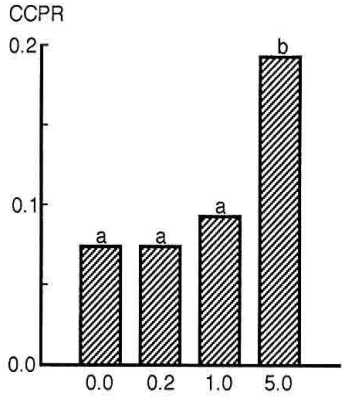

Figure 1: Effect of n-butyric acid on crypt cell production rate (CCPR) of pig colonic mucosa in short-term culture: mean CCPR (cells produced per 100 crypts per hour), n = 4. Means bearing different letters differ significantly ($p < 0.03$).

of biopsied human colon within a few hours as well as that of SAKATA & ENGELHARDT (1983) that SCFA instilled into temporarily isolated colonic segment increased the mitotic and labeling indices of the digestive tract epithelia (but not the near epidermis) within a few hours. The increase in CCPR by n-butyric acid in the present study indicated that the acid increased the rate of cells to enter into the mitotic phase in each crypt. Considering that length of DNA-synthesizing phase (S-phase) of the cell cycle of 6 to 9 hours and that of mitotic phase (M-phase) of 0.4 to 0.7 hours in hindgut epithelium (APPLETON et al., 1980), the effect of butyric acid should be not on the resting phase (G0/G1-phase) but on late S-phase or on the G2-phase just after the synthesis of DNA. However, the present results on the effect of n-butyric acid do not agree with our previous results (SAKATA, 1987) that the stimulatory effect of SCFA took 1 to 3 days to appear and required higher dose suggesting the effect of SCFA on G0/G1-phase of the cell cycle.

These two lines of results seem to suggest that the trophic effect of SCFA could be mediated by different mechanisms: rapidly activated local mechanism, rapidly activated systemic mechanism, and slowly activated and long-lasting systemic mechanism.

Methodologically, measurable CCPR and response to butyric acid clearly indicated that the tissue retained its proliferative activity and responsiveness to external chemical stimuli. These results seem to encourage the use of short-term culture of pig colonic mucosa for the study of local trophic effect of SCFA.

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Expression of estrogen and androgen receptor in the bovine gastrointestinal tract

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Summary

Reproductive and maturational nutritive needs are examples for situations in which alterations in circulating concentrations of sex steroids are associated with changes in gastrointestinal function. In order to investigate whether there is a causal relationship between sex steroids and gastrointestinal function, we aimed to investigate the responsiveness for androgens and for estrogens of the bovine gastrointestinal tract.

Using Northern blot analysis, estrogen receptor (ER) mRNA was detected in rumen tissue. Comparing the ER expression in rumen from females of different reproductive stages, we found that no differences related to cycle stage, pregnancy or parturition could be detected. In contrast, the ER expression rates in the uterus of the respective animals showed the same dependency of reproductive stage as demonstrated earlier for the ER protein, indicating that there might be a tissue specific regulation of ER. By in-situ hybridization of rumen tissue sections the expression of ER was localized in the epithelium of the papillae. In the muscular layer no positive signals for ER mRNA were observed.

Above rumen, the presence of ER and androgen receptor (AR) mRNA was determined in various intestinal tissues using reverse transkription (RT) and polymerase chain reaction (PCR). Primers were selected from the bovine androgen and estrogen receptor sequence to amplify parts of the sequence coding for the hormone binding part of the respective receptor. The PCR amplificates were subsequently electrophoresed on 1 % agarose gels and visualized by ethidium bromide staining.

ER mRNA expression was demonstrated in reticulum, omasum, abomasum, duodenum, jenunum, ileum, caecum and colon. AR mRNA expression was not determined in the forestomaches, but was present in all intestinal segments investigated.

To assess AR and ER as functional proteins, ligand binding studies were performed in the cytosolic fraction of gastrointestinal tissues using ^{3}H-methyltrienolone or ^{3}H-estradiol as ligands in a charcoal assay system. Specific binding for estradiol was observed in rumen and abomasum as well as in duodenum, ileum and colon; the concentrations measured were between 0.4 and 2.2 fmol/mg cytosolic protein. AR was present in all intestinal segments investigated (0.2 to 0.7 fmol/mg cytosolic protein). These concentrations are comparable to those observed in skeletal muscle. The functional relevance of sex steroid hormone receptors in the gastrointestinal tract as well as their regulation remains to be elucidated.

