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RESEARCH PAPER

Colitis affects the smooth muscle and neural response to motilin in the rabbit antrum

Inge Depoortere, Theo Thijs, Sara Janssen, Betty De Smet and Jan Tack

Centre for Gastroenterological Research, Catholic University of Leuven, Leuven, Belgium

Background and purpose: The underlying mechanisms of gastric dysfunction during or after an episode of intestinal inflammation are poorly understood. This study investigated the effects of colitis on the contractile effects of motilin, an important endocrine regulator of gastric motility, in the antrum.
Experimental approach: Myeloperoxidase (MPO) activity, NF-κB activity and motilin receptor density were determined in the antrum of rabbits 5 days after the induction of 2,4,6-trinitrobenzenesulphonic acid colitis. Smooth muscle and neural responses to motilin were studied in antral smooth muscle strips *in vitro*.
Key results: Colitis did not affect MPO activity, but increased NF-κB activity in the antrum. Motilin receptor density in the antrum was not affected. Under control conditions, motilin induced a slowly developing tonic smooth muscle contraction. Five days post-inflammation, tonic contractions to motilin were reduced and preceded by a rapid initial contraction. Other kinases were recruited for the phosphorylation of myosin light chain (MLC) (a multi-functional MLC kinase), and for the inhibition of MLC phosphatase (Rho kinase in addition to protein kinase C) to mediate the motilin-induced contractions during inflammation. Colitis potentiated the cholinergic neural on-contractions in the antrum. This was associated with a hyper-reactivity to motilin and an increased muscle response to ACh.
Conclusions and implications: Colitis altered the course of the motilin-induced smooth muscle contraction in the antrum. This involved changes in the kinases phosphorylating MLC. Increased cholinergic excitability to motilin in the antrum may play a role in the pathogenesis of inflammation-associated gastric motility disorders.
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Keywords: motilin; TNBS colitis; antrum; smooth muscle contraction; neural contraction; hyper-reactivity; myosin light chain kinase

Abbreviations: BSA, bovine serum albumin; DTT, dithiothreitol; EFS, electrical field stimulation; IL-1β, interleukin-1β; MLC, myosin light chain; MLCK, myosin light chain kinase; MPO, myeloperoxidase; NF-κB, nuclear factor kappa-light-chain enhancer of activated B cells; NK, neurokinin; PMSF, phenylmethylsulphonyl fluoride; SP, substance P; TNBS, 2,4,6-trinitrobenzenesulphonic acid

Introduction

In inflammatory bowel disease, the inflammatory process not only affects the intestinal mucosa, but also the deeper layers of the intestinal wall, including nerves and muscles (Collins, 1996). As a result, changes in intestinal motor activity develop in the gut segment affected by inflammation. This has been illustrated in patients with ulcerative colitis (Snape *et al.*, 1980; 1991; Marzio *et al.*, 1990; Reddy *et al.*, 1991) and in animal models of inflammation induced by chemicals, such as 2,4,6-trinitrobenzenesulphonic acid (TNBS) or enteric parasites (Crosthwaite *et al.*, 1990; Grossi *et al.*, 1993; Martinolle *et al.*, 1997; Depoortere *et al.*, 1999).

Correspondence: Dr Inge Depoortere, Centre for Gastroenterological Research, Gasthuisberg, O&N 1, box 701, B-3000 Leuven, Belgium. E-mail: inge.depoortere@med.kuleuven.be
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Motor dysfunction can also occur in segments beyond the site of inflammation. Indeed, delayed gastric emptying (Grill *et al.*, 1985; Gryboski *et al.*, 1992; Annese *et al.*, 1995), as well as changes in orocaecal transit (Rao *et al.*, 1987) and small bowel motility (Manousos and Salem, 1965) have been observed in patients with inflammatory bowel disease. Two other functional disorders associated with inflammation are post-infectious functional dyspepsia and post-infectious gastroparesis, in which symptoms develop acutely after a presumed infectious illness often characterized by vomiting and fever (Spiller, 2004). These patients experience impaired gastric accommodation or delayed gastric emptying.

Motility changes in non-inflamed regions of the gastrointestinal tract have also been observed in animal models of inflammation. TNBS-induced colitis in rats was associated with abnormal myoelectrical activity in the non-inflamed ileum (Aube *et al.*, 1999) and with delayed gastric emptying (McHugh *et al.*, 1993). Studies of *in vitro* contractility

demonstrated that acute ileitis results in both smooth muscle cell and neuronal dysfunction of the non-inflamed gastric fundus (Moreels *et al.*, 2001). In rats infected with *Trichinella spiralis*, decreased contractility has been observed in the worm-free ileum (Marzio *et al.*, 1990).

The mechanisms underlying enteric neuromuscular dysfunction of remote, non-inflamed regions of the gastrointestinal tract during or after intestinal inflammation are poorly understood. Both systemic effects (Marzio *et al.*, 1990; Jacobson *et al.*, 1995) and a role for an intrinsic or extrinsic reflex pathway have been put forward (Moreels *et al.*, 2001; De Schepper *et al.*, 2007).

Motilin, an important endocrine regulator of gastrointestinal motility, is involved in the regulation of the interdigestive migrating motor complex (Itoh *et al.*, 1976; Vantrappen *et al.*, 1979). Motilin and the motilin agonist, erythromycin-A, accelerate gastric emptying in patients with diabetic gastroparesis (Janssens *et al.*, 1990; Peeters *et al.*, 1992), and in other conditions of gastric stasis (see De Smet *et al.*, 2009). Studies *in vitro* confirmed the importance of motilin as a gastroprokinetic agent, and showed that motilin was more potent in increasing neural responses in the rabbit antrum than the 5-HT_4 agonist, tegaserod (Jarvie *et al.*, 2007).

A role for motilin in patients with Crohn's disease and ulcerative colitis has also been put forward, because plasma motilin levels are increased in these patients (Besterman *et al.*, 1983; Greenberg *et al.*, 1989). We have previously shown that, in a rabbit model of TNBS-induced colitis, motilin-induced contractility in the colon was decreased due to a down-regulation of motilin receptors (Depoortere *et al.*, 2001), but nothing is known on changes in motilin-induced contractility in the stomach, the primary site of action of motilin, during inflammation.

In view of the important effects of motilin on gastric motility, the aim of the present study was to determine the effect of TNBS-induced colitis on motilin-induced contractions in the rabbit antrum. As the gastroprokinetic effects of motilin in humans involve activation of both smooth muscle and neural motilin receptors (Coulie *et al.*, 1998; Cuomo *et al.*, 2006), the effect of colitis on both types of responses was studied. In order to elucidate the mechanisms involved, changes in the downstream effectors of the contractile apparatus were also investigated.

Methods

Induction of colitis

All animal care and experimental procedures were approved by the Ethical Committee for Animal Experiments of the University of Leuven. The model for the induction of colitis in rabbits has been optimized and characterized in a previous study (Depoortere *et al.*, 1999). New Zealand white rabbits (bred in our animal house) of either sex (2.3–3 kg) were used. Colitis was induced in 21 rabbits; 24 rabbits served as control. The rabbits were anaesthetized (35 mg·kg^{-1} ketamine, 5 mg·kg^{-1} xylazine), and a Foley catheter was inserted approximately 15 cm into the rectum and inflated with 3 mL of air. Gentle withdrawal of the catheter caused faecal pellets to be expelled. A dialysis bag (7.5 mm diameter, Spectrum Medical Industries, Houston, TX, USA) filled with 130 mg·kg^{-1} TNBS (Fluka, Buchs, Switzerland) in 50% ethanol was inserted into the distal colon for 1 h, and then removed. The rabbits were killed by a blow on the neck, 5 days after the induction of colitis. The antrum and the distal colon were removed and rinsed with 0.9% NaCl.

