Deficiency of intramuscular ICC increases fundic muscle excitability but does not impede nitricergic innervation

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Submitted 16 March 2007; accepted in final form 6 December 2007

Huizinga JD, Liu LW, Fitzpatrick A, White E, Gill S, Wang X-Y, Zarate N, Krebs L, Choi C, Starret T, Dixit D, Ye J. Deficiency of intramuscular ICC increases fundic muscle excitability but does not impede nitricergic innervation. Am J Physiol Gastrointest Liver Physiol 294: G589–G594, 2008. First published December 20, 2007; doi:10.1152/ajpgi.00130.2007.—The motility of the gastrointestinal tract is generated by smooth muscle cells and is controlled to a large extent by an intrinsic neural network. A gap of ~200 nm usually separates nerve varicosities from smooth muscle cells, which suggests that direct innervation of the smooth muscle by synapses does not occur. Enteric nerves do make synapse-like contact with proposed regulatory cells, the interstitial cells of Cajal (ICC), which in turn may be in gap junction contact with smooth muscle cells. The role played by ICC in enteric innervation is controversial. Experimental evidence has been presented in vitro for the hypothesis that nitricergic inhibition innervation is strongly reduced in the absence of ICC. However, in vivo data appear to dispute that. The present report provides evidence that explains the discrepancy between in vivo and in vitro data and provides evidence that inhibitory neurotransmitters can reach smooth muscle cells without hindrance when ICC are absent. The fundic musculature shows increased responses to substance P-mediated innervation and shows marked spontaneous activity, which is consistent with increased muscle excitability.

interstitial cells of Cajal

CONTROLLED INHIBITORY AND excitatory innervation to the musculature of the gut is critical for normal gastrointestinal function (2). Inhibitory innervation regulates gastric accommodation and timely opening of the sphincters, and it is a critical part of segmentation motor patterns in the intestine. Excitatory innervation is the primary inducer of contractile activity for mixing and aboral movement of gut content. The classic concept of gut innervation is that intrinsic nerves form nerve varicosities along their fibers from which neurotransmitters are released that diffuse to muscle cell receptors without an intermediary specialized junction. An alternative pathway for innervation of the gut musculature may occur via interstitial cells of Cajal (ICC), already established as intestinal pacemaker cells (17) and now implicated in gut innervation (21).

Within the gut musculature, many nerve varicosities form neuromuscular junction-like contacts with intramuscular ICC (ICC-IM), and each ICC-IM has gap junction contact with many smooth muscle cells (4, 8, 18, 21). Hence ICC are ideally suited to regulate neuronal traffic to and from the gut musculature as suggested by Santiago Ramón y Cajal when he described ICC for the first time in 1911 (11). Burns et al. (1) and Ward et al. (20) provided evidence that ICC-IM are the principal pathway from inhibitory nerves to smooth muscle cells, making the argument that neurotransmitters, in particular nitric oxide, released from nerve endings would not reach smooth muscle cells, because the distance would be sufficient to cause neurotransmitter inactivation. However, in vivo studies came to the conclusion that an inhibitory reflex mediated by nitric oxide, namely relaxation of the lower esophageal sphincter (LES) on swallowing, was still present in mutant mice (W/Wv) that have an abnormal c-kit gene and lack ICC-IM in the LES (15).

To resolve the controversy, our first objective was to structurally assess innervation with and without ICC-IM. We used tissue from the fundic part of the stomach of Ws/Ws mutant rats that lack a normal c-kit gene, and as expected, the number of ICC-IM based on quantification of c-kit immunopositivity within the fundic musculature was strongly reduced to 10 ± 1% of the value in wild-type rats (Fig. 1), similar to the antrum (9). Our ultrastructural studies showed that in both wild-type and Ws/Ws rats, nerve varicosities were apposed to smooth muscle cells by gaps of 200 nm or more; in addition, close structural associations of ~50 nm distance were seen (Fig. 1c). Such contacts have been observed previously in the human stomach (5). Only in wild-type rats were ICC-IM commonly associated with nerve varicosities through synapse-like structures (Fig. 1b). The ultrastructural features of the remaining ICC-IM in the Ws/Ws animals were very similar to those of ICC-IM in control tissue, except that the typical caveolae and a clear basal lamina were not commonly seen. Importantly, it was extremely rare to find a synapse-like structure between the remaining ICC-IM and nerve varicosities. In the Ws fundus, fibroblast-like cells (FLC) appeared to have replaced ICC-IM, but synapse-like structures between FLC and nerve varicosities were not observed. Structural evidence therefore suggests that nerve varicosities appose smooth muscle cells at a distance of 50–200 nm. This should not be a barrier for diffusion (16, 22).

