

RESEARCH PAPER

Control of enteric neuromuscular functions by purinergic A₃ receptors in normal rat distal colon and experimental bowel inflammation

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BACKGROUND AND PURPOSE

Adenosine A₃ receptors mediate beneficial effects in experimental colitis, but their involvement in enteric neuromuscular functions during bowel inflammation is undetermined. This study investigated the regulatory role of A₃ receptors on colonic motility in the presence of experimental colitis.

EXPERIMENTAL APPROACH

Colitis was induced in rats by 2,4-dinitrobenzenesulfonic acid. A₃ receptors and adenosine deaminase (ADA, adenosine catabolic enzyme) mRNA were examined by RT-PCR. Tissue distribution of A₃ receptors was detected by confocal immunofluorescence. The effects of 2,3-ethyl-4,5-dipropyl-6-phenylpyridine-3-thiocarboxylate-5-carboxylate (MRS1523) (MRS, A₃ receptor antagonist), 2-chloro-N₆-(3-iodobenzyl)-adenosine-5'-N-methyluronamide (2Cl-IB-MECA) (CIB, A₃ receptor agonist), dipyrindamole (DIP, adenosine transport inhibitor) and ADA were assayed on contractile responses evoked by electrical stimulation (ES) or carbachol in colonic longitudinal muscle preparations (LMP).

KEY RESULTS

RT-PCR showed A₃ receptors and ADA mRNA in normal colon and their increased level in inflamed tissues. Immunofluorescence showed a predominant distribution of A₃ receptors in normal myenteric ganglia and an increased density during colitis. MRS enhanced ES-induced cholinergic contractions in normal LMP, but was less effective in inflamed tissues. After pretreatment with dipyrindamole plus ADA, to reduce extracellular adenosine, CIB decreased cholinergic motor responses of normal LMP to ES, with enhanced efficacy in inflamed LMP. A₃ receptor ligands did not affect carbachol-induced contractions in LMP from normal or inflamed colon.

CONCLUSIONS AND IMPLICATIONS

Normally, adenosine modulated colonic cholinergic motility via activation of A₃ receptors in the myenteric plexus. A₃ receptor-mediated tonic inhibitory control by adenosine was impaired in inflamed bowel, despite increased density of functioning and pharmacologically recruitable A₃ receptors.

Abbreviations

2Cl-IB-MECA, 2-chloro-N₆-(3-iodobenzyl)-adenosine-5'-N-methyluronamide; ADA, adenosine deaminase; DNBS, 2,4-dinitrobenzenesulfonic acid; dNTP, deoxynucleotide triphosphate mixture; ES, electrical stimulation; FSPCX, 8-cyclopentyl-3-N-[3-((3-(4-fluorosulphonyl)benzoyl)-oxy)-propyl]-1-N-propylxanthine; GR159897,

5-fluoro-3-[2-[4-methoxy-4-[(R)-phenylsulphonyl]methyl]-1-piperidinyl]ethyl]-1H-indole; IBDs, inflammatory bowel diseases; L-732138, N-acetyl-L-tryptophan 3,5-bis(trifluoromethyl)benzyl ester; LMP, longitudinal muscle preparations; MMLV, Moloney murine leukemia virus; MRS1523, 2,3-ethyl-4,5-dipropyl-6-phenylpyridine-3-thiocarboxylate-5-carboxylate; MRS1754, 8-(4-[(4-cyanophenyl)carbamoylmethyl]oxy]phenyl)-1,3-di(n-propyl)xanthine; NK, neurokinin; NPA, N^ω-propyl-L-arginine; RT-PCR, reverse transcription-polymerase chain reaction; SB218795, (R)-[[2-phenyl-4-quinolinyl]carbonyl]amino]-methyl ester benzenecetic acid; TBS, Tris-buffered saline; ZM241385, 4-(2-[7-amino-2-(2-furyl)[1,2,4]triazolo[2,3-a][1,3,5]triazin-5-ylamino]ethyl)phenol

Introduction

Intestinal motor dysfunctions are regarded as a prominent cause for the development of digestive symptoms (diarrhea and/or constipation, visceral pain) in patients with inflammatory bowel diseases (IBDs), both during episodes of inflammatory activity and in periods of remission, severely undermining their quality of life (De Schepper *et al.*, 2008).

Bowel inflammatory disorders are associated with complex structural and/or functional changes in the enteric nervous system (Neunlist *et al.*, 2003) and smooth muscle cells (Wells and Blennerhassett, 2004), which can contribute to severe disturbances of gastrointestinal motility. A number of reports have demonstrated that, in the setting of experimental colitis, changes in the neurophysiology of enteric neural circuits can account for alterations of motor functions occurring in the inflamed gut (De Man *et al.*, 2001; Blandizzi *et al.*, 2003; Depoortere *et al.*, 2010), and there is evidence that such abnormalities depend on mutual interactions between the enteric nervous system and cells of the immune/inflammatory system (Mazzone and Farrugia, 2007). However, most of the mechanisms through which intestinal inflammation can affect the enteric neurotransmission and bowel neuromuscular contraction remain to be clarified.

Adenosine, an endogenous purine nucleoside, is involved in the control of several physiological functions via specific receptors, designated as A₁, A_{2A}, A_{2B} and A₃ (Burnstock, 2006; nomenclature follows Alexander *et al.*, 2009), and it plays a pivotal role as modulator of innate immune responses in the presence of inflammatory reactions (Antonioli *et al.*, 2008a; Haskó *et al.*, 2008). Recent observations have demonstrated a remarkable involvement of A₃ receptors in the anti-inflammatory actions of adenosine (Gessi *et al.*, 2008; Ochaion *et al.*, 2009). These findings have channelled pharmacological research towards the synthesis of selective A₃ receptor agonists, which have been shown to exert promising beneficial effects in several models of inflammation, including experimental colitis (Mabley *et al.*, 2003; Fishman *et al.*, 2006; Guzman *et al.*, 2006; Bar-Yehuda *et al.*, 2007; Ochaion *et al.*, 2008).

Besides the pivotal role played by adenosine in the regulation of inflammatory responses, several lines of evidence indicate a significant involvement of this mediator in the regulation of gastrointestinal neuromuscular functions. Such a control appears to depend either on the modulation of enteric neurotransmitter release via A₁, A_{2A} or A_{2B} receptors in rodents (Christofi *et al.*, 1990; Christofi and Wood, 1993; Duarte-Araújo *et al.*, 2004; Antonioli *et al.*, 2006; Zizzo *et al.*, 2009) and humans (Fornai *et al.*, 2009), or on a direct influence on gut smooth muscle cells via A_{2B} receptors (Fozard *et al.*, 2003). Recently, Bozarov *et al.* (2009) demonstrated the involvement of A₃ receptors in the modulation of reflexes driven by histamine and involving cholinergic activity in guinea-pig colon. However, there is a lack of knowledge about the possible contribution of A₃ receptors in the regulation of enteric neuromotor functions and their possible implication in the pathophysiology of gut dysmotility associated with bowel inflammation. In particular, based on the evidence that the pharmacological stimulation of this receptor subtype can ameliorate bowel inflammation, the characterization of a role played by A₃ receptors in the control of gut motility could pave the way to the development of novel therapeutic tools endowed with both anti-inflammatory properties and the ability to counteract enteric dysmotility associated with gut inflammation. Based on these considerations, the present study was designed to investigate the expression of A₃ receptors in the neuromuscular compartment of rat distal colon and to characterize their functional role in the control of colonic neuromuscular activity, both under normal conditions and in the presence of experimental colitis.

Methods

Animals

All animal care and experimental procedures were in accordance with the provisions of the European Community Council Directive 86-609, recognized and adopted by the Italian Government. Albino male Sprague-Dawley rats, 200–250 g body weight, were used throughout the study. The animals were

fed standard laboratory chow and tap water *ad libitum* and were not employed for at least 1 week after their delivery to the laboratory. They were housed, three in a cage, in temperature-controlled rooms on a 12 h light cycle at 22–24°C and 50–60% humidity.

Induction and assessment of colitis

Colitis was induced as described by Fornai *et al.* (2006). Animals were anaesthetized with isoflurane (3% in air), and 30 mg of 2,4-dinitrobenzenesulfonic acid (DNBS) in 0.25 mL of 50% ethanol was administered intrarectally with a polyethylene catheter inserted 8 cm proximal to the anus. Control animals received 0.25 mL of vehicle. Animals underwent subsequent experimental procedures 6 days after DNBS injection, in order to allow a full development of histologically evident colonic inflammation. At that time, the animals were killed by overdose of isoflurane and the distal colon was excised and processed for macroscopic damage score, recording of contractile activity, histology, reverse transcription-polymerase chain reaction (RT-PCR) or immunofluorescence analysis, as reported below. Care was taken to focus all experiments on distal colon, as DNBS-induced inflammation develops mostly in this gut region. Moreover, it has been previously appreciated that, as compared with distal colon, both proximal and middle colon exhibit different patterns of spontaneous motility as well as different contributions of enteric neurotransmitters to evoked motor responses (Suthamnatpong *et al.*, 1993).

