Effects of gap junction inhibition on contraction waves in the murine small intestine in relation to coupled oscillator theory

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ABSTRACT

Waves of contraction in the small intestine correlate with slow waves generated by the myenteric network of interstitial cells of Cajal (ICC). Coupled oscillator theory has been used to explain step-like gradients in the frequency (frequency plateaux) of contraction waves along the length of the small intestine. Inhibition of gap junction coupling between oscillators should lead to predictable effects on these plateaux and the wave dislocation (wave drop) phenomena associated with their boundaries. It is these predictions that we wished to test. We used a novel multi-camera diameter mapping system to measure contraction along 25-30 cm lengths of the murine small intestine. There were typically 2-3 plateaux per length of intestine. Dislocations could be limited to the wave fronts immediately about the terminated wave, giving the appearance of a three pronged fork - a fork dislocation; additionally, localised decreases in velocity developed across a number of wave fronts, ending with the terminated wave which could appear as a fork - slip dislocations. The gap junction inhibitor carbenoxolone increased the number of plateaux and dislocations and decreased contraction wave velocity. In some cases there was a reversal of the usual frequency gradient, with a plateau at a higher frequency than its proximal neighbour, and thus fork dislocations were inverted and the direction of propagation reversed. Heptanol had no effect on the frequency or velocity of contractions, but did reduce their amplitude. To understand intestinal motor patterns, the ICC pacemaker network is best evaluated as a system of coupled oscillators.

KEYWORDS
Small intestine; gap junction; coupled oscillator; frequency plateau; dislocation.
INTRODUCTION

In the small intestine waves of contraction travel distally from the gastroduodenal junction to the terminal ileum at a regular frequency of 8-12 per minute in humans (17) and 30-40 per minute in mice (39). This rhythm originates with the interstitial cells of Cajal (ICC) of the myenteric plexus which form a continuous network along the whole length of the small intestine (39, 72, 75). ICC generate electrical "slow" waves that in turn drive contraction of the muscle. Electrical slow waves, and thus contraction waves, can be explained by two mechanisms - phase waves or trigger waves (10, 61). With trigger waves there is a single oscillator (pacemaker ICC) and waves spread from this point by cycles of excitation and refraction. With phase waves every ICC oscillates by itself, but the phase and frequency of this oscillation can be influenced by neighbours, as described by the phase response curve and entrainment respectively. The result of this interaction (coupling) between oscillators is that waves can appear to propagate, though this only represents a coordinated phase difference between oscillators.

Coupled oscillator theory (phase waves) has been used to explain a number of phenomena related to contraction waves in the small intestine. As early as the mid nineteenth century it was noticed that the frequency of contractions is higher in the duodenum than the ileum (3, 4). Diamant and Bortoff showed that the gradient was usually step-like with plateaux in frequency separated by sharp boundaries (19, 20). They, together with Nelsen and Becker, suggested that frequency plateaux could be explained by frequency entrainment (19, 20, 54). There would be a population of coupled oscillators along the length of the gut (since shown to be the network of ICC) with a gradient in their natural frequency, the frequency at which each oscillates in isolation. Plateaux result from frequency entrainment, whereby one oscillator pulls up to its own natural frequency (or higher) a series of distal oscillators of lower natural frequency. Plateau boundaries occur where the difference in frequencies is too great for pulling. At these boundaries waves must be lost (dropped) - if neighboring plateaux have contraction intervals of 1.4 and 1.3 seconds then every fourteenth wave must be dropped. The end of the dropped wave is known alternately as a dislocation (56), terminology borrowed from crystallography, or phase singularity (76) because phase at the endpoint is indeterminate. Mathematical models of chains of coupled oscillators have shown dislocations to have a forked shape (37, 53, 55). Also such models predict a waxing and waning in the amplitude of the contraction wave at the plateau boundary (7, 53, 55). Both fork dislocations and waxing-waning occur at frequency plateau boundaries in the small intestine (9, 20, 44). They also occur in physical wave phenomena where there is a gradient in frequency, notably vortex sheets shed from tapered rods (7, 30, 55, 74) and plasma waves (50, 53).

