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Disturbance of the prejunctional modulation of cholinergic neurotransmission during chronic granulomatous inflammation of the mouse ileum

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1 The effect of chronic granulomatous inflammation of the intestine was studied on the prejunctional modulation of cholinergic nerve activity in the mouse ileum.

2 Contractions to carbachol (0.01–0.3 μM) and to electrical field stimulation (EFS, 0.25–8 Hz) of enteric neurons were higher in inflamed ileum as compared to control ileum. However, when the neurally-mediated contractions to EFS were expressed as percentage of the direct smooth muscle contraction to carbachol, the responses to EFS were similar in control and inflamed ileum.

3 Atropine (1 μM) abolished all contractions to EFS and carbachol in control and inflamed ileum. DMPP (3–30 μM), a nicotinic receptor agonist, induced concentration-dependent contractions that were more pronounced in inflamed ileum as compared to control ileum. Hexamethonium (100 μM), a nicotinic receptor blocker, significantly inhibited the contractions to EFS in inflamed ileum but not in control ileum.

4 In control ileum, histamine (10–100 μM) and the histamine H_1 receptor agonist HTMT (3–10 μM) inhibited the contractions to EFS concentration-dependently without affecting the contractions to carbachol. The inhibitory effect of histamine and HTMT was prevented by the histamine H_1 antagonist mepyramine (5–10 μM) but not by the H_2- and H_3-receptor antagonists cimetidine and thioperamide (both 10 μM). In chronically inflamed ileum however, histamine (10–100 μM) and HTMT (3–10 μM) failed to inhibit the contractions to EFS. The histamine H_2 and H_3 receptor agonists dimaprit and R(−)-α-methylhistamine did not affect the contractions to EFS in control and inflamed ileum.

5 The α_2-receptor agonist UK 14.304 (0.01–0.1 μM) inhibited the contractions to EFS in control and inflamed ileum without affecting the contractions to carbachol. The effect of UK 14.304 was reversed by the α_2-receptor antagonist yohimbine (1 μM). The inhibitory effect of UK 14.304 on contractions to EFS was of similar potency in control and inflamed ileum.

6 Our results suggest that the prejunctional modulation of cholinergic nerve activity by nicotinic and histaminic H_1 receptors is disturbed during chronic intestinal inflammation whereas the modulation by α_2-receptors is preserved. Such a disturbance of cholinergic nerve activity may contribute to the motility disturbances that are often observed during chronic intestinal diseases in humans.
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Keywords: Cholinergic nerves; enteric nervous system; gastrointestinal tract; granuloma; histamine; ileum; inflammation; *Schistosoma mansoni*; smooth muscle contractility; presynaptic inhibition

Abbreviations: CCh, carbachol; EFS, electrical field stimulation; HTMT, (6-[2-4-imidazolyl)ethylamino]-N-(4-trifluoromethyl-phenyl) heptanecarboxamide; TTX, tetrodotoxin

Introduction

It is well known that intestinal inflammation results in a disturbed gastrointestinal motility. At the smooth muscle level, inflammation may induce functional and structural changes of the smooth muscle cells leading to alterations in smooth muscle contractility. Although the origin of altered smooth muscle contractility during inflammation is still under study, it is hypothesized that the immune system may play a crucial role. Activation of mast cells and macrophages during the immune response may start a cascade of events that disturb directly and indirectly the normal activity of gastrointestinal smooth muscle (for review see Collins, 1996). Inflammation of the gastrointestinal tract also involves the enteric nervous system. Enteric nerves may play a role in the initiation and maintenance of intestinal inflammation but in a later stage inflammation may also alter the normal function of enteric nerves (for review see Sharkey & Parr, 1996). Immuno-histochemical studies showed that inflammatory bowel diseases in humans are associated with nerve fibre hypertrophy and with architectural alterations in the nerve

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plexuses and nerve cell bodies in the enteric nervous system (for review see Geboes & Collins, 1998). Patients with ulcerative colitis show an enhanced density and size of colonic substance P positive nerve fibres (Watanabe *et al*., 1997). Functional studies in animals demonstrated alterations of sympathetic and parasympathetic nerve function during acute jejunal inflammation (Collins *et al*., 1989; Swain *et al*., 1991; Venkova *et al*., 1999) and during chronic intestinal inflammation (Venkova *et al*., 2000). An upregulation of electrical and metabolic activities of jejunal myenteric neurons was observed in a model of acute intestinal inflammation, (Palmer *et al*., 1998). These results suggest that inflammation of the gastrointestinal tract may lead to a functional reorganization of intrinsic and extrinsic neuronal function. However, the underlying mechanisms of such a reorganization remain to be elucidated. In an animal model of chronic intestinal inflammation, we previously found a reduced transit of a semiliquid meal through the small intestine and an enhanced spontaneous activity of isolated muscle strips of the ileum (De Man *et al*., 1999; Moreels *et al*., 2001) suggesting a disturbed gastrointestinal motility. In addition, we also found an increased number of mast cells in the ileum and the presence of inflammatory cells in close proximity to nerve cell bodies in the myenteric plexus (Bogers *et al*., 2000). This correlates well with findings in the gastrointestinal tract of humans suffering from inflammatory bowel diseases (Stead *et al*., 1989; Weston *et al*., 1993; O'Sullivan *et al*., 2000). In some cases, these diseases are also associated with an enhanced release of inflammatory mediators in the intestine (Fox *et al*., 1990; Nolte *et al*., 1990; Knutson *et al*., 1990) and these mediators may interact with enteric nerves and smooth muscles.

In the gastrointestinal tract, acetylcholine is the primary neural regulator of gastrointestinal motility and modulation of cholinergic nerve activity directly affects intestinal smooth muscle contractility (Burks, 1994). It is well known that cholinergic nerves in the enteric nervous system contain a variety of prejunctional receptors. Activation of these receptors may inhibit or facilitate the release of acetylcholine (Wood, 1994). In physiological conditions, this allows a fine tuning of acetylcholine release and therefore the presynaptic modulation of neurotransmitter release is an important mechanism that directly modulates intestinal motility. However, in pathological conditions, inflammatory mediators released from immune cells may act on neuronal receptors and disrupt the normal modulation of enteric cholinergic nerve activity. Such interactions between inflammatory mediators and enteric cholinergic nerves may contribute to the motility disturbances that are observed during chronic inflammatory diseases of the intestine. To investigate this, we have studied the prejunctional modulation of cholinergic nerve activity in the mouse ileum and investigated whether this is disturbed during chronic granulomatous inflammation.

Methods

Schistosoma mansoni *infection*

The maintenance of the *Schistosoma mansoni* life cycle and the transcutaneous infection of mice with *Schistosoma mansoni* were previously described (Bogers *et al*., 2000). In brief, male Swiss mice (age: 7 weeks) were put in groups of five animals in a plastic tank filled with 1 cm of aquarium water. The mice were allowed to adapt for 15 min. Then infectious cercariae of a Puerto Rican strain of *Schistosoma mansoni* were added in the tank in a ratio of 100 cercariae per mouse and the mice were spontaneously infected *via* the transcutaneous route. The animals were kept in the tank for 60 min after which they were transferred back to their cages. Intestinal granuloma formation in mouse ileum occurs from the 12th week after *Schistosoma mansoni* infection resulting in a disturbed gastrointestinal motility (Moreels *et al*., 2001). After 16 weeks of infection, the mice were sacrificed and the *in vitro* contractility of the ileum was investigated. The local Ethics Committee of the University of Antwerp approved all experiments.

Tissue preparation

After the mice were sacrificed, the small intestine and the caecum were rapidly removed and put in ice-cold aerated Krebs-Ringer solution ((mM) NaCl 118.3, KCl 4.7, $MgSO_4$ 1.2, KH_2PO_4 1.2, $CaCl_2$ 2.5, $NaCHO_3$ 25, CaEDTA 0.026 and glucose 11.1). A $\sim$10 cm long segment of the distal ileum, located 0.5 cm proximal to the ileocolonic junction, was used for further preparation. The ileal segment was used in all experiments in the following way. The most distal $\sim$2.5 cm was used for the myeloperoxidase activity assay. The middle $\sim$5 cm was used to prepare longitudinal muscle strips for pharmacological experiments. The most proximal $\sim$2.5 cm was used for histological examination.

Histology

Immediately after the mice were sacrificed, tissue samples from the distal ileum were fixed in 4% formaldehyde and embedded in paraffin. Routine 4 μm hematoxylin-eosin stained sections perpendicular to the wall were examined.