Our second objective was to study inhibitory innervation. Hence we blocked the presumed dominant cholinergic innervation with atropine (2 × 10−6 M), blocked adrenergic nerves with guanethidine (2 × 10−6 M), and then electrically stimulated the enteric nerves. Stimulation at 10 Hz resulted in a marked reduction in tone in the wild-type rat fundus muscle strip preparations (Fig. 2, A and C). Hence in control tissue after blockade of cholinergic and adrenergic innervations, inhibitory innervation was dominant, causing reduction in tone. This inhibition was mediated in part by nitric oxide (Fig. 2, A

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and C) and was in part apamin sensitive (Fig. 2, A and C), likely mediated by ATP (7, 10). When fundic muscle preparations from the Ws/Ws rat were studied under identical conditions, usually no reduction in tone was observed (Fig. 2, B and C). At first sight, this appeared to be consistent with the study in the mouse fundus referred to earlier, which led to the conclusion that ICC-IM were essential for inhibitory innervation (1). However, in Ws/Ws fundic muscle strips, in contrast to control tissue, phasic contractile activity developed with very low resting tone. Nerve stimulation in the Ws/Ws fundic muscle resulted in marked reduction in phasic contractile activity, indicating significant inhibitory activity (Fig. 2B). Hence the lack of reduction in tone was not due to the inability of neurotransmitters to reach the smooth muscle cells but rather due to lack of active tone. Interestingly, in the W/Wv mice in vivo, a low LES resting tone was observed (15). In contrast to responses in control tissue, N6-nitro-l-arginine (l-NNA) not only reduced the inhibitory effect of nerve stimulation but revealed a strong noncholinergic excitatory innervation, revealed by marked contraction of the musculature in response to nerve stimulation (Fig. 2, B and C). This revealed another reason why nerve stimulation in Ws/Ws rats did not show a strong inhibitory response: in contrast to control tissue, nerve stimulation excited noncholinergic excitatory nerves that masked the effect of inhibitory nerves. Hence the absence of ICC allowed strong neural excitation, possibly restricted by ICC in wild-type animals.

To corroborate the mechanical evidence that nitrergic inhibition was not impaired, we also measured the electrical events following nerve stimulation at 10 Hz in the presence of atropine and guanethidine. The resting membrane potential of control and Ws/Ws fundus smooth muscle cells was –53.7 ± 1.1 mV and –46.5 ± 0.9 mV, respectively (P < 0.05). Electrical stimulation of enteric nerves evoked inhibitory junction potentials (IJPs) consisting of a fast and slow component (see METHODS) in both wild-type and Ws/Ws fundic muscle cells (Fig. 2E). The slow component was primarily mediated by nitric oxide and was similar in wild-type and Ws/Ws rats (Table 1); the fast component was apamin sensitive, hence likely mediated by ATP, and was also similar in wild-type and Ws/Ws rats. Hence both nitric oxide- and ATP-mediated electrical responses in smooth muscle cells were not hampered by the absence of ICC. Both nitric oxide and ATP apparently survive the time it takes to diffuse to smooth muscle cells.

The marked contractile activity observed in response to nerve stimulation at 10 Hz seen in the Ws/Ws animals after blockade of cholinergic and nitrergic pathways was unexpected and led us to examine inhibitory innervation after blockade of neurokinin 1 (NK1) receptors. Furthermore, we investigated the possibility that nitrergic nerves were more specifically stimulated at lower stimulus frequencies. Figure 3 shows that at 1 and 4 Hz after blockade of muscarinic, adrenergic, and NK1 receptors, strong inhibition of mechanical activity was observed in both control and Ws/Ws tissues. l-NNA strongly inhibited the responses. A 1-Hz stimulation, after blockade of cholinergic, adrenergic, and NK1 pathways, appeared to predominantly stimulate nitrergic nerves. Addition of l-NNA showed conclusively that in wild-type as well as Ws/Ws fundus, nitrergic innervation is prominent.

