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RESEARCH PAPER

Relative contribution of ecto-ATPase and ecto-ATPDase pathways to the biphasic effect of ATP on acetylcholine release from myenteric motoneurons

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Background and purpose: The relative contribution of distinct ecto-nucleotidases to the modulation of purinergic signalling may depend on differential tissue distribution and substrate preference.
Experimental approach: Extracellular ATP catabolism (assessed by high-performance liquid chromatography) and its influence on [^{3}H]acetylcholine ([^{3}H]ACh) release were investigated in the myenteric plexus of rat ileum *in vitro*.
Key results: ATP was primarily metabolized via ecto-ATPDase (adenosine 5′-triphosphate diphosphohydrolase) into AMP, which was then dephosphorylated into adenosine by ecto-5′-nucleotidase. Alternative conversion of ATP into ADP by ecto-ATPase (adenosine 5′-triphosphatase) was more relevant at high ATP concentrations. ATP transiently increased basal [^{3}H]ACh outflow in a 2′,3′-O-(2,4,6-trinitrophenyl)adenosine-5′-triphosphate (TNP-ATP)-dependent, tetrodotoxin-independent manner. ATP and ATPγS (adenosine 5′-[γ-thio]triphosphate), but not α,β-methyleneATP, decreased [^{3}H]ACh release induced by electrical stimulation. ADP and ADPβS (adenosine 5′[β-thio]diphosphate) only decreased evoked [^{3}H]ACh release. Inhibition by ADPβS was prevented by MRS 2179 (2′-deoxy-N^6-methyl adenosine 3′,5′-diphosphate diammonium salt, a selective $P2Y_1$ antagonist); blockade of ADP inhibition required co-application of MRS 2179 plus adenosine deaminase (which inactivates endogenous adenosine). Blockade of adenosine A_1 receptors with 1,3-dipropyl-8-cyclopentyl xanthine enhanced ADPβS inhibition, indicating that $P2Y_1$ stimulation is cut short by tonic adenosine A_1 receptor activation. MRS 2179 facilitated evoked [^{3}H]ACh release, an effect reversed by the ecto-ATPase inhibitor, ARL67156, which delayed ATP conversion into ADP without affecting adenosine levels.
Conclusions and implications: ATP transiently facilitated [^{3}H]ACh release from non-stimulated nerve terminals via prejunctional P2X (probably $P2X_2$) receptors. Hydrolysis of ATP directly into AMP by ecto-ATPDase and subsequent formation of adenosine by ecto-5′-nucleotidase reduced [^{3}H]ACh release via inhibitory adenosine A_1 receptors. Stimulation of inhibitory $P2Y_1$ receptors by ADP generated alternatively via ecto-ATPase might be relevant in restraining ACh exocytosis when ATP saturates ecto-ATPDase activity.
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Abbreviations: 2-MeSADP, 2-methylthio-adenosine diphosphate; α,β-MeATP, α,β-methylene adenosine 5′-triphosphate; ADA, adenosine deaminase; ADPβS, adenosine 5′[β-thio]diphosphate; ARL 67156, 6-N,N-diethyl-D-β,γ-dibromomethylene-D-adenosine-5-triphosphate; ATPase, adenosine 5′-triphosphatase; ATPDase, adenosine 5′-triphosphate diphosphohydrolase; ATPγS, adenosine 5′-[γ-thio]triphosphate; DMSO, dimethylsulphoxide; DPCPX, 1,3-dipropyl-8-cyclopentyl xanthine; EGTA, ethylene glycol-bis(2-aminoethylether)-N,N,N′,N′-tetraacetic acid; IMP, inosine monophosphate; MRS 2179, 2′-deoxy-N^6-methyl adenosine 3′,5′-diphosphate diammonium salt; PPADS, pyridoxal phosphate-6-azo(benzene-2,4-disulphonic acid) tetrasodium salt; R-PIA, R-N^6-phenylisopropyl adenosine; RB-2, reactive blue-2; TNP-ATP, 2′,3′-O-(2,4,6-trinitrophenyl)adenosine-5′-triphosphate; TTX, tetrodotoxin

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Introduction

ATP contained in synaptic vesicles is released from stimulated nerve terminals as well as from non-neuronal stressed cells (see Bodin and Burnstock, 2001), and it can act either as a fast neurotransmitter or as a presynaptic neuromodulator in most synapses (see Cunha and Ribeiro, 2000). Purine nucleotides influence both motor and secretor functions in the gastrointestinal tract (Giaroni *et al.*, 2002). ATP generally produces hyperpolarization and relaxation of the gut; however, conflicting results showing ATP contribution to excitatory neurotransmission in some regions of the gut from various animal species have been reported (e.g. Giaroni *et al.*, 2002; Barthó *et al.*, 2006; Zizzo *et al.*, 2007). $P2Y_1$ receptors were identified in enteric neurons and smooth muscle cells by immunohistochemistry; reactivity to $P2Y_2$ receptors sensitive to uridine nucleosides was also found in a smaller number of neurons (Giaroni *et al.*, 2002). Electrophysiological studies indicate that ATP mediates slow excitatory post-synaptic potentials (sEPSP) in NO synthase-containing descending interneurons in the myenteric plexus through a yet unidentified P2Y receptor, while the ADP-sensitive $P2Y_1$ receptor seems to be involved in sEPSP in submucosal S neurons (Galligan, 2002). Additionally, ATP is the predominant non-cholinergic fast synaptic transmitter in murine enteric neurons through the activation of $P2X_2$ and $P2X_3$ subunit-containing receptors (Galligan, 2002). Homomeric $P2X_2$ receptors were described in S type neurons while heteromeric $P2X_2/P2X_3$ receptors exist in AH type neurons (Galligan and North, 2004).

The study of nucleotide receptors and their functions is complicated by the presence on the cell surface of enzymes that rapidly break down extracellular nucleotides into nucleosides, the ecto-nucleotidases (Zimmermann, 2000). One nucleoside in particular, adenosine, directly activates P1 receptors located on smooth muscle fibres (Nicholls *et al.*, 1996) and enteric neurons, where it modulates the release of excitatory neurotransmitters, like substance P (Moneta *et al.*, 1997) and acetylcholine (Duarte-Araújo *et al.*, 2004a). The myenteric plexus contains the enzymes responsible for the formation of adenosine from ATP released from activated smooth muscle cells (Nitahara *et al.*, 1995) as well as from stimulated myenteric neurons (White and Leslie, 1982). Although the extracellular catabolism of ATP via the ecto-nucleotidase pathway contributes only partially to the total interstitial nucleoside concentration in the myenteric plexus (Correia-de-Sá *et al.*, 2006), it might be relevant under pathological conditions (e.g. intestinal ischaemia, chronic inflammation) when extracellular ATP levels become increased (Marquardt *et al.*, 1984; Bogers *et al.*, 2000).

Among the nucleotidases, four members of the ecto-nucleoside triphosphate diphosphohydrolase (E-NTPDase) family, namely NTPDase1, NTPDase2, NTPDase3 and NTPDase8, and two members of the ecto-nucleotide pyrophosphatases/phosphodiesterases (E-NPP) family, NPP1 and NPP3, are located at the plasma membrane and hydrolyse extracellular nucleotides (Zimmermann, 2000; Kukulski *et al.*, 2005; Stefan *et al.*, 2005). NTPDases dephosphorylate a variety of nucleoside triphosphates (like, ATP and UTP) and diphosphates (like, ADP and UDP) with different substrate specificity and ability. NTPDase1 [also called CD39, ATPDase (adenosine 5′-triphosphate diphosphohydrolase) or apyrase, EC 3.6.1.5] dephosphorylates ATP directly to AMP, removing one phosphate at a time with almost no release of the intermediate ADP. NTPDase2 [CD39L1, ATPase (adenosine 5′-triphosphatase), EC 3.6.1.3] conversely is a preferential nucleoside triphosphatase and hydrolyses ADP 10 to 15 times less efficiently than ATP, leading to minimal AMP accumulation (Matsuoka and Ohkubo, 2004). NTPDase3 (CD39L3 or HB6) and NTPDase8 (hepatic ATPDase) are described as functional intermediates between NTPDase1 and NTPDase2 (Kukulski *et al.*, 2005). Because their involvement in physiological processes, namely blood clotting, vascular inflammation, immune reactions and certain types of cancer, NTPDases are now considered as potential new drug targets (Gendron *et al.*, 2002). As for NPP1 and NPP3 (EC 3.6.1.9), they release nucleoside 5′-monophosphate from a variety of nucleotides and nucleotide derivatives, but intriguingly, their phosphorylated product (e.g. AMP) bind to NPPs with a higher affinity than their substrates do, and thus inhibit catalysis (Stefan *et al.*, 2005). Finally, AMP is hydrolysed to adenosine and inorganic phosphate by ecto-5′-nucleotidase (CD73, EC 3.1.3.5), a glycosylphosphatidylinositol-anchored enzyme located at the cell surface. It is interesting to note that ecto-5′-nucleotidase activity is concentrated in the myenteric smooth muscle cell layer (Nitahara *et al.*, 1995).

This sequential degradation not only terminates ATP signalling but also generates intermediates with distinct signalling properties. The relative contribution of distinct ecto-nucleotidase species to the modulation of purinergic signalling may depend on differential tissue and cell distribution, regulation of expression, targeting to specific membrane domains, but also on substrate availability and substrate preference. Our results showed that ATP is converted preferentially into AMP, which is then sequentially dephosphorylated into adenosine by ecto-5′-nucleotidase at the longitudinal muscle-myenteric plexus of the rat ileum; alternative conversion of ATP into ADP becomes relevant upon increasing the extracellular concentration of ATP. Understanding the effective contribution of the ADP-shunt is of central importance to predict when purinergic neuromodulation mediated by ADP-sensitive P2 receptors gains functional relevance. It has been reported that purinergic neurotransmission may be mediated by cholinergic neurons (Matsuo *et al.*, 1997; Barthó *et al.*, 2006). In the present work, we studied the pattern of extracellular ATP catabolism with particular emphasis on the relative contribution of ecto-ATPase (forming ADP) and of ecto-ATPDase (bypassing ADP formation) pathways, in order to assess their role in controlling [^{3}H]acetylcholine ([^{3}H]ACh) release from the myenteric plexus of the rat ileum.

