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Inhibitory responses to exogenous adenosine in murine proximal and distal colon

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1 The aims of the present study were firstly, to characterize pharmacologically the subtypes of P_1 purinoreceptors involved in the inhibitory effects induced by exogenous adenosine in longitudinal smooth muscle of mouse colon, and secondly, to examine differences in the function and distribution of these receptors between proximal and distal colon.

2 Adenosine (100 μM–3 mM) caused a concentration-dependent reduction of the amplitude of spontaneous contractions in the proximal colon, and muscular relaxation in the distal colon. In the proximal colon, adenosine effects were antagonized by a selective A_1 receptor antagonist, 1,3-dipropyl-8-cyclopentylxanthine (DPCPX, 10 nM), but were not modified by 3,7-dimethyl-1-propargylxanthine (DMPX, 10 μM) or by 9-chloro-2-(2-furanyl)-5-((phenylacetyl)amino)- [1,2,4]triazolo[1,5-c]quinazoline (MRS 1220, 0.1 μM), selective A_2 and A_3 receptor antagonists, respectively. In the distal colon, adenosine effects were antagonized by DPCPX, DMPX, and by a selective A_{2B} receptor antagonist, 8-[4-[((4-cyanophenyl)carbamoylmethyl)oxy]phenyl]-1,3-di(*n*-propyl) xanthine (MRS 1754, 10 μM), but not by 8-(3-chlorostyryl)-caffeine (CSC, 10 μM), a selective A_{2A} receptor antagonist, or by MRS 1220.

3 Tetrodotoxin (TTX 1 μM), the nitric oxide (NO) synthase inhibitor, N_ω-nitro-L-arginine methyl ester (L-NAME, 100 μM), or 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (10 μM), an inhibitor of soluble guanylyl cyclase, reduced adenosine effects only in distal colon. In addition, L-NAME induced a further reduction of adenosine relaxation in the presence of DPCPX, but not in the presence of MRS 1754.

4 From these results we conclude that, in the murine proximal colon, adenosine induces inhibitory effects *via* TTX-insensitive activation of A_1 receptor. In the distal colon, adenosine activates both A_1 and A_{2B} receptors, the latter located on enteric inhibitory neurons releasing NO.
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Abbreviations: CSC, 8-(3-chlorostyryl)-caffeine; DMPX, 3,7-dimethyl-1-propargylxanthine; DPCPX, 1,3-dipropyl-8-cyclopentylxanthine; L-NAME, N_ω-nitro-L-arginine methyl ester; MRS 1754, 8-[4-[((4-cyanophenyl) carbamoylmethyl) oxy] phenyl]-1,3-di (*n*-propyl)xanthine; MRS 1220, 9-chloro-2-(2-furanyl)-5-((phenylacetyl)amino)-[1,2,4]triazolo [1,5-c]quinazoline; NO, nitric oxide; ODQ, 1H-[1,2,4] oxadiazolo [4,3-a]quinoxalin-1-one; PPADS, 4-[[4-formyl-5-hydroxy-6-methyl-3-[(phosphonooxy) methyl]-2-pyridinyl]azo]-1,3-benzenedisulfonic acid; SNP, sodium nitroprusside; TTX, tetrodotoxin

Introduction

Adenosine 5′-triphosphate (ATP) and related purine derivatives are known to act as neurotransmitters or neuromodulators in the central and peripheral nervous systems (Burnstock, 1997). ATP released from enteric nerves has been reported to mediate inhibition of muscular activity in different regions of the gastrointestinal tract from various animal species *via* activation of P_2 receptors, mainly the P2Y family (Koh *et al*., 1997; Zagorodnyuk & Maggi, 1998; Xue *et al*., 1999; De Man *et al*., 2003a; Mulè & Serio, 2003; Serio *et al*., 2003b; Van Crombruggen & Lefebvre, 2004; Mulè *et al*., 2005). Adenosine, formed by the breakdown of ATP by ectoenzymes or released by enteric nerves, can influence gastrointestinal motility directly, by activating receptors located in smooth muscle (Serio *et al*., 1990; Nicholls *et al*., 1996; Kadowaki *et al*., 2000; Woods *et al*., 2003), or indirectly, by regulating the neurotransmitter release from enteric neurons (Tomaru *et al*., 1995; Moneta *et al*., 1997; Lee *et al*., 2001; Storr *et al*., 2002).

Adenosine receptors are designated as P_1 receptors and further subdivided in A_1, A_2 (A_{2A} and A_{2B}) and A_3 receptors by the relative order of potency of agonists and antagonists and by the coupled transduction mechanisms (Collis & Hourani, 1993; Ralevic & Burnstock, 1998). Their localization and function vary depending on the intestinal region or animal species. A_1 receptors have been localized on excitatory nerve endings, reducing neurotransmitter release, in rat ileum and in guinea-pig ileum and distal colon (Kadowaki *et al*., 2000; Lee *et al*., 2001; Storr *et al*., 2002), and on smooth muscle, inducing direct muscular inhibition, in rat duodenum and ileum (Nicholls *et al*., 1996; Nicholls & Hourani, 1997). Moreover, A_1 receptors have been shown to mediate also adenosine

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excitatory effects in several tissues (Bailey *et al*., 1992; Murthy *et al*., 1995; Shim *et al*., 2002). Postsynaptic inhibitory A_2 receptors have been observed in rat colon (Bailey & Hourani, 1992), guinea-pig distal colon (Kadowaki *et al*., 2000) and possum duodenum (Woods *et al*., 2003). Lastly, postsynaptic inhibitory A_3 receptors have been found in the possum duodenum (Woods *et al*., 2003).

