Institute of Veterinary Physiology
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Modulation of Na and acetate transport by heat shock proteins (Hsp) across the rumen epithelium of sheep

Thesis submitted for the partial fulfillment of a doctoral degree in Veterinary Medicine (Dr. med. vet.) at the Free University of Berlin

submitted by
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Aus dem Institut für Veterinär-Physiologie
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Dedicated to

My parents for their continuous support

and unselfish love

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Abbreviations

AE	Anion exchanger
cAMP	Cyclic adenosine monophosphate
ANOVA	Analysis of variance
CA	Carbonic anhydrase
CHO	Carbohydrate
CHX	Cycloheximide
CLA	Conjugated linoleic acid
DMSO	Dimethylsulfoxide
ENaC	Epithelial sodium channel
ER	Endoplasmic reticulum
cGMP	Cyclic guanosine monophosphate
G_t	Tissue conductance ($mS \cdot cm^{-2}$)
HSCFA	Undissociated short chain fatty acids
Hsc	Heat shock cognate protein
HSE	Heat shock element
HSF	Heat shock factor
Hsp	Heat shock proteins
IDV	Integrated density values
IREC	Isolated rumen epithelial cells
I_{sc}	Short circuit current ($\mu eq \cdot cm^{-2} \cdot h^{-1}$)
J_{ms}	Unidirectional ion transport in mucosal to serosal direction ($\mu eq \cdot cm^{-2} \cdot h^{-1}$)
J_{sm}	Unidirectional ion transport in serosal to mucosal direction ($\mu eq \cdot cm^{-2} \cdot h^{-1}$)
J_{net}	Net-transport of Ions ($\mu eq \cdot cm^{-2} \cdot h^{-1}$)
LPS	Lipopolysaccharide
MCT	Monocarboxylate transporter
MSD	Membrane spanning domain
N	Number of experimental animals
NBC	Sodium bicarbonate co-transport
NSCC	Non selective cation conductance
n	Number of tissues/epithelia
NHE	Na^+/H^+-exchanger
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffer saline

PD_t	Transepithelial potential difference (mV)
PKA	Proteins kinase A
PKCα	Protein kinase C alpha
R_c	Cellular resistance
R_s	Shunt resistance (paracellular resistance)
R_t	Tissue resistance
SARA	Subacute ruminal acidosis
S3226	3-[2-(3-guanidino-2-methyl-3-oxo-propenyl)-5-methyl-phenyl]-N-isopropylidene-2-methyl-acrylamide dihydrochloride
SCFA	Short chain fatty acids
SDS	Sodium dodecyl sulphate
SLC	Solute carrier
$\bar{x}$	Arithmetic mean

Chapter 1

General Introduction and literature review

1.1 Introduction:

The rumen is the largest compartment of the forestomachs in ruminants and provides an environment conducive for the complex ecosystem that exists to facilitate the digestion of forage. The highly characteristic digestive physiology of the rumen has attracted the attention of researchers all over the world, and numerous studies have been devoted to the motility of the reticulorumen, its regulation, and its effect on digestion. Similarly, the biochemical aspects of microbial digestion and its consequences for nutrition and metabolism in ruminants have been thoroughly studied. Furthermore, the forestomach epithelia exhibit a variety of transport mechanisms for ions and nutrients.

The reticulorumen is subdivided into several compartments by various folds of its wall; this has an effect of increasing the surface area contacting the digesta. The surface area of the rumen is further increased by the presence of numerous conical or tongue-shaped papillae. The distribution, size, and number of these papillae depend on the species and on diet. The papillae and the entire inner rumen wall are covered with a mucous membrane composed of a keratinized multilayered epithelium and a richly vascularized connective tissue, crossed by nerve fibers and lymph ducts. The epithelium is composed of four cell layers: the stratum basale, the stratum spinosum, the stratum granulosum, and the stratum corneum. The cells of the stratum basale appear to be the metabolically most active since they contain numerous mitochondria and free ribosomes (Steven and Marshall, 1972). A large proportion of the assimilation and metabolism of substances absorbed from the rumen thus occurs in this cell layer.

Ruminants acquire the main fraction of their energy from short chain fatty acids (SCFA), which are the major product of microbial fermentation (Bergman, 1990; Calsamiglia *et al.*, 2007) and constitute 50-75% of the energy supply (Faverdin, 1999), whereas amino acids are obtained from microbial protein synthesized in the rumen and from feed proteins that escape ruminal degradation. The mucosa of the reticulorumen is well known to possess an absorptive function as, according to Bruce *et al.* (1966), about 90% of the digested cellulose disappears from the stomach, whereas the remainder is fermented and taken up from the large intestines. However, significant progress has only recently been made in the quantitative evaluation of the absorption processes, through the use of the isotopic dilution technique and the development of methods for ruminal blood flow measurement and ruminal vein catheterization (Remond *et al.*, 1993), which make it possible to measure net fluxes of metabolites across the ruminal wall. The parallel use of *in vitro* techniques (Martens and

Harmeyer, 1978) has generated essential information for understanding the mechanisms involved in these transepithelial transport mechanisms.

SCFA produced by microbial fermentation are known to cause a drop in the pH of the rumen and ruminal epithelium (Gabel *et al.*, 1991b; Rowe *et al.*, 1993); this drop in pH is particularly rapid when the animals are concentrate-fed after being starved or without prior adaptation (Goad *et al.*, 1998; Owens *et al.*, 1998). One consequence of feeding excessive amounts of rapidly fermentable carbohydrates to ruminants, in conjunction with inadequate fiber, is sub-acute ruminal acidosis (SARA), which is characterized by high concentrations of SCFA, periods of low ruminal pH, depressed feed intake, and subsequent health problems (Nagaraja and Titgemeyer, 2007). Dairy cattle, feed-lot cattle, and feed-lot sheep are at high risk for developing this condition with an incidence of over 20%. SARA is a major concern within the dairy industry (Krause and Oetzel, 2005) as the economic losses caused by this condition result in decreased milk production, premature culling, and increased death loss. Costs of SARA resulting from loss of production alone have been estimated to be $ 1.12/d per cow (Stone, 1999). Animals suffering from SARA have been documented to show an increase in SCFA concentration that not only causes a drop in pH, but also an increase in temperature and osmolarity (AlZahal *et al.*, 2008). These changes have to be limited and reversible so as to maintain a conducive environment for the microbial population, which is highly diverse and pH sensitive (Owens *et al.*, 1998). Moreover, these changes have a diurnal pattern, and the rumen epithelium has to protect itself from such short-term challenges as it is unable to secrete a protective mucous layer as can the abomasum (Franco *et al.*, 1993; Masot *et al.*, 2007).

Heat shock proteins (Hsp), also called stress proteins, are induced by sub-lethal stresses and a variety of other factors (Akerfelt *et al.*, 2007; Anckar and Sistonen, 2007; Feder and Hofmann, 1999; Kumar *et al.*, 2009) and can play an important role in the protection of epithelial functions such as transport and barrier formation (Dokladny *et al.*, 2006; Ikari *et al.*, 2002; Musch *et al.*, 1999). Hsp are thought to be able to modify the functions of the rumen epithelium to enable it to cope more easily with diurnal changes of fermentation pattern, such as pH, osmotic pressure, and temperature. Therefore, the aim of the present study has been to investigate possible protective mechanisms of Hsp predominantly involved in the acid recovery of the rumen epithelium. Particular emphasis has been paid on the transport physiology of sheep rumen epithelium including molecular aspects of acid stress recovery, namely, Hsp70 expression and NHE (Na^+/H^+ exchanger) activity.

1.2 Review of literature:

1.2.1 Anatomy of the rumen:

The forestomachs of ruminants are composed of four compartments: rumen, reticulum, omasum, and abomasum (Habel, 1975) (Figure 1-1). The rumen, the largest of the compartments with a volume of 60 – 70 l in cows and 8 -10 l in sheep (approx. 10 % of body weight; Reynolds *et al.*, 2004), occupies most of the left portion of the abdominal cavity and is partially divided internally into sacs by muscular pillars. It receives its ***blood supply*** from the branches of the coeliac artery (Grossman, 1975; May, 1970), whereas the gastric vein is formed by the union of two trunks: the right ruminal vein and the confluence of the omaso-abomasal, left ruminal, and reticular veins (Grossman, 1975; May, 1970). The compound stomach of the ruminant is ***innervated*** by the vagus nerve, which has a dorsal and ventral trunk. The dorsal vagal trunk sends branches to the rumen, reticulum, and the greater curvature of the omasum and abomasum, whereas the ventral vagal trunk innervates the cardia, left surface of the rumen, reticulum, parietal surface of the rumen, and right surface and lesser curvature of the reticulum (Habel, 1975; May, 1970).

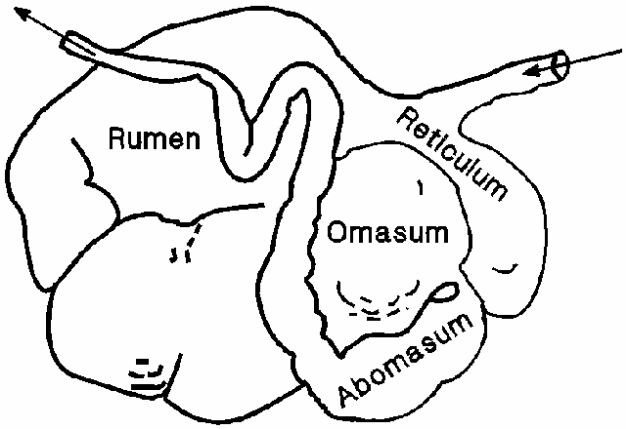


Figure 1-1: Topographic anatomy of the forestomachs.

The entire surface of the rumen is covered with tongue-shaped papillae formed by an aglandular-stratified squamous epithelium, which is keratinized and increases the absorptive surface area. Four distinct cell layers in the rumen epithelium can be distinguished. These are, from the luminal side, the *stratum corneum* (several layers of electron-dense cells that are classified cytologically into three types: flattened cells (R5A), balloon cells (R5C), and

intermediate cells (R5B) (Henrikson, 1971; Zitnan *et al.*, 1999)), the *stratum granulosum* (a layer of granular type A cells containing developing cytoplasmic keratin aggregates), the *stratum spinosum* (arranged in two layers and containing more filamentous material), and the *stratum basale* (either cuboidal or columnar with 2-3 cell layers and abundant metabolic organelles (Lavker *et al.*, 1969; Steven and Marshall, 1972). The rumen has proven to be accessible to a variety of procedures useful for the study of microbial digestive processes, and its epithelium has provided a non-glandular tissue for studies of transport mechanisms and the transport and metabolism of SCFA.

1.2.2 Rumen fermentation:

The rumen serves as a large fermentation vat in which micro-organisms (bacteria and protozoa), via extra-cellular enzyme complexes, break down ingested food stuff including cellulose or other complex carbohydrates, into SCFA, ammonia, CO_2, and methane (Gabel and Aschenbach, 2002). SCFA are generally produced in large amounts in the gut of herbivorous animal species, especially in the forestomach of ruminants (Allen, 1997), in the lower digestive tract of humans, and in all animal species in which intestinal fermentation resembles that occurring in the rumen (Mortensen and Clausen, 1996; Roy *et al.*, 2006). In the rumen, both non-structural and structural carbohydrates are hydrolyzed to monosaccharides or disaccharides by microbial enzymes (Bergman, 1990; Nafikov and Beitz, 2007). The resulting mono- and disaccharides are assimilated into microbial biomass or mainly fermented into SCFA. The principal SCFA in either the rumen or large intestine are acetate, propionate, and butyrate and are produced in a ratio varying from approximately 75:15:10 to 40:40:20 (Bergman, 1990). Protein is hydrolyzed, by microbial enzymes, into peptides and amino acids which are subsequently transported across the microbial cell wall for incorporation into cell biomass (Chalupa, 1978). In situations in which nitrogen for microbial growth is in excess, protein and its derivatives can also be fermented to produce energy, yielding ammonia (Chalupa, 1978; Dixon and Nolan, 1982; Russell and Strobel, 1987). Lipids, which are otherwise inert in the rumen, are partly hydrolyzed and hydrogenated, and glycerol, if present in the lipid, is fermented (Nafikov and Beitz, 2007). High levels of lipid, particularly unsaturated lipid, in the rumen are thought to poison microbes and suppress fermentation activity (Faverdin, 1999). Minerals are absorbed by microbes and are necessary for their growth, whereas microbes in turn synthesize many vitamins, such as cyanocobalamin, in quantities often large enough to sustain the ruminant, even when vitamins are deficient in the diet (Durand and Komisarczuk, 1988).

The fermentation in the rumen causes a drop in pH, which is continuously buffered by the secretion of HCO_3^- and PO_4^- rich saliva. However, this slightly acidic pH favors the absorption of SCFA via lipid diffusion (Charney *et al.*, 1998; Gabel *et al.*, 2002; Sehested *et al.*, 1999; von Engelhardt, 2005).

1.2.3 Transport mechanisms across the rumen:

Epithelia in the gastrointestinal tract exhibit, both *in vivo* and *in vitro*, a transepithelial potential difference (PD_t): the difference across the apical (PD_a) and basolateral (PD_b) membranes; the rumen has a PD_t of 20 mV to 60 mV (blood-side positive; (Ferreira *et al.*, 1966; Lang and Martens, 1999; Sehested *et al.*, 1996). These electrical gradients in combination with the chemical gradient influence the movement of various substances via one of two pathways: transcellular (through the cell) and/or paracellular (between the cells) (Edmonds and Mackenzie, 1984; Kermode and Edmonds, 1980). With regard to the transcellular pathway, substances can be actively or passively transported by means of pumps, carriers, or channels to circumvent the apical and basolateral membranes. Nevertheless, uncharged fatty-soluble substances have the ability to cross the cell membrane passively by non-ionic diffusion, as for example undissociated short chain fatty acids (HSCFA) or NH_3.

The resistance of the apical and basolateral membranes constitutes together the cellular resistance (R_c), whereas the paracellular resistance or shunt resistance (R_s) is mainly determined by tight junctions. The tissue resistance (R_t) comprises both cellular and paracellular resistances.

$$Rt = \frac{Rc \cdot Rs}{Rc + Rs}$$

According to their resistances, epithelial tissues can be divided into leaky (when R_t is < 1000 $\Omega.cm^2$) or tight (when R_t is > 1000 $\Omega.cm^2$). In tight epithelia, the tissue conductance (G_t), which is the reciprocal of the resistance ($G_t = 1/R$), can be increased by decreasing R_t via the activation of channels or pumps found in the membrane of epithelial cells (Powell, 1981)

1.2.3.1 Transport of SCFA:

An adult sheep can produce up to 3 to 6 mol of SCFA per day , and a high-production dairy cow more than 100 mol (Allen, 1997). The accumulation of SCFA in the rumen causes a drop in pH and an increase in osmolarity. Removal of SCFA from rumen is therefore of extreme importance for proper fermentation and digestion. Various studies have shown that up to 87% of intra-ruminally produced acetate disappears from the bovine reticulorumen, whereas the rest pass into the lower digestive tract (Gäbel, G., and Martens H., 1991; Hauffe and von Engelhardt, 1975; Peters *et al.*, 1990). Thus, the rumen must have efficient mechanisms to transport the product of microbial fermentation, especially SCFA (Gabel and Sehested, 1997; Muller *et al.*, 2002; Sehested *et al.*, 1999; Sehested *et al.*, 1996; Stevens and Stettler, 1966). Under physiological conditions, ruminal pH is maintained slightly on the acidic side (< 6.4) because of the production of SCFA, which in turn favors its uptake via non-ionic lipid diffusion as it is converted into the undissociated form, HSCFA (pKa of SCFA: 4.8) (Gabel *et al.*, 2002; Martens *et al.*, 1991a; Sehested *et al.*, 1999b). However, the absorption of HSCFA represents a threat to the cytosolic milieu since, during transfer through the epithelial layers, HSCFAs function as carriers of protons with an accompanying risk of intracellular acidification. This acidification is compensated by the activity of Na^+/H^+ exchangers (NHE1 + NHE3), monocarboxylate transporter (MCT1), the bicarbonate-importing system (Gabel and Aschenbach, 2002), and the proton pump (vHATPase) (Schweigel and Martens, 2003). Ali *et al.* (2006) have demonstrated an interaction between acetate transport and Na fluxes across the omasal epithelium of sheep and suggested that an uptake of acetate in an undissociated form occurs across the apical membrane, with an intracellular release of protons and activation of NHE. Despite the concentration gradient for SCFA between the forestomach content and the blood, which favors passive transport, further transport mechanisms such as anionic exchange systems are involved in SCFA permeation across the apical membrane (Aschenbach *et al.*, 2009; Gabel and Sehested, 1997). The extrusion of SCFA across the basolateral membrane is still under debate, but a recently demonstrated large anion conductance in isolated ruminal cells offers a suitable mechanism for the basolateral exit of SCFA ions (Stumpff *et al.*, 2009).

Because of the intensive intraepithelial metabolism of *n*-butyrate (Muller *et al.*, 2002), studies in portal-drained viscera do not provide useful data on the absolute absorption rate of n-butyrate. Most of the butyrate is converted to ketone bodies or CO_2 by the epithelial cells and

nearly all of the remainder is removed by the liver. Propionate is similarly removed by the liver and is largely converted to glucose (Kristensen and Harmon, 2004).

1.2.3.2 Transport of Na:

Since large quantities of Na^+ enter the rumen as sodium bicarbonate and phosphate in saliva (~ 50 mol/day in cattle (Erdmann, 1988) and 2.5 to 3 mol/day in sheep (Kay, 1960) without sodium deficiency), it is imperative for the animal's health for Na to be reabsorbed. The rumen has a great absorptive capacity for Na, as about 50% of the salivary Na is reabsorbed before arrival in the duodenum (Gäbel and Martens, 1991). The high extra-cellular (luminal) concentration of Na (20-160 $mM \cdot l^{-1}$), (Dobson, 1959) and apical cell membrane polarity (inside negative), (Martens *et al.*, 1991a) create an electrochemical gradient for Na entry into the cells. Na transport has been divided into *electrogenic* and *electroneutral* (Martens and Blume, 1987; Martens and Gabel, 1988; Stevens, 1964).

Electrogenic Na transport across the rumen epithelium occurs via a non-selective cation conductance (NSCC) in the luminal membrane, that increases when divalent cations (Mg^{++} and/or Ca^{++}) are removed from the mucosal solution (Leonhard-Marek *et al.*, 2005; Stumpff and Martens, 2007a) and requires the Na^+/K^+ pump and K^+ recycling through Ba^{++}-sensitive K^+ conductance in the basolateral membrane (Leonhard-Marek, 2002). Electrogenic Na transport across isolated ruminal epithelium in Ussing chamber studies is represented by a small positive short circuit current, which is not affected by Cl^- replacement or amiloride (Martens and Gabel, 1988), but is PD-dependent (Lang and Martens, 1999) and modulated by cytosolic Mg^{++} (see details in the review of Stumpff and Martens (2007a). Hence, electrogenic Na transport is not mediated by ENaC (epithelial Na channels) as in the colon, collecting duct of the kidney or frog skin (Dahlmann *et al.*, 2003). However, replacement of Na or the application of ouabain (serosal side) eliminates this current (Chien and Stevens, 1972; Harrison *et al.*, 1975).

In all *in vitro* studies, net Na transport is considerably higher than the short-circuit current suggesting that most of the Na is transported by an electrically silent mechanism working in parallel with electrogenic transport. Therefore, approximately 80-90% of the Na transport occurs electroneutrally through the Na^+/H^+ exchange and only 10-20% electrogenically (Martens and Gabel, 1988) (at high Na concentration). Na transport via the NHE is stimulated by mucosal SCFA or low luminal pH (Gäbel *et al.*, 1991b; Sehested *et al.*, 1996) and can be blocked by high doses of amiloride (1.0 mM, (Martens *et al.*, 1991a; Sehested *et al.*, 1996). The stimulation of Na transport via SCFA was first explained in a model by Petersen *et al.*

(1981) who proposed that, after the entry of undissociated SCFA (HSCFA) into the cell, H^+ is delivered by dissociation, which is recycled via the NHE, hence enhancing Na transport. The extrusion of Na across the basolateral membrane is mediated by the Na^+/K^+ ATPase, which can be regulated by cAMP and/or Ca^{++} (Moule *et al.*, 1987). Furthermore, a pH-dependent HCO_3^-/acetate- exchanger and HCO_3^--independent acetate uptake coupled to a protein and not MCT have also been recently reported (Gäbel, G. *et al.*, 2002) and is blocked by ouabain (Harrison *et al.*, 1975).

The transport of Na is modulated by the presence of other ions studied *in vivo* and *in vitro* (Table 1-1). An increase in the ruminal SCFA concentration or K concentration stimulates Na absorption, whereas an increase in the extracellular H^+ concentration (Gaebel *et al.*, 1989) or in the intra-ruminal osmolarity inhibits Na absorption.

Table 1-1: Modulators of Na transport across rumen epithelium.

Modulator	Effect on Na transport	Effect on I_{sc}	Reference
SCFA	↑ NHE	-	(Gabel *et al.*, 1989)
CO_2	↑ NHE	-	(Gäbel, G. *et al.*, 1991b; Sehested *et al.*, 1996)
NH_3/NH_4^+	↓ NHE (pH 7,4) ↑ NHE (pH 6,4)	-	(Abdoun *et al.*, 2003)
cAMP	↓ NHE	↓	(Gabel *et al.*, 1999; Wolffram *et al.*, 1989)
K^+	↑ Electrogenic Na	↑	(Lang and Martens, 1999)
Divalent cations	↓ Electrogenic Na	↓	(Leonhard-Marek, 2002)
Osmotic Pressure ↑	↓ NHE	-	(Schweigel *et al.*, 2005a)

1.2.4 Consequences of improper fermentation and absorption:

1.2.4.1 Acute ruminal acidosis:

Ruminants rely heavily on microbial fermentation to meet their energy requirement, and the physiological balance between acid production, buffering of SCFA, and absorption from the reticulo-rumen is extremely delicate. This is of vital importance as ruminal microbial species are highly diverse and pH-sensitive. For instance, a ration consisting mainly of forage diet leads to the increased development of species of cellulytic microbes, which are more stable at pH range 6.0 – 6.8, whereas a concentrate-rich diet leads to a pH range of 5.5–6.0 and hence an amylolytic milieu (Dirksen, 1985; Dirksen *et al.*, 1992; Owens *et al.*, 1998). Under physiological conditions, bacteria capable of lactic acid production, i.e., *S. bovis* and *Lactobacilli* spp. are not competitive. Grain engorgement and subsequently a fall in pH shifts the microbial population from lactic acid utilizers to lactic acid producers (population of *S. bovis* significantly increases) (Newbold and Wallace, 1988), causing an increase in the lactic acid (D^+ lactate) concentration in the rumen. Lactic acid is a 10 times stronger acid than SCFA (*pKa*: 3.8 vs. 4.8) and causes a rapid drop in ruminal pH (Figure 1-2).

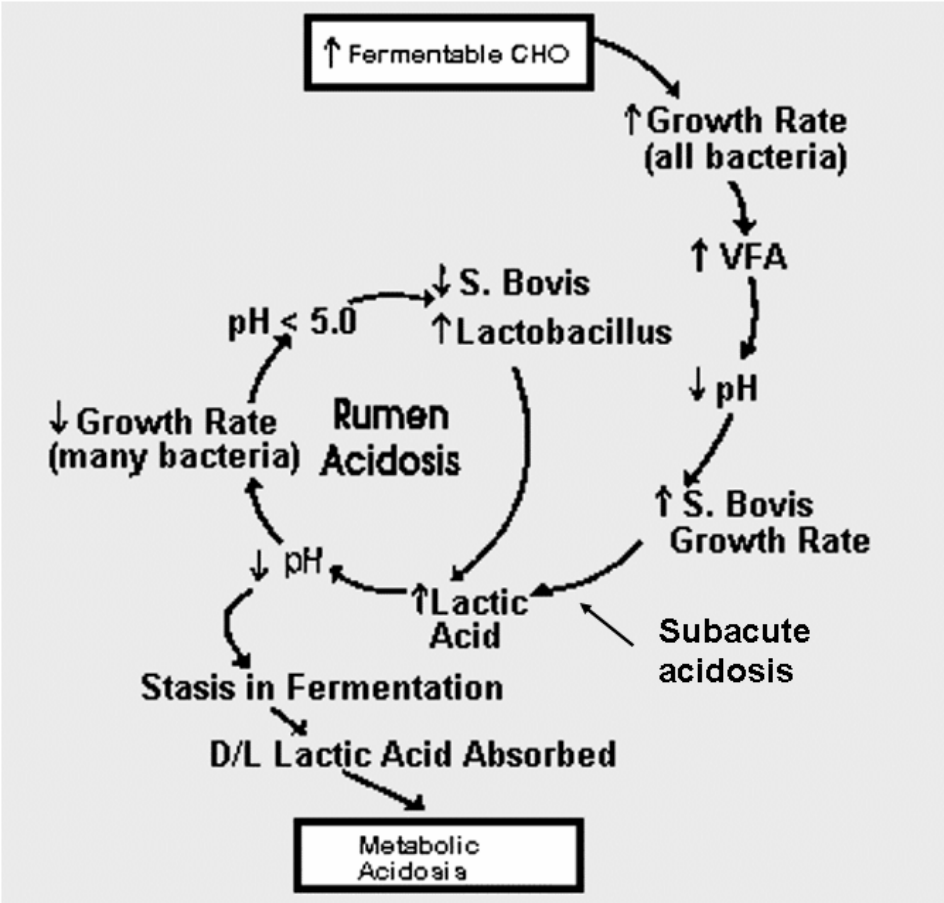


Figure 1-2: Scheme of pathophysiology of ruminal acidosis (Hodges *et al.*, 2006)

To gain maximum output, feed-lot economics dictate that highly fermentable rations are fed to deliver a proper energy supply to high-production animals. However, the complexity of managing a large herd does not always deliver the necessary intake of feedstuffs high in energy on the one hand and effective fiber on the other hand, thereby leading to an imbalance between acid production, buffering and the removal of SCFA from the reticulo-rumen. Consequently, a number of subtle changes in feed, availability of free glucose to the ruminal microbes, and change in environment or management can tip the balance, causing ruminal acidosis (Johnson, 1991).

Acidosis is a decrease of base excess in body fluids, resulting from insufficient capacity of physiological buffers. This is caused by an accumulation of acid, by a decrease of the alkaline reserve itself, or as the sequel of an underlying pathologic process to which the pH-regulation mechanism cannot adapt (Blood, 1999). Acute ruminal acidosis, also referred to as lactic acidosis (Dawson *et al.*, 1988; Underwood, 1992), is a disease originating from the fermentative disorder of the rumen and is manifested clinically. Classic acute acidosis is usually associated with individual animals accidentally taking up a large amount of concentrate, being not adapted to a diet high in concentrates, or having been off-feed for a certain period of time (see Figure 1-2) (Braun *et al.*, 1992; Dirksen, 1986).

