RESEARCH PAPER

Role of the A_{2B} receptor–adenosine deaminase complex in colonic dysmotility associated with bowel inflammation in rats

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BACKGROUND AND PURPOSE

Adenosine A_{2B} receptors regulate several physiological enteric functions. However, their role in the pathophysiology of intestinal dysmotility associated with inflammation has not been elucidated. Hence, we investigated the expression of A_{2B} receptors in rat colon and their role in the control of cholinergic motility in the presence of bowel inflammation.

EXPERIMENTAL APPROACH

Colitis was induced by 2,4-dinitrobenzenesulfonic acid (DNBS). Colonic A_{2B} receptor expression and localization were examined by RT-PCR and immunofluorescence. The interaction between A_{2B} receptors and adenosine deaminase was assayed by immunoprecipitation. The role of A_{2B} receptors in the control of colonic motility was examined in functional experiments on longitudinal muscle preparations (LMPs).

KEY RESULTS

A_{2B} receptor mRNA was present in colon from both normal and DNBS-treated rats but levels were increased in the latter. A_{2B} receptors were predominantly located in the neuromuscular layer, but, in the presence of colitis, were increased mainly in longitudinal muscle. Functionally, the A_{2B} receptor antagonist MRS 1754 enhanced both electrically-evoked and carbachol-induced cholinergic contractions in normal LMPs, but was less effective in inflamed tissues. The A_{2B} receptor agonist NECA decreased colonic cholinergic motility, with increased efficacy in inflamed LMP. Immunoprecipitation and functional tests revealed a link between A_{2B} receptors and adenosine deaminase, which colocalize in the neuromuscular compartment.

CONCLUSIONS AND IMPLICATIONS

Under normal conditions, endogenous adenosine modulates colonic motility via A_{2B} receptors located in the neuromuscular compartment. In the presence of colitis, this inhibitory control is impaired due to a link between A_{2B} receptors and adenosine deaminase, which catabolizes adenosine, thus preventing A_{2B} receptor activation.

Abbreviations

DNBS, 2,4-dinitrobenzenesulfonic acid; ES, electrical stimulation; IBDs, inflammatory bowel diseases; LMP, longitudinal smooth muscle preparation; MPO, myeloperoxidase; rES, recurrent trains of ES; RT-PCR, reverse transcription PCR; sES, single trains of ES

Introduction

The reciprocal and finely tuned interaction between the enteric nervous system and immune cells represents a critical aspect for the maintenance of physiological homeostasis in the gut (Ben-Horin and Chowers, 2008). In the presence of inflammatory bowel diseases, this complex interplay is markedly disturbed, with consequent upsetting of neurally driven gut functions and the occurrence of abdominal pain, cramping, faecal urgency and/or constipation (Lakhan and Kirchgessner, 2010). At present, despite the considerable progress made in understanding several pathophysiological features of inflammatory gut diseases, the mechanisms underlying enteric motor dysfunctions associated with intestinal inflammation have not been elucidated. Therefore, several issues remain unresolved in this field, where there is a high unmet clinical need for effective drugs to manage enteric motor disorders associated with gut inflammation.

Over recent years, increasing attention has been paid to the adenosine system, which represents a crucial link between the enteric neuromuscular layer and the immune components of the gut (Antonioli *et al.*, 2008a). In this regard, a large body of evidence highlights an active role of this nucleoside in the modulation of gastrointestinal homeostatic functions (Burnstock, 2008; Christofi, 2008; Antonioli *et al.*, 2013). In the presence of inflammation, the adenosine system undergoes a number of dynamic changes in expression pattern and/or function of receptors. For instance, the experimental induction of colitis is associated with a marked rearrangement of the adenosine pathway in the enteric neuromuscular compartment, involving enhanced inhibitory control driven by A_{2A} receptors as well as a concomitant loss of modulatory activity exerted by A₁ and A₃ receptors (Antonioli *et al.*, 2006; 2010; 2011a).

The pivotal role adenosine receptors have in the modulation of immune/inflammatory systems has spurred the scientific community towards the development of innovative pharmacological entities acting on the adenosine pathways, which are currently under active preclinical and clinical investigations for possible therapeutic use in a variety of inflammatory disorders (Haskó *et al.*, 2009; Fredholm *et al.*, 2011; Antonioli *et al.*, 2011b). With regard to the gastrointestinal tract, current data have demonstrated potential anti-inflammatory effects exerted by adenosine A_{2B} receptor ligands in experimental models of colitis, although with conflicting results (Kolachala *et al.*, 2008a,b; Frick *et al.*, 2009). However, little attention has been paid to the involvement of A_{2B} receptors in the pathophysiology of the enteric dysmotility associated with intestinal inflammation.

Based on this background, the present study was designed to investigate the expression of A_{2B} receptors in the neuromuscular compartment of rat colon and to characterize their functional role in the control of colonic motility in the presence of experimentally-induced colitis.

Methods

Animals

Male albino Sprague Dawley rats, 200–250 g body weight, were used throughout the study. The animals were fed stand-

ard laboratory chow and tap water *ad libitum* and were allowed at least a week to acclimatize after their delivery to the laboratory. They were housed three in a cage in a temperature-controlled room on a 12-h light/dark cycle at 22–24°C and 50–60% humidity. Their care and handling were in accordance with the provisions of the European Community Council Directive 86–609, recognized and adopted by the Italian Government. The experiments were approved by the Ethical Committee for Animal Experiments in the University of Pisa. All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010).

