Peristalsis and propulsion of colonic content can occur after blockade of major neuroneuronal and neuromuscular transmitters in isolated guinea pig colon

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Sia TC, Brookes SJ, Dinning PG, Wattchow DA, Spencer NJ. Peristalsis and propulsion of colonic content can occur after blockade of major neuroneuronal and neuromuscular transmitters in isolated guinea pig colon. Am J Physiol Gastrointest Liver Physiol 305: G933–G939, 2013. First published October 10, 2013; doi:10.1152/ajpgi.00257.2013.—We recently identified hexamethonium-resistant peristalsis in the guinea pig colon. We showed that, following acute blockade of nicotinic receptors, peristalsis recovers, leading to normal propagation velocities of fecal pellets along the colon. This raises the fundamental question: what mechanisms underlie hexamethonium-resistant peristalsis? We investigated whether blockade of the major receptors that underlie excitatory neuromuscular transmission is required for hexamethonium-resistant peristalsis. Video imaging of colonic wall movements was used to make spatio-temporal maps and determine the velocity of peristalsis. Propagation of artificial fecal pellets in the guinea pig distal colon was studied in hexamethonium, atropine, ω-conotoxin (GVIA), ibodutant (MEN-15596), and TTX. Hexamethonium and ibodutant alone did not retard peristalsis. In contrast, ω-conotoxin abolished peristalsis in some preparations and reduced the velocity of propagation in all remaining specimens. Peristalsis could still occur in some animals in the presence of hexamethonium + atropine + ibodutant + ω-conotoxin. Peristalsis never occurred in the presence of TTX. The major finding of the current study is the unexpected observation that peristalsis can occur after blockade of the major excitatory neuroneuronal and neuromuscular transmitters. Also, the colon retained an intrinsic polarity in the presence of these antagonists and was only able to expel pellets in an aboral direction. The nature of the mechanism(s)/neurotransmitter(s) that generate(s) peristalsis and facilitate(s) natural fecal pellet propulsion, after blockade of major excitatory neurotransmitters, at the neuroneuronal and neuromuscular junction remains to be identified.

preisstalsis; hexamethonium resistance; colon; acetylcholine; ω-conotoxin (GVIA); ibodutant; nicotinic; muscarinic

THE MECHANISMS UNDERLYING distension-evoked colonic peristalsis have been the subject of much investigation over the past century, but they remain incompletely understood. It is clear that the enteric nervous system plays an essential role (5, 8, 26). Major advances have recently been made with regard to the cell types that are required for distension-evoked peristalsis (34). It was once thought that the mucosa, and release of 5-HT from the mucosa, was necessary to initiate distension-evoked peristalsis (3, 10, 15, 16). However, recent studies performed in vitro have shown that distension-evoked peristalsis (17, 32) and colonic motor complexes (35) are not abolished when endogenous 5-HT is chemically depleted from enteric nerves and the mucosa, which contains the largest store of 5-HT in the body, is physically removed. Similar findings have been reported in mice with genetic mutations to block synthesis of mucosally derived 5-HT (42). On the basis of recent findings, it seems more likely that endogenous 5-HT plays a modulatory, but clearly not an essential, role in intestinal motility.

While endogenous 5-HT is not required for peristalsis (17, 32, 34) in vitro or gastrointestinal transit in vivo (42), it is clear from earlier work that nicotinic receptor activation is essential for peristalsis (5, 6). This is highly consistent with the major role of fast nicotinic synaptic potentials recorded from most myenteric neurons (40, 41). Despite extensive evidence that nicotinic receptor activation plays a major role in enteric neuronal transmission (2, 8), recent work has shown that, after an acute blockade of peristalsis with hexamethonium, intestinal (1) and colonic (26) peristalsis can recover in the continued presence of nicotinic antagonists. In the colon, it was shown that hexamethonium-resistant peristalsis persisted following further exposure to antagonists of neurokinin (NK)-3 receptors, 5-HT3 receptors, and P2 purinoreceptors (26). This raises the fundamental question: what are the intrinsic mechanisms/neurotransmitters that underlie hexamethonium-resistant peristalsis in the guinea pig distal colon?

