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ATP and nitric oxide: inhibitory NANC neurotransmitters in the longitudinal muscle-myenteric plexus preparation of the rat ileum

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1 The nature of neurotransmitter(s) involved in non-adrenergic non-cholinergic (NANC) relaxations induced by electrical stimulation (10 s trains, 1-8 Hz) was investigated in the precontracted longitudinal muscle-myenteric plexus preparation of the rat ileum.

2 Electrical stimulation of the tissue induced complex responses, consisting of a primary contraction, a primary relaxation, an off-relaxation and a rebound contraction, which were all tetrodotoxin(TTX)-sensitive.

3 Vasoactive intestinal polypeptide (VIP) and carbon monoxide (CO) did not induce relaxations. α-Chymotrypsin did not reduce the relaxations induced by electrical stimulation, while zinc protoporphyrin IX had non-specific effects.

4 Nitric oxide (NO) induced concentration-dependent relaxations. N^G-nitro-L-arginine methylester (L-NAME) abolished the primary contractions and off-relaxations, while it partially reduced the primary relaxations.

5 ATP induced relaxations and ATP-desensitization of the tissues partially reduced the primary relaxations. Suramin and reactive blue 2 did not consistently influence the primary relaxations.

6 The ATP-induced relaxations were not influenced by L-NAME or TTX. The inhibitory effect of ATP-desensitization and L-NAME did not summate.

7 The cyclic AMP content of the tissue did not increase upon electrical stimulation or after addition of NO or ATP. The cyclic GMP content of the tissue increased upon electrical stimulation and addition of NO, but not after addition of ATP.

8 It is concluded that the relaxation induced by electrical stimulation consists of two types of responses. The off-relaxation is completely nitrergic, while the primary relaxation is mediated by NO, ATP and an as yet unknown transmitter which is not VIP or CO.

Keywords: Nitric oxide; ATP; NANC transmission; rat ileum

Introduction

Inhibitory non-adrenergic non-cholinergic (NANC) neurones play an important role in the physiological control of gastrointestinal motility. They are involved in gastric receptive relaxation, in peristaltic movements throughout the gastrointestinal tract and in relaxation of tonically contracted sphincters (Abrahamsson, 1986; Otterso & Sarr, 1992; Zenilman, 1993). The nature of the transmitter(s) involved in NANC relaxation of gastrointestinal preparations is species- and tissue-dependent and several candidates have been proposed, the most cited being ATP (Burnstock, 1990), vasoactive intestinal polypeptide (VIP; Fahrenkrug *et al.*, 1978; Makhlouf & Grider, 1993) and nitric oxide (NO; Rand, 1992; Sanders & Ward, 1992; Brookes, 1993; Lefebvre, 1993; 1995; Rand & Li, 1995). Recently carbon monoxide (CO) was also proposed as an inhibitory NANC neurotransmitter in the gastrointestinal tract (Rattan & Chakder, 1993).

Functional experiments with rat ileum longitudinal muscle revealed only the involvement of NO (Kanada *et al.*, 1992; Li *et al.*, 1994) and not VIP and ATP in inhibitory NANC neurotransmission (Yagasaki *et al.*, 1983; Manzini *et al.*, 1986). However, morphological studies showed the presence of VIP-immunoreactive nerve cell bodies and fibres in the rat ileum (Schültzberg *et al.*, 1980) and more recently, coexistence of ATP and NO was reported in myenteric neurones of the rat ileum (Belai & Burnstock, 1994).

The aim of the present study was to reinvestigate the nature of the inhibitory NANC neurotransmitter(s) of the rat ileum, using a precontracted longitudinal muscle-myenteric plexus (LMMP) preparation. We compared the effect of the putative transmitters NO, ATP, VIP and CO with that of electrical field stimulation and further investigated the influence of inhibitors of the putative transmitters on electrically induced NANC relaxation. In addition we investigated the effect of electrical stimulation and ATP and NO addition on the cyclic nucleotide content of the tissue. A preliminary account of these results has been given (Smits *et al.*, 1995).

Methods

Male Wistar rats of 3 to 4 months (mass 300-430 g) were obtained from the Centre for Experimental Animals of the University of Leuven, Belgium. They were killed by a blow to the neck and bled. After laparotomy, a segment of 20 cm of the distal part of the ileum (terminal 10 cm not included) was removed rapidly and a maximum of 4 LMMP preparations of 1.5 cm were prepared as follows. After rinsing the lumen, the ileum was mounted on a glass rod and the longitudinal muscle with the myenteric plexus was gently wiped off with cotton wool soaked in Krebs solution. The strips were suspended under a load of 0.6 g in 7.5 ml organ baths containing Krebs solution (with the following composition in mM: NaCl 118.5, KCl 4.8, $CaCl_2$ 1.9, KH_2PO_4 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 25.0 and glucose 10.1) held at 37°C and gassed with 95% O_2/5% CO_2. Changes in isometric tension were recorded via a Grass force-displacement transducer FT03 on a Graphtec linearcorder F WR3701. Transmural electrical stimulation was performed via two platinum plate electrodes (22 × 7 mm, 6 mm apart) by means of a Grass S88 stimulator with a constant voltage unit (supramaximal voltage, 0.3 ms duration,

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10 s trains). After an equilibration period of 60 min (rinsing every 15 min) the optimal load for each tissue was determined; the contraction to 3×10^{-7} M methacholine was recorded for different loads ranging from 0.6 to 1.6 g, increasing the load stepwise by 0.2 g. The strips were then held at their optimal load, where they had shown the highest contraction to methacholine. For the rest of the experiment atropine (10^{-6} M) and guanethidine (4×10^{-6} M) were present in the Krebs solution in order to work under NANC conditions. To study relaxant responses, tone was raised by addition of 3×10^{-7} M prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) and when a stable plateau was reached relaxant stimuli were tested. A maximum of 4 $PGF_{2\alpha}$ additions were made to one tissue with a minimum washout period of 60 min in between. At the end of the experiments, the tissues were weighed.

