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RESEARCH PAPER

Regional- and agonist-dependent facilitation of human neurogastrointestinal functions by motilin receptor agonists

J Broad[1], S Mukherjee[1], M Samadi[1]*, JE Martin[1], GE Dukes[2] and GJ Sanger[1]

[1]*Neurogastroenterology group, Blizard Institute, Barts & The London School of Medicine and Dentistry, Queen Mary University of London, London, UK, and* [2]*Academic DPU, GlaxoSmithKline, Research Triangle Park, NC, USA*

Correspondence
Gareth J. Sanger, National Centre for Bowel Research and Surgical Innovation, 1st Floor, Abernethy Building, 2 Newark Street, London E1 2AT, UK. E-mail: g.sanger@qmul.ac.uk

*Present address: School of Biomedical Sciences, University of Edinburgh, Edinburgh, UK.

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BACKGROUND AND PURPOSE

Delayed gastric emptying is poorly managed. Motilin agonists are potential treatments but inadequate understanding into how enteric nerve functions are stimulated compromises drug/dose selection. Resolution is hampered by extreme species dependency so methods were developed to study human gastrointestinal neuromuscular activities and the neurobiology of motilin.

EXPERIMENTAL APPROACH

Protocols to study neuromuscular activities were developed for different regions of human stomach and intestine (71 patients) using circular muscle preparations and electrical field stimulation (EFS) of intrinsic nerves. Other tissues were fixed for immunohistochemistry.

KEY RESULTS

EFS evoked contractions and/or relaxations via cholinergic and nitrergic neurons, with additional tachykinergic activity in colon; these were consistent after 154 min (longer if stored overnight). Motilin 1–300 nM and the selective motilin agonist GSK962040 0.1–30 μM acted pre-junctionally to strongly facilitate cholinergic contractions of the antrum (E_{max} ≈ 1000% for motilin), with smaller increases in fundus, duodenum and ileum; high concentrations increased baseline muscle tension in fundus and small intestine. There were minimal effects in the colon. In the antrum, cholinergic facilitation by motilin faded irregularly, even with peptidase inhibitors, whereas facilitation by GSK962040 was long lasting. Motilin receptor immunoreactivity was identified in muscle and myenteric plexus predominantly in the upper gut, co-expressed with choline acetyltransferase in neurons.

CONCLUSIONS AND IMPLICATIONS

Motilin and GSK962040 strongly facilitated cholinergic activity in the antrum, with lower activity in fundus and small intestine only. Facilitation by motilin was short lived, consistent with participation in migrating motor complexes. Long-lasting facilitation by GSK962040 suggests different receptor interactions and potential for clinical evaluation.

LINKED ARTICLE

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Abbreviations

ChAT, choline acetyltransferase; EFS, electrical field stimulation; E_{max}, maximum response to agonist; L-NAME, N_{ω}-nitro-L-arginine methyl ester; MMC, migrating motor complex; NK, neurokinin; TTX, tetrodotoxin

Introduction

In humans, the gastrointestinal (GI) hormone motilin is found mostly in the duodenum, jejunum, and to a lesser extent, gastric antrum (Polak *et al.*, 1975), where it is secreted from the same cells as ghrelin (Wierup *et al.*, 2007) to mediate phase III of the gastric migrating motor complex (MMC; Vantrappen *et al.*, 1977; Sarna, 1985; also dogs: Lee *et al.*, 1983) and perhaps, influence hunger in both dogs and humans (Itoh *et al.*, 1975; Ang *et al.*, 2008). However, the mechanisms by which motilin stimulates GI motility are unclear. Firstly, although motilin receptor binding sites have been detected on both muscle and intrinsic nerves in the human gastric antrum (Miller *et al.*, 2000), most studies have focused on its ability to directly contract GI muscle in several different species (Lödtke *et al.*, 1989; Van Assche *et al.*, 2001; Sanger, 2008) and only recently have interactions with the GI nervous system begun to be investigated (e.g. Coulie *et al.*, 1998; Dass *et al.*, 2003). Secondly, major species differences in functions and expression of motilin and its receptor are now apparent (Sanger *et al.*, 2011) creating considerable doubt over the translational value of animal experiments. Finally, recent experiments with rabbit stomach suggest that the antibiotic drug and motilin agonist erythromycin (used to enhance gastric emptying in patients with gastroparesis; Maganti *et al.*, 2003) interacts with motilin receptors in a way which differs from motilin, generating markedly different abilities to increase enteric cholinergic activity (Dass *et al.*, 2003). These mechanism-, species- and ligand-dependent differences, described below, create a critical need to investigate the human GI neuromuscular biology of motilin.

The idea that erythromycin acts in two different, dose-dependent ways was suggested by experiments with healthy volunteers, where intravenous erythromycin (200 mg) evoked non-propagating, atropine-resistant contractions of the stomach (implying direct action on the muscle), whereas a lower dose (40 mg) stimulated propagating gastric motility, prevented by atropine (Coulie *et al.*, 1998). The possibility of a neuronally mediated activity was previously suggested by experiments with chicken isolated proventriculus (analogous to part of the mammalian stomach; Kitazawa *et al.*, 1997) and then in mammals by experiments with rabbit isolated stomach. In the latter, low concentrations of motilin and erythromycin greatly facilitated cholinergically mediated contractions evoked by electrical field stimulation (EFS), whereas higher concentrations contracted the muscle (Van Assche *et al.*, 1997; Dass *et al.*, 2003; Jarvie *et al.*, 2007). Interestingly, the effect of motilin faded quickly, even in the presence of peptidase inhibitors, whereas the activity of erythromycin was sustained for longer (Dass *et al.*, 2003; Jarvie *et al.*, 2007). The short-lasting activity of motilin seemed consistent with its role in mediating phase III MMC activity, but if motilin agonists are to be useful gastro-prokinetic drugs, they might need to mimic the longer-lasting action of erythromycin and not the short actions of motilin. However, before extrapolating rabbit data to humans, it is essential to appreciate the extreme species-dependent variations in motilin receptor functions and expression.