Mice are becoming increasingly important subjects for investigating gastrointestinal motility because of the availability of mutants and the advent of gene-targeting technology. However, so far a full description of the functions of purines, and in particular of adenosine, in the mouse gastrointestinal tract is not yet available. Giaroni *et al*. (2002) reported that adenosine induced relaxation in all regions of the mouse gastrointestinal tract, suggesting the presence of inhibitory P_1 receptors throughout the tract. In addition, postjunctional A_1 receptors appear to be present in mouse jejunum (De Man *et al*., 2003a), and neuronal A_1 receptors modulate cholinergic nerve activity in mouse ileum (De Man *et al*., 2003b). So far, there are no studies concerning the subtypes of adenosine receptors in mouse colon.

The aims of our study were to characterize the subtypes of P_1 purinoreceptors, A_1, A_2 (A_{2A} and A_{2B}) or A_3 receptors, involved in the inhibitory effects induced by adenosine in the longitudinal smooth muscle of mouse colon, and to examine differences in the function and distribution of the receptors between proximal and distal colon. In addition, possible interactions with the nitric oxide (NO) system, the main inhibitory system in murine intestine, have been evaluated.

Methods

Animals

All animal procedures complied with the regulations of the Ministero della Sanità (Italy), for animal welfare. Adult male mice of the C57BL/10SnJ strain (27 ± 0.3 g body weight; 15 weeks old) were obtained from Charles River Laboratories (Calco-Lecco, Italy) and were maintained in a light (12 h dark/12 h light) and temperature (23°C) controlled environment with free access to food and water. Tissues were taken from animals killed by cervical dislocation. The abdomen was immediately opened, the colon was rapidly removed, and placed in a dissecting dish filled with oxygenated Krebs solution and its contents gently flushed out. Then, segments (about 12 mm in length) were obtained either from proximal (immediately distal to the caecum) or distal (about 5 mm proximal to the anus) colon and suspended in 10 ml organ baths containing oxygenated (95% O_2 and 5% CO_2) Krebs solution maintained at 37°C.

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 a A/D converter, visualized, recorded, and analyzed on a personal computer using the PowerLab/400 system (Ugo Basile, Italy). Atropine (1 μM) and guanethidine (1 μM) were added to the Krebs solution at the beginning of the experiment to establish non adrenergic, non cholinergic (NANC) conditions. Longitudinal preparations were subjected to an initial tension of 200 mg and were allowed to equilibrate for at least 30 min. Spontaneous contractions of varying amplitude developed in all preparations. At the end of the equilibration period, the preparations were challenged with isoprotenerol (0.1 μM) to check that they were able to relax. Proximal and distal colon were relaxed by isoprotenerol equally (in proximal colon the relaxation was 193.1 ± 9.0 mg, while in distal colon it was 225.4 ± 19.5 mg; $n = 8$, $P > 0.05$).

Experimental protocols

After the equilibration time, concentration-dependent curves for adenosine were constructed by noncumulative addition of the drug before and after the different drugs used. Adenosine was applied for approximately 3 min at 20 min intervals. All the antagonists were allowed to maintain contact with the tissue for at least 30 min before repeating the doses of the agonist. The interval between the two assays was at least 1 h. Each preparation was tested with a single antagonist, except when otherwise stated. Time control experiments showed that a second curve to the agonist was reproducible. Concentrations of the drugs used were determined from the literature.

Solution and drugs

The composition of the Krebs solution was (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 following drugs were used: adenosine, atropine sulfate, 8-(3-chlorostyryl)-caffeine (CSC), 9-chloro-2-(2-furanyl)-5-((phenylacetyl) amino)-[1,2,4]triazolo[1,5-c]quinazoline (MRS 1220), 8-[4-[((4-cyanophenyl) carbamoylmethyl) oxy] phenyl]-1,3-di (*n*-propyl)xanthine (MRS 1754), 3,7-dimethyl-1-propargylxanthine (DMPX), 1,3-dipropyl-8-cyclopentylxanthine (DPCPX), guanethidine monosulfate, isoprotenerol, N_ω-nitro-L-arginine methyl ester (L-NAME), 1H-[1,2,4] oxadiazolo [4,3-a]quinoxalin-1-one (ODQ), 4-[[4-formyl-5-hydroxy-6-methyl-3-[(phosphonooxy) methyl]-2-pyridinyl]azo]-1,3-benzenedisulfonic acid tetrasodium salt (PPADS), sodium nitroprusside (SNP), theophylline, tetrodotoxin (TTX) (Sigma-Aldrich Inc., St Louis, U.S.A.). Adenosine, CSC, DPCPX, ODQ, MRS 1220, MRS 1754 were dissolved in dimethyl sulfoxide and further diluted in Krebs solution; DMPX was dissolved in ethanol. All the other drugs were dissolved in distilled water. The maximal final concentration of ethanol and dimethyl sulfoxide in the organ bath was 0.5%, which did not affect the contractility of the colonic segments.