Myeloperoxidase activity

Myeloperoxidase (MPO) is a marker of neutrophil infiltration. Measurement of MPO activity is generally used to monitor the degree of inflammation. Full thickness ileal segments were blotted dry, weighed and placed in a potassium phosphate buffer pH 6.0 containing 0.5% hexadecyltrimethylammonium bromide *a rato* of 5 g tissue per 100 ml buffer. The samples were placed on ice, minced and homogenized for 30 s (PRO 200, PRO Scientific Inc., Monroe, CT, U.S.A.). The homogenate was subjected to two sonication and freeze–thawing cycles. The suspension was centrifuged at $15{,}000 \times g$ for 15 min at 4°C. 0.1 ml aliquots of the supernatant were added to 2.9 ml of an *o*-dianisidine solution (16.7 mg of *o*-dianisidine in 1 ml methyl alcohol, 98 ml 50 mM potassium phosphate buffer pH 6.0 and 1 ml of a 0.05% H_2O_2 solution as a substrate for the MPO enzyme). The change in absorbance was read at 460 nm over 60 s using a Spectronic Genesys 5 Spectrophotometer (Milton Roy, Rochester, NY, U.S.A.). One unit of MPO activity was defined as the quantity able to convert 1 μmol H_2O_2 to H_2O per min at 25°C and was expressed in units per gram tissue.

Pharmacological studies

Tissue preparation After removal of the mucosa by sharp dissection under a stereomicroscope, longitudinal muscle strips of the distal ileum from control mice and from infected mice were mounted in organ baths (5 ml) filled with Krebs-Ringer solution, maintained at 37°C and aerated with a mixture of 5% CO_2 and 95% O_2.

Isometric tension recording The muscle strips were positioned between two platinum ring electrodes (distance in between rings: 10 mm, diameter of rings: 3 mm) that were mounted on a plexiglas rod. The lower end of the muscle strip was fixed and the other end of the muscle strip was connected to a strain gauge transducer (Scaime, France) for continuous recording of isometric tension. After an initial equilibration period of 20 min, muscle strips were gradually stretched until the amplitude of a contraction to 0.1 μM carbachol was maximal. This point was taken as the point of optimal length-tension relationship (Pelckmans *et al*., 1989). Muscle strips were then allowed to equilibrate for 60 min before experimentation. During the equilibration period the muscle strips were washed every 15 min with fresh Krebs-Ringer solution.

Experimental protocols In preliminary experiments, full concentration-response curves to carbachol (1 nM – 10 μM) were constructed to investigate the maximal responses to carbachol in control and inflamed ileum. During construction of a second and third full dose-response curve to carbachol in a single muscle strip, it was noticed that the responses to carbachol were diminished. To assure that the contractions to carbachol were reproducible, dose-response curves in a more narrow range of concentrations (0.01 – 0.3 μM) of carbachol were constructed in all following experiments. These dose-response curves were reproducible in time.

In a first series of experiments, frequency-response curves to electrical field stimulation (EFS, 0.25 – 8 Hz, 1 ms pulse duration, 10 s pulse trains, 40 V) and concentration-response curves to carbachol (0.01 – 0.3 μM) were first obtained in control conditions (saline) in separate muscle strips from control ileum and inflamed ileum. Then the curves to EFS and carbachol were re-investigated in the presence of the muscarinic receptor antagonist atropine (1 μM) or the nicotinic receptor antagonist hexamethonium (100 μM). In addition, the contractile effect of the nicotinic receptor agonist DMPP (3 – 30 μM) was investigated on separate muscle strips from control and inflamed ileum. Since nicotinic receptors are rapidly desensitized by DMPP, DMPP was added non-cumulatively. The response to DMPP was rapid and transient and reached its maximum in less than 30 s. To avoid desensitization as much as possible, DMPP was left in contact with the muscle strip during exactly 60 s after which the bath solution was rapidly rinsed with pre-warmed Krebs solution. The time between the different DMPP-induced contractions was 25 min during which the Krebs solution was refreshed every 5 min.

In a second series of experiments, frequency-response curves to EFS and concentration-response curves to carbachol were first obtained in control conditions (saline) in separate muscle strips from control and inflamed ileum. Then the curves to EFS and carbachol were re-investigated in the presence of histamine (10 – 100 μM) or in the presence of the specific agonists of histamine H_1, H_2 and H_3 receptors HTMT (3 – 10 μM), dimaprit (10 μM) and R(−)-α-methylhistamine (10 μM) respectively (pre-incubation time of compounds was 10 min). The different compounds were investigated on separate muscle strips. Since histamine and HTMT inhibited the contractions to EFS in control ileum but not in inflamed ileum, the effect of histamine in control ileum was further studied on neuronally-mediated contractions to repetitive electrical stimulation at 1 Hz in the absence and presence of histamine H_1, H_2 and H_3 antagonists (each 10 μM, pre-incubation time was 10 min) and in the absence and presence of the blocker of NO synthase L-NOARG (100 μM, pre-incubation time was 10 min).

Finally, the effect of the α_2-adrenoceptor agonist UK 14.304 (0.01 – 0.1 μM) in the absence and the presence of the α_2-adrenoceptor antagonist yohimbine (1 μM) was investigated on the contractions to EFS and carbachol in control and inflamed ileum. The pre-incubation time of the α_2-adrenoceptor agonist and antagonist was 10 min.

The effects of atropine, DMPP, hexamethonium, UK 14.304, histamine agonists and antagonists were always investigated on separate muscle strips. All experiments were performed in parallel with a muscle strip that served as time control receiving saline instead of the compound under study. The contractions to EFS (0.25 – 8 Hz) and to carbachol (0.01 – 0.3 μM) remained constant over the time course of the experiment.

Drugs used

The following drugs were used: tetrodotoxin (TTX) (Alomone Labs, Jerusalem, Israel); cimetidine, dimaprit dihydrochloride, R(−)-α-methylhistamine dihydrochloride, thioperamide, UK 14.304 (RBI, Natick, MA, U.S.A.); atropine sulphate, carbachol, 1-1-dimethyl-4-phenyl-piperazinium (DMPP), hexamethonium, histamine, L-nitro arginine (L-NOARG), mepyramine, yohimbine (Sigma-Aldrich, St. Louis, MO, U.S.A.); HTMT (6-[2-(4-imidazolyl)ethylamino]-N-(4-trifluoromethylphenyl) heptanecarboxamide (Tocris Cookson, Bristol, U.K.). All drugs were dissolved in distilled water except HTMT which was dissolved in ethanol. The final concentration of ethanol in the organ bath (0.1%) had no effect by itself on the contractions to EFS and carbachol or on the spontaneous activity of the muscle strip.

Presentation of results and statistical analysis

Contractions were calculated as the peak response induced by EFS, carbachol and DMPP. Since there is hypertrophy and hyperplasia of the smooth muscle during *Schistosoma mansoni*-induced chronic intestinal inflammation (Bogers *et al*., 2000) and since it was our aim to investigate neuronal responses to EFS and DMPP, the direct smooth muscle contraction to the muscarinic agonist carbachol was used as an internal control contraction. The cholinergic nerve-mediated contractions to EFS and DMPP were therefore expressed as a percentage of the cholinergic direct smooth muscle contraction to 0.3 μM carbachol. This concentration of carbachol (0.3 μM) was chosen since it was reproducible in time: higher concentrations of carbachol (>1 μM) were found to induce early fatigue of the smooth muscle strip and to

reduce the reproducibility of the concentration-response curve to carbachol. In control ileum, the contraction to 0.3 μM carbachol was 71±2% ($n=6$) of the maximal response to carbachol (reached at 3 μM). In chronically inflamed ileum, the contraction to 0.3 μM carbachol was 69±2% ($n=6$) of the maximal response to carbachol (reached at 3 μM) ($P>0.05$, unpaired Student's t-test). Values are shown as mean±s.e.mean for the number (n) of mice indicated. For statistical analysis, Student's t-test for paired and unpaired values, and one-way analysis of variance followed by Dunnett's test was used when appropriate. P values of less than 0.05 were considered to be significant.

Results

Histology and MPO activity

The ileum of infected mice showed typical signs of chronic inflammation: granulomas were abundantly present throughout the bowel wall. The mucosa of the ileum of infected mice showed blunted villi and an increased crypt depth (results not shown). The ileal MPO activity was significantly increased from 1.5±0.5 U mg^{-1} tissue in control mice ($n=8$) to 10.2±1.6 U mg^{-1} tissue in infected mice ($n=6$).