Induction and assessment of colitis

Colitis was induced by 2,4-dinitrobenzenesulfonic acid (DNBS) as previously described by Antonioli *et al.* (2010). A set of experiments was performed at days 3, 6 and 12 after DNBS administration in order to evaluate the time-course of colitis. Thirty rats were used in the control groups and 34 rats were treated with DNBS. After the animals had been killed using isoflurane anaesthesia followed by cervical dislocation, inflammation severity was assessed both macroscopically and histologically as well as through the evaluation of tissue myeloperoxidase (MPO) and TNF levels (Antonioli *et al.*, 2007). The macroscopic attributes assessed were as follows: presence of adhesions between colon and other intra-abdominal organs; consistency of colonic faecal material (indirect marker of diarrhoea); thickening of colonic wall; presence and extension of hyperaemia; and macroscopic mucosal damage (assessed with the aid of a ruler). Histological evaluations were carried out by light microscopy on sections (stained with haematoxylin and eosin) obtained from whole-gut specimens taken from a region of inflamed colon immediately adjacent to the gross macroscopic damage and fixed in cold 4% neutral formalin diluted in PBS. Histological features assessed included degree of mucosal architecture changes, cellular infiltration, external muscle thickening, presence of crypt abscess and goblet cell depletion. All parameters of macroscopic and histological damage were recorded and scored for each rat by two observers blinded to the treatment. An additional set of experiments was carried out to evaluate the alterations in colonic motility by means of tests on *in vivo* propulsive colonic motility in the absence and presence of bowel inflammation. Based on data on the time-course of colonic inflammation and related parameters, we decided to perform all the subsequent experimental procedures at day 6 after DNBS administration, as at this time inflammation was fully developed. Thus, at day 6, the colon was excised and processed for the evaluation of contractile activity and subjected to reverse-transcription (RT)-PCR, immunoprecipitation, Western blot and immunofluorescence analysis, as described below.

Determination of tissue MPO

MPO levels in colonic tissues were determined as previously reported by Antonioli *et al.* (2007) and used as a quantitative index to estimate the degree of mucosal infiltration by polymorphonuclear cells. Briefly, colonic tissue samples (300 mg) were homogenized 3 times (30 s each) at 4°C with a polytron

homogenizer (Cole-Parmer, Vernon Hills, IL, USA) in 1 mL of ice-cold 50 mmol·L⁻¹ phosphate buffer (pH 6.0) containing 0.5% hexadecyltrimethylammonium bromide to prevent pseudoperoxidase activity of haemoglobin as well as to solubilize membrane-bound MPO. The homogenate was sonicated for 10 s, freeze-thawed 3 times and centrifuged for 20 min at 18 000 g. The supernatant was then recovered and used for determination of MPO by means of a kit for ELISA (Bioxytech, Oxis International Inc., Portland, OR, USA). All samples were assayed within 2 days from collection. The results are expressed as ng of MPO 100 mg⁻¹ tissue.

TNF assay

TNF levels in colonic tissues were determined by means of an ELISA kit (Biosource International, Camarillo, CA, USA). For this purpose, as described by Antonioli *et al.* (2007), tissue samples previously stored at -80°C were weighed, thawed and homogenized in 0.3 mL of PBS (pH 7.2) 100 mg⁻¹ tissue at 4°C, and centrifuged at 13 400 g for 20 min. Aliquots, 100 µL, of the supernatants were then used for the assay. Tissue TNF levels were expressed as pg mg⁻¹ tissue.

Distal colonic propulsive motility

Distal colonic propulsive motility was evaluated according to Broccardo *et al.* (1999). In brief, a single 5-mm-diameter glass bead was inserted 3 cm into the distal colon of each rat. The time required for expulsion of the glass bead was determined to the nearest 0.1 min in both control and DNBS-treated animals. The decrease in colonic propulsion was measured as the increase in mean expulsion time of the glass bead compared with that of control rats.

RT-PCR

The expression of mRNA coding for A_{2B} receptors, adenosine deaminase and CD73 was assessed by RT-PCR. The analysis was performed on 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 an RT reaction with Moloney murine leukaemia virus (MMLV). RT was performed using specific primers based on the nucleotide sequences of the rat genes for A_{2B} receptors, adenosine deaminase and CD73 under previously reported conditions (Ralevic and Burnstock, 1998; Antonioli *et al.*, 2010; 2011a). PCR was carried out with a DNA Engine PCR thermocycler (Bio-Rad, Hercules, CA, USA). Untranscribed RNA was included in PCR reactions to verify the absence of genomic DNA. RT-PCR efficiency was evaluated with 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, quantified by densitometric analysis with the Kodak Image Station programme (Eastman Kodak, Rochester, NY, USA) and normalized to β-actin.

Immunofluorescence imaging

Immunofluorescence was performed on frozen distal colonic sections (8 µm thick) embedded in optimal cutting tempera-

ture compound as previously described (Antonioli *et al.*, 2010). Briefly, sections from control and DNBS-treated rats were fixed in 4% formaldehyde and incubated with 0.05 M NH₄Cl. Sections were then washed and incubated with rabbit anti-A_{2B} receptor (1:100; Alpha Diagnostic, San Antonio, TX, USA), or with either biotin-labelled rabbit anti-adenosine deaminase (1:100; Alpha Diagnostic), mouse biotin-labelled mouse anti-HuC/D (1:40; Molecular Probes, Eugene, OR, USA; HuC/D is a specific neuronal marker) or mouse anti-glial fibrillary acidic protein (1:500; Millipore, Milan, Italy; glial fibrillary acidic protein, GFAP, is a specific glial marker) for 1 h at room temperature. Sections were then rinsed and incubated with labelled goat anti-rabbit IgG (1:1000; Life Technologies, Milan, Italy) and goat anti-mouse IgG or streptavidin labelled with Alexa Fluor 488 (Life Technologies) for 1 h at room temperature. Negative controls were prepared by incubating sections with isotype-matched control antibodies at the same concentration as the primary antibody and/or pre-incubating each antibody with 200-fold molar excess of the corresponding blocking peptide. Stained tissue sections were imaged with a Leica TCSNT/SP2 confocal microscope (Leica Microsystems, Wetzlar, Germany) by collecting fluorescence sequentially to avoid fluorescence cross-talk using a 40× or 63× oil immersion objective (Leica Microsystems, Wetzlar, Germany). Images of a number of colon areas, corresponding to the longitudinal smooth muscle layer (LM), circular smooth muscle layer (CM) and myenteric ganglia (MG), were taken using identical camera settings in order to allow the number and intensity of pixels to reflect the differences in their protein expression. Protein levels of A_{2B} receptors were determined by measuring the area (number of pixels) and fluorescent intensity (average intensity of pixels) of staining from 24 images captured randomly in the colonic neuromuscular compartment from each control and inflamed tissue sample. Simultaneously, the background intensity outside the slices was acquired. Image analysis and quantification of the fluorescence intensity of A_{2B} receptors were performed using US National Institute of Health ImageJ Software v.1.48a.