The working solutions were prepared fresh on the day of the experiment by diluting the stock solutions in Krebs solution and were added to the organ bath.

Statistical analysis

All data are given as means $\pm$ s.e.m.: '*n*' in the results section refers to the number of animal preparations on which observations were made. In proximal colon, inhibitory effects induced by adenosine were estimated as the decrease in the amplitude of the spontaneous contraction and reported as a percentage of the maximal effect induced by 3 mM adenosine, corresponding to total suppression of spontaneous contrac-

tions. In distal colon, inhibitory effects induced by adenosine were estimated as the decrease in basal tone and reported as a percentage of the maximal effect induced by 3 mM adenosine, corresponding to the maximal relaxation obtained. Adenosine responses in the absence or in the presence of the different antagonists were fitted to sigmoid curves (Prism 4.0, GraphPAD, San Diego, CA, U.S.A.), and EC_{25} 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.

Results

Isolated segments of mouse colon displayed spontaneous activity consisting of phasic contractions with an amplitude of 401.7 ± 60.8 mg and a frequency of 5.54 ± 0.77 c.p.m. in the proximal region ($n = 32$), and with an amplitude of 254.6 ± 22.4 mg and a frequency of 5.3 ± 0.55 c.p.m. in the distal region ($n = 36$).

Adenosine (100 μM–3 mM) caused concentration-dependent inhibitory effects on longitudinal muscle of both proximal and distal colon. In proximal colon, the response to adenosine consisted of a reduction of the amplitude of spontaneous contractions, up to their complete disappearance at a concentration of 3 mM (Figure 1). In distal colon, adenosine caused a concentration-dependent relaxation (Figure 1). The actual amplitude of the relaxation observed in the distal colon in response to 3 mM of adenosine was 231.2 ± 18.9 mg ($n = 27$).

In both proximal and in distal colon, theophylline (0.1 mM), a nonselective P_1 receptor blocker, significantly antagonized the inhibitory effects induced by adenosine (Figure 1). On the contrary, PPADS (50 μM), a nonselective P_2 purinoceptor antagonist, had no effect on the inhibitory effects of adenosine (Figure 1, Table 1), indicating that adenosine exerts its effects by acting at P_1 receptors.

As P_1 purinoreceptors are divided into A_1, A_2 (A_{2A} and A_{2B}) and A_3 subclasses, we tested the effects of selective antagonists on adenosine-induced inhibitory effects, to characterize the receptor subtype(s) involved in the observed response. DPCPX (10 nM), a selective A_1 receptor antagonist, which *per se* did not modify spontaneous activity, significantly reduced the adenosine induced effects in both portions of colon, indicating the presence of A_1 receptors in both regions (Figure 2, Table 1). The A_2 receptor antagonist, DMPX (10 μM), did not modify the spontaneous mechanical activity and had no effect on the inhibition induced by adenosine in proximal colon, while it significantly antagonized the relaxation in the distal portion. Application of DPCPX and DMPX, together, to samples of distal colon showed additive effects (Figure 2). MRS 1220