Myeloperoxidase (MPO) activity

MPO, a marker for the number of neutrophils in tissue, was measured in the mucosa of the antrum and the colon with the procedure described by Bradley *et al.* (1982). Activity is expressed as units where 1 unit of MPO = 1 μmol H_2O_2 broken down by MPO.

NF-κB electromobility shift analysis (EMSA)

Nuclear extracts were prepared from antral tissue of rabbits with or without colitis according to the method described by Kako *et al.* (1998). Oligonucleotide probes containing the consensus sequence for NF-κB (5′-AGT TGA GGG GAC TTT CCC AGG C-3′) were 5′ end-labelled with T_4 polynucleotide kinase (Promega, Madison, WI, USA) and [γ-^{32}P] ATP (MP Biomedicals, Irvin, CA, USA). Binding reactions were performed by pre-incubating 5 μg of nuclear proteins in 10 mM Tris (pH 7.5), 50 mM NaCl, 5% glycerol, 1 mM EDTA, 1 mM dithiothreitol (DTT), 0.2 mg·mL^{-1} bovine serum albumin (BSA), 50 μg·mL^{-1} poly(dI-dC).poly (dI-dC) at room temperature for 10 min followed by the addition of ^{32}P-labelled oligonucleotide (10 000 cpm) and a second incubation at room temperature for 20 min. The complexes were fractionated on 4% polyacrylamide gels in TBE buffer. The gels were dried and exposed to Kodak film at −70°C. The specificity of the observed bands was evaluated by the addition of an excess of unlabelled NF-κB (1.75 pmol) or by addition of an excess (1.75 pmol) of non-specific competitor, SP1 (5′-ATT CGA TCG GGG CGG GGC GAG-C-3′).

Motilin receptor-binding studies

Antral smooth muscle tissue, freed from mucosa, was finely minced and homogenized in Tris–sucrose buffer (50 mM Tris–HCl–buffer pH 7.4, 250 mM sucrose, 25 mM KCl and 10 mM $MgCl_2$) with inhibitors (1 mM iodoacetamide, 1 μM pepstatin, 0.1 mM phenylmethylsulphonylfluoride (PMSF), 0.1 g·L^{-1} trypsin inhibitor, 0.25 g·L^{-1} bacitracin). Binding of ^{125}I-[Nle^{13}]-motilin was studied in washed centrifuged fractions of tissue homogenates as previously described (Bormans *et al.*, 1986). Briefly, the membranes were incubated with ^{125}I-Nle^{13}-porcine motilin (specific activity 1500 cpm·fmol^{-1}, final concentration 50 pM) in 50 mM Tris buffer (pH 8.0, 1.5% BSA, 10 mM $MgCl_2$) for 60 min. The reaction was stopped by adding cold buffer, and membrane-bound motilin was separated by centrifugation at 1000× *g*. All data were corrected for non-specific binding determined in the presence of an excess of unlabelled Nle^{13}-porcine motilin. Displacement curves were obtained by adding increasing amounts (10^{-11} to 10^{-6} M) of Nle^{13}-porcine motilin. The dissociation constant (K_d) and the maximal number of binding sites (B_{max}) were calculated from the displacement curves fitted to the equation of Akera and Cheng

(1977) by computer. The protein concentration was determined by the method of Lowry *et al*. (1951).

Contractility measurements
Circular strips, freed from mucosa (0.2 × 2.5 cm), were cut from the antrum and suspended along their circular axis in a tissue bath filled with Krebs buffer (NaCl: 120.9 mM; NaH_2PO_4: 2.0 mM; $NaHCO_3$: 15.5 mM; KCl: 5.9 mM; $CaCl_2$: 1.25 mM; $MgCl_2$: 1.2 mM; glucose: 11.5 mM) gassed with 95% O_2/5% CO_2.

Smooth muscle responses. After equilibration at optimal stretch (3 g) for 1 h, a response to 60 mM KCl was obtained, and strips were pre-incubated with 3 μM tetrodotoxin (TTX) for 30 min before application of motilin (0.1 μM), ACh (100 μM) or substance P (SP; 1 μM). The effect of kinase inhibitors on motilin-induced responses was tested by pre-incubation of strips in the presence of 3 μM TTX with the Rho kinase inhibitor HA1077 (10 μM), protein kinase C inhibitor GF109203X (10 μM), myosin light chain kinase (MLCK) inhibitor ML-7 (10 μM), or Ca^{2+}-calmodulin kinase II inhibitor, KN-62 (10 μM) before application of 0.1 μM motilin.

Neural responses. After equilibration at optimal stretch (3 g), electrical field stimulation (EFS) was applied via two parallel platinum rod electrodes with a Grass S88 stimulator (Grass, Quincy, MA, USA). Frequency spectra (1, 2, 4, 8, 16 Hz) were obtained by pulse trains (pulse 1 ms, train 10 s, 5 V). Voltage was kept at 5 V with a Med Lab Stimu-Splitter II (Med Lab, Loveland, CO, USA). Each consecutive pulse train was followed by a 90 s interval.

When a stable response was obtained at all frequencies, the frequency spectrum was repeated in the presence of motilin (1 nM). Contractions were measured with an isometric force transducer/amplifier (Harvard Apparatus, South Natick, MA, USA), recorded on a multicorder and sampled for digital analysis using the Windaq data acquisition system and a DI-2000 PGH card (Dataq Instruments, Akron, OH, USA).

The response was calculated as the mean response during (on-contraction) and after (off-contraction) the stimulation period, and was expressed in g·mm^{-2}. The cross-sectional area of the strip was calculated using the following equation: cross section (mm^2) = tissue wet weight (mg)/[tissue length (mm) × density (mg/mm^3)]. The density of the smooth muscle was assumed to be 1.05 mg·mm^{-3}.

Statistics
All values are represented by mean ± SEM with n denoting the number of tissue preparations tested, and N the number of animals used. The effect of inflammation on MPO activity; motilin receptor density/affinity; and the smooth muscle response to ACh, SP and motilin in the absence and presence of inhibitors was evaluated by one-way analysis of variance (ANOVA) analysis. The effect of motilin on EFS-evoked changes in muscle contractility in antral tissue from inflamed and control rabbits was evaluated by two-way ANOVA analysis, with one repeated measures factor (frequency). In case of significant factor effects, tests with contrasts were performed to locate pairs of factor levels with significant differences in the examined variables. Data were analysed with Statistica 6.0 (StatSoft, Inc., 2001), and a value of $P < 0.05$ was considered as statistically significant.

Materials

Motilin was custom synthesized by Eurogentec (Seraing, Belgium) and labelled with the lactoperoxidase method. DTT, ACh, SP, hexadecyltrimethyl ammonium bromide, *O*-diansidine dihydrochloride, H_2O_2 (for MPO assays), iodoacetamide, bacitracin and PMSF were all from Sigma Aldrich (St Louis, MO, USA). Sources of other materials were as follows: BSA, pepstatin and trypsin inhibitor: Serva (Heidelberg, Germany); the kinase inhibitors HA 1077 and ML-7 Calbiochem (Nottingham, UK); GF 109203X and KN-62 Tocris (Ellisville, MO, USA). TTX was from Carl Roth GmbH (Karlsruhe, Germany), ketamine (Ketalar, Pfizer, Surrey, UK) and xylazine (Rompun, Bayer, Leverkusen, Germany). NF-κB and SP-1 were from Promega, and poly (dI-dC).poly (dI-dC) from Amersham-Pharmacia Biotech (Piscataway, NJ, USA).