Since peristalsis can occur following blockade of major excitatory neuroneuronal transmitters, we were particularly interested in whether hexamethonium-resistant colonic peristalsis would still occur when major receptors for excitatory neuromuscular transmitters on the smooth muscle (acetylcholine and tachykinins) are also blocked. On the basis of findings from earlier studies in the guinea pig small intestine, it has been shown that atropine abolishes hexamethonium-resistant peristalsis (1); however, it is unclear whether this phenomenon exists in the colon. A major aim of the current study was to determine whether hexamethonium-resistant peristalsis in guinea pig distal colon requires activation of muscarinic receptors and, if peristalsis persists in the presence of hexamethonium and atropine, whether it would occur after subsequent blockade of NK-2 tachykininergic receptors, which mediate the major component of noncholinergic neuromuscular transmission (39). We have also investigated the mechanisms underlying the intrinsic polarity of hexamethonium-resistant aboral propulsion of colonic content.

METHODS

Preparation of tissues. Adult guinea pigs (250–400 g body wt) were killed by a blow to the occipital region and exsanguinated in a manner approved by the Animal Welfare Committee of Flinders

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University. The terminal 12–15 cm of distal colon, taken ~4 cm from the anus, was removed and placed in warmed Krebs solution, which was continuously bubbled with carbogem gas (95% O2:5% CO2). Endogenous pellets were naturally expelled. The isolated distal colon from each animal was further segmented to yield two preparations, each ~6 cm long. Video images of colonic wall movements and mechanical recordings were obtained from the circular muscle (see Experimental protocol).

Experimental protocol. Spatiotemporal maps were generated from peristaltic contractions propagating an acutely inserted natural fecal pellet coated in epoxy resin. Expulsion and reinsertion of the pellets were maintained throughout the experiment in 5-min intervals. A period of 20 min was allowed before effects of the administered antagonist drugs were recorded, allowing for more than three bath volume changes. Pellets were continually inserted during this equilibration period.

Mechanical recordings from circular muscle during peristalsis and fecal pellet propulsion. We recorded the force generated during each peristaltic contraction by inserting an artificial fecal pellet (with ligature attached) into the oral end of the colon and allowing it to naturally propagate midway along the length of colon. The pellet was fixed at this site, so that we could record peristaltic waves with an isometric recording transducer (FT-03C, Grass Instruments, Quincy, MA) connected to the ligature. Data were recorded from the force transducers onto a computer running LabChart 6 (ADInstruments, Bella Vista, NSW, Australia) via two custom-made preamplifiers (Biomedical Engineering, Finders University) and a Powerlab data acquisition system (model 4/30, ADInstruments). These experiments are described in RESULTS as the maintained-distension experiments conducted by a fixed pellet, since the pellet was not free to move along the entire length of the preparation.

Video imaging of peristalsis and generation of spatiotemporal maps. Circular muscle contractions of the gut wall were recorded using the Gastrointestinal Motility Monitoring system (Med-Associates, Saint Albans, VT). Briefly, the colon preparations were illuminated from below, and a digital video camera was used to record the propagation of a single fecal pellet along the length of the colon. Spatiotemporal maps were constructed from the digital videos that were acquired from individual pellet runs.

Measurements and statistics. The propagation velocity of inserted pellets was determined from spatiotemporal maps. Propagation of fecal pellets along the colon was characterized as 1) continuous propulsion (i.e., pellets did not pause along the length of the preparation at any point); 2) staggered propagation (i.e., pellets remained stationary for ≥1 period ≥5 s before propagation was resumed and pellets were expelled, which usually occurred as multiple interruptions in the continuous propulsion of the fecal pellet down the length of the colon and typically led to a prolonged delay of expulsion from the colon); 3) propagation with incomplete expulsion of the pellet; or 4) the absence of propagation (i.e., inserted pellets did not move from the site of insertion). Only preparations that showed complete expulsion of pellets were included in analysis of propagation velocity. Values are means ± SE; n refers to the number of preparations on which observations were made. A maximum of two preparations of distal colon were obtained from any one animal. Data sets were considered statistically significant where P < 0.05. Statistical analysis of velocities was conducted for all experiments using within-fields/repeated-measures analysis on Statistical Package for Social Sciences (SPSS, IBM), given the nature of experiments using repeated addition of receptor antagonists.

Drugs and solutions. Krebs solution contained (in mM) 118 NaCl, 4.7 KCl, 1.0 NaH2PO4, 2H2O, 25 NaHCO3, 1.2 MgCl2·6H2O, 11 d-glucose, and 2.5 CaCl2·2H2O. Hexamethonium (500 μM), atropine (1 and 3 μM), and TTX (1 μM) were purchased from Sigma (St. Louis, MO) and ω-conotoxin (GVIA, 0.1 μM) from Alomone Labs (Jerusalem, Israel). Iboduant (MEN-15596, 1 and 3 μM) was a gift from Dr. Vladimir Zagorodnyuk, sourced from the Menarini Group (Florence, Italy).