Methods

Preparation and experimental conditions

Animal handling and experiments followed the guidelines of the International Council for Laboratory Animal Science (ICLAS). Rats (Wistar, 150–200 g) of either sex (Charles River, Barcelona, Spain) were kept at a constant temperature (21°C) and a regular light (06.30–19.30 h) dark (19.30–06.30 h) cycle

with food and water *ad libitum*. The animals were killed after stunning followed by exsanguination. The experiments were performed on myenteric plexus-longitudinal muscle preparations from the rat ileum (Paton and Vizi, 1969) superfused with gassed (95% O_2 and 5% CO_2) Tyrode's solution containing (mmol·L^{-1}): NaCl 137, KCl 2.7, $CaCl_2$ 1.8, $MgCl_2$ 1, NaH_2PO_4 0.4, $NaHCO_3$ 11.9, glucose 11.2 and choline 0.001, at 37°C.

Kinetic experiments and high-performance liquid chromatography (HPLC) analysis

For kinetic experiments of nucleotide or nucleoside catabolism, the preparations were mounted in a 3 mL organ bath and superfused with gassed (95% O_2 and 5% CO_2) Tyrode's solution kept at 37°C. Because enzymatic activity might not be linearly related to the weight of the preparations, care was taken to use preparations of similar weight (26.7 ± 1.6 mg, $n = 19$). After a 30 min equilibration period, the organ bath was emptied, and 2 mL of a 30 μmol·L^{-1} solution of ATP or ADP in Tyrode's solution was added to the preparations at zero time. Samples of 75 μL were collected from the organ bath at different times up to 45 min for HPLC (L-6200 intelligent pump with L-4000 UV Detector, Hitachi, Germany) analysis of the variation of substrate disappearance and product formation (see Cunha and Sebastião, 1991). The actual concentrations of ATP, ADP, AMP, adenosine, inosine and hypoxanthine were expressed in μmol·L^{-1}. Concentrations of the substrate and products were plotted as a function of time (progress curves). The following parameters were analysed for each progress curve: half-degradation time of the initial substrate, time of appearance of the different concentrations of the products, concentration of the substrate or any product remaining at the end of the experiment. When modification of the extracellular catabolism of an initial substrate by an inhibitor was tested, the preparations were first incubated for at least 15 min with the modifiers before starting the kinetic experiment still in the presence of the modifier (see Cunha and Sebastião, 1991). In all experiments, the concentration of products at the different times of sample collection was corrected by subtracting the concentration of products in samples collected from the same preparation incubated without adding substrate. At the end of experiments, the remaining incubation medium was collected and used to quantify the lactate dehydrogenase (EC 1.1.1.27) activity. The negligible (0.12 ± 0.01 U·mL^{-1}, $n = 20$) activity of lactate dehydrogenase in bath samples collected at the end of the experiments is an indication of the integrity of the cells during the experimental period. Incubation of the preparations for 45 min with the bathing solution (i.e. with no added substrate) only produced spontaneous release of variable amounts of inosine monophosphate (IMP), which were never higher than 2 μmol·L^{-1}. The spontaneous degradation of adenine nucleotides and adenosine at 37°C in the absence of the preparation was negligible (0–5%) over 45 min.

[^{3}H]-acetylcholine release experiments

The procedures used for labelling the preparations and measuring evoked [^{3}H]ACh release were previously described (Duarte-Araújo *et al.*, 2004a) and used with minor modifications. Longitudinal muscle-myenteric plexus strips were mounted in vertical perfusion chambers of 3 mL capacity heated at 37°C. After a 30 min equilibration period, the preparations were incubated in Tyrode's solution containing 1 μmol·L^{-1} [^{3}H]choline (specific activity 2.5 μCi·nmol^{-1}) for 40 min. During the loading period the preparations were continuously stimulated with supramaximal square wave pulses of 1 ms duration delivered at 1 Hz frequency through an SD9 stimulator (Grass Instrument, Quincy, USA), and two platinum electrodes placed above and below the suspended muscle strip (electrical field stimulation; EFS). Following loading, muscle strips were washed by superfusion (15 mL·min^{-1}) with Tyrode's solution containing hemicholinium-3 (10 μmol·L^{-1}), which remained in the bathing medium until the end of the experiment to prevent choline uptake. After a 60 min period of washout, bath samples (2 mL) were automatically collected every 3 min by emptying and refilling the organ bath with the solution in use, by using a fraction collector (Gilson, FC 203B, France) coupled to a peristaltic pump (Gilson, Minipuls3, France) programmed device. Aliquots (0.5 mL) of the incubation medium were added to 3.5 mL of Packard Insta Gel II (USA) scintillation cocktail. Tritium content of the samples was measured by liquid scintillation spectrometry (% counting efficiency: 40 ± 2%) after appropriate background subtraction, which did not exceed 5% of the tritium content of the samples. The radioactivity was expressed as disintegrations per minute (dpm) per gram of wet weight of the tissue, determined at the end of the experiment. After the loading and washout periods, the preparation contained (10648 ± 324) × 10^3 dpm·g^{-1}, and the resting release was (115 ± 18) × 10^3 dpm·g^{-1} in 3 min ($n = 8$). When the fractional release was calculated, this value was 1.08 ± 0.14% of the radioactivity present in the tissue in the first collected sample.

[^{3}H]-acetylcholine release was evoked by EFS, starting in the twelfth (S_1) and thirty-ninth (S_2) minute after beginning of the release period, each consisting of 200 square wave pulses of 1 ms duration delivered at a 5 Hz frequency. The method of stimulation was the same as during the labelling period, except for the stimulation rate. Others have used this methodology to study the contractile responses induced by electrical stimulation of the myenteric plexus (see De Man *et al.*, 2003). In some experiments [^{3}H]ACh release was induced by 3 min application of ATP: only in S_2 [EFS in S_1 and ATP (1–300 μmol·L^{-1}) in S_2] or in either period [without EFS, ATP (100 or 300 μmol·L^{-1}) in S_1 and S_2]. As 97% of the radioactivity release in response to EFS is [^{3}H]ACh (Kilbinger and Nafziger, 1985), no attempt was made to separate labelled choline from ACh. Prevention of tritium outflow in the absence of external calcium [Ca^{2+}0 + EGTA (ethylene glycol-bis(2-aminoethylether)-N,N,N′,N′-tetraacetic acid), 1 mmol·L^{-1}] and in the presence of 1 μmol·L^{-1} tetrodotoxin (TTX) (Duarte-Araújo *et al.*, 2004a) indicates that evoked [^{3}H]ACh release results from vesicle exocytosis of depolarized nerve terminals. In both cases, evoked [^{3}H]ACh release was calculated by subtracting the basal from the total tritium outflow during the stimulation period.

Test drugs were added 15 min before S_2 and were present up to the end of the experiments. The change in the ratio between the evoked [^{3}H]ACh release during the two stimulation periods (S_2/S_1) relative to that observed in control situa-

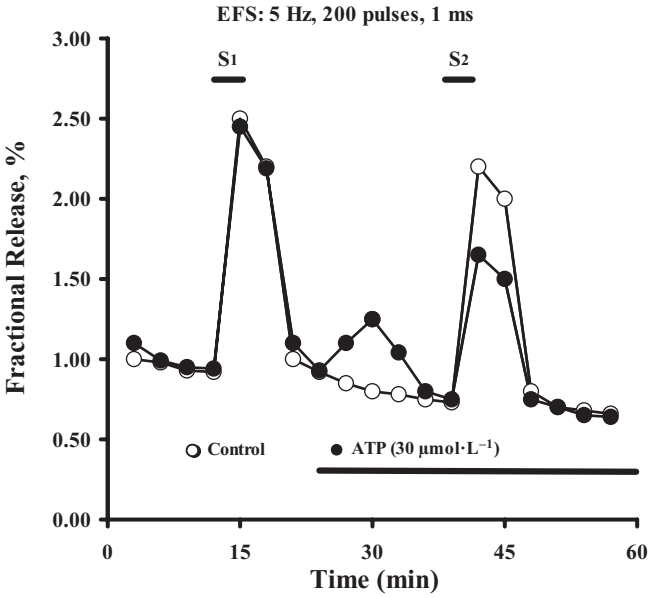

Figure 1 Effect of ATP (30 µmol·L^{-1}) on tritium outflow from the longitudinal muscle-myenteric plexus of the rat ileum. The time course of tritium outflow taken from a typical experiment is shown. Tritium outflow (ordinate) is expressed as a percentage of the total radioactivity present in the tissue at the beginning of the collection period (Fractional Release, %). The release of [^{3}H]acetylcholine ([^{3}H]ACh) in response to electrical field stimulation (EFS; 200 pulses of 1 ms duration delivered at a 5 Hz frequency) was elicited twice during the periods indicated (S_1 and S_2). ATP (30 µmol·L^{-1}) was applied 15 min before S_2 (as represented by the horizontal bar). The time course of tritium outflow in a control situation, that is, in the absence of ATP, is also shown for comparison. Note that ATP (30 µmol·L^{-1}) transiently increased the resting tritium outflow, but decreased the release of [^{3}H]ACh from the stimulated myenteric plexus.

tions (in the absence of test drugs) was taken as a measure of the effect of the tested drugs. When we evaluated the modifications of the effect of tested drugs by a modifier, this modifier was applied 15 min before starting sample collection and hence was present during S_1 and S_2. When the same drug was present in S_1 and S_2, the S_2/S_1 ratios were not significantly ($P > 0.05$) different from those obtained in control conditions, that is, without addition of drugs. None of the drugs, with the exception of ATP, changed significantly ($P > 0.05$) basal tritium outflow (see Figure 1).

Materials and solutions

Adenosine, ADA (type VI, 1803 U·mL^{-1}, EC 3.5.4.4), ADP, ADPβS, 2-MeSADP, α,β-MeATP, ATP, ATPγS, choline chloride, EGTA, DPCPX, hemicholinium-3, PPADS, RB-2 and R-PIA were from Sigma (St Louis, MO, USA). ARL 67156, MRS 2179 and TNP-ATP were from Tocris Cookson Inc. (UK); [methyl-^{3}H]choline chloride (ethanol solution, 80 Ci·mmol^{-1}) was from Amersham (UK).

DPCPX was made up in a 5 mmol·L^{-1} stock solution in 99% DMSO/1% NaOH 1 mol·L^{-1} (v·v^{-1}). R-PIA was made up in a 50 mmol·L^{-1} stock solution in DMSO. ADPβS, MRS 2179 and PPADS were made up as 3 mmol·L^{-1}, while 2-MeSADP and TNP-ATP were made up as 10 mmol·L^{-1} stock solutions in distilled water. All the other compounds were dissolved in Tyrode's solution. RB-2 was kept protected from the light to prevent photodecomposition. All stock solutions were stored as frozen aliquots at –20°C.