Responses to electrical stimulation and addition of putative neurotransmitters

The responses to electrical stimulation (10 s trains at 1, 2, 4 and 8 Hz; minimum interval in between dependent on the recovery of tone after a stimulation train) and addition of putative transmitters (NO: 10^{-7}, 10^{-6} and 10^{-5} M, 2 min interval in between; ATP: 10^{-5} and 10^{-4} M, only one concentration tested per $PGF_{2\alpha}$-plateau; CO: 10^{-4} M; VIP: 10^{-7} M) was investigated. Only one type of putative neurotransmitter was tested per $PGF_{2\alpha}$-induced plateau. In addition the responses to NO (10^{-7} M), ATP (10^{-4} M) and α-β-methylene ATP (α-β-MeATP; 3×10^{-5} M) was also investigated when the tissues were not contracted. The neurogenic nature of the responses to electrical stimulation was investigated by use of 3×10^{-6} M tetrodotoxin (TTX; incubation time 15 min).

Influence of inhibitors and ATP-desensitization

To investigate the involvement of neurotransmitters, electrical stimulation (see above) was studied first in the absence and then in the presence of inhibitors of the putative transmitters or the solvent of these inhibitors (control). The responses in control tissues were reproducible unless otherwise indicated. The inhibitors investigated were N^G-nitro-L-arginine methyl ester (L-NAME; 3×10^{-4} M), suramin (5×10^{-5} M), reactive blue 2 (5×10^{-5} M), α-chymotrypsin (10 u ml^{-1}) and Zn protoporphyrin IX (ZnPPIX; 10^{-3} M). The equilibration period for each antagonist was 30 min, with the exception of suramin and ZnPPIX, which were each allowed to equilibrate for 90 and 15 min respectively. The relaxant responses to ATP were studied in the absence of the purinoceptor antagonists. Possible non-specific effects of the purinoceptor antagonists and ZnPPIX were investigated by studying relaxant responses to 10^{-5} M NO in their absence and presence. Electrical stimulation was also investigated before and after ATP-desensitiza-

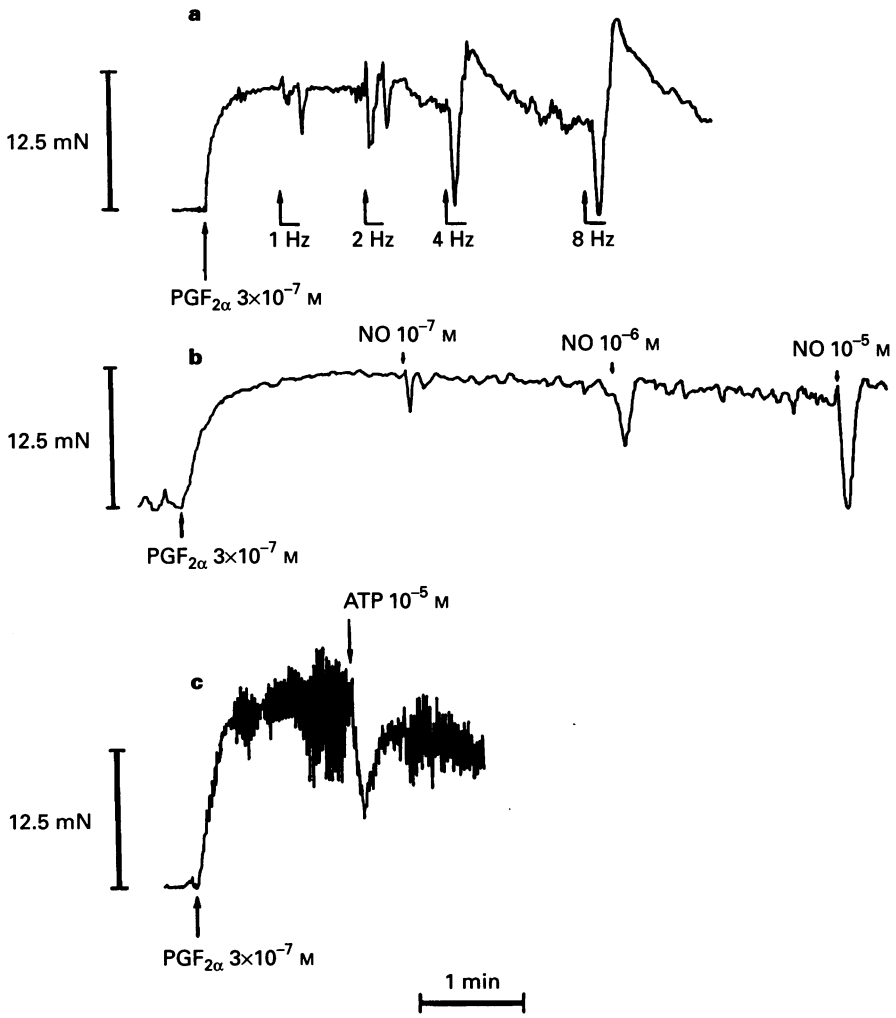

Figure 1 Representative experiments performed under NANC conditions showing the effects of electrical stimulation (supramaximal voltage, 0.3 ms duration, 10 s trains; a) and of addition of NO (b) and ATP (c) on the precontracted longitudinal muscle-myenteric plexus preparation of the rat ileum.

tion. Muscular desensitization to ATP was established by incubating 10^{-4} M ATP for 30 min before increasing tone with $PGF_{2\alpha}$ and was tested by investigating the responses to 10^{-4} M ATP and 10^{-5} M NO. The possible interaction between the nitrergic and purinergic component was investigated first by studying the effect of L-NAME (3×10^{-4} M) and TTX (3×10^{-6} M) on ATP (10^{-5} M)-induced relaxations and second by investigating the responses to electrical stimulation before and after ATP-desensitization in the presence of L-NAME (3×10^{-4} M).

