Neurotransmission in lower esophageal sphincter of W/W<sup>v</sup> mutant mice

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Zhang Y, Carmichael SA, Wang XY, Huizinga JD, Paterson WG. Neurotransmission in lower esophageal sphincter of W/W<sup>v</sup> mutant mice. Am J Physiol Gastrointest Liver Physiol 298: G14–G24, 2010. First published October 22, 2009; doi:10.1152/ajpgi.00266.2009.—To address the controversy surrounding the role of interstitial cells of Cajal (ICC) in nitrergic neurotransmission to gastrointestinal smooth muscle, circular smooth muscle from the lower esophageal sphincter (LES) of W/W<sup>v</sup> wild-type and mutant (ICC-deficient) mice were studied by using intracellular and tension recordings in vitro. Resting membrane potential was more negative, and the spontaneous unitary potentials diminished in mutant mice. In wild-type mice, nerve stimulation induced a biphasic inhibitory junction potential (IJP) consisting of a fast initial IJP followed by a long-lasting slow IJP (LSIJP). The IJP was markedly impaired in a significant proportion of mutant mice, whereas in others it was normal. Pharmacological studies in the mice with markedly impaired IJPs revealed that cholinergic and purinergic components of the nerve-mediated responses appeared intact. In wild-type mice, caffeine hyperpolarized smooth muscle cells, inhibited the initial fast IJP, and completely abolished the LSIJP. In mutant mice, caffeine depolarized smooth muscle cells and abolished the impaired LSIJP but did not affect the initial fast IJP. Immunohistochemical staining for c-Kit confirmed deficiency of ICC in mutant mice with a normal nitrergic IJP. Rings of LES circular smooth muscle from W/W<sup>v</sup> mutant mice generated significantly less spontaneous tone than controls. When tone was restored with carbachol, normal nitric LES relaxation was recorded. These data suggest that 1) there is significant variability in the generation of nitrergic neurotransmission in the LES of W/W<sup>v</sup> mutant mice, whereas purinergic and cholinergic neurotransmission are intact; 2) the altered nitrergic responses appear to be associated with abnormal Ca<sup>2+/-</sup>-dependent signaling initiated by spontaneous Ca<sup>2+</sup> release from sarcoplasmic reticulum in smooth muscle cells; and 3) c-Kit-positive ICC are not essential for nitrergic neurotransmission in mouse LES smooth muscle.

Recent studies in both ICC-deficient mice (26) and rats (1, 8, 15) challenged the concept that ICC play a primary role in the inhibitory innervation to GI smooth muscle, a position that is also supported by a recent comprehensive review (24). We have recently characterized distinct regional differences in the inhibitory innervation to the murine lower esophageal sphincter (LES) (40). The clasp fibers of the right side of the LES have a biphasic inhibitory junction potential (IJP) with an initial fast IJP followed by a unique long-lasting slow IJP (LSIJP). The former has both purinergic and nitrergic components, whereas the latter is purely nitrergic. The purinergic IJP is due to opening of apamin-sensitive small-conductance K<sup>+</sup> channels (SK), whereas the nitrergic IJP may be mediated by closing of Ca<sup>2+/-</sup>-activated Cl<sup>-</sup> channels (Cl<sub>Ca</sub>) (40, 43, 46). Basal activity of Cl<sub>Ca</sub> involves a Ca<sup>2+/-</sup>-dependent signaling pathway that is primed by spontaneous Ca<sup>2+/-</sup> release from the sarcoplasmic reticulum (SR) (44). Because of these distinct features of nitrergic innervation, clasp fibers provide a unique model to further characterize and potentially resolve the controversy surrounding the role of ICC in LES neurotransmission.

METHODS

Animal preparation. All animal procedures were approved by the Animal Care Committee of Queen’s University. WBB6F1/J homozygous mice (wild-type, control) and WBB6F1/J-kir<sup>2</sup>/kir<sup>2</sup>−/− mice (W/W<sup>v</sup> mutant mice) were used. All mice were purchased from Jackson Laboratories (Bar Harbor, ME). Mice of either sex, weighing between 20 and 30 g, were killed by decapitation following isoflurane anesthesia. The distal esophagus with attached stomach was removed en bloc via an incision in the diaphragm.

