Muscular Innervation of the Proximal Duodenum of the Guinea Pig

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Summary. We investigated the muscular structure and innervation of the gastroduodenal junction in the guinea pig. In the gastroduodenal junction, the innermost layer of the circular muscle contained numerous nerve fibers and terminals. Since this nerve network continued onto the deep muscular plexus (DMP) of the duodenum, we surmised that the numerous nerve fibers in the gastroduodenal junction were specialized DMP in the most proximal part of the duodenum. The innermost layer containing many nerve fibers was about 1000 \( \mu \)m in length and 100 \( \mu \)m in thickness in the proximal duodenum. This layer contained numerous connective tissue fibers composed of collagen and elastic fibers. Five to 30 smooth muscle cells lay in contact with each other and were surrounded by fine connective tissue. The nerve fibers in the proximal duodenum contained nerve terminals immunoreactive for choline acetyltransferase, dynorphin, enkephalin, galanin, gastrin-releasing peptide, nitric oxide synthase, substance P, and vasoactive intestinal polypeptide. Adrenergic fibers which contained tyrosine hydroxylase immunoreactivity were rare in the proximal duodenum. In the innermost layer of the proximal duodenum, there were numerous c-Kit immunopositive cells that were in contact with nerve terminals. This study allowed us to clarify the specific architecture of the most proximal portion of the duodenum. The functional significance of the proximal duodenum in relation to the electrical connection and neural cooperation of the musculature between the antrum and the duodenum is also discussed.

Intensive research has been conducted on the gastroduodenal junction by anatomists and physiologists. Anatomical studies on the muscular structure of the gastroduodenal junction have been performed in man and various mammals including the rabbit, cat, dog, pig, ox, horse and guinea pig (HORTON, 1928, 1931; TORGERSSEN, 1942; YAMAGAMI, 1955; CAI and GABELLA, 1984). These studies have demonstrated the occurrence of a proper anatomical sphincter at the end of the gastric antrum, and this has been identified as a thickening of the circular muscle layer. A connective tissue septum has been often recognized in the circular muscle layer between the stomach and the duodenum to divide the pyloric sphincter from the duodenum.

Muscular innervation of the stomach and the small intestine has been extensively studied (for review see FURNESS and COSTA, 1987). In the circular and longitudinal muscle layers, the small nerve bundles running parallel to the long axis of the muscle fibers have been named circular and longitudinal muscle plexuses, respectively. In the small intestine, a deep muscular plexus (DMP) consisting of a dense nerve fiber network is found at the inner part of the circular muscle layer. The DMP separates the innermost thin part of the circular layer from the outer thick one. The innermost part of this layer is formed by special smooth muscle cells (small dark cells) which are smaller in diameter and more electron-dense than the bulk smooth muscle cells existing in the outer, thick part of the layer (GABELLA, 1974, 1989). Nerve bundles of the DMP form a continuous meshwork around and along the small intestine. However, the stomach lacks the DMP and the innermost part of the circular layer (FURNESS and COSTA, 1987), this being a critical difference in the musculature between the stomach and the small intestine.

CAI and GABELLA (1984) showed by electron microscopy that in the guinea pig the innervation density was higher in the pylorus than either the antrum or duodenum. They revealed that the pyloric sphincter in the guinea pig was similar to that in other mammals and suggested it to be a suitable model for the experimental study of the pyloric sphincter. Physiological studies have shown that the pyloric region is richly supplied with both excitatory and inhibitory nerves and that this specialized nerve supply affects the gastroduodenal motility (for review see PAPASOVA, 1989). To explain the gastroduodenal function,
one must know the full innervation pattern of the
gastroduodenal junction.

In this study, we carefully investigated the muscu-
lar structure and muscular innervation in the transi-
tion between the antrum and the proximal duodenum
of the guinea pig, paying particular attention to the
innermost part of the circular layer. To clarify the
nerve character, we used double immunohistochem-
istry for neuronal markers. We further investigated
the interstitial cells of Cajal/special smooth muscle
cells related to the pacemaker function and/or neuro-
transmission (SANDERS, 1996; NAHAR et al., 1998;
KOMURO et al., 1999). As the receptor tyrosine kinase
c-Kit expressed in the gastrointestinal tract is one of
the markers for the interstitial cells of Cajal (BURNS
et al., 1997), the distribution of the c-Kit-immuno-
positive cells could be observed in the gastroduodenal
junction.