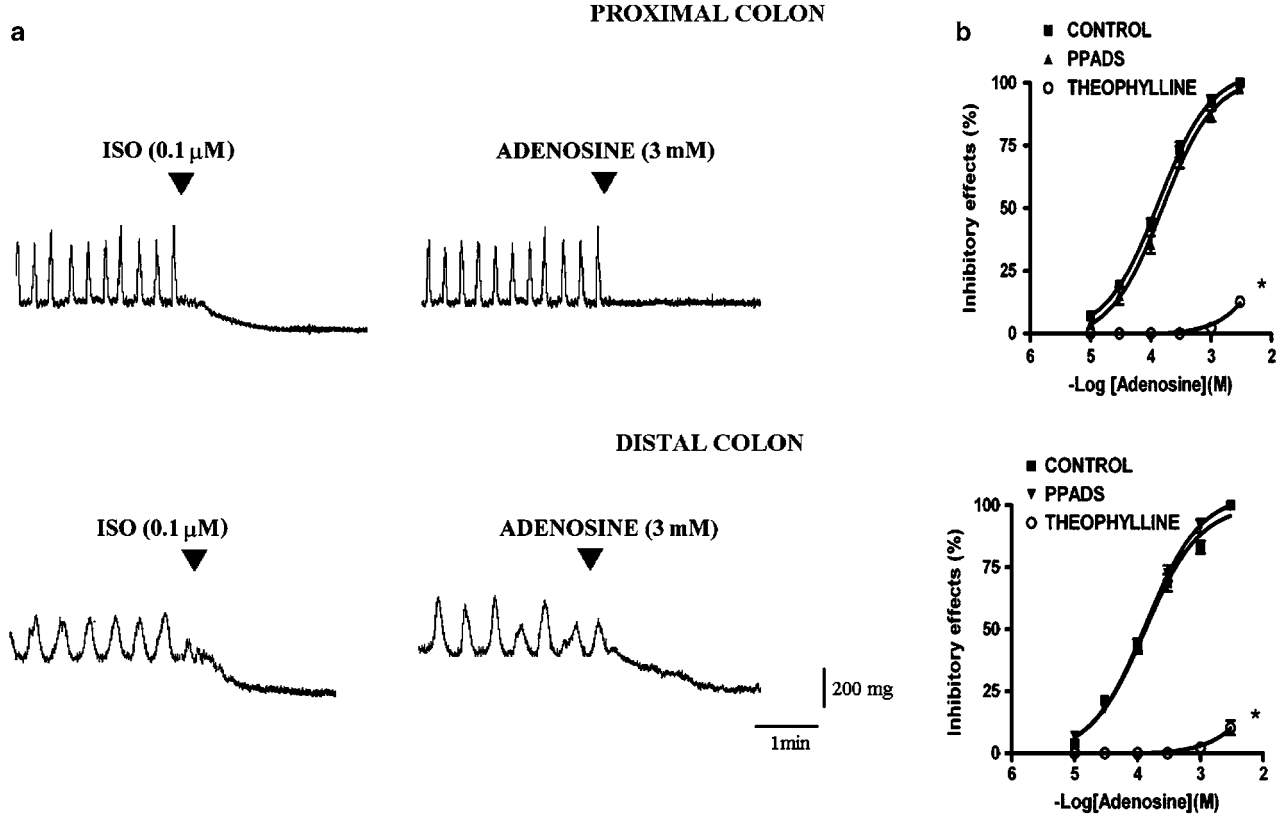

Figure 1 (a) Original recordings showing the inhibitory responses evoked by isoprotenerol or adenosine in proximal and in distal longitudinal colonic muscular preparations. (b) Concentration–response curves to adenosine in proximal and in distal colon in the absence or in the presence of theophylline (0.1 mM, $n = 4$) or PPADS (50 μM, $n = 4$). Data are means $\pm$ s.e.m. and are expressed as percentages of the maximal effect induced by 3 mM adenosine. The values for the control curves are the means of the control data obtained before each treatment ($n = 8$). $^*P < 0.05$ when the concentration–response curves were compared to those obtained in the respective control condition.

Table 1 EC_{25} values for adenosine-induced inhibitory effects before and after different pharmacological treatments

	Proximal colon			*Distal colon*		
	EC_{25} (mM)	*95% CLs* (mM)	n	*EC_{25} 95%* (mM)	*CLs* (mM)	n
Control	0.044	0.038–0.050	4	0.040	0.035–0.044	4
PPADS	0.058	0.048–0.072	4	0.043	0.036–0.052	4
Control	0.045	0.031–0.068	5	0.043	0.031–0.060	5
DPCPX	0.336*	0.256–0.440	5	0.069*	0.039–0.120	5
Control	0.040	0.033–0.070	6	0.045	0.031–0.075	6
DMPX	0.051	0.034–0.075	6	0.920*	0.650–1.297	6
Control	—	—		0.042	0.026–0.098	4
DPCPX + DMPX	—	—		3.365§	2.786–4.055	4
Control	—	—		0.046	0.028–0.074	5
CSC	—	—		0.059	0.027–0.130	5
Control	—	—		0.044	0.030–0.071	5
MRS 1754	—	—		0.596*	0.354–0.881	5
Control	0.041	0.031–0.075	4	0.043	0.030–0.077	4
MRS 1220	0.046	0.034–0.064	4	0.051	0.032–0.079	4
Control	0.035	0.027–0.046	6	0.042	0.031–0.076	6
TTX	0.049	0.027–0.090	6	0.780*	0.052–1.112	6
Control	—	—		0.050	0.032–0.089	4
DPCPX	—	—		0.084*	0.047–0.150	4
DPCPX + TTX	—	—		1.343§	0.960–1.875	4
Control	—	—		0.051	0.029–0.108	5
MRS 1754	—	—		0.483*	0.260–0.898	5
MRS 1754 + TTX	—	—		0.583*	0.335–1.106	5
Control	0.049	0.030–0.078	6	0.044	0.029–0.066	6
L-NAME	0.067	0.040–0.111	6	0.710*	0.590–0.850	6
Control	—	—		0.034	0.024–0.048	4
DPCPX	—	—		0.073*	0.034–0.156	4
DPCPX + L-NAME	—	—		1.076§	0.863–1.346	4
Control	—	—		0.047	0.026–0.085	4
MRS 1754	—			0.454*	0.221–0.665	4
MRS 1754 + L-NAME	—	—		0.501*	0.356–0.704	4
Control	—	—		0.044	0.027–0.075	4
ODQ	—	—		0.530*	0.480–0.620	4

*$P<0.05$ when compared to the respective control. §$P<0.05$ when compared to DPCPX alone.

