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P2 receptors in the murine gastrointestinal tract

C. Giaroni [a], G.E. Knight [b], H.-Z. Ruan [b], R. Glass [b], M. Bardini [b], S. Lecchini [a], G. Frigo [a], G. Burnstock [b,*]

[a] *Department of Internal Medicine and Therapeutics, Section of Pharmacology, University of Insubria and University of Pavia, via O. Rossi 9, I-21100, Varese, Italy*

[b] *Autonomic Neuroscience Institute, Royal Free and University College Medical School, Royal Free campus, Rowland Hill Street, London NW3 2PF, UK*

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Abstract

The actions of adenosine, adenosine 5′-triphosphate (ATP), 2-methylthio adenosine diphosphate ADP (2-MeSADP), 2-methylthio ATP (2-MeSATP), α,β-methylene ATP (α,β-meATP) and uridine triphosphate (UTP) on isolated segments of mouse stomach (fundus), duodenum, ileum and colon were investigated. The localization of $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2X_1$ and $P2X_2$ receptors and neuronal nitric oxide synthase (NOS) were examined immunohistochemically, and $P2Y_1$ mRNA was examined with in situ hybridization. The order of potency for *relaxation* of longitudinal muscle of all regions was: 2-MeSADP ≥ 2-MeSATP > α,β-meATP > ATP = UTP = adenosine. This is suggestive of $P2Y_1$-mediated relaxation and perhaps a further P2Y receptor subtype sensitive to α,β-meATP. As ATP and UTP are equipotent, the presence of a $P2Y_2$ receptor is indicated. ATP responses were inhibited by the $P2Y_1$-selective antagonist MRS 2179, and suramin. $P2Y_1$ receptors were visualized immunohistochemically in the smooth muscle of the ileum and in a subpopulation for myenteric neurones, which also stained for NOS. $P2Y_1$ mRNA was localized in neurones in both myenteric and submucosal ganglia in the ileum. Taken together, these results suggest that ATP was acting on non-adrenergic, non-cholinergic inhibitory neurons, which release both nitric oxide (NO) and ATP. Reduced relaxations to 2-MeSADP by tetrodotoxin and N^{ω}-nitro-L-arginine methyl ester, are consistent with this possibility. Adenosine acts via P1 receptors to relax smooth muscle of the mouse gut. Segments of mouse colon (in contrast to the stomach and small intestine) were *contracted* by nucleotides with the potency order: 2-MeSATP > α,β-meATP > ATP; the contractions showed no desensitization and were antagonized by suramin and PPADS, consistent with responses mediated by $P2X_2$ receptors. Immunoreactivity to $P2X_2$ receptors was demonstrated on both longitudinal and circular muscle of the colon, but not in the other regions of the gut, except for a small subpopulation of myenteric neurones. In summary, neuronal $P2Y_1$ receptors appear to mediate relaxation, largely through NO in all regions of the mouse gut, and to a lesser extent by $P2Y_1$, $P2Y_2$ and a novel P2Y receptor subtype responsive to α,β-meATP in smooth muscle, while $P2X_2$ receptors mediate contraction of colonic smooth muscle.

Keywords: Mouse; Adenosine 5′-triphosphate; P2 receptors; Stomach; Duodenum; Ileum; Colon

1. Introduction

Purine nucleotides and nucleosides have long been known to have effects on the gastrointestinal (GI) tract, influencing both motor and secretory functions. In 1970, Burnstock and colleagues proposed that adenosine 5′-triphosphate (ATP) was a transmitter involved in non-adrenergic, non-cholinergic (NANC) nerve-mediated responses of smooth muscle in the gut (Burnstock et al., 1970). Since then evidence has accumulated in support of the hypothesis that ATP is a NANC transmitter in the enteric nervous system (Burnstock, 2001). High concentrations of ATP in subpopulations of myenteric neurons in different regions of the gut of the guinea-pig, rabbit and rat have been reported (Crowe and Burnstock, 1981; Belai and Burnstock, 1994). In addition, after application of an appropriate stimulus, ATP is released from enteric nerves (Burnstock et al., 1978; McConalogue et al., 1996) and the subsequent activation of specific receptors

* Corresponding author. Tel.: +44-20-7830-2948; fax: +44-20-7830-2949.

E-mail address: g.burnstock@ucl.ac.uk (G. Burnstock).

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on enteric neurons and muscle cells may evoke either excitation or inhibition of smooth muscle function (see Burnstock, 2001).

Burnstock (1978) proposed a formal classification of receptors for adenosine and ATP, collectively called purinoceptors. Receptors selective for adenosine and adenosine monophosphate were designated as P1-purinoceptors and those selective for ATP and adenosine diphosphate (ADP) called P2-purinoceptors. This classification set the stage for further subdivisions of P1 receptors, into A_1, A_{2A}, A_{2B} and A_3 and of P2 receptors into P2X and P2Y families (Burnstock and Kennedy, 1985; Abbracchio and Burnstock, 1994; Ralevic and Burnstock, 1998). P2X receptors are ionotropic while P2Y receptors are G protein-coupled, each with their own subtypes, $P2X_{1-7}$ and $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$ and $P2Y_{13}$ (see King et al., 2000; Khakh et al., 2001; Nicholas, 2001). At present there are few selective agonists and antagonists that discriminate clearly between families of P2X and P2Y receptors, or between subtypes of receptors within each family, although 2-methylthio ADP (2-MeSADP) and MRS 2179 have been claimed to be selective agonists and antagonists, respectively, for the $P2Y_1$ receptor (Camaioni et al., 1998). The use of characteristic agonist potency profiles and effective antagonists, immunostaining and in situ hybridization for receptor subtypes can be used to help identify specific P2 receptor subtypes.

Both adenosine and ATP receptors have been suggested to play important roles in the modulation of motility in the GI tract. Adenosine can directly activate receptors located on smooth muscle (Nicholls et al., 1996; Kadowaki et al., 2000) or act prejunctionally, suppressing the release of excitatory neurotransmitters such as acetylcholine (ACh) and substance P (Moneta et al., 1997; Kadowaki et al., 2000). In most species, ATP activates P2 receptors on smooth muscle to produce relaxation of smooth muscle in the GI tract, although there are some examples where ATP also produces contraction of smooth muscle of some regions of the gut (Burnstock, 2001).

Although there is good evidence to suggest that purines can influence motility in the GI tract, a clear characterization of the different subtypes of P2 receptors involved has not yet been established. This is certainly true of the mouse GI tract, where there is very little information concerning the activity of purine compounds and the receptors they activate. The present investigation was carried out to characterize P2 receptors in the mouse gut, using pharmacological and morphological approaches. To this end, we studied the effect of purinoceptor agonists and antagonists on the longitudinal muscle of the stomach fundus, duodenum, ileum and colon. Further, the presence of $P2Y_1$ receptor mRNA was investigated in the ileum by in situ hybridization and $P2Y_1$, $P2Y_2$, $P2Y_4$ and $P2X_2$ receptors and nitric oxide synthase (NOS) were studied with immunohistochemical methods in the ileum, and $P2X_1$ and $P2X_2$ receptors were studied in the colon. This study was carried out on the GI tract of the mouse, partly because only scarce information is available concerning the role of purinergic transmission in the gut of this species, but also because the increasing availability of P2 receptor knockout mice makes such control information desirable.

2. Materials and methods

2.1. Animals

Principles of good laboratory animal care were followed and animal experimentation was in compliance with specific national (UK) laws and regulations. Adult male mice (strain C57/BL10) weighing 20–28 g, were killed by exposure to an increasing concentration of carbon dioxide and death was ensured by cervical dislocation according to Home Office (UK) regulations covering Schedule One procedures. After the abdominal cavity had been opened, the gut was rapidly removed and placed in a beaker with a physiological saline solution.

2.2. Pharmacological studies

The stomach was opened along the longitudinal axis of the greater curvature, pinned flat and longitudinal smooth muscle strips (1–2 mm in width, 5 mm in length) were dissected from the anterior fundus wall (upper part). The duodenum was dissected out at the base of the pylorus and a length of 1 cm was used; 1 cm segments of distal ileum, approximately 3 cm oral to the ileo-caecal junction and distal colon were also removed. Intestinal segments were flushed and cleared of connective tissue. Silk ligatures were applied to each end of the strip; one end was attached to a rigid support and the other to a Grass FT03C force displacement transducer. Tissues were mounted in 10-ml organ baths, continually gassed (95% O_2/5% CO_2) and containing Krebs solution of the following composition (mM): NaCl, 133; KCl, 4.7; $NaHCO_3$, 16.4; $MgSO_4$, 0.6; NaH_2PO_4, 1.4; glucose, 7.7 and $CaCl_2$, 2.5; pH 7.3. Experiments were carried out at 32 ± 1 °C, as this temperature reduced spontaneous activity. Mechanical activity was displayed on a Grass ink-writing oscillograph. An initial load of 0.5 g was applied to the stomach fundus and 0.75 g for the other regions; tissues were allowed to equilibrate for 60 min prior to the start of experiments.

