Absence of peristalsis in the ileum of \(W/W^v\) mutant mice that are selectively deficient in myenteric interstitial cells of Cajal

Tadashi NAKAGAWA\(^1\), Hiromi MISAWA\(^1\), Yoshiyuki NAKAJIMA\(^2\) and Miyako TAKAKI\(^1\)

\(^1\)Departments of Physiology II, 
\(^2\)Surgery, Nara Medical University School of Medicine, 840 Shijo-cho, Kashihara, Nara 634-8521, Japan

Abstract

It is well known that the enteric nervous system plays a key role in the generation of gastrointestinal peristaltic movements. Recently, the networks of interstitial cells of Cajal (ICC) have been found to be essential in the generation of spontaneous gastrointestinal movements. However, the role of ICC in the mechanisms involved in the generation of peristaltic movements is still controversial. The aim of the present study was to reveal how pacemaker myenteric ICC (ICC-MY) and the enteric nervous system contribute to the mechanisms involved in the generation of intestinal peristalsis. We compared spontaneous peristaltic movements of the ileum in wild type (WT) mice with those in \(W/W^v\) mutant mice which are selectively deficient in ICC-MY. Simultaneous recordings were made from both the circular and longitudinal muscle of a 4-cm long segment of ileum under hydrostatic pressure of 0–0.5 cm H\(_2\)O. Mechanical activity and continuous video-images of the ileum were compared between WT and \(W/W^v\) mutant mice under control conditions, in the presence of N-nitro-L-arginine methyl ester (L-NAME) and after tetrodotoxin (TTX). In the WT mouse ileum, peristaltic waves to propagate from the oral to the anal end were frequently observed. The frequency of these peristaltic waves and their associated synchronous longitudinal and circular muscle contractions was increased by L-NAME. The peristaltic waves were abolished by TTX. In the \(W/W^v\) mutant mouse ileum, no peristaltic waves to propagate from the oral to the anal end were observed in control and even after L-NAME, although the local spontaneously generated longitudinal and circular muscle contractions were enhanced by L-NAME. These local contractions were not abolished by TTX. The results presented here suggested that ICC-MY are essential for the generation of spontaneous intestinal peristaltic movements. It is conceivable that ICC-MY may determine the polarity of the excitation of the intestine such that longitudinal and circular muscle contractions propagate from the oral to the anal end of the intestinal segments, although the question of why ICC-MY are necessary for the neural pathways remains unresolved.

Key words: interstitial cells of Cajal, myenteric neurons, peristalses, \(W/W^v\) mouse
Introduction

Phasic activity of gastrointestinal smooth muscle involves the generation of rhythmic depolarization of the membrane forming slow waves that are often associated with spike potentials (Tomita, 1981). The interstitial cells of Cajal (ICC) distributed in the myenteric region, between the circular and longitudinal smooth muscle layers, are likely to be responsible for generation of the pacemaker activity. This is because impairment of the development of ICC by inhibiting the expression of c-Kit receptor proteins induces gastrointestinal disorders including the disappearance of the rhythmic activity of the smooth muscle (Huizinga et al., 1995, 1997; Sanders, 1996, 2001; Sanders et al., 1999). ICC also drive the rhythmic activity of smooth muscle in the guinea-pig stomach (Dickens et al., 1999). Furthermore, spontaneous inward currents are detected in isolated ICC, but not in gastrointestinal smooth muscle cells, when they are both maintained under cell culture conditions (Koh et al., 2002). The cellular mechanisms involved in this spontaneous activity in gastrointestinal tissues remain unclear, but are likely to involve cationic (Koh et al., 2001, 2002), and/or Cl currents (Tokutomi et al., 1995; Huizinga et al., 2002). The spontaneous activity is not inhibited by several-type of organic Ca\(^{2+}\)-antagonists such as verapamil (Golenhofen and Lammel, 1972), diltiazem (Ishikawa et al., 1985) and nifedipine (Dickens et al., 1999; Ishikawa et al., 2004; Liu et al., 1995). These results indicate that the influx of Ca\(^{2+}\) through voltage-gated L-type Ca\(^{2+}\)-channels may not be the main factor in the initiation of this activity (Sanders, 2001; Takaki, 2003). However such channels may be involved in the ICC of the submucosal region (ICC-SM) in the murine proximal colon (Yoneda et al., 2002).