As the coupling between oscillators decreases in strength, the velocity of phase waves is predicted to decrease as the phase lag between oscillators increases (21). Also plateaux should become shorter and more numerous as the reduction in coupling reduces the ability for entrainment (73). Investigated by electron...
microscopy, the ICC-MP network appears to have infrequent gap junctions (63) and connexin 43 immunohistochemistry does not reveal a significant presence (16). Nevertheless, dye coupling experiments have left little doubt that every ICC-MP is a pacemaker cell coupled by gap junctions to its neighbors (57). Gap junction inhibitors have been shown to disturb calcium wave propagation between ICC (57) but there is no data on their effect on frequency plateaux and other phase wave related phenomena in the small intestine. We wished to address this by investigating the effects of gap junction blockers on contraction wave propagation in the isolated, ex vivo whole murine small intestine.
METHODS

Intestine Preparation

All procedures were approved and carried out in accordance with regulations from the Animal Ethics Board of McMaster University. Small intestines of 14 week old CD1 mice were prepared in chilled and oxygenated Krebs containing (mM): 120 NaCl, 3 KCl, 15.5 NaHCO3, 1.2 NaH2PO4, 0.1 citric acid, 0.1 aspartic acid and 1.2 MgCl2. All the mesentery was cut away and a "cannula head" was inserted into the lumen at the gastro-duodenal junction. The cannula head consisted of a 12 mm long metal tube (fashioned from a 16 gauge needle) terminated by a plastic collar (Fig. 1 C, right). The intestine was slipped over the collar and tied just behind it, the collar preventing the intestine from slipping off. The intestine was cut to 25-30 cm length distal from the gastro-duodenal junction. Typically this would remove 5-10 cm of terminal ileum. The cannula head was connected to a reservoir of saline (135 NaCl, 5 KCl) and the intestinal contents were flushed out. It was essential to keep the level of the reservoir at most 10 cm above the intestine. The exertion of larger pressures prevented strong circular contractions from developing during the experiment. After flushing, the ileal end of the intestine was also connected to a cannula head.

Organ Bath and Multi-camera Rig

The organ bath consisted of a plastic wallpaper tray (54 x 12 cm; Fig. 1 B). Velcroed to the bottom of this was a manifold made of metal gutter covering. This manifold held a U-bend of 9 mm bore Tygon tubing connected to a water circulator, for heating the bath. Parallel to this ran a length of PE-190 tubing, perforated and closed at one end, for oxygenation. The bath was filled with 1.5 L of Krebs, containing (mM): 120 NaCl, 5.9 KCl, 15.5 NaHCO3, 1.2 NaH2PO4, 0.1 citric acid, 0.1 aspartic acid, 2.5 CaCl2, 1.2 MgCl2 and 6 glucose. Inside the U-bend sat a "kit-kat", a plastic bar 58 x 5 cm with five lengthwise square grooves or lanes, 5 mm wide and deep (Fig. 1 B and D). Each lane of the kit-kat held a single intestine. The intestine was held in place by inserting its cannula heads (one on either end) into a cannula receiver which squeeze-fitted into the lane (Fig. 1 C and D). The cannula receiver had either a tube to a saline reservoir (gastro-duodenal end) or an open ended tube above the level of the bath (ileal end).

Two brackets, one at either end of the bath, held two lengths of clear plastic that partitioned the solution surface at either side of the kit-kat (bubble covers; Fig. 1 B). This prevented surface bubbles (from the U-bend oxygenation tube) from passing over the field of the kit-kat. It was found that proper circulation of the bath solution was essential for the long term viability of intestines. Two aquarium air pumps (AW-20 aqualifter, Tom Aquarium Products, Shawnee, KS) were used with their outflow attached to the bracket at one end and their
inflow attached to the manifold, midway along the bath (Fig. 1 B). LED light wands at each end of the bath were used for shallow angle lighting. Illumination from above gave reflections and from the sides cast shadows in the lanes of the kit-kat, interfering with spatio-temporal mapping.

