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Contribution of ATP and nitric oxide to NANC inhibitory transmission in rat pyloric sphincter

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1 Changes in isometric tension were recorded from circular muscle strips of rat pyloric sphincter *in vitro*, in response to electrical field stimulation and exogenously applied muscle relaxants.

2 Concentration-response relationships were studied for relaxations to exogenously applied adenosine 5'-triphosphate (ATP) and two analogues, 2-methylthioATP (2-MeSATP) and α,β-methylene ATP (α,β-MeATP). These drugs evoked concentration-dependent relaxation of rat pyloric sphincter with an order of potency 2-MeSATP > ATP >> α,β-MeATP, indicating the presence of P_{2y}-purinoceptors. The IC_{50} value of each nucleotide was: 2-MeSATP, 5.0×10^{-8} M; ATP, 7.9×10^{-6} M; α,β-MeATP showed only slight activity at a concentration of 0.1 mM.

3 Frequency-response relationships for relaxations evoked by electrical field stimulation (EFS) were studied in the absence and presence of 10 μM N^G-nitro-L-arginine methyl ester (L-NAME, an inhibitor of nitric oxide (NO) synthesis) and 20 μM reactive blue 2 (a P_{2y}-purinoceptor antagonist). It was found that these substances significantly reduced the relaxant response of rat pyloric sphincter to EFS by 40% and 50% respectively. In the presence of both L-NAME and reactive blue 2 the responses were reduced by 75%.

4 Concentration-response relationships were studied for ATP and 2-MeSATP in the presence of L-NAME. It was found that L-NAME did not significantly inhibit the relaxant responses to these drugs.

5 Concentration-response relationships for ATP and noradrenaline were studied in the presence of reactive blue 2 (20 μM); the P_{2y}-antagonist significantly inhibited the relaxant response to ATP, but not that to noradrenaline.

6 The distribution of nitric oxide synthase in rat pyloric sphincter was investigated immunohistochemically, with immunoreactive nerve fibres found throughout the circular muscle layer and myenteric plexus of the sphincter.

7 While abundant vasoactive intestinal polypeptide (VIP)-containing nerve fibres were demonstrated immunohistochemically in the pyloric sphincter, relaxations to VIP (1 nM–0.3 μM) were not observed in this preparation.

8 It is concluded that ATP, acting through P_{2y}-purinoceptors, and NO contribute to NANC inhibitory neurotransmission in rat pyloric sphincter. NO appeared to contribute to the later component of NANC relaxation. The action of ATP was not mediated by NO, and VIP did not contribute to the NANC inhibitory responses in this preparation.

Keywords: Pyloric sphincter; nitric oxide; ATP; NANC; inhibition

Introduction

A major part of the autonomic innervation to the gastrointestinal tract is by non-adrenergic non-cholinergic (NANC) neurones, although the precise nature of transmitters involved remains controversial. In sphincteric regions of the gut, this NANC innervation is mainly inhibitory and is physiologically important in the relaxation of sphincters for the passage of ingested solids.

Vasoactive intestinal polypeptide (VIP) has been proposed as a candidate mediator for NANC innervation in stomach (Grider *et al.*, 1985), pyloric sphincter (Allescher *et al.*, 1989) and internal anal sphincter (Biancini *et al.*, 1985). However VIP does not mimic the electrophysiological actions of the final NANC transmitter *in vitro* (Daniel *et al.*, 1986, 1991). Functional (Allescher *et al.*, 1989) and biochemical (Torphy *et al.*, 1986) evidence has been presented suggesting that VIP is not the final NANC inhibitory transmitter in pyloric and lower oesophageal sphincters.

