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Alteration of the purinergic modulation of enteric neurotransmission in the mouse ileum during chronic intestinal inflammation

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1 The effect of chronic intestinal inflammation on the purinergic modulation of cholinergic neurotransmission was studied in the mouse ileum. Chronic intestinal inflammation was induced by infection of mice with the parasite *Schistosoma mansoni* during 16 weeks.

2 *S. mansoni* infection induced a chronic inflammatory response in the small intestine, which was characterised by intestinal granuloma formation, increased intestinal wall thickness, blunted mucosal villi and an enhanced activity of myeloperoxidase.

3 In *control ileum* and in *chronically inflamed ileum*, electrical field stimulation (EFS) of longitudinal muscle strips induced frequency-dependent contractions that were abolished by tetrodotoxin (TTX) and atropine. Carbachol induced dose-dependent contractions that were not affected by TTX but abolished by atropine.

4 In *control ileum*, adenosine and ATP dose-dependently inhibited the contractions to EFS. Theophylline and 8-phenyltheophylline, P_1 and A_1 receptor antagonists respectively, prevented this inhibitory effect of adenosine and ATP. PPADS, DMPX and MRS 1220, antagonists of P_2, A_2 and A_3 receptors, respectively, did not prevent this inhibitory effect of adenosine and ATP. Adenosine and ATP did not affect the contractions to carbachol.

5 The inhibitory effect of adenosine and ATP on contractions to EFS in *control ileum* was mimicked by the stable adenosine analogue methyladenosine and by the A_1-receptor agonist *N*(6)-cyclohexyladenosine, but not by the A3 receptor agonist 2-Cl IB-MECA or by the ATP analogues $\alpha\beta$-methylene-ATP and ADPβS. The inhibitory effect of adenosine on contractions to EFS was lost after prolonged (90 min) treatment of *control ileum* with methyladenosine (100 μM).

6 In *chronically inflamed ileum*, adenosine, methyladenosine, *N*(6)-cyclohexyladenosine and ATP all failed to inhibit the cholinergic nerve-mediated contractions to EFS. Also theophylline, 8-phenyltheophylline, PPADS, DMPX and MRS 1220 had no effect on the contractions to EFS and carbachol. The loss of effect of adenosine and ATP was still evident after 52 weeks of infection.

7 These results indicate that in physiological conditions neuronal adenosine A_1 receptors modulate cholinergic nerve activity in the mouse ileum. However, during chronic intestinal inflammation, this purinergic modulation of cholinergic nerve activity is impaired. This suggests that chronic intestinal inflammation leads to a dysfunction of specific neuronal regulatory mechanisms in the enteric nervous system.

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Abbreviations: ADPβS, adenosine-5′-0-(2-thiodiphosphate) trilithium salt; ATP, adenosine triphosphate; CCh, carbachol; 2Cl-IB-MECA, 2-chloro-N^6-(3-iodobenzyl)-adenosine-5′-*N*-methyluronamide; DMPX, 3,7-dimethyl-1-propargyl-xanthine; EFS, electrical field stimulation; PPADS, pyridoxal-phosphate-6-azopheny1-2′,4′-disulphonic acid tetrasodium; TTX, tetrodotoxin

Introduction

A disturbed intestinal motility is frequently observed in patients with chronic granulomatous intestinal inflammation. Inflammation alters the contractile function of smooth muscle cells, and this may directly contribute to inflammation-induced changes in gastrointestinal motility. However, chronic inflammation may also affect the synaptic signalling in the enteric nervous system. A dysfunction of enteric neurotransmission during experimental chronic inflammation of the intestine is reported (Venkova *et al*., 2000; Balemba *et al*., 2001; 2002). We previously reported that chronic inflammation of the small

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intestine disturbs the modulatory role of histamine on cholinergic enteric neurotransmission (De Man *et al*., 2001). This may be related to an enhanced number of mast cells that are observed in the chronically inflamed intestine (De Jonge *et al*., 2002). However, the exact mechanisms by which chronic intestinal inflammation affects enteric neurotransmission remain unclear.

In the gastrointestinal tract, acetylcholine is regarded as the major excitatory neurotransmitter and the prime regulator of gastrointestinal motility. The release of acetylcholine from enteric cholinergic nerves is under a well-regulated presynaptic control, involving specific neuronal receptors. Among these are purinergic P1 and P2 receptors, which, upon activation, enhance or inhibit the release of acetylcholine, depending upon the purinoceptor subtype that is involved (Sawynok & Jhamandas, 1976; Vizi & Knoll, 1976; Gustafsson *et al*., 1978; Moody & Burnstock, 1982). Interestingly, P1 and P2 purinoceptors are also present on immune cells and there is evidence that adenosine and ATP, which are the natural ligands of P1 and P2 receptors, respectively, are generated at sites of inflammation (for a review see Cronstein, 1994). This suggests that purines may act as neuroimmune modulators. There is evidence that purines regulate the activity of immune cells (Cronstein *et al*., 1983; Marone *et al*., 1984; Church & Hughes, 1985; Ramkumar *et al*., 1993; McCloskey *et al*., 1999) and that they play a critical role in the limitation and termination of inflammatory responses (Ohta & Sitkovsky, 2001). There is recent evidence that blockade of adenosine kinase, which results in increased endogenous levels of adenosine, downregulates the inflammatory response in experimental colitis (Siegmund *et al*., 2001). In addition, Mabley *et al*. (2003) recently showed that inosine, a purine that is formed from the breakdown of adenosine, effectively reduced the inflammatory response in a murine model of colitis. These findings suggest that purines are potential mediators of immunosuppression in inflammatory bowel diseases and that they have therapeutic potential for the treatment of these diseases. However, it remains unknown to what extent the immune-modulatory role of purines during inflammation interferes with their neuromodulatory role in the enteric nervous system.

In the present study, we have therefore investigated the effect of the purines adenosine and ATP on enteric cholinergic neurotransmission in healthy mice and in mice with chronic granulomatous inflammation of the intestine. Chronic intestinal inflammation was induced by infection of mice with the parasite *S. mansoni*. The larval form of this parasite penetrates the skin of the host and enters the host's blood stream (for a recent review on the life cycle of schistosomes and on schistosomiasis, see El-Garem, 1998; Ross *et al*., 2002). After maturing, the male and female schistosome worms unite and the adult female *S. mansoni* worm starts to deposit eggs in the mesenteric veins of the infected host. The intravascularly deposited eggs migrate through the vessel wall into the bowel wall. Some eggs succeed in migrating through the bowel wall and entering the lumen, after which they are shed with the faeces. A significant part of the eggs, however, remains deposited in the bowel wall, thereby triggering an immune response that results in granuloma formation around entrapped eggs. This chronic immune response in the intestine represents an interesting experimental model that allows one to study the mechanisms of motility disturbances during chronic granulomatous intestinal inflammation (Domingo & Warren, 1969; Weinstock, 1992).

Methods

Schistosoma mansoni infection

The maintenance of the *S. mansoni* life cycle and the transcutaneous infection of mice with *S. 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 *S. 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. After 16 weeks of infection, the mice were killed and the *in vitro* contractility of the ileum was investigated and compared with age-matched controls. The local Ethics Committee of the University of Antwerp approved all experiments.

Tissue preparation

All mice were fasted for 24 h with free access to water. Then animals were anaesthetised with diethyl ether and exsanguinated from the carotid artery. The small intestine and the caecum of *S. mansoni*-infected mice and age-matched controls were rapidly removed and put in ice-cold aerated Krebs–Ringer solution (118.3 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 2.5 mM $CaCl_2$, 25 mM $NaCHO_3$, 0.026 mM CaEDTA and 11.1 mM glucose). An $\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 killed, tissue samples from the distal ileum were fixed in 4% formaldehyde and embedded in paraffin. Routine 4 μM haematoxylin–eosin-stained sections perpendicular to the wall were examined.

