Neurogastroenterol Motil (2009) **21,** 451–466 doi: 10.1111/j.1365-2982.2008.01213.x

A_1 and A_{2a} receptors mediate inhibitory effects of adenosine on the motor activity of human colon

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Abstract *Experimental evidence in animal models suggests that adenosine is involved in the regulation of digestive functions. This study examines the influence of adenosine on the contractile activity of human colon. Reverse transcription-polymerase chain reaction revealed A_1 and A_{2a} receptor expression in colonic neuromuscular layers. Circular muscle preparations were connected to isotonic transducers to determine the effects of 8-cyclopentyl-1,3-dipropylxanthine (DPCPX; A_1 receptor antagonist), ZM 241385 (A_{2a} receptor antagonist), CCPA (A_1 receptor agonist) and 2-[(p-2-carboxyethyl)-phenethylamino]-5′-N-ethyl-carboxamide-adenosine (CGS 21680; A_{2a} receptor agonist) on motor responses evoked by electrical stimulation or carbachol. Electrically evoked contractions were enhanced by DPCPX and ZM 241385, and reduced by CCPA and CGS 21680. Similar effects were observed when colonic preparations were incubated with guanethidine (noradrenergic blocker), L-732,138, GR-159897 and SB-218795 (NK receptor antagonists). However, in the presence of guanethidine, NK receptor antagonists and N^{ω}-propyl-L-arginine (NPA; neuronal nitric oxide synthase inhibitor), the effects of DPCPX and CCPA were still evident, while those of ZM 241385 and CGS 21680 no longer occurred. Carbachol-induced contractions were unaffected by A_{2a} receptor ligands, but they were enhanced or reduced by DPCPX and CCPA, respectively. When colonic preparations were incubated with guanethidine, NK antagonists and atropine, electrically induced relaxations were partly reduced by ZM 241385 or NPA, but unaffected by DPCPX. Dipyridamole or application of exogenous adenosine reduced electrically and carbachol-evoked contractions, whereas adenosine deaminase enhanced such motor responses. In conclusion, adenosine exerts an inhibitory control on human colonic motility. A_1 receptors mediate direct modulating actions on smooth muscle, whereas A_{2a} receptors operate through inhibitory nitrergic nerve pathways.*

Keywords *adenosine receptors, enteric nervous system, human colon, intestinal motility, nitric oxide.*

Abbreviations: CCPA, 2-chloro-*N*-6 cyclopentyladenosine; CGS 21680, 2-[(p-2-carboxyethyl)-phenethylamino]-5′-*N*-ethyl-carboxamide-adenosine; dNTP, deoxynucleotide triphosphate mixture; DPCPX, 8-cyclopentyl-1,3-dipropylxanthine; FSCPX, 8-cyclopentyl-3-*N*-[3-((3-(4-fluorosulphonyl)benzoyl)-oxy)-propyl]-1-*N*-propyl-xanthine; GR-159897, (5-fluoro-3-[2-[4-methoxy-4-[[(R)-phenylsulphinyl]methyl]-1-piperidinyl]ethyl]-1H-indole); L-732,138, (*N*-acetyl-L-tryptophan 3,5-bis(trifluoromethyl)benzyl ester); MMLV, Moloney murine leukemia virus; MRS 1334, 1,4-dihydro-2-methyl-6-phenyl-4-(phenylethynyl)-3,5-pyridinedicarboxylic acid 3-ethyl-5-[(3-nitrophenyl)methyl] ester; MRS 1706, *N*-(4-acetylphenyl)-2-[4-(2,3,6,7-tetrahydro-2,6-dioxo-1,3-dipropyl-1*H*-purin-8-yl) phenoxy] acetamide; nNOS, neuronal nitric oxide synthase; NPA, N^{ω}-propyl-L-arginine; RT-PCR, reverse transcription-polymerase chain reaction; SB-218795, ((R)-[[2-phenyl-4-quinolinyl)carbonyl]amino]-methyl ester benzeneacetic acid); SEM, standard error of mean; SNP, sodium nitroprusside; ZM 214385, 4-(2-[7-amino-2-(2-furyl)[1,2,4]triazole[2,3-a][1,3,5]triazin-5-ylamino]ethyl) phenol.

INTRODUCTION

Adenosine is involved in the regulation of several physiological functions in various districts, including

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Received: 21 December 2007
Accepted for publication: 31 August 2008

the gastrointestinal system, and exerts its biological actions through interaction with specific receptor subtypes, designated as A_1, A_{2a}, A_{2b} and A_3.[1–4] These receptors are expressed throughout the gastrointestinal tract, with changes in the localization and density, depending on the species and gut region considered.[5,6] In humans, adenosine receptors have been detected in the enteric nervous system, smooth muscle and epithelial cells of jejunum, ileum and colon, suggesting their involvement in the control of secretory and motor bowel functions.[6,7]

Adenosine participates to the control of intestinal motility, by acting on enteric nervous system[8,9] or smooth muscle.[5,10] In particular, the role played by A_1 and A_{2a} receptors on bowel motor activity has been examined in studies performed on rodents. A_1 receptors have been localized on excitatory nerve endings, where they reduce neurotransmitter release in rat ileum as well as guinea-pig ileum and colon.[5,11,12] A_1 receptors are also expressed on smooth muscle and mediate direct inhibitory effects of adenosine on contractile activity in rat duodenum and ileum.[13,14] In addition, these receptors have been shown to mediate excitatory effects in several gastrointestinal tissues.[15,16] The influence of A_{2a} receptors on rat colonic motility has been investigated by Antonioli *et al.*,[17] who found that these receptors contribute to the modulation of cholinergic contractions through the activation of inhibitory nitrergic pathways. However, Duarte-Araujo *et al.*[18] had previously observed that A_{2a} receptors mediate a prejunctional facilitatory control on acetylcholine release in rat ileum, suggesting that these receptors exert differential regulatory actions depending on the gut region examined.

Current information on the significance of adenosine pathways in the regulation of gut motor functions have been obtained from rodents, while data in humans are lacking. Accordingly, this study was designed to investigate the possible involvement of adenosine in the control of contractile activity of human distal colon, with particular regard for the role of A_1 and A_{2a} receptors. For this purpose, we examined the receptor gene expression as well as the *in vitro* effects of selective A_1 and A_{2a} receptor ligands in preparations of human colonic smooth muscle.

MATERIALS AND METHODS

Tissue excision and preparation

Colonic specimens were obtained from patients undergoing surgery for uncomplicated neoplastic conditions. Samples consisted of sections of distal colon from a macroscopically normal region taken at a distance of at least 10 cm from any visible lesion. Care was taken to verify the absence of alterations by histological examination. Only tissues excised intra-operatively within 60 min from skin incision were employed for subsequent experimental procedures. Portions of tissue were immediately snap-frozen in liquid nitrogen and stored at −80 °C, for subsequent reverse transcription-polymerase chain reaction (RT-PCR) or fixed in cold 4% paraformaldehyde, diluted in phosphate buffered saline, for routine histology. The remaining parts of tissues were placed into pre-oxygenated Krebs solution and transported on ice to laboratory. Circular muscle strips of approximately 3 mm width and 20 mm length were prepared.[19] Informed patient consent was obtained before surgery, and the experimental protocol was approved by the Ethics Committee of our University Hospital.

RT-PCR analysis

Colonic tissues were subjected to mucosa and submucosa removal. Total RNA was isolated from neuromuscular layers by Trizol® (Life Technologies, Carlsbad, CA, USA) and chloroform. One microgram of RNA served as template for single strand cDNA synthesis in a reaction using 2 μL random hexamers (0.5 μg μL^{-1}) with 200 U moloney murine leukemia virus (MMLV)-reverse transcriptase in manufacturer's buffer containing 500 μmol L^{-1} deoxynucleotide triphosphate mixture (dNTP) and 10 mmol L^{-1} dithiothreitol. PCR was performed by specific primers based on the nucleotide sequence of cloned human A_1 and A_{2a} receptor genes.[6] PCR, consisting of 2 μL of RT reaction, Taq polymerase 2.5 U, dNTP 100 μmol L^{-1} and primers 0.5 μmol L^{-1} in a final volume of 50 μL, was performed by a T-gradient thermocycler (Biometra, Goettingen, DE, USA). After 1 min of initial denaturation at 94 °C, PCR was performed by 35 cycles of: denaturation at 94 °C (1 min); annealing at 53 °C (30 s); extension at 72 °C (1 min); final extension at 72 °C for 7 min. If no band was detected, PCR was repeated on 2 μL of the first amplification product with fresh PCR reagents, as previously reported by Christofi *et al.*[6] Untranscribed RNA was included in PCR reactions to verify the absence of genomic DNA. RT-PCR efficiency was evaluated by primers for human β-actin. Amplified products were separated by 1.5% agarose gel electrophoresis and stained with ethidium bromide. cDNA bands were visualized by UV light.

Recording of muscle contractile activity

The contractile activity of colonic circular smooth muscle was recorded as previously described by Fornai *et al.*[19] Preparations were set up in organ baths containing Krebs solution at 37 °C bubbled with 95% O_2 + 5% CO_2, connected to isotonic transducers (Basile, Comerio, Italy) under a constant load of 1 g, and allowed to equilibrate for at least 30 min. Krebs solution had the following composition (mmol L^{-1}): NaCl 113, KCl 4.7, $CaCl_2$ 2.5, KH_2PO_4 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 25, glucose 11.5 (pH 7.4 ± 0.1). Circular muscle activity was recorded by polygraphs (Basile). Field electrical stimulation was delivered by a BM-ST6 stimulator (Biomedica Mangoni, Pisa, Italy). Stimuli were applied as 10-s single trains of square wave pulses (0.5 ms, 30 mA) at frequencies ranging from 1 to 30 Hz. The interval between successive periods of electrical stimulation was about 30 min. Each preparation was challenged with electrical stimulations, and experiments started when reproducible responses were obtained (usually after two to three stimulations).