LES preparation and conventional intracellular recordings. The LES, which is identifiable as a distinct thickening of circular muscle at the gastroesophageal junction, was dissected free from adjacent tissue under an anatomical microscope. The mucosal layer was removed, and strips (0.5–1 × 2–2.5 mm) from the right side of the LES were pinned with mucosa side facing upward on the bottom of a recording chamber (4-ml volume) covered by Sylgard (Dow Corning, Midland, MI) and perfused at 2 ml/min with preoxygenated Krebs solution at 36°C. Nifedipine (1 μM) was added to the bath for all experiments to prevent spikes and electrode dislodgement. Guanethidine (3 μM) and substance P (1 μM) were added to eliminate responses to adrenergic and tachykininergic innervation, respectively, and thereby optimize the recording of inhibitory responses (40). All experiments were done using the clasp fibers of the LES, since our previous studies have demonstrated that this region has a biphasic IJP with distinct nitrergic and purinergic components (40). Two pairs of silver wires were used to deliver transmural nerve stimulation to the muscle preparations using four square-wave pulses (20 Hz) with a duration of 0.3 ms and voltage of 70 V, and electrical activity was recorded by using conventional intracellular electrodes as previously described (38, 41).
Immunohistochemical c-Kit staining of ICC and quantification of c-Kit immunoreactivity. In another series of experiments, both electrophysiological characterization of the IJP as well as c-Kit staining of intramuscular ICC (ICC-IM) were carried out in clasp fibers of both W/W<sup>+</sup> mutant and wild-type LES. Muscle strips were pinned with serosa side facing downward on the bottom of a tissue culture dish covered by Sylgard and fixed by immersion in 100% acetone for 10 min at 4°C. These tissues were then transferred and stored in cold PBS (0.1 M PBS, pH 7.4, 4°C) for c-Kit immunohistochemical staining of ICCs by use of a previously described protocol (30). Briefly, blocking of nonspecific staining was achieved by immersing the tissue in 5% normal goat serum for 1 h before incubation with rat monoclonal anti-c-Kit (1:200) overnight at room temperature. On the second day, tissues were incubated with Cy3-coupled goat anti-rat IgG (1:800) for 1 h before incubation with rat monoclonal normal goat serum for 1 h before incubation with rat monoclonal anti-c-Kit (1:200) overnight at room temperature. On the second day, tissues were incubated with Cy3-coupled goat anti-rat IgG (1:800) for 1 h. Control staining was prepared by omitting primary antibody from the incubation solution. All of the antibodies were diluted with 0.05 mol/l PBS with 0.3% Triton X-100. Tissues were subsequently examined for the presence of c-Kit-positive ICC by one of us (X.-Y. Wang) who was blinded to the animal genotype and electrophysiological findings, thereby allowing for objective quantitative correlation between electrophysiological results and ICC-IM numbers in the exact same tissues. Examination was done with a confocal microscope (LSM 510; Zeiss) with an excitation wavelength appropriate for Cy3 (570 nm). Confocal micrographs were digital composites of Z-series sections of 20 optical sections through a depth of 15–20 μm. Final images were obtained with Carl Zeiss software.

c-Kit immunoreactivity was quantified with use of tissues from nine individual animals. In addition, quantification was performed by using Photoshop, version 7.0 (Adobe Systems, Mountain View, CA) as previously described (19). Kit-immunopositive cells were identified and highlighted by density slicing on color scale images. The area of immunopositive cells on each picture was measured and expressed as percentage of total area (19).

Tension recording experiments from intact LES muscular rings. To isolate intact rings of LES muscle for mechanical recordings, an incision was made along the greater curvature of the excised stomach up to but not including the gastroesophageal junction and the tissue was pinned to a dissecting tray, luminal side up. After the stomach contents were washed away, the mucosal and submucosal layers of the proximal stomach and gastroesophageal junction were removed with forceps and scissors under an anatomical microscope. The gastroesophageal junction was then excised intact from the remainder of the stomach and trimmed to remove remaining gastric and esophageal muscle tissue, leaving an intact ring of mouse LES smooth muscle for study.

Tension recordings were performed in 10-ml water-jacketed pharmacology baths maintained at 37°C and filled with Krebs solution that was continuously bubbled with 95% O<sub>2</sub>-5% CO<sub>2</sub>. After isolation, LES rings were tied with two pieces of surgical suture to produce two loops. The upper loop was attached to a Grass FT03 isometric force transducer situated above the bath and the lower loop to a hook at the bottom of the bath, such that tension recordings were made in the axis of the circular smooth muscle. Two platinum stimulating electrodes were positioned on either side of the tissue. An initial amount of tension (preload) of 0.7 g was applied and the tissue was allowed to equilibrate for 1 h before the experiment was begun. Successful isolation of the LES was confirmed by electrical field stimulation (10 Hz, 0.5-ms pulse duration, 5-s stimulation, and 100 V), which induced prompt relaxation of the muscular rings. The magnitude of tone (expressed as g/mg of tissue) generated by the tissue after the 1-h equilibration period was noted, following which 60 mM KCl was added. Following repeated washing and a further equilibration period, electrical field stimulation (EFS)-induced responses were recorded before and after the administration of 100 μM N<sup>-</sup>O-nitro-L-arginine methyl ester (L-NAME). Because preliminary experiments revealed that basal tone in LES rings from W/W<sup>+</sup> mutant mice was markedly diminished, which made it impossible to detect EFS-induced LES relaxation, carbacbol (1 μM) was administered to induce resting tone prior to studying the EFS-induced relaxation responses. These relaxation responses were then compared with the EFS-induced relaxations recorded in control mice in the presence of carbacbol. The magnitude of LES relaxation was expressed as a % of initial tone (i.e., the tone recorded when strips were initially hung in the bath and before preload was applied).