Assay of cyclic nucleotides

Strips were mounted in an isotonic setup, that allowed quick clamping of the tissues (see below). Changes in length were recorded via a HSE lever transducer B type 368 on a Graphtec linearcorder WR3500. The tissues were mounted under 0.75 g tension (mean optimal load). After equilibrating for 1 h, tone was raised with $PGF_{2\alpha}$ and the tissue was stimulated electrically (supramaximal voltage, 0.3 ms duration; 10 s train at 2 Hz) or pharmacologically with NO (2×10^{-6} M) or ATP (10^{-5} M). The tissue was quickly clamped between two liquid nitrogen cooled plates after 7 s of stimulation or 1 or 10 s after the end of a 10 s stimulation train or when maximal relaxation (NO and ATP) was obtained. These time points correspond with the electrically induced primary relaxation, off-relaxation and rebound contraction respectively (see Results). As a control, some tissues were clamped after induction of tone with $PGF_{2\alpha}$, without applying a relaxant stimulus. The tissue was homogenized first with a membrane dismembrator (B. Braun A.G. Melsungen, 100%) for 45 s and then with an ultrasonic probe (B. Braun Labsonic U, Melsungen) for 4 times 5 s in 6% trichloroacetic acid on ice. The homogenate was centrifuged for 20 min at 2600 *g* and the trichloroacetic acid was extracted from the supernatant 4 times with 5 volumes of water-saturated ether. The adenosine 3':5'-cyclic monophosphate (cyclic AMP) content was measured by a binding assay based on the method of Tovey *et al.* (1974). The cyclic GMP content was determined by radioimmunoassay. The protein content of the samples was determined by the method of Lowry *et al.* (1951) with bovine serum albumin used as standard.

Statistical analysis

Relaxations and contractions were expressed as percentage of the $PGF_{2\alpha}$-induced tone, except for the contractions induced by 3×10^{-7} M $PGF_{2\alpha}$ and the spontaneous activity, which were expressed as mN × (mg mass of the tissue)$^{-1}$. The cyclic nucleotide contents were expressed as pmol cyclic nucleotide mg^{-1} protein. Data are given as means ± s.e.mean, *n* refers to the tissues obtained from different animals unless otherwise indicated.

Statistical comparisons between responses in the absence and the presence of inhibitors in the same tissues were done by paired *t* test. When comparison between results obtained in parallel tissues was required, an unpaired *t* test was used. In all comparisons, a *P*-value less than or equal to 0.05 was considered to be statistically significant.

Substances

Adenosine-5'-triphosphate (ATP; Boehringer, Mannheim, Germany), atropine sulphate (Sigma), bovine serum albumin (Sigma), α-chymotrypsin (Sigma), guanethidine sulphate (Sigma), methacholine (Schuchardt, Munchen, Germany), α-β-methyleneadenosine-5'-triphosphate (Sigma), N^G-nitro-L-arginine methyl ester (Sigma), prostaglandin $F_{2\alpha}$ (Sigma), reactive blue 2 (Sigma), suramin (RBI, Natick, U.S.A.), tetrodotoxin (Janssen Chimica, Beerse, Belgium), VIP (CRB, Northwich, U.K.), Zinc(II) protoporphyrin IX (Aldrich, Milwaukee, U.S.A.).

The cyclic AMP ^{3}H-assay system and the cyclic GMP ^{125}I-RIA-kit was bought from Amersham (Buckinghamshire, U.K.) and DuPont Canada Inc. (Ontario, Canada), respectively. All drugs were dissolved in water, except Zinc(II) protoporphyrin IX, which was first dissolved in 0.2 M NaOH and then neutralized by adding 0.2 N HCl up to a concentration of 10^{-1} M. All drug solutions were made on the day of the experiment except for VIP and $PGF_{2\alpha}$ where stock solutions were made and kept at −20°C. Saturated CO and NO solutions were prepared from gas (Air Liquide, Belgium) as described by Rattan & Chakder (1993) and Kelm & Schrader (1990), respectively.

Results

Responses to electrical stimulation and addition of putative neurotransmitters

The mean optimal load was 0.75 ± 0.06 g ($n = 33$). Preliminary experiments demonstrated that $PGF_{2\alpha}$ (3×10^{-7} M) contracted the tissue by 73 ± 4% of the maximal contraction to $PGF_{2\alpha}$ ($n = 12$ out of 4 rats). The mean contraction to 3×10^{-7} $PGF_{2\alpha}$ was 0.69 ± 0.05 mN mg^{-1} tissue mass ($n = 33$). The responses to this concentration of $PGF_{2\alpha}$ added 4 consecutive times, with a washing period in between, did not differ significantly. The tissues showed some spontaneous activity. At basal tone the mean amplitude of the spontaneous contractions, measured 1 min prior to administration of $PGF_{2\alpha}$, was 0.074 ± 0.009 mN mg^{-1} tissue mass ($n = 33$).

