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Effect of Ca supply on Vitamin D receptor and calbindin D9k

Influence of different calcium supplies and a single vitamin D injection on vitamin D receptor- and calbindin D9k-immunoreactivities in the gastrointestinal tract of goat kids

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**ABSTRACT:** The purpose of this study was to investigate whether diets differing in calcium (Ca) concentration have an influence on vitamin D (VitD) receptor (VDR) and calbindin D9k-(Calb9k) immunoreactivities in the gastrointestinal tract of growing goats. In addition, the effect of a single VitD injection was studied, to clarify whether exogenous VitD would even more increase active Ca absorption mechanisms. The hypothesis of the study was that lower Ca intake leads to higher active Ca absorption, and with that, to higher amounts of VDR and Calb9k immunoreactivities. One group (Ca-nk, according to age requirements) received 2.5 to 6 g Ca/d, whereas a second group (Ca-lk, lower than requirements) received 1.5 to 4 g Ca/d from the 6th wk (weaning) until 15th wk (slaughtering). In addition, 5 and 6 goat kids, respectively, of each group (Ca-nk, Ca-lk), were injected with VitD (0.05 mg/kg BW cholecalciferol) in the 14th wk of life. Blood samples were taken in the 14th and 15th wk. Calcium and VitD (25-hydroxyvitamin D and 1,25-dihydroxyvitamin D) concentrations were determined in serum. Immediately after slaughter, duodenum (DD) and rumen (RU) were mounted in conventional Ussing chambers. Unidirectional flux rates of Ca across gastrointestinal tissues were measured. Additionally, tissue specimens of the gastrointestinal tract were collected and formaldehyde-fixed paraffin sections were used for VDR and Calb9k immunohistochemistry. In all kid groups, a net absorption in RU and a net secretion of calcium in DD were observed. Immunoreactions of VDR were highest in the duodenal mucosa, whereas Calb9k immunoreactions were observed in the forestomach and intestinal tissues. The highest expression was observed in the duodenal surface epithelium. Additionally, in the VitD injected groups, an immunoreaction occurred in the jejunal superficial and basal gland and the ileal superficial epithelium. In contrast, the other groups showed no Calb9k immunoreactions at these sites. In conclusion, there is clear evidence for the RU as a main site for Ca absorption. The results of this study also indicate that VDR and Calb9k are
highly expressed in the duodenal mucosa. The active absorption may not play such an important role in the DD as there was also active transport evident in the RU. However, Calb9k expression seems to be stimulated by VitD administration.

**Key words:** calcium, calbindin D9k, goats, vitamin D, vitamin D receptor

**INTRODUCTION**

In growing animals, calcium (Ca) is essential for normal growth, maintenance of bones and teeth and the prevention of metabolic bone diseases. The Ca balance is controlled by 3 organ systems: the gastrointestinal tract, bone and kidneys (Bronner, 1997). Normocalcemia is maintained through the actions of calcitonin, parathyroid hormone (PTH), and 1.25-dihydroxyvitamin D (1.25VitD), the active form of vitamin D (VitD; Russel, 2001). In monogastric animals, intestinal Ca absorption is assumed to be achieved by 2 mechanisms: an active, transcellular transport and a passive paracellular pathway (Bronner et al., 1986; Bronner 1998). Active Ca transport is mainly regulated by 1.25VitD via transcriptional activation of genes, 1.25VitD binds to its classical nuclear receptor (Erben et al., 2002), the VitD receptor (VDR). The transcellular Ca absorption in the intestine is divided into 3 steps: First, passive entry of ionised Ca into enterocytes via transient receptor potential vanilloid channel type 6 (TRPV6) (Peng et al., 1999; Wilkens et al., 2009); second, cytosolic transfer of Ca bound to calbindin D9k (Calb9k), a VitD-dependent calcium binding protein (Bronner, 1987 and 1991; Feher et al., 1992) and third, the extrusion of Ca across the basolateral membrane, where Ca pumps, predominantly plasma membrane Ca-ATPase (PMCA1b) and sodium Ca exchanger (NCX), transport Ca into the blood (van Abel et al., 2003; Wassermann, 2004). These transporters (TRPV6, Calb9k, PMCA1b and NCX) are considered to be essential for
transcellular Ca absorption. Additionally, in ruminants, Ca is absorbed in the rumen (Breves and Schröder, 2005). It is not known, how the ruminal Ca absorption influences Ca homoeostasis (Martens, 2005). Different studies showed different results on the permeability of Ca across ruminal epithelium (Pfeffer et al., 1970; Dillon and Scott, 1979; Schröder et al., 1995; 2001). Although it has clearly been shown that in sheep these Ca flux rates in rumen must be active (Schröder et al, 2001), it is still not clear how this transport is realized on the molecular basis. Moreover, the absorption of Ca from the intestines may not be identical, even between ruminant species. Liesegang et al. (2004, 2006, 2008) showed that dairy goats and dairy cows have different Ca and bone metabolism characteristics compared to milk sheep. Growing goats had a higher bone turnover than growing sheep (Liesegang and Risteli, 2005). Thus, the mechanisms in relation to Ca in ruminants are not completely understood, especially the active Ca absorption mechanisms in the different parts of the gastrointestinal tract. Generally the organism can adapt to different stages of reproduction and growth, but the mechanisms of the adaptation to changing Ca needs are currently unknown.

The goal of the present study was to test the hypothesis that VDR and Calb9k concentrations vary in the gastrointestinal tract dependent on Ca intake. Another hypothesis was that different Ca feeding regimes plus VitD injection are factors which influence the Ca absorption. It was of special interest to see whether changes occur according to Ca absorption in rumen.

