Functional Histology of Endocrine Cells in Bovine Intestine at Different Developmental Stages

（各発達段階の牛の腸における内分泌細胞の機能組織学的研究）

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Chapter 1

General introduction

1.1. Overview

Endocrine cells distributed in the gastrointestinal tract produce various kinds of signal materials to regulate diverse functions of gut such as secretion, secretion and motility (65, 78). Many studies have elucidated the distribution of various endocrine cells in the gastrointestinal tract of different mammals including domestic animals such as cat (40), cattle (39), sheep (12), horse (38), pig (36), water buffalo (8, 52), and camel (16). These studies have aimed to demonstrate their distribution and relative frequencies in different parts of the gastrointestinal tract and to understand their functional roles in the digestive system. Several peptides produced by endocrine cells were newly identified in recent decades. In addition, some kinds of gut peptides including PYY and GLP-1 were distinguished to participate in the control mechanism within the gut as well as outside it such as nutritional and food intake regulation (30) which has high significance in the domestic animal husbandry.

The ruminant has drastic changes twice in nutrition, from fetus to postnatal, and suckling to herbivorous. However, no study has been focusing on the distribution of gastrointestinal endocrine cells in different developmental stages both at the pre- and post-natal stages. The present study was aimed to update the data concerning the regional distribution and

relative frequency of endocrine cells in the gastrointestinal tract of cattle from early fetus to adult cow. Furthermore, it is intended to emphasize the regulatory role of the large intestine because ruminants are usually noticed on the stomach.

The present study investigated the distribution of endocrine cells in the gastrointestinal tract of cattle at pre- and post-natal stages with different developmental stages (Chapter 2). It was focusing on the large intestine by the precise investigations on the detailed segments of the convoluted gut (Chapter 3). Furthermore, the detail study was done on the developmental plasticity of the colocalization pattern of gut hormones (Chapter 4). The present study would play a key role to understand the control mechanism of the bovine digestive tract from the view points of the developmental plasticity of the regulatory systems.

1.2. Chemical Signals including Regulatory Peptides of the Gut

In this section, brief descriptions are made on the characteristics of chemical signal materials which are dealt in the present study.

Peptide YY (PYY) is a member of PP family along with pancreatic polypeptide (PP) and neuropeptide Y (NPY) (24). These three peptides share similar chemical structure based on the number of amino acids (36-amino-acid residue peptides). The regional distribution of PYY has been studied in a number of different species using immunohistochemistry and

radioimmunoassy of tissue extracts. Lundberg et al. (53) first localized PYY to a population of endocrine cells in the intestinal mucosa of a variety of species, including man. PYY immunoreactive cells are present in the distal small intestine, colon and rectum, but were rare and absent in the stomach and duodenum. Immunohistochemical and tissue extraction studies subsequently confirmed the distal pattern of distribution of PYY in a number of species (1, 21, 22, 23, 81). Recent studies have demonstrated that $PYY_{1\text{-}36}$ and $PYY_{3\text{-}36}$ are the major molecular forms of the peptide in tissue and in the circulation (15). PYY is present in high concentration in mucosal extracts of the human ileum, colon and rectum (1). PYY is a multifunctional hormone; the main function is to inhibit gastric emptying and mediates the effects of ileal fat on gastric motility as indicated in rodents. PYY has been identified as a peptide involved in the ileal brake (50, 64). The temporal pattern of PYY release is different from that of other gastrointestinal peptides with suggested roles in feeding control. Plasma levels of PYY gradually increase after meal initiation, reaching a peak at about 60 minutes, and high levels are maintained for a number of hours after a meal (61).

PP is a structurally related member of the PP family. PP is a 36 amino acid peptide predominantly expressed in the endocrine pancreas. However, rare PP-immunoreactive endocrine cells have been described in some but not all species (20). PP cells have been demonstrated immunohistochemically in the pancreas of a great variety of species (31, 43, 44, 54, 84). Beside pancreas,

PP cells are also present in the intestinal mucosa, especially in the ileum and colon (10, 47, 49). PP secretion is stimulated by food ingestion and exercise, and vagal tone is an important determinant regulating PP secretion in rodents and human subjects (14). The main function of PP is to inhibit pancreatic exocrine secretion and relax the gallbladder (54). PP is considered as food intake regulator (14) and also plays an important role in negative feedback control of the pancreas, inhibiting neurally mediated stimulation of this gland (54).

Glucagon-like peptide-1 (GLP-1) belongs to a large family of glucagon (34, 37), which is secreted from L-cells of the gastrointestinal mucosa (34, 37, 79). GLP-1-producing L-cells have been identified in the jejunum, ileum and colon, with the highest cell densities reported in the ileum and colon (34, 37,). GLP-1 is secreted after nutrient ingestion and stimulates insulin secretion in a glucose dependent manner (42, 56, 63). GLP-1 functions as a gut hormone that helps regulate blood glucose and feeding behavior in mammals. GLP-1 is involved in the ileal brake mechanism. Gastric inhibitory and intestinal motility actions of ileal nutrients are partially mediated through GLP-1 release. On the other hand, GLP-1 increase insulin release and reduce appetite in human (30). GLP-1 is considered to be a biologically and therapeutically interesting hormone, because of its potential anti-hyperglycemic effects in Type 2 diabetes (30).

Chromogranin (CG) is an acidic protein widely distributed in entero-endocrine cells and in other members of the paraneuron family. Therefore, CG has been claimed as a common "marker" of all neuro-endocrine cells (13, 25, 28). As to the gastro-entero-pancreatic (GEP) endocrine system, previous studies localized CG in a variety of endocrine cells (11, 13, 17, 25, 66, 85, 90). Evidence increases that CG in this location may serve as a precursor for other hormones to be identified (18, 19, 35, 41, 71, 70, 73, 81). The main physiological function of CG is not yet clearly understood, but studies suggested that CG and other related proteins might function in the organization of the granule matrix (e.g. binding of calcium), in the processing or packaging of peptide hormones or their precursors, and possibly in the regulatory mechanisms after secretion (41, 72, 91).

Serotonin (Ser) is a regulatory amine of mucosal enterochromaffin (EC) cells. EC cells constitute the largest endocrine cell population in the gastrointestinal tract and produce over 90% of all the Ser synthesized in the body (4). Ser plays an important role in the control of gastrointestinal smooth muscle contraction and epithelial secretion (26).

Somatostatin (Som) is a peptide hormone synthesized and secreted by endocrine D cells. Som cells are widely distributed in the gastrointestinal mucosa, pancreatic islets and in the autonomic and central nervous system (6). Som cells are particularly abundant in the corpus and antral regions of the stomach (30, 46). Som has predominantly inhibitory action on a variety of

biological activities in different parts of the body such as the secretion of pituitary hormones, release of a wide variety of peptides hormones from all regions of the gut and endocrine pancreas, gastric acid secretion, pancreatic exocrine secretion, intestinal absorption, proliferation and gastrointestinal motility (51, 57).

1.3. General Materials and methods

The present experiments on animals were carried out in accordance with the guidelines of the Committee of Animal Experiments of Obihiro University of Agriculture and Veterinary Medicine (No. 17-51, 20-95, 21-108, 23-46). Totally forty-eight Holstein cattle in different pre- and postnatal stages were used in this study. Prenatal animals were obtained at the autopsy of their euthanized mothers for pathological inspection. The prenatal animals (fetuses) were divided into 3 developmental stages according to their crown-rump length (CRL): early fetus (CRL: 20 - 40 cm), mid fetus (CRL: 41 - 70 cm), and late fetus (CRL: 71 - 100 cm). The postnatal animals were divided into four developmental stages according to their age: suckling calf (1 - 2-week-old and 5-7-week-old), 2. weaning calf (2-month-old), 3. weaned calf (7-month-old and 10-month-old), and 4. adult cattle (1 - 8-year-old) (Table 1. 1). The postnatal animals were exsanguinated from the carotid artery under the anesthesia (xylazine hydrochloride 0.3mg/kg and thiopental 7mg/kg) and dissected.

Tissue samples were taken from the whole gastrointestinal tract; esophagus (upper and lower part), rumen, reticulum, omasum, oxyntic (body) and pyloric parts of abomasum (glandular stomach), duodenum (cranial part), jejunum (mid part), ileum (terminal part), cecum (body), ascending colon (proximal loop, centripetal turns, central flexure, centrifugal turns, distal loop), transverse colon, descending colon, sigmoid colon (cranial to the rectal ampulla) and rectum (ampulla and just cranial to the anorectal line). The samples were then fixed in the Bouin's solution at room temperature (RT) for 24 hours (h).

The obtained samples were trimmed into small pieces and were dehydrated in ascending concentration of ethanol, cleared by xylene and embedded in paraffin (Paraplast Plus®, Kendall, MA, USA). The samples were then cut at 1-4μm thickness used sliding microtome (Leica SM 2000 R, Germany, with blades S35 Feather, Japan), collected on gelatin coated slides and kept overnight in the warming cupboard (40°C).

Hematoxylin and eosin (HE) staining was performed to investigate the general histological inspection of the gastrointestinal tract.

Immunohistochemical staining was applied for the detection of targeted endocrine cells with specific antibodies. The sections were deparaffinized and rehydrated. The endogenous peroxidase activity was blocked with 0.3% H_2O_2 in methanol, and washed three times by phosphate buffer saline (PBS, pH 7.4). In order to block nonspecific bindings, the sections were incubated with

normal goat serum (1:50, S-1000, Vector Laboratories Inc., Burlingame, CA, USA) for 30 min at RT. The sections were again washed by PBS and incubated with targeted primary antisera and kept overnight in refrigerator (approximately 5 °C). The dilution tests of primary antiserum were performed to get the optimal dilution for the best condition of specific immunoreactivity before applying. In the second day, the sections were washed by PBS and incubated with secondary anti-rabbit IgG raised in goat (1:200, BA-1000, Vector Laboratories Inc.) for 30 min at RT. After washing again three times by PBS, the sections were further incubated with the avidin-biotin peroxidasae complex (ABC) method using Vectastain *Elite* ABC kit (Vector Laboratories, Inc.). Immunoreaction sites were revealed by Tris buffer (pH7.4) containing 0.02% 3, 3'-diaminobenzidine tetrahydrochloride and 0.06% H_2O_2. The sections were lightly counterstained with hematoxylin and dehydrated in ascending concentration of ethanol, cleared by xylene and then mounted.

Each of immunoreative cells in different gastrointestinal portions were observed under the conventional light microscope and photomicrographs were taken by a digital camera (DS-5M, Nikon, Tokyo, Japan). For the cell counting, special counting chamber with the counting grid was fixed in the eyepiece of the microscope and the number of immunoreactive cells was estimated in ten random unit areas per section of each gastrointestinal part. The square for 1 unit area was measured as 25μm x 25μm=625μm^2. Cells

only with clear and identifiable nucleus were counted and recorded for further analyzing.

The obtained data were arranged and calculated by Microsoft Excel 2010, and the values were statistically analyzed by GraphPad Prism (version 5.00, GraphPad Software, San Diego, California, USA) using one-way ANOVA followed by Tukey's post hoc multiple comparison tests. The values were expressed as Mean ± SEM and differences were considered statistically significant at $P < 0.05$.

Table 1.1. The detail information of cattle used in the present study

Developmental stage		Number of animals	
	CRL/age	Chapter 2 and 4	Chapter 3
Early fetus	20 - 40 cm	9	
Mid fetus	41 - 70 cm	8	
Late fetus	71 - 100 cm	5	
Suckling calf	1 - 2 weeks	5	
	5-7 weeks		4
Weaning calf	2 months	6	
Weaned calf	7 months		3
	10 months	3	
Adult	1 - 8 years	5	
Total		41	7

CRL: Crown-rump length

Chapter 2

Immunohistochemical study on the ontogenetic development of the regional distribution of peptide YY, pancreatic polypeptide, and glucagon-like peptide-1 endocrine cells in bovine gastrointestinal tract

2.1. Introduction

Endocrine cells dispersed in the gastrointestinal tract comprise the largest endocrine organ of the body. It is composed of more than 20 different cell populations (78). These gastrointestinal endocrine cells synthesize and release various types of gastrointestinal hormones to regulate the digestive system and the body (78). In recent decades, peptide YY (PYY) and glucagon-like peptide-1 (GLP-1) have been proven to influence feeding mechanisms in rodents and human (24, 77, 93).

PYY is a member of the pancreatic polypeptide (PP) family (83), which includes neuropeptide Y (NPY) and PP, all consisting of 36 amino acids. NPY is found only within the brain and peripheral nervous system, while PYY and PP are found mostly within endocrine cells of the gastro-entero-pancreatic system. PYY is synthesized and released from the endocrine cells primarily located in the ileum, colon, and rectum and its level is considered to be higher in colon and rectum (78, 83). PP is synthesized and secreted by

populations of cells located at the periphery of pancreatic islets (58, 76). PP cells are also distributed at a low level in the exocrine pancreas and in the gastrointestinal tract, mainly in the colon and rectum (39, 78, 83).

GLP-1 is one of the members that are produced from proglucagon sequence (37) Large numbers of GLP-1 endocrine cells have been identified in jejunum, ileum, and colon (77, 93). It has been reported that PYY and GLP-1 have important roles in the regulation of appetite and feeding, and have an additive effect on feeding control in rodents and human (59, 77, 93). However, the physiological importance of these three peptides in ruminants is yet to be determined (61, 62). There have been no studies on the ontogenetic development of these three kinds of endocrine cells in domestic animals. The present study was conducted to reveal the regional distribution and relative frequencies of PYY, PP, and GLP-1 endocrine cells in the gastrointestinal tract of pre- and postnatal cattle.

2.2. Materials and methods

In this study, twenty-one Holstein cattle in the following seven ontogenetic stages were examined. The detail of animals and method of sampling were described in the section of general materials in Chapter 1. Brief explanations are follows: early fetus: 20-40 cm in crown-rump length (CRL, n=3), mid-fetus: CRL 41-70 cm (n=3), late fetus: CRL 71-100 cm (n=3), suckling calf (1-2-week-old, n=3), weaning calf (2-month-old, n=3), weaned

calf (10-month-old, n=3), and adult (1-8-year-old, n=3). Tissue samples were obtained from esophagus, rumen, reticulum, omasum, abomasum, duodenum (cranial part), jejunum (mid-portion), ileum (terminal portion), cecum (body), colon (central flexure), and rectum (terminal portion, just cranial to the anorectal line).

The detail method of paraffin section and immunohistochemistry were explained in the Chapter 1. The primary antisera used in this chapter were raised in rabbit against porcine PYY (1:10,000, IHC7173, Peninsula Lab. Inc., Belmont, USA), human PP (1:10,000, Y080, Yanaihara Institute, Inc., Shizuoka, Japan) and human GLP-1 (1:10,000, Y-320, Yanaihara Institute).

2.3. Results

The present study demonstrated the characteristic distribution of PYY-, PP-, and GLP-1-immunoreactive endocrine cells, whereas immunoreactivity for these peptides in the nervous system was not revealed. Immunoreactive endocrine cells were not found in the esophagus, rumen, reticulum, omasum, or abomasum of all seven ontogenetic groups. They were detected in the small and large intestines of all groups at different frequencies. The mentioned immunoreactive cells were mostly detected in the basal region of the intestinal crypt glands and a few numbers were also detected in the villus region, but none of them were detected in the duodenal glands. Most of the detected endocrine cells were open-type, which had spindle and oval shapes

with the presence of cytoplasmic processes ending with the lumen. Round and spherical shaped cells were also found rarely in different regions. The frequencies of PYY-, PP- and GLP-1-immunoreactive cells were vary depending on intestinal regions and perhaps food habits of animals at different developmental ages.

Dispersed PYY-immunoreactive cells were detected mostly in the large intestine, especially in the rectum, but only a few cells were detected in the small intestine at all stages. PYY-immunoreactive cells were detected in the basal regions of intestinal crypt glands and a few numbers were also detected in the villus glands. PYY-immunoreactive cells were mostly spindle shape with long cytoplasmic processes reached the intestinal lumen. Few cells with the oval, round and spherical shapes were also found in different regions.

PYY-immunoreactive cells were immunohistochemically detected abundantly in the prenatal (early, mid and early) stages. In early fetal stage, PYY-immunoreactive cells were not detected in the duodenum portion of the small intestine. They were detected from jejunum to rectum parts. The most abundant immunoreactivities were observed in the ileum (Fig. 2.1A) and rectum (Fig. 2.1B) parts. In mid fetal stage, PYY-immunoreactive cells were distributed almost similar to that of early stage, but their frequencies were higher in mid fetal stage. Very few PYY-immunoreactive cells were detected in the jejunum of mid fetal stage. PYY-immunoreactive cells were observed from moderate to numerous in the ileum (Fig. 2.2A), cecum (Fig. 2.2B), colon

(Fig. 2.2C) and rectum (Fig. 2.2D) parts of the mid fetal stage. PYY-immunoreactive cells were also detected in the late fetal stage; their frequencies in the late fetal stage were also similar to that of early and mid-fetal stages. However, in the late fetal stage, PYY-immunoreactive cells were also detected with a very low frequency in the duodenum portion.

The frequency and distribution of PYY-immunoreactive cells were low in the postnatal (suckling, weaning, weaned and adult) stages. PYY-immunoreactive cells were not detected in the duodenum of suckling, weaning, weaned and adult stages. Very rare PYY-immunoreactive cells were observed in the jejunum of suckling, weaning and weaned stages. However, PYY-immunoreactive cells were detected from the ileum to the rectum portions of all postnatal (suckling, weaning, weaned and adult) stages. In suckling stage, the distribution of PYY-immunoreactive cells was higher in the ileum (Fig. 2.3A) and rectum (Fig. 2.3B) portions. The distribution of PYY-immunoreactive cells was almost same in the jejunum, ileum, cecum and rectum of weaning, weaned and adult stages. However, high number of PYY-immunoreactive cells was observed in the rectum portions of weaned (Fig. 2.3C) and adult (Fig. 2.3D) stages.

The regional distribution of PYY-immunoreactive cells was significantly ($P < 0.05$) higher in the rectum at mid fetus (16.27 ± 1.71), suckling calf (3.76 ± 0.53), weaned calf (5.03 ± 0.33), and adult (4.20 ± 2.10) stages than in other intestinal segments (Table 2.1). However, no significant

differences among the intestinal segments were observed in early and late fetus and weaning calf stages (Table 2.1, Fig. 2.10-11).

