Investigation regarding the epithelial electrolyte and nutrient transport across the intestinal mucosa in the horse

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IN LOVING MEMORY OF MY MOTHER
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Table of Contents

1 INTRODUCTION .............................................................................................................. 1
   1.1 Intestinal electrolyte transport ..................................................................... 1
   1.2 Intestinal nutrient transport ........................................................................ 3
   1.3 Objectives.......................................................................................................... 5

2 PAPER I ............................................................................................................................ 6

3 PAPER II .......................................................................................................................... 7

4 PAPER III ......................................................................................................................... 8

5 DISCUSSION AND CONCLUSIONS .......................................................................... 33
   5.1 Animals and sampling procedure ................................................................. 33
   5.2 Enrichment and functional integrity of BBMV ............................................. 33
   5.3 Intestinal electrolyte transport ..................................................................... 34
       5.3.1 Cl\(^{-}\) transport across the jejunum ...................................................... 34
       5.3.1.1 Stimulation of electrogenic Cl\(^{-}\) secretion .................................. 34
       5.3.1.2 Cl\(^{-}\) channel inhibitors ................................................................. 34
       5.3.2 Ca\(^{2+}\) and P\(_{i}\) transport along the intestinal axis .............................. 36
   5.4 Nutrient transport.............................................................................................. 38
   5.5 Conclusions and outlook .............................................................................. 40

6 SUMMARY ....................................................................................................................... 41

7 ZUSAMMENFASSUNG (GERMAN) .............................................................................. 43

8 REFERENCES .................................................................................................................. 46

9 ACKNOWLEDGMENTS ................................................................................................. 56
List of abbreviations

AP - alkaline phosphatase
ATP - adenosine triphosphate
BBMV - brush border membrane vesicles
CaCC - Ca^{2+}-activated Cl⁻ channel
CFTR - cystic fibrosis transmembrane conductance regulator
DIDS - 4,4''-diisothiocyanato-stilbene-2,2''-disulfonic acid
GLUT 5 - glucose transporter 5
GLUT2 - glucose transporter 2
GlyH-101 - N-(2-naphtalenyl)-(3,5-dibromo-2,4-dihydroxyphenyl)methylene glycine hydrazide
Gₜ - transepithelial tissue conductance
Iₛₜ - short circuit current
Kₘ - Michaelis-Menten constant, substrate concentration at Vₘₐₓ/2
NCX1 - Na⁺/Ca^{2+} exchanger 1.
NKCC - Na⁺⁻K⁺⁻2Cl⁻ cotransporter
NPPB - 5-nitro-2-(3-phenylpropylamino) benzoate
ORCC - outward rectifying Cl⁻ channel
PepT1 - peptide transporter 1
PMCA1 - plasma membrane Ca^{2+} ATPase 1
PTH - parathyroid hormone
SGLT1 - Na⁺-dependent glucose transporter 1
TEA - tetraethylammonium
TRP - transient receptor potential
TRPV5 - transient receptor potential vanilloid member 5
TRPV6 - transient receptor potential vanilloid member 6
Vₘₐₓ - maximum velocity of the enzyme catalysed reaction
1 INTRODUCTION

The intestinal absorption and secretion of electrolytes, nutrients and water play a fundamental role in whole-body maintenance and macromineral homoeostasis (Chang 1994; Breves 2010). In the horse, the intestinal tract is the system most commonly affected by diseases, and imbalances in electrolyte homoeostasis are often associated with various disorders (Hintz et al. 1994; Tinker et al. 1997; Toribio et al. 2007; Hurcombe et al. 2009). The majority of studies on the equine intestine focus on predisposing factors, clinical symptoms, therapeutic approaches and etiological agents of disease (Whitlock 1986; Tinker et al. 1997; McConnico 2004). Very little information is available on the underlying epithelial transport mechanisms and the intestinal sites of electrolyte and nutrient absorption in horses (Schryver 1975; Dyer et al. 2002). However, this information is crucial for a better understanding of the pathogenesis of intestinal diseases and the improvement of feed management. In other species, the structure and activity of different epithelial transporters and ion channels have intensively been investigated (Hediger et al. 1987; Chen et al. 1999; Hoenderop et al. 1999; Peng et al. 1999; Klang et al. 2005).

1.1 Intestinal electrolyte transport

Regarding the Cl\textsuperscript{-} secretion, Cl\textsuperscript{-} enters the enterocytes via the Na\textsuperscript{+}-K\textsuperscript{+}-2Cl\textsuperscript{-} cotransporter (NKCC) at the basolateral membrane and leaves the cell at the apical membrane via Cl\textsuperscript{-} channels with the cystic fibrosis transmembrane conductance regulator (CFTR) being considered the most important intestinal Cl\textsuperscript{-} channel. However, published data of several authors demonstrate the existence of alternative Cl\textsuperscript{-} channels including Ca\textsuperscript{2+}-activated Cl\textsuperscript{-} channels (CaCC) and the outward rectifying chloride channel (ORCC) (Barrett et al. 2000). In order to distinguish between different types of chloride channels, various channel inhibitors such as 5-nitro-2-(3-phenylpropylamino)benzoic acid (NPPB), 4,4\textsuperscript{'}-diisothiocyanato-stilbene-2,2\textsuperscript{'}-disulfonic acid (DIDS), CFTR\textsubscript{inh}-172, N-(2-naphtalenyl)-(3.5-dibromo-2.4-dihydroxyphenyl)methylene glycine hydrazone (GlyH-101) and glibenclamide have been studied, and their effects on channel conductance have been established in different species (Diener et al. 1989; Ma et al. 2002; Muanprasat et al. 2004; Bleich et al. 2007; Leonhard-Marek et al. 2009). In the horse, there are limited data on electrolyte transport across the large intestine. Electroneutral absorption of Na\textsuperscript{+} and Cl\textsuperscript{-} and electrogenic secretion of Cl\textsuperscript{-} were determined across the proximal colon (Clarke and Argenzio, 1990). Across the small colon, electrogenic Cl\textsuperscript{-} secretion was identified (Clarke et al. 1992). Furthermore, an active transport of Cl\textsuperscript{-} and Na\textsuperscript{+} across the cecum was determined (Giddings et al., 1974). There are no data available on Na\textsuperscript{+} and Cl\textsuperscript{-} transport across the equine small intestine.

Ca\textsuperscript{2+} is transported across the intestinal wall by either paracellular or transcellular mechanisms. Paracellular Ca\textsuperscript{2+} transport along the entire intestinal tract is passive and dependent on an electrochemical gradient, the exposure time or the solvent drag
mechanism (Bronner et al. 1986). The latter drives water and solutes paracellularly as a consequence of an osmotic gradient induced by active transport of other substances, for example glucose (Karbach 1992). Transcellular Ca\textsuperscript{2+} transport is active, saturable and regulated by hormones such as calcitriol, PTH, estrogens, glucocorticoids, Klotho as well as Ca\textsuperscript{2+} availability (Hoenderop et al. 2005; Lambers et al. 2004). Several authors suggest that transcellular Ca\textsuperscript{2+} transport plays a major role when Ca\textsuperscript{2+} intake is low compared to Ca\textsuperscript{2+} demands (Bronner et al. 1999; Martin-Tereso et al. 2011). Transcellular Ca\textsuperscript{2+} absorption involves at least three steps including apical Ca\textsuperscript{2+} entry, cytosolic translocation by Ca\textsuperscript{2+} binding proteins, and extrusion at the basolateral membrane by transport proteins such as plasma membrane Ca\textsuperscript{2+} ATPase 1 (PMCA1) and Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger 1 (NCX1) (Hoenderop et al. 2005). Apical Ca\textsuperscript{2+} transport occurs via the transient receptor potential vanilloid member 5 (TRPV5) and 6 (TRPV6). They belong to the TRPV subfamily of the transient receptor potential (TRP) family, and have been identified in several species such as rabbit, rat, human, pig, and sheep (Hoenderop et al. 1999; Peng et al. 1999; Peng et al. 2000; Suzuki et al. 2000; Hinterding 2002; Wilkens et al. 2009). In the horse, TRPV5 and 6, the cytosolic Ca\textsuperscript{2+} binding protein calbindin-D\textsubscript{9k}, as well as PMCA1 and NCX1 have recently been identified by Rourke et al. (2010).

Horses absorb approximately 45% - 73% of the Ca\textsuperscript{2+} provided with the diet indicating a high intestinal absorptive capacity for Ca\textsuperscript{2+} in comparison to other species (Schryver 1975). Sheep and cattle of equivalent age groups and provided with equivalent Ca\textsuperscript{2+} in the diet absorb approximately 28% - 39% (Hansard et al. 1954; Braithwaite et al. 1971). In addition to a high capacity for intestinal Ca\textsuperscript{2+} absorption, the horse has some other distinctive characteristics in macromineral homoeostasis. Compared to other species, horses have high plasma concentrations of Ca\textsuperscript{2+} (Geiser and Faulk, 1989), low plasma concentrations of Pi (Elfers et al., 1986), high renal Ca\textsuperscript{2+} excretion (Toribio et al., 2007), low calcidiol and calcitriol concentrations (Breidenbach et al., 1998; Maenpaa et al., 1988) and a low parathyroid gland sensitivity to Ca\textsuperscript{2+} (Toribio et al., 2003). Although the concentrations of vitamin D metabolites are low, the clinical picture of osteomalacia is hardly seen in the horse (El Shorafa et al., 1979). Oral uptake of cholecalciferol does not lead to a distinct increase in plasma concentrations of Ca\textsuperscript{2+} and calcitriol as documented in other species, but results in a high increase in plasma concentration of Pi, and Ca\textsuperscript{2+} x Pi products resulting in enzootic calcinosis (Grabner et al., 1985; Harmeyer et al., 2004). As demonstrated in vitamin D toxicosis, imbalances in Ca\textsuperscript{2+} homeostasis are often closely associated with disturbances in Pi homeostasis in the horse (Harrington et al., 1983; Harmeyer et al., 2004).

Several pathologic conditions are associated with Ca\textsuperscript{2+} imbalances in horses including hypoparathyroidism (Couetil et al., 1998), hyperparathyroidism (Ronen et al., 1992), renal failure (Elfers et al., 1986), humoral hypercalcemia of malignancy (Rosol et al, 1994), vitamin D toxicity (Harrington et al. 1983), exercise-induced hypocalcemia (Aguilera-Tejero et al., 2001), and sepsis (Toribio et al., 2005). In critically ill horses, acute hypocalcemia is often reported, particularly in animals with gastrointestinal diseases (Garcia-Lopez et al. 2001; Toribio et al. 2001; Toribio et al.
Hypocalcemia may be caused by renal loss, impaired PTH secretion of the parathyroid gland, insufficient Ca\(^{2+}\) mobilization in response to PTH or impaired intestinal Ca\(^{2+}\) absorption. Data on PTH response and renal Ca\(^{2+}\) excretion in affected horses did not reveal the underlying cause of hypocalcemia in these horses. Fractional urinary clearance of Ca\(^{2+}\) was appropriately reduced as an expected homeostatic response to hypocalcemia, and PTH response to hypocalcemia varied in affected horses making identification of underlying pathophysiology difficult (Toribio et al., 2001). These findings indicate that intestinal Ca\(^{2+}\) transport may play a key role in maintaining macromineral homeostasis necessitating the need to investigate intestinal Ca\(^{2+}\) and Pi transport in more detail.