Immunoprecipitation and immunoblotting of adenosine deaminase and A_{2B} receptors

Immunoprecipitation and immunoblotting were used to assess the binding of adenosine deaminase with the A_{2B} receptor subtype (Herrera *et al.*, 2001). Briefly, colonic specimens were homogenized in RIPA lysis buffer containing Tris-HCl 50 mM (pH 7.4), NP-40 1%, sodium deoxycholate 0.25%, NaCl 150 mM, EDTA 1 mmol·L⁻¹, PMSF 1 mmol·L⁻¹, leupeptin 10 µg·mL⁻¹, aprotinin 10 µg·mL⁻¹, sodium fluoride 1 mmol·L⁻¹ and sodium orthovanadate 1 mmol·L⁻¹, using a polytron homogenizer (Cole-Palmer). Homogenates were spun by centrifugation at 33 500×g for 15 min at 4°C, and the resulting supernatants were then separated from pellets and stored at -80°C. Protein concentration was determined in each sample by the Bradford method (Protein Assay Kit, Bio-Rad). To ensure equal sample loading (30 µL), aliquots from each sample were taken before immunoprecipitation and analysed by Western blot for determination of β-actin protein expression, using anti-β-actin antibody (Sigma-Aldrich, Milan, Italy). To perform co-immunoprecipitate analysis, equivalent amounts of protein lysates (250 µg), diluted in a final volume of 750 µL with RIPA

buffer, were immunoprecipitated with an anti-adenosine deaminase antibody coupled to protein A/G agarose beads overnight at 4°C. Normal rabbit IgG coupled to protein A/G agarose beads was used as a negative control. Immunocomplex-bound beads were washed four times with RIPA buffer and resuspended in 25 µL of Laemmli buffer. Samples were boiled for 3 min, and proteins were separated by 8% SDS-PAGE for immunoblotting. After transfer onto a PVDF membrane, the blots were blocked for 2 h with 0.1% Tween-20 in Tris-buffered saline with Tween (TBS-T), and incubated overnight at room temperature with rabbit anti-adenosine deaminase antibody or rabbit polyclonal anti-A_{2B} receptor antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) (dilutions 1:1000). After repeated washings with TBS-T, a peroxidase-conjugated goat anti-rabbit antibody (dilution 1:10 000) was added for 1 h at room temperature. After repeated washings with TBS-T, immunoreactive bands were visualized by incubation with chemiluminescent reagents (Immobilon reagent, Millipore, Billerica, MA, USA) and processed with Kodak Image Station 440 for signal detection.

Recording of contractile activity

The contractile activity of colonic longitudinal smooth muscle was recorded as previously described by Antonioli *et al.* (2010). 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, glucose 11.5 (pH 7.4 ± 0.1). Each preparation was allowed to equilibrate for at least 30 min. The contractile activity was recorded by polygraphs (Gemini 7080, Basile, Comerio, Italy). A pair of coaxial platinum electrodes was positioned 10 mm from the longitudinal axis of each preparation to deliver electrical stimulation by a BM-ST6 stimulator (Biomedica Mangoni, Pisa, Italy). At end of the equilibration period, each preparation was repeatedly challenged with electrical stimuli, and experiments were started when reproducible responses were obtained (usually after two or three stimulations).

Preliminary experiments were performed in order to select the frequency of electrical stimulation and the carbachol concentration that elicited submaximal and supramaximal contractions, suitable for investigating the effects of adenosine receptor ligands. These experiments led to the selection of a frequency of 10 Hz and 1 µM carbachol, as both these settings elicited submaximal contractions suitable for evaluating the enhancing or inhibitory effects of adenosine A_{2B} receptor ligands. In particular, the 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. In addition, we performed a set of experiments using a supramaximal concentration (100 µM) of carbachol, which revealed the inhibitory effects of A_{2B} receptor ligands.

Design of experiments

In the first set of experiments, the effects of MRS 1754 (A_{2B} receptor antagonist, 0.001–1 µM) on sES-induced motor

responses of colon preparations maintained in standard Krebs solution were assayed.

The second set of experiments was designed to assay the effects of MRS 1754 (0.01 µM) on contractile responses elicited by sES directed mainly to excitatory cholinergic nerves. Therefore, to prevent non-cholinergic motor responses, colon preparations were maintained in Krebs solution containing guanethidine, L-732138 (NK₁ receptor antagonist, 10 µM), GR159897 (NK₂ receptor antagonist, 1 µM), SB218795 (NK₃ receptor antagonist, 1 µM) and N^ω-propyl-L-arginine (NPA; 0.01 µM).

In the third series, the effects of MRS 1754 (0.01 µM) were determined on cholinergic contractions elicited by direct pharmacological activation of muscarinic receptors located on smooth muscle cells. For this purpose, colon 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 of incubation with MRS 1754.

In the fourth series of experiments, the effects of 5'-N-ethylcarboxamidoadenosine (NECA; adenosine receptor agonist, 0.001–100 µM) were tested on rES-induced cholinergic contractions. Thus, colon preparations were maintained in Krebs solution with added dipyridamole (adenosine transport inhibitor, 0.5 µM) and adenosine deaminase (the enzyme responsible for adenosine catabolism, 0.5 U·mL⁻¹) to prevent the formation of extracellular levels of endogenous bioactive adenosine (Duarte-Araújo *et al.*, 2004; Antonioli *et al.*, 2006; Giron *et al.*, 2008). The effects of NECA were tested in the presence of 8-cyclopentyl-1,3-dipropylxanthine (DPCPX; 0.01 µM), ZM 241385 (0.01 µM) and MRS1523 (0.1 µM), which are selective A₁, A_{2A} and A₃ receptor antagonists, respectively, to ensure selective A_{2B} receptor activation.

