Examination of the role of cholinergic myenteric neurons with the impairment of neural reflexes in the ileum of c-kit mutant mice

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Abstract

Our previous study showed that impairment of ascending and descending neural reflexes in the ileum of the c-kit mutant, W/W⁰, mice is due to a loss of interstitial cells of Cajal present at the myenteric plexus region (ICC-MY) in the mutant. In the present study, cholinergic interneurons were thought to be involved in these pathways, since hexamethonium, an antagonist of the nicotinic ACh receptor, significantly inhibited both neural reflexes in wild type mice. Therefore, we examined whether the loss of ICC-MY affects cholinergic interneurons involved in these pathways. Immunohistochemistry with anti-choline acetyltransferase revealed that there was no difference in the numbers of immunopositive cells in the myenteric plexus region between the wild type and mutant mice. In addition, there was no difference in the extent of spontaneous and EFS-evoked ACh release from longitudinal muscle with myenteric plexus preparations between the wild type and mutant mice. Exogenously added nicotine induced contraction or relaxation of ileal circular muscle in the absence or presence of atropine, respectively, to a similar extent in both the wild type and mutant mice. These results suggest that loss of ICC-MY resulted in an impairment of the ascending and descending reflex pathways at the step before activation of cholinergic interneurons.

Key words: myenteric ACh neurons, ascending contraction, descending relaxation, ICC, c-kit mutant mouse

Introduction

A localized stimulus applied to the intestinal wall induces contraction on the oral side (ascending contraction) and relaxation on the anal side (descending relaxation) of the stimulated region, resulting in peristaltic reflex (Bayliss and Starling, 1899). Myenteric neurons
are known to contain neural pathways responsible for peristalsis of the intestine. Interstitial cells of Cajal (ICC) are present in various regions of the gastrointestinal tract and are known to initiate slow waves in smooth muscle cells (Sanders, 1966; Suzuki and Hirst, 1999; Edwards et al., 1999; Hirst and Ward, 2003; Takaki, 2003). We have previously reported that ascending and descending neural reflexes were impaired in the ileum of c-kit mutant mice, W/W (Fujita et al., 2004). In the study, since selective loss of ICC present in the myenteric (Auerbach’s) plexus (ICC-MY) was found, ICC-MY were hypothesized to have an essential role in inducing ascending contraction and descending relaxation in response to the localized stimulus. However, neurotransmissions from excitatory and inhibitory efferent motor neurons to smooth muscle cells were unaffected in the mutant mice. The responsiveness of ileal circular muscle to an excitatory mediator, acetylcholine (ACh) and to Nor-1, a donor of nitric oxide which is an inhibitory mediator, was also unaffected in the mutant mice. Furthermore, although neuronal nitric oxide synthase present within the myenteric ganglia was somewhat (30%) decreased in the mutant mice ileum, the almost complete loss of nitric oxide-mediated relaxation of longitudinal muscle of the mutant mouse ileum could not be attributed to this decrease (Takeuchi et al., 2004). Therefore, the question of where the impaired region is in ascending and descending neural pathways in the mutant mouse ileum remained unsolved.

Participation of intrinsic cholinergic neurons in ascending and descending reflexes was reported in the guinea pig midcolon (Grider and Makhlouf, 1986). Participation of the neurons in descending relaxation was also reported in the rat proximal colon (Hata et al., 1990) and ileum (Kanada et al., 1993). The role of cholinergic interneurons in an ascending excitatory neural pathway was also suggested in the guinea-pig distal colon by mechanical recording of peristalsis generated by slow filling of the colonic lumen with fluid (Smith and Robertson, 1998). In the guinea pig ileum, however, although studies recording the membrane potentials of smooth muscle cells suggested participation of cholinergic neurons in ascending excitation (Smith et al., 1990) and in transmission from sensory neurons (Johnson et al., 1996), a study recording mechanical responses suggested minimal participation of cholinergic neurons in ascending and descending excitatory reflexes (Spencer et al., 2000).