SAUERWEIN, H., M. PFAFFL, K. HAGEN-MANN, A. MALUCELLI und H. H. D. MEYER (1995): **Expression des Östrogen- und Androgen-Rezeptors im Gastrointestinaltrakt des Rindes.** Dtsch. tierärztl. Wschr. **102,** 164–168

Zusammenfassung

Der sich mit Reproduktion und Entwicklung verändernde Bedarf an Nährstoffen ist ein Beispiel für Situationen, in denen Veränderungen der Sexualsteroidhormonkonzentrationen in der Zirkulation mit Veränderungen der gastrointestinalen Funktionen einhergehen. Um zu prüfen, ob diese Zusammenhänge zwischen den Sexualsteroiden und der Gastrointestinalfunktion kausaler Natur sind, war es unser Ziel, die Ansprechbarkeit des bovinen Gastrointestinaltrakts für Östrogene und Androgene zu untersuchen.

Die mRNA des Östrogenrezeptors (ER) wurde in Pansengewebe mittels Northern blot Analyse nachgewiesen. Ein Vergleich der ER Expression im Pansen von weiblichen Tieren unterschiedlichen reproduktiven Status erbrachte keine Unterschiede zwischen verschiedenen Zyklusstadien, Gravidität und Postpartalphase. Im Gegensatz dazu war im Uterusgewebe dieser Tiere eine Abhängigkeit der ER mRNA Expression vom reproduktiven Status gegeben, die auch bereits in früheren Arbeiten für das ER Protein berichtet wurde; diese Befunde deuten auf eine gewebespezifische Regulation des ER. Durch in-situ-Hybridisierung wurde die ER Expression im Pansen lokalisiert: ER mRNA wurde ausschließlich im Zottenepithel beobachtet; in den Muskelschichten wurden dagegen keine ER mRNA Signale festgestellt.
Außer Pansen wurden auch andere gastrointestinale Gewebe auf die Anwesenheit von ER und Androgenrezeptor (AR) mRNA geprüft; dies geschah mittels reverser Transkription (RT) und anschließender Polymerasekettenreaktion (PCR). Aus der bovinen AR und ER Sequenz wurden Primer so gewählt, daß die Amplifizierung der Sequenzen, die jeweils für den hormonbindenden Teil des Rezeptormoleküls kodieren, gewährleistet war. Die PCR-Amplifikate wurden anschließend durch Agarosegelelektophorese getrennt und mit Ethidiumbromid sichtbar gemacht. Für AR mRNA wurden die Vormägen nicht untersucht; in den verschiedenen Darmabschnitten wurde AR mRNA durchgängig detektiert. Um AR und ER als funktionelle Proteine nachzuweisen, wurden in den Cytosolfraktionen aus den verschiedenen Geweben Ligand-Bindungsstudien mit ^{3}H-Methyltrienolon (für AR) und ^{3}H-Östradiol-17-β (für ER) in einem Aktivkohle-Testsystem durchgeführt. Spezifische Östrogenbindung wurde in Pansen, Labmagen, Duodenum und Colon beobachtet; die gemessenen Konzentrationen lagen zwischen 0,4 und 2,2 fmol/mg cytosolischem Protein. AR wurde in allen Darmabschnitten nachgewiesen (0,2–0,7 fmol/mg cytosolischen Protein). Diese Konzentrationen sind denen in Skelettmuskulatur vergleichbar. Zu klären bleibt die funktionelle Bedeutung von Sexualsteroidhormonrezeptoren im Magen-Darm-Trakt und deren Regulation.

There is increasing evidence that sex steroids do not only exert effects on those organs related to reproductive function but also on tissues that were not regarded as classical target tissues so far. Although the marked effects of gonadal steroids on the metabolic state of the animal i. e. on growth rates, protein accretion and muscle growth are well known and are used for example as anabolic agents to improve performance, many of these effects were believed to be indirect i. e. mediated by other hormones. Since the discovery of specific receptors for estrogens and androgens in tissues that were regarded to be only indirectly influenced by gonadal steroids, a direct mode of action has to be taken into consideration. This is for example true for skeletal muscle in which estrogen receptors (ER) and androgen receptors (AR) have been described in live stock (MEYER and RAPP, 1985; SAUERWEIN and MEYER, 1989; SNOCHOWSKI et al., 1981). More recently, not only intermediary metabolism but also absorption of nutrients, determined by gastrointestinal function has been related to potential influences of gonadal steroids. For estrogens, a number of intestinal functions seems to be affected by alterations in circulating estrogen concentrations (THOMAS and IBARRA, 1987; THOMAS et al., 1988; DAHIYA and BRASITUS, 1987; CHENG and BJERKNER, 1988). For androgens no such investigations have been done. We aimed to determine as to whether the gastrointestinal tract of ruminants does contain ER and AR both at the transcriptional as well as at the translational level. To localize ER and AR expression, different segments of the bovine gastrointestinal system were compared and in vitro hybridisation was applied to investigate ER expression in rumen.