Preliminary experiments were performed to select the appropriate frequency of electrical stimulation and carbachol concentration that elicited submaximal contractions, suitable to appreciate the stimulant or inhibitory effects of adenosine receptor ligands. For this purpose, colonic preparations were challenged with electrical stimuli at increasing frequencies, ranging from 1 to 30 Hz. Frequency–response curves were constructed under the different experimental conditions adopted in the study to test adenosine receptor ligands: (i) standard Krebs solution; (ii) Krebs solution added with guanethidine and NK receptor antagonists; (iii) Krebs solution containing guanethidine, NK receptor antagonists and N^{ω}-propyl-L-arginine (NPA). Concentration–response curves to carbachol were constructed at concentrations ranging from 0.001 to 100 μmol L^{-1} in the presence of tetrodotoxin (1 μmol L^{-1}).

Design of experiments on adenosine receptor agonists and antagonists

In the first set of experiments, the effects of 8-cyclopentyl-1,3-dipropylxanthine (DPCPX; A_1 receptor antagonist), ZM 241385 (A_{2a} receptor antagonist), 2-chloro-*N*-6 cyclopentyladenosine (CCPA; A_1 receptor agonist) and 2-[(p-2-carboxyethyl)-phenethylamino]-5′-*N*-ethyl-carboxamide-adenosine (CGS 21680; A_{2a} receptor agonist) were assayed on electrically evoked contractions of colonic preparations maintained in standard Krebs solution. Concentrations of adenosine receptor agonists and antagonists were selected on the basis of concentration–response curves to electrical stimulation. To verify that DPCPX-induced effects resulted specifically from A_1 receptor blockade, the selective and irreversible A_1 receptor antagonist 8-cyclopentyl-3-*N*-[3-(|3-(4-fluorosulphonyl)benzoyl)-oxy)-propyl]-1-*N*-propyl-xanthine (FSCPX; 1 μmol L^{-1})[20] was tested on electrically evoked contractions, either alone or in combination with DPCPX. To verify that ZM 241385 acted specifically on A_{2a} receptors, its effects were assayed under blockade of A_1, A_{2b} and A_3 receptors by incubation of colonic preparations with selective antagonists [FSCPX 1 μmol L^{-1} for A_1, *N*-(4-acetylphenyl)-2-[4-(2,3,6,7-tetrahydro-2,6-dioxo-1,3-dipropyl-1*H*-purin-8-yl) phenoxy] acetamide (MRS 1706) 0.01 μmol L^{-1} for A_{2b}, and 1,4-dihydro-2-methyl-6-phenyl-4-(phenylethynyl)-3,5-pyridinedicarboxylic acid 3-ethyl-5-[(3-nitrophenyl)methyl] ester (MRS 1334) 0.001 μmol L^{-1} for A_3 receptors]. The concentrations of these antagonists were selected on the basis of previous studies and were reported to be devoid of significant effects on other receptor subtypes.[20–22] Additional subset of experiments were performed to verify the receptor specificity of agonists at the selected concentrations. For this purpose, the effects of CCPA and CGS 21680 were tested either alone or in the presence of A_1 or A_{2a} receptor blockade with DPCPX or ZM 241385. Based on these experiments, the concentrations of 0.01 μmol L^{-1} for DPCPX, 4-(2-[7-amino-2-(2-furyl)[1,2,4]triazole[2,3-a][1,3,5]triazin-5-ylamino]ethyl) phenol (ZM 214385) and CGS 21680, and 0.1 μmol L^{-1} for CCPA, were adopted for subsequent experiments aimed at examining the pathways involved in the effects mediated by A_1 and A_{2a} receptors.

In the second series of experiments, the effects of adenosine receptor antagonists and agonists were evaluated on electrically induced contractions elicited under blockade of noradrenergic and tachykininergic pathways. For this purpose, colonic preparations were maintained in Krebs solution containing guanethidine (adrenergic blocker, 10 μmol L^{-1}), *N*-acetyl-L-tryptophan 3,5-bis(trifluoromethyl)benzyl ester (L-732,138; NK_1 receptor antagonist, 10 μmol L^{-1}), 5-fluoro-3-[2-[4-methoxy-4-[[(R)-phenylsulphinyl]methyl]-1-piperidinyl]ethyl]-1H-indole (GR-159897; NK_2 receptor antagonist, 1 μmol L^{-1}) and (R)-[[2-phenyl-4-quinolinyl) carbonyl]amino]-methyl ester benzeneacetic acid (SB-218795; NK_3 receptor antagonist, 1 μmol L^{-1}).

The third set of experiments was designed to assay adenosine receptor ligands on motor responses elicited by electrical stimulation delivered predominantly to excitatory cholinergic innervation. Accordingly,

colonic preparations were maintained in Krebs solution containing guanethidine (10 μmol L^{-1}), NPA [selective inhibitor of neuronal nitric oxide (NO) synthase (nNOS); 0.1 μmol L^{-1}], L-732,138 (10 μmol L^{-1}), GR-159897 (1 μmol L^{-1}) and SB-218795 (1 μmol L^{-1}), to prevent non-cholinergic responses.

In the fourth series of experiments, adenosine receptor ligands were assayed on cholinergic responses of colonic preparations evoked by direct activation of muscarinic receptors on smooth muscle cells. For this purpose, colonic preparations were maintained in Krebs solution containing tetrodotoxin (1 μmol L^{-1}), and they were stimulated with carbachol (muscarinic receptor agonist, 1 μmol L^{-1}).

In the fifth set of experiments, adenosine receptor antagonists were tested on electrically evoked relaxations obtained by incubation of colonic tissues in Krebs solution containing guanethidine (10 μmol L^{-1}), L-732,138 (10 μmol L^{-1}), GR-159897 (1 μmol L^{-1}), SB-218795 (1 μmol L^{-1}) and atropine (1 μmol L^{-1}).

An additional set of experiments was performed to evaluate the effects of A_{2a} receptor stimulation on relaxant responses evoked by exposure of smooth muscle cells to a pharmacological source of NO. For this purpose, colonic specimens were maintained in Krebs solution containing tetrodotoxin, and the effects of CGS 21680 were evaluated on relaxations induced by the NO donor sodium nitroprusside (SNP, 1 μmol L^{-1}).

In all experiments designed to assess the effects of adenosine receptor agonists, Krebs solution was added with dipyridamole (adenosine reuptake inhibitor, 0.5 μmol L^{-1}) and adenosine deaminase (the enzyme responsible for adenosine conversion into inosine, 0.5 U mL^{-1}) to abate extracellular levels of endogenous adenosine.[17] Under these conditions, the effects of adenosine receptor antagonists no longer occurred, thus confirming that the levels of endogenous adenosine were markedly reduced. Adenosine receptor antagonists were added to the bathing fluid 20 min before the application of electrical stimuli or carbachol, whereas agonists were applied 10 min before stimulations to avoid receptor desensitization. To verify whether the *ex-vivo* experimental procedures and use of A_1 and A_{2a} receptor agonists affected the receptor sensitivity, in preliminary experiments CCPA or CGS 21680 were applied to colonic preparations at high concentrations for 10 min and, after appropriate intervening washings, the same treatment was repeated every 30 min for 4–5 h. Under these conditions, no appreciable variations in the responses of colonic preparations to the agonists were detected, suggesting that our *ex-vivo* tissue handling and experimental timing was not associated with receptor desensitization.

Design of experiments on dipyridamole, adenosine deaminase and exogenous adenosine

A group of experiments was performed to evaluate the effects of dipyridamole and adenosine deaminase on the motor activity of colonic preparations. To pursue this goal, dipyridamole (0.5 μmol L^{-1}) and adenosine deaminase (0.5 U mL^{-1}) were tested, either alone or in combination, on contractions elicited by electrical stimulation (standard Krebs solution) or carbachol (Krebs solution added with tetrodotoxin 1 μmol L^{-1}). In a further set of experiments, exogenous adenosine was tested, either alone or in the presence of DPCPX or ZM 241385, on colonic contractions evoked by electrical stimulation (standard Krebs solution) or carbachol (in the presence of tetrodotoxin 1 μmol L^{-1}).

Drugs and reagents

Atropine sulphate, guanethidine monosulphate, carbachol chloride, TRIzol®, L-arginine, SNP, dipyridamole, adenosine, adenosine deaminase (Sigma Chemicals Co., St Louis, MO, USA). Tetrodotoxin, DPCPX, FSCPX, MRS 2179, ZM 241385, MRS 1706, MRS 1334, CCPA, CGS 21680, L-732,138, GR-159897, SB-218795, NPA (Tocris, Bristol, UK). Random hexamers, MMLV-reverse transcriptase, *Taq* polymerase, dNTP mixture, dithiothreitol (Promega, Madison, WI, USA). Adenosine receptor ligands were dissolved in dimethylsulphoxide, and further dilutions were made with saline solution. Adenosine was dissolved in saline solution. Dimethylsulphoxide concentration in organ bath never exceeded 0.5%.

Statistical analysis

Results are given as mean ± standard error of mean. The significance of differences was evaluated on raw data, prior to percentage normalization, by Student's *t*-test for paired data or analysis of variance (ANOVA) for repeated measures, followed by *post hoc* analysis with Dunnett test or Student–Newman–Keuls test, as appropriate. $P < 0.05$ was considered significant. Colonic preparations included in each test group were obtained from distinct patients, and therefore the number of experiments refers also to the number of patients assigned to each group. Calculations were performed by commercial software (GraphPad Prism™, version 3.0 from GraphPad Software Inc., San Diego, CA, USA).