Myeloperoxidase activity

Myeloperoxidase is a marker of neutrophil infiltration, and the measurement of myeloperoxidase 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 at *a ratio* of 5 g tissue per 100 ml buffer. The samples were placed on ice, minced and homogenised for 30 s (PRO 200, PRO Scientific Inc., Monroe, CT, U.S.A.). The homogenate was subjected to two sonication and freeze–thaw cycles. The suspension was centrifuged at 15,000 $\times$ g for 15 min

at 4°C. Aliquots (0.1 ml) 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 myeloperoxidase enzyme). The change in absorbency was read at 460 nm over 60 s using a Spectronic Genesys 5 Spectrophotometer (Milton Roy, Rochester, NY, U.S.A.). One unit of myeloperoxidase activity was defined as the quantity able to convert 1 μmol H_2O_2 to $H_2O\,min^{-1}$ at 25°C and was expressed in U^{-1} tissue.

Pharmacological studies: tissue preparation and isometric tension recording

The ileum was opened along the longitudinal axis and the mucosa was removed by sharp dissection under a stereomicroscope. Longitudinal muscle strips of ~6.0 mm 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. The muscle strips were carefully 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 30 min during which the strips were washed every 5 min, the muscle strips were contracted with 0.1 μM carbachol. After washout of carbachol, the strips were stretched (increments of 0.5 g), and when the basal tone of the muscle strips was stabilised, the strips were again contracted with 0.1 μM carbachol. This procedure was repeated until the contraction to 0.1 μM carbachol was maximal. This point was taken as the point of optimal length – tension relation (Pelckmans *et al*., 1989). Muscle strips were then allowed to equilibrate for 60 min before starting the experimentation. During the equilibration period, the muscle strips were washed every 15 min with fresh Krebs – Ringer solution.

Experimental protocols

The contractile effect of electrical field stimulation (EFS, 0.25 – 8 Hz, 40 V, pulse width: 1 ms, pulse train: 10 s), carbachol (0.01 – 0.3 μM) and KCl (50 mM) was investigated in control ileum and chronically inflamed ileum that was obtained from mice that were infected during 16 weeks with *S. mansoni*. The effect of the blocker of neuronal conductance TTX (1 μM) and atropine (1 μM) was studied on the contractions to EFS and carbachol in control ileum and in chronically inflamed ileum. The effect of the purines adenosine, methyladenosine, *N*(6)-cyclohexyladenosine, 2Cl-IB-MECA, ATP, $\alpha\beta$-methylene-ATP and ADPβS was investigated on the contractions to EFS and carbachol in control ileum and in chronically inflamed ileum. To investigate the time course of the effects of purinergic compounds on neuronal enteric transmission, the effect of adenosine and ATP (10 μM) on EFS-induced contractions was further investigated in ileum from mice that were infected with *S. mansoni* during 10 and 52 weeks. Thereafter, the effect of specific antagonists of purinergic P_1 and P_2 receptors and of A_1, A_2 and A_3 receptors was investigated on the inhibitory effect of adenosine and ATP on cholinergic twitch contractions to EFS. In addition, the effect of ATP on contractions to EFS was studied in the presence of hexamethonium, a nicotinic receptor antagonist, and in the presence of L-nitroarginine, a blocker of NO synthase. Finally, the effect of the adrenergic α_2-receptor agonist UK14304 was investigated on the contractions to EFS and carbachol in control ileum and chronically inflamed ileum.

Solutions and drugs

The following drugs were used: adenosine, ADPβS, ATP, atropine sulphate, carbachol, *N*(6)-cyclohexyladenosine, hexamethonium, L-nitroarginine, methyladenosine, $\alpha\beta$-methylene-ATP (Sigma-Aldrich, St Louis, MO, U.S.A.); diethyl ether (Merck, Darmstadt, Germany); 3,7-dimethyl-1-propargylxanthine (DMPX), 8-phenyltheophylline, pyridoxal-phosphate-6-azophenyl-2′,4′-disulphonic acid tetrasodium (PPADS), theophylline, UK14304 (RBI, Natick, MA, U.S.A.); MRS 1220, 2-Cl-IB-MECA (Tocris Cookson Ltd, Avonmouth, Bristol, U.K.); tetrodotoxin (Alomone Labs, Jerusalem, Israel). All drugs were dissolved in distilled water except for the following: 8-phenyltheophylline was dissolved in DMSO at 50 mM and further dilutions were made in distilled water. 2Cl-IBMECA and MRS 1220 were dissolved in DMSO. *N*(6)-cyclohexyladenosine was dissolved in 50% ethanol at 50 mM and further dilutions were made in distilled water. DMPX was dissolved in ethanol. The maximal final concentration of ethanol and DMSO in the organ bath was 0.2%, which did not affect the contractility of the ileal muscle strips.

Presentation of results and statistical analysis

Contractions were calculated as the peak response to EFS. The cholinergic nerve-mediated contractions to EFS were expressed as a percentage of the direct smooth muscle contraction to the muscarinic receptor agonist carbachol (0.3 μM) as described previously (De Man *et al*., 2001). Muscarinic receptor sensitivity was not changed in inflamed ileum because the ratio of the receptor-mediated response to 0.3 μM carbachol to the nonreceptor-media response to 50 mM KCl was comparable in control and inflamed ileum (see Results). The concentration of 0.3 μM carbachol was chosen as reference contraction since this concentration produced reproducible contractions. Higher concentrations of carbachol ($\geq$1 μM) induced early fatigue of the smooth muscle strip and reduced the reproducibility of the responses to EFS and carbachol.

Values are shown as mean $\pm$ s.e.m. for the number (n) of mice indicated. For statistical analysis, a Student's *t*-test for paired or unpaired values was used. To compare the relative effect of the purinergic agonists in control and inflamed ileum, a two-way ANOVA (two factors: inflammation and drug under study) was used. If statistical significance was reached, this was followed by an unpaired Student's *t*-test (to compare two groups of data) or by a one-way ANOVA plus Dunnett's *post hoc* test (to compare three or more groups of data). When experiments were performed in control ileum only, a one-way ANOVA was used. P values of less than 0.05 were considered to be significant.

Results

Histology and myeloperoxidase activity

Mice that were infected with *S. mansoni* during 16 weeks showed an increased thickness of the ileal wall and intestinal granuloma formation (Figure 1) confirming previously published results (Varilek *et al*., 1991; Bogers *et al*., 2000; Moreels *et al*., 2001). The mucosa of infected mice showed blunted villi and an increased crypt depth (Figure 1), which are characteristics of chronic intestinal inflammation. The ileal myeloperoxidase activity was significantly increased from $1.8 \pm 0.2\,U\,g^{-1}$ tissue in control ileum ($n=6$) to $10.1 \pm 0.9\,U\,g^{-1}$ tissue in chronically inflamed ileum ($n=6$).

Pharmacology: contractility of control ileum and chronically inflamed ileum

The spontaneous activity of longitudinal muscle strips was higher in chronically inflamed ileum as compared to control ileum (Figures 2-4). The blocker of neuronal conductance tetrodotoxin (TTX, 1 μM) did not affect the spontaneous activity of the muscle strips (data not shown). In control and inflamed ileum, electrical field stimulation (EFS, 0.25 – 8 Hz) induced frequency-dependent transient contractions (Figures 2, 3a, 4a and 5a) while the cholinergic receptor agonist carbachol (0.01 – 0.3 μM) induced concentration-dependent sustained contractions (Figures 3a, 4a and 5b). The amplitude of the contractions to EFS and carbachol was increased in inflamed ileum (Figures 3a, 4a and 5). KCl (50 mM) induced sharp contractions that were increased in inflamed ileum (increase of $283 \pm 31\%$: from 12.8 ± 0.7 mN in control ileum to 32.3 ± 1.4 mN in inflamed ileum, $n=20$). The increase of the nonreceptor-mediated contractions to KCl in inflamed ileum

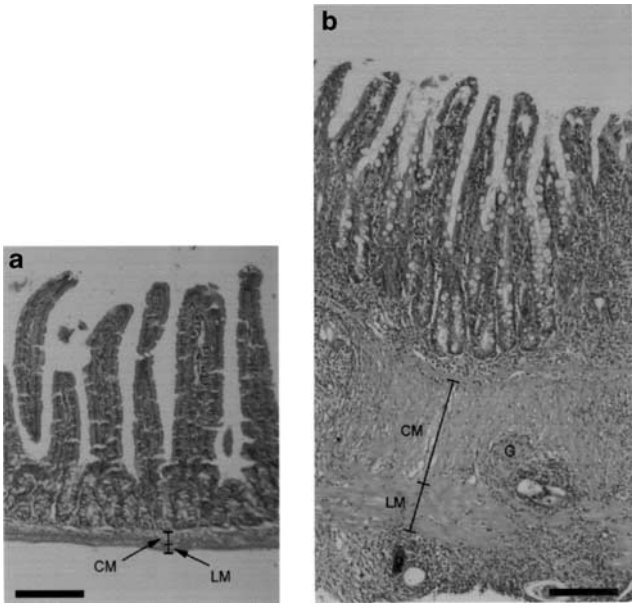

Figure 1 Representative photomicrographs of haematoxylin – eosin-stained cross-sections of (a) control ileum from uninfected mice and (b) chronically inflamed ileum of mice that were infected with *S. mansoni* during 16 weeks. Calibration bar represents 100 μm. LM, longitudinal muscle layer; CM, circular muscle layer; G, granuloma.

