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RESEARCH PAPER

Interaction between cannabinoid CB_1 receptors and endogenous ATP in the control of spontaneous mechanical activity in mouse ileum

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Background and purpose: Although it is well accepted that cannabinoids modulate intestinal motility by reducing cholinergic neurotransmission mediated by CB_1 receptors, it is not known whether the endocannabinoids are involved in more complex circuits and if they interact with other systems. The aim of the present study was to examine possible interactions between cannabinoid CB_1 receptors and purines in the control of spontaneous contractility of longitudinal muscle in mouse ileum.
Experimental approach: The mechanical activity of longitudinally oriented ileal segments from mice was recorded as isometric contractions.
Key results: The selective CB_1 receptor agonist, N-(2-chloroethyl)5,8,11,14-eicosaetraenamide (ACEA) reduced, concentration dependently, spontaneous contractions in mouse ileum. This effect was almost abolished by tetrodotoxin (TTX) or atropine. Inhibition by ACEA was not affected by theophylline (P1 receptor antagonist) or by P2Y receptor desensitization with adenosine 5′[β-thio]diphosphate trilithium salt, but was significantly reversed by pyridoxal phosphate-6-azo(benzene-2,4-disulphonic acid) (P2 receptor antagonist), by P2X receptor desensitization with α,β-methyleneadenosine 5′-triphosphate lithium salt (α,β-MeATP) or by 8,8′-[carbonylbis(imino-4,1-phenylenecarbonylimino-4,1-phenylenecarbonylimino) bis(1,3,5-naphthalenetrisulphonic acid)] (P2X receptor antagonist). Contractile responses to α,β-MeATP (P2X receptor agonist) were virtually abolished by TTX or atropine, suggesting that they were mediated by acetylcholine released from neurones, and significantly reduced by ACEA.
Conclusion and implications: In mouse ileum, activation of CB_1 receptors, apart from reducing acetylcholine release from cholinergic nerves, was able to modulate negatively, endogenous purinergic effects, mediated by P2X receptors, on cholinergic neurons. Our study provides evidence for a role of cannabinoids in the modulation of interneuronal purinergic transmission.
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Keywords: cannabinoids; CB_1 receptors; purines; ATP; cholinergic transmission; P2X receptors; enteric nervous system

Abbreviations: ACEA, N-(2-chloroethyl)5,8,11,14-eicosaetraenamide; ACPA, N-(cyclopropyl)-5Z,8Z,11Z,14Z-eicosatetraemamide; ADPβS, adenosine 5′[β-thio]diphosphate trilithium salt; α,β-MeATP, α,β-methyleneadenosine 5′-triphosphate lithium salt; EFS, electrical field stimulation; NF279, 8,8′-[carbonylbis (imino-4,1-phenylenecarbonylimino-4,1-phenylenecarbonylimino) bis(1,3,5-naphthalenetrisulphonic acid)]; PPADS, pyridoxal phosphate-6-azo(benzene-2,4-disulphonic acid) tetrasodium salt; SR 141716A, (N-piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide hydrochloride; TTX, tetrodotoxin

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Introduction

Over the past decade, the identification of a system of endogenous cannabinoids has given the functional basis for the effects of the active constituents of *Cannabis sativa*. The

endocannabinoid system contains specific receptors, specific ligands and specific enzymes for synthesis and degradation of the endocannabinoids (De Petrocellis *et al.*, 2004). There are at least two established cannabinoid G-protein-coupled receptors, CB_1 and CB_2 receptors (nomenclature follows Alexander *et al.*, 2008). CB_1 receptors are highly expressed in the central and peripheral nervous systems, although non-neuronal expression sites have been also described (Cota *et al.*, 2003). CB_2 receptors are predominantly, but not exclusively, present in immune cells, suggesting that endocannabinoids may play a role as immunomodulators (Samson *et al.*, 2003).

The involvement of the cannabinoid system in the regulation of different gastrointestinal functions has been well established (see Izzo and Coutts, 2005; Massa *et al.*, 2005; Massa and Monory, 2006). In fact, several regulatory functions have been related to cannabinoids, including gut motility, ion transport, gastric and intestinal secretion, epithelial wound healing, feeding behaviour (Gomez *et al.*, 2002; MacNaughton *et al.*, 2004; Izzo and Coutts, 2005; Massa *et al.*, 2005; Wright *et al.*, 2005; Massa and Monory, 2006). CB_1 receptors have been identified on enteric nerves and their activation results in inhibition of excitatory transmission *in vitro* (Coutts and Pertwee, 1997; Croci *et al.*, 1998; Izzo *et al.*, 1998; Storr *et al.*, 2004; Mulè *et al.*, 2007a,b) and intestinal propulsion *in vivo* (Colombo *et al.*, 1998; Izzo *et al.*, 1999; 2001; Pinto *et al.*, 2002; Casu *et al.*, 2003; Carai *et al.*, 2006).