Published data on intestinal Ca\(^{2+}\) and Pi transport in the horse are limited. Schryver et al. (1970) investigated the site of Ca\(^{2+}\) and Pi absorption by determining the degree of radiolabelled Ca\(^{2+}\) absorption from in vivo intestinal sacs, by calculating the apparent absorption in various regions using Cr\(_2\)O\(_3\) as an unabsorbable marker, and by comparing the degree of absorption of radiolabelled Ca\(^{2+}\) either deposited directly in the fistulated cecum or the stomach of ponies. Results of these studies suggested that the proximal part of the small intestine may be the major site of Ca\(^{2+}\) absorption followed by the distal part and the large intestine. Regarding Pi transport, Schryver et al. (1972) postulated a Pi absorption in the distal small intestine, the dorsal and the small colon and a Pi secretion in the proximal small intestinal tract and the cecum. These studies have improved our understanding of Ca\(^{2+}\) and Pi absorption in the horse. However, the complexity of whole body balance studies would not reveal the processes operative at the epithelial level. Regarding Pi transport, there are no published data on underlying epithelial transport mechanisms in horses. In other species, a secondary active Na\(^+\)/Pi transport has been identified for Pi absorption with the NaPiib and the PiT1 being the major underlying transporters (Berner et al. 1976; Shirazi-Beechey et al. 1988; Schröder et al. 1995; Schröder et al. 1996; Virkki et al., 2007; Muscher et al. 2009; Giral et al. 2009). In small ruminants, an additional H\(^+\)-dependent, secondary active Pi transport mechanism has been observed (Huber et al. 2002).

1.2 Intestinal nutrient transport

The horse is a nonruminant herbivore with fermentation of plant fiber taking place in the large bowel providing a substantial fraction of the horse’s energy requirements in respect to maintenance. In order to meet energy requirements for breeding and performance, horses are often fed diets that contain high levels of concentrates which are digested and absorbed in the small intestine as long as its limits of digestive and absorptive capacities are not exceeded (Dyer et al. 2002). These distinct characteristics of the equine intestine limit the ability of transferring data on nutrient transport mechanisms from other species to the horse necessitating in vitro and in vivo investigations in the horse.

Different intestinal transporters for glucose, amino acids and peptides such as the Na\(^+\)-dependent glucose cotransporter 1 (SGLT 1), the glucose transporter 2 (GLUT
and 5 (GLUT 5), several Na\(^+\)-dependent and Na\(^+\)-independent amino acid transport systems, and the peptide transporter 1 (PepT1) have been investigated in the intestinal epithelium in many species (Christensen 1990; Chen et al. 1999; Winckler et al. 1999; Klang et al. 2005). In the horse, the SGLT 1 and the GLUT 5 have been identified in the small intestine confirming the capacity of the equine small intestine to absorb glucose and fructose (Dyer et al. 2002; Merediz et al. 2004). However, there is no information available on the mechanisms of amino acid and peptide transport across the equine intestine. In other species, the importance of intestinal peptide absorption to whole-body nutrition and as well as its nutritional significance is now widely accepted (Webb et al. 1992; Leibach et al. 1996; Adibi 1997). Intestinal peptide transport significantly contributes to amino acid intake, and the absorption of amino acids in the form of peptides was demonstrated to be an even faster route of uptake per time than the absorption of their constituent amino acid in the free form (Adibi et al. 1968; Adibi 1971; Cheng et al. 1971; Steinhardt et al. 1986; Rerat et al. 1992). The tertiary active H\(^+\)/peptide symporter is driven by an inwardly directed H\(^+\) gradient which is induced by apically located Na\(^+\)/H\(^+\) exchanger. The latter is driven by a Na\(^+\) gradient induced by the Na\(^+\)/K\(^+\)-ATPase at the basolateral membrane (Ganapathy and Leibach, 1983). The H\(^+\)-coupled, pH-dependent, rheogenic nature of peptide transport was confirmed using human intestinal epithelial Caco-2 cell monolayers (Ganapathy et al. 1983; Thwaites et al. 1993a; Thwaites et al. 1993b), and the underlying PepT1, a member of the H\(^+\)-coupled oligopeptide transporter superfamily, has been identified in several species including rabbits, sheep, pigs, and chickens (Fei et al. 1994; Pan et al. 2001; Daniel and Kottra 2004; Klang et al. 2005). The PepT1 is capable of transporting a wide range of dipeptides and tripeptides, some peptide mimetics, and pharmacological active substances which have a chemical structure similar to peptides (Leibach et al. 1996; Dantzig 1997). Intestinal peptide absorption can be influenced by dietary protein (Gilbert et al. 2008), diurnal rhythm (Pan et al. 2002), fasting and starvation (Shimakura et al. 2006), hormones (Thamotharan et al. 1999), and disease (Shu et al. 2002; Sekikawa et al. 2003). Although PepT1 is considered the major transporter for peptide absorption, several studies on intestinal peptide transport have revealed conflicting results on the mechanisms of intestinal peptide transport suggesting the existence of multiple dipeptide transporters with at least one acidic pH-preferring class (uptake driven by an inwardly H\(^+\) gradient) and one neutral pH-preferring class (Rubino et al. 1971; Ganapathy et al. 1985; Inui et al. 1988; Kato et al. 1989). Other alternative routes of peptide uptake are also discussed including paracellular movement and transport by cell-penetrating peptides (CCP) which are capable of moving cargo to the inside of the cell either by direct penetration of the lipid bilayer or by endocytosis (Gilbert et al. 2008).
1.3 Objectives

The objectives of this study were to

i) adapt in vitro techniques for investigating intestinal transport mechanisms in horses including the Ussing chamber technique, radioisotope tracer technique, preparation of brush border membrane vesicles (BBMV) and uptake studies into BBMV

ii) to characterize the transport of Cl⁻, amino acids, dipeptides and glucose across the jejunum of healthy horses

iii) to localise the major sites of transepithelial Ca²⁺ and Pi transport along the intestinal axis of healthy horses

iv) to demonstrate the existence of an intestinal peptide transport mechanism in the horse and to identify its underlying driving force in regard to a H⁺- or Na⁺-mediated dipeptide cotransport.
2 PAPER I

Electrophysiological characterization of electrolyte and nutrient transport across the small intestine in horses


The contribution of Anja Cehak to the article is evaluated according to the following scale:

A - The PhD student has contributed to collaboration (0 - 33%)
B - The PhD student has contributed significantly (34 - 66%)
C - The PhD student has essentially performed this study independently
   (67 - 100%)

1. Design of experimental study     B
2. Laboratory work       B/C
3. Analysis of experiments      C
4. Scientific writing and presentation at congress   C
3 PAPER II

**In vitro studies on intestinal calcium and phosphate transport in horses**

A. Cehak, M. R. Wilkens, M. Guschlbauer, N. Mrochen, B. Schröder, K. Feige, G. Breves


The contribution of Anja Cehak to the article is evaluated according to the following scale:

- A - The PhD student has contributed to collaboration (0 - 33%)
- B - The PhD student has contributed significantly (34 - 66%)
- C - The PhD student has essentially performed this study independently (67 - 100%)

1. Design of experimental study
   - C
2. Laboratory work
   - B/C
3. Analysis of experiments
   - C
4. Scientific writing and presentation at congress
   - C

Ad 2.) M. Wilkens and N. Mrochen carried out RNA-isolation, RT-PCR and Westernblot analysis for identifying TRPV6, calbindin-D_{28k} and PMCA1.
4  PAPER III

In vitro studies on intestinal peptide transport in horses
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The contribution of Anja Cehak to the article is evaluated according to the following scale:

A - The PhD student has contributed to collaboration (0 - 33%)
B - The PhD student has contributed significantly (34 - 66%)
C - The PhD student has essentially performed this study independently (67 - 100%)

5. Design of experimental study  C
6. Laboratory work  C
7. Analysis of experiments  C
8. Scientific writing and presentation at congress  C
In vitro studies on intestinal peptide transport in horses

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ABSTRACT: Published data on the physiology of nutrient transport across the equine intestine is limited, and the existence and relevance of peptide transporters are still unknown in the horse. In the present study, the equine intestinal peptide transport was investigated by Ussing chamber experiments using the radioisotope tracer technique and by uptake studies into brush border membrane vesicles (BBMV). Jejunal mucosae of 16 healthy adult horses were used. Tissue samples were mounted in Ussing chambers, and electrophysiological parameters as well as unidirectional flux rates of the radiolabelled dipeptide glycyl-glutamine (Gly-Gln) were determined. The short-circuit current (I_{sc}) response to the luminal addition of Gly-Gln was significantly greater compared to the I_{sc} response to glycyl-sarcosine (Gly-Sar) addition (P < 0.01). Positive net flux rates were determined indicating absorption of the dipeptide. The addition of Gly-Sar reduced the flux rates significantly (P < 0.01) suggesting that both peptides compete for the same transport system. The flux rates were not affected by changes in luminal pH value. Uptake studies into BBMV demonstrated an uphill transport in both the absence and the presence of an inwardly directed H^+-gradient with the H^+-mediated uphill transport being significantly greater than the transport under equilibrium conditions (P < 0.001). A Na^+-gradient did not cause an uphill transport. Gly-Gln uptakes displayed Michaelis-Menten kinetics with the K_m value for the H^+-dependent Gly-Gln uptake being significantly different from the K_m value for the Gly-Gln uptake under equilibrium conditions (P < 0.05). In conclusion, the study demonstrated for the first time that dipeptides are transcellularly transported across the equine small intestine. The results indicate the presence of at least two transport systems for peptide absorption the horse: One secondary active H^+-mediated cotransport, and one that is capable of an uphill transport energised by a mechanism other than a H^+- or a Na^+-gradient.

Keywords: glycyl-glutamine, glycyl-sarcosine, horse, intestine, peptide, transport
INTRODUCTION

The importance of intestinal peptide absorption to whole-body nutrition has been demonstrated in many species (Leibach and Ganapathy, 1996; Adibi, 1997; Webb et al., 1992). Intestinal peptide transport significantly contributes to AA intake, and the absorption of AA in the form of peptides was demonstrated to be an even faster route of uptake per unit of time than the absorption of their constituent AA in the free form (Adibi and Phillipis, 1968; Adibi, 1971; Cheng et al., 1971; Steinhardt and Adibi, 1986; Rerat et al., 1992). A broad range of dipeptides and tripeptides, some peptide mimetics, and pharmacological active compounds with a chemical structure similar to peptides can be transported across the intestinal wall by peptide transporters (Leibach and Ganapathy, 1996; Dantzig, 1997). Their absorption is influenced by dietary protein, diurnal rhythm, fasting and starvation, hormones, and disease (Thamotharan et al., 1999; Pan et al., 2002; Shu et al., 2002; Sekikawa et al., 2003; Shimakura et al., 2006; Gilbert et al., 2008).

