RESEARCH PAPER

Adenosine negatively regulates duodenal motility in mice: role of A_1 and A_{2A} receptors

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BACKGROUND AND PURPOSE
Adenosine is considered to be an important modulator of intestinal motility. This study was undertaken to investigate the role of adenosine in the modulation of contractility in the mouse duodenum and to characterize the adenosine receptor subtypes involved.

EXPERIMENTAL APPROACH
RT-PCR was used to investigate the expression of mRNA encoding for A_1, A_{2A}, A_{2B} and A_3 receptors. Contractile activity was examined *in vitro* as changes in isometric tension.

KEY RESULTS
In mouse duodenum, all four classes of adenosine receptors were expressed, with the A_{2B} receptor subtype being confined to the mucosal layer. Adenosine caused relaxation of mouse longitudinal duodenal muscle; this was antagonized by the A_1 receptor antagonist and mimicked by N^6-cyclopentyladenosine (CPA), selective A_1 agonist. The relaxation induced by A_1 receptor activation was insensitive to tetrodotoxin (TTX) or N^ω-nitro-L-arginine methyl ester (L-NAME). Adenosine also inhibited cholinergic contractions evoked by neural stimulation, effect reversed by the A_1 receptor antagonist, but not myogenic contractions induced by carbachol. CPA and 2-*p*-(2-carboxyethyl) phenethylamino-5′-*N*-ethylcarboxamidoadenosine hydrochloride hydrate (CGS-21680), A_{2A} receptor agonist, both inhibited the nerve-evoked cholinergic contractions. L-NAME prevented only the CGS-21680-induced effects. *S*-(4-Nitrobenzyl)-6-thioinosine, a nucleoside uptake inhibitor, reduced the amplitude of nerve-evoked cholinergic contractions, an effect reversed by an A_{2A} receptor antagonist or L-NAME.

CONCLUSIONS AND IMPLICATIONS
Adenosine can negatively regulate mouse duodenal motility either by activating A_1 inhibitory receptors located post-junctionally or controlling neurotransmitter release via A_1 or A_{2A} receptors. Both receptors are available for pharmacological recruitment, even if only A_{2A} receptors appear to be preferentially stimulated by endogenous adenosine.

LINKED ARTICLE
This article is commented on by Antonioli *et al.*, pp. 1577–1579 of this issue. To view this commentary visit http://dx.doi.org/10.1111/j.1476-5381.2011.01529.x

Abbreviations
CCh, carbachol; CGS-21680, 2-*p*-(2-carboxyethyl) phenethylamino-5′-*N*-ethyl carboxamidoadenosine hydrochloride hydrate; CPA, N^6-cyclopentyladenosine; DPCPX, 1,3-dipropyl-8-cyclopentylxanthine; EFS, electrical field stimulation; GI, gastrointestinal; IB-MECA, N^6-(3-iodobenzyl) adenosine-5′-*N*-methyluronamide1-deoxy-1-6-((3-iodophenyl)methyl)amino -9*H*-purin-9-yl-*N*-methyl-β-D-ribofuranuronamide; L-NAME, N^ω-nitro-L-arginine methyl ester; MRS 1220, 9-chloro-2-(2-furanyl)-5-((phenylacetyl) amino)- 1,2,4 triazolo 1,5-c quinazoline; NBTI, *S*-(4-nitrobenzyl)-6-thioinosine; TTX, tetrodotoxin; ZM 241385, 4-(2-[7-amino-2-(2-furyl) [1,2,4]triazolo [2,3-a][1,3,5] triazin-5-ylamino]ethyl)phenol

Introduction

Adenosine is an endogenous nucleoside that can regulate a large number of physiological and pathophysiological processes (Antonioli *et al.*, 2008a). The biological actions of adenosine are mediated by specific receptors that are currently distinguished into four subtypes: A_1, A_{2A}, A_{2B} and A_3 (Fredholm *et al.*, 2000; Klotz, 2000; nomenclature follows Alexander *et al.*, 2009). The ability of adenosine to regulate several biological functions is strictly related to its extracellular concentration. The levels of adenosine at its receptors are determined by a variety of mechanisms, which include intracellular and extracellular adenosine biosynthesis, as well as cellular adenosine release, re-uptake and metabolism (Noji *et al.*, 2004). Furthermore, extracellular production of adenosine is increased under adverse conditions, such as hypoxia or inflammation, and this nucleoside appears to be involved in mutual interactions between immune/inflammatory cells and the enteric nervous system, which are responsible for concomitant alterations of bowel motility (Antonioli *et al.*, 2008b). Several lines of evidence highlight a prominent role of adenosine in the complex interactions between enteric neurons and smooth muscle, with fine-tuning of motility effected by this nucleoside. However, the specific function of adenosine in the regulation of motility in different regions of the gastrointestinal (GI) tract and in different species has not been fully clarified. In addition, the location of adenosine receptors is still controversial. Specifically, for instance, in the small intestine, exogenous adenosine or related compounds were found to inhibit cholinergic or tachykininergic transmission via activation of prejunctional A_1 receptors (Shinozuka *et al.*, 1985; Christofi *et al.*, 1990; Broad *et al.*, 1992; Nitahara *et al.*, 1995; Tomaru *et al.*, 1995; Coupar, 1999; Lee *et al.*, 2001; Storr *et al.*, 2002; Duarte-Araújo *et al.*, 2004a; Duarte-Araújo *et al.*, 2009). The roles of A_2 and A_3 receptors in the control of neuromuscular functions in the small bowel are much less clear. Conflicting evidence has been obtained on A_{2A} receptors, which have been suggested either to reduce cholinergic motor responses in guinea-pigs and rats (Gustafsson *et al.*, 1985; Storr *et al.*, 2002), or to facilitate ACh release in the same species (Tomaru *et al.*, 1995; Duarte-Araújo *et al.*, 2004a,b). A_{2B} receptors have been shown to be located on the enteric inhibitory neurons that release NO in mouse distal colon (Zizzo *et al.*, 2006; Chandrasekharan *et al.*, 2009). A_3 receptors have recently been reported to be expressed in the myenteric ganglia of rat distal colon and participate in the tonic inhibitory control of excitatory cholinergic motor activity induced by adenosine (Antonioli *et al.*, 2010). In addition to the control on myenteric nerves, adenosine has been shown to exert direct effects on smooth muscle cells in the intestinal small bowel. Post-junctional inhibitory A_1 receptors have been reported to be present in mouse jejunum and in ileum (De Man *et al.*, 2003; Zizzo *et al.*, 2009) and in rat duodenum and ileum (Nicholls *et al.*, 1996; Nicholls and Hourani, 1997). In addition, stimulation of A_{2A} and A_3 receptors has been shown to inhibit spontaneous contractions in possum duodenum (Woods *et al.*, 2003), and A_{2B} receptors to mediate relaxation in rat duodenum (Nicholls *et al.*, 1992; 1996). Thus, our knowledge of the physiological function of adenosine is far from complete, and more research is required to improve our understanding of adenosine-mediated regulation of GI motility and, also, to consider the possible involvement of adenosine in the pathophysiology of intestinal disorders.