RESULTS

RT-PCR analysis

PCR amplification of cDNA coding for A_1 and A_{2a} receptors was performed under similar conditions to those adopted by Christofi *et al.*,[6] although a less stringent annealing temperature was allowed. After the first run of amplification, A_{2a} receptor cDNA was detected as a faint band in all colonic specimens examined ($n = 6$), while no cDNA bands could be visualized for A_1 receptor. Therefore, PCR products were subjected to a second run of amplification under the same conditions, and DNA bands of the expected size for both A_1 and A_{2a} receptors could be detected following electrophoretic separation (Fig. 1). The identity of DNA bands was confirmed by sequencing analysis.

Effects of adenosine agonists and antagonists on colonic motor activity

During equilibration, most colonic preparations displayed rapid spontaneous activity, which was low in amplitude and generally stable throughout the experiment. Electrically evoked responses consisted of phasic contractions followed, in some cases, by after-contractions of variable amplitude. Atropine abolished phasic contractions, or converted them into relaxations, and only after-contractions became evident (not shown). Tetrodotoxin abolished electrically induced responses (not shown). Frequency–response curves, obtained under different experimental conditions, allowed to select the frequency of 10 Hz, which elicited submaximal contractions suitable for further increments by adenosine receptor antagonists (Fig. 2A). Accordingly, all the subsequent experiments, designed to test the effects of adenosine receptor ligands on electrically evoked contractions, were performed at the frequency of 10 Hz.

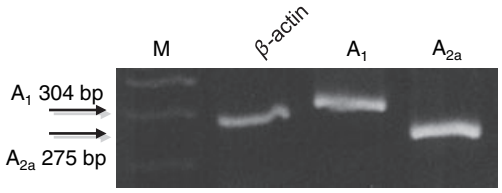

Figure 1 Reverse transcription-polymerase chain reaction analysis of adenosine A_1, A_{2a} and β-actin mRNA in the muscular layer of human distal colon. Representative agarose gel showing the amplification of adenosine A_1, A_{2a} and β-actin cDNAs. M, size markers.

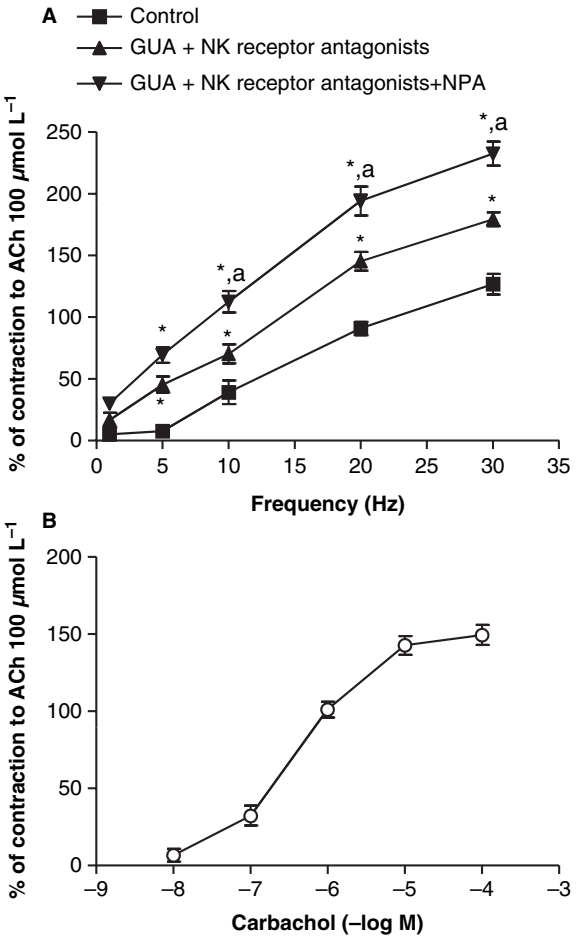

Figure 2 Preparations of colonic circular smooth muscle. (A) Effects of electrical stimulation (1–30 Hz) on the motor activity of smooth muscle maintained in standard Krebs solution (control), in the presence of guanethidine (GUA, 1 μmol L^{-1}) and NK receptor antagonists (*N*-acetyl-l-tryptophan 3,5-bis(trifluoromethyl)benzyl ester 10 μmol L^{-1}, 5-fluoro-3-[2-[4-methoxy-4-[[(R)-phenylsulphinyl]methyl]-1-piperidinyl]ethyl]-1H-indole (GR-159897; 1 μmol L^{-1}) and (R)-[[2-phenyl-4-quinolinyl]carbonyl]amino]-methyl ester benzeneacetic acid (SB-218795; 1 μmol L^{-1}) or in the presence of guanethidine, NK receptor antagonists and NPA (0.1 μmol L^{-1}). (B) Effects of increasing concentrations of carbachol (0.01–100 μmol L^{-1}) on the motor activity of smooth muscle maintained in Krebs solution containing tetrodotoxin (1 μmol L^{-1}). Each point represents the mean ± standard error of mean value obtained from 6–7 experiments. $^*P < 0.05$: significant difference *vs* control. $^aP < 0.05$: significant difference *vs* GUA+NK receptor antagonists.

In tissues maintained in standard Krebs solution, DPCPX (0.001–10 μmol L^{-1}) concentration-dependently increased electrically evoked contractions and a maximal increment of +75% was obtained at 1 μmol L^{-1} (Fig. 3). The irreversible blockade of A_1 receptors with FSCPX (1 μmol L^{-1}) resulted in an

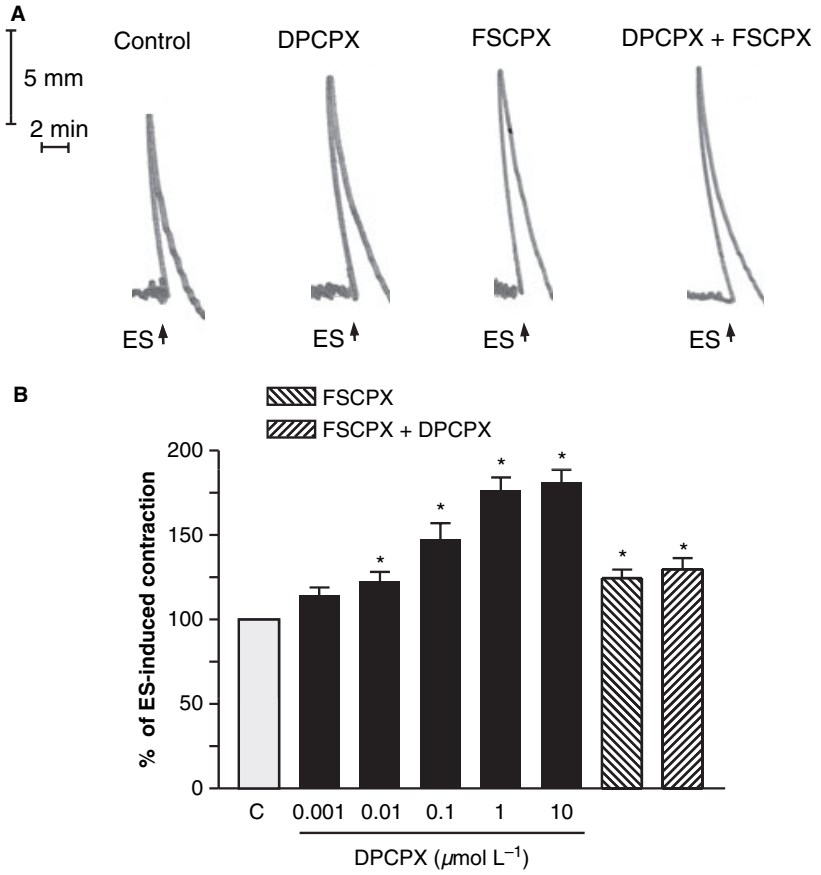

Figure 3 Preparations of colonic circular smooth muscle maintained in standard Krebs solution. (A) Representative tracings showing the effects of 8-cyclopentyl-1,3-dipropylxanthine (DPCPX; 0.01 μmol L^{-1}), 8-cyclopentyl-3-*N*-[3-((3-(4-fluorosulphonyl)benzoyl)-oxy)-propyl]-1-*N*-propyl-xanthine (FSCPX; 1 μmol L^{-1}) or DPCPX plus FSCPX on contractile responses of smooth muscle induced by electrical stimulation (ES, 10 Hz). (B) Effects of increasing concentrations of DPCPX (0.001–10 μmol L^{-1}), FSCPX (1 μmol L^{-1}) or FSCPX plus DPCPX (0.01 μmol L^{-1}) on contractile responses to electrical stimulation. Each column represents the mean ± standard error of mean value obtained from seven to eight experiments. $^*P < 0.05$: significant difference *vs* control.

increment of electrically evoked contractions, which was similar to that observed with DPCPX 0.01 μmol L^{-1} (+27% *vs* +22%, respectively). In addition, the enhancing effect of DPCPX 0.01 μmol L^{-1} was not modified by co-incubation with FSCPX 1 μmol L^{-1}, indicating that DPCPX 0.01 μmol L^{-1} was sufficient to ensure a specific and maximal blockade of A_1 receptors (Fig. 3). ZM 241385 (0.001–10 μmol L^{-1}) elicited also concentration-dependent increments of electrically evoked contractions, with a maximal effect of +58% observed at 1 μmol L^{-1} (Fig. 4A). The enhancing effect of ZM 241385 0.01 μmol L^{-1} (+48%) was not affected by preincubation of colonic preparations with the A_1, A_{2b} and A_3 antagonists FSCPX, MRS 1706 and MRS 1334 (+53%), suggesting that at this concentration ZM 241385 acted via selective inhibition of A_{2a} receptors (Fig. 4B). When colonic preparations were preincubated with ZM 241385 0.01 μmol L^{-1}, DPCPX 1 μmol L^{-1} increased further the electrically evoked contractions by 27% (Fig. 4C), suggesting that the enhancing effects exerted by DPCPX at high concentrations depend on a concomitant blockade of A_1 and A_{2a} receptors.