MATERIALS AND METHODS

Animals

Adult Hartley guinea pigs (body weight 250-400 g,
males) were used in this study. The use and treat-
ment of animals followed the Guide to Animal Use
and Care of the Nagoya University School of Medicine.
All guinea pigs were anesthetized with an intraperi-
toneal injection of sodium pentobarbital (50 mg/kg).

Histological and immunohistochemical studies of
paraffin sections

The abdomens of ten guinea pigs were opened along
a midline incision and the gastroduodenal junction
—including the gastric antrum and the proximal
duodenum—was dissected out. This segment was
immersed in the Bouin’s solution for 4 h. Fixed seg-
ments were dehydrated with a graded ethanol series,
treated with xylene, and embedded in paraffin
(Merck, Germany). Serial sections (5 μm thickness)
were cut on a microtome and mounted on gelatin-
coated glass slides. These sections were stained with
the azan method (HEIDENHAIN, 1915) or aldehyde-
fuchsin method (GOMORI, 1950).

For immunohistochemical staining, sections were
washed with methanol containing 0.3% H2O2. After
washing with 0.01 M phosphate buffered saline (PBS,
ph 7.2), they were incubated for 1 h in 10% normal
goat serum (diluted with PBS), then incubated with
antibodies (Table 1) for 12 h at room temperature.
The sections were washed three times with PBS, then
treated with biotinylated goat anti-rabbit IgG
(Vector, USA) for 1 h. After washing with PBS, they
were reacted with avidin-biotin-peroxidase complex
(Vector) for 1 h. The sections were incubated for 5
min with a solution containing 0.03% diaminobenz-
idine, and 0.005% H2O2 in 0.1 M Tris-HCl, pH 7.6.
These sections were weakly stained with hematoxy-
lin. All the sections were dehydrated with ethanol
and mounted in Entellan (Merck).

Immunohistochemical studies of frozen sections

The gastroduodenal junctions (five guinea pigs)
were immersed in 2% paraformaldehyde and 0.2% picric acid mixture in a 0.1 M phosphate buffer (PB,
ph 7.4) for 4 h. After washing with PBS, the speci-
mens were soaked overnight in 30% sucrose in 0.1 M
PB at 4°C, embedded in OCT compound (Miles, USA),
and frozen quickly. Fifteen-μm thick sections were
cut with a cryostat and thaw-mounted onto poly-L-
lysine-coated glass slides. Sections were incubated in
the mixture of monoclonal and polyclonal antibodies
(Table 1). Then sections were incubated for 1 h with
the mixture of TRITC (tetramethyl rhodamine iso-
thiocyanate)-conjugated swine anti-rabbit IgG (Dako,
Denmark) and FITC (fluorescein isothiocyanate)-
conjugated goat anti-mouse IgG (Sigma, USA). For
ChAT immunoreactivity, we used FITC-labeled don-
key anti-mouse IgG (Jackson Immunoresearch,
USA), biotin-labeled donkey anti-sheep IgG (Jackson
Immunoresearch) and Texas Red-streptavidin (Vec-
tor). These sections were examined with a confocal
laser scanning microscope MRC-1024 (Bio-Rad, USA)
using a Krypton-Argon laser.

Immunohistochemical studies of fresh frozen sections

Specimens of the gastroduodenal junction (five guinea
pigs) were embedded in OCT compound and frozen
quickly. Fifteen-μm thick fresh frozen sections were
fixed with acetone at 4°C for 10 min. The sections
were double immunostained as described above using
antibodies (Table 1), FITC-conjugated goat anti-rat
IgG (Jackson Immunoresearch), and TRITC-conju-
gated swine anti-rabbit IgG, and examined with a
confocal laser scanning microscope.

Transmission electron microscopic studies

Fifteen guinea pigs were transcardially perfused with
Ringer’s solution and then with a fixative containing
2.5% glutaraldehyde and 2% paraformaldehyde in
0.067 M PB. The gastroduodenal junctions were cut
into small pieces and immersed in the same fixative
for 4 h. These specimens were postfixed with 1% OsO4
and 0.8% potassium ferrocyanide in PB and
embedded in epoxy resin (Epok 812, Oken, Japan).
Ultrathin sections stained with uranyl acetate and
lead citrate were examined under a Hitachi H-7100
transmission electron microscope.
For elastic fiber staining of the ultrathin sections, we treated sections with tannic acid-uranyl acetate solution and lead citrate solution as described by KAJIKAWA et al. (1975).