A fifth set of experiments was performed to evaluate the effects of NECA on cholinergic contractions elicited by submaximal or supramaximal concentrations of carbachol (1 or 100 µM respectively). For this purpose, colonic tissues were maintained in Krebs solution containing dipyridamole plus adenosine deaminase and tetrodotoxin (1 µM). Again, the effects of NECA were assayed in the presence of selective A₁, A_{2A} and A₃ receptor antagonists.

In the final set of experiments, the effects of MRS 1754 on cholinergic colonic contractions evoked by electrical stimuli were tested in the presence of the adenosine deaminase inhibitor erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA; 0.5 µM) or the CD73 blocker adenosine 5'-(α,β-methylene) diphosphate (AOPCP; 200 µM). These experiments were also performed in the presence of selective A₁, A_{2A} and A₃ receptor antagonists in order to minimize the interaction between the exogenous adenosine and these receptor subtypes.

The effects of test drugs are expressed as % changes in control contractions elicited by ES or carbachol. The apparent potency of the A_{2B} receptor agonist is expressed as an EC₅₀ value. The % 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 A_{2B} receptor antagonist is expressed as a *K*_d value using the equation

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

where B is the molar concentration of the antagonist and DR is the ratio of equally effective concentrations of the agonist (EC_{50}) in the presence and absence of the antagonist.

Drugs and reagents

Atropine sulphate, guanethidine monosulphate, carbachol chloride, dipyridamole, DNBS, AOPCP, TRizol and adenosine deaminase were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Tetrodotoxin, MRS 1754 (N-(4-cyanophenyl)-2-[4-(2,3,6,7-tetrahydro-2,6-dioxo-1,3-dipropyl-1H-purin-8-yl)phenoxy]-acetamide), NECA, DPCPX, ZM 241385 (4-(2-[7-amino-2-(2-furyl)[1,2,4]triazolo[2,3-a][1,3,5]triazin-5-ylamino]ethyl)phenol), MRS 1523 (N-[9-chloro-2-(2-furanyl)[1,2,4]-triazolo[1,5-c]quinazolin-5-yl]benzene acetamide), EHNA, L-732 138 (N-acetyl-L-tryptophan 3,5-bis(trifluoromethyl)benzyl ester), GR-159897 (5-fluoro-3-[2-[4-methoxy-4-[[[R]-phenylsulphonyl]methyl]-1-piperidinyl]ethyl]-1H-indole), SB-218795 ((R)-[[2-phenyl-4-quinolinyl]carbonyl]amino]-methyl ester benzenecetic acid) and NPA were obtained from Tocris (Bristol, UK). Isoflurane was purchased from Abbott (Rome, Italy). Random hexamers, MMLV reverse transcriptase, *Taq* polymerase and dNTP mixture, and dithiothreitol were purchased from Promega (Madison, WI, USA). The A_{2B} antibody was purchased from Santa Cruz Biotechnology. For immunohistochemistry, anti- A_{2B} receptor and anti-adenosine deaminase were purchased from Alpha Diagnostic, whereas anti-HuCD and anti-GFAP were from Molecular Probes and Millipore respectively. Appropriate secondary antibodies were purchased from Life Technologies. Adenosine A_{2B} receptor ligands were dissolved in dimethyl sulphoxide, and further dilutions were made with saline solution. Dimethyl sulphoxide concentration in the organ bath never exceeded 0.5%.

Statistical analysis

Data are expressed as mean $\pm$ SEM. The significance of differences was evaluated for raw data, before percentage normalization, by performing Student's unpaired *t*-tests or by one-way ANOVA followed by *post hoc* Dunnett's test. $P < 0.05$ was considered significant. The colon preparations included in each test group were obtained from different animals, and therefore the number of trials was always the same as the number of animals assigned to the group. Calculations and

analyses were performed using GraphPad Prism 3.0 (GraphPad Software, San Diego, CA, USA).

Results

Assessment of intestinal inflammation and evaluation of distal colonic propulsive motility

At day 3 after DNBS administration, the distal colon was hyperaemic and oedematous, whereas at day 6 and 12 it appeared thickened and ulcerated, with evident areas of transmural inflammation. At days 6 and 12, adhesions were often present, and the bowel was occasionally dilated. Histologically, the colitis was characterized by an intense granulocyte infiltrate extending throughout the mucosa and submucosa (days 3, 6 and 12) and often involving the muscularis propria, which appeared thickened (days 6 and 12). Mean macroscopic and microscopic damage scores and tissue MPO and TNF levels estimated in colon samples are summarized in Table 1. The presence of experimentally-induced inflammation was also characterized by a significant impairment of distal colonic motility, which was already evident at day 3, but occurred mainly at days 6 and 12 after DNBS administration (Table 2).

RT-PCR

RT-PCR showed the expression of mRNA coding for A_{2B} receptors, adenosine deaminase and CD73 in colonic neuromuscular tissues from both control and DNBS-treated animals (Figure 1). The densitometric analysis demonstrated a significant increase in A_{2B} receptor, adenosine deaminase and CD73 mRNA expression in inflamed colonic tissues (Figure 1).

Immunofluorescence

Positive immunoreactivity for A_{2B} receptors was detected as a bright signal in the neuromuscular layers at the level of the myenteric ganglia and muscular layers of normal rat colon (Figure 2A). The induction of colitis was associated with a rearrangement of A_{2B} receptor distribution, with an increase of fluorescence intensity in the neuromuscular layer, largely at level of the longitudinal smooth muscle and, to a lesser

Table 1

Colonic inflammatory parameters in rats treated with vehicle (control) or DNBS (colitis) at days 3, 6 and 12

	Macroscopic damage score	Microscopic damage score	MPO (ng·100 mg ⁻¹ tissue)	TNF (pg·mg ⁻¹)
Vehicle day 3	1.3 $\pm$ 0.4	1.4 $\pm$ 0.3	6 $\pm$ 2	4.5 $\pm$ 2.2
DNBS day 3	3.4 $\pm$ 0.2 ^a	2.8 $\pm$ 0.6 ^a	17.4 $\pm$ 3.2 ^a	11.5 $\pm$ 3 ^a
Vehicle day 6	1.5 $\pm$ 0.2	1.2 $\pm$ 0.5	6.7 $\pm$ 1.7	5.2 $\pm$ 3.1
DNBS day 6	8.3 $\pm$ 1.5 ^a	5.1 $\pm$ 0.9 ^a	28.8 $\pm$ 5 ^a	20.8 $\pm$ 4.3 ^a
Vehicle day 12	1.5 $\pm$ 0.4	1.5 $\pm$ 0.2	5.8 $\pm$ 1.8	5.6 $\pm$ 2.4
DNBS day 12	7.8 $\pm$ 1.3 ^a	4.9 $\pm$ 0.7 ^a	26.4 $\pm$ 3.8 ^a	21.6 $\pm$ 5 ^a

^a $P < 0.05$ versus the respective group treated with vehicle.