There is substantial evidence that the endocannabinoid system is involved in the control of intestinal motility, in both the small and large intestines. However, besides the reduction of cholinergic excitatory neurotransmission through prejunctional CB_1 receptors, little is known about the possible involvement of endocannabinoids in complex circuits and on crosstalk with other systems. Recent work has provided evidence for a cannabinoid role in the modulation of GABA function in the myenteric plexus-longitudinal muscle preparation of guinea pig ileum (Begg *et al.*, 2002b), whereas Carai *et al.* (2006) reported that cannabinoid and opioid receptor systems did not interact in regulating gastrointestinal transit in mice. However, a crosstalk between κ-opioid and cannabinoid CB_1 receptors may take place under inflammatory conditions (Capasso *et al.*, 2008). Apart from a study by Begg *et al.* (2002a) showing that activation of presynaptic CB_1 receptors reduces the electrically-evoked release of adenosine in guinea-pig ileum, to our knowledge, to date no study has addressed the issue of the functional relationship between cannabinoids and purines in the regulation of intestinal contractility.

Purine nucleotides and nucleosides play an important role in the modulation of motor functions in the gastrointestinal tract and both adenosine and ATP receptors participate in such regulation. Adenosine acts through P1 receptors that have been further subdivided in A_1, A_{2a}, A_{2B} and A_3 receptors (Ralevic and Burnstock, 1998). Selective receptors for ATP and ADP, designated as P2 receptors, have been divided into two families: P2X ionotropic receptors; and P2Y metabotropic G-protein coupled receptors (Abbracchio and Burnstock, 1994). Seven mammalian P2X receptor subtypes ($P2X_1$ up to $P2X_7$) and eight mammalian P2Y receptor subtypes ($P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$, $P2Y_{13}$, $P2Y_{14}$) have been cloned and functionally defined as P2 receptors (Burnstock, 2007). The motor effects (contractions and/or relaxation) induced by purines are species- and region-specific in the gastrointestinal tract. In particular, purines may act through postjunctional receptors to affect directly the muscular contractility or through neuronal receptors, to modulate neurotransmitter release, prejunctionally or pre-synaptically (Cunha and Ribeiro, 2000; Kadowaki *et al.*, 2000; Galligan, 2002; Giaroni *et al.*, 2002; Woods *et al.*, 2003; Zizzo *et al.*, 2006; Bornstein, 2008).

Recently, we have demonstrated that longitudinal smooth muscle of mouse ileum is under an excitatory tonic influence by neuronal acetylcholine, which can be negatively modulated by CB_1 receptor activation (Baldassano *et al.*, 2008). Therefore, the present study was undertaken in the attempt to examine a possible interaction between purinergic system and cannabinoids in this experimental model, and to clarify if modulation of the ongoing release of acetylcholine can be the final target for both systems. Specifically, we analysed the effects of blocking P1 and P2 (P2X and P2Y) receptors on the responses induced by N-(2-chloroethyl)5,8,11,14-eicosaetraenamide (ACEA), a selective CB_1 cannabinoid receptor agonist.

Methods

Animals

All animal procedures were in conformity with the Italian D.L. no. 116 of 27 January 1992 and associated guidelines in the European Communities Council Directive of 24 November 1986 (86/609/ECC). Adult male mice (C57BL/10SnJ; weighing 25.5 ± 0.5 g), obtained from Charles River Laboratories (Calco-Lecco, Italy) were used for the study. Animals were housed in standard conditions under a constant light–dark cycle, constant temperature (22 ± 1°C) and humidity (55 ± 5%), with free access to food and water.

General

Animals were killed by cervical dislocation. The abdomen was immediately opened and the ileum was removed and placed in a modified Krebs solution of the following composition (mmol·L^{-1}): NaCl 119; KCl 4.5; $MgSO_4$ 2.5; $NaHCO_3$ 25; KH_2PO_4 1.2, $CaCl_2$ 2.5, glucose 11.1. Segments (20 mm in length) were suspended in a four-channel organ bath containing 8 mL of oxygenated (95% O_2 and 5% CO_2) Krebs solution maintained to 37°C. The distal end of each segment was tied to organ holders and the proximal end was secured with a silk thread to an isometric force transducer (FORT 25, Ugo Basile, Biological Research Apparatus, Comerio VA, Italy). Mechanical activity was digitized on an analog-to-digital converter, visualized, recorded and analysed on a personal computer using the PowerLab/400 system (Ugo Basile). Longitudinal preparations were subjected to an initial tension of 2 mN and were allowed to equilibrate for at least 30 min. Rhythmic spontaneous contractions developed in all preparations.

Experimental protocol
After equilibration responses of the preparations to cumulative concentrations of two CB_1 cannabinoid receptor agonists, ACEA (0.01–10 μmol·L^{-1}) or N-(cyclopropyl)-5Z,8Z,11Z,14Z-eicosatetraemamide (ACPA; 0.01–10 μmol·L^{-1}), were evaluated. The concentration-response curves to ACEA were repeated after theophylline, a non-selective P1 purinoceptor antagonist (10 μmol·L^{-1} for 30 min), pyridoxal phosphate-6-azo(benzene-2,4-disulphonic acid) (PPADS), a non-selective P2 receptor antagonist (50 μmol·L^{-1} for 30 min), desensitization of P2Y receptors with adenosine 5′[β-thio]diphosphate trilithium salt (ADPβS; 10 μmol·L^{-1} for 30 min), desensitization of P2X receptors α,β-methyleneadenosine 5′-triphosphate lithium salt (α,β-MeATP) (50 μmol·L^{-1} for 30 min) or 8,8′-[carbonylbis(imino-4,1-phenylenecarbonylimino-4,1-phenylenecarbonylimino) bis(1,3,5-naphthalenetrisulphonic acid)] (NF279), a P2X receptor antagonist (1 μmol·L^{-1} for 30 min). These agents were added to the organ bath before the agonists. ACEA was added to the bath at increasing concentrations in volumes of 80 μL. Each ACEA concentration was left in contact with the tissue for 10 min. Each preparation was tested with a single purinoreceptor inhibitor. The concentrations of the inhibitors used were determined from published data.