All drug/molecular target nomenclature used conforms to BJP's *Guide to Receptor and Channels* (Alexander *et al*., 2008).

Results

MPO measurement and activation of the NF-κB signal transduction pathway
Tissue MPO activity was assayed to monitor the neutrophil content in the antrum and colon 5 days after the induction of inflammation. TNBS treatment significantly ($P < 0.05$) increased the MPO activity in the colon from 0.29 ± 0.12 ($n = 10$, $N = 10$) to 1.17 ± 0.36 ($n = 8$, $N = 8$) U/mg tissue. In the antrum, no MPO activity was detected.

Activation of the transcription factor, NF-κB, was investigated by EMSA in nuclear extracts from the antrum after TNBS treatment. NF-κB DNA binding activity was significantly increased in nuclear extracts from antral tissue of rabbits with colitis (Figure 1A,B). The specificity of the result was confirmed by the addition of an excess of unlabelled NF-κB oligonucleotide probe and by addition of an excess of SP1 oligonucleotide with a binding site for an unrelated protein (Figure 1C).

Motilin receptor binding
Motilin receptor density was determined in membrane preparations from the antrum. No significant changes in the maximum number of binding sites were observed in antral tissue from rabbits after TNBS treatment (17.9 ± 5.6 fmol·mg^{-1} protein) compared to control tissue (19.9 ± 6.1 fmol·mg^{-1} protein; $n = 6$, $N = 6$ for both values). The competition binding experiments also revealed no changes in motilin receptor affinity (pK_d: control: 9.23 ± 0.03; TNBS: 9.20 ± 0.03; for both conditions, $n = 6$, $N = 6$). In contrast, motilin receptor density was decreased in the colon from

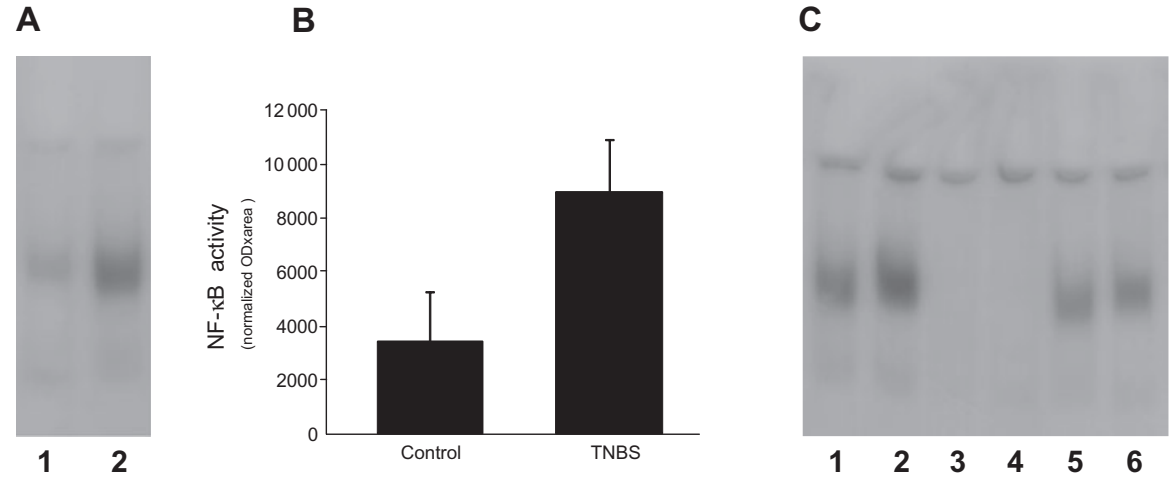

Figure 1 Effect of colitis on NF-κB activity in the antrum. (A) Nuclear extracts were prepared from the antrum of control rabbits (lane 1) or from rabbits 5 days after the induction of colitis with TNBS (lane 2), and EMSA was performed. (B) Densitometric analysis using Scion image software (Scion Corp., Frederick, MD, USA) of NF-κB activity in nuclear extracts prepared from the antrum of control ($n = 5$) and inflamed ($n = 8$) rabbits. (C) Competition analysis was performed by incubating nuclear extracts from tissue of rabbits with colitis with ^{32}P NF-κB and a 100-fold excess of unlabelled NF-κB oligonucleotide probe (lanes 3–4), or SP_1 oligonucleotide with a binding site for an unrelated protein (lanes 5–6). The results were compared to experiments to which only ^{32}P-NF-κB was added (lanes 1–2).

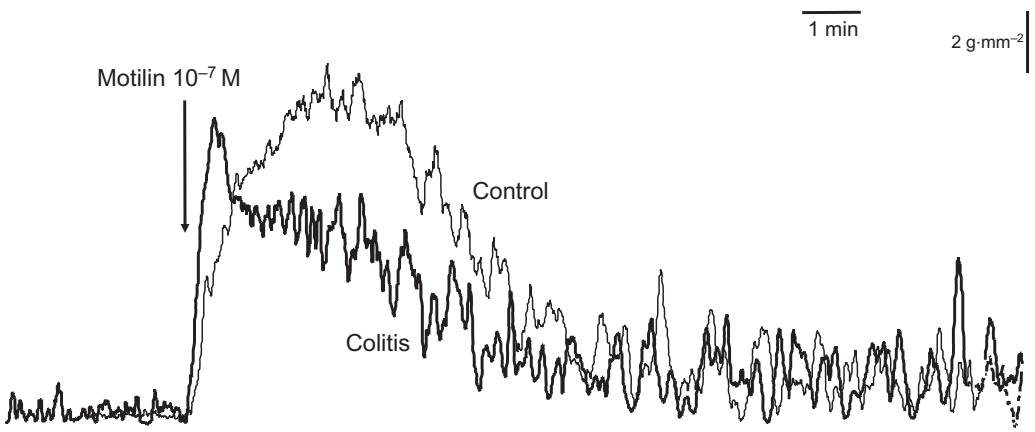

Figure 2 Effect of TNBS colitis on the smooth muscle response to motilin in the antrum. Strips from the antrum of control rabbits or from rabbits 5 days after the induction of colitis were stimulated with 10^{-7} M motilin in the presence of 3 μM TTX, and the contractile response was measured isometrically. Control strips responded to motilin with a slowly developing tonic contraction, while in strips from rabbits with colitis the tonic contraction was preceded by a rapid initial contractile response.

127.6 ± 20.9 to 36.7 ± 6.8 ($n = 6$, $N = 6$ for both) fmol·mg^{-1} protein 5 days post-inflammation.

Contractility studies

Smooth muscle responses. TNBS-induced colitis in rabbits did not affect the maximal smooth muscle response to motilin (10^{-7} M) in the antrum [control: 11.6 ± 1.2 ($n = 38$, $N = 23$), TNBS: 11.1 ± 1.7 ($n = 23$, $N = 16$) g·mm^{-2}]. On the other hand, the course of the motilin-induced contraction in the antrum was changed by the induction of colitis (Figure 2). In antral strips from control rabbits, the contraction to motilin was tonic with a tension of 10.2 ± 0.9 g·mm^{-2}, which developed slowly (maximal after 108 s for 72 s). The specificity of the motilin-induced contraction has been established by blocking the smooth muscle response to motilin (0.1 μM) with the motilin antagonist, GM109 (10 μM) (Van Assche *et al.*, 1997). Five days after TNBS treatment, the tension of the tonic contraction was reduced to 6.9 ± 0.8 g·mm^{-2}, and was preceded by a rapid initial contraction with a tension of 10.9 ± 1.7 g·mm^{-2}, which was maximal after 27 s and lasted for 5 s.

