British Journal of Pharmacology (2007) 151, 152–160
 0007–1188/07 $30.00
www.brjpharmacol.org

RESEARCH PAPER

Evidence that ATP or a related purine is an excitatory neurotransmitter in the longitudinal muscle of mouse distal colon

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Background and purpose: This study analysed the contribution of the purinergic system to enteric neurotransmission in the longitudinal muscle of mouse distal colon.
Experimental approach: Motor responses to exogenous ATP and to nerve stimulation *in vitro* were assessed as changes in isometric tension.
Key results: ATP induced a concentration-dependent contraction, reduced by 4-[[4-formyl-5-hydroxy-6-methyl-3-[(phosphonooxy)methyl]-2-pyridinyl]azo]-1,3-benzene disulphonic acid (PPADS), suramin, P2Y purinoreceptor desensitisation with adenosine 5′-O-2-thiodiphosphate (ADPβS), and atropine, but unaffected by P2X purinoceptor desensitisation with α,β-methylene ATP (α,β-meATP) and by 2,2-dimethyl-propionic acid 3-(2-chloro-6-methylaminopurin-9-yl)-2-(2,2-dimethyl-propionyloxymethyl)-propyl ester (MRS 2395), a $P2Y_{12}$ selective antagonist. The response to ATP was increased by 2′-deoxy-N^6-methyl adenosine 3′,5′-diphosphate (MRS 2179), a $P2Y_1$ selective antagonist, tetrodotoxin (TTX) or N_ω-nitro-L-arginine methyl ester (L-NAME). ADPβS, a P2Y-purinergic agonist, induced muscular contraction, with the same pharmacological profile as the ATP-induced contraction. ADP, a natural ligand for $P2Y_1$ receptors, induced muscular relaxation, antagonized by MRS 2179 and by TTX or L-NAME. Nerve stimulation elicited a transient nitrergic relaxation, followed by contraction. Contractile responses was reduced by atropine, PPADS, suramin, P2Y purinoceptor desensitisation, but not by P2X purinoceptor desensitisation, MRS 2179 or MRS 2395. None of the purinergic antagonists modified the nerve-evoked relaxation.
Conclusions and implications: In the longitudinal muscle of mouse distal colon, ATP, through ADPβS-sensitive P2Y purinoceptors, contributed to the excitatory neurotransmission acting directly on smooth muscle and indirectly via activation of cholinergic neurons. Moreover, P2Y1 purinoceptors appear to be located on nitrergic inhibitory neurons. This study provides new insights into the role of purines in the mechanism inducing intestinal transit in mouse colon.
British Journal of Pharmacology (2007) **151,** 152–160. doi:10.1038/sj.bjp.0707188; published online 12 March 2007

Keywords: ATP; P2Y receptors; enteric excitatory neurotransmission; mouse; colon; longitudinal muscle

Abbreviations: ADPβS, adenosine 5′-O-2-thiodiphosphate; EFS, electrical field stimulation; α,β-meATP, α,β-methylene ATP; L-NAME, N_ω-nitro-L-arginine methyl ester; MRS 2179, 2′-deoxy-N^6-methyl adenosine 3,5-diphosphate diammonium salt; MRS 2395, 2,2-dimethyl-propionic acid 3-(2-chloro-6-methylaminopurin-9-yl)-2-(2,2-dimethyl-propionyloxymethyl)-propyl ester; PPADS, 4-[[4-formyl-5-hydroxy-6-methyl-3-[(phosphonooxy)-methyl]-2-pyridinyl]azo]-1^3-benzene- disulphonic acid; 8-PT, 8-phenyltheophylline; TTX, tetrodotoxin

Introduction

Adenosine 5′-triphosphate (ATP) released from enteric nerves mediates muscular responses in different regions of gastrointestinal tract from various animal species via activation of P2 purinergic receptors (Burnstock, 2001). The P2 receptors are subdivided into two classes, the ligand-gated channels or P2X receptors and the G protein-coupled P2Y receptors (Abbracchio and Burnstock, 1994; Ralevic and Burnstock, 1998). In most species, ATP activates P2Y receptors to induce muscular inhibitory responses (Koh *et al.*, 1997; Zagorodnyuk and Maggi, 1998; Xue *et al.*, 1999; De Man *et al.*, 2003; Serio *et al.*, 2003; Van Crombruggen and Lefebvre, 2004; Mulè *et al.*, 2005). However, some reports indicate that ATP produces excitatory responses in some regions of the gut

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Received 28 July 2006; revised 23 October 2006; accepted 16 January 2007; published online 12 March 2007

(Bailey and Hourani, 1990; Matsuo *et al*., 1997; Murthy and Makhlouf, 1998; Zagorodnyuk and Maggi 1998; Sato *et al*., 1999) and that it mediates the non-cholinergic, non-tachykininergic component of the nerve-evoked excitatory responses (Shuba and Vladimorava, 1980; Huizinga *et al*., 1981; Zagorodnyuk and Maggi, 1998; Zhang and Paterson, 2005). The nature of the purinergic receptors that mediate muscular excitatory effects is uncertain. In guinea-pig ileal smooth muscle, the presence of excitatory P2X receptors on cholinergic nerve endings and of excitatory P2X and P2Y receptors on smooth muscle have been demonstrated (Matsuo *et al*., 1997; Sato *et al*., 1999). In rabbit gastric smooth muscle cells, ATP elicits contraction by activating preferentially P2Y receptors, which coexist with P2X receptors activated by high levels of ATP (Murthy and Makhlouf, 1998). In addition, contractile responses to ATP are mediated via P2Y receptors in rat colon muscularis mucosae (Bailey and Hourani, 1990), in rat gastric circular muscle (Otsuguro *et al*., 1996) and in guinea-pig colon (Zagorodnyuk and Maggi, 1998).