Hence, this study was undertaken to investigate whether and which mRNA of adenosine receptor subtypes is expressed in mouse duodenum and to examine the role of adenosine receptor activation on duodenal motor function. The results we obtained suggest that adenosine can negatively regulate duodenal motility, in mice, either activating A_1 inhibitory receptors located at the post-junctional level or controlling neurotransmitter release via A_1 or A_{2A} receptors located on enteric nerves.

Methods

All animal care and experimental procedures complied with the Italian D.L. no. 116 of 27 January 1992 and associated guidelines in the European Communities Council Directive of 24 November 1986 (86/609/ECC). Experiments were performed on adult male mice (C57BL/10SnJ; 25.5 ± 0.5 g body weight; 15 weeks old), obtained from Charles River Laboratories (Calco-Lecco, Italy) and maintained in a light- (12 h/12 h light) and temperature-controlled (23°C) environment with free access to food and water. Animals were killed by cervical dislocation, the abdomen was immediately opened and the duodenum was removed and placed in Krebs solution consisting of (mM): NaCl 119; KCl 4.5; $MgSO_4$ 2.5; $NaHCO_3$ 25; KH_2PO_4 1.2, $CaCl_2$ 2.5, glucose 11.1. The contents of the excised segments were gently flushed out with Krebs solution. Segments (20 mm in length) were suspended in a four-channel organ bath containing 10 mL of oxygenated (95% O_2 and 5% CO_2) Krebs solution maintained at 37°C.

RNA preparation and RT-PCR analysis

Total RNA was extracted from whole thickness duodenum and in a preparation devoid of mucosa layer, using PureLink™ RNA Mini Kit (Invitrogen, Paisley, UK) according to the manufacturer's instructions. After quantification by spectrophotometry, 1 µg of total RNA was reverse-transcribed in a final volume of 50 µL using the High Capacity c-DNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) and the following thermal cycle profile: 10 min at 25°C, 2 h at 37°C and 5 min at 85°C as described by the kit. The A_1, A_{2A}, A_{2B}, A_3 receptors, β-actin and sucrase isomaltase were amplified using 100 ng of cDNA per reaction. The oligonucleotide primers for mouse A_1, A_{2A}, A_{2B}, A_3 receptors, sucrase isomaltase and β-actin were as follows: A_1: (forward) 5′-TACATCTCGG CCTTCCAGG-3′, (reverse) 5′-AGTAGGTCTGTGCCCAATG-3′; A_{2A}: (forward) 5′-GTCCCTGGCCATCATCGT-3′, (reverse) 5′-GATCCTGTAGGCGTAGAT-3′; A_{2B}: (forward) 5′-TTCCACG GCTGCCTCTTC-3′, (reverse) 5′-CATCCCCCAGTTCTGTGC-3′; A_3: (forward) 5′-CGTTCCGTGGTCAGTTTG-3′, (reverse) 5′-CGCAGGCGTAGACAATAGG-3′; Sucrase Isomaltase: (forward) 5′-TTGATATCCGGTCCACGGTTCT-3′, (reverse) 5′-CAGGTGACATCCAGGTTGCATT-3′; β-actin: (forward) 5′-CCGCCCTAGGCACCAGGGT-3′, (reverse) 5′-GGCTGGGG TGTTGAAGGTCTCAAA-3′. PCR analysis was performed in triplicate and with the following thermal cycle profile: an initial step of 45 s at 95°C, an amplification cycle (94°C for 45 s, 55°C for 45 s and 72°C for 1 min) repeated 30 times and

a final step of 15 min at 72°C. The amplimers were separated on a 1% agarose gel containing 0.5 μg·mL^{-1} of ethidium bromide for visualization, and the gel was scanned under u.v. light. Expected length of the PCR products for A_1, A_{2A}, A_{2B}, A_3 receptors and β-actin and sucrase isomaltase were 208, 160, 238, 330, 300 and 143 bp respectively. The corrected band intensities of the specific adenosine receptors, obtained using the ImageJ software (U. S. National Institute of Health, Bethesda, MD, USA), were normalized with the corresponding β-actin mRNA expression. It was ensured that the band intensities of β-actin mRNA were similar before the comparison of the expression levels.

Recording of mechanical activity

The distal end of each segment was tied to an organ holder, and the proximal end was secured with a silk thread to an isometric force transducer (FORT 10, Ugo Basile, Biological Research Apparatus, Comerio VA, Italy). Mechanical activity was digitized on an A/D converter, visualized, recorded and analysed on a personal computer using the PowerLab/400 system (Ugo Basile, Italy). Longitudinal preparations were subjected to an initial tension of 200 mg and were allowed to equilibrate for at least 30 min. Rhythmic spontaneous contractions of varying amplitude developed in all preparations. After the equilibration time, preparations were challenged with 0.1 μM isoprenaline or with 10 μM carbachol (CCh) for 2 min, until stable responses were obtained. Concentration–response curves for adenosine or N^6-cyclopentyladenosine (CPA), an A_1 receptor agonist, were constructed by non-cumulative addition of the drugs. Adenosine or adenosine receptor agonists were applied for approximately 3 min at 20 min intervals.

The concentration–response curves to adenosine or to the adenosine receptor agonist were repeated in the presence of 1,3-dipropyl-8-cyclopentylxanthine (DPCPX), an A_1 purinoceptor antagonist, 4-(2-[7-amino-2-(2-furyl) [1,2,4]triazolo [2,3-a][1,3,5] triazin-5-ylamino]ethyl)phenol (ZM 241385), an A_{2A} purinoceptor antagonist, or 9-chloro-2-(2-furanyl)-5-((phenylacetyl) amino)-1,2,4 triazolo 1,5-c quinazoline (MRS 1220), an A_3 purinoceptor antagonist. Antagonists were in contact with the tissue for at least 30 min before repeating the dose–response curves for the agonists. Each preparation was tested with a single antagonist, except when otherwise stated. Time control experiments showed that a second curve to the agonists was reproducible. Concentrations of the drugs used were determined from literature and from previous experiments, where they have been shown, in mouse intestine, to be selective for the receptors examined (Serio *et al.*, 2003; Zizzo *et al.*, 2005a,b; 2009). The effects of tetrodotoxin (TTX) or N^{ω}-nitro-L-arginine methyl ester (L-NAME) were tested against a submaximal dose of adenosine or CPA.