1.2.4.2 Subacute ruminal acidosis (SARA):

SARA is defined as an intermittent fall of ruminal pH to non-physiological levels (< 5.5 for duration of 3-4 h) after concentrate uptake, because of mal-adaptation of the ruminal microflora and mucosa. SARA is one of the largest concerns, particularly to the dairy industry, because of the difficulty of diagnosis, on the one hand, and economic losses resulting from premature culling and increased casualties, on the other hand (Krause and Oetzel, 2005). It represents one of the most important metabolic disorders in intensive dairy farms and affects rumen fermentation, animal welfare (laminitis), productivity, and farm profitability (milk fat depression) (Kleen *et al.*, 2009; Morgante *et al.*, 2007). The onset of SARA is marked by the intake of a diet low in structure and high in energy at a time when the ruminal environment is not yet prepared to ferment it adequately (Kleen *et al.*, 2003). SARA leads to the development of clinically detectable consequences after a certain delay following the initial insult (Garrett *et al.* 1999; Nordlud, 1995; Oetzel, 2000). Studies suggest that a change in diet transiently causes a slight increase of passive permeability, which can be exacerbated by low pH (Emmanuel *et al.*, 2007; Gäbel *et al.*, 1987; Khafipour *et al.*, 2009) and an increase in osmotic pressure (Schweigel *et al.*, 2005b), which in combination can finally lead to clinical consequences attributable to the disintegration of the epithelium and penetration of bacteria. The penetration of bacteria and of endotoxins such as lipopolysaccharide (LPS) or histamine are all thus made easier and have recently been discussed in detail as possible pathogenic factors in SARA (Plaizier *et al.*, 2008). The most important and viable clinical sign of SARA is decreased dry matter intake, mainly because of decreased ruminal motility caused by bacterial endotoxins (Cottee *et al.*, 2004; Krause *et al.*, 2009). Another condition commonly associated with SARA is laminitis, which in turn leads to impaired performance by the animal. The exact etiology of laminitis is not yet clear, although vasoactive substances entering the blood stream from the rumen are suspected to cause detachment of the laminae, leading to discoloration, hemorrhages, abscessation, and ulcers in the hooves (Krause and Oetzel, 2005). The presence of SARA, therefore, should be suspected in herds with a high incidence of lameness or the lesions described above. Whereas intraruminal histamine does not affect healthy ruminants, histaminosis is apparent during ruminal acidosis as a low luminal pH (5.1) resulting in decreased intra-epithelial catabolic activity and increased pH-dependant movement of histamine across the isolated rumen epithelium of sheep (Aschenbach *et al.*, 2000).

SARA causes considerable economic losses to the dairy industry. However, the prevalence of SARA has not as yet been studied intensively. Garrett *et al.* (1999) have found in a survey of 15 Holstein herds in the U.S. that 19% of early-lactation cows and 26% of mid-lactation cows experience a ruminal pH of ~5.5 for around 3 h. In one of the herds, more than 40% of the cows were diagnosed as having SARA at the time of examination, whereas Kleen (2009) has recently described the overall prevalence of SARA to be 13.8% in a Dutch province.

A depression of milk fat percentage in cows affected by SARA has been documented (Dirksen, 1985; Oetzel., 2004), but as it usually occurs in individual animals, the decrease of milk fat remains undetected by bulk tank testing (Garrett *et al.*, 1999; Nocek, 1997). Milk fat depression has been found to be accompanied by a number of changes in the ruminal fermentation pattern and is commonly observed in ruminants fed highly fermentable diets or diets that contain high concentrations of poly-unsaturated fatty acids. The altered fermentation associated with these diets results in rumen outflow of unique biohydrogenation intermediates, some of which reduce lipid synthesis in the mammary gland. Trans-10, cis-12 conjugated linoleic acid (CLA) was the first of these bioactive intermediates to be identified, together with its impact on bovine lactation (Donnelly *et al.*, 2009). A comparison between acute and subacute acidosis is summarized in Table 1-2.

Table 1-2: Comparison of acute and subacute rumen acidosis in cattle*

Description	Acidosis	
	Acute	Subacute
Clinical signs	Present	Absent
Mortality	Yes	No
Ruminal pH	< 5.0	5.0 to 5.5
Total organic acids	Increased	Increased
Lactic acid	High (50 to 120 mM)	Normal (0 to 5 mM)
SCFA	Below normal (< 100 mM)	High (150 to 225 mM)
Gram-positive bacteria	Increased	No change
Blood pH	Decreased (< 7.35)	Normal to slightly decreased
Rumenitis	Yes	Yes
Laminitis	Yes	Yes
Liver abscess	Yes	Yes

1.2.5 Ruminal adaptation:

Adaptation of the rumen epithelium to dietary changes is of significant importance to domesticated ruminants (Etschmann *et al.*, 2009a) and has been well known for many years (Van Vuuren *et al.*, 1979). Insufficient adaptation during transition periods results in various disorders, including laminitis, ketosis, and SARA (Donovan *et al.*, 2004; Kleen *et al.*, 2003). Ruminal adaptation includes morphological and functional changes of the epithelium in response to diets rich in energy and protein. Morphological adaptation is characterized by the proliferation of the epithelial and subepithelial cells leading to an increase in the number and size of rumen papillae and, subsequently, to an augmentation in the epithelial absorptive surface area (Liebich *et al.*, 1987). The functional adaptation of the rumen epithelium to energy-rich diets has also been investigated in various ruminants, including sheep, goat, cows, and reindeer (Dirksen *et al.*, 1984; Gäbel *et al.*, 1991a; Shen *et al.*, 2004b; Storeheier *et al.*, 2003) and suggests an increase in the transport rates of minerals (Na, Mg, Ca) and SCFA both *in vivo* and *in vitro* (Dirksen *et al.*, 1984; Doreau *et al.*, 1997; Gäbel and Martens, 1991;

* Data modified from (Nagaraja and Chengappa, 1998)

Sehested *et al.*, 1997; Shen *et al.*, 2004b; Uppal *et al.*, 2003a). Most of these diet-dependent adaptations have been observed when a concentrate diet is fed over a period of several weeks; this does not allow a conclusion about the exact time scale of functional adaptation. In a recent study, Etschmann *et al.* (2009a) have demonstrated a rapid increase of Na transport via NHE; 73% of the increase of Na transport via NHE occurs during the first week after the changing of diet (Etschmann *et al.* 2009), a time in which morphological alterations have not been observed (Liebich *et al.*, 1987). This suggests that the adaptational changes at the cellular level are probably much faster than the relatively slow morphological alterations (Etschmann *et al.*, 2009b).

However, a daily variation in SCFA concentration and pH has also been documented in the rumen with or without adaptation (Nagaraja and Titgemeyer, 2007; Nocek *et al.*, 2002) (Figure 1-3).

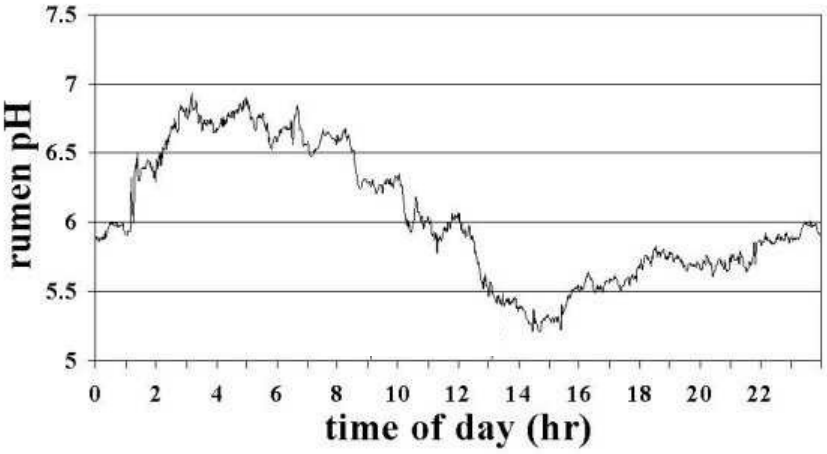


Figure 1-3: Diurnal variation in rumen pH (Nagaraja and Titgemeyer, 2007)

This demands a more prompt mechanism of epithelial adaptation to cope with these challenges via an increase in transport activity and stabilization of pH_i. The increase of transport activity per cell might be based upon the increased activity or number of transport proteins involved in nutrient absorption and/or regulation of pH_i. This, in turn, might be the result of alterations at the transcription level or protein translocation phenomena within the cell (Uppal *et al.*, 2003b). The mechanism that leads to the appearance of an increased number of active transport proteins at the surface of the rumen epithelium cell remains to be elucidated. The trafficking of vesicles and the insertion of the NHE into the apical membrane

have been demonstrated in a variety of tissues (Charney *et al.*, 2002), but this form of adaptive mechanism is still unknown in the rumen epithelium.

1.2.6 Stress recovery mechanisms and pH_i regulation:

The cellular stress response is a highly conserved defense mechanisms following exposure to high temperature or other environmental challenges and is characterized by the induction of the synthesis of heat shock proteins as a protective mechanism because any deviation from homeostatic conditions can be detrimental and provoke various cellular responses ranging from cell transformation to apoptosis (Bellamy *et al.*, 1995; Bromme and Holtz, 1996). Almost all intracellular processes, including cell metabolism, have a narrow pH range for optimal function, and this thus requires tight regulation (Busche *et al.*, 2002; Messonnier *et al.*, 2007). Many membrane proteins that form ion channels and transporters can detect chemical messengers such as growth factors and hormones and are also be influenced by changes in the intracellular H^+ concentration (Ben Ammar *et al.*, 2005; Lin *et al.*, 2005; Malo and Fliegel, 2006). The predominant threat to the rumen epithelium is the low luminal pH in the presence of SCFA and/or lactic acid. For example, a high concentration of lactic acid (80 $mM \cdot l^{-1}$) and a low pH (< 5.0) can lead to excessive desquamation and necrosis of the rumen epithelium (Chihaya *et al.*, 1988; Moller *et al.*, 1997; Steele *et al.*, 2009). Consequently, a variety of mechanisms are involved in the protection of the epithelium including transport mechanisms for various nutrients, mainly SCFA, and the regulation of pH_i in the rumen epithelium.

1.2.6.1 Anion exchanger and co-transporters:

As described in many cell types including the rumen (Chernova *et al.*, 2005; Chien and Stevens, 1972; Kopito, 1990; Kramer *et al.*, 1996), anion exchangers are involved in the electroneutral exchange of Cl^-/HCO_3^- and the regulation of intracellular pH and cell volume in either an Na-dependent or Na-independent manner (Alper, 2006). Anion exchangers (AEs) have evolved from two distinct gene families, viz., SLC4A (NBC, AE1, AE2 and AE3) and SLC26A (SLC26A3, SLC26A7, SLC26A9), with a wide range of differences including tissue distribution, apical or basolateral epithelial expression, electrogenicity, regulation, and physiological roles (Bonar and Casey, 2008). Although AEs are mainly involved in pH_i regulation, their interaction with SCFA, a role in intra-ruminal pH regulation, and an interaction with other anions such as chloride and bicarbonate has been proposed and demonstrated (Aschenbach *et al.*, 2009; Gäbel and Martens, 1991). Gabel *et al.* (1997) have

also mentioned other pH_i rescue mechanisms, such as a bicarbonate-importing system and H^+/monocarboxylate co-transport (MCT; exporting system) for the rumen epithelium (Gabel *et al.*, 2002).

The role of co-transporters in pH_i regulation is mainly attributed to the sodium bicarbonate co-transporter (NBC), which is expressed in many epithelia (Aalkjaer *et al.*, 2004; Gross and Kurtz, 2002) basolaterally or apically (Soleimani and Burnham, 2001) and is either electrogenic (NBC1 and NBC4) (Becker and Deitmer, 2004) or electroneutral (NBCn1) (Boron, 2001; Nejsum *et al.*, 2005). It is considered as one of the main pH_i regulatory mechanism in cultured ruminal cells depending on extracellular Na and not on intracellular Cl^- (Huhn *et al.*, 2003).

1.2.6.2 H^+ATPase:

The vacuolar type H^+-ATPase is capable of transporting protons against strong gradients across the cell membrane and has an important role in pH_i regulation in many cell types (Stevens and Forgac, 1997). The up-regulation of this protein has been observed during salinity stress in the lipid membrane of plant cells (Lopez-Perez *et al.*, 2009). A considerable amount of evidence is available regarding the presence of H^+-ATPase in the rumen epithelium (Albrecht *et al.*, 2008; Etschmann *et al.*, 2006; Schweigel and Martens, 2003). Albrecht *et al.* (2008) have studied the expression and localization of H^+-ATPase in the bovine rumen epithelium and conclude that this protein is present in abundance in the stratum spinosum and stratum granulosum, thereby indicating a vital task of H^+-ATPase in the pH_i recovery of rumen epithelial cells exposed more toward the luminal side. The role of H^+-ATPase in isolated ruminal epithelial cells has been described in detail by Etschmann *et al.* (2006) suggesting a 30% contribution of this proton pump to pH_i recovery. It has also been shown to modulate Mg^{++} and Cl^- transport in isolated ruminal epithelial cells (Schweigel and Martens, 2003).

1.2.6.3 Na^+/H^+ exchangers (NHE):

In sheep rumen epithelium, NHE is the predominant, electrically silent, Na^+ transport mechanism (Martens *et al.*, 1991a) and is inhibited by theophylline (Wolffram *et al.*, 1989), by an increase in cAMP (Gabel *et al.*, 1999) and by the mucosal addition of 1 mM amiloride (Martens *et al.*, 1991b). NHE activity has also been shown to be affected by extra-cellular osmolarity (Schweigel *et al.*, 2005b), bicarbonate concentration, pCO_2, and SCFA (Gabel *et al.*, 1991b; Muller *et al.*, 2000). Linkage of pH_i with SCFA and its regulation via NHE have

been suggested to be crucial to cell integrity and survival (Boron, 2001; Choi *et al.*, 2000; Soleimani and Burnham, 2001).

NHE extrudes H^+ from the cytosol by using the Na concentration gradient across the plasma membrane, generated by Na^+/K^+-ATPase, in an electroneutral manner [stoichiometry of 1:1 (Ohgaki *et al.*, 2005)], and is considered to be one of the most efficient pH_i regulators. In most cells, the NHE is virtually quiescent at around physiological pH_i (7.0-7.2) but is dramatically stimulated when the cell interior is acidified, even slightly, as dictated primarily by the H^+-sensitive modifier site (Aronson *et al.*, 1982; Wakabayashi *et al.*, 1994). NHE has also been shown to be activated by a number of other signaling molecules and second messengers, including protein kinase C alpha (PKCα) and Ca^{++} (Hodges *et al.*, 2006).

The NHE gene family is comprised of nine members (Table 1-3) that are categorized by their cellular localization (i.e., plasma membrane or intracellularly) (Zachos *et al.*, 2005) and that share 40-60% amino acid homology (Masereel *et al.*, 2003). In the gastrointestinal tract of many species, resident plasma membrane isoforms include NHE1 (basolateral) and NHE2 (apical) (Pizzonia *et al.*, 1998), recycling isoforms (NHE3 and NHE5), and intracellular isoforms (NHE6, 7, 9) (Zachos *et al.*, 2005). NHE3 is however the focus of this study, since it recycles between intracellular compartments and the plasma membrane and appears to interact with many signaling molecules. It is generally expressed on the luminal side of polarized epithelia and therefore appears to act as a first line of defense against acidosis (Claiborne *et al.*, 2008; Oehlke *et al.*, 2006).

Table 1-3: Types of NHE isoforms and their distribution*

Isoform	Cellular localization	Example of tissue
NHE1	PM (BLM)[1]	Expressed in virtually all cells types, tissues, and species
NHE2	PM (Ap[2]; BLM)	Stomach, colon, small intestine, adrenal gland, skeletal muscles, and kidney
NHE3	PM (Ap); RE[3]	Gastrointestinal tract and kidneys
NHE4	PM (BLM)	Gastrointestinal tract and liver
NHE5	PM; SV[4]; RE	Brain (hippocampus), spleen, testis, and skeletal muscle
NHE6	RE	Brain, skeletal muscle, and heart
NHE7	TGN[5]	Brain, skeletal muscles, pancreas, prostate gland, and gastrointestinal tract
NHE8	PM (Ap); TGN	Liver, kidneys, and gastrointestinal tract
NHE9	RE	Brain, heart, and skeletal muscle

* Modified from Masereel *et al.*(2003) and Donowitz M (2007).
[1] PM: Plasma membrane; BLM: Basolateral membrane.
[2] AP: Apical membrane.
[3] RE: Recycling endosome.
[4] SV: Seminal vesicles.
[5] TGN: Trans-Golgi network.

The kinetics of NHE for external Na follow a classical Michaelis-Menten model with a Hill coefficient of 1, suggesting a single binding site for the external Na (Levine *et al.*, 1995), which has been shown to interfere with extra-cellular cations other than K (Orlowski and Grinstein, 1997; Yun *et al.*, 1993). However, the activation of NHE by intracellular protons does not follow simple Michaelis-Menten kinetics (Aronson *et al.*, 1982), and the demonstrated Hill coefficient of 2 has led to the conclusion that the activation of NHE by intracellular protons is mediated by a modifier site (Aronson *et al.*, 1982).

1.2.6.3.1 NHE1:

NHE1 is ubiquitously expressed in all mammalian cells and is comprised of 813 to 822 amino acids with a molecular mass of ~ 91 kDa (Putney *et al.*, 2002). The regulation of this exchanger is mediated through interaction via a regulatory domain for stimulation by growth factors, protein kinase, fetal bovine serum and hyperosmolarity and also via a calmodulin binding domain in response to a rapid increase in intracellular Ca^{++} (Wakabayashi *et al.*, 1997; Yun *et al.*, 1995). However, this exchanger is not regulated by the recruitment of internal stores, as is the case for NHE3 (D'Souza *et al.*, 1998; Shrode *et al.*, 1998) and is highly sensitive to HOE642 (Bachmann *et al.*, 2007; Imahashi *et al.*, 2007). Because of its effects on pH_i homeostasis, cell volume, and the actin cortical network, NHE1 regulates a number of cell functions including adhesion, shape determination, migration, and proliferation (Putney *et al.*, 2002; Wakabayashi *et al.*, 1997; Yun *et al.*, 1995). The activity of NHE has been demonstrated in isolated ruminal epithelial cells (Schweigel and Martens, 2003) and according to Müller *et al.* (2000), 70% of pHi recovery in the cultured ruminal epithelial cells from acid load has been attributed to NHE1.

NHE1 is mainly expressed in the basolateral membrane of epithelia (Kennedy *et al.*, 2005; Maher *et al.*, 1996; Shin *et al.*, 2005; Zachos *et al.*, 2005). However, Graham et.al (2007) have recently proposed, on the basis of results obtained by immunostaining, that this exchanger is located in the stratum granulosum of bovine rumen epithelium with a progressive decrease toward the stratum basale; these authors have discussed the possible role of NHE1 in Na transport and interaction with SCFA (Graham *et al.*, 2007).

1.2.6.3.2 NHE3:

NHE3 was first cloned from rat, has been detected in the colon, small intestine, kidney, gall bladder, brain, and stomach, and has between 831-834 amino acids with a molecular weight

of ~93kDa. It consists of 12 putative encoded membrane-spanning domains (MSD), an extracellular N terminus, and an intracellular C terminus (Orlowski and Grinstein, 1997).

The N-terminal (~500 amino acids in the transmembrane transport domain) is necessary to catalyze the ion exchange, and the C-terminal domain is involved in regulation by growth factors, pH set point value, and protein kinase regulation (Donowitz and Li, 2007). MSD 4 and 9 contain sequences that are involved in determining sensitivity to amiloride and its analogs, whereas MSD 9 and 10 participate in Na^+ and H^+ transport (Wakabayashi *et al.*, 1992).

In polarized intestinal epithelial cells, the majority of NHE3 protein is localized at the apical membrane, where it can be found both on the microvilli and in the intervillus clefts (Cinar *et al.*, 2007; Janecki *et al.*, 1998). Intracellular NHE3 predominantly occurs in the endosomal pool, but its amount varies with cell line. For instance, rabbit ileal villus cells have ~70% of total NHE3 in a pool that co-localizes with the brush border, whereas in Caco-2 cells, ~80% of total NHE3 is in the brush border and ~20% has a diffuse supranuclear location (Janecki *et al.*, 1998). The differences in this distribution have important consequences for the regulation of NHEs by endocytosis/exocytosis. If a large subapical pool exists, any addition to the plasma membrane is likely to be an effective mechanism to increase NHE activity and its regulation. No general conclusion can be made about the differences in the types of cells that express higher versus lower percentage of NHE3 in the plasma membrane (Kumano *et al.*, 2008; Yip *et al.*, 1998). NHE3 plays an important role in Na re-absorption in the ileum upon meal stimulation and in net NaCl absorption and HCO_3^- secretion (Schultheis *et al.*, 1998). The NHE3-deficient mouse demonstrates impaired Na absorption in the intestine, and the lack of this exchanger results in diarrhea, increased fluid in the small intestine and colon, an enlarged diameter of the small intestine and colon, and a defect in acid-base balance and Na fluid homeostasis (Schultheis *et al.*, 1998). It has also been shown to play a vital role in the proximal tubule for Na and fluid absorption (Orlowski and Grinstein, 2004; Vallon *et al.*, 2000).

The activation of NHE3 might be controlled (*a*) by changes in turnover number or (*b*) by the traffic of the protein from the vesicular compartments into the apical membrane, with the opposite indicating inhibition (Yip *et al.*, 1998; Zhang and Melvin, 1996).

NHE activity *in vitro* has been determined by using techniques that measure either the cellular uptake of ^{22}Na or proton efflux by means of intracellular pH-sensitive dyes, e.g., 2´,7´bis (2-carboxyethyl)-5 (6)-carboxyfluorescein (BCECF-AM). Initial studies have suggested that NHE2 and NHE3 are regulated via changes in the H^+ affinity of the allosteric site or pH_i set

point. cAMP, cGMP, the activation of protein kinase A (PKA), and elevated intracellular calcium inhibit NHE3 activity.

NHE3 was previously assumed to mediate electroneutral Na transport across the rumen epithelium, by analogy to studies in other epithelia of the gut (Gawenis *et al.*, 2002; Musch *et al.*, 2007; Zachos *et al.*, 2005). However, this is now contradicted by the results of the recent study of Graham *et al.* (2007).

1.2.7 Heat shock proteins (Hsp):

The ability to respond to acute or harmful conditions and to repair sub-lethal damages caused by such circumstances is common to all cells. Cellular stress can be defined as a situation that results in a large extent of protein unfolding, misfolding, or aggregation and in marked changes in cell physiology. Following injury or stress of any type, cells undergo a stress response, involving the cessation of general protein synthesis and the up-regulation of heat shock proteins (Hsp), which have been implicated in promoting cell survival and repair (Tetzlaff *et al.*, 2007). Such a stress response was first discovered by Ritossa (1966) who observed a new pattern of chromosomal puffing in isolated *Drosophila* salivary glands exposed to a higher temperature, a pattern that correlated to changes in protein synthesis. Investigators later found that a range of stimuli induced the increased expression of Hsp, including anoxia, ethanol, heavy metal ions, ischemia, trauma, and brain injury (Ritossa, F., 1996; Welch, 1992). In addition to the role that Hsp play during stress, many of these proteins are constitutively expressed and function in normal protein folding, transport, regulation, and degradation in unstressed cells suggesting that they are involved in important tasks in cell physiology and that they act as “molecular chaperones” (Frydman *et al.*, 1994; Glover and Tkach, 2001; Haslbeck, 2002; Naylor and Hartl, 2001). Molecular chaperones have the ability to recognize the hydrophobic residues of nascent polypeptide molecules and to interact reversibly with them in an ATP-dependent or independent manner in order to stabilize and facilitate their folding.

The up-regulation of Hsp in response to stress is primarily controlled at the transcriptional level mediated by a transcription factor, called **heat shock factor (HSF)**, a leucine zipper family of transcription factors that bind to a *cis*-acting conserved element found in the promoter of heat-inducible genes, termed the **heat shock element (HSE)**.

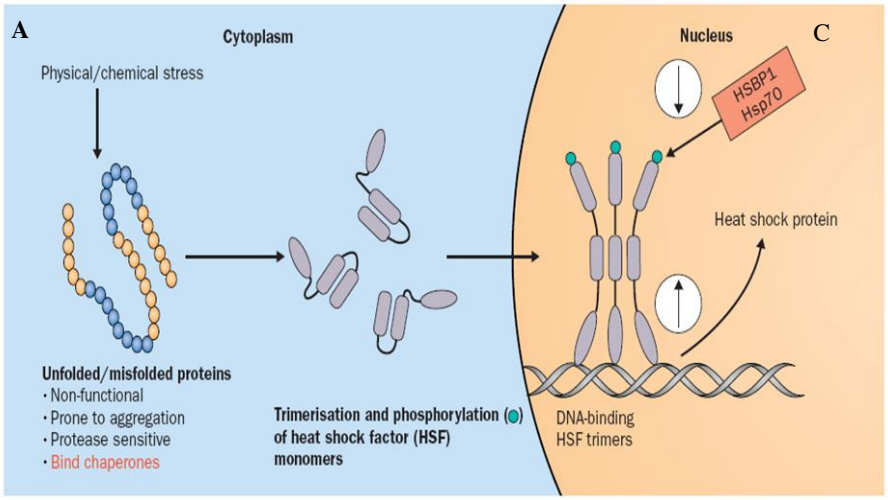


Figure 1-4: Mechanism of activation of heat shock factor (HSF) and subsequent production of Hsp. A) Within the cytoplasm, the HSF is maintained as monomers by certain Hsp. Upon stress, the Hsp are released from the HSF and can form trimers. B) The trimeric form of HSF can then activate the heat shock response by binding the heat shock element to DNA in the nucleus leading to the transcription of mRNA and finally to the synthesis of Hsp.

A number of HSF family members have been identified in various eukaryotes, such as frog, chicken, mouse, rat, and human cells, each sharing similarities in their DNA-binding specificity and homologies in their DNA-binding and oligomerization domains (reviewed in Pirkkala *et al.*, 2001). HSF1 is present in all eukaryotic cells and is the main HSF that responds to pathophysiological stresses, environmental stresses, and other non-stressful conditions discussed above (Morimoto, 1998). Other HSFs (HSF2, 3, 4) are either specific to some organisms, restricted to specific tissues, or are activated by other conditions (Akerfelt *et al.*, 2007).

