P2X3 RECEPTORS PARTICIPATE IN PURINERGIC INHIBITION OF GASTROINTESTINAL SMOOTH MUSCLE

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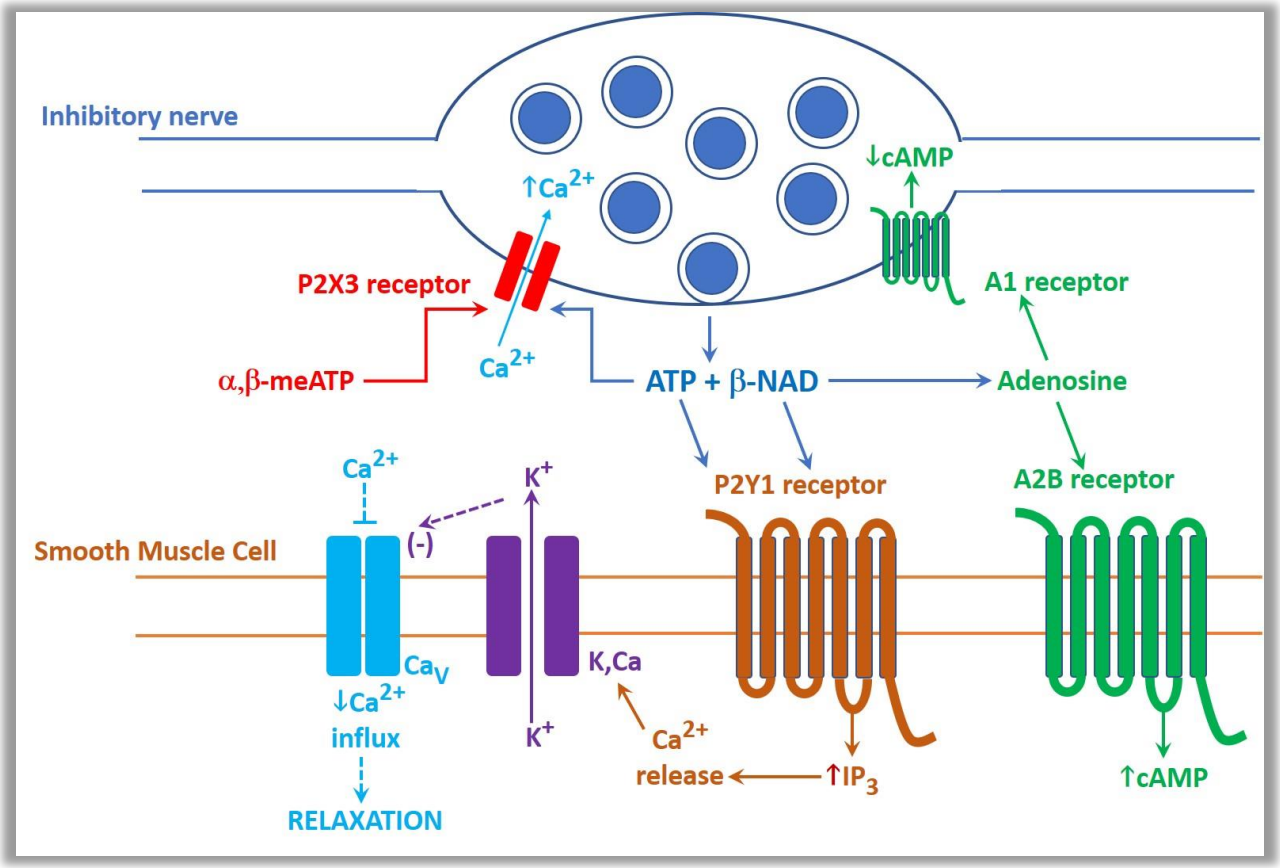
Highlights

- *Inhibitory nerves control the contractility of gastrointestinal smooth muscle*
- *ATP and β-NAD are released from inhibitory nerves and target P2Y1 receptors*
- *A synthetic nucleotide α,β-meATP mimics ATP and β–NAD, but cannot directly activate P2Y1 receptors*
- *Instead, α,β-meATP activates P2X3 receptors on inhibitory nerves*
- *P2X3 are Ca^{2+}-permeable ion channels which can release neurotransmitters*

Graphical Abstract



Abstract

The ATP analogue α,β-meATP is a potent relaxant of gastrointestinal smooth muscle, but its molecular target is uncertain inside the gut. α,β-meATP relaxed the carbachol-precontracted guinea-pig taenia coli in a concentration-dependent manner (EC_{50}, 2.0±0.1 μM). A luciferase-based assay confirmed that α,β-meATP solutions were minimally contaminated with ATP. α,β-meATP-evoked relaxations were inhibited by the competitive P2Y1 antagonist MRS2179 (pA_2 = 5.36), but also by the competitive P2X3 antagonist, A-317491 (pA_2 = 5.51). When MRS2179 and A-317491 were applied together, residual α,β-meATP responses converted from brief to prolonged relaxations. Sodium nitroprusside (a nitric oxide donor) also caused prolonged relaxations. Immunohistochemistry revealed that P2X3 receptors were present in myenteric ganglion cells and their varicose nerve terminals. The amplitude of α,β-meATP responses was not inhibited by TTX (NaV channel blocker) and ωCgTx (N-type CaV channel blocker). However, responses to α,β-meATP were inhibited by TEA (non-selective K^+-channel blocker), indicating that relaxations involved opening K^+-channels. The findings of this study are consistent with the conclusion that α,β-meATP stimulates Ca^{2+}-permeable P2X3 receptors on varicose nerve terminals to release inhibitory nucleotides: 1) ATP and β-NAD release results in P2Y1-mediated brief relaxations; 2) another released transmitter (possibly NO) results in prolonged relaxations. Prejunctional P2X3 receptors represent a purinergic feed-forward mechanism to augment the action of inhibitory nerves on gut motility. This positive feed-forward mechanism may counter-balance the known negative feedback mechanism caused by adenosine and prejunctional A1 receptors on inhibitory motor nerves. (232 words)

Keywords : Gastrointestinal Tract - purinergic transmission- ATP - P2X3 receptor - P2Y1 receptor

Abbreviations

A1, adenosine receptor (Type 1); A2B, adenosine receptor (Type 2B); A-317491, competitive P2X3 antagonist; [^{3}H]-ACh, tritiated acetylcholine; ATP, adenosine 5´-triphosphate; α,β-meATP, alpha,beta-methylene-ATP; EC_{50}, estimate of agonist potency; ICC, Interstitial Cells of Cajal; IJP, inhibitory junction potential; MRS2179, competitive P2Y1 antagonist; [^{3}H]-NA, tritiated noradrenaline; β-NAD, beta-nicotinamide adenine dinucleotide; NANC, nonadrenergic, noncholinergic; n_H, Hill co-efficient (slope of C/R curve); NO, nitric oxide; pA_2, estimate of antagonist efficacy; PDGFR, platelet-derived growth factor receptor, RLU, relative light units; SK channels, small-conductance Ca^{2+}-activated K^+-channels; SMCs, smooth muscle cells; SNP, sodium nitroprusside; TEA, tetraethylammonium chloride; TTX, tetrodotoxin; ωCgTx, omega conotoxin GV1A.