MATERIALS AND METHODS

Animals and Experimental Design
The trial included 22 goat kids (Saanen breed). Animals were housed in pens bedded with sawdust. During feeding, the animals were maintained in single pens and during the rest of the time, they were allowed to stay in groups. After weaning (6 wk post natal (pn)) goat kids were fed individually for 9 wk. The lower Ca kid group (Ca-lk) received 1.5 to 4 g Ca/d and the normal Ca kid group (Ca-nk) received 2.5 to 6 g Ca/d from the 6\textsuperscript{th} to 15\textsuperscript{th} wk of life, respectively (Figure 1). All animals received a diet which was balanced in energy and protein. The diet consisted of hay and concentrate (Table 1). The diet contained 1,500 IU/kg DM VitD in accordance with recommendations of Swiss Federal Research Station for Animal Production (RAP, 1999). Additionally, the animals had free access to water and a salt block (sodium chloride, Agrosal, Heilbronn, Germany). Seven days prior to slaughter (14\textsuperscript{th} wk pn) 5 or 6 goat kids of each group (Ca-lk and Ca-nk) were injected once i.m. with VitD (cholecalciferol, 0.05 mg/kg BW i.m., vitamin D\textsubscript{3} “S” ad us. vet., Streuli, Switzerland). The groups were then defined as Ca-lkVitD and Ca-nkVitD. Blood samples to describe the VitD status (1.25 VitD and 25-hydroxyvitamin D (25VitD)) were taken before VitD injection (14\textsuperscript{th} wk) and at the 15\textsuperscript{th} wk. During the whole trial blood samples were collected to measure Ca concentrations in serum.

\textit{Collection of Blood Samples}

Blood samples were collected from the external jugular vein (Vacutainer; 9 or 6 mL, without additives, Vacuette Greiner Bio-One Vacuette, St. Gallen, Switzerland). Blood was centrifuged (1,580 x g for 10 min) within 30 min of collection. Two tubes of serum were stored at -20 °C and one tube at -80 °C until analyses were performed.

\textit{Analysis of Serum Samples}
Serum was analyzed for Ca and 1.25VitD as previously described (Liesegang et al., 2000; Liesegang and Risteli, 2005). Serum 25VitD measurements were performed using a kit from Chromsystems Instruments and Chemicals GmbH (Munich, Germany), which allows the chromatographic determination of 25VitD using a simple isocratic high performance liquid chromatographic system employing an ultraviolet detector (HP-1100, Agilent Technologies, Palo Alto, CA, USA). The intra- and interassay CV were 4.4 and 5.6%, respectively (Pérez-Llamas et al., 2008).

**Tissue Sampling and Processing**

Within 10 min after slaughter, pieces of rumen (RU) and duodenum (DD) were collected for the Ussing chamber analyses and within 15 min 5 cm long tubular pieces of DD, jejunum (JJ), ileum (IL), caecum (CC) and colon (CO) and pieces of RU, reticulum (RET), omasum (OMA) and abomasum (ABO) were collected and fixed in neutral buffered 4% formaldehyde solution for 26 h. The piece of the DD was taken directly after pancreatic duct entrance and JJ, IL and CC probes were taken from the central part of these segments. The segment of the colon was taken 40 cm cranial of the anus. The pieces from the RU, RET and OMA were taken from the base and finally a piece of the ABO was taken in the middle of the greater curvature. When necessary, gastrointestinal samples were washed with physiological saline. After fixation, all gastrointestinal probes were cut into pieces of 1 cm in length and rinsed in tap water for 24 h. This procedure was followed by dehydration in graded ethanol (70%, 80%, 96% and 100%), xylene, and paraffin (60°C) and finally embedding in paraffin (Histowax, Leica). Transverse sections of 5 µm in thickness were cut, mounted on Superfrost plus adhesive slides (Menzel-Gläser, Braunschweig, Germany) and dried at 60°C overnight. Sections were stained with
hematoxylin and eosin (Romeis, 1989) to exclude animals possibly exhibiting pathological changes of the intestine.

**Ussing Chamber Technique**

Tissue specimens of DD and RU were mounted in a modified Ussing chamber (Ussing and Zehran, 1951) and bathed with a volume of 3.5 mL buffer solution on both sides of the intestinal wall. The Parson buffer solution contained (in mmol•L⁻¹): NaCl, 107; KCl, 4.5; NaHCO₃, 25; Na₂HPO₄, 1.8; NaH₂PO₄, 0.2; CaCl₂, 1.25; MgSO₄, 1; and glucose, 12.2; and was gassed with 5% CO₂ in 95% O₂ and kept at 37°C; pH was adjusted to 7.4. The epithelium was continuously short-circuited by an automatic voltage-clamp device (Aachen Microclamp, AC Copy Datentechnik, Aachen, Germany) with correction for solution resistance. Tissue conductance ($G_t$) was measured by recording the voltage resulting from bipolar current pulses (±100 mA) applied across the tissue at 1 min intervals and calculated according to Ohm’s law. The values for $G_t$ and the continuously applied short-circuit current ($I_{sc}$) were recorded every min. 10 min after mounting the tissues in the chambers, 10 µL $^{45}$Ca²⁺ was added to mucosal side (to measure mucosal to serosal calcium fluxes) or the serosal side (to measure serosal to mucosal fluxes) of the intestinal wall (labelled side). After additional 60 min to allow isotope flux rates to reach a steady state and $I_{sc}$ to stabilize, unidirectional ion flux rates were determined in sequential 20-min periods. From the measured unidirectional net flux rates ($J_{ms} = $ flux rate from mucosa to serosa; $J_{sm} = $ flux rate from serosa to mucosa), net ion flux rates ($J_{net}$) were calculated according to $J_{net} = J_{ms} - J_{sm}$ (nmol/h/cm²) from the mean unidirectional flux rates.