HSF1 exists in unstressed cells as a latent monomer and is transiently activated upon stress (Baler *et al.*, 1993; Kroeger *et al.*, 1993; Sarge *et al.*, 1993; Westwood *et al.*, 1991; Wu *et al.*, 1987) resulting in the formation of homotrimers associated with high affinity binding for the HSE and activation of the transcriptional activation domain (Figure 1-4). Furthermore, HSF1 has been demonstrated to be critical for the maintenance of cellular integrity after heat stress, and cells from HSF1$^{-/-}$ mice lack the ability to develop thermo-tolerance (Yan *et al.*, 2005; Zhang, Y. *et al.*, 2002).

1.2.7.1 Classification of Hsp:

Hsp are a diverse group of proteins that are divided into families based on molecular weight. Major families of Hsp include small chaperones and ubiquitin, Hsp60, Hsp, Hsp90 and Hsp100 and are briefly summarized in Table 1-4. The main emphasis of this study, however, will be on the Hsp70 family, its mechanism of interaction, and possible role in stress scenarios.

Table 1-4: Major heat shock proteins in mammals

Proteins	Localization	Function/feature
Small Hsp (Hsp28)	Cytosol, nucleus	Molecular chaperone, thermotolerance, helps refolding of denatured proteins
Hsp40 family (Hsp40)	Cytosol	Thermotolerance; molecular chaperone; associated with Hsp, molecular chaperone activity.
Hsp60 family (Hsp60)	Mitochondria	Molecular chaperone, possible role in auto-immune diseases. Maintain polypeptides in unfolded state
Hsp70 family (Hsp70) (Hsc70)	Cytosol, nucleus Cytosol, nucleus	Molecular chaperone, thermotolerance Constitutive and heat inducible, translocation of proteins across intracellular membranes, removal or refolding of denatured proteins
BiP (Grp78), (Grp75)	Endoplasmic reticulum Mitochondria	Facilitate the assembly of monomeric proteins into larger complexes
Hsp90 family (Hsp90)	Cytosol	Stabilize inactive hormone receptors; interact with certain protein kinases and molecular chaperone, escort polypeptides to their cellular compartments.
Large Hsp (Hsp110)	Cytosol, nucleus, nucleolus	Molecular chaperone, thermotolerance, helps the recovery of nucleolar transcription after stress

1.2.7.2 Hsp70 family:

Of all the Hsp gene families, Hsp70 is the most thoroughly studied and best characterized. This multi-gene family of highly conserved proteins contains constitutive (Hsc) and stress-inducible (Hsp70) members that play a critical role in normal cellular physiology, repair and protection after stress. These proteins were found in most cellular compartments including nuclei, mitochondria, chloroplasts, endoplasmic reticulum (ER), and cytosol (Craig *et al.*, 1994). Hsp70 protein consists of three functional domains: an N-terminal ATP-binding domain, a substrate binding domain (Wang *et al.*, 1993), and a carboxyl-terminal domain, which is important in the regulation of ATPase activity (Freeman *et al.*, 1995) (Figure 1-5).

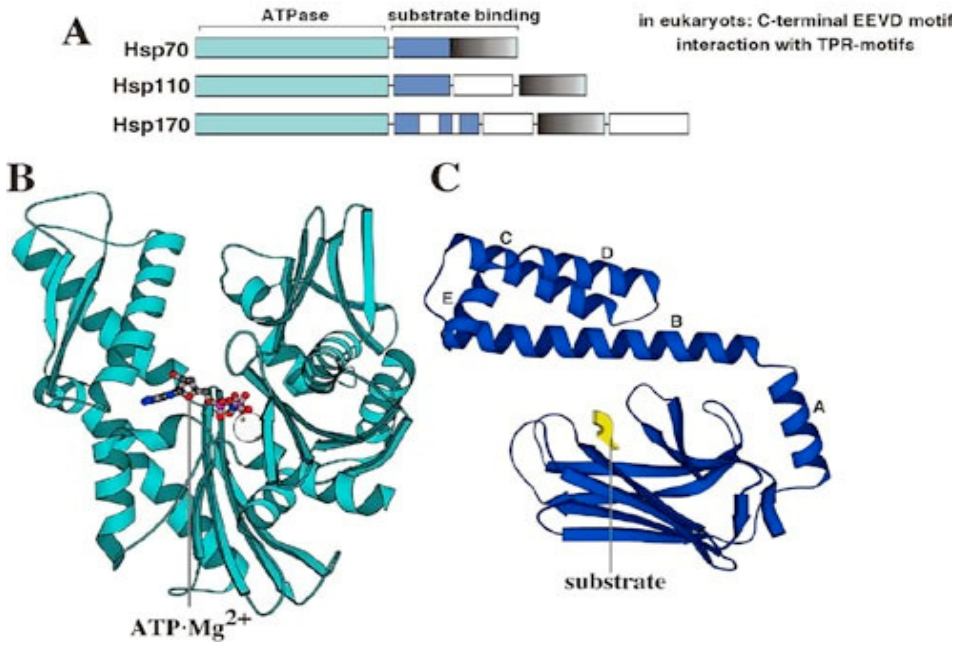


Figure 1-5: Molecular structure of Hsp70. A) Comparison of ATPase and substrate-binding domain among various Hsp families. B) ATPase-binding domain. C) Substrate-binding domain.

The Hsp70 appears to fulfill a variety of chaperone functions and works in cooperation with other proteins, particularly Hsp40 and Hsp90 (Frydman and Hartl, 1996). They stabilize unfolded precursor proteins (polypeptides) prior to their assembly into macromolecular complexes in the cytosol and/or translocation into the ER and mitochondria, interact with polypeptides through an extended unfolded conformation by recognizing peptide segments with a net hydrophobic character (Blond-Elguindi *et al.*, 1993; Flynn *et al.*, 1991), and execute a regulated cycle of peptide binding and release, which facilitates the acquisition of

the active conformation of polypeptides. Members of the Hsp70 family also facilitate the dissociation of protein complexes consisting of folded proteins.

1.2.8 Hsp70 and acidosis:

Following stress or shock, mammalian cells experience a rapid drop in intracellular pH, a decrease in the level of ATP, and an increase in cytosolic calcium levels (reviewed by Welch, 1992), which leads to the expression of heat shock or stress proteins (Hsp70) that function to restore homeostasis and help with cell survival (Lindquist and Craig, 1988; Morimoto, 1998). Hsp70 are proposed to limit injury resulting from diverse environmental stresses, including acidosis, but direct metabolic evidence for such a cytoprotective function in vertebrates has been largely limited to studies of cultured cells. For instance, the effect of chronic metabolic acidosis has been observed in proximal renal tubular cells; this occurs because of chronic renal failure. On the basis of the suggestion that chronic acidosis leads to an increased generation of reactive oxygen species, a model of chronic acidosis in renal cells has been developed to measure markers of oxidative stress and metabolism. Higher levels of reactive oxygen species and Hsp70 are observed at pH 7.0 (Wang and Luthe, 2003).

Particularly in the case of myocardial cells, severe ischemia has been noted to lead to a possible over-production of intracellular H^+ and a decrease pH_i. Dillmann *et al.* (1986) have demonstrated that the occlusion of the left descending coronary artery in dogs or rabbits activates the expression of Hsp70 protein (Western analysis), and that these changes take place 2 h after ischemic insult. A similar activation of Hsp70 occurs after cerebral ischemia, which induces increased amounts of Hsp70 in rat brain (Dienel *et al.*, 1986).

In vivo studies with tissues exposed to various stress scenarios have also been documented. Martin *et al.* (1998) have demonstrated a correlation between the expression of Hsp70 and resistance to acidosis in fish: lower resistance to acidosis is associated with the lowest level of Hsp70. A similar type of correlation between lactate concentration and Hsp70 has been observed by Poso *et al.* (2002) who have demonstrated the expression of Hsp70 mRNA in horse muscles after exercise; they conclude that acidosis, rather than energy depletion, is the major inducer of Hsp70 expression after exercise of moderate intensity.

As described earlier, acidosis is a condition that occurs as a result of an increase in SCFA concentration in the rumen followed by a drop in ruminal pH and an increase in intra-ruminal temperature resulting from high metabolism. In mice, lactic acidosis in heart tissues creates an intracellular environment that leads to the selective activation of genes, the production of new mRNA, and the synthesis of a typical group of stress proteins (Hammond *et al.*, 1982).

Treatment of *Lactobacillus sanfranciscensis* (LB: sanfranciscensis CB1) with chloramphenicol during acid adaptation almost completely eliminates the protective effect, suggesting that the induction of protein synthesis is required for the acid-tolerance response (De Angelis *et al.*, 2001). Dexamethasone has also been found to inhibit the heat-shock-induced expression of the major Hsp, viz., Hsp70, 90, and 110. Thus, the inhibitory effect of dexamethasone appears to apply to most, if not all, genes regulated by heat shock transcription factor 1 (Wadekar *et al.*, 2001).

Conclusions and objectives for the present study

The feeding regime of dairy cows has been continuously changed during the last few decades. The predominant alteration is the increase of energy intake, mainly as starch, according to the requirement of the dairy cow for milk production. One important consequence of this high starch intake is the altered fermentation pattern in the rumen characterized by the increased SCFA concentration, osmotic pressure, and temperature up to 41°C, and the decrease in pH.

As a response, the rumen epithelium exhibits adaptation, which includes the proliferation of the rumen epithelium including the number and size of papillae, and an increase in the transport rates of ions (Na, Ca, Mg, SCFA) and of enzyme activities. This adaptability of the rumen epithelium requires at least a few days (Na transport) or even weeks (number and size of papillae; SCFA transport) to become functional. However, the mentioned changes of SCFA, osmotic pressure, temperature, and pH show a diurnal rhythm and must be considered as a challenge for the actual function and integrity of the rumen epithelium. Little or almost nothing is known about the reaction of the epithelium under these circumstances. The well-known stress protein Hsp70 might be involved in this acute adaptability of the epithelium.

The objectives of this study are: (see Figure 1-6).

- to induce/express heat shock proteins (Hsp70) "*in vitro*" in the rumen epithelium exhibiting the parameters of acidosis, i.e., changed temperature, pH, osmolarity and high SCFA concentration.
- to investigate epithelium performance in the presence or absence of Hsp70 by measuring Na (NHE) and acetate transport.
- to measure the *in vivo* expression of Hsp70 in ruminants subjected to a change in their feeding regime and then to investigate the transport properties of the rumen epithelium.

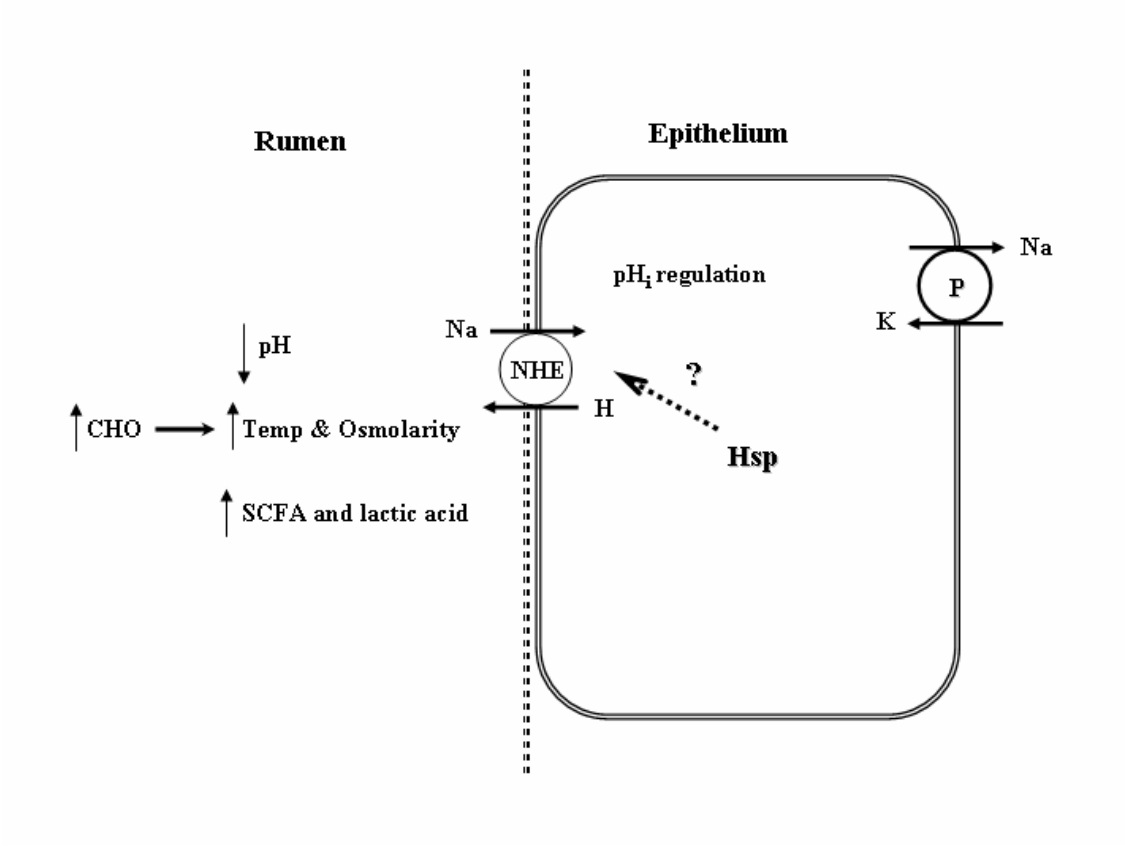


Figure 1-6: Sequence of events associated with ruminal acidosis and the proposed mechanism of cellular recovery. The increased fermentation of carbohydrates (CHO) in the rumen causes an increased concentration of SCFA, an elevation of intra-ruminal temperature, and a decrease of pH, which could compromise epithelial integrity and, therefore, has to be tackled. NHE is a well-known acid recovery mechanism for various cells and epithelia including rumen. However, its efficacy in short-term stress regulation and interaction with Hsp70 needs to be studied.

Chapter 2

Material and Methods

2.1 Experimental animals

Rumen epithelium was obtained from freshly slaughtered sheep (*Ovis aries*) of various breeds and sex. Weights of animals ranged between 30-40 kg at ages of approximately 9-14 months. The animals were fed with hay *ad libitum* or received an additional 800 g of concentrate in two portions (Table 2-1) according to the protocol of the experiment. The animals had free access to tap water and lick stones. For experiments with heat shock proteins, rectal and intra-ruminal temperature was recorded.

Table 2-1: Composition of fodder for the experimental animals

Parameter	Hay	Concentrate
	% of dry matter	
Dry matter	90.8	90.9
Ash	5.0	7.1
Crude protein	7.9	17.8
Crude fibre	34.1	8.91
Fat	1.3	2.88
Sugar	9.71	6.71
Starch	-	33.08
ADF (organic)	43.4	11.6
NDF (organic)	6.62	26.2
Ca	0.22	1.06
P	0.22	0.48
Mg	0.08	0.25
K	1.67	1.08
Na	0.01	0.48

2.1.1 Isolation, preparation, and handling of epithelial tissues/cells

Approximately two to three min after the sheep had been stunned and exsanguinated, their forestomachs were removed. The epithelial sheets were withdrawn from the ventral part of the rumen and sparingly washed with warmed (38ºC) and carbogen-gassed (95% O_2 - 5% CO_2) buffer solution until the fluid became clear. While immersed in the buffer solution, the muscle layer was carefully stripped from the epithelium. The epithelial sheets were then cut into

approx. 3 × 3 cm pieces for use in Ussing chambers. These pieces were subsequently transferred to warm buffer solution (37°C) to be transported to the laboratory. The term "epithelium" used throughout the study refer to the tissue obtained by this procedure.

Primary culture of ruminal epithelial cells (REC) were prepared as described by Galfi *et al.* (1981). Briefly, a piece of rumen mucosa was taken from cranial sac, washed three to four times with Ca^{++} and Mg^{++} free Dulbecco´s PBS (DPBS) containing 4% penicillin-streptomycin and stored at 4°C in this solution. After an incubation period of 45 min, the villi in the rumen were dissected from the remaining tissue in Ca^{++} and Mg^{++} free DPBS containing 1% penicillin-streptomycin and washed twice with antibiotic free DPBS. The villi were then cultivated in a process called "warm trypsinization", at 37°C with a trypsin-EDTA solution. This solution, together with the cells, was replaced every 30 min with fresh trypsin EDTA solution. Cells and the remnants of the plant components were

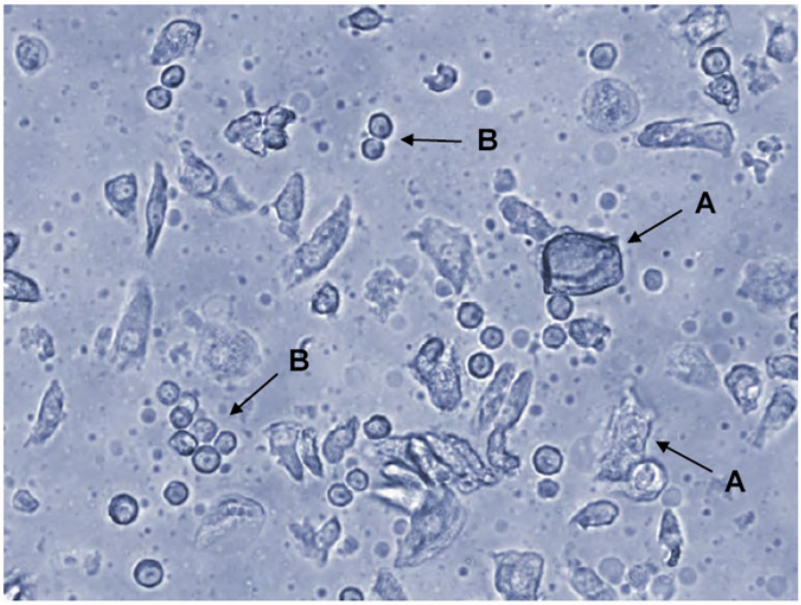


Figure 2-1: Microscopic image of a cell fraction obtained from the cell isolation procedure. The fraction contains both the horny cells (A) and the living cells from the stratum spinosum and stratum basale (B). (With permission from Inge Brinkmann 2006).

separated by filtration through gauze. Trypsinization was continued until non-epithelial cells appeared in the suspension. The fraction containing chiefly stratum spinosum and stratum basale cells (Figure 2-1) were pooled and the pool was washed three times in Ca^{++} and Mg^{++} free PBS at 4°C. After the assessment of cell viability by trypan blue exclusion, the cells

were re-suspended in minimal essential medium (MEM), 12% fetal bovine srerum, 2 $\mu mol \cdot ml^{-1}$ glutamine and 100 $\mu g \cdot ml^{-1}$ gentamycin. The cells were counted in a hemocytometer and nutrient medium was added to the suspension to adjust its cell count to $5 \times 10^5 \cdot ml^{-1}$. The medium was replaced initially after two days and subsequently twice weekly. The growth of monolayer usually took 10-15 days.

2.2 Ussing chamber technique:

All the experiments were carried out on the isolated ruminal epithelial tissue with the conventional Ussing chamber technique developed by the Danish scientist Hans Ussing (1949). This method has been modified many times for the investigations of rumen and other forestomach epithelial tissues (Ferreira *et al.*, 1972; Stevens, 1964). The chamber consists of two equal halves (Figure 2-2) between which the epithelia were mounted dividing the chamber into two equal spaces (luminal = apical = mucosal and blood side = basolateral = serosal). In these experiments, the exposed area of the epithelium was 3.14 cm^2. Silicon rings placed on both sides between the epithelium and the chamber were used to minimize edge damage. The usual procedure for all the experiments was to equilibrate the tissues under the short-circuited condition for not less than 30 min with the control buffer solution, so that all the electrophysiological values became relatively stable. After this incubation period, only those epithelial tissues that had a conductance (G_t) of not more than 8.0 $mS \cdot cm^{-2}$ and a short-circuit current (I_{sc}) of not less than 0.5 $\mu eq \cdot cm^{-2} \cdot h^{-1}$ were subsequently used in the experiments described. Under these conditions, the epithelial tissues remained stable for relatively longer period of time (Martens and Gabel, 1988). Epithelial tissues that deviated markedly from the above-mentioned electrophysiological values were considered to have been improperly prepared, transported, or handled. Once relatively stable electrophysiological parameters had been achieved, epithelial tissues with approximately the same electrophysiology (G_t and I_{sc}: less than 20%) were paired for the determination of unidirectional ion fluxes (J_{ms}: mucosal-serosal, or J_{sm}: serosal-mucosal). For each set of experiments, 12 epithelial pairs were available (when 4 clamps were used). Since the heterogeneous origin of sheep causes unavoidable variations in the electrophysiological values (Schultheiss and Martens, 1999), and because of variations among tissues within the same animal, control and experimental groups were performed with tissues from the same animal.

2.2.1 Electrical measurements

Electrical measurements were continuously obtained by a computer-controlled voltage-clamp device (AC Micro-Clamp, Aachen, Germany). The transepithelial potential difference (PD_t) was determined by using two AgCl half-cells and a millivoltmeter connected to the mucosal and serosal sides of the chambers by Agar gel bridges filled with 1.5M KCl solution. These bridges at the front were approximately 3 mm apart from the epithelium, whereas the rear bridges were 25 mm apart. The tissue conductance (G_t) was corrected for the conductance of the buffer solution (fluid resistance), and the PD_t was corrected for liquid junction potential measured before the epithelia were mounted. The conductance together with the PD_t and short-circuit current (I_{sc}) were displayed on the monitor at regular intervals (every 10 seconds).

2.2.1.1 Open circuit

In this method, current pulses of 100 µA amplitude (0.5 sec duration) were applied to cause a change in PD_t, and from this, the tissue conductance (G_t) could be calculated according to Ohm´s law. All the experiments began in this mode for at least for 30 min in order for the tissues to equilibrate.

$$Gt = \frac{\Delta I}{\Delta PDt}$$

where

G_t = Tissue conductance

ΔI = Current pulses

ΔPD_t = Change in transepithelial potential difference

2.2.1.2 Short-circuit

This technique is a particular form of the ***voltage clamp*** technique in which the transepithelial potential difference (PD_t) is clamped to 0.0 mV through the application of an external current. Under these conditions, the short-circuit current (I_{sc}) is equivalent to the sum of all electrogenic ion movement across the epithelial tissue. There was no electrical gradient across the epithelial tissue, and since the buffer solutions on both sides of the epithelial tissue were identical, there was also no chemical gradient across the epithelial tissue. When a net transport of an ion or nutrient was measured under these conditions, the transport mechanism of the ion was active or secondary active.

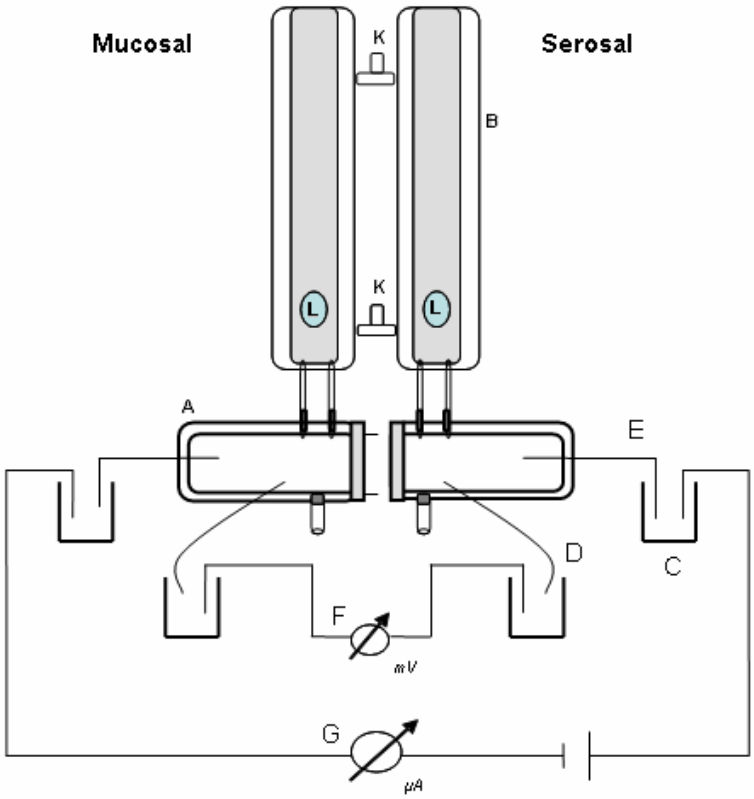


Figure 2-2: Representation of Ussing chamber technique. A = fiberglass chamber, B = Doubled wall glass, C = Electrode holder, D = Front Ag/AgCl bridges (Potential electrodes), E = Rear Ag/AgCl bridges (Current electrodes), F = Volt meter, G = Amp meter, K = Opening for water bath, L = Opening for gas.

2.3 Calculation of ion flux rates:

The specific radioactivity was calculated from the mean of the hot sample radioactivity [(H1+H2/2)]:

$$\textbf{Specific Radioactivity } (\mathbf{AK_{spec}}) = \frac{cpm_H}{V_H \cdot c}$$

where

$\mathbf{Cpm_H}$	=	Radioactivity of the hot sample (Cpm)
$\mathbf{V_H}$	=	Volume of the hot sample in ml
C	=	Concentration of the measured non-radioactive isotope in the buffer solution (mmol/l or µmol/l)

The unidirectional flux rate (J) per h and per cm^2 and J_{ms} (mucosal-serosal) and J_{sm} (serosal-mucosal) flux rate were calculated from the samples taken from the cold sides according to the following formula:

$$\mathbf{J} = \frac{P_2 \bullet V_b / V_s - \left[P_1 \bullet (V_b - V_s)/V_s\right]}{AK_{spec}.A.t}$$

where

J	=	Ion flux
$\mathbf{P_1}$	=	Counts per min at the beginning of flux period
$\mathbf{P_2}$	=	Counts per min at the end of the flux period
$\mathbf{V_b}$	=	Buffer volume (16ml)
$\mathbf{V_s}$	=	Sample volume (1.0ml)
$\mathbf{Ak_{spec}}$	=	Specific radioactivity in cpm/µM
A	=	Chamber free surface area ($3.14cm^2$)
t	=	Duration of the flux period (0.5 h)

The net flux rate was calculated from the unidirectional flux rate as follows:

$$\mathbf{J_{net} = J_{ms} - J_{sm}\ [\mu eq \cdot cm^{-2} \cdot h^{-1}]}$$

A positive net flux indicated absorption, whereas a negative net flux indicated secretion.

