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Functional evidence that ATP or a related purine is an inhibitory NANC neurotransmitter in the mouse jejunum: study on the identity of P2X and P2Y purinoceptors involved

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1 Conflicting views exist on whether ATP is a neurotransmitter in the enteric nervous system. We investigated the role of ATP in enteric transmission in circular muscle strips of the mouse jejunum.

2 On $PGF_{2\alpha}$-precontracted muscle strips and in the presence of atropine and guanethidine, electrical field stimulation (EFS, 1–8 Hz) of nonadrenergic noncholinergic (NANC) nerves induced transient relaxations that were abolished by the nerve-conductance blocker tetrodotoxin. The NO synthase blocker L-nitroarginine (L-NOARG) partially inhibited the NANC relaxations to EFS, but fast-twitch relaxations to EFS were still observed in the presence of L-NOARG.

3 In the presence of L-NOARG, ATP, the P2X receptor agonist $\alpha\beta$MeATP and the P2Y receptor agonist ADPβS relaxed jejunal muscle strips. Tetrodotoxin did not affect the relaxation to ATP and ADPβS, but inhibited that to $\alpha\beta$MeATP.

4 The L-NOARG-resistant NANC relaxations to EFS were almost abolished by apamin, a blocker of small-conductance Ca^{2+} activated K^+ channels, and by suramin and PPADS, blockers of P2 purinoceptors. Relaxations to ATP were almost abolished by apamin and suramin but not affected by PPADS.

5 Desensitisation of $\alpha\beta$MeATP-sensitive P2X receptors, the P2X receptor blocker Evans blue and the $P2X_{1,2,3}$ receptor blocker NF 279 inhibited the L-NOARG-resistant NANC relaxations to EFS and that to $\alpha\beta$MeATP without affecting the relaxation to ADPβS. Brilliant blue G, a $P2X_{2,5,7}$ receptor blocker, did not affect the relaxations to EFS.

6 Desensitisation of P2Y receptors and MRS 2179, a $P2Y_1$ receptor blocker, virtually abolished the L-NOARG-resistant NANC relaxations to EFS and the relaxation to ADPβS without affecting the relaxation to $\alpha\beta$MeATP.

7 Dipyridamole, an adenosine uptake inhibitor, or theophylline and 8-phenyltheophylline, blockers of P1 and A1 purinoceptors, respectively, did not affect the purinergic NANC relaxations to EFS.

8 Our results suggest that ATP or a related purine acts as an inhibitory NANC neurotransmitter in the mouse jejunum, activating P2 but not P1 purinoceptors. Relaxations to the purinergic NANC neurotransmitter mainly involve P2Y receptors of the $P2Y_1$ subtype that are located postjunctionally. Purinergic NANC neurotransmission also involves P2X receptors, most likely of the $P2X_1$ and $P2X_3$ subtype, located pre- and/or postjunctionally.
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Abbreviations: ADP, adenosine diphosphate; ADPβS, adenosine 5′-[β-thio]diphosphate trilithium salt; ATP, adenosine 5′-triphosphate; EFS, electrical field stimulation; GTN, glyceryl trinitrate; L-NOARG, L-nitroarginine; MRS 2179, N(6)-methyl-2′-deoxyadenosine-3′,5′-bisphosphate; NF 279, 8.8′-(carbonylbis(imino-4, 1-phenylenecarbonylimino-4,1-phenylenecarbonylimino)) bis(1,3,5-naphthalenetrisulphonic acid); PPADS, pyridoxalphosphate-6-azophenyl-2′,4′-disulphonic acid tetrasodium salt; TTX, tetrodotoxin; $\alpha\beta$MeATP, α-β methylene 5′-adenosine triphosphate

Introduction

The role of ATP in enteric neurotransmission is complex and much debated. There is good evidence that ATP mediates fast synaptic transmission at neuronal ganglia in the enteric nervous system (for a review, see Galligan *et al*., 2000). However. ATP may also act as a nonadrenergic noncholinergic (NANC) neurotransmitter in the gastrointestinal tract, as first proposed by Burnstock (Burnstock *et al*., 1970). Since then, the functional evidence that is reported in favour of ATP is well balanced by evidence reported against ATP being a NANC neurotransmitter in the gut. The strongest evidence in favour of ATP originates from electrophysiological studies: it is repeatedly reported that electrically induced inhibitory

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junction potentials in different intestinal tissues are sensitive to the bee venom apamin, as are the responses to exogenous ATP (Crist *et al*., 1992; Zagorodnyuk *et al*., 1996; Vogalis & Goyal, 1997; Fernandez *et al*., 1998; Pluja *et al*., 1999; Xue *et al*., 1999; Serio *et al*., 2003).

ATP activates P2 purinoceptors which are subdivided into ion-gated P2X receptors and G-protein-coupled P2Y receptors (for reviews, see Ralevic & Burnstock, 1998; North, 2002). Different subtypes of P2X receptors ($P2X_1$, $P2X_2$, $P2X_3$, $P2X_4$, $P2X_5$, $P2X_6$ and $P2X_7$) and P2Y receptors ($P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$ and $P2Y_{13}$) are recognised. The existence of these different subtypes of purinoceptors and the lack of specific inhibitors highly complicate a detailed study of purinergic neurotransmission.

The compounds apamin, suramin and PPADS are invaluable tools in the study of purinergic signalling. Apamin is a blocker of small-conductance Ca^{2+}-activated K^+ channels, which inhibits relaxations to ATP in different intestinal muscle preparations. Apamin may, however, also block nonpurinergic responses in the intestine (Lefebvre *et al*., 1991; Imoto *et al*., 1998; Rozsai *et al*., 2001). Suramin blocks P2 purinoceptors without differentiating between P2X and P2Y receptors. PPADS is reported to block certain but not all P2 receptors depending upon the region of the gut that is under study (Ralevic & Burnstock, 1998; North, 2002). Recently, more specific antagonists of purinoceptor subtypes became available. The compound NF 279 is a suramin analogue that preferentially blocks $P2X_1$ receptors (Rettinger *et al*., 2000), but also inhibits $P2X_2$ and $P2X_3$ receptor-mediated responses (Damer *et al*., 1998; Klapperstuck *et al*., 2000). The compound MRS 2179 is a selective blocker of $P2Y_1$ receptors (Boyer *et al*., 1998).