Dilutions of these stock solutions were made daily and appropriate solvent controls were used. No statistically significant differences between control experiments, made in the absence or in the presence of the solvents at the maximal concentrations used (0.5% v·v^{-1}), were observed. The pH of the superfusion solution did not change by the addition of the drugs in the maximum concentrations applied to the preparations.

Presentation of data and statistical analysis

The data are expressed as mean ± SEM, with *n* indicating the number of animals used for a particular group of experiments. Statistical analysis of data was carried out by using paired or unpaired Student's *t*-test or one-way analysis of variance (ANOVA) followed by Dunnett's modified *t*-test. A value of $P < 0.05$ was considered to represent a significant difference. The receptor nomenclature conforms to the British Journal of Pharmacology Guide to Receptors and Channels (Alexander *et al.*, 2008).

Results

Pattern of extracellular catabolism of adenine nucleotides and adenosine formation in the longitudinal muscle-myenteric plexus of the rat ileum

Figure 2 illustrates the time course of the extracellular catabolism of adenine nucleotides (ATP, ADP and AMP) in the longitudinal muscle-myenteric plexus of the rat ileum. ATP (30 µmol·L^{-1}) was catabolized with a half-degradation time of 6.9 ± 0.7 min ($n = 6$). The ATP metabolites detected in the bath were ADP, AMP, adenosine, inosine and hypoxanthine, whose concentrations increased with time. AMP was the first metabolite to appear in the bath, which reached a peak concentration of 6.86 ± 0.78 µmol·L^{-1} at 15 min (Figure 2A). The concentration of ADP attained a maximum of 3.98 ± 0.51 µmol·L^{-1} also at 15 min, whereas adenosine reached a maximum concentration of 7.85 ± 1.77 µmol·L^{-1} only 30 min after ATP (30 µmol·L^{-1}) application. The concentration of inosine increased continuously up to 45 min, reaching 13.71 ± 2.30 µmol·L^{-1}. The formation of hypoxanthine was almost negligible (1.99 ± 0.20 µmol·L^{-1} at 45 min).

As shown in Figure 2B, extracellular ADP (30 µmol·L^{-1}) was catabolized with a half-degradation time (6.6 ± 1.6 min, $n = 4$) comparable to that of ATP (30 µmol·L^{-1}) metabolism (see also, Figure 2D). ADP catabolism led rapidly to AMP formation, which was then sequentially metabolized into adenosine, inosine and hypoxanthine (Figure 2B). The maximum concentration of AMP (6.16 ± 0.88 µmol·L^{-1}) was obtained at 10 min. Adenosine reached a maximum concentration of 6.16 ± 0.39 µmol·L^{-1} at 15 min. The concentration of inosine increased continuously up to 45 min, reaching 8.02 ± 0.62 µmol·L^{-1}, while formation of hypoxanthine was still negligible (1.43 ± 0.23 µmol·L^{-1} at 45 min).

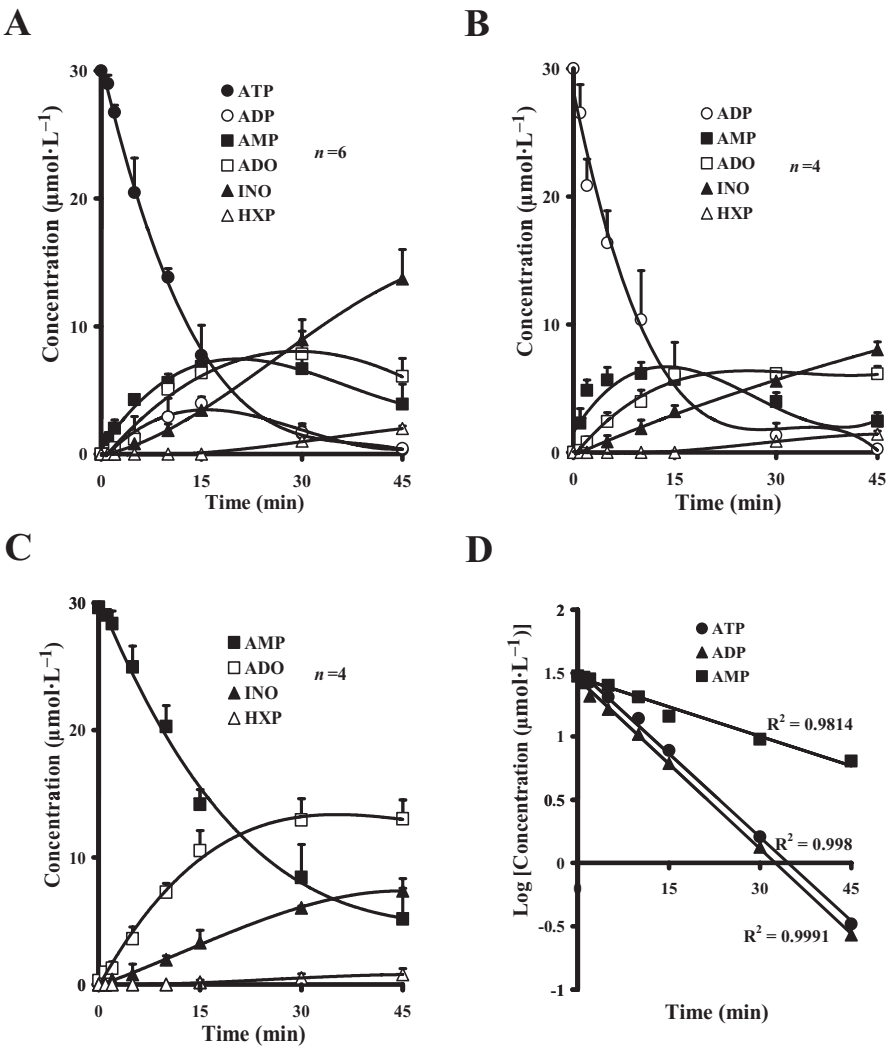

Figure 2 Time course of extracellular (A) ATP, (B) ADP and (C) AMP metabolism in the myenteric plexus of the rat ileum. Adenine nucleotides (30 μmol·L^{-1}) were added at time zero to the preparations, and samples (75 μL) were collected from the bath at indicated times on the abscissa. Each collected sample was analysed by high-performance liquid chromatography to separate and quantify ATP, ADP, AMP, adenosine (ADO), inosine (INO) and hypoxanthine (HXP). Averaged results obtained in (A) six, (B) four and (C) four experiments; the vertical bars represent SEM and are shown when they exceed the symbols in size. In control conditions, without the addition of ATP, ADP or AMP, only inosine monophosphate could be detected in the bath, reaching a maximum concentration (1.65 μmol·L^{-1}) at the end of 45 min. In (D), are represented the semi-logarithmic progress curves obtained by polynomial fitting of the catabolism of ATP (30 μmol·L^{-1}, filled circles), ADP (30 μmol·L^{-1}) and AMP (30 μmol·L^{-1}). Note that ATP, ADP and AMP linearly disappeared from the bath; the calculated half-degradation times appear in the text.

Extracellular AMP (30 μmol·L^{-1}) was catabolized with a half-degradation time of 15 ± 2.4 min. The AMP metabolites detected in the bath were adenosine, inosine and hypoxanthine, whose concentrations in the bathing fluid increased progressively reaching maximum values of 12.95 ± 2.21 μmol·L^{-1} at 30 min, 7.37 ± 0.96 μmol·L^{-1} at 45 min and 1.67 ± 0.92 μmol·L^{-1} at 45 min respectively (Figure 2C). Given the linearity of the kinetics of ATP, ADP and AMP degradation shown in Figure 2D, the analysis of the corresponding half-degradation time values clearly indicates that extracellular catabolism of AMP, through the ecto-5′-nucleotidase (EC 3.1.3.5), is the rate-limiting step in the generation of adenosine from exogenously added adenine nucleotides in the myenteric plexus.

The presence of *p*-nitrophenylphosphate in a saturating concentration (1 mmol·L^{-1}) did not alter the degradation kinetics of ATP, ADP or AMP, suggesting that the contribution of non-specific phosphatases, such as alkaline phosphatase (EC 3.1.3.1), to the extracellular catabolism of adenine nucleotides is negligible (data not shown).

Relative contribution of ecto-ATPase (forming ADP) and ecto-ATPDase (bypassing ADP formation) pathways for ATP catabolism in the longitudinal muscle-myenteric plexus

During the first 2 min of ATP degradation, there was a stoichiometric conversion of ATP into AMP without detectable formation of ADP (Figure 2A). These findings demonstrate the presence of ATPDase (ATP diphosphohydrolase or apyrase, EC 3.6.1.5) activity with a strong preference for nucleotide 5′-triphosphates in the rat myenteric plexus, through which ATP is hydrolysed directly into AMP with minimal sequential