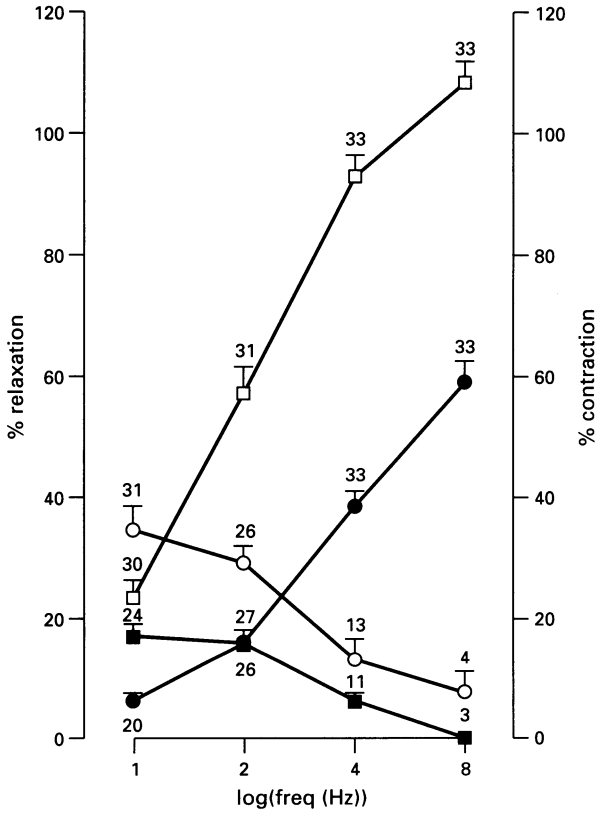

Figure 2 Mean frequency-response curves for the primary contraction (■), primary relaxation (□), off-relaxation (○) and rebound contraction (●) induced by electrical stimulation (supramaximal voltage, 0.3 ms duration, 10 s trains) under NANC conditions of the precontracted longitudinal muscle-myenteric plexus preparation of the rat ileum. Above the symbols, the number out of 33 tissues where the response was observed, is indicated. Contractions and relaxations were expressed relative to the contraction induced by 3×10^{-7} M $PGF_{2\alpha}$. Mean ± s.e.mean; $n = 33$.

Electrical stimulation of the precontracted preparations induced a complex response: at the beginning of the stimulation a fast contraction (primary contraction) was observed, followed by a relaxation (primary relaxation); during stimulation tone started to recover but only rarely increased above that present before stimulation; when the stimulation stopped a fast off-relaxation occurred following by a rebound contraction (Figure 1a where the full pattern can be seen for stimulation at 2 Hz). The amplitude of the primary contraction and off-relaxation decreased with increasing frequencies, while the amplitude of the primary relaxation and the rebound contraction increased with increasing frequencies (Figure 1a and 2). All these responses were abolished by TTX ($n=3$).

NO induced concentration-dependent, short-lasting relaxations (Figure 1b). The mean relaxations were 27 ± 9, 61 ± 6 and $94\pm5\%$ ($n=7$) for 10^{-7}, 10^{-6} and 10^{-5} M NO, respectively. In 2 out of 7 strips, an initial primary contraction was observed at 10^{-7} M NO; when adding the same NO concentration at basal tone a contraction was observed in 3 out of 7 preparations.

ATP induced short-lasting relaxations (Figure 1c), that attained 61 ± 3 and $58\pm5\%$ at 10^{-5} and 10^{-4} M, respectively ($n=6$). At basal tone, ATP induced small contractions attaining an amplitude of $14\pm2\%$ ($n=6$) at 10^{-4} M. α-β-MeATP (3×10^{-5} M) contracted the tissue at basal tone to $54\pm13\%$ ($n=4$).

VIP and CO did not induce any relaxation in precontracted preparations ($n=3$). In 1 out of 3 preparations a small contraction to 10^{-7} M VIP was observed.

Influence of inhibitors and ATP-desensitization

L-NAME (3×10^{-4} M) completely inhibited the primary contraction and the off-relaxation, while it reduced the primary relaxation significantly at a stimulation frequency of 4 to 8 Hz (Figure 3a and 4). The rebound contraction was not enhanced in the presence of L-NAME.

Reactive blue 2 (5×10^{-5} M) reduced the relaxant effect of 10^{-4} M ATP from $65\pm8\%$ to $25\pm10\%$ ($n=4$; $P<0.05$). Reactive blue 2 (5×10^{-5} M) influenced only the primary relaxa-