**Solutions and drugs.** The modified Krebs solution contained the following (in mM): NaCl 118.07, NaHCO<sub>3</sub> 25.00, d(+)-glucose 11.10, KCl 4.69, CaCl<sub>2</sub> 2.52, MgSO<sub>4</sub> 1.00, and NaH<sub>2</sub>PO<sub>4</sub> 1.01. All drugs were purchased from Sigma, except isoflurane (Baxter, Mississauga ON, Canada). The following drugs were used: nifedipine, atropine, guanethidine, amphetamine, substance P, N-nitro-L-arginine methyl ester (L-NAME), caffeine, cyclopiazonic acid (CPA). Nifedipine and CPA were dissolved in DMSO, caffeine was dissolved in Krebs solution, and all others in

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**Table 1. Average parameters of IJPs in wild-type and W/W<sup>+</sup> mutant mice**

<table>
<thead>
<tr>
<th></th>
<th>MP, mV</th>
<th>IJP Amplitude, mV</th>
<th>IJP Duration, ms</th>
<th>LSJP Amplitude, mV</th>
<th>LSJP Duration, s</th>
<th>Unitary Potential Spectrum, mV&lt;sup&gt;2&lt;/sup&gt;/Hz</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>n = 15</td>
<td>-43.8±1.1</td>
<td>18.5±2.4</td>
<td>703±44</td>
<td>5.5±0.7</td>
<td>11.6±1.7</td>
</tr>
<tr>
<td>W/W&lt;sup&gt;+&lt;/sup&gt; mutant</td>
<td>n = 35</td>
<td>-47.6±1.1*</td>
<td>10.0±1.2*</td>
<td>511±19*</td>
<td>2.3±0.3*</td>
<td>15.1±0.7 (n = 16)</td>
</tr>
</tbody>
</table>

Values are means ± SE. IJP, inhibitory junction potential; LSJP, long-lasting slow IJP. *P < 0.05; W/W<sup>+</sup> mutant vs. wild-type.

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**Fig. 1.** Typical inhibitory junction potentials (IJPs) recorded in W/W<sup>+</sup> wild and mutant mice. A: a typical IJP induced by 4 pulses (0.3 ms of duration) at 20 Hz in lower esophageal sphincter (LES) clasp of W/W<sup>+</sup> wild-type mouse consisted of an initial fast hyperpolarization following by a long-lasting slow hyperpolarization (LSIJP). B: IJPs in the LES clasp of W/W<sup>+</sup> mutant mice were variable, ranging from a normal IJP (a) to absence of the LSIJP (b).
distilled and deionized water. These were diluted to final concentrations with Krebs solution. Final concentration of DMSO in Krebs solution was no more than 1%, which did not produce any effect on the electrical activity of the tissue.

**Statistical analysis.** The following parameters were quantified: resting membrane potential (MP; mV), amplitude (mV), and duration at half-amplitude (ms) of both the initial IJP and LSIJP. The averaged power spectrum density between 0.1 and 0.6 Hz was used to quantify the amplitude (in mV²/Hz) of resting MP fluctuations, well described previously as unitary potentials (7). Data are shown as means ± SE, and n refers to number of animals. Only recordings in which a full protocol was completed in the same cell are included in the statistical analysis. Pre- and postdrug comparisons were made by use of the paired Student’s t-test, whereas comparison between basal tone in mutant vs. control muscle ring preparations was made by the unpaired Student’s t-test. A P value of < 0.05 was considered statistically significant.

**RESULTS**

**General properties of junction potentials evoked by nerve stimulation.** The electrical properties of the circular smooth muscle of the clasp fibers of the LES in wild-type and W/W <sup>v</sup> mutant mice are summarized in Table 1. In wild-type mice the MP averaged −43.8 mV (n = 15). It was characterized by unitary potentials of 1–4 mV (Figs. 1A, 2A, and 3A). Power spectrum density analysis of the unitary potentials revealed a mean amplitude of 0.415 mV²/Hz. These data were consistent with those of our previous publications in CD1 mouse (39, 42) and opossum (41, 44). Nerve stimulation induced a complex IJP consisting of an initial fast IJP followed by a LSIJP (Fig. 1A). Application of atropine (3 μM) significantly increased amplitudes of the initial IJP and LSIJP but did not affect the amplitude of unitary potentials (Table 2; Fig. 2, A and B).