Adenosine 5'-triphosphate (ATP) and nitric oxide (NO) have also been put forward as inhibitory transmitters in the gut. The evidence for ATP as a transmitter in the gut is substantial (Burnstock *et al.*, 1970; Hoyle & Burnstock, 1989). NO was first identified as endothelium-derived relaxing factor (EDRF) but, subsequently, has been proposed as a putative NANC inhibitory transmitter in a number of tissues (for review see Rand, 1992). NO formation is catalysed by nitric oxide synthase (NOS). Investigations of brain NOS show that the primary substrate is arginine, forming citrulline and NO, and requiring Ca^{2+} and reduced nicotinamide adenine dinucleotide phosphate (NADPH). Because of its instability, NO cannot be localized in tissues. The purification of NOS has allowed antisera to be raised, and permitted immunohistochemical localization of this enzyme (Bredt & Snyder, 1990). Enzymatic blockade of NO production with arginine methyl esters reduces or blocks the inhibitory NANC responses in a variety of tissues, including the lower oesophageal sphincter (Tottrup *et al.*, 1991), ileocolonic junction (Boeckxstaens *et al.*, 1990) and the canine pyloric sphincter (Allescher *et al.*, 1992). In the canine ileocolonic junction, it was shown that the relaxant action of ATP is mediated by NO or an NO-releasing substance (Boeckxstaens *et al.*, 1991). A recent study of the NANC inhibitory responses in the rabbit portal vein has shown that both NO and ATP are involved in the NANC inhibitory

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response (Brizzolara *et al.*, 1993), while studies in rat intestine have also proposed NO and ATP as cotransmitters (Belai & Burnstock, 1994).

In the present study the roles of ATP, VIP and NO in the NANC inhibitory innervation of rat pyloric sphincter were investigated. The receptor subtype mediating the effect of ATP in this tissue was also investigated and the possibility that the action of ATP is mediated through NO was examined. The distribution of the NO synthesizing enzyme NOS, and of VIP, was also studied immunohistochemically.

Methods

Male Sprague-Dawley rats (275–325 g) were killed by asphyxiation with CO_2. The gastric antrum and proximal duodenum were removed *en bloc*, opened along the greater curvature of the stomach and pinned flat. The junction between the gastric and duodenal mucosa was identified, the mucosa removed and a strip 2 mm wide was cut immediately proximal to this junction. This muscle strip was found to contain the thickened band of circular muscle, considered to be the anatomical representation of the pyloric sphincter and the only region to produce consistent relaxation to transmural electrical stimulation, as described by Anuras *et al.* (1974) in the opossum, cat and man. Silk ligatures were attached to each end of the muscle strip. One end was then tied to a rigid support and the other end to a force displacement transducer (Grass FT30C) to record isometric tension. The tissue was placed in a 5 ml organ bath containing modified Krebs-Ringer solution of the following composition (mM): NaCl 133, KCl 4.7, $NaHCO_3$ 16.4, $MgSO_4$ 0.6, $NaHPO_4$ 1.4, $CaCl_2$ 2.5 and glucose 7.7, aerated with 95% O_2-5% CO_2 at 37°C. Electrical field stimulation (EFS) was applied through 2 platinum wire rings 2.5 mm in diameter and 1 cm apart, through which the muscle preparation was threaded. Changes in tension were recorded on a Grass ink-writing oscillograph; 1 g of tension was applied. The tissue was allowed to equilibrate for at least 1 h. All strips were weighed and found to weigh within 2 standard deviations of the mean weight. Experiments with noradrenaline were performed at resting tension in the presence of atropine (2.3 μM). All other experiments were performed at resting tension and in the presence of atropine (2.3 μM), phentolamine (3.55 μM) and propranolol (3.8 μM).

Frequency-response relationships

The relationship between frequency of nerve stimulation (100 V, 0.25 ms, 0.5–32 Hz, 30 s stimulation every 5 min) and tissue response was studied in the absence, then presence of N^G-nitro-L-arginine methyl ester (L-NAME) (10 μM) or reactive blue 2 (20 μM). Frequency-response relationships constructed in the presence of L-NAME were repeated in the presence of L-arginine (1 mM). Finally, experiments were repeated in the presence of tetrodotoxin (3 μM).

Concentration-response relationships

The relationship between drug-concentration and tissue-response was investigated for noradrenaline, VIP, ATP and 2-methylthioATP (2-MeSATP) adding each agonist in a cumulative fashion. The concentration-response relationship for the purinoceptor agonist α,β-methylene ATP (α,β-MeATP) was studied under non-cumulative conditions, allowing at least 20 min between doses to prevent desensitization. Experiments with ATP were repeated in the presence of L-NAME (10 μM) and reactive blue 2 (20 μM); experiments with noradrenaline were repeated in the presence of reactive blue 2 (20 μM).