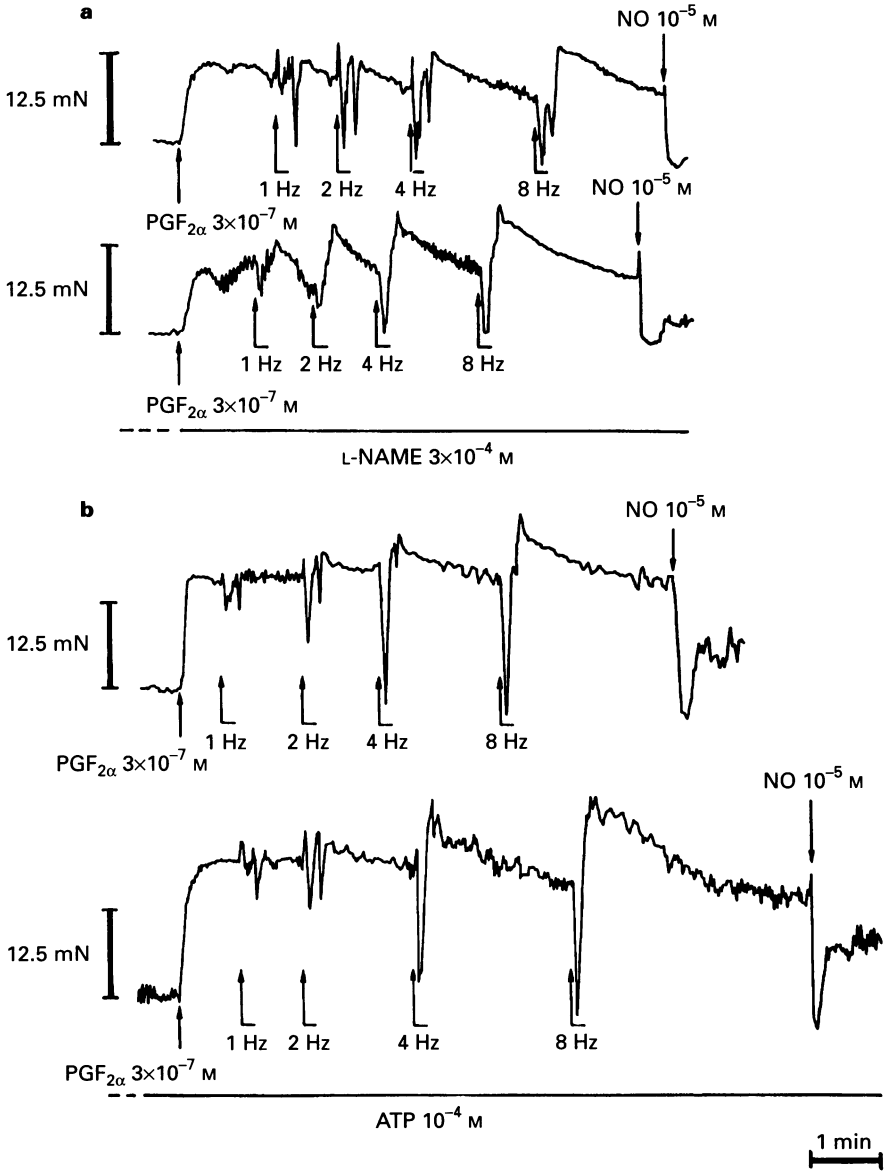

Figure 3 Representative experiments performed under NANC conditions showing the effect of L-NAME (a) and ATP-desensitization (b) on the responses to electrical stimulation (supramaximal voltage, 0.3 ms duration, 10 s trains) of the precontracted longitudinal muscle-myenteric plexus preparation of the rat ileum.

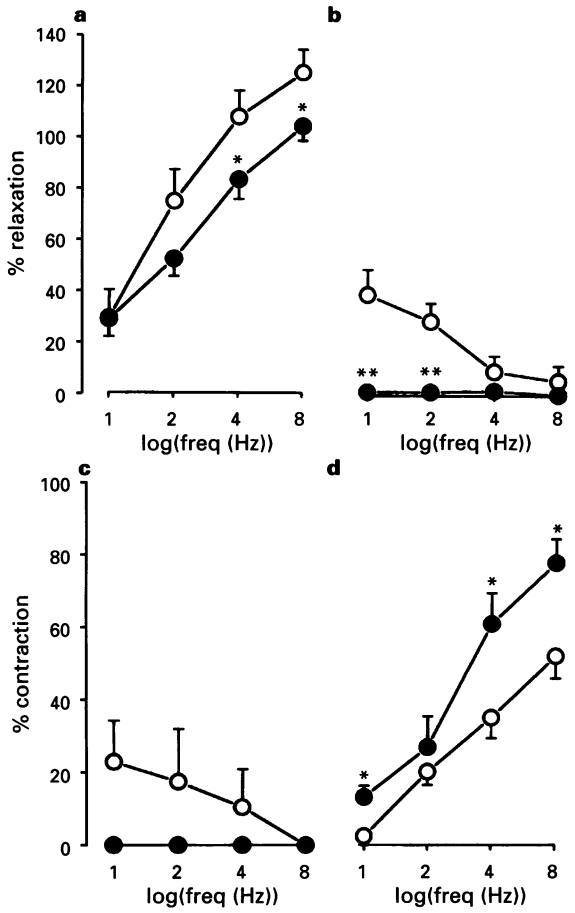

Figure 4 Mean effect of 3×10^{-4} M L-NAME on the primary relaxation (a), the off-relaxation (b), the primary contraction (c) and the rebound contraction (d) induced by electrical stimulation (supramaximal voltage, 0.3 ms duration, 10 s trains) under NANC conditions of the precontracted longitudinal muscle-myenteric plexus preparation of the rat ileum. Responses to electrical stimulation before (○) and after (●) administration of L-NAME. Mean ± s.e.mean; $n=9$. $^*P<0.05$, $^{**}P<0.01$; paired t test.

tion in an inconsistent way, increasing it at stimulation frequencies of 1 and 2 Hz, and decreasing it at 8 Hz ($n=5$) (Figure 5a). At 10^{-4} M, reactive blue 2 clearly decreased the $PGF_{2\alpha}$-induced contractions so that this concentration was not tested further. Suramin (5×10^{-5} M) did not influence the relaxation induced by 10^{-4} M ATP ($n=5$). It also had no effect on the primary contraction, the off-relaxation and the rebound contraction while it reduced the primary relaxation at 8 Hz ($n=5$; $P<0.05$; Figure 5b). At 10^{-4} M, suramin depressed the $PGF_{2\alpha}$-induced contraction. TTX (3×10^{-6} M) and L-NAME (3×10^{-4} M) did not influence the ATP (10^{-5} M)-induced relaxations ($n=5$). ATP-desensitization did not influence the NO (10^{-5} M)-induced relaxation but decreased the primary relaxation (Figure 3b and 6). In the control tissues for this series (Figure 6a), the primary relaxation also declined but the decrease after ATP-desensitization (Figure 6b) was significantly more pronounced that in the control tissues at 1, 2 and 4 Hz ($P<0.05$, unpaired t test). The other electrically induced responses (primary contraction, off-relaxation and rebound contraction) were not consistently changed. The influence of

Table 1 The cyclic AMP and cyclic GMP content in control conditions and after electrical stimulation ($n=5$-6; supramaximal voltage, 0.3 ms duration, 2 Hz; clamped after 7 s (ES1) of stimulation or 1 (ES2) OR 10 (ES3) s after a 10 s train) or after addition of 10^{-5} M ATP or 2×10^{-6} M NO ($n=3$–4; clamped at maximal relaxation)

	Cyclic AMP (*pmol mg^{-1}*)	*Cyclic GMP* (*pmol mg^{-1}*)
Control	6.08 ± 1.01	0.239 ± 0.041
ES1	7.51 ± 1.00	0.476 ± 0.091*
ES2	6.75 ± 1.07	0.411 ± 0.067*
ES3	6.90 ± 0.74	0.310 ± 0.043*
NO	4.04 ± 0.84	11.18 ± 2.44**
ATP	3.75 ± 0.89	0.240 ± 0.020

Values are mean ± s.e. mean $^*P<0.01$, $^{**}P<0.05$, significantly different from control value, unpaired t-test.