Contractility of control ileum and chronically inflamed ileum

The spontaneous basal activity in chronically inflamed ileal muscle strips was higher as compared to controls (Figure 1). The blocker of neuronal conductance tetrodotoxin (TTX, 1 μM) did not affect the spontaneous basal activity in control and in inflamed ileum. In control and inflamed ileum, electrical field stimulation (EFS, 0.25–8 Hz) induced frequency-dependent transient contractions (Figures 1 and 2A). These contractions to EFS were abolished by TTX (1 μM, $n=6$, results not shown) indicating that they were of neuronal origin in control and inflamed ileum. The muscarinic receptor agonist carbachol (1 nM–10 μM) induced concentration-dependent sustained contractions in control and inflamed ileum (Figures 1 and 2B). The contractions to carbachol were not affected by TTX (1 μM, $n=6$, results not shown) in control and inflamed ileum indicating that these contractions resulted from a direct activation of smooth muscle cells. The contractions to EFS and carbachol were

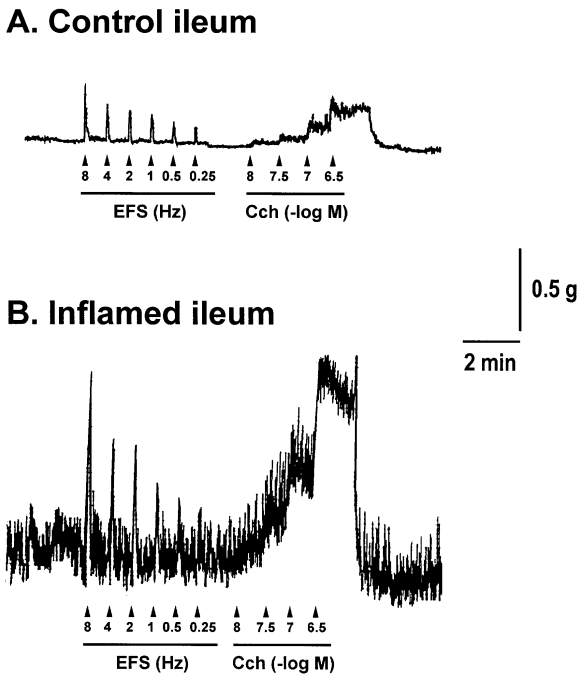

Figure 1 Typical tracings of longitudinal muscle from the mouse distal ileum showing the spontaneous basal activity and the contractions to electrical field stimulation (EFS) and carbachol (Cch) in normal ileum from control mice (A) and in chronically inflamed ileum from infected mice (B).

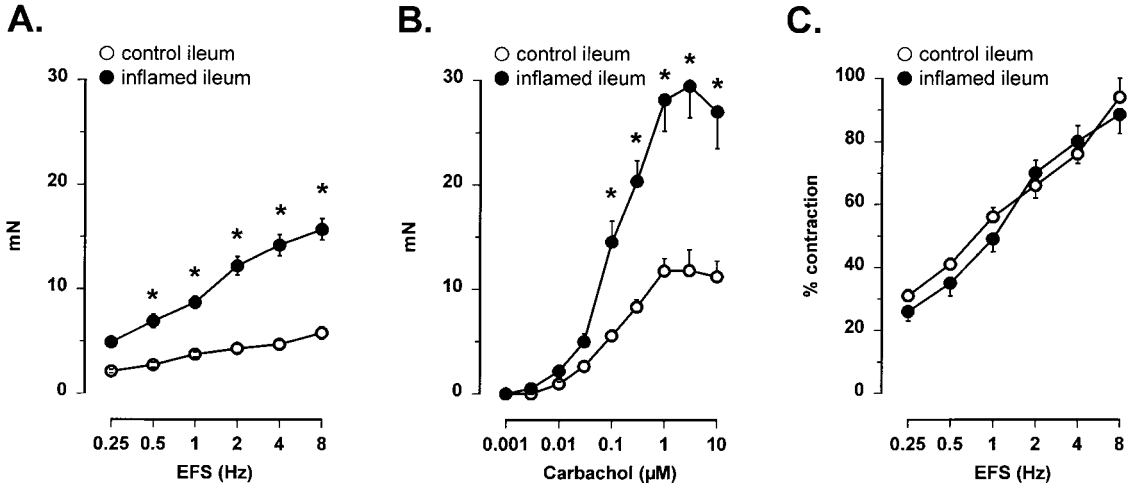

Figure 2 (A) shows the cholinergic nerve-mediated contractions to electrical field stimulation (EFS) and (B) shows the cholinergic direct smooth muscle-mediated contractions to the muscarinic agonist carbachol in control ileum and in inflamed ileum. Results are expressed in mN. (C) shows the responses to EFS in control ileum and in inflamed ileum after expressing the amplitudes of the contractions to EFS as percentage of the internal control contraction to 0.3 μM carbachol. Results are shown as mean±s.e.mean for $n=6-8$ experiments. *$P<0.05$. Student's t-test for unpaired values.

significantly higher in inflamed ileum as compared to control ileum (Figures 1 and 2A, B). Consecutive challenge of smooth muscle strips with high concentrations of carbachol (>1 μM) reduced the reproducibility of the contractions to carbachol. In all further experiments the concentration-response curve to carbachol was therefore limited to concentrations ranging from 0.01 to 0.3 μM. These concentration-response curves were reproducible in time. In control ileum, the contraction to 0.3 μM carbachol was 71±2% ($n=6$) of the maximal response to carbachol (Figure 2B). This was not different from inflamed ileum where the contraction to 0.3 μM carbachol was 69±2% ($n=6$) of the maximal response to carbachol (Figure 2B). Since our aim was to investigate inflammation-induced changes at the neuronal level, the neuronally-mediated contractions to EFS in all following experiments were expressed as a percentage of the direct smooth muscle contraction to 0.3 μM carbachol. This resulted in a frequency-response curve to EFS that was similar in control ileum and in chronically inflamed ileum (Figure 2C). This indicates that the enhanced contractile activity to EFS and carbachol that was observed in inflamed ileum, resulted from changes at the level of the smooth muscle and not at the level of the enteric neurons.

Effect of cholinergic receptor agonists and antagonists in control ileum and chronically inflamed ileum

The muscarinic receptor blocker atropine (1 μM, $n=4$) completely abolished the contractions to EFS and carbachol in control and inflamed ileum indicating that these contractions were cholinergic in origin (results not shown).

In control and in inflamed ileum, non-cumulative addition of the nicotinic receptor agonist 1,1, dimethyl-4-phenyl-piperazinium (DMPP, 3–30 μM, $n=4-6$) induced concentration-dependent contractions which were rapid in onset, transient and abolished by hexamethonium (100 μM) and by TTX (1 μM) (results not shown). This indicates that DMPP acted at neuronal nicotinic receptors and not at smooth muscle receptors. When the neuronally-mediated contractions to DMPP were expressed as percentage of the direct smooth muscle contraction to carbachol, the contractions to DMPP were significantly higher in chronically inflamed ileum as compared to those in control ileum (Figure 3).

In control ileum, the nicotinic receptor blocker hexamethonium (100 μM, $n=6$) had a small but non-significant inhibitory effect on the EFS-induced contractions (Figure 4A). In chronically inflamed ileum, hexamethonium (100 μM, $n=6$) induced a significant inhibition of the contractions to EFS (Figure 4C). Similar to TTX, hexamethonium did not affect the contractions to the muscarinic agonist carbachol in control and inflamed ileum (Figure 4B, D).

Effect of histamine, histamine receptor agonists and antagonists in control ileum

In ileal muscle strips from control animals, 10 and 100 μM histamine induced small transient contractions of 13.8±1.6% and 15.1±1.6% respectively ($n=8$) (Figure 5). The contractions to histamine, which disappeared spontaneously in less than 30 s, were not affected by TTX (1 μM), atropine (1 μM) or hexamethonium (100 μM) indicating that they were not neuronal or cholinergic in origin. The histamine H_1

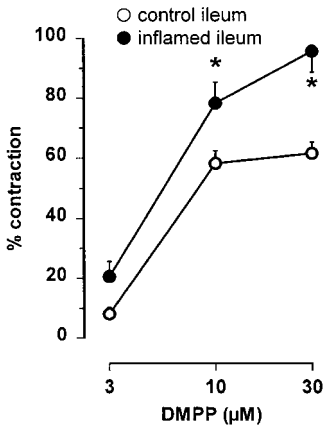

Figure 3 Contractile effect of the nicotinic receptor agonist DMPP in control ileum and in chronically inflamed ileum. Results are expressed as percentage of the internal control contraction to 0.3 μM carbachol and shown as mean±s.e.mean for $n=4-6$ experiments. *$P<0.05$, significantly different from control ileum. Student's t-test for unpaired values.

