Induction of rhythmic transient depolarizations associated with waxing and waning of slow wave activity in intestinal smooth muscle

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Pawelka AJ, Huizinga JD. Induction of rhythmic transient depolarizations associated with waxing and waning of slow wave activity in intestinal smooth muscle. Am J Physiol Gastrointest Liver Physiol 308: G427–G433, 2015. First published December 24, 2014; doi:10.1152/ajpgi.00409.2014.—Cannon described in 1902 the segmentation motor activity of the small intestine (Canon WB. J Med Res 7: 72–75, 1902). This motor pattern can arise when low-frequency transient depolarizations are evoked in the interstitial cells of Cajal associated with the deep muscular plexus (ICC-DMP) network, which then affect the omnipresent slow wave activity: changing its regular amplitude into a waxing and waning pattern. The objective of the present study was to investigate physiological stimuli that could induce the low-frequency component. Intracellular recordings were obtained from circular muscle with or without attached mucosa. Decanoic acid (1 mM) and butyric acid (10 mM) both evoked low-frequency transient depolarizations but through different mechanisms. Decanoic acid-induced waxing and waning was initiated by purely myogenic means when perfused onto exposed circular muscle. Butyric acid required the intact mucosa and uninhibited neural activity to elicit the low-frequency response. Evidence is provided that the transient rhythmic depolarizations occur in the absence of interstitial cells of Cajal associated with the myenteric plexus (ICC-MP). Onset of the slow transient depolarizations was stimulated by addition of Nω-nitro-L-arginine (L-NNA; 100 μM); thus the low-frequency component seems to be under chronic inhibition by nitric oxide. Excitatory tachykinergic stimulation induced the low-frequency component since substance P (0.5 μM) evoked it in the presence of neural blockade. In summary, interplay between two networks of myogenic pacemakers, neural activity, and nutrient factors such as fatty acids plays a role in the generation of the rhythmic low-frequency component that is essential for the development of the checked segmentation motor pattern.

interstitial cells of Cajal; pacemaking; intestinal motility; segmentation

AFTER A NUTRITIOUS MEAL, the predominant mechanical activity exhibited by the small intestine is the segmentation motor pattern, which consists of stationary circular muscle contractions dividing segments of the small intestine into rhythmic transient bead-like compartments (2). This motor pattern allows for optimal mixing of luminal content and exposure to digestive enzymes and to the villi for optimal absorption. In humans and rat models, infusion of fatty acids in the small digestive enzymes and to the villi for optimal absorption. In

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Tissue preparation. All animal procedures were approved by the Animal Research Ethics Board at McMaster University in accordance with the standards set by the Canadian Council on Animal Care. Female adult CD-1 mice (Charles River Laboratories, Wilmington, MA; ~14 wk of age) were euthanized by cervical dislocation. Small segments of the jejunum (1–2 cm) were prepared for intracellular recording. The mesenteric fat was carefully cut off with fine point scissors, and the segment was subsequently opened along the length of the mesenteric border and pinned flat on a Sylgard gel dish, mucosa facing upwards. The mucosa and submucosa were removed, and the electrode was positioned at the submucosal edge of the circular muscle layer. Penetration of a superficial smooth muscle cell was facilitated by injection of small short-lasting (5 ms) currents. A hemisected preparation (12) was utilized to determine how reagents added to the mucosa influence circular muscle electrical activity. The microelectrode required for intracellular recordings cannot penetrate the mucosa and submucosa layers to reach the muscle because the tip of the electrode will get plugged or brake, so a “hemissection” was necessary. A partitioned chamber was designed as described previously (12). In brief, a microscope mountable dish was separated into two halves by a plastic partition with a single 1-cm
wide gap at the center to allow the tissue sample to be pinned through. Two-centimeter segments of the jejunum were centered in the dish. The mucosa and submucosa were left intact on one side of the partition and excised on the other half. The seal was tested by the use of colored dye. Tissue was continuously perfused with oxygenated Krebs solution and maintained at room temperature until experimentation; then, the solution temperature was increased to 37°C for a 1-h equilibration period. To abolish contractile activity of the smooth muscle to enable cellular impalements, 0.5 μM of nicardipine were added to the Krebs solution.