To make sure that species differences were not the cause of the differences with data in the literature on W/Wv mice, we repeated some key studies on tissue from W/Wv mice. In wild-type mice, the fundic musculature underwent inhibition of contractile activity when enteric nerves were stimulated at 10 Hz in the presence of atropine and guanethidine (Fig. 4, A and E). Quantitatively, nerve stimulation in wild-type mice reduced contraction from 21.9 ± 3.2 to 15.8 ± 4.1 mN·mm⁻²·min⁻¹ (P < 0.01; n = 10). When the nerve stimulation was repeated in the presence of l-NNA, the contractile activity increased from 15.8 ± 2.1 to 25.0 ± 5.1 mN·mm⁻²·min⁻¹ (P < 0.01), revealing that noncholinergic enteric excitatory nerves were activated by the nerve stimulation, but this was only evident after blockade of nitrergic nerves. The additional presence of apamin increased contractile activity (Fig. 4A) from 15.3 ± 1.9 to 29.2 ± 6.4 mN·mm⁻²·min⁻¹ (P < 0.01). In W/Wv mice, fundic contractile activity before and after electrical stimulation in the presence of atropine and guanethidine was 24.4 ± 3.2 and 22.7 ± 3.1 mN·mm⁻²·min⁻¹ (n = 12), respectively; in...
some preparations, contractile activity was reduced up to 36%, whereas in other preparations the contractile activity increased up to 18%; because of this, the average responses were not different. Whereas noncholinergic excitation was revealed in wild-type tissues only after blockade of nitrergic innervation, it was already evident without block of inhibitory nerves in the Ws/Ws fundic muscle strips. When the nerve stimulation was repeated in the presence of L-NNA, Ws/Ws fundic muscle preparations that showed relaxation before addition of L-NNA showed contraction after addition of L-NNA. Other preparations showed increased contractile activity. The values were 19.1 ± 2.5 before and 23.5 ± 3.2 mN·mm²·min⁻¹ during nerve stimulation (P < 0.05). The presence of apamin caused a marked increase in contractile activity from 18.0 ± 3.5 to 27.6 ± 8.4 mN·mm²·min⁻¹ (P < 0.01) before and during nerve stimulation. Hence both nitric oxide-mediated and apamin-sensitive relaxation was normal in Ws/Ws mice. The noncholinergic excitation was inhibited by the NK1 antagonist SR-14033 (10⁻⁷ M) indicating the presence of substance P-mediated excitation in both wild-type and Ws/Ws tissue.

To study the excitatory innervation in W/Wv mice more specifically, further experiments were performed in the presence of L-NNA and apamin. Electrical stimulation of the enteric nerves caused marked contractile activity (Fig. 4, C and D). In wild-type gastric muscle, this was primarily an increase in tone. In W/Wv fundus, the increased tone was superimposed with phasic contractile activity. The contractile activity evoked in W/Wv mice on nerve stimulation was inhibited 51.4 ± 11% (n = 7) by the NK1 antagonist SK-1403, whereas it was inhibited only 8.7 ± 0.2% in control mice (Fig. 4, C and D). Hence in W/Wv mice, similar to Ws/Ws rats, the absence of ICC resulted in stronger substance P-mediated excitation. A likely explanation is that deficiency of ICC increases muscle excitability. One line of evidence is the marked spontaneous phasic contractile activity that is displayed by the muscle strips of the Ws/Ws fundus. Preliminary data from our laboratory show that development of phasic activity also occurs in wild-type fundic musculature but only after stimulation with KCl or carbachol (A. Fitzpatrick and J. D. Huizinga, unpublished observations). Another possible explanation is that ICC receive

Table 1. Characteristics of inhibitory junction potentials evoked by electrical nerve stimulation recorded in smooth muscle cells of the fundus in the presence of atropine and guanethidine