In W/W <sup>v</sup> mutant mice, the unitary potentials were markedly diminished (Table 1; Fig. 1B) and the MP and the IJP complex induced by nerve stimulation were highly variable. Mice could be divided into two groups based on the magnitude of the nitricergic IJP. In some animals the nitricergic IJP was virtually absent (Fig. 1Bb), whereas in others it was not significantly different from IJPs in wild-type animals (Fig. 1Ba). Of note, the amplitude of the IJP was consistent between recordings done in different cells in the same animal. Taking all data together, the smooth muscle cells were significantly more hyperpolarized and the magnitude of the nitricergic IJP significantly smaller in the W/W<sup>v</sup> mutant mice compared with wild-type controls (Table 1).

To further characterize the abnormal neurotransmission, additional experiments were performed on tissues with an impaired IJP defined as the nitricergic component of the IJP being < 2 mV in amplitude (n = 16/35); 2 mV was the smallest amplitude of IJPs recorded in wild-type mice. The resting MP in this group was more negative compared with that in wild-type (approximately −43.8 mV vs. approximately −49.8 mV, P < 0.05). Nerve stimulation evoked one of two kinds of responses, namely a fast IJP following by a small LSIJP (10/16) (Fig. 2D) or a fast IJP following by long-lasting excitatory junction potential (EJP) (6 of 16) (Fig. 2F). Bath application of atropine (3 μM) increased the amplitude of LSIJP (Fig. 2Dc) and converted the long-lasting slow EJP to a small long-lasting slow IJP (Fig. 2Fc). These data suggest that cholinergic innervation to smooth muscle is present in W/W<sup>v</sup> mutant mice. It also shows that cholinergic neurotransmission

### Table 2. Pharmacological characterization of LES clasp muscle electrophysiology in wild type and W/W<sup>v</sup> mutant mice with abnormal nitricergic IJP

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Control n</th>
<th>Amplitude, mV</th>
<th>Duration, ms</th>
<th>RMP, mV</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-NAME Control</td>
<td>6</td>
<td>43.2 ± 0.7</td>
<td>10.3 ± 1.7</td>
<td>102.1 ± 2.8</td>
</tr>
<tr>
<td>L-NAME Control</td>
<td>6</td>
<td>52.8 ± 0.5</td>
<td>11.9 ± 1.5</td>
<td>117.5 ± 3.1</td>
</tr>
<tr>
<td>Apamin</td>
<td>6</td>
<td>6.6 ± 1.9</td>
<td>5.5 ± 1.5</td>
<td>501.3 ± 7.6</td>
</tr>
<tr>
<td>Apamin</td>
<td>6</td>
<td>7.2 ± 2.0</td>
<td>6.6 ± 1.6</td>
<td>601.3 ± 8.6</td>
</tr>
<tr>
<td>Atropine</td>
<td>6</td>
<td>10.2 ± 1.0</td>
<td>7.5 ± 1.1</td>
<td>1002.0 ± 15.0</td>
</tr>
<tr>
<td>Atropine</td>
<td>6</td>
<td>11.1 ± 1.1</td>
<td>8.6 ± 1.6</td>
<td>1100.0 ± 14.0</td>
</tr>
<tr>
<td>Apamin + L-NAME</td>
<td>6</td>
<td>13.2 ± 2.2</td>
<td>9.6 ± 1.6</td>
<td>1300.0 ± 14.0</td>
</tr>
<tr>
<td>Apamin + L-NAME</td>
<td>6</td>
<td>14.3 ± 2.6</td>
<td>10.7 ± 1.7</td>
<td>1400.0 ± 15.0</td>
</tr>
<tr>
<td>Apamin + L-NAME</td>
<td>6</td>
<td>15.4 ± 2.9</td>
<td>11.8 ± 1.8</td>
<td>1500.0 ± 16.0</td>
</tr>
<tr>
<td>Apamin + L-NAME</td>
<td>6</td>
<td>16.5 ± 3.0</td>
<td>12.9 ± 1.9</td>
<td>1600.0 ± 17.0</td>
</tr>
</tbody>
</table>

Values are means ± SE. RMP, resting membrane potential; L-NAME, N<sub>ω</sub>-nitro-arginine methyl ester; N.M, not measured or not measurable. *P < 0.05 before and after drug application. †P < 0.05 comparison between W/W<sup>v</sup> control and mutant mice.
masks nitrergic neurotransmission in this type of in vitro experiment. The remaining experiments conducted in the W/Wv mutant mice were performed in the presence of 3 μM atropine.