In order to evaluate the efficacy of purinergic antagonists or desensitizing procedures, the effects induced by adenosine – a P1 purinoceptor agonist (1–100 μmol·L^{-1}), ATP – a P2 purinoceptor agonist (10 μmol·L^{-1}–1 mmol·L^{-1}), and α,β-MeATP – a P2X purinoceptor agonist (10–100 μmol·L^{-1}), were examined before and after the respective antagonists or desensitizing agents. Agonists were added into the bath at increasing concentrations in volumes of 80 μL and were applied for 3 min at 20 min intervals. α,β-MeATP (10–100 μmol·L^{-1}) was tested also in the presence of TTX (1 μmol·L^{-1}), atropine (1 μmol·L^{-1}) or ACEA (10 μmol·L^{-1}). These agents were added to the organ bath 30 min before the agonists.

In a different set of experiments to further explore interactions between CB_1 and P2X receptors in the modulation of cholinergic motor neurons, the effects of ACEA (10 μmol·L^{-1}) on the contractions evoked by electrical field stimulation (EFS) were evaluated in the absence and presence of NF279 (1 μmol·L^{-1}). EFS was applied by an S88 square-wave pulse generator (Grass Medical Instruments, Quincy, MA, USA) coupled via a stimulus isolation unit (Grass SIU5) to a pair of platinum electrodes placed in parallel on either side of the segments. EFS (0.5 ms duration, supramaximal voltage, in trains of 5 s, 8 Hz) was performed at 5 min intervals and stable and reproducible responses were observed for hours. The evoked contractions were abolished by the muscarinic receptor antagonist atropine (1 μmol·L^{-1}) or by the neuronal blocker TTX (1 μmol·L^{-1}) suggesting that they were mediated by cholinergic nerves.

Lastly, ACEA (0.01–10 μmol·L^{-1}) was tested on ileum precontracted by carbachol (1 μmol·L^{-1}).

Statistical analysis
Mean amplitude of spontaneous contractions was measured prior to and following drug administration after a new steady state was reached. Results are expressed as the changes in mean amplitude of the phasic contractions and reported as percentages of the values obtained in the control (e.g. –100% corresponds to the abolition of spontaneous activity). Relaxant and contractile effects induced by purinergic agonists were expressed respectively as a percentage of the maximal response. All data are expressed as mean values ± standard error of the mean. The letter *n* indicates the number of experimental animals. Statistical analysis was performed by means of two-way analysis of variance, followed by Bonferroni's *post hoc* test. A probability value of less than 0.05 was regarded as significant.

Drugs
The following drugs were used: ACEA, ACPA, adenosine, ATP, α,β-MeATP, ADPβS, atropine sulphate, carbachol, PPADS, theophylline, (Sigma Chemical Corp., St. Louis, MO, USA); NF279 (Tocris-Bioscience, Bristol, UK); TTX (Alomone Labs Ltd., Jerusalem, Israel). All drugs were dissolved in distilled water, except ACEA, adenosine and NF279, which were initially dissolved in dimethyl sulphoxide, and ACPA in ethanol. The working solutions were prepared freshly on the day of the experiments by diluting the stock solutions with Krebs solution. Control experiments using dimethyl sulphoxide alone, appropriately diluted, showed that it did not affect the contractility of the ileal segments.

Results

Isolated segments of mouse ileum displayed spontaneous activity, characterized by phasic contractions with amplitude of about 300 mg and frequency of about 30 cycles·min^{-1}. As previously described, these contractions were induced by acetylcholine released from neurones because they were reduced by atropine (1 μmol·L^{-1}) and increased by neostigmine (10 μmol·L^{-1}) (Figure 1) (Baldassano *et al.*, 2008).

CB_1 receptor activation and basal mechanical activity
The selective CB_1 receptor agonists, ACEA (0.01–10 μmol·L^{-1}) or ACPA (0.01–10 μmol·L^{-1}), produced concentration-dependent inhibitory effects, consisting in a decrease of the mean amplitude of spontaneous contractions, without changes in the resting tone. As previously shown (Baldassano *et al.*, 2008), the effects of ACEA were antagonized by SR141716A (0.1 μmol·L^{-1}), a CB_1 receptor antagonist, and it

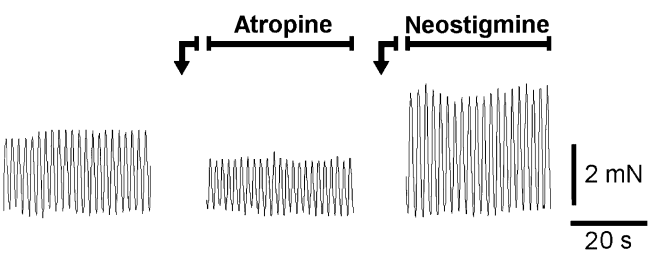

Figure 1 Typical tracings showing the effects of atropine (1 μmol·L^{-1}) or neostigmine (10 μmol·L^{-1}) on the spontaneous contractions of mouse ileum longitudinal muscle.