The change in the course of the motilin-induced smooth muscle contraction may be due to changes in the downstream effectors of the contractile apparatus as, in isolated smooth muscle cells, transient contractions involve Ca^{2+}-dependent mechanisms while sustained contractions represent a Ca^{2+}-independent phase (Murthy *et al.*, 2003). Therefore, the effect of Ca^{2+}-dependent MLCK inhibitors (ML-7, KN-62) involved in the phosphorylation of myosin light chain (MLC), leading to smooth muscle contraction, was investigated (Figure 3). In control tissue, motilin-induced tonic contractions were reduced by 57% ($P < 0.01$) by the calcium/calmodulin dependent MLCK inhibitor, ML-7, but not significantly by the multifunctional calcium/calmodulin dependent protein kinase II

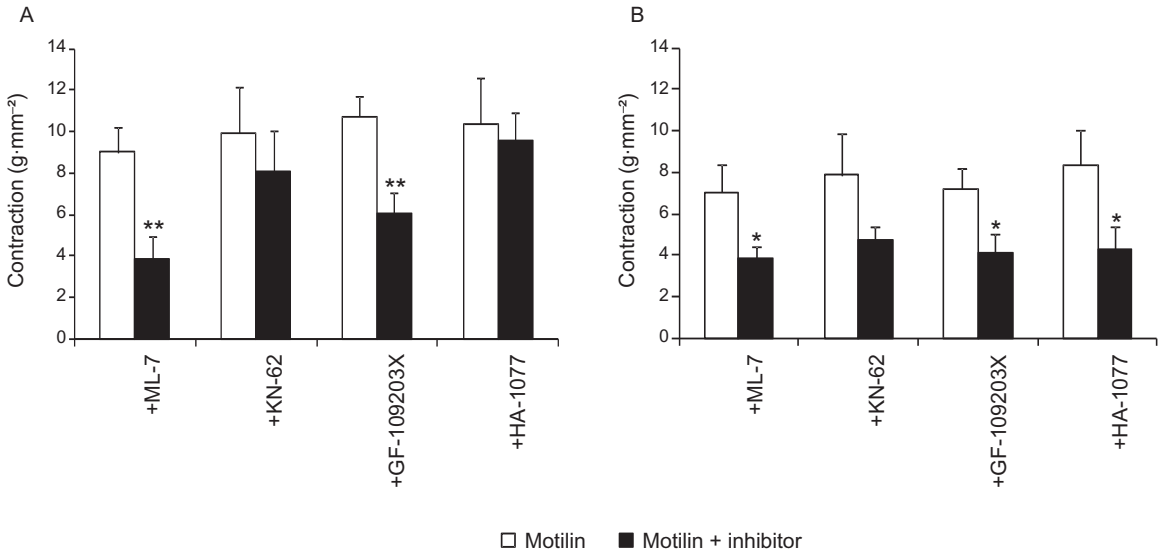

Figure 3 Effect of Ca^{2+}-dependent and Ca^{2+}-independent kinase inhibitors on the sustained smooth muscle contraction to motilin in the antrum from control rabbits (A) and from rabbits 5 days after the induction of TNBS colitis (B). Strips were pre-incubated in the presence of TTX (3 μM) with MLCK inhibitors (10 μM ML-7 or KN-62) involved in the phosphorylation of MLC, or with kinase inhibitors involved in the inhibition of MLC phosphatase (10 μM GF-109203X or HA-1077) before stimulation with motilin (10^{-7} M). The results are expressed as a percentage of the response to motilin in the presence of 3 μM TTX, but in the absence of inhibitors. The results are the mean ± SEM (control: $n = 7$, $N = 4$; colitis: $n = 5$, $N = 3$). *$P < 0.05$, **$P < 0.01$, significantly different from responses to motilin in the absence of inhibitors.

inhibitor, KN-62 (Figure 3A). The sustained contraction to motilin in antral tissue from rabbits with colitis was reduced to a similar extent by ML-7 (46%, $P < 0.05$) and tended to be reduced by KN-62, although this did not reach significance ($P = 0.09$) (Figure 3B). The role of a Ca^{2+}-independent pathway, which involves inhibition of MLC phosphatase through protein kinase C or Rho kinase, was also investigated (Figure 3). The protein kinase C inhibitor, GF-109203X, reduced the tonic contraction to motilin by 57% ($P < 0.01$) and 57% ($P < 0.05$) in antral tissue from control rabbits and rabbits with colitis respectively. The Rho kinase inhibitor, HA-1077, was ineffective in tissue from control rabbits, but decreased the tonic contraction by 51% in tissue from rabbits with colitis.

In the antrum of rabbits with TNBS-induced colitis, the rapid initial contraction to motilin, which preceded the tonic contraction, was reduced by 46% ($P < 0.01$) by KN-62, and by 38% ($P < 0.01$) by GF-109203X, but not by the other inhibitors (Figure 4).

Neural responses. EFS evoked changes in smooth muscle contractility that was frequency dependent and consisted of on- and off-contractions, observed during and after cessation of stimulation respectively. A representative tracing is shown in Figure 5A, and the changes in tension are summarized in Figure 6. The contractions were entirely neurogenic as they were blocked by TTX (3 μM). The on-contractions were significantly ($P < 0.001$) increased over the entire frequency spectrum in strips from the antrum of rabbits with colitis compared with strips from control rabbits (Figures 5A, 6). No significant changes in the off-contractions were observed. The effect of motilin on the neural responses was studied by the addition of motilin to the tissue bath at 10^{-9} M, 100-fold less than the concentration necessary to induce smooth muscle contractions. A representative tracing of the effect of motilin on neural responses elicited by EFS at 8 Hz in antral strips from control and inflamed rabbits is shown in Figure 5B. The effect of motilin on the neural responses elicited at different frequencies is summarized in Figure 7. Motilin enhanced EFS-induced on-contractions at 4 Hz ($P < 0.01$) in control strips (Figure 7A). In antral strips from rabbits with colitis, the change in tension induced by motilin was more pronounced than in control strips, and the effect was apparent between 1 and 8 Hz ($P < 0.001$) (Figure 7A). The marked potentiation of the off-contractions between 1 and 8 Hz by motilin ($P < 0.001$) in strips from control rabbits did not differ from the effect observed in strips from rabbits with colitis, except that it was only apparent at lower frequencies (1–4 Hz) ($P < 0.05$) (Figure 7B). The potentiation of the EFS-induced neural responses by motilin was blocked by GM109 (1 μM) (Van Assche *et al*., 1997; Depoortere *et al*., 2003).

Previous studies have shown that the effect of motilin on the on-contractions involves a cholinergic pathway, while the effect on the off-contractions is mediated by tachykinins (Depoortere *et al*., 2003). To study the involvement of changes in muscarinic or tachykinin-induced smooth muscle contractions, the responses to ACh and SP were studied. Colitis increased the maximal contraction to ACh by 18%, whereas the contraction to SP was reduced by 38% compared with controls (Figure 8). The phasic course of the ACh and SP-induced contraction was not affected by the induction of colitis. No changes in non-receptor mediated contractions mediated by 60 mM KCl were observed in antral tissue from rabbits with colitis.