A quantitative study of the smooth muscle cells and nerve bundles was carried out on the circular muscle layer of the pylorus, the innermost part of the circular layer, and the outer part of the circular layer of the proximal duodenum. After photographing, the number of muscle cells and nerve bundles were counted at a final magnification of ×4000 (Table 2).

**Scanning electron microscopic studies**

Five guinea pigs were fixed the same as for transmission electron microscopy. The gastroduodenal junctions were cut into small pieces and immersed in the same fixative for at least 1 week. The specimens were treated by the NaOH maceration method (TAKAHASHI-IWANAGA and FUJITA, 1986), then immersed in 2% tannic acid for 12 h and 1% OsO₄ for 4 h. After dehydration through an ethanol series, the tissues were critical-point dried using liquid CO₂ and evaporation-coated in an ion coater with gold-palladium. These specimens were examined using a Hitachi S-800 scanning electron microscope.

**RESULTS**

**General histology of the gastroduodenal junction (Fig. 1a)**

The gastric antrum and the proximal duodenum were clearly distinguishable by their mucosal structure (Fig. 1a, arrow). Glandular arrangement of the stomach was transformed to the villous arrangement of the small intestine. Although the lamina muscularis mucosae was continuous throughout the antrum,

<table>
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<th>Table 1. First antibodies</th>
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<tr>
<td><strong>Antigen</strong></td>
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<tr>
<td>Actin</td>
</tr>
<tr>
<td>α-Smooth muscle actin</td>
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<tr>
<td>Calcitonin gene-related</td>
</tr>
<tr>
<td>peptide (CGRP)</td>
</tr>
<tr>
<td>Choline acetyltransferase</td>
</tr>
<tr>
<td>(ChAT)</td>
</tr>
<tr>
<td>c-Kit</td>
</tr>
<tr>
<td>Dynorphin A</td>
</tr>
<tr>
<td>Enkephalin 8</td>
</tr>
<tr>
<td>Galanin</td>
</tr>
<tr>
<td>Gastrin-releasing peptide</td>
</tr>
<tr>
<td>(GRP)</td>
</tr>
<tr>
<td>Neuropeptide Y (NPY)</td>
</tr>
<tr>
<td>Nitric oxide synthase (NOS)</td>
</tr>
<tr>
<td>Protein gene product 9.5</td>
</tr>
<tr>
<td>(PGP 9.5)</td>
</tr>
<tr>
<td>S-100b protein (S100)</td>
</tr>
<tr>
<td>Substance P (SP)</td>
</tr>
<tr>
<td>Synaptophysin</td>
</tr>
<tr>
<td>Synaptobrevin 2</td>
</tr>
<tr>
<td>Tyrosine hydroxylase (TH)</td>
</tr>
<tr>
<td>Vasoactive intestinal</td>
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<td>polypeptide (VIP)</td>
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Fig. 1. Micrographs showing the structure of the gastroduodenal junction (anterior wall). a. Azan-stained tissue. The pyloric sphincter (PS) shows a muscular protuberance between the gastric antrum (ANT) and the duodenum (DUO). Arrow shows the boundary between the gastric and duodenal mucosa. b. PGP 9.5 immunoreactivity. In the initial part of the proximal duodenum, many nerve fibers are observed in the innermost part of the circular muscle layer (arrows). In the duodenum, a network of nerve fibers is found in the inner part of the circular muscle layer (deep muscular plexus, arrowhead). Inset shows a higher magnification of the proximal duodenum which contains numerous nerve fibers in the circular layer (arrows). Asterisks show myenteric ganglia. a and b: ×60, Inset: ×110