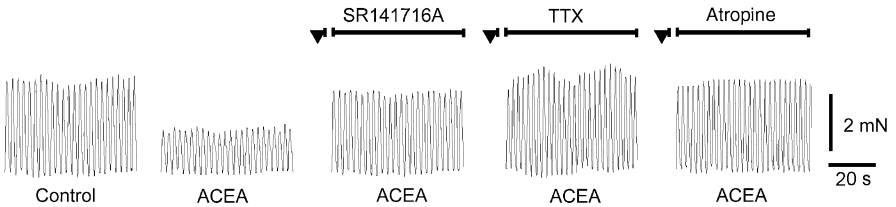

Figure 2 Typical tracings showing the inhibitory effects of the selective CB_1 receptor agonist N-(2-chloroethyl)5,8,11,14-eicosaetraenamide (ACEA; 10 μmol·L^{-1}) on the spontaneous mechanical activity of mouse ileal longitudinal muscle in control conditions or after SR141716A (100 nmol·L^{-1}), a CB_1 receptor antagonist, tetrodotoxin (TTX; 1 μmol·L^{-1}) or atropine (1 μmol·L^{-1}).

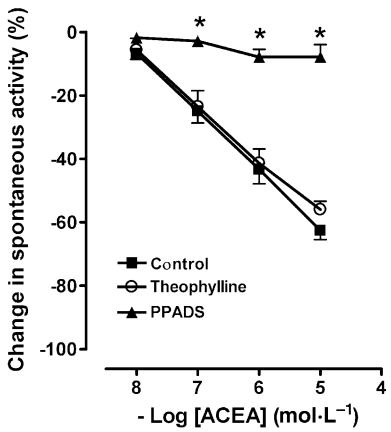

Figure 3 Concentration-response curves for the inhibitory effects induced by N-(2-chloroethyl)5,8,11,14-eicosaetraenamide (ACEA) in the control, in the presence of theophylline (10 μmol·L^{-1}), a P1 receptor antagonist or pyridoxal phosphate-6-azo(benzene-2,4-disulphonic acid) (PPADS; 50 μmol·L^{-1}), a P2 receptor antagonist. The inhibitory response is expressed as percent change of the resting activity (–100% corresponds to the abolition of spontaneous activity). All values are means ± standard error of the mean ($n = 5$ for each treatment). *$P < 0.05$ compared with control value.

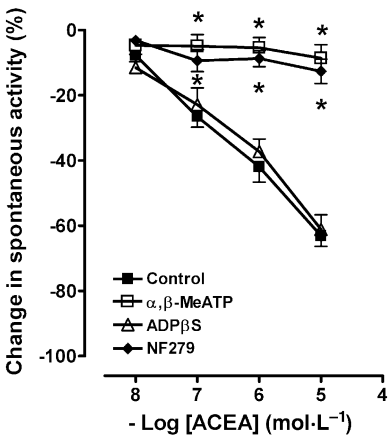

Figure 4 Concentration-response curves for the inhibitory effects induced by N-(2-chloroethyl)5,8,11,14-eicosaetraenamide (ACEA) in the control, after P2X receptor desensitization with α,β-methyleneadenosine 5′-triphosphate lithium salt (α,β-MeATP; 50 μmol·L^{-1} for 30 min), after P2Y receptor desensitization with adenosine 5′[β-thio]diphosphate trilithium salt (ADPβS; 10 μmol·L^{-1} for 30 min) or in the presence of NF279 (1 μmol·L^{-1}), a P2X receptor antagonist. The inhibitory response is expressed as percent change of the resting activity (–100% corresponds to the abolition of spontaneous activity). All values are means ± standard error of the mean ($n = 4$ for each treatment). *$P < 0.05$ compared with control value.

was almost abolished by the pretreatment with TTX (1 μmol·L^{-1}) or atropine (1 μmol·L^{-1}), suggesting that CB_1 receptor activation reduces an ongoing neural release of acetylcholine (Figure 2).

The inhibitory effects induced by ACEA (0.01–10 μmol·L^{-1}) were not affected by theophylline (10 μmol·L^{-1}), which per se did not affect the spontaneous mechanical activity, but they were almost abolished by PPADS (50 μmol·L^{-1} for 30 min), indicating that P2, but not P1, receptors were involved in the effects evoked by the selective CB_1 agonist (Figure 3).

In order to clarify the subtype of P2 receptors involved in the cannabinoid modulation of spontaneous activity, we tested the effects of ACEA after desensitization of P2Y or P2X purinoceptors . Desensitization of P2Y receptors with ADPβS (10 μmol·L^{-1} for 30 min) did not affect the spontaneous mechanical activity. A transient relaxation occurred on addition of ADPβS to the bath, after which the muscle recovered its basal tone. Desensitization of P2X purinoceptors with α,β-MeATP (50 μmol·L^{-1} for 30 min) produced a significant reduction in the amplitude of the phasic spontaneous contractions (43 ± 6%, $n = 5$; $P < 0.001$) that was more or less maintained as long as the drug was present. A transient contractile response occurred on addition of α,β-MeATP to the bath, after which the muscle recovered its basal tone.

