Localization of the sensory neurons and mechanoreceptors required for stretch-evoked colonic migrating motor complexes in mouse colon

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The pacemaker and pattern generator that underlies the cyclical generation of spontaneous colonic migrating motor complexes (CMMCs) has recently been identified to lie within the myenteric plexus and/or muscularis externa. Neither the mucosa, nor the release of substances from the mucosa were found to be required for the spontaneous generation of CMMCs. However, it is known that stretch applied to the colonic wall can also evoke CMMCs and since stretch of the gut wall is known to stimulate the mucosa, it is not clear whether release of substances from the mucosa and/or submucosal plexus are required for stretch-evoked CMMCs. Therefore, the aim of this study was to determine whether circumferential stretch-evoked CMMCs require the presence of the mucosa and/or submucosal plexus in isolated mouse colon. Spontaneous CMMCs were recorded from full length sheet preparations of colon in vitro. Graded circumferential stretch (at a rate of 100 μm/s) applied to a 15-mm segment of mid–distal colon reliably evoked a CMMC, which propagated to the oral recording site. Sharp dissection to remove the mucosa and submucosal plexus from the entire colon did not prevent spontaneous CMMCs and circumferential stretch-evoked CMMCs were still reliably evoked by circumferential stretch, even at significantly lower thresholds. In contrast, in intact preparations, direct stimulation of the mucosa (without accompanying stretch) proved highly inconsistent and rarely evoked a CMMC. These observations lead to the inescapable conclusion that the sensory neurons activated by colonic stretch to initiate CMMCs lie in the myenteric plexus, while the mechanoreceptors activated by stretch, lie in the myenteric ganglia and/or muscularis externa. Stretch activation of these mechanoreceptors does not require release of any substance(s) from the mucosa, or neural inputs arising from submucosal ganglia.

Keywords: migrating motor complex, colon, peristalsis, enteric, sensory neuron, pacemaker

INTRODUCTION

Colonic migrating motor complexes (CMMCs) are one of the major types of colonic motor pattern that occur spontaneously or, can be evoked by physiological stimuli in the large intestine of mammals and are thought to facilitate colonic transit (Sarna, 1991b; Bywater et al., 1998; Spencer et al., 1998a; Brierley et al., 2001; Spencer, 2001; Roberts et al., 2007, 2008; Heredia et al., 2010). In early in vivo studies, attempts to identify control mechanisms underlying CMMC generation proved challenging, probably due to the limitations of studying colonic motility in living animals (Sarna, 1986, 1991a,b; Karau et al., 1987). However, in the mid 1970s it was discovered that colonic motor complexes could be preserved during in vitro recordings made from the whole isolated colon of mice (Wood, 1973; Wood et al., 1986) and cats (Christensen et al., 1974). Since this discovery, many laboratories have recorded CMMCs in vitro and much has been learnt about the mechanisms underlying their cyclical generation (Bywater et al., 1998; Spencer et al., 1998a,b, 2005, 2007; Brierley et al., 2001; Roberts et al., 2007, 2008; Keating and Spencer, 2010).

One of the recent discoveries regarding spontaneous CMMCs has been the localization of the pacemaker and pattern generator underlying their cyclical generation (Keating and Spencer, 2010); and the basic intrinsic circuitry that underlies their generation and propagation (Spencer et al., 2005). It was found that careful removal of the mucosa and submucosal plexus from the entire mouse colon did not prevent spontaneous CMMCs (Keating and Spencer, 2010) – an observation verified by other laboratories (H. Sjovall, University of Gothenburg, personal communication). This important result showed that release of substances from the mucosa, or submucosal ganglia were not required for spontaneous CMMC generation or propagation.

Whilst much has now been learnt about the generation of spontaneous CMMCs, it is also known that CMMCs can be evoked by external stimuli, such as transmural electrical nerve stimulation (Spencer and Bywater, 2002; Powell et al., 2003), or circumferential stretch of the colon wall (Powell et al., 2003; Heredia et al., 2009). It is well known that stretch of the colonic wall potently activates intrinsic polarized neural pathways (Spencer and Smith,
We have previously reported that in order to preserve spontaneous care to prevent damage to the underlying myenteric plexus (Keating and Spencer, 2010). In this study, we removed the mucosa with carbogen gas (95% O₂/5% CO₂). Controlling temperature Krebs solution, which was constantly bubbled with carbogen gas (95% O₂/5% CO₂), the entire colon was removed from mice and placed in room temperature Krebs solution, which was constantly bubbled with carbogen gas (95% O₂/5% CO₂).