To date, only a motilin receptor pseudogene has been identified in rodents; attempts to identify motilin receptors in genomic DNA databases or clone the cDNA of motilin or its receptor have been unsuccessful (He *et al.*, 2010; Sanger *et al.*, 2011). This absence has been linked to evolution of specialized rodent gastric physiology, involving loss of an emetic reflex (Sanger *et al.*, 2011). Curiously, lagomorphs (exemplified by the rabbit, commonly used to study motilin) are the only other mammalian order lacking an emetic reflex. However, rabbits have an unusual reliance on coprophagia (re-ingestion of feces) for cellulose digestion, suggesting retention of motilin to help promote defecation of the hard fecal pellets, which follow the initial excretion and re-ingestion of partly digested feces (Costa *et al.*, 1997; Sanger *et al.*, 2009). This contrasts with humans where motilin agonists have inconsistent effects on lower bowel functions (Jameson *et al.*, 1992; Sharma *et al.*, 1995; Bassotti *et al.*, 1998; Emmanuel *et al.*, 2004; Venkatasubramani *et al.*, 2008). Together, these marked species differences in motilin functions, along with similarly marked differences in motilin potency at dog and human motilin receptors (Ohshiro *et al.*, 2008; Leming *et al.*, 2011), strongly suggest a need to define the activities, mechanisms and longevity of actions of motilin receptor agonists in humans.

Studies on motor nerve functions in human-isolated stomach are uncommon (Tonini *et al.*, 2000; Leclere and Lefebvre, 2002; Tomita *et al.*, 2007), so there was first a need to refine experimental protocols and minimize variations, inherent in research using human tissue. Further, instead of comparing the actions of motilin and erythromycin (a non-selective motilin receptor agonist, also inhibiting neuronal and purinergic P2X channel functions; Furness *et al.*, 1999; Zhao *et al.*, 2000), comparisons were made with the highly selective motilin receptor agonist GSK962040 (Sanger *et al.*, 2009; Leming *et al.*, 2011). The use of this molecule minimizes the likelihood that any differences in actions between motilin and GSK962040 can be attributed to mechanisms unrelated to the motilin receptor. The results reveal significant regional and agonist-dependent differences in how motilin agonists stimulate human GI cholinergic activity, and help define the potential clinical use of selective motilin agonists as treatments of disorders associated with delayed gastric emptying.

Methods

Nomenclature

The term 'motilin receptor' has been used and not the earlier names 'GPR38' or 'MTLR1', in accordance with Alexander *et al.* (2011).

Human tissues

Segments of stomach, terminal ileum and colon were obtained from patients undergoing surgery for obesity (stomach) or cancer (stomach, intestine). Tissues from cancer patients were macroscopically normal. The study was approved by the local ethics committee and written informed consent was obtained from all patients. Tissues were transferred to the laboratory within 2 h after resection in Krebs' solution (mM: NaCl 121.5, $CaCl_2$ 2.5, KH_2PO_4 1.2, KCl 4.7,

$MgSO_4$ 1.2, $NaHCO_3$ 25, glucose 5.6) equilibrated with 5% CO_2 and 95% O_2.

Immunohistochemistry and immunofluorescence

About 3 μm formalin-fixed segments of stomach and intestine were used. Peroxidase and avadin/biotin blocking was performed (Vector Laboratories, manufacturer's instructions; see Supporting Information Appendix S1 for details) and sections were incubated overnight at room temperature with the motilin receptor (GPR38) rabbit anti-human primary antibody (1:1200) (MBL International Corporation, MA, USA), alongside no primary antibody and blocking peptide controls. In dual-labelling experiments, sections were then incubated for 40 min with neurofilament protein 2F11 mouse anti-human primary antibody (1:800; Dako, Ely, UK). Peroxidase activity was developed using Nova red substrate kit (Vector Laboratories, Peterborough, UK) and 3′-diaminobenzidine detection kits (Biogenex, CA, USA) respectively. For immunoflourescence, the sections were incubated overnight at 4°C with the motilin receptor primary antibody (1:1000) and sheep anti-human choline acetyltransferase (ab18736; Abcam; 1:1000). The slides were then incubated with Alexa Fluor 568 donkey anti-rabbit IgG (Dako; diluted 1:400) and Dy Light 488 conjugated Affini pure donkey anti-sheep IgG (Jackson Immunoresearch, West Grove, PA, USA; 1:2000) for 30 min. The slides were treated with 4′,6-diamidino-2-phenylindole (Sigma, Gillingham, UK) and visualized with an epifluorescence microscope (Leica Microsystems, Bucks, UK).

Functional studies

Tissues were used immediately or after overnight storage at 4°C in fresh, oxygenated Krebs' solution. On arrival in the laboratory, the mucosa, muscularis mucosa and submucosal plexus, was removed by blunt dissection and discarded. Strips (3–5 × 15 mm) were cut approximately parallel to the circular muscle fibres (from intertaenia regions in colon) and mounted in tissue baths containing Krebs' solution at 37°C, gassed with 5% CO_2 in O_2. Changes in muscle tension were recorded using pre-calibrated isometric force transducers (AD Instruments, Chalgrove, UK) on a data acquisition system (Biopac Inc., CA, USA). The strips were given 2 g tension and allowed to recover for 60 min, changing bath solutions every 15 min. Thereafter, the strips were stimulated via two parallel platinum ring electrodes connected to a stimulator (STG2008, Scientifica, Uckfield, UK). Stimulation parameters were 50 V (c. 200 mA), 0.5 ms bipolar pulse duration, 5 Hz (unless otherwise specified), given for 10 s every 1 min. EFS was applied continuously until consistent responses were obtained (bath solution changed every 15 min). Following establishment of consistent responses, frequency–response curves were constructed in most tissues (1, 2, 5, 10, 15, 20 Hz, each delivered for 10 s every minute). After changing bath solutions, consistent responses to 5 Hz were re-established before further intervention. In other experiments to determine the muscle tension under which maximum responses to EFS were obtained, consistent responses to EFS were first established, before lowering the muscle tension to zero and re-establishing consistent responses to EFS; this procedure was then repeated for 1, 1.5, 2, 3 and 5 g tensions.

The effects of motilin and GSK962040 were investigated using single concentrations in each strip. Changes in amplitude of responses to EFS were determined by measuring at least three EFS-induced responses at a given time point, expressed as a percentage of the mean of at least three pretreatment EFS-induced responses. Changes in baseline tension were expressed as a % of the pretreatment EFS-induced contraction. To investigate effects on contractions evoked by carbachol, a preliminary concentration–effect curve was established (not shown) and 1 μM carbachol was selected as the concentration causing ~50% of maximum contraction. After obtaining consistent responses with 1 μM carbachol (5 min contact, repeated at 15 min intervals), motilin or GSK962040 was added 15 min before the last application.

Data analysis

Data are expressed as medians and ranges or means ± SEM; n values are numbers of patients. Differences between medians were determined using the Mann–Whitney U-test and differences between means were determined using Student's t-test for unpaired observations; $P < 0.05$ was considered statistically significant.