Many studies have attempted to identify the enteric P2 purinoceptors that are activated by exogenous ATP. However, as a consequence of the controversial role of ATP in enteric neurotransmission, the subtypes of P2 purinoceptors that may be activated by endogenously released ATP are hardly explored. In an electrophysiological study on isolated human jejunal muscle strips, Xue *et al*. (1999) reported that the ATP-mediated part of the inhibitory junction potential is mediated by P2Y but not P2X receptors. Similar findings were recently reported for mouse colon (Serio *et al*., 2003). Interestingly, however, there is recent immunohistochemical evidence that the mouse colon contains P2X and P2Y receptors, which can be activated by exogenously added purines (Giaroni *et al*., 2002). This suggests that there is a discrepancy between the functional effects of endogenously released and exogenously added purines, and supports the hypothesis that endogenous and exogenous purines activate different subtypes of purinoceptors (Matsuo *et al*., 1997; Heinemann *et al*., 1999).

The aim of the present study was to clarify these discrepancies by investigating whether ATP plays a role as an inhibitory neurotransmitter of NANC nerves in the mouse jejunum and by identifying the subtypes of P2 purinoceptor, which are activated by endogenously released and exogenously added purines in the mouse jejunum.

Methods

Tissue preparation

All mice were fasted for 24 h with free access to water. Then the animals were anaesthetised with diethyl ether and exsanguinated from the carotid artery. The small intestine from the stomach to the caecum was rapidly removed and put in ice-cold aerated Krebs–Ringer solution (118.3 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 2.5 mM $CaCl_2$, 25 mM $NaCHO_3$, 0.026 mM CaEDTA and 11.1 mM glucose). An $\sim$10 cm long segment of the jejunum, located $\sim$7 cm distal from the ligament of Treitz, was used for further preparation. All procedures and experiments were approved by the Ethics Committee of the University of Antwerp.

Pharmacological studies: tissue preparation and isometric tension recording

The jejunal segment was cut open longitudinally along the mesenteric border and the mucosal layer was removed. Jejunal muscle strips were cut in the circular direction. A silk thread was attached at both ends of the strips, after which they were mounted in organ baths (volume 5 ml) filled with Krebs–Ringer solution (37°C, aerated with 5% CO_2/95% O_2). The muscle strips were carefully positioned between two platinum ring electrodes (distance between the rings: 10 mm, diameter of the rings: 3 mm) that were mounted on a plexiglas rod. One end of the muscle strip was fixed and the other end was attached to a strain gauge transducer (Scaime Transducers, Annemasse, France) for continuous recording of isometric tension. After an initial equilibration period of 30 min during which the strips were washed every 10 min, the strips were contracted with 0.1 μM carbachol. After washout of carbachol, the strips were stretched (increments of 0.5 g) and when the basal tone of the muscle preparations was stabilised, 0.1 μM carbachol was added again. This procedure was repeated until the contraction to 0.1 μM carbachol was maximal. This point was taken as the point of optimal length–tension relationship (Boeckxstaens *et al*., 1990a, b). The strips were then allowed to equilibrate for 60 min before starting the experiment. During the equilibration period, the preparations were washed every 15 min with fresh Krebs–Ringer solution.

Experimental protocols

All experiments were performed in the presence of atropine and guanethidine to block cholinergic and adrenergic responses. Muscle strips were precontracted with 0.3 μM prostaglandin $F_{2\alpha}$ and relaxations were induced by electrical field stimulation (EFS) of intramural NANC nerves, ATP, $\alpha\beta$MeATP, ADPβS and nitroglycerin. Relaxations were studied first in the absence and then in the presence of blockers of nitrergic and purinergic transmission.

In preliminary experiments, the effect of the NO synthase blocker L-NOARG (15 min preincubation) was investigated on the NANC relaxations to EFS.

In a first series of experiments, the effect of apamin. a blocker of ATP-sensitive small-conductance Ca^{2+}-activated K^+ channels, and of suramin and PPADS, blockers of purinergic P2 receptors, was investigated on the L-NOARG-resistant relaxations to NANC nerve stimulation and on relaxations to ATP and nitroglycerin. The preincubation time of apamin, suramin and PPADS was 30 min.

In a second series of experiments, the effect of P2X receptor desensitisation was investigated on the L-NOARG-resistant NANC relaxations to EFS and on relaxations to purinoceptor agonists. P2X receptor desensitisation was achieved by a

30 min incubation of muscle strips with 10 μM $\alpha\beta$MeATP, according to Serio *et al*. (2003). The effects of Evans blue, a P2X receptor blocker in the intestinal tissue (Bultmann *et al*., 1996), NF 279, a blocker of $P2X_1$, $P2X_2$ and $P2X_3$ receptors in the rat (Damer *et al*., 1998; Klapperstuck *et al*., 2000; Rettinger *et al*., 2000) and Brilliant blue G, which blocks rat $P2X_2$ receptors (King *et al*., 1997), rat and guinea-pig $P2X_7$ receptors (Hu *et al*., 2001; Sperlagh *et al*., 2002) and human $P2X_5$ receptors (Jiang *et al*., 2000; Bo *et al*., 2003) was investigated. The preincubation time of Evans blue, NF 279 and Brilliant blue G was 30 min.

In a third series of experiments, the effect of P2Y receptor desensitisation was investigated on the L-NOARG-resistant NANC relaxations to EFS and on relaxations to purinoceptor agonists. P2Y receptor desensitisation was achieved by a 30 min incubation of muscle strips with 10 μM ADPβS according to Serio *et al*. (2003). Additionally, the effect of MRS 2179, a $P2Y_1$ receptor blocker (Boyer *et al*., 1998), was investigated. The preincubation time of MRS 2179 was 30 min.