Miniature CCD board cameras (1/3" SONY Super HAD CCD; 700 TVL; SONY Effio-E DSP), with 50 mm focal length lens (F2.0; AOV 9°) were purchased from Security Camera 2000 (Hong Kong). A row of ten cameras were mounted 65 cm above the organ bath by means of a horizontal L-bar supported by two clamp stands (Fig. 1 A). At the mounted height, the field of each camera was 38 x 52 mm (480 x 960 pixels; 0.079 x 0.054 mm/pixel) so that arranged in a row with slightly overlapping fields the total field was 36 x 5.2 cm. Note that the higher resolution orthogonal to the row, did not result from barrel distortion in the lens but from the format of the camera's analogue (TV) signal. The high resolution across the width of the intestine (54 μm/pixel) is not excessive with small diameter intestines from small animals - the murine contractions were at most 0.6 mm in amplitude (see Results). Camera signals were recorded by a sixteen channel digital video recorder at 30 frames per second (Vonnic K4116HMF, Markham, Ontario).

All experiments were carried out at 36 °C and in the presence of 0.5 mM lidocaine and 9 μM indomethacin. Intestinal intraluminal pressure was approximately 2.5 cmH₂O (1.87 mmHg; 250 Pa), as gauged by the height of the cannula reservoir solution surface above the height of the organ bath solution surface. As the organ bath was a closed system (unperfused), drugs had to be added directly as stock solutions or weakly diluted from these. When 18β-glycyrrhetic acid in an ethanol stock was added to the bath it immediately came out of solution. Carbenoxolone and 1-heptanol were both prepared as 1 mL stock solutions at x 1500 the maximum concentration required (40 μM and 2 mM respectively). Carbenoxolone was dissolved in 1 mL ethanol and 425 μL 1-heptanol was added to 375 μL ethanol. The 1 mL stock solution was then added to the bath (1.5 L volume) piecewise (1/8, 1/8, 1/4, 1/2) to reach the maximum concentration.

**Mapping and Analysis**

Calculation of diameter maps (Dmaps) and all other analysis was carried out with custom plugins written for ImageJ (NIH, Bethesda, MD). Dmaps were calculated by a thresholding algorithm. This was automated so that with two mouse clicks the movie output from one experiment (15+ hours of total recording from 10 cameras) could be converted into Dmaps overnight by the computer. Dmaps from neighboring cameras were stitched together after registering their border overlap by difference minimisation.

Dmaps are shown as images with time running horizontally and distance along the intestine running vertically from proximal (top) to distal (bottom). The intensity of the Dmap is the width ("diameter") of the intestine from black (contracted) to white (relaxed). Positive velocities are proximal to distal and negative velocities are distal to proximal.
The direction of features in an image - "local image orientation" - is an important subject in image analysis and computer vision. For instance in a Dmap such as Fig. 2 A we need the computer to measure the direction of contraction waves and thereby their velocity. Orientation is typically measured by calculating the derivatives of the image along the $x$ and $y$ axes. The orientation at any point in the image is equal to the arctangent of the ratio between $x$ and $y$ derivatives at that point. The derivatives can be calculated by convolution of the image with one-dimensional derivative kernels such as the Prewitt, Sobel and Savitsky-Golay (66). The image is convolved with the kernel and its transpose ($90^\circ$ orientated version) to calculate the $x$ and $y$ derivatives, respectively. The small size of one dimensional kernels makes them vulnerable to image noise without a pre-smoothing of the image or other noise removal technique. A recent solution has been the use of "steerable filters" (29). These are two dimensional derivative kernels and so are naturally less sensitive to noise - they sample more of the pixels around a point. A common steerable filter consists of the derivative of a 2D Gaussian (29). If the kernel/filter is represented as a matrix (square array of numbers), $G$, then the elements of the kernel ($g_{ij}$) are,

$$g_{ij} = \frac{1}{(2\pi\sigma^2)^{-1}} \exp\left[-\frac{(x^2 + y^2)}{2\sigma^2}\right]$$  \hspace{1cm} (eq.1)

$$x = i - 0.5n$$  \hspace{1cm} (eq.2)

$$y = j - 0.5n$$  \hspace{1cm} (eq.3)

where $n$ is the width of the kernel in pixels. $G$ has the appearance of a dumbbell, with one negative lobe and one positive (Fig. 2 A inset).