Therefore, the involvement of cholinergic interneurons in the ascending and descending neural pathways in the mouse ileum was examined in the present study. In addition, the question of whether cholinergic interneurons are impaired in the ileum of c-kit mutant mice was examined in an attempt to understand why the ascending and descending responses are lost in the mutant ileum (Fujita et al., 2004). We therefore first examined localization of choline acetyltransferase (Ch-AT) within myenteric ganglia by an immunohistochemical method in wild type and mutant mouse ileum. We next measured the release of ACh from longitudinal muscle with myenteric plexus (LMMP) preparations obtained from the ileum of wild type and mutant mice, since LMMP preparations of guinea pig ileum have been widely used for studying ACh release from myenteric neurons. Then, we examined the effect of nicotine in the absence or presence of atropine on wild type and mutant mouse ileum to clarify whether neurotransmission from nicotinic ACh receptors, present on some interneurons (or motor neurons), to smooth muscle cells via excitatory or inhibitory motor neurons is impaired in the mutant mouse ileum.
Methods

The c-kit mutant mice, WBB6F1-W/W and wild-type mice (+/+) (8-week-old) were purchased from Japan SLC (Shizuoka, Japan). The origin of parental strains has been described in detail (Go et al., 1980; Yokoyama et al., 1982). The animal experiments were performed using procedures approved by the institutional Animal Use and Care Committee at the Osaka Prefecture University. The mice were lightly anaesthetized with ether and then stunned by a blow to the head and bled via the carotid arteries. Segments of the ileum were removed and placed in Tyrode solution consisting of (mM) NaCl 137, KCl 2.7, CaCl₂ 1.8, MgCl₂ 1.1, NaH₂PO₄ 0.42, NaHCO₃ 11.9 and glucose 5.6. The contents of the excised segments were gently flushed out with Tyrode solution; the segments were cut in 2.5 cm in length, and equilibrated at 37°C for at least 30 min before the experiment in 20 ml of Tyrode solution aerated with 95% O₂ and 5% CO₂. Drugs were added to the organ bath in volumes less than 1% of the bathing solution (< 200 μl); these volumes did not affect the spontaneous contractile activity or muscle tone.

Recording of ascending contraction and descending relaxation in response to the stimulus of distension

The methods to record ascending contraction and descending relaxation were similar to that described in the previous study (Fujita et al., 2004). In brief, mouse ileal segments were held horizontally with the side adherent to the mesentery at the bottom in a specially designed organ bath. For the balloon to dilate the intestine, we used the ARASHI dilation catheter (Terumo Corporation, Tokyo, Japan) aimed for improving myocardial coronary blood flow in the localized stenotic lesion. The balloon (3.0 mm in diameter, 8.0 mm in length) was introduced into the lumen and positioned at the middle of the segment. The balloon was inflated with 0.1 or 0.15 ml air from the syringe to produce slightly greater local distension than that produced by a faecal bolus. The duration of distension was 15 s. To record ascending contraction, the mechanical response of the circular muscle about 1.0 cm oral to the balloon was recorded, by connecting a frog heart clip to a small area of the wall opposite to the anchor and then connecting the clip via a thread to an isotonic transducer (TD-112A, Nihonkohden, Tokyo, Japan). To record descending relaxation, the mechanical response of the circular muscle about 1 cm anal to the balloon was recorded. Both ends of the segment were free. This arrangement allowed preferential recording of the response of the circular muscle. The circular muscle was subjected to a resting load of 0.5 g.

Immunohistochemical study of c-Kit protein

Immunohistochemical study was carried out by the method described previously (Fujita et al., 2001, 2003). Briefly, short segments of the intestine were inflated and the mucosa was removed with a small razor, and the remaining strips (5 x 5 mm) were pinned to the silicon rubber. The tissues were fixed for 10 min at 4°C with 4% paraformaldehyde in 0.1 M phosphate buffer for 24 h. Following fixation, whole-mount preparations were washed three times with phosphate-buffered saline (PBS) and then placed in PBS containing 0.5% Triton X-100, 1% bovine serum albumin, and 10% normal donkey or goat serum for 1 h at room temperature to avoid
nonspecific staining. The preparations were then incubated with anti-choline acetyltransferase (Ch-AT) or -glial fibrillary acidic protein (GFAP) (1:2000) antibody in PBS at 4°C for 24 h. Immunoreactivity was detected using fluorescein isothiocyanate (FITC)-conjugated anti-goat or anti-rabbit IgG secondary antibody (Jackson Immuno Research Laboratories, West Grove, PA, USA). Confocal images were obtained under a laser scanning microscope (MRC-1024; Bio-Rad, Hertfordshire, UK).