In a different set of experiments to explore the role of adenosine receptors in the modulation of cholinergic motor neurons, electrical field stimulation (EFS) was applied by an S88 square-wave pulse generator (Grass Medical Instruments, Quincy, MA, USA) coupled via a stimulus isolation unit (Grass SIU5) to a pair of platinum electrodes placed in parallel on either side of the segments. Stimuli of 0.5 ms duration at a frequency of 4 Hz for 10 s were applied at 5 min intervals. The voltage was set to produce a submaximal contraction. In these conditions, the responses to EFS were stable and reproducible for hours. The evoked contractions were abolished by the muscarinic receptor antagonist, atropine (1 μM), or by the neuronal blocker, TTX (1 μM), suggesting that they were mediated by cholinergic nerve activation. The effects of the purinoceptor agonists, CPA, 2-*p*-(2-carboxyethyl) phenethylamino-5′-*N*-ethyl carboxamidoadenosine hydrochloride hydrate (CGS-21680) or N^6-(3-iodobenzyl) adenosine-5′-*N*-methyluronamide1-deoxy-1- 6-((3-iodophenyl)methyl) amino -9*H*-purin-9-yl-*N*-methyl-β-D-ribofuranuronamide (IB-MECA), A_1, A_{2A} and A_3 receptor agonists, respectively, on the EFS- evoked contractions were evaluated in the absence and presence of their respective receptor antagonists.

In order to increase the extracellular levels of adenosine, preparations were challenged for 20 min with *S*-(4-nitrobenzyl)-6-thioinosine (NBTI), a nucleoside uptake inhibitor. The effects of NBTI on the neural-evoked contractions were evaluated in the presence of adenosine receptor antagonists and L-NAME.

Lastly, the effects of the adenosine agonists and antagonists were assessed on cholinergic contractions elicited by direct pharmacological activation of muscarinic receptors located on smooth muscle cells. Then, colonic preparations were challenged with CCh (1 μM) in the continuous presence of TTX (1 μM) in the absence or presence of the different drugs tested.

Statistical analysis

All data are given as means ± SEM; *n* in the results section refers to the number of animal preparations on which observations were made. The amplitude of the relaxation induced by adenosine or by CPA was measured from the baseline (an ideal midline between the spontaneous changes of activity) to the lowest point reached and reported as a percentage of the effect induced by 0.1 μM isoprenaline. Adenosine or CPA responses in the absence or presence of the different antagonists were fitted to sigmoid curves (Prism 4.0, Graph-PAD, San Diego, CA), and EC_{50} values with 95% confidence limits (CLs) were determined from these curves. Statistical analysis was performed by means of Student's *t*-test or by means of analysis of variance followed by Bonferroni's test, when appropriate. A probability value of 0.05 was regarded as significant.

Solution and drugs

The drug/molecular target nomenclature (e.g. receptors, ion channels and so on) used in the present study conforms to British Journal of Pharmacology's *Guide to Receptors and Channels* (Alexander *et al.*, 2009). The following drugs: adenosine, CCh, CGS-21680, DPCPX, NBTI, L-NAME, MRS 1220 were all purchased from Sigma-Aldrich, Inc. (St. Louis, MO, USA). IB-MECA, CPA and ZM 241385 were purchased from Tocris Bioscience (Bristol, UK), and TTX was from Alomone Labs Ltd (Jerusalem, Israel). Adenosine, CGS-21680, CPA, DPCPX, IB-MECA, MRS1220, NBTI and ZM 241385 were dissolved in dimethyl sulphoxide (DMSO) and further diluted in Krebs. All the other drugs were dissolved in distilled water. The maximal final concentration of ethanol and DMSO in the organ bath was 0.5%, which did not affect the contractility of the duodenal segments. The working solutions were prepared fresh on the day of the experiment by diluting the stock solutions in Krebs and were added to the organ bath.

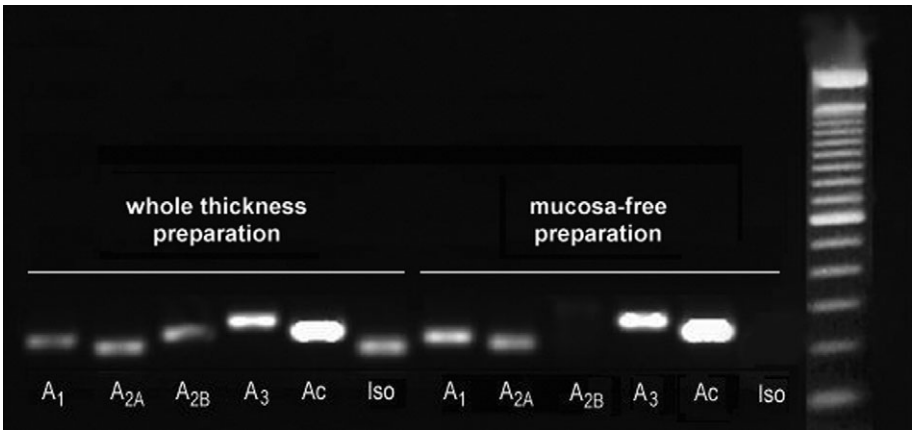

Figure 1

Expression of transcripts encoding the adenosine receptor subtypes, A_1, A_{2A}, A_{2B} and A_3, was analysed in the whole thickness duodenum and in the mucosa-free preparation. Transcripts encoding each of the four adenosine receptors were detected in the whole thickness preparation. In the preparations devoid of mucosa layer only, transcripts encoding for A_1, A_{2A} and A_3 receptor were detected. The mucosal epithelial marker sucrase–isomaltase was studied to assess the potential contamination of mucosa-free preparation with RNA from the mucosal epithelium. β-Actin primer was used as a control for cDNA integrity. A 100 bp DNA ladder was used as marker (M).

Results

Transcripts encoding adenosine receptors in mouse duodenum

RT-PCR was used to investigate the expression of mRNA encoding for A_1, A_{2A}, A_{2B} and A_3 receptors on whole thickness duodenum and in a mucosa-free preparation. Transcripts encoding each of the four adenosine receptors were found in the whole thickness preparations (Figure 1). Indeed, the preparations devoid of mucosal layer were positive only for the expected A_1, A_{2A} and A_3 receptor specific products (Figure 1). To assess that the preparation was not contaminated by mucosa, the presence of transcripts encoding the mucosal epithelial marker sucrase–-isomaltase was also investigated. As shown in Figure 1, transcripts encoding the sucrase–isomaltase were detected only in the whole thickness sample. The observation that transcripts encoding A_{2B} receptor was only observed in whole thickness samples is consistent with the possibility of the presence of A_{2B} receptor subtype exclusively in the mucosal layer.