**Pharmacological characterization of LES clasp muscle electrophysiology in controls and W/Wv mutants with abnormal nitrergic IJPs.** Apamin abolishes the purinergic fast IJP by blockade of SK channels, and L-NAME eradicates the nitrergic slow IJP by inhibition of nitric oxide synthase. These two drugs were therefore used to isolate the purinergic and nitrergic components of the IJP. Results are summarized in Table 2. In wild-type mice apamin (300 nM) depolarized the smooth muscle cells by 6.5 mV over control and decreased the amplitude of the initial fast IJP but did not change the power spectrum density of the unitary potentials or amplitude of the LSIJP (Fig. 3, A and B; Fig. 6B). In W/Wv mutant mice apamin produced a similar depolarization of 6.7 mV over control and markedly reduced all responses evoked by nerve stimulation (Fig. 3, C and D). In contrast to wild-type mice, apamin reduced the power spectrum density of the unitary potentials that was already truncated in W/Wv mutant mice (Fig. 3D, a and b; Fig. 6B).

Administration of L-NAME (200 μM) in the presence of apamin (300 nM) abolished the apamin-resistant initial IJP and LSIJP in wild-type mice (Fig. 4, A and B). L-NAME also abolished the small residual LSIJP in W/Wv mutant mice (Fig. 4, C and D). The data imply that purinergic innervation is persistent and that nitrergic innervation appears selectively impaired in this subset of W/Wv mutant mice.

**Effects of agents that interfere with SR function on LES clasp muscle electrophysiology in controls and W/Wv mutant mice with abnormal nitrergic IJPs.** We have previously demonstrated that the nitrergic IJP is blocked by drugs that interfere with either SR function or the Ca2+-calmodulin-dependent protein kinase II (CaMKII) signaling cascade (39, 41, 42, 43, 44), raising the possibility that these pathways are impaired in smooth muscle cells of the W/Wv mutant mice. Studies were therefore conducted using pharmacological interventions to interrupt this Ca2+-dependent signaling axis. Agents were added to the bath consecutively. In wild-type mice, caffeine (5 mM), which depletes SR Ca2+ by inducing massive Ca2+ release, rapidly hyperpolarized LES smooth muscle cells (Fig. 5A) and abolished both unitary potentials (Fig. 6A) and the nitrergic IJP, but it had no effect on the purinergic IJP (Fig. 5B) (Table 2). However, in W/Wv mutant mice caffeine induced a brief hyperpolarization followed by a sustained depolarization (Fig. 5C) and abolished the small LSIJP but left the purinergic IJP...
intact (Fig. 5D). Moreover, the amplitude of the unitary potentials was also diminished (Fig. 6A).

In wild-type mice, CPA (10 μM), a SR ATPase inhibitor that depletes store Ca2+, depolarized the smooth muscle cells (Fig. 7A) and abolished both the unitary potentials and the biphasic nitrergic IJP (Fig. 7C). CPA caused only depolarization and had no effect on the unitary potentials or the purinergic IJP in W/Wv mutant mice (Fig. 7B and D).

Immunohistochemical c-Kit staining of ICC in W/Wv mutant and wild-type mice. To examine a relationship between ICC and neurotransmission, experiments were performed in which immunohistochemical c-Kit staining was carried out on LES clasp muscle preparations from both wild-type and W/Wv mutant mice that were first characterized electrophysiologically. Figure 8A displays c-Kit-positive ICC-IM in the LES clasp. Figure 8B depicts the original recordings of the normal and impaired patterns of the IJP in the LES clasp of W/Wv mutant mice 2 and 8. Table 3 summarizes the electrophysiological parameters of IJPs that correspond to individual mice in Fig. 8A, mice 1-4 and 7-9. Significant ICC-IM staining in the LES clasp preparations were observed in wild-type mice, whereas only sparse ICC-IM of the LES clasp were visible in W/Wv mutant mice. Sparse ICC in LES was present both in animals with a markedly impaired IJP and a normal IJP, and there was no correlation between the magnitude of the nitrergic IJP and the percentage of c-Kit-positive area (Fig. 8C).

Tension recording studies in circular smooth muscle rings. As depicted in Fig. 9A, circular smooth muscle rings from W/Wv mutant mice generated significantly less spontaneous and KCl-induced tone than controls. Because of the low basal tone, EFS-induced LES relaxation was difficult to discern; therefore tone was generated pharmacologically by the addition of 1 μM carbachol. As depicted in Fig. 9B, in the presence of carbachol, EFS-induced LES relaxation was recorded that was of comparable magnitude to the LES relaxation recorded in control mice in the presence of carbachol. The EFS-induced LES relaxation was markedly inhibited by application of L-NAME.