<table>
<thead>
<tr>
<th></th>
<th>Atropine + Guanethidine</th>
<th>+ L-NNA</th>
<th>%Inhibition</th>
<th>+ Apamin</th>
<th>%Inhibition</th>
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<tr>
<td>Wildtype</td>
<td></td>
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<td>Duration of slow component, s</td>
<td>10.8 ± 0.7</td>
<td>2.2 ± 0.2 s</td>
<td>80</td>
<td>1.2 ± 0.1</td>
<td>9</td>
</tr>
<tr>
<td>Amplitude of fast component, mV</td>
<td>27.2 ± 1.3 mV</td>
<td>22.5 ± 1.2</td>
<td>17</td>
<td>12.4 ± 1.0</td>
<td>63</td>
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<tr>
<td>Ws/Ws</td>
<td></td>
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<tr>
<td>Duration of slow component, s</td>
<td>14.1 ± 1.7</td>
<td>2.5 ± 0.2</td>
<td>82</td>
<td>1.8 ± 0.2</td>
<td>5</td>
</tr>
<tr>
<td>Amplitude of fast component, mV</td>
<td>12.6 ± 1.2 mV</td>
<td>10.3 ± 1.1</td>
<td>18</td>
<td>3.2 ± 0.5</td>
<td>64</td>
</tr>
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%Inhibition due to apamin refers to control data in the atropine + guanethidine column. L-NNA, N⁵-nitro-L-arginine.
preferential innervation from substance P-containing nerves and that ICC usually reduce excitation of the musculature by these nerves. Absence of ICC would give the musculature unrestricted access to substance P innervation. Another possible explanation of excessive substance P innervation is that lack of development of ICC may actually increase substance P innervation, suggested by preliminary data on another \( \text{W}^\text{+/+} \) mutation, \( \text{W}^\text{+/+} \) mice, that showed a 219\% increase in substance P innervation in the intestine (6). Therefore we studied substance P innervation by using immunohistochemistry but did not find any difference (\( n = 5 \)) in the fundus of wild-type vs. \( \text{Ws/Ws} \) rats (Fig. 4F) or wild-type vs. W/Wv mice (not shown).

The question whether or not ICC are involved in neurotransmission has also been investigated by using the hypothesis that neural activation by field stimulation would release neurotransmitters onto ICC and this information would be transmitted to smooth muscle cells via gap junctions. Experimental evidence showed that blockade of gap junctions did not alter neurotransmission (3, 14). This is consistent with our view that neurotransmitters are secreted from nerve varicosities directly to smooth muscle cells.

FLC have always been seen to be closely associated with ICC (13). There are few structural data but no functional data on the innervation of FLC, although the hypothesis has been put forth that FLC might be involved in neurotransmission (19). In our study, direct contact between FLC and nerve varicosities was rarely seen, consistent with studies on human tissue (12). If present, our hypothesis would be that this would signify innervation of FLC and not necessarily (indirect) innervation of smooth muscle cells.

In summary, no evidence was found for any impediment in nitrergic innervation to smooth muscle in the absence of ICC-IM. Compared with wild-type tissue, field stimulation of enteric nerves yielded much stronger activation of substance P-mediated nerves, suggesting that ICC-IM might normally
protect the musculature from excessive excitation or that function of ICC musculature in the absence of ICC has increased excitability, a notion supported by the presence of marked spontaneous phasic contractile activity in Ws/Ws fundus.

**METHODS**

**Animals and contraction studies.** W/Wv mice were purchased from Jackson Laboratories (Bar Harbor, ME), and Ws/Ws rats were house bred; all animals were killed by cervical dislocation. The small intestine was exposed by a midline abdominal incision, and circular muscle strips of the fundus were taken and hung in organ baths through ring electrodes that provided electrical nerve stimulation. Tissues were equilibrated in 60 ml of 37–38°C Krebs solution saturated with 95% O₂-5% CO₂ for 1 h. Contraction changes were amplified by using Grass amplifiers (model 7 P122 D).

**Electron microscopy.** Tissues were fixed with 2% paraformaldehyde, 2.5% glutaraldehyde, 3% sucrose, and 1.25 mM CaCl₂ in 0.05 M cacodylate buffer (pH 7.4) at 4°C overnight. They were then postfixed in 2% osmium tetroxide (OsO₄) for 1 h, stained en bloc with 2% aqueous uranyl acetate for 40 min, dehydrated, infiltrated, and embedded in Epon-Araldite resin. Ultrathin sections were cut and stained with lead citrate for 5 min before being viewed with a transmission electron microscope (JEOL 1200EX Biosystem; Japanese Electron Optics Laboratories, Tokyo, Japan).

**Electrical stimulation of enteric nerves.** The influence of neurotransmitters on stomach muscle function is best studied through the electrical stimulation of the enteric nerves, causing release of neurotransmitter in situ, and measurement of the subsequent response of mechanical and electrical muscle activity. The stimulus parameters were 1, 4, and 10 Hz, 0.5-ms pulse duration for 30 s, at a stimulus strength of 20 V/cm.