**Experimental technique.** Electrical activity from individual circular smooth muscle cells was recorded by impalement with borosilicate glass micropipettes (fire polished; length: 7 cm; outer diameter: 1.5 mm; inner diameter: 0.86 mm) filled with 3 M KCl and fabricated to yield resistance between 30 and 70 MΩ. Signals were recorded using a MultiClamp 700B amplifier (Axon Instruments; Molecular Devices, Sunnyvale, CA). Signals were digitized at an acquisition rate of 2 kHz using a Digidata 1322A acquisition system (Axon Instruments). The zero current mode ($I_{0}$) of the current clamp of the Axon amplifier was used to record membrane potentials of circular smooth muscle cells near the level of the deep muscular plexus. The electrode was positioned at the edge of the circular muscle, and care was taken to only record from the most superficial cells.

**Solutions, drugs, and reagents.** Tissue preparations were kept in oxygenated (95% O$_2$-5% CO$_2$) Krebs solution (118.1 mM NaCl, 1.0 mM NaH$_2$PO$_4$, 1.2 mM MgSO$_4$, 2.5 mM CaCl$_2$, 4.8 mM KCl, 11.1 mM glucose, and 25 mM NaHCO$_3$, pH 7.35). All drugs and reagents were dissolved in deionized water except for decanoic acid in dimethyl sulfoxide (DMSO), Nω-nitro-L-arginine (L-NNA) in 50 μM HCl$_{aq}$, and nicardipine in 50% ethanol. DMSO never exceeded 0.1% total volume during experimentation. Drugs were dissolved daily as required except nicardipine and TTX, which were both stored at 20°C.

**Data analysis and statistics.** For continuous wavelet transformation (CWT) analysis, ~4 min per sample of continuous uninterrupted intracellular recordings were reduced to a sampling rate of 2 kHz and imported into Matlab (MathWorks, Natick, MA). The voltage time series were analyzed by the software to produce a frequency spectrum and a two-dimensional frequency over time contour plot. For continuous wavelet transform (CWT) analysis of control slow wave activity oscillated between 8.4 ± 1.5 min and a low-frequency component of 2.6 ± 0.2 Hz/min. With the use of hemidissected preparations, decanoic acid (1 mM) applied to the mucosa did not induce a waxing and waning pattern ($n = 8$; Fig. 3A) and no low-frequency com-
ponent was detected by CWT. Reagents did not cross the barrier or come in contact with the exposed circular muscle being recorded from, tested using 50 mM methylene blue dye. In three experiments, when decanoic acid applied to the mucosa did not produce a response within 40 min, the reagent was then perfused onto the circular muscle half of the chamber as a positive control. This resulted in a visible waxing and waning pattern in all preparations studied.

Fig. 2. Frequency components isolated from recordings of a standard slow wave and the waxing and waning phenomenon by continuous wavelet transformation (CWT) analysis. A and D: absolute membrane potential (AMP): time parameters of intracellular electrical activity recorded from circular muscle expressing slow wave (A) and waxing and waning (D) activity. B and E: power spectrum-frequency distribution of source depicts strength of frequency components. C and F: time-frequency contour plot of mean power displays the strength of the oscillatory components in each recording through time. In this and all subsequent figures, the highest strength is denoted in white, followed by yellow, red, and black.