DISCUSSION

The present study demonstrates that in LES clasp fibers of W/Wv mutant mice there is significant variability in the amplitude of unitary potentials and the nitrergic component of the IJP. The nitrergic component was normal in 19 of 35 mice, but markedly diminished (<2 mV) in the remainder. The small nitrergic component in these cells contrasted with the apamin (ATP or a related nucleotide mediated) and atropine-sensitive
(cholinergic) components of the nerve-stimulated junction potentials, which remained within their normal range. There was no relationship between the number of immunohistochemically defined ICC-IM and the amplitude of the nitrergic IJP; that is, in tissue with normal nitrergic IJPs there was a lack of ICC-IM similar to tissues with low-amplitude IJPs. Furthermore, in rings of LES circular smooth muscle from W/Wv mutant mice, normal nerve-mediated LES relaxation was recorded that was markedly inhibited by nitric oxide synthase inhibition. Therefore, ICC-IM are not responsible for neurally evoking nitrergic IJPs in LES smooth muscle cells.

The selective variability of the nitrergic IJP is unlikely due to a difference in number of nitrergic nerves in the W/Wv mutant mouse, since density of the nitrergic nerve endings is normal in these mice (5, 6). It is more likely that variability in properties of the smooth muscle cells of W/Wv mutant mice, and specifically variability in the Ca2+-dependent signaling pathway involving spontaneous Ca2+ release from the SR and Ca2+-activated chloride channels, is responsible. Such heterogeneity in certain smooth muscle properties is not uncommon (9, 21). In addition, W/Wv mutant mice have multiorgan dysfunction (2, 18, 22, 25, 28); hence it should be considered possible that smooth muscle cells are affected by the kit mutation, especially since smooth muscle cells and ICC share the same precursor cells (37). The marked nitrergic innervation of ICC as documented by immunohistochemical staining (5, 6) is likely specifically affecting ICC function, which will indirectly have an impact on smooth muscle activity.

Edwards et al. (7) first described ongoing electrical discharge superimposed on the resting MP in circular smooth muscle of guinea pig antrum and identified this as unitary potentials. The unitary potentials also exist in circular smooth muscle cells of opossum and mouse LES (39, 41, 42, 44) and mouse stomach (3). Based on studies showing that Cl channel blockers or drugs that affect SR function abolish the unitary potentials (11, 41, 44), it has been proposed that spontaneous Ca2+ release from the SR activates Ca2+-activated Cl channels, which contributes to the resting MP and the unitary potentials. Niflumic acid has been reported to markedly inhibit the unitary potentials and nitrergic IJPs in the circular smooth muscle of murine LES (39, 40, 42). However, others have reported a lack of effect of other chloride channel blockers, DIDS and 9-AC, on the unitary potentials in murine gastric fundus smooth muscle (3). The reason for this discrepancy is unknown. The abolition of unitary potentials and nitrergic IJPs by caffeine and CPA in the present study is consistent with a previous study in the opossum (44). It is unlikely that the caffeine-induced inhibition of the nitrergic IJP was due to a nonspecific effect of the induced hyperpolarization, because

![Image of Figure 4](http://apjpi.physiology.org/DownloadedFrom/10.1152/ajpgi.00079.2009)
other agents that cause comparable effects on membrane potential do not inhibit the nitrergic IJP (45).

Previous investigators have found decreased unitary potentials in the murine gastric fundus of ICC-deficient mice and have proposed that ICC-IM generate these unitary potentials (3). The present study also shows that unitary potentials are either decreased or absent in the LES of \textit{W/Wv} mutant mice (Table 1), but here we suggest that this is due to interruption of Ca\textsuperscript{2+}/H\textsuperscript{+}-dependent signaling in smooth muscle cells of the \textit{W/Wv} mutants to varying degrees. The following evidence supports this hypothesis: 1) resting MP is more negative in the mutant mice than in the wild type (Table 1); 2) caffeine hyperpolarizes smooth muscle cells and abolishes the unitary potentials in wild-type mice (Fig. 5, \textit{A} and \textit{B}) but depolarizes smooth muscle cells in mutant mice (Fig. 5, \textit{C} and \textit{D}); 3) CPA abolishes the unitary potentials in wild-type mice (Fig. 7, \textit{A} and \textit{C}) but does not have any observable effect on unitary potentials in the mutant group; and 4) purinergic IJPs, which are unaffected by drugs that affect the SR or Ca\textsuperscript{2+}/CaMKII-myosin light chain kinase pathway, are intact in the mutant mice with impaired nitrergic IJPs. On the other hand, nitrergic IJPs, which appear to be uniquely dependent on the Ca\textsuperscript{2+} signaling pathway, are impaired.