In several species, a carrier-mediated process energized by a transmembrane H⁺-gradient has been identified as the major mechanism of peptide absorption (Ganapathy and Leibach, 1983). The H⁺-coupled, pH-dependent, rheogenic nature of peptide transport was confirmed using human intestinal epithelial Caco-2 cell monolayers (Thwaites et al., 1993a; Thwaites et al., 1993b). The underlying PepT1, a member of the H⁺-coupled oligopeptide transporter superfamily, has been identified in birds and mammals including rabbits, sheep, pigs, and chickens (Fei et al., 1994; Pan et al., 2001; Daniel and Kottra, 2004; Klang et al., 2005). Studies on regional differences of intestinal PepT1 along the intestinal axis in rats revealed the highest expression of PepT1 transcripts in the jejunum, followed by ileum, distal colon, duodenum, and proximal colon. At protein level, strongest staining of PepT1 was found in the jejunum, followed by duodenum and ileum (Jappar et al., 2010).

Besides the PepT1, other transport mechanisms for peptide absorption have been reported. Studies on intestinal rabbit brush border membrane vesicles (BBMV) suggest the existence of multiple dipeptide transporters with at least one acidic pH-prefering class (uptake driven by an inwardly H⁺-gradient) and one neutral pH-prefering class (Rubino et al., 1971; Ganapathy and Leibach, 1985; Inui et al., 1988; Kato et al., 1989). Other alternative routes of peptide transport have been described including paracellular movement and transport by cell-penetrating peptides which are capable of moving cargo to the inside of the cell either by direct penetration of the lipid bilayer or by endocytosis (Gilbert et al., 2008).

In the horse, the existence and importance of peptide transporters are still unknown. Intestinal AA and peptide absorption however are of considerable nutritional significance, particularly in breeding and performance horses (Hintz, 1994; Hintz and Cymbaluk, 1994). Glade (1983) calculated that crude protein levels correlated positively with time to finish in race horses. Administration of an AA mixture lowered plasma lactate response and improved oxidative capacity (Glade, 1991). Surveys indicate that many race horses are fed significantly more protein than the NRC estimates of requirements (Southwood et al. 1993, Gallagher et al.1992, Winter and
Hintz 1981). In contrast Meyer (1987) suggested that high dietary protein intakes may have detrimental effects on performance because of increased water requirements due to urinary nitrogen excretion, elevated urea levels in the plasma resulting in higher urea entrance into the intestinal lumen and thus increased risk of enterotoxemia, higher energy costs due to nitrogen excretion, and increased ammonia load in the barn increasing the incidence and severity of respiratory diseases. The effect of dietary protein on metabolic changes has been documented by Miller-Graber et al. (1991) who demonstrated a significant effect of dietary protein levels on lactate/pyruvate ratio in performance horses.

Impaired peptide absorption may also be an important consequence in horses affected by intestinal disorders which are the most frequent pathologic conditions in horses (Tinker et al., 1997). The most important cause of equine death is colic with the small intestine being the second most commonly affected bowl segment involved in colic (White, 1990; Hintz and Cymbaluk, 1994).

The major site of protein digestion in the horse is the small intestine (Hintz et al., 1971). The importance of hind-gut produced microbial protein for the horses has been discussed, however published data indicates that microbial protein contributes only little to the amino acid pool of the horse (McMeniman et al., 1987).

The purpose of the present study was to demonstrate the existence of an intestinal peptide transport mechanism in the equine jejunum and to identify the underlying driving force of peptide uptake into the enterocytes in regard to a H\(^+\) - or Na\(^+\)-mediated dipeptide cotransport.

**MATERIALS AND METHODS**

**Animals and collection of tissue**

The animal handling regimen was approved by the Animal Welfare Commissioner of the University of Veterinary Medicine Hannover in accordance with the German Animal Welfare Law.

Sixteen adult horses (*Equus caballus*) were included in the study. 9 Warmblood Horses, 3 Trotters, 1 Thoroughbred Horse, 1 Trakehner Horse, 1 Arabian Horse, and 1 Haflinger Horse, 8 mares, 7 geldings, and 1 stallion with a mean age of 15 years and a mean BW of 523 kg were used. The horses were housed in paddocks at the Clinic for Horses, University of Veterinary Medicine Hannover. They were provided with hay *ad libitum* and had free access to water and mineral salt. All horses were free of intestinal diseases, had normal laboratory profiles, and were dewormed prior to the study.

Jejunal tissue samples were obtained during ventral laparotomy under general anaesthesia using the following standardised protocol: Horses were pre-medicated with 1.1 mg/kg of xylazine (CP-Pharma, Burgdorf, Germany). Anaesthesia was induced by 0.05 mg/kg diazepam (AbZ Pharma, Ulm, Germany) and 2.2 mg/kg ketamine (Vetoquinol, Ravensburg, Germany) and maintained with isoflurane (CP-Pharma) in 100% oxygen and continuous rate infusion of ketamine. After placing the
horses in dorsal recumbency, routine laparotomy was performed. Approximately 30 cm of the jejunum localized 1.5 m orally to the end of the ileocecal fold were resected, immediately rinsed with ice-cold saline and placed in ice-cold Krebs-Henseleit-solution (KHS) aerated with carbogen (95% O₂ / 5% CO₂). After surgery, horses were euthanized by administering 60 mg/kg pentobarbital without regaining consciousness. The intestine was opened along the mesenteric line and the mucosa was separated from the underlying muscle and serosal layers. Light microscopy confirmed the removal of both longitudinal and circular muscle layers. Tissue samples were either used for determination of electrophysiological parameters and transepithelial flux rates in Ussing chambers (n = 11) or for uptake studies into BBMV (n = 5). The latter ones were snap frozen in liquid nitrogen immediately after resection and stored at -80 °C until further processing.

**Ussing chamber technique, calculation of electrophysiological parameters and transepithelial flux rates**

In the first trial, the intestinal peptide transport was investigated by determining the short-circuit current (Isc) response to a concentration-dependent addition of peptides to the luminal side of the mucosa using Ussing chambers. Either glycyl-glutamine (Gly-Gln) (Hartmann Analytic GmbH, Braunschweig, Germany) or glycyl-sarcosine (Gly-Sar) (Moravek Biochemicals Inc., Brea, California) was added to the luminal side at increasing concentrations ranging from 2.5 mmol·l⁻¹ to 30 mmol·l⁻¹ (n = 5). Gly-Gln and Gly-Sar were chosen because they are considerably stable against brush-border hydrolases compared to dipeptides possessing other N-terminal AA. Furthermore they have been approved in studies on peptide transport in other monogastric and ruminant animals with a similar study design and the same in vitro techniques being used as in the present study allowing interpretation of results in the context of published data (Wolffram et al., 1997; Winckler et al., 1999).

In the second trial, the unidirectional flux rates of radiolabelled Gly-Gln (Hartmann Analytic GmbH, Braunschweig, Germany) were calculated in the absence and in the presence of the unlabeled dipeptide Gly-Sar in order to determine whether both dipeptides compete for the same transport system (n = 5).

In the third trial, the effect of an acidic luminal pH on peptide transport was investigated by determining the unidirectional flux rates of Gly-Gln at two different luminal pH values (pH 7.4 and pH 6.0, n = 5).

Ussing chambers with an exposed tissue area of 1.13 cm² were used. The chambers were connected to a computer-controlled voltage clamp device (K. Mußler, Aachen, Germany). Transepithelial potential differences (PDₜ) were measured using KHS agar bridges connected to calomel electrodes in 3 mol·l⁻¹ KCl. Tissue conductances (Gₜ) were determined from the changes in PDₜ caused by bipolar current pulses of 100 µA·cm⁻² of 200 ms duration. The currents were passed through 3 mol·l⁻¹ KCl buffer solution agar bridges connected to Ag/AgCl electrodes. Fluid resistances and junction potentials were measured before mounting the mucosa and were corrected during the experimental period. After an equilibration period of 15 min, tissues were short-circuited, and Isc and Gₜ were continuously monitored.
Buffer solutions for the serosal side of the tissues contained (in mmol·l⁻¹): NaCl 113.6, KCl 5.4, HCl 0.4, MgCl₂ 1.2, CaCl₂ 1.2, NaHCO₃ 21.0, Na₂HPO₄ 1.2, NaH₂PO₄ 0.3, glucose 10.0 and mannitol 23.0. The mucosal side of the tissue was incubated in a glucose-free buffer with the same composition, but with mannitol being substituted for glucose to ensure the same osmolarity. The osmolarity of the solutions was about 300 mosm·l⁻¹. For determining the effect of a pH gradient on unidirectional Gly-Gln flux rates, the luminal buffer solution was modified as following (in mmol·l⁻¹): NaCl 113.6, KCl 5.4, HCl 0.2, MgCl₂ 1.2, CaCl₂ 1.2, NaHCO₃ 1.0, Na₂HPO₄ 0.1, NaH₂PO₄ 1.4, Na gluconate 21.6, and mannitol 32.9, thus ensuring a luminal pH of 6.0. Each side of the tissue was bathed in 10 ml of buffer solution at 37 °C. The buffers were continuously stirred with a carbogen supply gas-lift system which maintained the pH at 7.4. Indomethacin (10 µmol·l⁻¹; Sigma-Aldrich, Taufkirchen, Germany) was added to all solutions to prevent a pre-stimulation of the cAMP pathway by endogenous production of prostaglandins (Clarke and Argenzio, 1990; Smith et al., 1981). Furthermore all buffer solutions contained amastatine (10 µmol·l⁻¹; Sigma-Aldrich) to prevent hydrolysis of dipeptides (Daniel and Adibi, 1994; Winckler et al., 1999).

Flux rates were determined under short-circuited conditions. After an equilibration period of 30 min, unidirectional flux rates of Gly-Gln were determined using the radioisotope [³H]-Gly-Gln as a tracer. 185kBq of [³H]-Gly-Gln (specific activity 370 GBq/mmol) were added to one side of the mucosa in each chamber and mucosal-to-serosal (J_msw) and serosal-to-mucosal (J_smw) flux rates were calculated from radiotracer appearance on the other side of the mucosa using standard equations (Schultz and Zalusky, 1964). Radioactivity was measured using a liquid scintillator counter with a counting accuracy > 95% (Packard Tricarb liquid scintillation analyzer, Dreieich, Germany). The differences between J_msw and J_smw (each 3 tissue samples per intestinal segment) were defined as the net flux rates (J_net).

To confirm tissue viability, glucose (10 mmol·l⁻¹; Sigma-Aldrich) was added to the mucosal compartment of each chamber to induce an increment of the I_sc based on electrogenic Na⁺-glucose cotransport at the end of each experimental period.