**Measurement of ACh released from LMMP preparations of mouse ileum**

The LMMP preparations of the mouse ileum were made as described previously in the experiments in guinea pigs (Takeuchi et al., 1991). Spontaneous and electrical field stimulation (EFS)-induced ACh releases were measured by the method described previously (Takeuchi et al., 2001). In brief, the preparations were equilibrated for 30 min by perfusion with Tyrode solution containing physostigmine salicylate (5 μM) and choline chloride (1 μM). Then, perfusion was stopped and the bathing medium was replaced by fresh Tyrode solution at intervals of 4 min. After a sample was collected for the measurement of spontaneous release of ACh, the preparations were stimulated by EFS. The stimulation was performed in trains of 60 s at 10 Hz. Bathing fluid was collected after a further 180 s period as shown previously. The parameters for EFS were as follows: supramaximal voltage (50 V), pulse duration of 0.5 ms, 600 pulses at 10 Hz. ACh release due to EFS was calculated by subtracting the output of the immediately preceding spontaneous release from the total output during the periods of stimulation. ACh released in the medium was assayed by HPLC (Yanaco, Kyoto), using a postcolumn enzyme (acetylcholinesterase plus choline oxidase) reactor (Eicom AC-Enzymap; Eicom, Kyoto).

**Antibody**

Rabbit polyclonal antisera against GFAP was purchased from DAKO Japan (Kyoto, Japan). Goat polyclonal antisera against Ch-AT (AB 144P) was purchased from Chemicon (Temecula, CA, USA).

**Drugs**

Hexamethonium, tetrodotoxin, atropine sulphate and nicotine were purchased from Wako Pure Chemical, Osaka, Japan. N⁵-Nitro-L-arginine (L-NOARG) and L-arginine were purchased from Sigma Chemical Co., St. Louis, USA.

**Results**

**Examination of the presence of cholinergic interneurons in ascending and descending neural pathways in wild type and W/W⁵ mice**

Ileal segments were held horizontally to record ascending and descending responses to local distension in a specially designed organ bath. At rest, the circular muscle of the ileal segments prepared from wild type mice exhibited spontaneous rhythmic contractions, amplitude of which was variable among the segments, while that prepared from W/W⁵ mice
exhibited the contractions with significantly greater amplitude, compare with the wild type mice (Fujita et al., 2004). We first examined whether cholinergic interneurons are present in the ascending and descending neural pathways in the mouse ileum by using the nicotinic receptor antagonist, hexamethonium. The rationale for this experiment is that if cholinergic interneurons are involved in the reflex pathways, ACh released from the neurons must activate nicotinic receptors present on other neurons to activate the pathways. Hexamethonium at a concentration of 500 μM, which effectively inhibited descending relaxation in the rat proximal colon (Hata et al., 1990), inhibited the ascending contraction in all 5 preparations examined in the wild type mice (Fig. 1A). Hexamethonium also inhibited the descending relaxation in all 4 preparations examined (Fig. 1B). Thus, it appears that cholinergic interneurons are involved in both neural pathways.

We next examined whether cholinergic interneurons are impaired in W/W' mice, since both ascending contraction and descending relaxation were lost in the ileal segments from the mutant mice (Fujita et al., 2004). The presence of cholinergic neurons within myenteric plexus region was examined by immunohistochemical staining with anti-Ch-AT antibody. Numerous cells stained with the antibody were observed in the myenteric plexus region: cells in ganglia and their network were densely stained, suggesting the presence of numerous cholinergic neurones in the myenteric plexus region of the ileum of wild type mice (Fig. 2A). In the mutant mice, numerous immunopositive cells were also observed in the same region (Fig. 2A). There was no difference between the numbers of immunopositive cells to the antibody in the wild type and mutant mice: 43.7 ± 5.8% and 45.3 ± 6.2% (n=4, 10 ganglia were examined in preparations obtained from 4 animals) of cells in myenteric ganglia of the wild type and mutant mice were stained, respectively. The network of myenteric plexus neurons was also examined by the immunostaining with anti-GFAP antibody, because GFAP is known to be a specific marker for

![Myenteric ACh neurons in neural reflexes](image-url)
glial cells which are present with the myenteric neuronal network. There was also no difference between overall observations of the network stained with the antibody in both mice types (Fig. 2B).