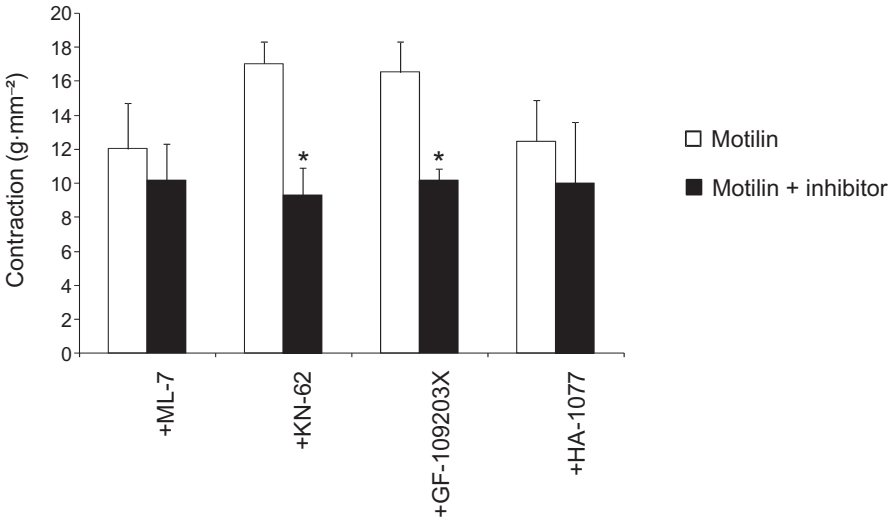

Figure 4 Effect of kinase inhibitors involved in the phosphorylation of myosin light chain on the rapid initial smooth muscle contraction to motilin in the antrum from rabbits after the induction of TNBS colitis. Strips were pre-incubated in the presence of TTX (3 μM) with MLCK inhibitors (10 μM ML-7 or KN-62) involved in the phosphorylation of MLC, or with inhibitors of MLC phosphatase (10 μM GF-109203X or HA-1077) before stimulation with motilin (10^{-7} M). The results are compared to the response to motilin in the presence of TTX, but in the absence of inhibitors. The results are the mean ± SEM ($n = 5$, $N = 3$). *$P < 0.01$, significantly different from responses to motilin in the absence of inhibitors.

Discussion

The underlying mechanisms of gastric dysfunction during or after an episode of intestinal inflammation are poorly understood. The present study was focused on the effects of colitis on the contractile effects of an important endocrine regulator of gastric motility, motilin. Colitis did not affect the magnitude of the smooth muscle contraction to motilin in the gastric antrum, but changed the pattern of the motilin-induced contractile response from a definite tonic contraction to an initial transient contraction, which was followed by a sustained contraction. In addition, the excitatory effect of motilin on the cholinergic on-contractions, induced by EFS, was enhanced.

The MPO activity measurements revealed a significant infiltration of neutrophils in the colon, but not in the antrum of rabbits, 5 days after the induction of colitis. However, the activity of the transcription factor, NF-κB, was markedly increased in antral tissue from rabbits with colitis. NF-κB is a component of the downstream signalling pathway of many cytokines, and may affect the transcription of a number of genes involved in the inflammatory process. It is quite likely that circulating cytokines could prime the activation of NF-κB.

Unlike the colon, motilin receptor density in the antrum was not changed by the inflammatory process, and this was also reflected in the maximal contractile response to motilin which was unaffected by the induction of colitis. The change in type of the motilin-induced smooth muscle contraction was rather specific for motilin because the phasic smooth muscle contraction to both ACh and SP remained unaltered by the inflammatory process.

It is well known that an increase in intracellular cytosolic Ca^{2+}, either through Ca^{2+} influx and/or release of Ca^{2+} from intracellular stores, leads to activation of MLCK, which subsequently phosphorylates MLC, resulting in muscle contraction. In addition to raising the cytosolic concentration of Ca^{2+}, G-protein-coupled receptor agonists also evoke Ca^{2+}-independent contractions mediated by inhibition of MLC phosphatase. The pathways that lead to inhibition of MLC phosphatase vary with the agonist, but they usually involve phosphorylation of the regulatory subunit (MYPT1) of MLC phosphatase via Rho kinase and/or phosphorylation of CPI-17, an endogenous inhibitor of MLC phosphatase via protein kinase C. Recent studies in isolated smooth muscle cells have shown that the initial transient contractile response to an agonist involves Ca^{2+}-dependent mechanisms, while the sustained contraction represents a Ca^{2+}-independent phase (Murthy *et al.*, 2000; 2003). In order to elucidate which of these post-receptor events were involved in the motilin-induced smooth muscle contractions, we compared the effect of different kinase inhibitors, involved in the phosphorylation of MLC, on the smooth muscle contraction to motilin in the antrum from control and inflamed rabbits. The motilin-induced tonic contraction was mediated by phosphorylation of MLC by MLCK, and by inhibition of MLC phosphatase through protein kinase C in the absence and presence of inflammation. In addition, Rho kinase was also involved in the inhibition of MLC phosphatase under inflammatory conditions. The rapid initial contraction that was only apparent in strips from rabbits with colitis involved phosphorylation of MLC through another MLCK, the multifunctional calcium-/calmodulin-dependent protein kinase II, but also inhibition of MLC phosphatase through protein kinase C. Thus, in general, we could not confirm the difference in Ca^{2+} dependency between phasic and tonic contractions, but could only show that during inflammation other kinases are recruited for the phosphorylation of MLC and for the inhibition of MLC phosphatase. Our results in antral strips from control rabbits, where we only observed a sustained contraction, are at