2.3.1 Measurement of Na and acetate fluxes

Since the Ussing chamber technique only allows the measurement of unidirectional fluxes, i.e., mucosal to serosal (J_{ms}) or serosal to mucosal (J_{sm}), careful pairing of the epithelia is important: hence, tissues were paired with differences of G_t < 20%. For flux measurements, 70 kBq of the radioactive isotope ^{22}Na (Amersham Buchler, Braunschweig) and/or 60 kBq of ^{14}C-acetate were added to the marked side (mucosal for J_{ms} and serosal for J_{sm}), called the "hot side". The other side was referred to as the "cold side". At 15 min after the addition of the radioisotope, 100-µl aliquots were taken from the hot side (referred to as "H1"), and this was repeated at the end of the flux periods (called the "H2"). These were used to calculate the specific activity. Periodic samples of 1 ml from the cold side were taken at an interval of 30 min, at least 3 times (called fluxes). The same volume was replaced in the reservoir with the respective experimental buffer to maintain a constant volume. ^{22}Na and ^{14}C were assayed by using a well-type crystal counter (LKB; Wallace-Perkin Elmer, Überlingen, Germany)

2.4 Estimation of heat shock proteins (Hsp70)

2.4.1 Incubation and collection of tissues/cells:

Rumen epithelium was equilibrated in the Ussing chamber under the short-circuit condition for approximately 30 min until all the electrophysiological parameters were stable. Thereafter, the temperature was raised gradually to the desired level by adjusting the temperature of the water bath. Similarly, buffer on the mucosal side was substituted with various concentrations of SCFA, pH, and osmolarity for 30 min (stress period). After the induction of various stress scenarios, the "stress" buffer on the mucosal side was exchanged, and the tissues were once again incubated under normal conditions for another 1.5 h (recovery period). At the end of this recovery period, the tissues were removed from the Ussing chambers, cut into small pieces, collected in RNA later solution (Ambion®), and stored at -80°C for further analysis (Figure 2-3).

Isolated rumen epithelial cells (IREC) were similarly subjected to the various stresses described above and again incubated for 1.5 h under normal conditions (recovery period). However, the only difference was that these cells were not short-circuited. The cells were then carefully scrapped off the culture dishes and collected in Falcon tubes, centrifuged at 4°C to form a pellet, and stored for later investigations.

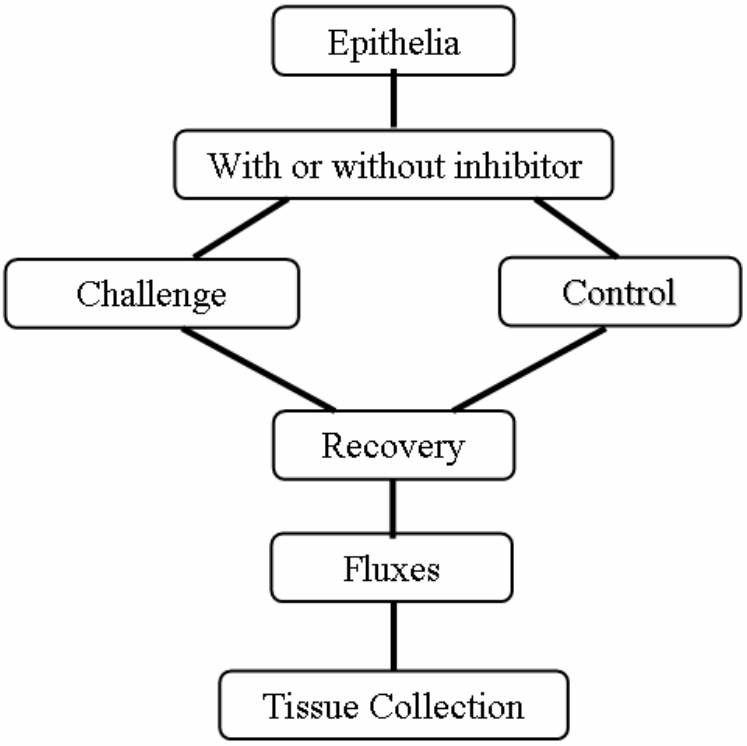


Figure 2-3: Representation of the experimental design. Challenge means incubation of epithelia at high temperature, low pH, high SCFA concentration or high osmolarity.

2.4.2 Western blot:

Samples from the rumen epithelium were mechanically crushed and homogenized (homogenizer, Retsch) by using metallic beads in a phosphate buffer saline (PBS) and centrifuged at 10,000 g for 1 min to remove tissue debris. Supernatant was collected, and the protein concentration was measured with the Bradford protein assay reagent (Bio-Rad). Equal amounts of protein (10 µg) for each sample were separated by 7.5% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and a 4% stacking gel by using the mini PROTEAN system (Bio-Rad laboratories Inc. USA). Samples were initially run at 150 V for 10 min, followed by 200 V for 60 min. The gel was later transferred to nitrocellulose membrane which was then blocked for 1 h with 5% skimmed milk. The membrane was incubated for 1 h with a primary antibody against Hsp70 followed by a 10-min wash and

further a 1-h incubation with a secondary antibody conjugated to horseradish peroxidase (HRP) at room temperature. The membrane was then washed for 30 min and exposed to chemiluminescence. Densitometric analysis (Chemilmager 5500®) was performed to quantify the signal intensity.

2.4.3 Reverse transcription with quantitative polymerase chain reaction (RT-qPCR):

Total RNA was isolated by using a commercially prepared system (Nucleospin RNA II kit, Macherey & Nagel®), and any fragments of genomic DNA that could contaminate the RNA were removed by incubating the preparation with DNase (DNase I reaction mixture) for 15 min. RNA integrity was determined by using a commercially prepared kit (RNA 6000 Nano kit, Agilent) which allocated an integrity number to each RNA sample (RNA integrity number, RIN). RNA samples with a RIN (Table 8-9) of not less than 8.5 were treated with deoxyribonuclease and reverse-transcribed with reverse transcriptase to prepare complementary DNA (cDNA) in a commercially available system (iScript cDNA synthesis kit, Bio-Rad®). Oligonucleotide primer pairs for Hsp70 were as follows:

5´-CGA CCT CAA CAA GAG CAT CA-3´ (antisense)
3´-GCT GGA GTT GTT CTC GTA GT-5´ (sense).

For the qPCR, samples were analyzed by using a commercially available system (iQ SybrGreen Supermix, Bio-Rad) that contained *Taq*-polymerase, deoxyriboneuclotide, SybrGreen, $MgCl_2$ (3 mM), and reaction buffer. A 12.5-µl sample of this master mix was added to 5 µl cDNA, 0.5 µl primer pairs, and 6.5 µl water and incubated in the thermocycler at an annealing temperature of 58°C for 35 cycles. To control for non-specific amplification, PCR were also carried out in the absence of cDNA (negative control).

2.5 Buffer solutions

All the chemicals used in the preparation of buffer solutions were of analytic grade and purchased from Sigma Chemicals Co. (St. Louis, MO, USA). The buffers were always prepared fresh for each experiment by using distilled water, and their various compositions are summarized in section 8.1. The osmolarity of the buffers was adjusted to 300 ± 10 mosmol/l (according to the principle of lowering the freezing point), and the pH was adjusted to various values, according to the protocol of the experiment, by using HCl or TRISMA.

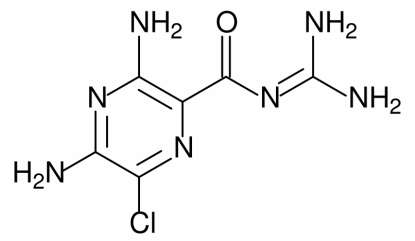
Bicarbonate-containing buffers were continuously gassed with carbogen (95% O_2 - 5% CO_2), and the temperature was adjusted to ~38°C or according to the protocol.

2.6 Inhibitors

All the inhibitors were handled carefully and dissolved in DMSO (dimethylsulfoxide) shortly before use in the experiments. Adequate time was given to the epithelium after the addition of inhibitors for the desired affect to be achieved.

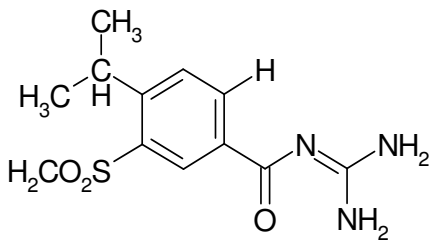
2.6.1 Amiloride

Amiloride is a potassium-sparing diuretic exerting its effect by blocking the epithelial Na channel (ENaC) at low concentrations (micromolar) and the non-specific NHE at high concentrations (> 0.1 $mmol \cdot l^{-1}$). It was generally used at a concentration of 1.0 $mmol \cdot l^{-1}$ on the mucosal side of the epithelium.



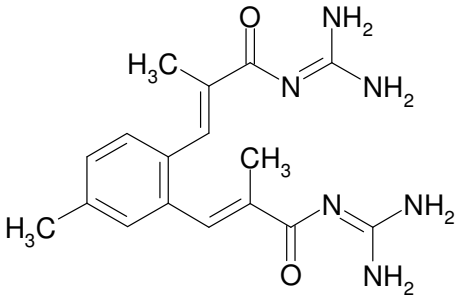
2.6.2 Cariporide (HOE 642)

Cariporide is a specific inhibitor of NHE1 with an IC_{50} of 0.03 and 0.34 $\mu mol \cdot l^{-1}$ (Masereel *et al.*, 2003). It was used at a concentration of 30 $\mu mol \cdot l^{-1}$ in all experiments.



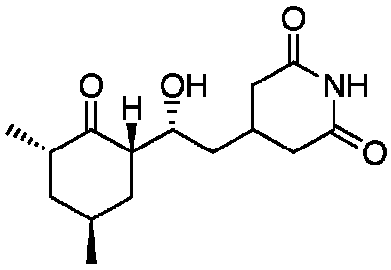
2.6.3 S3226

S3226 [3-{2-(3-guanidino-2-methyl-3-oxo-propenyl)-5-methyl-phenyl}-N-isopropylidene-2-methyl-acrylamide dihydrochloride] is a specific inhibitor of the NHE3 isoform and can inhibit electroneutral Na transport via NHE3 at a dose of 1.0 µmol·l^{-1}.



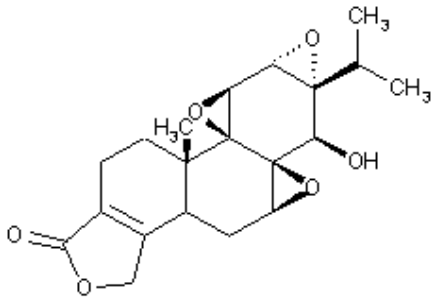
2.6.4 Cycloheximide

Cycloheximide (CHX), an inhibitor of protein synthesis, exerts its effect by interference at the translocation step during protein synthesis, thus blocking translocational elongation. Its effects can be reversed by simply removing it from the medium. In this study, cycloheximide was used at a concentration of 3.0 mmol·l^{-1}.



2.6.5 Triptolide

This natural product, isolated from the medicinal vine *Tripterygium wilfordii* Hook F (''Thunder God Vine''), is a potent inhibitor of nuclear factor kappa B (NF-κB) and NF-AT-mediated transcription and therefore leads to the down-regulation of many gene products necessary for the inflammatory response or cellular growth. Triptolide potently inhibits the heat shock induction of Hsp70 expression by interfering with the activity of the C-terminal transactivation domain of HSF1 and blocking its transcriptional activity (Powers and Workman, 2007). A concentration of 100 $nmol \cdot l^{-1}$ of this inhibitor was used during this study.



2.7 Data analysis

In this study, several samples of epithelial tissues were obtained from each animal to be used in different Ussing Chambers. "**N**" refers to the number of experimental animals, whereas "**n**" refers to the number of epithelial tissues per treatment group.

Each experimental protocol was tested on at least 3 experimental animals. The obtained data were then statistically evaluated by using Sigma Stat (3.0.1) and Sigma Plot program version 8.0 for Windows (SPSS Inc, Chicago, Illinois, USA).

The comparison between the treatment-groups were carried out by the Student t-test and a repeated measurement of the analysis of variance (ANOVA) with a two factorial model without interaction (flux rate = Attempt + Treatment + Rest). A significant effect of treatment was considered to have occurred at $p < 0.05$. The results are given as means ± SD (standard deviation). Microsoft Excel was used for the analysis of qPCR data.

Chapter 3

Results

3.1 *In vitro* Induction of Hsp70:

The heat shock response is a highly conserved mechanism that allows cells to withstand a variety of stress conditions (Winklhofer *et al.*, 2001). The activation of this response is characterized by an increased synthesis of heat shock proteins (Hsp), which protect cellular proteins from stress-induced denaturation (Duina *et al.*, 1998). Among the different families of Heat shock proteins, Hsp70 is regarded as a stress inducible heat shock protein that is up-regulated earlier than the other members (Nishimura and Dwyer, 1995).

In most of the studies performed in the past, Hsp70 had been induced in cell lines and a few intact epithelia (Nelson *et al.*, 2008; Pierzchalski *et al.*, 2009). Whether Hsp70 synthesis could be induced in rumen epithelium was unknown. The first step in this study was therefore to induce Hsp70 *in vitro* by using physiological parameters of acidosis such as high temperature, low pH, increased SCFA concentration and high osmolarity.

Conventionally, Hsp70 is induced by high temperature and so the following experiments can be considered as a type of pilot study to optimize the induction of the synthesis of Hsp70.

3.1.1 Effect of temperature:

Isolated rumen epithelia were incubated in control buffer (Table 8-1) at normal temperature (38° C) for approximately 30 min until the I_{sc} and G_t had become stable. At the end of this period, the average I_{sc} and G_t were 1.35±0.15 $\mu eq \cdot cm^{-2} \cdot h \cdot r^{-1}$ and 3.16±0.24 $mS \cdot cm^{-2}$, respectively. For the treatment with temperature, the tissues were exposed to 38°C or 42°C for a period of 30 min. The desired temperature of the water bath used for the regulation of the temperature of the Ussing chambers was obtained in approximately 5-6 min and then the tissues were exposed to the respective temperature for the planned time. Example of temperature-dependent changes of I_{sc} and G_t are given in Figure 3-1. An increase in temperature caused within ~15 min a ~90% (significant) increase of I_{sc} from 0.89±0.08 to 1.69±0.11 $\mu eq \cdot cm^{-2} \cdot hr^{-1}$, and an increase of G_t from 2.41±0.06 to 3.63±0.12 $mS \cdot cm^{-2}$ was also observed.

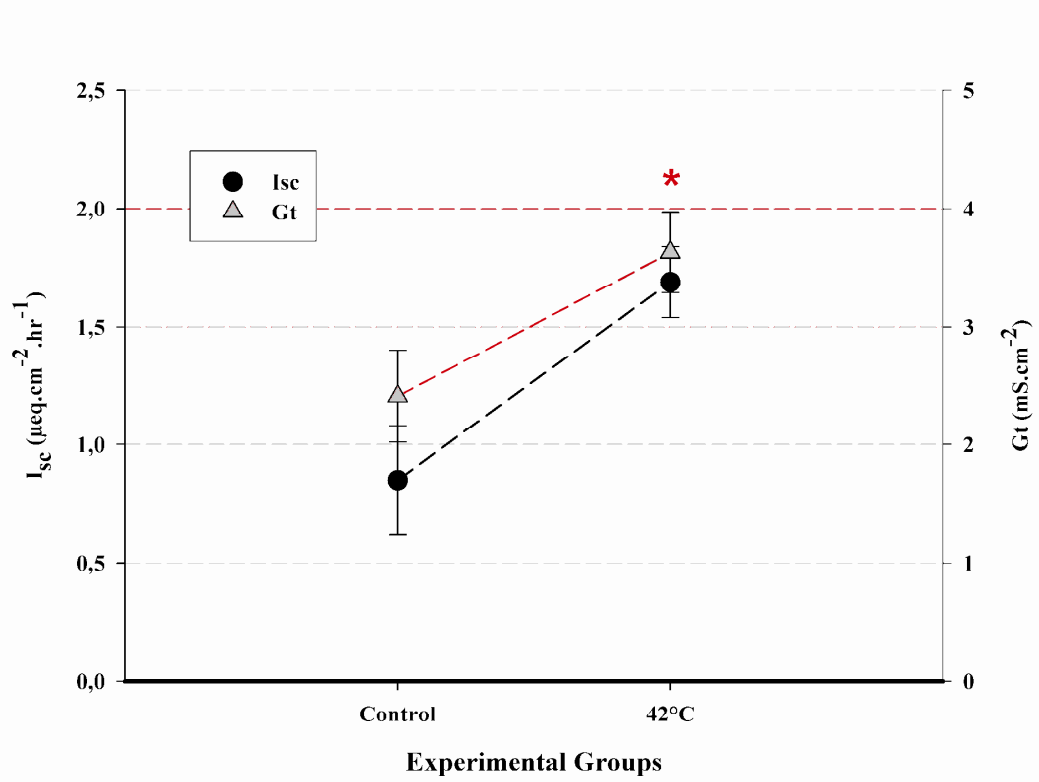


Figure 3-1: Effect of an increase of temperature from 38°C (control) to 42°C on short circuit current, I_{sc} and tissue conductance, G_t. Steady state values are shown, which were achieved after 15 min of change of temperature. Each point represents: Mean ± SD; N/n = 5/8. *p = 0.038 (I_{sc}) and 0.01 (G_t).

In order to confirm presence of Hsp70 in rumen epithelium, tissues from these experiments incubated at 42°C were analyzed by conducting qPCR. Figure 3-2 shows three replicates of tissues exposed to 42°C and then processed for qPCR.

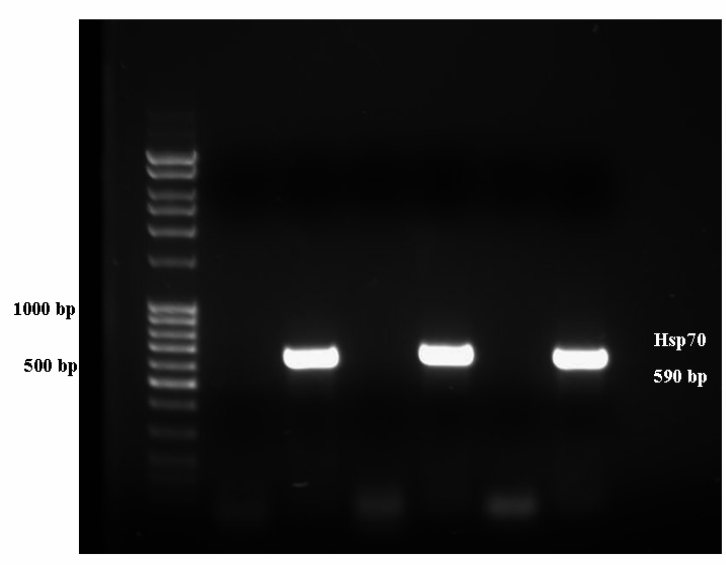


Figure 3-2: qPCR of three tissues obtained after 30 min of exposure to 42°C. Presence of Hsp70 gene was confirmed in rumen epithelium.

A similar kind of approach was adopted in experiments with isolated rumen epithelial cells (IREC), exposed to 37°C and 42°C for 30 min. However, in this set of experiments the electrophysiology could not be examined. The viability of the cells was monitored through cell counts and morphology by high resolution microscopy which suggested no apparent morphological differences between the groups. These cells were later analyzed for Hsp70 by using western blot and as seen in Figure 3-3, higher expression of Hsp70 was observed in cell culture exposed to 42°C for 30 min.

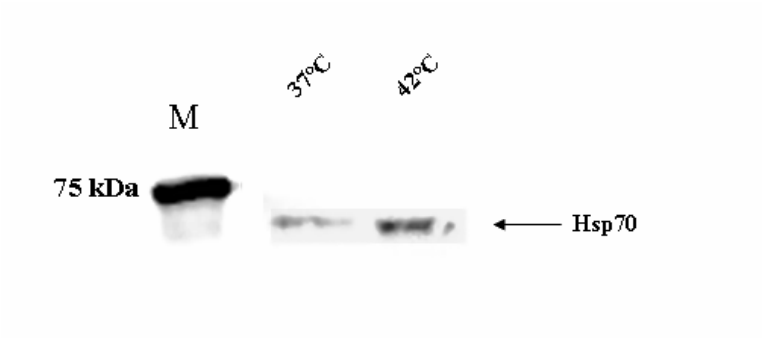


Figure 3-3: Effect of temperature on Hsp70 expression in isolated rumen epithelial cells (IREC). Cells were exposed to 37°C and 42°C for a period of 30 min and proceed immediately for Western blot.

Replicates from three different sheep used to obtain IREC were subjected to the same protocol for Hsp70 induction. Table 1 summarizes the out-come of Hsp70 expression in terms of integrated density values (IDVs) following Western blot. An increase of approximately 23% in Hsp70 expression occurred in cells exposed to 42°C for 30 min. (Table 3-1).

Table 3-1: Effect of increasing temperature (30 min) on Hsp70 expression in IREC. Mean ± SD;

Treatment	IDVs × 1000	N
37°C	187.45±17.15	3
42°C	230.63±19.59*	3

* $p = 0.04$

The above study clearly demonstrated the expression and synthesis of Hsp70 at increased temperature. In order to determine the effect of exposure time on Hsp70 expression, isolated ruminal epithelial tissues exposed to 37°C and 42°C were incubated for various durations i.e. 30, 60 and 120 min. The tissues from both temperature groups were then subjected to Western blot, which revealed both a time and temperature-dependent change in Hsp70 expression. Figure 3-4 shows results obtained from three animals subjected to the same protocol. Hsp70 synthesis can be seen to have gradually increased from 30 to 120 min. Hsp70 expression was significantly higher in terms of integrated density values (IDVs $\times 10^3$) at 60 and 120 min at 42°C as summarized in Table 3-2.

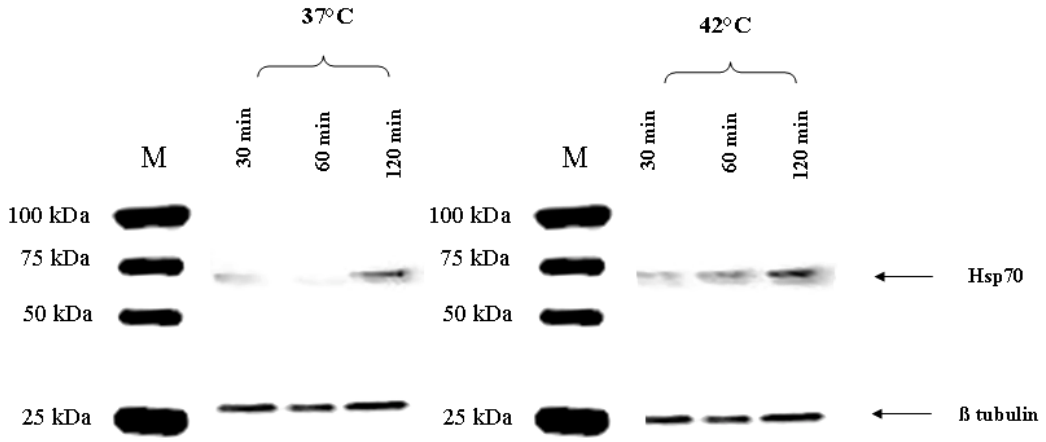


Figure 3-4: Time and temperature dependent increase of Hsp70 expression in rumen epithelium. Three tissues randomly selected from both treatment groups are shown collected immediately after the expiry of the defined incubation period.

An analysis of variance (ANOVA) from these experiments suggested a significant change in Hsp70 expression both within and between the treatment groups. The increase in temperature together with exposure time was generally observed to cause a significant increase in Hsp70 expression. Exposure to 42°C for 60 min resulted in an approximately 60% increase in Hsp70 expression, whereas a 120 min exposure at 42°C caused an increase of 146% in Hsp70 expression (Table 3-2).

Table 3-2: Effect of time and temperature on Hsp70 expression in rumen epithelium. Mean ± SD; N/n = 3/9

Treatment	IDVs × 1000		
	30 min	**60 min**	**120 min**
38°C	73.65±14.11[a]	81.89±18.25[1]	188.75±15.62[b,1]
42°C	86.24±11.58[a]	137.14±14.76[b,2]	212.75±17.58[c,2]

Different superscripts are significantly different at $p = < 0.05$

a, b and c (time-dependent) 1 and 2 (temperature-dependent)

Following this preliminary approach of inducing Hsp70 expression in rumen epithelia of sheep with the Ussing chamber method, an improved technical protocol was applied for the induction of Hsp70. For the observation of a wider range of temperature, the experiments were designed to incubate the tissues at 37°, 39°, 41° and 43° C followed by 1.5 hours of recovery under control conditions (38°C) because the expression of Hsp70 requires a sufficient period of recovery from this sub-lethal stress under controlled conditions (Chang *et al.*, 2007; Ma *et al.*, 2009).

Under these conditions, the observed changes of I_{sc} and G_t proceeded proportionally to the magnitude of the temperature stress.