**Release of ACh from LMMP preparations obtained from wild type and W/W' mice**

At rest, the spontaneous ACh release from LMMP preparations of the wild type mouse ileum was 319 ± 35 pmol/g tissue/min (n=6). EFS at 10 Hz evoked a significant increase in ACh release: 5,523 ± 983 pmol/g tissue/min (n=6). In the mutant mice, spontaneous and evoked ACh release were 383 ± 57 and 4,962 ± 1,331 pmol/g tissue/min (n=6), respectively. Thus, there was no significant difference between the values of both mice types in both the spontaneous and evoked release of ACh, suggesting that cholinergic interneurons present within the ascending and descending neural pathways are not changed in the mutant mice.

**Examination of activation of nicotinic receptors involved in the reflex neural pathways in wild type and W/W' mice**

Since cholinergic interneurons and ACh release from LMMP preparations were found to be unchanged in the mutant mice as shown above, the pathways present downstream from cholinergic neurons were examined. Activation of nicotinic receptors by nicotine resulted in contraction or relaxation of circular muscle of the ileum in the absence or presence of atropine (1 μM), respectively, in wild type mice (Fig. 3). The nicotine-induced contraction (data not shown) and relaxation were completely inhibited in the presence of tetrodotoxin (Fig. 3). In our
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Fig. 3. Effects of tetrodotoxin on nicotine-induced relaxation in wild type mouse ileum. Nicotine (1 μM) induced contraction or relaxation in the absence or presence of atropine at 1 μM, respectively. Nicotine-induced relaxation was abolished after treatment of the segments with tetrodotoxin (TTX, 1 μM) for 10 min. The lines indicate the presence of drugs. These records are typical of those from 3 preparations.

Fig. 4. Effects of L-NOARG and L-arginine on nicotine-induced relaxation of circular muscle of wild type mouse ileum. Atropine (1 μM) was added throughout the experiments. Relaxation was induced by nicotine (1 μM) in the absence or presence of L-NOARG (100 μM) without or with L-arginine (1 mM). The lines indicate the presence of drugs.

previous study, it was reported that ACh and nitric oxide were mediators of ascending contraction and descending relaxation, respectively, in the mouse ileum (Fujita et al., 2004). In the present study, nicotine-induced contraction was changed to relaxation in the presence of atropine. The nicotine-induced relaxation was almost abolished by the treatment of the segments with L-NOARG at 100 μM (1.7 ± 0.9% of the control, n=3) and the inhibition was effectively reversed by 1 mM L-arginine (77.8 ± 2.1% of the control, n=3) (Fig. 4). Therefore, nicotine appeared to activate the ascending and descending neural pathways. In the mutant mouse ileum, nicotine also induced contraction or relaxation in the absence or presence of atropine, respectively (Fig. 5). Hexamethonium (500 μM) completely inhibited these nicotine-induced responses in both mice (data not shown; n=3). Thus, it appeared that the neural pathways downstream of cholinergic interneurons are not impaired in the mutant mice.
Fig. 5. Effects of nicotine in the absence or presence of atropine on circular muscle of W/W\textsuperscript{v} mouse ileum. Nicotine (1 \mum) induced contraction or relaxation in the absence or presence of atropine (1 \mum), respectively. The lines indicate the presence of drugs. These records are typical of those from 3 preparations.

Discussion

It has been reported that ICC-MY were selectively lost in the W/W\textsuperscript{v} mouse ileum (Ward \textit{et al}., 1994; Huizinga \textit{et al}., 1995; Takeuchi \textit{et al}., 2004) and that ascending contraction and descending relaxation in response to localized distension were lost in the mutant mouse ileum (Fujita \textit{et al}., 2004). Despite these losses, neurotransmission from efferent excitatory and inhibitory motor neurons is not affected in responsiveness of the circular muscle to exogenously added ACh or Nor-1 (Fujita \textit{et al}., 2004). Thus, it appeared that after activation of efferent motor neurons neural pathways remain intact in the mutant mouse ileum. In the present study we further examined the pathway prior to efferent motor neurons to clarify the losses of the neural reflexes in the mutant mouse ileum.

