Neural regulation of slow-wave frequency in the murine gastric antrum

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Forrest, Abigail S., Tamás Ördög, and Kenton M. Sanders. Neural regulation of slow-wave frequency in the murine gastric antrum. Am J Physiol Gastrointest Liver Physiol 290: G486–G495, 2006. First published September 15, 2005; doi:10.1152/ajpgi.00349.2005.—Gastric peristaltic contractions are driven by electrical slow waves modulated by neural and humoral inputs. Excitatory neural input comes primarily from cholinergic motor neurons, but ACh causes depolarization and chronotropic effects that might disrupt the normal proximal-to-distal spread of gastric slow waves. We used intracellular electrical recording techniques to study cholinergic responses in stomach tissues from wild-type and W/W^v mice. Electrical field stimulation (5 Hz) enhanced slow-wave frequency. These effects were abolished by atropine and the muscarinic M_2-receptor antagonist 4-diphenylacetoxymethylpiperidine methiodide. ACh released from nerves did not depolarize antral muscles. At higher rates of stimulation (10 Hz), chronotropic effects were mediated by ACh and a noncholinergic transmitter and blocked by muscarinic antagonists and neurokinin (NK_1 and NK_2)-receptor antagonists. Neostigmine enhanced slow-wave frequency, suggesting that the frequency of antral pacemakers is kept low by efficient metabolism of ACh. Neostigmine had no effect on slow-wave frequency in muscles of W/W^v mice, which lack intramuscular interstitial cells of Cajal (ICC-IM). These muscles also showed no significant chronotropic response to 5-Hz electrical field stimulation or the cholinergic agonist carbachol. The data suggest that the chronotropic effects of cholinergic nerve stimulation occur via ICC-IM in the murine stomach. The capacity of gastric muscles to metabolize ACh released from enteric motor neurons contributes to the maintenance of the proximal-to-distal slow-wave frequency gradient in the murine stomach. ICC-IM play a critical role in neural regulation of gastric motility, and ICC-IM become the dominant pacemaker cells during sustained cholinergic drive.

Pacemaker; interstitial cells of Cajal; enteric nervous system; tachygastria; functional bowel disorders; gastric emptying

INTERSTITIAL CELLS of Cajal (ICC), located in the plane of the myenteric plexus (ICC-MY) or along the submucosal surface of the circular muscle in the colon, are responsible for the generation of electrical slow waves in the gastrointestinal (GI) tract (for review see Ref. 36). The role of ICC in pacemaker activity was discovered from studies on mutant animals (W/W^v and Sl/Sld^v mice) and a variety of other models in which ICC populations are depleted (20, 30–32, 41). In addition, many studies have shown that isolated smooth muscle cells lack spontaneous electrical activity (16, 19), although ICC display pacemaker activity (26, 27, 39). Electrical coupling of ICC-MY to circular smooth muscle cells allows generation of phasic contractions via activation of voltage-dependent Ca^{2+} channels in smooth muscle cells (9, 11, 31, 32). Phasic contractions initiated by slow waves are the basis for gastric peristalsis (22).

Slow waves propagate from the greater curvature of the orad corpus to the pylorus to produce gastric peristalsis. The normal pattern of slow-wave propagation is the result of a gradient in intrinsic frequencies along the longitudinal axis of the stomach. The orad corpus is the dominant pacemaker in the human stomach and in animal models such as the dog and mouse (14, 22, 29), because it generates slow waves at the fastest rate. The antrum is separated from the corpus, slow-wave frequency in the antral segment is phase locked to the corpus (e.g., ~8 min^{-1} in the mouse). Conditions that increase the intrinsic pacemaker frequency in the antrum or produce arrhythmic activity in this region can result in a breakdown in functional coupling between the corpus and antrum and interfere with normal gastric emptying (8, 23).