Materials and Methods

Animals and tissue sampling

Rumen tissue samples were collected from 11 heifers at different stages of the oestrous cycle, from one pregnant cow (second trimester, estimated by crown-rump length of the fetus and additional criteria according to SINOWATZ and RÜSSE, 1991) and from 3 cows during the post partum period, i. e. within 1 week after parturition.

The cyclic stage of the heifers was determined by macroscopic evaluation of the ovaries and the uterus as described by IRELAND et al. (1978). Additionally, samples from the entire gastrointestinal tract were taken from 4 eleven months old males, 2 of them being castrated within the first week of life.

All animals were slaughtered at the local abbatoir by stunning with a captive bold and subsequent exsanguination. The gastrointestinal tract was removed within 25 min after killing the animals. Segments (≈ 10 cm^2) were dissected from rumen, reticulum, omasum, abomasum, duodenum, jejunum, caecum, colon and rectum and were thoroughly washed with cold water. The segments were then cut into small pieces (≈ 1 cm^2) and were snap-frozen in liquid nitrogen. Until further processing the samples were kept at –80 °C.

For in-situ-hybridization studies small pieces (0.5 cm^2) were cut from rumen tissue and were fixated in 4 % paraformaldehyde for several hours. The tissue slices were then washed twice in PBS (pH 7.2) for 5 minutes and were incubated overnight in PBS/0.5 M sucrose at 4 °C. After embedding in OCT Medium (Slee) at –18 °C the tissue samples were stored at –20 °C.

Preparation of RNA from gastrointestinal tissues

RNA was extracted from GuSCN tissue homogenates by stepwise ethanol precipitation according to the protocol from CHIRGWIN et al. (1979).

Northern analysis of estrogen receptor (ER) mRNA

10 µg of tissue RNA were electrophoresed on 0.8 % agarose in the presence of 1.9 % (v/v) formaldehyde. The RNA was then blotted onto a Nylon membrane (Nylon N$^+$, Amersham) by capillary transfer (PAPANDRIKOPOULOU and HAHN, 1991) and baked for 2 hours at 80 °C. Standard prehybridization and hybridization conditions were employed (SAMBROOK et al., 1989). Briefly, hybridization was carried out using a 1184 bp PCR generated fragment of the bovine ER sequence (details ar given below), which was labeled with [^{32}P] deoxy-CTP (Amersham) using random priming (Multi Prime DNA labeling kit, Amersham). After hybridization overnight at 42 °C, the blots were washed two times with 2 × SCC/0.1 % sodium dodecyl sulfate (SDS) for 20 minutes at 42 °C and two times with 1 × SSC/0.1 % SDS for 20 minutes at 37 °C. The blots were then exposed to X-ray film using intensifying screens at 4 °C for 2 to 7 days. The autoradiographs were evaluated by laser densitometry.

Reverse transcriptase-polymerase chain reaction (RT-PCR)

For the detection of ER transcripts from RNA samples by RT-PCR, a 140 bp fragment was selected for amplification based on the published partial sequence of the bovine ER (EMBL, X66841). This fragment is equivalent to exon 5 encoding the amino acids 366 to 412 of ER that are part of the hormone binding domaine of the ER molecule. A computer algorithm (OLIGO, MedProbe, Norway) was used to choose the sense (5' GGC TTT GTG GAT TTG ACC CTC-3') and antisense (5'CTG TCC AAG AGC AAG TTA GGA-3') primers framing this region; the obligonucleotides were synthesized commercially (MWG Biotech, Ebersberg, Germany). Additionally, a 1164 bp ER fragment was amplified from bovine uterine RNA and was used as a ER probe in the Northern blot analyses. This fragment spans exons 1 to 5, representing amino acid 18 to 412, that form the highly conserved hormone binding part of the ER. The sequences of the sense and the antisense primer were 5'-ATC CAA GGG AAC GAG CTG GA-3' and 5'-CTG TCC AAG AGC AAG TTA GGA-3', respectively.