Preparation of BBMV, estimation of protein and determination of the activity of alkaline phosphatase

Jejunal BBMV were prepared from mucosal scrapings by a modification of the Mg²⁺-EGTA precipitation method (Biber et al., 1981; Binder and Murer, 1986; Schröder and Breves, 1996). Each mucosal scraping was obtained from a different horse, and BBMV were prepared from each sample separately (n = 5). Frozen jejunal mucosa (~25 g) was thawed on ice in buffer solution (300 mmol·l⁻¹ mannitol, 12 mmol·l⁻¹ Tris, and 5 mmol·l⁻¹ EGTA, pH 7.1) with 1 g tissue per 2 ml buffer solution used. All subsequent procedures were carried out at 4°C. Enterocytes were separated from the remaining intestinal tissue by a vibration procedure for 10 min using a vibro mixer type E1 (Chemap, Volketswil, Switzerland). The cell suspension was diluted with distilled water (4 ml distilled water per 1 ml buffer solution) and homogenised for 3 x 1 min in a blender (Turboblender D70, Moulinex, Germany). The volume of the homogenate was determined, and aliquots were taken for estimation of protein and
enzyme markers. For precipitation of membranes, MgCl₂ was added to a final concentration of 10 mmol·l⁻¹, and the homogenate was stirred on ice for 15 min. After centrifugation of the suspension (18 min, 3295 x g, 4°C), the pellet was discarded and the supernatant was again centrifugated (40 min, 26890 x g, 4°C). The pellet was resuspended in 35 ml of buffer solution (60 mmol·l⁻¹ mannitol, 5 mmol·l⁻¹ EGTA, pH 7.1) using a Potter/Elvehjem hand-held homogeniser (S30, Braun, Melsungen, Germany). The addition of MgCl₂ and the subsequent two-step centrifugation were repeated and followed by resuspension of the pellet in 35 ml of buffer solution which contained either 100 mol/l mannitol, 100 mmol/l KCl, 10 mmol/l HEPES (pH 7.4) (for glucose uptake studies) or 100 mol/l mannitol, 100 mmol/l KCl, 35 mmol/l HEPES, and 1 mmol/l MgSO₄ (pH 7.8) (for Gly-Gln uptake studies). The suspension was centrifugated (45 min, 34540 x g, 4°C) and the final pellet containing purified brush-border membranes was resuspended in 2 ml of the latter buffer solution by passing it through a 26 gauge needle several times. It was divided into aliquots, snap frozen in liquid nitrogen, and stored at -80 °C until further use.

Protein concentration was determined using a Coomassie blue kit (Bio-Rad, Munich, Germany) with γ-globulin as a standard. Enrichment of BBMV was determined by measuring the activity of alkaline phosphatase spectometrically from the hydrolysis of p-nitrophenyl phosphate.

**Functional integrity of BBMV and uptakes of Gly-Gln**

Functional integrity of equine BBMV was confirmed by Na⁺-coupled glucose uptake into BBMV using the radioisotope [³H]-D-Glucose as radiotracer (PerkinElmer, Rodgau, Germany). Glucose transport into BBMV was measured with and without an inwardly directed Na⁺-gradient as a function of time. A glucose concentration of 0.05 mmol·l⁻¹ was used, and incubation was stopped after 15 s, 25 s, 30 s, 40 s, 1 min, 2 min, 5 min, 10 min, 1 h, 2 h, 3 h, and 4 h.

The uptake of Gly-Gln into BBMV as a function of time was performed with either an inwardly directed H⁺-gradient (pH of the extravesicular medium: pHₑₓ = 5.9; pH of the intravesicular medium: pHᵢᵢ = 7.8) or with an inwardly directed Na⁺-gradient (Na⁺-concentration of the extravesicular medium = 100 mmol·l⁻¹; absence of Na⁺ in the intravesicular medium, n = 5). Both experiments were conducted with a simultaneous determination of Gly-Gln uptake into BBMV under equilibrium conditions (pHₑₓ/ex = 7.8; absence of Na⁺). All time course experiments were performed using a Gly-Gln concentration of 0.025 mmol·l⁻¹, and incubation was stopped after 10 s, 15 s, 20 s, 25 s, 30 s, 1 min, 2 min, 5 min, 10 min, 1 h, 2 h, and 3 h.

The uptake of Gly-Gln into BBMV was determined as a function of extravesicular substrate concentration either with an inwardly directed H⁺-gradient or with an inwardly directed Na⁺-gradient. All concentration dependent uptakes were performed with a simultaneous determination of Gly-Gln uptake under equilibrium conditions (pHₑₓ/ex = 7.8; absence of Na⁺). An incubation time of 10 s was applied, and Gly-Gln concentrations ranging from 0.25 to 10 mmol/l were used (n = 5).
The kinetic parameters $V_{\text{max}}$ (nmol·mg$^{-1}$ protein·10s$^{-1}$) and $K_m$ (mmol·l$^{-1}$) for the Gly-Gln uptake into jejunal BBMV were calculated using the Michaelis-Menten equation (Schröder and Breves, 1996).

Uptakes of glucose and Gly-Gln were quantified by the rapid filtration technique described by Kaune (1992) and Schröder and Breves (1996). A BBMV suspension (20 µl) was incubated in buffer solution (80 µl) containing 37 kBq (1 µCi) of either $[^3\text{H}]$-D-glucose or $[^3\text{H}]$-Gly-Gln and non-labelled glucose (Sigma-Aldrich) or Gly-Gln (Sigma-Aldrich). The buffer solution used for measuring uptake of glucose contained either 100 mmol·l$^{-1}$ NaCl or 100 mmol·l$^{-1}$ KCl along with 100 mmol·l$^{-1}$ mannitol and 10 mmol·l$^{-1}$ HEPES (pH 7.4). The incubation buffer used for uptake of Gly-Gln consisted of (mmol·l$^{-1}$) 100 KCl, 100 mannitol, 1 MgSO$_4$, 15 HEPES, and 50 MOPS (pH 5.9) or 100 KCl, 100 mannitol, 1 MgSO$_4$, and 35 HEPES (pH 7.8) or 100 NaCl, 100 mannitol, 1 MgSO$_4$, and 35 HEPES (pH 7.8, for uptake studies with a Na$^+$-gradient). All buffer solutions used for Gly-Gln uptake studies contained 10 µmol·l$^{-1}$ amastatine to prevent hydrolysis of dipeptides (Daniel and Adibi, 1994; Winckler et al., 1999). The suspension was incubated at 21°C, and incubation was stopped at specific time points by the addition of 1 ml ice-cold stop buffer solution. The stop buffer solution consisted of either (mmol·l$^{-1}$) 150 KCl and 10 HEPES (pH 7.4) (for glucose uptake studies) or 100 mannitol, 100 KCl, 35 HEPES, and 1 MgSO$_4$ (pH 7.8) (for Gly-Gln uptake studies). The solution was immediately filtered by vacuum suction, and filters were washed twice with 4 ml stop buffer solution to remove extravesicular radioactivity. Blanks were obtained from incubation of respective buffer solutions without vesicular membranes. Total radioactivity was counted in 80 µl buffer solution at the end of each experiment. All measurements were performed in triplicate. Filters were dissolved in scintillation fluid (PerkinElmer) for at least 30 min before radioactivity was measured using a liquid scintillator counter with a counting accuracy >95% (Packard Tricarb liquid scintillation analyzer, Dreieich, Germany).

**Statistical analysis**

Statistical analysis was performed using GraphPad Prism Version 5 for Windows (GraphPad Software, San Diego, California, USA). Data are expressed as means SEM, with $n$ = number of animals. Data were analyzed by Student’s $t$ test for paired observations or by 2-way repeated measures ANOVA with Bonferroni’s postest. Significance was set at $P<0.05$. Kinetic values $V_{\text{max}}$ and $K_m$ for Gly-Gln uptake as a function of substrate concentration were calculated by fitting Gly-Gln uptake rates versus respective concentrations in extravesicular buffer solution with a Graph Pad algorithm to a rectangular hyperbola relationship obtained from the law of mass action (Schröder and Breves, 1996).
RESULTS

Electrophysiological tissue properties and transepithelial flux rates of Gly-Gln
A glucose-induced $I_{sc}$ response confirmed the tissue viability at the end of each experiment. No significant changes in $I_{sc}$ and $G_t$ during flux rate measurements verified constant electrical conditions and adequate tissue integrity (data not shown). A concentration-dependent increase in $I_{sc}$ was determined after the addition of dipeptide indicating the existence of a peptide transport system. The $I_{sc}$ response differed between two dipeptides with Gly-Gln inducing a significantly greater $I_{sc}$ increase than Gly-Sar (Fig. 1).

The unidirectional flux rates of Gly-Gln in the absence and in the presence of Gly-Sar are presented in Fig. 2. In all experiments, $J_{ms}$ flux rates were greater than in the opposite direction ($J_{sm}$) resulting in positive $J_{net}$ indicating absorption of Gly-Gln. In the presence of Gly-Sar, $J_{ms}$ and $J_{net}$ flux rates of Gly-Gln were significantly decreased ($P < 0.05$ and $P < 0.01$, respectively; Fig. 2). A decrease in luminal pH did not affect the unidirectional flux rates of Gly-Gln (Fig. 3).

Enrichment and functional integrity of jejunal BBMV
The activity of jejunal brush-border alkaline phosphatase was about 14-fold enriched in equine BBMV compared to the initial tissue homogenate of mucosal scrapings indicating adequate purity of isolated BBMV.

The Na$^+$-dependent glucose uptake as a function of time showed a rapid glucose accumulation within the vesicular compartment above equilibrium values (overshoot phenomenon) within 1 min of incubation confirming functional integrity of BBMV. Glucose uptake in the absence of Na$^+$ increased slowly with time without any transient glucose accumulation within the vesicles, thus indicating a pure diffusion pathway (Fig. 4).

Uptake of Gly-Gln into jejunal BBMV
An overshoot phenomenon of Gly-Gln uptake into jejunal BBMV was demonstrated in both the presence and the absence of an inwardly directed H$^+$-gradient with the transient accumulation of Gly-Gln inside the BBMV being significantly greater in the presence of a H$^+$-gradient compared to equilibrium condition (Fig. 5 A).

An inwardly directed Na$^+$-gradient did not result in an overshoot of Gly-Gln uptake, but uptake increased only slightly with time. In contrast to this, an overshoot could be shown in these experiments in the absence of a Na$^+$-gradient (Fig. 5 B).

For the concentration-dependent Gly-Gln uptake, an incubation time of 10 s was applied because uptake of Gly-Gln was reasonably linear up to 25 s (Fig. 6). Gly-Gln uptake as a function of extravesicular substrate concentration displayed Michaelis Menten type saturation kinetic. For the H$^+$-dependent Gly-Gln uptake, $V_{max}$ was $2.81 \pm 0.18$ and $K_m$ was $1.21 \pm 0.26$. Regarding the Gly-Gln uptake under equilibrium conditions, $V_{max}$ was $2.77 \pm 0.21$ and $K_m$ was $3.14 \pm 0.60$. $V_{max}$ values did not differ significantly, however the $K_m$ values were significantly different ($P < 0.05$, Student’s $t$ test for paired observations).
DISCUSSION

To our knowledge, this is the first study demonstrating the existence of an intestinal peptide transport in the horse using the radioisotope tracer technique and uptake measurements into equine jejunal BBMV.

In the Ussing chamber experiments, the $J_{ms}$ and $J_{net}$ flux rates of Gly-Gln were significantly decreased in the presence of Gly-Sar suggesting that both dipeptides compete for the same transport system. Comparing the $I_{sc}$ response to the luminal addition of both peptides, Gly-Gln induced a significantly greater $I_{sc}$ response than Gly-Sar indicating a greater transport capacity for Gly-Gln. This confirms data of Winckler et al. (1999) with pig jejunal BBMV where similar affinity constants for both dipeptides were found, but with a higher apparent transport capacity for Gly-Gln compared to Gly-Sar.