With regard to ontogenetic stage, on the other hand, the frequency of PYY-immunoreactive cells was significantly ($P < 0.05$) higher in the ileum of the early fetus (7.60 ± 0.25) and the rectum of the mid fetus (16.27 ± 1.71) than at other stages, while in other regions of the gut, such a marked tendency was not observed (Table 2-1, Fig. 2. 12-13).

Open-type PP-immunoreactive cells were detected in the intestinal crypt gland with the spindle, oval and spherical shapes. In the small intestine, PP-immunoreactive cells were not detected in the duodenum part. However, very few PP-immunoreactive cells were detected in the jejunum of prenatal (early, mid and late fetal) and postnatal (suckling calf and weaned calf) stages. PP-immunoreactive cells were not detected in the jejunum of weaning calf and adult stages. PP-immunoreactive cell were mostly detected from the ileum to the rectum portions of all pre- and postnatal stages.

In the prenatal stages, the distribution of PP-immunoreactive cells was higher in the mid and late fetal stages. However, in the postnatal stages, they were detected with very low number from the ileum to the rectum portions. In the suckling stage, PP-immunoreactive cells were clearly observed in the ileum, colon and rectum portions (Fig. 2.4A-C). PP-immunoreactive cells, in the weaned and adult stages were mostly observed in the colon and rectum (Fig. 2.5A-C) portions. No significant differences of frequencies of PP-

immunoreactive cells were observed between any intestinal segments at different developmental stages (Table 2.1, Fig. 2.10-13).

Relatively abundant GLP-1-immunoreactive cells were mostly detected in the crypt glands of small and large intestines at all developmental stages. GLP-1-immunoreactive cells were all open-type with the spherical and spindle shapes, which had long cytoplasmic processes ended with the lumen similar to PYY-immunoreactive cells.

The localization of GLP-1-immunoreactive cells was higher in the prenatal (early, mid and late fetus) stages than in that of postnatal (suckling, weaning, weaned and adult) stages. GLP-1-immunoreactive cells, in the early and mid-fetal stages, were more numerous in the duodenum (Fig. 2.6A-B) and rectum (Fig. 2.6C) portions. GLP-1-immunoreactive cells in the late fetal stage were detected in all portions. However, they were more numerous in the small intestine (Fig. 2.7A-B).

GLP-1-immunoreactive cells were also moderately detected in all portions of the postnatal (suckling, weaning, weaned and adult) stages. Their immunohistochemical distribution was almost similar in the suckling and weaning stages. However, GLP-1-immunoreactive cells were observed higher in the duodenum of the suckling calf stage (Fig. 2.8A). In the weaned stage, GLP-1-immunoreactive cells were higher in the small intestine (Fig. 2.8B-D). GLP-1-immunoreactive cells in the adult stage were also moderately detected in all portions (Fig. 2.9A-B).

The regional distribution of GLP-1-immunoreactive cells was significantly ($P < 0.05$) increased in the duodenum (6.00 ± 0.70) and rectum (5.66 ± 1.25) of suckling calf, rectum (5.56 ± 2.01) of weaning calf, duodenum (3.80 ± 0.96), ileum (3.63 ± 0.53), and rectum (3.77 ± 0.50) of weaned calf, and rectum (5.63 ± 1.67) of adult compared with those of other intestinal regions (Table 2.1, Fig. 2.10-11). The distribution of GLP-1-immunoreactive cells was also significantly ($P<0.05$) increased ontogenetically at mid fetus stage (13.33 ± 1.77) in the duodenum, mid and late fetus stages (8.26 ± 1.33, 9.06 ± 2.03) in the jejunum, mid fetus stage (11.6 ± 2.03) in the ileum, late fetus stage (7.73 ± 2.36) in the cecum, mid and late fetus stages (9.46 ± 1.75, 8.93 ± 2.46) in the colon, and mid fetus stage (15.73 ± 1.54) in the rectum (Table 2.1, Fig. 2.12-13).

2.4. Discussion

In the present study, the regional distributions of PYY-, PP-, and GLP-1-immunoreactive cells were for the first time investigated in the gastrointestinal tract of cattle at different ontogenetic stages. The present results indicate that PYY, PP, and GLP-1 endocrine cells were absent in the esophagus and stomach. However, they were widely distributed in the small and large intestines of all cattle with different relative frequencies depending on regions and developmental stages.

Different endocrine cells have been studied in the gastrointestinal tract of various mammals including human (74), cat (40), cattle (39), sheep (12), horse (38), pig (36), water buffalo (8, 52) and camel (16) to demonstrate their distribution and relative frequencies in different parts of the gastrointestinal tract and to understand their functional roles in the digestive system. However, no detail studies on distribution of PYY, PP and GLP-1 endocrine cells in different pre and postnatal stages of neither human and rodents nor ruminants are reported yet. Thus, the present study could be a key paper for the distributions of mentioned endocrine cells in the gastrointestinal tract of cattle. The distribution and frequencies of PYY, PP and GLP-1 endocrine cells will be individually discussed with the available previous studies on ruminant species.

In the present study, PYY-immunoreactive cells were detected in all portions of the intestine with different regional frequencies; they were more numerous in the large intestine. Previous studies demonstrated PYY-immunoreactive cells mostly in the distal portions of the small intestine and in the large intestine (2, 3, 8, 52, 62). In cattle, PYY-immunoreactive cells were much less abundant in the ileum than in the large intestine, such as in colon and rectum, as has been reported in the intestine of babirusa (2), water buffalo (8, 52) and sheep (62). PYY-immunoreactive cells were not detected in any portion of the small intestine of the mouse deer (78) and carabao

(Philippine water buffalo) (8), while they were numerous in the large intestine, especially in the rectum of carabao (8).

The role of PYY peptide in the gastrointestinal tract has been widely investigated in monogastric species, especially in human (7). The nutrient infusion into the ileum inhibits jejunal motility (75, 87) and decreases antral and duodenal peristaltic pressure wave (27), the so-called "ileal brake". Moreover, it has been reported that colonic food infusion reduced ileal motility (88) and pancreatic secretion (32) with increasing PYY and GLP-1 in the colon (88). In rats, cecal nutrient infusion reduced food intake more than ileal infusion (55), suggesting the importance of the large intestine for the regulation of food intake. Onaga et al. (62) reported controversial findings for the ileal brake in sheep. At the same time, however, they reported the increasing abundance of PYY content in the distal large intestine. In cattle, it is assumed that abundant PYY-immunoreactive cells in the distal portions of small intestine and in the large intestine might be involved, at least in part, in the distal-to-proximal intestinal feedback. However, in ruminants, foregut fermenters, actual regulatory mechanisms are still unclear (61, 62).

In the bovine ontogenetic results, PYY-immunoreactive cells were decreased in postnatal stages. This decreasing tendency in the postnatal period is consistent with results of a previous study on the ontogeny of gut endocrine cells in water buffalo (52). They found that the frequency of PYY-immunoreactive cells was much higher in the intestine of younger (2-day-old)

buffalo than in the 5-month and 5-year-old buffalo. It is suggested that other intestinal epithelial cells such as goblet and absorptive epithelial cells show a greater increase in number with intestinal development after birth. The decreasing tendency of PYY-immunoreactive cells was prominent at the weaning period as well as nursing. The adaptation of digestive system to the herbivorous nature may also have drastic influence in the regulatory system of the gut.

At all developmental stages of the cattle, PP-immunoreactive cells were sparsely distributed in different intestinal portions. Similar to PYY-immunoreactive cell distribution, PP-immunoreactive cells also tended to be increased in the large intestine. However, no significant differences were observed among any intestinal regions of the seven stages examined in the present study. Similar to the present study, small numbers of PP-immunoreactive cells in the distal small intestine and relatively large numbers in the large intestine were reported in calf and cow (39), as well as sheep (12). However, intestinal PP-immunoreactive cells seem to differ depending on the animal species. In human colon and rectum, few PP-immunoreactive cells were detected (74). Moreover, rat PP-immunoreactive cells were transiently expressed for a short time in the endocrine cells of colon at the postnatal stage (20). It is thus assumed that the functional roles of PP may be different among species. In human and rodents, PP is thought

to reduce food intake and may induce long term suppression of appetite (74). However, the main role of PP in ruminants including cattle remains unclear.

In the present study, numerous GLP-1-immunoreactive cells were detected in all parts of the small and large intestines at all developmental stages. GLP-1-immunoreactive cells were more numerous at the prenatal stages than at calf and adult stages. The relative frequencies of GLP-1-immunoreactive cells tended to be higher in the duodenum and rectum of the intestine. The general tendency of the distribution of GLP-1-immunoreactive cells in the large intestine was basically similar to those of glicentin-immunoreactive cells in calf and cow (39), as well as sheep (12). This may be related to the fact that GLP-1 and glicentin are derived from the same precursor molecule (37).

GLP-1 is secreted in the distal small intestine and colon by stimulation of carbohydrate and fat, and inhibits gastroduodenal motility (69) and gastric acid secretion (89). Therefore, it is considered that GLP-1 also has an important role in ileal brake and/or distal-to-proximal feedback, like PYY, and the two peptides may act cooperatively (59, 89). The reasons for the decreased frequency of GLP-1-immunoreactive cells with development may be the same as those for PYY-immunoreactive cells.

The present study demonstrated the regional distribution and relative frequencies of PYY-, PP-, and GLP-1-immunoreactive cells in the intestine of cattle at different ontogenetic stages. It was shown that the localization and

frequency of these endocrine cells vary among different intestinal segments and at different developmental stages in cattle. These differences of PYY-, PP-, and GLP-1-immunoreactive cell distribution might be due to the stage-dependent changes in intestinal growth, secretion, motility, and diet. Further studies on the physiological functions of these three kinds of hormones – PYY, PP, and GLP-1 – in ruminants are needed. The present results on the ontogenetic distribution of three types of endocrine cells in the gastrointestinal tract of cattle should provide the basis for future extensive physiological and endocrinological studies on ruminants.

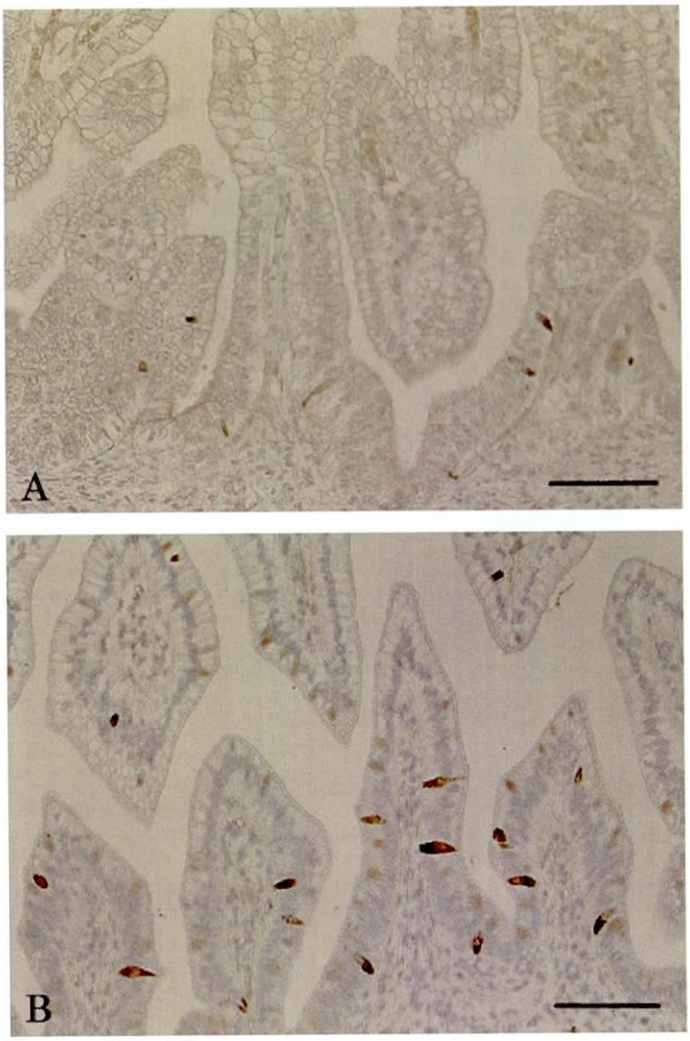

Fig. 2.1. Peptide YY-immunoreactive cells in the ileum (A) of early fetus (CRL 26 cm) and rectum (B) of early fetus (CRL 26 cm). Bar: 100 μm.

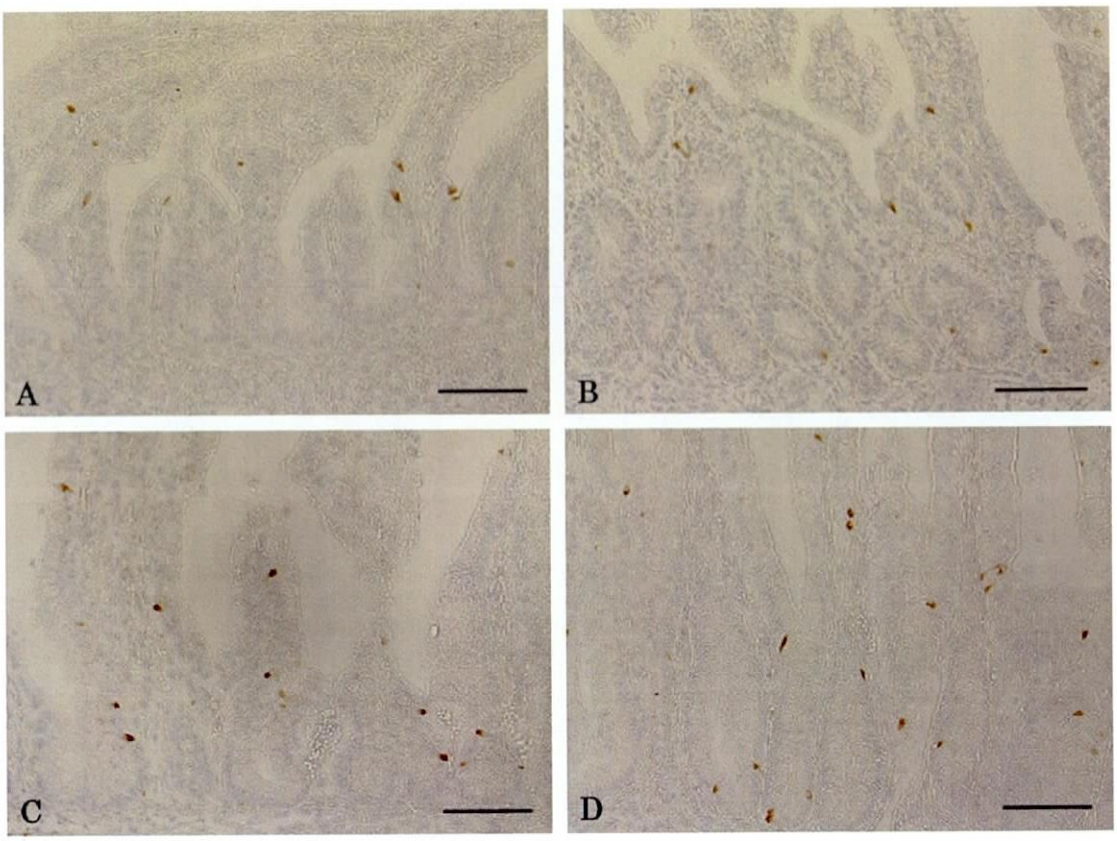

Fig. 2.2. Peptide YY-immunoreactive cells in the ileum (A), cecum (B), colon (C) and rectum (D) of mid fetus (CRL 55 cm). Bar: 100 μm.

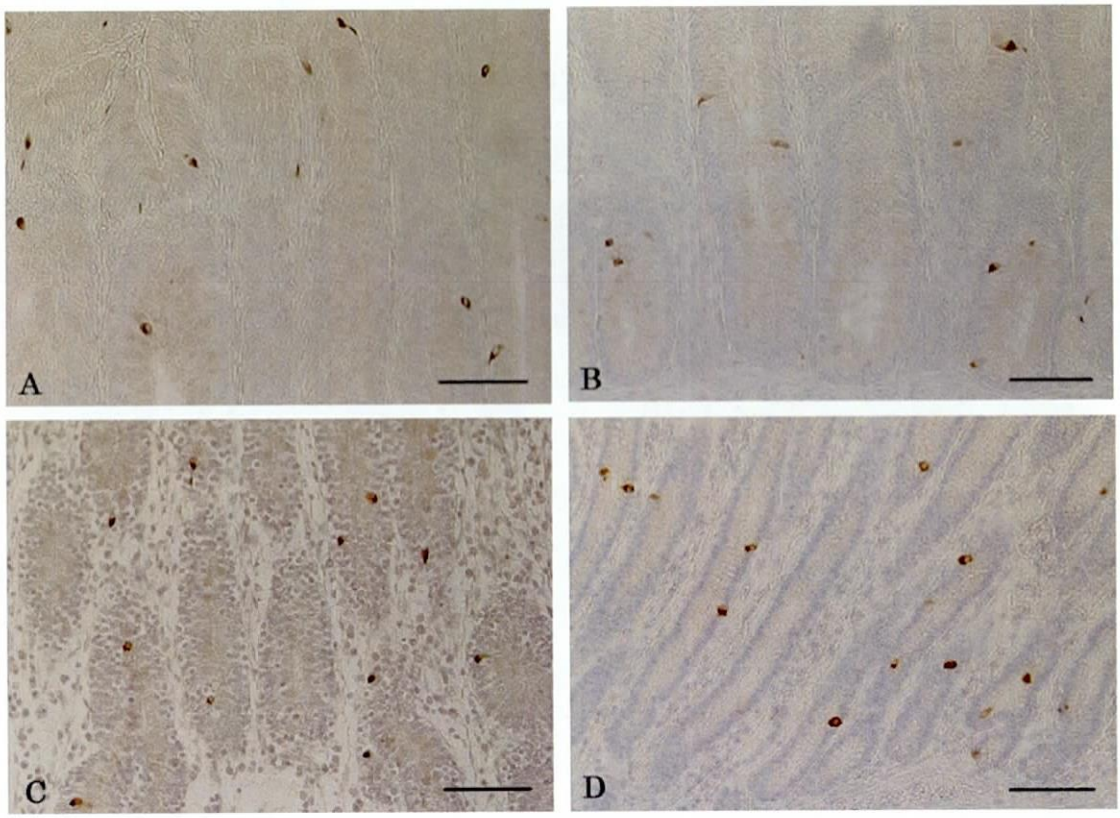

Fig. 2.3. Peptide YY-immunoreactive cells in the intestine of cattle, A: ileum of suckling calf (12-day-old), B: rectum of suckling calf (12-day-old), C: rectum of weaned calf (10-month-old), D: rectum of cow. Bar: 100 μm.

