A Smooth Muscle Nodule Producing 10–12 Cycle/Min Regular Contractions at the Mesenteric Border of the Pacemaker Area in the Guinea-Pig Colon*

Shigeru KOBAYASHI¹, Jalal Uddin CHOWDHURY², Hiroyuki TOKUNO², Niru Shamsun NAHARI¹ and Satoshi IINO¹

Departments of Anatomy¹ and Physiology², Nagoya University School of Medicine, Nagoya, Japan

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Summary. At the boundary between the proximal and distal divisions of the colon in the guinea-pig is a ring-like section which rhythmically contracts. HUKUHARA and his co-researchers demonstrated that antiperistaltic movements in the proximal colon start from this ring-like section, the so-called pacemaker area. Tissue specimens, 0.1–0.3 mm in width/height x 4–7 mm in length, were prepared from various parts of this area. Significantly, in the circular muscle at the mesenteric border, a nodular structure spontaneously producing 10–12 cycle/min regular mechanical contractions was found. Moreover, histological investigations after physiological recording revealed that the presence of the innermost and/or outermost portions of the circular muscle coat was not necessary for these spontaneous activities. Champy-Maillet (ZIO) staining showed that smooth muscle cells in this spontaneously contracting nodule were heavily innervated. Transmission electron microscopy showed that the smooth muscle tissue of this particular area was characterized by scanty interstitial elements such as fibroblasts. Plasma membranes of adjacent smooth muscle cells were frequently in direct contact with each other, forming many gap junctions. Scanning electron microscopy in the specimen prepared using a NaOH-maceration method revealed fine three-dimensional relationships between nerve terminals and smooth muscle cells. The nodular structure described in this paper may provide a useful experimental model for the investigation of colonic motility and its neural control.

MATERIALS AND METHODS

The cellular mechanism of intestinal motility has yet to be studied in detail. In the guinea-pig, the boundary between the proximal and distal divisions of the colon forms a characteristic flexure region (COOPER and SCHILLER, 1975). AIBA (1933) and MASUDA (1937) were the first to show that antiperistaltic activities described by ELLIOTT and BARCLAY-SMITH (1904), among others, started in this region, to gradually spread up the proximal colon. HUKUHARA and NEYA (1968), who termed this area the pacemaker area of the guinea-pig colon, showed that it sent waves, antiperistalses and peristalses, rhythmically in either the proximal or distal direction.

KOYAMALISHI et al. (1995) have recently shown that, in the canine proximal colon, the presence of an inner sublayer containing specialized smooth muscle cells was essential for pacemaker activities in the circular muscle coat. Thus, it seemed useful to re-examine whether in the guinea-pig colon any structural (or muscular) specialization might be present in the pacemaker area. A nodular structure consisting of a smooth muscle tissue producing 10–12 cycle/min regular spontaneous contractions was found in the circular muscle layer at the mesenteric border, and its histological features were examined. The purpose of the present paper is to preliminarily report the physiological, pharmacological and histological properties of this smooth muscle nodule.
(Nembutal; 100 mg/kg body weight) and stunned. The abdomen was opened along a midline incision and a segment of the colon containing the flexure region was removed. This segment contained the colonic pacemaker area, or sphincter coli, as described by HUKUHARA and co-researchers (see, HUKUHARA and NEYA, 1968).

**Physiological and pharmacological experiments**

The methods used for recording colonic activities were essentially the same as those described previously by CHIHARA and TOMITA (1987). Strips of the circular muscle coat about $0.1 \times 0.3\,\text{mm}$ in width/height (length: 4–7 mm) were cut parallel to muscle fibers. The muscle preparations were mounted horizontally in a small chamber (0.3 ml) by attaching one end with small pins to the bottom of the chamber and connecting the other end to a strain gauge force transducer system with a fine thread, to record tension development. The chamber was perfused with a

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**Fig. 1.** A schematic drawing of the guinea-pig colon showing the site of the colonic pacemaker area. A ring-like section at the boundary between the proximal and distal divisions of the colon is the pacemaker area designated by HUKUHARA and co-researchers (see, HUKUHARA and NEYA, 1968). We found that preparations of the circular muscles containing the mesenteric border of the pacemaker area produced 10–12 cycle/min regular mechanical contractions. **A.** Overview of the guinea-pig colon. The pacemaker area described by HUKUHARA and co-researchers is marked in red. **B.** Higher magnification of the colonic flexure region indicating the location of the smooth muscle nodule producing the constant rhythm (P-nodule; red). The distribution and arrangement of blood vessels visible to the naked eye are shown. The flexure region showed rhythmic hinge-like movements, pivoting around the P-nodule, when removed and kept in normal Krebs solution warmed to 25–30°C. **Dotted area** represents the mesenterium.
Krebs physiological salt solution (prewarmed to 35°C) at a rate of 2 ml/min. The normal Krebs solution contained (mM): NaCl 127, KHCO₃ 6, CaCl₂ 2.4, MgCl₂ 1.2, glucose 12, Tris-HCl 10, pH being adjusted to 7.4 at 35°C.