In colonic preparations maintained in Krebs solution containing dipyridamole and adenosine deaminase, the electrically evoked contractions were concentration dependently reduced by the A_1 receptor agonist CCPA or the A_{2a} receptor agonist CGS 21680 (Fig. 5A). The inhibitory effect exerted by CCPA (0.1 μmol L^{-1}) was blocked by DPCPX (0.01 μmol L^{-1}), but not ZM 241385 (0.01 μmol L^{-1}), whereas the inhibitory effect obtained with CGS 21680 (0.01 μmol L^{-1}) was prevented by ZM 241385 (0.01 μmol L^{-1}), but not DPCPX (0.01 μmol L^{-1}) (Fig. 5B).

When experiments were performed in the presence of Krebs solution added with guanethidine and NK receptor antagonists, electrical stimulation evoked phasic contractions which were prevented by atropine and, in most cases, they were converted into NPA-sensitive relaxations (not shown). Under these conditions, A_1 and A_{2a} receptor antagonists induced significant increments of electrically evoked contractions, which were similar to those recorded when colonic preparations were not exposed to guanethidine and NK receptor antagonists (+25% and +61%, respectively; Fig. 6A, B). In addition, both the A_1 and A_{2a}

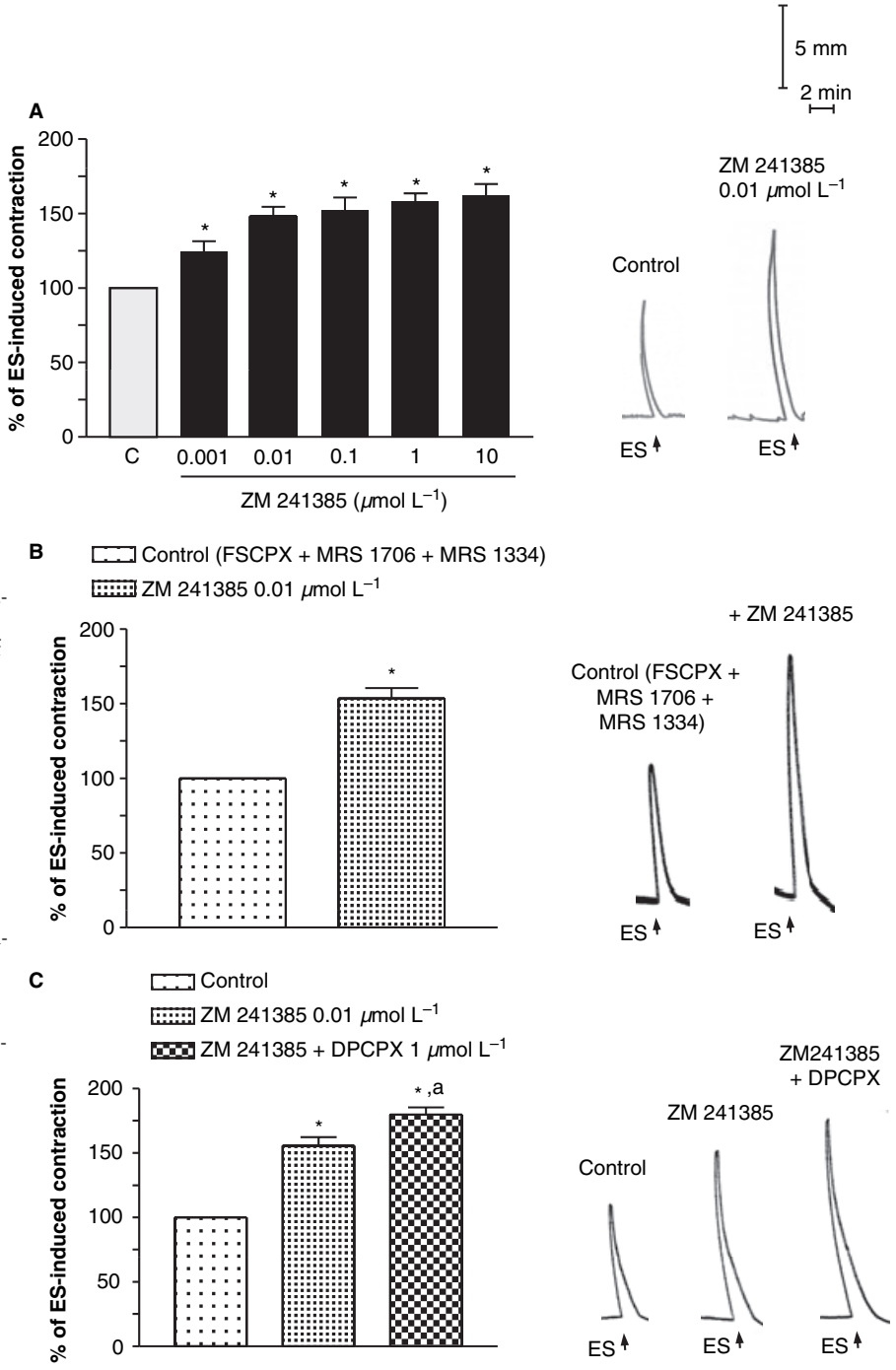

Figure 4 Preparations of colonic circular smooth muscle maintained in standard Krebs solution. (A) Effects of increasing concentrations of ZM 241385 (0.001–10 μmol L^{-1}) on contractions induced by electrical stimulation (ES, 10 Hz). (B) Effects of ZM 241385 (0.01 μmol L^{-1}) on contractile responses to ES in the presence of 8-cyclopentyl-3-*N*-[3-[(3-(4-fluorosulphonyl)benzoyl)-oxy)-propyl]-1-*N*-propyl-xanthine (FSCPX; 1 μmol L^{-1}), *N*-(4-acetylphenyl)-2-[4-(2,3,6,7-tetrahydro-2,6-dioxo-1,3-dipropyl-1H-purin-8-yl) phenoxy] acetamide (MRS 1706; 0.01 μmol L^{-1}) and 1,4-dihydro-2-methyl-6-phenyl-4-(phenylethynyl)-3,5-pyridinedicarboxylic acid 3-ethyl-5-[(3-nitrophenyl)methyl] ester (MRS 1334; 0.01 μmol L^{-1}). (C) Effects of ZM 241385 (0.01 μmol L^{-1}), alone or in combination with 8-cyclopentyl-1,3-dipropylxanthine (DPCPX; 1 μmol L^{-1}), on electrically evoked contractions. Each column represents the mean ± standard error of mean value obtained from six to seven experiments. $^{*}P < 0.05$: significant difference *vs* control; $^{a}P < 0.05$: significant difference *vs* ZM 241385.

receptor agonist decreased the motor responses induced by electrical stimuli (–23% and –49%, respectively; Fig. 6C, D).

In colonic preparations maintained in Krebs solution containing guanethidine, NK receptor antagonists and NPA, to prevent non-cholinergic responses,[19] electrical stimulation evoked phasic contractions which were abolished or markedly reduced by atropine (not shown). In this setting, DPCPX was still able to increase the contractile activity induced by electrical stimulation (+26%), whereas the enhancing effect of ZM 241385 no longer occurred (+7%) (Fig. 7A, B). The application of CCPA 0.1 μmol L^{-1} to the bathing fluid significantly reduced the electrically evoked contractions (–24%), whereas CGS 21680 was without effects (–2%) (Fig. 7C, D).