For each tissue, a concentration–response curve to carbachol (CCh) was constructed in order to obtain an EC_{50} concentration. Mean concentrations were found to be approximately 2 μM for the stomach fundus and 5 μM for the duodenum, ileum and colon. Concentration–

response curves for the different purinoceptor agonists were constructed cumulatively, or when rebound contractions were observed, non-cumulatively, on CCh-precontracted tissue. Curves to ATP were repeated in the presence of the P1 receptor antagonist 8-*p*-sulphophenyltheophylline (8-*p*SPT, 30 μM), the non-selective P2 receptor antagonists suramin (100 μM) and pyridoxalphosphate-6-azophenyl-2′,4′-disulphonic acid (PPADS, 30 μM) and the $P2Y_1$-selective antagonist MRS 2179 (1 μM). The curves to 2-methylthio ATP (2-MeSATP) were also repeated in the presence of suramin (100 μM) and PPADS (30 μM). The effect of MRS 2179 (1 μM) was also tested against 2-MeSADP and α,β-methylene ATP (α,β-meATP). Curves to 2-MeSADP were repeated in the presence of the NOS inhibitor N^{ω}-nitro-L-arginine methyl ester (L-NAME, 100 μM), then in the presence of L-NAME and L-arginine (L-Arg, 5 mM); the effect of a submaximal dose of ATP (300 μM), 2-MeSADP (3 μM) and α,β-meATP (30 μM) was evaluated in the absence and presence of the neurotoxin tetrodotoxin (TTX, 1 μM). The effect of a submaximal dose of ATP (300 μM) was evaluated in the absence and presence of either L-NAME (100 μM) or the prostanoid synthesis inhibitor, indomethacin (10 μM).

In colonic specimens, contraction concentration–response curves to ATP, 2-MeSATP and α,β-meATP were constructed in a cumulative fashion on basal tone. The curves to ATP and 2-MeSATP were repeated in the presence of suramin (100 μM) and PPADS (30 μM). The effect of atropine (1 μM) was tested against a single concentration of α,β-meATP (30 μM). All antagonists were equilibrated for 20 min before concentration–response curves or single doses of agonists were repeated.

2.3. In situ hybridization

2.3.1. Tissues

Tissues were dissected and immediately put in ice-cold Hanks' balanced salt solution, pH 7.5 (Gibco BRL, Scotland). Unfixed tissues were embedded in Tissue-Tek (Sakura Finetek, Netherlands) and frozen in isopropanol precooled in liquid N_2. Cryostat sections (10 μm) were placed on poly-L-lysine coated slides.

2.3.2. Probes

Antisense oligonucleotide probes 45 nucleotides in length for rat $P2Y_1$, were obtained from either Genosys (UK) or MWG Biotech (UK). Probes were labelled at their 3′-end with the digoxigenin (DIG) oligonucleotide tailing kit (Roche Diagnostics; Palo Alto, CA) according to the manufacturer's instructions. The sequence of the probe was: 5′-AGG TGG CAT AAA CCC TGT CGT TGA AAT CAC ACA TTT CTG GGG TCT-3′. The specificity of the probe was confirmed by screening the Genbank database. The sequence was found to detect mouse $P2Y_1$ receptor mRNA with high specificity.

2.3.3. Hybridization

In situ hybridization was carried out as previously described (Glass et al., 2000). Briefly, slides were dehydrated in ethanol, air-dried and incubated in prehybridization buffer (50% formamide; saline sodium citrate buffer (SSC); Denhardt's medium; 1 mg/ml denatured, sheared salmon sperm DNA (Sigma Chemical Co., Poole, UK) and 1 mg/ml tRNA type X from bakers' yeast (Sigma)) for 1 h at 37 °C in a humidified chamber. This was followed by incubation in hybridization buffer (1 ng labelled probe in 1 μl prehybridization buffer) at 37 °C overnight. Washing of unhybridized probe was performed as follows: 2 × 5min in SSC at room temperature, 2 × 15min in SSC at 37 °C, 2 × 15min in SSC at 37 °C, with a final stringency wash of 2 × 30min in SSC at 37 °C. DIG-labelled probes were detected by anti-DIG-alkaline phosphatase labelled Fab-fragments (Boehringer Mannheim). Negative controls included competing labelled probe with an excess (100×) of unlabelled probe.

2.4. Immunohistochemistry

Sections (12 μm) of mouse ileum and colon were cut using a cryostat (Reichert Jung CM1800) and collected on gelatine-coated slides and air-dried at room temperature. The slides were stored at −20 °C.

The avidin-biotin (ABC) technique was employed according to the protocol described by Llewellyn-Smith et al. (1993). The sections were left at room temperature for at least 10 min and then fixed in 4% formaldehyde (0.1 M phosphate buffer, pH 7.4) containing 0.2% saturated solution of picric acid for 2 min.

Endogenous peroxidase was blocked with 50% methanol containing 0.4% hydrogen peroxide for 10 min. Non-specific binding sites were blocked by a 20-min incubation with 10% normal horse serum (NHS) in phosphate-buffered saline (PBS) containing 0.05% merthiolate.

The $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2X_1$ and $P2X_2$ receptor antibodies (rabbit) were diluted to 2.5 μg/ml with 10% NHS. Primary antibodies were kindly donated by Roche Bioscience (Palo Alto, CA; Oglesby et al., 1999). The specimens were then incubated overnight at room temperature with the primary $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2X_1$ and $P2X_2$ antibodies. The secondary antibody was biotinylated donkey anti-rabbit immunoglobulin G (IgG) diluted 1:500 for 30 min, followed by the ExtrAvidin peroxidase conjugate used at 1:1000 for 30 min. After washing, a nickel-diaminobenzidine enhancement technique was used to visualize the reaction product. The specimens were dehydrated with xylene and mounted in Eukitt (BDH; Poole, UK).

The following control experiments were performed to establish a specific immunoreaction: omission of the primary antibody, replacement of the primary antibodies

with rabbit preimmune IgG or absorption of the primary antibodies with an excess of their homologous peptide antigen.

Whole mount preparations of the mouse ileum were prepared following the method of Toole et al. (1998) and double-stained for $P2Y_1$ receptors and NOS. The secondary antibody for $P2Y_1$ receptor staining was FITC-conjugated goat anti-rabbit and the secondary antibody for neuronal NOS was Cy-3-conjugated donkey anti-rabbit. Whole mount preparations were also stained for $P2X_2$ receptors using the ABC technique, described above.

Photographs were taken using a Leica DC200 digital camera attached to a Zeiss Axioplan microscope (Zeiss, Oberkochen, Germany) and pictures were processed using Adobe-Photoshop 5.0 software on an Apple Power-Macintosh G3. Prints were made with an Epson Stylus Photo 700 printer.

2.5. Drugs

L-Arg, α,β-meATP, ATP, atropine, carbamyl-β-methyl choline chloride (carbachol), ExtrAvidin-horseradish peroxidase, glucose oxidase, hydrogen peroxide, indomethacin, 2-MeSADP, 2-MeSATP, L-NAME, NHS, PPADS, saturated picric acid solution, merthiolate (thimerosal), 8-*p*SPT, suramin, TTX and uridine triphosphate (UTP) were purchased from Sigma; MRS 2179 was obtained from Tocris, Bristol, UK. Formaldehyde stabilized with 10% methanol was obtained from Analar, BDH; rabbit anti-NOS, biotinylated donkey anti-rabbit and goat anti-rabbit IgG were obtained from Jackson ImmunoResearch, PA, USA. Stock solutions were prepared in distilled water; indomethacin in 0.2 M $NaHCO_3$. The volume added to the organ bath to produce the final concentration was not in excess of 100 μl.