Drugs used

The following drugs were used: adenosine 5'-triphosphate (ATP); α,β-methylene ATP (α,β-MeATP); N^G-nitro-L-arginine methyl ester (L-NAME); L-arginine; noradrenaline bitartrate salt; vasoactive intestinal polypeptide (VIP); tetrodotoxin and reactive blue 2 (all obtained from Sigma Pharmaceuticals, U.K.); 2-methylthioATP (2-MeSATP) (Research Biochemicals Inc., U.K.); atropine sulphate (Phoenix Pharmaceuticals, U.K.); phentolamine mesylate (Ciba, U.K.) and propranolol hydrochloride (ICI, U.K.). All drugs were administered as aqueous solutions in microliter volumes and were prepared on the day of experimentation. Solutions of noradrenaline contained 300 μM ascorbic acid. The stock solution of tetrodotoxin (1 mM, in citrate buffer, pH 4.8) was stored at −20°C.

Preparation of results and statistical analysis

Results are shown as the % relaxation compared to the maximal relaxation of the tissue to EFS. Sodium nitroprusside (SNP) was initially used to determine maximal relaxa-

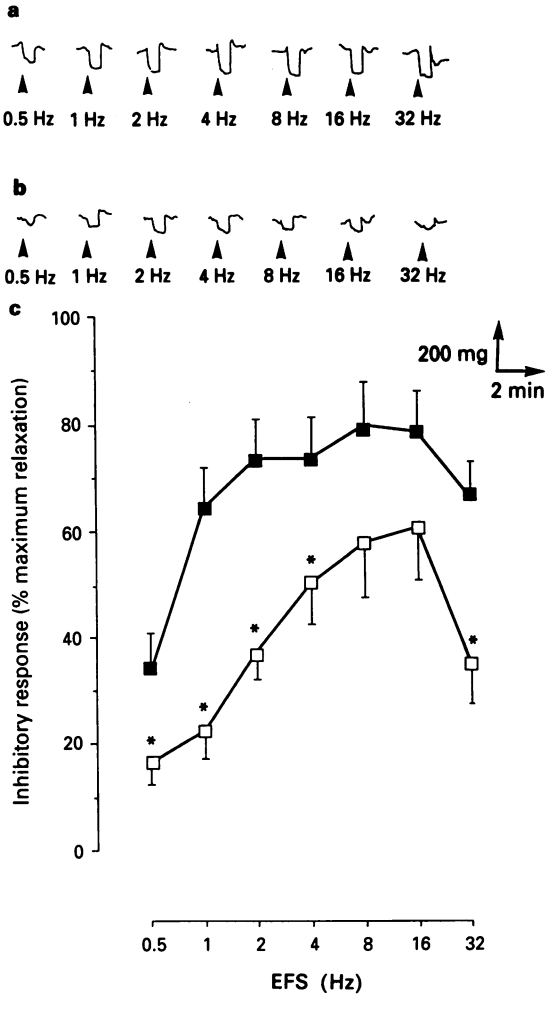

Figure 1 The effect of N^G-nitro-L-arginine methyl ester (L-NAME, 10 μM) on relaxations of rat pyloric sphincter to electrical field stimulation (EFS). (a) Before the application of L-NAME (10 μM) EFS caused relaxation of the sphincter. (b) A record taken 20 min after the application of L-NAME showing a reduction in relaxations to EFS. Arrowheads indicate the start of stimulation. (c) Frequency-response relationships representative of the effect of L-NAME on EFS. Responses in the absence (■, $n = 10$) and presence (□, $n = 10$) of L-NAME (10 μM) are shown. Data show mean response ± s.e. mean expressed as the % of the maximal relaxant response. *Indicates significance against the control group; $P \leq 0.05$.

tion. However EFS was found to produce a greater relaxant response than SNP in this preparation. Results are shown as mean ± standard error (s.e.) mean for the number of preparations indicated. For statistical analysis a paired Student's *t* test was used. *P* values of less than 0.05 were considered to be significant.