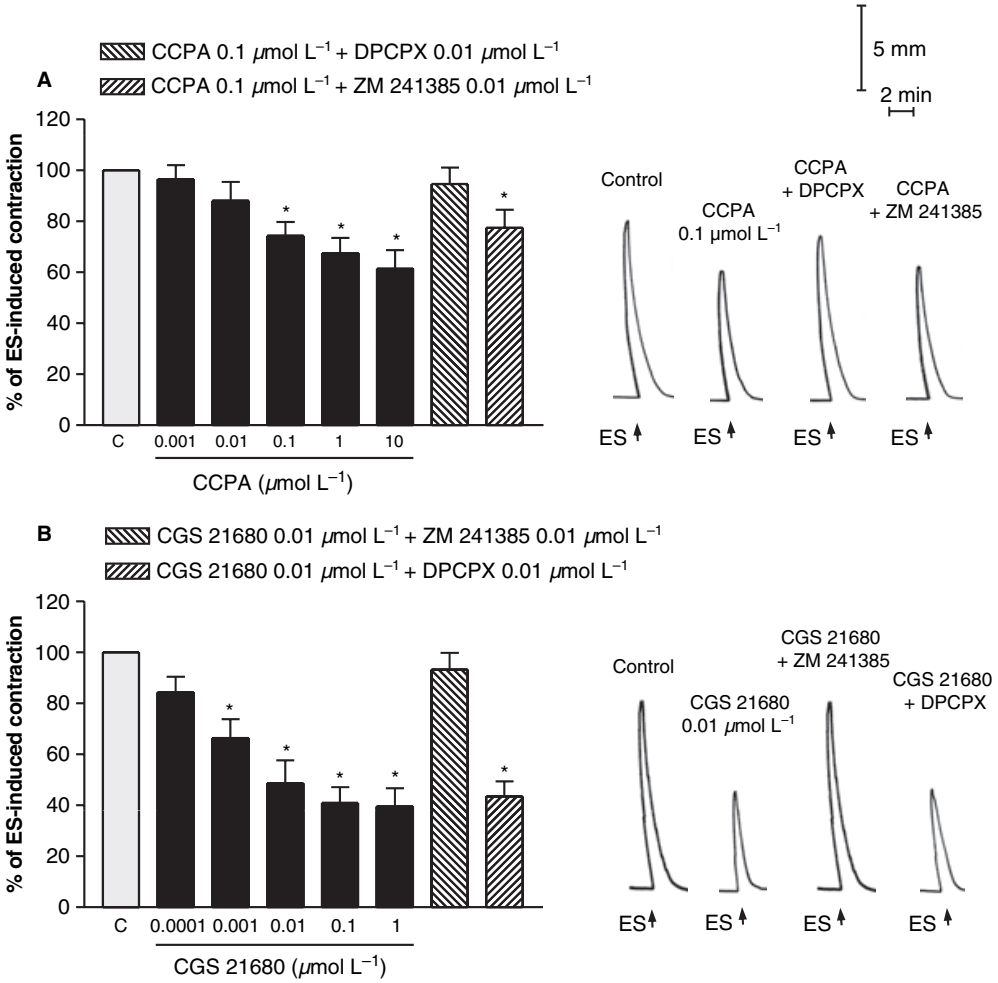

Figure 5 Preparations of colonic circular smooth muscle maintained in standard Krebs solution. (A) Effects of increasing concentrations of 2-chloro-*N*-6 cyclopentyladenosine (CCPA; 0.001–10 μmol L^{-1}), CCPA (0.1 μmol L^{-1}) plus 8-cyclopentyl-1,3-dipropylxanthine (DPCPX; 0.01 μmol L^{-1}) or CCPA (0.1 μmol L^{-1}) plus ZM 241385 (0.01 μmol L^{-1}) on contractions induced by electrical stimulation (ES, 10 Hz). (B) Effects of increasing concentrations of 2-[(p-2-carboxyethyl)-phenethylamino]-5′-*N*-ethyl-carboxamide-adenosine (CGS 21680; 0.0001–1 μmol L^{-1}), CGS 21680 (0.01 μmol L^{-1}) plus 4-(2-[7-amino-2-(2-furyl)[1,2,4]triazole[2,3-a][1,3,5]triazin-5-ylamino]ethyl) phenol (ZM 214385; 0.01 μmol L^{-1}) or CGS 21680 (0.01 μmol L^{-1}) plus DPCPX (0.01 μmol L^{-1}) on contractions induced by ES. Each column represents the mean ± standard error of mean value obtained from 8–10 experiments. $^*P < 0.05$; significant difference *vs* control.

The exposure of colonic preparations to increasing concentrations of carbachol (0.01–100 μmol L^{-1}), in the presence of tetrodotoxin, resulted in phasic contractions which were prevented by atropine (not shown). The construction of concentration–response curves allowed to select the submaximal concentration of 1 μmol L^{-1} for subsequent experiments with adenosine receptor ligands (Fig. 2B). The A_1 receptor antagonist significantly enhanced carbachol-induced contractions (+27%), whereas the A_{2a} receptor antagonist was without effects (Fig. 8A, B). When assaying the effects of agonists, the contractile responses induced by carbachol were significantly reduced by CCPA (–26%), but they were not affected by CGS 21680 (Fig. 8C, D).

In colonic tissues maintained in Krebs solution containing guanethidine, NK receptor antagonists and atropine, the application of electrical stimuli induced relaxant responses which were significantly reduced, but not abolished, by NPA, and were unaffected in the presence of NPA plus L-arginine (Fig. 9B). The residual NPA-insensitive relaxant responses to electrical stimulation were almost completely abolished by incubation with the $P2Y_1$ receptor antagonist MRS 2179 (data not shown), indicating a significant involvement of adenosine triphosphate (ATP). Under these

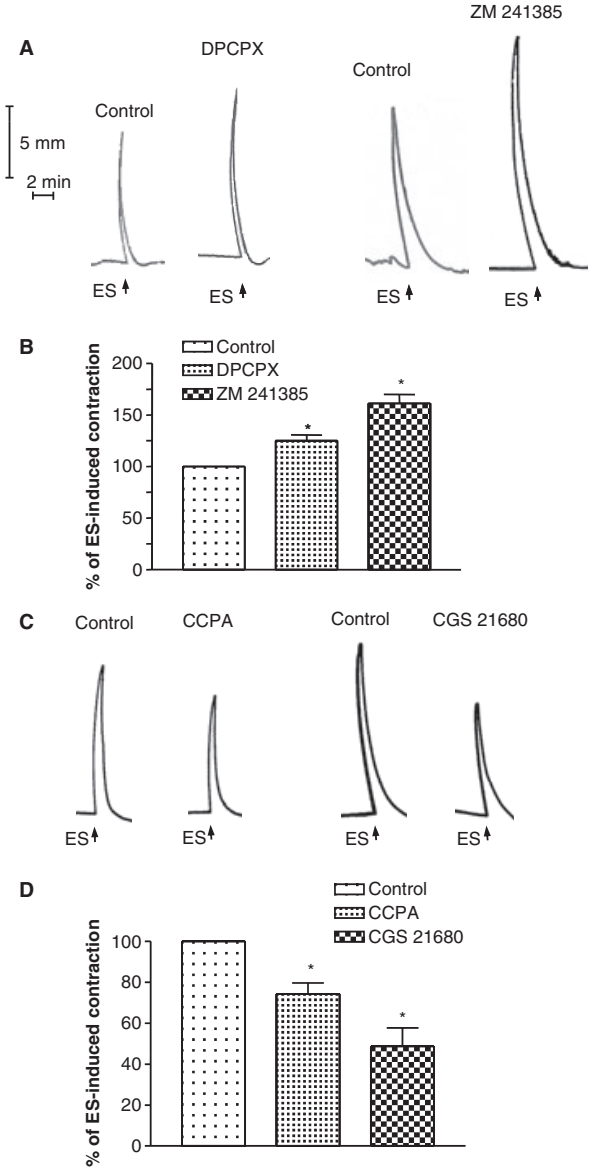

Figure 6 Preparations of colonic circular smooth muscle maintained in Krebs solution containing guanethidine and NK receptor antagonists. (A) Representative trace recordings showing the contractions induced by electrical stimulation (ES, 10 Hz), either in the absence (control) or in the presence of 8-cyclopentyl-1,3-dipropylxanthine (DPCPX; 0.01 μmol L^{-1}) or ZM 241385 (0.01 μmol L^{-1}). (B) Effects of DPCPX (0.01 μmol L^{-1}) or ZM 241385 (0.01 μmol L^{-1}) on contractions induced by ES. (C) Representative trace recordings showing contractions induced by ES, either in the absence (control) or in the presence of 2-chloro-*N*-6 cyclopentyladenosine (CCPA) (0.1 μmol L^{-1}) or 2-[(p-2-carboxyethyl)-phenethylamino]-5′-*N*-ethyl-carboxamide-adenosine (CGS 21680; 0.01 μmol L^{-1}). (D) Effects of CCPA (0.1 μmol L^{-1}) or CGS 21680 (0.01 μmol L^{-1}) on contractions induced by ES. Each column represents the mean ± standard error of mean value obtained from seven to nine experiments. *$P < 0.05$; significant difference *vs* control.