Recently, purinergic neurotransmission has been addressed in the gastrointestinal tract of mouse, an increasingly important animal model for investigating gastrointestinal motility because of the availability of mutants and the advent of gene-targeting technology. Immunohistochemical studies demonstrated the presence of P2 receptors throughout the entire gastrointestinal tract of the mouse (Giaroni *et al*., 2002). Functional studies reported ATP as an inhibitory neurotransmitter acting via P2Y receptors in gastric and colonic circular muscle preparation (Mulè and Serio, 2003; Serio *et al*., 2003) and via P2Y and, to a lesser extent, via P2X receptors in the jejunal circular muscle (De Man *et al*., 2003). In addition, ATP can induce contraction via activation of P2X receptors, located pre-synaptically on excitatory neurons in the stomach (Mulè *et al*., 2005) and at muscular level in the longitudinal muscle of the colon (Giaroni *et al*., 2002).

The aim of the present study was to analyse, in the longitudinal muscle of mouse colon, the contribution of the purinergic system to the enteric neurotransmission. We analysed, at first, the responses to exogenously applied purines and the subtypes of purinoreceptors involved and, subsequently, we examined whether activation of purinoreceptors is an essential step in the genesis of the nerve-evoked responses.

Methods

All animal procedures complied with the regulations of the Ministero della Sanità (Rome, Italy), for animal welfare. Adult male mice of the C57BL/10SnJ strains (25.5 ± 0.5 g body weight; we need 15 weeks old) were obtained from Charles River Laboratories (Calco-Lecco, Italy) and were maintained in a light (12h/12h; dark–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 distal colon (from 5 mm proximal to the anus) was rapidly removed, placed in a dissecting dish filled with oxygenated Krebs solution and the contents gently flushed out. Then, two segments (about 12 mm in length) of distal colon from each animals were 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 analysed on a personal computer using the PowerLab/400 system (Ugo Basile, Italy). Electrical field stimulation (EFS) was applied from a Grass S88 electrical stimulator (Grass Instruments Co., Quincy, MA, USA) through a stimulus isolation unit (SIU5) using direct coupling. Stimuli (0.5 ms, 4 Hz, supramaximal voltage) were delivered in 10-s train via a pair of platinum plate electrodes. 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.

Experimental design

After the equilibration time, concentration-dependent curves for the responses to purinergic agonists were constructed by non-cumulative addition of the drug before and after the different antagonists used. Agonists were 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 curve of the agonist. The concentration range used for ATP was 10μM–1 mM and for ADPβS was 0.3–100 μM. To limit costs, just in few control experiments a higher dose of ATP or ADPβS was tested to verify the maximal obtainable response. 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 literature.

In a second set of experiments, EFS was performed by stimulating the tissues with individual trains delivered at 15 min intervals. Subsequently, antagonists were added to the organ baths and, after a period of 20–30 min, EFS was repeated. Time control experiments showed no decay in the amplitude of the response to EFS for several hours.

Solutions and drugs

The composition of Krebs solution was (mM) 119 NaCl, 4.5 KCl, 2.5 $MgSO_4$, 25 $NaHCO_3$, 1.2 KH_2PO_4, 2.5 $CaCl_2$ and 11.1 glucose.

The following drugs were used: adenosine 5′-diphosphate (ADP), ATP, adenosine 5′-*O*-2-thiodiphosphate (ADPβS), atropine sulphate, 2′-deoxy-N^6-methyl adenosine 3′,5′-diphosphate diammonium salt (MRS 2179), 2,2-dimethyl-propionic acid 3-(2-chloro-6-methylaminopurin-9-yl)-2-(2,2-dimethyl-propionyloxymethyl)-propyl ester (MRS

2395), α,β-methylene ATP (α,β-meATP), N_ω-nitro-L-arginine methyl ester (L-NAME), 4-[[4-formyl-5-hydroxy-6-methyl-3-[(phosphonooxy)methyl]-2-pyridinyl]azo]-1,3-benzenedisulphonic acid tetrasodium salt (PPADS), 8-phenyltheophylline (8-PT), sodium nitroprusside (SNP), suramin and tetrodotoxin (TTX) (Sigma-Aldrich, Inc., St Louis, MO, USA). All the drugs were dissolved in distilled water except 8-PT, which was dissolved in dimethylsulphoxide and further diluted in Krebs. The maximal final concentration of dimethylsulphoxide in the organ bath was 0.5%, which did not affect the contractility of the colonic segments. The working solutions were prepared freshly on the day of the experiment by diluting the stock solutions in Krebs.