The inhibition of the spontaneous contractions induced by ACEA (0.01–10 μmol·L^{-1}) were not affected by P2Y receptor desensitization with ADPβS, whereas they were almost abolished by P2X receptor desensitization with α,β-MeATP (Figure 4), indicating that P2X receptors are involved in these effects of ACEA. To corroborate this finding we tested the effects of NF279, a P2X receptor antagonist. NF279 (1 μmol·L^{-1}) markedly and significantly decreased the ACEA-induced inhibitory responses (Figure 4). In addition NF279 per se caused a concentration-dependent decrease of the spontaneous contractions, which was prevented by TTX (1 μmol·L^{-1}) or atropine (1 μmol·L^{-1}) (Figure 5). Moreover, NF279 (10 μmol·L^{-1}), abolished the increase of the spontaneous contraction amplitude induced by neostigmine (10 μmol·L^{-1}) (Figure 5).

Purine-mediated effects

Adenosine (1–100 μmol·L^{-1}) caused a concentration-dependent reduction of the amplitude of spontaneous contractions, which was significantly antagonized by theophylline (10 μmol·L^{-1}) (Figure 6).

ATP (10 μmol·L^{-1}–1 mmol·L^{-1}) evoked a rapid and transient concentration-dependent relaxation, which was followed

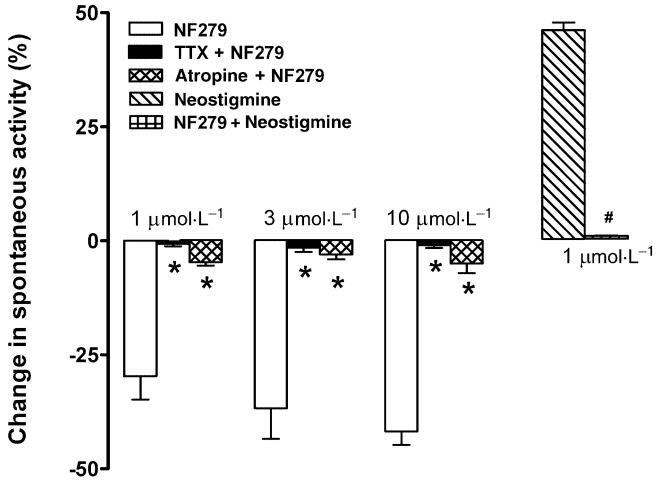

Figure 5 Inhibitory effects induced by NF279, on the amplitude of the spontaneous contractions in the control ($n = 8$), in the presence of tetrodotoxin (TTX; 1 μmol·L^{-1}) ($n = 4$) or in the presence of atropine (1 μmol·L^{-1}) ($n = 4$) and on the increase of the spontaneous contractions induced by neostigmine (10 μmol·L^{-1}) ($n = 4$). The ordinate shows the amplitude of the contractions expressed as percent change of the resting activity. All values are means ± standard error of the mean. *$P < 0.05$ compared with control value. #$P < 0.05$ compared with neostigmine.

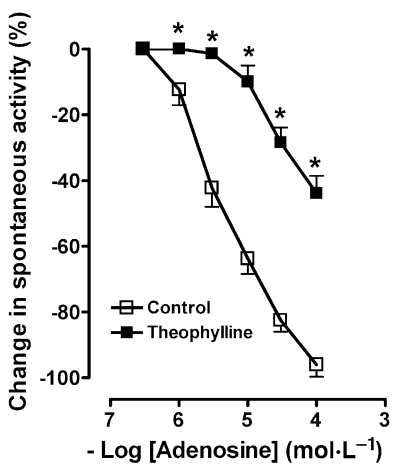

Figure 6 Concentration-response curves for the inhibitory effects induced by adenosine, a P1 receptor agonist, in the control or in the presence of theophylline (10 μmol·L^{-1}), a P1 receptor antagonist. The inhibitory response is expressed as percent change of the resting activity (–100% corresponds to the abolition of spontaneous activity). All values are means ± standard error of the mean ($n = 4$). *$P < 0.05$ compared with control value.

at the highest concentrations (0.1–1 mmol·L^{-1}) by a contraction. ATP responses were significantly reduced in the presence of PPADS (50 μmol·L^{-1}). The relaxation to ATP was abolished by desensitization of P2Y receptors with ADPβS (10 μmol·L^{-1} for 30 min), whereas the contractile responses were blocked by desensitization of P2X receptors with α,β-MeATP (50 μmol·L^{-1} for 30 min) (Figure 7). α,β-MeATP (10–100 μmol·L^{-1}), evoked a concentration-dependent contractile response, which was abolished by desensitization of P2X receptors with this agonist (50 μmol·L^{-1} for 30 min) and significantly antagonized by NF279 (10 μmol·L^{-1}). Moreover, the contraction induced by α,β-MeATP (10–100 μmol·L^{-1}) was significantly reduced by TTX (1 μmol·L^{-1}) or atropine (1 μmol·L^{-1}), suggesting the involvement of neuronal acetylcholine (Figure 8). Lastly, the contractile responses to α,β-MeATP were significantly reduced by ACEA (10 μmol·L^{-1}) (Figure 9).