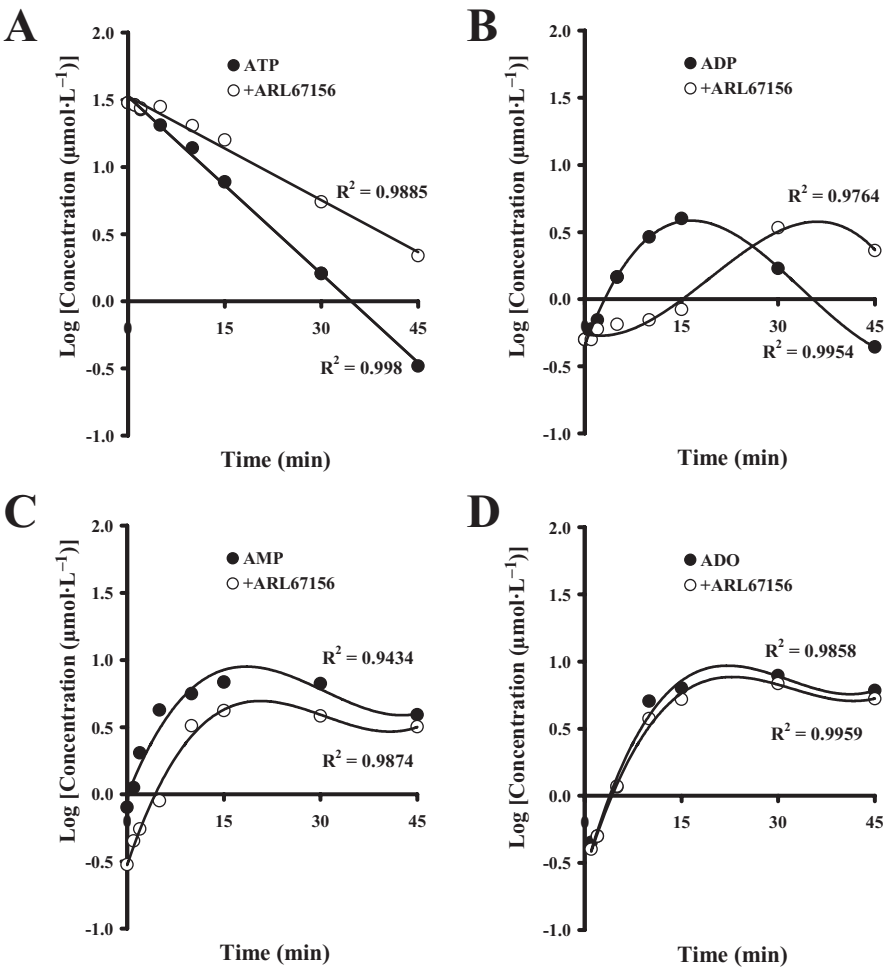

Figure 3 Semi-logarithmic progress curves obtained for the extracellular catabolism of ATP in the absence and in the presence of the ecto-ATPase (adenosine 5′-triphosphatase) inhibitor, ARL 67156 (6-N,N-diethyl-D-β,γ-dibromomethylene-D-adenosine-5-triphosphate) (100 µmol·L^{-1}). ATP (30 µmol·L^{-1}) was added at time zero to the preparations in the absence and in the presence of ARL 67156 (100 µmol·L^{-1}). Samples (75 µL) were collected at the times indicated on the abscissa and retained for high-performance liquid chromatography to separate and quantify (A) ATP, (B) ADP, (C) AMP and (D) adenosine (ADO). Both progress curves were obtained from the same preparations; in the absence of ARL 67156 (100 µmol·L^{-1}), time-matched results did not significantly ($P > 0.05$) differ from the control situation (Figure 2A). Semi-logarithmic curves were obtained by polynomial fitting from an average of six experiments; for the sake of clarity bars representing SEM are not shown. Note that ARL 67156 (100 µmol·L^{-1}) reduced (A) ATP catabolism and delayed (B) the formation of ADP, without affecting the profile of (C) AMP and (D) ADO generation. In control conditions, without the addition of ATP, only inosine monophosphate could be detected in the bath, reaching a maximum concentration (1.15 µmol·L^{-1}) at the end of 45 min.

breakdown to form ADP and then AMP is not hydrolysed by ecto-ATPase (EC 3.6.1.3) (see Figure 9 later).

To evaluate the relative contributions of these two pathways, we investigated the influence of the structural analogue of ATP that selectively inhibits ecto-ATPase activity, ARL 67156 (6-N,N-diethyl-D-β,γ-dibromomethylene ATP, formerly known as FPL 67156) (Crack *et al.*, 1995), on the kinetics of ATP (30 µmol·L^{-1}) catabolism in the rat myenteric plexus. When applied in a concentration (100 µmol·L^{-1}) near the IC_{50} value to inhibit ecto-ATPase activity, ARL 67156 moderately decreased ATP hydrolysis (Figure 3A), without significantly ($P > 0.05$) affecting the generation profile of AMP (Figure 3C) and adenosine (Figure 3D). The way ARL 67156 affected ATP catabolism was to delay ADP formation, that is, the concentrations of ADP reach maximum values (~4 µmol·L^{-1}) at 15 min and 30 min of incubation in the absence and in the presence of ARL 67156 (100 µmol·L^{-1}) respectively (Figure 3B).

Figure 4 illustrates the kinetics of ATP breakdown and metabolite formation, with increasing initial concentrations of the substrate (10–100 µmol·L^{-1}). The rate of ATP hydrolysis leading to ADP, AMP and adenosine formation was the same, irrespective of the initial concentration of ATP applied to the incubation fluid (Figure 4A). Interestingly, dephosphorylation of ATP leading to ADP formation increased progressively, compared with the direct conversion of ATP into AMP when the concentration of ATP applied to the bath increased from 10 to 100 µmol·L^{-1} (Figure 4B). The absolute amount of AMP detected following 15 min incubation with ATP (10–100 µmol·L^{-1}) remained essentially unchanged (~7 µmol·L^{-1}). Taken together these results support the idea that the ADP-generating ecto-ATPase pathway may function as an alternative for ATP breakdown whenever the substrate concentration rises to levels high enough to saturate the preferential ecto-ATPDase pathway.

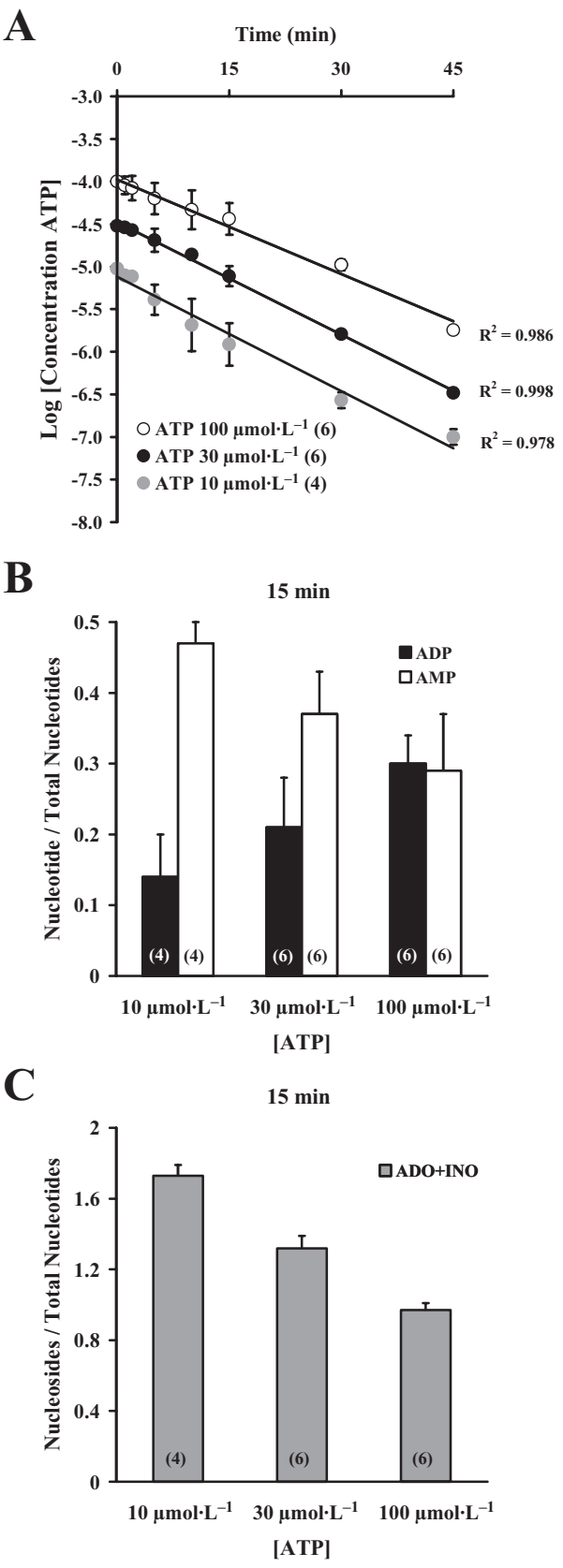

Figure 4 Kinetics of extracellular ATP breakdown and metabolite formation upon increasing the initial concentration of the substrate. ATP (10–100 µmol·L^{-1}) was added at time zero to the preparations. Samples (75 µL) collected at the times indicated on the abscissa were retained for high-performance liquid chromatography to separate and quantify (A) ATP, and its metabolites (B) ADP or AMP and (C) adenosine (ADO) plus inosine (INO). In (A), semi-logarithmic progress curves were obtained by polynomial fitting of the catabolism of ATP (10–100 µmol·L^{-1}). Note that the kinetics of ATP breakdown was not affected by the concentration of the initial substrate. In (B), shown is the relative amount of ADP and AMP as compared with the total amount of nucleotides [(ATP + ADP + AMP)] in the bath following 15 min incubation with ATP (10–100 µmol·L^{-1}). In (C), the ratio [nucleosides] [total nucleotides]$^{-1}$ following 15 min incubation with ATP (10–100 µmol·L^{-1}) as a direct measure of the activity of ecto-5′-nucleotidase is shown. Data shown are pooled from the number of experiments shown in parentheses. The vertical bars represent SEM and are shown when they exceed the symbols in size.

Formation of extracellular adenosine from the catabolism of adenine nucleotides is carried out by ecto-5′-nucleotidase (EC 3.1.3.5), which may be subject to 'feed-forward' inhibition by ATP and/or ADP (Naito and Lowenstein, 1985; reviewed by Cunha, 2001). This inhibition can be evaluated by quantifying the ratio ([Nucleosides] : [Total nucleotides]), which is a direct measure of the activity of ecto-5′-nucleotidase. Figure 4C shows that this ratio decreased when the concentration of ATP increased from 10 to 100 µmol·L^{-1}. In such circumstances, adenosine formation was delayed indicating that ATP did inhibit ecto-5′-nucleotidase in this preparation. Exposure of the myenteric plexus to a higher concentration of AMP (100 µmol·L^{-1}) did not alter its rate of hydrolysis or the pattern of appearance of its metabolites (data not shown).

ATP transiently facilitates [^{3}H]ACh release due to the activation of P2X receptors on myenteric nerve terminals

Exogenously applied ATP caused a dual effect on [^{3}H]ACh release from myenteric motoneurons of the rat ileum (Figure 1). ATP (1–300 µmol·L^{-1}) transiently increased spontaneous [^{3}H]ACh release in a concentration-dependent manner (see also, Figure 5A); tritium outflow returned to the prestimulation levels by 9–12 min after ATP application. Following 15 min of application, ATP (30 µmol·L^{-1}) decreased [^{3}H]ACh release from stimulated (EFS, 200 pulses of 1 ms duration applied at a 5 Hz frequency) myenteric motoneurons (Figure 1).