The evaluation of intestinal inflammation severity was performed both macroscopically and histologically, in accordance with the criteria previously reported by Antonioli *et al.* (2007). The criteria for macroscopic scoring of colonic damage were as follows: (i) presence of adhesions between colonic tissue and other organs (0 none, 1 minor and 2 major adhesions); (ii) consistency of colonic faecal material (0 formed, 1 loose and 2 liquid stools); and (iii) presence of ulceration (0 none, 1 hyperaemia, 2 ulceration without hyperaemia, 3 ulceration with inflammation at 1 side, 4 \geq 2 sites of ulceration and inflammation, 5 major sites of damage, 6 major sites of damage extending >2 cm). The score was increased by 1 unit for each millimetre of colonic wall thickness. Microscopic evaluations were carried out by light microscopy on haematoxylin- and eosin-stained sections obtained from whole-gut specimens, taken from a region of inflamed distal colon immediately adjacent to the gross macroscopic damage and fixed in cold 4% neutral formalin diluted in phosphate-buffered saline. Histological criteria included: mucosal architecture

loss (0–3), cellular infiltrate (0–3), muscle thickening (0–3), crypt abscess (0, absent; 1, present) and goblet cell depletion (0, absent; 1, present). All parameters of macroscopic and histological damage were recorded and scored for each rat by two observers, unaware of the treatment.

Reverse transcription-polymerase chain reaction

Expression of mRNA coding for A₃ receptors and adenosine deaminase was assessed by RT-PCR. The analysis was performed on distal colonic specimens excised as reported above, subjected to mucosa and submucosa removal by sharp dissection, snap-frozen in liquid nitrogen and stored at –80°C. Total RNA was extracted from colonic specimens by TRIzol® (Life Technologies, Carlsbad, CA, USA). Total RNA (2 µg) served as a template for single-strand cDNA synthesis in a reverse transcription (RT) reaction based on 2 µL random hexamers (0.5 µg·µL⁻¹) with 200 U Moloney murine leukemia virus (MMLV)-reverse transcriptase in manufacturer's buffer containing 500 µmol deoxynucleotide triphosphate mixture (dNTP) and 10 mM dithiothreitol. Polymerase chain reaction (PCR) was performed using specific primers based on the nucleotide sequence of A₃ and adenosine deaminase rat gene under previously reported conditions (Jackson *et al.*, 2002; Liu *et al.*, 2007). PCR, consisting of 2 µL RT products, Taq polymerase 2.5 U, dNTP 100 µmol and primers 0.5 µmol, was carried out by a PCR thermocycler DNA Engine (Biorad, Hercules, CA, USA). Untranscribed RNA was included in PCR reactions to verify the absence of genomic DNA. RT-PCR efficiency was evaluated by primers for rat β-actin. Amplified products were separated by 1.5% agarose gel electrophoresis and stained with ethidium bromide. cDNA bands were visualized by UV light, quantitated by densitometric analysis with Kodak Image Station programme (Eastman Kodak, Rochester, NY, USA) and normalized to β-actin.

Immunofluorescence imaging

Immunofluorescence was performed on OTC-embedded frozen distal colon sections (8–10 µm) as previously described (Giron *et al.*, 2008). Briefly, sections from control and DNBS-treated rats were fixed in 4% formaldehyde and incubated with 0.05 M NH₄Cl. Sections were washed in Tris-buffered saline (TBS, 0.05 M), treated for 30 min with 2% normal goat serum in TBS and incubated with anti-A₃ receptor antibody (1:400) for 1 h at room temperature. Sections were then rinsed three times for 10 min each with TBS before incubation with goat anti-rabbit IgG fluorescein/isothiocyanate-labelled

(Alexa Fluor 488, 1:1000; Invitrogen, Milan, Italy) in TBS for 1 h at room temperature. Non-specific binding was controlled by incubating tissue sections with non-specific isotype corresponding to anti-A₃ receptor antibody at the same dilution. Stained tissue sections were imaged with Leica SP2 confocal microscope (Leica Microsystems, Wetzlar, Germany).

Recording of contractile activity

The contractile activity of distal colonic longitudinal smooth muscle was recorded as previously described by Fornai *et al.* (2006). Segments of colon, excised as reported above, were placed into cold pre-oxygenated Krebs solution, opened along the mesenteric insertion and the mucosal/submucosal layer removed. Specimens were then cut along the longitudinal axis into strips of approximately 3 mm width and 20 mm length. The preparations were set up in organ baths containing Krebs solution at 37°C, bubbled with 95% O₂ + 5% CO₂ and connected to isotonic transducers (constant load = 1 g). Krebs solution had the following composition (mM): NaCl 113, KCl 4.7, CaCl₂ 2.5, KH₂PO₄ 1.2, MgSO₄ 1.2, NaHCO₃ 25 and glucose 11.5 (pH 7.4 ± 0.1). Each preparation was allowed to equilibrate for at least 30 min, with washing at 10 min intervals. The contractile activity was recorded by polygraphs (Gemini 7080, Basile, Comerio, Italy). A pair of coaxial platinum electrodes was positioned at a distance of 10 mm from longitudinal axis of each preparation to deliver electrical stimulation (ES) by a BM-ST6 stimulator (Biomedica Mangoni, Pisa, Italy). At end of the equilibration period, each preparation was repeatedly challenged with electrical stimuli (see below), and experiments started when reproducible responses were obtained (usually after two or three stimulations).

Preliminary experiments were performed to select the appropriate frequency of ES and carbachol concentration that elicited submaximal contractions, suitable for assessment of the effects of test drugs. For this purpose, colonic preparations, obtained from either normal or inflamed animals, were challenged with electrical stimuli at increasing frequencies, ranging from 1 to 20 Hz (0.5 ms, 30 mA). Frequency–response curves were constructed against maximal contractions elicited by acetylcholine (100 µM) under the different *in vitro* experimental conditions adopted in the present study: (i) standard Krebs solution; (ii) Krebs solution, containing guanethidine (adrenergic neurone blocker) and tachykinin NK₁, NK₂ and NK₃ receptor antagonists; and (iii) Krebs solution containing guanethidine, tachykinin NK₁, NK₂ and NK₃ receptor antagonists, and N^ω-propyl-L-arginine (NPA,

selective inhibitor of neuronal nitric oxide synthase). Concentration–response curves to carbachol were constructed against acetylcholine (100 µM) at concentrations ranging from 0.01 to 1000 µM in the presence of tetrodotoxin (1 µM). These preliminary experiments allowed the selection of a frequency of 10 Hz and a concentration of 1 µM carbachol, as both these settings elicited submaximal contractions suitable for the evaluation of the effects exerted by adenosine A₃ receptor ligands. Moreover, the ratios of control motor responses, elicited by electrical stimuli at 10 Hz or carbachol at 1 µM, over acetylcholine-induced maximal contractions were similar in preparations from both normal and inflamed colon (Figure 1). Electrical stimuli were applied as follows: (i) 10-s single trains (sES), consisting of square wave pulses (0.5 ms, 30 mA, 10 Hz); and (ii) recurrent trains (rES) of square wave pulses (0.5 ms, 30 mA, 10 Hz) applied for 5 s every 60 s. Different patterns of ES were adopted to test the effects of A₃ receptor ligands, as inhibitory effects associated with A₃ receptor activation were more obvious after repeated trains of ES.

Design of the experiments

In the first set of experiments, the effects of 2,3-ethyl-4,5-dipropyl-6-phenylpyridine-3-thiocarboxylate-5-carboxylate (MRS1523, A₃ receptor antagonist, 0.001–1 µM) were assayed on sES-induced motor responses of colonic preparations maintained in standard Krebs solution. To verify that MRS1523 acted specifically on A₃ receptors, its effects were evaluated also under blockade of A₁, A_{2A} and A_{2B} receptors by incubation of colonic preparations with selective antagonists [8-cyclopentyl-3-N-[3-((3-(4-fluorosulphonyl)benzoyl)-oxy)-propyl]-1-N-propylxanthine (FSCPX) 1 µM, ZM241385 0.01 µM and MRS1754 0.01 µM respectively] (Fredholm *et al.*, 2001; Bozarov *et al.*, 2009).

In the second series of experiments, the effects of MRS1523 on sES-evoked contractions were assessed in colonic preparations maintained in Krebs solution containing guanethidine (10 µM), N-acetyl-L-tryptophan 3,5-bis(trifluoromethyl)benzyl ester (L-732138) (NK₁ receptor antagonist, 10 µM), 5-fluoro-3-[2-[4-methoxy-4-[(R)-phenylsulphonyl]methyl]-1-piperidinyl]ethyl-1H-indole (GR159897) (NK₂ receptor antagonist, 1 µM) and (R)-[2-phenyl-4-quinolinyl]carbonyl]amino]-methyl ester benzenecetic acid (SB218795) (NK₃ receptor antagonist, 1 µM) (Drew *et al.*, 2005; Bozkurt and Sahin-Erdemli, 2008; Ianowski *et al.*, 2008), in order to prevent the recruitment of adrenergic and tachykinergic pathways.