RESULTS

In total, 32 preparations of distal colon were removed from 16 guinea pigs. In 32 of 34 (94%) preparations, insertion of a single fecal pellet into the oral end of isolated distal colon elicited a peristaltic wave, which propagated continuously along the colon, leading to expulsion of the pellet from the anal end. In the remaining two specimens, a staggered propulsion of the fecal pellet led to complete expulsion (see Fig. 3A). The average velocity of fecal pellets during these continuous runs was recorded at 1.98 ± 0.05 mm/s (205 runs, n = 32).

Hexamethonium-resistant peristalsis. Hexamethonium (500 μM) was applied to 16 preparations (n = 16). Ten of the 16 preparations (63%) displayed a continuous propagation along the colon (Figs. 1Ai, 2, and 3A). In 3 of the 16 preparations (19%), the presence of hexamethonium induced a staggered propulsion that led to complete expulsion of pellets from the colon (Fig. 3A). In addition, in 13 of the 16 preparations, the mean velocity in hexamethonium was 2.59 ± 0.1 mm/s compared with controls at 1.92 ± 0.08 mm/s (70 runs, n = 13, P = 0.09; Fig. 4A). In the remaining three preparations (19%), initial staggered propulsion was evident: pellets were propelled a short distance along the colon, then stopped and failed to expel (Fig. 3A).

Effect of atropine on hexamethonium-resistant peristalsis. We were particularly interested in whether atropine would inhibit or abolish hexamethonium-resistant peristalsis, as has been demonstrated in the guinea pig ileum (1). We were surprised to find that peristalsis and propulsion of fecal pellets persisted in the presence of atropine (1 μM) and hexamethonium (500 μM). Of a total of 13 preparations, none showed continuous expulsion of fecal pellets along the colon. In 7 of the 13 preparations, propulsion was staggered, leading to expulsion; in 4 preparations the pellets did not run at all, and 2 preparations the pellets ran in a staggered fashion but were not expelled. Overall, in the presence of atropine and hexamethonium, the mean propagation velocities were significantly diminished to 0.42 mm/s (27 runs, n = 13, P = 0.02; Figs. 3A and 4A). In 2 of these 13 preparations (15%), the pellets were somewhat propelled along the colon but were never expelled and remained within the lumen for the entire experiment (Fig. 3A); in the remaining 4 preparations (31%), the pellets did not move from the oral site of insertion. In the presence of hexamethonium and atropine, we further tested whether the N-type Ca2+ channel blocker ω-conotoxin (0.1 μM) would abolish peristalsis. In the presence of hexamethonium, atropine, and ω-conotoxin, peristalsis could still occur. In all five preparations tested, staggered propulsion was preserved, and, remarkably, pellets could propagate along the full length of the colon, albeit at very slow velocities (Fig. 3A). No significant differences in velocity were observed compared with hexamethonium and atropine alone (7 runs, n = 5, P = 0.25; Fig. 4A).

Effects of NK-2 receptor blockade on hexamethonium- and atropine-resistant peristalsis. When the NK-2 receptor antagonist iboduant (1 μM) was applied to the colon alone, peristalsis was unaffected (Fig. 1Bii). The mean propagation velocity was recorded at 2.36 ± 0.1 mm/s compared with controls at 1.92 ± 0.11 mm/s. This was not statistically significant (28 runs, n = 8, P = 0.62; Fig. 4B). When
hexamethonium and atropine were added to the colon segments, peristalsis was abolished in five of eight preparations (63%). In the remaining three preparations, staggered propulsion led to expulsion of the pellets (Fig. 3B), with velocities of 0.05 ± 0.01 mm/s (6 runs, n = 3, P = 0.015; Fig. 4B). Further addition of ω-conotoxin had no effect on the remaining active specimens (0.1 ± 0.02 mm/s, 6 runs, n = 3, P = 0.11; Fig. 4B).

We tested whether 3 μM ibodutant would lead to differences in peristalsis compared with 1 μM ibodutant. In four preparations, 3 μM ibodutant resulted in no difference in propagation velocity of pellets (1.62 ± 0.22 and 1.19 ± 0.34 mm/s for control and ibodutant, respectively, n = 4, P = 0.33). This higher dose resulted in staggered propulsion in one of four preparations, with other preparations showing continuous pro-
pulsion with no changes in the characteristics of propulsion. Furthermore, these four preparations were subjected to a higher concentration of atropine (3 μM). In two of these four preparations, propulsion occurred, although it was staggered, with velocities averaging 0.045 mm/s (n = 2, P = 0.18). In the other two preparations, peristalsis was abolished.