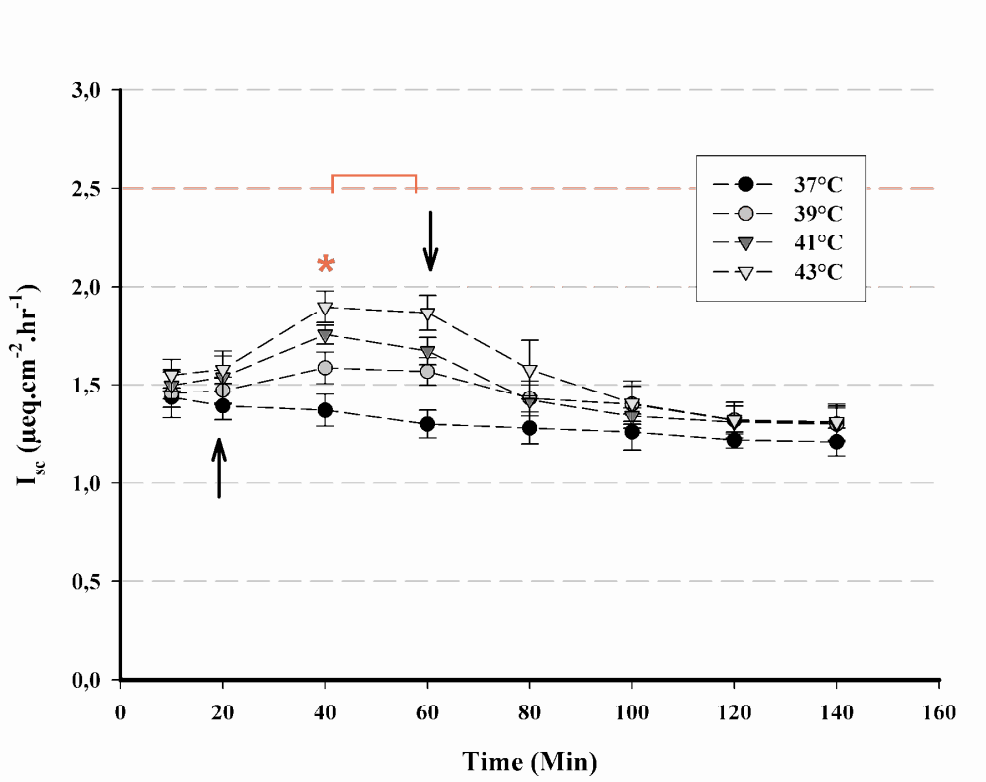


Figure 3-5: Effect of increasing the temperature on short circuit current (I_{sc}). The temperature was increased at the end of the equilibration period (↑) for 30 min and then returned back to normal (↓) i.e. from time 60 onwards. Each point represents Mean ± SD. N/n = 4/13

The I_{sc} of the control group exhibited the usual time dependent decrease from 1.44±0.03 µeq·cm^{-2}·h^{-1} to 1.21±0.02 µeq·cm^{-2}·h^{-1}. The change of temperature (43°C for instance) caused a temperature dependent increase of I_{sc} from 1.55±0.04 µeq·cm^{-2}·h^{-1} to 1.90±0.05 µeq·cm^{-2}·h^{-1} which remained steady for the duration of the stress period (Bracket in Figure 3-5). The decrease of temperature (back to 38°C) at time 60 min reduced the I_{sc} and at 100-110 min the I_{sc} (1.41±0.05 µeq·cm^{-2}·h^{-1}) was not significantly different from control group.

A linear correlation was obtained between temperature and I_{sc} when the peak of I_{sc} was plotted (Figure 3-6).

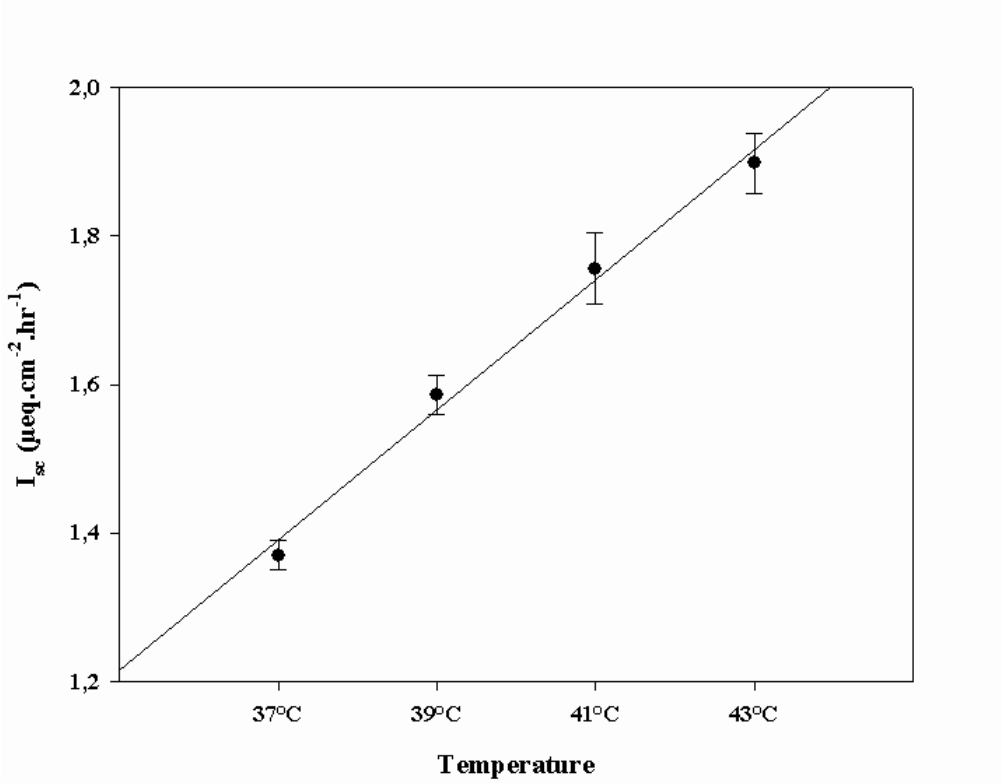


Figure 3-6: Effect of increasing temperature on short circuit current (I_{sc}) exhibiting a linear correlation (r^2 = 0.97) with the new steady state after change of temperature. Each point represents: Mean ± SD, N/n = 5/8-12.

The temperature dependent changes of G_t followed a similar pattern (Figure 3-7) as observed for I_{sc}. A significant increase in tissue conductance occurred at 41°C and 43°C. However, increase of temperature to 39°C did not cause any significant increase in G_t.

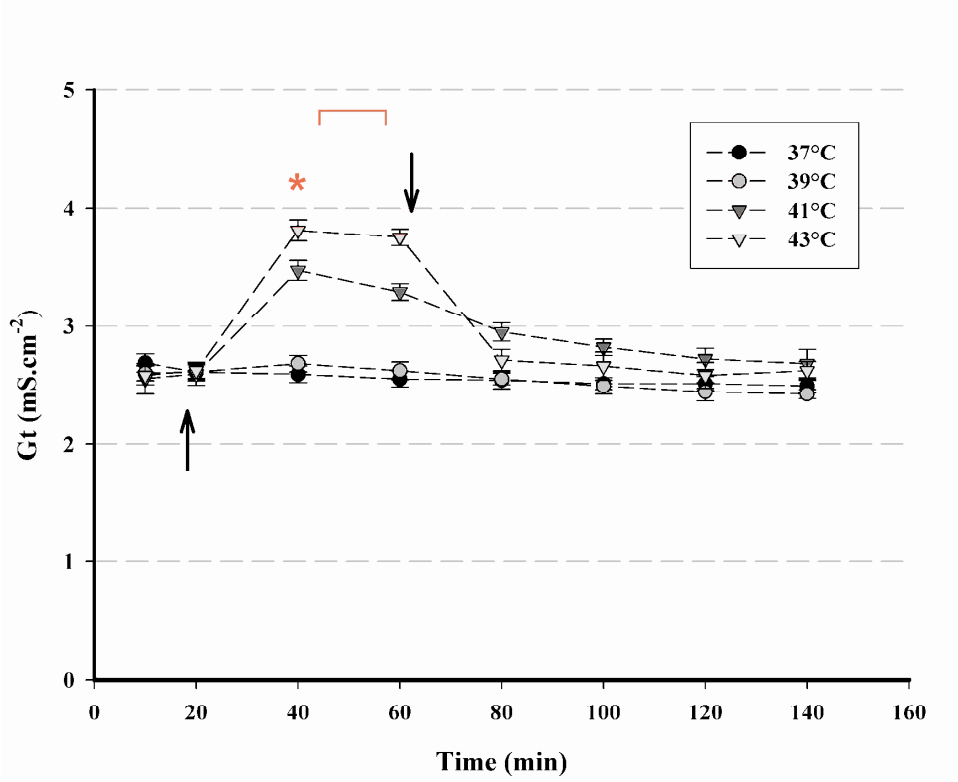


Figure 3-7: Effect of increasing temperature on tissue conductance (G_t). The tissues were incubated as described in Material and Methods and at the end of the equilibration period (time 0) the temperature was increased (↑) for 30 min an then returned to normal (↓). Each point represents: Mean±SD, N/n = 5/8-12

Western blot carried out from these experiments obviously revealed a more uniform and consistent method for the induction of Hsp70 in rumen epithelium (Figure 3-8). The highest expression of Hsp70 was observed at 41-42°C. However, interestingly when the temperature was raised to 43°C or 45°C the expression of Hsp70 declined.

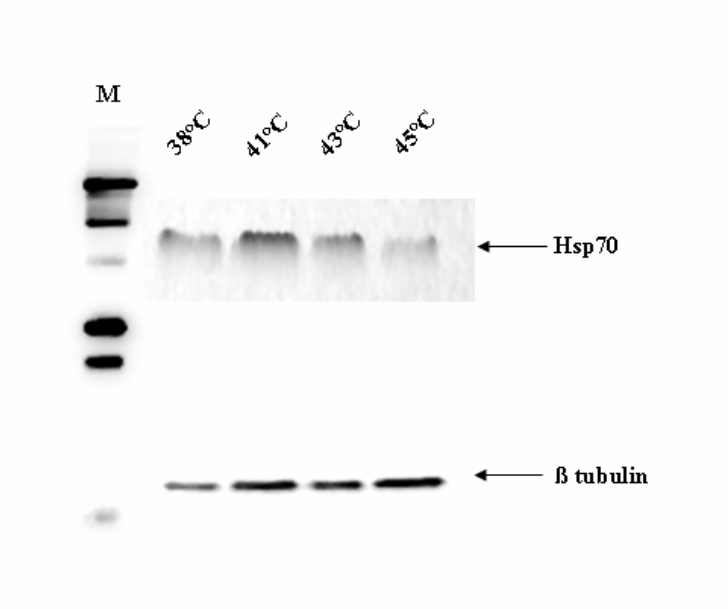


Figure 3-8: Effect of different temperature in Ussing Chamber on Hsp70 expression. Tissues were incubated with increasing temperatures for 30 min followed by 1.5 hr of recovery under 38°C.

Table 3-3 summarizes the Hsp70 expression in terms of IDV after treatment of tissues with various temperatures (30 min at elevated temperature and recovery of 90 min at control temperature).

Table 3-3: Hsp70 expression in terms of integrated density values after treatment of tissues with various temperatures (30 min at elevated temperature and recovery for 90 min at control temperature). Mean ± SD.

Treatment	Integrated Density Values (IDV ×1000)	N/n
38°C	70.67±10.27	8/21
41°C	106.58±15.55[a]	7/21
43°C	84.65±11.78	7/18
45°C	67.86±11.40[b]	8/16

(a) $p = 0.03$ (compared with control) (b) $p = 0.01$ (compared with 41°C)

The results show that the induction of Hsp70 is temperature and time-dependent. Furthermore, optimal results regarding the synthesis of Hsp70 protein were observed when the exposed tissue recovered for 90 min after the challenge by temperature.

3.1.2 Effect of pH:

In these experiments, tissues were incubated in either transport buffer (without SCFA) or with SCFA buffer (80 mM) on the mucosal side. In each case, the tissues were divided into four groups; pH 7.4, 6.4, 6.0 and 5.8. After an equilibration period of ~30 min with the control buffer (without SCFA), the pH on the mucosal side was changed by titration with HCl until the desired value was achieved (this procedure took no longer than 5 min). In the case of experiments with mucosal SCFA buffer, the buffer (without SCFA) was replaced (with SCFA) at the end of the equilibration period followed immediately by the pH change. This pH challenge on the mucosal side was maintained for a period of 30 min followed by a recovery period of about 1.5 hours with the control buffer (pH 7.4; without SCFA).

A decrease in mucosal pH without SCFA buffer resulted in an increase in I_{sc} whereas, the opposite effect was observed for the buffer with SCFA (Figure 3-9).

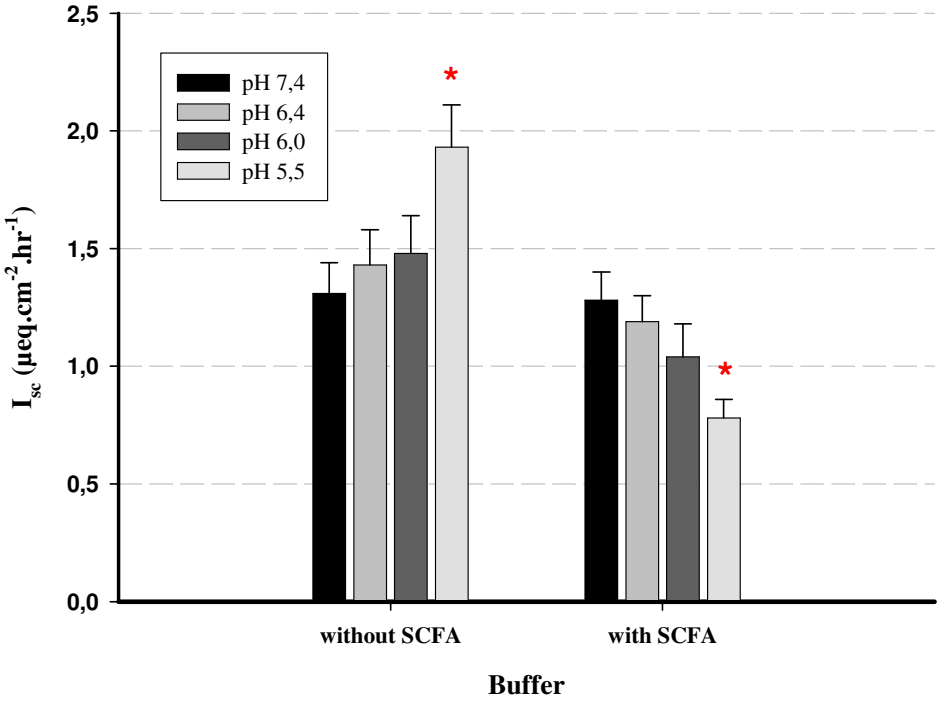


Figure 3-9: Effect of mucosal pH on I_{sc} in the absence or presence of SCFA. At the end of equilibration (30 min) the mucosal pH was changed by addition of HCl. The I_{sc} is the mean of the incubation period with the corresponding pH for 30 min. Each bar: Mean ± SD, N/n = 5/8-12. *p = 0.01 vs pH 7.4.

The magnitude of change in I_{sc} occurred in parallel to the drop in pH. For instance, a change of pH from 7.4 to 6.4 caused a 2% increase in I_{sc} whereas a pH drop from 7.4 to 5.8 caused a 31% increase. A similar but opposite effect on I_{sc} was observed for buffers with SCFA, a pH change from 7.4 to 6.4 caused a 3% decrease of I_{sc} whereas, a decrease of approximately 46% was observed at pH 5.8.

The effects of pH on electrophysiological parameters from these experiments are summarized in Table 3-4.

Table 3-4: Effect of mucosal pH on I_{sc} and G_t in the presence or absence of SCFA. Mean ± SD.

Treatment	Without SCFA		With SFCA		N/n
	I_{sc} $\mu eq \cdot cm^{-2} \cdot hr^{-1}$	G_t $mS \cdot cm^{-2}$	I_{sc} $\mu eq \cdot cm^{-2} \cdot hr^{-1}$	G_t $mS \cdot cm^{-2}$	
pH 7.4	1.39±0.36	3.07±0.31	1.34±0.24	2.79±0.44	5/6
pH 6.4	1.43±0.24	2.88±0.48	1.29±0.41	2.36±0.73	4/8
pH 6.0	1.49±0.48	3.13±0.22	1.05±0.59	2.30±0.38	4/8
pH 5.8	1.83±0.61[a]	3.78±0.46	0.72±0.45[b]	2.37±0.42	5/7

Significantly different from control (pH 7.4): (a) $p = 0.005$; (b) $p = 0.01$

Western blots of samples from these experiments revealed an increased expression of Hsp70 at pH 6.4 in SCFA free buffer. Moreover, when the pH was decreased to 5.8, Hsp70 expression was observed to decline (Table 3-5). In the presence of SCFA buffer on the mucosal side, the highest Hsp70 expression was observed at pH 6.0 and this increase in expression was almost two fold compared with the control tissues (without SCFA). Importantly, a similar pattern of down-regulation of Hsp70 expression was observed when the pH was further reduced to 5.8 (Figure 3-10)

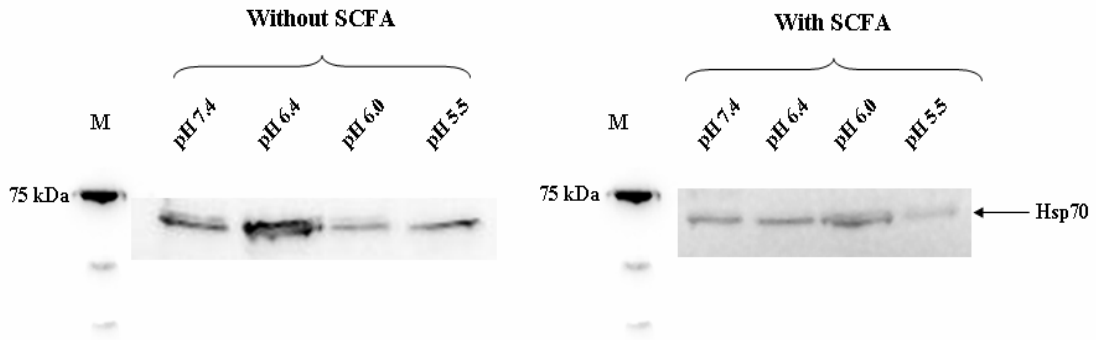


Figure 3-10: Hsp70 expression in rumen epithelium at various mucosal pH in the presence and absence of SCFA.

Table 3-5 summarizes the Hsp70 expression in terms of IDV with Mean±SD being compared by ANOVA. Hsp70 expression almost doubled when the pH was reduced on the mucosal side to 6.4 (in the absence of SCFA) and to 6.0 (in the presence of SCFA).

Table 3-5: Effect of mucosal pH on integrated density values of Hsp70 in the presence and absence of mucosal SCFA. Mean ± SD.

Treatment	Without SCFA	With SCFA	N/n
	IDV ×1000		
pH 7.4	36.51±5.41^{a,1}	11.45±2.89^{b,1}	5/6
pH 6.4	65.23±7.81^{c,2}	14.87±3.77^{d}	4/8
pH 6.0	23.77±2.76^{e,3}	23.59±3.47^{f,2}	4/8
pH 5.8	18.54±3.64^{g,4}	10.12±1.87^{h}	5/7

Different superscripts are significantly different from control $p = < 0.05$

letters (SFCA dependent); numerals (pH dependent)

3.1.3 Effect of SCFA concentration and mucosal pH

A total number of 5 animals were used in this set of experiments. Mucosal SCFA concentration at 10, 40, 70 and 100 mM were used at a mucosal pH of 7.4 (N = 2) or 6.4 (N = 3).

After an initial incubation of tissues with control buffer (without SCFA, pH 7.4), the buffer on the mucosal side was replaced with the respective SCFA concentrations and the tissues were

exposed for 30 min to this buffer followed by a recovery period with 40 mM SCFA buffer and pH 7.4 for 1.5 hours. A change from buffer without SCFA to buffer with 10 mM SCFA caused a significant increase in I_{sc} and G_t; this was also observed for 40 mM SCFA (10 mM > 40 mM for I_{sc}). However, concentrations of 70 mM and 100 mM SCFA on the mucosal side caused no significant change in electrophysiological parameters (Figure 3-11).

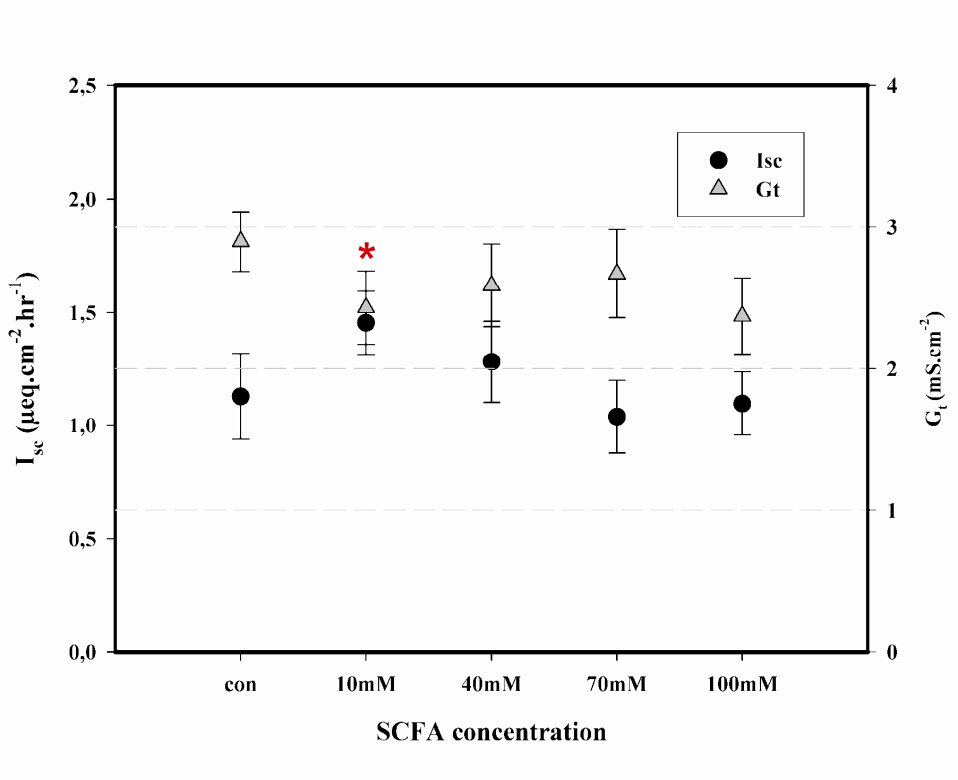


Figure 3-11: Effect of SCFA concentration on I_{sc} and G_t of sheep rumen epithelium. A change of buffer on the mucosal side from control (without SCFA) to 10 or 40 mM SCFA (pH 6.4) caused a significant increase in I_{sc} and G_t (p = 0.008). No significant change was seen for higher concentrations (70 and 100 mM, respectively). Each point represents Mean ± SD, N/n = 5/14.

Figure 3-12 shows that the up-regulation of Hsp70 depends on the SCFA concentration and on the pH. Furthermore, at a pH of 6.4 and 100 mM SCFA, Hsp70 expression appears to be slightly depressed. The results (Mean±SD) are compared by using ANOVA in Table 3-6.

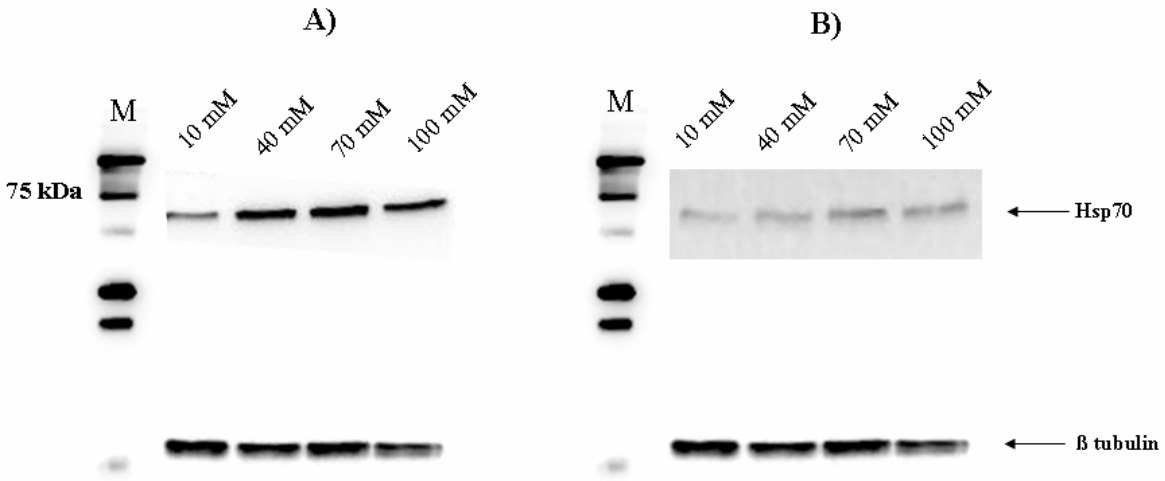


Figure 3-12: Hsp70 expression with various mucosal concentration of SCFA. A) pH 6.40 B) pH 7.4.

Table 3-6: Effect of pH at increasing SCFA concentration on Hsp70 expression. Mean ± SD; N/n = 4/10.

Treatment	IDVs × 1000 SCFA concentration (mM)			
	10	**40**	**70**	**100**
pH 7.4	15.56±9.87	18.26±8.87	32.17±15.78^{a}	19.45±13.77
pH 6.4	20.22±11.47	38.68±11.98$^{a,\alpha}$	57.52±14.52 $^{a,\alpha}$	31.45±16.78 $^{\alpha}$

Different superscripts (Roman letters within concentration and Greek letters within a column) are significantly different (p = < 0.05) from control (10 mM) or pH 7.4.

3.1.4 Effect of mucosal osmolarity:

The tissues were mounted in the usual way, and at the end of the equilibration period, the isoosmotic mucosal buffer solution was replaced by a solution with an osmotic pressure of 380 mosmol/l (Table 8-5 and addition of mannitol) and a pH of 7.4. A change of mucosal buffer to 380 mOsm·l^{-1} at pH 7.4 was observed to caused a 38% decrease of PD_t from 13.1 to 8.4 mV, whereas the G_t increased by 40% from 2.5 to 3.5 mS·cm^{-2}.

Table 3-7: Effect of mucosal osmotic pressure on I_{sc} and G_t. There was a significant change in electrophysiological parameters compared with the control. Mean ± SD.

Treatment	I_{sc} $\mu eq \cdot cm^{-2} \cdot hr^{-1}$	G_t $mS \cdot cm^{-2}$	PD mV	N/n
Control	1.25±0.15	2.53±0.19	13.18±0.34	2/5
380 mOsm·l^{-1} (pH 7.4)	0.97±0.18[a]	3.58±0.12[a]	8.47±0.21[a]	2/8
380 mOsm·l^{-1} (pH 6.4)	1.48±0.24	3.24±0.39[a]	11.45±2.54	2/6

Different superscripts in the same column are significantly different from control; $p = < 0.05$

Western blot carried out on samples from these tissues revealed an over expression of Hsp70 at high osmotic pressure. This expression was more pronounced at a mucosal pH of 6.4 compared with the control.

Hence, in summary, Hsp70 could be induced in the rumen epithelium by exposing the tissue to various stress scenarios. The highest expression of Hsp70 was observed at a mucosal pH of 6.4 (65.23±8.22), 70 mM SCFA (51.52±11.13) and high osmolarity at pH 6.4 (43.25±4.85).

3.1.5 Effect of inhibitors:

After the successful establishment of a protocol for the induction of Hsp70, further experiments were carried out to inhibit its expression in order finally to compare epithelial transport in the presence or absence of Hsp70. Two inhibitors, viz., Cycloheximide (3.0 mM) and triptolide (100 nM) were used to inhibit Hsp70 expression and were added on both the mucosal as well as the serosal side.

To avoid any non-specific effects of inhibitors on the tissue, its electrophysiology was carefully observed after their addition. Neither CHX nor triptolide produced any significant effect on the short circuit current and tissue conductance after addition (Table 3-8).