The third set of experiments was designed to assay MRS1523 on contractile responses elicited by

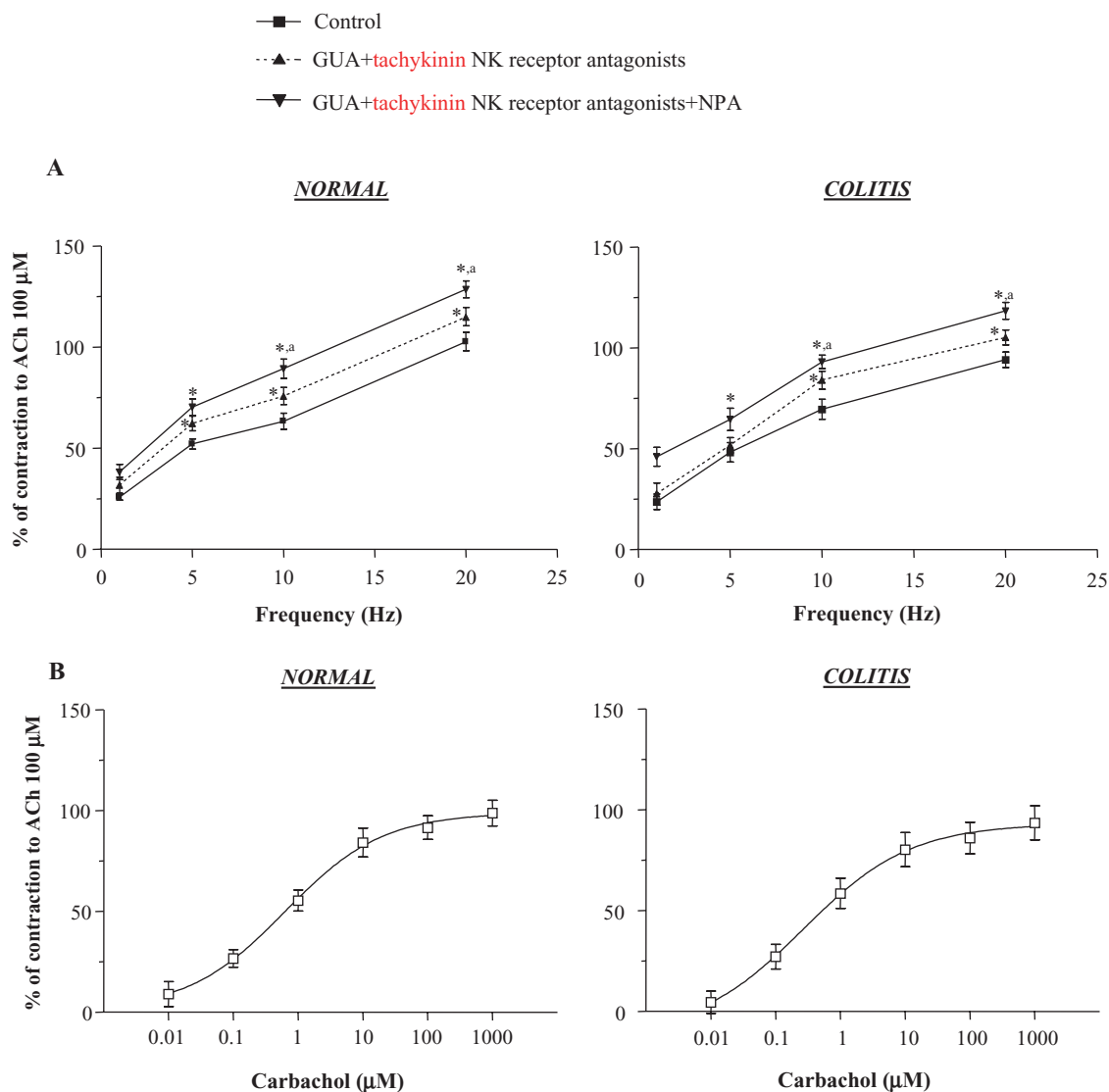


Figure 1

Preparations of longitudinal smooth muscle isolated from normal or inflamed colon. (A) Effects of electrical stimulation (1–20 Hz) on the motor activity of smooth muscle maintained in standard Krebs solution (control), in the presence of guanethidine (GUA, 10 μM) and tachykinin NK₁, NK₂ and NK₃ receptor antagonists (L-732138 10 μM, GR159897 1 μM and SB218795 1 μM), or in the presence of guanethidine, tachykinin NK₁, NK₂ and NK₃ receptor antagonists and NPA (0.1 μM). (B) Effects of increasing concentrations of carbachol (0.01–1000 μM) on the motor activity of smooth muscle maintained in Krebs solution containing tetrodotoxin (1 μM). Each point represents the mean ± SEM obtained from 6–8 experiments. **P* < 0.05, versus control. ^a*P* < 0.05, versus GUA + NK receptor antagonists. ACh, acetylcholine; GR159897, 5-fluoro-3-[2-[4-methoxy-4-[[[(R)-phenylsulphonyl]methyl]-1-piperidinyl]ethyl]-1H-indole]; L-732138, N-acetyl-L-tryptophan 3,5-bis(trifluoromethyl)benzyl ester; NK, neurokinin; NPA, N^ω-propyl-L-arginine.

sES directed mainly to excitatory cholinergic nerves. Therefore, to prevent non-cholinergic motor responses, colonic preparations were maintained in Krebs solution containing guanethidine, L-732138, GR159897, SB218795 and NPA (0.01 μM).

In the fourth series, MRS1523 was assayed on cholinergic contractions elicited by direct pharmacological activation of muscarinic receptors located on smooth muscle cells. For this purpose, colonic preparations were maintained in Krebs solution

containing tetrodotoxin (1 μM) and stimulated twice with carbachol (1 μM). The first stimulation was applied in the absence of other test drugs, whereas the second one was applied after 20 min incubation with MRS1523.

In the fifth series of experiments, the effects of 2-chloro-*N*₆-(3-iodobenzyl)-adenosine-5'-*N*-methyluronamide (2Cl-IB-MECA, A₃ receptor agonist, 0.001–100 μM) were tested on rES-induced cholinergic contractions. Thus, colonic preparations

were maintained in Krebs solution containing dipyrindamole (adenosine transport inhibitor, 0.5 µM) and adenosine deaminase (the enzyme responsible for adenosine catabolism, 0.5 U·mL⁻¹) to reduce the extracellular levels of endogenous bioactive adenosine (Duarte-Araújo *et al.*, 2004; Antonioli *et al.*, 2006; Giron *et al.*, 2008). Moreover, the Krebs solution also contained A₁, A_{2A} and A_{2B} adenosine receptor blockers to ensure that recording of 2Cl-IB-MECA-induced effects resulted from A₃ receptor activation.

A sixth set of experiments was performed to evaluate the effects of 2Cl-IB-MECA on cholinergic contractions elicited by carbachol (1 µM). For this purpose, colonic tissues were maintained in Krebs solution containing dipyrindamole plus adenosine deaminase, tetrodotoxin (1 µM), FSCPX (1 µM), ZM241385 (0.01 µM) and MRS1754 (0.01 µM). In both the fifth and sixth series, the effects of 2Cl-IB-MECA were assessed either in the absence or in the presence of MRS1523.

In the last set of experiments, exogenous adenosine was tested, either alone or in the presence of MRS1523, on colonic contractions evoked by electrical stimuli (standard Krebs solution) or carbachol (in the presence of tetrodotoxin 1 µM).

The effects of test drugs were expressed as percent changes of control contractions elicited by ES or carbachol. The apparent potency of the A₃ receptor agonist was expressed as EC₅₀ (concentration of the agonist that produces 50% of its own maximal response). The percent maximum inhibition of control motor responses (E_{\max}) was also estimated. Both parameters were calculated from concentration–response curves and then averaged. The apparent potency of the A₃ receptor antagonist was expressed as K_d values from the equation:

$$K_d = [B]/(DR - 1)$$

where B is the molar concentration of the antagonist and DR is the ratio of equi-effective concentrations of the agonist (EC₅₀) in the presence and in the absence of the antagonist.

Statistical analysis

Data are expressed as mean ± SEM. The significance of differences was evaluated on raw data, before percentage normalization, by performing unpaired Student's *t*-tests or by one-way ANOVA followed by *post hoc* Dunnett's test. $P < 0.05$ was considered significant. Colonic preparations included in each test group were obtained from distinct animals, and therefore the number of experiments refers also to the number of animals assigned to each group. Cal-

culations and analyses were performed using Graph-Pad Prism 3.0 (San Diego, CA, USA).