Effects of adenosine and adenosine receptor agonists on spontaneous mechanical activity in mouse duodenum

Isolated segments of mouse duodenum displayed spontaneous activity characterized by phasic contractions with an amplitude of 281.4 ± 25.1 mg (n = 30) and a frequency of 38.3 ± 2.8 cpm (n = 30), not modified by TTX (1 μM) or by atropine (1 μM). Adenosine (0.3–300 μM) produced a relaxation that persisted throughout the contact time (Figure 2). The effect enhanced with the increase in the concentration and the maximal response at the dose of 300 μM consisted of a muscular relaxation with an amplitude 70% of the relaxation induced by 0.1 μM isoprenaline (EC_{50}, 2.9 μM, 95% CL 2.0–4.4 μM, n = 30) (Figure 2). TTX (1 μM), a blocker of neuronal voltage-dependent Na^+ channels, or L-NAME (100 μM), a blocker of NO synthase, failed to affect the inhibitory effects induced by a submaximal dose of adenosine (30 μM), indicating that adenosine-induced relaxation was not dependent on neural action potentials or on endogenous NO production (Figure 2).

To investigate the receptor(s) responsible for the adenosine inhibitory effects, among the subtypes identified by RT-PCR in the neuromuscular compartment, we tested the effect of antagonists for A_1, A_{2A} and A_3 purinoceptors, DPCPX (10 nM), ZM 241385 (10 nM) and MRS 1220 (0.1 μM), respectively. Only DPCPX was able to significantly antagonize the relaxation induced by adenosine, shifting to the right the dose–response curve to adenosine to the right (EC_{50}, 20.8 μM, 95% CL 9.4–46.9 μM in the presence of DPCPX, n = 5) (Figure 2). Thus, in our preparation, A_1 receptors mediate the relaxation induced by exogenous adenosine. No additive effect was observed in the combined presence of A_1, A_{2A} and A_3 purinoceptor antagonists.

The inhibitory response to adenosine was mimicked by the selective A_1 receptor agonist CPA, but not by the selective A_{2A} and A_3 receptor agonists, CGS-21680 (up to 5 μM) and IB-MECA (up to 10 μM), respectively. The inhibitory effects of CPA (30 nM–30 μM) were antagonized by the selective A_1 receptor antagonist, DPCPX (10 nM) (Figure 3). As for adenosine, neither TTX (1 μM) nor L-NAME (100 μM) affected the inhibitory effects induced by a submaximal dose of CPA (3 μM) (Figure 3).

It is noteworthy that none of the antagonists used had any effect on the spontaneous contractile activity.

Effects of adenosine and adenosine-receptor agonists on the cholinergic contractions evoked by EFS and by CCh in mouse duodenum

EFS (0.5 ms, 4 Hz, submaximal voltage for 10 s) elicited an early high in amplitude (393.2 ± 15.4 mg, n = 22) cholinergic contraction. To assess the effect of adenosine on neurally-

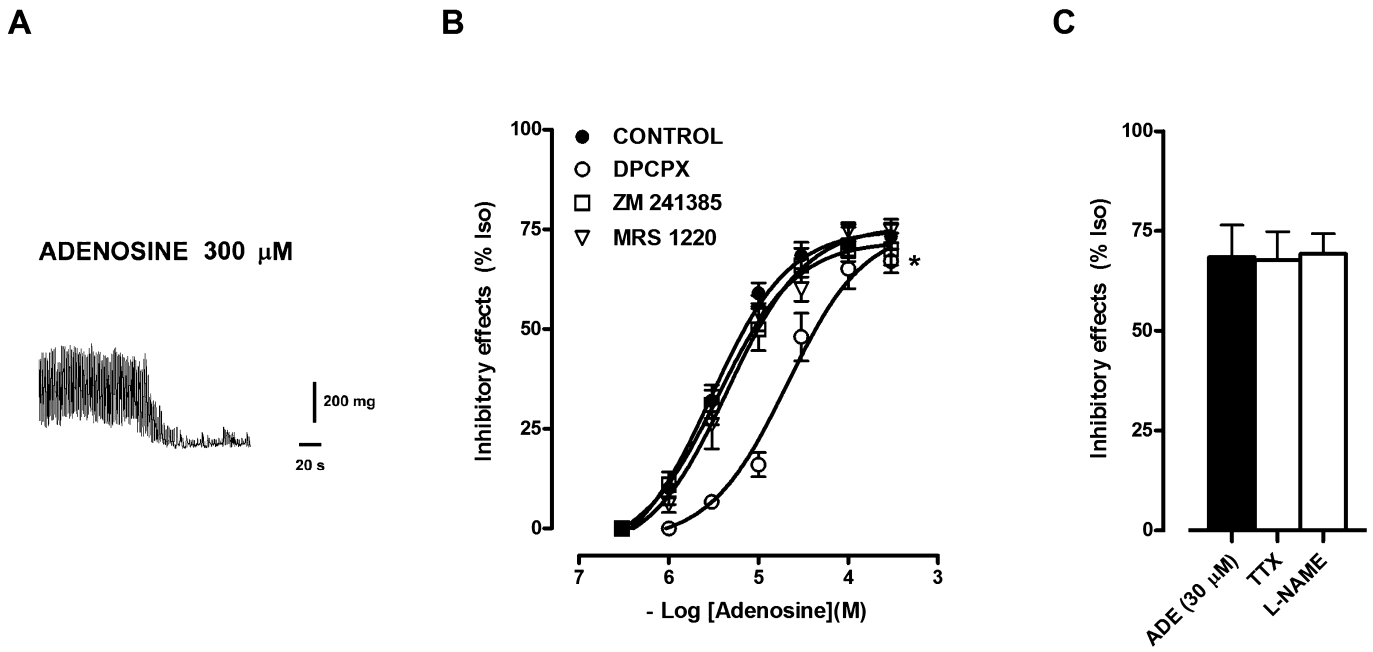

Figure 2

(A) Original recordings showing the inhibitory response evoked by adenosine (300 μM) in longitudinal duodenal muscular preparations. (B) Concentration–response curves to adenosine (0.3–300 μM), in the longitudinal duodenal muscular preparations, in the absence or in the presence of DPCPX (10 nM, n = 5), ZM 241385 (10 nM, n = 4) or MRS 1220 (0.1 mM, n = 4), A_1, A_{2A} and A_3 receptor antagonists, respectively. (C) Histograms showing the effects of TTX, a Na^+ voltage-gated neural channel blocker (1 μM, n = 4), or L-NAME, an NO synthase inhibitor (100 μM, n = 5), on the response induced by adenosine (30 μM). Data are means ± SEM and are expressed as percentage of the effect induced by 0.1 μM isoprenaline (Iso). The values for the control curves are the means of the control data obtained before each treatment (n = 13). *$P \leq 0.05$ when the concentration–response curves were compared with those obtained in the respective control condition.