Table 3-8: Mucosal and serosal addition of cycloheximide (3.0 mM) and triptolide (100 nM) did not change I_{sc} or G_t. The values were taken before (control) and 20 min after the addition of inhibitor. Mean ± SD.

Treatment	I_{sc} $\mu eq \cdot cm^{-2} \cdot hr^{-1}$	G_t $mS \cdot cm^{-2}$	N/n
Control	1.39±0.05	3.81±0.09	4/8
Cycloheximide	1.36±0.08	3.89±0.12	3/9
Control	1.76±0.04	3.27±0.03	3/8
Triptolide	1.71±0.02	3.44±0.05	4/10

Tissues from these experiments were subjected to Western blot for the evaluation of Hsp70 expression and possible inhibition. A 50% and 70% inhibition was observed for CHX and triptolide respectively (Figure 3-13).

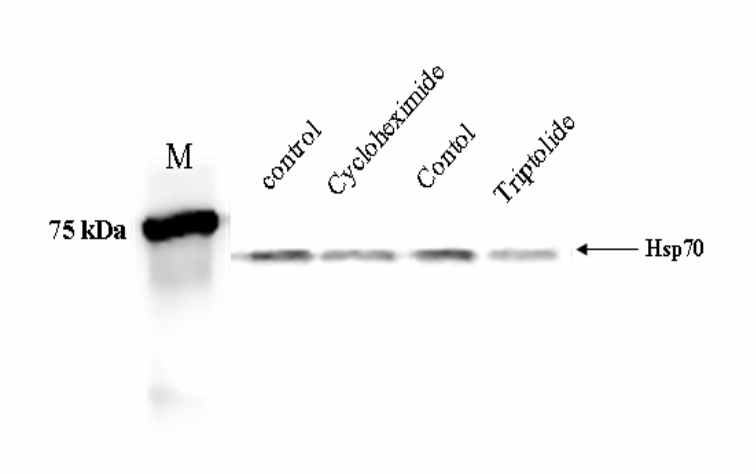


Figure 3-13: Inhibition of Hsp70 by cycloheximide (3.0 mM) or triptolide (100 nM)

Table 3-9: Effect of cycloheximide and triptolide on Hsp70 expression. Cycloheximide (3.0 mM) and triptolide (100 nM) both significantly reduced the expression of Hsp70. Mean ± SD

Treatment	IDVs × 1000	N/n
Control	31.89±4.35	4/8
Cycloheximide	14.36±2.08[a]	3/7
Control	48.76±6.04	3/7
Triptolide	11.62±3.02[b]	4/9

Compared with control in both cases (a), $p = 0.02$ (b), $p = 0.01$

The results obtained from these studies clearly suggest that Hsp70 can be induced under a variety of conditions and that its expression can be modulated by the application of inhibitors (Table 3-9). CHX and triptolide were equally effective and did not produce any undesirable effects on I_{sc} and G_t. in control tissues.

3.2 Measurement of Na flux rates:

3.2.1 Introduction:

Section 3.1 demonstrated that the induction and inhibition of Hsp70 expression in rumen epithelium could be achieved by using the Ussing chamber technique. This knowledge was then applied to the study of transport mechanisms across the rumen epithelium. Since NHE is involved in pH_i regulation and as its role in SARA is the main interest of this study, experiments were conducted to investigate electroneutral Na^+ transport via NHE. The isoforms of NHE discussed in the rumen epithelium include NHE1, 2, 3 and 8 (Graham *et al.*, 2007; Schweigel *et al.*, 2005a). NHE1 is generally expressed in the basolateral membrane of the polarised epithelia (Kennedy *et al.*, 2005). However, Graham *et al.* (2007) have concluded from immunostaining experiments that NHE1 is expressed in the apical membrane of the bovine rumen epithelium and have proposed this NHE isoform as the carrier for electroneutral Na transport. This finding is not in accordance with previous studies suggesting the basolateral expression of NHE1 in epithelia of the gut (Kennedy *et al.*, 2005; Maher *et al.*, 1996; Zachos *et al.*, 2005). Experiments were therefore carried out to characterize the isoform of NHE expressed in the apical membrane of the rumen epithelium by the application of NHE inhibitors: Amiloride (1.0 $mmol \cdot l^{-1}$) is a non-specific NHE inhibitor and was used in comparison with the NHE1 specific inhibitor (HOE642, 30.0 $\mu mol \cdot l^{-1}$) or NHE3 specific inhibitor (S3226, 1.0 $\mu mol \cdot l^{-1}$)

3.2.2 Effect of NHE inhibitors:

Isolated rumen epithelia of sheep were incubated with 80 mM SCFA buffer on the mucosal side at pH 7.4 to stimulate the Na^+/H^+ antiport. Mucosal amiloride (1.0 $mmol \cdot l^{-1}$) caused a decrease of J_{ms} and J_{net} Na indicating the inhibition of NHE. The NHE3 blocker S3226 (1.0 $\mu mol \cdot l^{-1}$) significantly reduced J_{ms} Na from 3.14±0.76 to 1.70±0.43 $\mu eq \cdot cm^{-2} \cdot h^{-1}$ and, hence, J_{net} from 1.96±0.85 to 0.86±0.52 $\mu eq \cdot cm^{-2} \cdot h^{-1}$. I_{sc} and J_{net} Na were not significantly different in the presence of mucosal S3226. However, the NHE1 inhibitor HOE642 (30 $\mu mol \cdot l^{-1}$; IC_{50} 0.08 $\mu mol \cdot l^{-1}$) failed to cause any significant inhibition of Na transport (Table 3-10).

Table 3-10: Effect of NHE inhibitors, viz., amiloride (1.0 mmol·l^{-1}), HOE642 (30.0 µmol·l^{-1}) and S3226 (1.0 µmol·l^{-1}) added to the mucosal side on electrophysiological parameters and unidirectional Na transport across the ruminal epithelium of sheep. Mean ± SD.

Treatment	J_{ms}^{Na}	J_{sm}^{Na}	J_{net}^{Na}	I_{sc}	G_t	N/n
	µeq·cm^{-2}·hr^{-1}				mS·cm^{-2}	
Control	3.14±0.76	1.12±0.27	2.02±0.85	1.07±0.22	3.56±0.15	3/9
Amiloride	2.24±0.51[a]	1.09±0.24	1.15±0.59[a]	0.98±0.15	4.49±0.47	3/9
HOE642	2.90±0.57	1.13±0.17	1.77±0.60	1.07±0.15	3.72±0.31	3/9
S3226	1.70±0.43[b]	0.83±0.26[a]	0.87±0.52[b]	0.74±0.14[b]	3.51±0.40	3/9

[a] $p = 0.03$, [b] $p = 0.02$,

Amiloride (1.0 mmol·l^{-1}) caused an 8% and S3226 (1.0 µmol·l^{-1}) significantly a 30% drop in I_{sc}, whereas the NHE1-specific inhibitor, HOE642 (cariporide (30.0 µmol·l^{-1})), did not cause a change in the short circuit current (Figure 3-14).

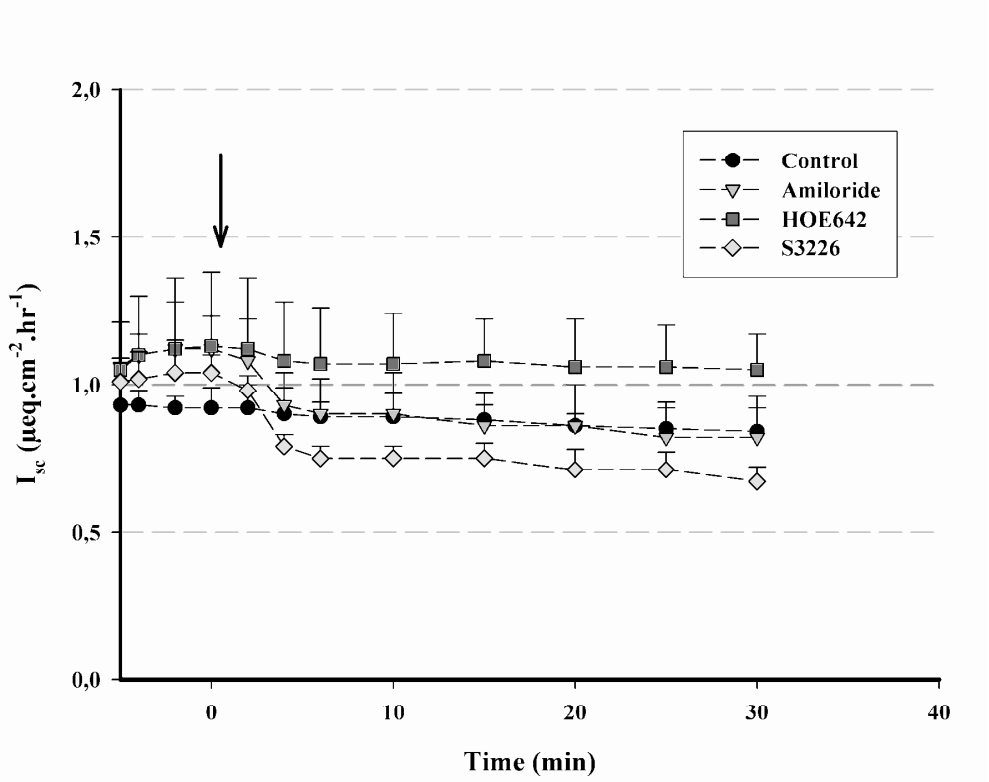


Figure 3-14: Changes of I_{sc} after mucosal application of NHE inhibitors (↓). NHE3 specific inhibitor (S3226) reduced the I_{sc} (~30%) significantly (p = 0.007) whereas, NHE1 specific inhibitor (HOE642) failed to produce any significant effect on I_{sc}. Each point: Mean ± SD, N/n = 3/6.

Interestingly, when the tissues obtained from these experiments were subjected to western blotting, a higher expression of Hsp70 was observed in the epithelia that were treated with NHE3 specific inhibitor on the mucosal side (Figure 3-15).

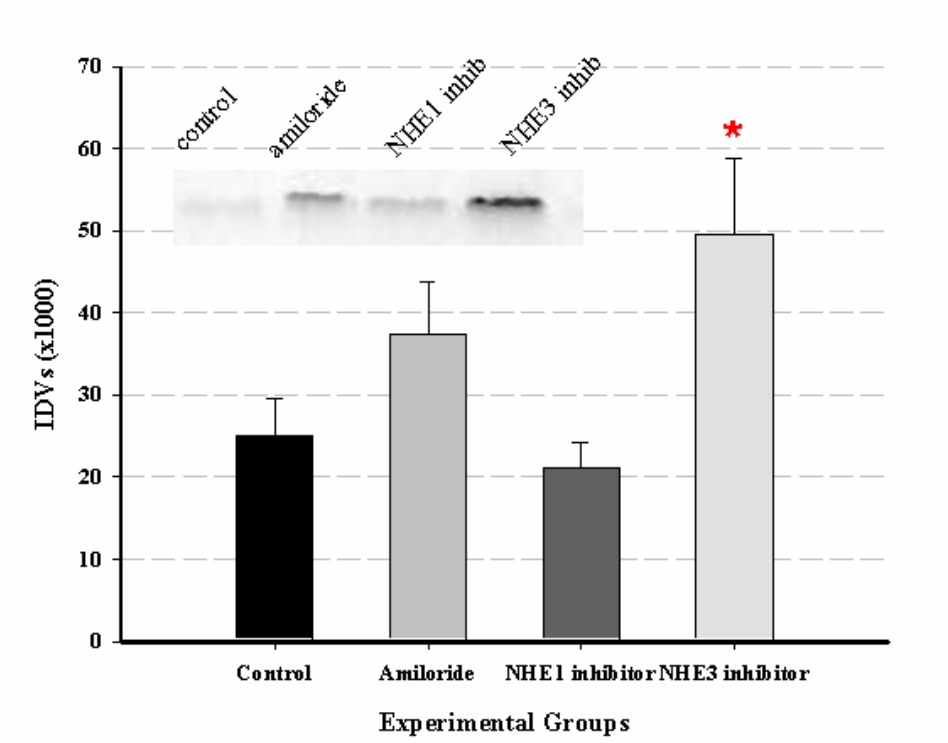


Figure 3-15: Effect of NHE inhibitors on Hsp70 expression. Mucosal addition of NHE3 specific inhibitor (1.0 µmol·l^{-1}) caused a significant over-expression of Hsp70, *p = 0.028. Each bar: Mean ± SD, N/n = 2/4.

3.2.3 Effect of Hsp70 inhibitor:

To evaluate the effect of Hsp70 on the Na transport across the rumen epithelium, tissues were incubated with or without Hsp70 inhibitor to render protection (without inhibitor and with Hsp70 being expressed) or to make them prone to stress (with inhibitor and with Hsp70 being suppressed). After an equilibration period of ~30 min with 80 mmol·l^{-1} SCFA buffer on mucosal side, the pH was reduced to 6.4 for 30 min to induce Hsp70 (see section 3.1.2) followed by a recovery period of 1.5 hours. In parallel, the pH of another group of epithelia was reduced to 6.4 for 30 min after the addition of CHX (3.0 mmol·l^{-1}) both on the mucosal and on the serosal side succeeded by a recovery period for 1.5 hours. Following this procedure, the induction or inhibition Hsp70 in the tissues was possible (see section 3.1.5) and after the recovery period, the unidirectional flux rates of Na were measured.

Under control conditions (pH 7.4), Na was transported in mucosal-serosal direction with a flux rate of 4.61±0.50 µeq·cm^{-2}·h^{-1}. Because J_{sm} Na was much lower (1.16±0.32 µeq·cm^{-2}·h^{-1}), a net transport (absorption) of 3.41±0.62 µeq·cm^{-2}·h^{-1} was observed. The flux rates of Na under control condition and in the presence of CHX (3.0 mmol·l^{-1}) were slightly changed, but CHX did not cause significant differences. The induction of Hsp70 caused a significant increase of J_{ms} Na from 4.61±0.50 µeq·cm^{-2}·h^{-1} (control) to 9.32±1.74 µeq·cm^{-2}·h^{-1} (protected). Because J_{sm} Na was not influenced by treatment, J_{net} Na was significantly enhanced (p = 0.008) from 3.45±0.62 (control) to 7.69±1.82 µeq·cm^{-2}·h^{-1} (protected). Suppression of Hsp70 synthesis by CHX (unprotected) caused a significant decrease of J_{ms} and J_{net}. (6.37±0.88 and 4.88±1.02 µeq·cm^{-2}·h^{-1} respectively). However, the flux rates under these conditions were still higher than those in the control group.

Table 3-11: Na transport rates under control conditions and after induction of Hsp70 with (Unprotected) or without cycloheximide (Protected). Mean ± SD.

Treatment	J^{Na}_{ms}	J^{Na}_{sm}	J^{Na}_{net}	I_{sc}	G_t	N/n
	µeq/cm^2/hr				mS·cm^{-2}	
Control	4.61±0.50	1.16±0.32	3.45±0.62	1.25±0.17	2.32±0.06	3/8
Control CHX	4.04±1.17	1.69±0.38	2.35±0.87	1.69±0.14	3.32±0.09	3/9
Protected	9.32±1.74[a]	1.63±0.38	7.69±1.82[a]	1.10±0.33	2.78±0.08	3/9
Unprotected	6.37±0.88[b]	1.49±0.39	4.88±1.02[b]	1.31±0.23	2.96±0.08	3/9

Different superscripts indicate significant differences: a (from control) p = 0.008.
b (from protected) p = 0.013.

CHX is an inhibitor of protein biosynthesis in eukaryotic organisms and hence, unspecific effects cannot be excluded despite the fact that flux rates in control tissues (see Table 3-11) are not significantly changed. Recent studies have shown that triptolide can be used for the inhibition of Hsp70 expression (Saluja and Dudeja, 2008). Corresponding experiments were therefore repeated with this inhibitor (100 nmol·l^{-1}) and the data are shown in Table 3-12 (below). The results are in agreement with the effects of CHX. Triptolide did not change the Na flux rates under control conditions (table). Induction of Hsp70 increased J_{ms} and J_{net} fluxes significantly, and this increase was significantly reduced by triptolide

Table 3-12: Na transport rates under control conditions and after induction of Hsp70 with (Unprotected) or without triptolide, Trp (Protected). Mean ± SD; N = number of animals; n = number of epithelia.

Treatment	J^{Na}_{ms}	J^{Na}_{sm}	J^{Na}_{net}	I_{sc}	G_t	N/n
	µeq·cm^{-2}·h^{-1}				mS·cm^{-2}	
Control	7.54±1.85	1.56±0.41	5.98±1.96	1.05±0.17	3.69±0.04	3/6
Control Trp	7.04±1.39	1.59±0.41	5.45±1.47	1.29±0.14	3.64±0.03	3/6
Protected	11.09±1.62[a]	2.22±0.62	8.86±1.84[a]	1.02±0.33	3.84±0.12	3/7
Unprotected	8.59±0.75[b]	2.06±0.34	6.54±0.83[b]	0.81±0.23	4.24±0.11	3/8

Different superscripts indicate significant differences: a) from control; $p = 0.03$.
b) from protected; $p = 0.04$

The epithelia from these experiments were evaluated for Hsp70 expression and showed an up-regulation of Hsp70, which was reduced by CHX or triptolide (Figure 3-16).

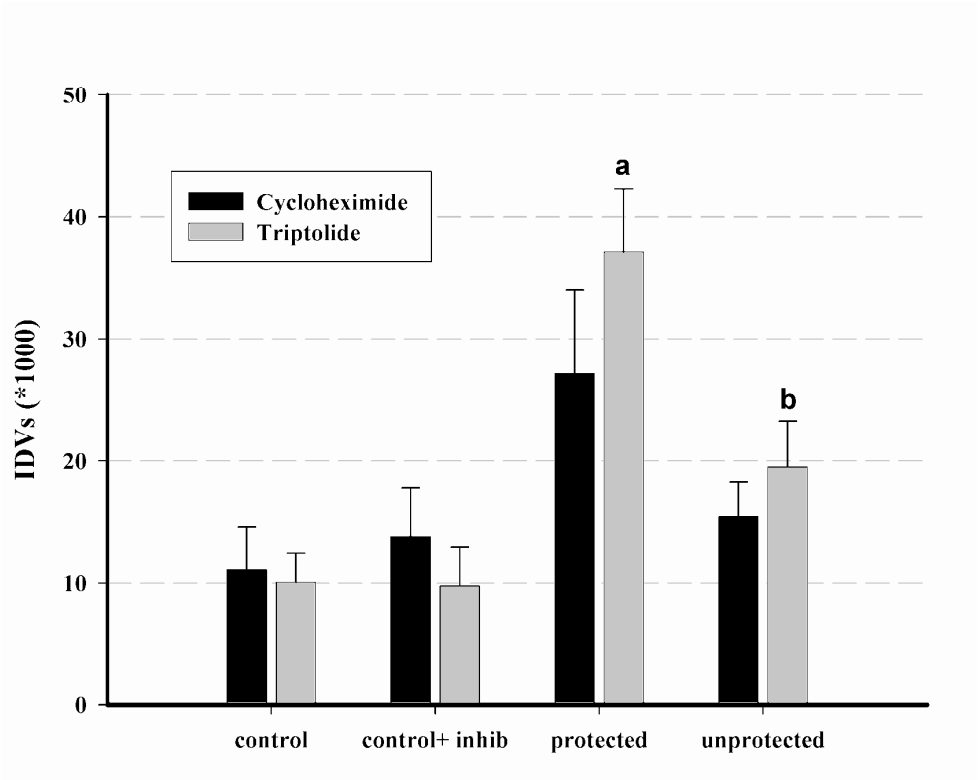


Figure 3-16: Hsp70 expression from the tissues exposed to CHX or TRP. There was a significant (a; $p = 0.03$) up-regulation of Hsp70 after challenge (protected) and down-regulation after the addition of inhibitors (b; $p = 0.04$). Each bar = Mean ± SD; N/n = 3/8

3.3 Measurement of acetate fluxes:

3.3.1 Introduction:

Interactions between NHE and SCFA flux rates have been shown in many tissues (Busche *et al.*, 1997) including the rumen (Gabel *et al.*, 2002). The stimulation of electroneutral Na transport after the induction of Hsp70 suggests possible effects on SCFA transport.

Effect of Hsp70 inhibitor on acetate fluxes:

After the incubation of tissues at mucosal pH 6.4 and 80 $mmol \cdot l^{-1}$ SCFA, with or without Hsp70 inhibitor for 30 min followed by 1.5 hours of recovery, acetate fluxes were measured under control conditions i.e. mucosal pH 7.4 and 40 mM SCFA in order to evaluate the role of Hsp70 on its transport. Only m-s fluxes were measured. The results summarized in Table 3-13 indicate that the induction of Hsp70 significantly enhanced acetate fluxes from 2.17±0.85 to 4.12±0.94 $\mu eq \cdot cm^{-2} \cdot hr^{-1}$.

Table 3-13: Acetate transport rates under control conditions and after induction of Hsp70 with or without the inhibitor, CHX. Mean ± SD.

Treatment	J_{ms}^{Ac} $\mu eq \cdot cm^{-2} \cdot hr^{-1}$	I_{sc} $\mu eq \cdot cm^{-2} \cdot hr^{-1}$	G_t $mS \cdot cm^{-2}$	N/n
Control	2.17±0.85	1.05±0.17	2.51±0.13	3/6
Control+inhibitor	2.21±0.48	1.29±0.14	2.69±0.06	3/7
Protected	4.12±0.94[a]	1.02±0.33	3.17±0.17[a]	3/8
Unprotected	2.71±0.28[b]	0.81±0.23	2.84±0.04	3/7

Compared with control (a) $p = 0.03$, compared with protected (b) $p = 0.04$

Since acetate fluxes are linked to the activity of NHE, 1.0 mmol·l^{-1} amiloride was applied to the mucosal side after the three fluxes to show possible interactions of NHE with acetate transport. Interestingly, acetate fluxes were significantly reduced, even in the protected group suggesting a role of NHE on its transport (Figure 3-17).

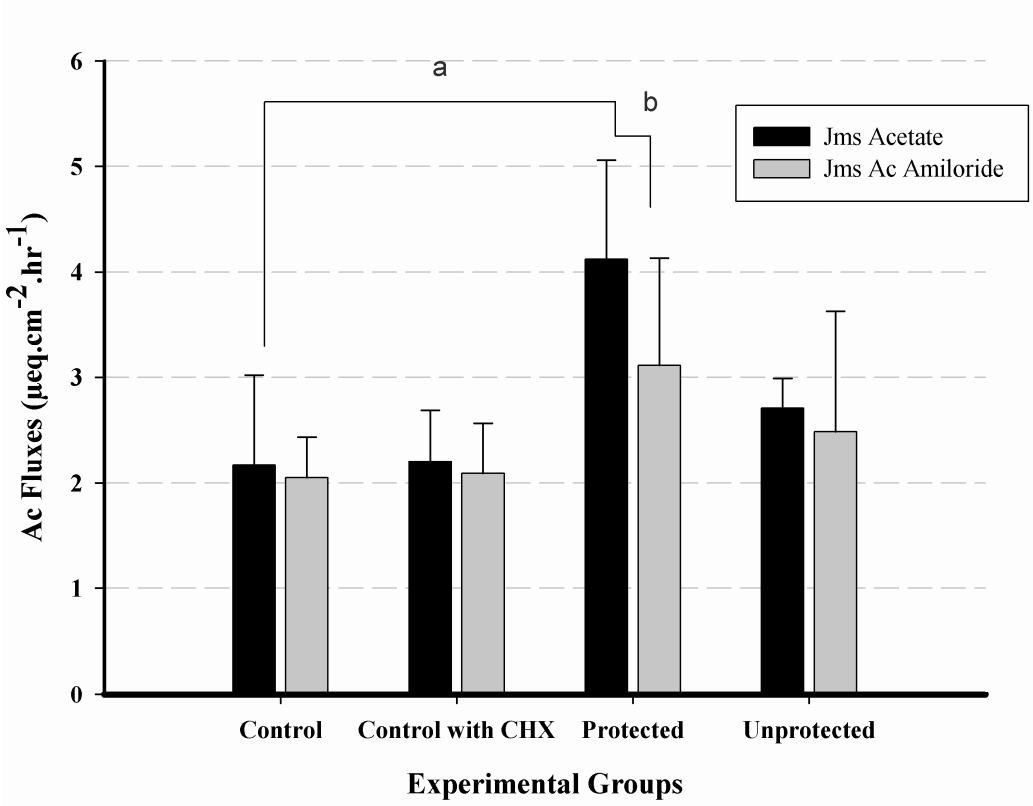


Figure 3-17: Effect of 1.0 mmol·l^{-1} amiloride on acetate transport across rumen epithelium of sheep applied after three flux periods (light bars) and compared with the fluxes of acetate before amiloride application (dark bars). Each bar = Mean ± SD, N/n = 2/9. [a] p = 0.03, [b] p = 0.04

3.4 In vivo induction of Hsp70:

3.4.1 Introduction:

In the previous *in vitro* experiments the induction of Hsp70 was tested under a variety of conditions that simulated the *in vivo* situation in the rumen, but that involve a change in only one parameter (pH, SCFA etc.) for a clear cut correlation with the possible factors. *In vivo* pH, SCFA concentration, temperature and osmotic pressure all change at the same time. Hence, possible changes of Hsp70 expression cannot be related only to one parameter; the alteration of several rumen parameters might be associated with Hsp70 expression. We chose for this purpose a simple approach. We measured rectal temperature immediately before slaughter and rumen temperature and ruminal pH 2 – 3 min later after the removal of the forestomach from the abdomen from sheep fed with hay ad libitum + 800g concentrate diet per day. 400 g of the concentrate was fed 3 h before slaughter. No significant differences were seen between rectal and ruminal temperature (39.2°C±0.08 and 38.8°C±0.18 respectively). However, we found a reasonable (r^2 = 0.61) correlation between rumen pH and temperature (Figure 3-18).