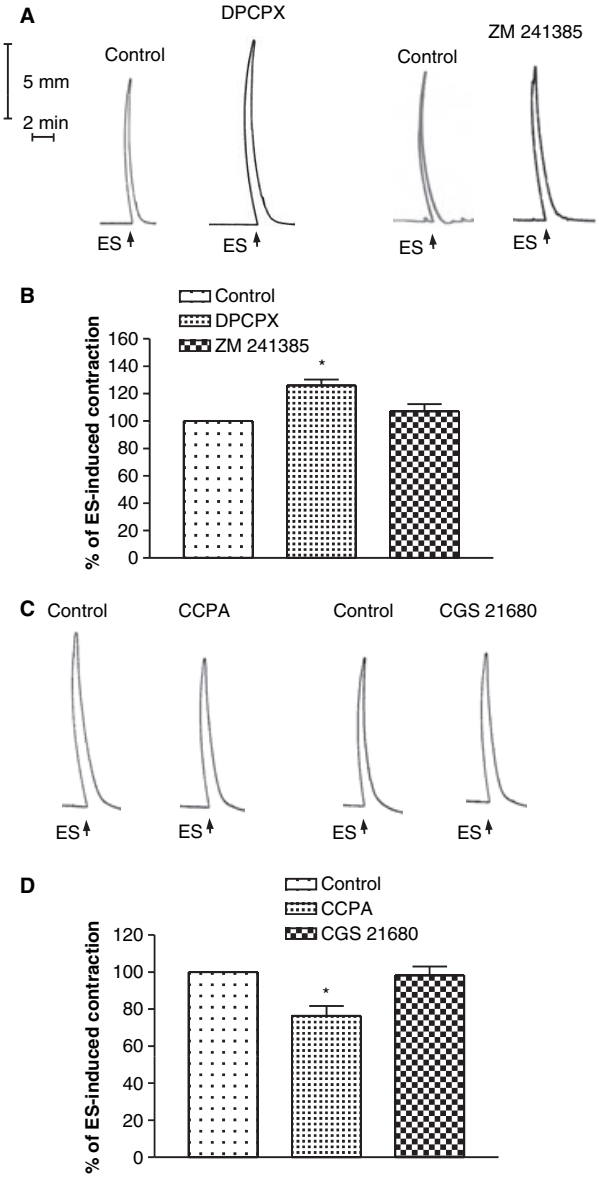

Figure 7 Preparations of colonic circular smooth muscle maintained in Krebs solution containing guanethidine, NK receptor antagonists, and NPA. (A) Representative trace recordings showing the effects of 8-cyclopentyl-1,3-dipropylxanthine (DPCPX; 0.01 μmol L^{-1}) or ZM 241385 (0.01 μmol L^{-1}) on contractions evoked by electrical stimulation (ES, 10 Hz). (B) Effects of DPCPX (0.01 μmol L^{-1}) or ZM 241385 (0.01 μmol L^{-1}) on contractions induced by ES. (C) Representative trace recordings showing the contractions induced by ES, either in the absence (control) or in the presence of 2-chloro-*N*-6 cyclopentyladenosine (CCPA; 0.1 μmol L^{-1}) or 2-[(p-2-carboxyethyl)-phenethylamino]-5′-*N*-ethyl-carboxamide-adenosine (CGS 21680; 0.01 μmol L^{-1}). (D) Effects of CCPA (0.1 μmol L^{-1}) or CGS 21680 (0.01 μmol L^{-1}) on contractions induced by ES. Each column represents the mean ± standard error of mean value obtained from 9–10 experiments. *$P < 0.05$; significant difference *vs* control.

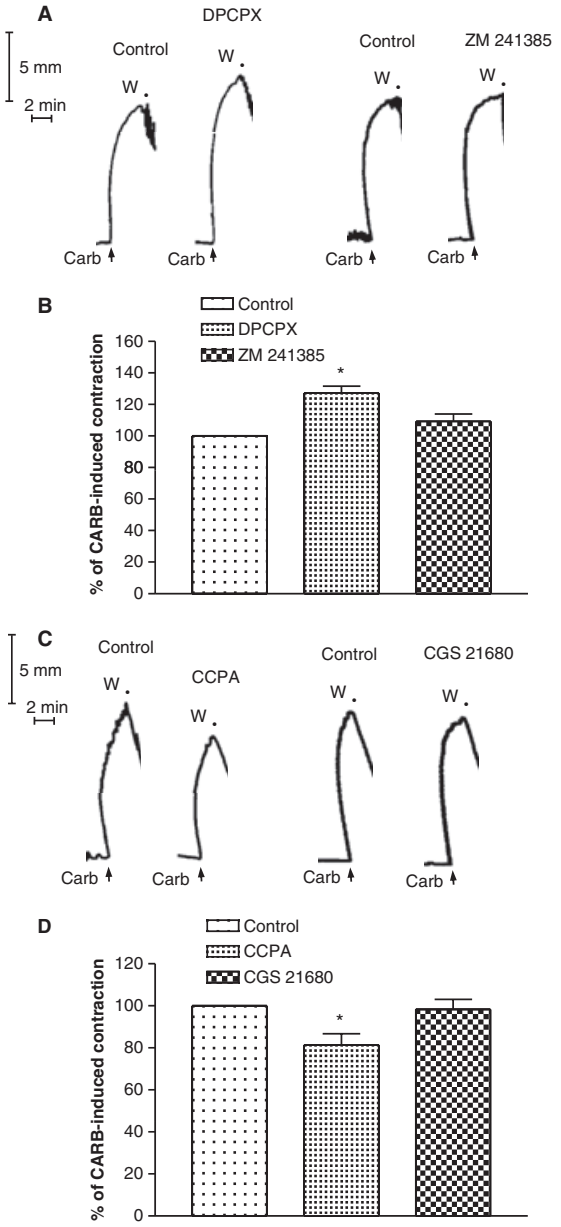

Figure 8 Preparations of colonic circular smooth muscle maintained in Krebs solution containing tetrodotoxin. (A) Representative trace recordings showing the effects of 8-cyclopentyl-1,3-dipropylxanthine (DPCPX; 0.01 μmol L^{-1}) or ZM 241385 (0.01 μmol L^{-1}) on contractions evoked by carbachol (CARB, 1 μmol L^{-1}). (B) Effects of DPCPX (0.01 μmol L^{-1}) or ZM 241385 (0.01 μmol L^{-1}) on contractile contractions evoked by CARB. (C) Representative trace recordings showing the effects of 2-chloro-*N*-6 cyclopentyladenosine (CCPA) (0.1 μmol L^{-1}) or 2-[(p-2-carboxyethyl)-phenethylamino]-5′-*N*-ethyl-carboxamide-adenosine (CGS 21680; 0.01 μmol L^{-1}) on contractions evoked by CARB. (D) Effects of CCPA (0.1 μmol L^{-1}) or CGS 21680 (0.01 μmol L^{-1}) on CARB-evoked contractions. Each column represents the mean ± standard error of mean value obtained from seven to eight experiments. *$P < 0.05$; significant difference *vs* control. W, washing.

conditions, incubation of colonic tissues with DPCPX did not affect electrically evoked relaxations, either alone or in combination with NPA or NPA plus L-arginine (Fig. 9A). By contrast, the application of ZM 241385 to colonic preparations induced a significant reduction of electrically evoked relaxant responses. In the presence of NPA, the A_{2a} receptor antagonist did not further reduce the relaxations induced by electrical stimulation (Fig. 9B, C). In colonic tissues maintained in Krebs solution containing tetrodotoxin, SNP evoked relaxant responses, which were not affected by the A_{2a} receptor agonist CGS 21680 (not shown).

Effects of dipyridamole, adenosine deaminase and exogenous adenosine on colonic motor activity

Under incubation in standard Krebs solution, electrically evoked contractions were significantly decreased by dipyridamole (–60%), whereas they were enhanced by adenosine deaminase, alone or in combination with dipyridamole (+70% and +85%, respectively) (Fig. 10A). Carbachol-evoked contractions (in the presence of tetrodotoxin) were reduced by dipyridamole (–36%). By contrast, adenosine deaminase, alone or in combination with dipyridamole, enhanced the contractile activity elicited by carbachol (+24% and +28%, respectively (Fig. 10B).

The application of adenosine (0.1–1000 μmol L^{-1}) to colonic preparations, maintained in standard Krebs solution, evoked concentration-dependent inhibitions of electrically induced contractions (Fig. 11A). Under these conditions, incubation of colonic preparations with DPCPX (0.01 μmol L^{-1}) or ZM 241385 (0.01 μmol L^{-1}) counteracted the inhibitory effects of adenosine (Fig. 11A). Exogenous adenosine (0.1–1000 μmol L^{-1}) concentration-dependently reduced also carbachol-evoked contractions (in the presence of tetrodotoxin) (Fig. 11B). In this setting, DPCPX (0.01 μmol L^{-1}), but not ZM 241385 (0.01 μmol L^{-1}), counteracted the inhibitory effects exerted by adenosine (Fig. 11B).

DISCUSSION

The ability of adenosine to modulate gut motor activity has been recognized as the end of 1920s.[2,3] Following these initial observations, several studies were performed to characterize the expression and function of adenosine receptors in the digestive tract of various animal species.[1,5,6,17] The expression and locations of adenosine receptors throughout the human gastrointestinal tract have been extensively documented by Christofi *et al.*,[6] but no data concerning