A pH gradient between the luminal and the mucosal side of the Ussing chambers did not affect the transport of Gly-Gln. The lack of effect of an acidic luminal pH on the peptide transport may be a consequence of an effective protection of the microclimate at the brush border membrane by the mucus layer covering the mucosal surface. The luminal mucin layer causes a stable unstirred layer and may act as an ampholyte for protection of the epithelium from luminal acid (Shiau et al., 1985; Rechkemmer et al., 1986). A stable microclimate at the surface of the jejunal epithelium has been demonstrated in other species including rat, guinea pig, and man (Lucas and Blair, 1978; Lucas et al., 1978; Daniel et al., 1985; Rechkemmer et al., 1986). Rechkemmer (1986) demonstrated that the pH-microclimate at the surface of the epithelium was stable and independent of changes in the luminal bulk phase pH. An acidic luminal pH invokes mucosal defense mechanisms including changes in mucosal blood flow, mucus and $\text{HCO}_3^-$ secretion. The latter one may play a role in vivo (Akiba and Kaunitz, 2011), but it did not affect peptide transport in the present study.

To investigate the peptide transport across the brush border membrane of enterocytes, BBMV were prepared from equine jejunum. The purity of BBMV was in accordance with data from similar studies in rats (Ghishan, 1992), rabbits (Shirazi-Beechey et al., 1988), pigs (Kaune et al., 1992), and horses (Dyer et al., 2002). Functional viability and compartment integrity of the BBMV was demonstrated by an overshoot uptake of glucose into the BBMV supporting data on glucose transport across the equine small intestine published by Dyer et al. (2002).

In the present study an overshoot for Gly-Gln uptake into BBMV was demonstrated. The existence of an overshoot indicates that peptides are not solely transported by passive diffusion, but peptide uptake is mediated by an active transport mechanism. Published data on the ability of inducing an overshoot phenomenon in peptide uptake is controversial. Several studies failed to find a transient accumulation inside the vesicles using rabbit, rat, and human intestinal tissue (Rajendran et al., 1987; Wilson et al., 1989). Adibi (1997) even stated that intestinal BBMV may not be a suitable
model for studying time-dependent uptakes of oligopeptides. Other studies on peptide uptake into bovine, rabbit, rat, and guinea pig BBMV demonstrated an overshoot driven by a H⁺-gradient (Ganapathy and Leibach, 1983; Himukai et al., 1983; Said et al., 1988; Wolffram et al., 1998). Explanations for these conflicting results may include species-specific differences in lipid composition of the brush border membrane, tightness and purity of BBMV, type of substrate and substrate concentration (Wolffram et al. 1998). The results of the present study confirm data published by Wolffram et al. (1998) who postulated that a low substrate concentration (0.015 – 0.025 mmol/l) may be important for demonstrating an overshoot phenomenon in peptide uptake.

In the present study, a transient accumulation of Gly-Gln inside the vesicular compartment was not only documented in the presence of a H⁺-gradient, but also without any H⁺-or Na⁺-gradient being applied. Considering that the intra- and extravesicular buffer solutions in these experiments were of the same composition and the experimental set up was identical, the driving force for this overshoot remains unclear. Extravesicular K⁺ may play a role because the capability of inducing an overshoot was lost when uptake was determined with a K⁺ free incubation buffer. The latter buffer was used to determine the Gly-Gln uptake in the presence of a Na⁺-gradient, and its composition was identical to the other extravesicular buffer solution except that K⁺ was replaced by Na⁺ in order to create a Na⁺-gradient. An inwardly directed Na⁺-gradient did not result in a transient accumulation of substrate inside the vesicles, but the Gly-Gln concentration only increased slowly and to a comparatively low level. This finding shows that intestinal peptide transport in horses is not coupled with Na⁺-transport. It supports data from other species which demonstrate that peptide transport is not mediated by a Na⁺-cotransport mechanism (Webb et al., 1992; Wolffram et al., 1998).

Investigations of the H⁺-dependent Gly-Gln uptake as a function of substrate concentration revealed the existence of a saturable transport process with a Kₘ of 1.21 mmol/l. This finding is comparable to published data on Gly-Gln uptake into a cell line derived from human intestine (Caco-2) and into human intestinal BBMV identifying a saturable peptide transport with a Kₘ of 0.90 mmol/l and 0.64 mmol/l, respectively (Thamotharan et al., 1996; Adibi, 1997). These Kₘ values are in line with a low-affinity, high capacity transporter. This makes sense in a physiological matter considering that the digestion of protein in the intestinal lumen may result in di-/tripeptide concentrations up 100 mmol/l (Ganapathy, 2006). Regarding the concentration-dependent Gly-Gln uptake in the absence of both, a H⁺- and a Na⁺-gradient, a saturable transport process was demonstrated with a Kₘ value differing from that calculated for the H⁺-dependent Gly-Gln uptake. These findings indicate the existence of two different peptide transport mechanisms in the horse and support data that suggest that at least two classes of peptide transporters occur, one acidic pH-preferring class and one neutral pH-preferring class (Rubino et al., 1971; Ganapathy and Leibach, 1985; Inui et al., 1988; Kato et al., 1989).
Once peptides are transported across the apical membrane, they are either transported into portal circulation as intact peptides by a basolateral peptide transporter or they are hydrolyzed to their constituent AA within the cell (Terada et al., 1999; Irie et al., 2004). Free AA may be transported into the blood stream contributing to the body’s AA pool or they may be used by intestinal mucosal cells for energy metabolism, protein synthesis, nucleosides, polyamides, and maintenance of the intestinal immune system (Gilbert et al., 2008). In other species, AA, particularly glutamin, have been documented to be the preferred energy source in the intestine (Reed et al., 2000). In the horse, glutamin has been demonstrated to facilitate mucosal restitution in the large intestine (Rötting et al., 2004). Transporting Gly-Gln across the apical membrane of the enterocytes, as demonstrated in the present study, may serve as a mechanism to supply the mucosal cells with glutamin. Since ruminal bacteria are capable of peptide uptake which is suggested to be mainly an energy-yielding rather than N-supplying mechanism (Broderick, 1988; Armstead and Ling, 1993; Wallace, 1996), peptide uptake in the mammalian intestine may have a similar purpose.

Demonstrating the capability of the horse to absorb peptides in the small intestine contributes to a better understanding of equine nutrition. So far, the focus of research has been either on sole protein content or a limited number of amino acids in the horse (Graham et al., 1993; Saastomoinen and Kostinen, 1993; Hintz and Cymbaluk, 1994). In some other species the transport of AA in the form of peptides (hydrolyzed soy or milk protein) was demonstrated to be a faster route of uptake per unit of time than their constituent AA in the free form or the intact protein with the same respective AA composition (Adibi and Philips, 1968; Rerat et al., 1988). Knowing that an intestinal peptide transport mechanism exists in the horse will therefore provide a new aspect to equine nutrition, particularly in performance horses, in which dietary formulations are used to improve performance. However before concrete application possibilities can be provided, further research needs to elucidate more details of the underlying mechanisms of peptide absorption including the factors modifying absorption kinetics as well as its potential nutritional significance. In vivo studies may complement the in vitro findings taking into account that many influencing factors of the in vivo situation still need to be considered.

In summary, an intestinal peptide transport was firstly documented in the horse. The uptake of Gly-Gln into equine BBMV was established as an appropriate experimental set up for investigating equine peptide transport. Besides demonstrating the existence of a H+-mediated dipeptide transport, a second peptide transport mechanism driven by a mechanism other than a H+- or a Na+-gradient was found.

**LITERATURE CITED**


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Figure 1. Short circuit currents (I_{sc}) response to the addition of dipeptides. The I_{sc} response to the addition of glycyl-glutamine (Gly-Gln) was significantly higher compared to the addition of glycyl-sarcosine (Gly-Sar). ΔI_{sc} is defined as the maximum I_{sc} response after the addition of each substance minus baseline I_{sc} prior to the addition of dipeptides. Data are expressed as mean ± SEM, 2-way repeated measures ANOVA (Dipeptide P < 0.0001, Concentration P = 0.0024, Interaction P = 0.007), n = 5.
Figure 2. Mucosal-to-serosal ($J_{ms}$), serosal-to-mucosal ($J_{sm}$) and net flux rates ($J_{net} = J_{ms} - J_{sm}$) of glycyl-glutamine (Gly-Gln) across stripped jejunal tissues in Ussing chambers in the absence (-) and presence (+) of glycyl-sarcosine (Gly-Sar). Data are expressed as mean ± SEM, n = 5, Student's t-test, n.s. = not significant.
Figure 3. Effect of mucosal acidic pH on mucosal-to-serosal ($J_{ms}$), serosal-to-mucosal ($J_{sm}$) and net flux rates ($J_{net} = J_{ms} - J_{sm}$) of glycyl-glutamine (Gly-Gln) across stripped jejunal tissues in Ussing chambers. Data are expressed as mean ± SEM, $n$ = 5, Student's t-test, n.s. = not significant.

![Graph showing flux rates of Gly-Gln at different pH levels](image-url)
Figure 4. Typical time course of Na\(^+\)-dependent and Na\(^+\)-independent glucose uptake into equine jejunal BBMV. An overshoot phenomenon was observed in the presence of an inwardly directed Na\(^+\)-gradient confirming functional integrity of the BBMV. Each point represents the average of triplicate measurements.
Figure 5. H⁺-dependent (A) and Na⁺-dependent (B) glycyl-glutamine (Gly-Gln) uptake into equine jejunal BBMV as a function of time. An overshoot phenomenon was observed in the presence of an inwardly directed H⁺-gradient (A). To a lesser extent, a transient accumulation inside the BBMV also occurred in the absence of a H⁺-gradient (2-way repeated measures ANOVA, Buffer P < 0.0001, Time P < 0.0001, Interaction P < 0.0001). An inwardly directed Na⁺-gradient did not result in an intravesicularly accumulation (B) contrasting an overshoot phenomenon in the absence of a Na⁺-gradient (2-way repeated measures ANOVA, Buffer P < 0.0001, Time P < 0.0001, Interaction P < 0.0001). Data are expressed as mean ± SEM, n = 5.
Figure 6. Initial uptake of glycyl-glutamine (Gly-Gln) into equine jejunal BBMV. The uptake was reasonably linear up to 25 s. Data are expressed as mean ± SEM, n = 5.
5 DISCUSSION AND CONCLUSIONS

5.1 Animals and sampling procedure

Horses used in the study were adult, healthy horses of varying breeds and age kept at maintenance level. The horses used for investigating Ca\textsuperscript{2+} and Pi transport were rather old (mean age of 19 years). Considering that requirements of Ca\textsuperscript{2+} and Pi depend on age, physiologic status and physical activity (Toribio, 2011), this may have contributed to a respective low intestinal transport in these horses. However, the impact of senior age on the results should not be overestimated considering that Schryver et al. (1974) did not identify any differences in efficiency of Ca\textsuperscript{2+} and Pi absorption between young mature and old horses fed adequate amounts of Ca\textsuperscript{2+} and Pi in the diet.