evoked cholinergic contractions, a concentration of 1 μM was chosen since this did not cause a significant reduction of the spontaneous phasic contraction. Adenosine, at this concentration, was able to inhibit the amplitude of cholinergic nerve-mediated contractions (Figure 4), without affecting the direct smooth muscle cholinergic contractions elicited by 10 μM CCh in the presence of TTX (774.8 ± 18.2 mg before adenosine, 772.9 ± 17.3 mg after adenosine, n = 3, $P > 0.05$). Adenosine-induced inhibition of the cholinergic contractions to EFS was reversed only by the A_1 receptor antagonist DPCPX (10 nM) (Figure 4). CPA at the concentration of 0.1 μM, which did not significantly reduce spontaneous activity, was able to mimic the inhibitory effects induced by adenosine, and its effect was antagonized by DPCPX (10 nM), an A_1 receptor antagonist. To further exclude that the possibility that the effects of adenosine or CPA on cholinergic contractions may be the result of an effect on the baseline tension, EFS-induced contractions were evoked in the presence of 1 nM isoprenaline, which *per se* affected the mechanical activity induced by 1 μM adenosine or 0.1 μM CPA. In such a condition, cholinergic contractions to EFS were not affected (from 381.5 ± 10.2 mg to 368.0 ± 9.1 mg in the presence of 1 nM of isoprenaline, n = 3, $P > 0.05$). Furthermore, CPA did not affect cholinergic contractions elicited by 10 μM CCh in the presence of TTX (780.1 ± 17.6 mg before CPA, 777.6 ± 20.5 mg after CPA, n = 3, $P > 0.05$). Indeed, CGS-21680, an A_{2A} receptor agonist, which *per se* did not affect the spontaneous activity, was inhibited the amplitude of the EFS-induced cholinergic contractions concentration-dependently with the maximum effect occurring with 5 μM, whilst IB-MECA (up to 3 μM) was once more without any effect (Figure 4). CGS-21680-induced inhibitory effects were specifically antagonized by the A_{2A} receptor antagonist, ZM 241385 (10 nM) (Figure 4). Moreover, the NO synthase inhibitor, L-NAME (100 μM), which *per se* hardly affected neurally-evoked activity, attenuated only the CGS-21680-induced inhibitory effects (Figure 5). CGS-21680 did not affected the cholinergic contractions elicited by 10 μM CCh in the presence of TTX (791.1 ± 16.6 mg before CGS-21680, 800.3 ± 17.2 mg after CGS-21680, n = 3, $P > 0.05$). Lastly, none of the adenosine receptor antagonist *per se* affected the cholinergic contractions induced by either CCh or EFS.

Effects of a nucleoside uptake inhibitor

In order to increase the extracellular levels of adenosine, preparations were challenged with NBTI, a nucleoside uptake inhibitor. NBTI (10 μM) *per se* did not significantly modify the spontaneous mechanical activity but reduced the amplitude of nerve-evoked cholinergic contractions (Figure 6). The direct smooth muscle contractions to 10 μM CCh in the presence of TTX were not modified by NBTI (783.4 ± 12.8 mg before and 775.7 ± 15.6 mg after NBTI, n = 3, $P > 0.05$). NBTI-induced inhibitory effects on neural cholinergic contraction were reversed by pretreatment with the A_{2A} receptor antagonist ZM 241385 (10 nM), but not by DPCPX (10 nM) or MRS 1220 (0.1 μM), A_1 and A_3 receptor antagonists,