Briefly, the RT reaction was conducted in the presence of total RNA (0.5 µg), antisense primer (20 pmol), RNAsein (20 U) and 50 U M-MLV reverse transcriptase (Gibco) in a final volume of 20 µl for 30 min at 52 °C for both the 1184 bp and the 140 bp fragment. The enzyme was then inactivated by heating for 1 min at 99 °C. PCR reagents, Taq DNA polymerase (2.5 U; Perkin Elmer Cetus, Überlingen, Germany) and sense primer (20 pmol) werde added to a final volume of 80 µl. The 1184 bp ER fragment was amplified in 40 cycles (1 min 10 s 94 °C, 1 min 55 °C, 2 min 72 °C); for the 140 bp ER fragment 35 cycles (1 min 93 °C, 30 sec 58 °C, 30 sec 72 °C) were performed.

To detect androgen receptor (AR) transcripts, a 172 bp fragment was selected for amplification based on the published sequence of the bovine AR (EMBL 69932). The primer sequences were 5'-TTG ATT TTT CAG CCC ATC CAC TGG A-3' for the antisense primer and 5'-CCT GGT TTT CAA TGA GTA CCG CAT G-3' for the sense primer. The selected AR fragment spans Exon F framed by parts of Exon E and G. The amino acids 766 to 823 that are part of the hormone binding domain are encoded by this particular fragment. For AR RT-PCR, the same reagents were used as described for ER; the RT reaction was performed for 30 min at 47 °C. For the PCR reaction 30 cycles (1 min 94 °C, 30 s 58 °C, 30 s 72 °C) were applied. The 140 bp ER and the 172 bp AR amplificates were visualized by electrophoresis on 1 % agarose gels stained with ethidium bromide.

In situ hybridization

6 to 9 µm sections of rumen tissue were used for in situ hybridization (AUSUBEL et al., 1991). Sections were mounted on polylysine coated slides; after incubation in 4 % paraformaldehyde (in PBS) for 20 min, the slides were washed three times in PBS and were then incubated for 1 min in ascending ethanol concentrations (30 %, 60 %, 80 %, 97 %). The so treated sections can be stored at –20 °C. Before hybridization sections were rehydrated, denaturated at 55 °C in 2 x SSC for 20 min and were then treated with proteinase-K and acetylated with acetic anhydride. After these treatments, the sections were dehydrated through a series of ethanol. The [^{35}S] CTP ER antisense cRNA probe was synthesized using a reaction mixture that contained T7 RNA polymerase, a linearized cDNA template (1184 bp) and [^{35}S] CTP αS (Amersham). The control sense probe was similarily synthesized but with the SP6 polymerase. Hybridization with an antisense or sense probe was carried out at 50 °C overnight. After washing and ethanol dehydration the slides were coated with Ilford autoradiographic emulsion, dried and then stored at 4 °C. After developing, the slides were counterstained with Harris hematoxylin.

Estrogen and androgen binding in cytosols prepared from gastrointestinal tissues

Cytosolic fractions were prepared from frozen tissue sections as described by SAUERWEIN & MEYER (1989). For ER analysis the cytosols were incubated with 2nM ^{3}H-Estradiol-17β (Amersham); for AR analysis 2nM ^{3}H-Methyltrienolone (R-1881, NEN) were used to determine total binding. Nonspecific was measured in parallel incubations with a 100-fold surplus of the respective ligand. After incubation overnight at 4 °C, unbound ligand was separated by charcoal treatment (SAUERWEIN & MEYER, 1989) and aliquots of the cytosols were either counted directly in Xylofluor (Baker Chemicals), or were further analysed after adsorption to Heparin-Sepharose as described by SAUERWEIN and MEYER (1989).