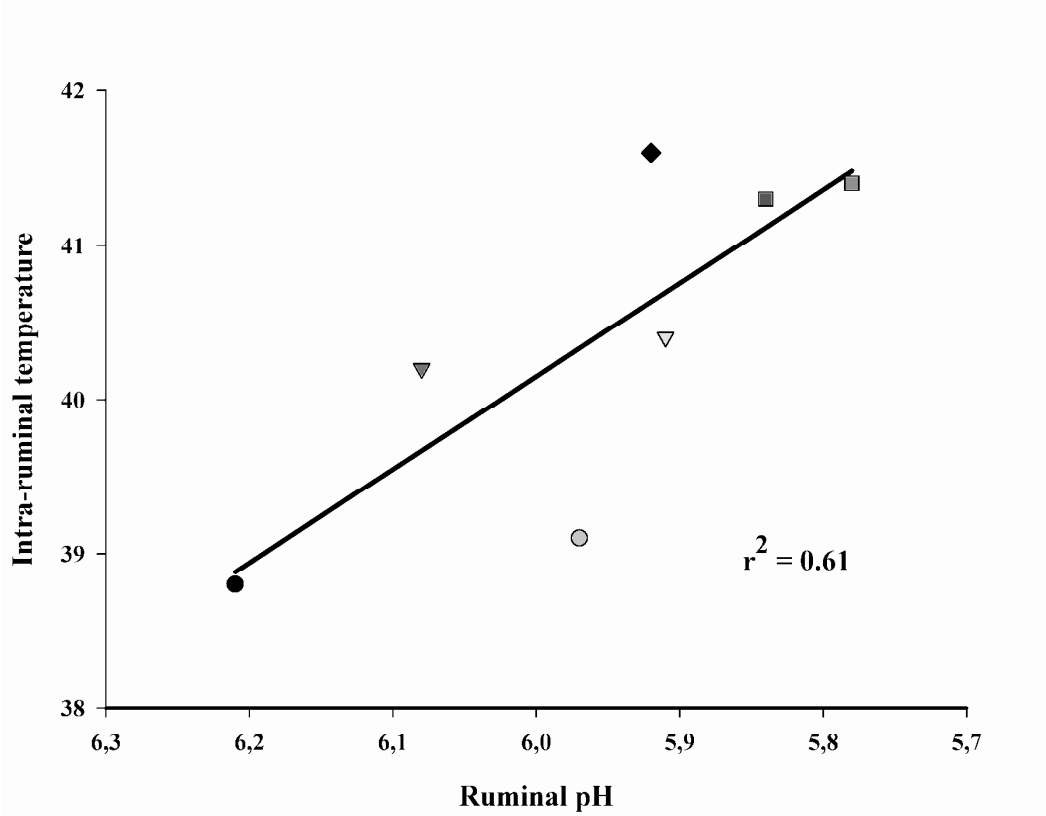


Figure 3-18: Correlation between rumen pH and rumen temperature taken from seven different sheep. The animals were offered 400 g of concentrate diet three hours before slaughter. The variation in rumen pH and temperature might be attributable to the variable amount of concentrate diet ingested by each animal.

3.4.2 Measurement of Hsp70 expression:

To evaluate Hsp70 expression, the tissues obtained after slaughter from these concentrate fed animals were subjected directly to Western blot and were not incubated in the Ussing chamber as for the previous protocols. The following figure presents the results obtained from these tissues. As can be seen, Hsp70 was up-regulated in animals fed with a concentrate diet and slaughtered after three hours.

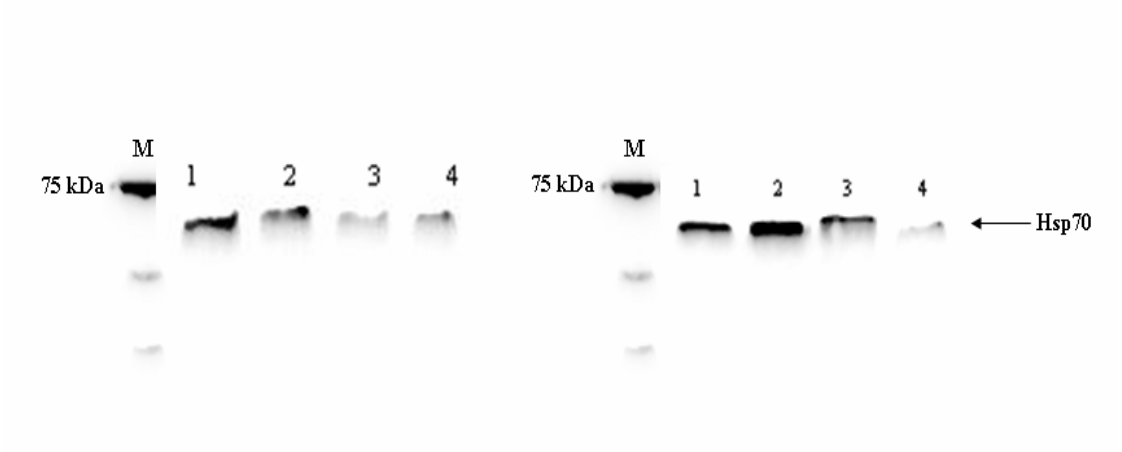


Figure 3-19: In vivo expression of Hsp70 obtained from eight animals. Four were fed a concentrate diet at least three hours before slaughter (1, 2) whereas the other four were hay fed (3, 4).

The following table summarizes the expression of Hsp70 (IDVs×1000) comparing the two feeding regimes. A significant 51% increase occurred in Hsp70 expression.

Table 3-14: Hsp70 expression in terms of integrated density values (IDVs ×1000). A significant difference can be seen between the tissues obtained immediately after slaughter of hay fed (HF) and concentrate fed (CF) animals. Mean ± SD.

Treatment	(IDV ×1000)	N/n
HF	70.67±10.27	8/11
CF	106.58±15.55[a]	7/11

$p = 0.01$

3.4.3 Measurement of Na and acetate fluxes:

The tissues obtained for these experiments were from animals that received concentrate diet 3 hours before slaughter. As described above, some of the tissue samples were taken immediately for Western blot, the rest of the tissue being used in Ussing chamber to study Na and acetate fluxes. After an initial incubation period for equilibration with 40 mM SCFA buffer on both the mucosal and the serosal side, three flux periods were obtained for Na and acetate. A significant increase in J_{ms}^{Na} and J_{net}^{Na} was noted for concentrate-fed animals compared with those that had been hay-fed (Table 3-15).

Table 3-15: Na transport rates of tissues of hay fed (HF) and concentrate fed (CF) animals. Mean ± SD.

Treatment	J^{Na}_{ms}	J^{Na}_{sm}	J^{Na}_{net}	I_{sc}	G_t	N/n
	µeq.cm^{-2}.hr^{-1}				mS.cm^{-2}	
HF	6.77±1.95	2.14±0.76	4.62±2.09	1.14±0.41	2.44±0.75	2/12
CF	10.94±2.49[a]	3.31±1.05	7.63±2.70[a]	1.68±0.31	3.45±1.26	3/18

$p = 0.02$

A similar increase in acetate transport was observed in concentrate-fed animals, as summarized in Table 3-16.

Table 3-16: Acetate transport rates tissues of hay-fed (HF) and concentrate-fed (CF) animals. Mean ± SD.

Treatment	J^{Ac}_{ms}	J^{Ac}_{sm}	J^{Ac}_{net}	I_{sc}	G_t	N/n
	µeq.cm-2.hr-1				mS.cm-2	
HF	2.87±0.85	3.06±0.85	-0.21±1.55	1.87±0.03	3.38±0.47	2/12
CF	5.94±1.72[a]	6.11±1.41[a]	-0.17±1.28	2.36±0.18[a]	4.45±0.86[a]	3/18

$p = 0.01$

Chapter 4

General Discussion and Conclusion

There is a substantial amount of evidence concerning the functional and morphological adaptation of rumen epithelium to changes in feed (Etschmann *et al.*, 2009b). Such a change usually takes days or weeks and is of significant importance for proper digestibility and animal health particularly during high energy intake (Van Vuuren *et al.*, 1979). Failure of dietary adaptation may lead to various metabolic problems including sub-acute ruminal acidosis (SARA) which imposes great economic losses on the dairy industry (Krause and Oetzel, 2005; Nagaraja and Titgemeyer, 2007). There is a growing recognition for ration formulation and proper management during these periods of adaptation (see review van Knegsel (2005).

However, independent of the diet-dependent changes of fermentation mentioned above, which have been discussed in the literature for decades [(see Dirksen *et al.* (1984) or Etschmann *et al.* (2009a)], a considerable daily variation in the SCFA concentration and pH in the rumen after feeding has also been reported (Nagaraja and Lechtenberg, 2007). Therefore, certain mechanisms must exist at the epithelial level to cope with these acute and short term challenges. Studies of acute changes in the pH of the luminal buffer solution have been performed *in vivo* (Gaebel *et al.*, 1987) and *in vitro* (Abdoun *et al.*, 2005; Gaebel *et al.*, 1989) and have shown that a moderate decrease in pH stimulates for example Na or SCFA transport. These positive effects on transport mechanisms have been primarily considered under the aspect of the availability of H^+ for NHE or the increase of the undissociated form of SCFA and hence the enhanced non-ionic diffusion of SCFA. Possible intraepithelial mechanisms were not previously discussed as they were unknown at that time. However, a growing body of evidence now exists indicating that heat shock proteins (Hsp) modulate epithelial transport and barrier function upon challenge by osmotic pressure, temperature or SCFA (see below). Hence, it was hypothesized that the Hsp70 may be up-regulated during these short-term stress scenarios. The aim of this study has therefore been to investigate short- term protective mechanisms of Hsp70 on transport mechanisms of the rumen epithelium. The following questions have been addressed:

1. Is it possible to induce Hsp70 *in vitro* in rumen epithelium with parameters of SARA i.e. high SCFA and low pH?
2. Does Hsp70 influence Na and acetate transport across the isolated rumen epithelium?
3. Is there a similar pattern of changes of Hsp70 expression based on diet and duration *in vivo*?

The results discussed in chapter 3 concerning the induction of Hsp70 with the parameters resembling SARA, i.e., high temperature, increased concentration of SCFA, high osmolarity and low pH, suggested that Hsp70 can be over-expressed when the afore-mentioned stressors are artificially induced. However, a much more cautious approach is required in terms of control tissues as some tissues that have not been subjected to any stressor (control group) exhibit over-expressed Hsp70 (Figure 8-1). The reason for this observation could be that Hsp70 are stress proteins and can be over-expressed if the animal or tissue is subjected to any stress (before slaughtering, after tissue preparation or during transportation of tissue to the laboratory). A careful monitoring of experimental animals and proper tissue preparation is, therefore, indispensable.

4.1 Induction of Hsp70:

Temperature: In this study, a variety of experimental protocols were devised to induce Hsp70 expression in the rumen epithelium *in vitro*.

As a first step, tissues were subjected to *various temperatures* mainly because many previous studies employed heat treatment chiefly in isolated cells to express Hsp70 (Charng *et al.*, 2007; McKenzie *et al.*). However, this approach was not successful in all cases. The incubation of tissue at increasing temperatures and times and the determination of Hsp *immediately at the end of exposure time* did not show consistent results. The induction of Hsp finally proved to be possible and reproducible when the tissues were exposed to a higher temperature for 30 min and immediately afterwards were incubated under control conditions for a *recovery period of 90 min*. This procedure is in agreement with observations in the literature and has been applied in a variety of tissues/cells (Brown *et al.*, 1992; Diller, 2006; Hinzpeter *et al.*, 2006). Therefore, this method was used for the induction of Hsp 70 in order to evaluate the role of Hsp for Na and acetate transport.

During the incubation of the tissues at higher temperatures in the Ussing chambers, the temperature could not be changed exclusively at the mucosal side, which should be the case in rumen fermentation but rather, the situation resembled whole body hyperthermia (WBH). The induction of Hsp70 using this approach is thus in agreement to the studies done by Xi *et al.* (2001).

The increase of temperature caused an increase of tissue conductance and short-circuit current. This might be attributed to increased fluidity of the epithelial membranes or faster movements of ions and generally follows the Q_{10} rule. The ionic base of the increased I_{sc} is not known but probably represents primarily electrogenic Na transport (Lang and Martens,

1999; Leonhard-Marek *et al.*, 2005). The parallel raise of I_{sc} and G_t suggested to apply the method of Yonath and Civan (1970) for the estimation of E_{Na}. The underlying assumptions of this model include a decrease of resistance to Na entry into the transporting cell of the epithelium and consequently proportional changes in I_{sc} and G_t. The ratio of these changes, i.e., in I_{sc} and G_t allows the calculation of E_{Na}. In this study, we have observed a linear relationship ($r^2 = 0.98$) between I_{sc} and G_t with the increase in temperature and the slope of this linear regression being 0.05, i.e., E_{Na} 20±0.8 mV (see Figure 4-1). An identical approach was taken by Schultheiss and Martens (1999) in studies with sheep omasum. Removal of divalent cations (Ca^{++} and Mg^{++}) from the muscosal solution increased I_{sc} and G_t and gave the value of E_{Na} 62±1.0 mV, in agreement with the E_{Na} in rabbit colon (65.4 mV; (Turnheim *et al.*, 1987). The much lower value of E_{Na} in the present study suggests that in addition to Na transport, other ions and conductances are involved. Possible candidates are either enhanced Cl absorption or K secretion, because corresponding conductances have been demonstrated for these ions (Stumpff and Martens, 2007b; Stumpff *et al.*, 2009). A further characterization of the ionic basis of increased I_{sc} was beyond the scope of this study. Nevertheless, it is important to mention here that the tissue conductance and short circuit current almost returned to pre-stress level when the temperature was brought back to normal during the recovery period.

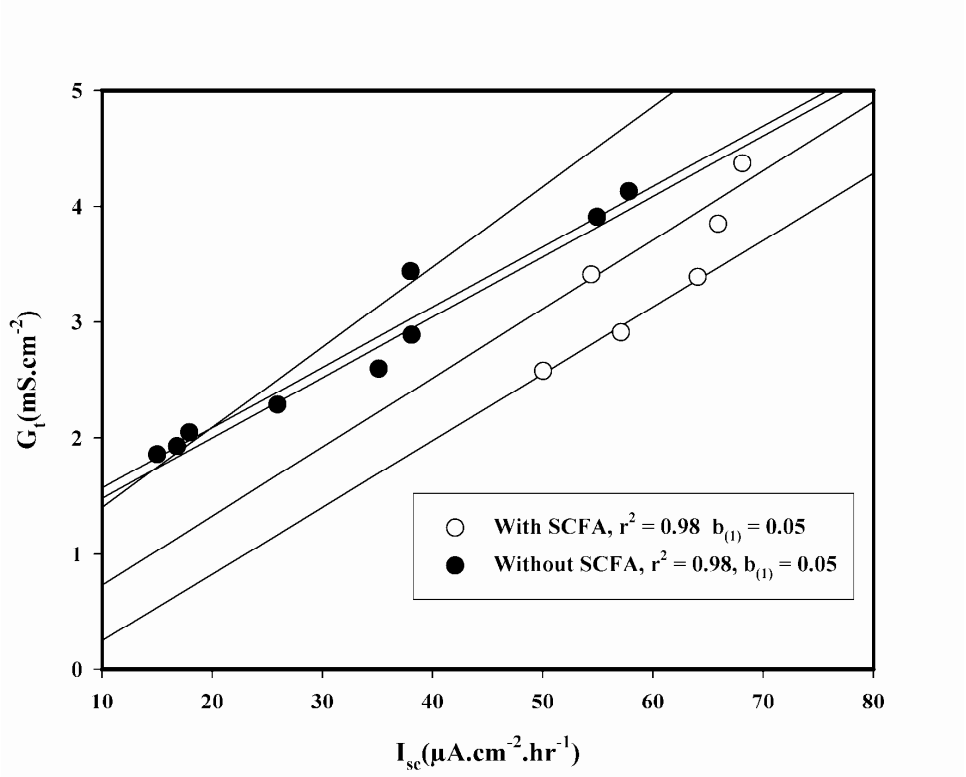


Figure 4-1: Plot of I_{sc} and G_t corresponding to the proposal of Yonath and Civan (1970) for the estimation of E_{Na}. For further details, see text. The slope of this linear regression was 0.05, i.e., E_{Na} = 20±0.8 mV. The figure shows the changes of I_{sc} and G_t after an increase of temperature until a new steady state is attained (see also Figure 3-1).

pH: Induction of Hsp70 with a protocol of low pH was successful, and the highest expression level of Hsp70 was observed at pH 6.4-6.2 in a control buffer without SCFA. In the presence of SCFA Hsp expression was generally lower but was also pH-dependent. Maximal expression with SCFA buffer was observed at pH 6.0-5.8. This approach resembles diurnal variation of pH in the rumen and is in agreement with previous reports of Hsp70 induction by using low pH in a variety of tissues and cell lines (Chopp *et al.*, 1989; Narasimhan *et al.*, 1996; Poso *et al.*, 2002).

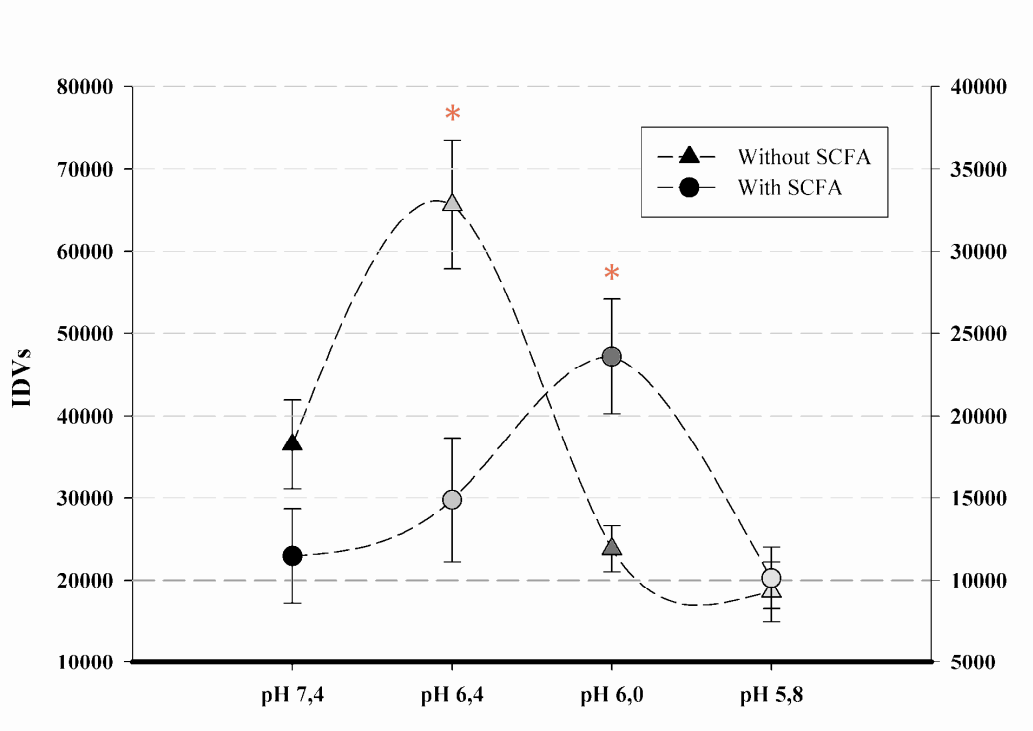


Figure 4-2: Effect of pH with or without SCFA on Hsp70 expression. Each point: Mean ± SD, N/n = 5/8. * p = < 0.05

Interestingly, a further decrease of mucosal pH caused the inhibition of Hsp70 (Figure 4-2) suggesting that the stress is no longer sub-lethal and jeopardizes even the synthesis of Hsp70. These observations are in contrast to the finding of Narasimhan et.al. (1996) who have reported an up-regulation of Hsp70 mRNA after a brief exposure of astrocytes to pH 5.2. The discrepancy in these findings might be attributed to the different behavior of the epithelium compared to isolated cells (astrocytes).

The generally lower expression of Hsp in the presence of SCFA indicates that incubation of rumen epithelium without SCFA induces Hsp expression and can be considered as a "stress" factor for the epithelium.

SCFA: Use of buffers at constant pH (7.40 and 6.40) with various SCFA concentrations also influenced the expression of Hsp70. A more intense expression of Hsp70 was observed at pH 6.4 and 70 mM SCFA. The pH in these experiments was reduced to simulate *in vivo* conditions. SCFA concentration and intra-rumen pH are related (Gabel *et al.*, 1991a). Again the response to the SCFA was bell shaped, similar to that recorded for pH exposure, showing the lowest expression of Hsp70 at low and extremely high SCFA concentrations (Figure 4-3). Low concentrations of SCFA might deprive cells of energy (particularly n-butyrate) and influence protein synthesis whereas very high concentrations of SCFA might cause a drastic drop in intra-cellular pH (pH_i). This relationship is in agreement with the findings of Shen *et al.* (2004b) (effect and importance of n-butyrate) and Gäbel *et al.* (2002) (effect of intracellular pH to SCFA influx).

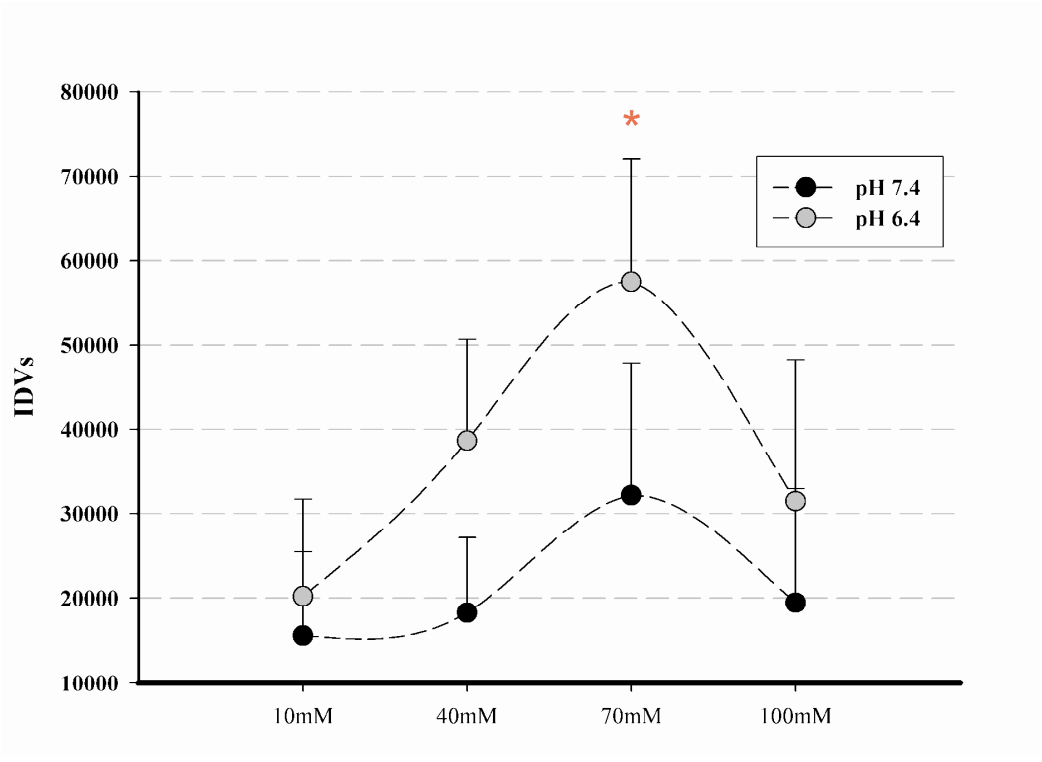


Figure 4-3: Effect of pH and SCFA concentration on Hsp expression. Each point: Mean ± SD, N/n = 5/8, * p = <0.05

4.2 Modulation of Hsp70 expression by inhibitors:

The intention of this study has been to investigate the possible role of Hsp70 on transport functions of the rumen epithelium. One precondition for this type of study is the reproducible induction of Hsp expression on the one hand, which has been optimized according to the parameters of (Table 4-1), and the inhibition of Hsp expression, on the other hand. It was possible to inhibit Hsp70 synthesis, when desired, by the use of cycloheximide (CXH), an inhibitor of proteins synthesis at the translocational step, used in a concentration of 3.0 $mM \cdot l^{-1}$. The justification of using this inhibitor is that it does not interfere with the previously formed protein molecules, e.g., transport proteins and exchangers. Secondly its use does not affect the electrophysiological parameters, I_{sc} and G_t (Table 3-11) suggesting no undesirable side effects. Similarly, a more specific inhibitor, triptolide causes the suppression of Hsp70 synthesis when used at a concentration of 100 nM/l. Again these inhibitors have always been compared and monitored for any undesirable effects by using them in control group for all the experiments performed.

The obtained data with respect to the optimization of Hsp expression are summarized in table 4-1

Table 4-1: Pilot studies for the expression of Hsp in rumen epithelium Except for protocol no. 1, tissues were allowed to recover for a period of 1.5 hours.

No	Title	Protocol	N/n	Hsp (*)	Rel	Rep
1	**Different durations**	38°C and 42°C for 30,60 and 120 min	3/9	++	+	-
2	**Different temperatures**	38°C, 41°C, 43°C and 45°C for 30 min	7/18	++	++	++
3	**Different pH**	7,4 6,4 6,0 and 5,8 for 30 min	4/8	+++	+++	++
4	**Various SCFA concentrations**	10, 40, 70 and 100 mM (30 min)	4/10	++	++	+
5	**High osmotic pressure**	390 mOsmol.l^{-1} (30 min)	2/5	+	+	+
6	**Hsp70 inhibitors**	CHX (3.0 mM) & TRP (100 nM)	3/8	--	++	++

(*) **+++** **Excellent**
++ **Good**
+ **Fair**
- **Poor**
Rel **Reliability**
Rep **Reproducibility**

4.3 Hsp and transport physiology

The effects of Hsp on ion transport have been studied in a variety of tissues and is summarized in Table 4-2. It is generally accepted that Hsp protect cells, tissues or organs against various stress (Feder and Hofmann, 1999) and not only exert manifold effects on epithelial transport functions and integrity (Ikari *et al.*, 2002; Musch *et al.*, 1999) but also protect these functions against injury. Up-regulation of Hsp prevents the heat-induced disruption of intestinal tight junctions (Dokladny *et al.*, 2006) and has positive effects on a range of transport mechanisms and ion channels.

Table 4-2: Effect of Hsp on transport mechanisms in cells or epithelia.

No	Transport properties	Cells/tissues type	Family of Hsp involved	Reference
1	↑ Cation channel	Artificial lipid bilayer	Hsc70	(Arispe and De Maio, 2000)
2	↑ ClC2	Human embryonic kidney-293 cells	Hsp70 & Hsp90	(Hinzpeter *et al.*, 2006)
3	↑ Ca^{++}	C6 glioma cells	Hsp70	(Kagaya *et al.*, 2000)
4	↑ Ca^{++}	HEK 293 cells	Hsp70	(Krieger *et al.*, 2006)
5	↑ K^{+} channels	Human promonocyte U937	Hsp70	(Negulyaev *et al.*, 1996)
6	$^{*}\leftrightarrow$ NHE	Human placental brush border	Hsp70	(Silva *et al.*, 1995)
7	↑ SGLT	Renal tubules cells	Hsp70	(Brown *et al.*, 1992)

* Indicates some interaction (not clear whether the activity is increased or decreased).