**Immunohistochemistry for Vitamin D Receptor**
The method was described previously (Liesegang et al., 2008). A biotinylated rat monoclonal antibody (9A7γ, Neo-Markers, P. H. Stehelin & Cie AG, Basel, Switzerland) was used to label VDR in gastrointestinal sections. This antibody was used at a 1:200 dilution. Negative controls were performed using tris-buffered saline (TBS) instead of primary antibody and positive controls employing duodenal cross-sections of pigs (cross-reactivity proven and giving identical results; Milde et al., 1989; Schröder et al., 2001). In every tissue specimen, nuclear staining intensities of 500 cells were recorded from the following cell types each: basal glandular cells (BG), intermediate glandular cells (IG), superficial glandular cells (SG) and surface epithelial cells (SE). The staining intensities (SI) of the nuclei were scored as negative = 0, very weak = 0.5, weak = 1, intermediate = 2 or strong = 3, correlating to absence of brown (i.e. blue counterstain only), light brown, brown or dark brown staining, respectively (i.e. SI 0 to SI 3). The semiquantitative evaluation of histochemical reactions for VDR was performed as described previously, i.e., an immunoreactive score (IRS) was calculated for the colored intensity (CI) using this formula (Boos et al., 2007; Liesegang et al., 2008):

\[
VDR-IRS = 0 \times n(CI_0) + 0.25 \times n(CI_{0.5}) + 1 \times n(CI_1) + 4 \times n(CI_2) + 9 \times n(CI_3).
\]

\(n = \) number of cells; VDR-IRS = vitamin D receptor immunoreactice score

**Immunohistochemistry for Calbindin D9k**

The method was performed using an immunohistochemical protocol. A CalbindinD9k polyclonal rabbit antibody was used for the immunohistochemistry (swant, Bellinzona, Switzerland). All subsequent steps were carried out at room temperature. Negative controls were performed using TBS instead of primary antibody and positive controls employing intestinal cross sections of pigs. For microscopic analysis, of the slides a light microscope (Leica DMLB, Leica Microsystems AG, Glattbrugg, Switzerland) was used. Following histomorphological
examination, the slides were assessed with a computerized digital camera (Colorview 12, Leica Microsystems AG, Glattpugg, Switzerland) analyzing the mean color intensity – i.e., grey scale values – of cells or tissues. The software used was analySIS Pro (version 5.0; Soft Imaging System GmbH, Münster, Germany). This procedure was always performed under standardized conditions, thus eliminating primarily errors normally occurring during visual grading of histochemical color intensities. However, this does, not eliminate interassay variations, which are caused by the use of slides processed histochemically in several succeeding batches (Grube, 2004; Taylor and Levenson, 2006). Digitalized pictures were taken, white balancing performed (i.e. measurement in a tissue-free district of the section thus representing 100% transmission), randomly selected regions of interest were defined containing immunopositive structures such as SE, SG, and BG, and color intensity was measured. The mean color intensity – i.e. grey scale value – of a selected structure within a region of interest was measured employing 256 different color levels, whereby zero represents black and 255 represents white. This means that high grey scale values correspond to lower immunoreaction intensities. After assessing 5 tissue areas within the tissue for each immunopositive structure per slide, a mean value per structure and animal was calculated. Transmission ($T$) values were transformed to extinction ($E$), which is proportional to dye concentrations at the level of the sections ($E = \lg 1/T$). This value was used for further statistical analysis.

**Statistical Analyses**

The results are presented as mean ± SEM. A multivariate ANOVA for repeated measures was performed with group as a cofactor included in the model to test difference of the time dependent patterns between the groups. The factor “cell type” was included in the model for the
VDR-IRS, i.e., location of the cells within the mucosa. To avoid false conclusions due to a
violation of the assumption of compound symmetry, a Huynh–Feldt correction was performed.
Furthermore, the difference between groups was tested with the Mann–Whitney U test (non-
parametric) to limit the influence of extreme values. The level of significance was set at $\alpha = 0.05$
for all tests. All statistical analyses were performed by use of SYSTAT for Windows (Version
11.0, SPSS Inc. Chicago, IL).

**RESULTS**

*Ca and Vitamin D concentrations in serum*

Mean serum Ca concentrations were similar (2.9 ± 0.7 mmol/L) in both groups over the
whole trial (reference value goats: 2.2 to 2.7 mmol/L; Tschuor et al., 2008). However,
differences were seen in the VitD concentrations in serum: in the 14th wk the 25VitD
concentrations were 30.7 ± 1.3 ng/mL in the Ca-nk group, 30.2 ± 4 ng/mL in the Ca-lk group,
31.7 ± 1.8 ng/mL in the Ca-nkVitD group, and 32.5 ± 1.3 ng/mL in the Ca-lkVitD group. In the
15th wk, 1 wk after VitD injection, the mean 25VitD concentrations showed an increase ($P <
0.001$) in the Ca-nkVitD group (38.7 ± 1.2 ng/mL) and the Ca-lkVitD group (46.7 ± 3.3 ng/mL)
compared to the Ca-nk group (29.9 ± 1.8 ng/mL) and the Ca-lk group (27.7 ± 2.2 ng/mL). The
1.25VitD concentrations in serum showed no difference within the groups (14th wk 158 ± 28
pmol/L and 15th wk 162 ± 16 pmol/L).

*Calcium Flux Rates*
Mucosal to serosal Ca flux rates ($J_{ms}$) exceeded respective flux rates in the opposite direction ($J_{sm}$) in the RU. This resulted in net Ca flux rates ($J_{net} = J_{ms} - J_{sm}$) ranging between +6 to +7 nmol/cm²/h in RU. In the Ca-nk group, the $J_{ms}$ and $J_{sm}$ flux rates were higher compared to the Ca-lk group in RU, but no group effect was indentified. In the DD of the kids without VitD injection, net Ca flux rates ranged between -9 and -5 nmol/cm²/h, and in kids with VitD injection, mean flux rates were around +0.5 nmol/cm²/h. The net flux rates were not different between the groups in RU ($P = 0.927$) and in DD ($P = 0.190$) (Table 2). The net flux rates were positive in RU in all groups, indicating Ca absorption, whereas the net flux rates in DD were negative or weakly positive, respectively, indicating a net secretion or only sparse absorption. No difference between the groups was observed ($P > 0.05$).