Tissue samples used for Ussing chamber experiments and uptake studies into BBMV were either harvested within 5 min post mortem from horses euthanized for reasons unrelated to this project or obtained during ventral laparotomy under general anesthesia. Intestinal tissues isolated from slaughtered animals could not be used for any of the studies due to an inevitable time delay in sample taking and subsequent negative impact on tissue viability. Similar observations were made by Dyer et al. (2002) who pointed out that there is a risk of epithelium shedding and proteolysis in equine small intestine obtained from animals slaughtered by conventional methods at the local abattoir.

5.2 Enrichment and functional integrity of BBMV

BBMV were successfully prepared from mucosal scrapings by a modification of the Mg\textsuperscript{2+}-EGTA precipitation method (Biber et al. 1981; Binder et al. 1986; Schröder et al. 1996). In order to confirm that prepared BBMV originated from the brush border membrane, the specific activity of the brush-border marker enzyme alkaline phosphatase (AP) was determined. A 14-fold enrichment in AP activity in BBMV compared to the initial tissue homogenate of mucosal scrapings confirmed adequate purity of isolated BBMV. Similar results for BBMV enrichment are reported in horses and other species (Shirazi-Beechey et al. 1991; Kaune et al. 1992; Dyer et al. 2002; Merediz et al. 2004; Zurich 2009).

In order to exclude contamination with basolateral membranes, an enrichment of the Na\textsuperscript{+}/K\textsuperscript{+}-ATPase activity, a marker enzyme for the basolateral membrane, may additionally be determined (Biber et al. 1981; Binder et al. 1986; Schröder et al. 1996). Enrichment factors up to a maximum of 4 are reported for Na\textsuperscript{+}/K\textsuperscript{+}-ATPase activity in BBMV preparation of satisfactory quality in other species (Shirazi-Beechey et al. 1989; Schröder et al. 1996; Schröder et al. 2003; Zurich 2009). In the present study, enrichment factors for the Na\textsuperscript{+}/K\textsuperscript{+}-ATPase were about 1.5 in a few horses, however, in most samples, activity of Na\textsuperscript{+}/K\textsuperscript{+}-ATPase could not be properly detected at all. Similar findings are reported by other authors indicating that a determination of
NA⁺/K⁺-ATPase activity in equine BBMV does not provide an additional value in respect to BBMV purity (Dyer et al. 2002; Merediz et al. 2004; Shirazi-Beechey, personal communication, 2009). The functional integrity of the BBMV was confirmed by Na⁺-dependent glucose uptake into the vesicles. Glucose uptakes showed a characteristic overshoot phenomenon (rapid glucose accumulation within the vesicular compartment above equilibrium values) verifying that the vesicles were intact and capable of substrate uptake. Time courses of Na⁺-dependent glucose uptakes were similar to the ones published by Dyer et al. (2002) who demonstrated a Na⁺-dependent glucose uptake into equine BBMV in line with cloning and sequencing the cDNA encoding equine SGLT1.

5.3 Intestinal electrolyte transport

5.3.1 Cl⁻ transport across the jejunum

5.3.1.1 Stimulation of electrogenic Cl⁻ secretion
Electrogenic Cl⁻ secretion has been studied in several species using the Ussing chamber technique (Bijman et al. 1991; Schultheiss et al. 1998; Schröder et al. 2004). In conformity with published data, tissues were preincubated with amiloride, TEA and Ba²⁺ in order to block apical Na⁺ and K⁺ channels, and thus eliminate movement of quantitatively important ions except for Cl⁻ ions (Benos 1982; Latorre et al. 1983). Under these circumstances $I_{sc}$ corresponds directly to electrogenic Cl⁻ secretion (Holtug et al. 1991; Bronsveld et al. 2000). For stimulation of Cl⁻ secretion, carbachol was used to stimulate Ca²⁺-dependent Cl⁻ secretion, and forskolin was administered to activate cAMP-dependent Cl⁻ secretion (Brown et al. 1990; Barrett et al. 2000). In conformity with published data, carbachol induced a rapid increase in $I_{sc}$ followed by a decline back to baseline, and forskolin addition resulted in a rapid increase in $I_{sc}$ slightly declining to a plateau above baseline (Isaacs et al. 1976; Leonhard-Marek et al. 1997). In line with the $I_{sc}$ increase, transepithelial tissue conductance ($G_t$) was elevated in response to forskolin addition. This finding is consistent with data in pig and mice, and it can be explained by the increased apical Cl⁻ conductance (Bleich et al., 2007; Leonhard-Marek et al. 2009).

Carbachol is a an acetylcholine analogue that induces a Ca²⁺ release from intracellular stores via muscarinergic M3 receptors resulting in K⁺ channel opening with subsequent cellular hyperpolarisation causing an electrical gradient favourable for Cl⁻ secretion (Barrett et al. 2000). Furthermore, carbachol increases the protein kinase C levels resulting in a direct activation of CFTR (Bronsveld et al. 2000). Forskolin activates the adenylate cyclase, increases cAMP, and thus activates CFTR and ORCC (Seamon et al. 1981; Brown et al. 1990).

5.3.1.2 Cl⁻ channel inhibitors
In order to discriminate between the different Cl⁻ channels, it is crucial to identify Cl⁻ channel inhibitors which effectively block Cl⁻ channels in equine tissue. So far, there are no published data on Cl⁻ secretion in horses that allow a differentiation between
the Cl⁻ channels being involved. In the present study, the effect of different Cl⁻ channel inhibitors on stimulated Cl⁻ secretion across the jejunum is firstly published in horse. Several substances which have well been established as Cl⁻ channel inhibitors in other species were analysed (Schultz et al. 1999; Walsh et al. 1999; Barrett et al. 2000; Liu et al. 2007). Irrespective of the dose, none of the Cl⁻ channel inhibitors changed the I_{sc} responses to carbachol and forskolin in the present study.

DIDS does not interact with CFTR, but with nearly all other types of Cl⁻ channels including Ca²⁺-activated Cl⁻ channels (Cabantchik et al. 1992; Schultz et al. 1999). The lack of I_{sc} response to the addition of DIDS after incubation with carbachol is conform with data published on Cl⁻ secretion in porcine jejunum indicating that directly Ca²⁺-activated Cl⁻ conductance may not exist in the jejunum (Leonhard-Marek et al. 2009). In fact, the potential contribution of Ca²⁺-activated Cl⁻ channels to Cl⁻ secretion across the intestine is controversially discussed (Clarke et al. 1994). Gruber et al. (1998) and Bronsveld et al. (2000) suggest that Ca²⁺-activated Cl⁻ channels exist in the intestine in humans and mice. Leonhard-Marek et al. (2009) however demonstrated that I_{sc} response induced by carbachol is caused by a K⁺ outflow and subsequent augmented electrical driving force, but not by directly Ca²⁺-activated Cl⁻ channels in porcine jejunum. Insensitivity to DIDS may also have been caused by a more dominant Cl⁻ secretion from other Cl⁻ channels such as CFTR which could have masked the smaller Cl⁻ current of Ca²⁺-activated and other alternative Cl⁻ channels.

In order to identify a potent CFTR blocker for equine intestine, the efficiency of different CFTR inhibitors to inhibit forskolin-stimulated Cl⁻ secretion was analysed. The highly specific CFTR blockers CFTRinh-172 and the GlyH-101 and the less specific anion channel blockers NPPB and glibenclamide were used. All of them have extensively been studied in detail in other species (Schultz et al. 1999), and none of them changed the I_{sc} responses to forskolin in the present study.

CFTRinh-172 and the GlyH-101 have found to be highly specific for CFTR in rats and mice (Ma et al. 2002; Muanprasat et al. 2004; Thiagarajah et al. 2004). In contrast, CFTRinh-172 was less effective in inhibiting Cl⁻ secretion across respiratory epithelia in pig and ferret, and it did not show any inhibiting effects on CFTR Cl⁻ conductance in porcine intestine (Liu et al. 2007; Leonhard-Marek et al. 2009). GlyH-101 is effective in humans, pig, and ferret. Regarding its potency in mice, differing efficiency is documented depending on the type of tissue investigated (Liu et al. 2007; Muanprasat et al. 2004).

Glibenclamide effectively inhibited forskolin-induced Cl⁻ currents in respiratory epithelia of mice, humans, and ferret, but not in porcine tissue (Liu et al. 2007). Besides its ability to interact with ATP-sensitive K⁺ channels, which is used for treatment of non-insulin dependent diabetes mellitus, glibenclamide directly blocks CFTR channels from the intracellular side by interacting with multiple binding sites of the channel (Sheppard and Welsh 1992). Furthermore, it blocks Ca²⁺-activated channels and ORCCs (Rabe et al. 1995).

The effect of NPPB varies in respect to species, type of tissue (respiratory and intestinal epithelia), intestinal site, and administration to either mucosal or serosal
side. NPPB has proven to be almost equally effective in respiratory epithelia of mice, man, ferret, and pig (McCarty 2000; Bleich et al. 2007; Liu et al. 2007; Sommer et al. 2007; Leonhard-Marek et al. 2009). It is effective in blocking basal chloride currents as well as a forskolin or carbachol stimulated intestinal Cl\(^-\) secretion in rat, and it significantly blocks forskolin induced \(I_{sc}\) response in porcine and murine intestine (Diener et al. 1989; Bleich et al. 2007; Leonhard-Marek et al. 2009). It inhibits CFTR channels by occluding the pore at an intracellular site, but it also blocks other Cl\(^-\) channels such as ORCC and Ca\(^{2+}\)-activated Cl\(^-\) channels as well as basolateral K\(^+\) channels (Schultz et al. 1999; Walsh et al. 1999). In the present study, the effect of NPPB on stimulated Cl\(^-\) secretion was investigated in the presence of DIDS ruling out a direct effect on ORCC and Ca\(^{2+}\)-activated channels (Bleich et al. 2007; Leonhard-Marek et al. 2009). NPPB may therefore interact directly with CFTR or may indirectly effect CFTR Cl\(^-\) secretion via a decrease in basolateral K\(^+\) efflux (Schultz et al. 1999). The lack of \(I_{sc}\) response to NPPB across equine jejunum may have been due to a dose of NPPB too low to block enough CFTR to be reflected in a change in \(I_{sc}\). However, the NPPB doses applied in the present study were similar to the ones described by other authors using comparable experimental settings (Greger et al. 1991; Bleich et al. 2007; Toth et al. 2008; Leonhard-Marek et al. 2009). The nonspecificity of NPPB complicates interpretation of the lacking NPPB effect on \(I_{sc}\) (Schultz et al. 1999). NPPB does not only influence Cl\(^-\) secretion, but has an impact on various intra- and transcellular mechanisms including modulation of intracellular Ca\(^{2+}\) concentration, blockage of Na\(^+\) and Cl\(^-\) absorption, inhibition of glucose transport and activation of ATP-sensitive K\(^+\) channels (Diener et al. 1989; Keeling et al. 1991; Kirkup et al. 1996). The lack of \(I_{sc}\) response in the present study is consistent with published data by Diener et al. (1989) who demonstrated that serosal addition of NPPB had no effect on \(I_{sc}\) across rat colon. They showed that mucosal administration of NPPB inhibited forskolin-induced serosal-to-mucosal flux of radiolabelled Cl\(^-\) completely in line with a decrease in \(I_{sc}\) of only 60%. An administration of NPPB to the serosal side of the mucosa was chosen in the present study, because previous studies demonstrated that NPPB enters the enterocytes across the apical membrane only with difficulties possibly due to the mucus covering the luminal side. This may even be complicated by enhanced mucus production caused by carbachol addition (Greger et al. 1991; Lindqvist et al. 1998; Asfaha et al. 2001; Joo et al. 2002).