Results

Regulation of rumen ER mRNA by alterations of endogenous steroid concentrations

Northern blot analysis of rumen RNA showed the presence of a single transcript with an ap-

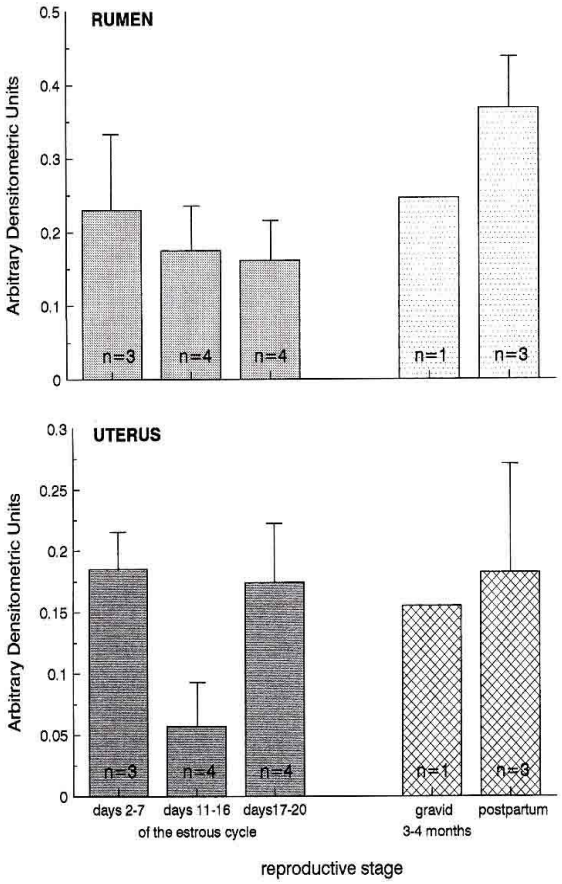

Fig. 1: Quantitation of ER mRNA expression in rumen and uterine tissue from heifers and cows at different reproductice stages. Northern blot analyses were performed as described in Materials and Methods, the densiotometric units obtained from the autoradiogram are given. Animals were grouped into three different stages of the estrous cycle; day 0 is defined as being the day of estrous.

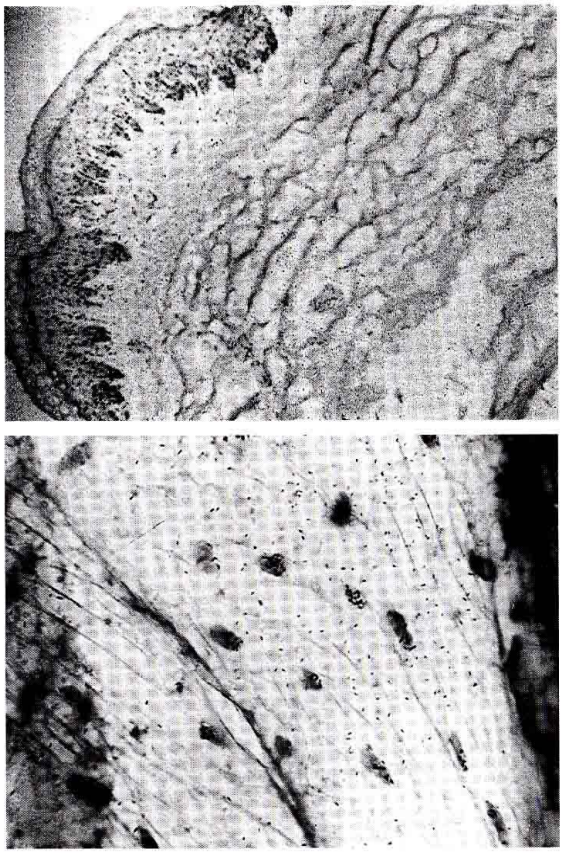

Fig. 2a: In situ hybridization of Er mRNA in rumen tissue sections. Survey of the kryosection.
Fig. 2b: Magnification of the border between tunica mucosa and tunica submucosa; ER mRNA positive grains are visible in the cytosols and the nuclei.