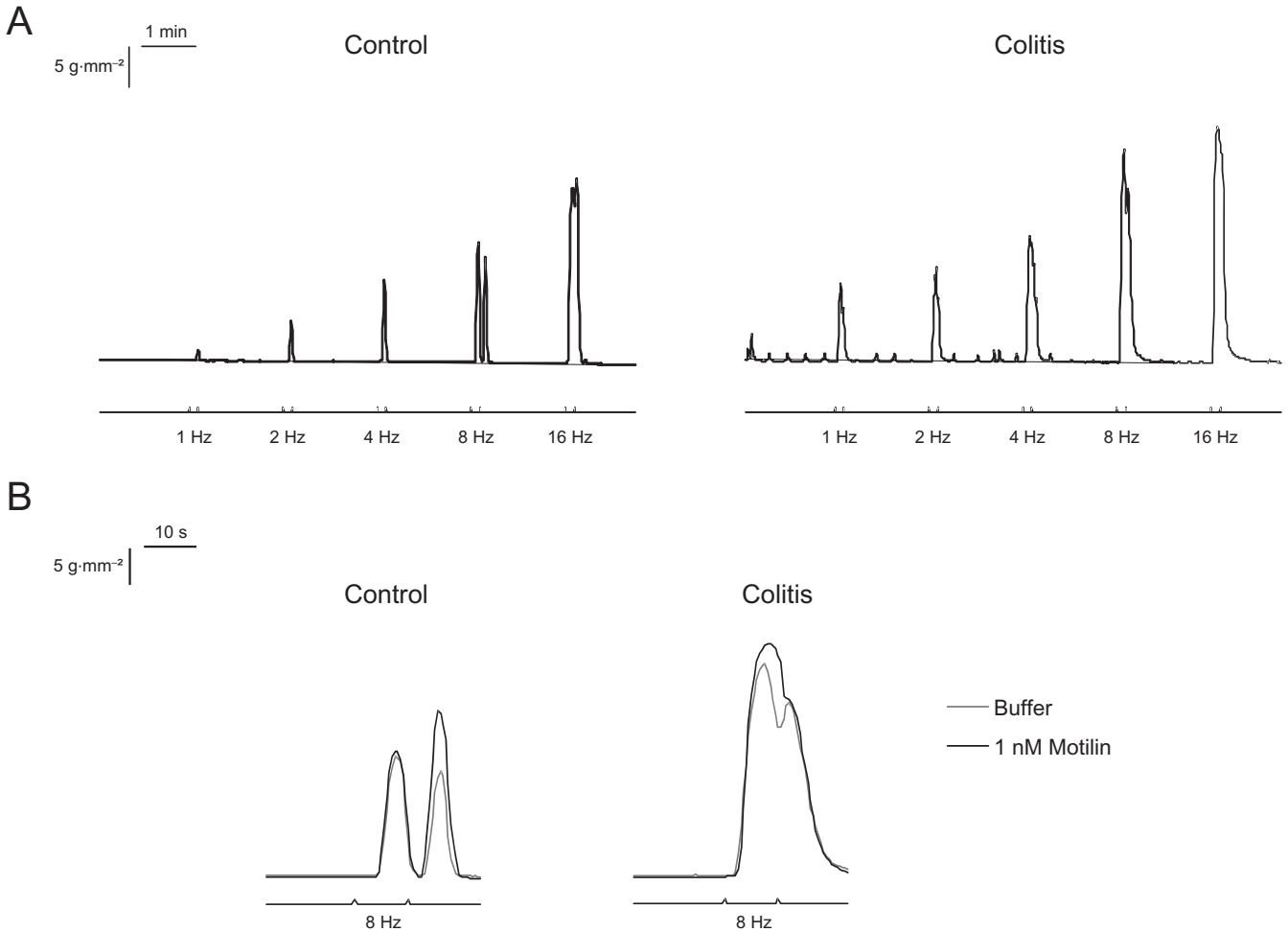

Figure 5 Representative tracings showing the different responses to EFS in antral strips from a control and a TNBS colitis rabbit (A), and the effect of motilin on each response (B). (A) Antral strips from a control rabbit (left) and a rabbit 5 days after the induction of TNBS colitis were stimulated by EFS at increasing frequencies (1–16 Hz). (B) Effect of motilin (1 nM) on neural responses elicited by EFS at 8 Hz of antral strips from a control rabbit (left) and a rabbit with TNBS colitis (right).

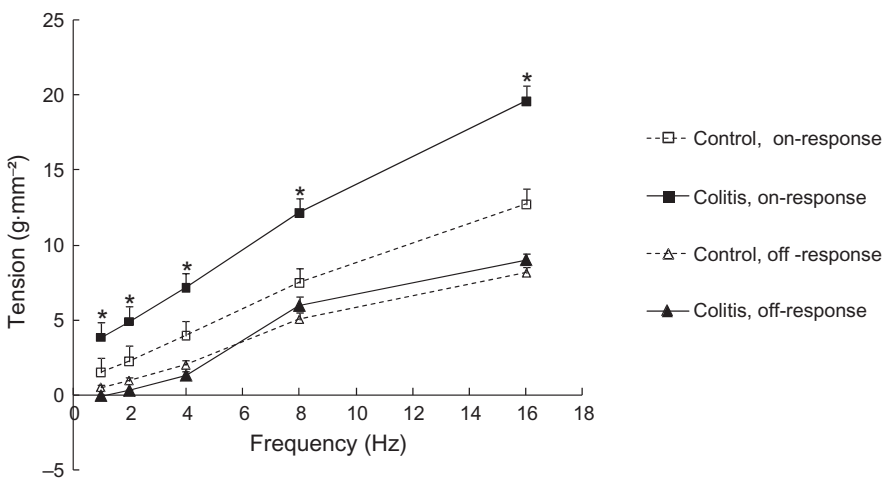

Figure 6 Effect of TNBS-induced colitis on the neural contractions in the antrum. Strips from the antrum of control rabbits or from rabbits 5 days after the induction of colitis were stimulated by EFS, and the mean response during (on-contraction) and after (off-contraction) the stimulation period at different frequencies (1–16 Hz) was determined and expressed in g·mm^{-2}. The results are the mean ± SEM (control: $n = 48$, $N = 24$; colitis: $n = 42$; $N = 21$). *$P < 0.001$, significantly different from on-contraction in strips from control rabbits.

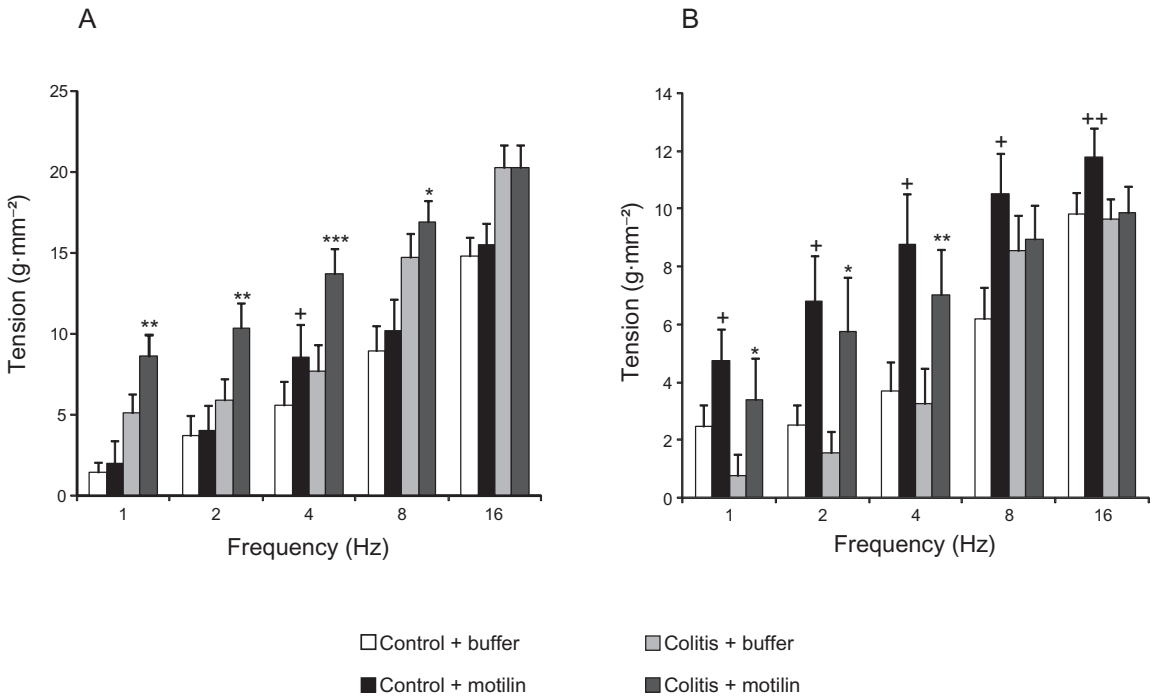

Figure 7 Effect of TNBS-induced colitis on the neural contractile response to motilin in the antrum. Strips from the antrum of control rabbits or from rabbits 5 days after the induction of colitis were stimulated by EFS in the absence or presence of 1 nM motilin, and the mean response during (on-contraction) (A) and after (off-contraction) (B) the stimulation period at different frequencies (1–16 Hz) was determined and expressed in g·mm^{-2}. The results are the mean ± SEM (control: $n = 10$, $N = 10$; colitis: $n = 12$, $N = 10$). +$P < 0.01$, ++$P < 0.001$, significantly different from responses in strips from control rabbits in the absence of motilin. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, significantly different from responses in strips from rabbits with colitis in the absence of motilin.