Statistical analysis

All data are given as means ± s.e.m.: '*n*' in the results section refers to the number of animal preparations on which observations were made. Contractile responses were expressed in absolute values (mg). Inhibitory effects were calculated as a percentage reversal of the initial level of active force. A 100% inhibition corresponded to the total suppression of spontaneous contractions, whereas a percentage higher than 100% indicated muscular relaxation. Responses to the agonists in the absence or in the presence of the different antagonists were fitted to sigmoid curves (Prism 4.0, GraphPAD, San Diego, CA, USA) and effector concentration for half-maximum concentration (EC_{50}) values (with 95% confidence interval (Cls)) for the agonists were determined from these curves. Statistical analysis was performed by means of Student's *t*-test or by means of analysis of variance (ANOVA) followed by Bonferroni's test, when appropriate. A probability value of >0.05 was regarded as significant.

Results

Motor responses to ATP

Isolated segments of mouse distal colon displayed spontaneous activity consisting of phasic contractions, with amplitude of about 230 mg and frequency of about 5 c.p.m. At basal tone, ATP induced concentration-dependent contractions that reached the maximal obtainable amplitude at the concentration of 1 mM and with an EC_{50} of 165.4 μM (95% CIs 103.1–265.6 μM, $n=20$) (Figure 1). Responses to ATP showed desensitization and an interval of 20 min between two consecutive applications was necessary. Occasionally at the higher concentrations, an early relaxation followed by the contractile response was observed. To examine the possible relaxant effects induced ATP, we tested this drug also in tissues precontracted with 30 mM KCl. In this condition, ATP still induced contractile effects and no relaxation was unmasked.

The contractions induced by ATP were significantly reduced by PPADS (500 μM) and by suramin (100 μM), non-selective P2 receptor antagonists, causing an approximately three-fold displacement to the right of the concentration–response curve to ATP and reducing the response to 1 mM ATP by about 50%, and thus indicating that ATP induced the

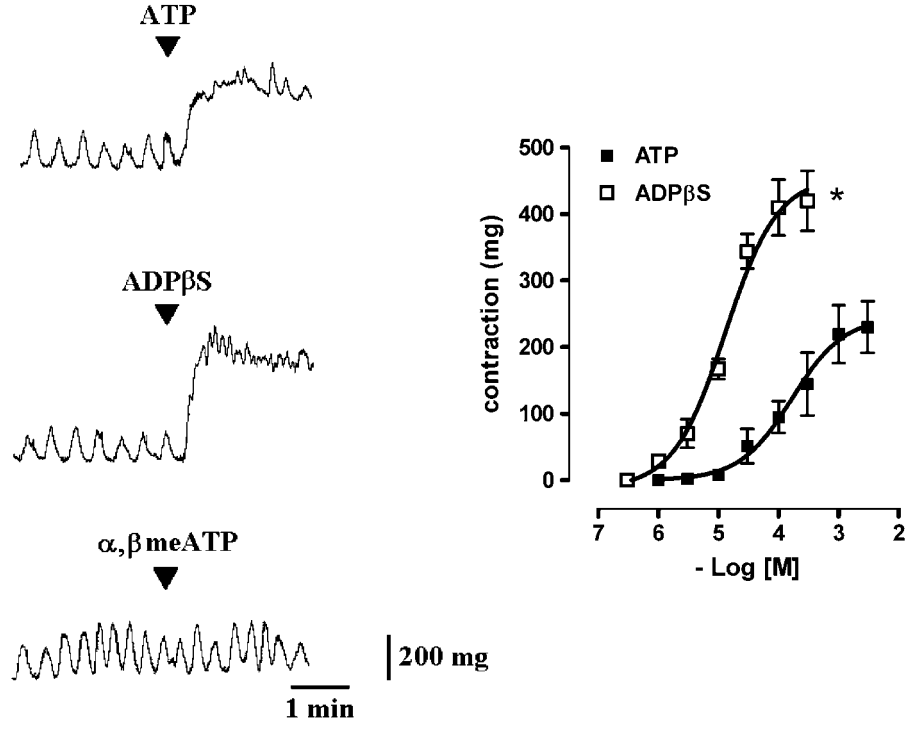

Figure 1 Left: original tracing showing the effects of ATP (1 mM), ADPβS (100 μM) and α,β-meATP (100 μM) on spontaneous mechanical activity of longitudinal muscle of mouse distal colon. Right: concentration–response curves to ATP ($n=20$ except for 3 mM ATP that is from four animals) and ADPβS ($n=20$ except for 300 μM ADPβS that is from four animals) in longitudinal muscle of mouse distal colon. Data are means ± s.e.m. and are expressed in absolute values. *$P<0.05$ when the concentration–response curves were compared.