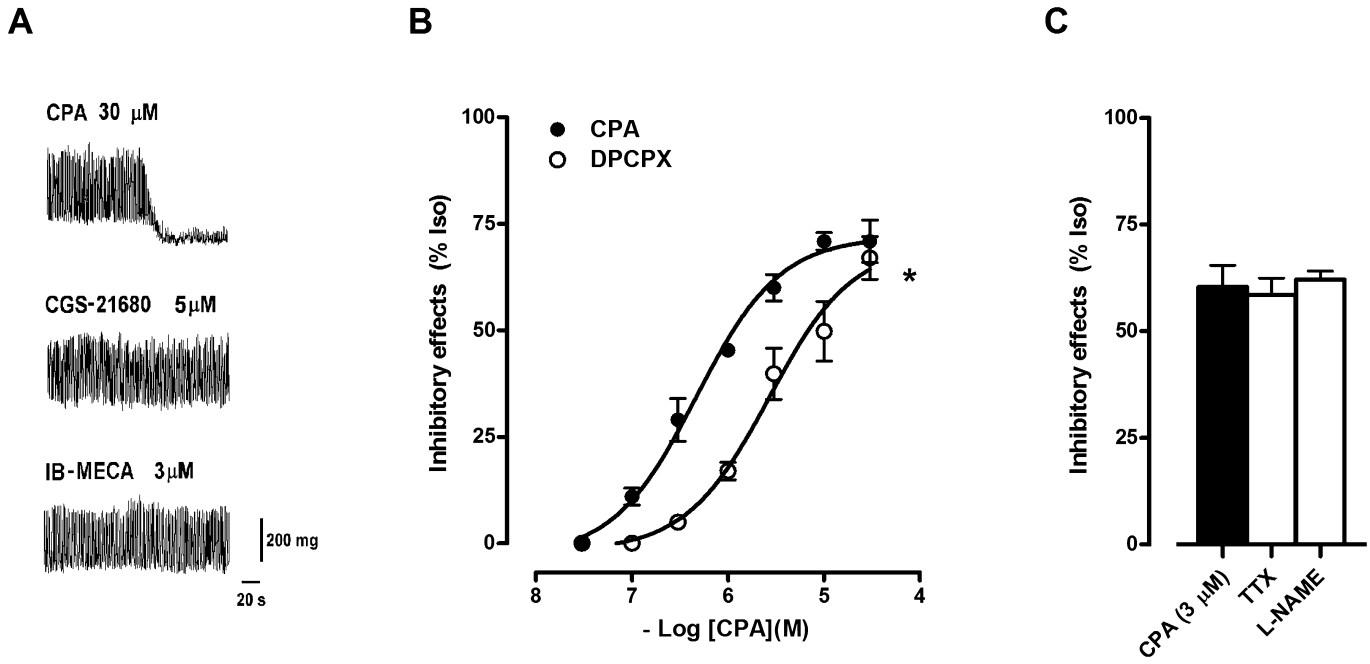

Figure 3

(A) Original tracing showing the effects of the A_1, A_{2A} and A_3 receptor agonists, CPA (30 μM), CGS-21680 (5 μM) and IB-MECA (3 μM), respectively, on spontaneous mechanical activity in the longitudinal muscle of mouse duodenum. (B) Concentration–response curves to CPA in the absence or in the presence of the A_1 receptor antagonist, DPCPX (10 nM, n = 5). (C) Histograms showing the effects of TTX, a Na^+ voltage-gated neural channel blocker (1 μM, n = 4), or L-NAME, an NO synthase inhibitor (100 μM, n = 5), on the response induced by CPA (3 μM). Data are means ± SEM and are expressed as percentage of the effect induced by 0.1 μM isoprenaline (Iso). *$P \leq 0.05$ when the concentration–response curves were compared with those obtained in the respective control condition.

respectively (Figure 6). The NO synthase inhibitor, L-NAME (100 μM), prevented the inhibition of EFS-induced cholinergic contractions induced by NBTI (Figure 6).

Discussion and conclusions

Adenosine, released by itself from neuronal endings or generated by breakdown of ATP, is considered to be an important modulator of intestinal motility in different animal species (Antonioli *et al.*, 2008b). Adenosine can influence gastrointestinal motility either directly, by activating receptors located on smooth muscle (Serio *et al.*, 1990; Nicholls *et al.*, 1996; Kadowaki *et al.*, 2000; Woods *et al.*, 2003; Fornai *et al.*, 2009), or indirectly, by regulating the neurotransmitter release from enteric neurons (Kadowaki *et al.*, 2000; Lee *et al.*, 2001; Storr *et al.*, 2002; Duarte-Araújo *et al.*, 2004a,b; Antonioli *et al.*, 2006; Zizzo *et al.*, 2006). The biological actions of adenosine are mediated by G-protein-coupled receptors currently distinguished into four subtypes: A_1, A_{2A}, A_{2B} and A_3 (Fredholm *et al.*, 2000; Klotz, 2000). These receptors are expressed throughout the gastrointestinal tract, with changes in the localization and density, depending on the species and gut region considered (Kadowaki *et al.*, 2000; Christofi *et al.*, 2001).

Data from our experiments indicate that in mouse duodenum, the transcripts for all four classes of adenosine receptors are expressed, and that the A_{2B} receptor subtype is confined to the mucosal layer. This is in agreement with the postulated role of adenosine A_{2B} receptors in the autocrine and paracrine regulation of gastrointestinal secretion (Strohmeier *et al.*, 1995; Christofi *et al.*, 2001). Whether or not activation of mucosal A_{2B} receptor may indirectly affect duodenal motility in the mouse was not investigated in the present study.

In addition, our results highlight a functional role played by A_1 and A_{2A} receptors in the regulation of mouse duodenal contractility by adenosine. Endogenous adenosine actions may be restricted to the release/production region at the intrinsic neural synapse leading to an inhibition of cholinergic transmission, while exogenously added adenosine seems to activate preferentially extrajunctional inhibitory A_1 receptors.

Longitudinal muscle from mouse duodenum, when mounted in an organ bath, shows spontaneous mechanical activity, which, differently from ileal segments (Baldassano *et al.*, 2008; 2009), is not under an excitatory tonic influence by neurally released ACh. Exogenously added adenosine caused a concentration-dependent relaxation of mouse longitudinal duodenal muscle that was antagonized by the selective A_1 receptor antagonist, DPCPX. Our results accord with those observed in other intestinal preparations, in which DPCPX at nanomolar concentrations competitively antagonizes A_1-mediated responses (Nicholls *et al.*, 1996; Kadowaki *et al.*, 2000). Moreover, CPA, a selective A_1 agonist, was able to mimic the effects of adenosine in a DPCPX-sensitive manner. Altogether these results indicate that A_1 receptors are involved in the adenosine-induced relaxation of mouse