**Vitamin D Receptor**

Vitamin D receptor immunoreactivities were exposed as brown staining in the nuclei in all segments of the intestinal tissues (DD, JJ, IL, CC and CO; Figures 2 and 3). Goblet cells were always devoid of any specific immunoreaction and thus not considered in the present study. This brown coloration was of varying degree and thus indicative of more or less high VDR concentrations within all other cell types present within the epithelium of crypts (BG, IG, SG) or the intestinal luminal SE. Nuclear immunostaining was present in all segments of the intestine, whereby immunoreactivities were highest in the DD compared to the more distal intestinal segments. Immunoreactive score of the Ca-nk group did not differ from data obtained from the Ca-lk group. Nor did the VitD injection show any effect on the immunoreactivities of VDR ($P = 0.454$). Intestinal VDR-IRS of BG decreased from higher levels in DD and JJ to lower levels in IL, CC, and CO. Mean VDR-IRS (BG+IG+SG+SE/4) differed between all segments (DD > JE >
CO > IL > CC; \( P \leq 0.05; \) Table 3). Differences between the cell types – basal, intermediate and superficial glandular/crypt epithelial cells and surface epithelial cells – within an intestinal segment were for DD (BG, IG > SG > SE; \( P \leq 0.05 \)), JE and IL (BG, IG > SG > SE; \( P \leq 0.05 \)) and CC, CO (IG > BG, SG > SE; \( P \leq 0.05 \)). No VDR immunoreactivity was observed in the forestomach in the tissues of goat kids.

**Calbindin D9k**

Calbindin9k was histochemically detectable and assessed quantitatively in tissues, in forestomach, i.e., RU, OMA, RET, ABO, and in intestinal tissues, i.e., DD, JJ and IL (Figures 4 and 5). There were no immunohistochemical reactions in the large intestine, i.e., CC and CO,. In the forestomach, the reaction always was in the superficial epithelium, and no difference was detected between the groups. In the intestines, immune reactions were not evident in all intestinal sections. However, reactions were present in all DD and in part JJ groups. The DD showed a higher expression than the JJ (\( P = 0.003 \)). There were differences between groups (\( P = 0.023 \)) and between different parts of intestinal sections (\( P < 0.0001 \)). The highest immunoreactivities were found in the duodenal surface epithelium. Between the different layers of DD differences were obtained (\( P < 0.0001 \)): DD SE > DD SG > DD BG. This was also true for the jejunum: JJ SE > JJ SG > JJ BG (SE/SG and SE/BG \( P = 0.0001 \); SG/BG \( P = 0.012 \)). Differences were also observed in JJ SG (\( P = 0.007 \)) and JJ BG (\( P = 0.010 \)) between the different kid groups.

The Ca-lk group showed differences in the JJ SG and JJ BG compared to the Ca-lk group with VitD injection (\( P = 0.037 \)). Different values in JJ SE (\( P = 0.043 \)) of the Ca-nk group were also measured in comparison to the Ca-nk group with VitD injection. The most important
differences were obtained in the JJ SG, JJ BG and IL SE due to the fact that only the groups with VitD injection showed an immunoreaction ($P = 0.013$; Table 4).

**DISCUSSION**

In all kid groups, a high positive net flux rate (net absorption) for Ca was measured in RU in the Ussing chambers. It can be concluded that the RU is a main site for active Ca transport mechanisms in growing goat kids. Previous studies described Ca net absorption before the DD in single- and multi-fistulated ruminants (Pfeffer et al., 1970; Dillon and Scott, 1979; Wylie et al., 1985). Höller et al. (1988) and Schröder et al. (1997, 1998) also observed active Ca transport mechanisms in caprine and ovine rumen by Ussing chamber technique. Furthermore, in sheep RU, active positive Ca net flux rates were observed, but there was no influence of Ca supply or 1.25VitD (Schröder et al., 2001). In contrast, studies in monogastric animals have shown that active Ca absorption is stimulated in response to Ca intake and that this effect is mediated by 1.25VitD (Nemere and Norman, 1991; Breves et al., 1991, 1995). Lower dietary Ca intake led to a stimulation of active Ca absorption by more than 50% in the RU of growing goats, but not in sheep (Schröder et al., 1997). In contrast, the current study demonstrates no increase of Ca net flux rates in dietary Ca depleted growing goats. In addition, the VitD injection showed no effect on Ca absorption in RU of goat kids. In DD, negative net flux rates were measured in groups not injected with VitD. From the present study, there is evidence of lower Ca absorption in DD in goat kids with VitD injection, but this has to be further investigated.

The reason for the single VitD pulse treatment 7 d before slaughtering was to simulate a therapy after calving to prevent parturient paresis which is used in dairy cattle practice 1 wk before calving. Hollis et al. (1976) showed a 1-wk lag in the conversion of cholecalciferol to
25(OH)VitD in dairy cattle. Horst and Littledike (1981) recommend administration 10 x 10^6 IU of VitD about 1-wk before the expected date of parturition and to repeat the injections, if necessary. The effectiveness of this latter procedure apparently relies on the transient increase in plasma 1.25VitD that follows the VitD injection. The additional VitD injection should then increase intestinal Ca absorption (Collins and Norman, 1991).

In monogastric species, e.g., pigs (Schröder et al., 1998) and rats (Brommage et al., 1995), active Ca absorption from the upper small intestines was stimulated by 1.25VitD. Schröder et al. (1997) observed an increase of net Ca absorption independent of Ca supply after VitD injection in the DD of goat kids. It is possible that 1.25VitD changes Ca absorption in the upper small intestine. Schröder et al. (1995) assumed that the duodenal mucosa is an optional target organ of 1.25VitD in goats. However, the mechanisms of Ca absorptions in RU are still unclear.