We have previously reported that spontaneous rhythmic contractions are observed in the proximal colon of Ws/Ws rats, where there is a deficiency of both ICC in the myenteric region (ICC-MY) and of ICC-SM (Yoneda et al., 2001). Furthermore, it has been reported that smooth muscle preparations of the small intestine (duodenum, jejunum or ileum) of W/W\(^v\) mutant mice show a variety of electrical activities (Malyasz et al., 1996), longitudinal cyclic contractions (Daniel et al., 2002) and distension-induced peristalsis (Huizinga et al., 1998). In the small intestine of W/W\(^v\) mutant mice, while ICC-MY are absent, the ICC of the deep muscular plexus region (ICC-DMP) are present (Malyasz et al., 1996; Takayama et al., 2001, 2002). Recent studies reported the presence of synapse-like contacts between enteric nerve terminals and intramucosal ICC (ICC-IM) in the stomach, where ICC-MY have been shown to be the pacemaker ICC (Ward et al., 2000, 2004). Furthermore, there is a loss of enteric motor neurotransmission in the stomach of Sl/Sl\(^e\) mice lacking ICC-IM (Beckett et al., 2002). We have recently reported that in the small intestine of W/W\(^v\) mutant mice, spontaneous electrical and mechanical activities are present and are modulated by nitrergic enteric nerves via ICC-DMP (Nakagawa et al., 2005).

The aim of the present study was to reveal how pacemaker myenteric ICC (ICC-MY) and the enteric nervous system contribute to the mechanisms generating intestinal peristalsis. We compared the spontaneous mechanical activity of the ileum of the wild type (WT) mouse with that of the W/W\(^v\) mutant mouse which is selectively deficient in ICC-MY. While spontaneous peristaltic movements were not observed in the W/W\(^v\) mutant mouse ileum under a hydrostatic
pressure of 0–5 cm H₂O, peristaltic waves propagating spontaneously from the oral to the anal end occurred in the WT mouse ileum, even under a hydrostatic pressure of 0–0.5 cm H₂O. The present results suggest that ICC-MY are essential for the generation of spontaneous intestinal peristaltic movements. It is conceivable that ICC-MY may determine the polarity of the excitation of the intestine such that longitudinal and circular muscle contractions propagate from the oral to the anal end of the intestinal segments, although the question of why ICC-MY are necessary for the neural pathways remains unresolved.

Methods

Male mice (WBB6F1-W/W¹ and +/+), weighing 25–30 g, were anesthetized with pentobarbital sodium 50 mg/kg i.p. and intestinal segments excised. The animals were treated ethically according to the guidelines for the Care and Use of Animals approved by the Physiological Society of Japan.

Segments (4.0 cm in length) of the terminal ileum were placed in a 50 ml bath containing Tyrode’s solution (37°C) and bubbled with oxygen. The composition of the Tyrode’s solution was as follows: NaCl 145, KCl 2.6, CaCl₂ · 2H₂O 1.5, MgCl₂ · 6H₂O 0.73, NaHCO₃ 4.8, NaH₂PO₄ · 2H₂O 0.33, glucose 11.1 (mM) (Takemasa, 1957). The segment was cannulated at both ends, as previously reported (Takaki, 2003; Nakagawa et al., 2003, 2005). Longitudinal smooth muscle contractions were recorded with an isometric force transducer (Nihon Kohden force-displacement transducer TB-651T), while circular smooth muscle contractions were recorded with a pressure transducer (Life Kit DX-312, Nihon Kohden, Tokyo, Japan) held under a hydrostatic pressure of 0–0.5 cm H₂O, although longitudinal and circular muscle contractions are not completely independent. The mean interval was obtained by averaging the mean intervals between each peak force of each group of longitudinal contractions (n=6~10) in 4 (WT) and 5 (W/W¹) segments from different mice. Mechanical activity was also recorded by a digital video camera (SONY, DCR-PC101NTSC, Tokyo, Japan) and continuous video images were subsequently analyzed.