Fig. 2. Histological characteristics of the proximal duodenum (a-d, posterior wall) compared with the gastric antrum (e-h) and duodenum (i-l). Immunoreactivity for α-SM actin (c, g, k) and synaptobrevin (b, f, j). Azan stain (a, e, i) and aldehyde fuchsin stain (d, h, l). The inner part of the circular layer of the proximal duodenum shows characteristic features such as numerous collagen fibers (a), nerve terminals (b), and elastic fibers (d). From the same sections, regions about 1 cm oral (gastric antrum, e-h) and 1 cm anal (duodenum, i-l) from the proximal duodenum are photographed at the same magnification. No specialized layers are observed except for a deep muscular plexus (arrowhead) in the duodenum (j). N solitary nerve cell in the nerve fiber, asterisks submucosa, arrows connective tissue septa. ×220
Fig. 2. Legend on the opposite page.
Fig. 3 a–f. Transmission electron micrographs of the submucosal surface of the circular layer in the gastroduodenal junction. These six photographs are taken from one tissue block. a to f are a series of the pyloric sphincter to the duodenum. a shows the pyloric sphincter just oral to the proximal duodenum. b–e show the musculature of the proximal duodenum. (Continued on the next page.)
f shows the musculature of the duodenum just anal to the proximal duodenum. Nerve fiber bundles are shown in yellow. b and e are the inner layer of the circular muscle, which contains numerous nerve fiber bundles. d and e show the inner and outer (asterisks) circular layers. In f, a deep muscular plexus divides circular muscle into inner and outer (asterisks) layers. a-f: ×1,700
Brunner's glands in the proximal duodenum penetrated it to extend into the submucosa. In the terminal antrum, the circular muscle layer thickened and formed a muscular mass, the pyloric sphincter. The thickness of the musculature peaked in the pyloric sphincter, and radically decreased just anal to the pyloric sphincter. The circular muscle of the antrum, pyloric sphincter, and proximal duodenum shifted gradually with no specific border. The circular musculature of the antrum, pyloric sphincter, and initial portion of the proximal duodenum contained connective tissue septa composed of collagen fibers. These septa ran perpendicularly to the long axis of the gastroduodenal junction and broke the circular muscle into muscular blocks. The outer longitudinal muscle was always continuous from the pylorus to the proximal duodenum. Sometimes the musculature was divided by the vessels at the proximal duodenum. A shallow circumferential groove lay between the pyloric sphincter and the proximal duodenum at the outer surface of the gastroduodenal junction.

Muscular innervation

Using the anti-PGP 9.5 antibody on paraffin sections (Fig. 1b), we could readily observe the general innervation pattern of the gastroduodenal junction. The myenteric plexus with nerve cell bodies continued throughout the gastroduodenal junction. The longitudinal muscle plexus was composed of fine nerve bundles running parallel to longitudinal muscle cells. The circular muscle plexus was found throughout the thickness of the circular muscle layer in the gastroduodenal junction. In the small intestine, a network of nerve bundles known as the DMP formed a line parallel to the submucosal surface of the circular layer in the section materials (Fig. 1b, arrowhead). Though this DMP was not observed in the antrum, it appeared surprisingly at the transition between the antrum and the proximal duodenum. At the anal region of the thickest portion of the pyloric sphincter, the inner part of the circular layer contained many nerve fibers (Fig. 1b, arrows). In these nerve bundles, solitary nerve cells were rarely recognizable. These nerve fibers networks continued to the DMP of the duodenum. In this region, nerve fibers at the DMP level increased to be situated in strata in the inner part of the circular layer. This thick DMP was about 1000 μm in length and 100 μm in thickness. The whole circumference of the proximal duodenum revealed this thick DMP.

Using the anti-synaptobrevin antibody (Fig. 2b) allowed clear observation of nerve terminals in the musculature as synaptobrevin is distributed on the synaptic vesicles (SÜDHOF, 1995). Synaptobrevin-immunopositive terminals were distributed with the same pattern as PGP 9.5-immunoreactive nerve fibers. In the initial part of the proximal duodenum, a thick DMP contained numerous immunopositive nerve terminals among the muscle cells.

Under the electron microscope (Fig. 3), the innervation pattern of the inner part of the circular muscle showed profound change from the antrum to the duodenum. The DMP in the duodenum (Fig. 3f) was easily observed between the inner thin and outer thick part of the circular layer, whereas the antrum (Fig. 3a) showed only the circular muscle plexus. The initial segment of the proximal duodenum (Fig. 3b, c) revealed numerous nerve bundles in the inner portion of the circular layer, and these nerves formed the thick DMP as was shown by the light microscopic observations. The ratio of nerve bundles to smooth muscle cells in the inner part of the proximal duodenum showed 3–4 times and 7 times as many as those of the bulk circular muscle in the antrum and the duodenum, respectively (Table 2).