After ingestion of food into the stomach, intrinsic nerves release ACh, which serves as the major excitatory transmitter to enhance the force of phasic contractions. As a result of cholinergic stimulation, the small-amplitude phasic contractions coupled to slow waves during the interdigestive period can become powerful, nearly occlusive peristaltic contractions that serve to grind antral contents and aide gastric emptying (1, 28). ACh also has chronotropic effects on antral slow-wave frequency when it is applied to the solution bathing the muscle (15, 24). Kim and co-workers (24) showed that exogenous ACh and carbachol (CCh) increased the frequency of slow waves in cultured ICC-MY and that these effects were abolished by the muscarinic M_3-receptor antagonist 4-diphenylacetoxymethylpiperidine methiodide (4-DAMP). The increase in antral slow-wave frequency in response to cholinergic stimulation suggests that there is a tendency, during the postprandial period, for antral pacemakers to escape from the dominance of the corpus pacemaker. This tendency could be accentuated in human patients and in animal models with disrupted ICC networks and reduced corpus-to-antrum coupling, as in diabetic gastroparesis (25, 29, 31).

Recent studies have shown that inputs from enteric motor neurons are directed at intramuscular ICC (ICC-IM), rather than at smooth muscle cells or the dominant pacemaker cells, ICC-MY (4, 18, 42). It is unclear whether physiological release of ACh from enteric motor neurons has chronotropic effects comparable to those of exogenous ACh and CCh. Thus we investigated whether continuous stimulation of cholinergic nerves, as might occur in the gastric phase of digestion, is capable of sustained elevation of antral slow-wave frequency. We have also sought to determine the role of ACh metabolism in regulating the frequency of antral slow waves. Finally, we
have used W/WV mice to determine whether the chronotropic effects of enteric excitatory nerves are dependent on ICC-IM.

MATERIALS AND METHODS

Animals and tissue preparation. The use of animals was approved for these experiments by the institutional care and use committee of the Univ. of Nevada. Balb/c mice (Charles River Laboratories, Wilmington, MA), WBB6F1/Ki6/Ki6 (W/WV) mice, and WBB6F1/J-Ki6/Ki6 (ME) wild-type controls (Jackson Laboratories, Bar Harbor, ME) of either gender were anesthetized using isoflurane inhalation and killed by cervical dislocation. The stomachs were removed and opened along the lesser curvature. The luminal contents were washed away using Krebs-Ringer bicarbonate solution (KRB), and the mucosa was removed by sharp dissection. The antral muscle region was cut away and pinned, with the mucosal aspect of the circular muscle facing upward, to the bottom of a recording chamber lined with Sylgard 184 estamer (Dow Corning, Midland, MI). Intrinsic nerves were stimulated by electrical field stimulation (EFS, 0.1-ms duration, 1–10 Hz, supramaximal voltage; S48 stimulator, Grass, Quincy, MA) delivered from platinum wires placed on either side of the muscle preparation in parallel with the longitudinal axis of the antrum. All EFS experiments were performed in the presence of 100 μM N-nitro-L-arginine (L-NA) to block nitricergic neurotransmission.

Solutions and drugs. The composition of the KRB used in these studies was (in mM) 118.5 NaCl, 1.2 MgCl2, 23.8 NaHCO3, 1.2 KH2PO4, 11.0 dextrose, and 2.4 CaCl2. The pH of the buffer was 7.3–7.4 when bubbled with 97% O2-3% CO2 at 37 ± 0.5°C. Atropine, CCh (carbamylcholine chloride), 4-DAMP, nesitigmine bromide, nifedipine, L-NA, tetrodotox (TTX), and WIN-62577 were purchased from Sigma. SR-48968 was a gift from Sanofi-Synthelabo. Atropine, CCh, 4-DAMP, L-NA, neostigmine bromide, and TTX were dissolved in water; 4-DAMP and L-NA required additional sonication. Nifedipine and SR-48968 were dissolved in ethanol, and WIN-62577 was dissolved in DMSO. The final bath concentration of DMSO and ethanol did not exceed 0.1% (vol/vol), and neither solvent had an effect at this concentration. All drugs were applied via bath perfusion for 10–20 min.