In a fourth series of experiments, the effects of theophylline (P1 adenosine receptor blocker), 8-phenyltheophylline (P1 adenosine A1 receptor blocker). dipyridamole (adenosine uptake inhibitor) and hexamethonium (nicotinic receptor blocker) were investigated on the L-NOARG-resistant NANC relaxations to EFS and on relaxations to ATP.

Solutions and drugs

The following drugs were used: nitroglygerin (Merck, Darmstadt, Germany); MRS 2179, NF 279, PPADS tetrasodium salt, suramin hexasodium salt (Tocris Cookson Ltd, Avonmouth, Bristol, U.K.); α-β methylene 5′adenosine triphosphate lithium salt ($\alpha\beta$MeATP), adenosine 5′-[β-thio]diphosphate trilithium salt (ADPβS), adenosine 5′triphosphate (ATP); Evans blue, Brilliant blue G, hexamethonium chloride (Sigma-Aldrich, St Louis, MO, U.S.A.); tetrodotoxin, apamin (Alomone Labs, Jerusalem, Israel).

Presentation of results and statistical analysis

Relaxations were calculated as the maximal inhibitory response to EFS or agonists under study. Values are calculated as % inhibition of the prostaglandin $F_{2\alpha}$-induced contraction. Results are shown as mean $\pm$ s.e.m. for the number (n) of mice indicated. For statistical analysis, Student's t-test for paired values was used. P-values of less than 0.05 were considered to be significant.

Results

Effect of P2 receptor antagonists on relaxations to EFS and ATP

On $PGF_{2\alpha}$-precontracted circular jejunal muscle strips and in the presence of 1 μM atropine and 3 μM guanethidine, EFS (1–8 Hz, 10 s) induced frequency-dependent NANC relaxations. All relaxations to EFS were abolished by the nerve-conductance blocker tetrodotoxin (1 μM, results not shown). The blocker of NO synthase L-nitroarginine (L-NOARG, 300 μM) significantly reduced the NANC relaxations to EFS (Table 1)

Table 1 Effect of L-NOARG (300 μM) on the NANC-nerve mediated relaxations to electrical field stimulation (EFS, 1–8 Hz) in mouse circular jejunal muscle strips.

EFS (Hz)	*Control (%)*	*L-NOARG (%)*
1	57.2 $\pm$ 4.6	29.7 $\pm$ 4.1*
2	73.4 $\pm$ 2.9	47.5 $\pm$ 3.5*
4	82.7 $\pm$ 3.7	60.2 $\pm$ 2.7*
8	83.8 $\pm$ 2.7	74.9 $\pm$ 3.3*

Results are expressed as % relaxation of a prostaglandin $F_{2\alpha}$-induced contraction, and are shown as mean $\pm$ s.e.m. for $n = 7$ experiments. *$P < 0.05$, significantly different from control ileum, paired Student's t-test.

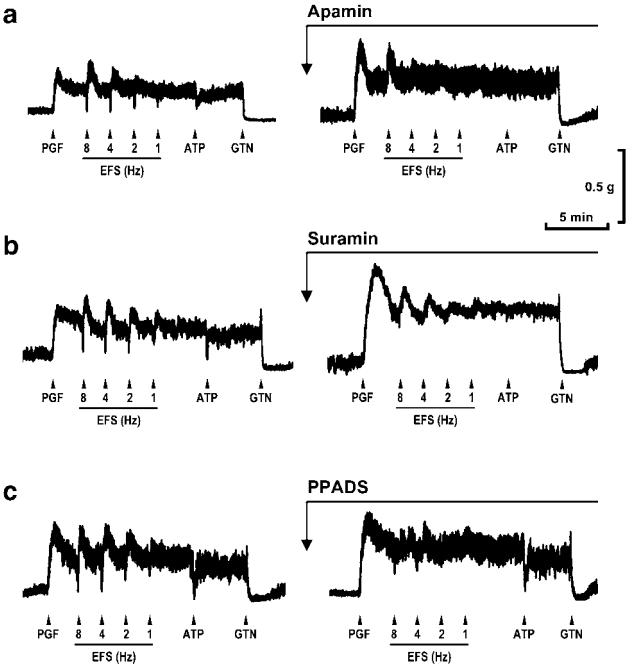

Figure 1 Typical tracings of three different muscle strips showing the nerve-mediated L-NOARG-resistant NANC relaxations to EFS (1–8 Hz) and the relaxations to ATP (10 μM) and nitroglycerin (GTN, 0.3 μM) before (left panels) and after (right panels) treatment of the strips with 1 μM apamin (tracing (a)), 250 μM suramin (tracing (b)) and 10 μM PPADS (tracing (c)).

but did not abolish them. The L-NOARG-resistant relaxations to EFS were fast in onset and of short duration (Figure 1). Apamin (1 μM), which blocks ATP-sensitive K^+ channels, almost abolished the L-NOARG-resistant fast NANC relaxations to EFS (Figures 1 and 2). P2 receptor blockade with suramin (250 μM) also almost abolished the L-NOARG-resistant relaxations to EFS, while PPADS (10 μM) strongly inhibited them (Figures 1 and 2).

In the presence of L-NOARG, ATP (10 μM) induced a transient relaxation of the circular jejunal muscle strips (Figure 1). Apamin (1 μM) and suramin (250 μM) virtually abolished the relaxations to ATP, whereas PPADS (10 μM) did not affect them (Figures 1 and 3). Apamin, suramin and PPADS did not affect the relaxations to the NO donor nitroglycerin (Figure 1).