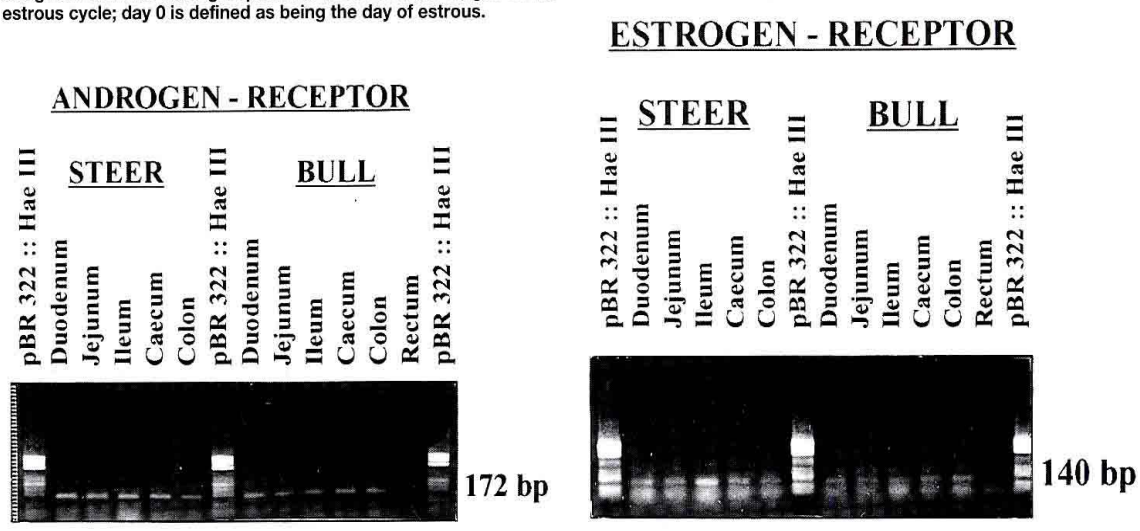

Fig. 3: Analysis of bovine ER and AR mRNA in various intestinal tissues from one bull and one steer.
20 µl of the respective RT-PCR reaction were electrophoresed in agarose gels and visualized with ethidium bromide.

proximate molecular size of 4.0 kb. This transcript was detectable in rumen tissue of all samples investigated i. e. in all physiological stages. Densitometric analysis of the transcript showed no significant differences between the different stages of the estrous cycle. In rumen from postpartal cows (i. e. 1–4 days postpartum) the ER expression rates were higher than in animals of the mid and late cycle stage ($p \leq 0.05$; Fig. 1a). This is in contrast to parallel observations in uterine tissue of the respective animals, were a marked dependency of cyclus stage was evident (Fig. 1 b). There were no differences between the ER expression rates in uterine tissue of animals in the early, the late cyclus stage and during the postpartal phase. In contrast, the ER transcription was significantly reduced during days 11 to 16 of the estrous cycle compared to all other reproductive stages investigated.

Histological localization of ER mRNA in rumen tissue

In situ hybridization of rumen sections with ^{35}S antisense RNA Probe showed that ER expression is limited to the inner mucosal propria as classified according to GRAU & WALTER (1967), whereas in the muscular layer no ER signals were observed (Fig. 2a and b). The specifity of the hybridization signal was confirmed by the sense probe that yielded no stain.

Table 1: ER and AR concentrations* in cytosols prepared from different segments of the bovine gastrointestinal tract.

Tissue	ER (fmol/mg cytosolic protein)	AR (fmol/mg cytosolic protein)
rumen	1.0	n.i.
reticulum	n.d.	n.i.
omasum	n.d.	n.i.
abomasum	0.4	n.i.
duodenum	2.0	0.2
jejunum	n.d.	0.7
ileum	1.5	0.2
caecum	n.d.	0.4
colon	2.2	0.6
rectum	n.i.	0.38

***Receptor concentrations were determined by calculating the difference between total binding (B_o) and nonspecific binding (NSB) determined by incubation with ^{3}H-Estradiol-17β or ^{3}H-Methyltrienolone alone (B_o) or including a 100-fold surplus of the respective unlabelled ligand (NSB).**
n. d.: not detectable
n. i.: not investigated

Expression of ER and AR mRNAs in different segments of the bovine gastrointestinal tract

ER mRNA was detectable by RT-PCR in RNA prepared from rumen, reticulum, omasum, abomasum as well as from duodenum, jejunum, caecum and colon from young bulls and steers, AR mRNA could be detected by RT-PCR in duonenum, jejunum, caecum, colon and rectum. In Fig. 3 representative agarose gels with the electrophoresed ER and AR RT-PCR amplificates from intestinal tissues from one bull and one steer are shown.

Estrogen and androgen binding in cytosols prepared from different segments of the bovine gastrointestinal tract

ER and AR concentrations in different gastrointestinal segments from a 11 months old steer are summarized in Table 1. Androgen receptor concentrations were determined in intestinal sections only. ER was detectable in rumen and abomasum, whereas no significant specific binding could be detected in reticulum and omasum. Low but significant amounts of specific androgen binding were determined in all intestinal segments investigated.