2.6. Statistical analysis

Relaxant responses were expressed as mean percentage reduction of the CCh-induced contraction (at EC_{50} concentration) ± standard error of the mean (SEM) of (*n*) animals and contractile responses were expressed as mean maximum tension developed in mg ± SEM (*n*). The potency of the agonists in causing relaxation or contraction was expressed as the negative $\log_{10}$ of the molar concentration of the agonist producing 25% of the response ($p[A]_{25}$), calculated by non-linear regression analysis of the individual log concentration–response curves, by means of the software PRISM 3.0 (GRAPHPAD Software, Inc., San Diego, CA, USA). Significance was tested by analysis of variance (ANOVA) followed by Bonferroni's test, or paired Student's *t*-test. A probability level of $P < 0.05$ was taken as significant for all statistical analyses.

3. Results

3.1. Relaxation

Adenosine, ATP, 2-MeSATP, 2-MeSADP, α,β-meATP and UTP relaxed all regions of the CCh-contracted mouse GI tract. The order of potency for the stomach fundus and colon was: 2-MeSADP = 2-MeSATP > α,β-meATP > UTP = ATP = adenosine (Figs. 1(a) and 4(a), respectively). The order of potency for the duodenum and ileum was: 2-MeSADP = 2-MeSATP > α,β-meATP > UTP = ATP = adenosine (Figs. 2(a) and 3(a), respectively). As none of the concentration–response curves from any of the regions of the GI tract reached a maximum response, pD_2 values could not be calculated. As such the concentration of agonist that produced 25% relaxation was calculated ($p[A]_{25}$) and data are shown in Table 1.

Concentration–response curves to ATP were significantly ($P < 0.001$ or $P < 0.0001$) inhibited by 8-*p*SPT (30 μM) in stomach fundus (Fig. 1(b)), duodenum (Fig. 2(b)), ileum (Fig. 3(b)) and colon (Fig. 4(b)). MRS 2179 (1 μM) significantly ($P < 0.001$ or $P < 0.0001$) inhibited responses to ATP in each region of the gut examined (Figs. 1(c), 2(c), 3(c) and 4(c)). Similarly, responses to 2-MeSADP in each region of the GI tract examined were inhibited by MRS 2179 (1 μM; $P < 0.001$ or $P < 0.0001$) (Figs. 1(d), 2(d), 3(d) and 4(d)). PPADS (30 μM) did not affect ATP responses in any

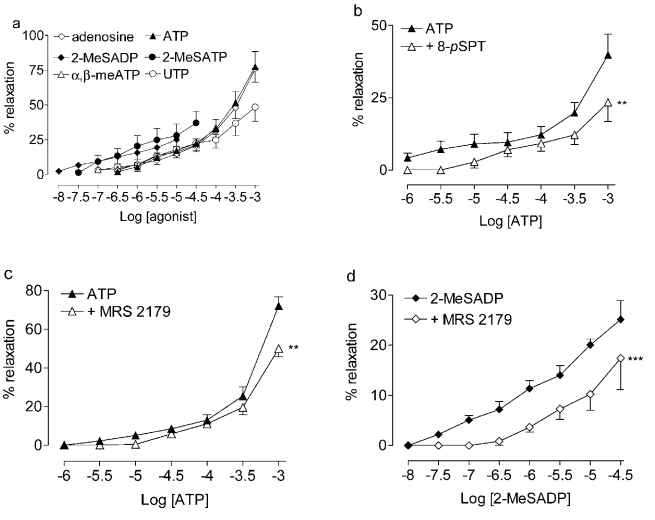


Fig. 1. Cumulative concentration–response curves on the CCh-precontracted (EC_{50} concentration) mouse GI longitudinal muscle of the gastric fundus. (a) Concentration–response curves for adenosine, ATP, 2-MeSATP, 2-MeSADP, α,β-meATP and UTP. (b) Concentration–response curve for ATP in the absence and presence of 8-*p*SPT (30 μM). (c) Concentration–response curve for ATP in the absence and presence of MRS 2179 (1 μM). (d) Concentration–response curve for 2-MeSADP in the absence and presence of MRS 2179 (1 μM). All symbols represent mean % relaxation ± SEM (unless masked by symbol; $n = 5$–10 for each agonist). Significance was tested using a two-way ANOVA; $**P < 0.01$, $***P < 0.001$.

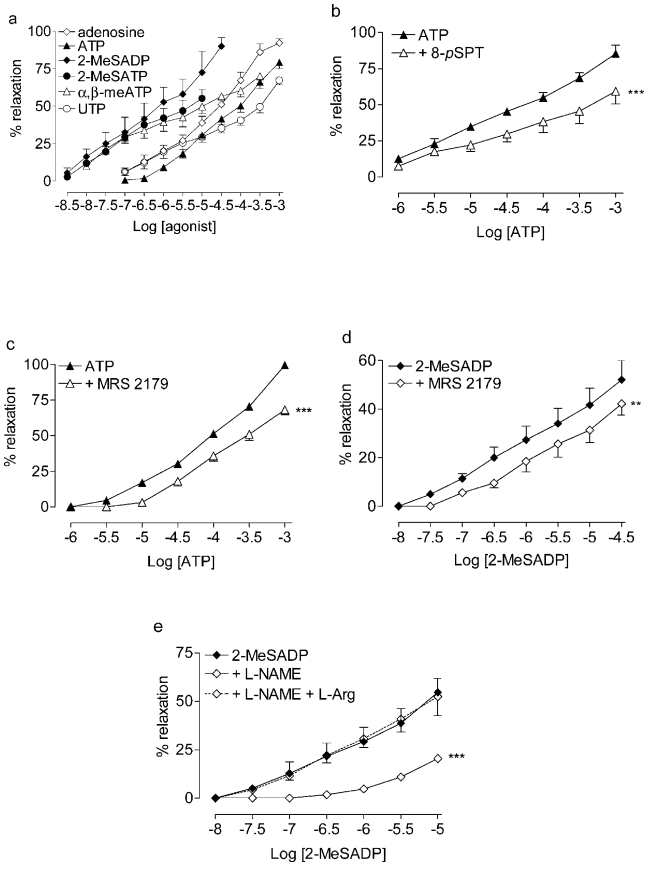


Fig. 2. Cumulative concentration–response curves on the CCh-precontracted (EC_{50} concentration) mouse GI longitudinal muscle of the duodenum. (a) Concentration–response curves for adenosine, ATP, 2-MeSATP, 2-MeSADP, α,β-meATP and UTP. (b) Concentration–response curve for ATP in the absence and presence of 8-*p*SPT (30 μM). (c) Concentration–response curve for ATP in the absence and presence of MRS 2179 (1 μM). (d) Concentration–response curve for 2-MeSADP in the absence and presence of MRS 2179 (1 μM). (e) Concentration–response curve for 2-MeSADP in the absence and presence of L-NAME (100 μM), and finally in the presence of L-NAME (100 μM) and L-arginine (5 mM). All symbols represent mean % relaxation ± SEM (unless masked by symbol; n = 4–10 for each agonist). Significance was tested using a two-way ANOVA; $**P < 0.01$, $***P < 0.001$.

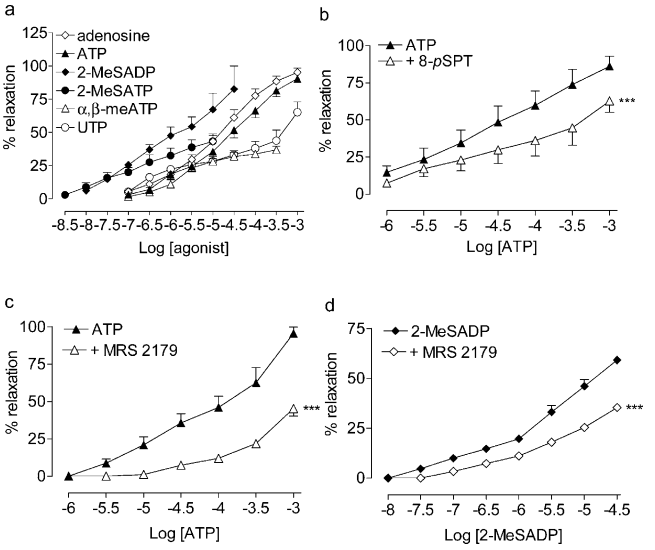


Fig. 3. Cumulative concentration–response curves on the CCh-precontracted (EC_{50} concentration) mouse GI longitudinal muscle of the ileum. (a) Concentration–response curves for adenosine, ATP, 2-MeSATP, 2-MeSADP, α,β-meATP and UTP. (b) Concentration–response curve for ATP in the absence and presence of 8-*p*SPT (30 μM). (c) Concentration–response curve for ATP in the absence and presence of MRS 2179 (1 μM). (d) Concentration–response curve for 2-MeSADP in the absence and presence of MRS 2179 (1 μM). All symbols represent mean % relaxation ± SEM (unless masked by symbol; n = 5–10 for each agonist). Significance was tested using a two-way ANOVA; $***P < 0.001$.