Analysis of data. Values are means ± SE; n represents the number of animals from which muscle strips were obtained. SigmaStat Statistical Software for Windows version 2.03 (SPSS Science, Chicago, IL) was used for statistical analyses. Before tests of significance were performed, data were examined for normality and equal variance to determine whether parametric or nonparametric tests should be employed. Paired and unpaired t-tests and one-way ANOVA (repeated measures) followed by multiple comparisons against the control (Tukey’s test) were used for statistical comparisons. P < 0.05 was used as a cutoff for statistical significance in all statistical procedures. P values quoted for ANOVA are the values for the individual post hoc test. Values for numbers of experiments (n) refer to the number of animals from which muscles were taken for experiments.

RESULTS

Regulation of slow-wave frequency by excitatory nerves in wild-type gastric antrum. Spontaneous, regular slow-wave activity could be recorded in all tissues. Because blockade of smooth muscle contractions with 1 μM nifedipine had no effect on slow-wave parameters (Table 1), all subsequent recordings were made in the presence of this drug to facilitate the maintenance of cell impalements during sustained nerve stimulation. No significant difference was found in the presence of nifedipine for any of the parameters measured (n = 11, P > 0.05 by unpaired t-test).

Continuous low-frequency EFS (1 Hz, 0.1-ms pulses, 5 min; see MATERIALS AND METHODS) had no significant effect on the frequency of slow waves in antral muscles of Balb/c mice (n = 7): 3.7 ± 0.3 and 3.9 ± 0.3 min−1 during control and EFS, respectively. Atropine, added during stimulation, did not significantly modify the response to EFS (4.1 ± 0.3 min−1 after atropine, n = 7, P > 0.05 by ANOVA; Fig. IA).

At 5-Hz EFS (0.1-ms pulses, 5 min), the frequency of antral slow waves increased: from 3.2 ± 0.4 min−1 under control conditions to 5.2 ± 0.7 min−1 during EFS (n = 6, P = 0.016 by ANOVA). The increase in frequency was abolished by the selective ACh M3-receptor antagonist 4-DAMP (100 nM; 3.2 ± 0.4 min−1, P > 0.05 when tested against control, ANOVA). Atropine (100 nM), added in the continued presence of 4-DAMP, had no further effect on slow-wave frequency (3.1 ± 0.4 min−1, P > 0.05 by ANOVA; Fig. IB).

At 10-Hz EFS, slow-wave frequency increased: from 3.5 ± 0.4 to 5.4 ± 0.4 min−1 (n = 6, P < 0.011 by ANOVA). Atropine (100 nM) added during EFS reduced the frequency of slow waves to 4.1 ± 0.04 min−1 but did not fully restore the control frequency. To test whether part of the chronotropic effects of 10-Hz stimulation were due to recruitment of peptidergic responses via the release of neuropeptides (NK), we applied the NK1-receptor antagonist WIN-62577 (1 μM) and the NK2-receptor antagonist SR-48968 (1 μM) in the continued presence of atropine. These compounds reduced the frequency of slow waves to values not significantly different from control conditions (3.5 ± 0.4 min−1, P > 0.05 by ANOVA; Fig. IC).

Table 1. Slow-wave parameters in Balb/c gastric antral muscles and effects of nifedipine

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>Nifedipine (1 μM)</th>
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<tbody>
<tr>
<td>Slow-wave frequency, min−1</td>
<td>2.8 ± 0.4</td>
<td>2.6 ± 0.4</td>
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<tr>
<td>Membrane potential, mV</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Minimum</td>
<td>−68 ± 3</td>
<td>−70 ± 4</td>
</tr>
<tr>
<td>Maximum</td>
<td>−37 ± 2</td>
<td>−41 ± 3</td>
</tr>
<tr>
<td>Slow-wave duration, s</td>
<td>9.4 ± 0.9</td>
<td>9.0 ± 0.6</td>
</tr>
<tr>
<td>Slow-wave amplitude, mV</td>
<td>31 ± 1</td>
<td>28 ± 2</td>
</tr>
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</table>

Values are means ± SE; n = 11. No significant difference was found in the presence of nifedipine for any of the parameters measured (P > 0.05 by unpaired t-test).
may be the result of direct stimulation of muscarinic receptors expressed by smooth muscle cells, because these cells express nonselective cation channels activated by muscarinic stimulation (5, 21).