Materials

All drugs were freshly prepared before use. Motilin (Tocris, Abingdon, UK) was dissolved in distilled water at 100 μM. GSK962040 ((N-(3-fluorophenyl)-1-[(4-{[(3S)-3-methyl-1-piperazinyl]methyl}phenyl)acetyl]-4-piperidinamine) was dissolved in distilled water to 1 mM. Carbachol, atropine, N^{ω}-nitro-L-arginine methyl ester hydrochloride (L-NAME; Sigma) and tetrodotoxin (TTX) (Tocris, UK) were each dissolved in distilled water. L732138 (Tocris) was dissolved in DMSO (Sigma) to 10 mM; GR159897 (Tocris) and SB235375 (GSK) were dissolved in ethanol to 10 mM. Stock solutions of captopril and phosphoramidon (10 mM) and of thiorphan (5 mM) were prepared in distilled water and of chymostatin (2.5 mM) in DMSO (Sigma).

Results

Motilin receptor immunoreactivity

Motilin receptor immunoreactivity was identified in all regions of stomach and intestine, particularly the upper gut. Punctate labelling was observed in longitudinal and circular muscle, and in the myenteric plexus (Figure 1). In the myenteric plexus this was detected mostly as punctate labelling of neuronal cell body membranes, with some additional immunoreactivity within the cytosol. Not all neuronal cells were labelled and immunoreactivity was not detected in nerve axons labelled by neurofilament 2F11, either in the myenteric plexus or the muscle layers of the antrum (data not shown).

Within the myenteric plexus of the stomach, motilin receptor immunoreactivity was co-localized with choline acetyltransferase (ChAT) (Figure 1). Of the 53 cells positive for ChAT in the antrum, 35 (66%) also expressed motilin receptors (7 ganglia, $n = 3$). In the fundus 28/55 (51%) of cells positive for ChAT also expressed motilin receptors (9 ganglia, $n = 3$).

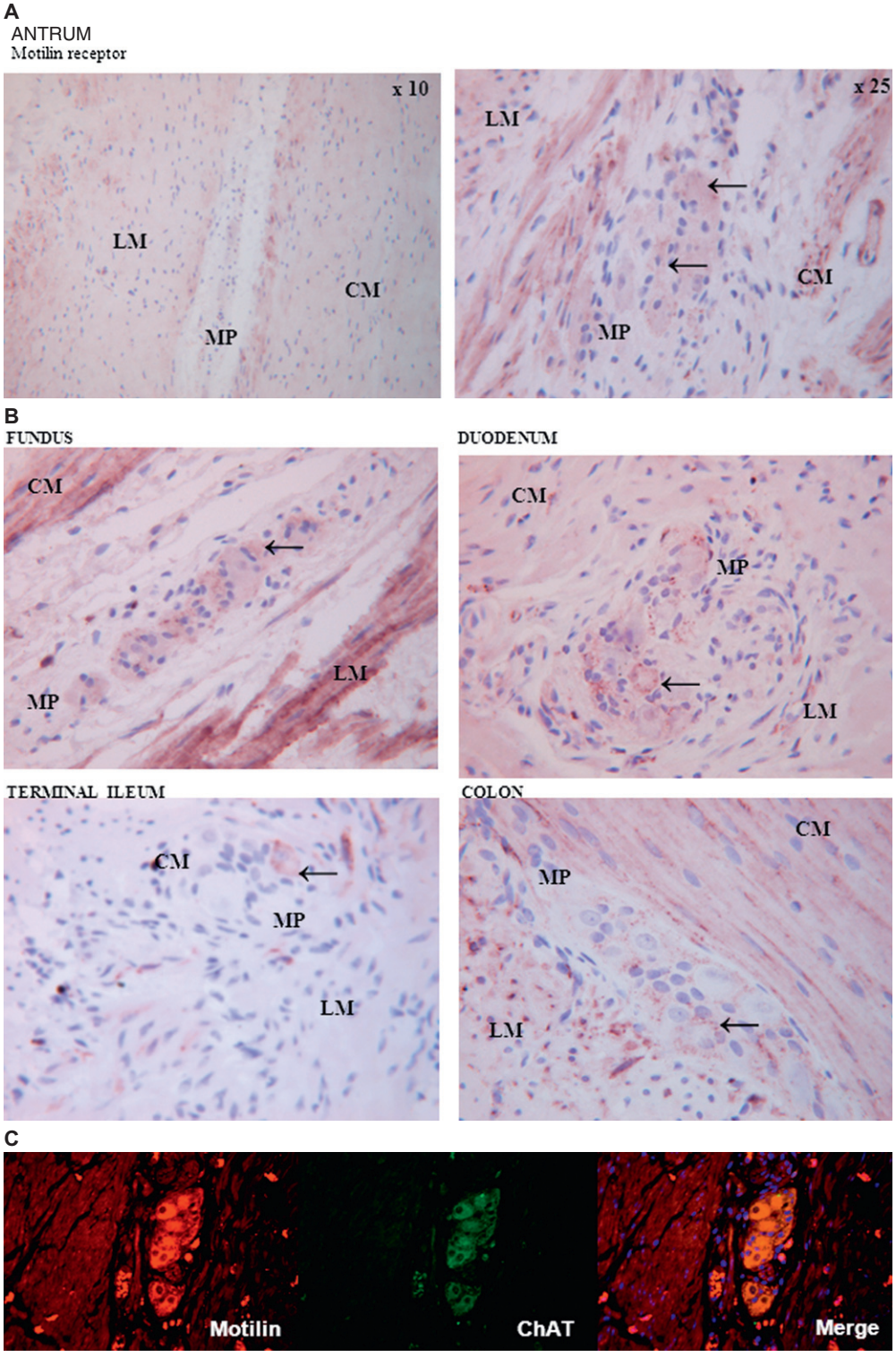

Figure 1

Motilin receptor immunoreactivity in the human gastrointestinal tract. The upper panel (A; immunohistochemical studies) illustrates the distribution of motilin receptors (stained in red) to the longitudinal and circular muscle layers of the antrum, and to the myenteric plexus. Illustrations are ×10 and ×25. Ganglion cells of the plexus show some granular peripheral staining for the receptor (examples highlighted by arrows). Staining was not observed with the negative control (no primary antibody; not shown). Panel (B) shows motilin receptor immunoreactivity in gastric fundus, duodenum, terminal ileum and colon. Illustrations are ×25 magnification. Panel (C; immunofluorescence studies) shows an expanded view of the co-localization of motilin receptors with ChAT immunoreactivity in the antrum. LM = longitudinal muscle, CM = circular muscle, MP = myenteric plexus.

Human neuropharmacology

Table 1 summarizes the patient details. For tissues studied on the day of surgery, consistent responses were achieved after 92–314 min; recovery times did not appear influenced by GI region. For tissues stored overnight, recovery was usually longer (103–425 min), but responses to EFS appeared similar. For example, contractions evoked by EFS in antrum from obese patients studied on the day of surgery or after overnight storage, generated respectively, 0.65 ± 0.12 and 0.45 ± 0.08 g tension; n = 9, 18; P = 0.17.