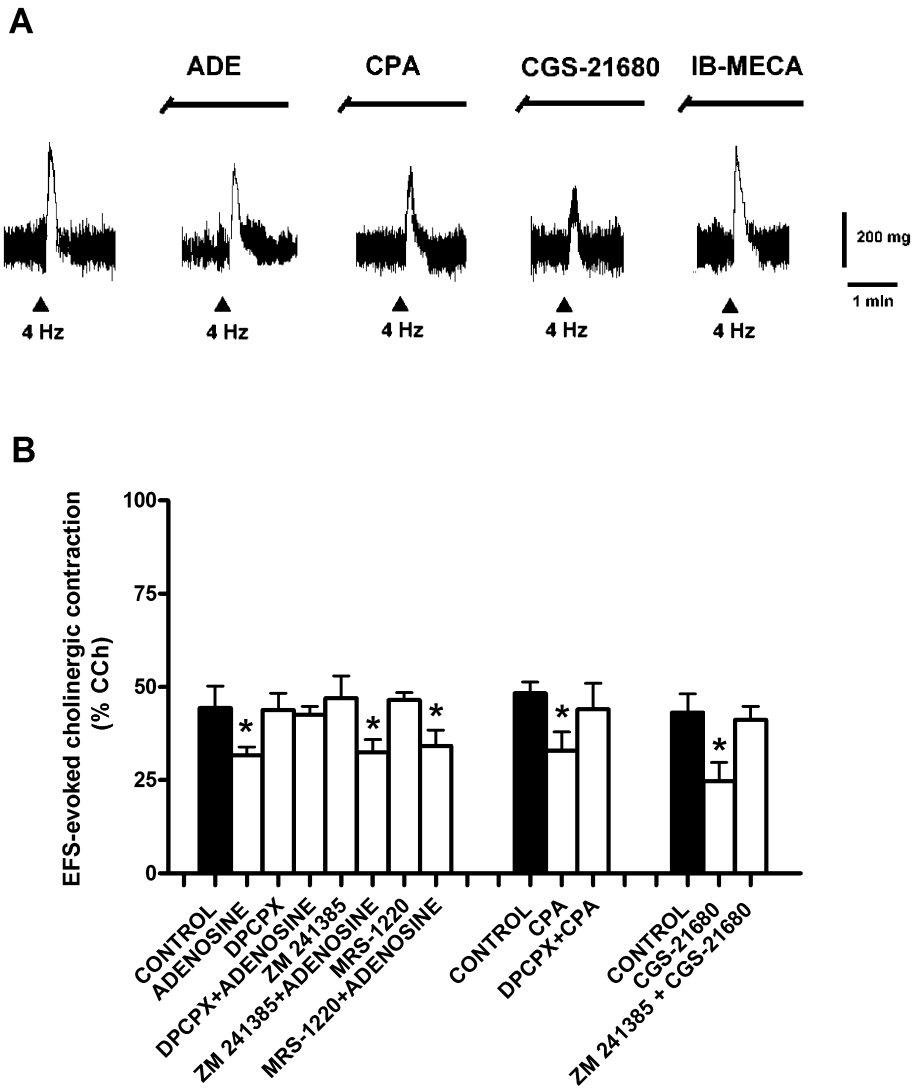

Figure 4

(A) Original tracing showing the effects of adenosine (1 μM) and the A_1, A_{2A} and A_3 receptor agonists, CPA (0.1 μM), CGS-21680 (5 μM) and IB-MECA (3 μM), respectively, on the neurally evoked cholinergic contraction (0.5-ms pulse, 4 Hz, submaximal voltage for 10 s) in the longitudinal muscle of mouse duodenum. (B) Histogram showing the effects induced on the neurally evoked cholinergic contraction by: (i) adenosine (1 μM) alone or in the presence of the A_1, A_{2A} and A_3 receptor antagonists DPCPX (10 nM, $n = 4$), ZM 241385 (10 nM, $n = 4$) and MRS 1220 (0.1 μM, $n = 3$), respectively; (ii) CPA (0.1 μM) alone or in the presence of DPCPX (10 nM, $n = 3$); (iii) CGS-21680 (5 μM) alone or in the presence of ZM 241385 (10 nM, $n = 4$). Data are means ± SEM and are expressed as a percentage of the amplitude of contraction induced by CCh (10 μM). *$P \leq 0.05$ when compared with the respective control.

longitudinal duodenal muscle. The relaxation induced by A_1 receptor activation was not dependent on neuronal action potentials or on NO synthesis, since it was not modified by TTX or L-NAME, indicating that A_1 receptors are probably present at the post-junctional level. Post-junctional inhibitory A_1 receptors have been reported to be present in rat duodenum and ileum (Nicholls *et al.*, 1996; Nicholls and Hourani, 1997), in murine ileum and colon (Zizzo *et al.*, 2006; 2009) and in the human colon (Fornai *et al.*, 2009). On the other hand, the relaxant response induced by adenosine was not modified by selective A_{2A} and A_3 receptor antagonists, *per se*, or in combination with an A_1 receptor antagonist, and it was not mimicked by the selective A_{2A} and A_3 receptor agonists. Thus, in contrast to the results obtained in possum duodenum (Woods *et al.*, 2003), A_{2A} and A_3 receptors are not involved in the modulation of spontaneous activity by exogenous adenosine in mouse duodenum under our experimental conditions.

Exogenously administered adenosine was also able to inhibit the EFS-induced cholinergic contractions. Once more, this effect is mediated via activation of A_1 receptors. Adenosine, and an A_1 receptor agonist, failed to affect the contractions induced by CCh in the presence of TTX, indicating that the sensitivity of the muscle to muscarinic activation is not changed and thus suggesting that the inhibition of EFS-induced cholinergic contractions by

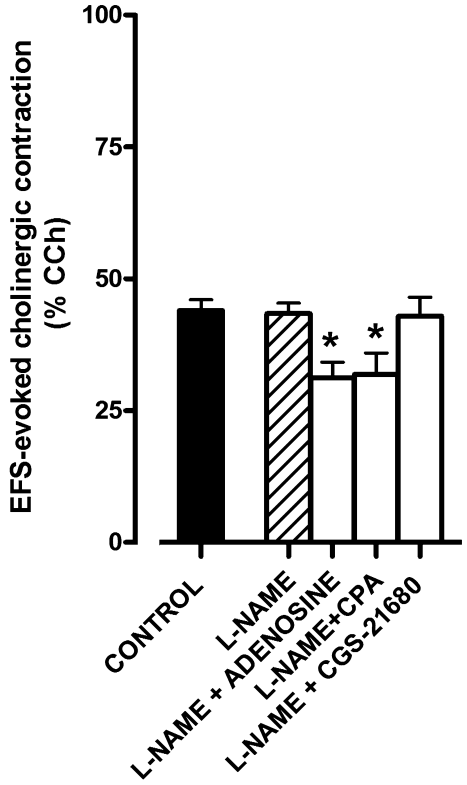

Figure 5

Histogram showing the effects of pretreatment with L-NAME, an NO synthase inhibitor (100 μM, $n = 5$), on the effect induced by adenosine (1 μM), CPA (0.1 μM) or CGS-21680 (5 μM) on the amplitude of cholinergic contractile responses evoked by EFS (0.5 ms pulse, 4 Hz, submaximal voltage for 10 s) in the longitudinal muscle of mouse duodenum. Data are means ± SEM and are expressed as a percentage of the amplitude of contraction induced by CCh (10 μM). The values for the control and for L-NAME are the means of the data obtained before each treatment. *$P \leq 0.05$ when compared with the respective control.