The following drugs and chemicals were used in the present experiment:


Histological methods

Histological methods used in the present study were also essentially the same as those used in our previous studies (KOBAYASHI et al., 1994, 1995). Thus, the Champy-Maillet (ZIO) staining for autonomic nerve terminals and related structures, scanning electron microscopy combined with NaOH-maceration, and conventional transmission electron microscopy using ultra-thin sections (70 nm thick) of epoxy-resin embedded materials were the major methodologies employed. Toluidine-blue stained semithin sections (1.0 μm thick) of resin embedded tissues were examined and photographed by light microscopy.

For both scanning and transmission electron microscopy, the colonic flexure region was selectively perfusion-fixed with 2.5% glutaraldehyde solution. We inserted a thin glass tube (200 μm in diameter) into a branch of the upper mesenteric artery irrigating the colonic flexure region, and through this perfused a Ringer solution into this region followed by the fixative (2.5% glutaraldehyde in a 0.1 M phosphate buffer, pH 7.4) using a syringe pump (STC-521, Termo Co., Tokyo) at a rate of 1.5 μl/min. Procedures of post-fixation in an osmium tetroxide solution, dehydration and resin-embedding were essentially the same as those described previously. The same facilities and procedures as were used in our previous experiments were used again (KOBAYASHI et al., 1995).

Tissue preparations after physiological recording were examined by light and electron microscopy as described previously (KOBAYASHI et al., 1995; NAHAR et al., 1996). Not perfusion fixation but immersion fixation procedures were employed for this purpose.

RESULTS

Physiological and pharmacological observations

Anatomy of the guinea-pig intestine was described by COOPER and SCHILLER (1975). The smooth muscle nodule to be described was located at the mesenteric border of the colon a few millimeters proximal to the center of the flexure region (Fig. 1). This represents a portion of the colonic pacemaker area described by HUKUHARA and co-researchers (see, HUKUHARA and NEYA, 1968).
Fig. 3. Physiological activities of the smooth muscle preparation whose cross section is shown in Figure 2. This preparation showed spontaneous mechanical contractions of 10–12 cycle/min with some irregularities. Atropine (1.0 μM) considerably removed those irregularities. L-NAME (50 μM) had no effect on the spontaneous contractions, whereas indomethacin (1.0 μM) increased their amplitude. Substance P (3 nM: Subst P) remarkably activated spontaneous activity. On the other hand, VIP (30 nM) suppressed spontaneous activity, and induced significant relaxation of the specimen. Leu-enkephalin (10 μM; Leu-Enk) and l-arginine (1.0 mM; L-Arg) had no effect on the spontaneous contractions, but caffeine (5.0 mM) induced a distinct tonic contraction followed by slight relaxation, even though this drug was given about 6 h after starting the experiment. At "L-NAME washed", L-NAME in the normal Krebs solution was removed. At "L-Arg washed", l-arginine in the normal Krebs solution was removed. Horizontal bars indicate time periods when drugs and chemicals were administered, whereas the vertical bar indicates the power of the contraction. Drugs were usually administrated for 4 min. It usually took 1 min for each drug to reach the tissue preparation in the chamber. The panels, 1-5, are continuous, running from the top left to the bottom right.
Tissue preparations used in the present physiological and pharmacological investigations usually contained the whole depth of the circular muscle coat together with the associated connective tissue of the submucosal and myenteric plexus layers (Fig. 2).

Shortly after fixing the preparations to the strain gauge force transducer system, the smooth muscle preparations were relatively inactive with only small but constant fluctuations. The start of rhythmical contractile activities varied from preparation to preparation. However, they usually began suddenly 40–120 min after starting the recording and continued at least 6 h or more. During this period of high rhythmical activities, all physiological and pharmacological investigations were performed.

Figure 3 illustrates a recording obtained from a muscle preparation producing typical spontaneous contractions continuing for longer than 3 h without intermission, and whose average frequency was about 10–12 cycle/min.