It appears that the facilitatory effect of ATP results from activation of ionotropic P2X receptors, because ATP-induced tritium outflow was nearly abolished in the presence of TNP-ATP (10 µmol·L^{-1}), which blocks preferentially P2X receptors in the micromolar concentration range without affecting P2Y receptor activity (Virginio *et al.*, 1998) (Figure 5B). The non-selective P2 receptor antagonist, PPADS (10 µmol·L^{-1}), also reduced ATP-induced [^{3}H]ACh release by 73 ± 8% (n = 6) (Figure 5B). α,β-methyleneATP (30 µmol·L^{-1}), a potent agonist of $P2X_1$ and $P2X_3$ receptors (Ralevic and Burnstock, 1998; North, 2002), was devoid of effect on the spontaneous [^{3}H]ACh release. The facilitatory effect of ATP (100 µmol·L^{-1}) on [^{3}H]ACh outflow was dependent on Ca^{2+} influx from the extracellular media, but was insensitive to blockade by TTX

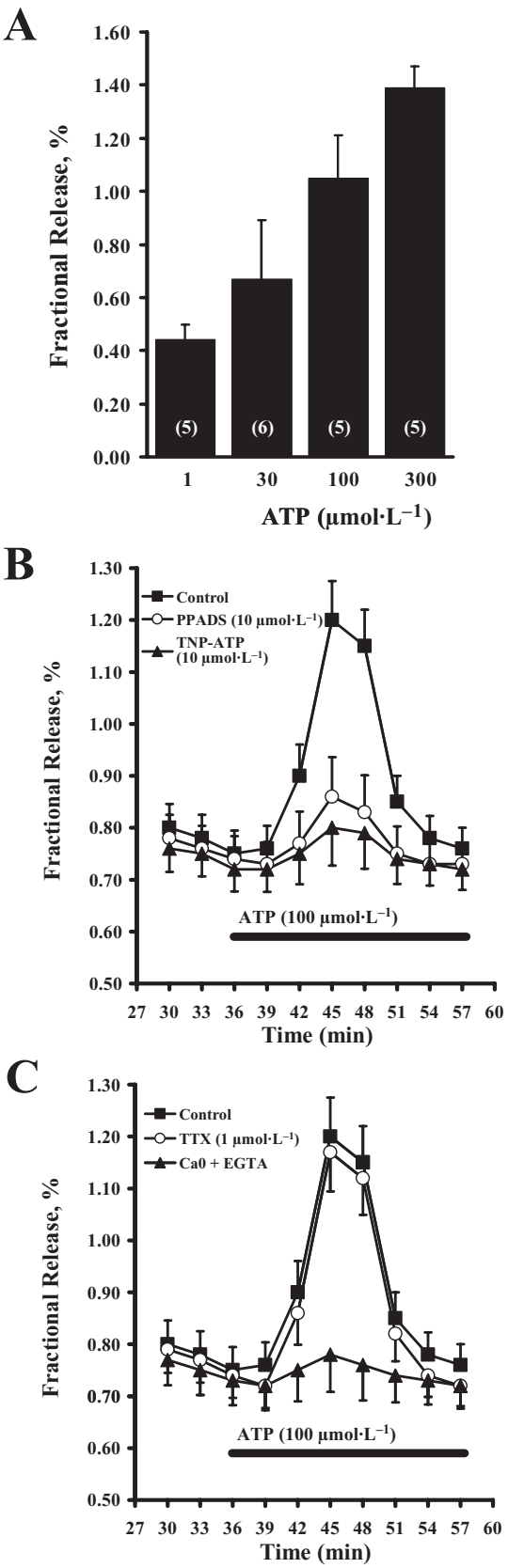

Figure 5 Extracellular ATP transiently activates P2X receptors mediating spontaneous [^{3}H]ACh release from myenteric nerve terminals. In (A), the ordinates represent spontaneous tritium outflow (as Fractional Release %; see *Methods*) induced by increasing extracellular ATP concentrations (1–300 µmol·L^{-1}). ATP was applied as indicated in Figure 1. Each column represents pooled data from five to six experiments. The vertical bars represent SEM. In (B) and (C), shown is the time course of [^{3}H]ACh release induced by ATP (100 µmol·L^{-1}, arrow) in the absence and in the presence of (B) two non-selective P2X antagonists, PPADS (10 µmol·L^{-1}) and TNP-ATP (10 µmol·L^{-1}), (C) TTX (1 µmol·L^{-1}, an action potential generation blocker) and Tyrode's solution without Ca^{2+} plus EGTA (1 mmol·L^{-1}). Tritium outflow (ordinates) is expressed as Fractional Release, %. The abscissa indicates the times at which the samples were collected. Each point is pooled data (±SEM) from five to six experiments. None of the drugs changed spontaneous tritium outflow. [^{3}H]ACh, [^{3}H]acetylcholine; EGTA, ethylene glycol-bis(2-aminoethylether)-N,N,N′,N′-tetraacetic acid; PPADS, pyridoxal phosphate-6-azo(benzene-2,4-disulphonic acid) tetrasodium salt; TNP-ATP, 2′,3′-O-(2,4,6-trinitrophenyl)adenosine-5′-triphosphate; TTX, tetrodotoxin.

(reduction by 3 ± 8%, $n = 6$) (Figure 5C), when this toxin was applied in a concentration (1 µmol·L^{-1}) that fully blocked tritium outflow caused by EFS (Duarte-Araújo *et al*., 2004b). Resistance to TTX (1 µmol·L^{-1}), which blocks Na^+ influx thereby blocking axonal conduction, indicates that the ionotropic P2X receptors are most probably located on cholinergic nerve terminals.

Adenine nucleotides inhibit [^{3}H]ACh release directly, through activation of P2Y$_1$ purinoceptor, and indirectly, by formation of adenosine leading to A$_1$ receptor activation

The inhibitory effect of ATP (30 µmol·L^{-1}, –25 ± 3%, $n = 6$) on stimulation-induced [^{3}H]ACh release was mimicked by the non-selective P2 agonist, ATPγS (30 µmol·L^{-1}, –22 ± 3%, $n = 6$), but not by the preferential P2X agonist, α,β-methyleneATP (30 µmol·L^{-1}) (Figure 6A). These findings suggest that ATP inhibition of evoked [^{3}H]ACh release from myenteric motoneurons is not due to fast desensitization of facilitatory prejunctional P2X purinoceptors operated by α,β-methyleneATP (30 µmol·L^{-1}). Exogenous ADP also decreased [^{3}H]ACh release from stimulated myenteric motoneurons respectively by 22 ± 4% ($n = 6$) and 37 ± 1% ($n = 6$), when the nucleotide was applied in 30 and 100 µmol·L^{-1} concentrations (see Figure 6B); ADP (30 and 100 µmol·L^{-1}) failed to increase spontaneous [^{3}H]ACh release. The inhibitory effect of ADP on evoked [^{3}H]ACh release was reproduced by its stable analogue, ADPβS (30 µmol·L^{-1}, –27 ± 3%, $n = 6$) (Figure 6B), but not by 2-MeSADP (30 µmol·L^{-1}). This might be due to the chemical instability of 2-MeSADP (30 µmol·L^{-1}) (Von Kugelgen and Wetter, 2000), as this drug is extensively hydrolysed (more than 80% following a 15 min period of incubation) in the longitudinal muscle-myenteric plexus (data not shown). It is worth noting that none of the stable ATP analogues used in this study affected the activity of NTPDases (Yegutkin and Burnstock, 2000).

The inhibitory effect of ADP (100 µmol·L^{-1}, –37 ± 1%, $n = 6$) was partially reduced by MRS 2179 (0.3 µmol·L^{-1}, –24 ± 4%, $n = 5$, a selective P2Y$_1$ antagonist), by ADA, the enzyme that inactivates endogenous adenosine (0.5 U·mL^{-1}, –26 ± 5%, $n = 6$), and by DPCPX, a selective adenosine A$_1$

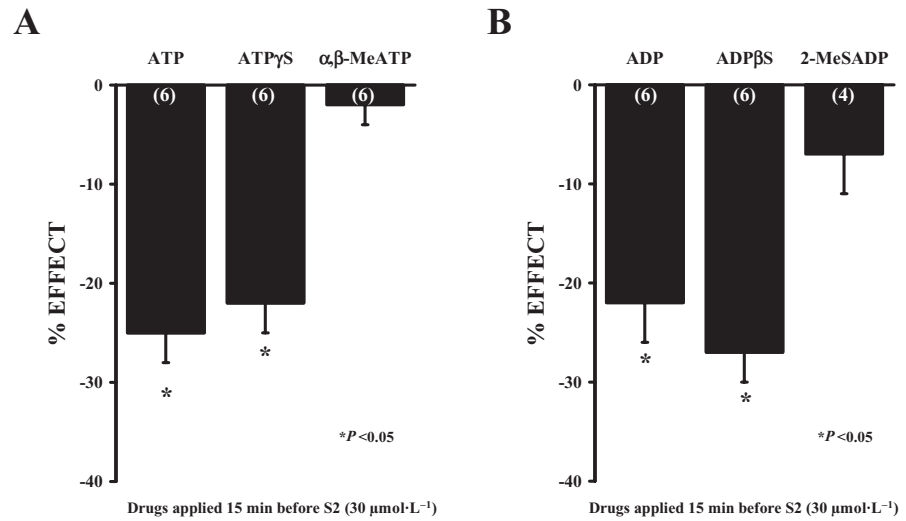

Figure 6 Effects of exogenously added adenine nucleotides on [^{3}H]ACh release from myenteric neurons stimulated with EFS. The release of [^{3}H]ACh in response to EFS (200 pulses of 1 ms duration delivered at a 5 Hz frequency) was elicited twice (S_1 and S_2). (A) Nucleotide triphosphates (ATP, ATPγS and α,β-MeATP) and (B) diphosphates (ADP, ADPβS and 2-MeSADP) were added 15 min before S_2 in a 30 μmol·L^{-1} concentration. The ordinates are percentage changes in S_2/S_1 ratios compared with controls. Each column represents pooled data (±SEM) from an *n* number of experiments shown in parentheses. *$P < 0.05$ (one-way ANOVA followed by Dunnett's modified *t*-test) when compared with zero percentage of change. 2-MeSADP, 2-methylthio-adenosine diphosphate; [^{3}H]ACh, [^{3}H]acetylcholine; EFS, electrical field stimulation; α,β-MeATP, α,β-methylene adenosine 5′-triphosphate; ADPβS, adenosine 5′[β-thio]diphosphate; ATPγS, adenosine 5′-[γ-thio]triphosphate.

antagonist (10 nmol·L^{-1}, –28 ± 7%, $n = 5$). The non-selective P2 antagonists, PPADS (10 μmol·L^{-1}) and RB-2 (30 μmol·L^{-1}), failed to modify ADP (100 μmol·L^{-1}) inhibition of evoked [^{3}H]ACh release. Co-application of MRS 2179 (0.3 μmol·L^{-1}) plus ADA (0.5 U·mL^{-1}) completely abolished ADP inhibition of [^{3}H]ACh release from stimulated myenteric motoneurons (Figure 7A). These findings suggest that ADP acts directly, via ADP-sensitive $P2Y_1$ purinoceptors, and indirectly, via adenosine formation leading to adenosine A_1 receptor activation, to reduce the evoked release of [^{3}H]ACh. A similar pattern of the ability of ATP to inhibit evoked [^{3}H]ACh release from myenteric motoneurons was shown by using MRS 2179 (0.3 μmol·L^{-1}) and ADA (0.5 U·mL^{-1}). In these conditions, the inhibitory $P2Y_1$ receptors activated by ADP, generated via ecto-ATPase, play a major role compared with the adenosine A_1 receptor, when the concentration of ATP was increased from 30 to 100 μmol·L^{-1}. That is, attenuation of the inhibitory effect of ATP (100 μmol·L^{-1}, –30 ± 3%, $n = 5$) was greater in the presence of MRS 2179 (0.3 μmol·L^{-1}, –19 ± 2%, $n = 6$) than in the presence of ADA (0.5 U·mL^{-1}, –24 ± 5%, $n = 4$), while both drugs had a similar effect (reduction by ~19%) with 30 μmol·L^{-1} ATP.