<table>
<thead>
<tr>
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<th>Nerve bundles (N)</th>
<th>Muscle cells (M)</th>
<th>Ratio (N/M)</th>
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<tbody>
<tr>
<td>Pylorus</td>
<td>239</td>
<td>2996</td>
<td>0.080</td>
</tr>
<tr>
<td>Proximal duodenum (inner)</td>
<td>865</td>
<td>3029</td>
<td>0.286</td>
</tr>
<tr>
<td>Proximal duodenum (outer)</td>
<td>114</td>
<td>2991</td>
<td>0.038</td>
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Pylorus: circular muscle of the pyloric sphincter
Proximal duodenum (inner): innermost layer of the circular muscle of the proximal duodenum
Proximal duodenum (outer): outer layer of the circular muscle of the proximal duodenum

Number of muscle cells and nerve bundles counted in 25,000 μm²–30,000 μm² areas.
Fig. 4 a–c. Transmission electron micrographs of the innermost part of the circular layer in the proximal duodenum. a–c show connective tissue distribution in the musculature with tannic acid-uranyl acetate and lead citrate staining. In these micrographs, elastic fibers (arrows) are shown to be amorphous materials with high electron density, and collagen fibers are electron dense thin structures. The inner circular layer contains numerous elastic fibers and collagen fibers around the smooth muscle cells. The outer circular layer (asterisks) contains a few connective tissues. N shows nerve bundles. d and e show interstitial cells (IC) in the musculature. These cells are distributed in the spaces among the smooth muscle cells and often locate near the nerve bundles (N). S Schwann cell. a: $\times 1,560$, b: $\times 8,400$, c: $\times 23,000$, d: $\times 5,600$, e: $\times 8,600$
Muscular specialization

We treated the consecutive sections with azan stain, aldehyde-fuchsin stain, actin immunohistochemistry, and synaptobrevin immunohistochemistry (Fig. 2). On the azan-stained sections (Fig. 2a), the inner part of the circular layer of the proximal duodenum was organized into numerous small groups of smooth muscle cells with fine connective tissue networks stained with aniline blue, whereas the outer part of the circular layer contained coarse connective tissue septa. In the antrum (Fig. 2e), numerous septa were observed in the circular layer. In the duodenum, a few septa were observed in the outer part of the circular layer. These connective tissue septa were composed of collagen and elastic fibers and often continued from the submucosal surface to the myenteric border of the circular layer. The anti-α-SM actin antibody clearly showed the muscular architecture of the proximal duodenum (Fig. 2c). In the inner part of the circular layer, a small number of cells formed a group, whereas a large number of cells in the outer part of the circular layer lay in contact with each other and formed a large muscular mass as seen in the antrum (Fig. 2g) and the duodenum (Fig. 2k). The inner part of the proximal duodenum, which showed fine connective tissue networks and numerous small bundles of smooth muscle cells, accorded with the rich nerve terminal area (thick DMP) revealed by synaptobrevin immunoreactivity. Under the electron microscope (Figs. 3, 4), about 5–30 smooth muscle cells appeared in contact with each other in the inner part of the circular layer. Many collagen fibers and nerve bundles were observed among these muscle cell groups (Fig. 4a).

Using aldehyde-fuchsin, violet-stained elastic fibers were demonstrated as thin filamentous structures at the connective tissue bundles in the muscle layers. In the inner part of the proximal duodenum (Fig. 2d), many violet fibers were distributed between the smooth muscle cells. These fibers ran along the circumference, parallel to the long axis of the smooth muscle cells, whereas elastic fibers in the connective tissue septa coursed randomly. In the duodenum (Fig. 2i), the inner part of the circular layer contained elastic fibers as did the proximal duodenum. In the antrum (Fig. 2h) and outer circular layer of the duodenum, the connective tissue septa contained numerous elastic fibers. Using a tannic acid-uranyl acetate staining method (Fig. 4a–c), the central component of the elastic fibers was easily observed as electron-dense structures by electron microscopy. Many elastic fibers were distributed around the smooth muscle cells in the inner part of the proximal duodenum, whereas the outer circular layer contained elastic fibers in the connective tissue septa and the perivascular space.