Immunohistochemistry

After dissection, the pyloric sphincter was fixed in 4% (w/v) paraformaldehyde in 0.1 M phosphate buffered saline (PBS) pH 7.3 for 4 h at 4°C. Tissues were then washed in 7% (w/v) sucrose in PBS containing 0.01% (w/v) sodium azide and stored at 4°C for at least 18 h in the same solution.

The tissues were mounted in embedding medium (Tissue-Tek OCT compound, Miles Inc., U.S.A.) and 10 μM longitudinal sections were cut on a cryostat (Reichart-Jung) at −25°C. The sections were thaw-mounted on gelatine coated slides and incubated in humid chambers at room temperature for 16 h with rabbit polyclonal antisera, diluted 1:3000, to NOS (Abbott, U.S.A.) or VIP (INC, U.K.). The preparations were washed three times in PBS and incubated with biotin-conjugated goat anti-rabbit immunoglobulin (Amersham, U.K.) at a dilution of 1:500 for 1 h, washed again with PBS, and then incubated with streptavidin-fluorescein isothiocyanate (FITC) conjugate (Amersham, U.K.) at a dilution of 1:250 for 1 h. Immunoreactivity was viewed with a Zeiss microscope and photographed with Kodak Tmax 3200 film.

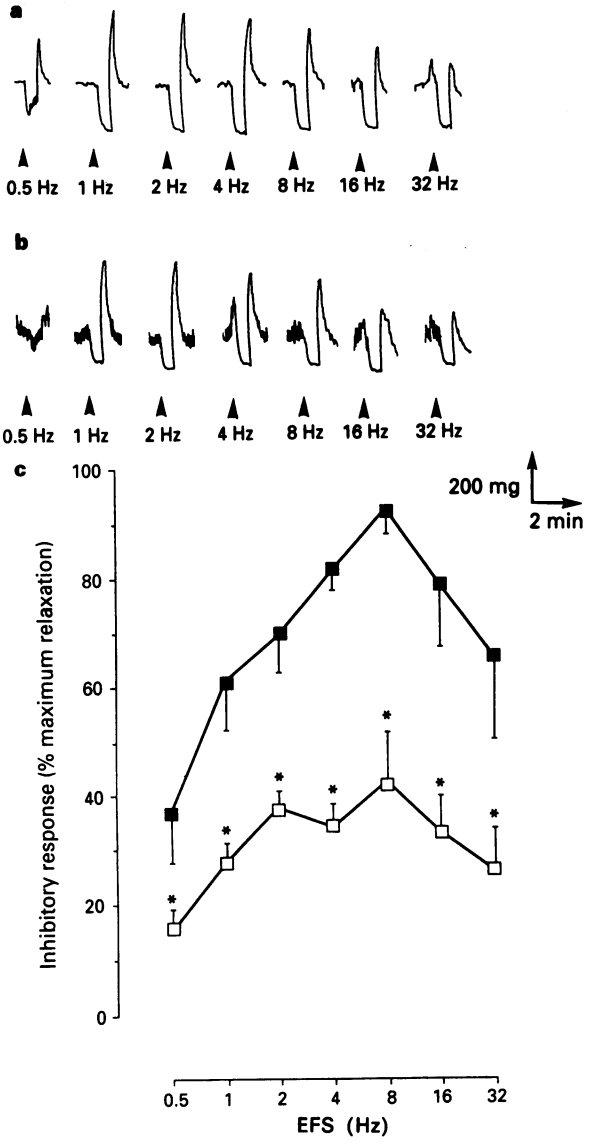

Figure 2 The effect of reactive blue 2 (20 μM) on relaxations of rat pyloric sphincter to EFS. (a) Before the application of reactive blue 2 (20 μM), EFS caused relaxation of the sphincter, followed by a rebound contraction. (b) 20 min after the application of reactive blue 2 there is a reduction in the relaxations to EFS. Arrowheads indicate the start of stimulation. (c) Frequency-response relationships showing the effect of EFS on rat pyloric sphincter. Responses in the absence (■, $n = 9$) and presence (□, $n = 9$) of reactive blue 2 (20 μM) are shown. Data show mean response ± s.e. mean expressed as the % of the maximal relaxation. *Indicate significance against the control group. $P \leq 0.05$.