1. Introduction

The inhibitory action of nucleotides on gastrointestinal motility is a deceptively complex process involving more than one P2 receptor subtype (see Burnstock, 2008, 2014; Goyal et al., 2013; King, 2015) and more than one effector in the microcircuitry of interconnected cell types in the wall of the gut (see Sanders et al., 2012, 2014; Baker et al., 2015). Also, it has been proposed that two nucleotides - β-NAD (beta-nicotinamide adenine dinucleotide) and ATP (adenosine 5´-triphosphate) - are co-released by myenteric motor nerves (Mutafova-Yambolieva, 2012) and that β-NAD is a better candidate than ATP as a NANC inhibitory neurotransmitter in the GI tract (Sanders, 2016; and this issue for: Sanders, 2021). Ultimately, the inhibitory action of nucleotides released from purinergic nerves is brought about by increasing the K^+-permeability of smooth muscle cells (SMCs), through an event called the fast inhibitory junctional potential (fast IJP) which is generated by opening small-conductance (SK-type) K^+-channels that are blocked by apamin, TEA and other drugs (see Burnstock, 2008; Sanders et al., 2014). Thus, inhibitory P2 receptors initiate two fundamental responses in SMCs: a hyperpolarization of the SMC membrane potential and an elongation of SMC length (Burnstock, 2008, 2014; Goyal et al., 2013; Sanders et al., 2014; Baker et al., 2015; King, 2015, 2021).

P2Y1 receptors for synaptically-released nucleotides appear to play an indispensable role in this complex purinergic inhibitory circuitry, because the apamin-sensitive fast IJP is absent in $P2Y1^{-/-}$ knockout mice (Gallego et al. 2012; Hwang et al., 2012). The P2Y1 receptor is a membrane-inserted heptahelical protein which couples to a heterotrimeric G-protein ($G_{q/11}$) and raises the activity of the intracellular signalling enzyme

phospholipase C (PLCβ) when expressed in host cells. In turn, PLCβ generates inositol trisphosphate (IP_3) and afterwards IP_3 releases Ca^{2+} from intracellular stores (Webb et al., 1993; Filtz et al., 1994; Simon et al., 1995). Inhibitory P2Y1 receptors in the gut work through the same $G_q\alpha$/PLCβ/IP_3/$Ca^{2+}{}_i$ signaling pathway which leads to the downstream activation of Ca^{2+}-dependent and apamin-sensitive K^+-channels (K_{Ca}, SK-type), followed afterwards by the closure of voltage-sensitive Ca^{2+}-channels (CaV, L-type) and the relaxation of smooth muscle tone (Beyder and Farrugia, 2012; Goyal et al., 2013; Burnstock, 2014; Sanders et al., 2014; Kennedy, 2015; King, 2015, 2021; Mader et al., 2016). Evidence supports the presence of SK2 and SK3 channels on SMCs (Klemm and Lang, 2002), with SK3 channels also present on intramuscular fibroblast-like *PDGFRα*$^+$-cells (Sanders et al., 2012, 2014; Baker et al., 2015), whereas L-type Ca^{2+}-channels are found on SMCs as well as on intramuscular ICC/*Kit*$^+$cells (Beyder & Farrugia, 2012).

A discrepancy was discovered when comparing the pharmacological profiles of recombinant P2Y1 receptors and inhibitory P2Y1 receptors of the gut (Burnstock, 2014; King 2015). The synthetic ATP analogue alpha,beta-methylene ATP (α,β-meATP) fails to activate any of the pharmacologically characterized recombinant P2Y1 receptors in avian and mammalian species, including: chick, turkey, bovine, guinea-pig, human, mouse, rat (Webb et al., 1993; Filtz et al., 1994; Henderson et al.,1995; Tokuyama et al., 1995; Schachter et al., 1996; Leon et al., 1997; Gao et al., 2006). However, α,β-meATP readily causes purinergic inhibition in the avian and mammalian gut and is widely regarded as a potent relaxant [Satchell and Maguire, 1975; Burnstock and Kennedy, 1985; Den Hertog et al. 1985; Cusack et al., 1987; Dudeck et al., 1995; Piper and Hollinsworth, 1995; Windscheif et al., 1995; Bültmann et al., 1996; Storr et al., 2000; De

Man et al., 2003; Van Crombruggen et al., 2007; King and Townsend-Nicholson, 2008; Zhang et al., 2010). One simple explanation for this discrepancy is that α,β-meATP does not work directly through P2Y1, but works instead through another receptor upstream of the P2Y1 signalling pathway (Burnstock, 2014; King, 2015).

The present study addresses the identity and location of the α,β-meATP receptor in the gut. One study has reported that α,β-meATP activates P2Y11 receptors on SMCs in the guinea-pig taenia coli (King and Townsend-Nicholson, 2008). However, P2Y11 is a pseudogene in rat and mice genomes and does not produce a functional protein in these species, even though α,β-meATP is a smooth muscle relaxant in the rat and mouse gut (Windscheif et al., 1995; Storr et al., 2000; De Man et al., 2003; Van Crombruggen et al., 2007; Zhang et al., 2010). There is another plausible target for α,β-meATP, namely ATP-gated P2X3 receptors which are known to be present on myenteric motoneurons (Poole et al., 2002; Xiang and Burnstock, 2004; Van Crombruggen et al., 2007; King and Townsend-Nicholson, 2008) and at which α,β-meATP is a potent agonist (Chen et al., 1995; Lewis et al., 1995; Khakh et al., 2001; North, 2002; Jarvis and Khakh, 2009; Illes et al., 2021).

2. Methods and materials

2.1 Organ-bath experiments

Animals were housed and maintained according to the U.K. Animals (Scientific Procedures) Act, 1986. Male guinea pigs (250-500 g, body weight) were euthanised by a Schedule 1 procedure and exsanguinated via the carotid arteries. The abdomen was opened, the caecum ligated and removed in its entirety, then placed in chilled Krebs solution. Segments of taenia coli were dissected from the guinea-pig caecum without breaching the caecal wall.

Smooth muscle strips (5x15 mm) were hung vertically in 5 ml organ baths. A lower silk ligature was tethered to a fixed glass rod and an upper silk ligature was attached to an isometric force transducer (FT03; Gould Instruments). Organ baths were filled with a modified Krebs solution, containing (mM): NaCl 133, KCl 4.7, $NaHCO_3$ 16.4, Na_2HPO_4 1.4, $MgSO_4$ 0.6, $CaCl_2$ 2.5 and glucose 7.7. The Krebs solution was gassed with 95% O_2/5% CO_2 and the bath temperature kept at 36-37°C. Muscle strips were preloaded with 1-gram tension and then allowed to rest for 45-60 min.