Following recovery, stimulation at 1–20 Hz evoked different responses in different regions (Figure 2), consisting of frequency-dependent, monophasic contractions (both stomach regions) and frequency-dependent relaxations and contractions followed by 'after-contraction' on termination of EFS (small and large intestines). In subsequent experiments only 5 Hz was used, because at this frequency responses were submaximal (stomach) or represented each phenotype observed throughout the frequencies examined. Data obtained using fundus from obese and cancer patients were pooled after analysis of contractions to 5 Hz EFS showed that similar tension was developed in each group [2.11 ± 1.08 g (g wet weight)$^{-1}$ of tissues from obese (0.32 ± 0.05 g wet weight) and 1.79 ± 0.40 g (g wet weight)$^{-1}$ in tissues from cancer patients (0.26 ± 0.02 g wet weight); n = 4 each, $P > 0.05$]. Analysis of contractions to EFS (5 Hz) under different baseline tensions showed that responses reached maximum at around 2 g in both fundus and antrum, so 2 g was used for all tissues (1.20 ± 0.33, 1.43 ± 0.75, 1.36 ± 0.34 g (g wet weight)$^{-1}$ was the tension developed under 2 g load in, respectively, antrum from obese patients, fundus from obese patients and fundus from cancer patients, n = 4, 4, 6).

In each region, responses to EFS were prevented by application of 1 μM TTX (respectively n = 4, 4, 3, 4, 3 for fundus, antrum, duodenum, terminal ileum, colon). In the fundus and antrum (Figure 3), the contractions were prevented by 1 μM atropine (n = 3 and 3 respectively) and increased by 300 μM L-NAME (respectively by 11 ± 7% and 57 ± 17%, n = 14 and 15). In duodenum and terminal ileum, contractions during EFS were prevented by 1 μM atropine (n = 2, 4) and relaxations during EFS were prevented by 300 μM L-NAME, a procedure which also facilitated or revealed contractions during EFS (n = 1, 4). In terminal ileum, the after-contractions that followed termination of EFS were prevented by atropine. In the colon (Figure 3), the large after-contractions that followed termination of EFS were clearly reduced by atropine (56 ± 12% n = 3) and then decreased further by the additional application of neurokinin (NK_{1-3}) antagonists L732138 1 μM, GR159897 0.1 μM and SB235375 0.1 μM (34 ± 19% n = 3).

Gastric antrum

Motilin (1–300 nM) concentration-dependently facilitated EFS-evoked contractions (Figures 4, 6), with a maximum response to agonist (E_{max}) of 1041 ± 592% and pEC_{50} of 7.5 ± 1.3 (n = 3–5, each concentration); these values were not significantly changed by addition of 300 μM L-NAME (E_{max} 623 ± 273%, pEC_{50} 7.5 ± 1.0; $P > 0.05$ each; n = 3–5). Having achieved maximal activity, the response to motilin at concentrations ≥10 nM faded quickly during its continuous presence [100 nM motilin achieved peak activity in 18.2 ± 5.1 min, fading by 50% of maximum ($t^1/_2$) in 12.8 ± 2.6 min; n = 5]; the presence of protease inhibitors (10 μM thiorphan, captopril and chymostatin plus 100 μM phosphoramidon) had no significant effects on this response (18.0 ± 4.2 and 9.6 ± 4.2 min, respectively, for time to peak and $t^1/_2$ for 100 nM motilin; n = 4; $P > 0.5$). Interestingly, at 300 nM motilin, the decline in contractile amplitude did not occur uniformly, with small contractions occurring in-between much larger contractions. The times taken for the maximum response induced by 300 nM to decline by 50% were 15 ± 4 min if the small contractions were measured, and 26 ± 4 min when measuring the larger contractions (n = 3).

At the higher concentrations of motilin (100, 300 nM), two other effects were observed. Firstly, baseline muscle tension increased, equivalent to, respectively, 68 ± 54% and 98 ± 74% of the contraction amplitude to EFS before the addition of motilin (n = 5, 3; Figure 4). Secondly, there was a small, short-lived decrease in magnitude of EFS during the first 3 min after addition of motilin (by 6.9 ± 4.9% initial EFS with 300 nM; n = 3; Figure 4) and before the much larger increase in contractions. This was not observed in the presence of 300 μM L-NAME (300 nM, n = 3). Finally, motilin (100 nM) had no significant effects on contractions evoked by carbachol (1 μM), which were 3.7 ± 7.9% greater than before motilin addition (n = 3, $P > 0.05$).

GSK962040 (0.1–30 μM) concentration-dependently increased EFS-evoked contractions (Figures 4, 5), with an apparent E_{max} of 810 ± 406% and estimated pEC_{50} of ~4.8 (n = 3–4, each concentration); poor compound solubility prevented testing higher concentrations. The time to reach maximum was slower than for motilin (35.0 ± 7.2 min) and the response did not consistently fade during the remainder of the experiment (70 min total time; Figures 4, 5). In the presence of 300 μM L-NAME, the estimated E_{max} (392 ± 229%; n = 3–4) and apparent pEC_{50} (~5.0) for GSK962040 were not significantly different to those observed in the absence of L-NAME.

No concentration of GSK962040 caused an initial reduction in amplitude of EFS-evoked contractions (e.g. during the first 3 min after addition of GSK962040 30 μM, contractions were –0.8 ± 7.1% of that caused by EFS before its addition; n = 3). The highest concentrations of GSK962040 did not consistently change baseline muscle tension (which were, respectively, –37 ± 62% and –39 ± 61% of the contractions to EFS at 10 and 30 μM; n = 4, 3) (Figure 4). GSK962040 (30 μM) had no effects on the contractions evoked by carbachol 1 μM (contractions were –1.3 ± 6.1% of those before addition of GSK962040, n = 4, $P > 0.05$).