The lack of \(I_{sc}\) response to the Cl\(^-\) channel inhibitors strongly supports findings of other authors suggesting the existence of substantial species-specific differences at the level of epithelial electrolyte transport, particularly in terms of CFTR structure and function (Liu et al., 2007; Leonhard-Marek 2009).

### 5.3.2 Ca\(^{2+}\) and P\(_{i}\) transport along the intestinal axis

Ca\(^{2+}\) net flux rates were significantly different among different intestinal sites and highest flux rates were determined across the duodenum. This clearly indicates that the duodenum has the greatest absorptive potential for Ca\(^{2+}\) in horses. These findings support data of Schryver et al. (1970) who firstly described Ca\(^{2+}\) and P\(_{i}\)
transport across the horse, and it is consistent with published data on other monogastric animals (Kaune et al. 1992; Schröder et al. 1993; Hoenderop et al. 2005). In regard to the major effective site of Ca\(^{2+}\) absorption in vivo, however, the comparatively long gut length of the jejunum should be taken into account because that compensates for the 10-fold higher Ca\(^{2+}\) net flux rates across the duodenum. In contrast to findings of Schryver et al. (1970) who postulated a Ca\(^{2+}\) secretion in the cecum, positive Ca\(^{2+}\) net flux rates were determined across the cecum. Factors influencing the in vivo situation, but are eliminated in the in vitro situation, may have accounted to these contrasting results. The Ussing chamber technique allows determination of transport at mucosal level without influencing factors such as total absorptive surface, transit time of ingesta, and gut length. Other factors, which might affect Ca\(^{2+}\) transport, include paracellular movement and solvent drag (Karbach et al. 1992).

Verapamil significantly inhibited Ca\(^{2+}\) net flux rates across the jejunum. TRPV6 whose existence has been documented in the present study and in the study of Rourke et al. (2010) is not directly inhibited by verapamil. Thus, the TRPV6 may have indirectly been inhibited by verapamil-induced changes in K\(^{+}\) conductance and subsequent membrane depolarization (Leonhard-Marek et al. 1996) or another verapamil-sensitive Ca\(^{2+}\) channel may exist in the jejunum of horses. Active Ca\(^{2+}\) transport across the intestine was demonstrated in TRPV6/calbindin-D\(_{9k}\) double-knockout mice, and the verapamil-sensitive L-type channel Ca\(^{2+}\) channel Cav1,3 has been documented in the jejunum of rat (Morgan et al. 2003; Benn et al. 2008).

Regarding the transport of P\(_i\), results of the present study indicate significant active P\(_i\) secretion across the jejunum. This finding partly supports data published by Schryver et al. (1972) who postulated P\(_i\) secretion across the proximal small intestine and across the cecum. Along the intestinal axis, the relatively small differences in unidirectional flux rates resulted in low net flux rates of P\(_i\), and thus no significant differences between intestinal segments could be detected. These findings are inconsistent with data of Schryver et al. (1972) who identified distinct differences in P\(_i\) transport along the intestinal axis suggesting that the dorsal colon may be the major site of P\(_i\) absorption.

Ouabain significantly reduced serosal-mucosal flux rates of P\(_i\) across the jejunum. Ouabain, an inhibitor of the Na\(^+\)K\(^+\)ATPase, is well established for testing for a Na\(^+\)-dependent P\(_i\) transport in the intestine (Schröder et al. 1995). Na\(^+\)-dependent P\(_i\) absorption relies on a mucosal-to-serosal Na\(^+\) gradient induced by the Na\(^+\)K\(^+\)ATPase allowing the transport of P\(_i\) from the luminal to the blood side. In the present study, ouabain affected P\(_i\) secretion which may be explained by other effects of ouabain. At low dose levels, ouabain alters potassium conductance and subsequently decreases the transmembrane potential which may have influenced the serosal-to-mucosal flux rates of P\(_i\) (Miura et al. 1978; Matsumoto et al. 2008).

In other species, a mechanism of P\(_i\) secretion can be found in the salivary glands (Vayro et al. 1991; Homann et al. 2005; Huber et al. 2007). Particularly in the salivary glands of ruminants, high amounts P\(_i\) are secreted into the parotid primary saliva despite a low concentration of P\(_i\) in the blood, thereby indicating the need of an
energy-requiring transport mechanism (Vayro et al. 1991). Although the NaPiIIb has
been identified in the salivary glands, the mechanisms which facilitate and control the
transport of $\text{P}_i$ from the blood, through the acinar cells into the primary saliva have
not been fully understood so far (Virkki et al. 2007).
Secretion of $\text{P}_i$ into the proximal intestine may be an important feature in equine
intestinal physiology. The horse is a hindgut fermenter, requiring appropriate buffer
capacities in order to maintain luminal pH and microbial homeostasis in the large
intestine. $\text{P}_i$ secreted across the jejunum may neutralize acids produced during the
fermentation process as documented in the rumen of cattle and sheep (Counotte et
al., 1979; Kohn et al., 1998).

5.4 Nutrient transport

The addition of glucose, L-alanine and glycyl-L-glutamine induced a significant
increase in $I_{sc}$ in the horse which was comparable to $I_{sc}$ responses to nutrients
described in other species, and thus suggests the existence of equivalent transport
mechanisms (Winckler et al. 1999; Breves et al. 2001; Toth et al. 2008). The
electrophysiological response to glucose substantiated published data on Na$^+$-
dependent glucose transport in horses (Dyer et al. 2002).
Contrasting the absent effect of an acidic luminal pH on amino acid and dipeptide
transport in rat jejunum (Toth et al. 2008), the $I_{sc}$ response to glucose, L-alanine and
glycyl-L-glutamine was significantly reduced at a luminal pH of 5.4 compared to a pH
of 7.4. Since unidirectional flux rates of glycyl-L-glutamine were not affected by a
decrease in luminal pH, the decrease in $I_{sc}$ response was not caused by reduced
transport of glycyl-L-glutamine, but it may have been a consequence of mucosal
defence mechanisms in response to the acidic luminal challenge (Akiba et al. 2011).
Rechkemmer et al. (1986) demonstrated that the pH-microclimate at the surface of
the epithelium was stable and independent of changes in the luminal bulk phase pH.
Such a stable microclimate at the surface of the jejunal epithelium has been
demonstrated in other species including rat, guinea pig, and man (Lucas and Blair,
1978; Lucas et al., 1978; Daniel et al., 1985; Rechkemmer et al., 1986).

An intestinal dipeptide transport was firstly documented in the horse. Transport of
glycyl-L-glutamine was electrogenic, saturable, stimulated by a H$^+$ gradient, and not
affected by an acidic luminal pH as long as transport occurred across the intact
mucosa being protected by a luminal mucin layer. The Michaelis-Menten constant
($K_m$) of the H$^+$-dependent glycyl-L-glutamine uptake (1.21 mmol/l) was comparable to
published $K_m$ values of glycyl-L-glutamine uptake into a cell line derived from human
intestine (Caco-2) and into human intestinal BBMV suggesting the existence of a
PepT1 in the horse (Thamotharan et al., 1996; Adibi, 1997).
The electrophysiological response to the luminal addition of dipeptides was
significantly different between glycyl-L-glutamine and glycyl-sarcosine. In conformity
with data published by Winckler et al. (1999) glycyl-L-glutamine induced a greater $I_{sc}$
response than glycyl-sarcosine. Winckler et al. (1999) performed his experiments in
the absence of amastatine, a well established peptidase inhibitor (Daniel et al. 1994), and he suggested that the higher maximal current response in case of glycyl-L-glutamine may be partly accounted to a release of glycine and glutamine and their mucosal uptake by electrogenic amino acid transport. He supported his thesis by demonstrating that amastatine causes a 20 % reduction in the maximal $I_{sc}$ response to glycyl-L-glutamine, but did not affect the $I_{sc}$ response to glycyl-sarcosine. The contribution of amino acid transport derived by glycyl-L-glutamine hydrolysis caused by brush border membrane peptidases is controversially discussed. In contrast Minami et al. (1992) documented that glycyl-L-glutamine is predominantly absorbed as an entire peptide in human intestine, and jejunal glycyl-L-glutamine transport in mice did not show any significant difference in $I_{sc}$ response to luminal glycyl-L-glutamine addition in the absence and presence of amastatine (Toth et al. 2008). Species-specific differences may account for these contrasting results. So far, there are no data on the effect of amastatine on peptide transport in the horse. Therefore, amastatine was used in all experiments excluding an interfering effect of amino acid transport with the dipeptide transport in the first place. Consequently, the higher current response to glycyl-L-glutamine addition compared to the response to glycyl-sarcosine addition indicates a greater transport capacity of the peptide transporter for glycyl-L-glutamine.

Glycyl-L-glutamine uptake studies into BBMV demonstrated an overshoot phenomenon. Published data on the ability of inducing an overshoot phenomenon in peptide uptake are controversial. In consistency with the findings in the horse, there are published data on peptide uptake into bovine, rabbit, rat, and guinea pig BBMV demonstrating an overshoot driven by a $H^+$-gradient (Ganapathy et al. 1983; Himukai et al. 1983; Said et al. 1988; Wolffram et al. 1998). Other studies failed to find a transient accumulation of substrate within the vesicles (Rajendran et al. 1987; Wilson et al. 1989). Adibi (1997) even suggested that intestinal BBMV may not be a suitable model for studying time-dependent uptakes of oligopeptides. Species-specific differences in lipid composition of the brush border membrane, tightness and purity of BBMV, type of substrate and particularly substrate concentration may all account for the ability of inducing an overshoot phenomenon (Wolffram et al. 1998).

Besides demonstrating a transient accumulation of glycyl-L-glutamine inside the vesicular compartment in the presence of a $H^+$-gradient, an overshoot was also documented in the absence of $H^+$ gradient. In this case, intra- and extravesicular buffer solutions were of the same composition and the experimental set up was identical to the set up used to investigate the $H^+$-dependent glycyl-L-glutamine uptake. Thus, the driving force for this overshoot remains unclear. Possibly extravesicular $K^+$ may play a role because in all experiments in which an overshoot was documented, the extravesicular buffer solutions contained $K^+$. In experiments, in which no overshoot was found, $K^+$-free extravesicular buffer solutions were used, since $K^+$ was replaced for $Na^+$ in order to test for the effect of a $Na^+$ gradient on glycyl-L-glutamine uptake.

The concentration-dependent glycyl-L-glutamine uptake in the absence of a $H^+$ gradient was a saturable transport process with a $K_m$ value differing from that
calculated for the H⁺-dependent glycyl-L-glutamine uptake indicating the existence of two different peptide transport mechanisms in the horse, and thus supporting the classification of peptide transporters into one acidic pH-preferring class and one neutral pH-preferring class (Rubino et al. 1971; Ganapathy et al. 1985; Inui et al. 1988).