Muscle strips were precontracted with carbachol (300 nM), which generated ~80% maximal tension (EC_{50}, 34±6 nM; Hill coefficient, 0.98±0.09; n = 4). Mechanical activity was recorded using a PowerLab-8/s interface and associated software PowerLab *Chart for Windows* (Version 4; AD Instruments, NSW Australia). Non-cumulative agonist concentration/response curves (C/R curves) were constructed using ATP (0.01 µM to 1 mM) and α,β-meATP (0.01 µM to 0.3 mM) applied for 1-5 minutes, afterwards followed by a 10-minute washout period. Routinely, adenosine deaminase (ADA; 2 $U.mL^{-1}$) was

added to bathing solutions to help degrade any locally generated adenosine, which otherwise would activate inhibitory A2B receptors in the taenia coli (Satchell and Maguire, 1975; Maguire and Satchell, 1979; Prentice and Hourani, 1997).

Statistical significance between data in C/R curves was tested by two-way analysis of variance (ANOVA) followed by a *post hoc* test (Tukey), using GraphPad *Prism* V.4.01 (GraphPad Software, Inc., Calif. USA). A probability of $p<0.05$ was considered significant in statistical tests.

2.2 Oocyte experiments

Xenopus laevis frogs were anesthetized with Tricaine (0.4% w/v, in tap water) and euthanised by decapitation, after which ovarian lobes were removed surgically. Collagenase-treated and defollicated oocytes (stages V and VI) were injected cytosolically with a cRNA for rat P2X3 (40 ng, 1 $mg.mL^{-1}$) and then stored in a modified Barth's solution at 18°C for 48 hours, and afterwards at 4°C for 2-5 days. Barth's solution contained (mM): NaCl 110, KCl, 1, Tris-HCl 7.5, $Ca(NO_3)_2$ 0.33; $CaCl_2$ 0.41, $MgSO_4$, 0.82, $NaHCO_3$, 2.4, mM; gentamycin sulphate 50 $\mu g.L^{-1}$; adjusted to pH 7.50.

Nucleotide-activated membrane currents were recorded from cRNA-injected oocytes using voltage-clamp techniques (Axoclamp-2A amplifier; Axon Instruments, Calif. U.S.A.). The voltage- and current-recording microelectrodes (0.5–2.0 MΩ) were filled with a filtered 3 M KCl solution. Oocytes were placed in a recording chamber (5.0 ml capacity) and superfused with a Ringer's solution (5 $mL.min^{-1}$ at 18°C). The Ringer's solution contained (mM): NaCl 110, KCl 2.5, HEPES 5, $BaCl_2$ 1.8, adjusted to pH 7.40.

Nucleotide-activated membrane currents were recorded at a holding potential of -30 mV. A-317491 was applied for 20 min prior to, and during, nucleotide applications (EC_{75} concentration, 3 μM). Increasing antagonist concentrations were applied and the degree of inhibition of nucleotide responses determined by normalising data to the mean current of three control applications of agonist at the EC_{75} concentration in the absence of the antagonist. Inhibition curves were drawn using GraphPad *Prism* V.4.01.

2.3 Immunohistochemistry

Full-thickness segments of the guinea-pig caecal wall were fixed 2% formaldehyde/ 0.2% picric acid in 0.1 M sodium phosphate buffer (pH 7.0). Tissue blocks of the taenia and underlying circular muscle were cryo-protected and embedded in OCT compound. Frozen tissues were sectioned (10 μm thick) in the transverse and longitudinal axes. Post-fixation, antigen retrieval was carried out for slide-mounted sections immersed in sodium citrate (10 mM, pH 6.0 at 80°C, for 10 min). Afterwards, sections were prepared for peroxidase immunohistochemistry using a rabbit anti-rat P2X3 antibody diluted 1:400 in PBS (Millipore Bioscience Research Reagents, Calif. USA), a donkey anti-rabbit biotinylated antibody diluted 1:200 (Millipore Bioscience), ExtrAvidin-Peroxidase diluted 1:100 (Sigma Aldrich, Gillingham UK) and 3% hydrogen peroxide. During incubations, sections were kept in a humid chamber at room temperature (20°-22°C).

2.4 Luciferase Assay

Contaminant ATP levels in drug solutions were measured using a commercially-available luciferase assay, designed foremost as a rapid detection system for bacterial ATP using the PD20 luminometer and LuciPac Pen kits (Kikkoman Company, Tokyo,

Japan) (King and Goodey, 2012). Single-use Pens contained a mixture of luciferin and a recombinant Firefly luciferase, together with an ATP-regenerating enzyme (pyruvate orthophosphate di-kinase), held in a sterile and sealed reaction chamber. Nucleotide solutions were added to this reaction chamber, shaken for 50 seconds and then the clear-walled chamber was inserted into an internally-calibrated PD20 luminometer, to provide a stable light reading within 10 seconds. Any degraded ATP in the reaction chamber was converted back to ATP by the included pyruvate orthophosphate di-kinase. Based on the internal calibration signal, readings were given as relative light units (RLUs) and provided a working range of 10-1,000,000 RLUs. The background "dark noise" of unopened reaction chambers was 10 RLUs. Pens were also tested against sterile and filtered water (Molecular Biology grade, Sigma-Aldrich). A linear calibration curve was established for a range of ATP concentrations (1 fM to 1 μM). Each nucleotide solution was tested a minimum of 6 times, with the same batch of Pens on the same day. Each test reading was stored in an internal memory chip, and then later downloaded onto a spreadsheet. The co-efficient of variation was <10% in multiple readings for each nucleotide concentration.

2.5 Drugs and solutions

ATP (disodium salt), α,β-meATP (lithium salt), adenosine deaminase (ADA), carbamylcholine chloride (Carbachol), collagenase (Type 1A), sodium nitroprusside, TEA (chloride salt) and TTX were obtained from Sigma-Aldrich (Gillingham, UK). Sterile water (W3500; Sigma Aldrich) was used in the luciferase assay. MRS2179 was obtained from Tocris-Cookson Ltd (Bristol, UK) and A-317491 was a gift from Abbvie Inc. (Illinois, USA). Conotoxin GVIA was a gift from Professor Annette Dolphin (NPP, UCL). All drugs were

made up in de-ionised, filtered and sterile water as stocks of either 10 mM or 100 mM, and stored at -20°C. Drugs were thawed just before use and diluted in a modified Krebs solution or Ringer's solution.

3. Results

3.1 Nucleotide-evoked relaxations

Nucleotides potently relaxed carbachol-precontracted muscle strips of the guinea-pig taenia coli. ATP-evoked relaxations were rapid, yet brief (<20 s duration), and these relaxations waned in the presence of this nucleotide (Fig. 1A). Similarly, α,β-meATP evoked rapid and brief relaxations (<60 s duration), as full in amplitude as those evoked by ATP, and these too waned in the presence of this nucleotide (Fig. 1A). The relaxations evoked by α,β-meATP were noticeably longer than ATP-evoked relaxations (Fig, 1A).

The possible contamination of α,β-meATP solutions by ATP was assessed using a luciferase assay (Fig. 1B). The averaged signal was 50 RLUs for a solution of α,β-meATP (0.1 μM), whereas the averaged signal was 20,000 RLUs for equimolar ATP (0.1 μM). By crude analysis, a signal ratio of 1:400 indicated that the level of ATP contamination was 0.25% of the reference 0.1 μM ATP solution (around 2.5×10^{-9} M). By detailed analysis, the level of ATP contamination was found to be much lower and determined as 10 picomolar (10^{-11}M) by extrapolation from a standard curve for ATP luminescence ([ATP]: 1 fM to 1 μM; RLU, 10-1,000,000).