Gastric fundus

In each patient tested, motilin (1–300 nM) caused a small concentration-dependent facilitation of EFS-evoked contractions (Figures 4, 5), with an E_{max} of 124 ± 70% and pEC_{50} of 6.7 ± 0.5 (n = 3–5, each concentration). This activity faded evenly during the continuous presence of motilin ($t^1/_2$ = 17 ± 5 min). There was no apparent difference between the responses to motilin in tissues from obese and cancer patients; for example, 300 nM motilin caused 63 and 96% enhancement of EFS in obese fundus (n = 2), and 9, 20 and 115% enhancement of EFS in cancer fundus (n = 3). The highest concentration of motilin increased muscle tension (300 nM; 124 ± 27% of EFS; n = 5). Decreases in amplitude of EFS-evoked contractions were not observed at any time. In

Table 1

Patients and tissues used in functional studies

Summary					
				Time (minutes) to achieve consistent responses	
Region (total *N*)	**Reason for removal**	**Male : female ratio**	**Age (years)**	**Used on day of surgery**	**Used after overnight storage**
Stomach (23)	Bariatric surgery for obesity	1:1.9	47 (25–86)	154 (92–314) (n = 17 patients, 128 strips)	222 (103–425) (n = 58 patients, 311 strips) $P < 0.01$ compared with tissues used on day of surgery
Stomach (25)	Esophagogastric cancer	1:0.5	63 (40–87)		
Small and large intestines (23)	Ileo-colonic resection for cancer	1:1.3	66 (18–88)		

Individual data						
Region (*N*)	**Disease**	**Fresh/overnight**	***N* (strips)**	**Male : female ratio**	**Age (years)**	**Recovery time (min)**
Fundus (34)	Obesity	Fresh	7 (25)	1:2.5	56 (43–75)	114 (86–230)
		Overnight	10 (25)	1:9	48 (27–57)	181 (123–315)
	Cancer	Fresh	1 (1)	F	85	309
		Overnight	18 (59)	1:0.4	65 (49–87)	247 (137–337)
Antrum (23)	Obesity	Fresh	9 (64)	1:2	51 (30–72)	163 (104–314)
		Overnight	17 (82)	1:1.3	47 (25–86)	188 (103–411)
	Cancer	Overnight	2 (7)	F	40, 75	337, 425
Duodenum (4)	Cancer	Overnight	4 (8)	1:1	74 (61–75)	336 (270–425)
Terminal ileum (7)	Cancer	Overnight	7 (44)	1:2.5	65 (29–77)	240 (215–355)
Ascending colon (8)	Cancer	Fresh	3 (13)	1:2	75 (67–77)	102 (92–137)
		Overnight	5 (19)	1:4	76 (60–88)	230 (191–299)
Transverse colon (1)	Cancer	Fresh	1 (3)	F	18	156
Descending colon (8)	Cancer	Fresh	2 (22)	1:1	53, 53	150, 164
		Overnight	6 (57)	1:0.2	70 (60–82)	179 (110–250)

Recovery times defined as the time to achieve consistent responses to 5 Hz EFS following suspension in tissue baths. Data expressed as medians (ranges in parenthesis). *N* = number of patients (numbers of strips in parenthesis); tissues from some patients were used on the day of surgery and after overnight storage. A minority of preparations failed to respond consistently to EFS and were discarded.

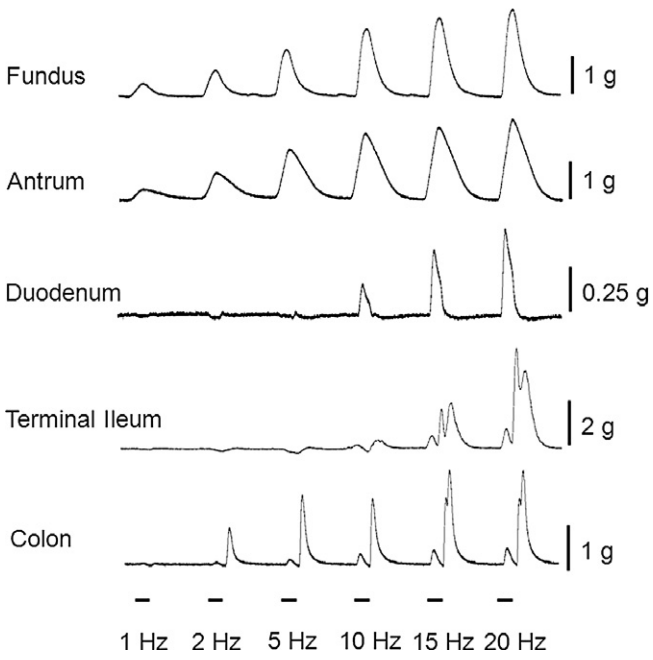

Figure 2

Circular muscle relaxations and contractions to EFS in human gastrointestinal tissues. Frequency-dependent, monophasic contractions were evoked during EFS in gastric fundus (110 strips; n = 34) and antrum (153 strips; n = 24). In duodenum and terminal ileum, relaxations (1–5 Hz; respectively 8/8 strips, n = 4 and 18/28 strips; n = 5) and contractions followed by relaxations (10–20 Hz; 5/8 strips; n = 4 and 22/28 strips; n = 5) were observed during EFS, followed by rapid 'after-contraction' on termination of EFS. In ascending and descending colon, contractions or relaxations occurred during EFS, usually followed by an after-contraction – for example, 5 Hz evoked contractions in 65% (ascending: 21/32 strips, n = 7/8) and 70% (descending: 60/79 strips, n = 8/8) of strips, and relaxations in 35% (ascending: 11 strips, n = 3/8) and 30% (descending: 19 strips, n = 4/8). After-contractions occurred in 27 (84%) and 67 (85%) of these strips. EFS (1–20 Hz, 50 V, 0.5 ms bipolar pulse duration) was applied for 10 s every 1 min, as indicated by the horizontal bar.

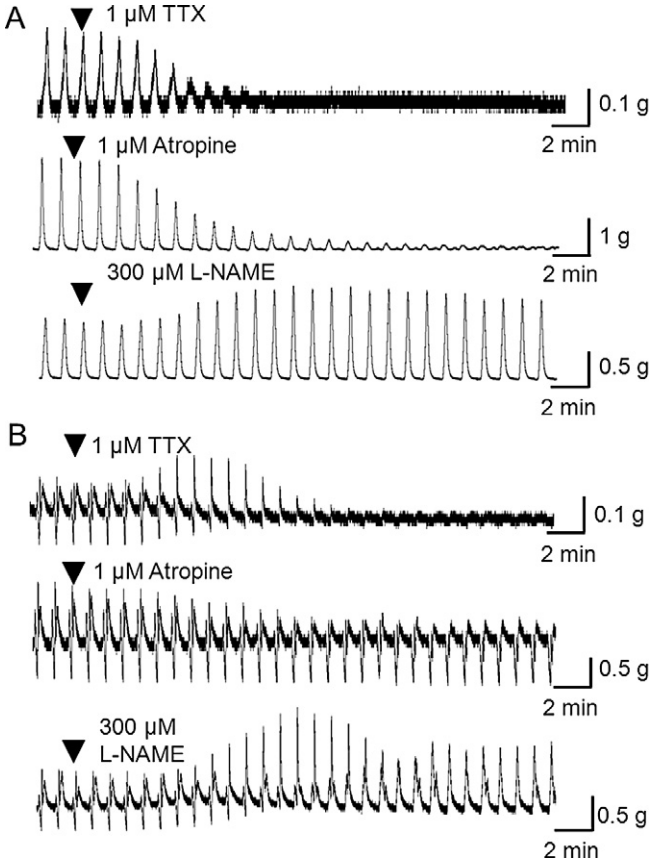

Figure 3

Experimental records illustrating the pharmacology of responses to EFS in human isolated gastric antrum and colon. All responses to EFS were prevented by 1 μM tetrodotoxin (TTX). In the antrum (A), the contractions were prevented by 1 μM atropine and increased by 300 μM L-NAME. In the colon (B), the contractions and relaxations during EFS were prevented by, respectively, 1 μM atropine or 300 μM L-NAME, the latter facilitating or revealing contractions. The minimum contact time for L-NAME, atropine and TTX was 30 min.