Fig. 3. Frequency components isolated from intracellular recordings postaddition of decanoic acid or butyric acid to the mucosa by CWT analysis. A: AMP: time recording of circular muscle electrical activity after addition of 1 mM decanoic acid to mucosa-intact half of partitioned chamber. Recording was obtained 10 min after addition of decanoic acid. D: voltage-time parameters of circular muscle activity after addition of 10 mM butyric acid to mucosa-intact half of partitioned chamber. B and E: power spectrum-frequency distribution of source depicts strength of frequency components relative to the power of the high-frequency component. C and F: time-frequency contour plot of mean power displays the strength of the oscillatory components in each recording through time.
To determine whether or not the low-frequency component was generated independently of the dominant high-frequency slow wave, slow wave activity from the ICC-MP networks needed to be abolished. To achieve this, circular muscle tissue preparations exhibiting slow wave activity were incubated with methylene blue, washed, and subsequently exposed to intense fiber optics illumination (17). There was no change to any slow wave parameter during the control period, methylene blue incubation, and methylene blue washout. Once the slow wave activity was completely inhibited by illumination (Fig. 4A), 1 mM decanoic acid (Fig. 4D) was added. In four out of five experiments, the low-frequency component (2.8 ± 0.5 counts/min; n = 4) was resolved and filtering of the raw data revealed the low-frequency component in the voltage time domain (Fig. 4D, inset). There was no marked effect of the methylene blue procedure on the resting membrane potential; a potential minor effect was difficult to determine since recording from the same smooth muscle cell for the duration of an experiment (before and after methylene blue) was not always possible.

Addition of 10 mM butyric acid to circular muscle preparations did not induce the waxing and waning phenomenon; however, it did have an effect on the slow wave exhibited by smooth muscle cells. The short chain fatty acid decreased the duration of the slow wave (n = 5; P < 0.05; from 1.74 ± 0.16 to 1.55 ± 0.11 s). There was no change to the frequency or amplitude parameters. In the presence of butyrate, the resting membrane potential was −49.6 ± 2.0 mV and the slow waves plateaus occurred at −32.8 ± 1.5 mV membrane potential.

Butyric acid (10 mM), perfused to the mucosa, induced an obvious change from regular slow wave activity to the waxing and waning pattern in five out of eight experiments (Fig. 3D). The time required for the change in pattern varied among preparations (n = 5, 19.9 ± 3.2 min). CWT analysis determined that perfusion of butyric acid induced a low-frequency component in all preparations studied (n = 8; Fig. 3F). The resting membrane potential was −56.3 ± 2.4 mV, the minimum and maximum at the point of maximal waning were −50.7 ± 2.0 and −42.3 ± 2.5 mV, and the plateaus of the most depolarized slow waves were at −36.7 ± 3.4 mV.

To determine whether the low-frequency component was under nitrergic inhibition, 200 μM L-NNa was perfused onto circular muscle, which resulted in waxing and waning from a steady slow wave in four out of five experiments (Fig. 5A). The time required to establish this pattern was 32.2 ± 3.3 min (n = 4). CWT analysis revealed that the addition of L-NNa resulted in the onset of the low-frequency component in all five experiments (Fig. 5C).

To investigate whether excitatory neurons could activate the slow component, the effect of substance P was studied, given the dense presence of neurokinin 1 (NK1) receptors on ICC-DMP (26). The experiments were conducted in the presence of nerve conduction blockade by lidocaine so that potential effects of substance P could be attributed to actions on the myogenic system. Lidocaine (100 μM) did not induce low-frequency activity after 40 min of perfusion (n = 3). Substance P (0.5 μM), added to the inflow reservoir after tissue had been exposed to lidocaine for a duration of 10 min, induced the onset of the low-frequency component (n = 4; mean 3.6 ± 0.6 counts/min) and induced the waxing and waning pattern (Fig. 5D). The time required to induce the waxing and waning was 22.2 ± 3.0 min. Substance P induced waxing and waning with a resting membrane potential of −56.4 ± 4.1 mV, and the
point of maximal waning had a low membrane potential of 
\(-50.6 \pm 3.5\) mV and a high of 
\(-42.4 \pm 4.1\) mV. The maximum value of the slow wave plateaus reached 
\(-38.6 \pm 3.7\) mV. In four other animals, substance P did not induce 
waxing and waning with a resting membrane potential of 
\(-55.6 \pm 3.2\) mV (\(N = 15\); \(n = 4\)) and the slow wave plateaus 
reached to \(-39.7 \pm 2.4\) mV.