(0.1 μM), a selective A_3 receptor antagonist, failed to modify the effects of adenosine in both portions (Figure 2, Table 1). In distal colon, in order to discriminate between A_{2A} and A_{2B} receptor subtypes we tested the effects of CSC or MRS 1754, which are selective A_{2A} and A_{2B} receptor antagonists, respectively, on the adenosine-induced relaxation. Relaxation was not modified by CSC (10 μM), but it was significantly antagonized by MRS 1754 (10 μM) (Figure 3). However, neither drug modified the spontaneous mechanical activity.

Tetrodotoxin (TTX; 1 μM), which blocks voltage-gated Na^+ channels in neurons, did not prevent the inhibitory effects of adenosine in proximal colon, while in distal colon, TTX partially reduced the relaxation evoked by adenosine. In distal colon, TTX caused a further inhibition of the response to adenosine remaining in the presence of DPCPX, but it was without effect on the response resistant to MRS 1754 (Figure 4, Table 1).

Interaction with the NO pathway

The inhibitory response to adenosine could be related to activation of the NO pathway in the colon. We therefore tested the effects of adenosine in the presence of drugs which interfere with nitrergic transmission. The NO synthase inhibitor, L-NAME (100 μM), significantly reduced adenosine effects in the distal colon, but did not affect responses to adenosine in the proximal colon (Figure 5, Table 1). A selective inhibitor of NO-stimulated soluble guanylyl cyclase, ODQ (10 μM), also induced a reduction of adenosine-induced inhibitory effects in distal colon (Figure 5, Table 1). Moreover, in this tissue, L-NAME reduced the response to adenosine remaining in the presence of DPCPX, but it was without any effects on the response after MRS 1754 (Figure 5, Table 1). The NO donor, sodium nitroprusside (SNP; 100 μM), induced a muscular relaxation that was insensitive to pretreatment with theophyl-

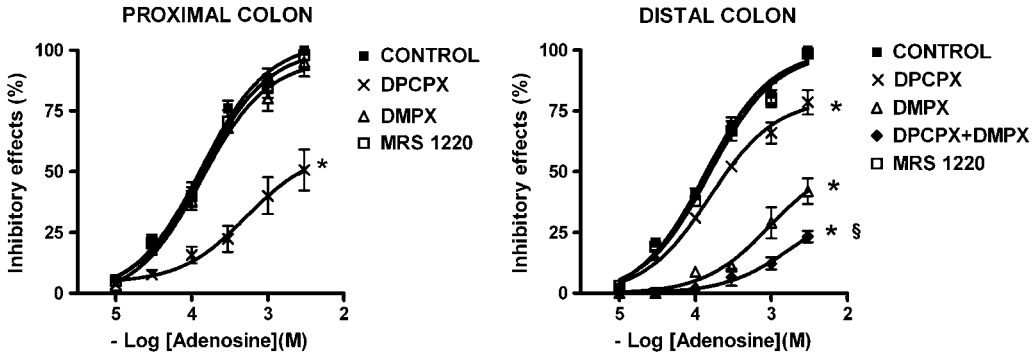

Figure 2 Concentration–response curves to adenosine before and after DPCPX (10 nM, $n=5$), DMPX (10 μM, $n=6$), MRS 1220 (0.1 μM, $n=4$) in proximal colon or DPCPX plus DMPX ($n=4$) in distal colon. Data are means$\pm$s.e.m. and are expressed as percentages of the maximal effect induced by 3 mM adenosine. The values for the control curves are the means of the control data obtained before each treatment (proximal colon: $n=15$; distal colon: $n=19$). $^*P<0.05$ when the concentration–response curves were compared to those obtained in the respective control condition. $^{\S}P<0.05$ when the concentration–response curves were compared to DPCPX alone.

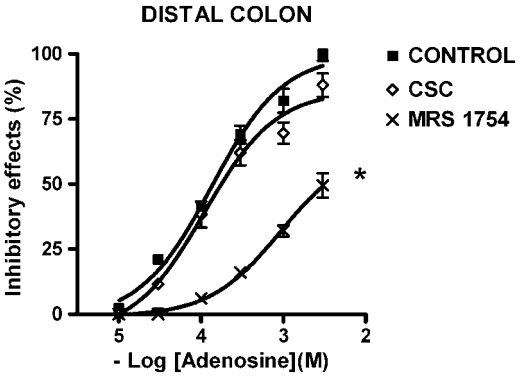

Figure 3 Concentration–response curves to adenosine before or after CSC (10 μM, $n=5$) or MRS 11754 (10 μM, $n=5$) in distal colon. Data are means$\pm$s.e.m. and are expressed as percentages of the maximal effect induced by 3 mM adenosine. The values for the control curve are the means of the control data obtained before each treatment ($n=10$). $^*P<0.05$ when the concentration–response curve was compared to that obtained in the control condition.

line (0.1 mM), DPCPX (10 nM) or DMPX (10 μM) (data not illustrated).