Does peristalsis recover after blockade with TTX? It is well established that TTX blocks colonic peristalsis (5). In our preparations, pellets could not be inserted into the colon in the presence of TTX (1 μM) because of increased constriction of the oral region. This precluded insertion of the pellet into the oral end. To circumvent this problem, we applied TTX to the colon when the pellet was already inserted and fixed at a point midway along the colon (see METHODS). Under these conditions, TTX consistently abolished any peristaltic activity induced by a fixed pellet. We were interested to see if peristalsis would recover following prolonged exposure to TTX, similar to our finding with hexamethonium. We found that peristalsis never recovered following maintained (>1 h) exposure to 1 μM TTX (n = 6).

Effects of ω-conotoxin on distension-induced peristalsis. N-type Ca2+ channels are known to play a major role in neurotransmitter release from enteric neurons (24, 25). We were interested in whether ω-conotoxin added alone (in the absence of hexamethonium) would affect peristalsis. We found that ω-conotoxin abolished peristalsis in three of eight preparations.
A pellet was inserted into the anal end and a contraction was elicited, at which point the pellet was immediately expelled. Attempts to insert a pellet from an anal direction encountered resistance in all conditions of control and drugs in all instances. In the presence of hexamethonium, atropine and ibodutant, no orally migrating peristalsis was triggered. In the presence of hexamethonium and atropine, the velocity of propulsion was further reduced to 0.04 ± 0.01 mm/s (P = 0.07; Fig. 4C).

**Does an intrinsic polarity underlie peristalsis in the presence of neuroneuronal and neuromuscular antagonists?** It is known that, in the isolated guinea pig colon, propulsion of fecal pellets can only occur in an oral-to-anal direction (5, 6, 26). We investigated whether peristalsis that persists in the presence of these neuroneuronal and neuromuscular antagonists would maintain an oral-to-anal directionality. In the presence of hexamethonium, atropine, ibodutant, and ω-conotoxin, fecal pellets inserted into the anal end of the colon never propagated orally (Fig. 5). In the same preparations, pellets inserted into the oral end were propelled in the anal direction. This nonnicotinic, nonmuscarinic, non-NK-2 receptor-mediated propagation did not occur in the presence of TTX (n = 6).

Attempts to insert a pellet from an anal direction encountered resistance in all conditions of control and drugs in all instances.

**DISCUSSION**

The major finding of this study is that peristalsis and propulsion of fecal content could occur not only in the presence of hexamethonium, but also following blockade of muscarinic receptors, NK-2 receptors, and ω-conotoxin-sensitive Ca²⁺ channels. While this study confirms that nicotinic and muscarinic receptors play a major role in enteric neurotransmission (25), ω-Conotoxin inhibits depolarization-evoked acetylcholine release (22) and, together with tachykinins, is inhibited at a prejunctional level (20). In the presence of hexamethonium and atropine, further addition of ω-conotoxin did not affect propagation velocity. This suggests that the mechanisms that underlie hexamethonium-, atropine-, and NK-2 receptor-resistant peristalsis do not require N-type Ca²⁺ channels. When applied in the absence of any other drug, ω-conotoxin blocked peristalsis.

We expected that fecal pellet propulsion would not occur in the presence of hexamethonium + atropine. We found that, in a proportion of animals where pellets were propelled in the presence of hexamethonium + atropine, the velocity of propulsion was significantly slower because of the highly staggered nature of propagating contractions. We further evaluated the mechanisms underlying this propulsion by observing propulsion in the presence of the NK-2 receptor antagonist ibodutant, hexamethonium, and atropine. It is known that NK-2 receptors are localized predominantly on the neuromuscular junction, mediating the excitatory reflex responses of the guinea pig colon (9, 21, 30). We found that ibodutant alone did not reduce the velocity of peristalsis. Interestingly, Lecci et al. (18) found that, in guinea pig colon in vivo, NK-2 receptor antagonists also did not block peristalsis and, in fact, significantly increased the speed of propulsion of an artificially inserted rectal balloon. While peristalsis has never been shown to occur in the presence of hexamethonium and atropine, in vivo or in vitro, a further novelty of our experiments is that peristalsis can occur in the presence of hexamethonium, atropine, and ibodutant (Fig. 3B).

In light of the fact that peristalsis could still occur in some preparations in hexamethonium and atropine, we were interested in whether this nonnicotinic, nonmuscarinic pathway would still require N-type Ca²⁺ channels, which are known to play a major role in enteric neurotransmission (25). ω-Conotoxin inhibits depolarization-evoked acetylcholine release (22) and, together with tachykinins, is inhibited at a prejunctional level (20). In the presence of hexamethonium and atropine, further addition of ω-conotoxin did not affect propagation velocity. This suggests that the mechanisms that underlie hexamethonium-, atropine-, and NK-2 receptor-resistant peristalsis do not require N-type Ca²⁺ channels. When applied in the absence of any other drug, ω-conotoxin blocked peristalsis.