The results of the present immunohistochemical study in growing goats demonstrated that VDR are present in all intestinal segments, as was also shown in earlier studies (Boos et al., 2007; Liesegang et al., 2008; Riner et al., 2008). The observed VDR-immunoreactivities were prominent in duodenal mucosa, lower in jejunum and colon, further declined in ileum and were lowest in caecum. The results indicate that VDR are highly expressed at the site of active, VitD-dependent intestinal Ca absorption. The level of Ca intake as well as VitD injections had no influence on VDR-immunoreactions. Interestingly there were no immunohistochemical reactions of VDR in the forestomach, but a clear active net flux rate of Ca was shown in the Ussing chamber. This active transport seems to be VitD-independent, but dependent on Calb9k, because it is expressed in the forestomachs.
To the authors’ knowledge, these are the first results presenting the expression of the Ca
binding protein Calb9k in caprine gastrointestinal tissues. Calbindin D9k was detected
immunohistochemically in the forestomachs and intestinal epithelium of growing goats.
Duodenum and jejunum exhibited an immunohistochemical staining for Calb9k in all kid groups.
Interestingly, goat kids with VitD injection showed an additional immunohistochemical reaction
in jejunum and ileum which was, however, not detectable in non-injected animals at these sites.
There were 2 differences in immunoreactive Calb9k staining: a proximal to distal decrease within
the intestinal segments based mainly on high Calb9k in surface epithelium and superficial
glandular to basal glandular epithelial cells decreases within the segment. The distribution
pattern of Calb9k protein demonstrated in the present study is in accordance with several reports
on Calb9k expression in the duodenal tissue of cows, mice, and goats (Schröder et al., 1998;
Yamagishi et al., 2002; van Abel et al., 2003; Yamagishi et al., 2006).

It was formerly suggested that the expression of VDR in the chicken intestine may be
correlated with the stage of differentiation of the enterocytes (Clemens et al., 1988). In this
study, VDR immunoreactivity was localized mainly in nuclei of epithelial cells and was more
abundant in the crypt than in the villus cells. Calbindin was found predominantly in villus-tip
cells in normal rat DD (Taylor et al., 1984; van Corven et al., 1985). Calcium transport was
approximately twofold higher in the villus-tip than in the crypt cell-fraction basolateral
membranes, although the affinity for the Ca uptake was similar in rats (Smith et al., 1985). It
may be concluded that Calb9k and ATP-dependent Ca-transport are most prominent in absorbing
villus cells and that the vitamin-D dependent Ca absorption primarily takes place here as well
(van Corven et al., 1985).
In the present study, the enterocytes displayed the highest VDR-IRS in the basal and intermediate glandular cells and the highest Calb9k reactivities in the surface epithelium. The surface epithelial cells have a very short lifetime, and on that account, the receptors in the surface epithelium would not be effective for long periods of time. The surface epithelial cells which develop from dividing stem cells in the crypts differentiate when they move to the neck of the glands. There, they are integrated into the surface epithelium, and are shed after apoptosis. Results of Walters and Weiser (1987) supported the opinion that mature enterocytes have the greatest capacity for transcellular movement of calcium. Furthermore, their findings showed that partially differentiated midvillus cells have the capacity to respond most rapidly to 1.25VitD. The villus tip cells have differentiated to a point where they are no longer able to synthesize additional proteins for Ca absorption (Walters and Weiser, 1987). Using in situ hybridization, Freeman et al. (1995) found the highest density of PMCA1 in the crypts and basal cells. Consequently, essential active Ca absorption is shown by coordinated actions of the ligand-active transcription factor VDR and the Ca pump PMCA1 (Freeman et al., 1995). In DD and JJ villus tip cells, TRPV6 has been shown to co-localize with Calb9k (Hoenderop et al., 1999; Peng et al., 2003). Furthermore, Weber et al. (2001) presented a co-localization of TRPV6 and Calb9k, suggesting that apical TRPV6 and intracellular Calb9k are interrelated in a system controlling Ca entry and intracellular Ca concentrations in intestinal epithelium (Weber et al., 2001).

In contrast, Benn et al. (2008) postulated an active intestinal Ca absorption in the absence of TRPV6 and Calb9k in mice. Akhter et al. (2007) supported an alternative pathway for intestinal Ca absorption and intracellular Ca diffusion that does not require intestinal calbindin. There is evidence that calbindin possibly may have other roles in the intestine, for example as a
modulator of Ca channel activity and/or as an intracellular Ca buffer (Benn et al., 2008). These investigations relating to monogastric animals may also explain a possible mechanism in the RU of ruminants. The assumption of VitD-sensitive Ca absorption is supported by the detection of VDR and Calb9k in goat small intestine (Schröder et al., 1995, 1997). In sheep, VDR and Calb9k were found in the JJ (Schröder et al., 2001). Additionally, the presence of TRPV5 and TRPV6 in DD and JJ could be demonstrated with in-situ hybridization technique (Wilkens et al., 2009). In this study, TRPV6 expression was higher in the jejunum than in the DD (Wilkens et al., 2009). It seems that the jejunum is more important than duodenum for active Ca absorption in sheep (Schröder et al., 1997). In contrast, our results and the results of Riner et al. (2008) show that the DD is more important for Ca absorption in goats. The treatment with 1.25VitD had no effect on the pre-intestinal Ca absorption. However, an increase of Calb9k was present upon injection of VitD in the small intestine, jejunum and ileum. These findings show that intestinal Calb9k transcription was stimulated by the binding of VitD to its receptor and the activation of the complex thereafter. The daily Ca intake seems to be important for active and passive mechanism, respectively. Active Ca absorption is dominant at conditions of lower Ca intake (Pansu et al., 1993), whereas the passive mechanism is more prominent when high Ca amounts are present in the feedstuff (Bronner and Pansu, 1999). It has to be considered that, in the present study, the Ca requirements decrease from the 2nd to the 3rd month in growing goats. For this reason, the animals fed the lower Ca diet were not really deficient compared to the animals that received the diet with the normal Ca contents. In addition, it seems that these young animals have a high ability to recover and cope with lower calcium amounts in their diet. This may be seen by the small differences in the VDR and Calb9k immunohistochemistry reactions at the age of 15 wk. In the present study the Ussing chamber results revealed an active Ca absorption in the
RU, but only Calb9k and no VDR were detected by immunohistochemical methods. Also in the DD there was evidence of active Ca absorption after VitD injection obtained with the Ussing chamber technique. Additionally, the immunohistochemical methods revealed intestinal VDR and Calb9k in goat kids. This study verified the results of Schröder and Breves (2006), that the proximal small intestines are major sites for controlled known active Ca absorption in monogastric animals and ruminants. The mechanisms related to the active transport in the rumen are not yet known, also seen in other studies (Schröder et al., 1995) and need to be addressed in further studies.