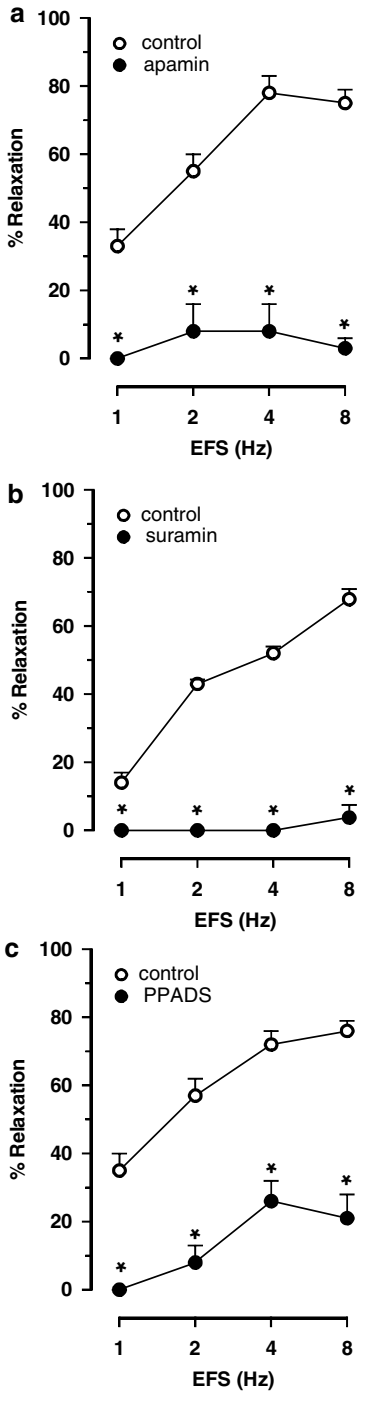

Figure 2 Effect of (a) apamin (1 μM), (b) suramin (250 μM) and (c) PPADS (10 μM) on the nerve-mediated L-NOARG-resistant NANC relaxations to EFS (1–8 Hz). Results are expressed as % relaxation of a prostaglandin $F_{2\alpha}$-induced contraction, and are shown as mean$\pm$s.e.m. for $n=4$–6 experiments. *$P<0.05$, significantly different from controls, paired Student's t-test.

Effect of P2X receptor agonists and antagonists

Circular muscle strips of the mouse jejunum relaxed to the P2X receptor agonist $\alpha\beta$MeATP. The amplitude of the relaxation to 10 μM $\alpha\beta$MeATP (71$\pm$4%, $n=7$) was comparable to that of 10 μM ATP (73$\pm$4%, $n=7$). The relaxation to $\alpha\beta$MeATP (10 μM) was inhibited from 71$\pm$4% in controls to 56$\pm$3% by tetrodotoxin (1 μM, $n=8$).

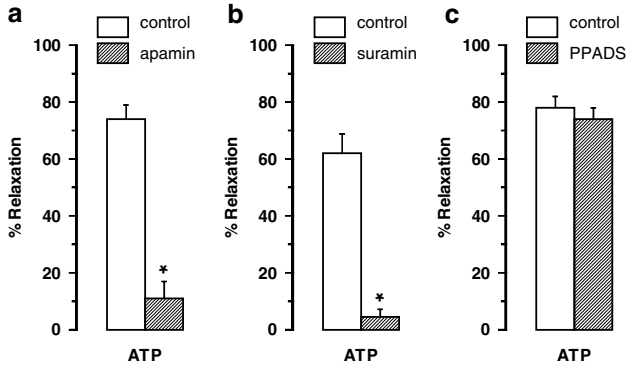

Figure 3 Effect of (a) apamin (1 μM), (b) suramin (250 μM) and (c) PPADS (10 μM) on the L-NOARG-resistant relaxations to ATP (10 μM). Results are expressed as % relaxation of a prostaglandin $F_{2\alpha}$-induced contraction, and are shown as mean$\pm$s.e.m. for $n=4$–6 experiments. *$P<0.05$, significantly different from controls, paired Student's t-test.

Desensitisation of P2X receptors, achieved by treating the muscle strips during 30 min with 10 μM $\alpha\beta$MeATP (Serio *et al*., 2003), significantly reduced the relaxation to $\alpha\beta$MeATP by 59$\pm$13% ($n=7$) (Figure 4a). Desensitisation of $\alpha\beta$MeATP-sensitive P2X receptors slightly reduced the L-NOARG-resistant NANC relaxations to EFS, and had no effect on the relaxations to ATP and ADPβS (Figure 4a).

Evans blue (10 μM), a putative P2X receptor antagonist in the intestinal muscle (Bultmann *et al*., 1996), slightly inhibited the L-NOARG-resistant relaxations to EFS and the relaxation to $\alpha\beta$MeATP, without affecting those to ATP and ADPβS (Figure 5a).

NF 279 (1 μM), which blocks rat $P2X_1$, $P2X_2$ and $P2X_3$ receptors (Damer *et al*., 1998; Klapperstuck *et al*., 2000; Rettinger *et al*., 2000), slightly inhibited the L-NOARG-resistant NANC relaxations to low-frequency EFS and those to $\alpha\beta$MeATP, but had no effect on NANC relaxations to higher frequency EFS or on the relaxations to ATP and ADPβS (Figure 5b).

Brilliant blue G (1–10 μM), which blocks $P2X_5$ and $P2X_7$ receptors (King *et al*., 1997; Jiang *et al*., 2000; Hu *et al*., 2001; Sperlagh *et al*., 2002; Bo *et al*., 2003), had no effect on the relaxations to EFS or ATP (results not shown).

Effect of P2Y receptor agonists and antagonists

Circular muscle strips of mouse jejunum relaxed to the P2Y receptor agonist ADPβS. The amplitude of the relaxation to 1 μM ADPβS (75$\pm$4%, $n=6$) was comparable to that of 10 μM ATP (73$\pm$4%, $n=7$). The relaxation to ADPβS (1 μM) was not affected by tetrodotoxin. Desensitisation of P2Y receptors, achieved by treating the muscle strips during 30 min with 10 μM ADPβS (Serio *et al*., 2003), potently inhibited the relaxation to ADPβS by 95$\pm$5% ($n=7$, Figure 4b) and the L-NOARG-resistant relaxations to NANC nerve stimulation (Figure 4b). P2Y receptor desensitisation also inhibited the relaxation to ATP by 27$\pm$7% ($n=6$), but not the relaxation to $\alpha\beta$MeATP (Figure 4b). Compared to P2Y receptor desensitisation, desensitisation of P2X plus P2Y receptors had no additional effect on the NANC relaxations to EFS (Figure 4c), but reduced the relaxation to ATP by 94$\pm$7% ($n=5$) (Figure 4c).