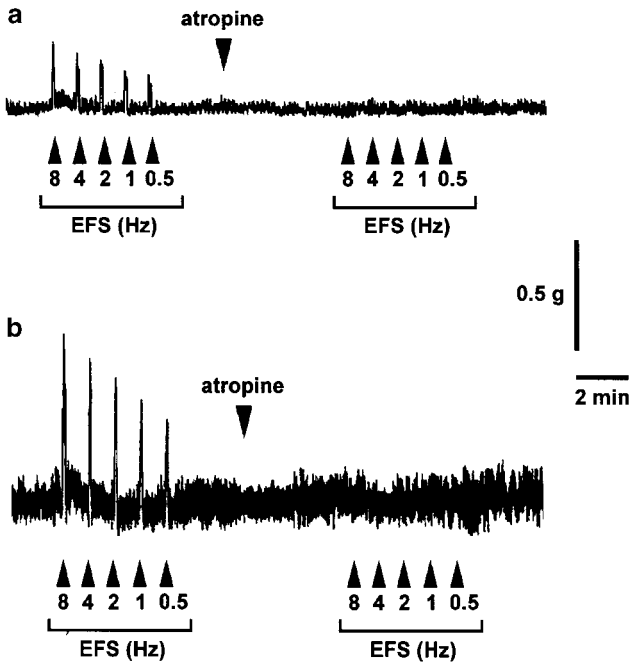

Figure 2 Typical tracings showing the effect of atropine (1 μM) on the frequency-dependent neurogenic contractions to electrical field stimulation (EFS, 0.5 – 8 Hz) in longitudinal muscle strips from *control ileum* (a) and from *chronically inflamed ileum* (b). Similar results were obtained in at least five other experiments.

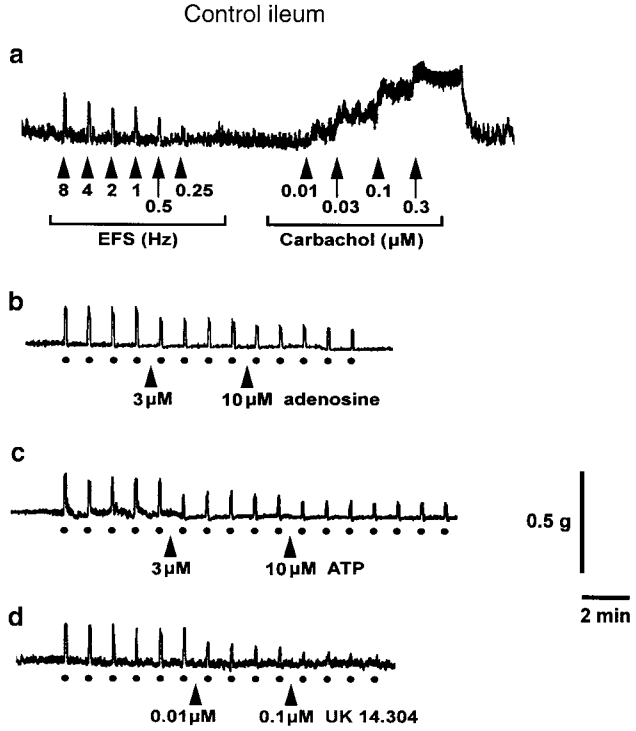

Figure 3 Typical tracings showing the activity of longitudinal muscle strips from *control ileum*. Tracing a shows the frequency-dependent contractions to electrical field stimulation (EFS, 0.25 – 8 Hz) and the concentration-dependent contractions to carbachol (0.01 – 0.3 μM). Tracings b, c and d show that adenosine, ATP and the α_2-adrenoceptor agonist UK14304 respectively inhibited the neurogenic contractile response to repetitive EFS at 1 Hz.

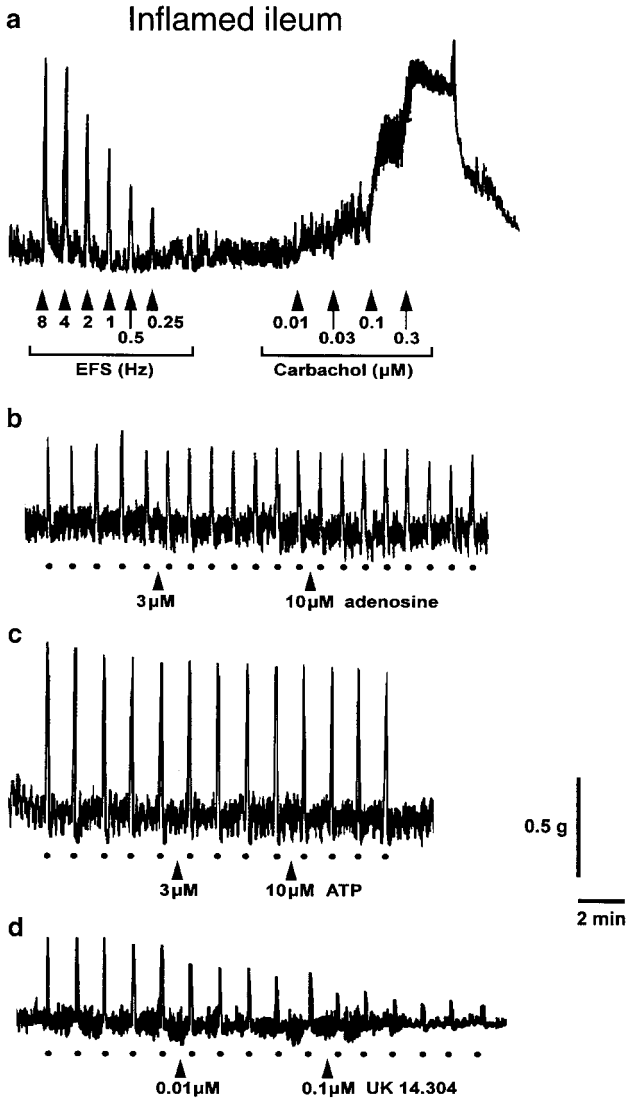

Figure 4 Typical tracings showing the activity of longitudinal muscle strips from *chronically inflamed ileum* (16-week infected mice). Tracing a shows the frequency-dependent contractions to electrical field stimulation (EFS, 0.25 – 8 Hz) and the concentration-dependent contractions to carbachol (0.01 – 0.3 μM). Tracings b and c show that adenosine and ATP, respectively, failed to inhibit the neurogenic contractile response to repetitive electrical stimulation at 1 Hz whereas the inhibition induced by the α_2-adrenoceptor agonist UK14304 was still evident (tracing d).

was comparable to the increase of the receptor-mediated contraction to 0.3 μM carbachol (increase of 277 $\pm$ 36%: from 8.2 $\pm$ 0.4 mN in controls to 21.2 $\pm$ 1.7 mN in inflamed ileum, $n = 20$).