Discussion

Our results demonstrate that specific receptors for estrogens and androgens are present in different segments of the gastrointestinal tract of a ruminant species i. e. in bovines.

In monogastric species the presence of estrogen receptors in the gastrointestinal tract has been reported in rat intestinal mucosal cells (ARJMANDI et al., 1993), in IEC-6 cells, i. e. a non-transformed line of cells isolated from rat small intestinal crypts (THOMAS et al., 1993), in rabbit upper intestinal mucosa (BOGDARIN, 1991), in baboon gastrointestinal tract (WINBORN et al., 1987) and in human gastric mucosal tissue (WU et al., 1990) and human colorectal mucosa specimens (SCAMBIA et al., 1991; MEGGOUH et al., 1991; DI LEO et al., 1992; RUSSO et al., 1992; MARUGO et al., 1985).

The concentrations of ER and AR protein we measured in the different segments of the bovine gastrointestinal tract were much lower than those documented for classical estrogen or androgen responsive tissues, e. g. bovine uterus (40–420 fmol/mg cytosolic protein; MEYER et al., 1988) or rat prostate (170 ± 20 fmol/mg cytosolic protein; KRIEG et al., 1976). Compared to the ER and AR concentrations measured in intestines of monogastric species, the ER concentrations in the ruminant gastrointestinal tract were in the same order of magnitude: HENDRICKSE et al. (1993) reported ER concentrations between 0.6–3 fmol/mg in human colorectal mucosa. In human gastric mucosal tissue ER and AR concentrations of 0.9 to 87.9 fmol/mg and 1.5 to 73.5 fmol/mg, respectively (WU et al., 1990). MEGGOUH et al. (1991) reported higher binding capacities for ER and AR in human colorectal mucosa (148 ± 67 fmol/mg and 19 ± 9 fmol/mg). However, the concentrations measured herein for ER and AR in the bovine gastrointestinal tract are comparable to those determined in bovine skeletal muscle (ER: 0.3–2 fmol/mg, MEYER & RAPP, 1985; AR: 0.2–0.8 fmol/mg, SAUERWEIN & MEYER, 1989).

The size of the ER transcript we observed in Northern blot analysis is in agreement with the ER transcript size demonstrated by PARL et al. (1987) for human, rat and calf uterus. The changes of ER mRNA concentrations reported herein for uterine tissue throughout the oestrous cycle are in parallel to those described by MEYER et al. (1988) for the receptor protein. Both the present and the quoted study demonstrate that the highest ER concentrations (mRNA and protein) are found at the beginning and at the end of the estrous cycle. A nadir is reached during the days 14 to 16 of the cycle. The lack of parallel changes in rumen ER mRNA expression rates during the estrous cycle indicates that ER might be differentially regulated in different tissues. This concept is further supported by the increased ER mRNA concentrations in rumen of postpartal animals, which is absent in uterus.

The results of the in-situ hybridization studies in rumen tissue slices demonstrate that ER expression is limited to the surface epithelium of the papillae, whereas in deeper layers no expression could be detected. This localization indicates that ER might be functional in absorbtive processes rather than in rumen motility. However, any notions on physiological, direct effects of estrogens on gastrointestinal tissue remain speculative; there is some evidence that estrogens might influence calcium transport in rats (ARJMANDI et al., 1993), possibly by affecting the intestinal responsiveness to 1,25-dihydroxyvitamin D_3 (GENNARI et al., 1990). Another aspect of estrogenic effects on the intestine is the absorption of free fatty acids which is altered by estrogen treatment; a concomitant change in free fatty acid binding protein levels has been reported in rabbit small intestine mucosa (BOGDARIN, 1990). In rats an increase of intestinal low density lipoprotein (LDL) receptor mRNA has been demonstrated after treatment with ethinylestradiol (STAELS et al., 1990). Concerning a potential role of androgens in regulating gastrointestinal function, no data are available for physiological conditions. Epidemiological and experimental studies suggest that androgens might influence colonic carcinogenesis, suppressing epithelial proliferative abnormalities (IZBICKI et al., 1990). However, all hypotheses on the functional role of sex steroids regulating gastrointestinal function have been developed in in-vivo experiments. In order to move on our understanding of the nature of alterations in gastrointestinal tissue induced by sex steroids, detailed investigations in vitro are necessary to identify the specific pathways that link receptor stimulation and physiological effect.

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