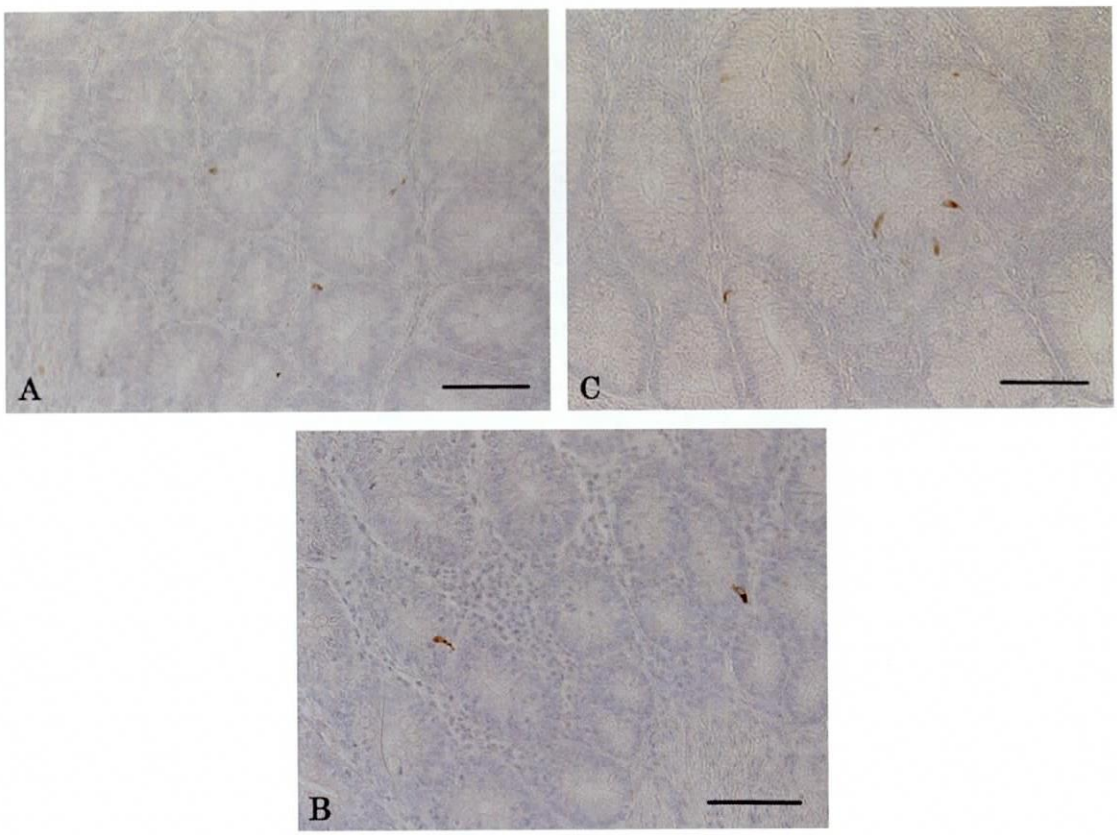

Fig. 2.4. Pancreatic polypeptide-immunoreactive cells in the intestine of cattle. A: ileum of suckling calf (12-day-old), B: rectum of suckling calf (12-day-old), C: colon of suckling calf (12-day-old). Bar: 100 μm.

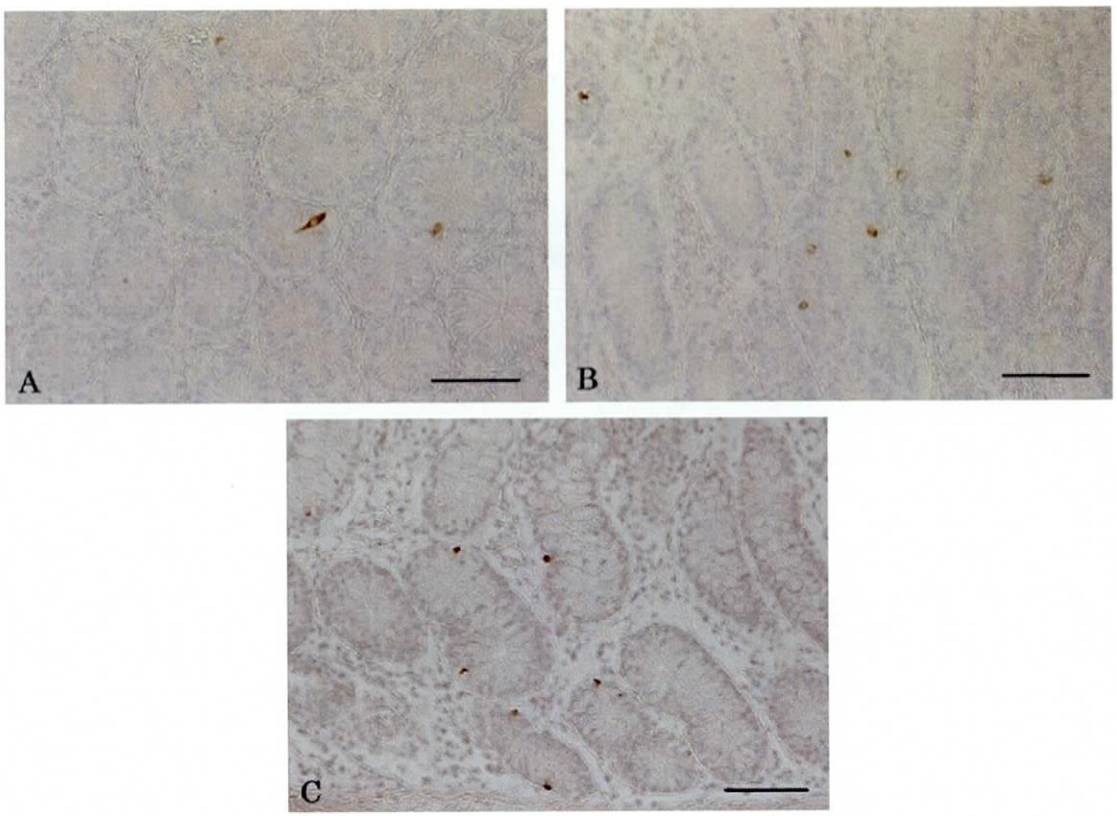

Fig. 2.5. Pancreatic polypeptide-immunoreactive cells in the intestine of cattle. A: colon of cow, B: rectum of cow, C: rectum of weaned calf (10-month-old). Bar: 100 μm.

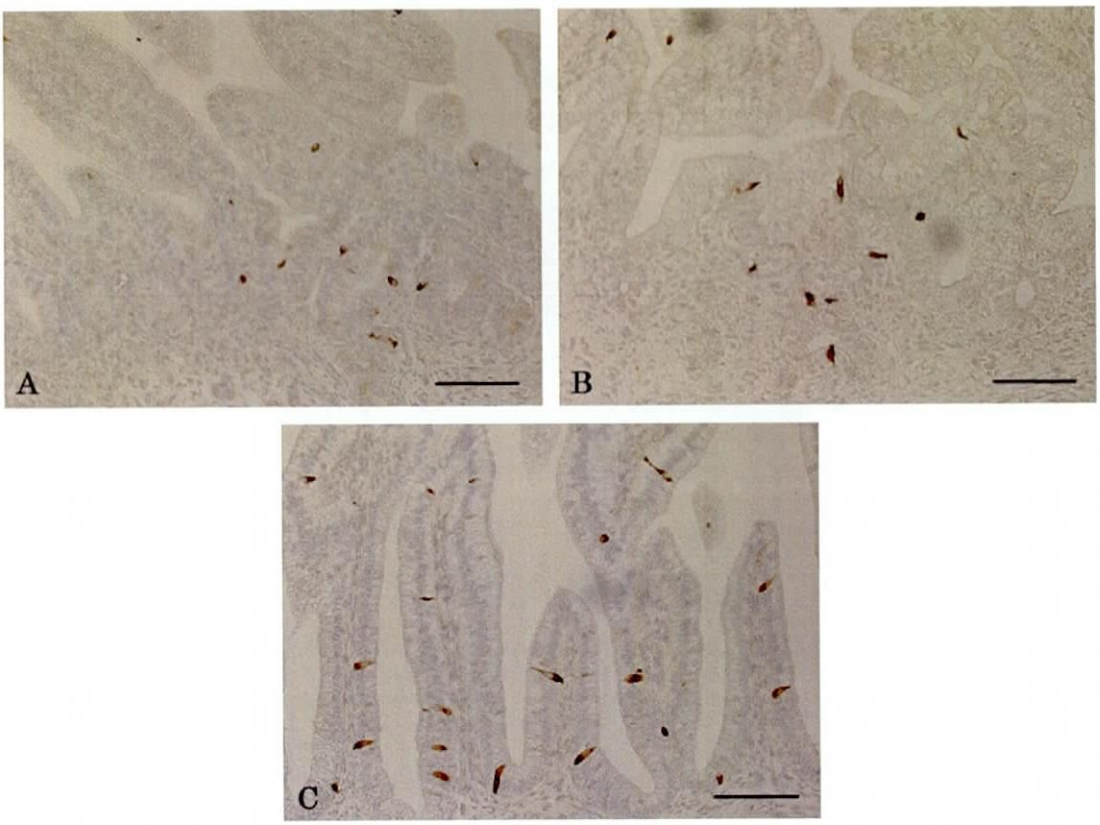

Fig. 2.6. Glucagon-like peptide-1-immunoreactive cells in the intestine of cattle. A: duodenum of mid fetus (CRL 55 cm), B: duodenum of early fetus (CRL 26 cm), C: rectum of early fetus (CRL 20 cm). Bar: 100 μm.

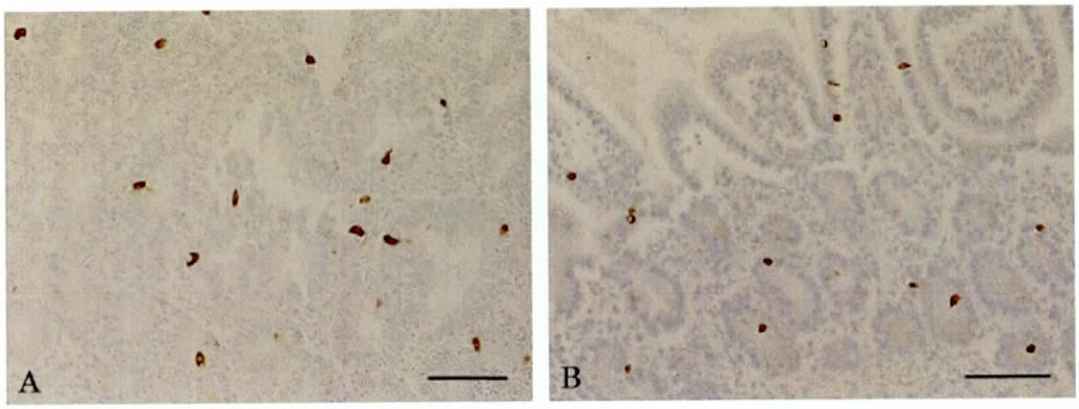

Fig. 2.7. Glucagon-like peptide-1-immunoreactive endocrine cells in the intestine of cattle. A: duodenum of late fetus (CRL 96 cm), B: jejunum of late fetus (CRL 90 cm). Bar: 100 μm.

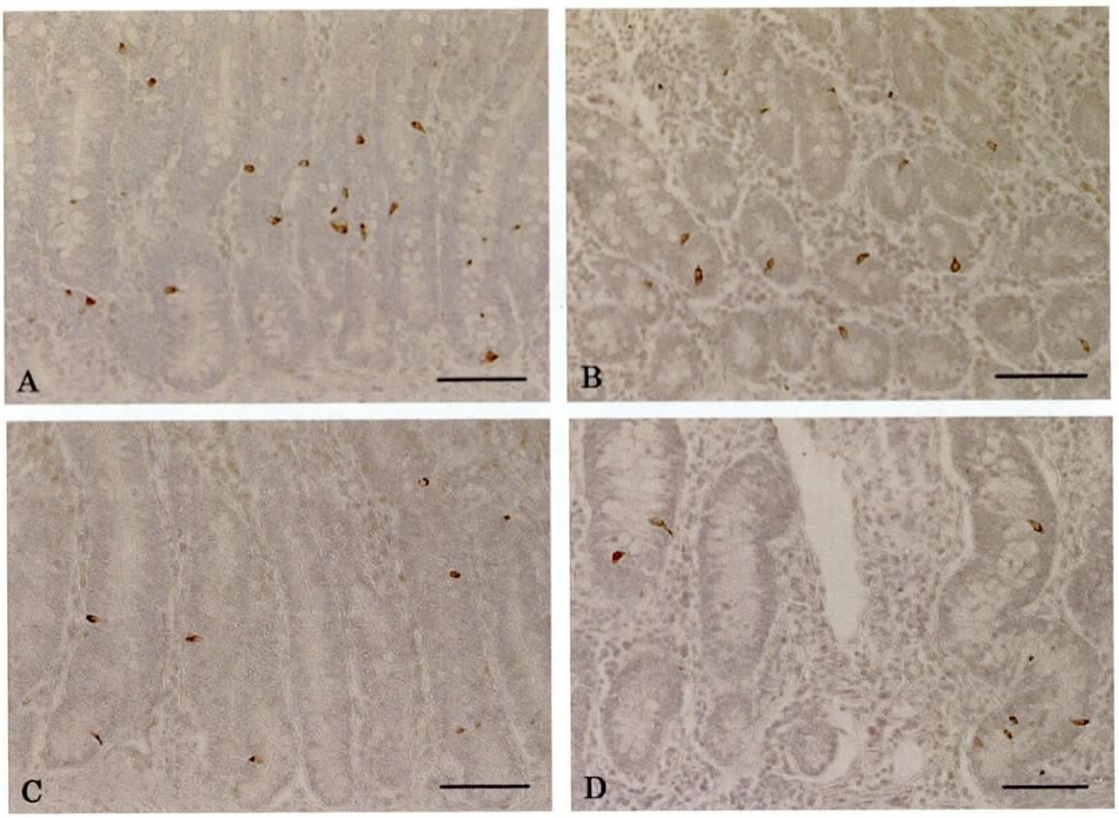

Fig. 2.8. Glucagon-like peptide-1-immunoreactive cells in the intestine of cattle. A: duodenum of suckling calf (12-day-old), B: duodenum of weaned calf (10-month-old), C: jejunum of weaned calf (10-month-old), D: ileum of weaned calf (10-month-old). Bar: 100 μm.

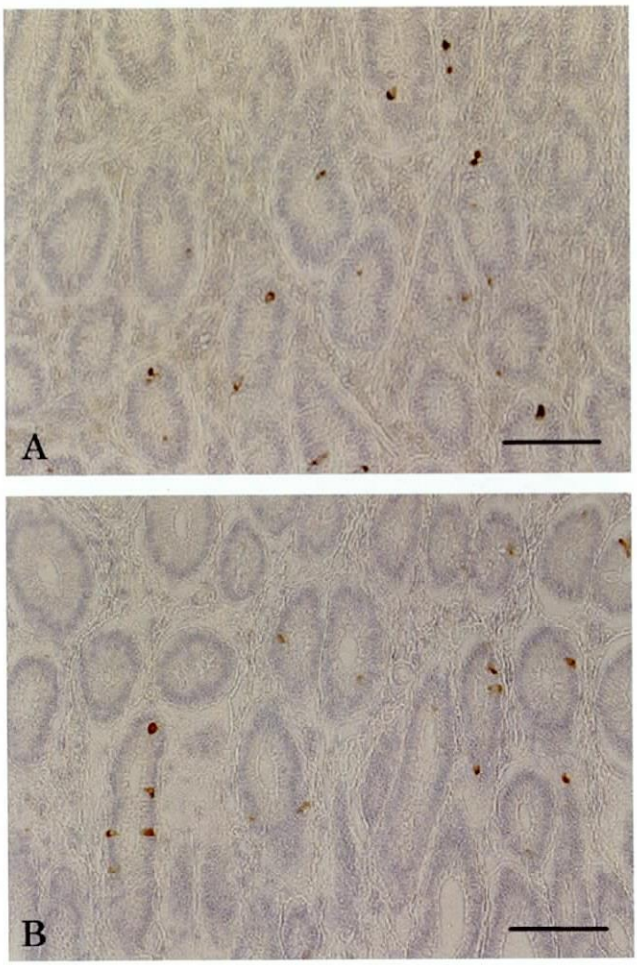

Fig. 2.9. Glucagon-like peptide-1-immunoreactive cells in the intestine of cattle. A: ileum of cow, B: rectum of cow. Bar: 100 μm.

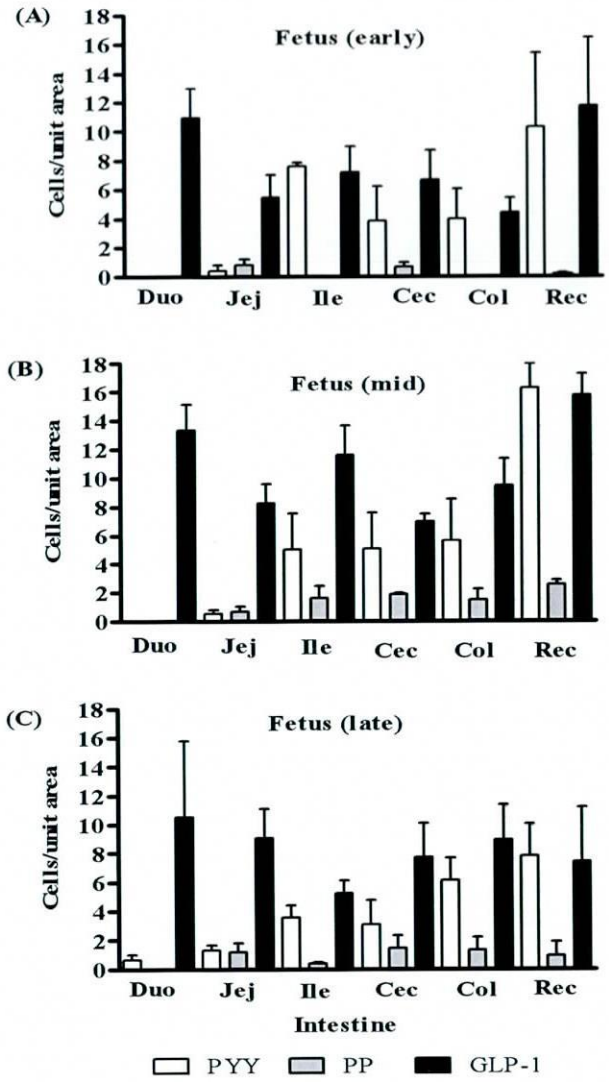

Fig. 2.10. Distribution and relative frequencies of endocrine cells per unit area (625 μm^2) in the intestine of prenatal (A: early fetus, B: mid fetus, and C: late fetus). PYY: peptide YY, PP: pancreatic polypeptide, GLP-1: glucagon-like peptide-1. Duo: duodenum, Jej: jejunum, Ile: ileum, Cec: cecum, Col: colon, Rec: rectum.