There is clear evidence for the RU as a main site for Ca absorption in growing goats, although the exact mechanisms related to this are not yet clarified. The VDR and Calb9k exist mainly in the intestinal tract of goat kids. The results of this study indicate that VDR are highly expressed at the site of expected maximal intestinal Ca absorption, in the duodenal mucosa. Nuclei of enterocytes stained positive for VDR with the strongest immunoreactions in the intermediate and basal glandular cells. Calbindin D9k revealed the most intensive staining in the surface epithelium in the forestomach and DD. Vitamin D injection increased Calb9k-expression in the more distal intestinal sections, i.e. JJ and IL, but there was no response to the diet in the gastrointestinal tract. Because there was clear evidence of active Ca absorption in the rumen, the active absorption may not play such an important role in the DD. In RU, Ca absorption seems to be VitD-independent.

LITERATURE CITED

Active intestinal calcium transport in the absence of transient receptor potential vanilloid type 6 and calbindin-D9k. Endocrinology 149:3196-3205.


Table 1. Daily allowance (kg of original substance) and nutrient intake (daily intake in g or MJ in dry matter) of the experimental diets; nk = normal calcium concentration; lk = lower calcium concentration

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Ca-nk 6pn-8pn</th>
<th>Ca-lk 6pn-8pn</th>
<th>Ca-nk 9pn-15pn</th>
<th>Ca-lk 9pn-15pn</th>
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</thead>
<tbody>
<tr>
<td><strong>Daily allowance</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hay&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.4</td>
<td>0.4</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>Concentrate&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.4&lt;sup&gt;b1&lt;/sup&gt;</td>
<td>0.4&lt;sup&gt;b2&lt;/sup&gt;</td>
<td>0.4&lt;sup&gt;b1&lt;/sup&gt;</td>
<td>0.4&lt;sup&gt;b2&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Nutrient intake</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DM</td>
<td>0.7</td>
<td>0.7</td>
<td>0.8</td>
<td>0.8</td>
</tr>
<tr>
<td>NEL&lt;sup&gt;c&lt;/sup&gt;, MJ</td>
<td>4</td>
<td>4.1</td>
<td>4.4</td>
<td>4.5</td>
</tr>
<tr>
<td>Absorbable protein</td>
<td>58</td>
<td>58</td>
<td>64</td>
<td>65</td>
</tr>
<tr>
<td>Calcium</td>
<td>5.4</td>
<td>2.9</td>
<td>5.8</td>
<td>3.3</td>
</tr>
<tr>
<td>Phosphorus</td>
<td>2.5</td>
<td>2.5</td>
<td>2.8</td>
<td>2.7</td>
</tr>
<tr>
<td>Magnesium</td>
<td>2.1</td>
<td>2.1</td>
<td>2.3</td>
<td>2.3</td>
</tr>
</tbody>
</table>
a Hay (barn-dried, balanced in grasses and leguminoses): 4.5 MJ NEL/kg DM, 76 g of AP/kg of DM, 4.3 g of Ca/kg of DM, 3.1 g of P/kg of DM

concentrate b1: 7 MJ of NEL/kg of DM, 88 g of AP/kg of DM, 9.8 g of Ca/kg of DM, 3.5 g of P/kg of DM

concentrate b2: 7.1 MJ of NEL/kg of DM, 89 g of AP/kg of DM, 3.5 g of Ca/kg of DM, 3.5 g of P/kg of DM

(KLIBA 2705b1, 2704b2, Provimi Kliba AG, Kaiseraugst, Switzerland)

calculated according to RAP (1999)

DM = dry matter; during 9 week-feeding trial (n = 11 for each group; nk = normal calcium concentration; lk = lower calcium concentration); pn = weeks post natal
Table 2. Electrical properties, unidirectional (sm = serosal-mucosal; ms = mucosal-serosal) and net flux-rates in nmol·cm⁻²·h⁻¹ ($J_{net} = J_{ms} - J_{sm}$); means ± SE (no group effect, $P > 0.05$)

<table>
<thead>
<tr>
<th></th>
<th>rumen</th>
<th>duodenum</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ca-nk</td>
<td>Ca-nkVitD</td>
</tr>
<tr>
<td>ms flux</td>
<td>14.18 ± 2.38</td>
<td>11.06 ± 2.85</td>
</tr>
<tr>
<td>sm flux</td>
<td>6.78 ± 2.68</td>
<td>3.74 ± 1.12</td>
</tr>
<tr>
<td>net flux</td>
<td>6.92 ± 2.23</td>
<td>7.31 ± 1.97</td>
</tr>
<tr>
<td></td>
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</tr>
</tbody>
</table>
Table 3. Vitamin D receptor immunoreactive scores (mean ± SE) of the various cell types within the epithelial layers of the different segments of goat kids’ intestines.