Materials

Acetylcholine bromide, atropine sulphate, guanethidine monosulphate, carbachol chloride, dipyrindamole, DNBS, FSCPX, TRIzol® and adenosine deaminase were purchased from Sigma Chemicals Co. (St. Louis, MO, USA). Tetrodotoxin, MRS1523, 2Cl-IB-MECA, 4-(2-[7-amino-2-(2-furyl)[1,2,4]triazolo[2,3-*a*][1,3,5]triazin-5-ylamino]ethyl)phenol (ZM241385), 8-(4-[(4-cyanophenyl)carbamoymethyl]oxy]phenyl)-1,3-di(n-propyl)xanthine (MRS1754), L-732138, GR159897, SB218795 and NPA were obtained from Tocris (Bristol, UK). Isoflurane was purchased from Abbott (Roma, Italy). Random hexamers, Moloney murine leukaemia virus-reverse transcriptase, *Taq* polymerase and dNTP mixture, dithiothreitol were purchased from Promega (Madison, WI, USA). The A₃ receptor antibody was purchased from Alpha Diagnostic (San Antonio, TX, USA). The goat anti-rabbit IgG fluorescein/isothiocyanate-labelled was purchased from Invitrogen (Milan, Italy). Adenosine receptor ligands were dissolved in dimethyl sulphoxide, and further dilutions were made with saline solution. Dimethyl sulphoxide concentrations in organ bath never exceeded 0.5%.

Results

Assessment of colitis

At day 6 after treatment with DNBS, the distal colon appeared thickened and ulcerated with evident regions of transmural inflammation. Adhesions were often present, and the bowel was occasionally dilated. Colitis was characterized by an intense granulocyte infiltrate extending throughout the mucosa and submucosa, sometimes involving the muscular layer. An increase in both macroscopic and microscopic damage score was observed (1.43 ± 0.41 and 1.67 ± 0.35 in normal rats; 12.7 ± 1.8 and 9.1 ± 1.1 in DNBS-treated animals respectively; $P < 0.05$ versus normal rats).

RT-PCR

RT-PCR revealed the expression of mRNA coding for A₃ receptors and adenosine deaminase in colonic neuromuscular tissues dissected from normal or DNBS-treated animals (Figure 2A). The densitometric analysis indicated a significant increase in A₃ receptor and adenosine deaminase mRNA expression in colonic tissues dissected from rats with colitis (Figure 2A).

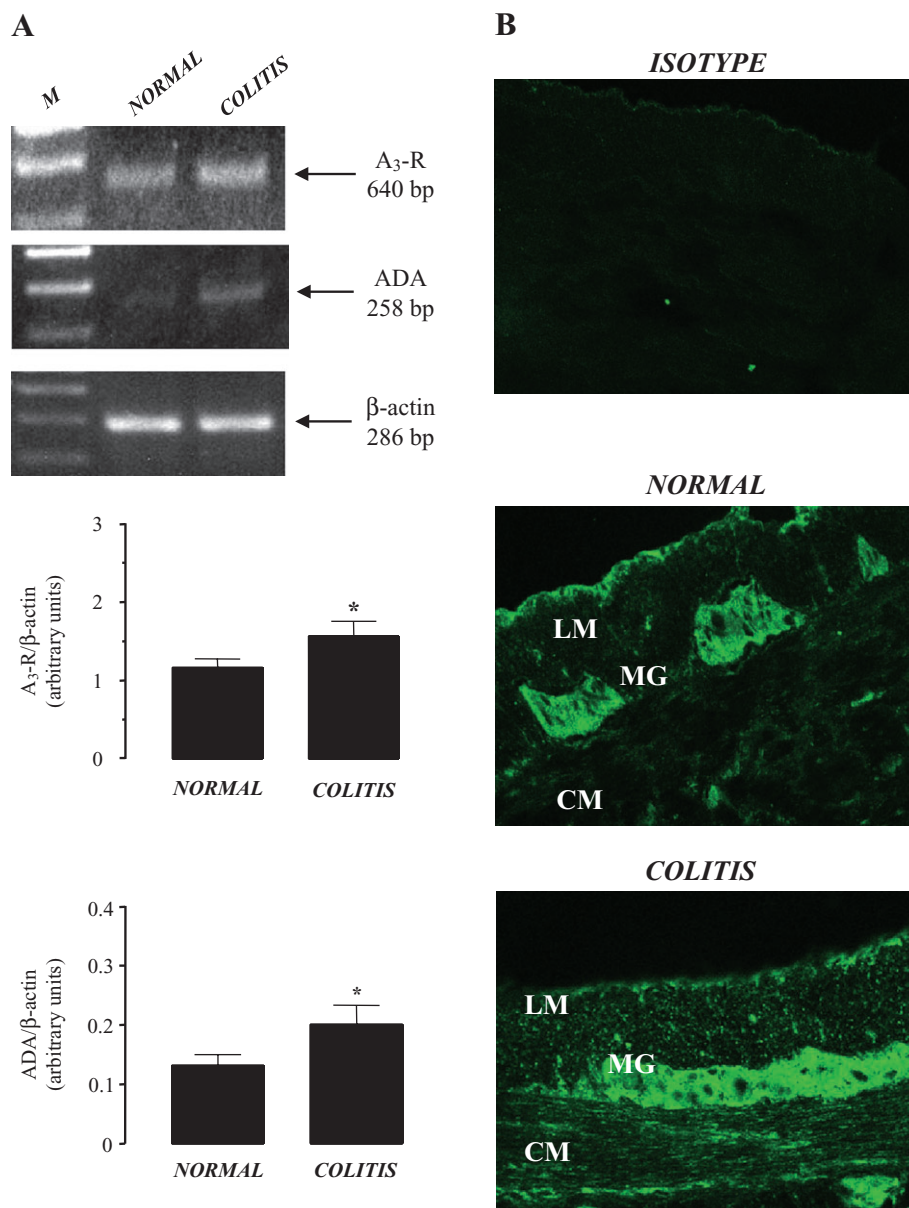


Figure 2

(A) RT-PCR analysis of A₃ receptor (A₃R) and adenosine deaminase (ADA) mRNA expression in the neuromuscular layer of distal colon, either in absence (normal) or in the presence of colitis. The panel displays representative agarose gels, referring to the amplification of cDNAs coding for A₃R, ADA and β-actin, and column graphs referring to the densitometric analysis of respective cDNA bands normalized to the expression of β-actin. Each column represents the mean value ± SEM obtained from four experiments. **P* < 0.05, versus normal. (B) A₃R immunofluorescence staining in colonic sections obtained from control (normal) and DNBS-treated (colitis) rats. Confocal micrographs showed the presence of A₃R in the colonic tissue stained with anti-A₃R antibody. Immunofluorescence analysis revealed a predominant localization of A₃R in myenteric ganglia of normal colon and highlighted an increase in their density, both at the ganglionic level and in smooth muscle layers, following the induction of colitis. Objective: 63×. ADA, adenosine deaminase; CM, circular muscle; DNBS, 2,4-dinitrobenzenesulfonic acid; LM, longitudinal muscle; M, size markers; MG, myenteric ganglia; RT-PCR, reverse transcription-polymerase chain reaction.

Immunofluorescence

Positive immunoreactivity for A₃ receptors was detected as a bright signal within neuromuscular layers at the level of myenteric ganglia of control rat colon (Figure 2B). The full development of

colitis was associated with an increased immunoreactivity for A₃ receptors in myenteric ganglia associated with their *de novo* appearance in both longitudinal and circular muscle layers (Figure 2B).

Contractile activity of colonic longitudinal smooth muscle

During the equilibration period in standard Krebs solution, some colonic preparations developed spontaneous contractile activity, which remained stable throughout the experiment and, in most cases, was low in amplitude and did not interfere with motor responses evoked by ES or carbachol. The development of spontaneous motor activity was observed with similar frequency and amplitude in preparations from normal or inflamed colon. Electrically evoked responses consisted of phasic contractions followed, in some cases, by after-contractions of variable amplitude. Atropine (1 μ M) abolished these phasic contractions, or converted them into relaxations, and only after-contractions became evident (data not shown; $n = 8$). Tetrodotoxin (1 μ M) abolished the electrically induced contractions (data not shown; $n = 8$).

Effects of A₃ receptor blockade

Under resting conditions, the A₃ receptor antagonist MRS1523 did not affect the spontaneous contractile activity of normal or inflamed colonic preparations. In normal colonic tissues maintained in standard Krebs solution, MRS1523 (0.001–1 μ M) concentration-dependently increased sES-evoked contractions, with a maximal enhancement of $29 \pm 3\%$ at 0.1 μ M, in tissues from normal rats (Figure 3A). Of note, in preliminary experiments MRS1523 (0.1 μ M) did not influence contractions elicited by electrical stimuli at 1 Hz (not shown; $n = 8$), while this A₃ receptor antagonist exerted enhancing effects only in few colonic preparations under ES at 5 Hz (data not shown; $n = 2$ of 8). In the presence of colitis, the enhancing effects of MRS1523 on sES-induced contractions no longer occurred (Figure 3B).

The stimulating effect of MRS1523 at the concentration of 0.1 μ M on normal colon was not affected by preincubation of preparations with A₁, A_{2A} and A_{2B} receptor antagonists ($27 \pm 4\%$ enhancement), suggesting that MRS1523 acted via selective blockade of A₃ receptors (Figure 4). Consistent with our findings, it was previously shown that MRS1523 0.1 μ M is devoid of significant effects on other adenosine receptors (Wolber and Fozard, 2005). Therefore, MRS1523 was employed at the concentration of 0.1 μ M in the subsequent sets of experiments.