The amplitude of evoked relaxations was concentration-dependent for both α,β-meATP and ATP, with the former being as efficacious as ATP and yet 3-fold more potent

(Fig. 1C). Agonist EC_{50} values and Hill coefficients were calculated from concentration/response (C/R) curves for 4 determinations for each nucleotide (n= 4): α,β-meATP, 2.0±0.1 μM, (n_H, 2.5±0.4); ATP, 5.7±0.4 μM (n_H, 1.2±0.1).

Transient relaxations evoked by α,β-meATP were converted into prolonged relaxations (>300 s duration) when inhibitory receptors were blocked by the P2Y1-selective antagonist, MRS2179 (Fig. 2A). MRS2179 (100 μM) produced a non-parallel and rightwards shift in the C/R curve for α,β-meATP (Fig. 2B). MRS2179 caused a fully surmountable antagonism in 4 experiments, with a 24-fold reduction in α,β-meATP potency (EC_{50} values: 47±1 μM *vs* 2.03 μM; n = 4). An apparent pA_2 value of 5.36 was calculated based on the observed dose ratio. However, MRS2179 changed the slope of the C/R curves (n_H: 2.5 *vs* 0.65; n = 4) and changed the duration of α,β-meATP-evoked relaxations, suggesting that the inhibitory action of MRS2179 was not solely due to competitive antagonism of P2Y1 receptors (see **Discussion**). In the presence of this P2Y1 receptor antagonist, sodium nitroprusside also evoked concentration-dependent and prolonged relaxations (Fig. 2A,C). The EC_{50} value for the NO-donor sodium nitroprusside (SNP) was 0.47±0.14 μM (n_H, 0.88±0.11; n = 6).

3.2 P2X3 receptors and NANC relaxations

Nucleotides potently activate recombinant P2X3 receptors expressed in RNA-injected *Xenopus laevis* oocytes. ATP and α,β-meATP evoked inward currents were rapid in onset, yet transient (<20 s duration), and rapidly waned in the presence of each nucleotide. Nucleotide-evoked inward currents were concentration-dependent; the EC_{50}

value for α,β-meATP was 1.91±0.09 μM (n_H, 0.69±0.12; n = 4) (Fig.3A). By comparison, the EC_{50} value for ATP was 1.30±0.15 μM (n_H, 0.80±0.11, n = 6).

The activity of the selective P2X3 antagonist, A-317491, was tested first at recombinant P2X3 receptors expressed in *Xenopus* oocytes (Fig. 3B). A-317491 inhibited the inward currents mediated α,β-meATP (3 μM) ~~by~~ in a concentration-dependent manner. The calculated IC_{50} value was 0.28±0.06 μM (n = 4-9) and P2X3 receptors were fully blocked by A-317491 at 100 μM.

The inhibitory activity of α,β-meATP at the guinea-pig taenia coli was reassessed in the presence of A-317491 (100 μM) (Fig. 3C). The P2X3-selective antagonist caused a parallel rightward shift in the C/R curve for α,β-meATP, reducing its potency by 33-fold (EC_{50} values: 65.8 μM *vs* 2.03 μM; n = 4), without significantly altering the slope of the C/R curve (n_H: 3.1±0.4 *vs* 2.5±0.4; n = 4). The parallel and rightwards shift of the C/R curve indicated that A-317491 acted as a competitive antagonist at inhibitory receptors, with an apparent pA_2 value of 5.51 at a P2X3-like receptor.

The blocking activity of MRS2179 (100 μM) was reassessed on α,β-meATP responses in the background presence of A-317491 (100 μM). Here, the P2Y1-selective antagonist had a modest inhibitory action on α,β-meATP potency (Fig. 3C), causing a further rightwards shift in the C/R curve (EC_{50} values: 78.1 μM *vs* 65.8 μM). The addition of MRS2179 caused an approximately 40-fold reduction (78.1 μM *vs* 2.03 μM), whereas A-317491 by itself had caused an approximately 30-fold reduction in α,β-meATP potency (65.8 μM *vs* 2.03 μM).

3.3 P2X3-immunopositive tissues

P2X3-positive labelling was observed in some but not all neurons in myenteric ganglia of the guinea-pig caecum (Fig 4A,B). Non-stained neurons in myenteric ganglia were surrounded by P2X3-labelled puncta which may represent immunopositive nerve terminals of interneurons (Fig. 4B). The taenia coli of the caecum also contained numerous P2X3-immunopositive striations, especially noticeable in the longitudinal axis of this muscle layer (Fig. 4C). Furthermore, P2X3-immunopostive labelling was observed in elongated cells (possibly fibroblasts) inside the muscle band of the taenia coli (Fig 4D).

Based on the likelihood that α,β-meATP activated P2X3 receptors to release transmitters from inhibitory motoneurons, the blocking action of neurotoxins was assessed on transient relaxations evoked by α,β-meATP (Fig. 4E). In 4 experiments tetrodotoxin (TTX; 1 μM), which blocks voltage-gated Na^+-channels (NaV), reduced transient relaxations to α,β-meATP by around 10% of their initial amplitude. In 4 experiments, omega-conotoxin GVIA (ωCgTX; 0.1 μM), which blocks voltage-dependent Ca^{2+}-channels (CaV; N-type), reduced transient relaxations by only 2% of their initial amplitude. In 4 experiments, the non-selective K^+-channel blocker tetraethylammonium (TEA; 0.6 mM) reduced α,β-meATP responses by 90% of their initial amplitude.

4. Discussion

For this tribute edition of *Autonomic Neuroscience* commemorating the scientific career of Geoffrey Burnstock, I have addressed a longstanding pharmacological conundrum involving the inhibitory activity in the gastrointestinal tract of the synthetic nucleotide, α,β-meATP. For many years this conundrum had deeply puzzled Geoff, who constantly advised me to investigate this issue and publish my results (see Figure 5). In the present study, one new finding of importance was α,β-meATP is only sparingly contaminated by ATP and, accordingly, its inhibitory actions must be due to α,β-meATP itself and not to background ATP present in drug solutions. This conclusion also was supported by the noticeably longer duration of α,β-meATP-evoked relaxations relative to ATP-evoked relaxations.