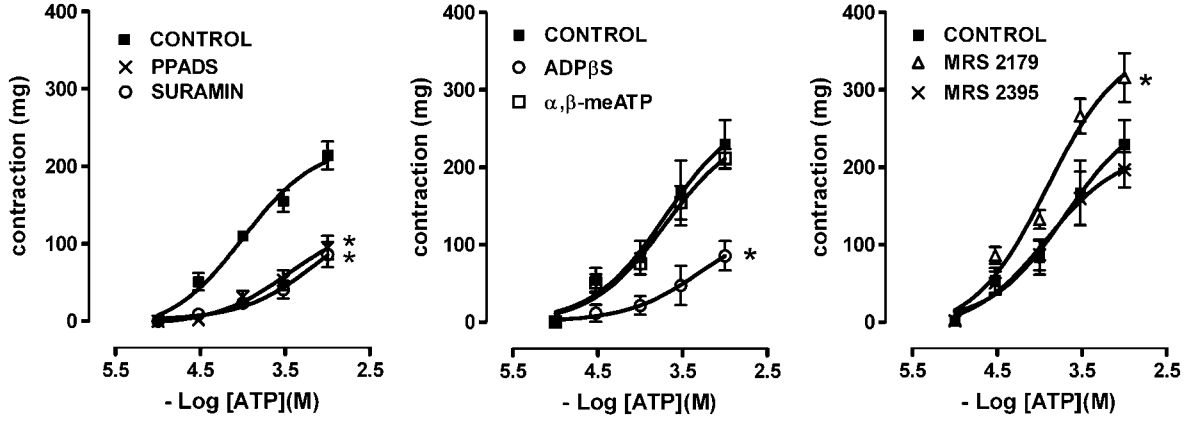

Figure 2 Concentration–response curves to ATP before and after PPADS (500 μM; $n=4$), suramin (100 μM; $n=6$), α,β-meATP (1 μM for 30 min, $n=4$), ADPβS (10 μM for 30 min; $n=5$), MRS 2179 (1 μM; $n=4$), MRS 2395 (1 μM; $n=4$) in longitudinal muscle of mouse distal colon. Data are means$\pm$s.e.m. and are expressed in absolute values. The values for the control curves are the means of the control data obtained before each treatment. *$P<0.05$ when the concentration–response curves were compared with those obtained in the respective control condition.

Table 1 Effects of the different pharmacological treatments on the contractions induced by 1 mM ATP or by 100 μM ADPβS

	ATP (1 mM) Δ%	n	*ADPβS (100 μM)* Δ%	n
PPADS	-55.5 ± 7.1*	4	-67.00 ± 5.80*	5
Suramin	-59.8 ± 7.7*	6	-58.50 ± 4.92*	6
ADPβS	-62.6 ± 8.3*	5	-75.0 ± 2.9*	4
MRS 2179	$+37.6\pm13.6$*	4	$+29.7\pm5.4$*	5
MRS 2395	-14.3 ± 9.8	4	-8.8 ± 9.6	4
α,β-meATP	-8.0 ± 5.4	4	$+1.4\pm6.3$	4
Indomethacin	-4.4 ± 8.0	6	-4.0 ± 5.9	6
Atropine	-27.1 ± 9.5*	6	-26.4 ± 8.4*	6
TTX	$+36.7\pm14.8$*	5	$+26.7\pm10.7$*	5
L-NAME	$+50.0\pm10.3$*	4	$+38.4\pm11.3$*	6

Data are expressed as the percentage of the decrease or increase in the response to ATP or ADPβS before pharmacological treatment, taken as 100%. A concentration of 1 mM of ATP or of 100 μM of ADPβS induced the maximal obtainable response. Values are means$\pm$s.e.m.
*$P<$ 0.05 when compared with the respective control.

contractile effect by activating P2 receptors (Figure 2, Table 1). The occasional relaxation observed at 1 mM ATP was not modified by the P2-purinergic antagonists, indeed it was abolished by 8-PT (10 μM), indicating an action of ATP on the P1 purinergic receptors. Desensitization of the P2Y purinoreceptors with ADPβS (10 μM for 30 min) reduced the contractions evoked by ATP causing an approximately 2-fold displacement to the right of the concentration–response curve to ATP and reducing the response to 1 mM ATP by about 60%, whereas P2X purinoceptor desensitisation, induced by addition of α,β-meATP (1 μM for 30 min) had no significant effects, suggesting an involvement of P2Y receptors in the evoked contractions (Figure 2, Table 1). Moreover, the contractile responses to ATP were increased by MRS 2179 (1 μM), a $P2Y_1$ selective antagonist, causing a shift to the left of the concentration–response curve to ATP and increasing the response to 1 mM ATP by about 40%, whereas they were not affected by MRS 2395 (1 μM), a P2Y12-selective antagonist (Figure 2, Table 1).

The contractile responses induced by ATP were not modified by indomethacin (10 μM), but they were partly antagonized by atropine (1 μM), a muscarinic antagonist (Figure 3, Table 1). In addition ATP-induced responses were significantly increased by TTX (1 μM), which blocks neuronal action potentials and by L-NAME (100 μM), a nitric oxide (NO) synthase inhibitor (Figure 3, Table 1). Multiple comparison showed that the increase in the response observed in the presence of TTX was comparable to that induced by L-NAME.

Motor responses to purinergic agonists

The P2Y-purinergic agonist, ADPβS, induced a concentration-dependent contraction of mouse colonic longitudinal muscle, which reached the maximal obtainable amplitude at the concentration of 100 μM with an EC_{50} of 13.0 μM (95% CIs 7.0–23.9 μM, $n=20$) (Figure 1). ADPβS was shown to be more potent than ATP (Figure 1, Table 1). The responses to ADPβS also showed desensitization, and an interval of 20 min between two consecutive applications was necessary. Indeed, the P2X-purinergic agonist, α,β-meATP, did not cause any significant changes of the muscular activity (Figure 1).

ADPβS-induced contractions were significantly reduced by PPADS (500 μM) and suramin (100 μM), by P2Y purinoreceptor desensitization with ADPβS (10 μM for 30 min), but were not modified by P2X purinoceptor desensitization with α,β-meATP (1 μM for 30 min) (Figure 4, Table 1). In addition, ADPβS-induced effects were increased in the presence of MRS 2179 (1 μM), a $P2Y_1$-selective antagonist (Figure 4, Table 1). Once more, MRS 2395 (1 μM), a $P2Y_{12}$-selective antagonist was without any effect on the concentration–response curve to ADPβS (Figure 4, Table 1). ADPβS responses were not modified by indomethacin (10 μM), but were partly antagonized by atropine (1 μM) and significantly increased by TTX (1 μM) and by L-NAME (100 μM) (Figure 5, Table 1). Once more, multiple comparison showed that the increase in the response observed in the presence of TTX was comparable to that induced by L-NAME.