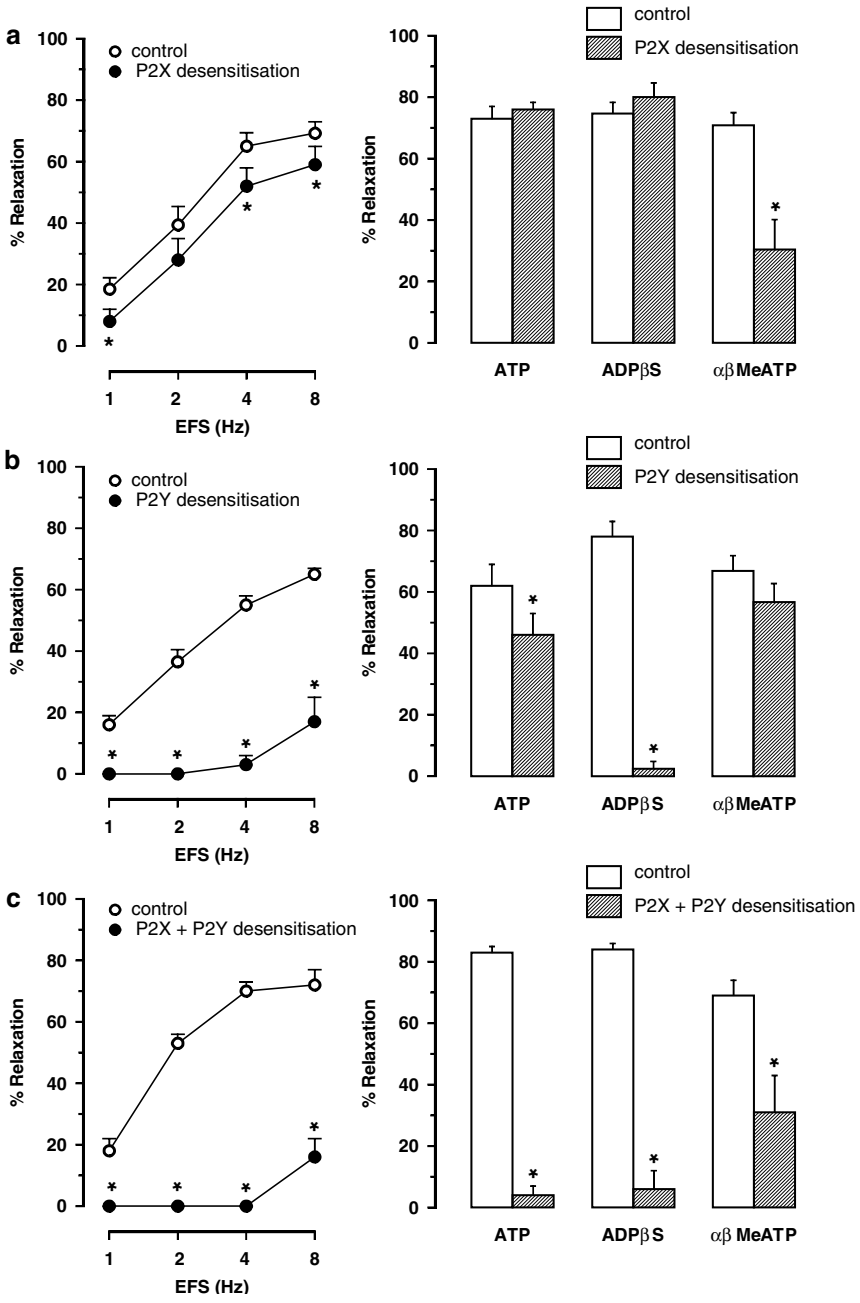

Figure 4 Effect of (a) P2X receptor desensitisation and (b) P2Y receptor desensitisation and (c) P2X + P2Y receptor desensitisation on the L-NOARG-resistant NANC relaxations to EFS (1–8 Hz) and on the relaxations to ATP (10 μM), ADPβS (1 μM) and $\alpha\beta$MeATP (10 μM). Results are expressed as % relaxation of a prostaglandin $F_{2\alpha}$-induced contraction, and shown as mean ± s.e.m. for $n = 6$ experiments in each group. *$P < 0.05$, significantly different from controls, paired Student's t-test.

The $P2Y_1$ receptor antagonist MRS 2179 (1 μM) (Boyer *et al*., 1998) strongly inhibited the L-NOARG-resistant NANC relaxations to EFS and the relaxation to ADPβS. MRS 2179 also slightly inhibited the relaxations to ATP and $\alpha\beta$MeATP, but significance was reached only for ATP (Figure 6).

Effect of compounds that modulate purinergic transmission

Theophylline (10 μM), a P1 purinoceptor blocker (results not shown), or 8-phenyltheophylline (10 μM), an adenosine A1 purinoceptor blocker, had no effect on the L-NOARG-resistant NANC relaxations to EFS, slightly inhibited the relaxation to ATP and abolished the relaxation to adenosine (Figure 7). Dipyridamole (10 μM), an adenosine uptake inhibitor, had no effect on the amplitude of the relaxations to ATP, but changed the shape of the relaxations from transient into sustained ones. Dypyridamole, however, did not affect the amplitude or the shape of the relaxations to EFS (results not shown). Hexamethonium (100 μM), a nicotinic receptor blocker, slightly but significantly inhibited the relaxations to EFS (relaxation to 2 Hz EFS was inhibited from 64 ± 2% in controls to 52 ± 5% in the presence of hexamethonium, $n = 5$, $P < 0.05$). Hexamethonium had no