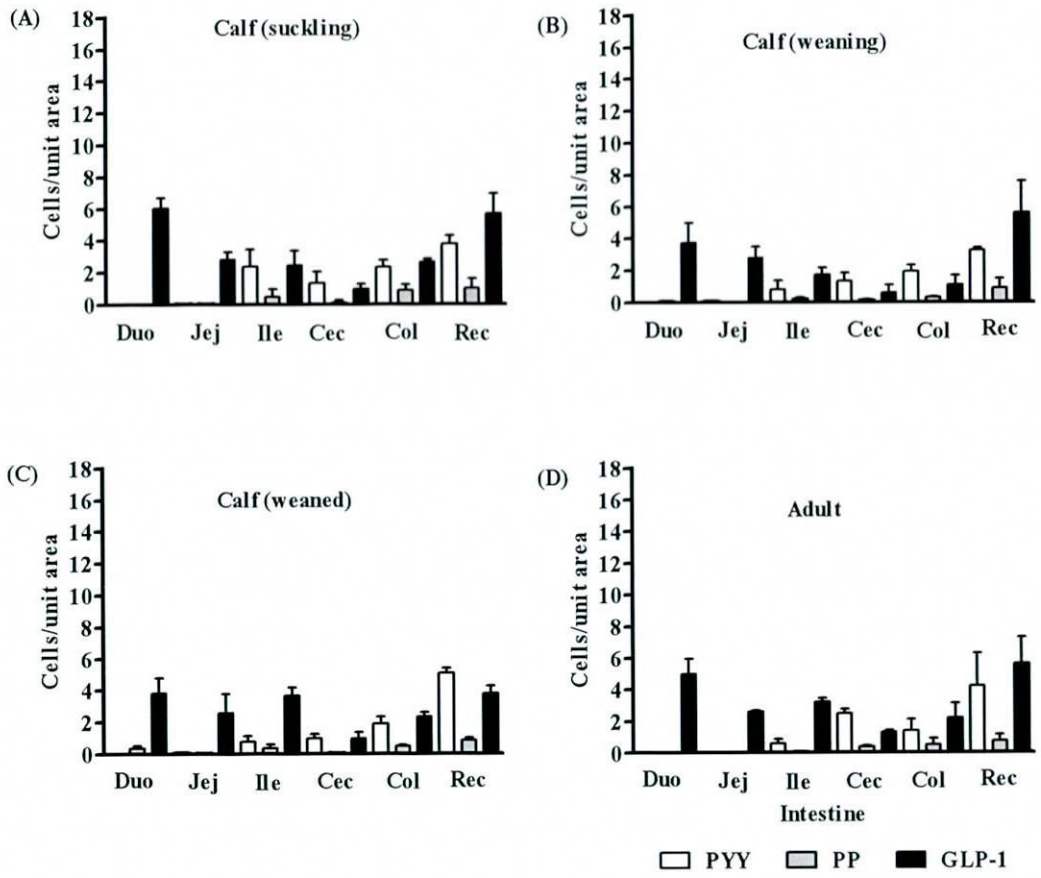

Fig. 2.11. Distribution and relative frequencies of endocrine cells per unit area (625 μm^2) in the intestine of postnatal (A: suckling calf, B: weaning calf, C: weaned calf, D: adult). PYY: peptide YY, PP: pancreatic polypeptide, GLP-1: glucagon-like peptide-1. Duo: duodenum, Jej: jejunum, Ile: ileum, Cec: cecum, Col: colon, Rec: rectum.

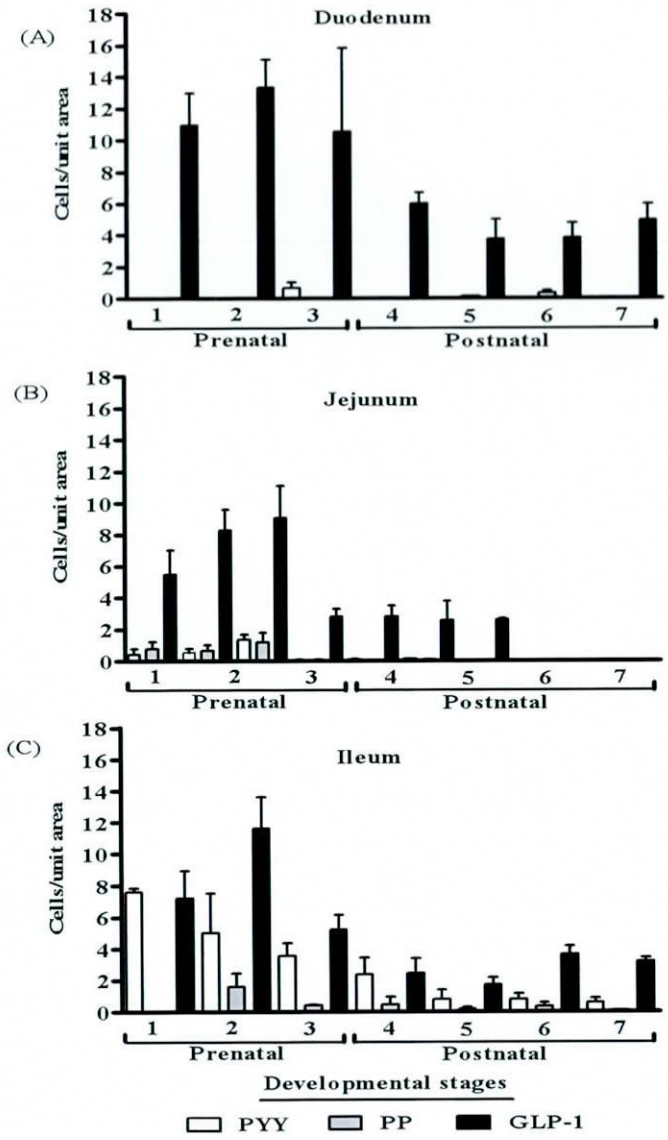

Fig. 2.12. Distribution and relative frequencies of endocrine cells per unit area (625 μm^2) in the small intestine (A: duodenum, B: Jejunum, and C: ileum) of seven developmental stages of cattle (1: early fetus, 2: mid fetus, 3: late fetus, 4: suckling calf, 5: weaning calf, 6: weaned calf and 7: adult). PYY: peptide YY, PP: pancreatic polypeptide, GLP-1: glucagon-like peptide-1.

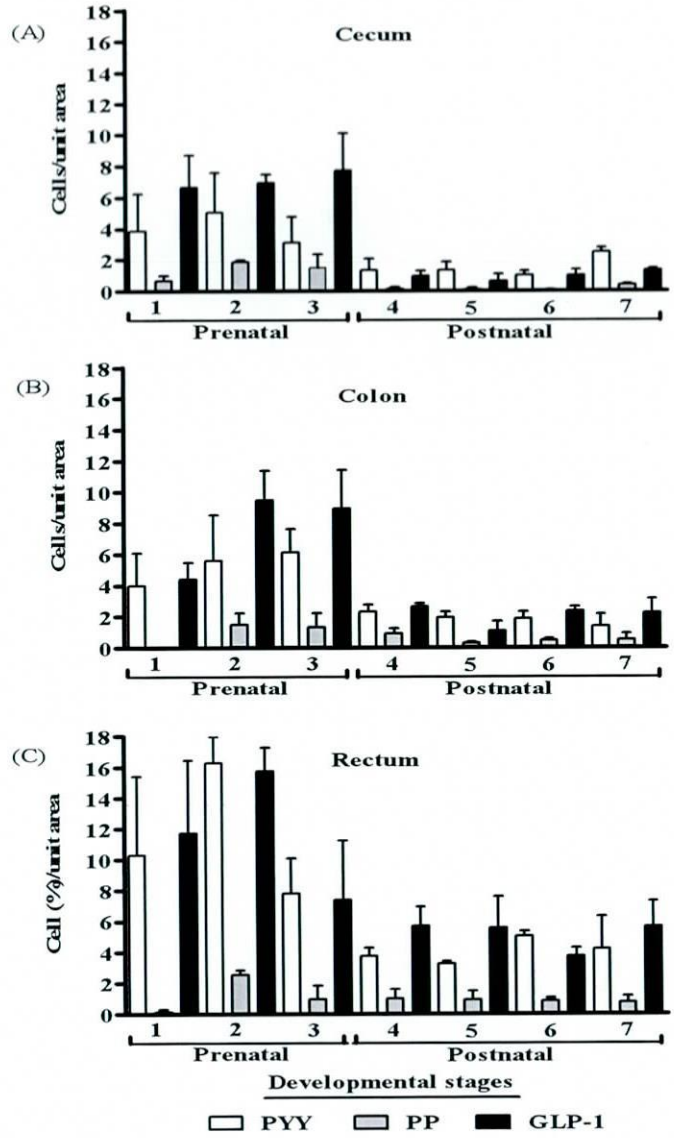

Fig. 2.13. Distribution and relative frequencies of endocrine cells per unit area (625 μm^2) in the large intestine (A: cecum, B: colon, and C: rectum) of seven developmental stages of cattle (1: early fetus, 2: mid fetus, 3: late fetus, 4: suckling calf, 5: weaning calf, 6: weaned calf and 7: adult). PYY: peptide YY, PP: pancreatic polypeptide, GLP-1: glucagon-like peptide-1.

Table 2.1. Distribution and relative frequency of PYY-, PP-, and GLP-1-immunoreactive cells in the bovine intestine at different developmental stages.

	Small intestine			Large intestine		
	Duo	Jej	Ile	Cec	Col	Rec
Fetus (early)						
PYY	ND	0.40 ± 0.40	7.60 ± 0.25	3.86 ± 2.37	4.00 ± 2.06	10.27 ± 5.14
PP	ND	0.80 ± 0.41	ND	0.66 ± 0.32	ND	0.13 ± 0.13
GLP-1	10.12 ± 2.04	5.46 ± 1.54	7.20 ± 1.75	6.66 ± 2.01	4.40 ± 1.04	11.73 ± 4.72
Fetus (mid)						
PYY	ND	0.53 ± 0.26	5.00 ± 2.54	5.06 ± 2.53	5.6 ± 2.91	16.27 ± 1.71[a, 9]
PP	ND	0.66 ± 0.35	1.60 ± 0.85	1.86 ± 0.12	1.46 ± 0.75	2.53 ± 0.31
GLP-1	13.33 ± 1.77[1]	8.26 ± 1.33[2]	11.6 ± 2.03[5]	6.93 ± 0.55	9.46 ± 1.75[7]	15.73 ± 1.54[10]
Fetus (late)						
PYY	0.75 ± 0.35	1.33 ± 0.33	3.56 ± 0.81	3.06 ± 1.68	6.13 ± 1.52	7.80 ± 2.27
PP	ND	1.20 ± 0.60	0.40 ± 0.05	1.46 ± 0.86	1.33 ± 0.88	0.93 ± 0.93
GLP-1	10.53 ± 5.27	9.06 ± 2.03[3]	5.23 ± 0.89	7.73 ± 2.36[6]	8.93 ± 2.46[8]	7.40 ± 3.81
Calf (suckling)						
PYY	ND	0.03 ± 0.03	2.36 ± 1.07	1.30 ± 0.73	2.30 ± 0.45	3.76 ± 0.53[b]
PP	ND	0.03 ± 0.03	0.46 ± 0.46	0.13 ± 0.13	0.90 ± 0.32	1.00 ± 0.57
GLP-1	6.00 ± 0.70[c]	2.80 ± 0.49	2.43 ± 0.95	0.93 ± 0.32	2.63 ± 0.20	5.66 ± 1.25[d]
Calf (weaning)						
PYY	ND	0.06 ± 0.06	0.80 ± 0.56	1.33 ± 0.52	1.93 ± 0.36	3.26 ± 0.14
PP	0.95 ± 0.03	ND	0.16 ± 0.12	0.10 ± 0.10	0.30 ± 0.05	0.90 ± 0.58
GLP-1	3.73 ± 1.25	2.80 ± 0.70	1.70 ± 0.46	0.60 ± 0.45	1.06 ± 0.61	5.56 ± 2.01[e]
Calf (weaned)						
PYY	ND	0.06 ± 0.06	0.76 ± 0.37	0.96 ± 0.29	1.87 ± 0.43	5.03 ± 0.33[f]
PP	0.33 ± 0.17	0.03 ± 0.03	0.33 ± 0.24	0.03 ± 0.03	0.46 ± 0.08	0.83 ± 0.18
GLP-1	3.80 ± 0.96[g]	2.57 ± 1.18	3.63 ± 0.53[h]	0.96 ± 0.38	2.33 ± 0.28	3.77 ± 0.50[i]
Adult						
PYY	ND	ND	0.56 ± 0.29	2.46 ± 0.29	1.36 ± 0.75	4.20 ± 2.10[j]
PP	ND	ND	0.06 ± 0.03	0.36 ± 0.08	0.50 ± 0.40	0.73 ± 0.40
GLP-1	4.96 ± 0.98	2.60 ± 0.05	3.20 ± 0.23	1.33 ± 0.08	2.20 ± 0.91	5.63 ± 1.67[k]

Data represent mean ± SEM; Duo: duodenum, Jej: jejunum, Ile: ileum, Col: colon, Rec: rectum.

PYY: peptide YY, PP: pancreatic polypeptide, GLP-1: glucagon-like peptide-1, ND: not detected.

The superscript letters (a-k) indicate significant differences of values within individual rows ($P<0.05$).

The Arabic numbers (1-10) indicate significant differences of values within individual columns ($P<0.05$).

Chapter 3

Quantitative Immunohistochemical Study of Endocrine Cell Distribution in the Bovine Large Intestine

3.1. Introduction

The gastrointestinal tract is the largest endocrine organ in the body (65). Gut hormones are chemical messengers which are localized in the endocrine cells distributed throughout the mucosa of glandular stomach and intestine (65, 68, 59). They are playing important roles in entire digestive tract to regulate many digestive functions, such as secretion, absorption and motility. Many studies have elucidated the regional distribution and relative frequencies of various kinds of endocrine cells in the gastrointestinal tract of different animals. However, detail studies on the distribution of endocrine cells in different parts of the large intestine especially in domestic animals have not been reported yet. The large intestine, as well as stomach, has important physiological function in the digestive process of ruminants (33). Previous reports on the endocrine cells in the ruminant large intestine examined only few portions (3, 8, 12, 39). The discriminative portions, which are characteristic to the spiral colon and rectum, were not examined at so many segments for endocrine cell distribution. The present study aimed to

clear the detail of endocrine cell distributions in eleven different portions of large intestine of cattle.

3.2. Materials and methods

Six calves in two groups (suckling and weaned) were used in this study. The age of the suckling calves were between 5 - 7 weeks, and weaned calves were 7 months. Tissue samples were taken from eleven parts of the large intestine detailed in Chapter 1 and processed for paraffin sections as described before. The primary antisera used in this chapter, in addition to those used in Chapter 2, were raised in rabbit against chromogranin (CG: 1:15,000, Code 20085, INCSTAR, Stillwater, MN, USA), serotonin (Ser: 15,000, Code sero-23, donated by Dr. Nishiitsutsuji-Uwo, Kyoto), and somatostatin (Som: 1:10,000, Code 20H2T, INCSTAR). Immunohistochemical procedures were mentioned in Chapter 1.

3.3. Results

Six types of endocrine cells were detected with the antisera against CG, Ser, GLP-1, PYY, PP and Som in eleven different portions of large intestine of suckling and weaned calves. The regional distribution and relative frequencies of these endocrine cells were different in each parts of large intestine (Fig. 3.10A-C and 3.11A-C, Table 3.1). However, no significant differences in the frequencies of mentioned endocrine cells were observed

between two groups, suckling and weaned. The distribution and relative frequencies of each immunoreactive endocrine cell will be discussed individually.

CG-immunoreactive cells were abundantly detected in all segments of the large intestine. The general distribution of CG-immunoreactive cells was higher in the most distal parts of the colon and two parts of the rectum (Fig 3. 1A-C and 3.2A-C). The frequency of CG-immunoreactive cells were significantly higher in the descending colon of both suckling and weaned groups (suckling: 19.03 ± 0.61, weaned: 18.53 ± 3.06), sigmoid colon of both groups (suckling: 22.90 ± 0.87, weaned: 18.37 ± 2.39), ampulla of rectum of both groups (suckling: 27.90 ± 1.55, weaned: 23.37 ± 5.79) and rectum just cranial to the anorectal line of both groups (suckling: 21.60 ± 0.86, weaned: 17.40 ± 0.78) (Fig. 3.10A, Table 3.1)

The general distribution of Ser-immunoreactive cells was almost similar to that of CG; higher in the most distal parts of the colon and two parts of the rectum (Fig 3.3A-C, and Fig. 3.4A-C). Significant differences ($P < 0.05$) of Ser-immunoreactive cells were observed in sigmoid colon of suckling group (19.33 ± 0.87), ampulla of rectum of suckling group (23.37 ± 0.81) and rectum just cranila to the anorectal line of both groups (suckling: 18.77 ± 1.03, weaned: 10.37 ± 2.60) (Fig 3. 10B, Table 3.1).

GLP-1-immunoreactive cells were rarely detected in the cecum and the proximal parts of the colon. However, their frequencies were high in the

distal parts of the colon and two parts of the rectum (Fig. 3.5A-C and Fig. 3.6A-C). No significant differences of GLP-1-immunoreactive cells were observed between any regions of the large intestine as well as two different developmental stages, suckling and weaned (Fig 3.10C, Table 3.1).

The frequencies of PYY-immunoreactive cells were varied among regions (Fig. 3.7A-C, 3.8A-C). They were few in the cecum and the proximal portions of the colon, but were significantly higher in the sigmoid colon (4.10 ± 0.40) and ampulla of rectum (3.60 ± 0.43) of the suckling group (Fig. 3.11A and Table 3.1).