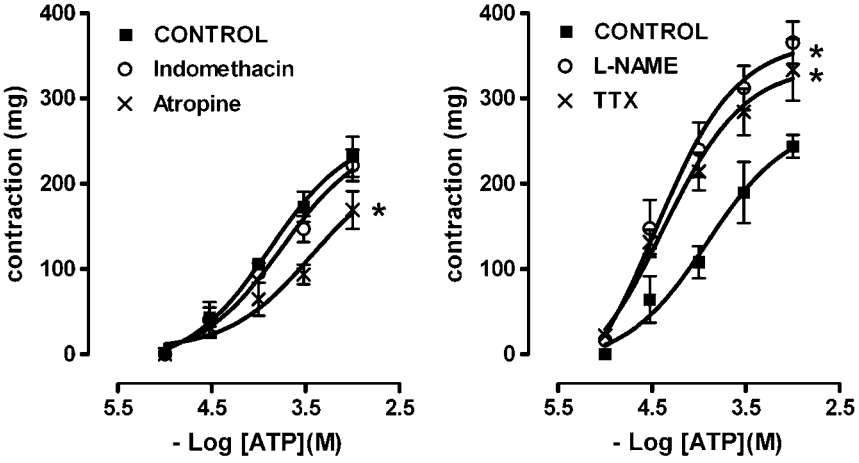

Figure 3 Concentration–response curves to ATP before and after atropine (1 μM; $n=6$) or indomethacin (10 μM; $n=6$), TTX (1 μM, $n=5$) or L-NAME (100 μM; $n=4$) in longitudinal muscle of mouse distal colon. Data are means$\pm$s.e.m. and are expressed in absolute values. The values for the control curves are the means of the control data obtained before each treatment. *$P<0.05$ when the concentration–response curves were compared with those obtained in the respective control condition.

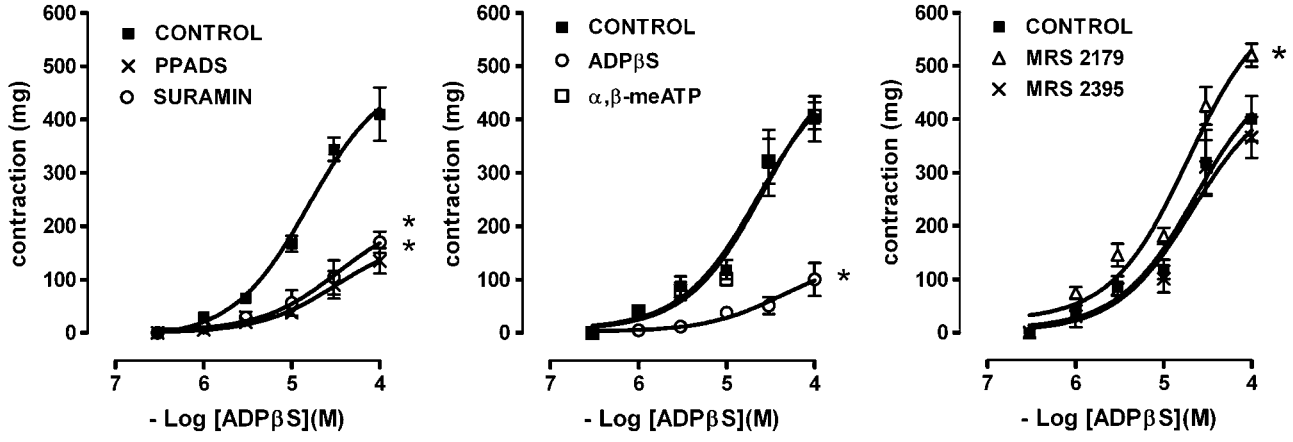

Figure 4 Concentration–response curves to ADPβS before and after PPADS (500 μM; $n=5$), suramin (100 μM; $n=6$), α,β-meATP (1 μM for 30 min, $n=4$), ADPβS (10 μM for 30 min; $n=5$), MRS 2179 (1 μM; $n=5$), MRS 2395 (1 μM; $n=4$) in longitudinal muscle of mouse distal colon. Data are means$\pm$s.e.m. and are expressed in absolute values. The values for the control curves are the means of the control data obtained before each treatment. *$P<0.05$ when the concentration–-response curves were compared with those obtained in the respective control condition.

ADP, the most potent natural ligand at $P2Y_1$ receptors (Ralevic and Burnstock, 1998), induced an inhibitory effect consisting of an initial reduction of the spontaneous mechanical activity until a sustained muscular relaxation was observed (Figure 6). Responses induced by ADP were greatly reduced by MRS 2179 (1 μM), a $P2Y_1$-selective antagonist, TTX (1 μM) and L-NAME (100 μM) (Figure 6).