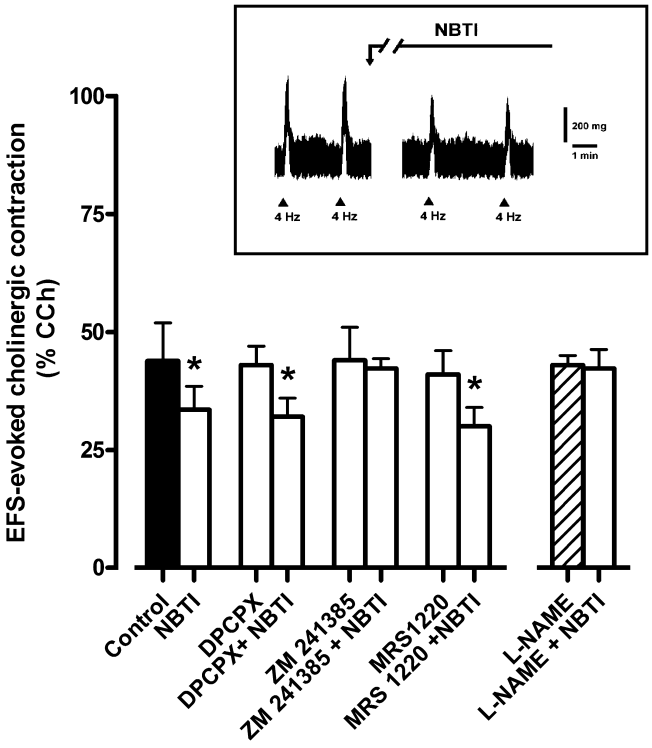

Figure 6

Histogram showing the effects induced by the nucleoside uptake inhibitor, NBTI (10 μM) alone or in the presence of the A_1, A_{2A} and A_3 receptor antagonists, DPCPX (10 nM, $n = 5$), ZM 241385 (10 nM, $n = 3$), MRS 1220 (0.1 mM, $n = 3$), or L-NAME, an NO synthase inhibitor (100 μM, $n = 4$), on the amplitude of cholinergic contractile responses evoked by EFS (0.5-ms pulse, 4 Hz, submaximal voltage for 10 s), in the longitudinal muscle of mouse duodenum. Insert: typical trace showing the effect of NBTI (10 μM) on the amplitude of cholinergic contractile responses evoked by EFS (0.5 ms pulse, 4 Hz, submaximal voltage for 10 s). Data are means ± SEM and are expressed as a percentage of the amplitude of contraction induced by CCh (10 μM). The values for the control and NBTI are the means of the data obtained before each treatment. *$P \leq 0.05$ when compared with the respective control.

adenosine involves a prejunctional mechanism. The observation that A_1 receptor activation is able to reduce EFS-evoked cholinergic contractions even in the presence of L-NAME suggests that A_1 receptors may be localized on excitatory cholinergic neurons, where they reduce neurotransmitter release, as shown in rat ileum as well as in guinea-pig ileum and colon and in mouse ileum (Kadowaki *et al.*, 2000; Lee *et al.*, 2001; Storr *et al.*, 2002; De Man *et al.*, 2003). Although the effects of exogenous adenosine, at the concentrations tested, were not antagonized by an A_{2A} receptor antagonist, activation of A_{2A} receptors inhibited the EFS-induced cholinergic contractions, without affecting the direct muscle cholinergic contraction. This inhibition of the EFS-induced cholinergic contraction by A_{2A} receptor activation was antagonized by an NO synthase inhibitor, indicating that, in mouse duodenum A_{2A} receptors exert their modulation of cholinergic excitatory pathways through the generation of NO. This conclusion is in agreement with the known ability of the nitrergic system to down-regulate ACh release from myenteric neurons through activation of guanylyl cyclase (Hebeiss and Kilbinger, 1996; 1998; Mang *et al.*, 2002), and increments in intracellular calcium concentration, required for nNOS activation, can be triggered by A_{2A} receptors via adenylyl cyclase/cAMP signalling (Fredholm *et al.*, 1994). Moreover, A_{2A} receptor modulation of cholinergic responses by recruitment of intrinsic inhibitory nitrergic nerves have also been demonstrated in rat and human colon (Antonioli *et al.*, 2006; 2011; Fornai *et al.*, 2009). The observation that none of the adenosine receptor antagonists had any effect on the spontaneous activity or on the EFS-induce cholinergic contractions suggests that in mouse duodenum, as reported in ileum and colon (Zizzo *et al.*, 2006; 2009), the endogenous adenosine level in basal conditions may be not sufficient to activate the receptors and thus to be involved in the maintenance of the spontaneous and evoked muscular activity.

Since the levels of adenosine at its receptors are determined by a variety of mechanisms, including cellular adenosine

re-uptake (Noji *et al.*, 2004), in order to increase the extracellular level of adenosine, preparations were treated with NBTI, an adenosine uptake inhibitor. In the presence of NBTI, the cholinergic contractile response to EFS was decreased, whilst the amplitude of the spontaneous activity was unchanged. These effects of NBTI were prevented by antagonism of A_{2A} receptors, but not of A_1 and A_3 receptors and by NO synthase inhibition. Moreover, the direct contractile response to activation of muscarinic Ach receptors was not modified in the presence of NBTI. Thus, an increased level of endogenous adenosine would exert a negative modulation on cholinergic transmission via activation of A_{2A} receptors probably on intrinsic nitrergic neurons. Recently, it has been reported that under conditions of bowel inflammation, recruitment of A_{2A} receptors by an increased production of endogenous adenosine enhances the inhibitory control of colonic motility (Antonioli *et al.*, 2011). Altogether, these findings indicate that nucleoside transporters play a role in maintaining the physiological level of endogenous adenosine limiting its extracellular concentration. Endogenous adenosine seems to preferentially activate only A_{2A} receptors, indicating that, according to the recent concept of 'purinome' (Schwiebert and Fitz, 2008; Volonté and D'ambrosi, 2009), the mechanism of nucleoside inactivation and the regional distribution of adenosine receptor subtypes may provide unique conditions for adenosine to control the excitability of myenteric neurons. The observation that in the presence of NBTI there was not observed any modification of spontaneous activity suggests that adenosine is just released during EFS by enteric interneurons at the synaptic regions to modulate nitrergic interneurons function and in turn cholinergic transmission, but not the functionality of nitrergic motoneurons direct to the muscle.

Lastly, although we also observed the expression of transcripts for A_3 receptors in our preparation, in contrast to findings in the rat distal colon (Antonioli *et al.*, 2010), such receptors do not seem to play any role in the control of intestinal motility in mouse duodenum under physiological conditions. The presence of mRNA for these receptor subtypes is probably because they are present on cells of the vascular and immune system that are also included in muscular preparation (Fredholm *et al.*, 2001). However, the possibility that A_3 receptors could have relevant roles in pathological conditions of the gut, including ischaemia and inflammation, cannot be excluded. Indeed, in inflammatory bowel disease, an increased density of functioning and pharmacologically recruitable A_3 receptors has been reported, suggesting that activation of adenosine A_3 receptors by selective agonists could represent suitable tools for the management of those phases of inflammatory bowel disease characterized by enhanced motor activity of the bowel and diarrhea (Antonioli *et al.*, 2010).

In conclusion, under normal conditions, adenosine can negatively regulate duodenal motility, in mice, either activating A_1 inhibitory receptors located at the post-junctional level or controlling neurotransmitter release via A_1 or A_{2A} receptors located on enteric nerves. In particular, A_1 receptors appear to act via a direct inhibition of enteric cholinergic nerves, whilst A_{2A} receptors exert their modulating function on cholinergic excitatory pathways through the synthesis of NO. Moreover, both receptors are available for pharmacological recruitment, even if only A_{2A} receptors appear to be preferentially stimulated by endogenous adenosine.

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Conflicts of interest

None.

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