Discussion

The results of the present study show that exogenous adenosine exerted an inhibitory effect on muscular contractility in mouse colon in conditions where both cholinergic and catecholaminergic transmission were blocked (NANC conditions). In proximal colon, adenosine effects were due to TTX-insensitive activation of A_1 purinoceptors, whereas in distal colon, adenosine activated A_1 and A_{2B} purinoceptors, the latter located on enteric inhibitory nerves, most probably generating NO.

Adenosine is considered as an inhibitory neurotransmitter of the enteric nervous system in different animal species (Ralevic & Burnstock, 1998). Adenosine can influence gastrointestinal motility directly, by activating receptors located on smooth muscle (Serio *et al*., 1990; Nicholls *et al*., 1996; Kadowaki *et al*., 2000; Woods *et al*., 2003), or indirectly, by regulating neurotransmitter release from enteric neurons. Giaroni *et al*. (2002) suggested the presence of P_1 receptors in mouse colon, but the receptors subtypes were not investigated. Our studies confirm that in mouse colon, adenosine induces inhibitory effects and, as our experiments have been performed in the presence of atropine and guanethidine (NANC conditions), they are not the consequence of a modulation of cholinergic or catecholaminergic transmission. The response of the tissue was observed in a range of adenosine concentrations similar to that observed by Giaroni *et al*. (2006) in mouse intestine, but quite high when compared to other animal preparations. This may reflect a species specificity in sensitivity of intestinal smooth muscle to adenosine. Interestingly, the responses to adenosine differ between proximal and distal colon. In proximal colon, adenosine plays a modulator role, causing only a decrease in the amplitude of the spontaneous contractions, although the proximal colon was able to relax in response to isoprotenerol. Indeed, in distal colon adenosine induces muscular relaxation. It may be that these different responses are related to the different motor patterns of the proximal and distal colon: the former functions as reservoir, whereas the latter acts as a propulsive conduit (Camilleri & Ford, 1998).

The observation that adenosine effects were prevented by theophylline, a nonselective P_1 purinoceptor antagonist, but not by PPADS, the nonselective P_2 purinoreceptor antagonist, indicated that adenosine was acting on P_1 purinoceptors. However, theophylline is known also as a nonselective inhibitor of cyclic nucleotide phosphodiesterase, so the marked reduction of the adenosine effects could be also due to an interference with the intracellular signalling pathways induced by activation of adenosine receptors.

The next step was to analyze the effects induced by more selective adenosine receptor antagonists in order to distinguish between the different receptor subtypes. Adenosine acts through different G-protein-coupled P_1 purinoreceptor subtypes classified as A_1, A_2 (further subdivided into A_{2A} and A_{2B}), and A_3 receptors (Collis & Hourani, 1993; Ralevic & Burnstock, 1998). A_1 receptors are known to mediate mainly adenosine excitatory effects (Bailey *et al*., 1992; Murthy *et al*., 1995; Shim *et al*., 2002), although A_1 relaxant receptors have been reported in rat duodenum and ileum (Nicholls *et al*., 1992; Nicholls & Hourani, 1997). Our results suggest the