On their own, the selective $P2Y_1$ receptor antagonist, MRS 2179 (0.3 μmol·L^{-1}), facilitated [^{3}H]ACh release from stimulated myenteric motoneurons by 28 ± 2% ($n = 6$), whereas the non-selective P2 antagonists, PPADS (10 μmol·L^{-1}) and RB-2 (30 μmol·L^{-1}), increased [^{3}H]ACh release only by 7 ± 3% ($n = 5$) and 2 ± 7% ($n = 4$) respectively. A higher concentration of MRS 2179 (1 μmol·L^{-1}) did not increase further the facilitatory action (28 ± 6%, $n = 4$) of the $P2Y_1$ receptor antagonist. Inhibition of ecto-ATPase with ARL 67156 (100 μmol·L^{-1}) prevented MRS 2179 (0.3 μmol·L^{-1})-induced facilitation (–9 ± 7%, $n = 6$) of evoked [^{3}H]ACh release, demonstrating that endogenous ADP tonically activates inhibitory $P2Y_1$ receptors on myenteric nerve terminals. MRS 2179 does not affect NTPDase activities in the concentration range commonly used to inhibit $P2Y_1$ receptors (Munkonda *et al.*, 2007). The magnitude of the $P2Y_1$ receptor-mediated inhibitory tonus might be positively correlated with the stimulation train length, that is, with the amount of ADP generated at the myenteric synapse. To confirm this hypothesis, we performed experiments increasing the number of pulses delivered to the preparation from 200 to 500, keeping constant the stimulation frequency (5 Hz) and the pulse width (1 ms). In these circumstances, facilitation of evoked [^{3}H]ACh release by MRS 2179 (0.3 μmol·L^{-1}) was increased to 36 ± 3% ($n = 4$).

Stimulation of inhibitory ADP-sensitive $P2Y_1$ purinoceptors may be cut short by sequential activation of adenosine A_1 inhibitory receptors on myenteric motoneurons

Figure 8 shows that two stable analogues of ADP and adenosine, respectively ADPβS (0.3–30 μmol·L^{-1}) and R-PIA (30–300 nmol·L^{-1}), both inhibited the release of [^{3}H]ACh in a concentration dependent manner. The selective $P2Y_1$ receptor antagonist, MRS 2179 (0.3 μmol·L^{-1}), prevented the inhibitory effect of ADPβS (30 μmol·L^{-1}, $n = 6$) on evoked [^{3}H]ACh release (Figure 8A). Blockade of the adenosine A_1 receptor with DPCPX (10 nmol·L^{-1}) significantly ($P < 0.05$) enhanced the inhibitory action of ADPβS (30 μmol·L^{-1}; $n = 6$), compared with control. Likewise, inactivation of endogenous adenosine with ADA (0.5 U·mL^{-1}) augmented ADPβS (30 μmol·L^{-1}; $n = 4$) inhibitory action to 51 ± 4% (M = 4) (data not shown). On the contrary, the selective A_1 receptor antagonist, DPCPX (10 nmol·L^{-1}), fully blocked inhibition of [^{3}H]ACh release caused by R-PIA (300 nmol·L^{-1}), whereas MRS 2179 (0.3 μmol·L^{-1}) was devoid of effect (Figure 8B).

Application of the adenosine A_1 receptor agonist, R-PIA (300 nmol·L^{-1}) during the whole assay, including S_1 and S_2,

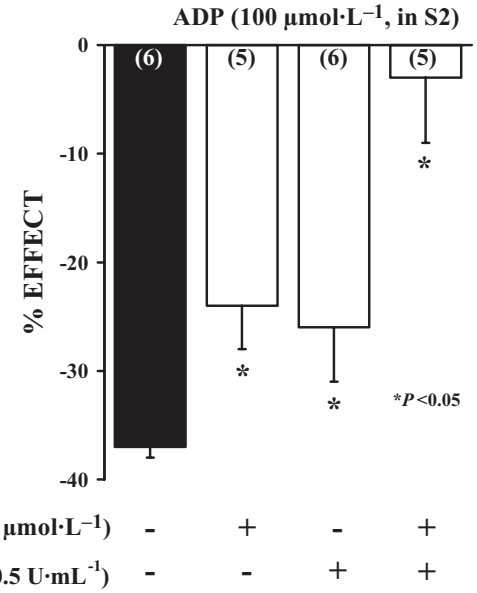

Figure 7 Effect of exogenously added ADP on [^{3}H]ACh release from myenteric neurons stimulated by EFS in the absence and in the presence of the selective $P2Y_1$ receptor antagonist, MRS 2179 (0.3 µmol·L^{-1}) and of ADA (0.5 U·mL^{-1}). The release of [^{3}H]ACh in response to EFS (200 pulses of 1 ms duration delivered at a 5 Hz frequency) was elicited twice (S_1 and S_2). ADP (100 µmol·L^{-1}) was added 15 min before S_2; MRS 2179 (0.3 µmol·L^{-1}) and/or ADA (0.5 U·mL^{-1}) were added to the incubation media at the beginning of the release period (time zero) and were present throughout the assay, including S_1 and S_2. The ordinates are percentage changes in S_2/S_1 ratios compared with controls. The average S_2/S_1 ratios in the presence of MRS 2179 (0.3 µmol·L^{-1}, 0.87 ± 0.04, $n = 6$) and of ADA (0.5 U·mL^{-1}, 0.85 ± 0.06, $n = 4$) (without ADP) were not significantly ($P > 0.05$) different from the control value (0.83 ± 0.11, $n = 4$; data not shown). Each column represents pooled data (±SEM) from an n number of experiments shown in parentheses. *$P < 0.05$ (one-way ANOVA followed by Dunnett's modified t-test), significant differences compared with the effect of ADP in the absence of MRS 2179 and/or ADA. [^{3}H]ACh, [^{3}H]acetylcholine; ADA, adenosine deaminase; EFS, electrical field stimulation; MRS 2179, 2′-deoxy-N^6-methyl adenosine 3′,5′-diphosphate diammonium salt.

attenuated the inhibitory action of ADPβS (30 µmol·L^{-1}) on [^{3}H]ACh release from stimulated myenteric motoneurons (Figure 8C). In contrast, pretreatment of the preparations with ADPβS (30 µmol·L^{-1}, in S_1 and S_2) failed to affect the inhibitory effect of R-PIA (300 nmol·L^{-1}) (Figure 8C). These results suggest that activation of inhibitory $P2Y_1$ purinoceptors may be cut short by subsequent activation of inhibitory A_1 receptors by adenosine.

Discussion and conclusions

Our data suggest that ATP transiently activates facilitatory P2X receptors mediating spontaneous [^{3}H]ACh release from myenteric motoneurons. P2X facilitation may be rapidly terminated by extracellular ATP catabolism into active adenine nucleotides, such as ADP, and adenosine. As a consequence of the rapid conversion of ATP directly into AMP, catalysed by ecto-ATPDase (EC 3.6.1.5), and subsequent AMP dephosphorylation to adenosine by the ecto-5′-nucleotidase (EC 3.1.3.5), the magnitude of ATP excitation may be ultimately regulated by the nucleoside adenosine activating presynaptic inhibitory adenosine A_1 receptors. In addition, activation of $P2Y_1$ purinoceptors by ADP generated alternatively via ecto-ATPase (EC 3.6.1.3) might be functionally relevant to restrain stimulation-induced ACh exocytosis, particularly when ATP accumulation reaches the V_{max} for ecto-ATPDase. Stimulation of ADP-sensitive inhibitory $P2Y_1$ purinoceptors regulating [^{3}H]ACh release may be cut short by sequential activation of inhibitory A_1 receptors by adenosine generated from the catabolism of released adenine nucleotides (Figure 9).

