain optimal conditions for ruminal fermentation, ruminal fluid needs to be buffered sufficiently to avoid acidic conditions, which involves buffering by bicarbonate (HCO_3^-) entering the rumen with saliva. Surprisingly, however, the buffering constants of the bicarbonate system have never been determined in ruminal fluid, compromising the ability to estimate the contribution of this system to ruminal buffering.

Furthermore, ammonia can accumulate to toxic levels in the rumen due to fermentational deamination of protein and non-protein nitrogen. In part, this ammonia can be incorporated into high-grade microbial protein, but amounts exceeding microbial capacity must be absorbed from the rumen, hepatically detoxified and renally excreted, with detrimental consequences both for the protein efficiency of the ruminant and for the environment. Therefore, interest in identifying and characterizing the efflux pathway for ammonia from the rumen is considerable and enhanced by the fact that ruminal efflux of ammonia has long been known to involve absorption of the ionic form (NH_4^+) through a cation channel, thus contributing to elimination of protons and ruminal pH homeostasis.

The present thesis aimed to answer two unsolved questions regarding to ruminal buffering:

1. What are the buffering constants for the bicarbonate buffering system in ruminal fluid?
2. What is the molecular identity of the transport protein that mediates the absorption of NH_4^+ from the rumen?

The first part of the thesis focussed on the determination of the buffering constants of the bicarbonate system in ruminal fluid (Paper 1, Hille et al., 2016). In this open buffer system, ruminal protons are mainly buffered by HCO_3^- that enters the rumen with saliva or via secretion of the ruminal epithelium. To calculate the buffer capacity of ruminal fluid, the solubility of CO_2 (α) and the equilibrium constant of CO_2/HCO_3^- (pK) needed to be known, neither of which have previously been determined for ruminal fluid. Accordingly, ruminal fluid from cattle fed either hay or concentrate diets was investigated using both the classical Astrup technique and a newly developed titration technique. In both feeding scenarios, values were found to be similar to the values known for Ringer solution and human blood. The data

show that $SCFA^-$ and HCO_3^- are the primary buffers in ruminal fluid and that at physiological levels of ruminal pH, HCO_3^- is almost completely converted to CO_2, which leaves with eructation. This mechanism ensures both an efficient elimination of protons from the rumen, and the maintenance of the concentration gradient driving HCO_3^- secretion across the ruminal epithelium. Finally, the concept of base excess was introduced for ruminal fluid for the first time.

The purpose of the second part of the present thesis was to identify and characterize possible transport proteins mediating the absorption of ammonium by the ruminal epithelium. Recent work shows that *in vitro*, ruminal transport of Na^+ and NH_4^+ can be stimulated by certain agonists of the transient receptor potential family (TRP), and that the ruminal epithelium expresses mRNA for the bovine analogue of TRPV3 (bTRPV3) as a suitable candidate gene. The current thesis demonstrates that these modulators also stimulate the absorption of the essential nutrient Ca^{2+} in isolated ruminal epithelium in a dose dependent manner (Paper 2, Rosendahl et al., 2016). In a second step, the bTRPV3 channel was overexpressed in HEK-293 cells and characterized via the whole cell and single channel configuration of the patch clamp technique and via intracellular calcium imaging (Paper 3, Schrapers et al., 2018). It emerged that this channel reflects the properties of the conductance found in the native ruminal epithelium: the bTRPV3 conducts various cations, and the conductance of monovalent cations is modulated by the divalent cations and by TRPV3 agonists such as menthol and thymol. The conductance of different cations was measured using both the whole cell and single channel patch clamp technique, which revealed decreasing permeabilities in the order of $NH_4^+ > Na^+ > Mg^{2+} > Ca^{2+} > NMDG^+$. Furthermore, the application of various TRPV3 agonists increased Na^+, NH_4^+ and K^+ currents in the whole cell patch clamp configuration. Using intracellular calcium imaging, an increase in calcium influx after the addition of menthol could subsequently be found. The bovine TRPV3 should thus play an important role in mediating the ruminal transport of physiologically relevant cations such as Na^+, K^+, NH_4^+ and Ca^{2+}.