Interestingly, caffeine induces depolarization in mutant mice and also during the hyperpolarization phase in the wild-type mice, whereas CPA produces depolarization in both groups. It is noteworthy that the time course of the depolarization evoked by caffeine is similar in wild-type and mutant mice (Fig. 5A, \textit{inset}). The mechanisms underlying depolarization by caffeine and CPA are unknown (20). It has been speculated that it can be attributed to activation of nonselctive cation channels (29).

Recently, Hwang et al. (17) reported that drugs affecting SR function also nonselctively block the TREK-1 channel and proposed that opening of this Ca\textsuperscript{2+}/H\textsuperscript{+}-insensitive K\textsuperscript{+} channel is responsible for the nitrergic IJP in gut smooth muscle. However, these interesting results need confirmation in different tissues and different species. It has been demonstrated that several chemically distinct drugs that affect either SR function or the Ca\textsuperscript{2+}-CaMKII-myosin light chain kinase pathway all block the nitrergic IJP (41, 42, 44). Furthermore, we have evidence that putative TREK-1 channel blockers do not antagonize the nitrergic IJP in mouse LES (45).

The \textit{W/Wv} mutant mouse has been used extensively as a model to study the role of ICC in pacemaking and neurotransmission in the gastrointestinal tract (13, 14, 23, 35, 36). Burns et al. (5) were the first to propose a role for ICC in mediating nitrergic neurotransmission to smooth muscle cells in murine stomach. These authors reported normal distribution of nitrergic inhibitory nerves in the stomachs of \textit{c-kit} mutants but found that nitric oxide-dependent inhibitory neurotransmission was absent. Subsequently, similar observations were reported in mouse LES, pyloric sphincter, and gastric antrum (27, 34).
Ward et al. (33) noted that in W/Wv mutant mouse LES preparations, the nitrergic component of the IJP was completely absent. This contrasts with our findings, where we found a normal nitrergic IJP in the majority of the W/Wv mutant LES preparations. In addition, when tone was pharmacologically induced in LES muscle rings from W/Wv mutants, a normal nerve-mediated LES relaxation could be recorded that was markedly inhibited by L-NAME. Consistent with our electrophysiological and tension recording studies, Sivarao et al. (26) showed that the resting LES pressure was significantly hypotensive in W/Wv mutant mice, but normal L-NAME-sensitive relaxation was documented. In vitro studies using the LES of ICC-deficient Ws/Ws rats provided further evidence that neural relaxation is mediated by both nitrergic and purinergic innervation, which is comparable to that seen in wild-type rats (8). In addition, Alberti et al. (1) reported that in the Ws/Ws rat colon, similar to our study, nitrergic neurotransmission is variable, ranging from normal amplitude to the complete absence of the nitrergic IJP. Finally, Huizinga et al. (15), studying gastric fundus smooth muscle from both ICC-deficient rats and mice, found the nitrergic neurotransmission to be intact but that this was obscured by an enhanced substance P-mediated excitatory innervation. Although possible, the abnormal nitrergic IJP recorded in the present studies is unlikely to be due to exaggerated excitatory responses because both cholinergic and tachykinergic neurotransmission were blocked by atropine and substance P tachyphylaxis, respectively. Hence, the observation that a normal nitrergic IJP can be recorded in W/Wv mice with histologically confirmed ICC-IM deficiency suggests that ICC-IM are not required for nitrergic neurotransmission to smooth muscle cells. It is possible that under normal circumstances ICC play a role in nitrergic neurotransmission, but when they are absent this neurotransmission is able to carry on via other mechanisms [e.g., directly between nerve and muscle (15, 24)].

The present evidence that the cholinergic EJP and purinergic IJP are persistent in W/Wv mutant mice does not support a role for ICC in cholinergic and purinergic neurotransmission, although a role for ICC in mediating excitatory neurotransmission in intestinal smooth muscle has been proposed (4, 31, 33).

The data on W/Wv mice have been presented into two categories based on the amplitude of the IJP. This is a somewhat arbitrary distinction, and one could argue that the data could be presented as a single group with simple reporting of
From this the conclusion would have been that IJPs are impaired in W/Wv mutant mice and associated with a lack of ICC-IM. It is possible that this strategy has resulted in such a conclusion in previous studies. Doing so, however, would have ignored the fact that in our study most W/Wv mutant mice have a normal IJP.