Extra-cellular recordings of electrical activity of longitudinal and circular muscle cells were made using a glass electrode filled with Tyrode’s solution, into which a platinum wire (200 µm) was inserted, along with a Dual-Channel Bioelectric Amplifier (MEG-2100) under filtration at LO CUT (0.08 Hz) and HI CUT (10 K)(Takaki, 2000; Nakagawa et al., 2005). The inside diameter of the electrode was 0.5 mm and its length was 2 cm. This electrode was gently placed on the serosa to suck the muscle layers of the segment by a weak negative pressure without stimulating the muscle (Takaki, 2003; Nakagawa et al., 2005). All data of the three activities were recorded on a personal computer (Fujitsu, Tokyo, Japan) through an A/D converter (DIGIDATA 1322A, Axon Instruments Inc., Foster City, CA, USA) at 6.667 KHz, filtered at 100 Hz, and analyzed with Axoscope 7 (Axon Instruments Inc., Foster City, CA, USA).

Drugs used were as follows: N-nitro-L-arginine methyl ester (L-NAME) and tetrodotoxin (TTX) was purchased from Sigma Chem. (USA). L-NAME and TTX were dissolved in distilled water as a stock solution, and diluted further with Tyrode’s solution to the desired concentrations (the ratios of the dilution were over 1: 1000). The dilution procedures did not
alter the pH of the Tyrode’s solution. Stock solutions were stored at 4°C and dissolved further with Tyrode’s solution just before use to obtain the desired concentrations.

Whole-mount preparations of the ileum from both WT and W/WV animals were fixed in either acetone (4°C, 5 min) or paraformaldehyde (4% w/v in 0.1 M PBS for 10 min at 4°C). After fixation, the preparations were preincubated in normal goat serum for 1 hr (10% in PBS, containing 0.3% (v/v) Triton-X 100 (PBS-TX)) before being incubated in primary antibodies. They were then incubated for 48 hours at 4°C in a rat monoclonal antibody raised against c-Kit protein (ACK2, 5 µg/ml in PBS-TX, Bioscience, San Diego, CA) or overnight at 4°C in a rabbit polyclonal antibody raised against protein gene product 9.5 (PGP 9.5; 1:3000 in PBS-TX, CHEMICON, Temecula, CA). Immunoreactivity for c-Kit and PGP 9.5 were detected using Alexa Fluor®488-conjugated secondary antibody (Alexa Fluor®488 goat anti-rat; Molecular Probes Inc., Eugene, OR; 1:200 in PBS for 2 hours in the dark at room temperature) and Texas Red-conjugated secondary antibody (Texas Red goat anti-rabbit; ICN Pharmaceuticals, Inc., Aurora, OH; 1:200 in PBS for 1 hour in the dark at room temperature), respectively.

For double-label immunostaining, the tissues were fixed in acetone (4°C, 5 min), then incubated in primary antibodies (followed by incubation in secondary antibodies) in a sequential manner. The preparations were examined with a Bio-Rad MRC 600 (Hercules, CA) confocal microscope. Confocal images are digital composites of Z-series scans of 10–15 optical sections through a depth of 10–15 µm. Final images were constructed with Comos software (Bio-Rad).

Measured values were expressed as the mean ± standard error (S.E.). Differences between values were tested using the paired Student t-test, and probabilities of less than 5% (P<0.05) were considered to be significant.

Results

Immunohistochemical studies
In the ileum of three WT mice, the ICC-MY were identified by their c-Kit immunoreactivity, while the myenteric plexus (MP) was identified by PGP9.5 immunoreactivity (Fig. 1A). In the ileum of six W/WV mice, there was no evidence of c-Kit positive immunoreactivity indicating the absence of ICC-MY, while the MP in these preparations was clearly observed (Fig. 1B). These results are similar to those reported in previous studies (Malysz et al., 1996; Takayama et al., 2001, 2002; Nakagawa et al., 2005).