We wished to measure orientation specifically along lines of contraction - the minima or valleys in the Dmap (Fig. 4 A). Minima in a function are defined by where its derivative goes from negative (downward stroke) to positive (upward stroke). The Dmap was convolved with $G$ to give its time derivative. In matrix notation,

$$D_{dt} = D \ast G$$  \hspace{1cm} (eq.4)

where $D$ is the Dmap; $D_{dt}$ is its time derivative and $\ast$ is the convolution operator. Contraction lines were defined as where $D_{dt}$ went from negative to positive (green lines in Fig. 4 A and B).

The simplest way to calculate orientation in the Dmaps would have been to convolve the Dmap with $G$ and its transpose ($G^T$ in mathematical notation) to calculate the $x$ and $y$ derivatives. However along the lines of contraction the $x$ and $y$ derivatives tend towards zero (by their definition as minima) and so the orientation, as a
ratio of these values, diverges (tends to be very large or very small). To put it another way, if you are stood in the valley bottom you can't measure the slope of the surrounding hills. However in the time derivative of the Dmap (D_{dt}) the contraction lines lie right on the middle of the slope from negative to positive (Fig. 4 B). Therefore orientation was calculated by convolving D_{dt}, rather than D, with G. Where O is the orientation (Figure 4 C),

\[ O = \tan^{-1} \frac{D_{dt} \ast G^T}{D_{dt} \ast G} \]  

(eq.5)

G was 21 pixels on both sides (0.7 s by 1.13 mm) and \( \sigma^2 \) (the Gaussian's variance) was 12.25 pixels (0.4 s or 0.97 mm). It can be seen (Fig. 4 D) that the contraction lines (green lines) lie between divergences in orientation (intense blue and red colored bulges).

The orientation as defined above is orthogonal (at right angles) to actual direction of the contraction waves (blue arrows in Fig. 4 F and G, respectively). Therefore the direction of the contraction waves was found by the function,

\[ f(o_{ij}) = \begin{cases} 
  o_{ij} + \pi / 2 & , \quad o_{ij} < 0 \\
  o_{ij} - \pi / 2 & , \quad o_{ij} > 0 
\end{cases} \]  

(eq.6)

where \( o_{ij} \) is an element of O (Fig. 4 E). This was then used to calculate velocity, V,

\[ v_{ij} = \frac{y_r}{x_r} \tan \left[ f(o_{ij}) \right] \]  

(eq.7)

where \( y_r \) was the spatial resolution (0.079 mm/pixel) and \( x_r \) was the temporal resolution (0.033 s/pixel). The set of velocities occurring at contraction lines were found and their distribution calculated.

Given equations 4 to 7 it can be shown that,

\[ V = -\frac{y_r}{x_r} \frac{D_{dt} \ast G}{D_{dt} \ast G^T} \]  

(eq.8)
RESULTS

Propulsive circular muscle contractions propagated from the gastroduodenal junction to the ileum with occasional dropping of waves at dislocations (Fig. 3). The proximal four to five centimeters usually had a marked degree of tone, giving a dark band along the top of the Dmaps. There were typically 2-3 frequency plateaux (Fig. 4 A), although in some intestines there were none (Fig. 4 A middle). The average change in frequency at plateau boundaries was $0.18 \pm 0.03 \text{ min}^{-1}$ (change in interval of $102 \pm 18 \text{ ms}; n = 7$). There was no simple pattern to contraction velocities (Fig. 4). There were often local gradients in velocity (Fig. 4 A) but there was no overall proximal-distal trend in velocity (Fig. 4 B).

Plateau boundaries were correlated with phase dislocations. Dislocations were variable in appearance (Fig 5). In some dislocations distortion was limited to the wave fronts immediately about the terminated wave, giving the appearance of a three pronged fork - a fork dislocation (Fig. 5 A-C). In others a localised decrease in velocity developed across a number of wave fronts, ending with the terminated wave which could appear as a fork - these we call slip dislocations (Fig. 5 D-H). In fork dislocations the phase singularity was clearly visible as the centre of the fork. However in slips it was often not clear where the singularity should be defined because as the dislocation proceeded, wave fronts often coalesced (Fig. 5 E-G). Commonly this took the form of interdigititation between wave fronts, giving a sawtooth pattern (Fig. 5 E and F). Generally dislocations were of a fixed position. However on rare occasions slips could be seen to change position or glide (Fig. 5 H). Fork dislocations didn't always repeat, whereas slip dislocations were cyclical (Fig. 5 J). In temporal profiles of width at the slip dislocation/plateau boundary there was a waxing and waning in contraction amplitude at the same frequency as the dislocation (Fig. 5 J). There was also a waxing-waning of amplitude at fork dislocations (Fig. 5 I).