**DISCUSSION**

The present study shows that the pattern of rhythmic 
waxing and waning of the amplitude of slow wave-driven 
contractions that is associated with segmentation (11) incor-
porates transient rhythmic depolarizations that can be 
evoked by mucosal stimulation as well as direct myogenic 
stimulation. The present study also provides evidence that 
the transient rhythmic depolarizations are inhibited by nitric 
oxide and can be evoked by the excitatory neurotransmitter 
substance P. Furthermore, evidence is provided that the 
ICC-MP are not the origin of the rhythmic transient depo-
larizations, indirectly supporting the hypothesis that this 
activity is generated by ICC-DMP.

**Induction of rhythmic transient depolarizations by mucosal 
or myogenic stimulation.** When 1 mM decanoic acid was 
perfused onto exposed circular muscle preparations, without 
the mucosa, a low-frequency component emerged and a rhythm-
ic waxing and waning pattern developed from a steady slow 
wave. Butyric acid (10 mM) on the same preparation did not 
evoke the low-frequency component nor induce waxing and 
waning. In contrast, when decanoic acid was perfused onto the 
mucosa no response was elicited. Exposure of the mucosa to 
butyric acid always induced the low-frequency component and 
evoked a visible waxing and waning pattern.

Butyric acid, a common component of cellular and luminal 
bacterial metabolism, is transported into enterocytes via non-
ionic diffusion (SCFA/HCO₃ exchanger) by a monocarboxy-
late transporter (MCT type 1) or through sodium coupled 
monocarboxylate transporters (SMCT or SLC5A8/12) (9). 
Kunze et al. (12) observed that a spritz of 5 mM butyrate on the 
mucosa in the rat colon induced a nonsynaptic burst of action 
potentials in primary afferent sensory (AH) neurons. Hence, 
AH neurons may play a role in stimulating the low-frequency 
component in response to butyric acid. Although it has been 
shown that AH neurons have functional communication with 
ICC-MP (30), there is no evidence yet that AH neurons are in 
direct contact with ICC-DMP.

Decanoic acid may induce the low-frequency component in 
ICC-DMP by stimulating phospholipase C (PLC) activity, 
similar to PLC activation being responsible for slow wave 
generation in ICC-MP (18, 28) since decanoic acid was shown 
to modulate paracellular permeability by increasing intracel-
ular calcium levels through activation of PLC (13, 14). This is 
consistent with recent calcium imaging data from our labora-
tory (11) showing that addition of 1 mM decanoic acid evoked 
a low-frequency oscillating pattern of calcium activity in ICC-
DMP. In vivo, medium chain fatty acids can permeate through 
a paracellular route in between epithelial cells (3); hence, 
decanoic acid will have access to the blood stream from which 
it can act on ICC and nerves.

Previously, we provided evidence that the waxing and waning 
pattern was due to interaction between the phase of the
rhythmic transient depolarizations and the amplitude of the slow wave activity (11): the phenomenon of phase amplitude coupling, as occurs between low- and high-frequency electrical activities in the brain (4). An alternative explanation is that the depolarization provided by the ICC-DMP reduces the amplitude of the slow waves transiently and rhythmically (20). Could the reduction in slow wave amplitude, the waning part of waxing and waning, simply be due to depolarization without the need for phase amplitude coupling? There is extensive literature on the effect of depolarization on slow wave activity (15, 19, 20, 25). Depolarization leads to a reduction in slow wave amplitude, with a slight further depolarization or no effect on the plateau phase of the slow wave. The plateau phase of the slow wave does not hyperpolarize upon depolarization. The assumption is that the average reversal potential of the ion channels that contribute to the plateau phase of the slow wave determines the plateau potential and that does not change much by depolarization. Depolarization experiments do not show the waxing and waning phenomenon as shown here in Figs. 2D, 3D, and 5D. The most depolarized state at maximal waning is strongly hyperpolarized compared with the maximal slow wave plateau state. Hence, the waxing and waning phenomenon is likely due to a combination of phase amplitude coupling (11) and depolarization (20).