Table 1
$p[A]_{25}$ values (concentration that induced 25% relaxation) of different purinoceptor agonist-induced relaxation in the adult mouse gut

	Stomach	Duodenum	Ileum	Colon
ATP	4.2 ± 0.2	5.2 ± 0.1	5.5 ± 0.2	5.5 ± 0.4
Adenosine	4.3 ± 0.1	5.8 ± 0.2	5.7 ± 0.2	5.3 ± 0.3
2-MeSATP	5.6 ± 0.6	7.0 ± 0.3	6.5 ± 0.4	6.7 ± 0.5
2-MeSADP	5.4 ± 0.3	7.2 ± 0.4	7.1 ± 0.1	6.8 ± 0.3
α,β-meATP	4.5 ± 0.3	7.0 ± 0.5	5.3 ± 0.2	6.7 ± 0.2
UTP	4.3 ± 0.5	5.5 ± 0.4	5.3 ± 0.4	5.5 ± 0.6

Values are means ± SEM of at least five experiments.

region of the gut (see Table 2). Responses to α,β-meATP were unaffected by MRS 2179 (1 μM) in any region of the GI tract tested (see Table 2). Suramin (100 μM) significantly inhibited responses to ATP and 2-MeSATP in each region of the gut tested (see Table 2). ATP (100 μM–1 mM) induced relaxations that were followed by a rebound contraction in the duodenum, ileum and colon but not in the stomach fundus. This rebound response was not influenced by addition of indomethacin (10 μM).

TTX (1 μM) significantly reduced the relaxation induced by a submaximal concentration of ATP (300 μM) and 2-MeSADP (3 μM), but had no effect against a submaximal concentration of α,β-meATP (30 μM) (see Table 3) in each of the regions of the gut tested. L-NAME (100 μM) caused a significant ($P < 0.0001$) inhibition of the concentration–response curve to 2-MeSADP in the duodenum; the inhibitory effect of L-NAME was reversed by the addition of L-Arg (5 mM) (Fig. 2(e)). In contrast, L-NAME (100 μM) failed to affect the relaxant response to ATP (300 μM) either in the stomach fundus, duodenum, ileum or colon.

3.2. Contraction

In the mouse colon longitudinal muscle only, ATP, 2-MeSATP and α,β-meATP induced a concentration-dependent contraction at basal tone (Fig. 4(e)). The order of potency was: 2-MeSATP > α,β-meATP > ATP. As

Table 2
Effect of the purinoceptor antagonists PPADS (30 μM), MRS 2179 (1 μM) and suramin (100 μM) on purine agonist $p[A]_{25}$ values (concentration that induced 25% relaxation) in the adult mouse gut

	Stomach	Duodenum	Ileum	Colon
ATP	3.6 ± 0.1	5.3 ± 0.4	5.5 ± 0.4	4.6 ± 0.6
+ PPADS	0.5 ± 0.1	5.4 ± 0.3	4.3 ± 0.4	4.5 ± 0.5
α,β-meATP	4.0 ± 0.01	5.4 ± 0.2	4.5 ± 0.2	5.7 ± 0.03
+ MRS	3.9 ± 0.01	5.2 ± 0.3	4.4 ± 0.1	5.2 ± 0.1
2179 ATP	3.8 ± 0.04	5.6 ± 0.1	5.4 ± 0.2	6.3 ± 0.1
+ Suramin	3.5 ± 0.1*	5.2 ± 0.2*	4.5 ± 0.3*	4.9 ± 0.2*
2-MeSATP	6.8 ± 0.4	7.1 ± 0.3	6.7 ± 0.4	7.6 ± 0.1
+ Suramin	5.8 ± 0.5*	6.0 ± 0.4**	5.9 ± 0.3*	6.1 ± 0.1*

Values are means ± SEM of at least five experiments. $*P < 0.05$, $**P < 0.01$ vs. agonist in the absence of the antagonist following a paired Student's *t*-test.

none of the concentration–response curves for the agonists reached a maximum response, pD_2 values were calculated following linear regression of the curves. These were calculated to be: ATP, 3.73 ± 0.28 ($n = 4$); 2-MeSATP, 5.40 ± 0.45 ($n = 4$) and α,β-meATP, 4.51 ± 0.27 ($n = 4$). PPADS (30 μM; Fig. 4(f)) and suramin (100 μM; Fig. 4(g)) caused a significant inhibition of the concentration–response curves to ATP ($P < 0.001$ and $P < 0.05$, respectively). In the presence of PPADS (30 μM), the contractile response to the highest concentrations of ATP (300 μM–1 mM) was completely abolished and a relaxation response prevailed at basal tone. Repeated administration of ATP (300 μM; $n = 3$) and 2-MeSATP (1 μM; $n = 3$) at intervals of 8 min, after which time the tension had returned to baseline, did not result in a reduction of the response to either agonist. The contractile response to a single concentration of α,β-meATP (30 μM) was not affected by atropine (1 μM).

3.3. In situ hybridization

Staining for $P2Y_1$ receptor mRNA was detected in the myenteric and submucosal plexus, in the intestinal glands (crypts) of the ileum and along the lumen of the villi (Fig. 5(a); phase contrast shown in Fig. 5(c)). Competition of labelled probe with an excess of unlabelled probe only resulted in a faint staining along the lumen of the villi (Fig. 5(b)).

3.4. Immunohistochemistry

$P2Y_1$ receptor immunoreactivity was present in the circular and longitudinal smooth muscle layers of the ileum and less intensely in a subpopulation of myenteric neurones (Fig. 5(d)). $P2Y_2$ receptor immunoreactivity was observed in the circular and longitudinal muscle layers of the ileum and was of a much lower intensity than that seen for $P2Y_1$ receptors (Fig. 5(e)). The staining was specific for $P2Y_1$ and $P2Y_2$ receptors since preabsorption of the primary antibody resulted in no observed immunoreactivity. No immunostaining for $P2Y_4$ receptors was observed.

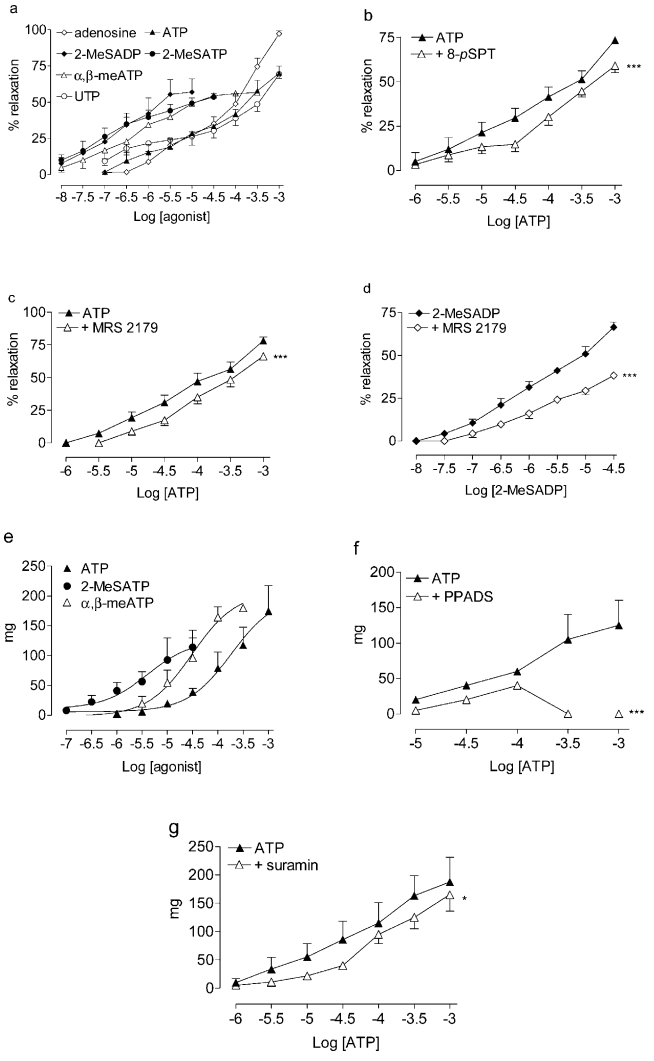
$P2X_2$ receptor immunoreactivity was present in the circular and longitudinal smooth muscle layers of the colon (Fig. 5(f)). $P2X_1$ receptor immunoreactivity was not found in the smooth muscle of the colon, but was observed on smooth muscle on blood vessels within the GI muscle (Fig. 5(g)). Immunostaining to $P2X_2$ receptors was observed on a small subpopulation of myenteric neurones of the ileum from whole mount preparations (Fig. 5(h)).