Results

Effect of L-NAME, reactive blue 2 and tetrodotoxin on NANC relaxations induced by electrical field stimulation

EFS for 30 s (100 V, 0.25 ms, 0.5–32 Hz) induced relaxation in rat pyloric sphincter. These relaxations were fast in onset and were often followed by a rebound contraction. At high frequencies (≥ 16 Hz) the relaxation often extended beyond the period of stimulation. Tetrodotoxin (3 μM) abolished relaxations induced by EFS. L-NAME (10 μM; Figure 1) significantly reduced relaxations induced by EFS; the maximum reduction, 41.8% ± 6.0, occurred at 1 Hz. Relaxant responses were reduced by 22.0% ± 11.2 and 17.9% ± 14.9 at 8 and 16 Hz respectively, however these reductions were not significant. Reactive blue 2 (20 μM; Figure 2) significantly ($P \leq 0.05$) reduced relaxations induced by EFS at all frequencies, the relaxant response at 8 Hz was reduced by 50.1% ± 5.8. In the presence of both L-NAME and reactive blue 2 the relaxant response to EFS at 8 Hz was reduced by 75% ± 6.48 ($n = 6$).

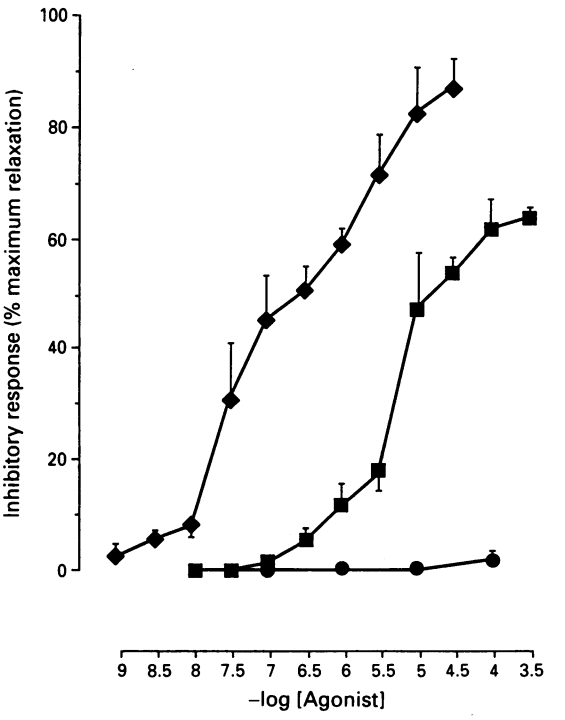

Figure 3 Concentration-response relationships showing the effect of ATP (■, $n = 8$), 2-MeSATP (◆, $n = 8$) and α,β-MeATP (●, $n = 8$) on isolated pyloric sphincter strips from rat. Data show mean response ± s.e mean expressed as the % of the maximal relaxation. *Indicates significance against the control group; $P \leq 0.05$. For abbreviations, see text.

Effect of L-arginine on the inhibitory effect of L-NAME

L-Arginine (1 mM) reversed the inhibitory effect of L-NAME by 54.2% ± 19.3 at 8 Hz, this reversal was not significant.

Effect of exogenous application of ATP, 2-MeSATP, α,β-MeATP and VIP

Exogenous application of ATP and 2-MeSATP relaxed the pyloric sphincter in a concentration-dependent fashion (Figure 3). 2-MeSATP was the most potent agonist but did not reach a maximum at the concentrations used (1 nM–30 μM); from these data an IC_{50} value of 5.0×10^{-8} M was calculated. Relaxations to ATP (IC_{50} 7.9×10^{-6} M) reached a maximum at 0.3 mM. α,β-MeATP was only slightly active at a concentration of 0.1 mM, showing a low affinity for the P_2-purinoceptors (P_{2y}-subtype) involved. Exogenous application of VIP (1 nM–0.3 μM) elicited no response in this preparation.