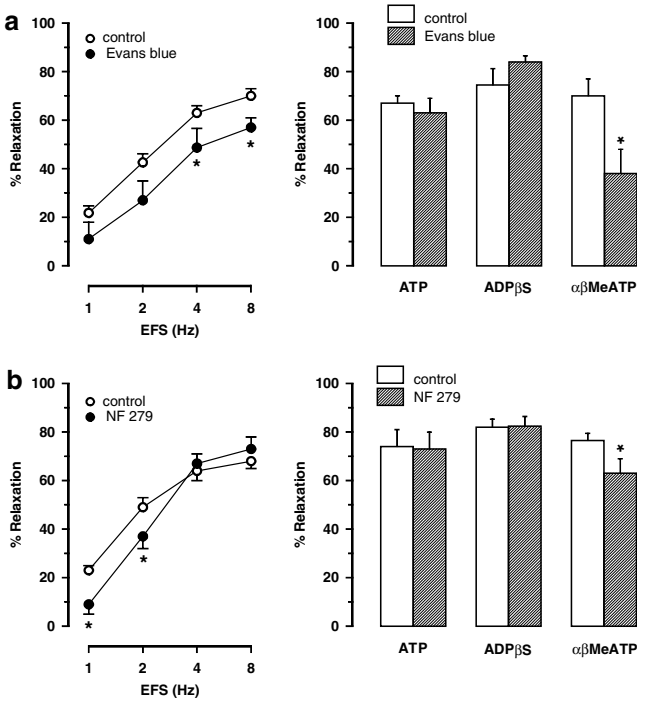

Figure 5 Effect of (a) Evans blue (10 μM, P2X receptor blocker) and (b) NF 279 (1 μM, $P2X_{1,2,3}$ receptor blocker) on the L-NOARG-resistant relaxations to EFS of NANC nerves (1–8 Hz), ATP (10 μM), ADPβS (1 μM) and $\alpha\beta$MeATP (10 μM). Results are expressed as % relaxation of a prostaglandin $F_{2\alpha}$-induced contraction, and are shown as mean$\pm$s.e.m. for $n=5$–8 experiments. *$P<0.05$, significantly different from controls, paired Student's t-test

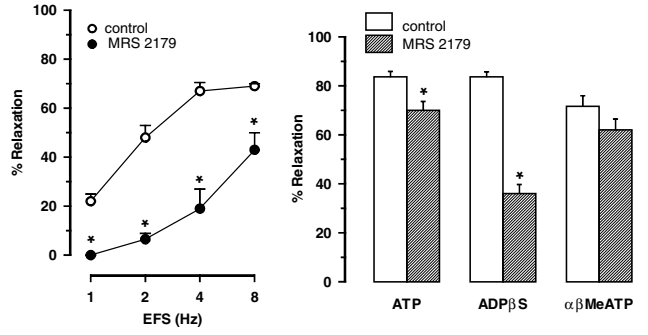

Figure 6 Effect of MRS 2179 (1 μM, $P2Y_1$ receptor blocker) on the L-NOARG-resistant relaxations to EFS of NANC nerves (1–8 Hz), ATP (10 μM), ADPβS (1 μM) and $\alpha\beta$MeATP (10 μM). Results are expressed as % relaxation of a prostaglandin $F_{2\alpha}$-induced contraction, and are shown as mean$\pm$s.e.m. for $n=6$ experiments. *$P<0.05$, significantly different from control ileum, paired Student's t-test.

effect on the relaxations to ATP (relaxation to 10 μM ATP was 67$\pm$7% in controls and 69$\pm$4% in the presence of hexamethonium, $n=6$, $P>0.05$).

Discussion

The present study presents evidence that ATP or a related purine mediates the fast component of the non-nitrergic

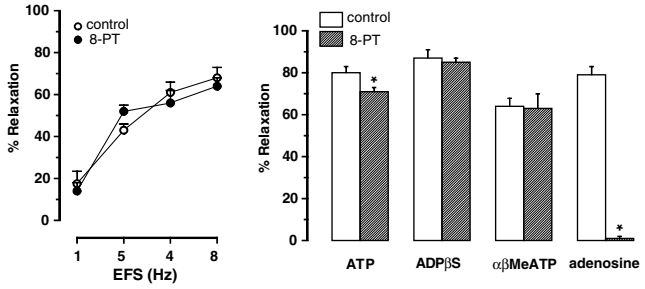

Figure 7 Effect of 8-phenyltheophylline (8-PT, 10 μM, adenosine A1 receptor blocker) on the L-NOARG-resistant relaxations to EFS of NANC nerves (1–8 Hz), ATP (10 μM), ADPβS (1 μM), $\alpha\beta$MeATP (10 μM) and adenosine (30 μM). Results are expressed as % relaxation of a prostaglandin $F_{2\alpha}$-induced contraction, and are shown as mean$\pm$s.e.m. for $n=5$ experiments. *$P<0.05$, significantly different from controls, paired Student's t-test.

relaxations to NANC nerve stimulation in the mouse jejunum. The relaxation to the purinergic NANC neurotransmitter is mainly mediated by $P2Y_1$ receptors located on the smooth muscle and by P2X receptors, possibly of the $P2X_1$ and/or $P2X_3$ subtype, located on enteric neurons as well as on smooth muscle cells.

ATP and enteric NANC neurotransmission

EFS of NANC nerves in jejunal circular muscle strips induced frequency-dependent transient relaxations. L-NOARG, a blocker of NO synthase, inhibited these relaxations, confirming that nitric oxide is an important neurotransmitter in the gut (Boeckxstaens *et al*., 1990a; Bult *et al*., 1990). However, in contrast to what we have observed in the mouse gastric fundus (De Man *et al*., 2001), L-NOARG did not completely abolish the response to NANC nerve stimulation in the mouse jejunum. A fast-twitch relaxation was still observed in the presence of L-NOARG. This L-NOARG-resistant NANC relaxation was almost abolished by apamin, a blocker of ATP-sensitive small-conductance Ca^{2+}-activated K^+ channels. Apamin is widely used as a tool to block responses to endogenously released and exogenous added purines. Electrophysiological studies on the gut smooth muscle indeed showed that the fast part of the inhibitory junction potential, induced by electrical stimulation of NANC nerves, is mimicked by exogenous ATP, and that both responses are blocked by apamin. It is likely that the L-NOARG-resistant fast response to EFS that we observed in isolated murine circular jejunal muscle strips is similar to the L-NOARG-resistant fast inhibitory junction potential that is repeatedly observed in different intestinal smooth muscle preparations. We found that apamin also blocked the response to exogenous ATP, indicating that the L-NOARG-resistant relaxations to EFS are mediated by an endogenously released purine. This is confirmed by the results obtained with the P2 purinoceptor antagonists suramin and PPADS. Suramin almost abolished the fast L-NOARG-resistant response to EFS, as well as the response to exogenous ATP, confirming that ATP or a related purine mediates the fast NANC relaxation to EFS. However, the effect of PPADS was not as straightforward: PPADS strongly inhibited the L-NOARG-resistant relaxation to EFS, but it did not affect the relaxation to exogenous ATP. A similar lack of effect of PPADS on relaxations to exogenous

ATP was reported by Giaroni *et al*. (2002) in the mouse small intestine.