4.1 Pharmacological activity of α,β–meATP

In the past, α,β-meATP has been used extensively in the study of gastrointestinal motility and often on the guinea-pig taenia coli, where Burnstock and his colleagues first established the concept of purinergic transmission which involved the release of ATP or a related nucleotide from inhibitory motor nerves (Burnstock, 1970, 2008, 2014). Since 1975, α,β-meATP and other methylene phosphonate derivatives were known to have a strong inhibitory action on the guinea-pig taenia coli (Satchell and Maguire, 1975). Since 1985, the agonist potency of α,β-meATP relative to ATP and 2-MeSATP remains part of the grounding principles for discriminating native P2 receptor subtypes (Kennedy and Burnstock, 1985). For native P2Y receptors, the agonist potency order was first described as 2-MeSATP>ATP>α,β-meATP and for native P2X receptors it was α,β-meATP>ATP=

2-MeSATP (Burnstock and Kennedy, 1985). In the mid-1990s and during the initial studies of cloned P2X receptors, α,β-meATP helped to define the pharmacological profiles of some recombinant P2X receptors and, afterwards, helped to differentiate between subtypes of recombinant P2X receptors (Buell et al., 1996). Group 1 P2X receptors respond well to α,β-meATP (P2X1 and P2X3), Groups 2 and 3 do not (P2X2, P2X4 P2X5 and P2X7), and Group 3 was then thought to show pore dilatation (P2X7) (Buell et al., 1996; MacKenzie et al. 1999). Currently, only three P2X receptor subtypes respond to α,β-meATP at the concentration range active at guinea-pig taenia coli: these are P2X1, P2X3 and heteromeric P2X2/3 receptors (Buell et al., 1996; Dunn et al., 2001; North, 2002; Jarvis and Khakh, 2009; Illes et al., 2021). Two of these P2X receptors are known to be sensitive to A-314791 at the concentration range used in this paper, namely P2X3 and P2X2/3 receptors (Jarvis et al., 2002; Jarvis and Khakh, 2009; Illes et al., 2021). Therefore, a working hypothesis for the inhibitory action of α,β-meATP in the GI tract involved the activation of Ca^{2+}-permeable homomeric P2X3 receptors on motor nerve endings to release ATP or a related nucleotide which subsequently activates P2Y1 receptors in the taenia coli (see Figure 5, and Burnstock, 2014; King, 2015). By coincidence, the EC_{50} value for α,β-meATP (2 μM) was the same for the activation of recombinant P2X3 receptors and relaxation of the taenia muscle in the present study.

4.2 Nucleotide-evoked relaxations

Both ATP and α,β-meATP were found to elicit brief relaxations even at maximum agonist concentrations. Relaxations were inhibited by as much as 90% by TEA a non-selective K^+-channel blocker which is less potent than apamin but still inhibits the

purinergic fast IJP without appreciably changing the resting membrane potential of SMCs (Bauer and Kuriyama, 1982). Relaxations to ATP were shorter in duration than α,β-meATP-evoked relaxations; this difference has already been reported for the taenia coli (Maguire and Satchell, 1979; Den Hertog et al., 1985; Windscheif et al., 1995). The observed difference in duration was difficult to reconcile at first, since both ATP and α,β-meATP are nearly equipotent at P2X3 receptors and should be equally capable of activating P2X receptors on motor nerve endings (Chen et al., 1995; Lewis et al., 1995; North, 2002; Jarvis and Khakh, 2009; Illes et al., 2021). However, ATP is rapidly broken down by nucleotidases in the guinea-pig taenia coli whereas α,β-meATP is not (Welford et al., 1986) and, accordingly, one plausible explanation for longer α,β-meATP relaxations is its longer duration of action at P2X receptors on motor nerve endings. The efficacy and potency of ATP, but not α,β-meATP, is potentiated in the circular muscle of rat colon by the nucleotidase inhibitor, ARL67156 (Van Crombruggen et al., 2007).

In the present study, α,β-meATP was approximately 3-fold more potent than ATP. This potency order ran counter to earlier reports on inhibitory P2Y receptors in the taenia coli, where normally ATP>α,β-meATP (Satchell and Maguire, 1975; Burnstock & Kennedy, 1985; Piper and Hollingsworth 1995; Windscheif et al., 1995). However, organ bath experiments in the present study were carried out with adenosine deaminase (ADA) in the bathing solution, whereas earlier studies had not done so. The action of ADA on lowering ATP potency suggests that, in those earlier studies of the guinea-pig taenia coli, nucleotidases had generated adenosine which then contributed via inhibitory A2B receptors to the inhibitory action attributed to ATP (Maguire and Satchell, 1979; Prentice and Hourani, 1997). In line with this proposal, the adenosine uptake inhibitor

dipyridamole acts to limit any fall in the local adenosine concentration and has been shown to greatly potentiate the efficacy and potency of ATP at the guinea-pig taenia coli, thereby establishing an agonist potency order of ATP>α,β-meATP (Maguire and Satchell, 1979).

4.3 Antagonism of α,β-meATP responses

In the present study, α,β-meATP-evoked relaxations were inhibited by the P2Y1-selective antagonist MRS2179. None of the characterised P2Y1 isoforms is activated by α,β-meATP (Webb et al., 1993; Filtz et al., 1994; Henderson et al.,1995; Tokuyama et al., 1995; Schachter et al., 1996; Leon et al., 1997; Gao et al., 2006)., whereas MRS2179 is a competitive antagonist for P2Y1 and no other P2Y receptor subtype with a pK_B of 6.99 (pA_2 of 6.8) (Boyer et al., 1998; von Kügelgen, 2006). By contrast, the pA_2 value for MRS2179 in the taenia was considerably lower at 5.36; this disparity between the expected and observed pA_2 values indicated that inhibition of α,β-meATP responses was not solely due to P2Y1 blockade. MRS2179 also is a weak antagonist at Group 1 P2X receptors, including P2X3 receptors with an IC_{50} of 13 μM (Brown et al., 2000). Coincidentally, α,β-meATP-evoked relaxations were inhibited by another Group 1 P2X receptor antagonist NF279, as well as by MRS2179, in the mouse jejunum (De Man et al., 2003) and in rat colon (Van Crombruggen et al., 2007). Crucially, α,β-meATP-evoked relaxations were potently inhibited in the present study with a pA_2 value of 5.51 by the highly-selective P2X3 antagonist A-314791. Thus, there is a strengthening argument for the involvement of Group 1 P2X receptors in α,β-meATP responses.

4.4 Distribution of P2X3 subunits in the caecum

In the present study, P2X3-like material was observed in myenteric ganglia lying between of longitudinal and circular muscle layers of the guinea-pig caecum. Some but not all myenteric neurons were strongly P2X3-positive, as observed in earlier studies of P2X3-positive neurons in myenteric and submucosal ganglia in all segments of the guinea-pig gut (Poole et al., 2002; Xiang and Burnstock, 2004). However, electrophysiological studies reveal that nearly all myenteric and submucosal neurons are excited by ATP (Barajas-Lopez et al., 1996; Zhou and Galligan, 1996) and, to this end, P2X3-like material was observed in darkened varicose nerve terminals surrounding all stained and unstained myenteric neurons of the caecum. The present results indicate that both interneurons and inhibitory motoneurons may be a potential target for ATP and α,β-meATP, as proposed earlier for inhibitory responses in the dog ileum (Boeckxstaens et al., 1991). Additionally, P2X3-positive material was observed in nerve fibres in transverse and longitudinal sections of the taenia muscle. This network of immunopositive material appears to correspond to the penetrating nerve fibres identified by light and electron microscopy and running between the muscle fascicles of the taenia (Bennett and Rogers, 1967).