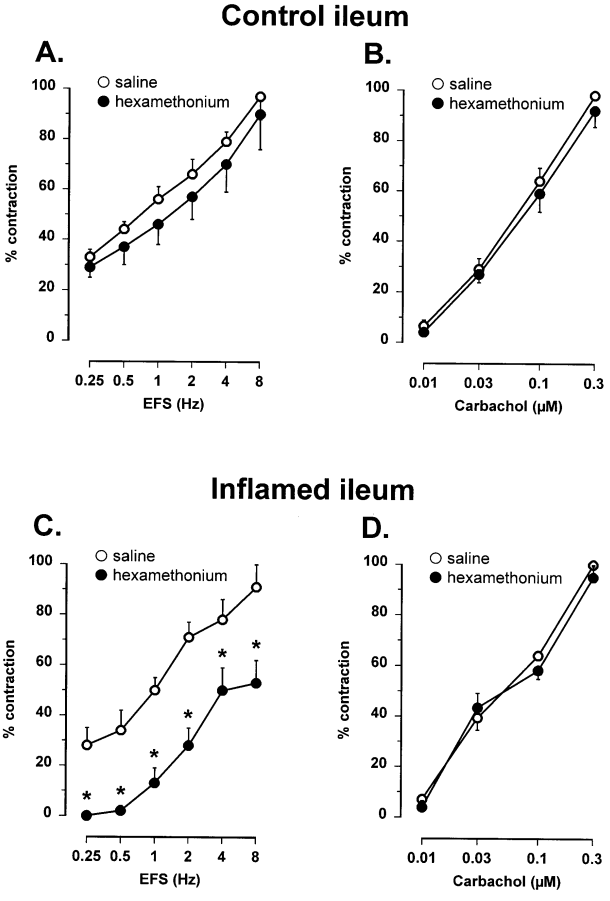

Figure 4 Effect of hexamethonium (100 μM) on the cholinergic nerve-mediated contractions to electrical field stimulation (EFS) and on the direct smooth muscle contractions to carbachol in control ileum (A and B) and in chronically inflamed ileum (C and D). The responses to EFS and to carbachol were obtained in the same muscle strip originating either from control ileum or from inflamed ileum. Results are expressed as percentage of the internal control contraction to 0.3 μM carbachol and shown as mean±s.e.mean for $n=6$ experiments. *$P<0.05$, significantly different from control values in saline. Student's t-test for paired values.

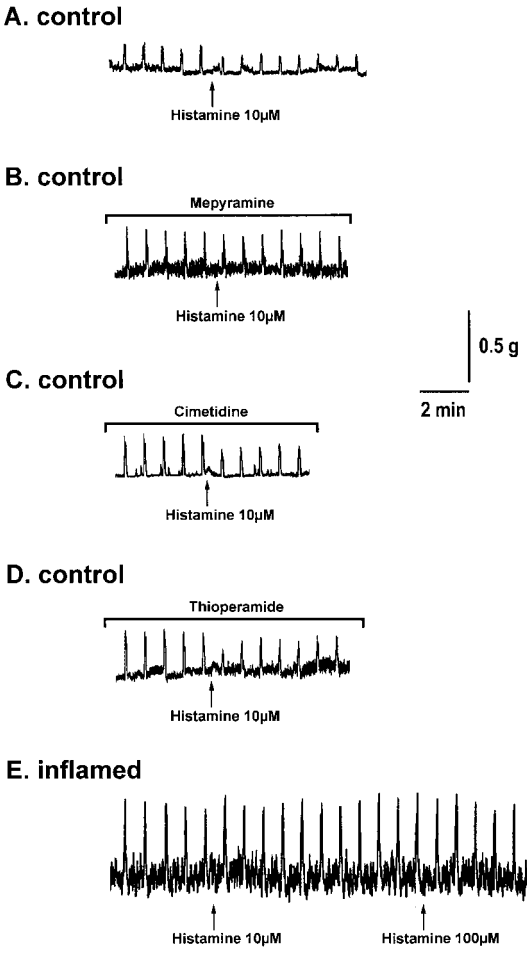

Figure 5 Typical tracings of longitudinal muscle from the mouse distal ileum showing the contractions to repetitive electrical field stimulation (EFS) at 1 Hz (1 ms pulse duration, pulse train of 10 s, one pulse train per min) in normal ileum from control mice (tracings A, B, C and D) and in chronically inflamed ileum from infected mice (tracing E). (A) shows the inhibitory effect of 10 μM histamine on contractions to EFS at 1 Hz. (B) shows that 10 μM histamine had no inhibitory effect in the presence of mepyramine (10 μM). (C) and (D) show that the inhibitory effect of 10 μM histamine was preserved in the presence of cimetidine (C, 10 μM) and thioperamide (D, 10 μM) respectively. (E) shows the absence of effect of histamine on contractions to EFS at 1 Hz in chronically inflamed ileum. Incubation time of the histamine receptor antagonists was 10 min.

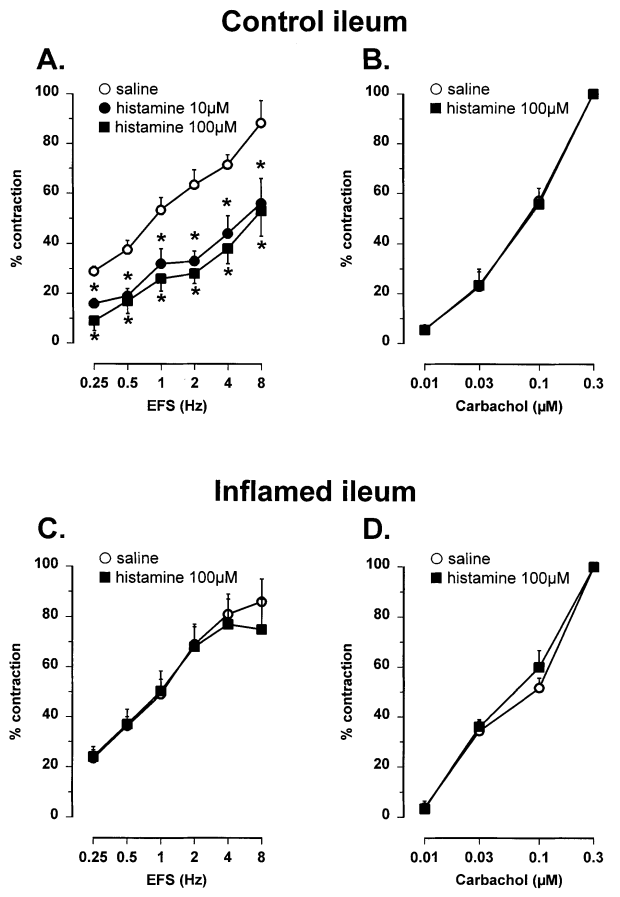

Figure 6 Effect of histamine (10 and 100 μM) on the cholinergic nerve mediated contractions to electrical field stimulation (EFS) and on the direct smooth muscle contractions to carbachol in control ileum (A and B) and in chronically inflamed ileum (C and D). The responses to EFS and to carbachol were obtained in the same muscle strip originating either from control ileum or from inflamed ileum. Results are expressed as percentage of the internal control contraction to 0.3 μM carbachol and shown as mean$\pm$s.e.mean for $n=4-6$ experiments. *$P<0.05$, significantly different from control values in saline, one-way ANOVA followed by Dunnett's test for multiple comparisons.

antagonist mepyramine (10 μM) abolished these contractions to histamine whereas the histamine H_2 and H_3 antagonists cimetidine and thioperamide (both 10 μM) had no effect (Figure 5). In the presence of histamine (10–100 μM), the nerve-mediated cholinergic contractions to EFS were significantly inhibited whereas the contractions to carbachol were not affected (Figures 5 and 6A, B). The effect of histamine persisted in the presence of the blocker of nitric oxide synthase L-nitroarginine (L-NOARG). L-NOARG (100 μM) by itself significantly enhanced the contractions to 1 Hz EFS from 54$\pm$3 to 68$\pm$4%, ($n=4$) but in the presence of L-NOARG, histamine (10 μM) still significantly inhibited the contractions to 1 Hz EFS from 68$\pm$4 to 50$\pm$4% ($n=4$). This indicates that NO was not involved in the inhibitory effect of histamine on contractions to EFS.