Fig. 5. Insertion of fecal pellets into the anal end of isolated colon failed to induce orally migrating peristalsis. A: in the presence of hexamethonium + atropine, a pellet was inserted into the anal end and a contraction was elicited, at which point the pellet was immediately expelled. B: in a separate preparation from a different animal, the same result was obtained in hexamethonium + atropine + ω-conotoxin. C: in another preparation, in the presence of hexamethonium + atropine + ibodutant, no orally migrating peristalsis was triggered.
in three of eight preparations. The significant effect of ω-conotoxin suggests that N-type Ca\textsuperscript{2+} channel antagonists play a major role in the generation of peristalsis, but their activation is not a prerequisite for peristalsis.

Is hexamethonium-, atropine-, and NK-2 receptor-resistant peristalsis mediated by a neural phenomenon? While peristalsis could still occur in the presence of hexamethonium, atropine, an NK-2 receptor antagonist, and ω-conotoxin, it did not occur in the presence of TTX. If peristalsis in the presence of these antagonists is truly neural in origin, then the mechanism by which the enteric motor neurons can still be excited to cause muscle contraction is particularly interesting. It seems difficult to believe that the enteric motor neurons can excite smooth muscle after blockade of the major neuroneuronal and neuromuscular transmitters. If the enteric nervous system is responsible for the generation of peristalsis in the presence of these antagonists, the neurotransmitters responsible must activate enteric excitatory motor neurons via synaptic potentials that do not require nicotinic, muscarinic, or NK-2 receptors. It is possible that peristalsis that persists in the presence of these antagonists is myogenic in origin and that TTX abolishes this motor pattern by acting on TTX-sensitive Na\textsuperscript{+} channels in the smooth muscle, in addition to enteric neurons. In support of this notion, there is abundant evidence that TTX-sensitive Na\textsuperscript{+} channels are present in mouse vas deferens (12), sheep lymphatics (11), rabbit pulmonary artery (28), rat uterus (27), and human colon (29, 36). Interestingly, there is also recent evidence suggesting TTX-resistant peristalsis (6).

Why is there an intrinsic polarity underlying peristalsis in the presence of hexamethonium, atropine, NK-2 receptor antagonists, and ω-conotoxin? A major finding of our current study is that, in the presence of hexamethonium, atropine, ibodutant, and ω-conotoxin, fecal pellets were always expelled in an oral-to-anal direction. We also noted that pellets inserted into the anal end never propagated orally. The reason why pellets never propagated orally is not clear. It is clear that the interstitial cells of Cajal are localized within various anatomic layers of the gastrointestinal wall (14, 33, 37) and act as myogenic pacemaker cells that generate phasic contractility in the gut wall (7, 13, 19, 38). Also, there is abundant evidence that a gradient in slow-wave frequency due to interstitial cells of Cajal exists along the length of the gastrointestinal tract (4, 23, 31). While the mechanism underlying the preservation of an intrinsic polarity in the colon in the presence of all antagonists is unclear, one could postulate that a gradient in myogenic activity generated by interstitial cells of Cajal plays an important role.

Conclusion. The major finding of the current study is that peristalsis and the propulsion of fecal pellets can occur following blockade of the major neuroneuronal and neuromuscular transmitters in the distal colon of guinea pigs. The mechanisms underlying this hexamethonium-resistant peristalsis were abolished by TTX, which suggests that a neurally mediated phenomenon is essential. Alternatively, a myogenic process may underlie peristalsis under these conditions but require TTX-sensitive Na\textsuperscript{+} channels in smooth muscle or other cell types. Of particular interest is why an intrinsic polarity prevails after blockade of nicotinic, muscarinic, and NK-2 receptors. This will form the basis of future investigations.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

T.C.S., S.J.B., P.G.D., D.A.W., and N.J.S. are responsible for conception and design of the research; T.C.S. performed the experiments; T.C.S. analyzed the data; T.C.S., S.J.B., P.G.D., D.A.W., and N.J.S. interpreted the results of the experiments; T.C.S. prepared the figures; T.C.S. and N.J.S. drafted the manuscript; T.C.S., D.A.W., and N.J.S. edited and revised the manuscript; T.C.S. and N.J.S. approved the final version of the manuscript.

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