**MATERIALS AND METHODS**

**PREPARATION OF TISSUES**

C57BL/6 mice (30–90 days of age) of either sex were euthanized by isoflurane inhalation overdose, followed by cervical dislocation, as approved by the animal welfare committee at Flinders University. The entire colon was removed from mice and placed in room temperature Krebs solution, which was constantly bubbled with carbogen gas (95% O₂/5% CO₂).

**DISSECTION TO REMOVE THE MUCOSA AND SUBMUCOSA IN SHEET PREPARATIONS OF COLON**

We have previously reported that in order to preserve spontaneous CMMCs following sharp dissection to remove the mucosa requires care to prevent damage to the underlying myenteric plexus (Keating and Spencer, 2010). In this study, we removed the mucosa and submucosa by firstly making a longitudinal incision along the mesenteric border and pinning the entire colon as a flat sheet preparation, with the mucosal surface facing uppermost. We then pinched the mucosal surface with fine dissecting forceps (see below) and physically cut off the mucosa and underlying submucosa from the myenteric plexus with fine surgical scissors. In general, peeling the mucosa and submucosa off the underlying myenteric plexus rarely preserved CMMCs. The forceps we used for dissections to pinch small segments of mucosa were: catalog # 5SF inox (code: 11252-00) or 55SF (code: 11255-20) from Fine Science Tools, Canada. The scissors we used to cut off the mucosa and submucosa were Vannas, Tubingen, Germany; code # 15003-08, which were 8.5 cm in length. The dissection process to remove the mucosa and submucosal mucosa was performed in a small Petri dish containing ice cold Krebs solution that was constantly bubbled with carbogen gas (95% O₂/5% CO₂).

**METHOD FOR RECORDING MOTOR ACTIVITY FROM THE CIRCULAR MUSCLE LAYER AND APPLYING CONTROLLED LEVELS OF CIRCUMFERENTIAL STRETCH**

Colonic preparations used for recording and evoking CMMCs in response to stretch ranged in length from 36 to 48 mm (under resting slack conditions). This distance equated to approximately 70% of the entire length of colon. The terminal region of anal sphincter was not attached to the colon. To record motor activity from the circular muscle layer, two independent hooks were used (see Figure 1) to record and stretch the colon from a single site in the mid to distal region of colon. One hook (12 mm long) was applied to the mid–distal region of colon, while the proximal hook was 9 mm long and was used only for recording spontaneous changes in muscle tension. A distance of 6–9 mm separated the closest oral to anal edges of the two hooks. A basal resting tension was applied to the distal hook of 300 mg and 400–500 mg to the proximal hook (see Figure 1). Circumferential stretch was applied at a rate of 100 μm/s to a level that measured as a 30% increase in circumferential diameter, calculated for each preparation to take into account preparation variability. This level of stretch was maintained for 5 s by a stepper motor controlled tissue stretcher. The circumferential diameter of each preparation slack varied between 7 and 8 mm. The tissue stretcher used to apply controlled stretches was set to apply stretch at an time point that was 20% shorter than the spontaneously occurring CMMCs. In intact control colons, stretch was applied approximately every 50–60 s and every 80–90 s for colonic preparations devoid of mucosa and submucosal plexus.
**PROTOCOL FOR MUCOSAL STIMULATION**

The protocol used for stimulation of the mucosa was identical to that used in our previous study on guinea-pig distal colon (Spencer et al., 1999). In brief, the isolated whole mouse colon was pinned mucosal side uppermost and mechanical recordings made simultaneously from the proximal mid and distal regions. The mucosal surface was stimulated by three consecutive brush strokes, applied via a fine artists paint brush, over an area ∼7 mm in length across the full circumference, applied ∼2–4 mm from the anus.

**MEASUREMENTS AND STATISTICS**

Measurements of the half duration and peak amplitude of CMMCs were measured from tension recordings, as was the interval between CMMCs. The propagation velocity of CMMCs was not possible to ascertain with only two recording sites, as the actual direction of propagation was not possible to determine with confidence. Data in the Section “Results” are presented as means ± SEM. The use of “N” in the Section “Results” refers to the number of animals on which examinations were made. Data sets were considered statistically significant if *P* values < 0.05 were reached. Student’s unpaired *t*-test were used for comparison of data.

**DRUGS AND SOLUTIONS**

The Krebs solution used contained (in mM): NaCl, 118; KCl, 4.7; NaHPO₄·2H₂O, 1.0; NaHCO₃, 25; MgCl₂·6H₂O, 1.2; d-Glucose, 11; CaCl₂·2H₂O, 2.5. Hexamethonium was obtained from Sigma Chemical Co., MO, USA and made up as a stock solution of 100 mM in deionized water.