Table 2

Relationship between tissue MPO levels and colonic motility in rats

	Normal	DNBS day 3	DNBS day 6	DNBS day 12
MPO (ng 100 mg ⁻¹ tissue)	6.5 ± 1.2	16 ± 3.2 ^a	30.4 ± 4.1 ^a	32.6 ± 3.8 ^a
Mean expulsion time (min)	4.4 ± 0.3	7.4 ± 0.8 ^a	14.3 ± 1.4 ^a	13.5 ± 1.1 ^a

^a*P* < 0.05 versus control animals. **Figure 1**

RT-PCR analysis of A_{2B} receptor (A_{2B-R}), adenosine deaminase (ADA) and CD73 mRNA expression in the neuromuscular layer of distal colon, either in the absence (normal) or in the presence of colitis. This figure displays representative agarose gels for the amplification of cDNAs coding for the A_{2B} receptor, adenosine deaminase, CD73 and β-actin, as well as column graphs showing the results of the densitometric analysis of the respective cDNA bands normalized to the expression of β-actin. Each column represents the mean value ± SEM obtained from six trials. **P* < 0.05 versus normal. M, size markers.

Figure 2

(A) *A_{2B}* receptor immunofluorescence staining in colon sections obtained from control (normal) and DNBS-treated (colitis) rats. Confocal micrographs show the presence of *A_{2B}* receptors in the neuromuscular layer of colonic tissues stained with anti-*A_{2B}* receptor antibody. Boxed areas are displayed at a higher magnification in the right panels. LM, longitudinal muscle; CM, circular muscle; MG, myenteric ganglia. Scale bars: 75 µm (left panels); 37.5 µm (right panels). (B) Levels of fluorescence intensity, expressed as arbitrary units (AU), in the longitudinal smooth muscle, circular smooth muscle and myenteric ganglia of the colonic neuromuscular layer, either in the absence (normal) or in the presence of colitis. **P* < 0.001 versus colitis.

Figure 3

(A) Double-staining immunohistochemistry showing the distribution of the neuronal marker HuC/D (green) and A_{2B} receptors (red) in the myenteric plexus of colonic cryosections from control (normal) and DNBS-treated (colitis) rats. Scale bar: 36.5 μm. (B) Double immunostaining showing the expression of the glial marker GFAP (green) and A_{2B} receptor (red) in myenteric plexus of colonic cryosections from control (normal) and DNBS-treated (colitis) rats. LM, longitudinal muscle; CM, circular muscle; MG, myenteric ganglia. Scale bar: 37.5 μm.

extent, in the myenteric ganglia and circular smooth muscle layer (Figure 2A). The analysis of fluorescence intensity revealed a significant increase in A_{2B} receptor expression in all layers of the neuromuscular compartment, with a marked predominance in the longitudinal muscle (Figure 2B).

Double-label immunofluorescence of normal colon showed that A_{2B} receptors were mainly localized in HuC/D⁺ neurons (Figure 3A), but were also present in GFAP⁺ glial cells (Figure 3B). Colitis was associated with an increase in A_{2B} receptor immunoreactivity in the neuromuscular layer, with more intense staining for GFAP and a fainter Hu immunoreactivity signal in myenteric ganglia (Figure 3A and B).

Contractile activity of colonic longitudinal smooth muscle

During the equilibration period in standard Krebs solution, colon preparations, obtained from control animals or those with colitis, 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 electrically evoked responses consisted of phasic contractions followed, in some cases, by after-contractions of variable amplitude. Atropine (1 μM) abolished these phasic contractions or con-

Figure 4

Effects of increasing concentrations of MRS 1754 (0.001–1 μM) on contractions evoked by sES (0.5 ms, 10 Hz, 30 mA, 10 s) in colon preparations, maintained in standard Krebs solution, from normal (A) and inflamed (colitis) (B) rats. Each column represents the mean $\pm$ SEM obtained from eight trials. * $P < 0.05$ versus control.

verted them into relaxations, and only after-contractions were then evident (data not shown). Tetrodotoxin (1 μM) abolished the electrically induced contractions (data not shown).

Effects of A_{2B} receptor blockade

Under resting conditions, the A_{2B} receptor antagonist MRS 1754 did not affect the spontaneous contractile activity of normal or inflamed colonic tissues. In normal colon preparations maintained in standard Krebs solution, MRS 1754 (0.001–1 μM) concentration-dependently increased sES-evoked contractions, with a maximal effect of $+35 \pm 3.7\%$ occurring at 0.01 μM (Figure 4A). However, in the presence of colitis, the enhancing effects of the A_{2B} receptor antagonist on

sES-induced contractions no longer occurred (Figure 4B). The response patterns of MRS 1754 at a concentration of 0.01 μM were not affected by pre-incubation with A_1 , A_{2A} or A_3 receptor antagonists in preparations from either normal or inflamed colon, indicating that MRS 1754 acted via selective blockade of A_{2B} receptors (data not shown).

In colonic tissues maintained in Krebs solution supplemented with guanethidine (10 μM), NPA (0.01 μM), L-732138 (NK_1 receptor antagonist, 10 μM), GR159897 (NK_2 receptor antagonist 1 μM) and SB218795 (NK_3 receptor antagonist, 1 μM), sES evoked phasic contractions that were prevented by atropine (not shown). Under these conditions, MRS 1754 (0.01 μM) was able to enhance the electrically evoked contractions in normal tissues, while in the presence of colitis this potentiating effect no longer occurred (Figure 5A).