Glycyrrhetic acid (enoxolone) is one of the most commonly used gap junction inhibitors. It is however poorly water soluble which made it unsuitable for use in our organ bath (see Methods). Therefore we used carbenoxolone, a close analogue of glycyrrhetic acid, which in contrast is highly water soluble. The concentration of carbenoxolone was doubled every ten minutes over the range $5 \mu\text{M to } 40 \mu\text{M}$. Carbenoxolone broke contractions up into regions of oppositely directed wave fronts (Fig. 6 A). Positions of dislocations, corresponding to plateau boundaries, observed under control conditions were usually preserved with the addition of carbenoxolone (Fig. 6 B and E), but sometimes were not (lower boundary, Fig. 6 B). Carbenoxolone induced a large number of dislocations at new positions (Fig. 6 B and E). These induced dislocations could be aligned, forming new frequency plateaux, or scattered (Fig. 6 B and E).

Prior to carbenoxolone, frequency always decreased (waves were dropped) distally, and so fork dislocations (pure or ending a slip) always had the same polarity (dark grey points, Fig. 6 B and E). In the presence of carbenoxolone frequency sometimes increased distally (Fig. 6 F) and so waves were dropped proximally, corresponding to forks of the opposite polarity to control (light grey points, Fig. 6 E). Stable lines of
dislocations, corresponding to plateau boundaries, usually consisted of forks of a single polarity but sometimes were of mixed polarity (Fig. 6 E). More often forks of opposite polarity intermingled in close proximity. Between lines of dislocations of opposing polarity, corresponding to hills or valleys in frequency, wave front direction changed (Fig. 6 G). This follows from their geometry. Forks of opposite polarity must run in the opposite direction given that waves are dropped and not created. Therefore the wave front must change direction an odd number of times between forks of opposite polarity.

Carbenoxolone changed the velocity of contractions (Fig. 6 A, D and G). To quantify this, local orientation velocities were calculated along contraction wave fronts (see Methods). Distally directed waves have positive velocity and proximally directed waves have negative velocity. The distribution of velocities was changed by carbenoxolone, shifting down the peak of positive velocities and increasing the proportion of negative velocities (Fig. 7 A). The largest change was from control to 5 μM carbenoxolone, which shifted the peak of positive contraction velocities from 0.91±0.03 to 0.56±0.02 cm/s ($p = 1.04 \times 10^{-4}$; Fig. 7 B). Above 5 μM this peak shifted little more (Fig. 7 B), though the proportion of negative velocities continued to increase (Fig. 7 A).

Before glycyrrhetic acid and carbenoxolone were first reported as gap junction inhibitors (18), long chain alcohols and fatty acids were used and still commonly are. The concentration of 1-heptanol was doubled every ten minutes over the range 250 μM to 2 mM. Heptanol initially formed droplets on the surface of the organ bath solution. However after a few seconds the droplets dissolved and then the bath shimmered for a few seconds. This was likely due to oscillations in surface tension and convection cells driven by the heptanol, known as the Marangoni effect (8, 31). Heptanol had no effect on contraction velocity or pattern (Fig. 8) but as the concentration reached 0.5 mM and above contractions were weakened in amplitude (Fig. 8 A). At 2 mM contractions had disappeared in all intestines tested ($n = 6$).
DISCUSSION

We have shown that frequency plateau boundaries in the small intestine are associated with a wide variety of fork and slip dislocations and that gap junction inhibition increases the number of boundaries (dislocations) and decreases wave velocity. The occurrence of these phenomena indicates that the pacemaker system underlying the contraction patterns is a system of coupled oscillators. This study also shows that regulation of gap junction conductance alone can be a powerful tool to reduce long propulsive contractions and hence appears to promote mixing and absorption. Understanding the mechanisms of motor pattern generation in the intestine, will require further knowledge about the behaviour of coupled oscillators.