The contractions to EFS in control and inflamed ileum were abolished by TTX (1 μM) and by the muscarinic receptor blocker atropine (1 μM) (Fig. 2), indicating that the response to EFS is of neuronal cholinergic origin. The contractions to carbachol were abolished by atropine (1 μM) but not affected by TTX (1 μM) (data not shown), indicating that these contractions are not neurogenic in origin but result from a direct activation of muscarinic receptors on the smooth muscle. The cholinergic nerve-mediated contractions to EFS were then normalised to the cholinergic smooth muscle contraction to carbachol. The normalised frequency – response curves to EFS were similar in control and chronically inflamed ileum as shown in Figure 5c.

Effect of purines on contractions to EFS and carbachol in control ileum

In *control ileum*, adenosine (3 – 30 μM) induced a dose-dependent inhibition of cholinergic nerve-mediated contractions to EFS (Figures 3b and 6a) without affecting the direct smooth muscle contractions to carbachol (Figure 7a). The inhibitory effect of adenosine was mimicked by the stable adenosine analogue methyladenosine (3 – 30 μM) (Figure 6b) and by the specific A_1 purinoreceptor agonist *N*(6)-cyclohexyladenosine (0.01 – 0.1 μM) (Figure 6c). Adenosine acts on purinergic P_1 receptors which are subdivided in adenosine A_1, A_2 and A_3 receptor subtypes. The P_1 purinoceptor antagonist theophylline (10 μM) prevented the inhibitory effect of adenosine on the contractions to EFS (Figure 8a) while PPADS (10 μM), a P_2 purinoceptor antagonist, had no effect (Figure 8e). The inhibitory effect of adenosine on contractions to EFS was also prevented by the A_1 receptor antagonist 8-phenyltheophylline (10 μM, Figure 8b), but not by the A_2 and A_3 receptor antagonists DMPX (10 μM) and MRS 1220 (0.5 μM), respectively (Figure 8c and d). Higher concentrations of MRS 1220 (1 – 3 μM) partially reversed the inhibitory effect of adenosine on cholinergic twitch contractions, but at these concentrations MRS 1220 also reversed the inhibitory action of the A_1 receptor agonist *N*(6)-cyclohexyladenosine. This suggests that MRS 1220 also blocks A_1 receptors when used at higher ($\geqslant$ 1 μM) concentrations. To rule out a possible role of A_3 receptors in the purinergic modulation of murine enteric neurotransmission, the effect of the specific A_3 receptor agonist 2Cl-IB-MECA was investigated. 2Cl-IB-MECA (0.1 – 1 μM) had no effect on the cholinergic twitch contractions to EFS (Figure 6d) or on the contractions to carbachol (0.01 – 0.3 μM, results not shown).

In *control ileum*, ATP (3 – 30 μM) dose-dependently inhibited the cholinergic nerve-mediated contractions to EFS (Figures 3c and 9a) but did not affect the myogenic contractions to carbachol (Figure 7b). The effect of ATP was not mimicked by $\alpha\beta$-methylene-ATP and ADPβS which are stable ATP analogues: $\alpha\beta$-methylene-ATP (0.1 – 30 μM) and ADPβS (0.1 – 10 μM) had no inhibitory effect on the cholinergic nerve-mediated contractions to EFS (Figure 9b and c). The inhibitory effect of ATP on contractions to EFS was blocked by theophylline (10 μM, Figure 10a) and by 8-phenyltheophylline (10 μM, Figure 10b) but not by DMPX (10 μM, Figure 10c), MRS 1220 (0.5 μM, Figure 10d) or PPADS (10 μM, Figure 10e). Since ATP activates nitrergic neurons in some species, we also investigated the effect of ATP in the presence of the blocker of nitric oxide synthase L-nitroarginine. L-Nitroarginine (300 μM) by itself significantly enhanced the cholinergic nerve-mediated contraction to EFS 1 Hz from 47 $\pm$ 2 to 59 $\pm$ 3%. However, after blockade of NO synthase with L-nitroarginine, ATP still significantly inhibited the contraction to EFS from 59 $\pm$ 3 to 37 $\pm$ 4% by 3 μM ATP and from 59 $\pm$ 3 to 27 $\pm$ 3% by 10 μM ATP ($P < 0.05$, one-way ANOVA followed by Dunnett's test).

Adenosine and ATP still induced an inhibition of neurogenic contractions to EFS in the presence of the nicotinic receptor blocker hexamethonium (100 μM, Figure 11).

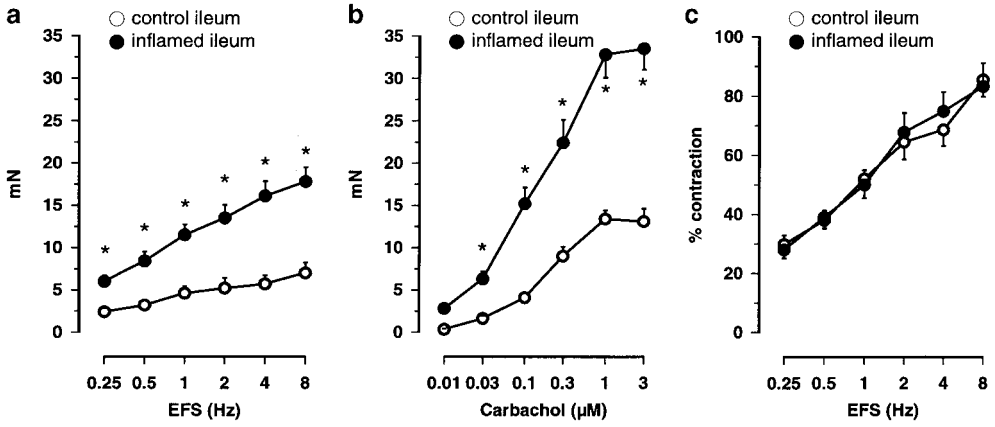

Figure 5 Panel a shows the cholinergic nerve-mediated contractions to electrical field stimulation (EFS, 0.25 – 8 Hz) and panel b shows the cholinergic direct smooth muscle-mediated contractions to carbachol (0.01 – 0.3 μM) in control ileum and in chronically inflamed ileum. Results are expressed in mN. Panel c shows the frequency – response curve to EFS in control ileum and in chronically inflamed ileum after expression of the nerve-mediated contractions to EFS as a percentage of the direct smooth muscle contraction to 0.3 μM carbachol. Results are shown as mean $\pm$ s.e.m. for $n = 6 - 8$ experiments. *$P < 0.05$, significantly different from control ileum, unpaired Student's *t*-test.

Effect of purines on contractions to EFS and carbachol in chronically inflamed ileum

In *chronically inflamed ileum* from 16-week infected mice, adenosine and methyladenosine failed to inhibit the cholinergic nerve-mediated contractions to EFS (Figures 4b, 6e and f). *N*(6)-cyclohexyladenosine induced a slight but significant inhibitory effect but only at the highest concentration used (Figure 6g), and this effect was less pronounced as compared to control ileum (0.1 μM *N*(6)-cyclohexyladenosine inhibited the contraction to 1 Hz EFS by 15.6 $\pm$ 3.7% in inflamed ileum and by 57.8 $\pm$ 5.7% in control ileum, $n = 6 - 8$, $P < 0.001$ unpaired Student's *t*-test). Similar to control ileum, 2Cl-IB-MECA (0.1 – 1 μM) had no effect on the neurogenic contractions to EFS (Figure 6h).

In contrast to control ileum, ATP (3 – 30 μM) did not inhibit the cholinergic contractions to EFS in *chronically inflamed ileum* (Figures 4c and 9d). Also $\alpha\beta$-methylene-ATP and ADPβS had no effect on these contractions in inflamed tissue (Figure 9e and f). Adenosine, methyladenosine, *N*(6)-cyclohexyladenosine, ATP, $\alpha\beta$-methylene-ATP also had no effect on the myogenic contractions to carbachol in chronically inflamed ileum (shown in Figure 7c and d for adenosine and ATP). Since adenosine or ATP did not inhibit the contractions to EFS in inflamed ileum, the effect of the purinoceptor antagonists was not investigated in the presence of adenosine or ATP in inflamed ileum.