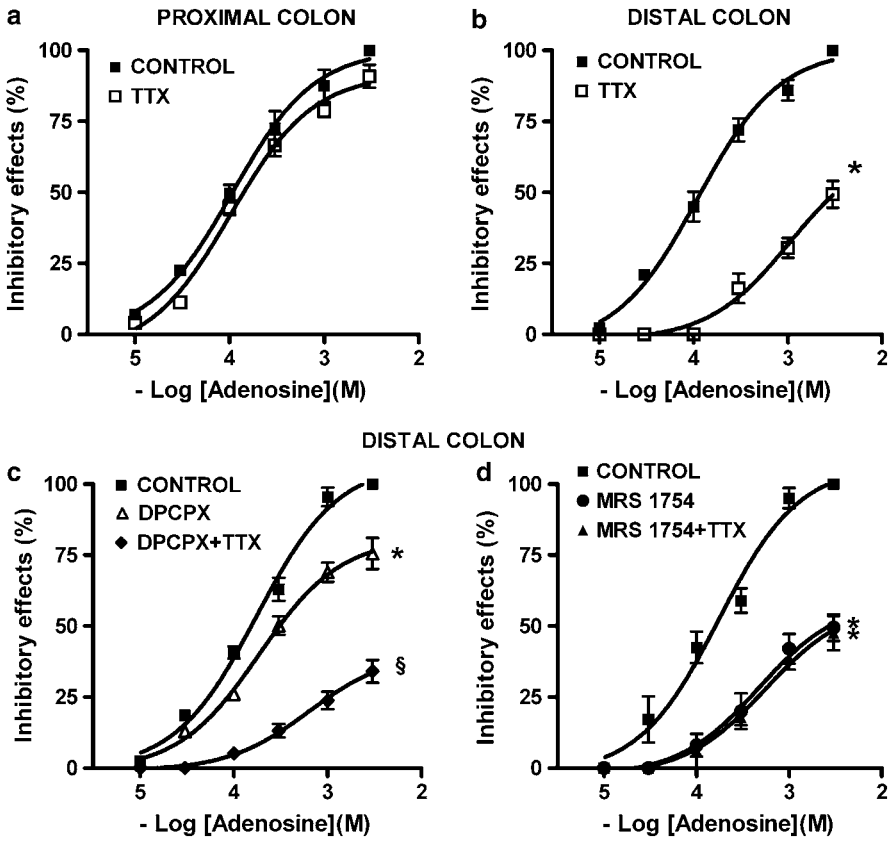

Figure 4 (a, b) Concentration–response curves to adenosine before ($n=6$) and after treatment with TTX (1 μM, $n=6$) in proximal and distal colon. (c) Concentration–response curves to adenosine before ($n=4$) and after DPCPX (10 nM, $n=4$) or DPCPX plus TTX ($n=4$) in distal colon. (d) Concentration–response curves to adenosine before ($n=5$) and after MRS 1754 (10 μM, $n=5$) or MRS 1754 plus TTX ($n=5$) in distal colon. Data are means $\pm$ s.e.m. and are expressed as percentages of the maximal effect induced by 3 mM adenosine. $^{*}P<0.05$ when the concentration–response curves were compared to those obtained in the respective control condition. $^{\S}P< 0.05$ when the concentration–response curves were compared to DPCPX alone.

presence of relaxant A_1 adenosine receptors also in mouse colon because DPCPX, a selective A_1 receptor antagonist, at the concentration we used (Coupar, 1999) inhibited the response to adenosine in both regions. In distal colon, the relaxation remaining in the presence of DPCPX was antagonized by DMPX, a selective A_2 receptor antagonist (Fredholm *et al*., 1994; De Man *et al*., 2003b), suggesting that, in this part of the colon, both A_1 and A_2 adenosine receptors were present and mediated the relaxation. This finding is in agreement with the general idea that A_2 receptors mediate adenosine-induced muscular relaxation (Bailey & Hourani, 1992; Nicholls *et al*., 1996; Kadowaki *et al*., 2000; Woods *et al*., 2003). Furthermore, our experiments indicated the presence of functional A_{2B} receptors involved in the adenosine-induced relaxation in murine distal colon, as this relaxation was antagonized by MRS 1754, a selective A_{2B} receptor antagonist, but not by CSC, a selective A_{2A} receptor antagonist. Postjunctional inhibitory A_{2B} receptors have been demonstrated in other animal preparations, such rat ileum and colon (Bailey & Hourani, 1992; Nicholls *et al*., 1996), or guinea-pig colon (Kadowaki *et al*., 2000). In addition, data from our experiments would not support a role for A_3 receptors in the inhibitory effects induced by adenosine in mouse colon, as suggested in the possum duodenum (Woods *et al*., 2003). Lastly, the observation that none of the adenosine receptor antagonists used had any effect on the spontaneous activity rules out an involvement of endogenous adenosine in the maintenance of the basal muscular activity in murine colon, again in contrast to results from possum duodenum (Woods *et al*., 2003).

TTX was unable to reduce adenosine effects in proximal colon indicating that, in this region, the inhibitory response to adenosine is independent of neuronal action potentials, and suggesting that the A_1 adenosine inhibitory receptors may be localized postjunctionally. However, it cannot be excluded that prejunctional A_1 receptors may directly induce neurotransmitter release from nerve terminals.

On the other hand, in distal colon, TTX reduced the effects of adenosine, suggesting that in this region some receptors are localized on enteric nerves, where adenosine would act causing neurotransmitter release. The observation that TTX was able to further antagonize adenosine effects persisting after A_1 receptor blockade suggests the presence of functional A_1 receptors that, as it was observed in proximal colon, would be more likely to be postjunctional. Indeed, TTX did not modify adenosine relaxation in the presence of the A_{2B} receptor antagonist indicating that the A_{2B} receptors were located presynaptically on inhibitory neurons. Although the presence of neuronal A_1 receptors has been reported in the gastrointestinal tract (Kadowaki *et al*., 2000; Lee *et al*., 2001; Storr *et al*., 2002; De Man *et al*., 2003b), to our knowledge this is the first report of A_2 receptors located on enteric inhibitory