In a separate study, we also tested whether the effects of 5-Hz EFS were inhibited by TTX, a general neurotoxin. In these experiments, slow-wave frequency under control conditions averaged $2.5 \pm 0.5 \text{ min}^{-1}$, which increased to $4.5 \pm 0.7$

**Fig. 1.** Effect of enteric motor nerve stimulation on slow-wave frequency. A: slow waves from Balb/c gastric antral muscle under control conditions and during 1-Hz electrical field stimulation (EFS, 0.1 ms, 150 V) in the absence and presence of 100 nM atropine (left) and summary of effects of EFS on slow-wave frequency in 7 experiments (right). B: slow waves under control conditions and during 5-Hz EFS (0.1 ms, 150 V) in the absence and presence of 100 nM 4-diphenylacetoxy-N-methylpiperidine methiodide (4-DAMP) and 100 nM atropine (left) and summary of slow-wave frequencies under these conditions in 6 experiments (right). C: slow waves under control conditions and during 10-Hz EFS (0.1 ms, 150 V) in the absence and presence of 100 nM atropine and atropine + neurokinin (NK)-receptor antagonists SR-48968 (SR) and WIN-62577 (WIN) at 1 μM each (left) and summary of slow-wave frequencies under these conditions in 6 experiments (right). *Significantly different from control ($P < 0.05$ by 1-way ANOVA). Electrical traces become noisy during EFS because of stimulus artifacts.
min⁻¹ during EFS (n = 5, P = 0.019 by ANOVA). Addition of TTX during EFS reduced slow-wave frequency to 2.4 ± 0.4 min⁻¹ (Fig. 2). TTX had no effect on the duration or amplitude of slow waves or on membrane potential.

Enteric motor neurons have been reported to be spontaneously active in some GI muscles (35). If there is spontaneous release of ACh in murine antral muscles, its effects must be neutralized by endogenous acetylcholinesterases (AChE), because neither TTX (1 μM) nor atropine (1 μM) affects basal slow-wave frequency (4). Thus we tested the effects of AChE blockade by neostigmine on basal slow-wave frequency. Neostigmine (1 μM) caused a significant increase in basal slow-wave frequency: from 3.1 ± 0.4 min⁻¹ during control recording to 4.3 ± 0.5 min⁻¹ (P < 0.001 by ANOVA). Atropine (100 nM) reversed the increase in frequency caused by neostigmine, returning frequency to 3.1 ± 0.5 min⁻¹ (P > 0.05 compared with control; Fig. 3A). TTX (100 nM) also abolished the increase in frequency caused by neostigmine (from 4.5 ± 1.0 min⁻¹ during control recording to 8.8 ± 1.5 min⁻¹ in the presence of neostigmine, P = 0.026) and TTX returned frequency to 4.7 ± 0.3 min⁻¹ (P > 0.05 compared with control, n = 5; Fig. 3B). l-NNA (100 μM) did not affect the increased frequency of antral slow waves produced by 1 μM neostigmine (P > 0.05, n = 4; Fig. 3C).

We also tested the effects of neostigmine on the chronotropic effects of 5-Hz EFS. In these experiments, 5-Hz EFS increased slow waves from 2.7 ± 0.3 to 6.0 ± 0.7 min⁻¹ (P < 0.001). Neostigmine (1 μM), added during continuous EFS, did not further increase slow-wave frequency (i.e., 6.4 ± 0.7 min⁻¹, P > 0.05 by ANOVA). Application of 100 nM atropine under these conditions reduced slow waves to 2.8 ± 0.2 min⁻¹, which was not significantly different from control (P > 0.05; Fig. 3D).