4.4 Na and acetate transport:

The results of the present study are in agreement with the findings of many previous studies of Na transport in rumen epithelium: J_{net} Na in the mucosal-serosal direction and $J_{net} > I_{sc}$ (Ferreira *et al.*, 1972; Gabel *et al.*, 1991a; Martens *et al.*, 1991a; Schweigel *et al.*, 2005a; Sehested *et al.*, 1996; Stevens, 1964) and the stimulation of J_{net} Na by SCFA (Gabel *et al.*, 1991a; Sehested *et al.*, 1996). Furthermore, the inhibition of Na transport by amiloride and S3226 supports the early conclusion that electroneutral Na transport is mediated via NHE (Chien and Stevens, 1972; Martens *et al.*, 1991a). The pronounced or missing effects of various inhibitors of NHE give further insight into the isoform of NHE that is probably involved. SCFA stimulates Na transport (Gabel and Sehested, 1997; Gabel *et al.*, 1991a; Sehested *et al.*, 1996) and this increase is inhibited by amiloride or S3226 further supporting the conclusion that electroneutral Na transport is mediated via NHE and via the isoform NHE3.

HOE642 as a specific inhibitor for NHE1 has been found to be ineffective in all experiments. By contrast, S3226 as a specific inhibitor of NHE3 significantly reduces J_{ms} Na and abolishes electroneutral Na transport, because the remaining J_{net} Na is not significantly different from I_{sc}, despite some variations. The used concentrations of the inhibitors (30 µM HOE642 and 1 µM S3226) are far higher than the IC_{50} for the respective NHE isoform according to the studies of Schwark *et al.* (1998), but much lower than the concentrations that probably causes the non-specific effects on other NHE isoforms (Schwark *et al.*, 1998).

This observation is in contrast to the studies of Graham *et al.* (2007) who have demonstrated, by RT-PCR, the presence NHE1−3 and NHE8 in bovine rumen and who conclude, from immunostaining, that NHE1 is "predominantly localized to the stratum granulosum with a progressive decrease toward stratum basale". Consequently, the authors discuss this isoform with regard to the well-known interaction between the non-ionic diffusional uptake of SCFA, the intracellular release of protons, the activation of NHE1, and Na influx. A further observation supports the conclusion that transepithelial Na transport is mediated by NHE3 and not by NHE1. Schweigel *et al.* (2005a) have shown that an increase in luminal osmotic pressure causes a significant inhibition of J_{ms} and J_{net} Na; they discuss in detail that this inhibition of J_{ms} and J_{net} Na is caused by an effect of osmotic pressure on NHE3 activity. By contrast, hyperosmolarity causes a rapid alkalinization in cells indicating increased NHE1 exchange activity (Schweigel *et al.*, 2005a). The assumption of NHE3-mediated electroneutral transport is further supported by the observation that cAMP inhibits Na transport across the

rumen epithelium (Gabel *et al.*, 1999; Wolffram *et al.*, 1989). cAMP is well established as inhibiting NHE3 via the activation of PKA and not NHE1 (see review by Orlowski and Grinstein (2004).

The expression (challenge) and/or inhibition (inhibitors) of Hsp70 allows the demonstration of the effects of Hsp on Na and acetate transport across isolated rumen epithelium. Transport of Na and acetate is significantly higher when Hsp70 is over-expressed. The effects of Hsp70 on Na and acetate transport are summarized in Table 4-3.

Table 4-3: Effect of Hsp70 on Na and acetate transport

Treatment	J_{ms}^{Na}	J_{net}^{Na}	J_{ms}^{Ac}	N/n
Without Hsp70	6.37±0.88	4.88±1.02	2.71±0.28	3/8
With Hsp70	9.32±0.74	7.70±0.82	4.12±0.94	3/7
Δ%	68	63	65	

Mechanism of Hsp: The exact mechanisms involved in Hsp70 rendered transport physiology are not clear. The results of this study however, are in agreement with the findings of Brown *et al.* (1992) who have observed a positive effect of Hsp on glucose and sulfate flux in flounder renal tubules. The authors also observed a higher survival rate for the microvilli after a mild heat stress. This so called "net prosurvival effect" by Hsp70 (Giffard *et al.*, 2008) has been attributed to various interactions of Hsp with cellular molecules.

The link between Hsp expression and phenotypic effects are still under discussion. Kirkegaard *et al.* (2010) has recently propose that Hsp can offer cell protection by inhibiting lysosomal membrane permeabilization by binding to an endosomal anionic phopholipid (BMP). Similarly Hsp expression plays an important role in recovery from Ca^{++} impairment via protease activity in C6 cells (Kagaya *et al.*, 2000) whereas according to Krieger *et al.* (2006) Hsp70 binding might act as an adaptor for Ca^{++}-dependent targeting of PKC to Ca^{++} channels. This is in line with previous findings suggesting a protective role for SCFA in induced DNA damage and intracellular Ca^{++} concentration by oxidative stress in distal colon cells (Abrahamse *et al.*, 1999). The binding of NHE1 and Hsp70 has been reported previously

(Konstantinidis *et al.*, 2009), suggesting possible interactions. The regulation of NHE via Hsp70 is complex and includes multiple protein-protein interactions. Hence, a possible link between Hsp70 expression and NHE3 activity in our study is proposed, but the exact mechanism of this interaction is still unknown. The higher acetate fluxes, however, are possibly attributed to increased NHE activity (Gabel *et al.*, 2002; Sehested *et al.*, 1996; Xuhang *et al.*, 2004) (see Figure 4-4 for detail).

Although Hsp70 has been studied in detail *in vitro*, the importance of these chaperone proteins can be imagined *in vivo,* particularly in ruminants experiencing a daily variation in fermentation parameters in the rumen (Figure 1-3). The positive correlation between rumen pH and intra-ruminal temperature (Figure 3-18) and the *in vivo* expression of Hsp70 due to these parameters (Figure 3-19) clearly indicates a role of Hsp70 in ruminant physiology. However, more studies need to be performed in order to determine the exact induction time and threshold for protection by Hsp70.

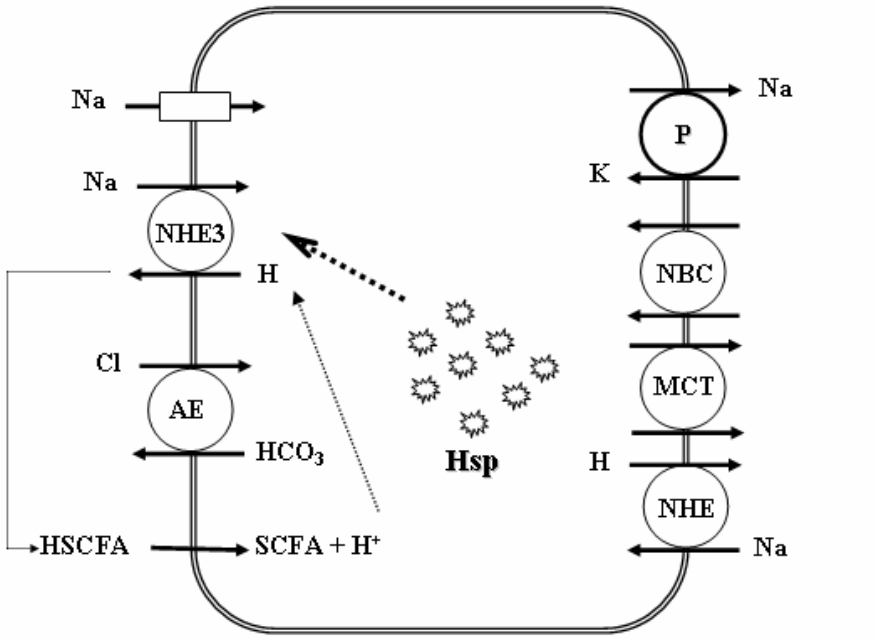


Figure 4-4: Proposed model of pH_i recovery in rumen epithelium and possible role of Hsp70 induced NHE activity. The complex mechanisms previously proposed for the Hsp mediated NHE3 activity probably also hold true for rumen epithelium and suggested a protein-protein interaction between NHE and Hsp. Higher SCFA fluxes are related to H^+ recycling via NHE.

In conclusion, the chaperone Hsp70 is well known as a stress protein with a variety of protective effects on cells, epithelia and organs. The expression of Hsp can be induced by ruminal fermentation parameters such as pH, SCFA concentration, osmotic pressure, and temperature. The observed effects of Hsp70 over-expression and its influence on epithelial transport indicate a short-term protective mechanism for rumen epithelium from diurnal changes in the fermentation pattern. This protection is particularly important for the rumen, since it is unable to secrete a protective layer to avoid acid stress during digestion, as in the case of abomasum. The results of the *in vitro* studies, which can be considered a model, are in agreement with the *in vivo* observations and support the working hypothesis that Hsp modulates the transport properties of the rumen epithelium. Hence, a novel rapid mechanism of rumen adaptation has been described.

The protection offered by the Hsp70 *in vitro* has a narrow range beyond which the stress is no longer physiological and leads to a suppression of Hsp synthesis (see above, Figure 4-2 and Figure 4-3). However, these findings must be considered with caution because the epithelium without blood flow *in vitro* is probably more prone to stress than epithelium *in vivo*. Hence, more research is needed in order to investigate the detailed mechanism of Hsp70-induced protection, its role in epithelial transport, and particularly its expression *in vivo* under harsh SARA conditions.

Chapter 5

Summary

It has been known from previous studies that the rumen epithelium can adapt, both morphologically and functionally, to changes in diet. This process may take days or weeks however, a daily variation in ruminal pH, predominantly after meals, has also been documented particularly in case of SARA that causes a low ruminal pH (<5.5) for a period of 3-4 hours per day. This diurnal variation in rumen pH and SCFA concentration puts the rumen epithelium in a stressful scenario and demands a rescue mechanism from these short term challenges.

It was therefore, the intention of this study to investigate the protective role of heat shock proteins and NHE in the rumen epithelium exposed on the luminal side to changes of pH, SCFA concentration, temperature or osmotic pressure. Using conventional Ussing chamber technique, *in vitro*, Na and acetate transport across the rumen epithelium were carried out to evaluate these protective effects whereas, concentrate fed animals with ruminal fermentation pattern resembling SARA were used to study the Hsp70 expression *in vivo*. The following results were obtained:

1. Characterization of electroneutral Na transport via NHE clearly showed that the isoform NHE3 is mediating electroneutral Na transport. Application of the specific NHE3 blocker S3226 reduced J_{ms} Na and J_{net}, which was not different from I_{sc}, indicating that S3226 abolished electroneutral Na transport.

2. Incubation of isolated rumen epithelium in the Ussing chamber simulating the conditions of SARA i.e. low mucosal pH (6.4-6.0) and/or high SCFA (70-80mmol·l^{-1}) concentrations, increasing temperature or osmotic pressure induced an over-expression of Hsp70. This expression was reduced when the stress conditions exceeded beyond a certain limit. For instance, an increase in temperature (>44°C), SCFA concentration on mucosal buffer (>100mmol·l^{-1}) and decrease in pH on mucosal side (<5.5) reduced Hsp70 expression.

3. Hsp70 expression could be reduced using a non specific (cycloheximide) or specific inhibitor (triptolide) thus allowing further studies in the presence or absence of Hsp70. Cycloheximide was most effective in a concentration of 3.0 mmol·l^{-1} while Triptolide was used in a concentration of 100 nmol·l^{-1}. The use of these inhibitors did not affect the I_{sc} or G_t and Na or acetate flux rates under control conditions.

4. Electroneutral Na as well as acetate transport were significantly higher in the tissues after an over expression of Hsp70. This increase of flux rates was significantly reduced by treatment with cycloheximide or triptolide.
5. Induction of Hsp expression by low rumen pH and an increase of temperature was confirmed *in vivo* by feeding concentrates. The tissues of these sheep exhibited higher Na transport rates.

It is concluded that the diurnal variation of fermentation pattern are inducing mechanisms which help to protect the tissue against theses challenges. Hps 70 is obviously an integral part of this acute adaptation and enhances NHE3 activity. Extrusion of protons taken up at increasing rates at higher SCFA concentrations and low pH must be considered as a first line of protection against SARA conditions of the rumen fluid. Although the exact mechanism between Hsp expression and NHE3 activity is not clear, Hsp is obviously a protective mechanism in the rumen epithelium too.

Zusammenfassung

Modulation des Na- und Acetat-Transports durch Heat Shock Proteine im Pansenepithel des Schafes

Zahlreiche Studien belegen, dass das Pansenepithel sich sowohl in funktioneller, als auch in morphologischer Hinsicht an Veränderungen in der Fütterung anpassen kann. Dieser Prozess kann Tage oder Wochen dauern; jedoch werden auch kurzfristige tägliche Schwankungen im pH des Pansens beobachtet, welcher insbesondere nach den Mahlzeiten und im Falle von SARA für 3-4 Stunden auf sehr niedrige Werte (<5.5) absinken kann. Diese tägliche Variation im pH Wert und in der Fettsäurekonzentration im Pansen stellt eine starke Belastung für das Pansenepithel dar und protektive Mechanismen sind erforderlich, um diesen akuten Belastungsschüben zu begegnen.

Es war daher die Absicht dieser Studie, die Schutzfunktion eines Heat Shock Proteins (Hsp70) und des NHEs an Epithelien des Pansens zu untersuchen, welche auf der mukosalen Seite Veränderungen im pH, in der Konzentration kurzkettiger Fettsäuren, in der Temperatur oder dem osmotischen Druck ausgesetzt worden waren. Um die protektive Funktion dieser Proteine zu untersuchen, wurde der Transport von Na und Azetat über das Pansenepithel in konventionellen Untersuchungen in der Ussingkammer in vitro gemessen. Begleitend wurde an Tieren, bei welchen durch Konzentratfütterung im Pansen SARA-ähnliche Fermentationsbedingungen induziert worden waren, die in vivo Expression von Hsp70 untersucht. Die folgenden Ergebnisse wurden erhalten:

1. Die Charakterisierung des elektroneutralen Natriumtransportes durch NHE zeigte klar, dass die Isoform NHE3 den elektroneutralen Natriumtransport vermittelt. Applikation des spezifischen NHE3 Blockers S3226 reduzierte J_{ms} Na und J_{net}, welche sich danach nicht signifikant vom gleichbleibenden I_{sc} unterschieden. Dieses weist darauf hin, dass S3226 den elektroneutralen Natriumtransport hemmt.
2. Die Inkubation von isoliertem Pansenepithel in der Ussingkammer unter SARA ähnlichen Bedingungen mit niedrigem pH (6.4-6.0) und/oder hoher Konzentration kurzkettiger Fettsäuren (70-80 $mmol \cdot l^{-1}$), ansteigender Temperatur oder Zunahme des osmotischen Druckes induzierte eine Überexpression von Hsp70. Diese Expression wurde reduziert wenn die Stressoren ein bestimmtes Maß überschritten. So kam es z.B.

bei einer Zunahme der Temperatur >44ºC, einer Konzentration von kurzkettigen Fettsäuren >100 mM und einem Abfall des pHs auf der mukosalen Seite >5.5 zu einem Abfall der Hsp70 Expression.

3. Die Expression von Hsp70 konnte durch einen nicht-spezifischen (Cycloheximid) oder spezifischen (Triptolid) Inhibitor abgesenkt werden, wodurch weitere Untersuchungen in der Anwesenheit und Abwesenheit von Hsp70 ermöglicht wurden. Cycloheximid war am effektivsten in einer Konzentration von 3.0 $mmol \cdot l^{-1}$, während Triptolid in einer Konzentration von 100 $nmol \cdot l^{-1}$ eingesetzt wurde. Unter Kontrollbedingungen hatte der Gebrauch dieser Inhibitoren weder auf den Kurzschlussstrom (I_{sc}) noch auf die Leitfähigkeit (G_t), die Na- oder die Azetatfluxe einen Einfluss.
4. Der elektroneutrale Transport von Na und der Transport von Azetat waren signifikant höher nach Überexpression von Hsp70. Diese Zunahme der Fluxraten wurde durch die Behandlung mit Cyclohexamid oder Triptolid signifikant reduziert.
5. Die Induktion von Hsp70 Expression in vivo durch einen niedrigen pH des Pansens und durch eine Zunahme der Temperatur wurde durch die Untersuchung von Geweben von Schafen mit unterschiedlicher Fütterung bestätigt. Epithelien von Schafen, die eine hohe Kraftfutterration erhalten hatten, zeigten auch höhere Na Transportraten.

Diese Ergebnisse führen zu der Schlussfolgerung, dass die tägliche Variation des Fermentationsmusters Mechanismen induziert, die das Gewebe vor der damit verbundenen Belastung schützt. Die Induktion von Hsp70 ist offenbar ein integraler Bestandteil dieser akuten Anpassungsreaktion und erhöht die Aktivität des NHE. Die Ausschleusung von Protonen, die mit zunehmender Fettsäurekonzentration und sinkendem pH vermehrt aufgenommen werden, muss als eine erste Schutzmaßnahme gegen die bei SARA vorherrschenden Bedingungen in der Pansenflüssigkeit gelten. Obwohl die genaue Natur des Zusammenhangs zwischen Hsp Expression und NHE3 Aktivitität nicht klar ist, stellen Hsp offenbar einen protektiven Mechanismus im Pansenepithel dar.

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Appendix

8.1 Buffer solutions:

Table 8-1: Transport buffer/control buffer solution

Substance	**mM·l^{-1}**
NaCl	115.00
KCl	5.00
$NaHCO_3$	25.00
$NaH_2PO_4 \cdot H_2O$	0.40
$Na_2HPO_4 \cdot H_2O$	2.40
Glucose ($C_6H_{12}O_6 \cdot H_2O$)	5.00
$CaCl_2 \cdot 2H_2O$	1.20
$MgCl_2 \cdot 6H_2O$	1.20
Gas	Carbogen (5% O_2 + 95% CO_2)

Table 8-2: Experimental buffer solution (40mM SCFA).

Substance	**mM·l^{-1}**
NaCl	20.00
$NaHCO_3$	25.00
Na Acetate	24.00
Na Propionate	12.00
Na Butyrate	4.00
Glucose ($C_6H_{12}O_6 \cdot H_2O$)	10.00
$CaCl_2 \cdot 2H_2O$	1.00
$MgCl_2 \cdot 6H_2O$	1.00
KH2PO4	1.00
K2HPO4	2.00
Na Gluconate	60.00
Gas	Carbogen (5% O_2 + 95% CO_2)

Table 8-3: Challenge buffer solution (80 mM)

Substance	**$mM \cdot l^{-1}$**
NaCl	20.00
$NaHCO_3$	25.00
Na Acetate	48.00
Na Propionate	24.00
Na Butyrate	8.00
Glucose ($C_6H_{12}O_6 \cdot H_2O$)	10.00
$CaCl_2 \cdot 2H_2O$	1.00
$MgCl_2 \cdot 6H_2O$	1.00
KH2PO4	1.00
K2HPO4	2.00
Na Gluconate	20.00
Gas	Carbogen (5% O_2 + 95% CO_2)

Table 8-4: Different concentration of SCFA for mucosal side.

Substance	**10 mM**	**40 mM**	**70 mM**	**100mM**
NaCl	20.00	20.00	20.00	20.00
$NaHCO_3$	25.00	25.00	25.00	25.00
Na-Acetate	6.00	24.00	42.00	60.00
Na Propionate	3.00	12.00	21.00	30.00
Na Butyrate	1.00	4.00	7.00	10.00
$KH_2PO_4 \cdot H_2O$	1.00	1.00	1.00	1.00
$K_2HPO_4 \cdot H_2O$	2.00	2.00	2.00	2.00
Glucose	10.00	10.00	10.00	10.00
$CaCl_2 \cdot H_2O$	1.00	1.00	1.00	1.00
$MgCl_2 \cdot 6H_2O$	1.00	1.00	1.00	1.00
Na Gluconate	90.00	60.00	30.00	-
Gas	Carbogen (5% O_2 + 95% CO_2)			

Table 8-5: Buffer with high osmolarity (380 mOsmol/l)

Substance	**$mM \cdot l^{-1}$**
NaCl	20.00
$NaHCO_3$	25.00
Na Acetate	48.00
Na Propionate	24.00
Na Butyrate	8.00
Glucose ($C_6H_{12}O_6 \cdot H_2O$)	10.00
$CaCl_2 \cdot 2H_2O$	1.00
$MgCl_2 \cdot 6H_2O$	1.00
KH2PO4	1.00
K2HPO4	2.00
Na Gluconate	60.00
Gas	Carbogen (5% O_2 + 95% CO_2)

8.2 Inhibitors:

Table 8-6: Concentrations of various inhibitors

Substance	**Concentration ($mM \cdot l^{-1}$)**
Indomethacin	0.001
Amiloride	1.00
Cariporide (HOE642)	0.03
S3226	0.001
Cycloheximide	3.00
Triptolide	0.0001

8.3 Electrophysiology

Table 8-7: Average electrophysiology of randomly selected epithelia during equilibration period (~30 min)

No. of experiments	I_{sc}	G_t	PD
1	1.58±0.03	3.31±0.07	14.51±0.07
2	1.38±0.02	3.72±0.05	12.92±0.11
3	1.19±0.02	2.53±0.03	12.71±0.23
4	1.66±0.03	3.14±0.04	14.22±0.18
5	1.12±0.02	3.21±0.03	13.44±0.08

Table 8-8: Effect of stress on electrophysiology.

Parameters	High temperature			Low pH			High osmolarity		
	Before	After	change	Before	After	change	Before	After	change
PD (mV)	13	8	38%	16	26	62%	19	17	10%
I_{sc} ($\mu eq.cm.hr^{-1}$)	1.2	0.97	16%	1.6	2.0	25%	1.2	1.4	16%
G_t ($mS.cm^2$)	2.6	3.3	26%	2.2	2.0	9%	1.7	2.2	29%

8.4 Molecular biology

Table 8-9: RNA integrity number (RIN) of various samples.

Sample No	RIN
1	9.0
2	8.8
3	8.9
4	8.9
5	9.0
6	8.9
7	8.7
8	8.8
9	9.0
10	9.4
11	9.4
12	8.7

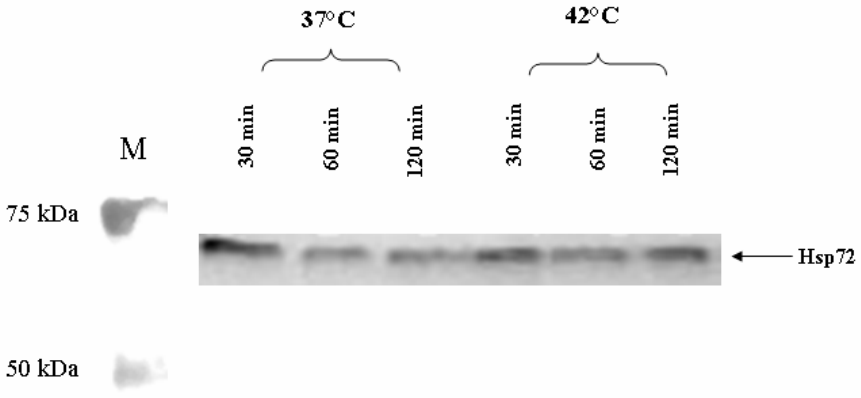

Figure 8-1: Varying expression of Hsp70 with temperature and duration

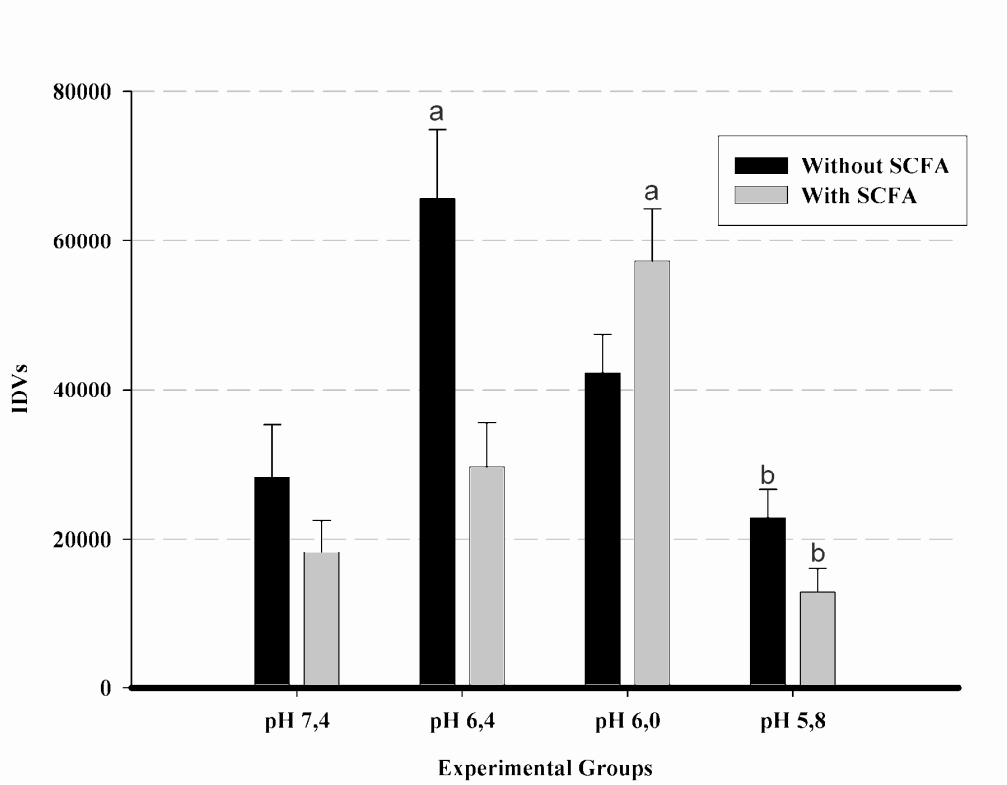

Figure 8-2: Hsp70 expression with different mucosal pH using control and SCFA buffer. (a) is significantly different from pH 7.4 (p = 0.0079; (b) is significantly different from pH 6.4 (p = 0.003). Each point: Mean ±SD, N/n = 5/8-12

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Selbständigkeitserklärung zur Dissertation

Hiermit bestätige ich, dass ich die vorliegende Arbeit selbständig angefertigt habe. Ich versichere dass ich ausschließlich die angegebenen Quellen und Hilfen in Anspruch genommen habe.
Ferner erkläre ich, dass die Arbeit bisher in keinem anderen Promotionsverfahren angenommen oder abgelehnt worden ist.

Imtiaz Rabbani

05.11.2010