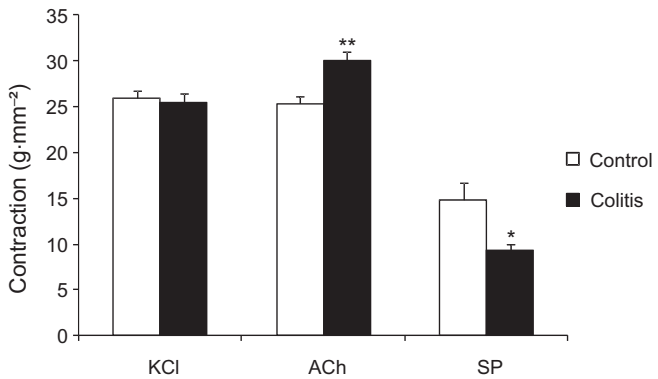

Figure 8 Effect of TNBS-induced colitis on the smooth muscle response to ACh, SP or KCl in the antrum. Strips from the antrum of control rabbits or from rabbits 5 days after the induction of colitis were stimulated with 100 μM ACh, 1 μM SP or 60 mM KCl, and the contractile response (g·mm^{-2}) was measured isometrically. The results are the mean ± SEM (control: $n = 16$, $N = 7$; colitis: $n = 16$, $N = 7$). *$P < 0.05$, **$P < 0.001$, significantly different from smooth muscle contractions in strips from control rabbits.

variance with the study of Huang *et al.* (2005), who showed that in isolated rabbit gastric smooth muscle cells, motilin initiated a transient contraction involving MLCK-dependent MLC phosphorylation and a sustained contraction involving Rho kinase- and protein kinase C-dependent inhibition of MLC phosphatase. In fact, the pattern of motilin-induced contractions observed in isolated smooth muscle cells was mimicked in our contractility studies with strip preparations from rabbits with colitis.

Studies with the motilin agonist, erythromycin A, have shown that the antral motor effects of erythromycin A *in vivo* in humans are mediated via a 'high affinity' receptor located on nerves, probably cholinergic in nature, and a 'low affinity' receptor located on smooth muscle cells (Coulie *et al.*, 1998). As both effects may contribute to the acceleration of gastric emptying, we also studied the effect of TNBS colitis on the neural responses to motilin in the rabbit antrum.

Studies *in vitro* confirmed that also in the rabbit antrum, motilin interacts at high doses directly with a smooth muscle receptor, whereas at low doses it activates a neural receptor and enhances cholinergic and tachykinin-mediated neurotransmission (Van Assche *et al.*, 1997). Pharmacological studies showed that the neural on-contractions, elicited during EFS, were completely blocked by atropine, while the off-contractions were only partially blocked (Depoortere *et al.*, 2003). The atropine-resistant off-contractions were blocked by NK_1 and NK_2 antagonists, suggesting that tachykinins synergize with acetylcholine in the transmission process of the off-contractions. In the present study, colitis potentiated the on-contractions, but not the off-contractions in the antral strips, suggesting hyper-reactivity of cholinergic myenteric neurons in the antrum. In these preparations, the excitatory effect of motilin on the cholinergic on-contractions was enhanced, but not on the off-contractions. We also observed an up-regulation of muscarinic, and down-regulation of tachykinin receptors, as receptor-mediated contractile responses to ACh and SP were increased and decreased, respectively, whereas non-receptor-mediated contractile

responses to KCl in the gastric antrum were not affected. The up-regulation of muscarinic receptors may enhance the on- and off-responses, but in the latter case the cholinergic hyper-reactivity is probably balanced by a decrease in the tachykinin-mediated response, due to the down-regulation of tachykinin receptors. In addition to changes in receptor density, the release of ACh and SP from nerves may be altered as well. Suppressed release of ACh and enhanced release of SP have been observed in the myenteric plexus of the rat jejunum after infection with *T. spiralis* (Collins *et al.*, 1989; Swain *et al.*, 1992). The inhibition of ACh release from the rat myenteric plexus was mimicked *in vitro* by interleukin (IL)-1β (Main *et al.*, 1993). In addition, changes in acetylcholinesterase activity and, thus, in the bioavailability of ACh have also been reported. Decreased acetylcholinesterase activity has been demonstrated in the *T. spiralis*-infected jejunum of rat and guinea pig (Palmer and Koch, 1995; Davis *et al.*, 1998), and Osinski and Bass (1993) showed that decreased acetylcholinesterase activity caused cholinergic supersensitivity in the denervated rat jejunum *in vivo*. So far, there are no reports of changes in ACh release or acetylcholinesterase activity in remote, non-inflamed regions of the gastrointestinal tract during intestinal inflammation.

In conclusion, colitis affected motilin-induced contractions in the antrum. The motilin-induced tonic contraction was reduced and preceded by a rapid intial contraction. This process involved changes in the kinases that led to the phosphorylation of MLC, which resulted in contraction. The increased motilin content in the duodenum of rabbits with TNBS colitis (Depoortere *et al.*, 2001) and the neural hyper-reactivity to motilin in the antrum may affect gastric emptying and may play a role in the pathogenesis of inflammation-associated gastric motility effects.

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Conflict of interest

None.

References

Akera T, Cheng VK (1977). A simple method for the determination of affinity and binding site concentration in receptor binding studies. *Biochim Biophys Acta* **470**: 412–423.

Alexander SPH, Mathie A, Peters JA (2008). Guide to Receptors and Channels (GRAC). *Br J Pharmacol* **153** (Suppl. 2): S1–S209.

Annese V, Bassotti G, Napolitano G, Frusciante V, Bruno M, Conoscitore P *et al.* (1995). Gastric emptying of solids in patients with nonobstructive Crohn's disease is sometimes delayed. *J Clin Gastroenterol* **21**: 279–282.

Aube AC, Cherbut C, Barbier M, Xing JH, Roze C, Galmiche JP (1999). Altered myoelectrical activity in noninflamed ileum of rats with colitis induced by trinitrobenzene sulphonic acid. *Neurogastroenterol Motil* **11**: 55–62.

Besterman HS, Mallinson CN, Modigliani R, Christofides ND, Pera A, Ponti V *et al.* (1983). Gut hormones in inflammatory bowel disease. *Scand J Gastroenterol* **18**: 845–852.

Bormans V, Peeters TL, Vantrappen G (1986). Motilin receptors in rabbit stomach and small intestine. *Regul Pept* **15**: 143–153.

Bradley PP, Priebat DA, Christensen RD, Rothstein G (1982). Measurement of cutaneous inflammation: estimation of neutrophil content with an enzyme marker. *J Invest Dermatol* **78**: 206–209.

Collins SM (1996). The immunomodulation of enteric neuromuscular function: implications for motility and inflammatory disorders. *Gastroenterology* **111**: 1683–1699.

Collins SM, Blennerhassett PA, Blennerhassett MG, Vermillion DL (1989). Impaired acetylcholine release from the myenteric plexus of *Trichinella*-infected rats. *Am J Physiol* **257**: G898–903.

Coulie B, Tack J, Peeters T, Janssens J (1998). Involvement of two different pathways in the motor effects of erythromycin on the gastric antrum in humans. *Gut* **43**: 395–400.

Crosthwaite AI, Huizinga JD, Fox JA (1990). Jejunal circular muscle motility is decreased in nematode-infected rat. *Gastroenterology* **98**: 59–65.

Cuomo R, Vandaele P, Coulie B, Peeters T, Depoortere I, Janssens J *et al.* (2006). Influence of motilin on gastric fundus tone and on meal-induced satiety in man: role of cholinergic pathways. *Am J Gastroenterol* **101**: 804–811.

Davis KA, Masella J, Blennerhassett MG (1998). Acetylcholine metabolism in the inflamed rat intestine. *Exp Neurol* **152**: 251–258.

Depoortere I, Van Assche G, Thijs T, Geboes K, Peeters TL (1999). Differential changes in ACh-, motilin-, substance P-, and K(+)-induced contractility in rabbit colitis. *Am J Physiol* **277**: G61–G68.