Under basal conditions, neostigmine had no effect on the membrane potential: −74.1 ± 2.6 and −72.5 ± 2.8 mV in control and in the presence of neostigmine, respectively (P > 0.05 by ANOVA). However, although EFS alone had no effect on membrane potential (i.e., −69.7 ± 7.0 and −72.2 ± 8.1 mV in control and during EFS, respectively, P > 0.05 by ANOVA), addition of neostigmine during EFS resulted in significant depolarization (i.e., −55.2 ± 9.6 mV), which was abolished by 100 nM atropine (i.e., −72.8 ± 6.5 mV).

Effects of CCh and excitatory nerve stimulation on slow-wave frequency in W/W° gastric antrum. We also compared responses of antral muscles from W/W° mutants and strain-matched wild-type mice to CCh and enteric nerve stimulation. CCh was used as a muscarinic agonist for these studies. CCh (100 nM) increased the slow-wave frequency in strain-matched control mouse antral muscles: from 5.0 ± 0.8 min⁻¹ under control conditions to 7.8 ± 1.8 min⁻¹ in the presence of CCh (P = 0.04 by ANOVA; Fig. 4, C and E). In addition, there was significant depolarization of membrane potential of antral muscles from strain-matched WBB6F1/J-Kit−/Kit+ mice from −61 ± 4 mV in control recordings to −45 ± 7 mV in the presence of CCh (P = 0.0076 by ANOVA). The maximal level of depolarization during slow waves increased: from −41 ± 5 mV in control recordings to −32 ± 4 mV in the presence of CCh (P = 0.0189 by ANOVA, n = 7). These effects were entirely reversed by the application of 100 nM atropine: RMP returned to −65 ± 8 mV in the presence of atropine, and the maximum level of depolarization during slow waves was −41 ± 7 mV after atropine (n = 7; Fig. 4A). The amplitude of the slow waves was significantly reduced in the presence of CCh (from 21 ± 3 to 13 ± 5 mV, P = 0.0034 by ANOVA, n = 7), and this change was reversed by 100 nM atropine to a value not significantly different from control (22 ± 5 mV, P > 0.05 by ANOVA). The duration of the slow waves was unaffected by CCh, and atropine also had no effect on this parameter (P > 0.05 by ANOVA; Fig. 4C).

In W/W° mice, 100 nM CCh also depolarized the membrane potential: from −72 ± 7 mV in control to −63 ± 9 mV in the presence of CCh (P = 0.002 by ANOVA). CCh also increased the maximal level of depolarization reached during the peaks of slow waves: from −54 ± 5 mV in control to −45 ± 6 mV in the presence of CCh (P = 0.002 by ANOVA). These responses were completely inhibited by 100 nM atropine: RMP in the presence of atropine was −71 ± 7 mV, and the maximal level of slow-wave depolarization was −56 ± 6 mV (n = 7, both P > 0.05 compared with control values). The frequency of slow waves in W/W° antral muscles was not affected by CCh: from 5.2 ± 1.3 min⁻¹ in control to 5.6 ± 1.4 min⁻¹ in the presence of carbachol (P > 0.05 by ANOVA). Similarly, atropine had no effect on slow-wave frequency (4.8 ± 1.4

Fig. 2. A: slow-wave activity from a Balb/c antral muscle under control conditions, during 5-Hz EFS, and during 5-Hz EFS after addition of 100 nM TTX. B: summary of effects of TTX on EFS-induced increases in slow-wave frequency in 5 experiments. TTX restored control slow-wave frequency. *Significantly different from control (P < 0.05 by 1-way ANOVA). Electrical traces during EFS become noisy because of stimulus artifacts.
min⁻¹ in the presence of atropine; Fig. 4, D and E). Also, the amplitudes of the slow waves were not significantly affected by CCh or atropine: 18 ± 5, 17 ± 5, and 15 ± 4 mV for control, in the presence of CCh, and in the presence of atropine, respectively (P > 0.05 by ANOVA). As in the control tissues, the durations of slow waves were unaffected by CCh, and atropine also had no effect (Fig. 4, B and D; P > 0.05).