A differential effect of PPADS on endogenous and exogenous ATP was previously also observed in the guinea-pig ileum (Matsuo *et al*., 1997; Heinemann *et al*., 1999). It was suggested that endogenous and exogenous ATP activate different subsets of P2 receptors. PPADS blocks certain but not all subtypes of P2 receptors, depending upon the tissue that is studied (Ralevic & Burnstock, 1998; North, 2002), and this may explain the discrepant effect of PPADS on the relaxations to the endogenously released purinergic neurotransmitter and exogenously added ATP. An alternative explanation for the discrepant effect of PPADS is that the enteric purinergic neurotransmitter is not ATP, but an ATP-related purine such as ADP. In favour of this hypothesis is that the ADP analogue ADPβS clearly relaxed jejunal muscle strips and that desensitisation of ADPβS-sensitive receptors potently inhibited the purinergic NANC relaxation to EFS. On the other hand, the finding that apamin and suramin potently inhibited the relaxations to ATP as well as those to EFS, suggests that ATP is the main mediator of the inhibitory purinergic NANC neurotransmission in the murine small intestine.

It is not likely that purinergic NANC neurotransmission in the mouse jejunum is mediated by adenosine. Adenosine may be generated from the phosphorylation of ATP. We found that the adenosine uptake inhibitor dipyridamole turned the transiently shaped relaxations to exogenous ATP into sustained relaxations. The P1 purinoceptor blocker theophylline and the adenosine A1 receptor blocker 8-phenyltheophylline slightly inhibited the relaxations to exogenous ATP and expectedly abolished relaxations to adenosine. This indicates that a minor part of the response to exogenous ATP is mediated by adenosine, acting on P1 purinoceptors. However, dipyridamole, theophylline or 8-phenyltheophylline had no effect on the purinergic NANC relaxations to EFS. This suggests that adenosine does not play a significant role in the signal transduction pathway of the endogenously released purinergic neurotransmitter in the mouse jejunum.

We found evidence that purinergic NANC neurotransmission is under presynaptic modulation, since hexamethonium partially inhibited the purinergic NANC relaxations to EFS without affecting the direct smooth muscle response to ATP. This suggests that purinergic nerve signalling in the mouse jejunum is modulated at nicotinic synapses.

Both the P2X receptor agonist $\alpha\beta$MeATP and the P2Y receptor agonist ADPβS relaxed the jejunal circular muscle strips, indicating that P2X and P2Y receptors are functionally active in this tissue. Furthermore, the relaxation to $\alpha\beta$MeATP but not that to ADPβS was partially inhibited by the nerve-conductance blocker tetrodotoxin. This is in contrast with the findings in rat ileum (Storr *et al*., 2000), but confirms findings in the mouse ileum (Vial & Evans, 2001), and suggests that the response to $\alpha\beta$MeATP in the mouse small intestine is partially of neuronal origin.

Purinergic NANC neurotransmission and P2Y receptors

Expectedly, desensitisation of $\alpha\beta$MeATP-sensitive P2X receptors reduced the response to $\alpha\beta$MeATP, but not that to ADPβS, while desensitisation of P2Y receptors reduced the response to ADPβS without affecting that to $\alpha\beta$MeATP. This shows that there was no crossdesensitisation between P2X and P2Y receptors after the respective desensitisation procedures. P2X receptor desensitisation only slightly inhibited the purinergic NANC relaxations to EFS, while desensitisation of P2Y receptors virtually abolished them. This strongly suggests that the purinergic transmitter that is released upon stimulation of enteric NANC nerves mainly activates P2Y receptors. Most likely, these P2Y receptors are located on the smooth muscle because the relaxations to the P2Y receptor agonist ADPβS had no neuronal component, as shown by the absence of the effect of tetrodotoxin. It is highly likely that the P2Y receptors that are involved in purinergic NANC neurotransmission are of the $P2Y_1$ subtype, because MRS 2179, a specific $P2Y_1$ receptor blocker (Boyer *et al*., 1998), potently inhibited both the neuronal purinergic response to EFS as well as the direct smooth muscle response to ADPβS. These findings are in good agreement with recent immunohistochemical data reporting intense immunoreactivity for $P2Y_1$ receptors in the circular and longitudinal muscle layers of the mouse small intestine (Giaroni *et al*., 2002). Interestingly, P2Y receptor desensitisation only moderately inhibited relaxations to exogenous ATP, while it almost abolished the purinergic NANC relaxations to EFS. This discrepant effect confirms the hypothesis, discussed above, that exogenous and endogenous purines may activate different subtypes of purinoceptors. Our study shows that relaxations to exogenous ATP are insensitive to PPADS, Evans blue. NF 279 and P2X receptor desensitisation, and only partially sensitive to MRS 2179 and P2Y receptor desensitisation. Interestingly, relaxations to ATP were significantly inhibited after P2X plus P2Y receptor desensitisation. Since ATP-induced relaxations were tetrodotoxin-insensitive, these results suggest the involvement of smooth muscle P2X and P2Y receptors in the relaxation to exogenous ATP.