Among the smooth muscle cells lay interstitial cells of Cajal/specialized smooth muscle cells, fibroblasts/fibroblast-like cells and macrophages (Fig. 4d, e). In the ultrathin sections, the interstitial cells of Cajal revealed their oval or slender cell bodies. The perinuclear region had a narrow cytoplasm with few organelles. The elongated processes of the cells contained many mitochondria, a Golgi apparatus, endoplasmic reticulum, and ribosomes. A few caveolae were recognized along the cell surfaces. They were surrounded by basal laminae. These cells lay near the nerve bundles and were often in direct contact with nerve terminals. In the interstitial space, there were fibroblasts/fibroblast-like cells which possessed flattened cell bodies with numerous rough endoplasmic reticulum and a Golgi apparatus (Komuro et al., 1999), and macrophages which contained many lysosomal dense granules and vacuoles. These types of cells lacked basal laminae around their surfaces. Schwann cells were also observed in the interstitial spaces (Fig. 4d). These cells were distinguished from other cells by their thin processes surrounding the nerve fibers.

Immunohistochemical characterization of nerves (Fig. 5)

To characterize the immunohistochemical phenotype of the nerves in the thick DMP of the proximal duodenum, we employed the double immunolabeling

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**Fig. 5 a–l.** Immunohistochemical demonstrations of the nerve fibers supplying the innermost part of the circular layer in the proximal duodenum. Green colored structures show synaptophysin immunoreactive nerve terminals. Red shows immunoreactivity to the neuronal markers or glial marker as shown below. Yellow structures are double-labeled nerve terminals. *Asterisks* show submucosa. **a:** S-100b protein immunoreactivity (red) which shows Schwann cells, **b:** choline acetyltransferase immunoreactivity (ChAT), **c:** CGRP immunoreactivity, **d:** dynorphin immunoreactivity (Dyn), **e:** enkephalin immunoreactivity (Enk), **f:** galanin immunoreactivity (Gal), **g:** GRP immunoreactivity, **h:** NOS immunoreactivity, **i:** NPY immunoreactivity, **j:** substance P immunoreactivity (SP), **k:** TH immunoreactivity, **l:** VIP immunoreactivity. ×140.
Fig. 5. Legend on the opposite page.
method. To visualize all the nerve terminals, we examined the immunoreactivity for synaptophysin, which is a synaptic vesicle protein (SÜDHOF, 1995). Synaptophysin immunoreactivity and S-100β immunoreactivity (Fig. 5a), which shows the framework of the enteric nervous system (KOBAYASHI et al., 1986), revealed the general innervation pattern of the muscle layer as observed by the PGP 9.5 immunoreactivity. ChAT immunoreactivity (Fig. 5b), which was a marker of cholinergic nerves, was distributed in numerous nerve terminals in the inner part of the circular layer. Almost all nerve bundles contained ChAT-immunoreactive nerves. Nerve fibers immunopositive for dynorphin (Fig. 5d), enkephalin (Fig. 5e), galanin (Fig. 5f), GRP (Fig. 5g), NOS (Fig. 5h), substance P (Fig. 5j) and VIP (Fig. 5l) were recognized in most nerve bundles of the inner part. CGRP (Fig. 5c), NPY (Fig. 5i) and TH (Fig. 5k) immunopositive nerves were rare in the muscle layer. The DMP in the duodenum showed the same spectrum of immunohistological types with this thick DMP.

c-Kit-immunoreactive cells and nerve fibers (Fig. 6)

C-Kit-immunoreactive cells were distributed among the smooth muscle cells in the musculature of the gastroduodenal junction (Fig. 6a, c). They possessed a multipolar shape and long processes. Most of the c-Kit-immunopositive cells were associated with synaptobrevin-immunopositive nerve terminals (Fig. 6b, d). The c-Kit-immunopositive cells and synaptobrevin-immunopositive nerve terminals were distributed richly in the innermost part of the proximal duodenum. In the antrum and outer circular layer of the proximal duodenum, c-Kit- and synaptobrevin-immunopositive structures were fewer than in the innermost layer of the proximal duodenum. In the duodenum, many c-Kit-immunopositive cells were observed to be associated with DMP and myenteric plexus.
Scanning electron microscopic observation (Fig. 7)

Three-dimensional relationships among the smooth muscle cells, nerve fibers and interstitial cells were demonstrated along the submucosal surface of the circular layer of the proximal duodenum. Smooth muscle cells were clearly observed with their long and wide processes. Among the circular muscle cells, interstitial cells which had spindle-shaped or triangular-shaped perinuclear cytoplasm were observed. These cells had long slender cytoplasmic processes that were associated with each other and formed a network. Nerve bundles with many varicosities also formed a network among these cells.