As observed in muscles of Balb/c mice, 5-Hz EFS increased the frequency of slow waves in strain-matched wild-type mice: from 4.3 ± 0.3 min⁻¹ in control to 6.1 ± 0.6 min⁻¹ during EFS (n = 5, P = 0.002). The effects of EFS were entirely blocked by 100 nM atropine: slow-wave frequency was 4.7 ± 0.3 min⁻¹ in the presence of atropine (P > 0.05 compared with control, n = 5; Fig. 5, A and C). EFS affected neither the amplitude nor the duration of slow waves and did not cause depolarization of membrane potential (Fig. 5A).

In contrast to effects in wild-type mice, 5-Hz EFS did not affect the frequency of slow waves in antral muscles of W/W°...
mice: 4.4 ± 0.3 and 4.5 ± 0.3 min\(^{-1}\) in control and during EFS, respectively (\(P > 0.05, n = 8\)). Atropine (100 nM) had no effect on slow-wave frequency when added during EFS (4.3 ± 0.3 min\(^{-1}\), \(P > 0.05, n = 8\); Fig. 5, B and C). EFS had no effect on the amplitude or duration of the slow waves, and nerve stimulation had no effect on RMP (Fig. 5 B).

EFS at 10 Hz caused an increase in the frequency of slow waves in \(W/W^v\) antral muscles: from 5.1 ± 1.0 min\(^{-1}\) in control to 7.6 ± 1.2 min\(^{-1}\) during EFS (\(P = 0.029, \text{ANOVA}, n = 5\)). This increase was reduced to levels not significantly different from control values by a combination of the NK antagonists SR-48968 (1 \(\mu\)M) and WIN-62577 (1 \(\mu\)M). Atropine (100 nM) caused no further reduction in slow-wave frequency (Fig. 6, B and C). A small, but significant, depolarization in membrane potential was also observed: from −75 ± 11 mV in control to −65 ± 12 mV during 10-Hz EFS (\(P = 0.029\) by ANOVA). This depolarization was also inhibited by the NK antagonists SR-48968 (1 \(\mu\)M) and WIN-62577 (1 \(\mu\)M), and atropine caused no further change in membrane potential after the NK antagonists. No alteration in the amplitude or duration of the slow waves was observed during 10-Hz EFS (Fig. 6, A and B).

Neostigmine (1 \(\mu\)M) increased slow-wave frequency in strain-matched control muscles: from 5.1 ± 1.2 min\(^{-1}\) in control to 7.2 ± 1.0 min\(^{-1}\) (\(P = 0.042, \text{ANOVA}, n = 8\); Fig. 7, A and C). In contrast, neostigmine failed to produce a significant increase in the basal frequency of slow waves in \(W/W^v\) muscles: from 6.5 ± 0.8 min\(^{-1}\) in control to 7.7 ± 1.3 min\(^{-1}\) during EFS (\(P > 0.05\) by ANOVA, \(n = 5\); Fig. 7, B and C). Membrane potentials depolarized in strain-matched control and \(W/W^v\) muscles in response to neostigmine, but there was no effect on the amplitudes or durations of the slow waves. The depolarizations in \(W/W^v\) muscles and strain-matched control
muscles and changes in slow-wave frequency in strain-matched control muscles were abolished by 100 nM atropine (Fig. 7, A and B).

**DISCUSSION**

In the present study, we sought to determine whether the chronotropic effects of cholinergic stimulation of antral muscles observed with exogenous ACh occur during sustained release of ACh from enteric motor neurons. Our data provide further evidence for the important role of ICC-IM in the regulation of gastric motility by enteric neurons (3, 4, 18, 42). We found that electrical responses to ACh released from enteric motor neurons are poorly simulated by addition of exogenous ACh (24) and CCh to solutions bathing gastric muscles. We made the novel observation that metabolism of ACh released from enteric motor neurons through the action of AChE not only restricts cholinergic signals to neuro-ICC-IM synapses (3, 42) but also participates in the regulation of intrinsic slow-wave frequency and contributes to maintenance of the proximal-to-distal frequency gradient in murine gastric muscles. Finally, our data suggest that ICC-IM become the dominant pacemaker cells in murine antral muscles during sustained cholinergic drive.