The inflammatory granulomatous response in the intestine starts from the 8th week of infection with *S. mansoni* (Varilek *et al*., 1991; Bogers *et al*., 2000), and the number of mast cells in the muscle layers is significantly enhanced from the 12th week of infection (Bogers *et al*., 2000; De Jonge *et al*., 2002). To investigate further the time course of the loss of the neuromodulatory effect of purinergic agonists during inflammation, additional experiments were performed on ileal muscle strips from mice that were with *S. mansoni* during 10 and 52 weeks. In the ileum of 10-week infected mice, adenosine and ATP still significantly inhibited the contractions to EFS (1 Hz) (Table 1). The potency of inhibition was comparable to that observed in control (uninfected) mice (Table 1). However, in the ileum of 52-week infected mice, adenosine and ATP failed to inhibit the contractions to EFS (Table 1). This loss of effect was similar to that observed in 16-week infected mice (Table 1).

Effect of prolonged treatment of control ileum with methyladenosine

In order to investigate the possible mechanism of the disturbed A_1 purinoceptor-mediated modulation during inflammation, we studied whether desensitisation of A_1 receptors occurs after prolonged contact with an A_1 receptor agonist. Therefore, the inhibitory effect of 10 μM adenosine on contractions to EFS was investigated before and after a 90 min treatment of the strips with the stable adenosine analogue methyladenosine (100 μM). After the 90 min treatment, muscle strips were washed two times with fresh Krebs – Ringer solution to remove methyladenosine from the organ bath. Prolonged treatment of the muscle strips with methyladenosine did not affect the amplitudes of the contractions to EFS (Figure 12), indicating that the contractility of the smooth muscle was not affected. However, after methyladenosine treatment, the ability of adenosine to inhibit the neurogenic contractions to EFS was significantly reduced (Figure 12). This lack of inhibitory effect of adenosine in treated muscle strips persisted 90 min after methyladenosine treatment. In time control strips, which were treated with saline for 90 min, contractions to EFS were still fully sensitive to inhibition by adenosine (data not shown).

Effect of α_2-adrenoceptor activation in control and chronically inflamed ileum

To investigate whether there was a general loss of prejunctional modulatory mechanisms during intestinal inflammation, the effect of the specific agonist of adrenergic α_2-receptors UK14304 was studied. Similar to what we reported before (De Man *et al*., 2001), UK14304 (0.01 μM) inhibited the cholinergic nerve-mediated contraction to 1 Hz EFS with similar potency in control and inflamed ileum (contraction to 1 Hz EFS was inhibited from 47.6 $\pm$ 4.8 to 28.0 $\pm$ 5.6% ($n = 6$) in *control ileum*

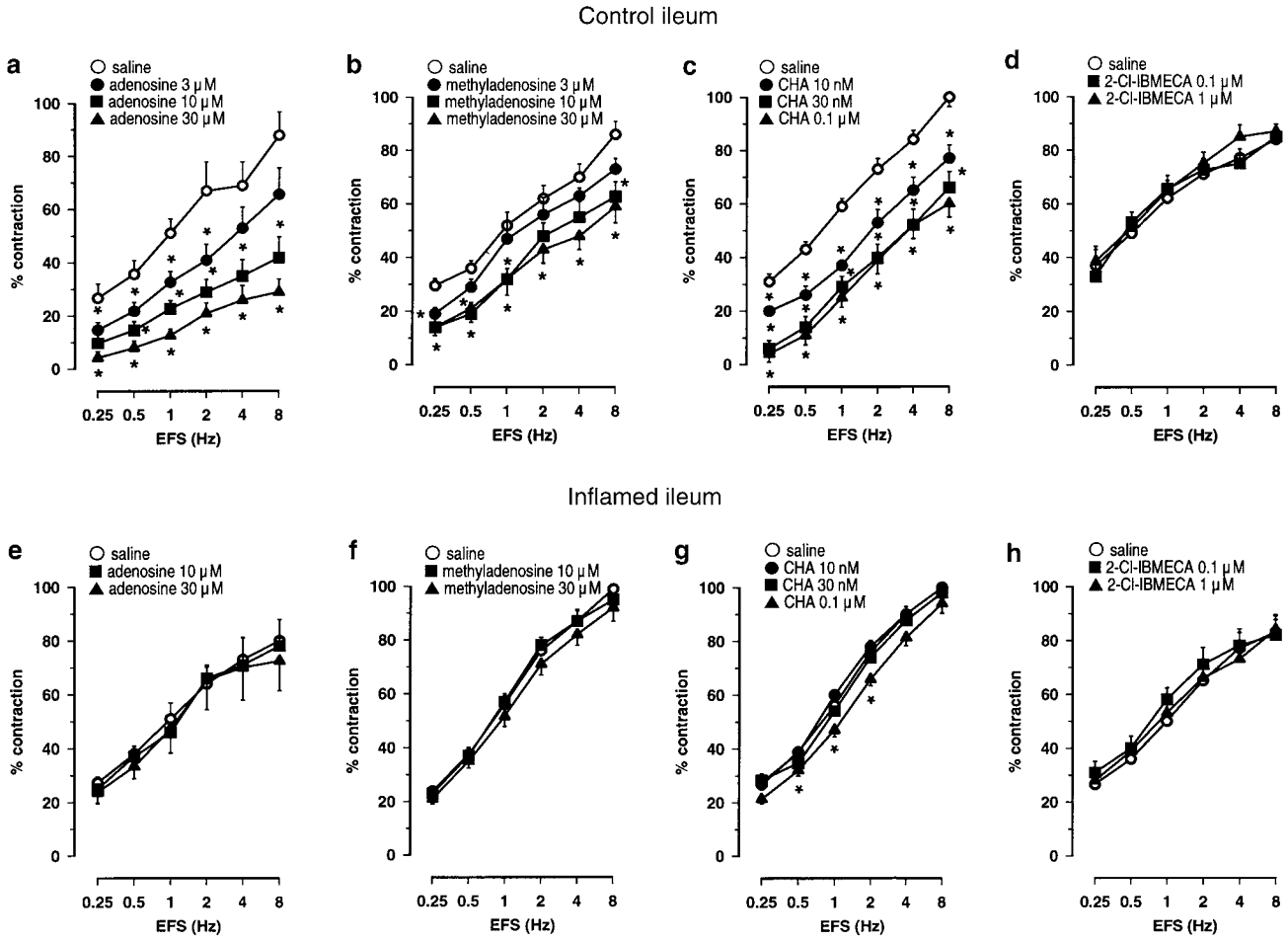

Figure 6 Effect of adenosine, methyladenosine, *N*(6)-cyclohexyladenosine (CHA) and 2-Cl-IB-MECA on the frequency – response curves to electrical field stimulation (EFS) in *control ileum* (upper panels, a – d) and in *chronically inflamed ileum* (lower panels, e – h). Results are expressed as a percentage of the internal control contraction to 0.3 μM carbachol and shown as mean $\pm$ s.e.m. for $n = 6 - 8$ experiments. $^*P < 0.05$, significantly different from control values in saline, two-way ANOVA followed by one-way ANOVA plus Dunnett's *post hoc* test.

and from 49.80 ± 6.2 to $32.3 \pm 5.8\%$ ($n = 6$) in *chronically inflamed* ileum ($n = 6$) by 0.01 μM UK14304).

Discussion

There is compelling evidence that chronic intestinal inflammation disturbs gastrointestinal motility, but the exact mechanisms of these disturbances are largely unknown. It is hypothesised that the immune response during chronic inflammation of the gut may directly affect the normal function of the enteric nervous system, but this is not fully investigated. Our present results show that chronic intestinal inflammation significantly impairs the purinergic control of enteric cholinergic neurotransmission in the mouse ileum. Chronic intestinal inflammation was induced by infecting mice with the parasite *S. mansoni*. This induces a chronic granulomatous inflammation of the small intestine that may last for more than 12 months depending upon the severity of infection (Domingo & Warren, 1969; Weinstock, 1992). Histology showed a diffuse inflammatory infiltrate in the mucosal and smooth muscle layers of the inflamed ileum, a gross thickening of the bowel wall, granuloma formation, blunted villi and an enhanced MPO activity. This confirms previous findings (Domingo & Warren, 1969; Varilek *et al*., 1991; Weinstock, 1992; Bogers *et al*., 2000) and shows that murine schistosomiasis represents an interesting experimental model of chronic granulomatous intestinal inflammation.