In the present study, cholinergic interneurons were suggested to be involved in the neural reflex pathways, since ascending and descending neural reflexes were inhibited by hexamethonium which blocks ACh-mediated transmission within a myenteric neural network. Recently, a special synapse-like junction between the ICC-DMP and myenteric cholinergic neurons was observed in the human small intestine (Wang \textit{et al}., 2003). In contrast, the intracellular recording study of inhibitory junction potentials (i,j.ps) in the rat colon suggested that ACh is the primary neurotransmitter from sensory neurons to inhibitory motor neurons, so that the cholinergic sensory neurons have long descending projections to inhibitory motor neurons (Bian \textit{et al}., 2003).

We, therefore, next examined whether cholinergic interneurons involved in the reflex pathways were impaired in the mutant mice. Microscopic observation by immunostaining of ileal tissue with anti-GFAP antibody revealed that ganglion and the network of the myenteric plexus remained unaffected in the mutant mouse ileum. Cholinergic interneurons were
immunostained with anti-Ch-AT antibody in both the wild type and mutant mouse ileum. No appreciable difference was observed in both tissues. Although microscopic observation is not necessarily quantitative, the present results can not attribute the complete loss of ascending and descending neural reflexes in the mutant mouse ileum to undetected changes in cholinergic interneurons.

The LMMP preparations of the guinea pig ileum have been widely used for studying ACh release from myenteric neurons and the regulatory mechanisms of release. EFS-induced ACh release from LMMP preparations obtained from the mouse ileum was studied to examine whether ACh release from cholinergic neurons was affected in the mutant mice. There was no difference in the extent of spontaneous and EFS-evoked ACh release between the wild type and the mutant mice. These results indicate ACh release from myenteric cholinergic neurons is not affected in W/W<sup>v</sup> mice. Since there are many cholinergic motor neurons, in addition to cholinergic myenteric interneurons, within the ileal tissue, the possible change in ACh release from the interneurons might be overlooked due to the large amount of ACh released from the motor neurons. However, the similar values of released ACh in the wild type and the mutant mice in the present study could not explain the complete loss of the neural reflexes.

The presence of cholinergic interneurons in the neural pathways for ascending and descending reflexes indicates that released ACh from the interneurons activates nicotinic ACh receptors present on other interneurons. Therefore, the neural pathways could be summarized as shown in Fig. 6. Immunohistochemistry with anti-Ch-AT antibody suggests that step (1) is unchanged, measurement of released ACh suggests that step (2) is unchanged, and nicotine-induced responses suggest that the final step (3), from activation of nicotinic ACh receptors to induction of contraction or relaxation of smooth muscle cells, is unchanged in the mutant ileum. Taking into account these and our previous results (Fujita et al., 2004), the pathways after cholinergic interneurons seem to be intact and impaired region(s) seems to be present before the cholinergic interneurons involved in the pathways in the W/W<sup>v</sup> mouse ileum. Thus, ICC-MY participate in the ascending and descending reflex pathways at the region of activation of afferent sensory neurons to cholinergic interneurons.

Although the region at which ICC-MY participate in the neural reflexes has been narrowed down, as discussed above, an important question remained unsolved. That is, specialized contacts between ICC-DMP and myenteric neurons were reported in the murine (Zhou and...
Komuro, 1992; Komuro and Seki, 1995; Wang et al., 1999; Kobilo et al., 2003) and human (Faussone-Pellegrini, 1983; Torihashi et al., 1999; Wang et al., 2003) small intestine. Specialized contacts between ICC-IM and the neurons were also reported in the murine gastric fundus (Ward et al., 2000) and antrum (Mitsui and Komuro, 2002), and colon (Wang et al., 2000). However, a structural connection between ICC-MY and myenteric neurons has not been reported. Therefore, the structural as well as functional association of ICC-MY with ascending and descending reflex pathways appears to be the pivotal focus of future studies.

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