Whole mount preparations of ileum were dual stained for $P2Y_1$ receptors and neuronal NOS. Immunoreactivity to $P2Y_1$ receptors was observed in a subpopulation of myenteric neurones (Fig. 6(a)). Immunoreactivity to NOS was also observed in a subpopulation of myenteric neurones (Fig. 6(b)). A number of neurones within the myenteric plexus were found to be labelled for both

Table 3
Effect of TTX (1 μM) on % relaxation induced by ATP (300 μM) and 2-MeSADP (3 μM) and α,β-meATP (30 μM) in the adult mouse gut. Note that TTX inhibits responses to ATP and 2-MeSADP, but not to α,β-meATP

	Stomach	Duodenum	Ileum	Colon
ATP	33.8 ± 0.6	61.8 ± 4.6	45.7 ± 8.5	56.0 ± 6.0
+ TTX	23.2 ± 6.5**	49.3 ± 5.6*	28.4 ± 5.3*	36.3 ± 4.2*
2-MeSADP	19.3 ± 3.4	56.8 ± 7.1	40.4 ± 5.5	55.3 ± 6.4
+ TTX	5.3 ± 1.8*	30.0 ± 3.7*	19.4 ± 2.2**	25.7 ± 5.3**
α,β-meATP	14.1 ± 2.7	26.0 ± 2.6	20.4 ± 4.1	50.3 ± 2.7
+ TTX	15.5 ± 3.7	25.7 ± 3.4	19.4 ± 1.8	57.5 ± 4.2

Values are means ± SEM of at least five experiments. $*P < 0.05$, $**P < 0.001$ vs. agonist in the absence of TTX following a paired Student's *t*-test.



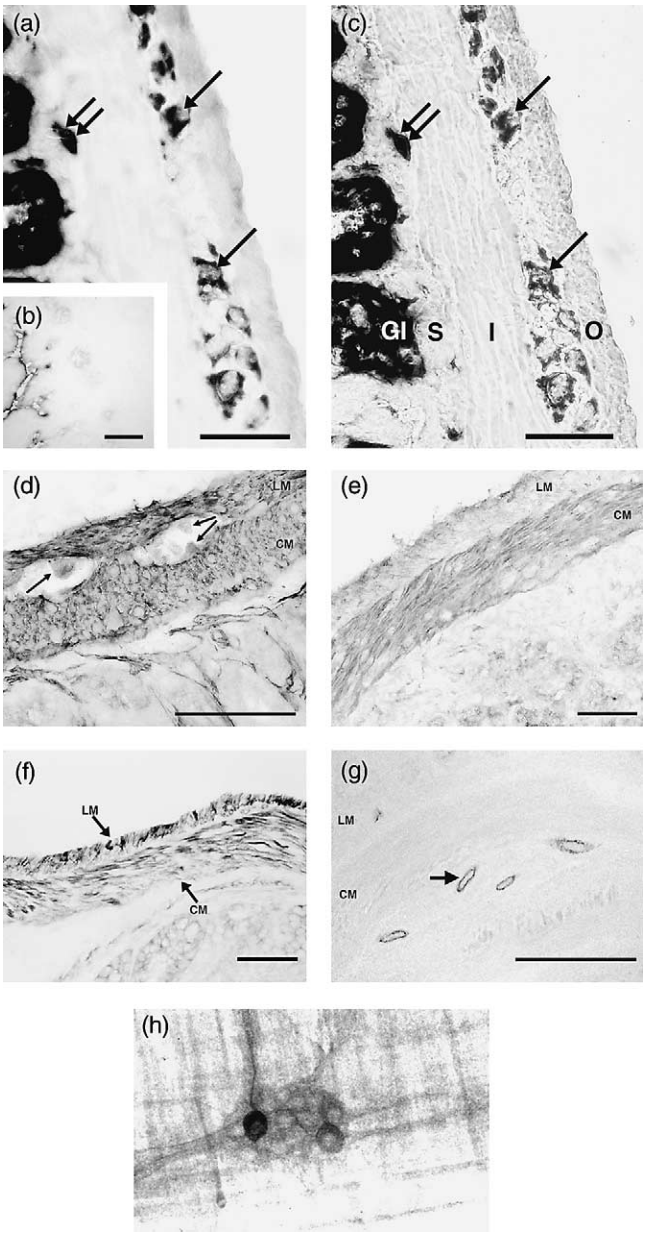


Fig. 4. Cumulative concentration–response curves for adenosine, ATP, 2-MeSATP, 2-MeSADP, α,β-meATP and UTP on the CCh-precontracted (EC_{50} concentration) and non-cumulative, concentration–response curves for ATP, 2-MeSATP and α,β-meATP on basal tone of the mouse colon longitudinal muscle. (a) Concentration–response curves for adenosine, ATP, 2-MeSATP, 2-MeSADP, α,β-meATP and UTP. (b) Concentration–response curve for ATP in the absence and presence of 8-*p*SPT (30 μM). (c) Concentration–response curve for ATP in the absence and presence of MRS 2179 (1 μM). (d) Concentration–response curve for 2-MeSADP in the absence and presence of MRS 2179 (1 μM). All symbols represent mean % relaxation ± SEM (unless masked by symbol; n = 4–10 for each agonist). Significance was tested using a two-way ANOVA; $***P < 0.001$. (e) Concentration–response curves for ATP, 2-MeSATP and α,β-meATP. (f) Concentration–response curve for ATP in the absence and presence of PPADS (30 μM). (g) Concentration–response curve for ATP in the absence and presence of suramin (100 μM). All symbols represent mean contraction in mg ± SEM (unless masked by symbol; n = 4–6 for each experiment). Significance was tested using a two-way ANOVA; $*P < 0.05$, $***P < 0.001$.

$P2Y_1$ receptors and NOS (Fig. 6(c)) although neurones labelled with $P2Y_1$ receptor immunoreactivity alone or NOS immunoreactivity could also be observed.

4. Discussion

This study has characterized multiple P2 receptors on the longitudinal muscle of the mouse GI tract. The predominant effect of stimulation of these receptors is relaxation, with the exception of the colon longitudinal muscle, which also possesses a contractile P2 receptor.

In the mouse stomach fundus, duodenum, ileum and colon, 2-MeSADP, 2-MeSATP, α,β-meATP, ATP, UTP and adenosine all relaxed the longitudinal muscle in a concentration-dependent manner. 2-MeSADP and 2-MeSATP were equipotent and more potent than α,β-meATP, ATP or UTP. The high activity of 2-MeSADP and 2-MeSATP suggests the involvement of $P2Y_1$ recep-

Fig. 5. Visualization of P2Y and P2X receptors in the mouse GI tract. (a) Light micrograph of in situ hybridization for $P2Y_1$ receptor mRNA in the mouse ileum, showing staining in the myenteric plexus (single arrows) and in the submucosal plexus (double arrows); GI glands (crypts) are also labelled. The perinuclear regions of cell bodies of myenteric and submucosal ganglia were found to express $P2Y_1$ receptor mRNA. (b) Competition of labelled probe with an excess of unlabelled probe prevents staining of submucosal and myenteric ganglia and of intestinal glands, leaving only some background staining along the luminal site of the villi. (c) Phase contrast picture of panel (a), illustrating the tissue morphology: **O**, outer muscularis (containing longitudinal muscle); **I**, inner muscularis (containing circular muscle); **S**, submucosal layer; **GI**, intestinal glands. Scale bars: a and c, 50 μm; b, 100 μm. (d) Section of mouse ileum showing immunopositive staining for $P2Y_1$ receptors in the longitudinal (**LM**) and circular (**CM**) layers and in a subpopulation of myenteric neurones (arrows). (e) Section of mouse ileum showing immunopositive staining for $P2Y_2$ receptors in the longitudinal (**LM**) and circular (**CM**) muscle layers of smooth muscle. (f) Section of mouse colon showing immunopositive staining with $P2X_2$ antibodies in the longitudinal (**LM**) and circular (**CM**) layers. (g) Section of mouse colon showing immunopositive staining for $P2X_1$ receptors in the smooth muscle of blood vessels within the GI wall only (arrows). Scale bars are equivalent to 50 μM. (h) Whole mount preparation of ileum showing low intensity staining for $P2X_2$ receptors in a subpopulation of myenteric neurones; Magnification ×170.

tors (Harden et al., 1998; King et al., 1998). The $P2Y_1$ receptor, which promotes phospholipase C-catalysed generation of inositol phosphate and subsequent mobilization of intracellular calcium, is a receptor for endogenous ADP and ATP, with ADP being the most potent natural agonist (Harden et al., 1998; Ralevic and Burnstock, 1998). The presence of a $P2Y_1$ receptor is further supported by the sensitivity of both ATP and 2-MeSADP to the selective $P2Y_1$ antagonist MRS 2179 (Camaioni et al., 1998).