PP-immunoreactive cells were detected rarely in all portions of the large intestine. (Fig 3.9A-C). The PP-immunoreactive cells were very few in the cecum and proximal parts of the colon, increased in the middle portions, descending colon, sigmoid colon and ampulla of rectum (Fig. 3.11B, Table 3.1).

Som-immunoreactive cells were only detected with a very few number in the transverse colon of the suckling stage. They were not detected in any other portions of both suckling and weaned groups (Fig. 3.11C, Table 3.1).

3.4. Discussion

In chapter 1, the regional distribution and relative frequencies of three types of endocrine cells, PYY-, GLP-1- and PP-immunoreactive cells were revealed in the small and large intestine of cattle at different developmental stages (fetus, calf, adult). These three types of endocrine cells were more

numerous in the large intestine of all developmental groups. This fact leads the present study to investigate the distribution in more detailed topography of bovine large intestine. The relative frequencies of CG-, Ser-, GLP-1-, PYY-, PP- and Som-immunoreactive endocrine cells were evaluated in eleven different portions of large intestine in order to understand the characteristics of the tortuous intestine of the ruminant. The distinguishing differences in distributions of these six types of endocrine cells were found in each portions of large intestine.

General distribution of endocrine cells represented by CG-immunoreactive cells indicates the distal abundance in the bovine gut. In addition, each type of endocrine cells immunoreactive for Ser, GLP-1, PYY, and PP also showed similar tendency to be more abundant in the distal portions. Som-immunoreactive cells were too low in frequency to discuss here.

The present study demonstrated the detail distribution of endocrine cells along with the tortuous large intestine of ruminant. Previous studies on the ruminant have so far examined only three portions in the large intestine (cecum, central flexure of the spiral colon, terminal rectum) of cattle (39), sheep (12), water buffalo (8), and mouse deer (3). Although these results showed the tendency of the abundance of endocrine cells in the rectum, it was not compared in detail with the rest of the large intestine. The eleven portions of the intestine examined in the present study are the peculiar points which are the characteristics of ruminant. The present study certainly

distinguished the distal distribution of endocrine cells in the bovine large intestine. It is suggesting that the huge numbers of endocrine cells in the large intestine especially distal portions of colon and rectum might be related to the physiological importance of those portions of the digestive system in ruminants. This emphasize again that the distal-to-proximal feedback regulation (59, 89) may play important roles in the digestive tract of the ruminant. It is necessary to make detail physiological studies on the linkage between different portions of the large intestine of ruminant and gut hormones.

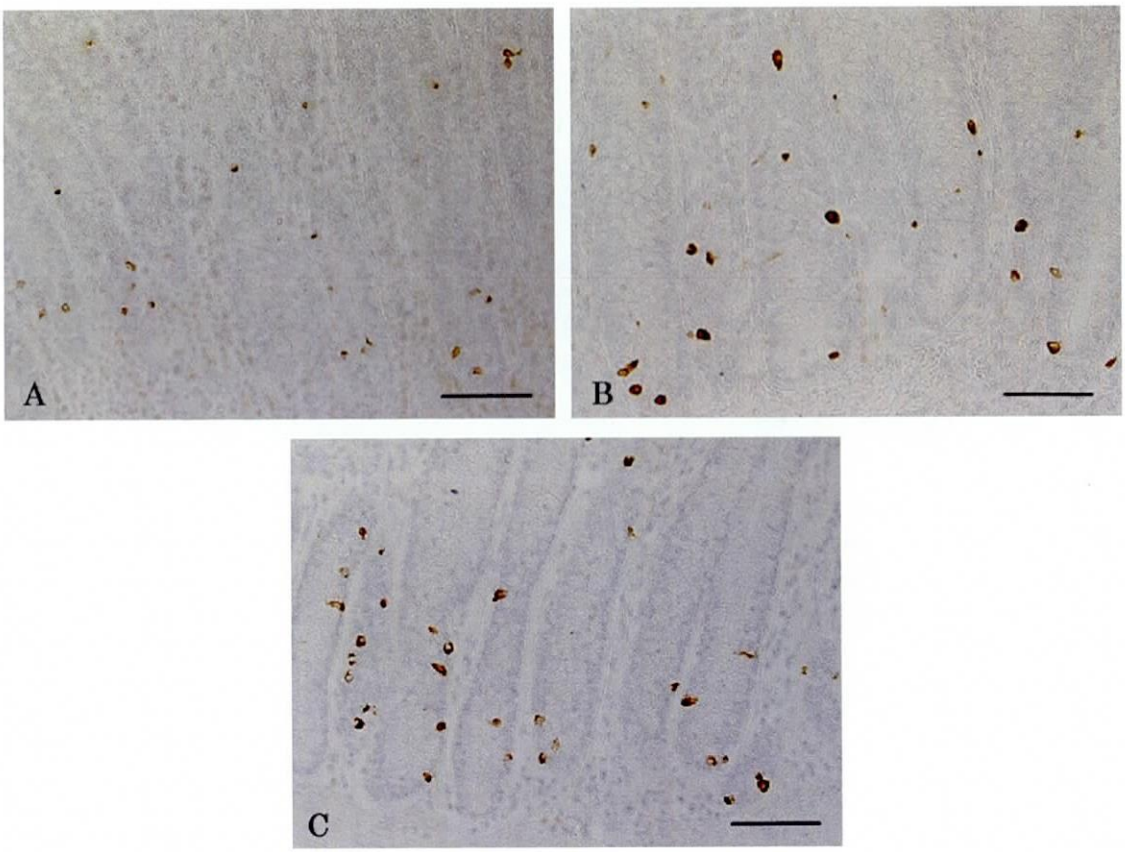

Fig. 3.1. Chromogranin-immunoreactive cells in the large intestine of suckling and weaned calves. (A) cecum of the weaned calf, (B) centripetal turns of colon of the suckling calf, (C) transverse colon of the weaned calf. Bar = 100 μm.

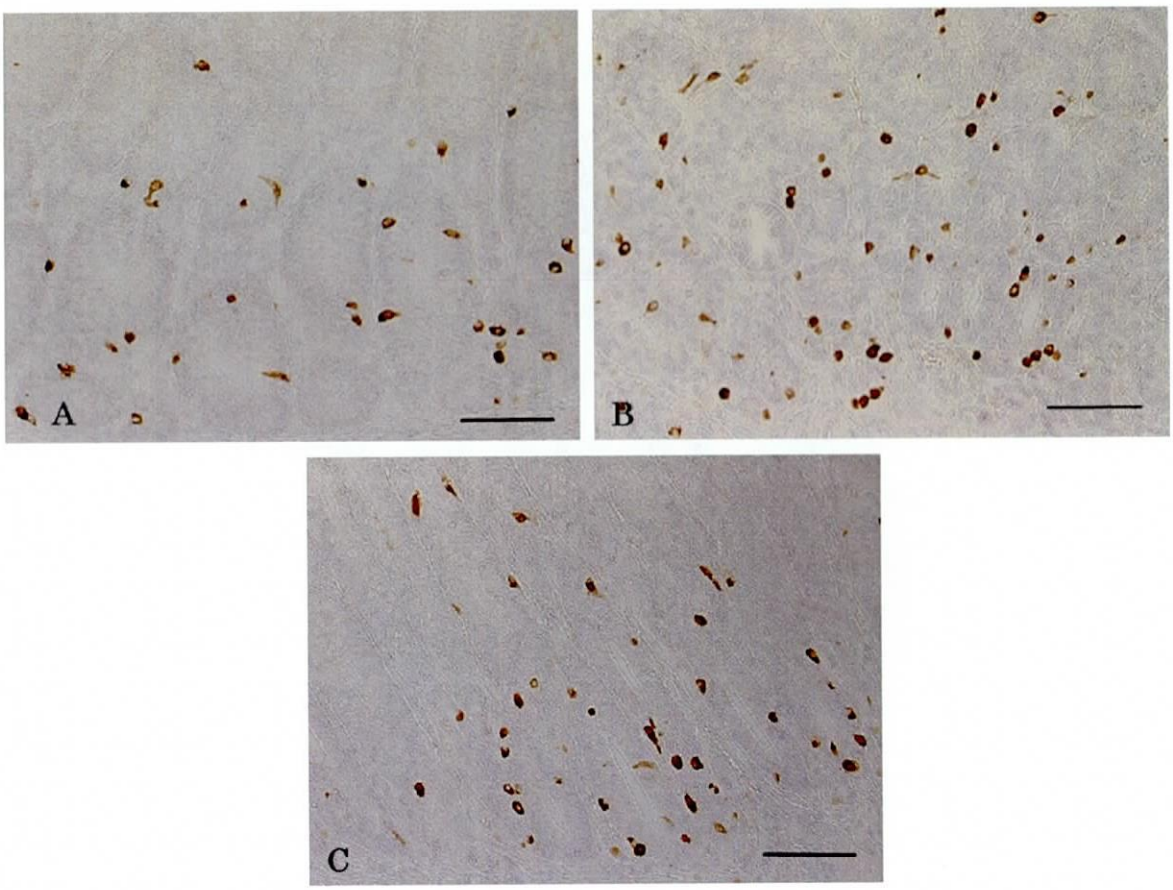

Fig. 3.2. Chromogranin-immunoreactive cells in the large intestine of suckling and weaned calves. (A) descending colon in the suckling calf, (B) ampulla of rectum in the suckling calf, (C) rectum just cranial to the anorectal line of the suckling calf. Bar = 100 μm.

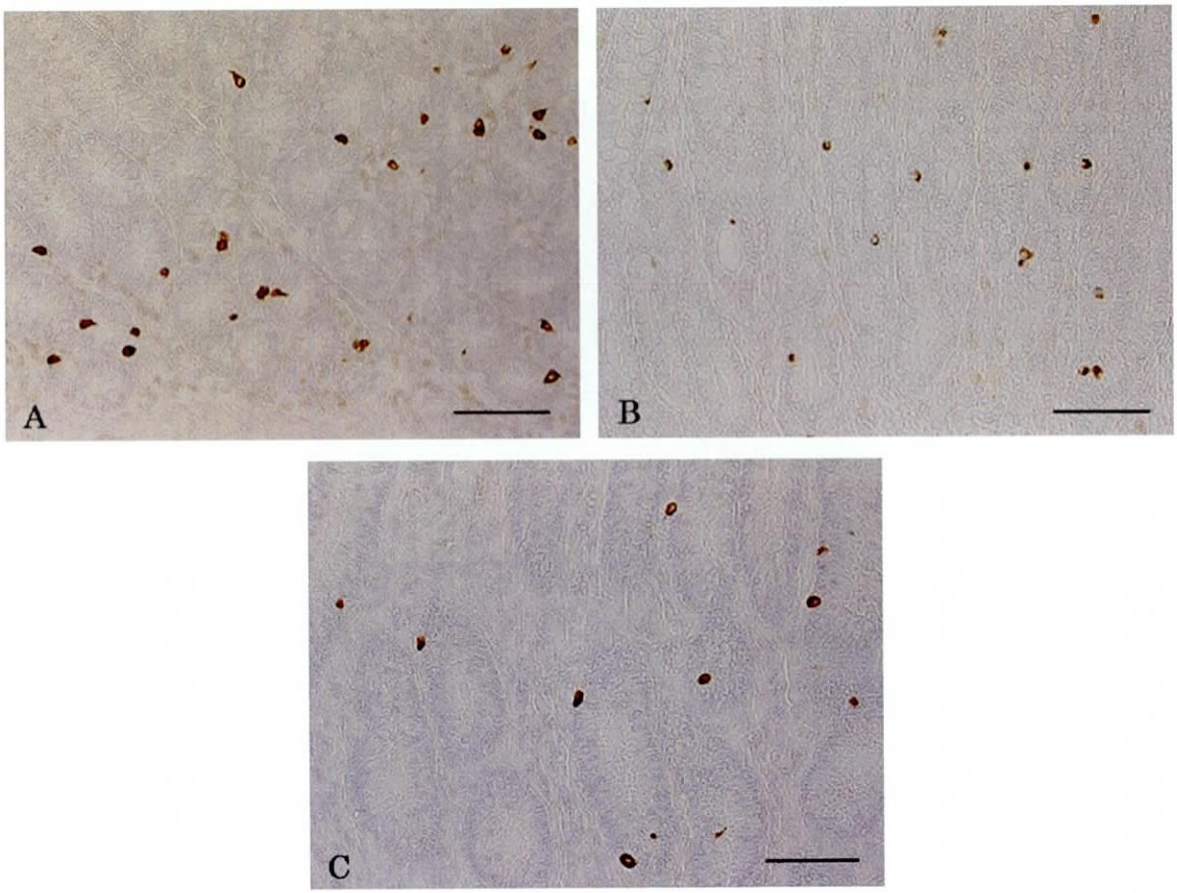

Fig. 3.3. Serotonin-immunoreactive cells in the large intestine of suckling and weaned calves. (A) cecum of the suckling calf, (B) proximal loop of colon in the weaned calf, (C) centripetal turns of colon in the weaned calf. Bar = 100 μm.

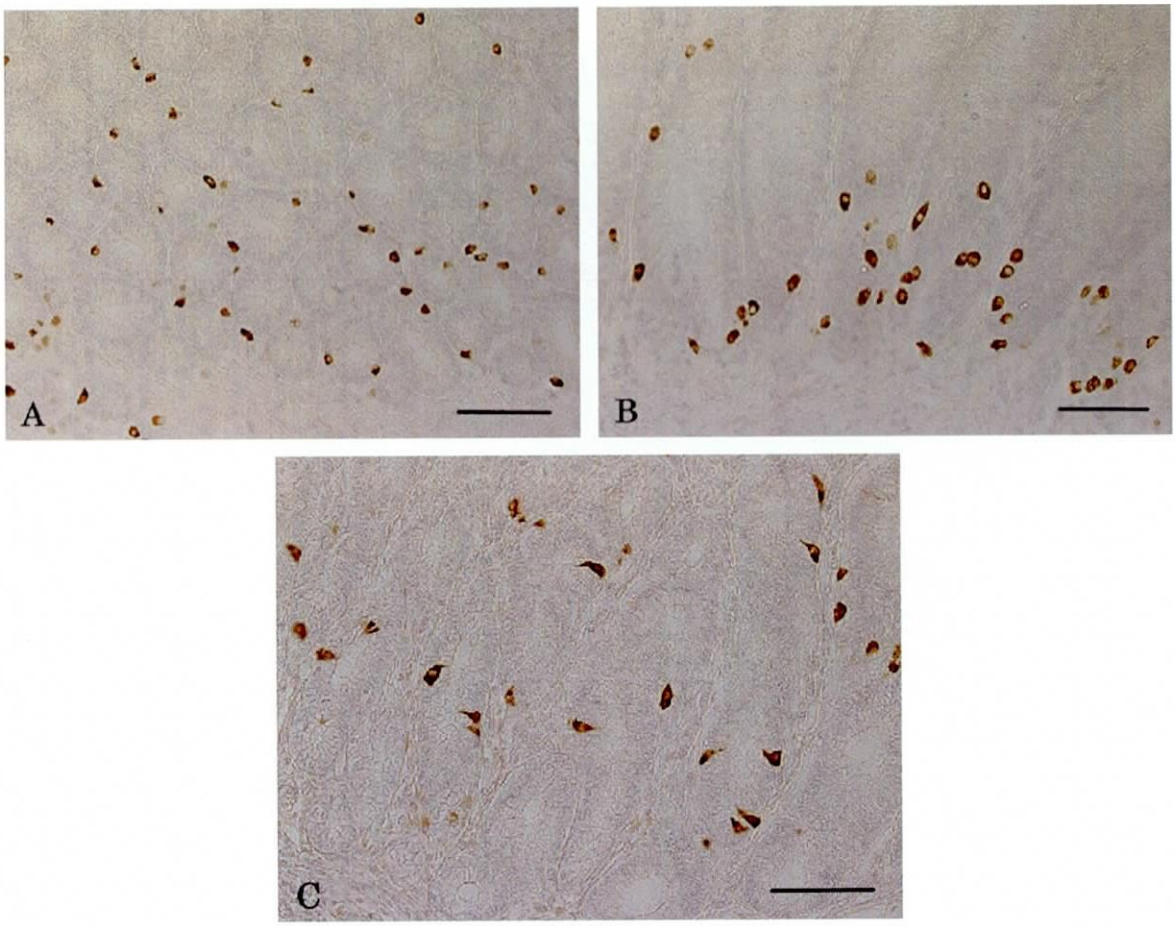

Fig. 3.4. Serotonin-immunoreactive cells in the large intestine of suckling and weaned calves. (A) descending colon of the suckling calf, (B) ampulla of rectum in the suckling calf, (C) rectum just cranial to the anorectal line in the suckling calf. Bar = 100 μm.