P2X3 receptors were first identified in the sensory ganglia of spinal dorsal roots (Chen et al., 1995; Lewis et al., 1995) and then later in all types of peripheral neurons including the enteric nervous system (see Dunn et al., 2001; North, 2002). P2X3 receptor activation of peripheral neurons results in action potential discharge at the cell body and transmitter release at nerve endings (see Dunn et al., 2001; North, 2002). In the present study, TTX (NaV channel blocker) and ωCgTx (N-type CaV channel blocker) barely affected α,β-

meATP-evoked relaxations and only reduced these relaxations by 10% or less. The results indicate that evoked relaxations in the guinea-pig taenia coli did not especially depend on action potential conduction from P2X3-positive myenteric neurons nor depended on the activation of voltage-gated Ca^{2+}-channels on P2X3-positive varicose nerve terminals. Instead, P2X3 receptors are known to be Ca^{2+}-permeable channels (as well as permeable to Na^{+} and K^{+}), and appear to be capable of conducting a Ca^{2+}-current large enough to trigger the release of neurotransmitters (see Khakh et al., 2001; North, 2002; Jarvis and Khakh, 2009; Illes et al., 2021). Recently, [^{3}H]-ACh release from motor nerve endings in human detrusor muscle was found to be regulated by α,β-meATP through prejunctional P2X receptors which were blocked by A-317491 and TNP-ATP (Silva-Ramos et al., 2020). The ability of P2X receptors to facilitate transmitter release actually occurs in a number of sites in the PNS and CNS, and this ability has been addressed in a wide-ranging and comprehensive review (Sperlágh et al., 2007). Of note, α,β-meATP enhanced the release of [^{3}H]-ACh and [^{3}H]-NA in the longitudinal muscle of guinea-pig ileum by a mechanism that was further potentiated by blocking A1 receptors with 8-phenyltheophylline (Sperlágh and Vizi, 1991). This release of [^{3}H]-NA in particular supports a prejunctional action for α,β-meATP, because only adrenergic nerve terminals occur in the longitudinal muscle whereas their cell bodies lie outside the gut wall and in prevertebral sympathetic ganglia.

4.5 Brief and prolonged α,β-meATP responses

One last complication in the present study was the transition of α,β-meATP-evoked relaxations from brief responses (<60 s) to prolonged responses (>300 s), when P2Y1

and P2X3 receptor antagonists were present in the bathing solution. At least 2 possible mechanisms may be at play here. For the first possibility, several studies have reported that two P2Y receptors exist in the muscle layers of the gastrointestinal tract (Dudeck et al., 1995; Piper and Hollingsworth, 1995; Windscheif et al., 1995; Bültmann et al., 1996; De Man et al., 2003; Van Crombruggen et al., 2007; King and Townsend-Nicholson, 2008; Zhang et al., 2010), with P2Y1 and P2Y11 being proposed as the two postjunctional targets in the guinea-pig taenia coli (King and Townsend-Nicholson, 2008). Of note, α,β-meATP has been shown to be a weak agonist at recombinant human P2Y11 receptors (Van der Weyden et al., 2000; King and Townsend-Nicholson, 2008). The full P2Y11 gene is absent in the rat and mouse genomes (Dreisig and Kornum, 2016; Kennedy, 2017), despite α,β-meATP being inhibitory in the rodent GI tract and where another mechanism must be at work (Windscheif et al., 1995; Storr et al., 2000; De Man et al., 2003; Van Crombruggen et al., 2007; Zhang et al., 2010). For this second possibility, prejunctional P2X3 receptors activated by α,β-meATP may release another inhibitory transmitter. In this respect, α,β-meATP does not cross-desensitise with 2-MeSATP for their evoked relaxations of the guinea-pig taenia coli (Dudeck et al., 1995). One obvious non-nucleotide transmitter is nitric oxide (NO), since a nitrergic slow IJP follows the purinergic fast IJP in response to field stimulation of inhibitory nerves in the guinea-pig taenia coli (Shuttleworth et al., 1991, 1999; Bridgewater et al., 1995; Selemidis et al., 1997; Barthó et al., 1998). Electrically-evoked NANC relaxations in the taenia coli were shown to be partially inhibited by the NOS-inhibitor L-NNA and almost completely inhibited by the combination of apamin and L-NNA (Shuttleworth et al., 1999). However, NO-dependent relaxations to field electrical stimulation of inhibitory nerves first require

the full blockade of apamin-sensitive relaxations before they become fully developed (Selemidis et al., 1997). Perhaps this explains why only brief relaxations to α,β–meATP were observed prior to the addition of MRS2179 and A-314791 in the present study. Coincidentally, the NO-donor sodium nitroprusside also evoked large and prolonged relaxations when P2Y1-receptors and P2X3 receptors were blocked.

5 Conclusions

The present study has helped to explain how the synthetic nucleotide α,β-meATP causes relaxation of smooth muscle in the gastrointestinal tract, *in vitro*. The molecular target for this nucleotide is not P2Y1 but P2X3; however, α,β-meATP-evoked relaxations are dependent on competent P2Y1 receptors (see Figure 5). Burnstock (2014) had tentatively proposed that the real target for α,β-meATP was prejunctional P2X3 receptors which then promoted release of inhibitory nucleotides. The histochemical and pharmacological evidence presented here adds weight to this proposal. *In vivo*, prejunctional P2X3 receptors may operate as a facilitatory mechanism with a first phase of ATP release resulting in the release of more inhibitory nucleotides through a positive feedback mechanism. This positive feedback may partly explain why the amplitude of the fast IJP is so large (20-30 mV) to only a single electrical shock applied to purinergic nerves in the GI tract (Bennett et al., 1966; King, 2021). Afterwards, synaptic ATP and β-NAD are degraded enzymatically to adenosine which then downregulates further release of inhibitory nucleotides via prejunctional A1 receptors on purinergic nerves (King, 1994). Prejunctional inhibition may partly explain the phenomenon of IJP rundown, in response to stimulus trains of electrical shocks at frequencies as low as 1 Hz applied to inhibitory

nerves (Bennett et al., 1966; King, 1994). In gastrointestinal disease, the megacolon of Hirschsprung's patients is associated with a stark reduction of P2X3-positive material in aganglionic segments of the gut (Facer et al., 2001) and, as a result, the fast IJP is absent in the lower constricted rectosigmoidal colon where exogenous ATP fails to hyperpolarise SMCs (Zagorodnyuk et al., 1989). Thus, prejunctional P2X3 receptors along with postjunctional P2Y1 receptors play an interdependent and co-operative role in the inhibitory innervation of the gut – more so than ever realised.