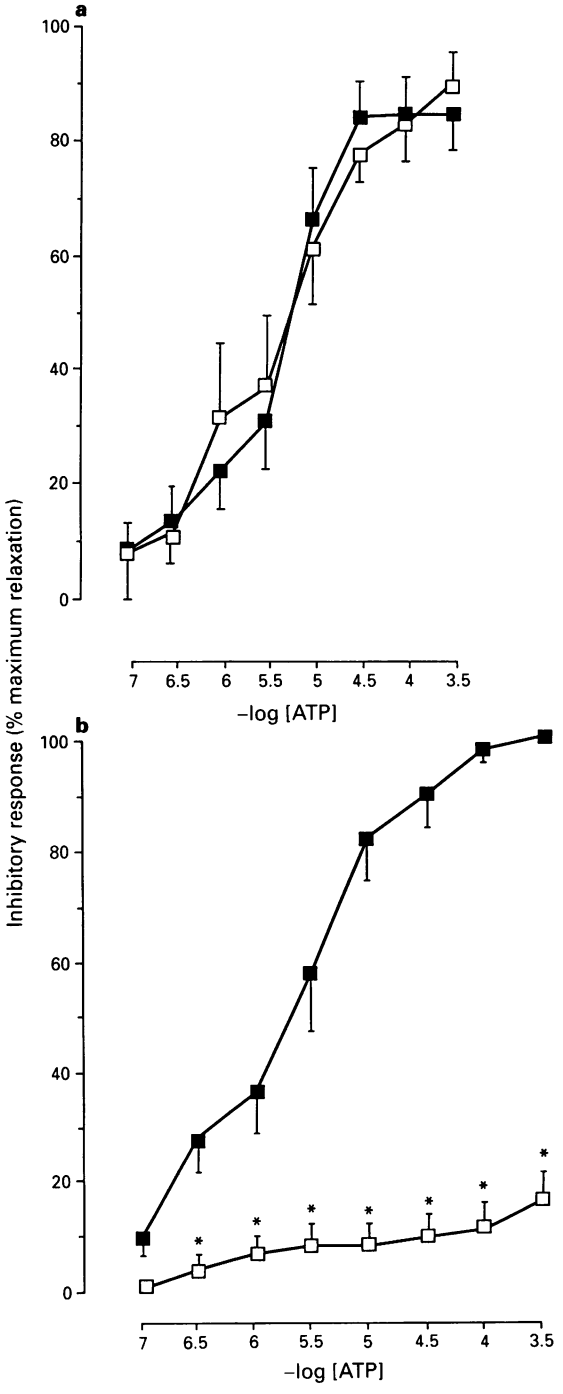

Figure 4 Concentration-response relationships showing the effect of ATP on isolated pyloric sphincter strips from rat. (a) The responses in the absence (■, $n = 10$) and presence (□, $n = 10$) of N^G-nitro-L-arginine methyl ester (L-NAME, 10 μM). (b) Responses in the absence (■, $n = 10$) and presence (□, $n = 10$) of reactive blue 2 (20 μM) are shown. Data show mean response ± s.e. mean expressed as the % of the maximal relaxation. *Indicates significance against the control group; $P \leqslant 0.05$.

Effect of L-NAME and reactive blue 2 on drug-induced relaxations

L-NAME did not significantly inhibit the relaxations evoked by exogenous application of ATP (Figure 4a), suggesting that ATP acts directly on smooth muscle and not indirectly through NO. Reactive blue 2 did significantly ($P \leqslant 0.05$) inhibit the relaxations evoked by exogenous application of ATP at concentrations above 0.3 μM. At the highest concentration (0.3 mM) the relaxant effect of ATP was inhibited by 84% ± 5 (Figure 4b). Reactive blue 2 had no inhibitory effect on the relaxations caused by exogenous application of noradrenaline (Figure 5), indicating its antagonistic actions were selective to ATP at this concentration.

Immunohistochemistry

The thickened circular muscle region of the pyloric region was found to have numerous NOS-like immunoreactive nerve fibres (Figure 6a). Immunoreactive fibres and nerve cell bodies were also seen in the myenteric plexus of the sphincter. The region also contained an abundant supply of VIP-like immunoreactive fibres within the circular muscle of the sphincter (Figure 6b). The myenteric plexus contained many VIP-like immunoreactive nerve fibres but no immunoreactive nerve cell bodies were seen.