EFS and carbachol induced frequency- and dose-dependent contractions in control and chronically inflamed ileum. The contractions to EFS were of neuronal cholinergic origin since they were abolished by TTX and atropine. The contractions to carbachol resulted from a direct activation of muscarinic receptors on the smooth muscle since these contractions were not affected by TTX but abolished by atropine.

Chronically inflamed ileum showed an enhanced contractile activity for EFS, carbachol and KCl. The cholinergic nerve-mediated contractions to EFS and the cholinergic myogenic contractions to carbachol were enhanced to a comparable degree. Also the nonreceptor-mediated contraction to KCl was increased to a comparable degree as the contraction to carbachol. This indicates that the hypercontractile activity of chronically inflamed ileum did not result from a change in muscarinic receptor function. Most likely, the hypercontracti-

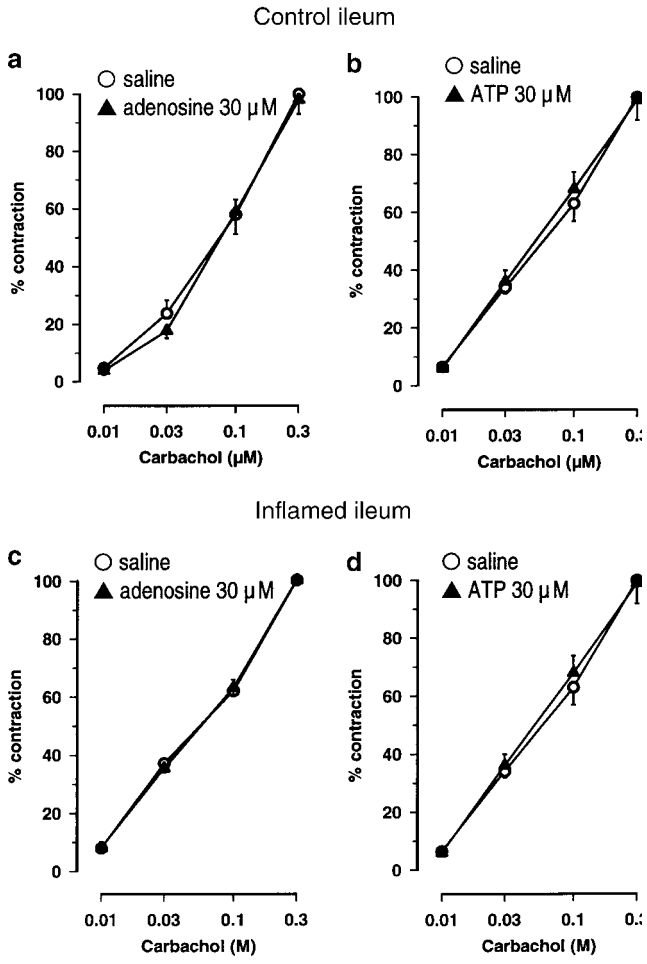

Figure 7 Effect of adenosine and ATP on the direct smooth muscle contractions to carbachol in *control ileum* (upper panels, a and b) and in *chronically inflamed ileum* (lower panels, c and d). Results are expressed as percentage of the internal control contraction to 0.3 μM carbachol and shown as mean $\pm$ s.e.m. for $n = 6-8$ experiments. Two-way ANOVA did not show any significant differences.

lity results from smooth muscle cell hypertrophy and hyperplasia that is observed during murine intestinal schistosomiasis (Bogers *et al*., 2000; Moreels *et al*., 2001). However, chronic intestinal inflammation may also induce changes at the level of the enteric nerves since we found a disturbed purinergic control of cholinergic nerve activity in inflamed ileum.

In the gastrointestinal tract, purinoceptors are present on many different cell types including smooth muscle cells, enteric nerves, immune cells and glial cells. Purinoceptors are divided into P_1 and P_2 receptors. P_1 receptors are activated by adenosine and they are subdivided in adenosine A_1, A_2 and A_3 receptors. P_2 receptors are activated by ATP and they are subdivided in several different subtypes. Two distinct classes of P_2 subtypes are recognised: P_{2X} receptors that are activated by $\alpha\beta$-methylene-ATP and P_{2Y} receptors that are activated by ADPβS (Bultmann *et al*., 1996; Zagorodnyuk & Maggi, 1998) (for a recent review on P_1 and P_2 purinergic receptors, see Burnstock & Williams, 2000; Fredholm *et al*., 2001).

Purinergic modulation of cholinergic neurotransmission in normal ileum

It is well established in several species that purines modulate the release of acetylcholine from enteric cholinergic nerves (Sawynok & Jhamandas, 1976; Vizi & Knoll, 1976; Gustafsson *et al*., 1978; Moody & Burnstock, 1982). Whether this also holds true for murine small intestine was not investigated yet, but our results in normal mouse ileum show that adenosine dose-dependently inhibited the nerve-mediated cholinergic contractions to EFS. This inhibition resulted from a prejunctional action since adenosine did not affect the direct smooth muscle contractions to carbachol. The inhibitory effect of adenosine on cholinergic nerve activity was prevented by the P_1 antagonist theophylline but not by the P_2 antagonist PPADS, and by the A_1 antagonist 8-phenyltheophylline but not by the A_2 antagonist DMPX and the A_3 antagonist MRS 1220. In addition, the inhibitory effect of adenosine was mimicked by the stable adenosine analogue methyladenosine and by the A_1 specific agonist *N*(6)-cyclohexyladenosine, but not by the A_3 agonist 2Cl-IB-MECA. These results strongly suggest that adenosine inhibits cholinergic nerve activity in the mouse ileum by activation of neuronal P_1 receptors of the A_1 subtype. Our results in the mouse ileum correlate well with previous findings of A_1 receptor-mediated inhibition of cholinergic responses and acetylcholine release in other species (Moody & Burnstock, 1982; Christofi *et al*., 1992; Coupar, 1999; Lee & Parsons, 2000; Lee *et al*., 2001).

Similar to adenosine, ATP inhibited cholinergic nerve-mediated contractions to EFS in normal mouse ileum. This effect of ATP was still evident after inhibition of nitric oxide synthase, indicating that nitrergic nerves are not involved in this mechanism of prejunctional inhibition. The inhibitory effect of ATP was not blocked by A_2, A_3 and P_2 receptor antagonists but it was prevented by P_1 and A_1 purinoceptor antagonists. This was surprising since ATP is supposed to act on P_2 receptors. Most likely, exogenous ATP is rapidly dephosphorylated to adenosine that in turn activates P_1 receptors. Such a dephosphorylation is reported to occur rapidly in intestinal tissue (Nicholls & Hourani, 1997). In support of this is the finding that the stable ATP analogues $\alpha\beta$-methylene-ATP and ADP-β-S, which are less susceptible to dephosphorylation, did not mimic the effect of exogenous ATP as they did not inhibit the neurogenic twitch contraction to EFS. This is in contrast with guinea-pig ileum where P_2 receptors modulate neurogenic contractions to EFS (Bartho *et al*., 1997). In this tissue, P_2 receptors also modulate intestinal peristalsis (Heinemann *et al*., 1999). Although our results do not exclude P_2 receptors from being involved in intestinal peristalsis in the mouse, they do suggest that the activity of murine cholinergic motor nerves, innervating the longitudinal muscle, is not modulated by P_2 receptors.