<table>
<thead>
<tr>
<th>Layer</th>
<th>Ca-nk</th>
<th>Ca-nkVitD</th>
<th>Ca-lk</th>
<th>Ca-lkVitD</th>
<th>Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>DD BG</td>
<td>1190 ± 209</td>
<td>1439 ± 133</td>
<td>1432 ± 193</td>
<td>1576 ± 229</td>
<td>1412 ± 92&lt;sup&gt;1,a&lt;/sup&gt;</td>
</tr>
<tr>
<td>DD IG</td>
<td>1102 ± 202</td>
<td>1395 ± 132</td>
<td>1335 ± 209</td>
<td>1407 ± 243</td>
<td>1315 ± 95&lt;sup&gt;1,a&lt;/sup&gt;</td>
</tr>
<tr>
<td>DD SG</td>
<td>316 ± 120</td>
<td>288 ± 66</td>
<td>243 ± 54</td>
<td>263 ± 77</td>
<td>276 ± 37&lt;sup&gt;2,a&lt;/sup&gt;</td>
</tr>
<tr>
<td>DD SE</td>
<td>59 ± 9</td>
<td>138 ± 36</td>
<td>94 ± 30</td>
<td>110 ± 47</td>
<td>102 ± 17&lt;sup&gt;2,a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Mean DD</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>776 ± 53&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>JJ BG</td>
<td>831 ± 159</td>
<td>797 ± 175</td>
<td>1288 ± 213</td>
<td>1086 ± 301</td>
<td>1004 ± 109&lt;sup&gt;1,a&lt;/sup&gt;</td>
</tr>
<tr>
<td>JJ IG</td>
<td>878 ± 214</td>
<td>589 ± 141</td>
<td>1144 ± 216</td>
<td>1017 ± 308</td>
<td>903 ± 112&lt;sup&gt;1,b&lt;/sup&gt;</td>
</tr>
<tr>
<td>JJ SG</td>
<td>201 ± 78</td>
<td>128 ± 12</td>
<td>315 ± 86</td>
<td>248 ± 76</td>
<td>223 ± 35&lt;sup&gt;2,b&lt;/sup&gt;</td>
</tr>
<tr>
<td>JJ SE</td>
<td>52 ± 16</td>
<td>87 ± 26</td>
<td>172 ± 54</td>
<td>60 ± 21</td>
<td>96 ± 19&lt;sup&gt;3,b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Mean JJ</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>557 ± 65&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>IL BG</td>
<td>143 ± 30</td>
<td>224 ± 70</td>
<td>237 ± 55</td>
<td>260 ± 116</td>
<td>221 ± 35&lt;sup&gt;1,b&lt;/sup&gt;</td>
</tr>
<tr>
<td>IL IG</td>
<td>137 ± 21</td>
<td>242 ± 40</td>
<td>306 ± 63</td>
<td>263 ± 90</td>
<td>245 ± 30&lt;sup&gt;1,b&lt;/sup&gt;</td>
</tr>
<tr>
<td>IL SG</td>
<td>62 ± 13</td>
<td>118 ± 14</td>
<td>97 ± 22</td>
<td>88 ± 23</td>
<td>94 ± 10&lt;sup&gt;2,b&lt;/sup&gt;</td>
</tr>
<tr>
<td>IL SE</td>
<td>33 ± 8</td>
<td>104 ± 29</td>
<td>36 ± 8</td>
<td>91 ± 53</td>
<td>68 ± 16&lt;sup&gt;3,b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Mean IL</td>
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<td></td>
<td></td>
<td></td>
<td>157 ± 20&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>CC BG</td>
<td>72 ± 22</td>
<td>51 ± 23</td>
<td>159 ± 59</td>
<td>89 ± 28</td>
<td>94 ± 20&lt;sup&gt;1,k&lt;/sup&gt;</td>
</tr>
<tr>
<td>CC IG</td>
<td>219 ± 44</td>
<td>138 ± 39</td>
<td>355 ± 87</td>
<td>212 ± 65</td>
<td>232 ± 34&lt;sup&gt;2,c&lt;/sup&gt;</td>
</tr>
<tr>
<td>CC SG</td>
<td>83 ± 10</td>
<td>126 ± 15</td>
<td>130 ± 52</td>
<td>102 ± 18</td>
<td>112 ± 15&lt;sup&gt;1,a&lt;/sup&gt;</td>
</tr>
<tr>
<td>CC SE</td>
<td>17 ± 11</td>
<td>39 ± 15</td>
<td>43 ± 29</td>
<td>38 ± 17</td>
<td>35 ± 9&lt;sup&gt;3,b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Mean CC</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>118 ± 18&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>CO BG</td>
<td>93 ± 33</td>
<td>191 ± 84</td>
<td>269 ± 91</td>
<td>170 ± 38</td>
<td>185 ± 36&lt;sup&gt;1,b&lt;/sup&gt;</td>
</tr>
<tr>
<td>CO IG</td>
<td>252 ± 54</td>
<td>603 ± 171</td>
<td>582 ± 152</td>
<td>394 ± 63</td>
<td>470 ± 68&lt;sup&gt;2,b&lt;/sup&gt;</td>
</tr>
<tr>
<td>CO SG</td>
<td>105 ± 12</td>
<td>243 ± 82</td>
<td>160 ± 22</td>
<td>144 ± 41</td>
<td>166 ± 26&lt;sup&gt;1,a&lt;/sup&gt;</td>
</tr>
<tr>
<td>CO SE</td>
<td>17 ± 11</td>
<td>30 ± 11</td>
<td>27 ± 12</td>
<td>5 ± 2</td>
<td>20 ± 5&lt;sup&gt;3,c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Mean CO</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>210 ± 30&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>
BG, IG, SG = basal, intermediate, superficial glandular cells; SE = surface epithelium; DD = duodenum; JJ = jejunum; IL = ileum; CC = caecum; CO = colon; Mean values with different letters within the right column, i.e. between the different intestinal segments differ ($p \leq 0.05$); Mean values with different ciphers, i.e. between the different epithelia differ ($p \leq 0.05$); No significant group effect was observed ($P > 0.05$); nk normal calcium concentration, $n = 5$; nkVitD= normal calcium concentration and VitD injection, $n = 6$; lk = lower calcium concentration, $n = 6$; lkVitD = lower calcium concentration and VitD injection, $n = 5$. 
Table 4. Cytoplasmic Calbindin 9k extinction values (mean ± SE) of the various cell types within the epithelial layers of the different segments in gastrointestinal tract in goat kids.