In summary, this thesis

- determined the buffering constants for the bicarbonate buffer system in ruminal fluid for the first time,
- demonstrated that TRP channel agonists can stimulate transport of Ca^{2+} across the native ruminal epithelium *in vitro* and
- characterized the bovine representative of TRPV3 as an ion channel suitable for mediating the transport of Na^+, K^+ and NH_4^+ and Ca^{2+} across the rumen.

Zusammenfassung

Funktionelle und molekulare Untersuchung von Mechanismen, welche zur Entfernung von Protonen aus dem Pansen beitragen

Der Pansen von Rindern und Schafen ist eine große Fermentationskammer, in der symbiotische Mikrobiota sonst unverdauliche Faserbestandteile des Futters zu kurzkettigen Fettsäuren (SCFA) abbauen. Diese stellen die wichtigste Energiequelle für Wiederkäuer dar. Um optimale Bedingungen für die Fermentation sicherzustellen, muss die Pansenflüssigkeit ausreichend gepuffert werden. Dies geschieht durch die Sekretion von Bicarbonat (HCO_3^-), das über das Pansenepithel oder den Speichel in den Pansen gelangt. Bisher fehlt jedoch eine Bestimmung der Pufferkonstanten des Bikarbonat-Puffersystems in der Pansen-flüssigkeit, so dass der Beitrag des Systems zur Pufferung des Pansens nicht abgeschätzt werden kann.

Ferner kann sich Ammoniak durch die fermentative Desaminierung von Protein und Nicht-Protein-Stickstoff im Pansen anreichern, wobei toxische Konzentrationen erreicht werden. Ein Teil dieses Ammoniaks kann in hochwertiges mikrobielles Protein eingebaut werden. Überschreitet die Ammoniakkonzentration aber die mikrobielle Kapazität, muss Ammoniak aus dem Pansen resorbiert, in der Leber entgiftet und renal ausgeschieden werden. Dieses hat negative Folgen sowohl für die Proteineffizienz des Wiederkäuers als auch für die Umwelt, weshalb die Identifizierung und Charakterisierung des Resorptionsweges von Ammoniak bedeutend ist. Es ist seit langem bekannt, dass der Efflux von Ammoniak aus dem Pansen als Ion (NH_4^+) durch einen Kationenkanal erfolgt, wodurch Protonen eliminiert und die pH-Homöostase im Pansen gefördert wird.

Mit der vorliegenden Arbeit sollen zwei ungelöste Fragen, die für die Pansenpufferung relevant sind, beantwortet werden:

1. Was sind die Pufferkonstanten für das Bikarbonat-Puffersystem in der Pansen-flüssigkeit?
2. Welche molekulare Identität hat das Transportprotein, welches die Resorption von NH_4^+ aus dem Pansen vermittelt?

Für den ersten Teil der Arbeit wurden Pufferkonstanten des Bikarbonatsystems in der Pansenflüssigkeit bestimmt (Paper 1, Hille et al., 2016). In diesem offenen Puffersystem werden die Protonen hauptsächlich durch HCO_3^- gepuffert, welches mit dem Speichel in den Pansen gelangt oder vom Pansenepithels sezerniert wird. Um die Pufferkapazität der Pansenflüssigkeit zu berechnen, musste die Löslichkeit von CO_2 (α) und die Gleichgewichtskonstante von CO_2/HCO_3^- (pK) bekannt sein. Beide Konstanten wurden bisher nicht für die Pansenflüssigkeit bestimmt. Für diese Studie wurde Pansenflüssigkeit von mit