5.5 Conclusions and outlook

Summarizing the results of this thesis, in vitro techniques such as the Ussing chamber technique, radioisotope tracer technique and uptake studies into BBMV have been successfully used to investigate electrolyte and nutrient transport across the intestine in horses.

For the first time intestinal dipeptide transport was described in the horse. Besides demonstrating an overshoot in glycyl-L-glutamine uptake studies, the existence of a H⁺-mediated dipeptide transport was confirmed and a second dipeptide transport mechanism driven by a mechanism other than a H⁺- or a Na⁺-gradient was found. Regarding Ca²⁺ transport, results indicate that the duodenum is the focus of interest in respect to active Ca²⁺ transport for in vitro investigations in the horse in future; however, the jejunum should also be considered important in respect to clinically relevant Ca²⁺ uptake in the in vivo situation. In respect to TRPV6, the use of more specific inhibitors of intestinal Ca²⁺ transport, such as ruthenium red, as well as structural investigations of regulatory proteins such as Calmodulin, Calbindin, Klotho, BSPRY, FGF23 may provide further details on intestinal Ca²⁺ transport in the horse. Furthermore, findings of this thesis point to alternative Ca²⁺ channels (e.g. Caᵥ1.3) in the horse.

The capability of the equine jejunum to actively secrete Pᵢ into the intestinal lumen was documented. Results suggest that Na⁺ is involved in the transport. However, the exact mechanism of Pᵢ transport from the blood to the luminal side is not fully elucidated so far.

Regarding the Cl⁻ transport, the sole determination of electrophysiological parameters of stimulated Cl⁻ transport across the jejunum in the presence of commonly used Cl⁻ channel inhibitors has shown not to be satisfactory. Thus, there is a need to expand the investigations to transepithelial flux measurements of radiolabelled Cl⁻ and to other intestinal sites in order to identify underlying mechanisms of Cl⁻ secretion functionally at epithelial level in horses.
6 SUMMARY

Anja Cehak

Investigation regarding the epithelial electrolyte and nutrient transport across the intestinal mucosa in the horse

Transepithelial transport mechanisms play a key role in regulating the absorption and secretion of electrolytes and nutrients in the gastrointestinal tract. In the horse, intestinal disorders are the most common pathologic conditions, often associated with imbalances in macromineral homeostasis. Published data on equine intestinal transport are limited, and the existence and the relevance of most underlying transport mechanisms are still unknown.

The aim of the present study was to characterize the transport mechanisms of electrolytes and nutrients across the intestine in healthy horses using the Ussing chamber technique, the radioisotopes tracer technique, and uptake studies into BBMV. Transport of glucose, alanine and glycyl-L-glutamine across the jejunum was analysed by determining electrophysiological parameters in response to nutrient addition in Ussing chambers. The effect of different luminal pH values (7.4 and 5.4) on nutrient transport was determined. Cl⁻ secretion was analysed by determining the effect of different stimulants and inhibitors on electrogenic Cl⁻ secretion. Transepithelial transport of Ca²⁺ and Pi transport along the intestinal axis was investigated by calculating unidirectional flux rates of Ca²⁺ and Pi at different intestinal segments. The effect of verapamil on jejunal Ca²⁺ transport and the influence of ouabain on jejunal Pi transport were documented. The existence and characteristics of jejunal dipeptide transport were investigated by determining unidirectional flux rates of glycyl-L-glutamine in Ussing chambers and by uptake studies into BBMV.

For Ussing chamber experiments the stripped mucosa was mounted in Ussing chambers and Iₛ𝑐 and Gᵢ were continuously monitored. The addition of glucose, L-alanine and glycyl-L-glutamine induced an increase in Iₛ𝑐 that was significantly greater at pH 7.4 than at pH 5.4. For blocking apical Na⁺ and K⁺ channels, tissues were preincubated with amiloride, TEA and barium. The latter induced a significant increase in Iₛ𝑐 indicating that K⁺ currents contribute to basal Iₛ𝑐 in equine small intestine. The subsequent addition of carbachol and forskolin induced an Iₛ𝑐 increase representing stimulated Cl⁻ secretion. For differentiation of different Cl⁻ channels, the effect of DIDS, NPPB, glibenclamide, CFTRinh-172, and GlyH-101 on stimulated Cl⁻ secretion was analysed, and their dose-response effect was investigated. None of the Cl⁻ channel inhibitors changed Iₛ𝑐 significantly, irrespective of the dose applied.

Positive net flux rates of Ca²⁺ were determined in all intestinal segments indicating absorptive capacity for Ca²⁺ along the entire intestinal axis. Ca²⁺ transport was significantly different between intestinal segments with the duodenum showing the highest Ca²⁺ net flux rates. Duodenal Ca²⁺ net flux rates were 10-fold higher than
jejunal Ca\textsuperscript{2+} net flux rates, and flux rates were lowest in the dorsal colon. Jejunal Ca\textsuperscript{2+} transport was significantly inhibited by verapamil.

Regarding the P\textsubscript{i} transport, serosal-to-mucosal flux rates of P\textsubscript{i} across the jejunum were significantly higher than the mucosal-serosal flux rates indicating that the equine jejunum is capable of P\textsubscript{i} secretion. Along the intestinal axis, net flux rates of P\textsubscript{i} were low as a result of relatively small differences in unidirectional flux rates, and thus, no significant differences between intestinal segments were detected. Ouabain significantly inhibited jejunal P\textsubscript{i} transport.

Regarding the peptide transport, the I\textsubscript{sc} response to luminal addition of glycyl-L-glutamine was significantly greater compared to the I\textsubscript{sc} response to glycyl-sarcosine addition. Positive net flux rates of glycyl-L-glutamine were determined across the jejunum indicating absorption of the dipeptide. The addition of glycyl-sarcosine reduced the flux rates significantly suggesting that both dipeptides compete for the same transport system. The flux rates were not affected by changes in luminal pH.

For analysing dipeptide transport in more detail, BBMV were prepared from jejunal samples using a modified Mg\textsuperscript{2+}-EGTA-precipitation-method, and uptakes of glucose and glycyl-L-glutamine into BBMV were determined by rapid filtration technique. Functional integrity of the vesicles was confirmed by a Na\textsuperscript{+}-dependent glucose uptake into the vesicles. Uptakes of glycyl-L-glutamine into BBMV were performed in the presence and absence of an inwardly directed H\textsuperscript{+} or Na\textsuperscript{+} gradient. An overshoot phenomenon was demonstrated in both the absence and the presence of an inwardly directed H\textsuperscript{+}-gradient, with the H\textsuperscript{+}-mediated transport being significantly greater than the transport under equilibrium conditions. A Na\textsuperscript{+}-gradient did not cause an overshoot phenomenon. Glycyl-L-glutamine uptakes displayed Michaelis-Menten type saturation kinetic with a K\textsubscript{m} value for the H\textsuperscript{+}-dependent glycyl-L-glutamine uptake being suggestive of a PepT1. The K\textsubscript{m} value for the glycyl-L-glutamine uptake under equilibrium conditions was significantly different from the K\textsubscript{m} obtained for the H\textsuperscript{+}-dependent uptake indicating the existence of two different peptide transport mechanisms in the horse: one active H\textsuperscript{+}-dependent cotransport and one driven by a mechanism other than a H\textsuperscript{+}- or a Na\textsuperscript{+}-gradient.

In conclusion, the thesis demonstrates for the first time that peptides are transported across the equine small intestine. The duodenum was found to be most important for Ca\textsuperscript{2+} absorption, and a distinct feature of equine intestinal physiology, the capability of P\textsubscript{i} secretion in the jejunum was documented. In respect to the absent effects of Cl\textsuperscript{-} channel inhibitors on Cl\textsuperscript{-} transport, in vitro investigations on intestinal Cl\textsuperscript{-} secretion remain a challenge in horses.
7 ZUSAMMENFASSUNG (GERMAN)
Anja Cehak

Untersuchungen zum epithelialen Elektrolyt- und Nährstofftransport der intestinalen Mukosa des Pferdes


Die jejunalen Pi-Fluxraten von serosal nach mukosal waren signifikant höher als die Pi-Fluxraten von mukosal nach serosal, ein Hinweis darauf, dass Pi im Jejunum des Pferdes sezerniert wird. Entlang der Darmachse waren die Pi-Nettofluxraten in Folge der nur geringfügig unterschiedlichen unidirektionalen Pi-Fluxraten sehr niedrig, so dass keine signifikanten Unterschiede festgestellt werden konnten. Durch Zugabe von Ouabain wurde der Pi-Transport im Jejunum signifikant gehemmt.


Um die funktionellen Eigenschaften des Peptidtransportes besser zu evaluieren, wurden mittels modifizierter Mg\(^{2+}\)-EGTA-Präzipitationsmethode jejunale BSMV präpariert, und Aufnahmestudien zum Glukose- und Peptidtransport mit Hilfe der Schnellfiltrationstechnik durchgeführt. Die funktionelle Integrität der Vesikel wurde durch die Na\(^{+}\)-abhängige Glukoseaufnahme bestätigt. Die Aufnahme von Glycyl-L-Glutamin in die Vesikel wurde in An- und Abwesenheit eines einwärts gerichteten H\(^{+}\)- oder Na\(^{+}\)-Gradienten durchgeführt. Ein Overshoot-Phänomen (schnelle initiale Aufnahme von Glycyl-L-Glutamin in die Vesikel über den eigentlichen Konzentrationsausgleich zwischen extra- und intravesikulärem Raum hinaus) wurde sowohl in Anwesenheit als auch in Abwesenheit eines H\(^{+}\)-Gradienten beobachtet, wobei die H\(^{+}\)-abhängige Aufnahme signifikant höher war als die Aufnahme ohne Anwesenheit des H\(^{+}\)-Gradienten. Ein Na\(^{+}\)-Gradient induzierte kein Overshoot-Phänomen. Konzentrationsabhängige Aufnahmen von Glycyl-L-Glutamin in die Vesikel wiesen hyperbolische Verläufe auf, die der Michaelis-Menten-Kinetik entsprechen. Der K\(_{m}\)-Wert der H\(^{+}\)-abhängigen Glycyl-L-Glutamin Aufnahme weist auf die Existenz eines PepT1 beim Pferd hin. Der K\(_{m}\)-Wert für die Glycyl-L-Glutamin Aufnahme in Abwesenheit eines Gradienten unterschied sich signifikant von dem K\(_{m}\)-Wert der H\(^{+}\)-abhängigen Aufnahme, ein Hinweis für die Existenz zweier verschiedener Transportsysteme für die Peptidaufnahme beim Pferd.
Die Ergebnisse dieser Studie zeigen erstmalig, dass Peptide über die Darmwand des Pferdes transportiert werden. Das Duodenum erwies sich als wichtigster Darmabschnitt für die Ca\textsuperscript{2+}-Absorption, und eine für das Pferd spezifische transportphysiologische Eigenschaft des Intestinaltraktes, die P\textsubscript{T}-Sekretionsfähigkeit, wurde im Jejunum nachgewiesen. In Anbetracht fehlender Effekte der Cl\textsuperscript{-}-Kanal-Inhibitoren auf den Cl\textsuperscript{-}-Transport, bleibt die in vitro Untersuchung der intestinalen Cl\textsuperscript{-}-Sekretion des Pferdes eine Herausforderung.
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