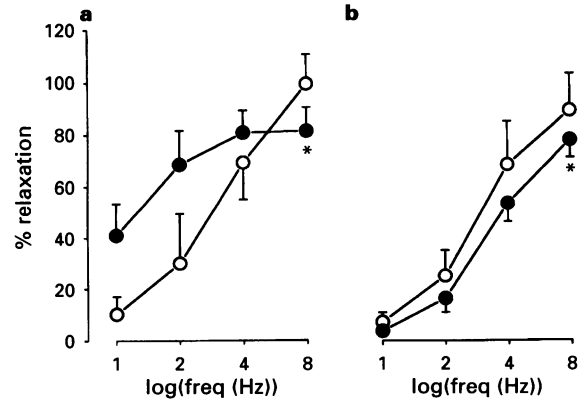

Figure 5 Mean effect of reactive blue 2 (5×10^{-5} M; a) and suramin (5×10^{-5} M; b) on the primary relaxation induced by electrical stimulation (supramaximal voltage, 0.3 ms duration, 10 s trains) under NANC conditions of the precontracted longitudinal muscle-myenteric plexus preparation of the rat ileum. Responses to electrical stimulation before (○) and after (●) administration of purinoceptor antagonist. Mean ± s.e.mean; $n=5$–6. $^*P<0.05$, paired t test.

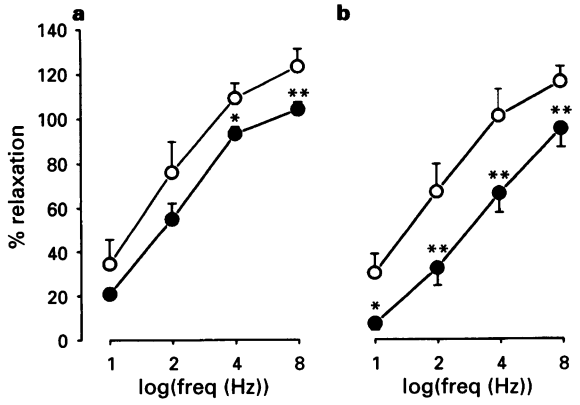

Figure 6 Mean effect of ATP-desensitization on the primary relaxation induced by electrical stimulation (supramaximal voltage, 0.3 ms duration, 10 s trains) under NANC conditions of the precontracted longitudinal muscle-myenteric plexus preparation of the rat ileum. (a) Represents the control tissues, where the responses were studied twice without ATP-desensitization (○, ●), while (b) represents the responses before (○) and after (●) ATP-desensitization. Mean ± s.e.mean; $n=7$. $^*P<0.05$, $^{**}P<0.01$, paired t test.

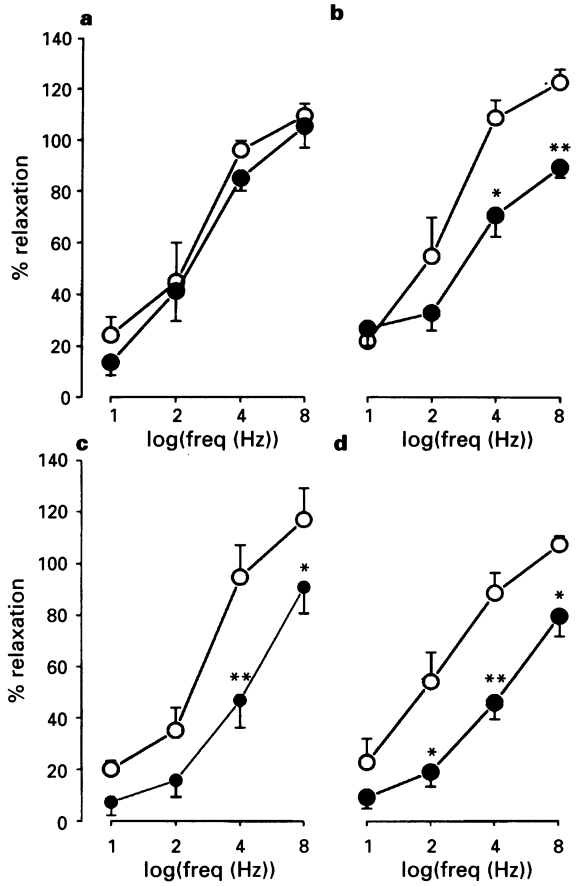

Figure 7 Mean primary relaxation induced by electrical stimulation (supramaximal voltage, 0.3 ms duration, 10 s trains) under NANC conditions of the precontracted longitudinal muscle-myenteric plexus preparation of the rat ileum before and after addition of L-NAME (b), after ATP-desensitization (c) and after a combination of both (d); (a) represents the control experiments where the responses to electrical stimulation were studied twice without addition of inhibitors in between. Primary relaxation before (○) and after (●) the treatment. Mean ± s.e.mean; $n=9$. $^{*}P<0.05$; $^{**}P<0.01$, paired t test.

ATP-desensitization on the primary relaxation was confirmed by experiments, where the responses in the control tissues were reproducible (Figure 7a and c). The inhibition of the primary relaxation by the combination of L-NAME and ATP-desensitization was not significantly higher (unpaired t test) compared to addition of L-NAME alone or ATP-desensitization alone (Figure 7).