The kinetics of the extracellular conversion of ATP into other metabolically active derivatives, namely ADP and adenosine, may be functionally relevant to their role in controlling synaptic efficiency. Histochemical studies have shown that the myenteric plexus contains the enzymes responsible for the catabolism of ATP into adenosine (Nitahara *et al.*, 1995; Sévigny *et al.*, 1998). Co-expression of ecto-NTPDase1 (CD39, ATPDase or apyrase, EC 3.6.1.5), causing dephosphorylation of ATP directly into AMP with only a modest formation of ADP, and ecto-NTPDase2 (CD39L1, ecto-ATPase, EC 3.6.1.3), generating sequentially ADP and AMP, has been shown in some nerve structures (Kegel *et al.*, 1997; Vlajkovic *et al.*, 2002), demonstrating that complex pathways regulate the extracellular metabolism of adenine nucleotides. Experimental data strongly indicate that ATPDase participates in the termination of P2 receptor-mediated signal transmission, whereas the function of ecto-ATPase remains a matter of speculation. Our results indicate that ATP (30 µmol·L^{-1}) is metabolized ($t½$ = 6.9 ± 0.7 min) predominantly into AMP, which is subsequently dephosphorylated into adenosine ($t½$ = 15.04 ± 2.42 min) by ecto-5′-nucleotidase (EC 3.1.3.5) in the myenteric plexus of the rat ileum; appearance of ADP in the incubation fluid was absent during the first 2 min (Figure 2A). The alternative pathway, conversion of ATP into ADP, becomes more relevant as the extracellular ATP concentration increased to 100 µmol·L^{-1}. In these circumstances, besides ADP accumulation in the extracellular milieu, we observed a delay of adenosine formation probably due to feed-forward inhibition by increased levels of extracellular ATP (Naito and Lowenstein, 1985; Cunha, 2001). These findings are in good agreement with the observations in porcine cortical synaptosomes (Kukulski and Komoszyński, 2003) and in C6 glioma cells (Grobben *et al.*, 1999), where the calculated K_m value for ecto-ATPDase in respect to ATP as a substrate was several (three) times lower in comparison with the corresponding values for ecto-ATPase. Because both enzymes have a similar molecular activity, these findings suggest that when ATP concentration in the extracellular space is low (close to the V_{max} for ecto-ATPDase), ATP will be hydrolysed preferentially by ecto-ATPDase than by ecto-ATPase. Ecto-ATPDase seems to have similar affinities with respect to ATP and ADP (Figure 2). The delay in ADP formation upon inhibiting ecto-ATPase with ARL 67156 (Crack *et al.*, 1995), without compensatory changes in AMP formation, suggests that the ecto-ATPase pathway works probably as an alternative pathway for inactivating ATP when its concentration reaches high levels in the myenteric synapse. Therefore, ecto-ATPase may be responsible for the removal of high, toxic, concentrations of ATP on the one hand and on the other hand for the production of a compensatory signal mol-

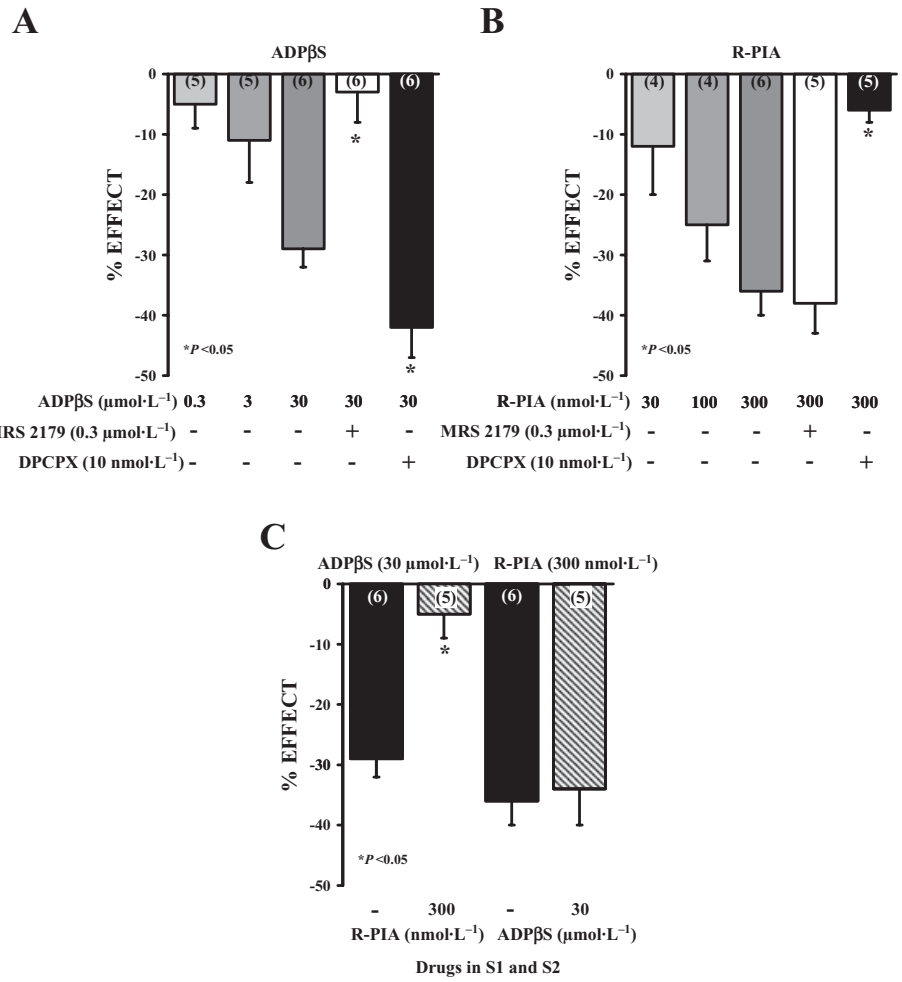

Figure 8 Crosstalk between inhibitory $P2Y_1$ and A_1 receptors regulating [^{3}H]ACh release from myenteric neurons stimulated by EFS. The release of [^{3}H]ACh in response to EFS (200 pulses of 1 ms duration delivered at a 5 Hz frequency) was elicited twice (S_1 and S_2). The ordinates are percentage changes in S_2/S_1 ratios compared with controls. (A) ADPβS (0.3–30 μmol·L^{-1}) and (B) R-PIA (30–300 nmol·L^{-1}) were added 15 min before S_2; MRS 2179 (0.3 μmol·L^{-1}) and DPCPX (10 nmol·L^{-1}) were added to the incubation media at the beginning of the release period (time zero) and were present throughout the assay, including S_1 and S_2. The average S_2/S_1 ratios in the presence of MRS 2179 (0.3 μmol·L^{-1}, 0.87 ± 0.04, $n = 6$) and of DPCPX (10 nmol·L^{-1}, 0.89 ± 0.07, $n = 6$) alone were not significantly ($P > 0.05$) different from the control value (0.83 ± 0.11, $n = 4$; data not shown). Each column represents pooled data (±SEM) from an n number of experiments shown in parentheses. *$P < 0.05$ (one-way ANOVA followed by Dunnett's modified t-test), significant differences compared with the inhibitory effects of (A) ADPβS (30 μmol·L^{-1}) and (B) R-PIA (300 nmol·L^{-1}) in control conditions respectively. In (C), ADPβS (30 μmol·L^{-1}) was applied 15 min before S_2 in the absence and in the presence of R-PIA (300 nmol·L^{-1}, applied in S_1 and S_2), and vice versa. Each column represents pooled data (±SEM) from the number of experiments shown in parentheses. *$P < 0.05$ (one-way ANOVA followed by Dunnett's modified t-test), significant differences compared with the inhibitory effects of ADPβS (30 μmol·L^{-1}) or R-PIA (300 nmol·L^{-1}) in the absence of the other modulator respectively. [^{3}H]ACh, [^{3}H]acetylcholine; ADPβS, adenosine 5′[β-thio]diphosphate; DPCPX, 1,3-dipropyl-8-cyclopentyl xanthine; EFS, electrical field stimulation; MRS 2179, 2′-deoxy-N^6-methyl adenosine 3′,5′-diphosphate diammonium salt; R-PIA, R-N^6-phenylisopropyl adenosine.

ecule, ADP, which can control neuronal activity during sustained firing, hypoxia or ischaemia and inflammatory insults of the enteric nervous system.

In the myenteric plexus, all enteric neurons, except NO synthase (EC 1.14.13.39)-immunoreactive inhibitory muscle motoneurons, contain choline acetyltransferase (EC 2.3.1.6) (Furness, 2000), suggesting that myenteric neurons are mostly cholinergic and that ATP and ACh might be co-released. Here, we provided evidence indicating that cholinergic nerve terminals of the myenteric plexus of the rat ileum possess TNP-ATP-sensitive P2X receptors triggering the release of [^{3}H]ACh in the absence of action potential generation (see also Barthó *et al*., 2006). A common denominator to all P2X receptor subtypes is a Ca^{2+} influx through non-selective cation channels (also permeant to Na^+ and/or K^+) promoted by purines acting through the receptor channel itself (see Figure 5C). ATP can also inhibit the membrane potassium conductance in enteric neurons (Barajas-López *et al*., 1994). Via these mechanisms, ATP can cause membrane depolarization leading to a secondary opening of voltage-gated Ca^{2+} channels and to transmitter release. ATP-induced increase in basal [^{3}H]ACh release was dependent on extracellular Ca^{2+} but was resistant to blockade of axonal conduction by TTX, indicating that its action is likely to be independent on generation of action potentials and has to be mediated by P2X receptors present on myenteric nerve endings. Likewise, ATP can increase ACh

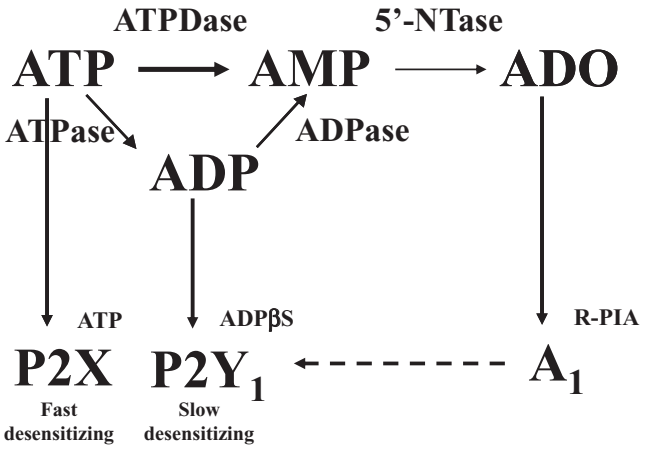

Figure 9 Schematic representation of the modulatory role of ATP and its metabolites on [^{3}H]ACh release from myenteric neurons of the rat ileum. The myenteric plexus contains the enzymes responsible for the catabolism of ATP released from activated smooth muscle cells as well as from stimulated myenteric neurons. ATP is converted preferentially into AMP via ecto-ATPDase (EC 3.6.1.5), which is then sequentially dephosphorylated into ADO by ecto-5′-nucleotidase (5′-NTase, EC 3.1.3.5); the alternative pathway, conversion of ATP into ADP via ecto-ATPase (EC 3.6.1.3), only becomes relevant upon increasing the extracellular concentration of ATP. Understanding the effective contribution of the ATP breakdown via ecto-ATPase (generating ADP) and via ecto-ATPDase (bypassing ADP formation) is of central importance to predict the fine tuning of purinergic control of gut motility. Data suggest that ATP transiently activates facilitatory P2X receptors mediating spontaneous [^{3}H]ACh release, while ATP metabolites, like ADP and ADO, interplay to control evoked transmitter release by activating inhibitory $P2Y_1$ and A_1 receptors respectively. [^{3}H]ACh, [^{3}H]acetylcholine; ADO, adenosine; ADPβS, adenosine 5′[β-thio]diphosphate; ATPase, adenosine 5′-triphosphatase; ATPDase, adenosine 5′-triphosphate diphosphohydrolase; R-PIA, R-N^6-phenylisopropyl adenosine.