Although the frequency gradient of the small intestine has been quantified by a number of authors since Walter Alvarez's pioneering studies a century ago (3, 4, 6, 14, 15, 17, 23, 34, 60) it was only with Diamant and Bortoff in 1969 that frequency plateaux were described (20) (though see Fig. 4 of (4)). This may have been due to a lack of spatial resolution or the averaging of data from different intestines. However our data and that of others (45, 70) show that plateaux simply do not exist in some preparations, that there is either no or a smooth frequency gradient (Fig. 4 middle). In the case of no gradient, there may be only a slight natural frequency gradient, so that the whole preparation is entrained to the same frequency (there is only one plateau). In the case of a smooth gradient, coupled oscillator chain models show that given a particular natural frequency gradient which would normally give frequency plateaux, the addition of a noise term to the phase coupling will instead produce a smooth frequency gradient (73). This noise could represent stochastic changes in gap junction conductance or membrane potential.

According to coupled oscillator theory the direction of the phase wave (sign of it velocity) is dependent on the frequency gradient, its speed (amplitude of its velocity) is not (this is dependent on coupling strength). Therefore there is no particular reason why there should be a relationship between frequency and velocity - a dispersion relationship. Neither we nor anyone else, as far as we are aware, have observed a velocity gradient in the small intestine of mice. However a velocity gradient has been observed along the small intestine in dogs and cats (1, 44, 45, 52, 68). In these cases velocity may be more dependent on slow wave amplitude (see Fig. 5 of (45)), slow wave upstroke rate and coupling strength, rather than frequency. We did see local changes in velocity. Some of these were associated with slip dislocations (Fig. 5). Others however did not occur at plateau boundaries. Sub-centimetre fluctuations in measured velocity may have resulted from the measured contraction line passing close to divergences in image orientation (Fig. 2 D; Methods). This would also explain the long tails in the velocity distributions (Fig. 7). Super-centimeter fluctuations (Fig. 4) appear to be real phenomena and may be just stochastic rather than having any particular significance in regards to coupled oscillator theory.
Fork dislocations have been seen in diameter maps of the rabbit distal colon (22) and rat small intestine (9) though neither study associated these with frequency plateau boundaries. A 240-electrode array study of the whole cat intestine (upwards of 100 cm) was the first to associate fork dislocations with plateau boundaries (44). By combining a very high spatial resolution with coverage of a long length of intestine, our novel multi-camera technique has allowed us to observe details of fork dislocations and has also revealed slip dislocations, not noted before in the small intestine. In both forks and slips, the first wave after the dropped wave usually had a (rotated) "V" indentation at the plateau boundary (Fig. 5). This gave the appearance of oral and aboral propagation from a pacemaker at the apex of the "V". In a sense this is correct - the distal plateau is entrained by the ICC at the boundary and so these ICC can be thought of as a "pacemaker". It is not correct in the sense of an ectopic or "peripheral" pacemaker that arises only transiently at the "V" then disappears again, as was proposed when this was seen in electrode array studies of the porcine and canine small intestine (5, 44, 45). Why would the pacemaker emerge for only one wave cycle? Why would the "V" wave from the pacemaker not propagate further in the proximal (oral) direction? In slip dislocations the "V" is preceded by a progressive (regular) slowing of velocity about the dislocation boundary and slip dislocations are repeated at regular intervals. None of this is explained by a peripheral or ectopic pacemaker but is a natural outcome of phase walk through - the oscillators at either side of the boundary are not entrained and so come in and out of phase according to their frequency difference (25). Forks with "V" indents arise naturally from coupled oscillator models (37, 53, 55).

The superposition (addition) of two waves of different frequency results in a wave with a frequency half way between the two and an amplitude that waxes and wanes at a frequency equal to their difference. This waxing-waning is known in acoustics as a "beat", combination tone, difference tone or Tartini tone (36, 51, 55). Waxing and waning of slow wave amplitude was observed by Bortoff in the cat small intestine (11, 12) and was found to be associated frequency plateau boundaries (20). This was explained as a superposition of different frequency slow waves either side of the boundary. However it is possible that waxing-waning might arise independently of superposition as it is seen at frequency plateau boundaries in a chain of van der Pol oscillators without superposition of signals (53). Another possibility is that waxing-waning results from amplitude modulation of a single slow wave signal by the phase of a signal at the waxing-waning frequency (38). However there is no evidence for such a low frequency signal outside the plateaux boundary.