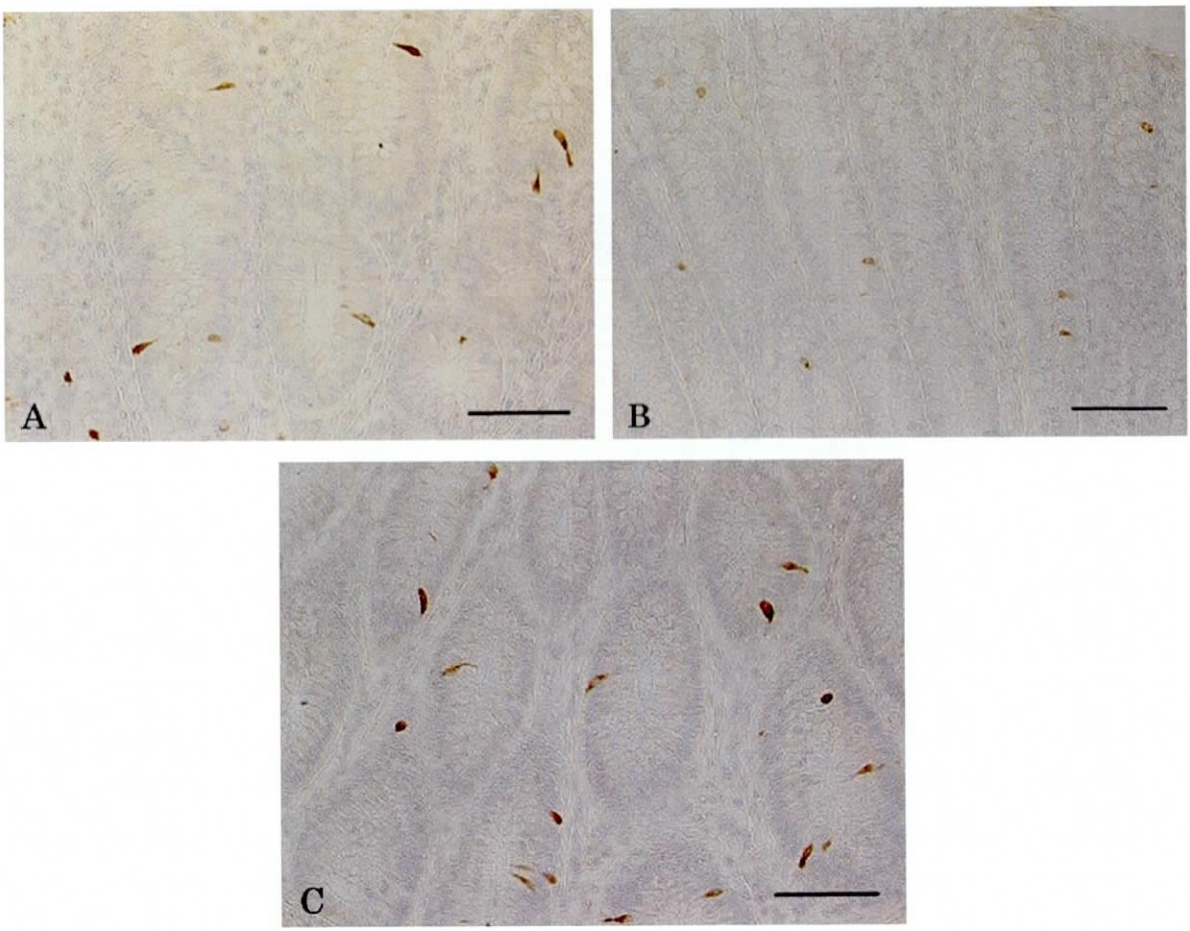

Fig. 3.5. Glucagon-like peptide-1-immunoreactive cells in the large intestine of suckling and weaned calves. (A) proximal loop of colon in the suckling calf, (B) transverse colon in the weaned calf, (C) descending colon in the suckling calf. Bar = 100 μm.

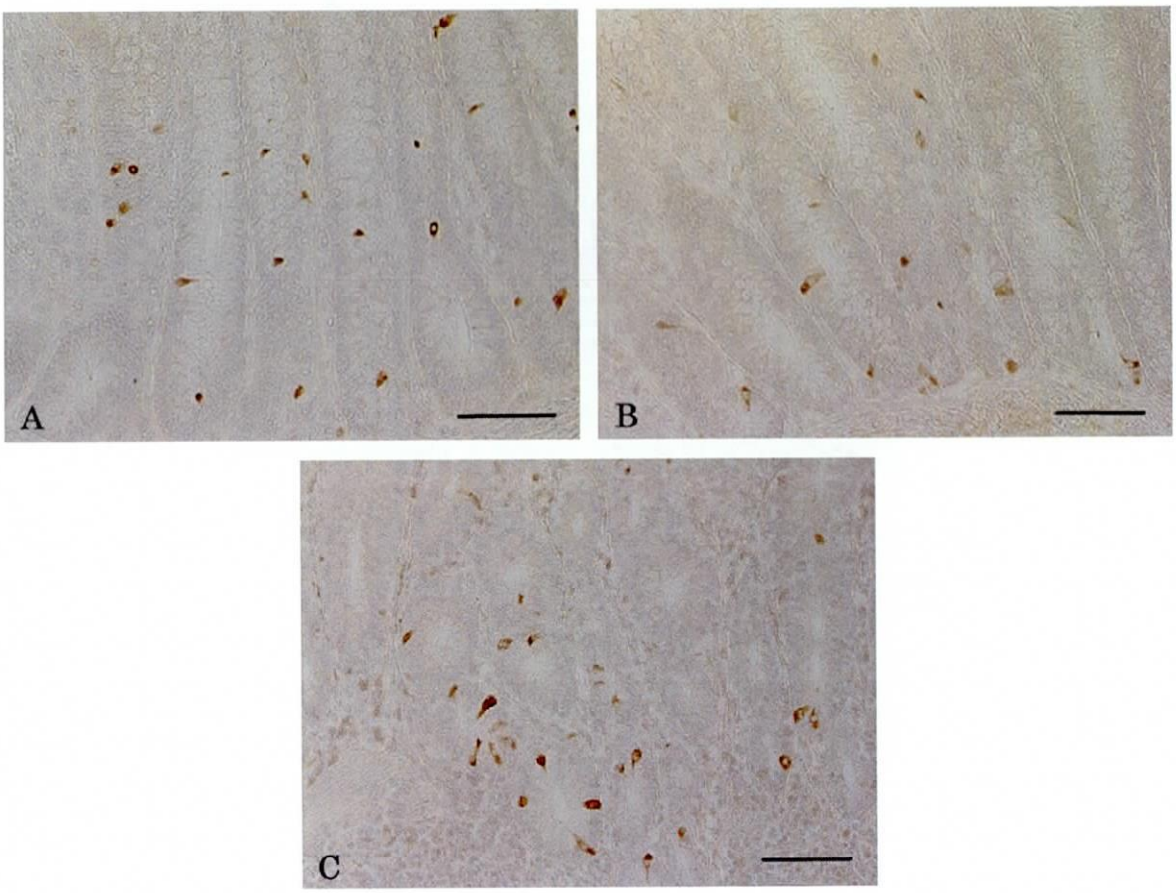

Fig. 3.6. Glucagon-like peptide-1-immunoreactive cells in the large intestine of suckling and weaned calves. (A) sigmoid colon in the suckling calf, (B) ampulla of rectum in the suckling calf, (C) rectum just cranial to the anorectal line in the suckling calf. Bar = 100 μm.

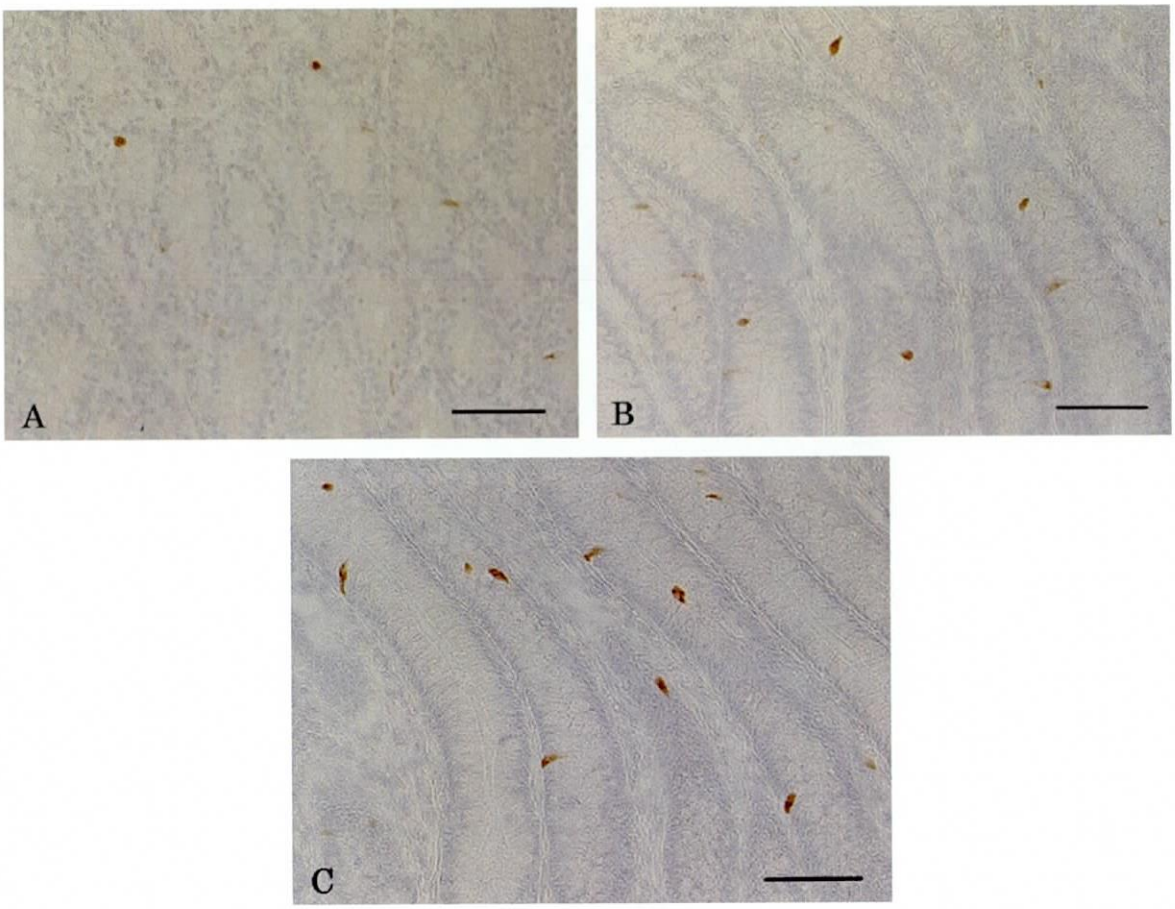

Fig. 3.7. Peptide YY-immunoreactive cells in the large intestine of suckling and weaned calves. (A) distal loop of colon in the weaned calf, (B) transverse colon in the suckling calf, (C) descending colon in the suckling calf. Bar = 100 μm.

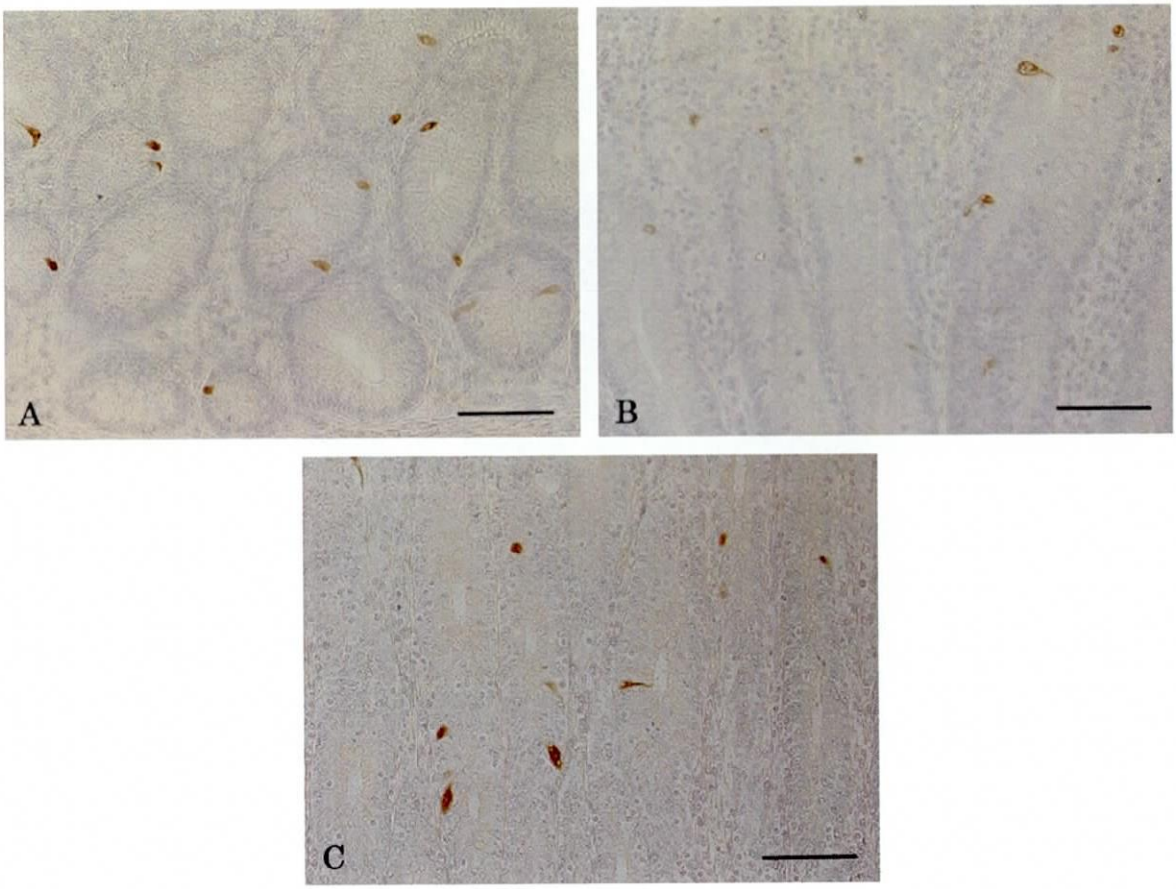

Fig. 3.8. Peptide YY-immunoreactive cells in the large intestine of suckling and weaned calves. (A) sigmoid colon in the suckling calf, (B) ampulla of rectum in the suckling calf, (C) rectum just cranial to the anorectal line in the weaned calf. Bar = 100 μm.

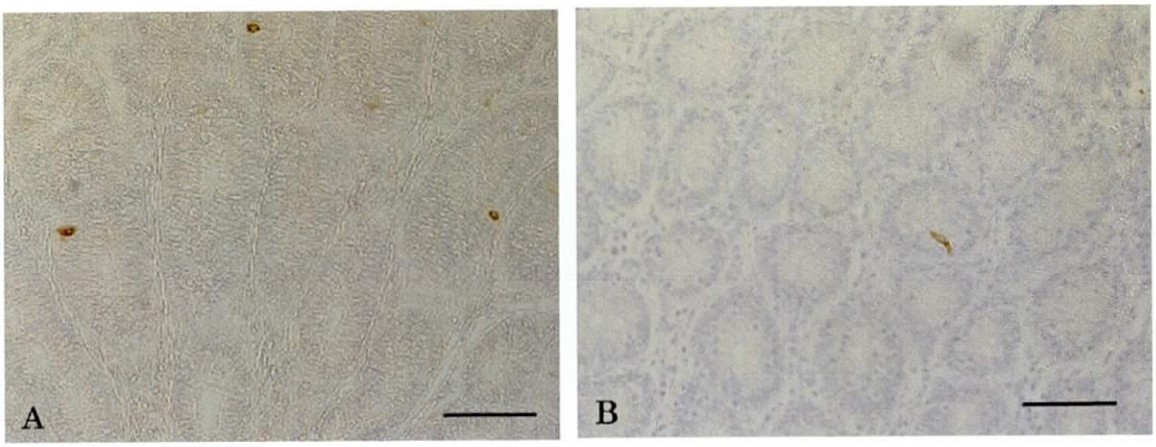

Fig. 3.9. Pancreatic polypeptide-immunoreactive cells in the large intestine of suckling and weaned calves. (A) centrifugal turns of colon in the weaned calf, (B) transverse colon of the suckling calf, Bar = 100 μm.

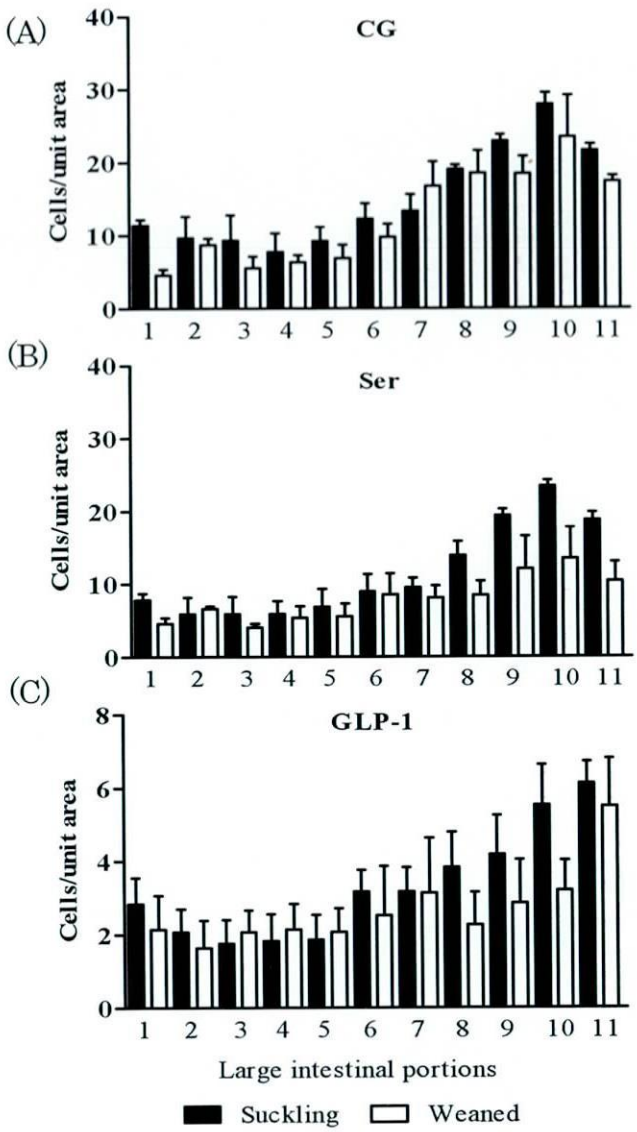

Fig. 3.10. Distribution and relative frequencies of endocrine cells per unit area (625 μm²) in the large intestine of suckling and weaned calves. CG: chromogranin, Ser: serotonin, GLP-1: glucagon-like peptide-1, 1: cecum, 2: proximal loop of colon, 3: centripetal turns of colon, 4: central flexure of colon, 5: centrifugal turns of colon, 6: distal loop of colon, 7: transverse colon, 8: descending colon, 9: sigmoid colon, 10: ampulla of rectum, 11: rectum just cranial to the anorectal line.