DISCUSSION

This study employed light and electron microscopy to provide evidence that the nerve fibers in the innermost part of the circular layer in the gastroduodenal junction outnumbered those in the surrounding region. It is known that nerve terminals in the inner part of the circular muscle of the small intestine are numerous; they are called the DMP; deep muscular plexus (FURNESS and COSTA, 1987). As the nerve fiber network observed in this study was continuous with the DMP, we considered it a specialized DMP and called it thick DMP. Since this thick DMP was recognized neither at the pyloric sphincter nor at the antrum, we regarded the part containing the thick DMP to be the most proximal, initial portion of the duodenum.

Muscular innervation

Our study showed that the innermost part of the circular layer contained the most abundant nerve fibers in the proximal duodenum. A number of investigators have reported that adrenergic fibers (COSTA and GABELLA, 1971; GILLESPIE and MAXWELL, 1971), nitrergic fibers (WARD et al., 1994, 1998; WANG et al., 1996; KUO et al., 1998) and peptidergic fibers containing enkephalin, substance P, and VIP (ALUMETS et
al., 1979; Edin, 1980; Ferré et al., 1989; Belai et al., 1995) in the gastroduodenal junction were more numerous than those in the antrum and the duodenum. Most of these reports did not pay attention to the innermost portion of the gastroduodenal junction except for the study by Kudo et al. (1998). We have also recognized numerous nerve terminals which showed immunoreactivity for PGP 9.5, as well as synaptic vesicle proteins such as synaptophysin and synaptobrevin (Sudhof, 1995) in the innermost part of the gastroduodenal junction of the mouse, rat, and canine (data not shown). It is likely that these nerve fiber networks in the innermost part of the circular layer are shared by mammalian species and essential for gastroduodenal motility.

From immunohistochemical studies in the small intestine of the guinea-pig, nerve fibers in the DMP are known to be immunoreactive for ChAT, dynorphin, enkephalin, GRP, NOS, NPY, SP and VIP (Furness and Costa, 1987; Furness et al., 1987; Steeke et al., 1991; Costa et al., 1992), whereas CGRP and TH-immunopositive nerves are rare in the DMP (Furness and Costa, 1987). In our study, an immunohistochemical pattern similar to the DMP was observed in the thick DMP of the proximal duodenum. These immunohistochemical findings also confirm the continuity between the thick DMP of the proximal duodenum and that of the duodenum.

Most of the nerve fibers in the DMP of the small intestine are intrinsic to the intestine and originate from myenteric neurons (Wilson et al., 1987). Myenteric neurons in the guinea pig ileum projecting to the circular muscle have been classified as excitatory neurons, which show ChAT and SP immunoreactivity, and inhibitory neurons, which show NOS and VIP immunoreactivity (Costa et al., 1996). ChAT/SP-immunopositive neurons project their axons orally and NOS/VIP-immunopositive neurons, anally. These findings are also obtained in the myenteric neurons in the duodenum (Clerc et al., 1998). In the guinea pig stomach, most myenteric neurons contain ChAT or NOS immunoreactivity, and these neurons often show SP or VIP immunoreactivity, respectively (Schemann et al., 1995; Vanden Berghe et al., 1999). In the gastroduodenal junction, although the immunohistochemical phenotype of the myenteric neurons has not been determined, ChAT/SP-immunopositive excitatory neurons, NOS/VIP-immunopositive inhibitory neurons, and other types of neurons in the myenteric ganglia may innervate the circular muscle, especially the innermost layer.

Muscular structure

Our study showed that the innermost part of the circular muscle in the guinea pig proximal duodenum was composed of small muscle bundles and contained many nerve fibers. Smooth muscles having these characteristics have been classified as multunit smooth muscles (Bozler, 1948). We consider that the inner circular muscles may act as a multunit smooth muscle consisting of numerous independent units. On the other side, the outer circular muscles of the proximal duodenum can be classified as unitary muscles which receive a few nerve fibers and behave like single units (Bozler, 1948). In the canine pylorus, it was demonstrated that the circular muscle consisted of small bundles of smooth muscle cells with an extensive connective tissue in the submucosal region (Vogalis et al., 1991; Ward et al., 1994). The smooth muscle cells of the submucosal region exhibited electrical properties different from the main circular muscles. These studies also suggested that the submucosal region which is divided into small bundles works like multunit smooth muscles, and outer part of the circular layer works as unitary smooth muscles.