The nicotinic receptor blocker hexamethonium did not block the inhibitory effect of adenosine and ATP on cholinergic nerve activity. This indicates that adenosine and ATP acted postsynaptically on cholinergic motor neurons that innervate the longitudinal ileal muscle. This postsynaptic action is in contrast with previous findings in the guinea-pig gastric antrum where purines inhibit cholinergic transmission at a presynaptic nicotinic receptor-dependent site (Christofi *et al*., 1992). Overall, these discrepancies indicate that the mechanisms of purinergic modulation of cholinergic transmis-

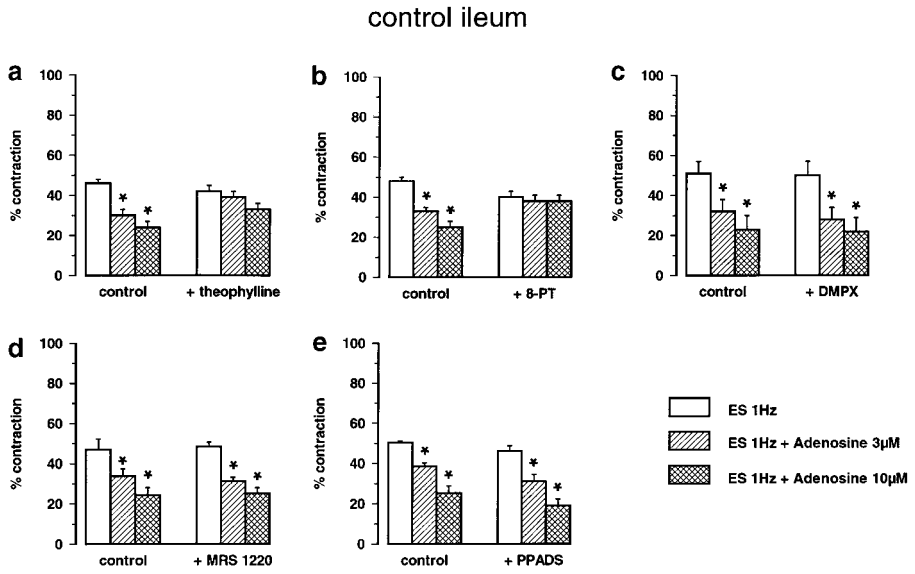

Figure 8 The inhibitory effect of adenosine (3 μM, hatched bars; 10 μM, crosshatched bars) on nerve-mediated contractions to 1 Hz electrical field stimulation (EFS) in *control ileum* was prevented by 10 μM theophylline (a) and by 10 μM 8-phenyltheophylline (8-PT, b) but not by 10 μM DMPX (c), 0.5 μM MRS 1220 (d) or 10 μM PPADS (e). Results are expressed as percentage of the internal control contraction to 0.3 μM carbachol and shown as mean + s.e.m. for $n=6$ experiments. $^*P<0.05$, significantly different from control values in saline (open bars), one-way ANOVA followed by Dunnett's *post hoc* test.

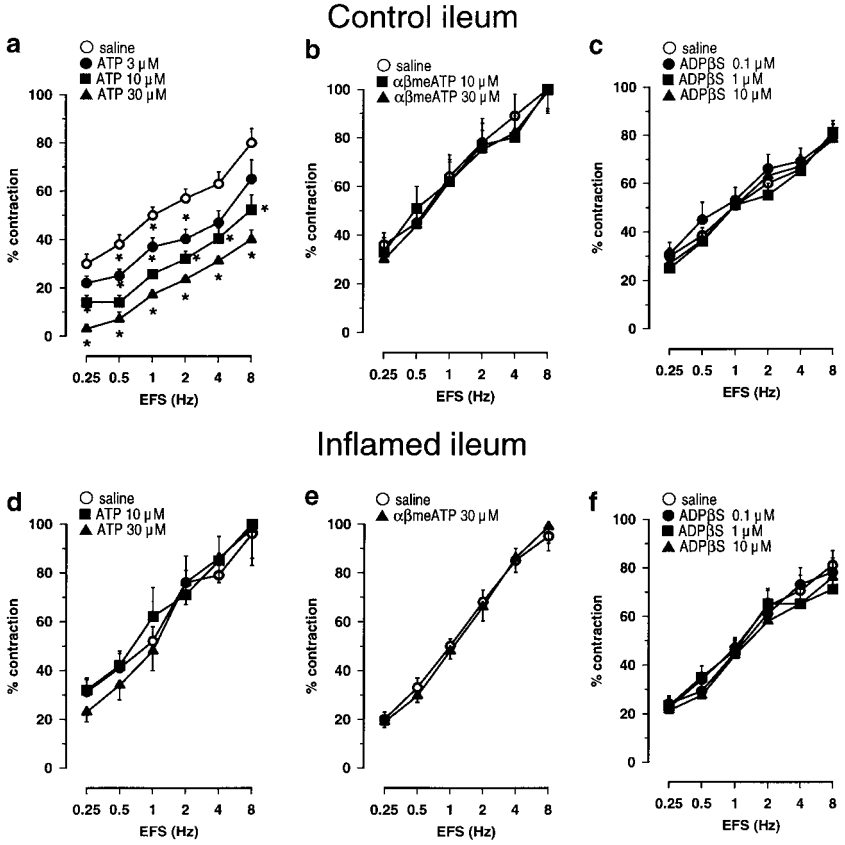

Figure 9 Effect of ATP, $\alpha\beta$-methylene-ATP and ADPβS on the frequency – response curves to electrical field stimulation (EFS) in *control ileum* (upper panels, a – c) and in *chronically inflamed ileum* (lower panels, d – f). Results are expressed as a percentage of the internal control contraction to 0.3 μM carbachol and shown as mean $\pm$ s.e.m. for $n=4-6$ experiments. $^*P<0.05$, significantly different from control values in saline, two-way ANOVA followed by one-way ANOVA plus Dunnett's *post hoc* test.

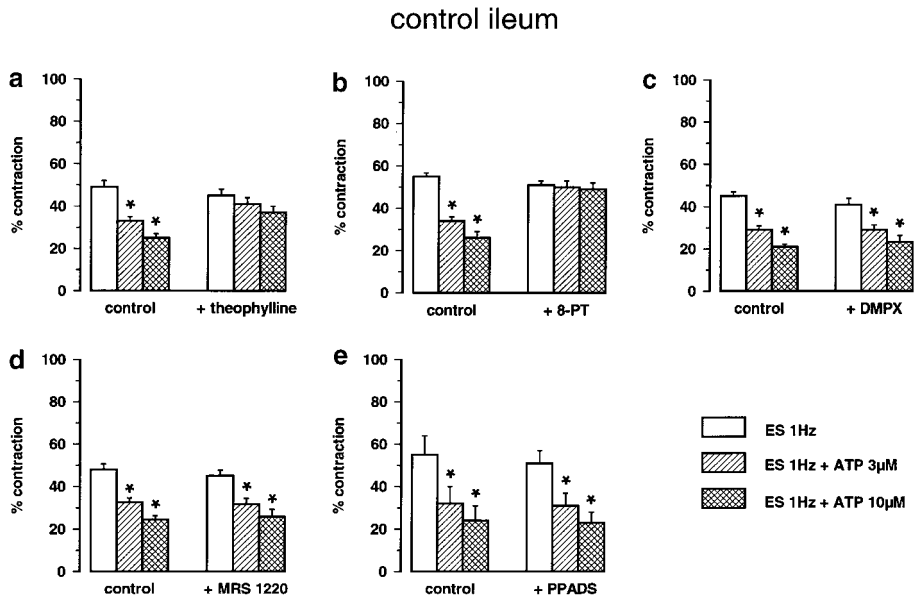

Figure 10 The inhibitory effect of ATP (3 μM, hatched bars; 10 μM, crosshatched bars) on nerve-mediated contractions to 1 Hz electrical field stimulation (EFS) in *control ileum* was prevented by 10 μM theophylline (a) and by 10 μM 8-phenyltheophylline (8-PT, b) but not by 10 μM DMPX (c), 0.5 μM MRS 1220 (d) or 10 μM PPADS (e). Results are expressed as a percentage of the internal control contraction to 0.3 μM carbachol and shown as mean$\pm$s.e.m. for $n=6$ experiments. *$P<0.05$, significantly different from control values in saline (open bars), one-way ANOVA followed by Dunnett's *post hoc* test.