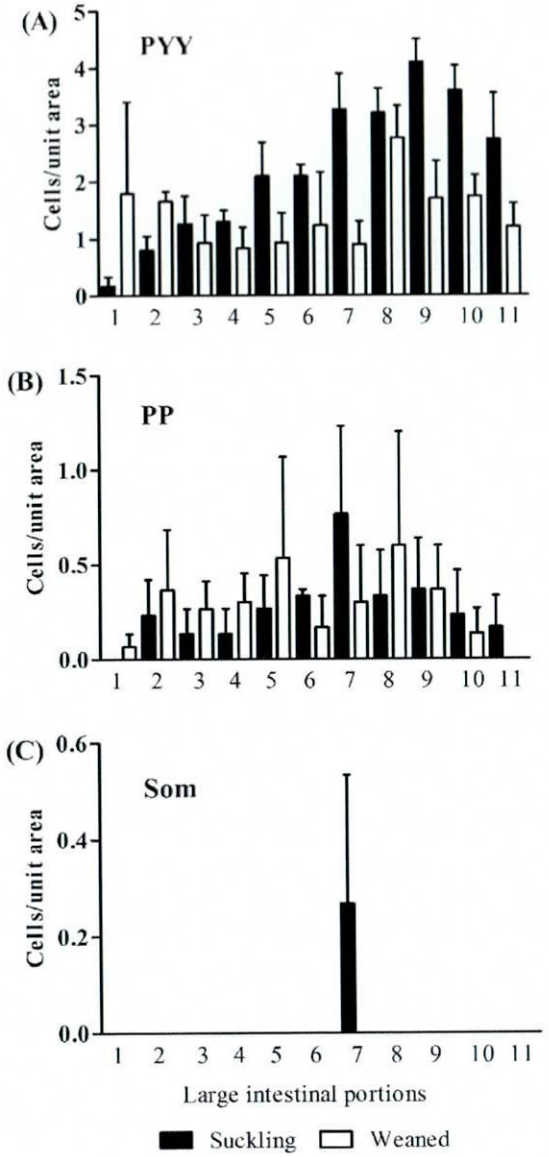

Fig. 3.11. Distribution and relative frequencies of endocrine cells per unit area (625 μm^2) in the large intestine of suckling and weaned calves. PYY: peptide YY, PP: pancreatic polypeptide, Som: somatostatin, 1: cecum, 2: proximal loop of colon, 3: centripetal turns of colon, 4: central flexure of colon, 5: centrifugal turns of colon, 6: distal loop of colon, 7: transverse colon, 8: descending colon, 9: sigmoid colon, 10: ampulla of rectum, 11: rectum just cranial to the anorectal line.

Table 3.1. Distribution and relative frequency of CG-, Ser-, GLP-1, PYY-, PP- and Som-immunoreactive cells in the bovine large intestine at suckling and weaned stages.

Intestinal portions	Endocrine cells					
	CG	Ser	GLP-1	PYY	PP	Som
1 (suckling)	11.40 ± 0.83	7.80 ± 0.90	2.83 ± 0.72	0.16 ± 0.16	ND	ND
1 (weaned)	4.56 ± 0.79	4.60 ± 0.70	2.13 ± 0.93	1.80 ± 1.60	0.06 ± 0.06	ND
2 (suckling)	9.70 ± 2.95	5.90 ± 2.25	2.06 ± 0.63	0.80 ± 0.25	0.23 ± 0.18	ND
2 (weaned)	8.70 ± 0.85	6.56 ± 0.33	1.63 ± 0.75	1.66 ± 0.16	0.36 ± 0.31	ND
3 (suckling)	9.30 ± 3.52	5.83 ± 2.42	1.76 ± 0.64	1.26 ± 0.49	0.13 ± 0.13	ND
3 (weaned)	5.56 ± 1.52	4.00 ± 0.55	2.06 ± 0.60	0.93 ± 0.48	0.26 ± 0.14	ND
4 (suckling)	7.73 ± 2.53	5.86 ± 1.73	1.83 ± 0.72	1.30 ± 0.20	0.13 ± 0.13	ND
4 (weaned)	6.26 ± 1.00	5.30 ± 1.65	2.13 ± 0.69	0.83 ± 0.37	0.30 ± 0.15	ND
5 (suckling)	9.16 ± 1.97	6.80 ± 2.47	1.86 ± 0.66	2.10 ± 0.58	0.26 ± 0.17	ND
5 (weaned)	6.80 ± 1.84	5.50 ± 1.75	2.06 ± 0.64	0.93 ± 0.52	0.53 ± 0.53	ND
6 (suckling)	12.33 ± 2.08	8.93 ± 2.42	3.16 ± 0.59	2.10 ± 0.20	0.33 ± 0.033	ND
6 (weaned)	9.73 ± 1.81	8.46 ± 2.94	2.53 ± 1.3	1.20 ± 0.93	ND	ND
7 (suckling)	13.30 ± 2.31	9.43 ± 1.37	3.16 ± 0.66	3.26 ± 0.63	0.76 ± 0.46	0.26 ± 0.26
7 (weaned)	16.73 ± 3.31	8.06 ± 1.65	3.13 ± 1.50	0.90 ± 0.40	0.30 ± 0.30	ND
8 (suckling)	19.03 ± 0.61[a]	13.83 ± 1.96	3.83 ± 0.95	3.20 ± 0.43	0.33 ± 0.24	ND
8 (weaned)	18.53 ± 3.06[1]	8.36 ± 1.94	2.26 ± 0.87	2.76 ± 0.56	0.60 ± 0.60	ND
9 (suckling)	22.90 ± 0.87[b]	19.33 ± 0.87[e]	4.16 ± 1.08	4.10 ± 0.40[h]	0.36 ± 0.27	ND
9 (weaned)	18.37 ± 2.39[2]	12.07 ± 4.37	2.86 ± 1.16	1.70 ± 0.65	0.36 ± 0.23	ND
10 (suckling)	27.90 ± 1.55[c]	23.37 ± 0.81[f]	5.53 ± 1.10	3.60 ± 0.43[i]	0.23 ± 0.23	ND
10 (weaned)	23.37 ± 5.79[3]	13.47 ± 4.23	3.20 ± 0.81	1.73 ± 0.37	0.13 ± 0.13	ND
11 (suckling)	21.60 ± 0.86[d]	18.77 ± 1.03[g]	6.13 ± 0.58	2.73 ± 0.81	0.16 ± 0.16	ND
11 (weaned)	17.40 ± 0.78[4]	10.37 ± 2.60[5]	5.50 ± 1.30	1.20 ± 0.416	ND	ND

Data represent mean ± SEM; 1: cecum, 2: proximal loop of colon, 3: centripetal turns of colon, 4: central flexure of colon, 5: centrifugal turns of colon, 6: distal loop of colon, 7: transverse colon, CG: chromogranin, Ser: serotonin, GLP-1: glucagon-like peptide-1, PYY: peptide YY, PP: pnacreatic polypeptide, Som: somatostatin, ND: not detected.

The superscript letters (a-i) indicate significant differences of values within individual column for suckling stage ($P<0.05$). The arabic numerals (1-5) indicate significant differences of values within individual columns for weaned stage ($P<0.05$).

Chapter 4

The developmental plasticity of colocalization pattern of peptide YY and glucagon-like peptide-1 in the endocrine cells of bovine rectum

4.1. Introduction

Representative gut hormones that are assumed to have roles in feeding control are peptide YY (PYY) and glucagon-like peptide-1 (GLP-1) (7, 76, 86). PYY is a 36 amino acid straight chain polypeptide belonging to the pancreatic polypeptide family (7, 54). GLP-1 is a peptide hormone belonging to glucagon super-family and produced from the same precursor as glucagon and glicentin (34, 37). PYY and GLP-1 are synthesized and released from a special type of entero-endocrine cells (7, 76). These cells are also referred as the L type of endocrine cells because of the large size of their secretory granules in most animal species (7, 60, 78). A tendency for an increase of L cells along the gut is reported in the large intestine, with the highest density in the rectum (37). Endocrine cells including glucagon-like immunoreactive cells and PYY-IR cells are abundant in the large intestine, particularly in the rectum (39, 78).

Colocalization of PYY and proglucagon-derived peptides (glicentin and GLP-1) in gut endocrine cells has been reported at different ratios in various

animal species (5, 7, 9, 37, 60, 78). However, detailed study on the colocalization of PYY and GLP-1 in the different ontogenetic stages and the plasticity of the colocalization has not been conducted yet. Therefore, evidence is needed to clarify the plasticity of their colocalization depending on the developmental stages of animals. Ruminants have two drastic changes in nutritional stages: from suckling to herbivore, which occurs from fetal to postnatal stages, and from suckling to herbivorous, respectively. The present study was aimed to investigate the colocalization patterns and developmental plasticity of PYY and GLP-1 in the bovine rectum at different ontogenetic stages using immunohistochemical methods

4.2. Materials and methods

Twenty one Holstein cattle at seven different developmental stages, early fetus: 20 - 40 cm in crown-rump-length (CRL, n=3), mid fetus: 41 - 70 cm (n=3), late fetus: 71 - 100 cm (n=3), suckling calf (1 to 2-week-old, n=3), weaning calf (2-month-old, n=3), weaned calf (10-month-old, n=3) and adult (1 to 8-year-old, n=3) were used in this study. Tissue samples from the rectum just proximal to the anorectal line were used. The detail of animals and tissue processing were described in Chapter 1. Consecutive thin (2 μm) sections were used in the immunohistochemistry. The primary antisera used were raised against PYY and GLP-1, which were same as those in Chapter 2 and 3.

4.3. Results

PYY- and GLP-1-immunoreactive cells were detected abundantly in the crypt, being relatively more abundant at the base of the crypt. They were more numerous in younger animals (as described in Chapter 1). The present chapter focused on the colocalization pattern of them. Three types of immunoreactive cells were observed in the present study (Fig 4-1); 1) cells showing immunoreactivities for both PYY and GLP-1 were expressed simply as PYY/GLP-1-immunoreactive cells, 2) cells showing immunoreactivity only for PYY and not for GLP-1 were expressed as PYY-immunoreactive cells, and 3) cells showing immunoreactivity only for GLP-1 not for PYY were expressed as GLP-1-immunoreactive cells. These three types of endocrine cells were observed in all developmental stages at different percentages (Fig. 4.2). The most remarkable differences were observed in PYY/GLP-1-immunoreactive cells among different developmental stages. The percentage of PYY/GLP-1-immunoreactive cells was significantly higher in the fetal (early, mid and late) and suckling stages (64-76%, average 72%) and decreased drastically in the weaning, weaned and adult stages (17-34%, average 26%). No significant differences of PYY/GLP-1-immunoreactive cells were found from the early fetal to the suckling stages and from the weaning to adult stages. The percentage of GLP-1-IR cells was low in the fetal and suckling stages (7-29%, average 18%) and increased in the herbivorous stages (weaning, weaned, adult; 49-58, average 54%). The percentage of PYY-immunoreactive cells was

higher in the period after suckling (17-25%, average 20%) in contrast to that in the period before weaning (6-19%, average 10%) (Fig. 4.2, Table 4.1).

4.4. Discussion

Colocalization of PYY and GLP-1 was proven in the endocrine cells of bovine rectum. Previous immunohistochemical studies on rodents and humans have reported the colocalizaiton of PYY with enteroglucagon or proglucagon-derived peptides such as glicentin and GLP-1 (5, 7, 9, 34, 60). In the present study, the percentages of colocalization changed with the development of cattle. It has been reported that cells expressing both PYY and glucagon appeared in the endocrine pancreas of rat at the transition stages (late fetus and early postnatal) of development (45). Such multihormonal cells were not detected in the mature pancreas. On the other hand, cells expressing both PYY and proglucagon-derived peptides were reported as typical subpopulations of enteroendocrine cells in the distal large intestine of mouse, rat, pig and human (5, 9, 67). It is tempting to associate the pathway variations of cellular differentiation of PYY/GLP-1 cells with the ontogenetic differentiation depending on the regional difference of gut.

However, no study has reported different patterns of PYY and GLP-1 colocalization of gut endocrine cells in different developmental stages. In the present study, PYY and GLP-1 immunoreactivities were abundantly colocalized in different bovine developmental stages. The PYY/GLP-1

colocalization was observed in over 70% of counted cells in the prenatal and suckling stages and had no significant differences throughout those stages. However, the percentage of PYY/GLP-1 endocrine cells was significantly decreased in the herbivorous (weaning, weaned and adult) stages. An interesting finding in this study is the high percentage of PYY/GLP-1 endocrine cells in the suckling stage in addition to the prenatal stage. It is possible that the regulatory mechanism of the gastrointestinal tract in the suckling stage is different from that of animals at the herbivorous stages. In ruminants, marked morphological and functional changes occur around the weaning transition. The percentages of PYY- and GLP-1-IR single endocrine cells were also different among developmental stages. GLP-1-IR single cells increased drastically after the suckling stage, whereas the changes of PYY-IR cells were not as conspicuous as those of PYY/GLP-1- and GLP-1-IR cells. The change and decrease of PYY/GLP-1 (colocalized)-IR cells at the weaning could be related to the adaptation of the regulatory mechanism for the herbivorous nature of the digestion. Functional differentiation may occur from PYY/GLP-1 cell to PYY and/or GLP-1 cells as developmental physiology. It is possible that the differentiation occurs actively at the weaning stage.

The present study revealed the developmental plasticity of colocalization pattern of PYY and GLP-1 in endocrine cells of the bovine rectum. PYY/GLP-1-IR cells might play different physiological roles depending on feeding habits and specific developmental stages of animals. In

this context, regulatory roles of these peptides in the feeding mechanism of ruminant are highly noteworthy.

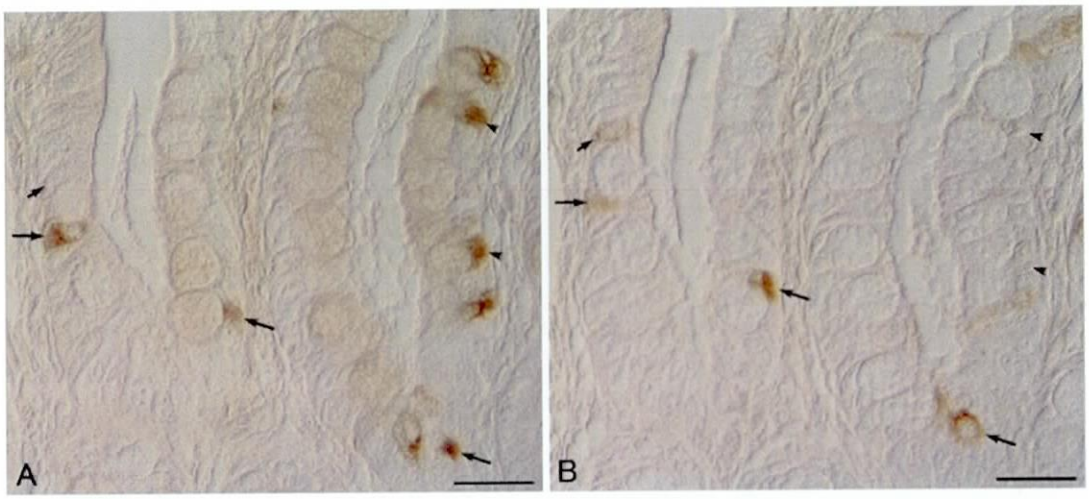

Fig. 4.1. PYY/GLP-1-immunoreactive cells (large arrows), PYY-immunoreactive cell (arrowheads), and GLP-1-immunoreactive cells (small arrows) in consecutive sections immunostained for PYY (A) and GLP-1 (B). Rectum of cattle fetus (CRL 66 cm). Bar: 20 μm.

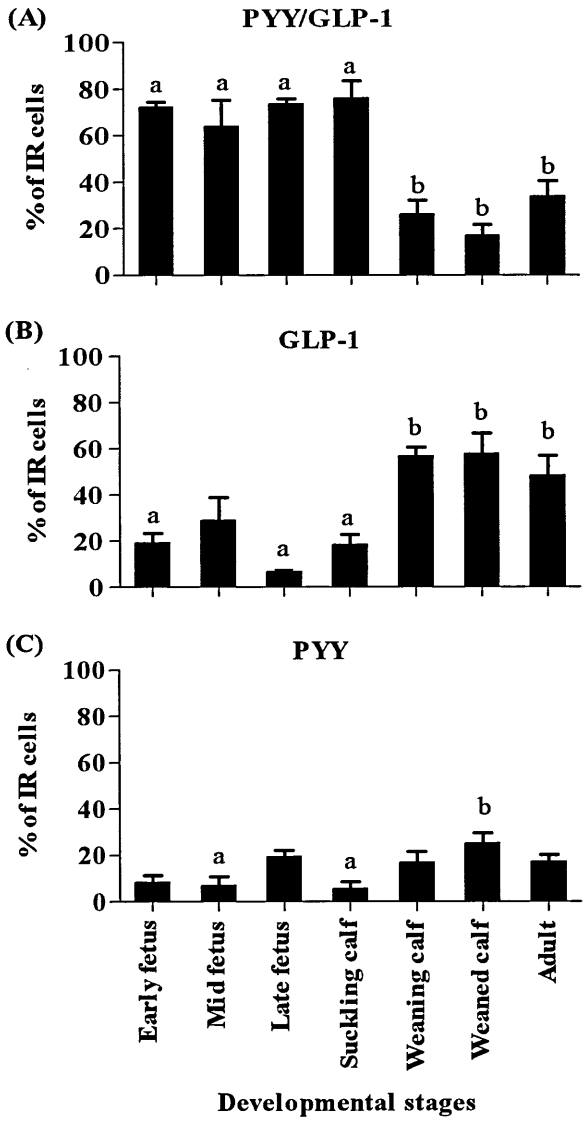

Fig. 4.2. Percentages of peptide YY (PYY) - and glucagon-like peptide-1 (GLP-1)-immunoreactive cells in the bovine rectum at different developmental stages. A: PYY/GLP-1 (colocalized)-immunoreactive cells, B: GLP-1-immunoreactive single cells, C: PYY-immunoreactive single cells. The data represent mean ± SEM, n= 21. Significant differences were observed between results labeled with different characters (a and b) ($P < 0.05$).

Table 4. 1. The percentage of PYY/GLP-1-, PYY- and GLP-1-immunoreactive cells in the rectum of cattle at different developmental stages.

	PYY/GLP-1	GLP-1	PYY
Fetus			
Early	72.33 ± 2.10[a]	19.3 ± 3.98[a]	8.37 ± 3.27
Mid	64.02 ± 11.35[a]	28.86 ± 10.21	7.12 ± 3.85[a]
Late	73.70 ± 2.18[a]	6.853 ± 0.80[a]	19.45 ± 2.77
Calf			
Suckling	76.06 ± 7.51[a]	18.28 ± 4.61[a]	5.663 ± 3.20[a]
Weaning	26.1 ± 6.29[b]	56.80 ± 3.65[b]	17.11 ± 4.60
Weaned	17.11 ± 4.59[b]	57.67 ± 8.97[b]	25.22 ± 4.40[b]
Adult			
Cow	34.1 ± 6.34[b]	48.5 ± 8.43[b]	17.39 ± 3.04

Superscript letters (a and b) shows significant differences within each column ($P < 0.05$), mean ± SEM. GLP-1: glucagon-like peptide-1, PYY: peptide YY.

Chapter 5

General discussion

The regional distribution and relative frequencies of endocrine cells were updated in the bovine digestive tract. The distribution and frequency of CG-, Ser-, PYY-, PP-, GLP-1- and Som-immunoreactive cells varied among different intestinal segments and at different developmental stages. The results were outlined as the conceptual schematic drawings (Fig. 5.1-3).