Mepyramine (10 μM, $n=9$) but not cimetidine or thioperamide (both 10 μM, both $n=6$) prevented the inhibitory effect of histamine on the nerve-mediated contractions to EFS (Figures 5 and 7). Mepyramine, cimetidine or thioperamide by themselves did not affect the spontaneous activity of the muscle strips or the contractions to EFS or carbachol in control ileum (Figures 5 and 8A–C).

Further experiments were performed with specific agonists of histamine H_1, H_2 and H_3 receptors. HTMT (3–10 μM), a specific agonist of histamine H_1 receptors, concentration-dependently inhibited the nerve-mediated contractions to EFS (0.25–8 Hz, $n=6$) (Figure 9A) but had no effect on the contractions to carbachol (0.01–0.3 μM, $n=6$) (contraction to 0.3 μM carbachol was 7.5$\pm$0.6 mN in the absence of HTMT and 7.7$\pm$0.9 mN in the presence of 10 μM HTMT, $n=6$, $P>0.05$). The inhibitory effect of 10 μM HTMT on neuronal responses to EFS was prevented after blockade of histamine H_1 receptors with mepyramine (5 μM, $n=5$) (Figure 10B) but not after blockade of histamine H_2 and H_3 receptors with cimetidine and thioperamide (both 10 μM,

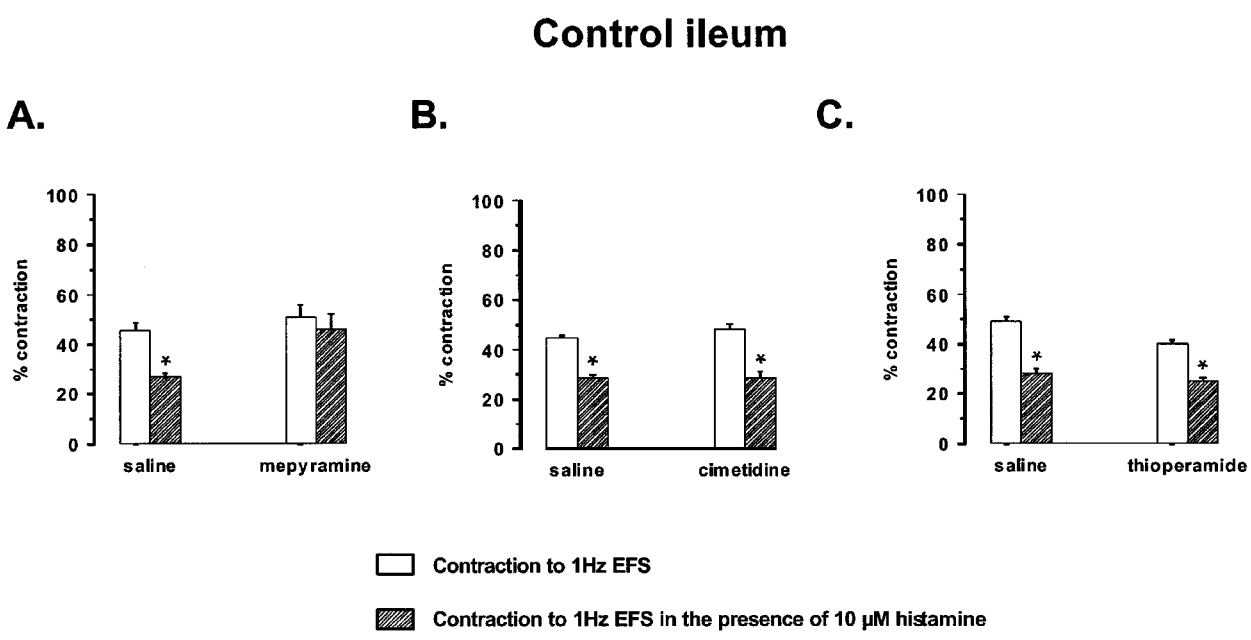

Figure 7 Effect of histamine (10 μM) on the cholinergic nerve mediated contractions to 1 Hz electrical field stimulation (EFS) in control ileum in the absence (saline) and presence of mepyramine (10 μM, A), cimetidine (10 μM, B) and thioperamide (10 μM, C), antagonists of H_1, H_2 and H_3 receptors respectively. Experiments with the histamine receptor antagonists were performed on separate muscle strips from control ileum. Results are expressed as percentage of the internal control contraction to 0.3 μM carbachol and shown as mean$\pm$s.e.mean for $n=6-9$ experiments. *$P<0.05$, significantly different from control values in saline, cimetidine or thioperamide. Student's t-test for paired values.

$n=5$) (Figure 10C). A small transient contraction to HTMT was observed only with higher concentrations of HTMT (30 μM, $n=2$) but these concentrations were not used in further experiments. The specific agonists of histamine H_2 and H_3 receptors, dimaprit (10 μM, $n=6$) and R(−)-α-methylhistamine (10 μM, $n=6$) respectively, did not affect the contractions to EFS (Figure 9B, C). Although R(−)-α-methylhistamine is a specific H_3 receptor agonist, its effect was further investigated in the presence of the H_1 receptor antagonist mepyramine (10 μM) and the H_2 receptor antagonist cimetidine (10 μM). However, also after blockade of H_1 and H_2 receptors, R(−)-α-methylhistamine did not affect the contractions to EFS (0.25–8 Hz) (contraction to 1 Hz was 44.5$\pm$4.1% in the presence of mepyramine and cimetidine (both 10 μM) and 41.7$\pm$4.1% in the presence of mepyramine and cimetidine plus R(−)-α-methylhistamine (all 10 μM) ($n=5$, $P>0.05$).

Effect of histamine, histamine receptor agonists and antagonists in chronically inflamed ileum

In chronically inflamed ileal muscle strips from infected animals, the transient contraction to 10 μM histamine was smaller as compared to control ileum (from 13.8$\pm$1.6% in controls to 6.4$\pm$3.2% in inflamed ileum, $n=7-10$, $P=0.042$) whereas the contraction to 100 μM histamine was preserved in inflamed muscle strips (15.2$\pm$1.6% in controls vs 14.6$\pm$3.2% in inflamed ileum, $n=7-10$, $P=0.873$). Similar to control ileum, the contractions to histamine were not affected by TTX (1 μM), atropine (1 μM), hexamethonium (100 μM), cimetidine or thioperamide (both 10 μM) but abolished by mepyramine (10 μM). In contrast to control ileum, histamine (10–100 μM, $n=6$) had no inhibitory effect on the nerve-mediated contractions to EFS in chronically inflamed ileum (Figures 5 and 6C, D). The specific antagonists of histamine H_1, H_2 and H_3 receptors mepyramine, cimetidine and thioperamide did not affect the contractions to EFS and carbachol or the spontaneous activity of chronically inflamed ileal muscle strips (Figure 8D–F).

In contrast to control ileum, the specific agonist of histamine H_1 receptors HTMT (10 μM, $n=6$) did not affect the contractions to EFS in chronically inflamed ileum (Figure 9D). Also the agonists of histamine H_2 and H_3 receptors dimaprit (10 μM, $n=6$) and R(−)-α-methylhistamine (10 μM, $n=6$) respectively did not affect the contractions to EFS in chronically inflamed ileum (Figure 9E, F).

Effect of adrenergic α_2-receptor agonist and antagonist in control and chronically inflamed ileum

The specific α_2-receptor agonist UK 14.304 had no effect on the spontaneous activity of control and chronically inflamed ileum. However, UK 14.304 (0.01–0.1 μM) concentration-dependently inhibited the nerve mediated contractions to EFS with a similar potency in control and in inflamed ileum ($n=6-8$) (Figure 11). The inhibitory effect of UK 14.304 on contractions to EFS was of neuronal origin because the direct smooth muscle contractions to carbachol were not affected (results not shown) in control or in chronically inflamed ileum. The inhibitory effect of UK 14.304 (100 nM) was completely reversed by the α_2-receptor antagonist yohimbine (1 μM, $n=4$) both in control and inflamed ileum (results not shown). Yohimbine by itself did not affect the contractions to EFS or carbachol or the spontaneous activity of the muscle strips.