Motor responses to EFS

In the longitudinal muscle of mouse colon, EFS (0.5 ms, 4 pps, supramaximal voltage for 10 s) elicited a TTX-sensitive response consisting in an initial, low in amplitude, muscular relaxation (218$\pm$25 mg, $n=6$) followed by an high in amplitude contraction (588$\pm$101 mg, $n=6$) (Figure 7). L-NAME (100 μM) abolished the relaxant response (Figure 7). The contractile response was antagonized by atropine (1 μM) and by PPADS (500 μM) and suramin (100 μM), suggesting the involvement of cholinergic and purinergic systems. The nerve-evoked contraction was significantly reduced by P2Y purinoceptor desensitization with ADPβS (10 μM for 30 min), but it was not affected by P2X purinoceptor desensitization with α,β-meATP (1 μM for 30 min) (Figure 7). Moreover, the contraction residual to atropine, was further reduced by P2Y purinoreceptor desensitisation with ADPβS (Figure 7). Indeed, MRS 2179 (1 μM), $P2Y_1$ antagonist, or MRS 2395 (1 μM), $P2Y_{12}$-selective antagonist, had no effects on the contractile response (Figure 7). None of the purinergic antagonists used modified the relaxant response to EFS (Figure 7).

Discussion

ATP released from enteric nerves has been reported to mediate muscular inhibitory/excitatory responses in differ-

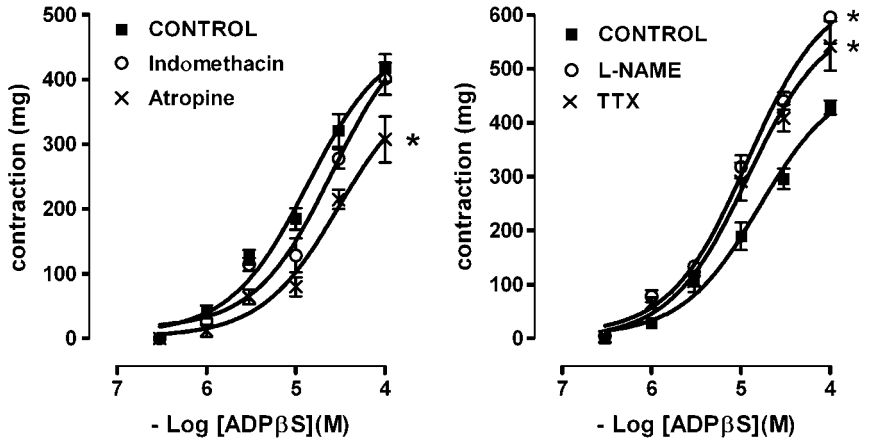

Figure 5 Concentration–response curves to ADPβS before and after atropine (1 μM; $n=6$) or indomethacin (10 μM; $n=6$), TTX (1 μM, $n=5$) or L-NAME (100 μM; $n=6$) in longitudinal muscle of mouse distal colon. Data are means$\pm$s.e.m. and are expressed in absolute values. The values for the control curves are the means of the control data obtained before each treatment. *$P<0.05$ when the concentration–response curves were compared with those obtained in the respective control condition.

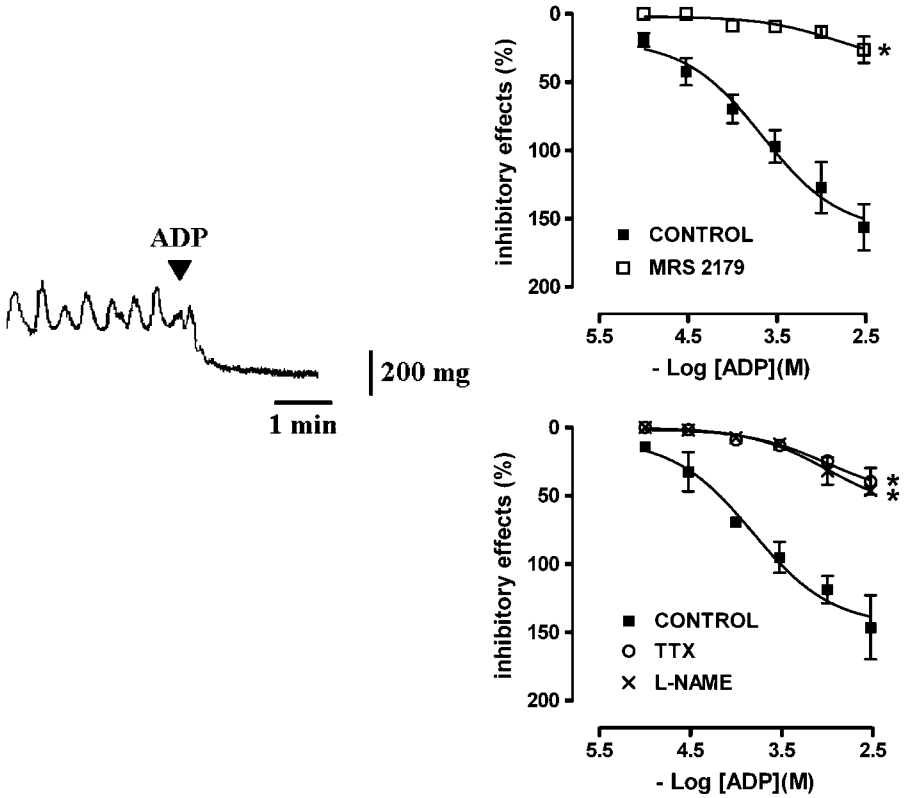

Figure 6 Left: original tracing showing the effects of ADP (3 mM) on spontaneous mechanical activity of longitudinal muscle of mouse distal colon. Right: concentration–response curves to ADP before and after MRS 2179 (1 μM; $n=4$), TTX (1 μM, $n=5$) or L-NAME (100 μM; $n=5$) in longitudinal muscle of mouse distal colon. Data are means$\pm$s.e.m. and are expressed as a percentage reversal of the initial level of active force, 100% inhibition corresponding to total suppression of spontaneous contractions. The values for the control curves are the means of the control data obtained before each treatment. *$P<0.05$ when the concentration–response curves were compared with those obtained in the respective control condition.