α-Chymotrypsin did not inhibit the primary contraction, the primary relaxation or the off-relaxation, while it reduced the rebound contractions significantly at 8 Hz ($P<0.05$; $n=4$). ZnPPIX (10^{-3} M) significantly reduced the primary relaxation at 4 and 8 Hz, but it also reduced the NO (10^{-5} M)-induced relaxations to the same extent (primary relaxation at 8 Hz from 89.8 ± 8.7 to 76.3 ± 4.2%, $n=6$; relaxation induced by NO from 112.0 ± 6.8 to 82.3 ± 9.2%, $n=6$).

Assay of cyclic nucleotides

The cyclic AMP- and cyclic GMP-content of the tissues before and after applying relaxant stimuli is given in Table 1. The cyclic AMP content was not altered by electrical stimulation or by addition of NO or ATP. The cyclic GMP content was significantly increased by a factor 2 after 7 s stimulation at 2 Hz. This interval has been selected to correspond with the electrically induced primary relaxation. One and 10 s after stimulating at 2 Hz for 10 s, the cyclic GMP content was still significantly increased but the increase was less pronounced at 10 s; these moments correspond to the functionally observed off-relaxation and rebound contraction. NO (2×10^{-6} M) increased the cyclic GMP content more than 40 times, while ATP (10^{-5} M) had no influence.

Discussion and conclusions

In this study we investigated the nature of the NANC neurotransmitter(s) involved in relaxations induced by electrical stimulation of the precontracted longitudinal muscle-myenteric plexus preparation of the rat ileum. Electrical stimulation induced TTX-sensitive complex responses comparable to the patterns reported by Barthó & Lefebvre (1995) in the same tissue but at basal level. The primary contraction, the primary relaxation, the off-relaxation and the rebound contraction correspond with phase 1, 2, 4 and 5, respectively, according to the terminology of Barthó & Lefebvre (1995). Phase 3 in the experiments of Barthó & Lefebvre (1995) is a contraction above the tone level present before stimulation, occuring after the primary relaxation, but during stimulation. Although tone also started to recover after the relaxation during stimulation in the present investigation, it rarely increased above the prestimulation level, probably because of the precontracted state of the tissue, favouring relaxations. The primary contraction decreased with increasing frequency while the primary relaxation increased, suggesting the predominance of the inhibitory transmitter(s) at higher frequencies. The off-relaxation decreased with increasing frequency; it might be swamped by the rebound contraction, which increases with frequency. This work concentrated on the neurotransmitter(s) involved in the relaxations but some of the inhibitors tested also influenced the contractile responses (see below).

Involvement of VIP and CO

Previous experiments with non-contracted rat ileum preparations did not provide evidence for an involvement of VIP in NANC relaxations (Yagasaki *et al.*, 1983). Also in contracted tissues no such evidence was obtained since: (1) VIP did not relax the tissues; (2) α-chymotrypsin, which cleaves VIP in non-active parts and has been shown to abolish the effects of exogenous VIP on smooth muscle preparations (De Beurme & Lefebvre, 1987; Ellis & Farmer, 1989), did not influence the relaxations induced by electrical stimulation; (3) upon electrical stimulation, the cyclic AMP-content of the tissues did not increase, while VIP in general acts via activation of adenylate cyclase (Said, 1991). VIP induced contraction in one preparation. Contractions to VIP have been described in longitudinal muscle of the guinea-pig ileum (Kusunoki *et al.*, 1986; Gordon *et al.*, 1990; Katsoulis *et al.*, 1992). Neuropeptides might be involved in the rebound contractions as α-chymotrypsin reduced this response at higher stimulation frequencies. The reduction of the rebound contraction by α-chymotrypsin cannot be related to the inhibition of tachykinins, as α-chymotrypsin cleaves peptides at the level of tyrosine, which is not present in the sequence of the tachykinins. Carbon monoxide formed from haem by the enzyme haem oxygenase, has been proposed as an inhibitory NANC neurotransmitter in the opossum internal anal sphincter (Rattan & Chakder, 1993). In the LMMP preparations, CO did not induce relaxations. The inhibitor of the haem oxygenase, ZnPPIX, reduced the primary relaxations but it also reduced the NO-induced relaxations, suggesting a non-specific inhibition of relaxations. Also other investigators have observed non-specific actions of ZnPPIX versus relaxant stimuli, not related to haem oxygenase inhibition (Ny *et al.*, 1995; Tøttrup *et al.*, 1995).

Involvement of NO

A nitrergic contribution was demonstrated by the observation that the inhibitor of NO-synthesis, L-NAME, partially inhibited the primary relaxations and abolished the off-relaxations and the primary contractions. The latter observation corroborates previous findings on nitrergic contractions (Barthó *et al.*, 1992); NO has indeed been shown to induce contractions at low concentrations and relaxations at high concentrations in this tissue (Barthó & Lefebvre, 1994) and this was confirmed in this study. The involvement of NO in the primary relaxation and the off-relaxation was confirmed by the increase in cyclic GMP, as NO is known to act via activation of soluble guanylate cyclase and to cause accumulation of cyclic GMP (Waldman & Murad, 1987). The increase in cyclic GMP by exogenous NO was much more pronounced than that produced by stimulation but a similar discrepancy has been found in other tissues (sheep urethral muscle, Garcia-Pascual & Triguero, 1994; pig gastric fundus, Lefebvre *et al.*, 1995). The increase in cyclic GMP content during the primary relaxation (as measured after 7 s of stimulation) and during the off-relaxation (as measured 1 s after a 10 s train of stimulation) was similar notwithstanding the clearly more pronounced amplitude of the primary relaxation. This corresponds to the incomplete blockade of the primary relaxation by L-NAME and suggests that other neurotransmitter(s) are involved in the primary relaxation. The incomplete inhibition by L-NAME cannot be attributed to the duration of incubation with L-NAME, as increase of this incubation period did not augment the inhibiting effect of L-NAME; other NO synthase inhibitors were equally unsuccessful (data not shown). The incomplete blockade of the primary relaxations by L-NAME contrasts with the complete blockade of the electrically induced relaxations by NO synthesis inhibitors in previous work (Kanada *et al.*, 1992; Li *et al.*, 1994). However Kanada *et al.* (1992) might be measuring off-relaxations (see Figure 4) and these are also completely blocked by NO-synthesis inhibitors in our experiments. The relaxations that Li *et al.* (1994) show (Figure 4) correspond to the primary relaxations in the present investigation. We have no explanation why these responses were completely blocked by NO synthesis inhibition but the differences in methodology and the absence of atropine might play a role.