In another series of experiments, the effects of A_{2B} receptor blockade were tested on contractions evoked by direct activation of muscarinic receptors on longitudinal smooth muscle. For this purpose, the effects of MRS 1754 (0.01 μM) on contractions evoked by carbachol (1 μM) in the presence of tetrodotoxin were assayed. In this setting, the carbachol-induced contractions were significantly increased by the A_{2B} antagonist in normal preparations, but not in inflamed tissues (Figure 5B).

Effects of A_{2B} receptor activation

The effects of increasing concentrations of the adenosine receptor agonist NECA were tested on rES-induced contractions in both normal and inflamed colon preparations, which were maintained in Krebs solution containing dipyrindamole and adenosine deaminase to minimize interference by endogenous adenosine, as well as DPCPX, ZM 241385 and MRS 1523 to prevent the activation of A_1 , A_{2A} and A_3 receptors by NECA. Under these conditions, cumulative application of NECA induced a decrease in rES-evoked cholinergic contractions of normal colonic tissues ($EC_{50} = 0.3 \pm 5.8$ μM; $E_{max} = -58.3 \pm 3.7\%$) (Figure 6). The magnitude, but not the apparent potency, of this inhibitory effect was significantly increased when the effects of NECA were assayed in preparations from rats with colitis ($EC_{50} = 0.27 \pm 6.4$ μM; $E_{max} = -79 \pm 4.8\%$) (Figure 6). The inhibitory effects of NECA on preparations from both normal and inflamed colon were similarly antagonized by MRS 1754 (K_d values: 1.7 ± 0.4 nM and 1.4 ± 0.7 nM respectively) (Figure 6).

Under the same experimental conditions, NECA reduced the contractions elicited by submaximal (1 μM) (Figure 7A) and supramaximal (100 μM) (Figure 7B) concentrations of carbachol in the presence of tetrodotoxin in preparations from control rats and, to a greater extent, also in preparations from inflamed animals.

Molecular and functional interaction between adenosine deaminase and A_{2B} receptors

Immunoprecipitation assay. The immunoprecipitation of adenosine deaminase from homogenized colonic neuromuscular tissues obtained from control or inflamed rats yielded a 37 kDa band corresponding to the A_{2B} receptor (Figure 8A). Analogously, the immunoprecipitation of A_{2B} receptors yielded a 43 kDa band corresponding to adenosine

Figure 5

(A) Effects of MRS 1754 (0.01 μ M) on contractions evoked by sES (0.5 ms, 10 Hz, 30 mA, 10 s) in colon preparations from normal and inflamed (colitis) rats, maintained in Krebs solution containing guanethidine (10 μ M), L-732 138 (10 μ M), GR-159897 (1 μ M), SB-218795 (1 μ M) and NPA (0.01 μ M). Each column represents the mean $\pm$ SEM obtained from eight trials. * P < 0.05 versus control. (B) Column graphs showing the effects of MRS 1754 (0.01 μ M) on contractions evoked by carbachol (CARB; 1 μ M) in normal or inflamed (colitis) colon preparations maintained in Krebs solution containing tetrodotoxin (1 μ M). Each column represents the mean $\pm$ SEM value obtained from six trials. * P < 0.05 versus control.

Figure 6

Effects of increasing concentrations of NECA (0.001–100 μ M), alone or in combination with MRS 1754 (0.01 μ M), on contractions evoked by rES (0.5 ms, 30 mA, 10 Hz) in colon preparations, from normal or inflamed rats, maintained in Krebs solution containing dipyrindamole (0.5 μ M), adenosine deaminase (0.5 U mL⁻¹), guanethidine (10 μ M), L-732 138 (10 μ M), GR-159897 (1 μ M), SB-218795 (1 μ M), NPA (0.01 μ M), DPCPX (0.01 μ M), ZM 241385 (0.01 μ M) and MRS 1523 (0.1 μ M). Each point represents the mean $\pm$ SEM of six trials. * P < 0.05 versus NECA alone.

deaminase (Figure 8A). This finding indicates that the A_{2B} receptor and adenosine deaminase form a molecular complex in the neuromuscular compartment of rat colon. As a further demonstration of an interaction between A_{2B} receptors and adenosine deaminase, the immunoprecipitation of proteins with the anti-adenosine deaminase antibody (Figure 8B) or with the anti-A_{2B} receptor antibody (not shown), stained with Coomassie blue, revealed the presence of two bands corresponding to the A_{2B} receptor and adenosine deaminase. To

Figure 7

Column graphs showing the effects of NECA (0.1 μM) on contractions evoked by submaximal (A) and supramaximal (B) concentrations of carbachol (CARB; 1 and 100 μM respectively) in colon preparations maintained in Krebs solution containing dipyridamole (0.5 μM), adenosine deaminase (0.5 $\text{U}\cdot\text{mL}^{-1}$), tetrodotoxin (1 μM), DPCPX (0.01 μM), ZM 241385 (0.01 μM) and MRS 1753 (0.01 μM). Each point represents the mean $\pm$ SEM of six trials. * $P < 0.05$ versus control.

confirm the immunoprecipitation data, a double immunofluorescence analysis was performed to evaluate the A_{2B} receptor and adenosine deaminase distribution in the colonic neuromuscular layer. Thus, immunoreactivity for adenosine deaminase was detected in the myenteric ganglia of normal rat colon, where it colocalized with A_{2B} receptors. In the presence of colitis, the adenosine deaminase staining increased markedly in myenteric ganglia and, to a lesser extent, in both the longitudinal and circular muscle layers, where it was found to colocalize mostly with A_{2B} receptors (Figure 8C).

Effect of adenosine deaminase and CD73 inhibition on colonic motility of longitudinal smooth muscle. The selective adenosine deaminase blocker EHNA (0.5 μM) decreased the sES-induced cholinergic contractions in preparations from control animals and this effect was enhanced in tissues from animals with colitis (Figure 9). MRS 1754 (0.01 μM) significantly antagonized the effects of EHNA (0.5 μM) in both normal and inflamed colon preparations (Figure 9).