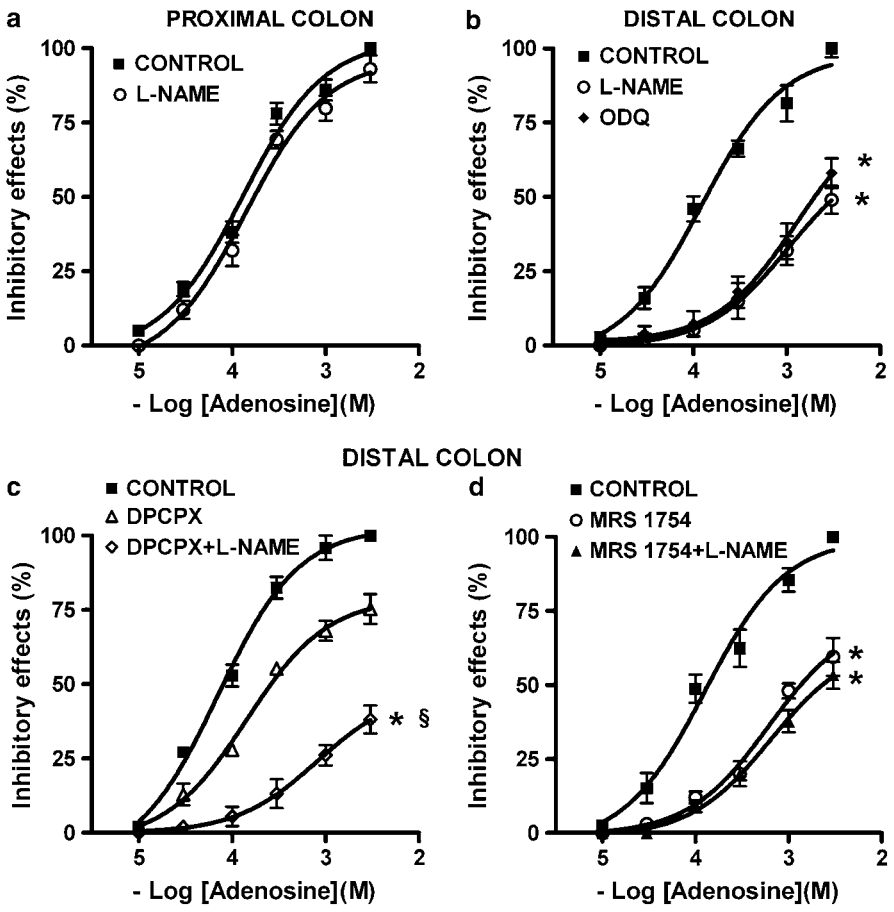

Figure 5 (a) Concentration–response curves to adenosine before ($n=6$) and after L-NAME (100 μM, $n=6$) in proximal colon. (b) Concentration–response curves to adenosine before ($n=10$, the values are the means of the control data obtained before each treatment) and after L-NAME (100 μM, $n=6$) or ODQ (1 μM, $n=4$) in distal colon. (c) Concentration–response curves to adenosine before ($n=4$) and after DPCPX (10 nM, $n=4$) or DPCPX plus L-NAME ($n=4$), in distal colon. (d) Concentration–response curves to adenosine before ($n=4$) and after MRS 1754 (10 μM, $n=4$) or MRS 1754 plus L-NAME ($n=4$), in distal colon. Data are means ± s.e.m. and are expressed as percentages of the maximal effect induced by 3 mM adenosine. $^{*}P<0.05$ when the concentration–response curves were compared to those obtained in the respective control condition. $^{\S}P<0.05$ when the concentration–response curves were compared to DPCPX alone.

neurons. However, prejunctional facilitatory A_2 receptors have been shown on cholinergic nerves in guinea-pig and rat ileum (Tomaru *et al*., 1995; Duarte-Araujo *et al*., 2004).

As NO is the main mediator released by inhibitory NANC nerves in various regions of the gastrointestinal tract from different animal species, including mouse (Olsson & Holmgren, 2001; Serio *et al*., 2003a; Ueno *et al*., 2004) and an interaction between NO and the purinergic system has been reported in gastrointestinal smooth muscle preparation of rodents (De Luca *et al*., 1999; Giaroni *et al*., 2002; Van Crombruggen & Lefebvre, 2004), we tested the possibility that nitrergic inhibitory nerves may be involved in the inhibition in mouse colon, evoked by adenosine. Inhibition of NO biosynthesis with L-NAME decreased the response to adenosine only in distal colon and not in proximal colon, suggesting that, in distal colon, adenosine acts also through NO production to induce the inhibitory response. This suggestion was further supported by the observation that ODQ, which is a potent and selective inhibitor of NO-stimulated soluble guanylyl cyclase, significantly reduced the effect of adenosine in distal colon. In this tissue, L-NAME was still able to antagonise the response to adenosine after block of A_1 receptors but not after block of A_{2B} receptors, indicating that adenosine was modulating NO release *via* A_{2B} receptors. Lastly, our study indicated that, in mouse distal colon, NO would not facilitate the release of adenosine from inhibitory nerve terminals. This conclusion is supported by our finding that the inhibitory effects induced by exogenous NO (derived from the NO donor, SNP) were not antagonized by the different adenosine receptor antagonists.

In conclusion, we provide evidence that exogenous adenosine acts as inhibitory modulator of the contractility of mouse longitudinal smooth muscle of proximal and distal colon by activating P_1 purinoceptors. In proximal colon, adenosine reduces spontaneous muscular activity *via* a mechanism not dependent on neuronal action potentials, activating exclusively A_1 receptors. In distal colon, adenosine induces muscular relaxation *via* activation of A_1 and A_{2B} receptors, the latter located on the enteric inhibitory neurons which release NO.

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