Neural regulation of rhythmic transient depolarizations. Circular smooth muscle cells responded to the addition of 200 μM l-NNa with the induction of the waxing and waning pattern. Inhibition of neuronal nitric oxide (NO) synthase prevents the production of nitric oxide. ICC-DMP express the intracellular receptor soluble guanylyl cyclase and can respond to NO stimulation (27). l-NNa can inhibit both neural nigeric stimulation as well as intracellular ICC production of nitric oxide. Similar to the low-frequency oscillations of the rat colon in ICC-MP (22), activity of the ICC-DMP in the mouse small intestine may be under tonic inhibition by NO. This result is not likely mediated by depolarization since neither l-NNa nor TTX or lidocaine had a significant effect on the resting membrane potential. It is possible therefore that the low-frequency component is inhibited by cGMP acting on ion channels in ICC other than the K channels responsible for the resting membrane potential.

Substance P (0.5 μM) induced the low-frequency component and produced the waxing and waning phenomenon in the presence of TTX. It is therefore likely that this occurs by direct stimulation of the NK1 receptors on ICC-DMP (8, 26).

Cellular origin of the transient rhythmic depolarizations. ICC-DMP have been suggested as the origin of the rhythmic transient depolarizations, based on the fact that these cells show rhythmic calcium transients in the same frequency range (11). To exclude ICC-MP as a possible source, methylene blue was used to abolish the high-frequency slow wave in a short time frame (24); we provided the control experiments and proof of ICC-MP ablation in earlier experiments (16, 17). There were no significant changes to any slow wave parameter during the addition of methylene blue in dark surroundings or the subsequent wash. Methylene blue exposure leads to dye accumulation in ICC-MP but not ICC-DMP (24). Subsequent exposure to light abolished slow wave activity, as shown previously (16, 17, 24). Once the slow wave was inhibited, no significant high- or low-frequency oscillatory activity was picked up with CWT analysis. Addition of decanoic acid produced a low-frequency component indicating that the ICC-MP network did not produce it.

Comparison with other theories on the origin of the waxing and waning pattern. An in vivo study by Ailiani et al. (1) on rat small intestine activity using MRI of whole jejunum found that both peristalsis and segmentation occurred at ~0.45 Hz, the slow wave frequency. Fast Fourier transform (FFT) analysis of peristalsis revealed one dominant high frequency while segmentation revealed two dominant high-frequency components. In both cases, low-frequency components found were dismissed as artifacts of slow changes in the position of the gastrointestinal tract. The present study suggests that the low-frequency components have physiological significance. Interestingly, these FFT results are very similar to those obtained by Suzuki et al. (23) studying waxing and waning in the rabbit intestine in vitro. Suzuki noted three conditions favoring waxing and waning: slight depolarization, variation in slow wave frequency at a point, and electrotonic coupling between muscle fibers. Diamant and Bortoff (6) originally presented the hypothesis that waxing and waning resulted from the interaction of two high-frequency pacemakers of slightly different frequencies. This, indeed, occurs at the plateau boundaries of a system of coupled oscillators exhibiting a frequency gradient (21).

It is important to note that FFT analysis of nonstationary signals can be problematic. FFT assumes the signal analyzed to be stationary; the algorithms provide power density spectrum information of only the frequency domain, thus time domain information is lost. In the present study FFT analysis often displayed two or more high-frequency peaks (not shown). However, CWT analysis did not show this; hence, sharp peaks of similar frequencies obtained by FFT analysis can be an artifact due to the fact that FFT does not take into account variation of frequencies over time.

In summary, the present study provides evidence that a myogenic mechanism orchestrates the waxing and waning pattern of slow wave activity and that it can be abolished or evoked by neural stimulation. Our data are, therefore, not in contradiction with the experimental data from Gwynne and Bornstein (10). The switch from peristalsis to segmentation occurs via the induction of rhythmic transient depolarizations. The present study shows that this phenomenon, essential for absorption of nutrients, can be evoked in multiple ways and is under both inhibitory and excitatory neural control.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: A.J.P. and J.D.H. conception and design of research; A.J.P. performed experiments; A.J.P. analyzed data; A.J.P. and J.D.H. interpreted results of experiments; A.J.P. prepared figures; A.J.P. drafted manu-
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