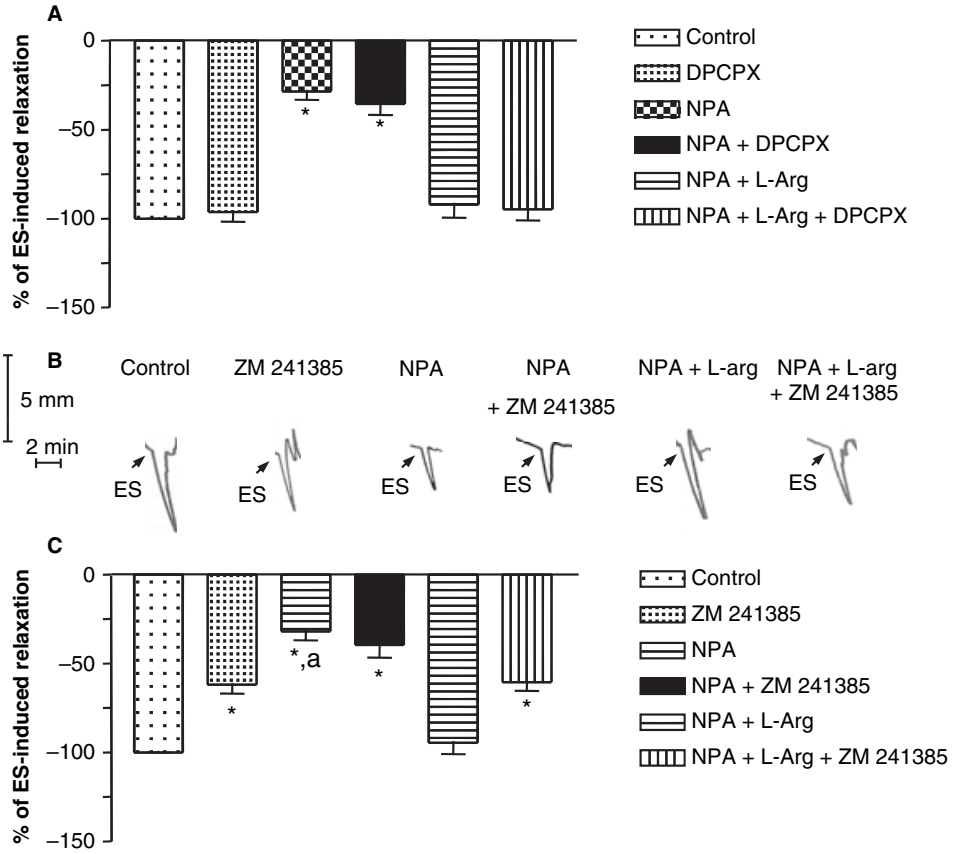

Figure 9 Preparations of colonic circular smooth muscle maintained in Krebs solution containing guanethidine, NK receptor antagonists and atropine. (A) Effects of 8-cyclopentyl-1,3-dipropylxanthine (DPCPX; 0.01 μmol L^{-1}), NPA (0.1 μmol L^{-1}), NPA plus DPCPX, NPA plus L-arginine (L-arg, 100 μmol L^{-1}), and NPA plus L-arginine plus DPCPX on relaxations evoked by electrical stimulation (ES, 10 Hz). (B) Representative trace recordings showing the effects of ZM 241385 (0.01 μmol L^{-1}), NPA (0.1 μmol L^{-1}), NPA plus ZM 241385, NPA plus L-arginine (L-arg, 100 μmol L^{-1}) and NPA plus L-arginine plus ZM 241385 on ES-evoked relaxations. (C) Effects of ZM 241385 (0.01 μmol L^{-1}), NPA (0.1 μmol L^{-1}), NPA plus ZM 241385, NPA plus L-arginine (L-arg, 100 μmol L^{-1}), and NPA plus L-arginine plus ZM 241385 on ES-evoked relaxations. Each column represents the mean ± standard error of mean value obtained from 8–10 experiments. $^{*}P < 0.05$; significant difference *vs* control; $^{a}P < 0.05$; significant difference *vs* ZM 241385 alone.

the influence of these receptors on human gut motility are currently available. In the present study, we have attempted to characterize the possible involvement of adenosine in the control of neuromuscular functions in distal regions of normal human colon. Evidence was indeed obtained that adenosine participates to the inhibitory modulation of colonic motility via activation of A_1 and A_{2a} receptors.

Prior to perform experiments on colonic contractions, care was taken to verify the expression of A_1 and A_{2a} receptors in the experimental model, and the presence of specific mRNAs coding for both receptors could be detected in circular muscle preparations of human colon by means of RT-PCR assays. These results corroborate previous observations on the presence of A_1 and A_{2a} receptors in the human colon, where A_1 receptors were found to be expressed in circular muscle, while A_{2a} receptors were detected in both circular muscle and myenteric plexus.[6]

The application of A_1 and A_{2a} receptor antagonists to colonic preparations concentration-dependently increased electrically evoked contractions of circular smooth muscle. Moreover, the incubation of colonic tissues with A_1 and A_{2a} receptor agonists reduced the amplitude of motor responses to electrical stimuli in a concentration-dependent fashion, and these effects were reversed by A_1 and A_{2a} receptor antagonists, suggesting that both receptors are implicated in the modulating actions of adenosine deputed to down-regulate the contractile activity of human distal colon driven by excitatory pathways. Of note, A_1 receptors influence the motor responses of human colonic tissue

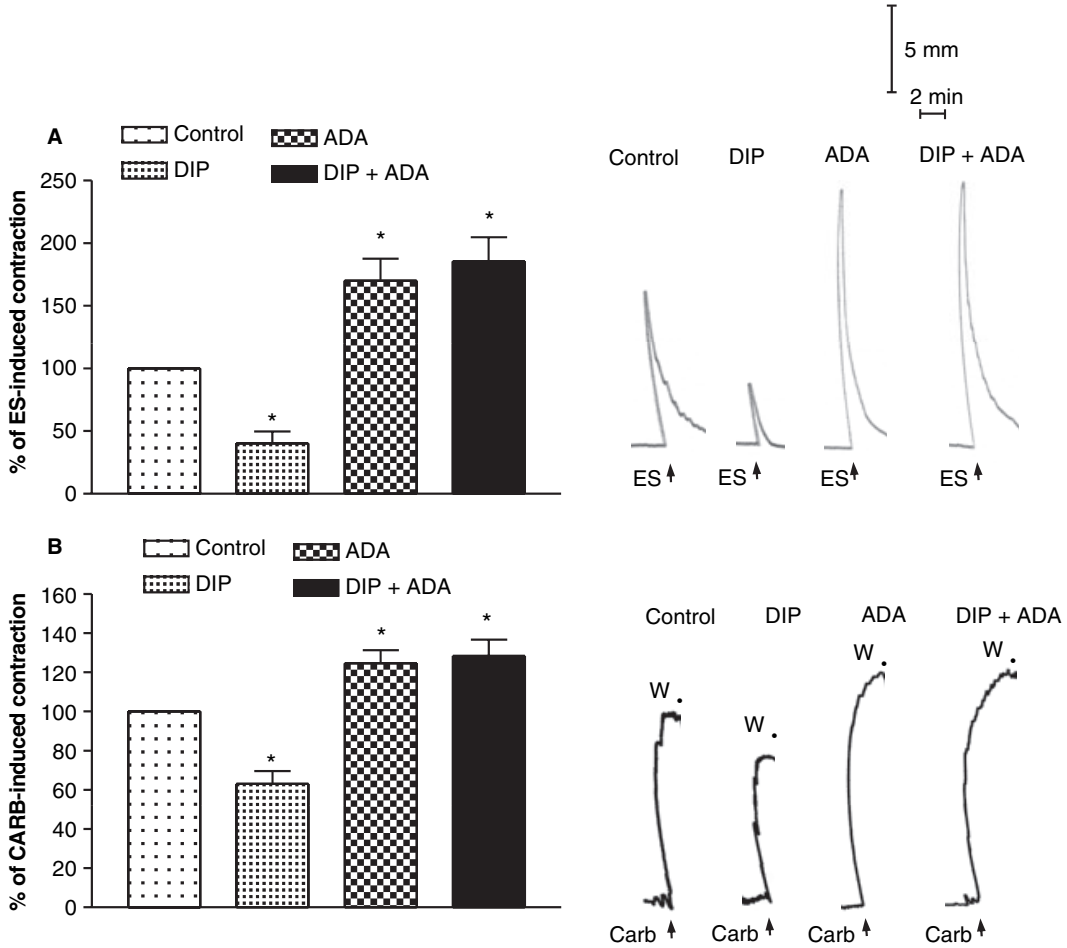

Figure 10 Preparations of colonic circular smooth muscle maintained in standard Krebs solution. (A) Effects of dipyridamole (DIP, 0.5 μmol L^{-1}), adenosine deaminase (ADA, 0.5 U mL^{-1}), and DIP plus ADA on contractions induced by electrical stimulation (ES, 10 Hz). (B) Effects of DIP, ADA or DIP plus ADA on contractions evoked by carbachol (CARB, 1 μmol L^{-1}) in the presence of tetrototoxin (1 μmol L^{-1}). Each column represents the mean ± standard error of mean value obtained from six to seven experiments. *$P < 0.05$: significant difference *vs* control.

with lower efficiency than A_{2a} receptors. In this regard, evidence from previous reports[18,24,25] indicates a great variability in the magnitude of inhibitory effects mediated by A_1 receptors on intestinal motility, which appear to depend not only on the species, but also on the gut region under examination. Thus, it is conceivable that the inhibitory responses mediated by A_1 receptors in human colon differ considerably from those observed in rodents, and that, according to our findings, the human colonic circular smooth muscle is predominantly modulated by A_{2a} receptors.

The application of adenosine deaminase to colonic preparations significantly enhanced the contractile responses, either alone or in combination with dipyridamole, thus confirming that, under our experimental conditions, extracellular adenosine exerted an inhibitory control on colonic motor activity. The observation that dipyridamole reduced the evoked colonic contractions suggests that nucleoside transporters play a relevant role in maintaining physiological levels of endogenous extracellular adenosine. Moreover, exogenous adenosine evoked DPCPX- and ZM 241385-sensitive inhibitory effects on colonic motor responses in the presence of standard medium, indicating that, although being tested against a background of endogenous adenosine, exogenously applied adenosine was still able to modulate the contractile activity of human colon.

The colonic tissues were studied under different conditions in an attempt to gain insight into the pathways modulated by adenosine and the underlying mechanisms. These experiments showed that the effects of adenosine receptor ligands could be appreciable also after blockade of noradrenergic and