When colonic preparations were maintained in Krebs solution containing guanethidine and tachykinin NK₁, NK₂ and NK₃ receptor antagonists, sES elicited phasic contractions that were prevented by atropine and, in most cases, were converted into NPA-sensitive relaxations (not shown; $n = 7$). Under these conditions, the effects of MRS1523 (0.1 μ M)

on sES-induced contractions in normal and inflamed colonic tissues were similar to those recorded in the presence of standard Krebs (normal colon: $27 \pm 4\%$ enhancement $P < 0.05$ vs. control; colitis: $9.2 \pm 4.4\%$; $P > 0.05$ vs. control).

In colonic tissues incubated in Krebs solution containing guanethidine, neurokinin (NK) receptor antagonists and NPA, to record cholinergic motor responses, sES-evoked phasic contractions were abolished or markedly reduced by atropine (data not shown; $n = 8$). In this setting, MRS1523 was able to enhance the electrically evoked contractions in normal tissues, while in the presence of colitis this potentiating effect no longer occurred (Figure 5).

In a set of experiments, the effects of A₃ receptor blockade were tested on contractions evoked by direct activation of muscarinic receptors on longitudinal smooth muscle. For this purpose, the effects of MRS1523 were tested on contractions evoked by carbachol (1 μ M) in the presence of tetrodotoxin. In this setting, carbachol-induced colonic contractions were not significantly affected by MRS1523 both in the absence and in the presence of colitis (Figure 6).

Effects of A₃ receptor activation

The effects of increasing concentrations of the A₃ receptor agonist 2Cl-IB-MECA were tested on rES-induced contractions in normal and inflamed colonic preparations, which were maintained in Krebs solution containing dipyrindamole and adenosine deaminase, to minimize the interference by endogenous adenosine, and FSCPX, ZM241385 and MRS1754 to prevent the activation of A₁, A_{2A} and A_{2B} receptors by 2Cl-IB-MECA. Under these conditions, the cumulative application of 2Cl-IB-MECA induced a decrease in rES-evoked contractions of normal tissues ($EC_{50} = 30 \pm 3.6$ nM; $E_{max} = 40 \pm 4\%$ decrease) (Figure 7A). The magnitude, but not the apparent potency, of such inhibitory effect was significantly increased when 2Cl-IB-MECA was assayed in preparations from inflamed colon ($EC_{50} = 26 \pm 2.8$ nM; $E_{max} = 60 \pm 5\%$ decrease) (Figure 7B). The inhibitory effects of 2Cl-IB-MECA in both normal and inflamed colonic tissues were antagonized by MRS1523 with similar patterns (K_d values: 10 ± 0.6 nM and 11 nM \pm 1.1 respectively) (Figure 7). Under the same conditions, the contractions elicited by carbachol (1 μ M) in the presence of tetrodotoxin were not affected by 2Cl-IB-MECA in preparations from both normal and inflamed colon (Figure 8).

Effects of exogenous adenosine

The application of adenosine (0.01–1000 μ M) to colonic preparations, maintained in standard Krebs solution, evoked concentration-dependent inhibitions of rES-induced contractions either in normal

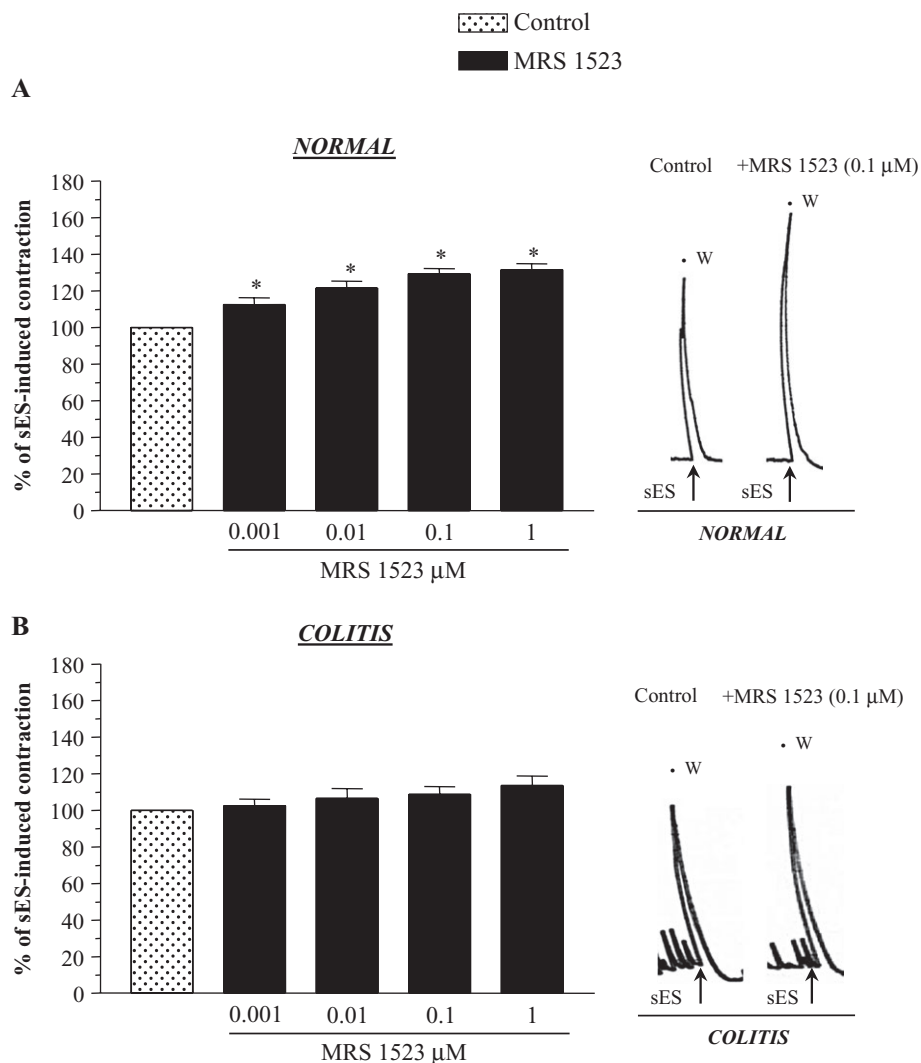


Figure 3

Preparations of longitudinal smooth muscle isolated from normal (A) or inflamed colon (colitis) (B). Effects of increasing concentrations of MRS1523 (0.001–1 μM) on contractions evoked by sES (0.5 ms, 10 Hz, 30 mA, 10 s) in colonic preparations maintained in standard Krebs solution. Each column represents the mean \pm SEM obtained from six experiments. * $P < 0.05$, versus control. ES, electrical stimulation; MRS1523, 2,3-ethyl-4,5-dipropyl-6-phenylpyridine-3-thiocarboxylate-5-carboxylate; W, washing.

or inflamed tissues (Figure 9). MRS1523 (0.1 μM) counteracted the inhibitory effects of exogenous adenosine, with a more pronounced antagonistic effect in preparations from the inflamed colon (Figure 9). Exogenous adenosine (0.01–1000 μM) concentration-dependently reduced also carbachol-evoked contractions (normal colon: by $36 \pm 4\%$; colitis: by $42 \pm 3\%$). In this setting, MRS1523 (0.1 μM) did not significantly counteract the inhibitory effects exerted by adenosine.

Discussion and conclusions

The involvement of adenosine pathways in the pathophysiology of enteric dysmotility associated

with bowel inflammation has been postulated, but available data are not exhaustive and in some cases they are even conflicting (Antonioli *et al.*, 2008b; Burnstock, 2008; Christofi, 2008). The present study was carried out to determine the expression and localization of A_3 receptors in the neuromuscular compartment of rat distal colon, as well as to characterize the effects of their pharmacological modulation on colonic motor activity. Particular care was taken to highlight the influence exerted by A_3 receptors on the regulatory pathways of colonic neuromotility following the induction of experimental colitis. Overall, our results point out two main novel concepts: (i) under normal conditions, A_3 receptors are expressed in the myenteric ganglia of colon and participate in a tonic inhibitory control on