CB_1 receptor activation and cholinergic evoked responses

EFS (supramaximal voltage, 0.5 ms pulse duration, in trains of 5 s, 8 Hz) of the isolated mouse ileum caused a cholinergically mediated twitch contraction that was completely abolished by atropine (1 μmol·L^{-1}) or by TTX (1 μmol·L^{-1}). ACEA (10 μmol·L^{-1}) markedly reduced the electrically evoked cholinergic contraction. This effect was significantly, but not completely, reduced in the presence of NF279 (1 μmol·L^{-1}), which per se reduced the EFS-induced contraction ($n = 5$) (Figure 10).

ACEA (0.01–10 μmol·L^{-1}) had no significant influence on the smooth-muscle contraction level stimulated by carbachol- (% change of tension – 2.3 ± 3.1, $n = 4$).

Discussion and conclusions

The results of the present study suggest that, in mouse ileum, activation of CB_1 receptors, apart from reducing acetylcholine release from cholinergic nerves, is able to modulate negatively an endogenous ongoing purinergic action on cholinergic neurons, mediated by P2X receptors.

In the gastrointestinal tract, acetylcholine is regarded as the major excitatory neurotransmitter and the prime regulator of gastrointestinal motility. The release of acetylcholine from enteric nerves is under a well-regulated pre-synaptic and/or pre-junctional control, involving specific neuronal receptors. These include cannabinoid CB_1 (Coutts and Pertwee, 1997; Croci *et al.*, 1998; Izzo *et al.*, 1998; Storr *et al.*, 2004; Mulè *et al.*, 2007b) and purinergic P1 and P2 receptors, which, upon activation, enhance or inhibit the release of acetylcholine, depending on the purinoceptor subtype involved (Sawynok and Jhamandasn, 1976; Vizi and Knoll, 1976; Kadowaki *et al.*, 2000; Lee *et al.*, 2001; De Man *et al.*, 2003a,b).

In longitudinal muscle of mouse ileum, functional evidence for an ongoing release of neuronal acetylcholine, which increases the spontaneous mechanical activity has been reported (Vial and Evans, 2001; Baldassano *et al.*, 2008). In this experimental model, activation of neuronal cannabinoid CB_1 receptors decreases the spontaneous mechanical contractions by modulating negatively the cholinergic excitatory influence (Baldassano *et al.*, 2008).

Our results suggest that the release of acetylcholine that sustains contraction through the activation of muscarinic receptors on the smooth muscle is facilitated by endogenous ATP, acting pre-synaptically on cholinergic neurons through P2X receptors. In fact, we performed experiments after prolonged exposure of the preparations to ADPβS or α,β-MeATP, which have been used as specific desensitizing agents to block P2Y or P2X receptor-mediated responses respectively (Ralevic and Burnstock, 1998; De Man *et al.*, 2003a; Serio *et al.*, 2003; Mulè *et al.*, 2005). Desensitization of P2X purinergic receptors

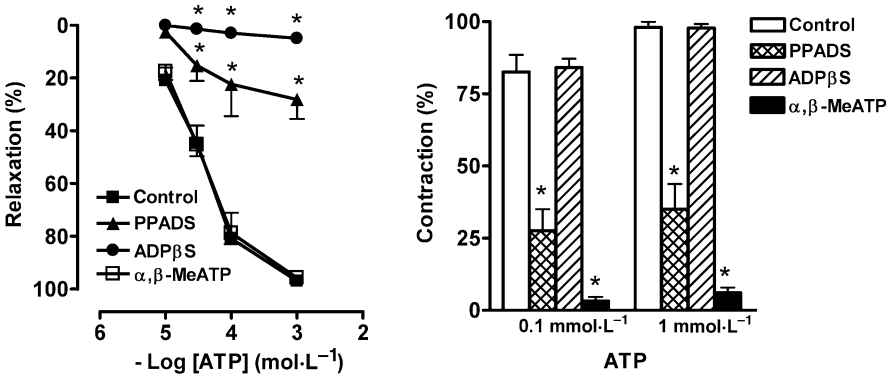

Figure 7 Relaxant or contractile effects evoked by ATP (10 μmol·L^{-1}–1 mmol·L^{-1}), a P2 receptor agonist, in mouse ileal longitudinal muscle in control and after pyridoxal phosphate-6-azo(benzene-2,4-disulphonic acid) (PPADS; 50 μmol·L^{-1}), a P2 receptor antagonist, desensitization of P2Y receptors with adenosine 5′[β-thio]diphosphate trilithium salt (ADPβS; 10 μmol·L^{-1} for 30 min) or desensitization of P2X receptors with α,β-methyleneadenosine 5′-triphosphate lithium salt (α,β-MeATP; 50 μmol·L^{-1} for 30 min). All values are means ± standard error of the mean. (n = 5 for each treatment) and are reported as a percentage of the maximal effect. *P < 0.05 compared with control value.