**RESULTS**

In isolated sheet preparations of whole mouse colon, mechanical recordings made simultaneously from the proximal and distal colon revealed spontaneous CMMCs occurred with a mean interval between of 78 ± 8 s (N = 8), where the mean peak amplitude of CMMC contractions was 3.9 ± 1.1 g (N = 8). When the mucosa and submucosal plexus were removed from the whole isolated colon, spontaneous CMMCs were still recorded (Figure 2). Histological staining of these preparations for Hematoxylin and Eosin (H and E) confirmed the removal of the submucosal ganglia and mucosa (Figure 3). Overall, there was no significant difference in the amplitude (control: 3.9 ± 1.1 g; N = 5, cf. mucosa removed: 2.4 ± 0.6 g.s) or area-under-contraction (control: 20.9 ± 4.9 g.s, cf. 16.9 ± 4.9 g.s; see Figure 4; *P* < 0.05; N = 5) of spontaneous CMMCs in intact preparations, compared with spontaneous CMMCs following removal of the mucosa and submucosal plexus (Figure 4). However, the interval between CMMCs was significantly longer. CMMCs in mucosa-free preparations occurred every 131 ± 11 s, which equated to an increase in interval of 68%, *P* < 0.005; N = 5, Figure 4, consistent with our previous study (Keating and Spencer, 2010).

In intact preparations, when graded increases in circumferential stretch (of 30% beyond resting slack width) were applied to the distal colon, at a controlled rate of 100 μm/s, a CMMC was evoked in the distal colon that propagated to the proximal colon (Figure 2). When the identical rate of circumferential stretch was applied to the same region of colon in mucosa and submucosal plexus-free preparations, the stretch threshold required to evoke a CMMC actually decreased by 23%, since the stretch threshold length in control preparations was 2.2 ± 0.1 mm (N = 5), but was 1.7 ± 0.1 mm (N = 5) in mucosa and submucosal plexus-free preparations (Figure 5; N = 5, *P* < 0.01). There was no significant difference in the area-under-contraction of evoked CMMCs in intact preparations compared with stretch-evoked CMMCs in mucosa and submucosal plexus-free preparations (Figures 4 and 5; N = 5). Hexamethonium (200 μM) was used to test the role of nicotinic transmission in spontaneous and evoked CMMCs. Hexamethonium always abolished spontaneous CMMCs (N = 5; Figure 2) and stretch-evoked CMMCs in intact preparations (N = 5; Figure 6), or preparations with their mucosa and submucosal plexus removed (Figure 7).

Overall, in intact preparations of colon, there were no significant differences in the mean area-under-contraction of evoked CMMCs (control: 30.6 ± 5.7 g.s; N = 5) compared with those...
evoked by stretch in preparations with mucosa and submucosal plexus removed (31 ± 9.6 g.s, N = 6; see Figure 5; P > 0.05; unpaired t-test). Similarly, there was no difference in the mean peak amplitude of CMMCs evoked in intact preparations, compared with those evoked following removal of the mucosa and submucosal plexus (control: 6.6 ± 1.7 g; Mucosa off 4.8 ± 1.4, NS). Taken together, these results suggest that once initiated by stretch, evoked CMMCs in intact segments of colon are indistinguishable from CMMCs evoked in preparations devoid of mucosa and submucosal plexus.

EFFECTS OF MUCOSAL STIMULATION IN THE ABSENCE OF CIRCUMFERENTIAL STRETCH

The experiments above showed that acute circumferential stretch of sheet preparations of colon, without direct compression of the mucosa, readily evokes CMMCs. However, we sought to determine if direct mucosal stimulation (without circumferential stretch) would also evoke CMMCs. To do this, we recorded spontaneous CMMCs in isolated full length sheet preparations of whole colon, with the mucosa present and facing uppermost. Once stable spontaneous CMMCs were recorded, we applied three brush strokes to the mucosa, using a fine artists paint brush, as previously described (Spencer et al., 1999). Overall, we found CMMCs rarely evoked by direct mucosal stimulation. In only 2 of 50 trials (N = 9) was a CMMC evoked in the distal colon which propagated to the proximal colon. Commonly no response was evoked (Table 1). In fact, in 32 of these 50 trials, no contractile response was elicited (see Table 1; N = 6). In the two trials where mucosal stimulation did evoke a CMMC, we removed the mucosa from the site of stimulation to expose the underlying circular muscle. In this case, reapplication of the identical mucosal stimulus to the circular muscle revealed that a CMMC could still be evoked, suggesting
that the initial response was unlikely to be due to the mucosal stimulation, but rather inadvertent stimulation of the underlying myenteric plexus or muscularis externa.