In the presence of carbenoxolone frequency sometimes increased distally, rather than decreased, with fork dislocations reversed in polarity. The simplest explanation for this is that carbenoxolone changed the natural frequency in places. Gap junction inhibition has been shown to increase calcium wave frequency in the bladder (33). This could occur through gap junction inhibition effecting calcium homeostasis in interstitial cells of Cajal and thus changing the frequency of the calcium clock (41). However such effects were often transient, as forks of opposite polarity often intermingled in close proximity.
The lack of effect of heptanol on smooth muscle conduction properties has been noted before (32, 77). Long chain alcohols and fatty acids do not specifically interact with or block the pore of the connexin channel but instead change the physical properties of the plasma membrane - primarily membrane fluidity - leading to a mechano-conformational response by the channel (42, 69, 71). The non-specificity of this mechanism means that many ion channels are inhibited by long chain alcohols and fatty acids at similar concentrations (42, 71), explaining the effect of heptanol on contraction amplitude. It also explains why heptanol does not always inhibit gap junctions (32, 77) as its effect is dependent on any number of physical factors - the composition of the plasma membrane, mechanical stress on the membrane, cytoskeletal anchoring of connexins. As Srinivas has suggested "actions of n-alkanols may depend on the cholesterol content of the membranes and on the localization of gap junctions in such membranes" (69). In Xenopus oocytes, Cx50 but not Cx46, was sensitive to octanol (26).

Through the 1970s and 80s a large number of publications modeled slow wave propagation with chains of coupled relaxation (van der Pol) oscillators (1, 21, 48, 54, 62, 64, 65). A major criticism of these models was their simplicity (usually just two ordinary differential equations) and therefore abstraction from biophysics, meaning they were not of much working use to gastrointestinal physiologists (59). This is certainly a fair criticism. In response to this criticism, by 1977 Derek Linkens introduced a model of coupled Hodgkin-Huxley oscillators (49, 58). The Hodgkin-Huxley and the related Fitzhugh-Nagumo oscillator, remain popular models of GI wave propagation (2, 47) because they produce much of the gross phenomena associated with contraction propagation whilst being practical to simulate systems with large numbers of elements. Nevertheless more detailed biophysical models (various ionic currents, calcium dynamics) of systems of coupled oscillators are also possible (13, 24, 27, 40, 41). The criticism of individual models does nothing to prejudice coupled oscillator theory as this is a general concept with which to understand or frame the data. All this concept says is that all the individual elements (cells) oscillate independently of each other and any external stimulus and that they can influence each other's phase (they are coupled). A model which satisfies these criteria, can be as biophysical or abstracted as one likes and if it allows for an external stimulus can be analysed in terms of phase response curves (40). The lack of prejudice against coupled oscillator theory, as against relaxation oscillator models, is reflected by the continued use of terms like entrainment, pulling phase advance and intrinsic frequency (13, 28, 35, 43) that necessarily imply coupled oscillator theory (they have no meaning in any other context) even if the theory is not explicitly mentioned.

Distinct from models, conceptually one can talk about slow wave (contraction) propagation in terms of excitation and refraction (trigger waves) or the phase response curve and entrainment (coupled oscillator theory and phase waves). However the phase response curve and excitation/refraction really describe the same thing - the response of a cell to a stimulus as a function of the time of the last excitation. So their biophysics will be much the same - interactions between membrane voltage, ion channels and intracellular calcium (24, 40, 41, 46). Therefore when it comes to the biophysical reality that we try to approach with more detailed models, the
distinction in terminology may dissolve. As Winfree suggested "the differences between these two approaches
[trigger and phase waves] are in part semantic differences and that a quantitative, rather than a verbal, biophysical
model may behave much the same way under either description" (76). This is borne out by the fact that the
slowing of velocity with gap junction inhibition, as shown here, is predicted by models of both coupled oscillators
(21) and trigger waves (67). Nevertheless there is a clear distinction between phase and trigger waves - all cells
oscillate independently versus only one cell (the pacemaker) oscillates. In this context coupled oscillator theory is
a better conceptual framework with which to understand contraction waves in the small intestine.