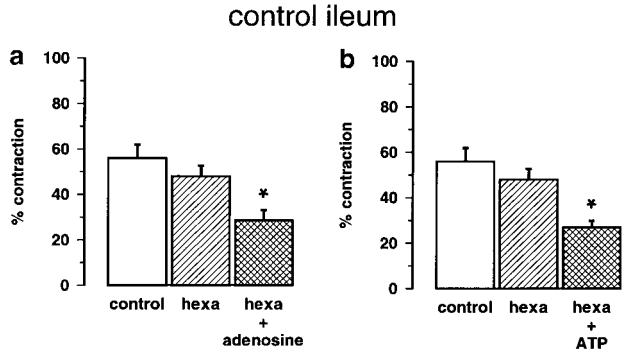

Figure 11 Blockade of nicotinic receptors with 100 μM hexamethonium (hexa) did not affect the inhibitory effect of 10 μM adenosine (a) and 10 μM ATP (b) on the cholinergic nerve-mediated contraction to electrical field stimulation (EFS, 1 Hz) in *control ileum*. Results are expressed as a percentage of the internal control contraction to 0.3 μM carbachol and shown as mean$\pm$s.e.m. for $n=7$ experiments. *$P<0.05$, significantly different from control values in saline, one-way ANOVA followed by Dunnett's *post hoc* test.

sion in the enteric nervous system may differ between species and between gastrointestinal tissues that are under study.

Purinergic modulation of cholinergic neurotransmission in chronically inflamed ileum

We next investigated the effect of purines on cholinergic nerve activity during chronic intestinal inflammation. In chronically inflamed ileum, adenosine, methyladenosine and ATP all failed to inhibit the cholinergic nerve-mediated contractions to EFS. The specific A_1 agonist *N*(6)-cyclohexyladenosine still slightly inhibited the contractions to EFS in chronically inflamed ileum. However, this effect was minor compared to control ileum and observed only at the highest dose used. These results indicate that the purinergic control of cholinergic nerve activity was significantly impaired during chronic intestinal inflammation.

The underlying mechanism of this disturbed purinergic neuromodulation is not clear. Depoortere *et al*. (2002) recently demonstrated that inflammation of the rabbit colon results in a loss of purinergic inhibitory neurotransmission through an unknown mechanism. Possibly, purines that are released during inflammation interfere with purinergic receptors on enteric nerves. During the inflammatory process, purines such as adenosine and ATP are released from mast cells (Marquardt *et al*., 1984). Purines also modulate mast cell degranulation (Marquardt *et al*., 1978; Church & Hughes, 1985; Ramkumar *et al*., 1993; Fozard *et al*., 1996) and they can potentiate their own effect on mast cells by a positive feedback mechanism (Marquardt *et al*., 1978). Purines are therefore thought to play a crucial role in the mast cell response to inflammation. In the present study, we found that the loss of the neuromodulatory role of adenosine and ATP was evident in mice that were infected during 16 and 52 weeks but not in mice that were infected during 10 weeks. Interestingly, the number of mast cells in the ileal muscle layer is significantly enhanced from the 12th week after *S. mansoni* infection of mice (Bogers *et al*., 2000; De Jonge *et al*., 2002). The majority of these mast cells are located in the vicinity of myenteric neurons (Bogers *et al*., 2000), which correlates well with findings in humans (Stead *et al*., 1989). It is hypothesised that mediators of immune cells such as mast cells are in the first line to disturb the function of the enteric nervous system during chronic inflammation. Therefore, the loss of the neuromodulatory role of adenosine during chronic intestinal inflammation may result from chronic exposure of enteric nerves to adenosine released during the inflammatory process. This is supported by our

Table 1 Effect of adenosine and ATP on the cholinergic nerve-mediated contractions to EFS (1 Hz) in control mice and in mice that were infected with *S. mansoni* during 10, 16 and 52 weeks

	EFS 1 Hz control (n=6)	*EFS 1 Hz 10 weeks (n=4)*	*EFS 1 Hz 16 weeks (n=6)*	*EFS 1 Hz 52 weeks (n=4)*
Saline	51 ± 4%	49 + 5%	51 ± 6%	54 ± 7%
Adenosine (10 μM)	23 ± 3%*	24 ± 6%*	49 ± 9%	48 ± 7%
Saline	63 ± 5%	53 ± 6%	52 ± 6%	52 ± 8%
ATP(10 μM)	31 ± 2%*	26 ± 8%*	61 ± 9%	55 ± 13%

Results are expressed as a percentage of the internal control contraction to 0.3 μM carbachol and shown as mean ± s.e.m. for the number (n) of experiments indicated.*$P<0.05$, significantly different from control values in saline, Student's t-test for paired values.

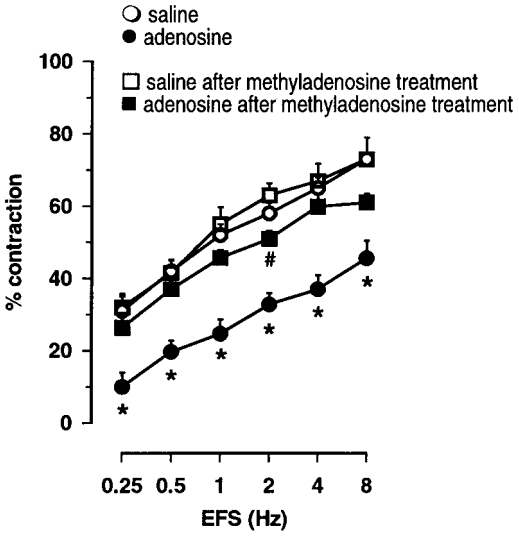

Figure 12 Effect of adenosine on the neurogenic contractions to EFS before and after prolonged treatment of ileal muscle strips with methyladenosine (100 μM, 90 min treatment). Before methyladenosine treatment, the contractions to EFS were significantly inhibited by 10 μM adenosine. After methyladenosine treatment, the contractions to EFS were not affected by 10 μM adenosine. Results are expressed as a percentage of the internal control contraction to 0.3 μM carbachol and shown as mean ± s.e.m. for $n=5$ experiments. $P<0.05$, significantly different from control values in saline (*) and from control values in saline after methyladenosine treatment (#), one-way ANOVA followed by Dunnett's *post hoc* test.

observation that prolonged treatment of muscle strips with methyladenosine abrogated the inhibitory effect of adenosine on the cholinergic nerve-mediated contractions to EFS. This suggests that purinoceptor desensitisation can occur during prolonged contact with purines, which is in accordance with previous observations in intestinal tissue (e.g. Burnstock *et al.*, 1970; Weston, 1973; Okwuasaba *et al.*, 1977). Such a desensitisation of adenosine receptors on mast cells is also observed when mast cells are chronically exposed to adenosine (Marquardt & Walker, 1987). This correlates well with our previous finding that the neuromodulatory function of histamine, a major mast cell mediator, is impaired during chronic intestinal inflammation (De Man *et al.*, 2001).

The consequence of the loss of purinergic modulation of enteric cholinergic neurotransmission during chronic inflammation remains unknown. Adenosine is regarded as a sophisticated modulator of enteric neurotransmission. Since acetylcholine is the prime regulator of gastrointestinal motility, a disturbed release of acetylcholine may result in significant alterations of coordinated intestinal propulsive patterns. We previously reported that chronic intestinal inflammation impairs the transit of a semiliquid meal through the small intestine (Moreels *et al.*, 2001). Although this disturbance may also result from inflammation-induced changes at the level of the smooth muscle, it indicates that the chronically inflamed intestine has a disturbed motility pattern to which impaired modulation of enteric neurotransmission may contribute.

It is unclear whether intestinal inflammation induces a general disturbance of enteric neuromodulatory mechanisms. Recently, Zhao *et al.* (2001) reported that also the β_3 adrenoceptor-mediated control of smooth muscle activity is impaired in the inflamed rat colon. In our model, the specific α_2 adrenoceptor agonist UK 14.034 had a comparable inhibitory effect on neurogenic cholinergic contractions in control and chronically inflamed ileum as reported before (De Man *et al.*, 2001) and confirmed in this study. This indicates that chronic inflammation disturbs the function of certain but not all prejunctional receptors in the enteric nervous system.

In conclusion, we demonstrated that chronic intestinal inflammation in mice disturbs the purinergic P_1 receptor-mediated modulation of cholinergic motor nerves innervating longitudinal muscle in the ileum. This suggests that chronic inflammation leads to a dysfunction of neuronal modulatory mechanisms in the enteric nervous system. Such dysfunctions may contribute to the gastrointestinal motility disturbances that are observed in patients with chronic inflammatory bowel disorders.

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