Purinergic NANC neurotransmission and P2X receptors

Our data suggest that purinergic NANC neurotransmission also involves P2X receptors, albeit to a lesser extent compared to P2Y receptors. Purinergic NANC relaxations to EFS were slightly but significantly inhibited by desensitisation of $\alpha\beta$MeATP-sensitive P2X receptors and by Evans blue, a blocker of P2X receptors in the intestinal tissue (Bultmann *et al*., 1996). NF 279, which preferentially blocks $P2X_1$ receptors (Damer *et al*., 1998; Klapperstuck *et al*., 2000) but also inhibits $P2X_2$ and $P2X_3$ receptors in the rat (Rettinger *et al*., 2000), inhibited the EFS-induced purinergic relaxations, but only those to low-frequency stimulation. Brilliant blue G, which blocks $P2X_7$-mediated responses in the myenteric plexus of guinea-pig small intestine and rat hippocampus (Hu *et al*., 2001; Sperlagh *et al*., 2002) and $P2X_5$ receptors in transfected kidney cells (Bo *et al*., 2003), had no effect on the relaxations to EFS. These results indicate that $P2X_1$, $P2X_2$ and/or $P2X_3$ receptors but not $P2X_5$ or $P2X_7$ receptors mediate a minor part of the purinergic NANC relaxations to EFS in the mouse jejunum.

The P2X receptor blocker Evans blue and P2X receptor desensitisation with $\alpha\beta$MeATP inhibited the purinergic NANC relaxation with similar potency, suggesting that purinergic neurotransmission involves rapidly desensitising P2X receptors such as $P2X_1$ or $P2X_3$ receptors (Ralevic & Burnstock, 1998; North, 2002). P2X receptor desensitisation only partially

inhibited the relaxation to $\alpha\beta$MeATP, suggesting that the relaxation to $\alpha\beta$MeATP involves additional purinoceptors that are relatively insensitive to desensitisation such as $P2X_2$ and $P2X_4$ receptors (Ralevic & Burnstock, 1998; North, 2002).

The lack of specific antagonists of purinoceptor subtypes makes it difficult to draw a clear conclusion on the subtypes of P2X receptors that mediate purinergic NANC neurotransmission. Our current findings together with previous reports in the literature may however, help to unravel some uncertainties. Although immunohistochemistry failed to show $P2X_1$ reactivity in the myenteric plexus of the mouse ileum (Vial & Evans, 2001; Giaroni *et al*., 2002), there is functional evidence for the presence of presynaptic $P2X_1$ receptors in the mouse enteric nervous system (Vial & Evans, 2001). This fits well with our current findings that NF 279 inhibits the relaxation to purinergic NANC nerve stimulation. $P2X_2$ immunoreactivity is observed only in a small subpopulation of myenteric neurones in the mouse ileum (Giaroni *et al*., 2002). These may be sensory nerves since $P2X_2$ receptors in the mouse gut seem to be located mainly on afferent nerve structures (Castelucci *et al*., 2003; Ma & Kirchgessner, 2003). If $P2X_2$ receptors are mainly located on afferent nerves, it seems unlikely that the efferent inhibitory motor nerve activity that we have studied is mediated directly by $P2X_2$ receptors. This is also supported by the observation that Brilliant blue G, which is reported to block $P2X_2$ receptor function at micromolar concentrations (King *et al*., 1997), did not affect the purinergic NANC relaxations to EFS. $P2X_3$ receptors are expressed on enteric nerves in the guinea-pig intestine, including inhibitory motor nerves (Poole *et al*., 2002; Van Nassauw *et al*., 2002), and $P2X_3$ receptors regulate intestinal peristalsis in the mouse (Bian *et al*., 2003). Our finding that NF 279, which also blocks recombinant rat $P2X_3$ receptors, inhibited the purinergic relaxations to NANC nerve stimulation points towards the involvement of $P2X_3$ receptors, next to $P2X_1$ receptors, in purinergic NANC neurotransmission in the mouse intestine. $P2X_4$ receptors are also present on enteric nerves, but they are insensitive to suramin and PPADS (Ralevic & Burnstock, 1998; North, 2002). We found that suramin and PPADS strongly inhibited the relaxation to purinergic NANC nerve stimulation, which argues against the involvement of $P2X_4$ receptors in purinergic NANC neurotransmission in the mouse intestine.

The relaxation to $\alpha\beta$MeATP had a tetrodotoxin-sensitive component, confirming the presence of P2X receptors on enteric nerves. Prejunctional P2X receptors may be involved in the presynaptic regulation of purinergic nerve activity, since purinergic NANC relaxations to EFS were slightly inhibited by P2X receptor desensitisation, Evans blue and NF 279. However, it has to be kept in mind that this inhibition may also result from blockade of postjunctional P2X receptors. Although such receptors are not yet described in the literature, we found that the major part of the relaxation to $\alpha\beta$MeATP was tetrodotoxin-insensitive. This suggests the presence of $\alpha\beta$MeATP-sensitive P2X receptors on intestinal smooth muscle cells. In contrast to prejunctional intestinal P2X receptors, which are well studied and postulated to mediate fast synaptic transmission in enteric nerves (for a review, see Galligan *et al*., 2000), postjunctional intestinal P2X receptors have raised little attention yet, and their exact role and identity remains to be elucidated. Although we found functional evidence that purinergic NANC neurotransmission in the mouse jejunum is mediated by P2X receptors, our results do not allow a conclusion on whether these P2X receptors are located on enteric nerves, smooth muscle cells and/or other cells such as interstitial cells of Cajal (Burnstock & Lavin, 2002).

Conclusion

In conclusion, we provided functional evidence that the fast relaxation to NANC nerve stimulation of the mouse jejunum is mediated by ATP or a related purine. This endogenously released purinergic neurotransmitter relaxes jejunal circular muscle mainly by activating $P2Y_1$ receptors located on the smooth muscle. P2X receptors, located on the smooth muscle and/or on enteric nerves, are also involved in purinergic enteric neurotransmission, but to a lesser degree. These P2X receptors most likely include $P2X_1$ and $P2X_3$, but not $P2X_5$ or $P2X_7$ receptors.

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