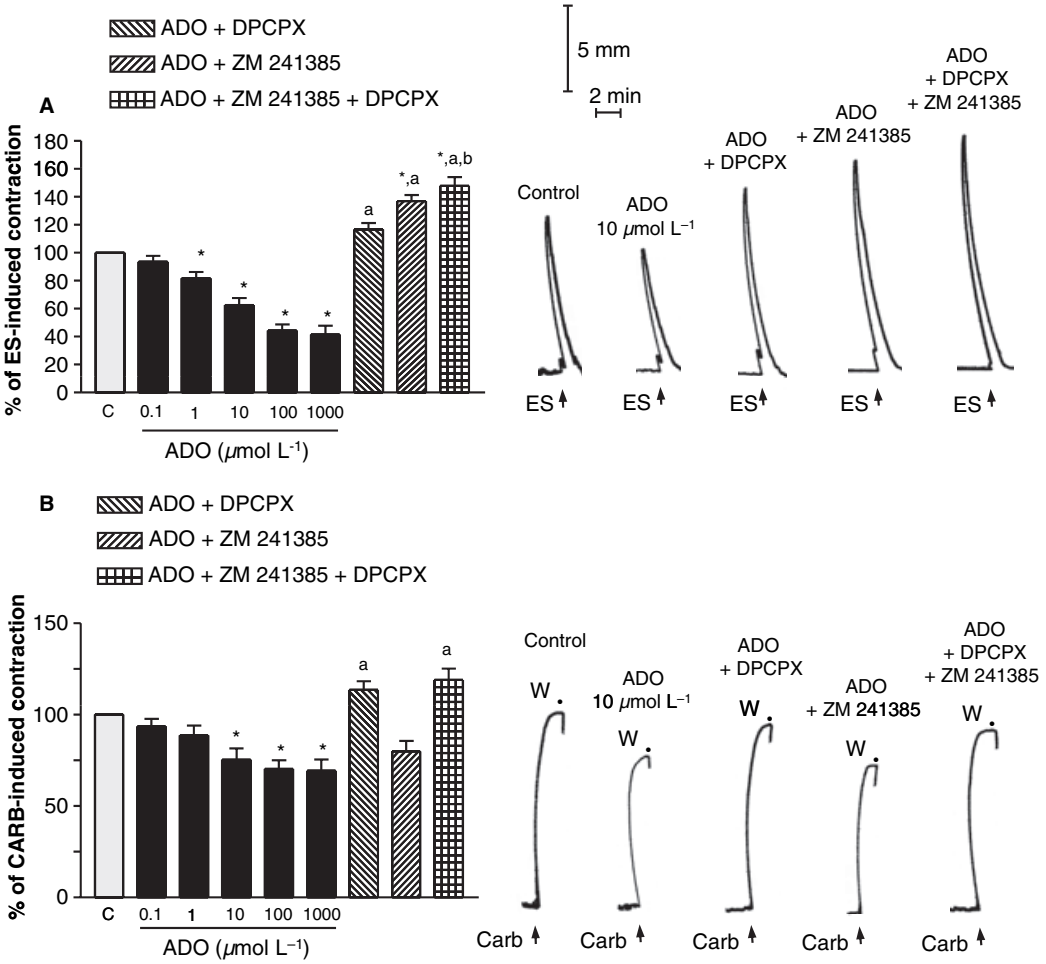

Figure 11 Preparations of colonic circular smooth muscle maintained in standard Krebs solution. Effects of increasing concentrations of adenosine (ADO, 0.1–1000 μmol L^{-1}) alone or in combination with 8-cyclopentyl-1,3-dipropylxanthine (DPCPX; 0.01 μmol L^{-1}), ZM 241385 (0.01 μmol L^{-1}), or DPCPX plus ZM 241385 on contractions induced by ES (A) or carbachol (CARB, 1 μmol L^{-1}) in the presence of tetrodotoxin (1 μmol L^{-1}) (B). Each column represents the mean ± standard error of mean value obtained from seven to eight experiments. $^{*}P < 0.05$: significant difference *vs* control; $^{a}P < 0.05$: significant difference *vs* ADO 10 μmol L^{-1}; $^{b}P < 0.05$: significant difference *vs* ADO 10 μmol L^{-1} plus DPCPX.

tachykininergic pathways. Moreover, when in concomitance with noradrenergic and tachykininergic ablation, the colonic preparations were subjected to blockade of nNOS, the effects exerted by A_1 receptor ligands were still evident, but the modulating actions mediated by A_{2a} receptors no longer occurred. It was also considered that adenosine ligands might have affected the evoked motor activity of human colon at neuronal and/or muscular sites, and therefore their effects were tested in the presence of direct activation of muscarinic receptors on smooth muscle by carbachol. Under carbachol stimulation, the contractile responses were enhanced by the A_1 receptor antagonist, but not affected by A_{2a} receptor blockade. In addition, the A_1, but not A_{2a}, receptor agonist significantly reduced the carbachol-evoked contractions. Taken together, these findings support the concept that A_1 and A_{2a} receptors exert their inhibitory control on human colonic motility through different mechanisms: A_1 receptors appear to act directly on colonic smooth muscle, where they reduce the contractile responses evoked by cholinergic receptor activation; A_{2a} receptors exert their modulating function at level of intrinsic neural pathways, where they are likely to stimulate the activity of inhibitory nitrergic neurons.

To the best of our knowledge, this is the first study providing a functional characterization of adenosine functions in human colon. Some lines of evidence concur with the present results to suggest that

adenosine regulates the myenteric neurotransmission and smooth muscle activity in normal digestive tissues, with different mechanisms and functional consequences depending on the gut region and species examined. Of note, Christofi *et al.*[6] observed that A_1 receptors are localized in the circular muscle layer, but not in myenteric ganglia, of human colon. These observations are consistent with our findings and strengthen the proposal that the functions mediated by A_1 receptors in human colon are unlikely to occur at neuronal level. In line with this view, previous studies have demonstrated that A_1 receptors exert direct inhibitory actions on the contractile activity of smooth muscle in rat duodenum and ileum.[13,14] However, other authors obtained evidence that these receptor subtypes mediate modulating actions of adenosine on cholinergic pathways at neuronal level in guinea-pig and rat ileum.[11,18]

Our observation that A_{2a} receptors act at neuronal level to modulate the colonic contractile activity is in agreement with the neuronal localization of these receptors described by Christofi *et al.*[6] in the human colon, while the lack of influence by A_{2a} receptors on carbachol-induced motor responses is in apparent discordance with the observation that these receptors are also expressed in the circular smooth muscle of human colon, as shown by Christofi *et al.*[6] However, our experiments with carbachol stimulation were designed to gain information on the contractile activity driven by muscular cholinergic receptors, and therefore the possibility that muscular A_{2a} receptors might mediate regulatory functions unrelated to the muscarinic receptor pathway can not be ruled out.

In this study, the hypothesis that A_{2a} receptors inhibit the contractile activity of human colon via stimulation of nitrergic neurons was subjected to further verification in a series of specific experiments, where relaxing responses were induced by electrical stimulation of inhibitory nerves in the presence of pharmacological blockade of cholinergic, tachykininergic and noradrenergic pathways. The results showed that these evoked relaxations were partly reduced by inhibition of nNOS, and that the residual NPA-insensitive relaxant responses were largely prevented by blockade of $P2Y_1$ receptors, indicating a contribution by both NO and ATP release. In line with this picture, blunting effects of NO blockers on electrically evoked relaxations as well as evidence of ATP-dependent relaxant activity in human colon have been previously reported.[26–29] In our experimental setting, the non-adrenergic non-cholinergic relaxations elicited by electrical stimulation were not affected by A_1 receptor blockade, while they were partly reduced by ZM 241385, thus confirming that A_{2a} receptors modulate the motility of human colon by recruitment of intrinsic inhibitory nerves. In addition, two lines of experimental evidence allowed us to conclude that A_{2a} receptors mediate an excitatory control on neuronal nitrergic pathways: (i) upon blockade of nNOS by NPA, the electrical stimulation evoked blunted, ATP-dependent relaxations which were not reduced further by A_{2a} receptor blockade; (ii) colonic relaxations elicited by SNP were insensitive to A_{2a} receptor activation by CGS 21680. Of note, these observations are in full agreement with the results of a previous study on isolated rat colon, in which A_{2a} receptors were shown to mediate inhibitory actions on colonic neuromuscular functions through activation of neuronal nitrergic pathways.[17]

In recent years, increasing attention is being paid to the role played by the adenosine system in the pathophysiology of intestinal inflammatory conditions.[30] Several lines of evidence suggest that the pharmacological modulation of adenosine receptors and enzymes driving adenosine catabolism can favorably affect the outcome of intestinal inflammation.[31–33] Moreover, adenosine receptors seem to be implicated in motor alterations associated with bowel inflammation, as previous evidence suggests that A_1 receptors are involved in the development of postoperative ileus,[34] and that the control of A_{2a} receptors on colonic motility is enhanced in the presence of experimental colitis in rats.[17] In this respect, the present findings could help to better understand the pathophysiological role of adenosine in the regulation of intestinal motor activity in the presence of inflammatory bowel disorders.

Although the present study was focused on high affinity A_1 and A_{2a} receptors, the possible involvement of low-affinity A_{2b} and A_3 receptors in the control of human colonic motility deserves some discussion. As observed by Christofi *et al.*[35], A_{2b} receptors are located in myenteric plexus and circular muscle, suggesting possible implications of these receptors in the regulation of human colonic motility. By contrast, no conclusive evidence is currently available on the expression of A_3 receptors in the neuromuscular compartment of human colon. In preliminary experiments, we have been able to detect mRNA coding for A_3 receptors in neuromuscular tissue from human colon as well as to verify that the blockade of A_{2b} and A_3 receptors marginally affects the evoked colonic motility (M. Fornai, L. Antonioli, R. Colucci, N. Ghisu, P. Buccianti, C. Blandizzi, M. Del Tacca, unpublished data). Although these preliminary

findings encourage more extensive investigations, it appears that low-affinity A_{2b} and A_3 receptors do not contribute significantly to the control of human colonic neuromuscular functions, while they could play relevant roles in the presence of gut pathological conditions, including ischemia and inflammation.[30,35]

In conclusion, the present results confirm that both adenosine A_1 and A_{2a} receptors are expressed in the neuromuscular compartment of human distal colon, and provide the first functional evidence that adenosine, acting at both muscular and neuronal sites, contributes to regulatory networks deputed to the inhibitory modulation of colonic motility. These findings can provide a basis to interpret the physiological roles played by adenosine in the control of gut motility under either normal or pathological conditions.

ACKNOWLEDGMENT AND DISCLOSURES

This work was supported by grants from the Italian Ministry of Education, University and Research (no. 2003052707_002) and University of Pisa.

COMPETING INTERESTS

The authors have no competing interests.

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