ent regions of gastrointestinal tract from various animal species via activation of P2Y and/or P2X receptors (see Lecci *et al*., 2002 for review). In mouse, ATP has been reported to induce inhibitory effects in all regions of the gut acting on P2Y receptors (Giaroni *et al*., 2002, 2006; Serio *et al*., 2003; Mulè *et al*., 2005), whereas excitatory effects owing to activation of P2X receptors located on excitatory nerves in the stomach (Mulè *et al*., 2005) or on smooth muscle in the longitudinal muscle of the colon have been shown (Giaroni *et al*., 2002).

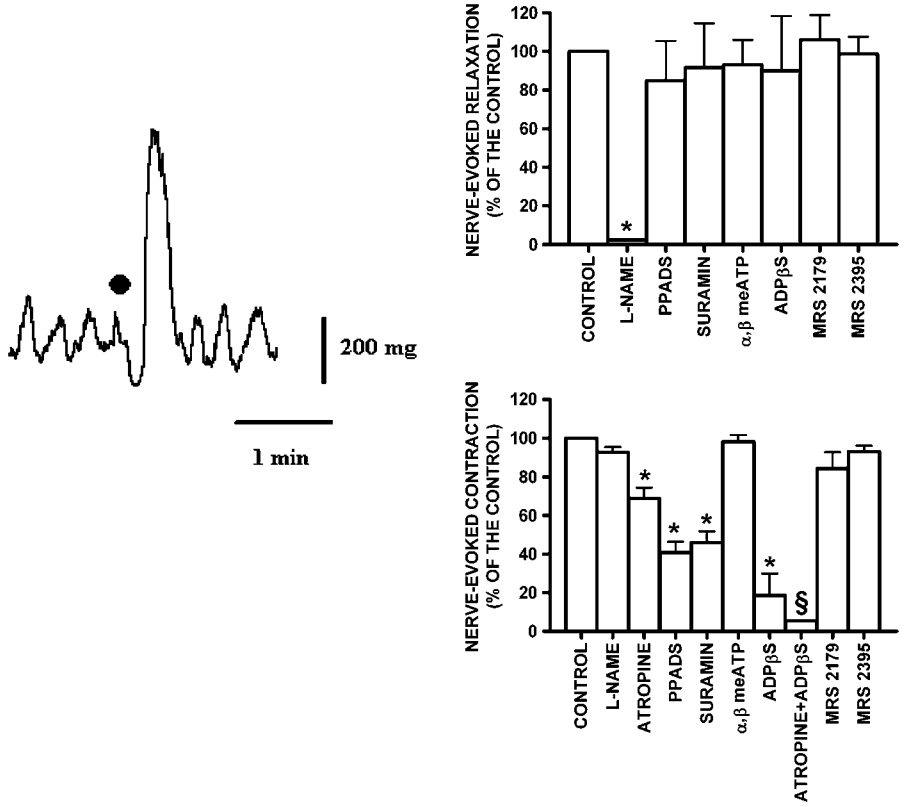

Figure 7 Non-adrenergic, non-cholinergic (NANC) responses evoked by electrical field stimulation (EFS) in longitudinal colonic muscle preparations. Left: typical tracings showing the NANC-evoked responses induced by EFS in mouse colonic segments (0.5 ms, 4 Hz, supramaximal voltage for 10 s). Right: histograms showing the effects of the different drugs on the amplitude of the relaxation and of the contraction evoked by EFS (0.5-ms 4 Hz, supramaximal voltage for 10 s) in the longitudinal muscle of distal mouse colon. Data are means ± s.e.m. ($n = 4$–6 for each antagonist) and are expressed as a percentage of the respective control taken as 100%. The values for the control are the means of the control data obtained before each treatment. *$P < 0.05$ when compared with the respective control. §$P < 0.05$ when compared with atropine.

Data from our experiments indicate that, in the longitudinal muscle of distal colon, ATP may induce excitatory effects via the activation of ADPβS-sensitive P2Y purinoceptors. In fact, the contractile effects induced by ATP and by its stable analogue, ADPβS, were antagonized by the non-selective P2 antagonists, PPADS or suramin. ADPβS was more potent than ATP, owing to either less susceptibility to degradation by nucleotidases or higher specificity for the receptors mediating contraction, most likely to be the P2Y purinoreceptor subtypes. This latter conclusion is strengthened by the observation that desensitization of P2Y receptors with ADPβS, but not by desensitization of P2X receptors with α,β-meATP, antagonized the excitatory response to both ATP and ADPβS itself. Moreover, α,β-meATP, a P2X receptor agonist, did not modify the mechanical activity. In addition, contractions to ATP or ADPβS show desensitization, further supporting the involvement of P2Y, but not of P2X purinoceptors (Ralevic and Burnstock, 1998).