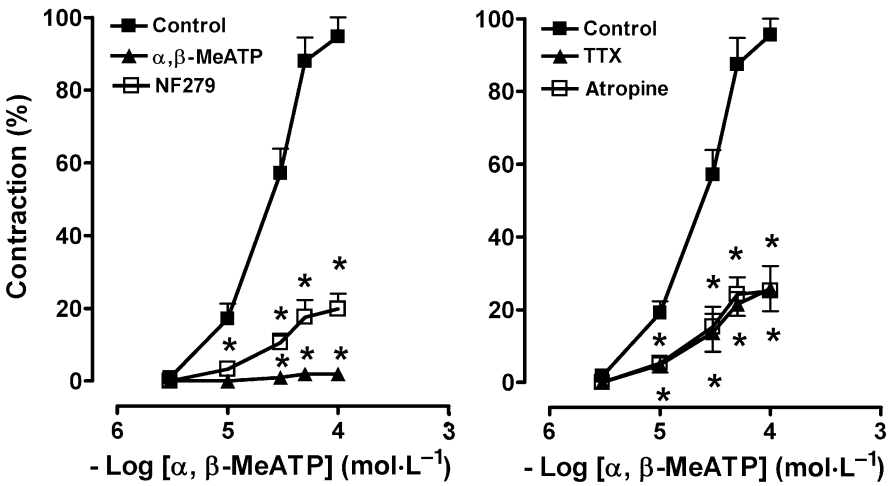

Figure 8 Concentration-response curves for the contractile effects induced by α,β-methyleneadenosine 5′-triphosphate lithium salt (α,β-MeATP), a P2X receptor agonist in the control, after P2X receptor desensitization with α,β-MeATP (50 μmol·L^{-1} for 30 min), in the presence of NF279 (1 μmol·L^{-1}), tetrodotoxin (TTX; 1 μmol·L^{-1}) or atropine (1 μmol·L^{-1}). All values are means ± standard error of the mean (n = 5 for each treatment) and are reported as a percentage of the maximal effect. *P < 0.05 compared with control value.

with α,β-MeATP, which antagonized the contraction to ATP or to α,β-MeATP in our preparations, reduced significantly the spontaneous contractions, whereas desensitization of P2Y receptors, which antagonized the relaxation evoked by ATP, failed to affect the amplitude of spontaneous contractions. These observations suggest a role for P2X receptors, but not for P2Y receptors, in control of the spontaneous motility in mouse ileum. On the other hand, theophylline, at a concentration effective in antagonizing the inhibitory effects on muscular contractions induced by adenosine, did not affect the spontaneous mechanical activity, ruling out a role for purinergic P1 receptors in the control of spontaneous contractility in the mouse ileum. In addition, NF279, a compound that preferentially blocks P2X1 receptors (Rettinger *et al.*, 2000), but also inhibits P2X2 and P2X3 receptor-mediated responses (Damer *et al.*, 1998; Klapperstuck *et al.*, 2000), reduced in a concentration-dependent manner, the spontaneous contractions and this action was no longer observed after block of nerve action potentials with TTX or antagonism of muscarinic receptors with atropine. NF279, which antagonized the contraction to α,β-MeATP, also abolished the increase of spontaneous contractions caused by neostigmine, which prevents the metabolic deactivation of acetylcholine, supporting our hypothesis that endogenous activation of P2X receptors facilitates the ongoing release of neuronal acetylcholine. On the other hand, the hypothesis that in our preparation P2X receptors are functionally expressed on cholinergic nerves is supported by the observations that α,β-MeATP, which activates homomeric P2X receptors composed of P2X1 or P2X3 subunits (North, 2002), caused a contractile response virtually abolished by TTX or atropine and the block of P2X receptors by NF279 reduced significantly the cholinergic contractile response to electrical nerve activation. The relatively high concentration of α,β-MeATP required to evoke a response may reflect restricted access of the agonist to the P2X receptors on enteric neurons. Although immunohistochemistry failed to show P2X1 receptor reactivity in the myenteric plexus of the mouse ileum (Vial

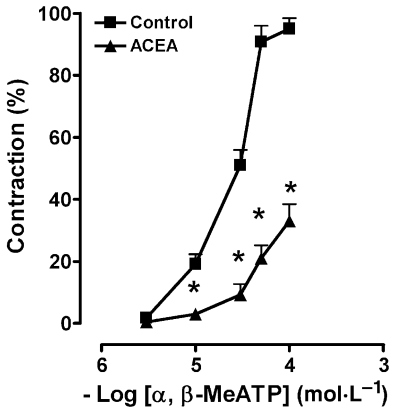

Figure 9 Concentration-response curves for the contractile effects induced by α,β-methyleneadenosine 5′-triphosphate lithium salt (α,β-MeATP), P2X receptor agonist in the control or after N-(2-chloroethyl)5,8,11,14-eicosaetraenamide (ACEA; 10 μmol·L^{-1}). All values are means ± standard error of the mean ($n = 4$) and are reported as a percentage of the maximal effect. *$P < 0.05$ compared with control value.