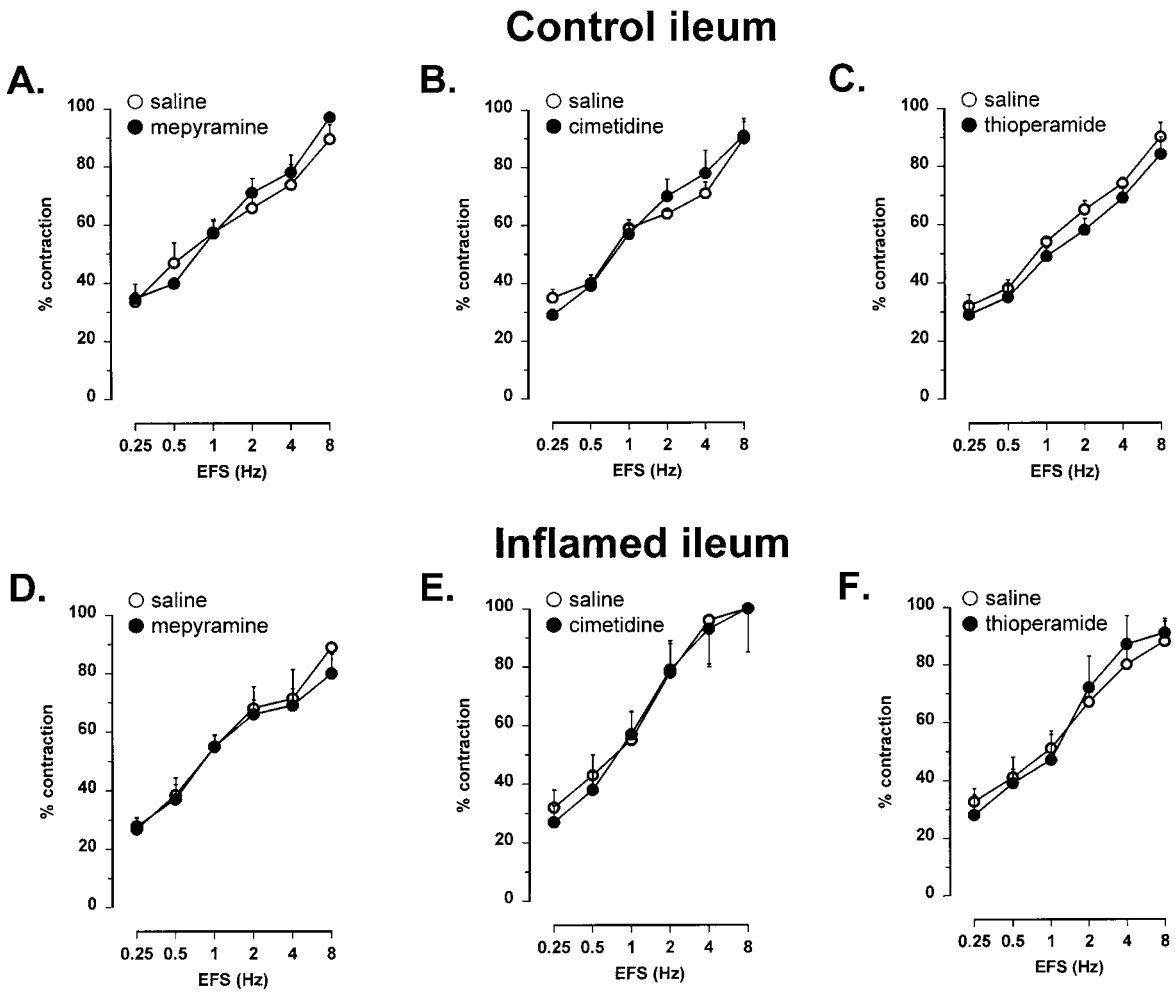

Figure 8 Effect of antagonists of histamine H_1, H_2 and H_3 receptors mepyramine (10 μM), cimetidine (10 μM) and thioperamide (10 μM) on the cholinergic nerve-mediated contractions to electrical field stimulation (EFS) in control ileum (A, B and C) and in chronically inflamed ileum (D, E and F). Experiments with the histamine receptor antagonists were performed on separate muscle strips originating either from control ileum or from inflamed ileum. Results are expressed as percentage of the internal control contraction to 0.3 μM carbachol and shown as mean $\pm$ s.e.mean for $n=6$ experiments. Student's t-test for paired values showed no statistical differences.

Discussion

The main finding of the present study is that the presynaptic modulation of cholinergic nerve activity in the ileum is disturbed during chronic granulomatous inflammation of the intestine. This disturbance involves nicotinic and histaminic H_1 receptors but not adrenergic α_2-receptors.

Chronic granulomatous inflammation of the mouse small intestine was induced by infecting mice during 16 weeks with the parasite *Schistosoma mansoni*. The small intestine of infected animals showed typical signs of chronic granulomatous inflammation as reported previously (Bogers *et al.*, 2000). Pharmacological studies on isolated muscle strips of the terminal ileum showed an enhanced spontaneous activity and an enhanced contractile activity to EFS and carbachol. In control as well as in inflamed ileum, the contractions to EFS resulted from activation of cholinergic enteric nerves because these contractions were completely blocked by the muscarinic receptor antagonist atropine and by the blocker of neuronal conductance tetrodotoxin. The contractions to carbachol were blocked by atropine but not affected by tetrodotoxin indicating that they resulted from a direct action at muscarinic receptors on the smooth muscle. Since our aim was to investigate the prejunctional modulation of cholinergic neurotransmission, the neurally-mediated responses to EFS were expressed as a percentage of the direct smooth muscle response to carbachol. The resulting frequency-response curve to EFS in inflamed ileum did not differ from that obtained in control ileum. Firstly, this indicates that the enhanced spontaneous activity and the enhanced contractile activity to EFS and carbachol in chronically inflamed ileum most likely result from changes at the smooth muscle level. Secondly, this indicates that the enteric cholinergic nerves in control and inflamed ileum had a similar sensitivity to activation by electrical field stimulation. However, marked differences were found in the sensitivity to the prejunctional modulation of cholinergic nerve activity.