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**Fig. 4.** Effects of electrical stimulation of enteric nerves in wild-type and W/Wv mouse fundic musculature. A: in wild-type mouse fundus, stimulation of enteric nerves at 10 Hz in presence of atropine and guanethidine caused decrease in tone of musculature. This relaxation was largely abolished by prior blockage of nitric oxide synthesis by L-NNA. Relaxation was further diminished in presence of apamin, and contractile activity develops (see C). B: in W/Wv mouse fundus, stimulation of enteric nerves in presence of atropine and guanethidine caused little or no relaxation. Nerve stimulation in presence of L-NNA caused abolishment of any relaxation and development of contractile activity, indicating that relaxation of excitatory nerves masked relaxation in absence of L-NNA. With addition of apamin, nerve stimulation caused further development of contractile activity (see D). C: in wild-type mouse fundus, stimulation of enteric nerves in presence of L-NNA (2 × 10⁻⁴ M) and apamin (10⁻⁶ M) caused development of contractile activity. This contractile activity experienced little effect from neurokinin 1 (NK1) receptor antagonist SR-14033 (10⁻⁶ M), but atropine markedly inhibited contractile activity induced by nerve stimulation. D: in W/Wv mouse fundus, stimulation of enteric nerves in presence of L-NNA and apamin caused development of contractile activity consisting of tone and high-amplitude superimposed phasic contractile activity. This contractile activity was strongly inhibited by NK1 receptor antagonist SR-14033 (10⁻⁶ M), and atropine further inhibited contraction. E: quantification of A and B, calculating changes in contractile activity as changes in area under curve before and during nerve stimulation (n = 10 each group). Statistical significance of drug effect at *p = 0.02 and #p = 0.01 is indicated as determined by 2-tailed Wilcoxon signed rank test for paired differences. F: immunohistochemistry using rabbit anti-substance P antibody. Substance P-containing nerves were evenly distributed throughout circular (CM) and longitudinal (LM) muscle layers of fundus. They were also seen in myenteric plexus. There was no difference between wild-type and Ws/Ws tissues.
The mechanical activity in response to nerve stimulation is studied by calculating the area under the curve of mechanical activity over a 15-s stimulation period during stimulation and then subtracting the control value calculated in the same way but before electrical stimulation. Electrical stimulation of enteric nerves evoked IJPs consisting of a fast and slow component in both wild-type and Ws/Ws fundic muscle cells. The slow component is best quantified by the duration of the IJP measured at the base of the IJP. The fast component is best quantified by the peak amplitude of the IJP.

Immunohistochemistry. Tissues were embedded in Tissue-Tek (Miles, Elkhardt, IN) and frozen in isopentane in a beaker submerged in liquid nitrogen. Frozen sections (8 mm) were cut with the cryostat and were mounted on the coated slides. Frozen sections were then fixed in 4% paraformaldehyde for 10–15 min at room temperature. After the nonspecific binding was blocked in 5% normal goat serum, tissues were incubated overnight at room temperature in rabbit anti-substance P antibody (1:2,000; Chemicon, Temecula, CA). Secondary antibody was biotin-conjugated goat anti-rabbit IgG packaged within a Vectastain ABC kit (Vector Laboratories, Burlingame, CA). 3,3'-Diaminobenzidine (0.05%) plus 0.01% H2O2 in 0.05M Tris-buffered saline (pH 7.6) was used as a peroxidase substrate. The primary antibody was diluted in 0.5% normal goat serum in 0.05 M PBS (pH 7.4) + 0.1% Triton X-100. Negative controls included the omission of primary or secondary antibodies.

ACKNOWLEDGMENTS

SR-140333 was provided by Sanofi-Synthelabo Recherche, Montpellier, France.

GRANTS

This research was supported by the Canadian Institutes of Health Research (CIHR). L. Liu was supported by a Clinician Scientist award from the CIHR and an operating grant from the Canadian Association of Gastroenterology (CAG). E. White was supported by a CIHR Ph.D. student award. N. Zarate received the CAG/CIHR-Novartis-Bristol Myers Squibb fellowship. S. Gill and L. Krebs were recipients of the Stephen Collins Summer student scholarship funded by Astra Zeneca.

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