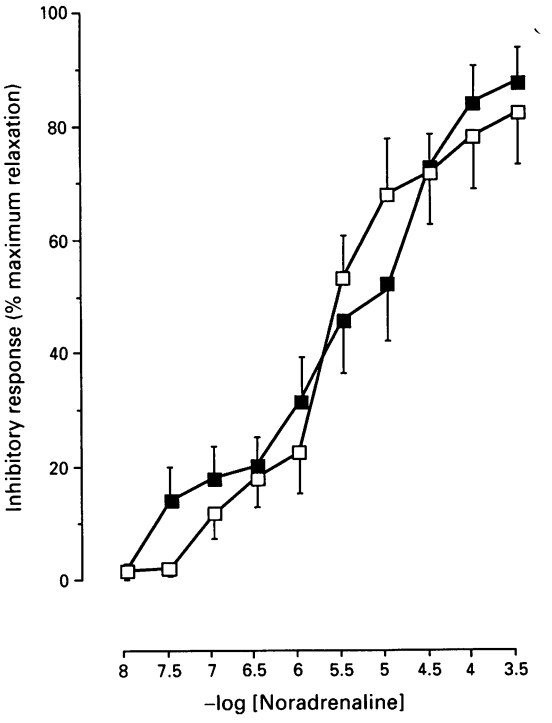

Figure 5 Concentration-response relationships showing the effect of noradrenaline on isolated pyloric sphincter strips from rat. Responses in the absence (■, $n = 10$) and presence (□, $n = 10$) of reactive blue 2 (20 μM) are shown. Data show mean response ± s.e. mean expressed as the % of the maximal relaxation. No significant difference between the points was found.

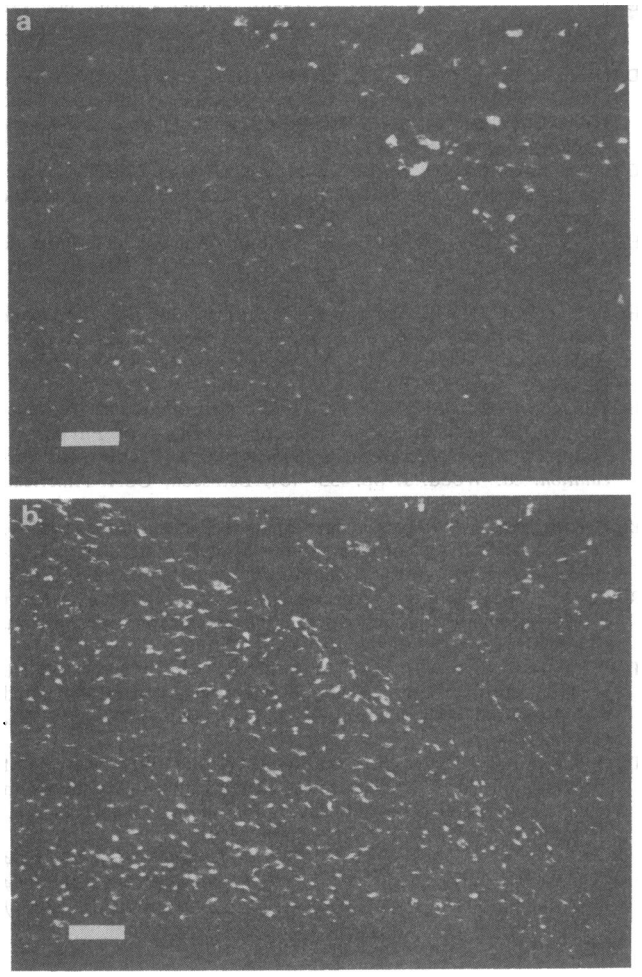

Figure 6 (a) Immunofluorescence micrograph of nitric oxide synthase (NOS)-like immunoreactivity in the pyloric sphincter region of rat. The circular muscle region of the sphincter shows fibres labelled with NOS-like immunofluorescence (calibration bar = 450 μm). (b) Immunofluorescence micrograph of vasoactive intestinal polypeptide (VIP)-like immunoreactivity in the pyloric sphincter region of rat. A dense distribution of VIP-like immunofluorescent fibres is seen within the circular muscle of the sphincter. (Calibration bar = 450 μm).