The presence of $P2Y_1$ receptors in the ileum was confirmed by the presence of $P2Y_1$ mRNA in neurones of the myenteric and submucosal plexus of the mouse small intestine. Further, both the longitudinal and circular smooth muscle coats were found to be intensely stained using $P2Y_1$ antibodies; in addition immunoreactivity to $P2Y_1$ receptors could be seen on a subpopulation of myenteric neurons in the ileum, colocalized with NOS. This finding that $P2Y_1$ receptors in the mouse small intestine are dually located on myenteric neurones and on the smooth muscle is consistent with the partial inhibition of 2-MeSADP- and ATP-induced relaxation by TTX, in all the regions studied. The high activity of 2-MeSADP and the dense immunoreactivity to $P2Y_1$ receptors suggest that $P2Y_1$ receptors are the principle receptor type mediating NANC relaxation in the gut mediated by NO and ATP. P2Y receptors mediating hyperpolarization of the mouse colon have been identified (Koh et al., 1997) and 2-MeSATP was found to increase an apamin-sensitive K^+ current in the mouse ileum (Vogalis and Goyal, 1997).

There is evidence that NO is a major mediator of NANC inhibitory neurotransmission in the canine gut

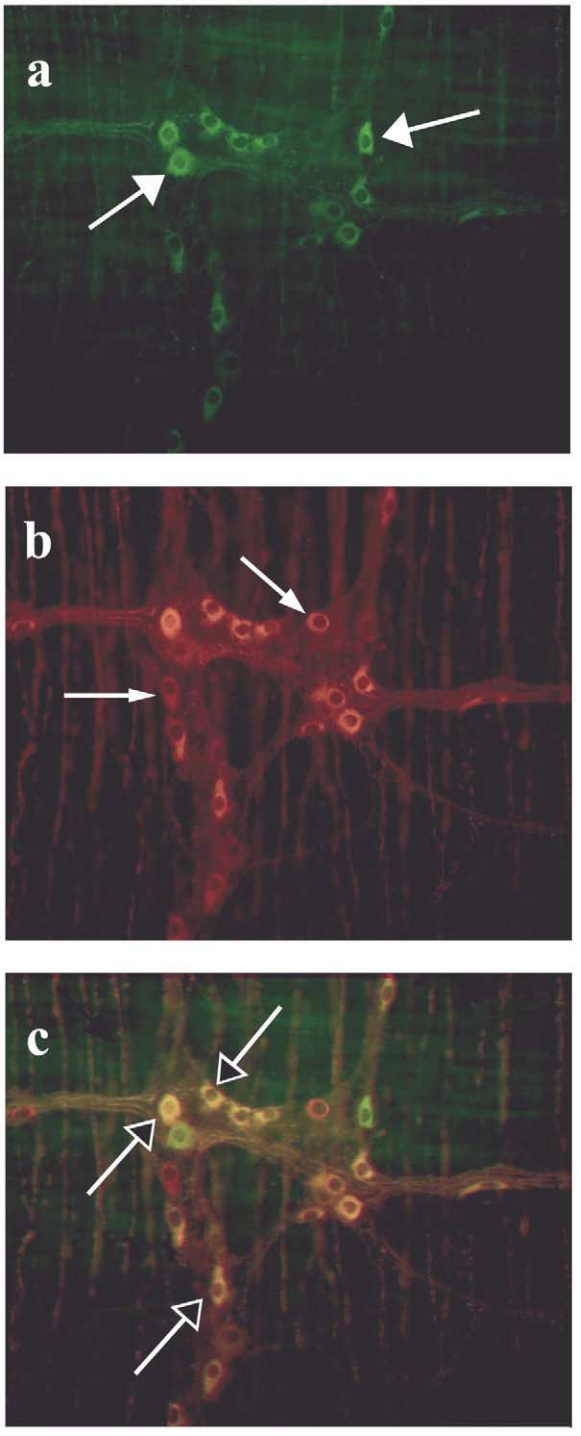


Fig. 6. Whole mount preparation of mouse ileum stained with both $P2Y_1$ receptor and neuronal NOS antibodies. (a) Whole mount preparation of ileum showing a subpopulation of myenteric neurones immunopositive for $P2Y_1$ receptors. Thick arrows indicate neurones stained for $P2Y_1$ receptors only. (b) Same whole mount preparation of ileum showing immunopositive neurones for neuronal NOS in a subpopulation of myenteric neurones. Thin arrows indicate neurones stained for NOS only. (c) Fig. 6(a) and (b) superimposed showing neurones stained for both $P2Y_1$ receptors and NOS (open arrows). Neurones immunopositive for $P2Y_1$ receptors only (green) and NOS only (red) are also visible. Magnification for each plate is ×170.

and that ATP may stimulate these neurones (Boeckxstaens et al., 1991). Relaxations to the selective $P2Y_1$ agonist, 2-MeSADP (although not to ATP), were reduced by both L-NAME, which inhibits the activity of NO synthase (Rees et al., 1990) and TTX. The presence of NOS immunoreactivity in neurones of the mouse ileum and the colocalization of $P2Y_1$ and NOS immunoreactivity support the suggestion that $P2Y_1$ receptors are located on neurons of the myenteric and submucosal ganglia, stimulating relaxation partly through the release of NO and also by $P2Y_1$ receptors on smooth muscle.

The four regions of the mouse GI tract were also found to relax to exogenously applied UTP and α,β-meATP, both of which are inactive at $P2Y_1$ receptors (Ralevic and Burnstock, 1998), indicating that further P2Y receptor subtypes may be present. The activity of UTP indicates the presence of $P2Y_2$ and/or $P2Y_4$ receptors. UTP is equipotent or more potent than ATP at $P2Y_2$ receptors (King et al., 2000) and in the mouse GI tract, ATP and UTP were found to be equipotent. Further, immunoreactivity to $P2Y_2$, but not $P2Y_4$ receptors was demonstrated in the circular and longitudinal muscle of the ileum.

The mouse GI tract was also found to relax in response to α,β-meATP. Since the relaxation was not inhibited by TTX in any region of the intestine studied, this would suggest the presence of the receptor on the intestinal smooth muscle. A P2Y receptor of an, as yet, unknown subtype has been described for the guinea-pig taenia coli (Bültmann et al., 1996). In other preparations such as the rat stomach and duodenum, α,β-meATP is highly potent in causing relaxations of the circular and longitudinal muscle, respectively (Johnson et al., 1996; Otsuguro et al., 1998). In the guinea-pig colon, α,β-meATP produces both relaxation and hyperpolarization of the circular muscle (Zagorodnyuk et al., 1996). In the mouse gut, this atypical α,β-meATP-sensitive receptor is not sensitive to the selective $P2Y_1$ receptor antagonist MRS 2179. A similar response to α,β-meATP has been reported for the rat ileum, resistant to the effects of atropine and TTX and therefore situated on the smooth muscle (Storr et al., 2000).

The pharmacological action of ATP is complicated by its breakdown by ecto-nucleotidases to adenosine, which retains its own effects by acting at P1 receptors (Moody et al., 1984; Kadowaki et al., 2000). Adenosine-induced relaxation suggests the presence of inhibitory P1 receptors in each of the regions examined. Indeed, the sensitivity of ATP-induced relaxations to the P1 receptor antagonist 8*p*SPT suggests that the effect of ATP may be partly mediated through P1 receptor activation.