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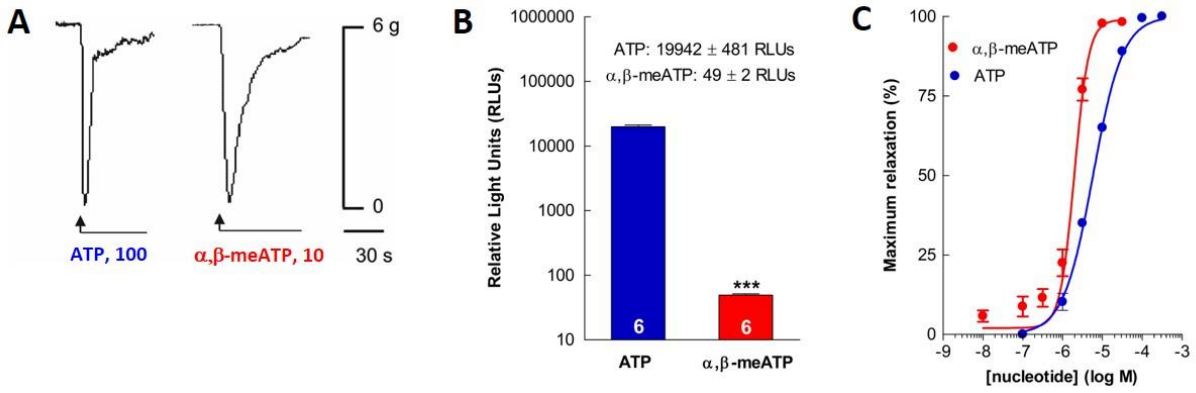

Fig 1. *Nucleotide-evoked relaxations of gastrointestinal smooth muscle.*

In A, maximal concentrations of ATP (100 μM) and α,β-meATP (10 μM) potently relaxed the guinea-pig taenia coli pre-contacted by carbachol (0.3 μM). Evoked relaxations were full in amplitude (zero tone). In B, α,β-meATP was found to be minimally contaminated with ATP as assessed by a luciferase assay in 6 experiments. The respective luminometry signals were 49±6 RLUs for α,β–meATP *versus* 19942±481 RLUs for ATP (n = 6; both nucleotides at 0.1 μM, $p < 0.001$). In C, α,β-meATP and ATP relaxed the taenia coli in a concentration-dependent manner, with a potency order α,β-meATP>ATP in 4 experiments. Both agonists fully relaxed the taenia coli. The C/R curves represent the fit of the Hill equation to the data (expressed as mean±SEM).

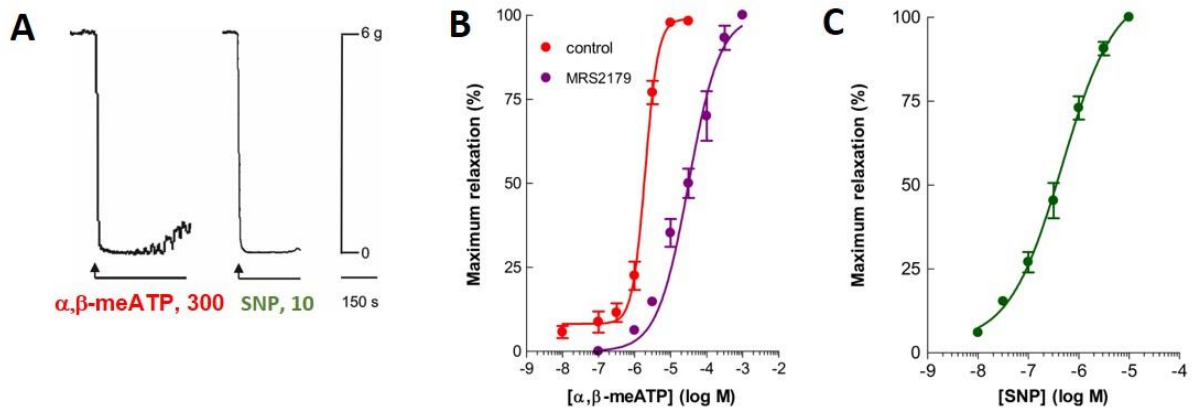

Fig 2. *Prolonged relaxations following P2Y1 receptor blockade.*

In A, maximal concentrations of α,β-meATP (300 μM) and sodium nitroprusside (SNP, 10 μM) evoked prolonged relaxations in the guinea-pig taenia coli when P2Y1 receptors were blocked by MRS2179 (100 μM). Evoked relaxations were full in amplitude (zero tone). In B, α,β-meATP relaxed the taenia coli in a concentration-dependent manner, under control conditions (red symbols) or in the presence of MRS2179 (purple symbols) in 4 experiments. In C, SNP also fully relaxed the taenia coli in a concentration-dependent manner in 4 experiments. The C/R curves represent the fit of the Hill equation to the data (expressed as mean±SEM).

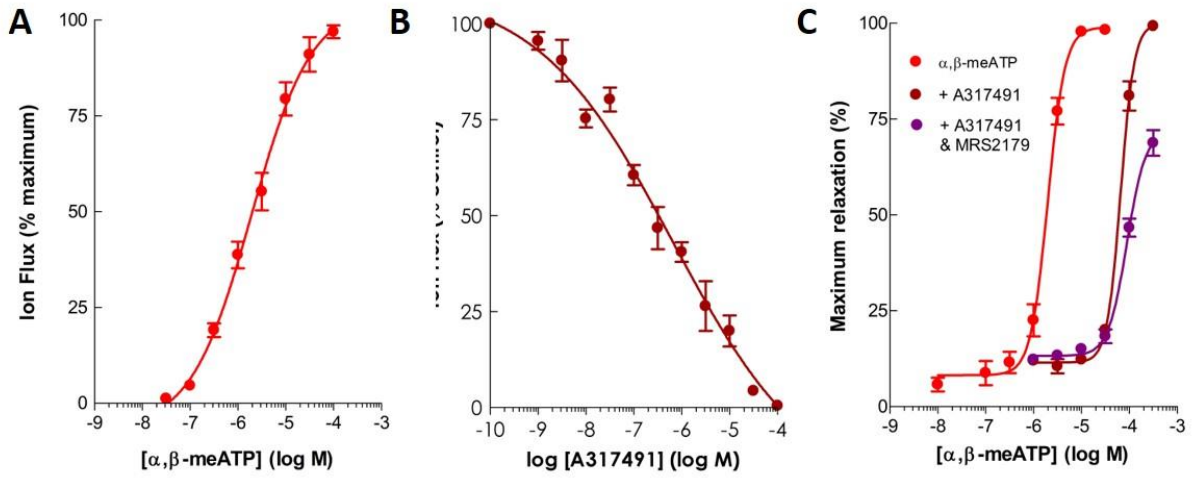

Fig 3. *Pharmacology of recombinant P2X3 receptors and native inhibitory P2 receptors*.

In A, α,β-meATP activated recombinant P2X3 receptors expressed in *Xenopus* oocytes in a concentration-dependent manner in 4 experiments. In B, A-317491 inhibited α,β-meATP-evoked inward currents mediated by P2X3 receptors in a concentration-dependent manner in 9 experiments. Using the Cheng-Prusoff equation, the K_i for A317491 was 109 nM. In C, α,β-meATP relaxed the taenia coli in a concentration-dependent manner, under control conditions (red symbols) or in the presence of A-317491 (100 μM) (brown symbols) in 4 experiments. α,β-meATP-evoked relaxations were further inhibited by the combination of A-317491 (100 μM) and MRS2179 (100 μM) (purple symbols) in 4 experiments. The C/R curves represent the fit of the Hill equation to the data (expressed as mean±SEM).