Discussion

Until recently, the most likely candidate for the inhibitory NANC response seen in the pyloric sphincter was considered to be VIP (Allescher *et al.*, 1989). However, the failure of VIP to produce consistent results *in vitro* (Daniel *et al.*, 1991) suggested that it was either not the final NANC transmitter or that some other NANC transmitter was present.

NO is synthesized from L-arginine by a cytosolic enzyme which appears to be related to NADPH-diaphorase in neuronal and endothelial cells. Recent studies in rat anococcygeus muscle (Li & Rand, 1989), opossum lower oesophageal sphincter (Tottrup *et al.*, 1991) and canine pyloric sphincter (Allescher *et al.*, 1992) suggested that NO or some related substance is involved in NANC responses *in vitro* and *in vivo*. This study shows that NO is a NANC neurotransmitter in rat pyloric sphincter, as has been previously demonstrated in canine pyloric sphincter *in vivo* and *in vitro* (Allescher *et al.*, 1992), on the basis of the reductions in relaxations to EFS induced by L-NAME and the reversal of this effect by L-arginine. However, this study showed that blockade of NO synthesis did not completely abolish relaxations of the sphincter to EFS.

ATP has been suggested as a NANC inhibitory transmitter in a wide variety of tissues (see Hoyle, 1992). Rat pyloric sphincter can be included in this list based on the reduction of relaxations to EFS in the presence of reactive blue 2 and the response to exogenous application of ATP and its analogues. The relative potency of ATP and its alkylthio derivative (2-MeSATP) is consistent with the classification for a purinoceptor of the P_{2y} subtype. The putative P_{2y} antagonist, reactive blue 2 (Burnstock & Warland, 1987), reduced relaxations of the sphincter to EFS and to exogenous application of ATP. Reactive blue 2 did not effect the relaxation caused by exogenous application of noradrenaline, implying that the action of reactive blue 2 is selective at the concentration used in this study and that there is a P_{2y}-purinoceptor mediated component to NANC relaxation in rat pyloric sphincter.

Studies of the canine ileocolonic junction have shown that NO is the final effector of NANC inhibitory neurotransmission in this tissue, mediating the action of EFS and exogenously applied ATP (Boeckxstaens *et al.*, 1991). The present study showed that relaxations evoked by ATP and EFS were not blocked fully by L-NAME, indicating that the action of ATP is not mediated by NO.

NOS-like immunoreactivity has been shown in several gut tissues in rat, including the ileum and colon (Belai *et al.*, 1992). The results of this study show that the pyloric sphincter of the rat also displays NOS-like immunoreactivity. The pyloric sphincter was also found to have a dense distribution of VIP-like immunoreactive nerve fibres as previously observed by Alumets *et al.* (1979), the absence of VIP-like immunoreactive nerve cell bodies may be due to a lack of colchicine pretreatment. Since exogenous application of VIP did not elicit a response the role of VIP in this preparation is unclear, but may involve neuromodulation of excitatory transmission as the basis for its reported inhibitory actions. Alternatively, it was found by Allescher *et al.* (1989) that injection of VIP into the canine gastroepiploic artery inhibited spontaneous and acetylcholine-induced pyloric contractions, but that *in vitro* muscle strips did not respond to exogenous application of VIP. It has also been shown that VIP is involved in slow inhibitory junction potentials in guinea-pig ileum but that this effect is masked by an excitatory potential mediated by a concomitant release of substance P (He & Goyal, 1993). Further investigation of rat pyloric sphincter may reveal an action of VIP *in vivo* or that inhibitory actions of VIP are masked by contraction mediated by release of substance P.

This study provides evidence that both ATP, acting directly on P_{2y}-purinoceptors, and NO contribute to the NANC inhibitory response of rat pyloric sphincter. Even though VIP-like immunoreactivity was present in the sphincteric region, exogenously applied VIP did not elicit a response in this preparation.

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