We hold the view that the nodule of special smooth muscle tissue producing the regular contractile activities is about 1 mm in diameter longitudinally, and about 3 mm in diameter in the circular direction. Though difficult to define the shape of this rhythm-producing structure, we felt that it was roughly oval in shape. The spontaneous mechanical activities of the smooth muscle tissue immediately adjacent to the small pacemaker nodule were significantly different from those of the latter. In tissue preparations taken from the muscular coat even a short distance from this small nodule, the spontaneous activities quickly became irregular and weaker. They then disappeared altogether in tissue taken from an area within a few millimeters from the nodule. However, description of the details of the regional differences in the spontaneous mechanical activities within the pacemaker area is not the purpose of the present paper.

As shown in Figure 2, we usually left a trace of the connective tissue of the submucosal and myenteric plexus layers. Therefore, those tissue preparations used for physiological and pharmacological investigations usually contained a trace of nerve elements of both the submucosal and myenteric plexuses. However, unlike the canine proximal colon reported previously (KOBAYASHI et al., 1995; NAHAR et al., 1996), the presence of neither the innermost nor outermost portions of the circular muscle coat was needed for the production of spontaneous contractions. The tissue preparations from which both the innermost and outermost portions of the circular muscle coat had been removed could produce spontaneous rhythms, though they were not completely identical to those obtained from the tissue preparations containing the whole depth of the circular muscle coat.

In the preparation shown in Figure 2, physiological and pharmacological properties of the pacemaker nodule were examined. Although the initial spontane-
ous mechanical contractions of 10-12 cycle/min were accompanied by some irregularities, an administration of atropine (1.0 μM), a well-known blocker of the muscarinic acetylcholine receptors, considerably removed these irregularities. L-NAME (Nω-L-arginine methyl ester; 50 μM), an inhibitor of NO-synthases, had no effect on the spontaneous contractions, whereas indomethacin (1.0 μM), a prostaglandin synthase inhibitor, increased their amplitudes. Substance P (3 nM), a neuropeptide, remarkably enhanced spontaneous activities. On the other hand, VIP (porcine vasoactive intestinal polypeptide; 30 nM), another neuropeptide, suppressed the spontaneous activities. Leu-enkephalin (10 μM), also a neuropeptide, and L-arginine (1.0 mM), the substrate of NO-synthases, had no effect on the spontaneous contractions, but caffeine (5.0 mM) showed a distinct stimulatory effect, even though this drug was given about 6 h after starting the experiment. Details of the experiments on the physiological and pharmacological properties of the rhythm-producing smooth muscle nodule will be reported elsewhere, since they are beyond the scope of the present paper.

Morphological observations

The interior of the colonic flexure region showed a distinct pattern of small furrows and folds. The flexure region was cut open along the antimesenteric border. When this colonic segment was left to contract naturally, it curved serosal-side-in and mucosal-side-out, forming a horse shoe shape. Several mucosal folds appeared in a longitudinal direction in the immediately proximal portion of the pacemaker area, whereas in its immediately distal portion, ridge-like elevations or foldings showed a tendency to arrange themselves in a circular direction. When the

Fig. 5. Innervation of the mesenteric border of the colonic pacemaker area in the guinea-pig. In this Champy-Maillet (ZIO) stained preparation, there are nerve terminal bundles within the muscular coat (CMP). Ganglia (asterisks) and nerve strands of the myenteric plexus are also seen. The big arrow indicates a fragment of a fairly thick artery irrigating the pacemaker area. Small arrowheads present in a vertical line indicate the site of mesenterium attachment. No serosa exists on the left hand side of the mesenterium attachment. ×50
specimens were perfusion-fixed in situ for electron microscopy, a circular valve or crista appeared on the mesenteric border side; we therefore used it as a landmark for deciding the location of the smooth muscle nodule producing regular spontaneous activities. The smooth muscle nodule was located on the serosal side of the crista, and both were within a few millimeters proximal to the center of the flexure region (refer to Figs. 1 and 4).

Nerve terminals in the rhythm-producing smooth muscle were observed in the whole-mount specimens of the Champy-Maillet (ZIO) stained intestine. Bundles of varicose nerve terminals in the smooth muscle of the pacemaker area were striking due to the density of their distribution, especially in the spontaneously contracting nodular area (Fig. 5). Transmission electron microscopy showed that, in addition to smooth muscle cells, nerve terminals, enteroglial (Schwann) cells and fibroblasts and like cells could be identified together with connective tissue fibers such as elastic fibers, and collagenous fibers of varying thickness (Fig. 6). Endothelial cells of blood capillaries in the circular muscles of the pacemaker area were non-fenestrated in type, as usually seen in the muscle coat throughout the alimentary canal. Plasma membranes of adjacent smooth muscle cells were frequently in direct contact. Thus, many gap junctions were present. Inter-