Depoortere I, Van Assche G, Peeters TL (2001). Motilin receptor density in inflamed and noninflamed tissue in rabbit TNBS-induced colitis. *Neurogastroenterol Motil* **13**: 55–63.

Depoortere I, Thijs T, Thielemans L, Robberecht P, Peeters TL (2003). Interaction of the growth hormone-releasing peptides ghrelin and growth hormone-releasing peptide-6 with the motilin receptor in the rabbit gastric antrum. *J Pharmacol Exp Ther* **305**: 660–667.

De Schepper HU, De Man JG, Van Nassauw L, Timmermans JP, Herman AG, Pelckmans PA *et al.* (2007). Acute distal colitis impairs gastric emptying in rats via an extrinsic neuronal reflex pathway involving the pelvic nerve. *Gut* **56**: 195–202.

De Smet B, Mitselos A, Depoortere I (2009). Motilin and ghrelin as prokinetic drug targets. *Pharmacol Ther* **123**: 207–223.

Greenberg GR, Buchan AM, McLeod RS, Preston P, Cohen Z (1989). *Gut* hormone responses after reconstructive surgery for ulcerative colitis. *Gut* **30**: 1721–1730.

Grill BB, Lange R, Markowitz R, Hillemeier AC, McCallum RW, Gryboski JD (1985). Delayed gastric emptying in children with Crohn's disease. *J Clin Gastroenterol* **7**: 216–226.

Grossi L, McHugh K, Collins SM (1993). On the specificity of altered muscle function in experimental colitis in rats. *Gastroenterology* **104**: 1049–1056.

Gryboski JD, Burger J, McCallum R, Lange R (1992). Gastric emptying in childhood inflammatory bowel disease: nutritional and pathologic correlates. *Am J Gastroenterol* **87**: 1148–1153.

Huang J, Zhou H, Mahavadi S, Sriwai W, Lyall V, Murthy KS (2005). Signaling pathways mediating gastrointestinal smooth muscle contraction and MLC20 phosphorylation by motilin receptors. *Am J Physiol Gastrointest Liver Physiol* **288**: G23–G31.

Itoh Z, Honda R, Hiwatashi K, Takeuchi S, Aizawa I, Takayanagi R *et al.* (1976). Motilin-induced mechanical activity in the canine alimentary tract. *Scand J Gastroenterol Suppl* **39**: 93–110.

Jacobson K, McHugh K, Collins SM (1995). Experimental colitis alters myenteric nerve function at inflamed and noninflamed sites in the rat. *Gastroenterology* **109**: 718–722.

Janssens J, Peeters TL, Vantrappen G, Tack J, Urbain JL, De Roo M *et al.*

(1990). Improvement of gastric emptying in diabetic gastroparesis by erythromycin. Preliminary studies. *N Engl J Med* **322**: 1028–1031.

Jarvie EM, North Laidler VJ, Corcoran S, Bassil A, Sanger GJ (2007). Differences between the abilities of tegaserod and motilin receptor agonists to stimulate gastric motility *in vitro*. *Br J Pharmacol* **150**: 455–462.

Kako K, Wakamatsu H, Hamada T, Banasik M, Ohata K, Niki-Kuroiwa T *et al.* (1998). Examination of DNA-binding activity of neuronal transcription factors by electrophoretical mobility shift assay. *Brain Res Brain Res Protoc* **2**: 243–249.

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951). Protein measurement with the Folin phenol reagent. *J Biol Chem* **193**: 265–275.

Main C, Blennerhassett P, Collins SM (1993). Human recombinant interleukin 1 beta suppresses acetylcholine release from rat myenteric plexus. *Gastroenterology* **104**: 1648–1654.

Manousos ON, Salem SN (1965). Abnormal motility of the small intestine in ulcerative colitis. *Gastroenterologia* **104**: 249–257.

Martinolle JP, Garcia-Villar R, Fioramonti J, Bueno L (1997). Altered contractility of circular and longitudinal muscle in TNBS-inflamed guinea pig ileum. *Am J Physiol* **272**: G1258–G1267.

Marzio L, Blennerhassett P, Chiverton S, Vermillion DL, Langer J, Collins SM (1990). Altered smooth muscle function in worm-free gut regions of *Trichinella*-infected rats. *Am J Physiol* **259**: G306–313.

McHugh K, Castonguay TW, Collins SM, Weingarten HP (1993). Characterization of suppression of food intake following acute colon inflammation in the rat. *Am J Physiol* **265**: R1001–R1005.

Moreels TG, De Man JG, De Winter BY, Timmermans JP, Herman AG, Pelckmans PA (2001). Effect of 2,4,6-trinitrobenzenesulphonic acid (TNBS)-induced ileitis on the motor function of non-inflamed rat gastric fundus. *Neurogastroenterol Motil* **13**: 339–352.

Murthy KS, Grider JR, Kuemmerle JF, Makhlouf GM (2000). Sustained muscle contraction induced by agonists, growth factors, and Ca(2+) mediated by distinct PKC isozymes. *Am J Physiol Gastrointest Liver Physiol* **279**: G201–210.

Murthy KS, Zhou H, Grider JR, Makhlouf GM (2003). Inhibition of sustained smooth muscle contraction by PKA and PKG preferentially mediated by phosphorylation of RhoA. *Am J Physiol Gastrointest Liver Physiol* **284**: G1006–1016.

Osinski MA, Bass P (1993). Chronic denervation of rat jejunum results in cholinergic supersensitivity due to reduction of cholinesterase activity. *J Pharmacol Exp Ther* **266**: 1684–1690.

Palmer JM, Koch TR (1995). Altered neuropeptide content and cholinergic enzymatic activity in the inflamed guinea pig jejunum during parasitism. *Neuropeptides* **28**: 287–297.

Peeters TL, Muls E, Janssens J, Urbain JL, Bex M, Van Cutsem E *et al.* (1992). Effect of motilin on gastric emptying in patients with diabetic gastroparesis. *Gastroenterology* **102**: 97–101.

Rao SS, Read NW, Brown C, Bruce C, Holdsworth CD (1987). Studies on the mechanism of bowel disturbance in ulcerative colitis. *Gastroenterology* **93**: 934–940.

Reddy SN, Bazzocchi G, Chan S, Akashi K, Villanueva-Meyer J, Yanni G *et al.* (1991). Colonic motility and transit in health and ulcerative colitis. *Gastroenterology* **101**: 1289–1297.

Snape WJ Jr, Matarazzo SA, Cohen S (1980). Abnormal gastrocolonic response in patients with ulcerative colitis. *Gut* **21**: 392–396.

Snape WJ Jr, Williams R, Hyman PE (1991). Defect in colonic smooth muscle contraction in patients with ulcerative colitis. *Am J Physiol* **261**: G987–G991.

Spiller RC (2004). Inflammation as a basis for functional GI disorders. *Best Pract Res Clin Gastroenterol* **18**: 641–661.

Swain MG, Agro A, Blennerhassett P, Stanisz A, Collins SM (1992). Increased levels of substance P in the myenteric plexus of *Trichinella*-infected rats. *Gastroenterology* **102**: 1913–1919.

Van Assche G, Depoortere I, Thijs T, Janssens JJ, Peeters TL (1997). Concentration-dependent stimulation of cholinergic motor nerves or smooth muscle by [Nle13]motilin in the isolated rabbit gastric antrum. *Eur J Pharmacol* **337**: 267–274.

Vantrappen G, Janssens J, Peeters TL, Bloom SR, Christofides ND, Hellemans J (1979). Motilin and the interdigestive migrating motor complex in man. *Dig Dis Sci* **24**: 497–500.