At the highest concentration tested, ATP induced a relaxation, which was followed by a rebound contraction of the duodenum, ileum and colon. Previous studies on guinea-pig taenia-coli have demonstrated that rebound contractions are mediated by the release of prostaglandins, since they were blocked by indomethacin, an inhibitor of prostaglandin synthesis (Burnstock et al., 1975). In our study of the mouse intestine, however, ATP is not inducing a contraction via the production of prostanoids since the rebound contraction was insensitive to indomethacin, as was also found for the circular muscle of the guinea-pig small intestine (Mitchell and Wood, 1976).

ATP-induced contractions were prominent in the colon. At basal tone, ATP, α,β-meATP and 2-MeSATP induced concentration-dependent contractions with 2-MeSATP being the most potent agonist. Contractions to 2-MeSATP and ATP did not show desensitization, suggesting the involvement of $P2X_2$ receptors (Ralevic and Burnstock, 1998). 2-MeSATP has been shown to elicit non-selective inward cation currents with high potency, whereas α,β-meATP is inactive as an agonist at the recombinant $P2X_2$ receptor (Brake et al., 1994). The lack of sensitivity of ATP and 2-MeSATP-induced contractions to TTX, suggests that a majority of excitatory P2 receptors are located on smooth muscle. This hypothesis is substantiated by the presence of immunoreactivity to $P2X_2$ receptors on smooth muscle cells both in the circular and longitudinal layer and the lack of immunoreactivity to $P2X_1$ receptor antibodies. The high potency displayed by α,β-meATP (which has no activity on $P2X_2$ receptors) suggests the participation of another P2X receptor, possibly of $P2X_1$ or $P2X_3$ subtype, in the contractile response to ATP in the mouse colon, although the lack of staining for $P2X_1$ receptors would seem to discount the participation of $P2X_1$ receptors. The mouse ileum was found to display immunoreactivity to $P2X_2$ receptors on a small subpopulation of myenteric neurones. This is consistent with the guinea-pig, which has been shown to possess $P2X_2$ receptor immunoreactivity on myenteric neurones identified as inhibitory motor neurons of the myenteric plexus innervating the circular smooth muscle (Vulchanova et al., 1996; Castelucci et al., 2002). In the mouse ileum, responses to ATP are mainly inhibitory; ATP was only observed to induce a contractile response at very high concentrations and only on limited occasions.

In the guinea-pig ileum, there is evidence of multiple P2 receptor subtypes involved in the contractile response of the longitudinal muscle (Matsuo et al., 1997): a receptor sensitive to α,β-meATP, possibly of the $P2X_1$ subtype, located on cholinergic enteric neurons; a 2-MeSATP-sensitive P2X receptor, located on smooth muscle cells and mediating a rapid phasic response and a 2-MeSATP-sensitive P2Y receptor, mediating a tonic long-lasting response (Kennedy and Humphrey, 1994; Sato et al., 1999). In the mouse colon, we have shown that a $P2X_1$ or $P2X_3$ receptor subtype is unlikely to be located on the cholinergic enteric neurons mediating the release of ACh, since atropine had no inhibitory effect on the responses to α,β-meATP.

In conclusion, pharmacological and morphological investigations have shown the presence of $P2Y_1$ receptors on myenteric and submucosal NANC inhibitory neurons colocalized with NOS-containing neurones, mediating relaxation of the longitudinal muscle in the mouse gut largely through the production of NO, and by ATP acting via $P2Y_1$ receptors on smooth muscle. $P2Y_2$ receptors have also been identified on the smooth muscle of the ileum although the lower intensity of staining and the lower potency of UTP indicate that $P2Y_1$ receptors are the dominant receptor subtype mediating relaxation in the gut. In addition, there is pharmacological evidence for an atypical, α,β-meATP-sensitive inhibitory P2Y receptor on smooth muscle, in addition to relaxation following metabolism of ATP to adenosine, acting via P1 receptors. The presence of $P2X_2$ receptors, mediating a contractile response in the mouse colon has also been demonstrated. The distribution of different purinoceptors mediating both relaxation and contraction in the mouse gut is schematically represented in Fig. 7.

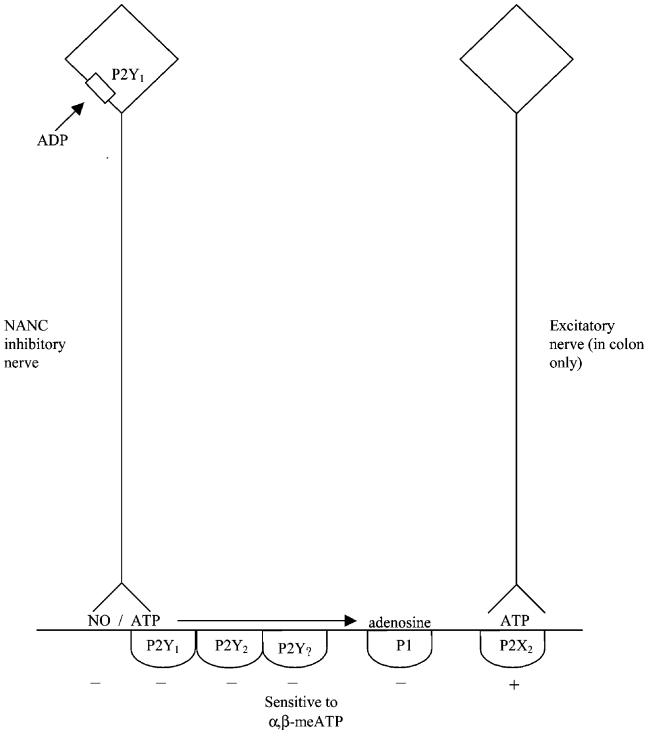


Fig. 7. A schematic representation of the distribution of different purinoceptor subtypes in the mouse intestine. ADP produces relaxation (−) by activation of $P2Y_1$ receptors located on a subpopulation of neurons in the myenteric and submucosal plexuses, stimulating the release of NO and ATP from NANC inhibitory nerves and by activation of $P2Y_1$ receptors on smooth muscle. In addition, smooth muscle relaxation is mediated by an unidentified P2Y receptor sensitive to α,β-meATP, by P1 receptors after breakdown of ATP to adenosine and $P2Y_2$ receptors. ATP is probably released as a cotransmitter from excitatory nerves in the colon; it produces contraction (+) by directly acting on $P2X_2$ receptors located on smooth muscle cells.

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References

Abbracchio, M.P., Burnstock, G., 1994. Purinoceptors: are there families of P2X and P2Y purinoceptors? Pharmacology and Therapeutics 64, 445–475.

Belai, A., Burnstock, G., 1994. Evidence for the coexistence of ATP and nitric oxide in non-adrenergic, non-cholinergic (NANC) inhibitory neurones in the rat ileum, colon and annococcygeus muscle. Cell and Tissue Research 278, 197–200.

Boeckxstaens, G.E., Pelkmans, P.A., Bult, H., De Man, J.G., Herman, A.G., Van Maercke, Y.M., 1991. Evidence for nitric oxide as mediator of non-adrenergic non-cholinergic relaxations induced by ATP and GABA in the canine gut. British Journal of Pharmacology 102, 434–438.

Brake, A.J., Wagenbach, M.J., Julius, D., 1994. New structural motif for ligand-gated ion channels defined by ionotropic ATP receptor. Nature 371, 519–523.

Bültmann, R., Dudeck, O., Starke, K., 1996. Evaluation of P2-purinoceptor antagonists at two relaxation-mediating P2-purinoceptors in guinea-pig taenia coli. Naunyn Schmiedeberg's Archives of Pharmacology 353, 445–451.

Burnstock, G., 1978. A basis for distinguishing two types of purinergic receptor. In: Straub, R.W., Bolis, L. (Eds.), Cell Membrane Receptors for Drugs and Hormones: A Multidisciplinary Approach. Raven Press, New York, pp. 107–118.

Burnstock, G., 2001. Purinergic signalling in gut. In: Abbracchio, M.P., Williams, M. (Eds.), Handbook of Experimental Pharmacology, vol. 151/II Purinergic and Pyrimidinergic Signalling II Cardiovascular, Respiratory, Immune, Metabolic and Gastrointestinal Tract Function. Springer, Berlin.

Burnstock, G., Kennedy, C., 1985. Is there a basis for distinguishing two types of P2-purinoceptor? General Pharmacology 16, 433–440.