<table>
<thead>
<tr>
<th>Layer</th>
<th>Ca-nk</th>
<th>Ca-nkVitD</th>
<th>Ca-lk</th>
<th>Ca-lkVitD</th>
<th>Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>RU SE</td>
<td>1.4 ± 0.1</td>
<td>1.4 ± 0.1</td>
<td>1.5 ± 0.1</td>
<td>1.6 ± 0.0</td>
<td>1.5 ± 0.0</td>
</tr>
<tr>
<td>RET SE</td>
<td>1.4 ± 0.1</td>
<td>1.5 ± 0.0</td>
<td>1.4 ± 0.0</td>
<td>1.5 ± 0.1</td>
<td>1.4 ± 0.0</td>
</tr>
<tr>
<td>OMA SE</td>
<td>1.4 ± 0.0</td>
<td>1.5 ± 0.1</td>
<td>1.4 ± 0.0</td>
<td>1.4 ± 0.0</td>
<td>1.4 ± 0.0</td>
</tr>
<tr>
<td>ABO SE</td>
<td>1.5 ± 0.1</td>
<td>1.4 ± 0.0</td>
<td>1.6 ± 0.1</td>
<td>1.6 ± 0.0</td>
<td>0.9 ± 0.2</td>
</tr>
<tr>
<td>DD SE</td>
<td>2.6 ± 0.2</td>
<td>3.0 ± 0.2</td>
<td>2.8 ± 0.2</td>
<td>3.0 ± 0.2</td>
<td>2.8 ± 0.1\a</td>
</tr>
<tr>
<td>DD SG</td>
<td>1.7 ± 0.1</td>
<td>2.0 ± 0.1</td>
<td>2.0 ± 0.1</td>
<td>2.0 ± 0.2</td>
<td>1.9 ± 0.1\b</td>
</tr>
<tr>
<td>DD BG</td>
<td>1.4 ± 0.0</td>
<td>1.4 ± 0.0</td>
<td>1.5 ± 0.1</td>
<td>1.5 ± 0.2</td>
<td>1.3 ± 0.1\b</td>
</tr>
<tr>
<td>JJ SE</td>
<td>1.5 ± 0.1</td>
<td>3.0 ± 0.2</td>
<td>1.9 ± 0.4</td>
<td>2.5 ± 0.4</td>
<td>1.7 ± 0.3\b</td>
</tr>
<tr>
<td>JJ SG</td>
<td>0</td>
<td>1.8 ± 0.1</td>
<td>0</td>
<td>1.8 ± 0.1</td>
<td>1.8 ± 0.0*</td>
</tr>
<tr>
<td>JJ BG</td>
<td>0</td>
<td>1.4 ± 0.0</td>
<td>0</td>
<td>1.6 ± 0.0</td>
<td>1.5 ± 0.0*</td>
</tr>
<tr>
<td>IL SE</td>
<td>0</td>
<td>1.3 ± 0.1</td>
<td>0</td>
<td>1.2 ± 0.0</td>
<td>1.3 ± 0.0*</td>
</tr>
<tr>
<td>IL SG</td>
<td>no immunohistochemical reaction in all groups</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>IL BG</td>
<td>no immunohistochemical reaction in all groups</td>
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<tr>
<td>CC, CO</td>
<td>no immunohistochemical reaction in all groups</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
BG = basal glandular cells; SG = superficial glandular cells; SE = surface epithelium; RU = rumen; RET = reticulum; OMA = omasum; ABO = abomasum; DD = duodenum; JJ = jejunum; IL = ileum; CC = caecum; CO = colon; Mean values with different letters within the right column, i.e. between the different intestinal segments differ (p ≤ 0.05); Mean values with different ciphers, i.e. between the different epithelia differ (p ≤ 0.05); * group effect (p ≤ 0.05).
nk  normal calcium concentration, n = 5; nkVitD=normal calcium concentration and VitD injection, n = 6; lk = lower calcium concentration, n = 6; lkVitD = lower calcium concentration and VitD injection, n = 5
Figure 1. Calcium intake of goat kids (mean ± SE) in g/d during the 9 week-feeding trial (n = 11 for each group); pn = post natal; different letters indicate differences between Ca-nk and Ca-lk (p ≤ 0.05); ■ = kids fed normal (recommended) amounts of calcium; □ = kids fed lower than normal amounts of calcium; * indicates significant (P<0.05) differences between the time point before to time point after; black bar indicates daily Ca-requirements: 5 g/d until week 12 pn, afterwards 4.5 g/d (RAP, 1999).

Figure 2. Vitamin D receptor immunohistochemistry in goat kids duodenum (DD; A, a) and jejunum (JJ; B, b); VDR immunoreaction is demonstrated as brown staining and contrasts well with the blue counterstaining of the nuclei. DD (A) and JJ (B) exhibit a clear gradient in VDR immunostaining. Strong reactions are visible in BG. IG, SG and SE and demonstrate a stepwise reduced immunostaining, respectively. Small inlets (a, b) show the BG marked with an asterisk at a higher magnification to underline the decreasing VDR immunoreaction along the length of the small intestine and the cytoplasmic staining in DD and JJ. SE = superficial epithelium; SG = superficial glandular cells; IG = intermediate glandular cells; BG = basal glandular cells; MM = lamina muscularis mucosae; M = tunica muscularis; V = intestinal villi; N = nucleus

Figure 3. Vitamin D receptor immunohistochemistry in goat kids ileum (C, c), caecum (D, d) and colon (E, e). C-E generally exhibit weaker immunoreactions as compared to DD and JJ. Goblet cells (G) (marked only in e) are always devoid of VDR; see Fig. 2 for key.

Figure 4. Calbindin D9k immunoreactions in duodenum (DD, A), jejunum (JJ, B) and ileum (IL, C) of a goat kid without vitamin D injection. Calb9k immunoreaction is demonstrated as brown staining without background staining. DD and JJ (A, B) exhibit a clear gradient in Calb9k immunostaining. Strong reaction is visible in SE. SG and BG demonstrate a stepwise reduce immunostaining, respectively. The IL showed no Calb9k immunoreaction. SE = superficial
epithelium; SG = superficial gland; BG = basal gland; MM = lamina muscularis mucosae; M = tunica muscularis; V = intestinal villi

**Figure 5.** Calbindin D9k immunoreactions in duodenum (DD, A), jejunum (JJ, B) and ileum (IL, C) of a goat kid with vitamin D injection. Calb9k immunoreaction is demonstrated as brown staining without background staining. In DD, JJ and IL (A, B, C) exhibits a clear gradient in Calb9k immunostaining. Strong reaction is visible in SE. SG and BG demonstrate a stepwise reduce immunostaining, respectively. See Fig. 4 for key. Additionally, Calb9k immunoreactions of the rumen (D) and omasum (E), as examples of the forestomachs, L = omasal leave
Fig. 1
Fig. 2
Fig. 4