Marked differences in electrical activity between the ileum of the W/WV and WT mouse
Electrical activity typically consisted of a regular rhythm of slow waves with superimposed spike potentials in the ileum of the WT mouse (Fig. 2A) and of an irregular rhythm of action potentials with spike potentials, but no slow waves, in the ileum of the W/WV mouse (Fig. 2B). Similar data were obtained in recordings from the ileum of each of the three WT mice and three W/WV mice studied.
Continuous recording of video-images and mechanical activity of spontaneous peristaltic waves in the ileum of the WT mouse

In a 1-cm long segment of the ileum of WT mice, rhythmic electrical slow waves with associated spike potentials were synchronized with contractions of both the longitudinal and circular muscle layers without any distension (Nakagawa et al., 2005). The mechanical activity was a mixed pattern of pendular and segmental movements, that did not form peristaltic waves, because these motilities did not propagate uni-directionally (Huizinga et al., 1998). Neither 0.1
µM TTX nor 100 µM L-NAME affected the frequency of the electrical slow waves or the synchronous longitudinal and circular muscle contractions (Nakagawa et al., 2005). Similarly in a 2-cm long segment of the ileum of WT mice, under 0–5 cm H₂O hydrostatic pressure, peristaltic waves propagating from the oral end to the anal end were not observed. However, in a 4-cm long segment of the ileum of WT mice, under 0–5 cm H₂O hydrostatic pressure, peristaltic contractions were frequently observed to propagate from the oral end to the anal end (Fig. 3A). As shown in Fig. 4, these peristalses involve synchronous contraction of the longitudinal and circular smooth muscle layers. Such contractions are essential for the transport of the intraluminal contents from the oral end to the anal end of the segment. L-NAME enhanced the occurrence of peristalses but TTX abolished them, leaving a mixed pattern of pendular and segmental movements (Fig. 4). In the other three segments, similar results were obtained. The mean interval of the contractions in the four segments from different WT mice was 62.2 ± 26.2 sec (n = 6–10 peristalses in each segment). However, after L-NAME, the mean interval of contractions in the same segments was significantly (P<0.05) decreased to 35.0 ± 8.7 sec (n = 6–10 peristalses in each segment). Thus the frequency of peristalses was significantly increased after NO-synthase (NOS) inhibition.

Continuous recording of video-images and mechanical activity in the ileum of the W/W' mouse

In 1-cm long segments of the ileum of W/W' mice, irregular and intermittent action
Ileal peristaltic activity in \( W/W^v \) mutant mouse

Potentials associated with quiescent periods were recorded, but electrical slow waves were not detected. Action potentials with superimposed spike potentials were recorded when strong circular muscle contractions occurred (Fig. 2B). Irregular and intermittent synchronous longitudinal and circular muscle contractions associated with quiescent periods were also observed (Nakagawa et al., 2005). After TTX or L-NAME, the quiescent periods were attenuated or abolished, and the frequency and thus the regularity of electrical and mechanical activities appeared to increase (Nakagawa et al., 2005). In the 4-cm long segments of the ileum of \( W/W^v \) mice, under 0–5 cm \( H_2O \) hydrostatic pressure, no peristaltic contractions to propagate from the oral to the anal end were observed (Fig. 3B). Instead, local and irregular synchronous circular and longitudinal muscle contractions were frequently observed (Fig. 5). Such a motility pattern would indicate that the intraluminal contents would not be transported from the oral end to the anal end of the segment. In the other four segments, similar results were obtained. The mean interval of contractions in five segments from different \( W/W^v \) mice was 54.4 ± 22.3 sec (\( n = 6–10 \) local and irregular contractions in each segment). After L-NAME, the mean interval of contractions in the same segments was significantly \( (P<0.05) \) decreased to 25.7 ± 4.6 (\( n = 6–10 \) local contractions in each segment). Thus the frequency of local longitudinal and circular muscle contractions was significantly increased by NOS inhibition, but still without peristalses. TTX largely decreased the amplitude but did not alter the frequency of these local contractions (Fig. 5), as reported by Nakagawa et al. (2003; 2005).
Discussion

Although Daniel et al. (2002) reported the presence of cyclic mechanical activity (slower in the ileum than jejunum) in the longitudinal and circular muscle of the small intestine in the W/W^v mutant mouse, we have recently shown that spontaneous electrical and mechanical activities of the longitudinal and circular muscle were significantly different between the ileum and jejunum in the W/W^v mutant mouse (Nakagawa et al., 2003, 2005). Furthermore, we found that tight synchronicity between longitudinal and circular muscle contraction was present in the segments of jejunum and ileum even in the W/W^v mouse. The present result obtained in the longer ileum of the W/W^v mouse is substantially the same as that of our recent studies (Nakagawa et al., 2003, 2005). This synchronicity could be due to the existence of extensive coupling between the circular and longitudinal smooth muscle layers (possibly through ICC) (Liu et al., 1998) and/or mechanical interaction between the muscle layers that transfers an active event from one layer to another (Wood and Perkins, 1970).