Burnstock, G., Campbell, G., Satchell, D.G., Smythe, A., 1970. Evidence that adenosine triphosphate or related nucleotide is the transmitter substance released by non-adrenergic inhibitory nerves in the gut. British Journal of Pharmacology 40, 668–688.

Burnstock, G., Cocks, T., Paddle, B., Staszewska-Barezak, J., 1975. Evidence that prostaglandin is responsible for the 'rebound contraction' following stimulation of non-adrenergic, non-cholinergic ('purinergic') inhibitory nerves. European Journal of Pharmacology 31, 360–362.

Burnstock, G., Cocks, T., Kasakov, L., Wong, H.K., 1978. Direct evidence for ATP release from non-adrenergic, non-cholinergic ("purinergic") nerves in the guinea-pig taenia coli and bladder. European Journal of Pharmacology 49, 145–149.

Camaioni, E., Boyer, J.L., Mohanram, A., Harden, T.K., Jacobson, K.A., 1998. Deoxyadenosine bisphosphate derivatives as potent antagonists at $P2Y_1$ receptors. Journal of Medicinal Chemistry 41, 183–190.

Castelucci, P., Robbins, H.L., Poole, D.P., Furness, J.B., 2002. The distribution of purine $P2X_2$ receptors in the guinea-pig enteric nervous system. Histochemistry and Cell Biology 117, 415–422.

Crowe, R., Burnstock, G., 1981. Comparative studies of quinacrine-positive neurons in the myenteric plexus of stomach and intestine of guinea-pig, rabbit and rat. Cell and Tissue Research 221, 93–107.

Glass, R., Townsend-Nicholson, A., Burnstock, G., 2000. P2 receptors

in the thymus: expression of P2X and P2Y receptors in the thymus of adult rats, an immunohistochemical and in situ hybridization study. Cell and Tissue Research 300, 295–306.

Harden, T.K., Barnard, E.A., Boeynaems, H.M., Burnstock, G., Dubyak, G., Hourani, S.M.O., Insel, P.A., 1998. P2Y receptors. In: Girdlestone, D. (Ed.), The IUPHAR Compendium of Receptor Characterization and Classification. IUPHAR Media, London, pp. 209–217.

Johnson, C.R., Charlton, S.J., Hourani, S.M., 1996. Responses of the longitudinal muscle and the muscularis mucosae of the rat duodenum to adenine and uracil nucleotides. British Journal of Pharmacology 117, 823–830.

Kadowaki, M., Takeda, M., Tokita, K., Hanaoka, K., Tomoi, M., 2000. Molecular identification and pharmacological characterization of adenosine receptors in the guinea-pig colon. British Journal of Pharmacology 129, 871–876.

Kennedy, I., Humphrey, P.P.A., 1994. Evidence for the presence of two types of P2 purinoceptor in the guinea-pig ileal longitudinal smooth muscle preparation. European Journal of Pharmacology 261, 273–280.

Khakh, B.S., Burnstock, G., Kennedy, C., King, B.F., North, R.A., Séguéla, P., Voight, M., Humphrey, P.P.A., 2001. International Union of Pharmacology. XXXIV. Current status of the nomenclature and properties of P2X receptors and their subunits. Pharmacological Reviews 53, 107–118.

King, B.F., Townsend-Nicholson, A., Burnstock, G., 1998. Metabotropic receptors for ATP and UTP: exploring the correspondence between native and recombinant nucleotide receptors. Trends in Pharmacological Sciences 19, 506–514.

King, B.F., Burnstock, G., Boyer, J.L., Boeynaems, J.M., Weisman, G.A., Kennedy, C., Jacobson, K.A., Humphries, R.G., Abbracchio, M.P., Miras-Portugal, M.T., 2000. The P2Y receptors. In: Girdlestone, D. (Ed.), The IUPHAR Compendium of Receptor Characterization and Classification. IUPHAR Media, London, pp. 306–320.

Koh, S.D., Dick, G.M., Sanders, K.M., 1997. Small-conductance Ca^{2+}-dependent K^+ channels activated by ATP in murine colonic smooth muscle. American Journal of Physiology 273, C2010–C2021.

Llewellyn-Smith, I.J., Pilowsky, P., Minson, J.B., 1993. The tungstate-stabilized tetramethylbenzidine reaction for light and electron microscopic immunocytochemistry and for revealing biocytin filled neurons. Journal of Neuroscience Methods 46, 27–40.

Matsuo, K., Katsuragi, T., Fujiki, S., Sato, C., Furukawa, T., 1997. ATP release and contraction mediated by different P2-receptor subtypes in guinea-pig ileal smooth muscle. British Journal of Pharmacology 121, 1744–1748.

McConalogue, K., Todorov, L., Furness, J.B., Westfall, D.P., 1996. Direct measurement of the release of ATP and its major metabolites from the nerve fibres of the guinea-pig taenia coli. Clinical and Experimental Pharmacology and Physiology 23, 807–812.

Mitchell, G.W., Wood, J.D., 1976. Effects of inhibitors of prostaglandin synthesis on rebound excitation of guinea-pig small bowel. European Journal of Pharmacology 40, 63–66.

Moneta, N.A., McDonald, T.J., Cook, M.A., 1997. Endogenous adenosine inhibits evoked substance P release from perfused networks of myenteric ganglia. American Journal of Physiology 272, G38–G45.

Moody, C.J., Meghji, P., Burnstock, G., 1984. Stimulation of P1-purinoceptors by ATP depends partly on its conversion to AMP and adenosine and partly on direct action. European Journal of Pharmacology 97, 47–54.

Nicholas, R.A., 2001. Identification of the $P2Y_{12}$ receptor: a novel member of the P2Y family of receptors activated by extracellular nucleotides. Molecular Pharmacology 60, 416–420.

Nicholls, J., Brownhill, V.R., Hourani, S.M., 1996. Characterization of P1-purinoceptors on rat isolated duodenum longitudinal muscle and muscularis mucosae. British Journal of Pharmacology 117, 170–174.

Oglesby, I.B., Lachnit, W.G., Burnstock, G., Ford, A.P.D.W., 1999. Subunit specificity of polyclonal antisera to the carboxy terminal regions of P2X receptors, $P2X_1$ through $P2X_7$. Drug Development and Research 47, 185–189.

Otsuguro, K., Ohta, T., Ito, S., Nazakato, Y., 1998. Two types of relaxation-mediating P2 receptors in rat gastric circular muscle. Japanese Journal of Pharmacology 78, 209–215.

Ralevic, V., Burnstock, G., 1998. Receptors for purines and pyrimidines. Pharmacological Reviews 50, 413–492.

Rees, D.D., Palmer, R.M.J., Schulz, R., Hodson, H.F., Moncada, S., 1990. Characterization of three inhibitors of endothelial nitric oxide synthase in vitro and in vivo. British Journal of Pharmacology 101, 746–752.

Sato, C., Tsujioka, Y., Katsuragi, T., 1999. Cross desensitizations on contractions by P2-agonists of the guinea pig ileum. Japanese Journal of Pharmacology 80, 311–317.

Storr, M., Franck, H., Saur, D., Schusdziarra, V., Allescher, H.-D., 2000. Mechanisms of α,β-methylene ATP-induced inhibition in rat ileal smooth muscle: involvement of intracellular Ca^{2+} stores in purinergic inhibition. Clinical and Experimental Pharmacology and Physiology 27, 771–779.

Toole, L., Belai, A., Burnstock, G., 1998. A neurochemical characterisation of the golden hamster myenteric plexus. Cell and Tissue Research 291, 385–394.

Vogalis, F., Goyal, R.K., 1997. Activation of small conductance Ca^{2+}-dependent K_+ channels by purinergic agonists in smooth muscle cells of the mouse ileum. Journal of Physiology 502, 497–507.

Vulchanova, L., Arvidsson, U., Riedl, M., Wang, J., Buell, G., Surprenant, A., North, R.A., Elde, R., 1996. Differential distribution of two ATP-gated ion channels (P_{2X} receptors) determined by immunocytochemistry. Proceedings of the National Academy of Sciences of the USA 93, 8063–8067.

Zagorodnyuk, V., Santicioli, P., Maggi, C.A., Giacchetti, A., 1996. The possible role of ATP and PACAP as mediators of apamin-sensitive NANC inhibitory junction potentials in circular muscle of guinea-pig colon. British Journal of Pharmacology 119, 779–786.