**DISCUSSION**

The aim of this study was to determine if the mucosa and submucosal plexus were required for the generation and propagation of CMMCs evoked by circumferential stretch. The major finding of the current study shows that circumferential stretch, but not mucosal stimulation, readily evokes CMMCs in isolated mouse colon; and that removal of the mucosa and submucosal plexus does not prevent their initiation or propagation following circumferential stretch. Mucosal stimulation alone, in the absence of

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**Table 1** | Proportion of mucosal stimuli that evoked a local or propagating contraction, or no response.

| Animal | Mucosa on | No contraction | Local contraction | Propagated to mid colon | Propagated to proximal colon |
|--------|-----------|----------------|------------------|------------------------|-----------------------------|
| Animal 1 | Mucosa on | 7  | 3  | 1   | 2   | 1  |
| Animal 2 | Mucosa on | 5  | 1  | 3   | 0   | 1  |
| Animal 3 | Mucosa on | 5  | 1  | 0   | 4   | 0  |
| Animal 4 | Mucosa on | 3  | 1  | 2   | 0   | 0  |
| Animal 5 | Mucosa on | 6  | 2  | 3   | 1   | 0  |
| Animal 6 | Mucosa on | 5  | 5  | 0   | 0   | 0  |
| Animal 7 | Mucosa on | 5  | 5  | 0   | 0   | 0  |
| Animal 8 | Mucosa on | 6  | 6  | 0   | 0   | 0  |
| Animal 9 | Mucosa on | 8  | 8  | 0   | 0   | 0  |
stretch rarely evoked CMMCs (2 of 50 trials) and proved highly unreliable.

**POSSIBLE MECHANISMS UNDERLYING EVOKED CMMCs**

Under normal physiological conditions *in vivo*, when an intraluminal distention stimulus is applied to the gut wall, it imposes at least two major types of stimuli to the gut wall. Firstly, it distorts and compresses the mucosa, which is known to stimulate release of many different substances such as 5-HT, and secondly, distension of the gut wall imposes stretch to the muscularis externa and enteric ganglia. Whether one, or both of these different types of stimuli are required for the initiation of distension-evoked CMMCs was unknown and had been difficult to reconcile. The experimental approach we employed in flat sheet preparations was chosen to test independently whether direct mucosal compression or stretch were able to evoke CMMCs.

**CURRENT UNDERSTANDING ABOUT 5-HT RELEASE FROM EC CELLS DURING INTESTINAL MOTOR PATTERNS**

It has been well characterized that endogenous 5-HT is released from EC cells in both the small intestine (Bullbring and Lin, 1958; Bertrand, 2006) and colon (Foxx-Orenstein et al., 1996; Grider et al., 1996) following mechanical stimulation of the mucosa. These findings have led to the popular conclusion that release of 5-HT from EC cells following luminal distension is responsible for the initiation of peristalsis in small intestine (Bullbring and Lin, 1958) and colon (Grider et al., 1996; Kadowaki et al., 1996; Jin et al., 1999) or CMMCs in mouse colon. Until recently, no studies had ever actually recorded release of 5-HT from the mucosa to verify these hypotheses. Recent studies have now used real time amperometry to record 5-HT release directly from the mucosa. When the first direct recordings of 5-HT release were made from the mouse colon (Keating and Spencer, 2010), it was found that 5-HT is released as a consequence of the contractions underling CMMCs and that any mucosal release of 5-HT is not their underlying cause. This was demonstrated when recent studies showed that removal of the mucosa abolished all 5-HT release, but did not abolish spontaneous CMMCs (Keating and Spencer, 2010). Similar conclusions have now also been demonstrated for peristalsis in the guinea-pig colon, where removal of the mucosa and submucosal plexus in tubular preparations abolished all release of 5-HT, but did not prevent peristalsis, nor the natural propulsion of fecal pellets along the colon (Spencer et al., 2011). These conclusions are entirely consistent with the work of Bertrand (2006) who also concluded that EC cells released 5-HT as a consequence of contraction induced during peristalsis and deformation of the ECs, rather than the EC cells releasing 5-HT to initiate peristalsis.