Under pharmacological blockade of A_1 , A_{2A} and A_3 receptors, MRS 1754 (0.01 μM) did not significantly affect the enhancing effect of AOPCP on the contractions evoked by sES

in normal colonic tissues (Figure 10A), while in the presence of colitis, AOPCP or MRS 1754, alone or in combination, did not modify the sES-induced motor responses (Figure 10B).

Discussion and conclusions

The evaluation of how and to what extent the adenosine pathway is involved in the pathophysiology of intestinal motor disorders represents an interesting matter of discussion and an intriguing field of investigation (Burnstock, 2008). In this context, increasing efforts are being focused on the exploration of the physiological role played by adenosine in the regulation of enteric functions as well as on the characterization of the molecular and functional alterations occurring in this system in the presence of intestinal inflammation (Antonioli *et al.*, 2008b).

The present study was designed to specifically investigate the expression and distribution of A_{2B} receptors in the neuromuscular compartment of rat distal colon, as well as to elucidate the contribution of this receptor subtype to the control of colonic neuromotility following the induction of inflammation. Our experiments highlighted three main

Figure 8

Representative images of immunoprecipitation (IP) with anti-adenosine deaminase (ADA) (A, upper panel) or anti-A_{2B} receptor antibody (A, lower panel) followed by immunoblotting (IB) for A_{2B} (A, upper panel) and ADA (A, lower panel) in colonic tissues from normal and DNBS-treated (colitis) rats. (B) Representative images of proteins immunoprecipitated with anti-adenosine deaminase antibody and stained with Coomassie blue. (C) Double-staining immunohistochemistry showing the distribution of ADA (green) and A_{2B} receptors (red) in the myenteric plexus of colonic cryosections from control (normal) and DNBS-treated (colitis) rats. LM, longitudinal muscle; CM, circular muscle; MG, myenteric ganglia. Scale bar: 75 μm.

Figure 9

Recording of motor activity from preparations of colonic longitudinal smooth muscle isolated from normal or inflamed (colitis) rats. Effects of EHNA (0.5 μ M) on cholinergic contractions evoked by sES (0.5 ms, 10 Hz, 30 mA, 10 s) either alone or when incubated with MRS 1754 (0.01 μ M). Each column represents the mean $\pm$ SEM obtained from six trials. * P < 0.05 versus control; § P < 0.05 versus EHNA alone.

novel findings: (i) under normal conditions, A_{2B} receptors are expressed in the myenteric ganglia as well as in the longitudinal and circular muscular layers of the colon, where they participate in the tonic inhibitory control of excitatory cholinergic motor activity, acting mainly at the level of smooth muscle cells; (ii) in the presence of colitis, a marked redistribution of A_{2B} receptors occurs, with an increase in A_{2B} fluorescence intensity in the neuromuscular layer, mainly at the level of the longitudinal muscle layer – in this setting, the tonic inhibitory control of A_{2B} receptors on excitatory cholinergic pathways is impaired, despite an evident up-regulation of these receptors, which retain a full sensitivity to the application of exogenous agonist; and (iii) the impairment of inhibitory control by A_{2B} receptors in the presence of colitis can be ascribed to a co-expression and molecular link of these receptors with the catabolic enzyme adenosine deaminase.

Data obtained from our molecular investigations demonstrated the presence of mRNA coding for A_{2B} receptors in the colonic neuromuscular layer under normal conditions. Subsequent immunofluorescence analysis confirmed the RT-PCR data, revealing an appreciable expression of A_{2B} receptors in the myenteric plexus as well as in the longitudinal and circular muscle layers. Our functional investigations allowed us

to demonstrate a significant involvement of A_{2B} receptors in the tonic inhibitory control by endogenous adenosine of colonic excitatory cholinergic motility. Moreover, in normal colonic tissues incubated with dipyridamole and adenosine deaminase to minimize interference by endogenous adenosine, the agonist NECA decreased the amplitude of cholinergic motor responses elicited by electrical stimuli in a concentration-dependent fashion. In addition, we also evaluated whether the effects of A_{2B} receptor ligands on normal colonic motor activity were mediated by receptors located at neuronal and/or muscular sites. For this purpose, the effects of the A_{2B} receptor agonist and antagonist on colonic contractions elicited by direct stimulation of muscular muscarinic receptors by carbachol were tested (in the presence of tetrodotoxin in order to prevent neurogenic responses). Under these conditions, the contractile responses were significantly enhanced by A_{2B} receptor blockade, while they were markedly decreased by A_{2B} receptor activation, thus indicating that these receptors perform their modulating action on colonic motility at the muscular level, where they are highly expressed. Consistent with our observations, the colonic expression of A_{2B} receptors was comparable to that recently observed in the normal rat ileum (Zoppellaro *et al.*, 2013) as well as in normal colonic tissues from mice

A NORMAL **B COLITIS** **Figure 10**

Effects of AOPCP (200 μM), alone or in combination with MRS 1754 (0.01 μM), on cholinergic contractions evoked by electrical stimulation (sES: 0.5 ms, 30 mA, 10 Hz) in colon preparations from normal (A) or inflamed rats (B) in the presence of DPCPX (0.01 μM), ZM 241385 (0.01 μM) and MRS 1753 (0.01 μM). Each column represents the mean ± SEM of six trials. **P* < 0.05 versus control.

(Chandrasekharan *et al.*, 2009), guinea pigs (Kadowaki *et al.*, 2000) and rats (Dixon *et al.*, 1996). In this regard, our study expands previous knowledge, providing novel insights into the distribution of A_{2B} receptors in the neuromuscular compartment of normal colon. This distribution pattern is consistent with the findings by Vieira *et al.* (2011), who detected the presence of A_{2B} receptors in longitudinal muscle-myenteric plexus preparations of rat ileum. In addition, our functional observations are in line with previous studies showing inhibitory actions of adenosine on colonic smooth muscle from guinea pigs and rats via A_{2B} receptor stimulation (Kadowaki *et al.*, 2000; Fozard *et al.*, 2003). A similar inhibitory modulation was also observed in mouse distal colon, although in this species the activation of A_{2B} receptors was not associated with an inhibition of excitatory cholinergic pathways, but rather with a facilitation of the

control of inhibitory nitroergic nerves (Zizzo *et al.*, 2006; Chandrasekharan *et al.*, 2009).