Heu- oder Kraftfutter-gefütterten Rindern verwendet. Mittels der klassischen Astrup-Technik und einer neu entwickelten Titrationstechnik wurden die Konstanten α und pK bestimmt. Bei Proben aus beiden Fütterungsregimen wurden Werte ermittelt, die den für Ringerlösung und menschliches Blut bekannten Werten ähnlich sind. Die Daten zeigen, dass $SCFA^-$ und HCO_3^- die primären Puffer in der Pansenflüssigkeit sind und dass HCO_3^- bei physiologischen pH-Werten fast vollständig in CO_2 umgewandelt wird, welches den Pansen durch Ruktus verlässt. Dieser Mechanismus gewährleistet sowohl eine effiziente Entfernung von Protonen aus dem Pansen als auch die Aufrechterhaltung des Konzentrationsgradienten, der die HCO_3^--Sekretion über das Pansenepithel antreibt. Schließlich wurde erstmals das Konzept der Basenabweichung für Pansenflüssigkeit vorgeschlagen.

Das Ziel des zweiten Teils der vorliegenden Arbeit war es, mögliche Transportproteine zu identifizieren und zu charakterisieren, die die Absorption von Ammonium über das Pansenepithel vermitteln. Vorherige Arbeiten zeigten, dass in isoliertem Pansenepithel der Transport von Na^+ und NH_4^+ durch Agonisten der transienten Rezeptorpotentialfamilie (TRP) stimuliert werden kann, während molekularbiologische Untersuchungen die Expression des bovinen TRPV3 (bTRPV3) im Pansenepithel als geeignetes Kandidatengen belegen.

Im Rahmen der vorliegenden Dissertation konnte zusätzlich gezeigt werden, dass die Agonisten die Absorption von Ca^{2+} im isolierten Pansenepithel dosisabhängig stimulieren (Paper 2, Rosendahl et al., 2016). Im Weiteren wurde der bTRPV3-Kanal in HEK-293-Zellen überexprimiert und mittels whole-cell und single-channel Konfiguration der Patch-Clamp-Technik und intrazellulärem Calcium-Imaging charakterisiert (Paper 3, Schrapers et al., 2018). Es stellte sich heraus, dass dieser Kanal die Eigenschaften zur Kationen-Leitfähigkeit des nativen Pansenepithels widerspiegelt: der bTRPV3 leitet verschiedene Kationen und die Leitfähigkeit für monovalente Kationen wird durch divalente Kationen und durch TRPV3-Agonisten wie Menthol und Thymol moduliert. Die Leitfähigkeit verschiedener Kationen wurde mit Hilfe der whole-cell und der single-channel Patch-Clamp-Technik gemessen. Dabei zeigte sich, dass die Permeabilität der Kationen in der Reihenfolge $NH_4^+ > Na^+ > Mg^{2+} > Ca^{2+} > NMDG^+$ abnimmt. Weiterhin steigerte die Applikation von TRPV3-Agonisten die Na^+-, NH_4^+- und K^+-Ströme bei whole-cell Patch-Clamp-Messungen. Mittels intrazellulärem Calcium-Imaging konnte zusätzlich gezeigt werden, dass die Applikation von Menthol einen Calciumeinstrom erzeugt. Der bovine bTRPV3 dürfte eine bedeutende Rolle bei der Absorption von physiologisch wichtigen Kationen wie Na^+, K^+, NH_4^+ und Ca^{2+} aus dem Pansen spielen.

Zusammenfassend wurde im Rahmen dieser Dissertation

- erstmalig die Pufferkonstanten für das Bikarbonat-Puffersystem in der Pansenflüssigkeit bestimmt,
- gezeigt, dass TRP-Kanal-Agonisten den Transport von Ca^{2+} über das native Pansenepithel *in vitro* stimulieren können und
- der bovine TRPV3 als geeignetes Kanalprotein charakterisiert, um den Transport von Na^{+}, K^{+}, NH_4^{+} und Ca^{2+} über das Pansenepithel zu vermitteln.

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