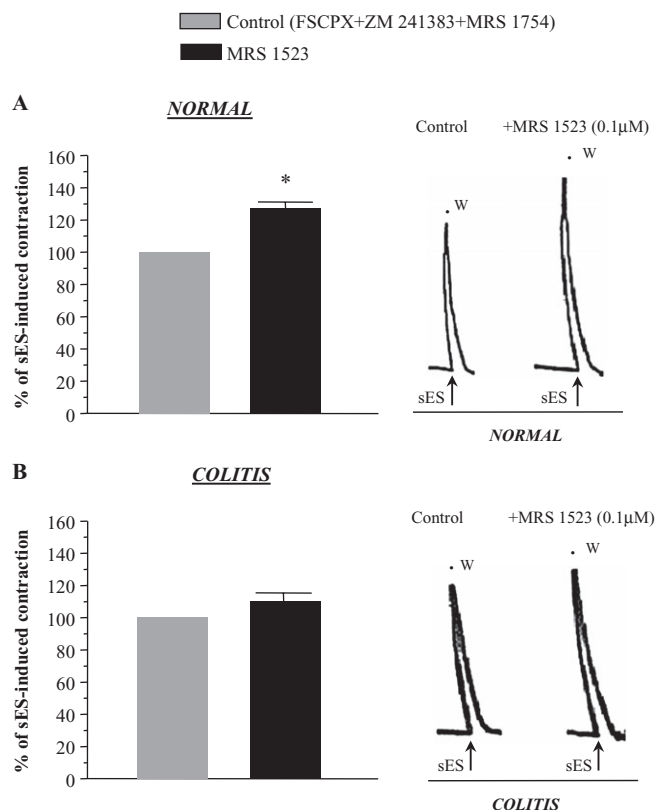


Figure 4

Preparations of longitudinal smooth muscle isolated from normal (A) or inflamed colon (colitis) (B). Effects of MRS1523 (0.1 μM) on contractile responses to sES (0.5 ms, 10 Hz, 30 mA, 10 s) in the presence of FSCPX (1 μM), ZM241385 (0.01 μM) and MRS1754 (0.01 μM). Each column represents the mean ± SEM obtained from six experiments. **P* < 0.05, versus control. ES, electrical stimulation; FSCPX, 8-cyclopentyl-3-N-[3-((3-(4-fluorosulphonyl)benzoyl)-oxy)-propyl]-1-N-propylxanthine; MRS1523, 2,3-ethyl-4,5-dipropyl-6-phenylpyridine-3-thiocarboxylate-5-carboxylate; W, washing; ZM241385, 4-(2-[7-amino-2-(2-furyl)[1,2,4]triazolo[2,3-*a*][1,3,5]triazin-5-ylamino]ethyl)phenol.

excitatory cholinergic motor activity, acting at neuronal level; and (ii) in the presence of bowel inflammation, the tonic inhibitory control of A₃ receptors on enteric cholinergic pathways is impaired, despite an evident up-regulation of A₃ receptors that maintain a full sensitivity to the application of exogenous agonists.

Under normal conditions, our molecular analysis identified the presence of mRNA coding for A₃ receptors in the neuromuscular layer of rat colon. Subsequent immunofluorescence investigations confirmed RT-PCR data, demonstrating the expression of A₃ receptors in ganglia of the myenteric plexus, while no appreciable immunoreactivity could be detected in muscle layers. Notably, a previous report by Guzman *et al.* (2006) demonstrated the presence of A₃ receptors in normal colonic

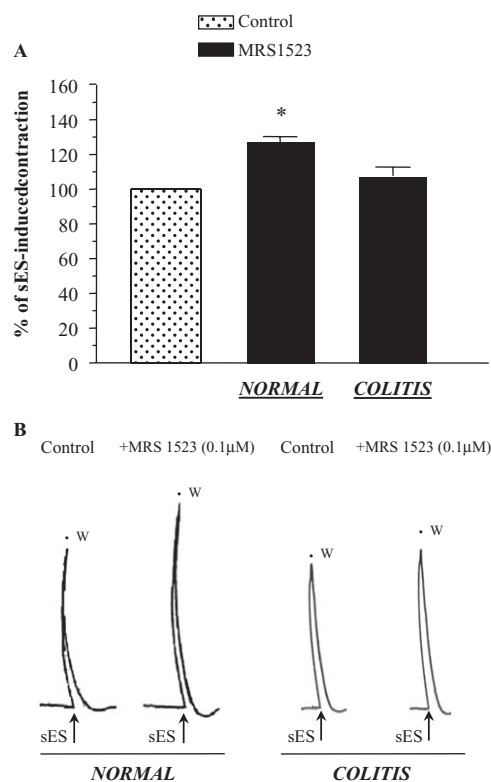


Figure 5

Preparations of longitudinal smooth muscle isolated from normal or inflamed colon (colitis). (A) Effects of MRS1523 (0.1 μM) on contractions evoked by sES (0.5 ms, 10 Hz, 30 mA, 10 s) in colonic preparations maintained in Krebs solution containing guanethidine (10 μM), L-732138 (10 μM), GR159897 (1 μM), SB218795 (1 μM) and NPA (0.01 μM). Each column represents the mean ± SEM obtained from 6–8 experiments. **P* < 0.05, versus control. (B) Representative tracings showing the contractions evoked by sES either alone (control) or in the presence of MRS1523. ES, electrical stimulation; GR159897, 5-fluoro-3-[2-[4-methoxy-4-[(R)-phenylsulphonyl]methyl]-1-piperidiny]ethyl]-1H-indole; L-732138, N-acetyl-L-tryptophan 3,5-bis(trifluoromethyl)benzyl ester; MRS1523, 2,3-ethyl-4,5-dipropyl-6-phenylpyridine-3-thiocarboxylate-5-carboxylate; NPA, N^ω-propyl-L-arginine; SB218795, (R)-[[2-phenyl-4-quinolinyl]carbonyl]amino]-methyl ester benzeneacetic acid; W, washing.

tissues by means of high-density oligonucleotide microarray analysis. In this respect, our findings add novel evidence to previous observations, providing insight into the distribution of these adenosine receptors in the neuromuscular compartment of normal colon. Functional investigations were then performed under different experimental conditions in an attempt to determine the enteric neuromotor pathways regulated by A₃ receptors and the underlying mechanisms. These experiments demonstrated a significant involvement of A₃ receptors in the inhibitory control of normal colonic neuromuscular contraction. In particular, the *in vitro* application of a selective A₃ receptor antagonist to colonic preparations enhanced the electrically evoked

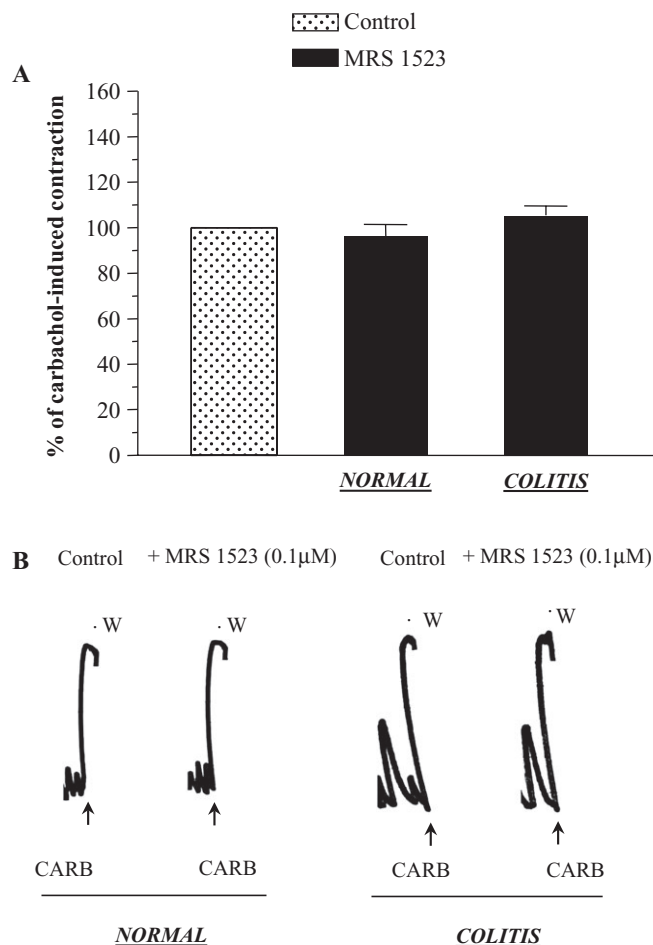


Figure 6

Preparations of longitudinal smooth muscle isolated from normal or inflamed colon (colitis). (A) Column graphs showing the effects of MRS1523 (0.1 μM) on contractions evoked by carbachol (1 μM) in colonic preparations maintained in Krebs solution containing tetrodotoxin (1 μM). Each column represents the mean ± SEM value obtained from six experiments. (B) Representative tracings showing the contractions induced by carbachol in the absence (control) and in the presence of MRS1523. CARB, carbachol; MRS1523, 2,3-ethyl-4,5-dipropyl-6-phenylpyridine-3-thiocarboxylate-5-carboxylate; W, washing.

cholinergic contractions of longitudinal smooth muscle. Moreover, in colonic tissues incubated with dipyrindamole and adenosine deaminase, to minimize the influence of endogenous adenosine, the A₃ receptor agonist 2Cl-IB-MECA reduced the amplitude of cholinergic motor responses to electrical stimuli in a concentration-dependent fashion. This inhibitory effect occurred in the presence of A₁, A_{2A} and A_{2B} receptor blockade, and it was reversed by MRS1523, thus substantiating the evidence that A₃ receptors are specifically involved in the regulatory actions through which adenosine down-regulates the contractile activity of normal distal colon driven by the cholinergic excitatory pathway. It was also