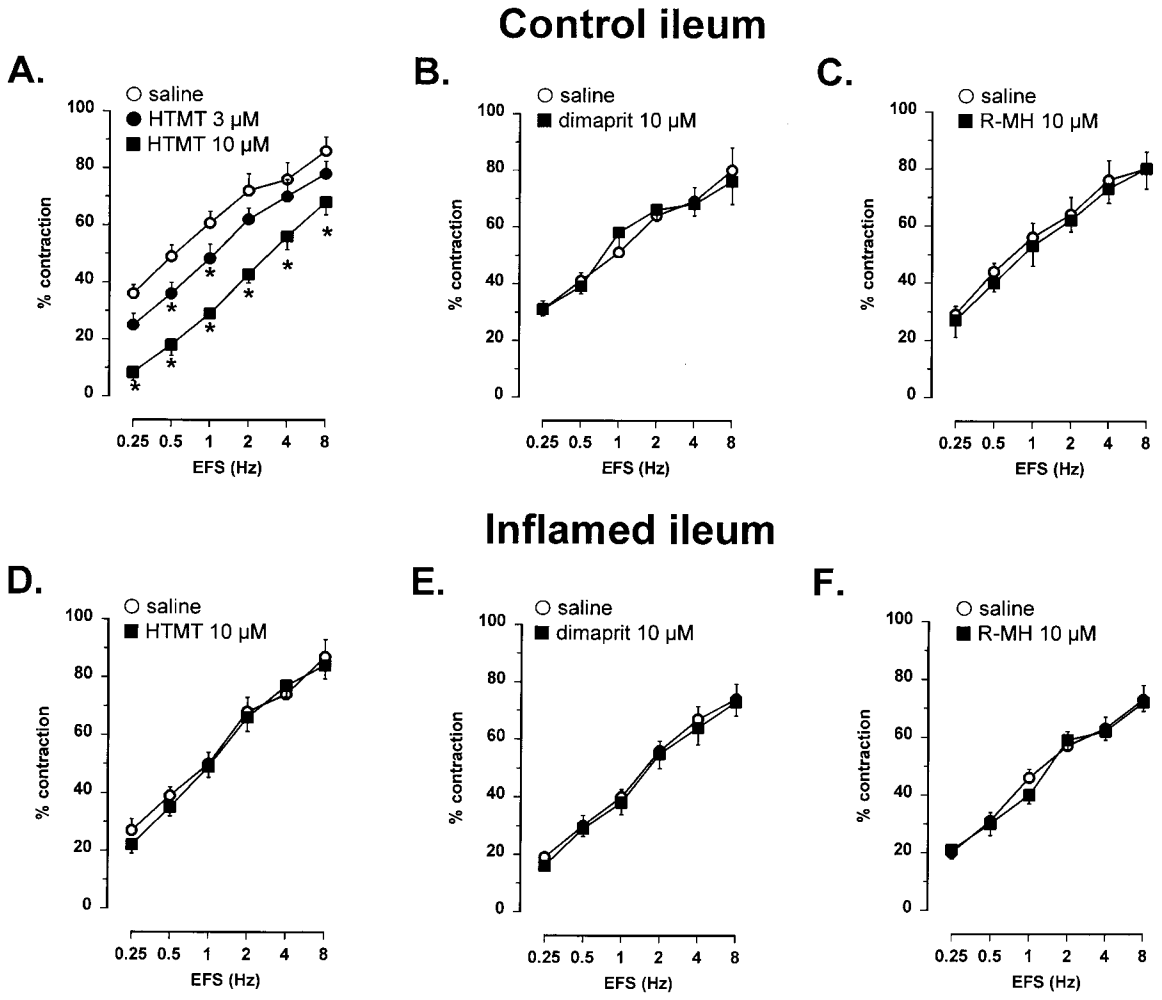

Figure 9 Effect of agonists of histamine H_1, H_2 and H_3 receptors HTMT (3–10 μM), dimaprit (10 μM) and R(−)-α-methylhistamine (R-MH, 10 μM) respectively on the cholinergic nerve-mediated contractions to electrical field stimulation (EFS) in control ileum (A, B and C) and in chronically inflamed ileum (D, E and F). Experiments with the histamine receptor agonists were performed on separate muscle strips originating either from control ileum or from inflamed ileum. Results are expressed as percentage of the internal control contraction to 0.3 μM carbachol and shown as mean ± s.e.mean for $n = 5–7$ experiments. $^*P < 0.05$, significantly different from control values in saline, one-way ANOVA followed by Dunnett's test for multiple comparisons (effect of HTMT) and Student's *t*-test for paired values (effect of dimaprit and R(−)-α-methylhistamine).

Nicotinic receptors

We first compared the effect of nicotinic receptor activation in control and chronically inflamed ileum. The nicotinic receptor agonist DMPP induced fast and transient contractions in control and inflamed ileum. DMPP acted specifically on neuronal nicotinic receptors because hexamethonium, which blocks nicotinic receptors, and TTX, which blocks the neuronal conductance, abolished the contractions to DMPP. This is in agreement with the presence of nicotinic receptors on cholinergic enteric neurons. However, the contractions to DMPP were significantly higher in chronically inflamed ileum as compared to control ileum, even when these neuronally-mediated contractions to DMPP were expressed as a percentage of the direct smooth muscle contraction to carbachol. This suggests that chronic inflammation results in a hyperreactivity of nicotinic receptors. To investigate this further, we studied the effect of hexamethonium on contractions to electrical stimulation. As pointed out above, the EFS-induced contractions are of neuronal cholinergic origin because they are abolished by TTX and by atropine. In control ileum, hexamethonium had a minor but nonsignificant inhibitory effect on these contractions. This is in agreement with observations in the guinea-pig ileum (Trzeciakowski, 1987). In chronically inflamed ileum however, hexamethonium significantly inhibited the contractions to EFS without affecting the direct smooth muscle responses to carbachol. This supports the hypothesis that there is a more sensitive modulation of cholinergic nerve activity by nicotinic receptors during chronic inflammation.

The underlying mechanism of this effect of inflammation on neuronal nicotinic receptors is unknown. Xia *et al.* (1999) recently demonstrated that the inflammatory cytokine interleukin(IL)-1β suppresses nicotinic neurotransmission in the submucous plexus of the guinea-pig ileum. IL-1β also excites enteric neurons and presynaptically inhibits cholinergic neurotransmission in the myenteric plexus of the guinea-pig ileum (Kelles *et al.*, 2000). These results indicate that

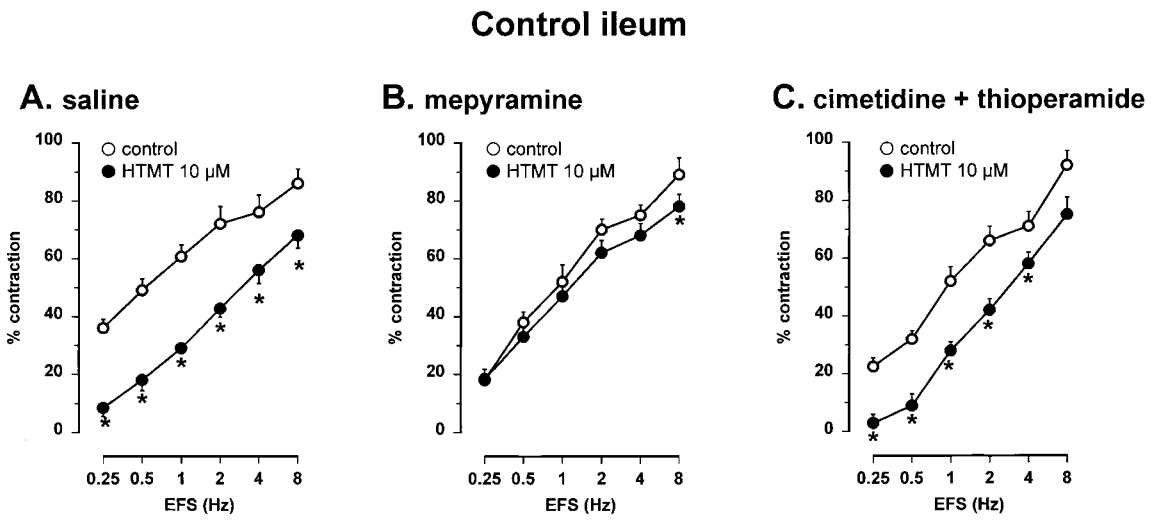

Figure 10 Effect of 10 μM HTMT on the cholinergic nerve-mediated contractions to electrical field stimulation (EFS) in control ileum. The contractions to EFS were obtained (A) in the presence of saline, (B) in the presence of the histamine H_1 receptor antagonist mepyramine (5 μM) and (C) in the presence of the histamine H_2 and H_3 receptor antagonists cimetidine and thioperamide (both 10 μM). Experiments in saline, mepyramine and cimetidine + thioperamide were performed on separate muscle strips. Results are expressed as percentage of the internal control contraction to 0.3 μM carbachol and shown as mean ± s.e.mean for $n = 5-6$ experiments. $^*P < 0.05$, significantly different from control values in saline, mepyramine or cimetidine + thioperamide, Student's *t*-test for paired values.

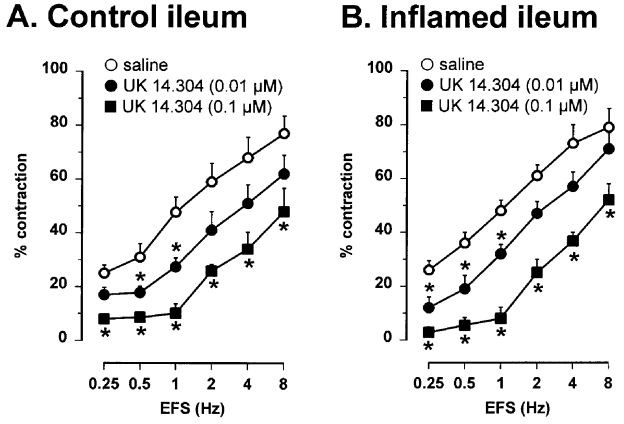

Figure 11 Effect of the α_2-adrenergic receptor agonist UK 14.304 (0.01 and 0.1 μM) on the contractions to electrical field stimulation (EFS) in control ileum (A) and in chronically inflamed ileum (B). Results are expressed as percentage of the internal control contraction to 0.3 μM carbachol and shown as mean ± s.e.mean for $n = 6-8$ experiments. $^*P < 0.05$, significantly different from control values in saline, one-way ANOVA followed by Dunnett's test for multiple comparisons.

inflammatory cytokines may disturb the normal modulation of enteric neurotransmission. Interestingly, we previously found an upregulation of IL-1β in the ileum of *Schistosoma mansoni* infected animals. However, this upregulation was observed only during the initial phase of the infection, whereas during the chronic granulomatous phase of the infection, the levels of IL-1β were normalized (Moreels *et al.*, 2001). Possibly, IL-1β may have a long-term effect on presynaptic neurotransmission that continues after normalization of IL-1β levels. Alternatively, other inflammatory mediators such as IL-6 may also be involved in this effect since IL-6 has a similar effect as IL-1β on cholinergic enteric neurotransmission (Xia *et al.*, 1999; Kelles *et al.*, 2000).