We found that repetitive stimulation of enteric motor neurons at ≥5 Hz caused a significant increase in the frequency of slow waves in wild-type murine gastric antral muscles. The chronotropic effects elicited by field stimulation with the parameters chosen were due to activation of enteric neurons, inasmuch as the responses were inhibited by TTX. The predominant effects were cholinergic in nature and totally blocked by atropine and, more specifically, by 4-DAMP, suggesting that ACh M3 receptors mediate the chronotropic effects. The involvement of ACh M3 receptors in chronotropic effects was also the conclusion of a previous study in which the effects of cholinergic agonists were studied on pacemaker currents in cultured gastric ICC (24).

Pharmacological studies showed that production of inositol trisphosphate in response to ACh M3 receptor stimulation may mediate the chronotropic effects in ICC. Stimulation of antral muscles at higher frequencies recruited supplemental chronotropic effects via the release of NK. Atropine failed to completely restore control slow-wave frequency during 10-Hz stimulation, but addition of NK1- and NK2-receptor antagonists restored prestimulation slow-wave frequency. Our data are consistent with a scenario in which ACh is the primary transmitter responsible for chronotropic effects at low stimulus frequencies, but higher frequencies of nerve stimulation produce summed effects from the release of ACh and NK.

In Balb/c and wild-type strain-matched control mice (i.e., WBB6F1/J-Kit+/Kit+), muscarinic stimulation via exogenous agonists caused depolarization and increased slow-wave frequency (24). These responses are likely to have resulted from stimulation of multiple cell types in gastric muscles that express a variety of cholinergic receptors. Indeed, the integrated response of antral muscles to exogenous muscarinic agonists could even have been due to release of additional neurotransmitters through the stimulation of enteric neurons via nicotinic and muscarinic receptors (17). Data in the present study show
that the chronotropic effects of muscarinic stimulation are similar whether ACh is released from enteric motor neurons or added to the bath. However, the effects of exogenous ACh or CCh on resting membrane potential may be primarily the result of stimulation of muscarinic receptors on smooth muscle cells or ICC-MY (24). ACh (24) and CCh (this study) depolarized membrane potentials of antral muscles, but this effect was not mimicked by neurally released ACh. Neostigmine also caused depolarization of wild-type and mutant muscles, but it affected the frequency of the slow waves only in antral muscles with ICC-IM. Thus smooth muscle cells do not appear to be accessible to ACh released from motor neurons unless the metabolism of ACh is inhibited (42). Our data strongly suggest that the signaling pathway(s) promoting chronotropic effects in antral muscles is localized in ICC-IM, because muscles of W/Wv mice lacked chronotropic responses even to exogenous CCh.

Neostigmine, an AChE inhibitor, prevents the metabolic deactivation of ACh released by motor neurons (33). In the murine antrum, neostigmine increased antral slow-wave frequency, and this effect was blocked by atropine. These data suggest ongoing release of ACh in the absence of applied nerve stimulation, but the metabolic capacity of gastric muscles in the immediate vicinity of the nerve terminals prevents spontaneous neural activity from affecting pacemaker activity. TTX blocked the effects of neostigmine, suggesting that spontaneous activity of enteric motor neurons and action potential invasion of nerve terminals are responsible for the release of ACh under basal conditions. Spontaneous activity of enteric motor neurons has been demonstrated previously (35, 36). The chronotropic effects of muscarinic stimulation might negatively impact the proximal-to-distal pacemaker frequency gradient in the stomach. Thus ACh metabolism may be an important mechanism for preserving the pacemaker frequency gradient and controlling pre- and postprandial gastric responses to facilitate gastric peristalsis. For example, if AChE expression or activity is reduced, higher-than-normal antral frequency responses during the gastric phase of digestion could lead to disruption in the corpus-to-antrum frequency gradient, cause functional uncoupling between corpus and antral pacemakers, and inhibit the normal spread of gastric peristaltic contractions. This sequence of events could adversely affect gastric emptying. Demonstration of the importance of ACh metabolism in the regulation of antral slow-wave frequency may provide a novel hypothesis for investigations into the causes of postprandial gastric dysrhythmias in human patients. It is interesting to note that treatment of myasthenia gravis with AChE inhibitors often results in nausea (12), which is possibly a manifestation of gastric dysrhythmias.