Involvement of ATP

Although no involvement of ATP in NANC relaxation of the rat small intestine was shown in a previous study in non-contracted segments (Manzini *et al.*, 1986), we reinvestigated the possible role of ATP as ATP has been colocalized with NO in the myenteric neurones of the rat ileum (Belai & Burnstock, 1994). When the tissues were not contracted with $PGF_{2\alpha}$, ATP induced contractions, confirming the observations of Manzini *et al.* (1986). ATP might thus exert its effects via two different receptors, one linked with the contractile apparatus and one linked with the relaxant apparatus. In many blood vessels, ATP exerts a dual action, relaxation via P_{2y}-receptors and contractions via P_{2x}-receptors (see e.g. rabbit portal vein, Kennedy & Burnstock, 1985; human small pulmonary arteries, Liu *et al.*, 1989). As α-β-methylene ATP is more potent than ATP at P_{2x}-receptors in the absence of breakdown inhibitors (Kennedy & Leff, 1995), the more pronounced contraction by α-β-methylene ATP in the rat ileum might correspond with the presence of P_{2x}-receptors. One can thus speculate that ATP acts as a relaxant agent via P_{2y}-receptors. Reactive blue 2 has been used in many studies as a P_{2y}-antagonist (Serio *et al.*, 1990; Matharu & Hollingsworth, 1992; Kennedy & Humphrey, 1994; Soediono & Burnstock, 1995). Although it reduced the ATP-induced relaxation, it had no consistent effect on the primary relaxation in the rat ileum. Suramin has been used previously to study the involvement of P_2-receptors in many tissues including the guinea-pig ileum (Kennedy & Humphrey, 1994) and the rat gastric fundus (Matharu & Hollingsworth, 1992). As the incubation time of suramin is important for its antagonistic actions (Leff *et al.*, 1990), it was incubated for 90 min but still, it did not reduce the ATP-induced relaxations. Therefore, it cannot be used to evaluate the role of ATP in the rat ileum. Previous studies indicated that ATP-desensitization did not influence the relaxations induced by electrical stimulation in the longitudinally mounted rat ileum under NANC conditions at basal tone (Yagasaki *et al.*, 1983; Manzini *et al.*, 1986). In our preparation ATP-densitization reduced the primary relaxation partially, but had no effect on the off-relaxation. This suggests that ATP is involved in the primary relaxation, as well as NO. As L-NAME did not influence the primary relaxation induced by electrical stimulation at 1 Hz, while ATP-desensitization did, ATP might be the most important transmitter at lower frequencies of stimulation.

The contradiction between our study and the previous ones, is probably again due to the fact that in the previous studies the influence of ATP-desensitization on off-relaxations was investigated (see Figure 2 in Yagasaki *et al.*, 1983; Figure 3 in Manzini *et al.*, 1986). ATP did not change the cyclic AMP or cyclic GMP content of the tissues; P_{2X}-purinoceptors are indeed thought to contain intrinsic ion channels while P_{2Y}-receptors in general act by activation of phospholipase C (Dalziel & Westfall, 1994).

Nitrergic-purinergic interaction

Our observation that in the rat ileum, NO and ATP play a functional role in the inhibitory NANC relaxation corresponds with the demonstration that NO and ATP coexist in myenteric neurones of the rat ileum (Belai & Burnstock, 1994). The combined involvement of NO and ATP as inhibitory NANC neurotransmitters has been proposed in other tissues such as the rat pyloric sphincter (Soediono & Burnstock, 1994), the rabbit portal vein (Brizzolara *et al.*, 1993) and the guinea-pig colon (Zagorodnyuk & Maggi, 1994; Briejer *et al.*, 1995). In these 3 tissues NO and ATP act independently and exert their effect directly on the smooth muscle. In the canine ileocaecal junction and the terminal ileum, relaxations induced by ATP were blocked by TTX and inhibitors of NO synthase, suggesting that ATP provokes the release of NO and that NO is the final neurotransmitter (Boeckxstaens *et al.*, 1991). In our tissues, the relaxations induced by ATP were not influenced by TTX nor by L-NAME excluding the release of NO by ATP. The reverse i.e. that NO induces the release of ATP is unlikely as ATP-desensitization did not influence the NO-induced relaxations. Still, the inhibitory effect of L-NAME and ATP-desensitization on the primary relaxation did not summate when a combination was tested. This suggests that both neurotransmitters, although not sequentially linked, need the other for full activity; it also leaves open the possibility that still other neurotransmitters are involved in the primary relaxation.

In conclusion electrical stimulation of the precontracted rat ileum LMMP preparation in NANC conditions induced a complex pattern consisting of a primary contraction, a primary relaxation, an off-relaxation and a rebound contraction. The primary contraction and off-relaxation were completely nitrergic in nature, while the primary relaxation was partly nitrergic, partly purinergic and partly mediated by another inhibitory NANC neurotransmitter which is not VIP or CO.

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