The pacemaker ICC-MY are selectively deficient in the ileum of the W/W^v mouse and thus rhythmic electrical slow waves and mechanical activities do not occur. Any electrical and mechanical activity that is present in the W/W^v mouse ileum is largely myogenic. The enteric nitricergic nerve-mediated inhibition of this mechanical and electrical activity which occurs via the ICC-DMP in the circular muscle layer is more prominent in the ileum than in the jejunum of the W/W^v mouse (Nakagawa et al., 2005). It is conceivable that enteric nitricergic nerves originate from the MP and distribute within the circular muscle. Therefore, ICC-DMP and

![Simultaneous recordings of spontaneous local irregular contractions composed of longitudinal and circular muscle contractions from the ileum of the W/W^v mouse under hydrostatic pressures of 0 and 0.5 cmH_2O.](image)

L: longitudinal muscle activity. C: circular muscle activity. L-NAME: N-nitro-L-arginine methyl ester (100 µM). TTX: tetrodotoxin (0.1 µM).
enteric nitrergic nerves would interact within the circular muscle layer of the ileum. The longitudinal muscle behaves in concert with the circular muscle, indicating the presence of a coupling between the longitudinal and circular muscle layers (possibly through ICC) (Liu et al., 1998) or mechanical interaction between the layers (Wood and Perkins, 1970) in the whole ileum preparation used in the present study.

It is well known that the intrinsic mucosal reflex in the intestine which is composed of an oral contraction and an anal relaxation (the peristaltic reflex) is evoked by the enteric nervous system (Hukuhara et al., 1961). The present results have shown that the frequency of peristaltic waves composed of longitudinal and circular muscle contractions were significantly increased following NOS inhibition. Therefore, it is conceivable that nitrergic nerves tonically inhibit the generation of peristalses in the WT ileum.

The most striking finding in the present study was that peristaltic waves did not occur in the ileum of the W/WV mouse. As previously reported (Malysz et al., 1996), the present immunohistochemical studies also revealed that the structure of the myenteric plexus in the ileum of the W/WV mouse appeared to be normal. In the present motility study, however, peristaltic waves could not be observed even after treatment with L-NAME in the ileum of the W/WV mouse. L-NAME enhanced the occurrence of spontaneous local circular and longitudinal muscle contractions in the W/WV mouse ileum in accordance with our previous reports (Nakagawa et al., 2003; 2005). This enhanced activity, however, did not trigger spontaneous peristaltic movements. These results suggest that even with the presence of the enteric nervous system, ICC-MY are essential for the spontaneous generation of intestinal peristaltic movements.

On the other hand, a recent study has revealed that in the W/WV mouse ileum, where ICC-MY are absent, the NO-mediated relaxation response evoked by electrical field stimulation (EFS) is not induced, suggesting that ICC-MY are essential for inducing the NO-mediated relaxation response (Takeuchi et al., 2004). Furthermore, the intestinal reflexes reported by Hukuhara et al. (1961) that can be caused by local distension with a small balloon, were not induced in the W/WV mouse ileum (Fujita et al., 2004). These studies also would support our conclusion, but leave unresolved as to why ICC-MY are necessary for the neural pathways to initiate the intestinal reflexes.

We conclude that ICC-MY are essential for the generation of spontaneous intestinal peristaltic movements even though the enteric nervous system is present. It is conceivable that ICC-MY determine the polarity of the excitation of the intestinal segment such that longitudinal and circular muscle contractions propagate from the oral to the anal end of the intestinal segments, although the question of why ICC-MY are necessary for the neural pathways remains unresolved.

Acknowledgments

This study was partly supported by Grant-in-Aid for Scientific Research 1437018, 14657311, 16650090 and 17300130 from the Ministry of Education, Science, Sports and Culture of Japan to M. Takaki.
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