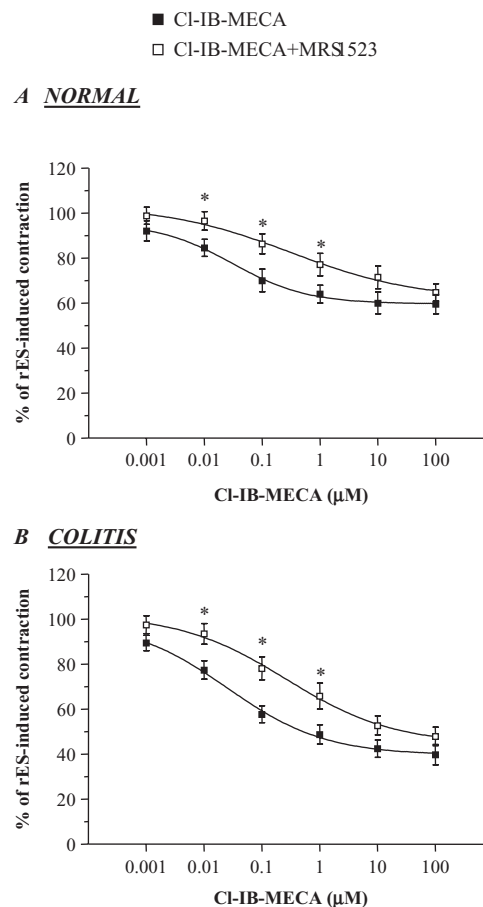


Figure 7

Preparations of longitudinal smooth muscle isolated from normal (A) or inflamed colon (colitis) (B). Effects of increasing concentrations of 2Cl-IB-MECA (0.001–100 μM), alone or in combination with MRS1523 (0.1 μM), on contractions evoked by rES (0.5 ms, 30 mA, 10 Hz, 5 s every 60 s) in colonic preparations maintained in Krebs solution containing dipyrindamole (0.5 μM), adenosine deaminase (0.5 U·mL⁻¹), guanethidine (10 μM), L-732138 (10 μM), GR159897 (1 μM), SB218795 (1 μM), NPA (0.01 μM), FSCPX (1 μM), ZM241385 (0.01 μM) and MRS1754 (0.01 μM). Each point represents the mean ± SEM of eight experiments. **P* < 0.05, versus 2Cl-IB-MECA alone. 2Cl-IB-MECA, 2-chloro-*N*-[3-(3-iodobenzyl)-adenosine-5'-*N*-methyluronamide]; ES, electrical stimulation; FSCPX, 8-cyclopentyl-3-*N*-[3-((3-(4-fluorosulphonyl)benzoyl)-oxy)-propyl]-1-*N*-propylxanthine; GR159897, 5-fluoro-3-[2-[4-methoxy-4-[[[(*R*)-phenylsulphonyl]methyl]-1-piperidinyl]ethyl]-1*H*-indole]; L-732138, *N*-acetyl-L-tryptophan 3,5-bis(trifluoromethyl)benzyl ester; MRS1523, 2,3-ethyl-4,5-dipropyl-6-phenylpyridine-3-thiocarboxylate-5-carboxylate; NPA, *N*^ω-propyl-L-arginine; ZM241385, 4-(2-[7-amino-2-(2-furyl)[1,2,4]triazolo[2,3-*a*][1,3,5]triazin-5-ylamino]ethyl)phenol.

considered that A₃ receptor ligands might affect the evoked motor activity of rat colon at neuronal and/or muscular sites, and therefore their effects were tested in the presence of a direct activation of muscarinic receptor on smooth muscle by carbachol. Under carbachol stimulation, the contractile responses of colonic preparations were not

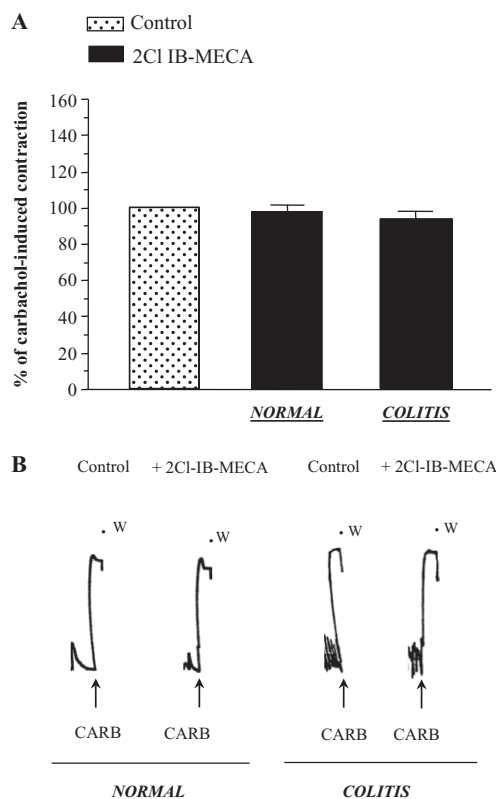


Figure 8

Preparations of longitudinal smooth muscle isolated from normal or inflamed colon (colitis). (A) Column graphs showing the effects of 2Cl-IB-MECA (0.1 μ M) on contractions evoked by carbachol (1 μ M) in colonic preparations maintained in Krebs solution containing dipyrindamole (0.5 μ M), adenosine deaminase (0.5 U·mL⁻¹), tetrodotoxin (1 μ M), FSCPX (1 μ M), ZM241385 (0.01 μ M) and MRS1754 (0.01 μ M). Each column represents the mean \pm SEM of six experiments. (B) Representative tracings showing the contractions induced by carbachol in the absence (control) and in the presence of 2Cl-IB-MECA. 2Cl-IB-MECA, 2-chloro-*N*-(3-iodobenzyl)-adenosine-5'-*N*-methyluronamide; CARB, carbachol; FSCPX, 8-cyclopentyl-3-*N*-[3-((3-(4-fluorosulphonyl)benzoyl)-oxy)-propyl]-1-*N*-propylxanthine; MRS1523, 2,3-ethyl-4,5-dipropyl-6-phenylpyridine-3-thiocarboxylate-5-carboxylate; W, washing; ZM241385, 4-(2-[7-amino-2-(2-furyl)[1,2,4]triazolo[2,3-*a*][1,3,5]triazin-5-ylamino]ethyl)phenol.

significantly affected by A₃ receptor activation or blockade, thus indicating that A₃ receptors do not operate at muscular sites to control colonic neuromuscular contraction. Taken together, the present molecular and functional findings concur in providing the first demonstration supporting an active role of A₃ receptors in the inhibitory control of colonic neuromuscular functions, through a modulation at level of the cholinergic nerve pathway. In line with our observation, an interesting study by Wunderlich *et al.* (2008) provided the proof of an inhibitory influence exerted by endogenous adenosine, via A₃ receptors, on cholinergic transmission in human submucosal plexus, thus demonstrating an active role played by these adenosine receptors in the

control of enteric secretomotor reflexes. Moreover, recent evidence suggests that A₃ receptors participate in the inhibitory modulation of short inter-plexus reflexes driven at colonic level by cholinergic, 5-hydroxytryptaminergic and peptidergic nerve circuits (Bozarov *et al.*, 2009).

A number of studies indicate adenosine as a prominent player in the pathophysiological mechanisms proposed to down-regulate activated immune cells and protect tissues from inflammatory injury via specific receptor subtypes expressed on immune/inflammatory cell populations (Antonioli *et al.*, 2008b). In this context, special attention has been recently paid to the role played by A₃ receptors in the regulation of inflammatory responses. Of note, pharmacological agents targeting this receptor pathway are being tested in preclinical models of inflammation or hypoxic damage with encouraging results (Ochaion *et al.*, 2008; Van der Hoeven *et al.*, 2008), and some A₃ receptor agonists have already entered the phase of clinical development for treatment of rheumatoid arthritis (Silverman *et al.*, 2008). Several lines of evidence suggest that the activation of A₃ receptors can favourably affect the outcome of intestinal inflammation, thus representing a promising target for the development of a novel therapeutic strategy for IBDs (Mabley *et al.*, 2003; Guzman *et al.*, 2006). However, there is a scarcity of information on the possible pathophysiological significance that A₃ receptors might have in the abnormalities of enteric neuromuscular functions associated with inflammatory bowel disorders. In order to address this issue, the second part of the present study was dedicated to investigate the influence of A₃ receptors on colonic neuromuscular activity in the presence of colitis. Under this pathological condition, our molecular analysis revealed a marked increment of A₃ receptor expression, particularly at the level of the myenteric ganglia, which occurred in concomitance with the appearance of A₃ receptor positivity also in the muscular compartment. These observations are in keeping with the report by Guzman *et al.* (2006), who found an enhanced expression of A₃ receptors in colonic tissues from rats with TNBS-induced colitis. However, the present changes, observed in the presence of bowel inflammation, correlated only in part with the patterns of evoked contractile activity recorded in functional experiments. Indeed, the enhancing effects, resulting from the blockade of A₃ receptors, on the electrically induced cholinergic contractions were no longer evident in the setting of colitis. On the other hand, when A₃ receptors were stimulated by exogenous application of a selective agonist or adenosine, the contractile responses evoked by electrical stimuli were inhibited with