It has been reported in several species that the pyloric sphincter and the proximal duodenum are divided by connective tissue septum and vessels (Horton, 1928, 1931; Torgersen, 1942; Yamagami, 1955). The guinea pig also has a septum of connective tissue spanning the full thickness of the circular muscle (Caia and Gabella, 1984). Most researchers have thought that these septa mark the border between the pyloric sphincter and the proximal duodenum. In our observations of the gastroduodenal junction, however, there were many connective tissue septa in the pyloric sphincter and a few septa in the proximal duodenum. On the other hand, we could not determine a distinct septum between the pyloric sphincter and the proximal duodenum. Therefore, we consider that the pyloric sphincter and the proximal duodenum cannot be divided by any of the connective tissue septa.

The innermost part of the circular layer contained numerous collagen and elastic fibers around smooth muscle cells. These characteristics are similar to the inner layer of the small intestine (Gabella, 1974, 1989). Therefore, these findings also confirm the continuity of the inner layer between the most proximal portion of the duodenum and the duodenum. The numerous collagen and elastic fibers in this layer are consonant with the idea that these fibers play a mechanical role in the transmission of force and can be regarded as constituting an intramuscular microtendon (Gabella, 1989). It is believed that small
bundles of smooth muscle cells in the proximal duodenum are connected by these microtendons and show sensitive movements regulated by the numerous nerves.

**Interstitial cells of Cajal**

In this electron microscopic study, we observed the interstitial cells of Cajal among the smooth muscle cells in the inner circular layer of the proximal duodenum. These cells were almost identical to the interstitial cells of Cajal in the circular layer of the stomach or in the DMP layer of the small intestine (Komuro et al., 1999). Most interstitial cells of Cajal are known to show c-Kit immunoreactivity in the guinea pig gastrointestinal tract (Burns et al., 1997). In the small intestine, c-Kit-immunopositive cells are located only in the DMP and myenteric plexus layers, whereas in the gastric antrum, they are distributed in the whole musculature. Using an anti-c-Kit antibody, we observed c-Kit-immunopositive cells in the whole musculature, especially the inner part of the circular layer containing numerous c-Kit-immunopositive cells.

The interstitial cells of Cajal associated with the myenteric plexus are thought to generate electrical slow waves, whereas interstitial cells associated with the circular muscle plexus and DMP are thought to act as modulators of the neurotransmission (Sanders, 1996; Komuro et al., 1999). Interstitial cells of Cajal in the circular layer are closely associated with cholinergic or nitrergic nerve terminals, and have been suggested to mediate the excitatory or inhibitory neurotransmission (Burns et al., 1996; Ward et al., 1998, 2000). In the proximal duodenum in this study, c-Kit-immunopositive cells were in contact with numerous nerve terminals and these terminals contained ChAT-immunoreactivity or NOS-immunoreactivity. It is likely that the numerous c-Kit immunopositive cells in the proximal duodenum are involved in neurotransmission rather than slow wave generation. These cells may receive excitatory or inhibitory input from the enteric neurons and conduct these stimuli to the adjoining smooth muscle cells.

**Function of the proximal duodenum**

Electrophysiological studies of the canine gastro-duodenal junction (Allescher et al., 1988; Sanders and Vogalis, 1989; Vogalis and Sanders, 1990; Vogalis et al., 1991; Bayguinov et al., 1992) have indicated spontaneous electrical slow waves being generated in the antrum and conveyed to the pyloric region via the circular muscle. Since in this study most circular muscle cells in the pylorus and the proximal duodenum were electrically quiescent, slow waves disappeared in the gastroduodenal junction. In the region just anal to the proximal duodenum, slow waves appeared again and increased with amplitude. These findings imply that the gastroduodenal junction is an electrical insulator that prevents slow-wave conduction from the stomach to the duodenum, and requires neural control for gastroduodenal motility. Our data indicating that the innermost part of the proximal duodenum is composed of a multiunit smooth muscle with a rare electrical connection and rich nerve fibers are in good accordance with this hypothesis. As Vogalis et al. (1991) suggested that the submucosal region of the canine gastro-duodenal junction is neurally regulated and shows contractile behavior different from the stomach, the proximal duodenum of the guinea pig may also show a behavior distinct from the stomach. We believe that electrical slow waves spreading from the distal antrum to the pyloric sphincter decrease in the gastroduodenal junction, especially the innermost part of the proximal duodenum, and that the proximal duodenum shows electrical properties different from the antrum. The proximal duodenum, moreover, may show a pattern of neural control similar to that of the duodenum.

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**REFERENCES**