**PREVIOUS HYPOTHESES REGARDING STRETCH-EVOKED CMMCs IN MOUSE COLON**

It was recently concluded that distension of the mouse colon activates CMMCs by releasing 5-HT from EC cells, and that this release of 5-HT was suggested to activate sensory nerve endings in the mucosa itself (Heredia et al., 2009). These conclusions were not supported by any recordings of 5-HT release (Heredia et al., 2009). If these conclusions are accurate, then removal of the mucosal layer would be expected to prevent distension or stretch from evoking CMMCs. We found that following removal of the mucosa and submucoosal plexus, circumferential stretch still readily evoked CMMCs. This suggests that the mucosa itself, or release of substances from the mucosa are not required for stretch-evoked or spontaneous CMMCs. In further support of this, when we applied direct mucosal stroking to the mouse colon (in the absence of acute stretch or distension), CMMCs were rarely evoked (2 of 50 trials from nine animals) and on the two occasions they were evoked, they could still be evoked by stimulating the same site after removal of the mucosa. Taken together, these results strongly suggest that distension of the mouse colon activates stretch-receptors in the myenteric plexus and/or muscularis externa and that activation of these stretch-receptors do not require release of substances from the mucosa, nor could it be possible that they require activation of sensory nerve endings in the mucosa, or submucosal ganglia. We confirmed that no mucosa was present in our preparations by immunohistochemical staining for H and E which showed that not only the mucosa, but the entire submucous plexus had been excised. In these preparations, we showed previously that all detectable release of 5-HT was prevented (Keating and Spencer, 2010). In support of our conclusions, the recent work of Gershon and colleagues has shown that selective blockade of 5-HT release from EC cells does not have any effect on gastric emptying or colonic motility in live mice (Yadav et al., 2010). Our recent findings in the guinea-pig colon are also highly consistent with our findings in this current study; and the work of Gershon and colleagues. In guinea-pig distal colon we also recently showed that removal of the mucosa or submucosal plexus did not prevent peristalsis, nor prevent fecal pellet propulsion evoked by natural distension (fecal pellets; Spencer et al., 2011).

We are unable to explain how mucosal stimulation in the past has been claimed to evoke CMMCs (Heredia et al., 2009). It is presumed that by stroking the mucosa with a brush activates only sensory nerve endings in the mucosa. However, forceful compression of the mucosa also inadvertently compresses the underlying myenteric ganglia, which in itself is known to be a potent stimulus for activating intrinsic neural circuitry (Spencer et al., 2003). We found that mucosal stimulation rarely evoked a CMMC and in the two of 50 trials where a CMMC was evoked, it continued to do so after removal of the mucosa and submucosal plexus when the stimulus was applied to the circular muscle. Taken together, we found no evidence that mucosal stimulation alone reliably evokes CMMCs.

**INTRINSIC SENSORY NEURONS AND EVOKED CMMCs**

A number of major types of mechanosensitive enteric neurons have been described, including myenteric and submucosal Dogiel type II neurons (Furness et al., 1998) and different classes of Dogiel type I neurons (Spencer and Smith, 2004; Mazzuoli and Schemann, 2009). Of these sensory neurons, Dogiel type II cells have been shown to project to the lamina propria and mucosa (Song et al., 1994) and are potently activated by exogenous 5-HT applied to their cell bodies (Wood and Mayer, 1979) and mucosal terminals, largely via 5HT1 receptors (Bertrand et al., 2000). The results of the present study suggest that the submucosal...
intrinsic primary afferent neurons are not required for the initia-
tion or propagation of CMMCs in mouse colon. It is likely they are more closely involved in reflexes activated by nutri-
ents (Gwynne and Bornstein, 2007) or in secretomotor reflexes. The sensory neurons that are activated by distension must be located within the outer muscle layers (myenteric plexus) of the colon, and are sufficient to trigger and maintain fully functional CMMCs. Whilst there is sound evidence that mucosally projecting Dogiel type II neurons in the myenteric plexus are intrinsic sen-
sory neurons (Bornstein et al., 2004; Bornstein, 2006), our results suggest that activation of the mucosally projecting processes of Dogiel type II neurons are clearly not required for spontaneous or evoked CMMC generation in mouse colon, nor the initia-
lation of colonic peristalsis in guinea-pig colon (Spencer et al., 2011).

CONCLUSION

The findings of the current study show that circumferential stretch, but not mucosal stimulation reliably evokes CMMCs in isolated mouse colon. The mechanisms that initiate CMMCs in response to circumferential stretch do not require neurons in the submu-
cosal plexus, release of substances from the mucosa, or activation of nerve endings in the mucosa. The intrinsic sensory neurons that initiate stretch-evoked CMMCs lie in the myenteric plexus; and their mecanoreceptive terminals lie either in the myenteric ganglia and/or muscularis externa.

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