In recent years, the involvement of A_{2B} receptors in the pathophysiological mechanisms underlying inflammatory intestinal injury has been a matter of heated debate and intense research. The discussion has been fostered by the findings that, following initial encouraging results supporting an ameliorative effect of A_{2B} receptor antagonists in mice with intestinal inflammation (Kolachala *et al.*, 2008a,b), these results were questioned by Frick *et al.* (2009), who reported a protective effect associated with A_{2B} receptor activation in the same model of colitis.

Our experiments on the time-course of colonic damage corroborate previous findings indicating a causal relationship between bowel inflammation and the changes in enteric motor activity (Collins, 1996; Sharkey and Kroese, 2001; Blandizzi *et al.*, 2003). In this context, when considering the mechanisms underlying the functional effect of A_{2B} receptors on the abnormalities of enteric neuromuscular functions associated with inflammatory bowel diseases, our study provides the first evidence that, following the induction of colitis, A_{2B} receptor immunoreactivity undergoes a marked up-regulation, particularly in the longitudinal muscle layer, and this is associated with an increased staining of glial cells and a fainter intensity of neuronal cell immunoreactivity. The present molecular observations regarding the expression of A_{2B} receptors are in keeping with a previous study by Kolachala *et al.* (2005), who reported a significant increase in this protein, driven by TNF, in inflamed colon from humans and mice, as assessed by immunofluorescence and Western blot analysis. Along the same lines, a recent paper demonstrated an increased immunoreactivity for A_{2B} receptors in the longitudinal and circular smooth muscle layer of rats infected with herpes simplex virus type 1 (Zoppellaro *et al.*, 2013).

Notably, the changes highlighted by our molecular investigations in the setting of bowel inflammation appear to be partly at odds with our functional experiments. Indeed, the potentiating effects of the A_{2B} receptor antagonist on colonic cholinergic contractions no longer occurred when the experiments were repeated in the presence of colitis, despite the increase in A_{2B} receptor density in the muscular compartment of inflamed colon. Nevertheless, the stimulation of A_{2B} receptors with the agonist NECA reduced, with enhanced efficacy, the cholinergic-mediated contractile responses evoked by electrical stimulation or the addition of carbachol. On this basis, and as also documented by molecular data, it is conceivable that the induction of colitis is associated with an increased expression of A_{2B} receptors, mainly at the muscular level, where they retain their full responsiveness to exogenous pharmacological activation, but no longer participate in the inhibitory control of colonic cholinergic motility mediated by endogenous adenosine.

An interesting novel point arising from the present study is the mechanism underlying the reduced interaction of A_{2B} receptors with endogenous adenosine despite their enhanced expression in the neuromuscular compartment of inflamed colon. Based on our previous findings (Antonioli *et al.*, 2010), we hypothesized that this condition arises from a reduced availability of endogenous adenosine in the A_{2B} receptor bio-phase as a consequence of an interaction between the catabolic enzyme adenosine deaminase and A_{2B} receptors, and

an increase in the expression of adenosine deaminase. This hypothesis was substantiated by the results from our immunoprecipitation/immunoblotting analysis, which revealed the occurrence of a molecular link between adenosine deaminase and A_{2B} receptors. In addition, the double-staining immunofluorescence analysis demonstrated the colocalization of A_{2B} receptors with adenosine deaminase, mainly at the level of the myenteric ganglia in normal tissue, but also in the smooth muscle layers in colitis. Notably, such interplay was found to occur at the functional level as well, as the increase in endogenous adenosine availability obtained through the pharmacological blockade of adenosine deaminase resulted in the restoration of A_{2B} receptor-mediated inhibitory activity in the presence of bowel inflammation. Furthermore, our experiments demonstrated that ecto-5'-nucleotidase is likely to represent a direct source of endogenous adenosine recruiting A_{2B} receptors in the neuromuscular compartment of normal rat colon, as the inhibition of this enzyme, as well as the blockade of A_{2B} receptors, resulted in a significant enhancement of electrically-induced contractions. However, in the presence of bowel inflammation, this enhancing effect no longer occurred – possibly as a consequence of increased endogenous adenosine catabolism by adenosine deaminase. A functional link between ecto-5'-nucleotidase and adenosine receptors, in particular the A_{2A} receptor, in the colonic neuromuscular compartment has previously been observed, although with different patterns of functional responses (Antonioli *et al.*, 2011a).

On the basis of several lines of evidence generated by studies on immune cells, the occurrence of molecular and functional interactions between purine receptors and metabolic enzymes is being increasingly acknowledged (Franco *et al.*, 1997; 1998; Herrera *et al.*, 2001). In particular, it is now thought likely that the mechanisms of nucleoside inactivation (i.e. adenosine deaminase and/or nucleoside transporters) can channel endogenous adenosine towards a compartmental recruitment of specific receptor subtypes, with the purpose of shaping the magnitude and duration of immune responses (Franco *et al.*, 1997; 1998; Herrera *et al.*, 2001; Antonioli *et al.*, 2012). In this context, the present study has demonstrated, for the first time, the existence of such a dynamic molecular network, recently designated as the 'enteric purinome' (Antonioli *et al.*, 2011c), in the neuromuscular compartment of the colon, where its functional task is to trigger, maintain and terminate purinergic signalling under different pathophysiological conditions.

In conclusion, our results add to the understanding of how adenosine pathways can influence gut neuromuscular functions under both normal and pathological conditions. In particular, the present findings suggest that A_{2B} receptors are profoundly involved in the regulation of colonic motility, pointing out, for the first time, the critical role played by adenosine deaminase in modulating A_{2B} receptor engagement by endogenous adenosine during inflammation. These observations, consistent with the emerging concept of the enteric purinome, open up new perspectives for the evaluation of purine metabolic enzymes and receptor subtypes as integrated molecular units responsible for the fine regulation of intestinal neuromuscular functions. Consequently, future investigations addressing the possible role of the enteric purinome in the processes of intestinal neuromuscular plasticity

could be of pivotal importance for the development of novel therapeutic tools for the management of motor disorders associated with bowel inflammation.

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Conflict of interest

None.

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