the presence of 300 μM L-NAME, the E_{max} (209 ± 120%) and pEC_{50} (7.4 ± 1.0) were not significantly different from those observed in its absence (n = 3–4).

In each patient tested, GSK962040 (0.1–30 μM) concentration-dependently increased EFS-evoked contractions (apparent E_{max} 98 ± 51%; estimated pEC_{50} ~4.8) without appearing to fade over time (n = 3–5, each concentration; Figures 4, 5). The highest concentration of GSK962040 also increased baseline muscle tension (by 63 ± 33% EFS at 30 μM; n = 5). In the presence of 300 μM L-NAME, the E_{max} (80 ± 84%) and estimated pEC_{50} (~4.8) were not significantly different from those observed in its absence (n = 3–4).

Small and large intestines

Only the actions of 300 nM motilin and 30 μM GSK962040 were evaluated. In the duodenum, motilin increased muscle tension (1272 ± 908% of the contractions evoked by EFS prior to addition of motilin), apparently increased EFS-induced relaxation (157 ± 41%), and induced the appearance of large-amplitude after-contractions; these actions of motilin faded over time during its continuous presence (20 ± 5 min; n = 3; Figure 6). The presence of L-NAME abolished relaxations to EFS, replacing these with contractions. Subsequent addition of motilin caused contraction of the muscle but now increased the contractions during EFS (Figure 6).

In terminal ileum, motilin evoked muscle contraction (by 365 ± 289% of EFS; n = 4), increased spontaneous contractile activity, and apparently increased EFS-induced relaxation (by 98 ± 66%, n = 4; Figure 6). These activities faded over time (10 ± 2 min, n = 4). The presence of L-NAME abolished relaxations to EFS (replacing with contractions; n = 6; Figure 6) and addition of motilin caused contraction of the muscle and increased EFS-evoked contractions (by 38 ± 16% of EFS; n = 3).

Motilin had no effect on responses to EFS in any region of the colon (n = 4, Figure 6). However, in 3/4 patients, motilin caused a small increase in baseline muscle tension (39 ± 23% of EFS in the three responders). GSK962040 30 μM did not

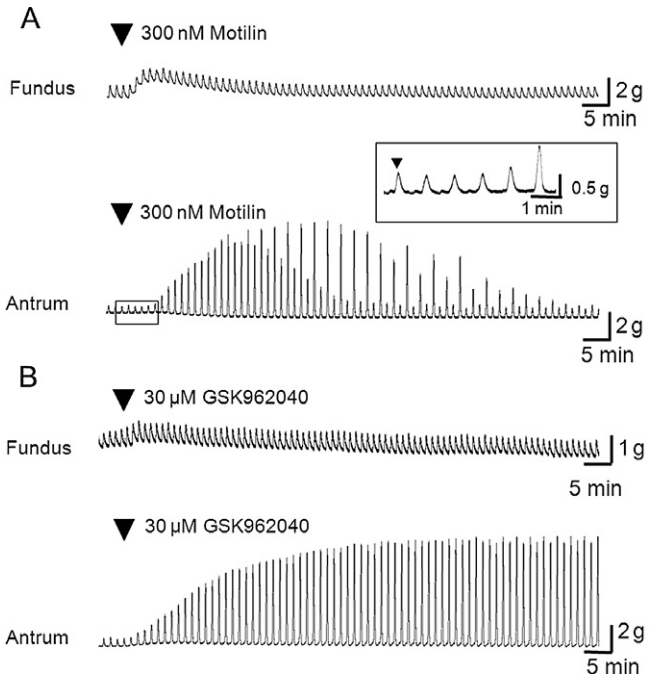

Figure 4

Experimental records showing the response of circular muscle strips from human gastric fundus and antrum to the continuous presence of motilin and GSK962040. The contractions are shown in response to EFS (50 V, 0.5 ms bipolar pulse duration, 5 Hz) given for 10 s, every 1 min. Inset: expanded trace showing initial inhibition of response and start of baseline contraction.

change baseline muscle tension or the EFS-evoked contractions (n = 4; not shown).

Discussion

Motilin receptor agonists have previously been shown to evoke short-lived muscle contractions (e.g. human stomach and colon; Lödtke *et al.*, 1989; Van Assche *et al.*, 2001). We report for the first time on a more potent, regionally dependent ability of motilin agonists to facilitate human GI neuromuscular functions. Further, the different motilin agonists acted differently, causing sustained or short-lived facilitation of cholinergic activity. Because such activity is likely to stimulate gastric motility, these findings have major implications in determining the type and dose of motilin agonist to treat patients with gastroparesis.

The study was conducted using human GI tissues, orientated in the direction of the usually thicker circular muscle, in which cholinergic-, nitrergic- and tachykininergic-mediated muscle contractions and relaxations were evoked by EFS. Critically, neuromuscular functions were shown to have recovered after surgery and collection. Further, the phenotypes of responses to EFS were shown to be consistent between tissues used immediately or after overnight storage. Others have successfully obtained functional responses in human GI tissues stored for 24 h but measurements of neuromuscular recovery times were not provided (experiments began after ~60–120 min, when muscle tone or spontaneous contractions were consistent; Gagnon *et al.*, 1972; Tonini *et al.*, 2000; Auli *et al.*, 2008). In the present study, consistent responses to EFS were achieved ~2.5 h after preparing tissues on the day of surgery or ~3.5 h for tissues stored overnight, irrespective of GI region.