The present study revealed the region specific distribution of endocrine cells in the bovine intestine. The huge numbers and various kinds of endocrine cells were detected in the large intestine especially distal portions of the colon and rectum. The eccentrically located abundance of endocrine cells in the distal portions of bovine large intestine would reflect the physiological importance of those portions for the regulation of the digestive system in ruminants. It is interesting to associate the characteristic distribution of endocrine cells in the bovine terminal gut with the particular mechanism such as the distal-to-proximal regulation (59, 89).

Furthermore, the colocalization pattern of regulatory peptides (PYY and GLP-1) showed the developmental plasticity. It is postulated that these changes are responsible to the adaptation for the developmental changes of the nutrition of the ruminant as herbivore. The distribution as well as the colocalization of the peptides showed the developmental plasticity.

Characteristic combinations of the distribution at each stage might represent the physiological significance at equivalent stage. For example, abundance of PYY/GLP cells in the suckling calf suggests that the co-secretion of PYY and GLP-1 may have peculiar roles in the coordination of digestive function at the juvenile stage.

It is suggested that PYY/GLP-1 (colocalized) cells might have different physiological roles depending on feeding habits, specific developmental stages of animals, and the physiological functions of its released hormones (PYY and GLP-1). The detail cytological and physiological studies are required to be conducted regarding different patterns of endocrine cell colocalization in different species and the physiological functions of the colocalization in the intestine.

Further studies on the physiological functions of endocrine cells especially on PYY and GLP-1 cells, in ruminants are needed. The present results would provide the basis for future extensive physiological and endocrinological studies on ruminants.

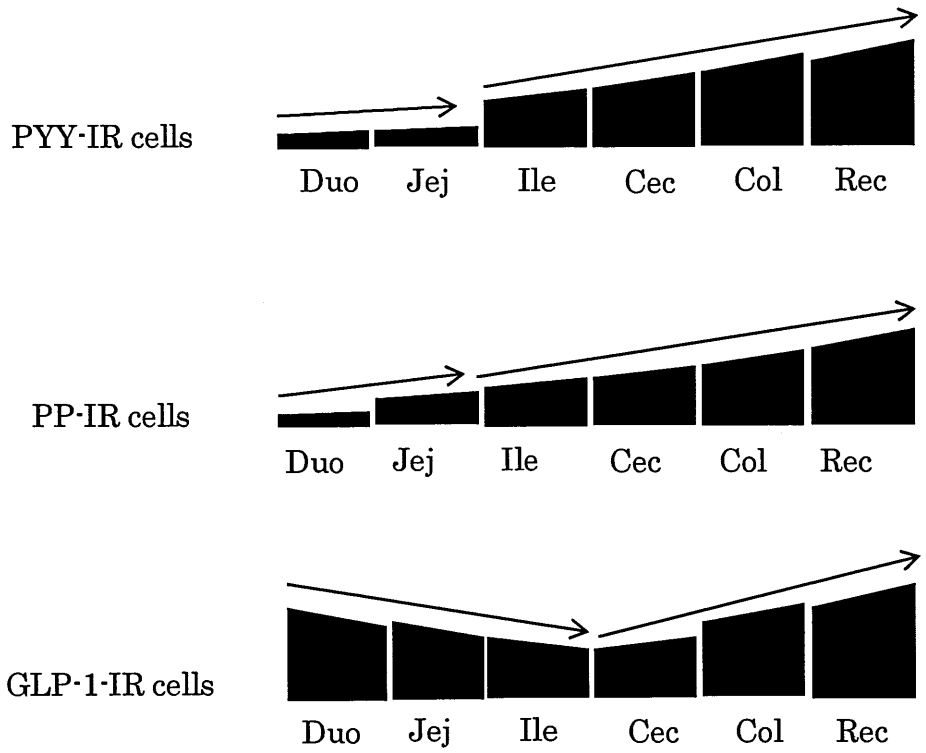

Fig. 5.1. The diagrammatical illustration showing the distributional changes of PYY-, PP- and GLP-1-immunoreactive cells in the small and large intestine of cattle. PYY: peptide YY, PP: pancreatic poly peptide, GLP-1: glucagon-like peptide-1. Duo: duodenum, Jej: jejunum, Ile: ileum, Cec: cecum, Col: colon, Rec: rectum.

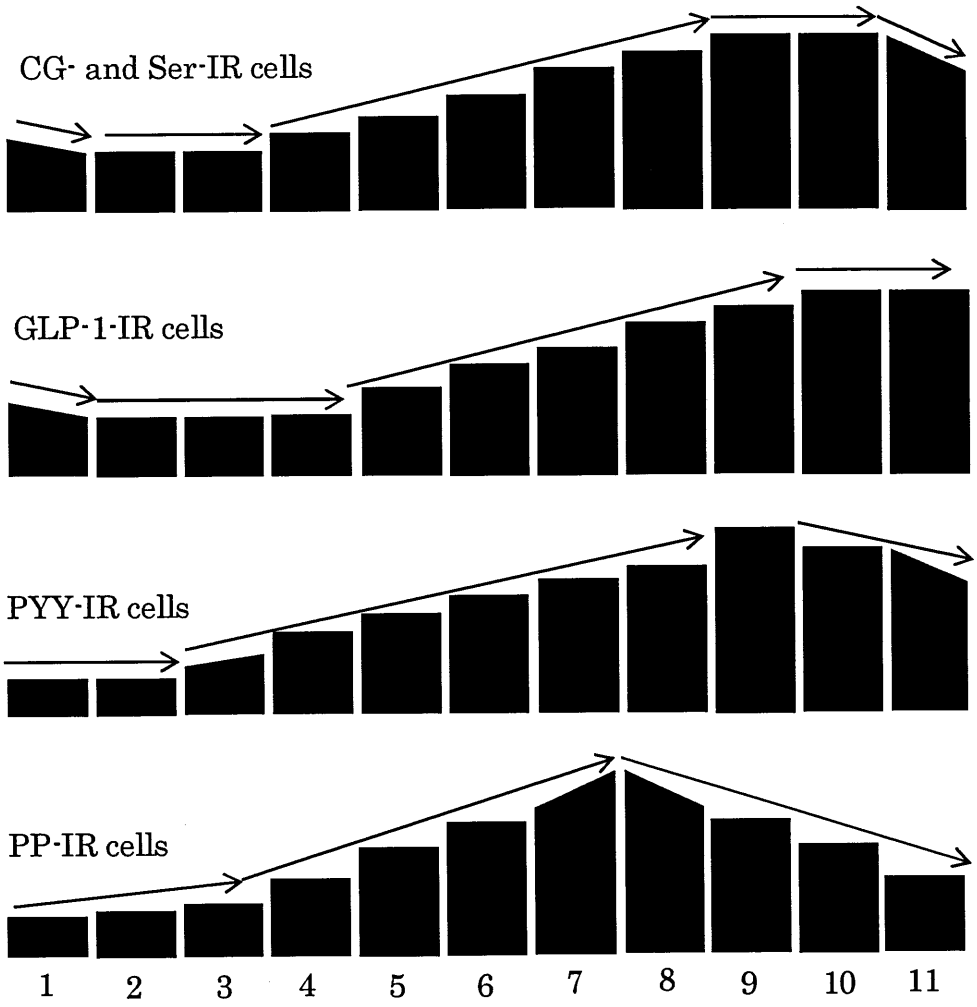

Fig. 5.2. Diagrammatic illustration of endocrine cell distribution in the 11 portions of the large intestine. 1. Cecum, 2. Proximal loop of colon, 3. Centripetal turns of colon, 4. Central flexure of colon, 5. Centrifugal turns of colon, 6. Distal loop of colon, 7. Transverse colon, 8. Descending colon, 9. Sigmoid colon, 10. Ampulla of rectum, 11. Rectum just cranial to the anorectal line.

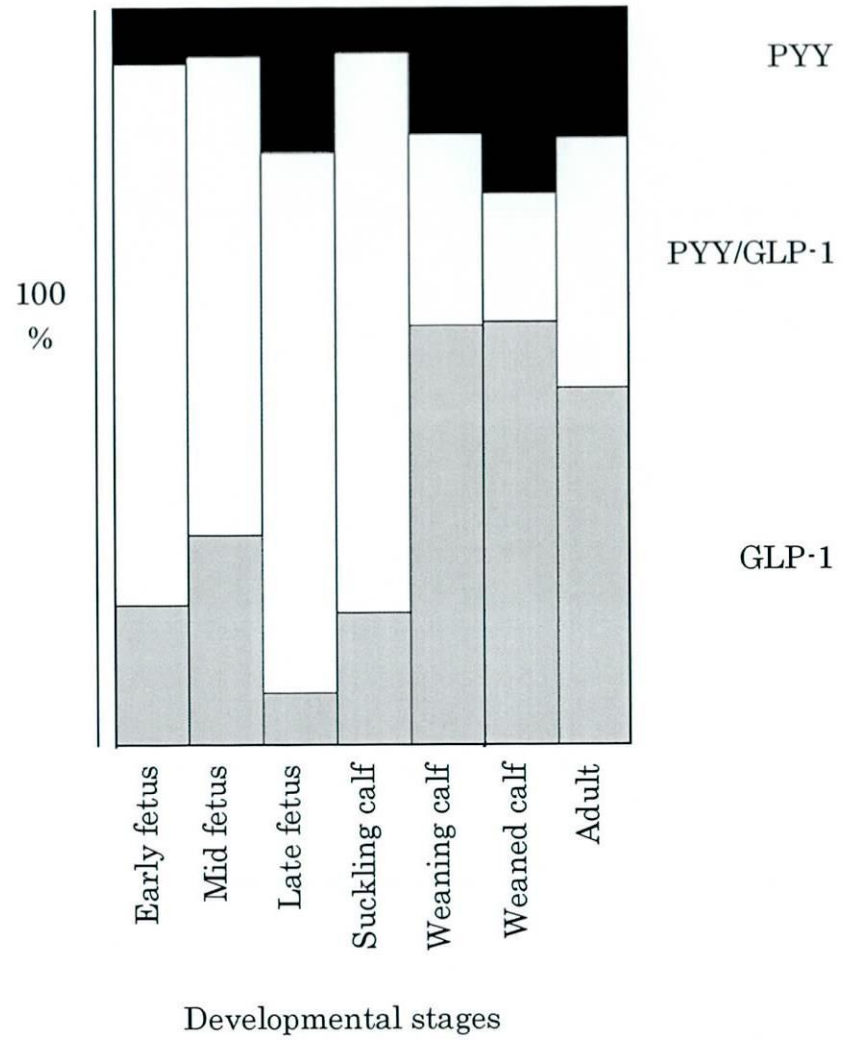

Fig. 5.3. The diagram shows different patterns and plasticity of colocalization of PYY and GLP in endocrine cells of the rectum of cattle at different developmental stages. PYY: cells showing immunoreactivity only for peptide YY, GLP-1: cells showing immunoreactivity only for glucagon-like peptide-1, PYY/GLP-1: colocalized cells with immunoreactivities for both PYY and GLP-1.

Functional Histology of Endocrine Cells in Bovine Intestine at Different Developmental Stages

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Abstract

The gastrointestinal tract is under the control of endocrine cells within. Gut hormones are chemical messenger synthesized and released from special types of endocrine cells in the epithelium of the gastrointestinal tract. Different endocrine cells produce various kinds of hormones for different functions of the gastrointestinal tract. The general distributions of these endocrine cells are various depending on gastrointestinal region, animal species, age, feeding habits and many other factors. The present study investigated the general distribution and relative frequencies of endocrine cells in the gastrointestinal tract of cattle at different developmental stages.

The regional distribution and relative frequency of peptide YY (PYY)-, pancreatic polypeptide (PP)-, and glucagon-like peptide-1 (GLP-1)-immunoreactive cells were determined immunohistochemically in the

gastrointestinal tract at seven ontogenetic stages of pre- and postnatal cattle. Different frequencies of PYY-, PP-, and GLP-1-immunoreactive cells were found in the intestines at all stages. They were not found in the esophagus and stomach. The frequencies varied depending on the intestinal segment and the developmental stage. The frequencies of PYY- and PP-immunoreactive cells were lower in the small intestine and increased from ileum to rectum, whereas GLP-1-immunoreactive cells were more numerous in duodenum and jejunum, decreased in ileum and cecum, and increased again in colon and rectum. The frequencies also varied according to pre- and postnatal stages. All three cell types were most numerous in fetus, and decreased in calf and adult groups, indicating that the frequencies of these three types of endocrine cells decrease with postnatal development. The results suggest that these changes vary depending on the adaptation of growth, secretion, and motility of intestine at different ontogenetic stages of cattle.

The regional distribution and relative frequencies of endocrine cells were also studied in eleven different portions of large intestine of suckling and weaned calves. Six types of endocrine cells were detected throughout the large intestine. They were chromogranin (CG), serotonin (Ser), GLP-1, PYY, PP and somatostatin (Som)-immunoreactive cells. These endocrine cells were found in all eleven portions of large intestine of suckling and weaned calves with different frequencies. These immunoreactive cells were more numerous

in the distal portions of colon and ampulla of the rectum than the rest portions of large intestine. No significant differences of these immunoreactive cells were observed between suckling and weaned groups. This study suggested that the huge numbers of endocrine cells in the large intestine especially distal portions of colon and rectum might be related to the physiological importance of those portions, such as distal-to-proximal feedback regulation, in the control of digestive system.

PYY- and GLP-1-immunoreactive cells are predominant component of endocrine cells in the bovine large intestine as revealed in the above mentioned parts. The present study examined the plasticity of colocalizatrion pattern of PYY and GLP-1 immunoreactivites in the bovine rectum which is revealed to have high concentrations of endocrine cells. Three kinds of cells were identified: 1) cells immunoreactive for both PYY/GLP-1 (colocalized cells), 2) cells immunoreactive only for PYY, 3) cells only immunoreactive for GLP-1. The percentage of PYY/GLP-1 cells was high in the prenatal (early, mid and late fetuses) and suckling stages (71%), however, they decreased in the postnatal (weaning, weaned and adult) stages (26%). On the other hand, PYY (10%) and GLP-1 (18%) single cells were low in the prenatal stages and increased in the postnatal stages (20% and 55%, respectively). This study indicates that the percentage of PYY/GLP-1 (colocalized) cells is significantly decreased in the herbivorous (weaning, weaned and adult) stages of cattle. An interesting finding in this study is the high percentage of PYY/GLP-1 endocrine cells in the suckling stage in addition to the prenatal stage. PYY/GLP-1 cell might be a

new type of endocrine cell which can adapt to the change of digestion depending on feeding habits and/or specific developmental stages of ruminant. This study demonstrated the developmental plasticity of colocalization percentage of gut hormones with the change of the nutritional transition. The changing phase in the combination of hormones which are co-secreted and/or independently secreted may strengthen the complexity and preciseness of the control.

The present study revealed the region specific distribution of endocrine cells in the bovine intestine. The characteristic might change depending on the developmental stages. Furthermore, the colocalization pattern of regulatory peptides showed the developmental plasticity. It is postulated that these changes are responsible to the adaptation for the developmental change of the nutrition of the ruminant as herbivore.

Functional Histology of Endocrine Cells in the Bovine Intestine at Different Developmental Stages

(各発達段階の牛の腸における内分泌細胞の機能組織学的研究)

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要　旨

消化管は消化管内分泌細胞の制御下にある。消化管ホルモンは消化管上皮にある特定の型の内分泌細胞によって合成と放出される化学的伝達物質である。各種内分泌細胞がそれぞれのホルモンを産生して消化管機能を多様に調整している。それら内分泌細胞の分布は消化管の領域，動物種，年齢，食性およびその他の要因により様々である。本研究では，各発達段階の牛の消化管における内分泌細胞の分布と頻度ならびに調節物質の共存関係の可塑性について検索した。

第１章では，消化管内分泌細胞に関するこれまでの知見について概説し，本研究で対象としている調節物質の化学的および機能的特徴ならびに本研究で使用した材料と方法について詳述した。

第２章では，胎子期を３段階（初期，中期，後期），生後を４段階（哺乳期，離乳期，離乳後，成体）に区分して，牛の消化管における peptide YY (PYY), pancreatic polypeptide (PP), and glucagon-like peptide-1 (GLP-1)の各含有

細胞の分布と出現頻度を免疫組織化学により明らかにした。PYY, PP, GLP-1 の各細胞は腸においてすべての時期で認められた。それらは食道と胃では観察されなかった。腸の部位および発達段階によって頻度が異なっていた。これら三種の細胞は胎子で最も多く，子牛と成体で減少した。この減少は各発達段階での発育や食性の変化に適応しているものと考えられた。

第３章では，内分泌細胞の分布と出現頻度に関して，哺乳子牛と離乳後子牛の大腸 11 ヶ所について詳細に検索した。6 種類の内分泌細胞 (Chromogranin: CG, Serotonin: Ser, GLP-1, PYY, PP, Somatostatin: Som)が大腸全体を通して観察されたが，遠位結腸と直腸において特に豊富に認められた。内分泌細胞が特に豊富に認められた消化管末端部は遠位-近位フィードバック機構での調整において重要な役割を果たしているものと考えられた。

第４章では，PYY 細胞と GLP-1 細胞がどの発達段階においても，大腸，特に直腸で豊富に認められたことから，内分泌細胞におけるそれらホルモンの共存関係について検索した。両者を持つPYY/GLP-1 細胞，どちらか一方だけを持っているPYY 細胞と GLP-1 細胞という 3 種が存在していることが確認された。PYY/GLP-1 細胞は胎子期と哺乳期において多く（72%），離乳期以降は大きく減少した（26%）。一方，PYY 細胞は胎子と哺乳期を通して 10%だったものが，離乳期以降は 20%になり，GLP-1 細胞は同じ発達期において 18%から 55%に増加した。離乳期以降のこれらの変化は消化管機能調整機構の草食への適応変化であると考えられた。発達に伴う共存関係に可塑性があることは，各発達段階によって単独分泌お

よび共分泌の組み合わせを変えることで調整機構の高度化ならびに精密化を行っているものと考えられた。

第５章では，総合考察を行った。本研究において，消化管部位特異性の消化管内分泌細胞分布があり，細胞の種類ならびに量ともに消化管末端部に多いことが強調された。さらに，消化管ホルモンの共存には強い可塑性があることが本研究によって明らかにされ，離乳期にホルモンの共存関係が大きく変わることが特筆された。以上のことから，消化管機能の調整機構は各発達段階に合わせた柔軟な対応ができるものと考えられた。

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