W/Wv mice carry mutations that decrease the tyrosine kinase activity of Kit receptors (6). Because Kit receptor function is required for normal development and maintenance of the ICC phenotype in GI muscles, populations of ICC are reduced in
tissues of \textit{W/Wv} mice. ICC-IM are lost from the stomachs of these animals \cite{7}, but ICC-MY are present in normal numbers along the greater curvature (site of our recordings) in the corpus and antrum \cite{30}. The presence of ICC-MY preserves the ability to generate electrical rhythmicity and provides a pathway for propagation of slow waves in stomachs of \textit{W/Wv} mice, but loss of ICC-IM greatly reduces responses to stimulation of inhibitory (nitrergic) and excitatory (cholinergic) motor neurons \cite{4, 7, 38, 42}. In stomachs of wild-type mice, stimulation of cholinergic nerves can elicit premature slow waves (i.e., phase advance the normal cycle), but the ability to stimulate premature slow waves is lost in antrums of \textit{W/Wv} mice \cite{4}. Data from the present study support the concept that ICC-IM mediate neural regulation due to cholinergic neurotransmission and supplement previous findings by showing a positive chronotropic effect of sustained cholinergic stimulation. We also found that higher frequencies of nerve stimulation supplement muscarinic chronotropic effects by recruitment of neurons that release NK. NK persist in eliciting chronotropic effects in the absence of ICC-IM. The site of slow-wave regulation by NK in \textit{W/Wv} antral muscles may be ICC-MY, because ICC-IM are missing.

ICC-IM generate random spontaneous electrical transients, referred to as unitary potentials (see Ref. 13 for quantitative description of this activity). Evidence suggesting that ICC-IM are responsible for unitary potentials comes from the observation that muscles lacking ICC-IM fail to generate these events \cite{2, 7}. Unitary potentials are random depolarizations that are likely to result from the discharge of transient inward currents in ICC-IM, which are scattered throughout bundles of antral muscles \cite{7, 13}. It is possible to enhance the probability of unitary potential discharge by stimuli such as depolarization, anode break (cessation of hyperpolarizing pulses), and neural stimulation \cite{13, 18}. The summation of unitary potentials in response to these stimuli has been called a “regenerative potential,” and these events are thought to be a means by which ICC-IM amplify pacemaker potentials initiated by ICC-MY in gastric muscles \cite{10, 13, 37}. Data from the present study suggest a more fundamental role for ICC-IM and unitary potentials during periods of high cholinergic stimulation in antral muscles. Our findings suggest that, even during continuous cholinergic stimulation, ICC-MY are not directly innervated or influenced by ACh released from motor neurons. During periods of high cholinergic drive, however, cholinergic stimulation of \textit{ACh M3} receptors on ICC-IM causes these cells to become rhythmic \cite{41} and to generate regular regenerative potentials at a rate higher than the intrinsic ICC-MY frequency. Thus, during sustained cholinergic stimulation, as might occur during the gastric phase of digestion, ICC-IM become the dominant pacemaker in antral muscles, and through this mechanism, enteric motor neurons accelerate the frequency of slow waves.

In summary, our data support the concept that ACh released from enteric neurons regulates the frequency of intrinsic pacemaker activity in antral muscles. This aspect of cholinergic regulation is also manifest when antral muscles are exposed to exogenous ACh, possibly through stimulation of receptors expressed by ICC-IM. Cholinergic regulation of slow-wave frequency occurs primarily via the innervation of ICC-IM. Sustained cholinergic stimulation of ICC-IM causes these cells to emerge as the dominant pacemaker cells in antral tissues. Our data suggest that basal release of ACh occurs in antral...
GRANTS

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REFERENCES