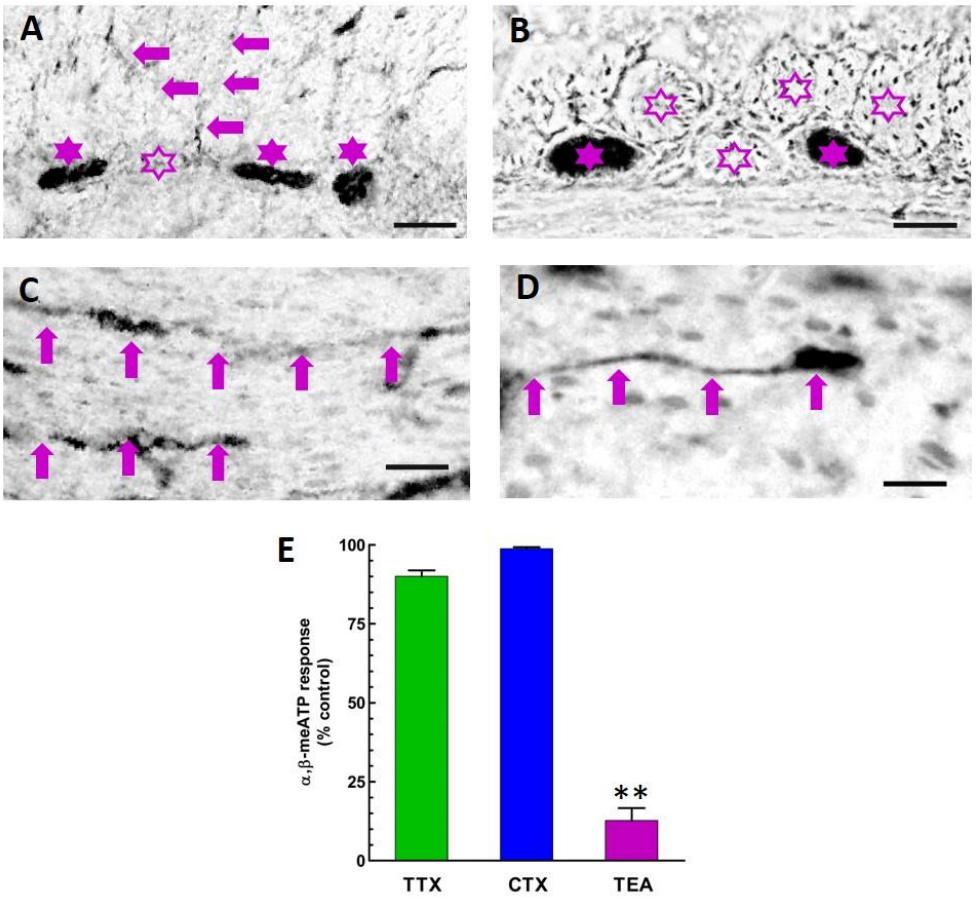

Fig 4. *P2X3-immunoreactivity in the guinea-pig taenia coli.*

In A, P2X3-positive material was observed in some neurons in a caecal ganglion (filled stars). A network of immunopositive material also was observed in the guinea-pig taenia coli above the caecal ganglion, sectioned in the transverse axis (filled arrows). In B, P2X3-positive material was seen in black puncta surrounding immunostained neurons (filled stars) and unstained neurons (open stars) in caecal ganglia. In C, immunopositive fibres were observed in the taenia sectioned in the longitudinal axis (filled arrows). In D, some immunopositive cells (possibly fibroblasts) were observed in longitudinal sections (filled arrows). Scale: 100 μm (A), 20 μm (B-D). In E, α,β-meATP-evoked relaxations were barely affected by the NaV inhibitor tetrodotoxin (TTX, 1 μM), or the N-type CaV

inhibitor, omega-conotoxin (ωCgTX, 0.1 μM) in 4 experiments, but were considerably inhibited (p<0.01) by the non-selective K^+-channel inhibitor tetraethylammonium (TEA, 0.6 mM) in 4 experiments (data expressed as mean±SEM).

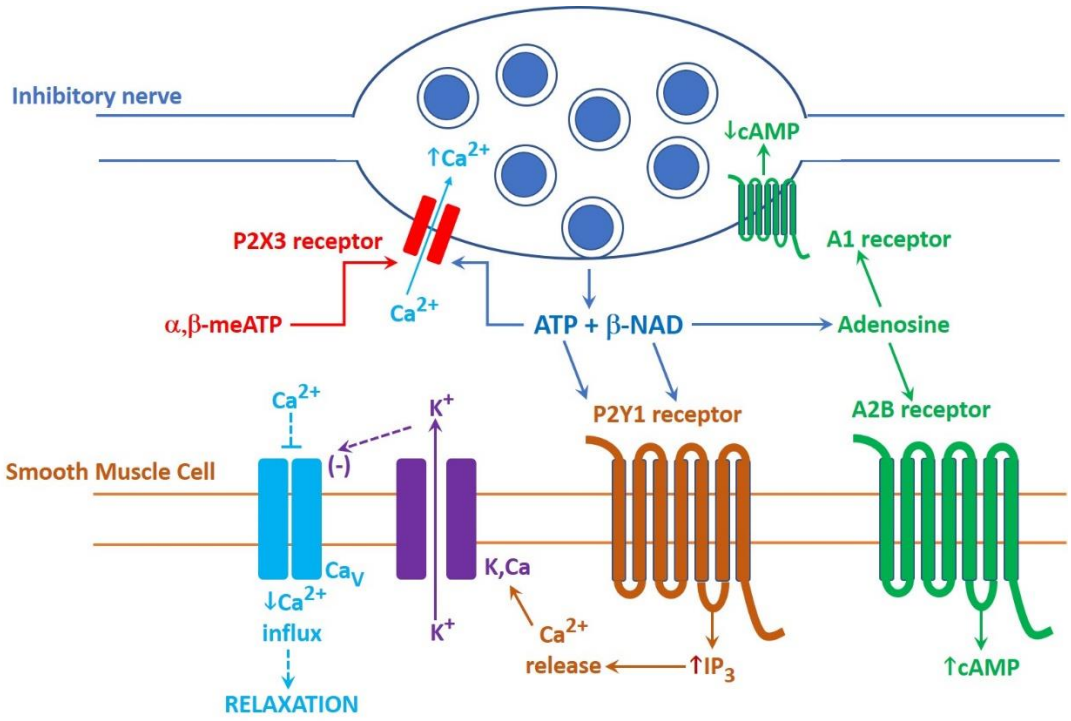

Fig 5. *Schematic diagram of purinergic neurotransmission in the gastrointestinal tract.*

Inhibitory myenteric nerves release ATP and β-NAD stored in dense-core vesicles at the active zone of varicose nerve endings via Ca^{2+}-dependent exocytosis. Extracellular ATP and β-NAD may activate P2Y1 receptors on smooth muscle cells, or can be degraded by ecto-nucleotidases into adenosine which then can activate A1 and A2B receptors. The P2Y1 signalling cascade leads to the opening of K^+-channels (K_{Ca}, SK-type) and the resulting IJP and hyperpolarisation leads to the closure of Ca^{2+}-channels (CaV, L-type), then finally smooth muscle relaxation. α,β-meATP targets Ca^{2+}-permeable P2X3 receptors on inhibitory nerves to release ATP and β-NAD which then activate the P2Y1 signalling cascade. Vesicularly-released ATP also may activate prejunctional P2X3 receptors to release more inhibitory nucleotides. Adapted from an earlier, less detailed schematic diagram (see: Kennedy, 2015).