An enhanced sensitivity of neuronal nicotinic receptors during chronic inflammation may directly alter normal motility patterns. Neurotransmitters modulate their own release by activation of autoreceptors that are located on the nerve terminals. In the intestine, nicotinic receptors are thought to be located on the cell soma and dendrites of the neurons in the myenteric plexus (Töröcsik *et al.*, 1991). There is however recent evidence that nicotinic receptors are also located at the nerve terminal (Galligan, 1999) where they may be involved in presynaptic modulation of neurotransmitter release (Schneider *et al.*, 2000). Therefore, a hyperreactivity of nicotinic receptors during chronic inflammation may result in an enhanced activity of cholinergic nerves and disturb normal gastrointestinal motility. As pointed out above, isolated muscle strips from inflamed ileum were not hyperreactive to electrical stimulation of cholinergic nerves. Possibly, such an hyperreactivity might be observed only in the intact organ in which the intramural neuronal network is preserved. In *in vivo* experiments we previously found that chronic intestinal inflammation inhibits the gastrointestinal transit of a semiliquid meal (De Man *et al.*, 1999; Moreels *et al.*, 2001) suggesting that there is a disturbed peristaltic rhythmicity in the intact inflamed small intestine.

Histamine receptors

Apart from a disturbed autoreceptor function, the enhanced sensitivity of nicotinic receptors during chronic inflammation may also disturb the enteric neuro-muscular response through other mechanisms. A histamine-dependent hyperexcitability of enteric nerves of the submucous plexus was

observed in a model of nematode-sensitized guinea-pig colon (Frieling *et al*., 1994). In this tissue, histamine acts on cholinergic nerves at nicotinic synapses (Tamura *et al*., 1988; Wood, 1992). The histamine induced inhibition of cholinergic nerve activity is well documented in the guinea-pig intestine but ignored in almost any other species. In the ileum of control mice, we found that histamine concentration-dependently inhibited the neuronally-mediated contractions to cholinergic nerve stimulation. This effect resulted from an action at the cholinergic neuron and not from an action at the smooth muscle since histamine did not affect the direct smooth muscle contractions to carbachol. The inhibitory effect of histamine on cholinergic nerve activity was prevented by the histamine H_1 antagonist mepyramine but not by the histamine H_2 antagonist cimetidine or the histamine H_3 antagonist thioperamide. In addition, the inhibitory effect of histamine on cholinergic-nerve mediated contractions was mimicked by the histamine H_1 receptor agonist HTMT but not by the histamine H_2 and H_3 receptor agonists dimaprit and R(−)-α-methylhistamine respectively. This suggests the involvement of H_1 receptors in the histamine-induced inhibition of cholinergic nerve activity. In the guinea-pig small intestine, there is evidence that histamine H_3 receptors are involved in the presynaptic inhibition of cholinergic nerve activity (Trzeciakowski, 1987; Tamura *et al*., 1988; Poli *et al*., 1991) but enteric synaptic transmission in this species is modulated by H_1, H_2 and H_3 receptors (Izzo *et al*., 1998). Histamine acting on neuronal H_1 receptors also modulates cholinergic nerve activity in the myenteric plexus of the rat small intestine and in the cat stomach (Sakai, 1979; Sim *et al*., 1989). This suggests that histamine H_1 receptors may play a role in enteric cholinergic neurotransmission but that their role and importance may depend upon the species and tissue under study.

Although histamine and the histamine H_1 receptor agonist HTMT inhibited cholinergic nerve activity in control ileum, they did not affect the contractions to cholinergic nerve stimulation in chronically inflamed ileum. This was surprising because there is evidence from electrophysiological studies that histamine inhibits cholinergic nerve activity at nicotinic synapses in the guinea-pig ileum (Tamura *et al*., 1988). Since we found an enhanced reactivity to nicotinic receptor activation during chronic inflammation, we also expected an enhanced reactivity to histaminic receptor activation in the inflamed ileum. However, we found an absence of effect of histamine and HTMT on cholinergic nerve activity in chronically inflamed ileum. It is possible that, unlike in the guinea-pig ileum, neuronal histamine receptors are not located solely at nicotinic synapses in the mouse ileum. Alternatively, there is a marked regional difference in sensitivity to histamine in the intestine (Leurs *et al*., 1991; Barker, 1985). Our experiments were performed in the terminal part of the ileum since granuloma formation was most evident in this region of the small intestine. Differences between studies may therefore also arise from different regions of the small intestine that are under study. It should also be noticed that the results of electrophysiological studies, which are performed on local enteric synapses, cannot be compared directly with the results of contractility experiments, which are performed on isolated muscle strips. This is illustrated by the fact that electrophysiological studies showed that the histamine-induced inhibition of cholinergic nerve activity in the guinea-pig ileum was reversed by nicotinic receptor blockade (Tamura *et al*., 1988) whereas this was not the case in contractility studies on isolated muscle strips of the same tissue (Hew *et al*., 1990).

The exact mechanism on the loss of the inhibitory effect of histamine and HTMT on cholinergic nerve activity during chronic inflammation remains to be investigated. We previously demonstrated an enhanced number of mast cells in chronically inflamed ileum (Bogers *et al*., 2000). Mast cells contain a vast number of neuroactive mediators and mast cell degranulation alters smooth muscle contractility (Fargeas *et al*., 1992). Mast cell mediators may exert their activity even if the mast cell is not degranulated (Gottwald *et al*., 1995; Stead, 1992). Therefore, during the chronic phase of inflammation, the continuous action of mast cell mediators such as histamine may result in a desensitization of histamine receptors. Our observation that the direct smooth muscle contraction to 10 μM histamine was lower in chronically inflamed ileum points towards a diminished reactivity of histamine receptors to exogenous histamine. In the intestine, there is evidence that histamine receptor desensitization after prolonged application of histamine is not likely to occur *in vivo* (Tamura & Wood, 1992) although such a desensitization is reported repeatedly in *in vitro* intestinal preparations (for instance: Bielkiewicz & Cook, 1984; Hishinuma & Uchida, 1988; Horio *et al*., 1990; Perez-Garcia *et al*., 1998). Specifically for the histamine H_1 receptor it is shown that desensitization results from a reduced affinity of the receptor for histamine (Horio *et al*., 1990) and from the internalization of the H_1 receptor (Hishinuma & Young, 1995) which may explain why the histamine receptor antagonists had no effect on the contractions to cholinergic nerve stimulation during chronic inflammation. The sensitivity to desensitization of the H_1 receptor may also depend upon the precise location of the receptor since we noticed that the direct contractile effect of 100 μM histamine on the smooth muscle was preserved in chronically inflamed ileum whereas the neuronally-mediated inhibitory effect of histamine on cholinergic nerve activity was lost.

Adrenergic α_2-receptors

To investigate whether the disturbance of the modulation of cholinergic nerve activity during chronic inflammation was a general feature of prejunctional receptors, we also studied the effect of α_2-adrenoceptor activation on cholinergic nerve activity. It is well known that activation of adrenergic α_2-receptors on cholinergic neurons in the enteric nervous system inhibits the release of acetylcholine (Drew, 1978; Wikberg, 1977). This was not investigated previously in the mouse ileum but we found that cholinergic contractions to electrical stimulation of the mouse ileum were concentration-dependently inhibited by the α_2-receptor agonist UK 14.304. Since UK 14.304 did not affect the direct smooth muscle responses to carbachol, these results indicate that UK 14.304 acted on cholinergic neurons and not on the smooth muscle. The effect of UK 14.304 was specific since the α_2-receptor antagonist yohimbine reversed the inhibitory effect of UK 14.304 on the contractions to EFS. In chronically inflamed ileum, the inhibitory effect of UK 14.304 on cholinergic nerve-mediated contractions was comparable with the effect of UK 14.304 in control ileum. These results indicate that the

modulatory role of α_2-receptors on cholinergic nerve activity is well preserved during chronic granulomatous inflammation of the mouse intestine in contrast to the modulatory role of nicotinic and histaminic receptors. This demonstrates that the disturbance of the prejunctional inhibition of cholinergic nerve activity during chronic intestinal inflammation is confined to certain but not to all prejunctional receptors and that this disturbance does not result from a non-specific damage of neuronal receptors.

Conclusion

Our results indicate that chronic granulomatous inflammation of the intestine results in a disturbed presynaptic modulation of cholinergic nerve activity. This involves neuronal nicotinic and histaminic H_1 receptors but not adrenergic α_2-receptors. This disturbance of neuromodulatory mechanisms in the enteric nervous system may contribute to the motility disturbances that are often observed during chronic inflammatory bowel diseases in humans.

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