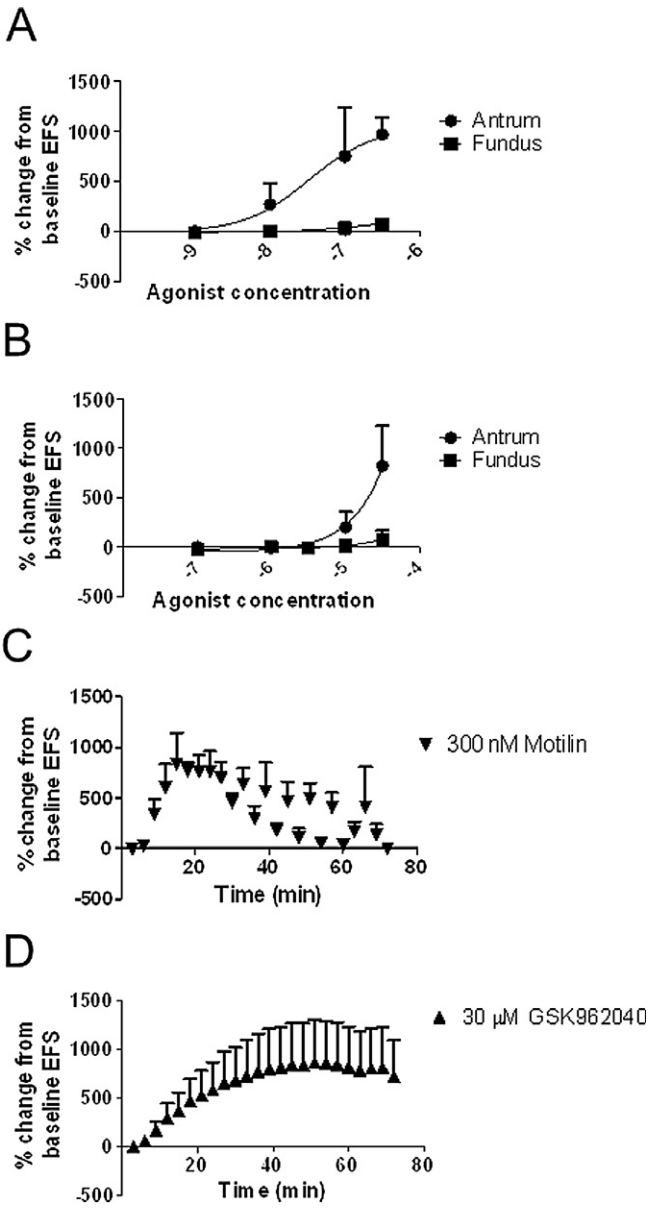

Figure 5

Effects of motilin and GSK962040 on contractions to EFS in circular muscle strips from human gastric antrum and fundus. Panels (A) and (B) show concentration–response curves for, respectively, motilin (1–300 nM) and GSK962040 (0.1–30 µM) (n = 3–5 each concentration). Panels (C) and (D) show the time-dependent changes in tension in response to EFS for the antrum following addition of, respectively, 300 nM motilin and 30 µM GSK962040 (n = 3). During fade of response to motilin, small contractions occurred in-between larger contractions. EFS (50 V, 0.5 ms bipolar pulse duration, 5 Hz) was given for 10 s, every 1 min. N = number of patients.

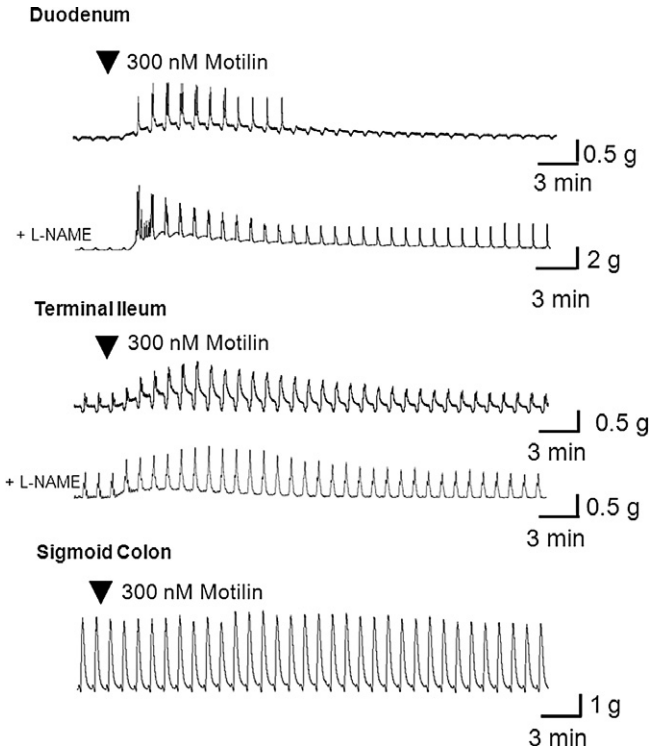

Figure 6

Experimental records of the effects of motilin in human duodenum, terminal ileum and sigmoid colon. Each experiment shows the effects of 300 nM motilin on muscle tension and on the responses of circular muscle strips to EFS (50 V, 0.5 ms bipolar pulse duration, 5 Hz, given for 10 s, every 1 min), in the absence or presence of L-NAME 300 μM.

Different regions of the GI tract responded differently to EFS. In the fundus and antrum, EFS evoked frequency-dependent, monophasic contractions caused by cholinergic activity dominating simultaneous activation of nitrergic inhibitory neurons. Tonini *et al.* (2000) found that EFS more readily activated the nitrergic system in the fundus, perhaps explained by the use of different stimulus intensities (respectively, EFS delivered over 10 s every min, compared with 5 s every 5 min). Regardless, both observations are broadly consistent with the dominant cholinergic and nitrergic presence in human fundus myenteric plexus (Pimont *et al.*, 2003). In the small and large intestines, EFS induced more complex, triphasic responses, via cholinergic, nitrergic and tachykininergic activity, consistent with previous reports (Maggi *et al.*, 1990; Cellek *et al.*, 2006; Auli *et al.*, 2008), although a small purinergic activation has also been observed in colon at low-frequency EFS (Auli *et al.*, 2008). The predominant cholinergic and nitrergic involvement is consistent with high numbers of cholinergic and nitrergic motor- and interneurons in human colon (Porter *et al.*, 2002).

For the first time, our experiments demonstrate that the major action of low concentrations of motilin is to facilitate cholinergically mediated contractions of human stomach circular muscle; broadly similar activity was previously reported using rabbit gastric antrum (Dass *et al.*, 2003), although some differences in actions were identified (see below). In the present study, motilin appeared to act at a pre-junctional site, as submaximally effective contractions to carbachol were unaffected. Notably, enhancement of cholinergic activity was considerably greater in the antrum (maximum ~1000%) compared with the fundus (~120%). This difference is consistent with the need for the antrum to generate more powerful phasic muscle contractions during gastric emptying and was not explained by the need for either region to be placed under difference initial tensions. Interestingly, the potency of motilin in the human gastric antrum was lower than that previously reported when tested at the human recombinant receptor (respectively, pEC_{50} values of 7.5 and 10.4; see Sanger *et al.*, 2009); perhaps this difference reflects the additional need for motilin to penetrate into the muscle and reach the motilin receptors on the cholinergic nerves.