release from isolated nerve terminals (synaptosomes) from the guinea-pig myenteric plexus (Reese and Cooper, 1982; but see, Barthó *et al.*, 2006). Murine enteric neurons express mainly $P2X_2$ and $P2X_3$ subunit-containing receptors (Galligan, 2002), although unusual purinoceptor subtypes sharing some properties with $P2X_4$ and $P2X_6$ may also be involved in ATP-induced currents in myenteric neurons (Barajas-López *et al.*, 1996). The involvement of the $P2X_3$ receptor is unlikely because there was almost no response to α,β-MeATP, which potently activates homomeric P2X receptors composed by $P2X_1$ or $P2X_3$ subunits and $P2X_{2/3}$ heteromeric receptors, but does not activate $P2X_2$ homomeric receptors (Ralevic and Burnstock, 1998; North, 2002; see also Zhou and Galligan, 1996); fast desensitizing ($\tau < 1$ s) $P2X_3$ receptors are only present in approximately 10% of myenteric neurons (Zhou and Galligan, 1996), which are mainly AH (intrinsic intestinal sensory) neurons but not S motoneurons and interneurons (Furness, 2000). Therefore, ATP-induced [^{3}H]ACh release from non-stimulated myenteric neurons of the rat ileum is most likely due to the activation of prejunctional receptors containing $P2X_2$ subunits, which are sensitive to micromolar concentrations of trinitrophenyl-substituted nucleotides, especially TNP-ATP (Virginio *et al.*, 1998), as well as to non-nucleotide compounds, like PPADS (North, 2002). Using electrophysiological, single-cell Ca^{2+} imaging and molecular biology techniques, Ohta *et al.* (2005) demonstrated that ATP mainly activates excitatory $P2X_2$ receptors resulting in Ca^{2+} influx in primary cultures of myenteric neurons isolated from the neonatal rat intestine. Our suggestion that homomeric $P2X_2$ receptors are expressed in myenteric motoneurons is supported by immunohistochemical studies in the guinea-pig intestine (Castelucci *et al.*, 2002) and by data derived from $P2X_2$ receptor knockout mice (Ren *et al.*, 2003).

Ionotropic P2X and metabotropic P2Y receptors are co-expressed in many cell types. The relative contribution of ecto-ATPase (forming ADP) versus ecto-ATPDase (bypassing ADP formation) pathway seems critical to define the extracellular inactivation of ATP (the natural ligand for both P2X and P2Y receptors) and formation of biologically active metabolites (e.g. ADP and adenosine) capable of regulating neurotransmitter release via P2Y and P1 receptor activation. Here, we showed that ATP and its stable analogue, ATPγS, but not α,β-MeATP, consistently inhibited electrically evoked [^{3}H]ACh release from myenteric neurons. Applied in similar experimental conditions, ADP and the enzymatically stable ADP analogue, ADPβS, mimicked the inhibitory action of ATP. We suspect that inhibition of electrically evoked transmitter release by adenine nucleotides may be mediated by $P2Y_1$ receptors, because the selective $P2Y_1$ antagonist, MRS 2179 (Von Kugelgen and Wetter, 2000), counteracted the inhibitory action of ADPβS. The non-selective P2 receptor antagonists, PPADS and RB-2, produced puzzling results versus ADP-induced inhibitory response. These antagonists are reported to block $P2Y_1$ receptors (Von Kugelgen and Wetter, 2000), but contrasting data have been published. For example, PPADS was ineffective as an antagonist at rabbit aortic endothelial $P2Y_1$ receptors (Ralevic and Burnstock, 1998). Sensitivity to MRS 2179 discounts an effect mediated by distinct ADPβS-sensitive $P2Y_{6,12,13}$ receptors. The involvement of α,β-MeATP-sensitive $P2Y_{2,4,11}$ receptors can also be dismissed, as this compound was devoid of effect on evoked [^{3}H]ACh release (see Abbracchio *et al.*, 2006). Electrophysiological, immunohistochemical and molecular studies have demonstrated inhibitory $P2Y_1$ receptors on myenteric and submucosal neurons of rodents (Galligan, 2002; Giaroni *et al.*, 2002; Hu *et al.*, 2003), as well as in man (Gallego *et al.*, 2006; Wunderlich *et al.*, 2008). Purinergic neurotransmission is known to occur between neurons in the enteric nervous system. Whether the $P2Y_1$ receptor inhibition of [^{3}H]ACh release is mediated via the production of NO from inhibitory nerves (Giaroni *et al.*, 2002; Zizzo *et al.*, 2007) requires further investigations.

Tonic activation of inhibitory $P2Y_1$ receptors might also be physiologically relevant during high levels of enteric nerve activity, as predicted from augmentation of [^{3}H]ACh release in the presence of MRS 2179, particularly when stimulation train duration was prolonged (from 200 to 500 pulses). In these circumstances, ATP released from stimulated myenteric neurons might reach such high levels that AMP generation via ecto-ATPDase is saturated and ATP catabolism is partially diverted to the formation of ADP via the ecto-ATPase pathway, with ADP being the most potent natural agonist of inhibitory $P2Y_1$ receptors (Ralevic and Burnstock, 1998). This hypothesis was further supported by the sensitivity of MRS 2179 facilitation of [^{3}H]ACh release from stimulated myen-

teric neurons to the inhibition of ecto-ATPase by ARL 67156. Up-regulation of ATPDase at the transcriptional level has been observed in response to prolonged stimulation of $P2Y_1$ receptors (Lu *et al.*, 2007). This up-regulation might reflect a feedback circuit to regulate excess of extracellular ATP under certain pathological conditions, such as ischaemic insults and chronic inflammation.

The inhibitory effect of ADP on evoked [^{3}H]ACh release was not fully blocked by MRS 2179 and complete blockade required co-application of the $P2Y_1$ receptor antagonist together with ADA. This contrasts with ADPβS, which does not generate adenosine. ADA rapidly removes adenosine in the bathing solution, and it may prevent the activation of inhibitory adenosine A_1 receptors present on myenteric neurons (Duarte-Araújo *et al.*, 2004a). The selective adenosine A_1 receptor antagonist, DPCPX (10 nmol·L^{-1}), also partially attenuated the inhibitory action of ADP (100 μmol·L^{-1}). These findings suggest that ADP acts directly, via ADP-sensitive $P2Y_1$ purinoceptors, and indirectly, via adenosine formation leading to adenosine A_1 receptor activation, to reduce the release of [^{3}H]ACh from stimulated myenteric motoneurons. The adenosine A_1 receptors have a high affinity for adenosine and are widely distributed in mammals (including man), particularly on nerve terminals. Adenosine exerts an inhibitory action in the enteric nervous system mediated by neuronal A_1 receptors sensitive to DPCPX (10 nmol·L^{-1}) (e.g. Nitahara *et al.*, 1995; Barajas-López *et al.*, 1996; De Man *et al.*, 2003; Duarte-Araújo *et al.*, 2004a; Correia-de-Sá *et al.*, 2006) (Figure 8). Thus, while the ecto-5′-nucleotidase pathway modulates the rate of adenosine formation from the catabolism of released ATP via AMP-forming ecto-ATPDase, alternative ATP hydrolysis via ecto-ATPase controls the amount of ADP generated at the myenteric synapse. The combined effect of both pathways, in parallel with feed-forward inhibition of ecto-5′-nucleotidase by high ATP amounts, might serve to regulate the local concentration of ADP and adenosine available to interact with inhibitory $P2Y_1$ and adenosine A_1 receptors respectively. It is also interesting to note that ecto-5′-nucleotidase activity is concentrated in the smooth muscle plasma membrane (Nitahara *et al.*, 1995; Sévigny *et al.*, 1998), which may lead to a delay in the accumulation of adenosine that is produced some distance away from the receptor sites. Heinemann *et al.* (1999) showed that the P1 receptor antagonist, 8-phenyltheophylline, attenuated the peristalsis-inhibiting effect of low (10 μmol·L^{-1}) ATP concentrations, but this antagonism was less robust upon increasing the concentration of the nucleotide. We showed a similar pattern on the ability of ATP to inhibit evoked [^{3}H]ACh release from myenteric motoneurons using ADA (0.5 U·mL^{-1}), suggesting that $P2Y_1$ receptors activated by ADP generated via ecto-ATPase play a greater role as ATP concentrations are raised.

Thus, our results are in line with previous observations suggesting that ATP and its metabolites, namely ADP and adenosine, form a 'purinergic cascade' leading to complex interactions between P1 and P2 receptors needed to control neurotransmission in several synapses (see Ralevic and Burnstock, 1998). Here, we demonstrated that activation of ADP-sensitive $P2Y_1$ purinoceptors may be cut short by sequential activation of inhibitory A_1 receptors by adenosine. Our results, showing that ADPβS and MRS 2179 were unable to change the inhibitory effect of R-PIA, which potently activates adenosine A_1 receptors, are in disagreement with the previously reported reduction in the adenosine A_1 ligand affinity observed in cells possessing $A_1/P2Y_1$ heterodimers (Yoshioka *et al.*, 2001). Although heteromerization between adenosine A_1 and $P2Y_1$ receptors may provide the molecular basis to explain hybrid pharmacology of these receptors, that is, atypical $P2Y_1$ receptors that are sensitive to adenosine A_1 receptor antagonists, this might not occur on cholinergic neurons of the rat myenteric plexus as DPCPX favoured, instead of decreasing, the ability of ADPβS to inhibit evoked [^{3}H]ACh release.

Through these complex interactions, gradients of ATP breakdown products (ADP and adenosine) may provide fine tuning of peristaltic motor performance in the gut during stressful situations, such as sustained neuronal activity, ischaemia and chronic inflammation, when extracellular ATP levels become increased (see also, Milusheva *et al.*, 1990; De Man *et al.*, 2003). Up to 1% of intracellular ATP may be released in response to stimuli. Given that intracellular ATP is around 3–6 mmol·L^{-1}, such an increase has marked consequences for the kinetics of P2 and P1 receptor activation, the latter being activated by secondary formation of adenosine through the ecto-nucleotidase pathway. Since the original purinergic receptor hypothesis and classification by Burnstock (1980), extensive investigation has established their indispensable role in cellular homeostasis and raised excitement over the potential of developing therapeutic targets in many pathological conditions. As our understanding of the underlying signalling and pathophysiological implications of the ecto-nucleotidase pathway evolves, the potential for new therapies will expand (cf. Gendron *et al.*, 2002).

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Conflict of interest

None.

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