In summary, whereas cholinergic and purinergic innervation to LES is preserved, nitrergic innervation is impaired in a subgroup of W/Wv mutant mice. These differences in response to nitric oxide cannot be explained by differences in the presence of intramuscular ICC nor by differences in the presence of nitrergic nerves (5, 6), although the latter was not confirmed in the present study. Furthermore, this cannot be explained by variations in the proximity of ICC-IM to the recorded smooth muscle, since the amplitude of the IJP was consistent between cells in tissue from the same animal; if the IJP was markedly abnormal, it was similarly abnormal in all cells recorded in a given animal. Given that the nitrergic IJP and unitary potentials appear to depend on activity of Ca$^{2+}$-activated Cl$^{-}$ channels, we speculate that in circular smooth muscle cells of the clasp fibers of W/Wv animals, there is a large variability in the number of Cl$^{-}$ channels and/or proteins associated with Ca$^{2+}$-dependent signal cascades regulating these channels. This requires confirmation by patch-clamp studies. Although variability in smooth muscle activity is not uncommon, it is obvious that the variability in W/Wv mice is much larger compared with wild-type mice. This maybe a direct consequence of the abnormal c-Kit signaling pathway although that too has to be confirmed. The depolarizing effect of CPA and caffeine in the mutant mice with abnormal IJPs suggests that other calcium-dependent signaling pathways to K$^{+}$ and/or nonselective cation channels may be affected as well.

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GRANTS

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REFERENCES

Table 3. IJP and ICC characteristics in mutant mice

<table>
<thead>
<tr>
<th>Individual Mouse</th>
<th>No. of Cells</th>
<th>MP, mV</th>
<th>IJP Amplitude, mV</th>
<th>IJP Duration, ms</th>
<th>LSIJP Amplitude, mV</th>
<th>LSIJP Duration, ms</th>
<th>Unitary Potential Spectrum, mV²/Hz</th>
<th>% of Positive Area*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>n = 3</td>
<td>-45.4±1.2</td>
<td>18.6±1.6</td>
<td>463±38</td>
<td>6.6±1.0</td>
<td>11,212±1,135</td>
<td>0.163±0.038</td>
<td>1.116896</td>
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<td>2</td>
<td>n = 5</td>
<td>-45.3±2.6</td>
<td>19.0±2.9</td>
<td>495±18</td>
<td>8.0±1.8</td>
<td>11,179±419</td>
<td>0.147±0.04</td>
<td>0.721089</td>
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<tr>
<td>3</td>
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<td>-44.4</td>
<td>21.9</td>
<td>584</td>
<td>4.9</td>
<td>11,967</td>
<td>0.017</td>
<td>3.839601</td>
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<tr>
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<td>21.7±1.2</td>
<td>477±10</td>
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<td>11,849±916</td>
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<tr>
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<td>11.5±2.0</td>
<td>379±14</td>
<td>1.8±0.2</td>
<td>13,497±1,734</td>
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<td>442±15</td>
<td>3.8±0.7</td>
<td>14,398±1,262</td>
<td>0.0270±0.0071</td>
<td>1.747254</td>
</tr>
</tbody>
</table>

Values are means ± SE. * % of total area with c-Kit staining on immunohistochemistry.

Fig. 8. Immunohistochemical c-Kit staining of interstitial cells of Cajal (ICC) in LES clasp in 7 different W/W' wild-type (A, mice 1–4 and 7–9) and 2 mutant mice 5–6 and representative electrophysiological recordings (B, mice 2 and 11). Normal c-Kit-positive ICC networks (in red) in the LES clasp fibers were present in wild-type mice 5 and 6. However, in W/W' mutant mice (mice 1–4 and 7–9), the number of c-Kit-positive intramuscular ICC (ICC-IM) were markedly decreased. Electrophysiological data showed both normal and impaired IJP patterns (B), suggesting that the ICC-IM are not essential for neurotransmission in the LES clasp fibers. Vertical dashed lines in electrophysiological recordings mark where time scale changes. Scale bars in A, mouse 8, represent 50 μm and apply to LES images. C: scatterplot of the amplitude of the LSIJP against percentage of c-Kit-positive area. There was no correlation between the amplitude of the LSIJP and percentage of c-Kit-positive area in the W/W' mutant mice.

Fig. 9. A: tension recording studies demonstrated that rings of LES circular smooth muscle from W/W\* mutant mice generated less spontaneous and KCl-induced tone than controls. Open bars in A represent spontaneous tone, whereas solid bars represent maximal tone induced by application of 60 mM KCl (\(n = 9\) for W/W\* mutants and \(n = 4\) for controls; *P < 0.01). B: when tone was restored in LES circular smooth muscle rings from W/W\* mutants by application of carbachol, L-NAME-sensitive LES relaxation was recorded that was no different than the LES relaxation recorded in control animals in the presence of carbachol (\(n = 5\) and 6 for W/W\* and control and mice, respectively; *P < 0.05, **P < 0.01 vs. relaxation in the absence of L-NAME).