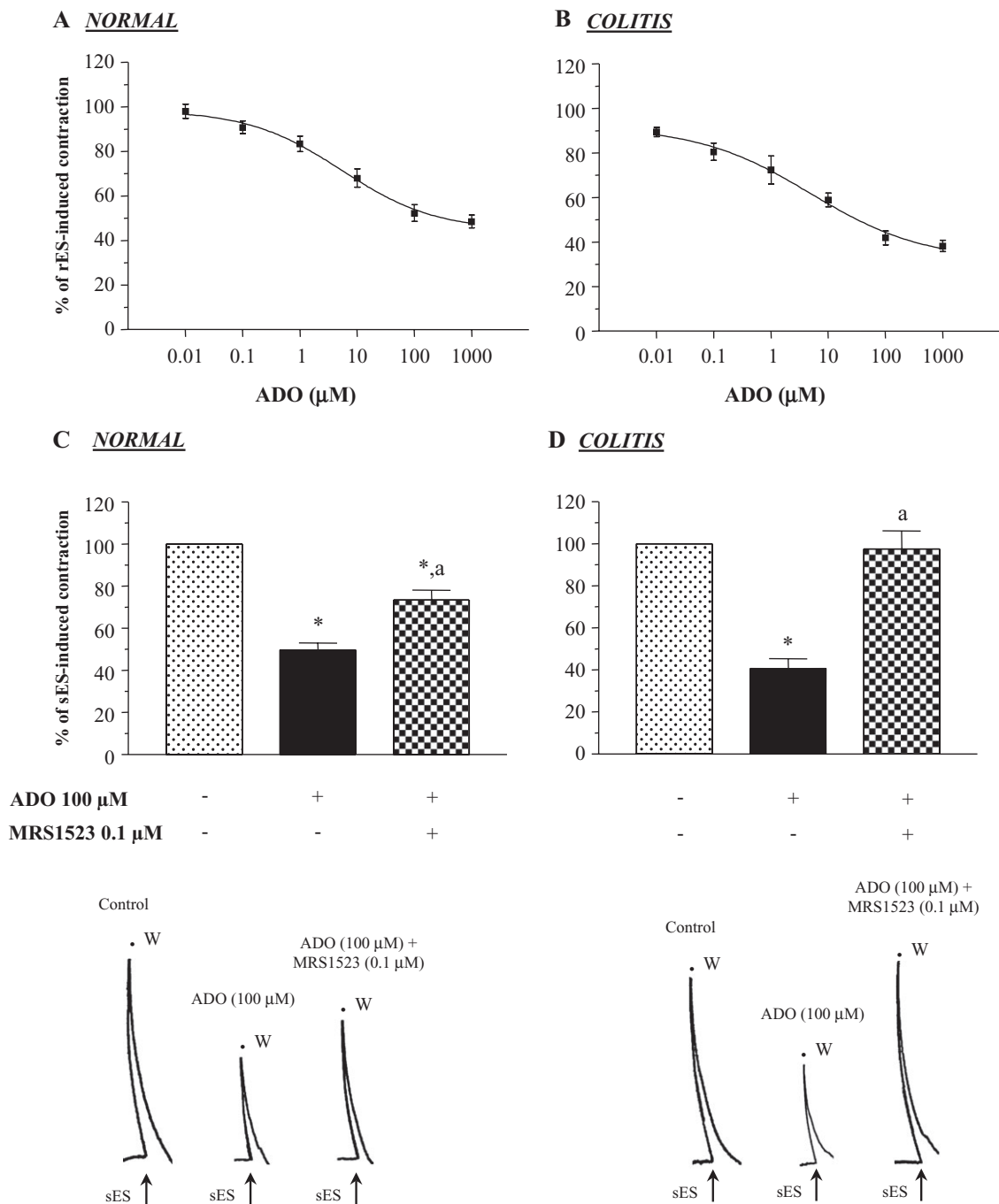


Figure 9

Preparations of colonic longitudinal smooth muscle isolated from normal or inflamed colon (colitis). (A, B) Effects of increasing concentrations of adenosine (ADO, 0.01–1000 μM) on contractions induced by rES (0.5 ms, 30 mA, 10 Hz, 5 s every 60 s) in colonic preparations maintained in standard Krebs solution. (C, D) Column graphs and tracings showing the effects of ADO (100 μM), alone or in combination with MRS1523 (0.1 μM) on contractions elicited by sES (0.5 ms, 10 Hz, 30 mA, 10 s). Each column represents the mean \pm SEM value obtained from eight experiments. * P < 0.05: significant difference versus control; ^a P < 0.05: significant difference versus ADO 100 μM alone. ES, electrical stimulation; MRS1523, 2,3-ethyl-4,5-dipropyl-6-phenylpyridine-3-thiocarboxylate-5-carboxylate; W, washing.

enhanced efficacy, as indicated by comparison with similar experiments in preparations from normal colon, and antagonized by MRS1523. Moreover, despite the induction of A_3 receptors in the muscu-

lar compartment of inflamed colon, as indicated by immunofluorescence analysis, no significant effects were recorded when testing the A_3 receptor agonist and antagonist on carbachol-induced cholinergic

contractions. Taken together these findings support the view that, in the presence of colitis, A₃ receptors, despite being overexpressed in the myenteric plexus and able to mediate enhanced inhibitory effects on colonic neuromuscular contraction following their pharmacological activation, lose their tonic modulating control on excitatory cholinergic nerve pathways. In this context, the *de novo* induction of A₃ receptors in smooth muscle layers of inflamed colon does not appear to contribute to the inhibitory control of this receptor pathway on colonic cholinergic motility, and its functional meaning remains undetermined. However, we cannot rule out the possibility that the A₃ receptor expression in the muscular compartment of inflamed colon might subserve regulatory functions unrelated to the cholinergic motor pathway or linked to other inflammatory pathophysiological events, independent of enteric motility. Moreover, as immunofluorescence analysis showed an increased expression of A₃ receptors in the circular muscle of the inflamed distal colon, it is conceivable that these receptors participate to the control of motor activity in this muscle layer, although the present study was specifically designed to examine the involvement of A₃ receptors in the regulation of longitudinal muscle motor activity.

A relevant issue, arising from the overall results of the present study, deals with the mechanisms underlying the enhanced neural expression of A₃ receptors in association with a reduced recruitment of this receptor pathway by endogenous adenosine at the level of the inflamed colon. There is evidence indicating an increased expression of A₃ receptors in the setting of inflammation and suggesting that such conditions may depend on the actions of various pro-inflammatory factors (Madi *et al.*, 2007). As an alternative explanation, the enhanced A₃ receptor expression might represent an up-regulatory response to compensate for a reduced availability of endogenous adenosine in the receptor biophase. This latter hypothesis appears to be supported by our RT-PCR assays, showing that the colonic expression of adenosine deaminase, the main enzyme responsible for adenosine catabolism, is significantly enhanced in rats with DNBS-induced colitis. In this regard, it has been previously shown also that extracellular adenosine levels can increase in the early phase of adverse conditions, while undergoing a subsequent decrease in the late phase through an induction of catabolic pathways driven by adenosine deaminase, which participates in the processes of innate adaptation to protect the organism from the detrimental effects of long term increase in adenosine concentration (Eltzschig *et al.*, 2006). The contention that the enhancement of A₃ receptor expression in the inflamed colon might

occur in response to an enhanced catabolic inactivation of adenosine is also consistent with proposals by other authors, who have suggested that, in the presence of inflammation, mechanisms of nucleoside inactivation (i.e. adenosine deaminase and/or nucleoside transporters) could channel endogenous adenosine into discrete microenvironments within the enteric neuromuscular layer (Duarte-Araújo *et al.*, 2004; Correia-de-Sá *et al.*, 2006; Timóteo *et al.*, 2008), thus leading to a compartmental recruitment of specific receptor subtypes. This dynamic molecular network, involving adenosine synthetic/catabolic enzymes, transporters and receptors, has been recently designated as “purinome”, to reflect its functional task, which consists of triggering, maintaining and terminating the purinergic signaling under different pathophysiological conditions (Schwiebert and Fitz, 2008; Volonté *et al.*, 2009). In view of these recent advances, future investigations should address a holistic evaluation of the enteric purinome, in order to gain detailed information on the roles played by adenosine pathways in the pathophysiology of intestinal motor disorders.

In conclusion, the present findings highlight a novel and interesting role of adenosine A₃ receptors in the regulation of colonic neuromuscular functions, both under normal conditions and in the presence of bowel inflammation. These observations, together with increasing knowledge relating A₃ receptors with the modulation of immune/inflammatory processes, might represent a promising basis for the development of novel pharmacological tools potentially useful for the therapeutic management of enteric dysmotility associated with bowel inflammation. In particular, the activation of adenosine A₃ receptors by selective agonists could represent suitable tools for the management of those phases of IBD characterized by enhanced bowel motor activity and diarrhoea.

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Statement of conflicts of interest

None.

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