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DISCLOSURES
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FIGURES

Figure 1
Multi-camera rig and organ bath. A row of ten cameras (A) were mounted 65 cm above the organ bath (B). kk, kit-kat; ht, heating tube (oxygenation tube runs in parallel); bc, bubble cover (lower cover not shown for clarity); co, circulation outflow; ci, circulation inflow. (C) Intestines were tied to cannula heads (ch) at the gastro-duodenal junction and 30 cm distal to this. The intestine was then held in a lane of the kit-kat by inserting the cannula heads into cannula receivers (cr) which fit tightly into the lane (D).

Figure 2
Detection of contractions and calculation of contraction velocity from Dmaps. Bold capitals in parentheses indicate matrix notation of images (see Methods). (A and B) A Dmap was convolved with a steerable filter kernel (insert, magnified) to give its time derivative. Contractions (green lines) are where the time derivative changes from negative to positive. (C and D) Convolution of the time derivative the kernel, gave the local orientation. Note the divergence of orientation between contraction lines. (E) The direction of the contraction, \( f(\alpha_0) \) is orthogonal to the local orientation. (F) Dmap with local orientation vectors. (G) Dmap with contraction direction vectors.

Figure 3
Dmap of a 20 cm length of small intestine. Duodenum (top) to distal (bottom). Note the dislocations as marked by arrows.

Figure 4
Contraction interval and velocity along the length of the small intestine. Interval was calculated as the average of the first five peaks in the temporal autocorrelation. Velocity was calculated by local orientation along contractions (see Methods). Distance is from the gastro-duodenal junction. (A) Three example intestines. (B) Mean velocity for six intestines over 2 cm distance bins.

Figure 5
Phase dislocations at frequency plateau boundaries. (A) Fork. (B) Asymmetric fork. (C) Two space-adjacent forks. (D) Slip. (E and F) Sawtooth slips. (G) Hard slip. (H) Gliding slip. (I and J) Waxing-waning associated with fork dislocations (I) and slip dislocations (J). Upper panels show contraction amplitude profile along dotted line in lower Dmaps. Wave drop times indicated by filled circles.
Figure 6

Effects of carbenoxolone. (A) Example Dmaps from one intestine. *Left to right*, control, 10 μM and 40 μM carbenoxolone. (B) Locations and times of wave drops at fork dislocations and ends of slip dislocations, in one intestine. Distance is from the gastro-duodenal junction. At wave drops the wave is lost distally (*dark grey*) or proximally (*light grey*). Dotted lines indicate addition of carbenoxolone (control, 5 and 10 μM). (C) Contraction interval along the length of the intestine. Control (*grey*) and 10 μM carbenoxolone (*black*). Interval was calculated as the average of the first five peaks in the temporal autocorrelation. (D) Contraction velocity along the length of the intestine with 10 μM carbenoxolone. (E - G) Same as (B - D) for another intestine.

Figure 7

Effect of carbenoxolone on contraction velocity. (A) Probability density of contraction velocities (see Methods). Positive velocities correspond to distal directed waves and negative velocities to proximal directed waves. Calculated from six intestines, over two minutes per intestine, with bins of 0.05 cm/s. (B) Mean and mode negative velocities. Paired t-tests with respect to control (*n* = 6 intestines, **p < 10⁻², ***p < 10⁻³, ****p < 10⁻⁴).

Figure 8

Effects of 1-heptanol. (A) Example Dmaps from one intestine. *Left to right*, control, 0.5 mM and 1 mM heptanol. (B) Probability density of contraction velocities (see Methods). Positive velocities correspond to distal directed waves and negative velocities to proximal directed waves. Calculated from six intestines, over two minutes per intestine, with bins of 0.05 cm/s. (C) Mean and mode negative velocities. Paired t-tests with respect to control showed no p < 0.05 (*n* = 6 intestines).
REFERENCES


fig. 1
Dmap ($\mathbf{D}$), $o_{ij}$

fig. 2
fig. 4
fig. 6
A

- cntrl
- 5 µM
- 10 µM
- 20 µM
- 40 µM

B

positive velocity (cm/s)

mean
mode

fig. 7
fig. 8