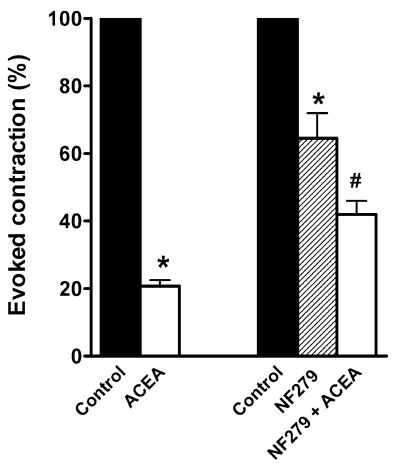

Figure 10 Inhibitory effects induced by N-(2-chloroethyl) 5,8,11,14-eicosaetraenamide (ACEA; 10 μmol·L^{-1}) on the contractions evoked by EFS (supramaximal voltage, 0.5 ms pulse duration, in trains of 5 s, 8 Hz) in the absence or in the presence of 8,8′-[carbonylbis(imino-4,1-phenylenecarbonylimino-4,1-phenylenecarbonylimino) bis(1,3,5-naphthalenetrisulphonic acid)] (NF279; 1 μmol·L^{-1}). All values are means ± standard error of the mean ($n = 5$) and are expressed as a percentage of the respective control taken as 100%. *$P < 0.05$ compared with control value. #$P < 0.05$ compared with ACEA or NF279.

and Evans, 2001; Giaroni *et al*., 2002), there is functional evidence for the presence of pre-synaptic P2X1 receptors in the mouse enteric nervous system (Vial and Evans, 2001) and P2X3 receptors regulate intestinal peristalsis in the mouse (Bian *et al*., 2003). Indeed, Vial and Evans (2001) showed that in mouse ileum the contractions to α,β-MeATP were partially blocked by TTX, but not by atropine, and ATP evoked relaxation but no contraction. The discrepancy with our results could be explained by considering the pre-load of the muscular segments. In our experiments, longitudinal preparations were subjected to an initial tension of 200 mg rather than 1 g. It is well accepted that cholinergic nerve terminals are easily damaged by excessive stretching and 1 g in mouse ileum could represent a considerable tension, which facilitates the revealing of relaxation, but impairs cholinergic pathways.

To assess if modulation of the ongoing release of acetylcholine can be the final target for both the cannabinoid and purine systems, we tested the inhibitory effects induced by ACEA in the presence of purinergic antagonists or desensitizing agents. We found that the response to the CB_1 agonist was virtually abolished by PPADS, desensitization of P2X receptors with α,β-MeATP or NF279 but not modified by theophylline or desensitization of P2Y receptors with ADPβS. These results suggest that there is a functional link between PPADS-sensitive P2X receptors and CB_1 receptors. It is possible that the activation of CB_1 receptors negatively modulates activity of purinergic neurones, which, in turn, *via* P2X receptors, triggers acetylcholine release from cholinergic neurones. Indeed, one might object that the reduction of the spontaneous contractions caused by the block of P2X with α,β-MeATP or NF279 could account for the inhibition of ACEA actions. However, this supposition can be ruled out because ACEA kept on inhibiting when the spontaneous mechanical activity was reduced by a sub-maximal concentration of adenosine (data not shown). To further support the hypothesis of a relationship between CB_1 and P2X receptors we tested ACEA on electrically induced cholinergic contractions in the absence and presence of NF279. The observations that the CB_1 agonist reduced the electrically evoked cholinergic responses and this effect was significantly, but not completely, reduced in the presence of NF279 would confirm that CB_1 receptor activation is able to modulate not only the prejunctional release of acetylcholine but also the pre-synaptic purinergic pathway, which in turn activates P2X receptors. On the other hand, the cannabinoid agonist failed to affect the ileal tone in segments contracted by carbachol, which acts by activating muscarinic receptors on the smooth muscle, ruling out an involvement of post-junctional mechanisms in its action.

So far, an interaction between CB_1 and P2X receptors has been documented only in nociceptive sensory neurons (Krishtal *et al*., 2006). To our knowledge, this is the first evidence for a possible role of cannabinoids in the modulation of enteric interneuronal purinergic transmission. Although recent data have ruled out an effect of anandamide on neuro-neuronal interaction in rodent small intestine (Yuece *et al*., 2007), our findings are in agreement with the studies by Lòpez-Redondo *et al*. (1997), who proposed, in guinea pig ileum, a neuro-neuronal action site of cannabinoids, because cannabinoid agonists were able to reduce neuronal excitatory postsynaptic potentials. Moreover, in the same preparation, Begg *et al*. (2002a) showed that, in addition to being able to reduce acetylcholine release, activation of pre-synaptic CB_1 receptors also reduces the electrically evoked release of adenosine. Therefore, cannabinoids can have a more complex spectrum of actions than has previously been supposed. However, the observation that – in our experiments – contractile responses to the P2X receptor agonist, α,β-MeATP, which are mediated by acetylcholine release, were significantly reduced by ACEA, suggests that CB_1 receptors are also located post-synaptically to inhibit cholinergic nerves, in line with previous observations suggesting a pre-junctional inhibitory role on the excitatory cholinergic neurotransnission (Coutts and Pertwee,

1997; Croci *et al*., 1998; Izzo *et al*., 1998; Storr *et al*., 2004; Mulè *et al*., 2007b).

In conclusion, in mouse ileum, cannabinoids, through CB_1 receptors, negatively modulate acetylcholine release, acting not only prejunctionally on cholinergic neurons, but also interfering with the purinergic system. In fact, endogenous ATP, through P2X receptors, would sustain the ongoing release of acetylcholine and this action can be influenced by CB_1 receptor activation. The proposed mechanism might participate in the suppression of gastrointestinal motor function following activation of the cannabinoid receptors in the bowel.

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Conflict of interest

None.

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