Fig. 6. A transmission electron micrograph of the smooth muscle tissue producing the 10–12 cycle/min spontaneous regular contractions at the mesenteric border of the colonic flexure region in the guinea-pig. There are smooth muscle cells, abundant nerve terminals (NT), profiles of blood capillaries (BC) and fibroblasts/fibroblast-like cells (F). In the area indicated by arrows, cell membranes of smooth muscle cells are in direct contact without intercalation of the basal lamina. This picture shows the submucosal surface portion of the circular muscle coat; thus, the outermost portion of the submucosal connective tissue (SM) is visible at the top. Notice that interstitial elements such as fibroblasts (F) and collagenous fibers are scanty in this smooth muscle tissue. ×6,000
stitial elements such as fibroblasts and collagenous fibers were few.

In scanning electron microscopy, a fine three-dimensional relationship between smooth muscle cells and other types of cells could be observed (Fig. 7). Fine nerve plexuses occurred in the space between the smooth muscle bundles. They consisted of a framework of enteroglial cells and bundles of varicose nerve terminals supported and partially enveloped by the enteroglial cell framework. In the interstice between smooth muscle cells and nerve terminal strands, fibroblasts/fibroblast-like cells and collagenous and elastic fibers could be identified, though these interstitial elements were limited in number.

**DISCUSSION**

We found the occurrence of a nodular structure of special smooth muscle tissue producing 10–12 cycle/min of steady spontaneous mechanical contractions within the mesenteric border of the pacemaker area, or sphincter coli, of the guinea-pig colon as described by HUKUHARA and NEYA (1968).

Previous authors, among them ELLIOTT and BARCLAY-SMITH (1904), AIBA (1933), MASUDA (1937), HUKUHARA and NEYA (1968) and HUKUHARA (1973), investigated the physiological and morphological properties of the colonic flexure region in the guinea-pig. They showed more or less that this region is the origin of the
antiperistalses and peristalses. However, none of the previous authors reported the occurrence of any structure producing such constant mechanical contractions as those illustrated in this present paper. This may be due to the following two improvements: 1) the recording system we used was sensitive enough to record the slight tension fluctuations of the preparations, and 2) the smooth muscle tissue producing such regular mechanical contractions is restricted in distribution, and so none of the previous authors examined this structure. We have investigated, in the present study, several kinds of tissue preparations obtained from various regions which HUKUHARA and NEYA (1968) included in the colonic pacemaker area. However, the smooth muscle tissue producing such regular contractions was located only in the small area described in the present paper. Tissues around this region, especially at the antimesenteric border, usually produced spontaneous contractions, although the constant mechanical rhythms could not be recorded outside of the mesenteric border of the pacemaker area.

The cellular origin of the spontaneous electrical and mechanical activities in the colon remains to be studied. Since THUNEBERG (1982), many authors have claimed that the intestinal pacemaker cells are the interstitial cells of Cajal. However, under this term at least three kinds of histological structures have been discussed: 1) Cajal’s originally described autonomic end-apparatus; 2) Thuneberg’s “interstitial cells of Cajal (ICCs)” which many electron microscopists regard as fibroblasts/fibroblast-like interstitial cells; and 3) the special smooth muscle cells in the inner sublayer of the circular muscle coat in the canine proximal colon where SANDERS and SMITH (1986) and DANIEL and BEREZIN (1992) reported recording pacemaker activities (see, KOBAYASHI, 1990; KOBAYASHI et al., 1995). The present study showed that the spontaneously contracting tissue in the colonic flexure region mainly contains smooth muscle cells with heavy autonomic innervations. The fact that stromal elements, such as connective tissue cells and fibers, were virtually non-existent may be of particular importance. We are proposing that the pacemaker activity should be sought in smooth muscle cells rather than in interstitial/stromal cells.

In our preliminary neuro-pharmacological experiments this smooth muscle tissue, unlike the inner sublayer of the inner circular muscle coat in the canine proximal colon (KOBAYASHI et al., 1995), was not susceptible to nitric oxide (NO) reagents such as L-NAME (50 µM) and L-arginine (1.0 mM). On the other hand, substance P (3 nM) remarkably enhanced muscle activity, whereas VIP (porcine; 30 nM) suppressed both the amplitude and frequency of the spontaneous contractions. Furthermore, Leu-enkephalin (10 µM) did not significantly affect the spontaneous contractions. The mesenteric border of the pacemaker area in the guinea-pig colon may also provide a new and useful experimental model for the investigation of the neural mechanism of the control of intestinal motility.

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