The functional observations contrast with the distribution of immunoreactive motilin receptors in both muscle layers of the fundus and antrum, with smaller distribution to neurons of the myenteric plexus; these observations were previously reported for human stomach (region not defined; Takeshita *et al.*, 2006) and human antrum (Ter Beek *et al.*, 2008). However, in the functional experiments, only relatively high concentrations of motilin caused contraction of the muscle. Because receptor function depends on efficiency of coupling to downstream effector mechanisms, these data suggest that motilin receptors expressed by GI cholinergic neurons are better coupled than those on the muscle and hence, play a greater role in mediating the GI actions of motilin.

The ability of motilin to facilitate cholinergic activity was not sustained during its continued presence, even with addition of peptidase inhibitors. Interestingly, the response to the highest concentration of motilin (300 nM) faded unevenly, with small EFS-induced contractions occurring in between larger contractions. This pattern was not observed in previous studies with the rabbit isolated gastric antrum (Dass *et al.*, 2003) or in the other human GI regions, and why it occurs only in the antrum and only during the fading influence of motilin, is unclear. Groups of interstitial cells of Cajal controlling electrical rhythm in different muscle bundles can move out of synchrony with each other (Lee *et al.*, 2007), so perhaps it can be speculated that motilin induces a similar uncoupling. Regardless of the reason for the manner by which the response to motilin fades, it is tempting to speculate that the inability to evoke a sustained response is consistent with the proposed role for motilin in mediating phase III gastric MMC activity. However, the latter activity terminates abruptly as it moves along the GI tract (Vantrappen *et al.*, 1977) and does not 'fade', as in the present experiments. Perhaps the difference indicates that other mechanisms must also be involved in regulating the actual propagation of the MMC (Nakajima *et al.*, 2010).

In the duodenum and terminal ileum, motilin increased muscle tension and enhanced after-contraction amplitudes, suggesting prokinetic activity of motilin. The results were consistent with expression of motilin receptors in muscle layers and in the myenteric plexus, as previously reported in human ileum and colon (Feighner *et al.*, 1999; Ter Beek *et al.*, 2008). In the human colon, co-localization of motilin receptor mRNA has also been reported with NO synthase and to a lesser extent, ChAT (Feighner *et al.*, 1999). In the present

studies, some motilin receptor immunoreactivity was identified in the colon, but motilin had no effects on responses to EFS and only small, inconsistent ability to increase muscle tension; GSK962040 had no effects. Studies with erythromycin or other motilin receptor agonists on human colonic motility *in vivo* are equivocal, suggesting no activity (Jameson *et al.*, 1992; Venkatasubramani *et al.*, 2008), increased colonic motility (Bassotti *et al.*, 1998) or shortening of colonic transit time (Sharma *et al.*, 1995). Nevertheless, motilin receptor agonists have been reported to facilitate neuronally mediated contractions in colon from patients with idiopathic chronic constipation (Chieppa *et al.*, 2000). Perhaps, therefore, motilin will have some direct effect on the colon in certain patients, so further studies are warranted.

GSK962040 acted similarly to motilin, considerably enhancing cholinergic function in the antrum and to a lesser extent, in the fundus. As for the experiments with motilin (see above), the potency of GSK962040 was lower than when tested at the recombinant human receptor (pEC_{50} values of 7.9 and ~4.8 respectively; see Sanger *et al.*, 2009), perhaps explained by the additional need to penetrate into the isolated tissue. Further, when compared with motilin in the present experiments, the effective concentrations of GSK962040 were higher, as predicted by the lower potency of this molecule for the human motilin receptor (Sanger *et al.*, 2009). However, in contrast to the fading response to motilin in the antrum, the effect of GSK962040 was long lasting. Similar long-lasting activity has previously been observed in rabbit antrum with GSK962040 and erythromycin (Dass *et al.*, 2003; Jarvie *et al.*, 2007; Sanger *et al.*, 2009). Together, these experiments argue for at least two different ways in which the human motilin receptor can be activated. One possible explanation is that different motilin receptor agonists bind to different sites on the receptor in its native environment, influencing different downstream mechanisms (Sanger, 2008). Another possibility relates to the existence of an agonist-dependent desensitization of the motilin receptor, as reported in both recombinant receptor systems and smooth muscle preparations (Thielemans *et al.*, 2005). However, desensitization studies using smooth muscle preparations do not predict the ability of erythromycin to facilitate cholinergic activity in a long-lasting manner (Dass *et al.*, 2003). Further, studies with the recombinant motilin receptor do not necessarily predict the ability of motilin receptor agonists to promote short- or long-lasting effects in man (Westaway and Sanger, 2009). As such, the mechanisms for different neuronal activities of different motilin receptor agonists remain unclear.

Finally, the results imply that optimal gastric prokinetic activity for motilin agonists will be observed at low doses, which minimally contract the muscle. This finds consistency with the use of lower doses of erythromycin to accelerate gastric emptying (~200 mg 3× daily) compared with those used when given as a broad-spectrum antibiotic (often 250–500 mg orally, 4× daily to adults). The latter doses supramaximally increase gastric emptying, are associated with higher incidence of nausea (Desautels *et al.*, 1995; Boivin *et al.*, 2003), and the actions tend to fade during repeat dosing (Richards *et al.*, 1993). By contrast, repeated, low doses of erythromycin are reported to maintain an improvement of symptoms associated with gastroparesis (DiBaise and Quigley, 1999; Dhir and Richter, 2004). A lack of tolerance or nausea has also been observed with repeat doses of GSK962040, measuring gastric emptying in healthy volunteers (Dukes *et al.*, 2010).

In summary, the present study has refined the methods needed to study neuromuscular functions in human GI tissues and shown regionally dependent abilities of motilin receptor agonists to facilitate cholinergic activity. A major difference in the activity caused by motilin and GSK962040 suggests that the motilin receptor in its native environment can respond in different ways to different motilin receptor agonists. Together, these experiments provide a method to model the actions of motilin agonists on human GI cholinergic functions and identify motilin receptor agonists most likely to exert clinical benefit.

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Conflict of interest

GED is employed by GlaxoSmithKline. GJS is a previous employee of GSK. The remaining authors disclose no conflicts.

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Supporting information

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Appendix S1 Supplementary methods

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