In the attempt to characterize the subtype of P2Y receptor mediating contraction, we tested ATP- and ADPβS-induced contractions in the presence of MRS 2179, a $P2Y_1$-selective antagonist. Interestingly, MRS 2179 enhanced the contractile responses to ATP and ADPβS, ruling out a role of $P2Y_1$ in the purinergic contraction. The increase induced by MRS 2179 could be explained assuming that ATP also acts on P2Y1 purinoreceptors mediating inhibitory effects, which would counteract its contractile effects. This hypothesis is corroborated by the observation that ADP, the most potent natural ligand at the $P2Y_1$ receptors, induced inhibitory effects in our preparations. Therefore, it is possible to conclude that ATP and ADPβS would act on two different P2Y receptors, one subserving the contractile response and one, probably $P2Y_1$ receptor subtypes, subserving inhibitory effects, which are normally overcome by the contraction. Data from our experiments do not allow us to identify the P2Y ADPβS-sensitive receptor subtype mediating contraction; however, excitatory P2Y receptors, producing contraction, activated by ADPβS have been reported in guinea-pig ileum and colon (Zagorodnyuk and Maggi, 1998; Sato *et al*., 1999), as well in non-intestinal smooth muscle (Najbar *et al*., 1996; Naramatsu *et al*., 1997). Our data seem to exclude the involvement of $P2Y_{12}$ receptors, because MRS 2395, a $P2Y_{12}$-selective antagonist, was not able to antagonise purine-induced contractions. Therefore, further experiments are required to address this issue.

Actually, biphasic effects, relaxation followed by contraction, induced by ATP have been described in the longitudinal muscle of the distal colon by Giaroni *et al*. (2002). In our experiments, even in the precontracted tissue, ATP was not able to relax colonic muscle, although sometimes it

induced a small relaxation at the highest concentration. However, this effect was abolished by P1 receptor antagonists, indicating that it was due to activation of these receptors. The presence of P1 receptors mediating relaxation and activated by adenosine has been already characterised in this tissue (Zizzo *et al*., 2006).

It is reported that purinergic contraction may be mediated by cholinergic neurons (Barthó *et al*., 1997, 2006; Matsuo *et al*., 1997) or by prostaglandin synthesis (Burnstock *et al*., 1975). In our preparation, purinergic contraction involves cholinergic step as shown by the sensitivity of the response to atropine, but it was not due to the release of prostaglandins, as it was insensitive to indomethacin. Indeed, purinergic contraction was enhanced in the presence of TTX suggesting the possibility that inhibitory $P2Y_1$ purinoreceptors are located at neuronal level. Block of NO synthesis with L-NAME increased the contractile responses to ATP and to ADPβS indicating that activation of P2Y1 receptors induces release of NO from inhibitory nerves, as suggested by Giaroni *et al*. (2002). An interplay between NO and purinergic system has been already reported in some gastrointestinal smooth muscle preparation of rodents (De Luca *et al*., 1999; Giaroni *et al*., 2002; Van Crombruggen and Lefebvre, 2004). Our conclusion is corroborated by the observation that ADP-induced relaxation was significantly antagonised by TTX or by L-NAME.

The responses to EFS consisted of an early relaxation, nitrergic in nature, followed by a muscular contraction. The contractile response was due to the activation of cholinergic and purinergic pathways, as it was antagonized by atropine and purinergic antagonists. In particular, the observation that it was reduced by the P2Y receptor desensensitization with ADPβS indicates a functional role of P2Y receptors in the nerve-evoked excitatory responses in the longitudinal muscle of the murine distal colon. Purinergic neurotransmission is known to occur between neurons in the enteric nervous system (Burnstock, 2001). However, considering the pharmacological profile of the response to exogenous ATP it is likely that P2Y receptors are located at post-junctional level. The contraction remaining after atropine was further reduced by P2Y desensitization suggesting that the purines, through P2Y receptors, may mediate the non-cholinergic component of the nerve-evoked response. Excitatory purinergic neurotransmission in the gut muscle has been reported also in other animal preparations (Zagorodnyuk and Maggi, 1998; Zhang and Paterson, 2005). The lack of the effect of MRS 2179 or of MRS 2395 on the relaxation or on the contraction indicates that $P2Y_1$ or $P2Y_{12}$ receptors are non-involved nerve-evoked responses.

Lastly, our previous researches demonstrated that ATP, in the circular muscle of mouse distal colon, caused only inhibitory responses (Serio *et al*., 2003), therefore purinoreceptor activation induces different effects on the two muscular layers: inhibitory on the circular muscle and excitatory on the longitudinal one. Because the two muscular layers show different behaviour during the propulsive activity, circular relaxation coupled with longitudinal contraction (Wood, 1998), our results would suggest a role of purines in the mechanism inducing the intestinal transit in mouse colon.

In conclusion, in the longitudinal muscle of mouse distal colon, ATP contributes to the excitatory neurotransmission acting directly on smooth muscle and indirectly via activation of cholinergic neurons. These effects are likely to be mediated by ADPβS-sensitive P2Y purinoceptors. In addition, P2Y1 purinergic receptors appear to be located on nitrergic inhibitory neurons, regulating the release of NO.

Acknowledgements

This work was supported by a grant from Ministero dell'Università e della Ricerca Scientifica, Italy.

Conflict of interest

The authors state no conflict of interest.

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