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Nitric oxide, an enteric nonadrenergic-noncholinergic relaxant transmitter: evidence using phosphodiesterase V and nitric oxide synthase inhibition

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1 The effects of N^G-nitro-L-arginine (L-NOARG), a nitric oxide synthase inhibitor, and SK&F 96231, a phosphodiesterase type V inhibitor, on electrical field stimulated (EFS) nonadrenergic noncholinergic (NANC) relaxations of rat fundal strips, guinea-pig isolated ileum longitudinal muscle with intact myenteric plexus, and guinea-pig taenia caeci were investigated.

2 Reproducible repeated control random EFS frequency-response curves were obtained for all three tissues.

3 Depending on the frequency of stimulation, L-NOARG ($10^{-4}-5\times10^{-3}$ M) caused either a complete or partial inhibition of the NANC-induced relaxations of the rat fundal strips and the guinea-pig isolated ileum longitudinal muscle with intact myenteric plexus, but not of the guinea-pig taenia caeci. The inhibitory action of L-NOARG was partially or totally reversed, depending on the tissue, by L-arginine (5×10^{-3} M).

4 SK&F 96231 ($10^{-6}-10^{-4}$ M) caused a concentration- and frequency-dependent potentiation of both the size and duration of the EFS-induced NANC relaxant response of rat fundal strips and guinea-pig isolated ileum longitudinal muscle with intact myenteric plexus, but not of the guinea-pig taenia caeci.

5 Zaprinast, another phosphodiesterase type V inhibitor ($10^{-6}-10^{-4}$ M) caused a concentration- and frequency-dependent potentiation of the NANC relaxant responses to EFS of rat fundal strips.

6 SK&F 96231 and zaprinast alone ($10^{-6}-10^{-4}$ M) caused a concentration-dependent relaxation of the agonist-induced tone of all three tissues with the maximum degree of relaxation found to be in the order stomach < ileum < caecum. This is the reverse order for ability of SK&F 96231 to potentiate relaxant responses to EFS.

7 These results suggest NO is involved in the NANC nerve-mediated relaxation of rat fundal strips and guinea-pig isolated ileum longitudinal muscle with intact myenteric plexus, but not the guinea-pig taenia caeci.

Keywords: Rat fundal strip; guinea-pig isolated ileum longitudinal muscle with intact myenteric plexus; guinea-pig taenia caeci; nonadrenergic noncholinergic (NANC) transmission; nitric oxide (NO); phosphodiesterase; guanosine 3':5'-cyclic monophosphate (cyclic GMP); SK&F 96231; zaprinast; N^G-nitro-L-arginine (L-NOARG)

Introduction

Nonadrenergic, noncholinergic (NANC) neurones play an important role in the inhibitory innervation of the gastrointestinal tract (Burnstock & Costa, 1973). The nature of the inhibitory transmitter of these NANC neurones has been the subject of much debate. In some tissues, vasoactive intestinal polypeptide (VIP) has been demonstrated to have inhibitory effects (Furness & Costa, 1982; D'Amato *et al.*, 1988; Li & Rand, 1990). It has also been shown that VIP is present in enteric nerves and is able to mimic NANC nerve stimulation when, for example, applied to the rat colon (Grider & Makhlouf, 1986) and guinea-pig taenia caeci (Grider *et al.*, 1985). Further support is supplied by reports that VIP antiserum is able to block, in part, neurogenic relaxations in a number of gastrointestinal tissues (Goyal *et al.*, 1980; Li & Rand, 1990; Grider & Rivier, 1990). In contrast, other studies have provided evidence that, in certain tissues, adenosine 5'-triphosphate (ATP) is the NANC inhibitory transmitter (Maguire & Satchell, 1981), for example in the guinea-pig taenia caeci (Satchell, 1981).

More recent work indicates a possible role for nitric oxide (NO) in NANC inhibitory transmission in the gastrointestinal tract (Boeckxstaens *et al.*, 1991a,b; Tøttrop *et al.*, 1991; Lefebvre *et al.*, 1992; Osthaus & Galligan, 1992). Unlike VIP, which elicits its actions in the gut by activating adenylyl cyclase, NO activates soluble guanylyl cyclase (Arnold *et al.*, 1977). Studies have shown that electrical field stimulation (EFS) increases guanosine 3':5'-cyclic monophosphate (cyclic GMP) levels in the lower oesophageal sphincter (Torphy *et al.*, 1986; Barnette *et al.*, 1989) and the internal anal sphincter (Josyln *et al.*, 1990; Grous *et al.*, 1991) and it has also been demonstrated that an increase in cyclic GMP results in the relaxation of a variety of smooth muscles (Bowman & Drummond, 1984; Torphy *et al.*, 1986). Evidence to support NO as an inhibitory transmitter is provided from the use of inhibitors of its biosynthesis e.g. N^G-monomethyl-L-arginine (L-NMMA) (Palmer *et al.*, 1988) and N^G-nitro-L-arginine (L-NOARG) (Mulsch & Busse, 1990). Boeckxstaens *et al.* (1990), using the canine ileocolonic junction, demonstrated that these compounds caused partial inhibition of NANC relaxations in response to electrical stimulation.

Cyclic nucleotides are inactivated by phosphodiesterases, of which there are at least seven different isoenzymes (Sonnenburg & Beavo, 1994). Some studies on NANC inhibitory innervation have employed an inhibitor of the cyclic GMP specific phosphodiesterase isoenzyme (phosphodiesterase V), zaprinast. A phosphodiesterase V inhibitor would be anticipated to potentiate the relaxant response to nerve stimulation if NO, acting on guanylyl cyclase, was a released transmitter. The results obtained with this inhibitor have been variable; Rajfer *et al.* (1992) found it potentiated electrically induced relaxations of the human corpus cavernosum at all frequencies while Gibson & Mirzazadeh (1989), using the mouse ano-

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coccygeus found potentiation of the responses to the lower frequencies only and Barbier & Lefebvre (1992) reported no potentiation of the NANC relaxations of the rat gastric fundus. These results may reflect the variable role that NO plays in the responses of different tissues.

In the present study the nature of the NANC inhibitory neurotransmitter, released by EFS, in three gastrointestinal smooth muscle preparations, namely stomach, small and large intestine, has been investigated. The use of two complementary approaches: (a) inhibition of NO biosynthesis by L-NOARG and (b) the use of SK&F 96231, a more specific phosphodiesterase V inhibitor than zaprinast (Murray, 1993), may allow a more definitive demonstration of the involvement of NO.

Methods

Rat fundal strips

Charles River Wistar rats of either sex weighing 250–350 g were fasted, with free access to water, for 24 h. They were killed by a blow to the head followed by exsanguination. The stomach was exposed via a midline incision and removed. Longitudinal muscle strips 2–3 mm wide and 20 mm long were prepared from the fundus by cutting parallel to the greater curvature. The muscle strips were mounted in 25 ml organ baths in modified Krebs-Ringer solution (composition, mM: NaCl 118.3, KCl 4.7, $MgSO_4$1.2, /KH_2PO_4 1.2, $CaCl_2$ 2.5, $NaHCO_3$ 25 and glucose 11.1) containing 10^{-6} M guanethidine and 10^{-6} M indomethacin. The baths were maintained at 37°C and aerated with 95% O_2:5% CO_2.

The muscle strips were mounted with one end attached to a hook at the base of the electrode. The other end was attached to a force displacement transducer (Dynamometer UF1). A tension of 1 g was applied and the tissues allowed to equilibrate for at least 45 min with washes three times every 15 min.

After the equilibration period the tissues were contracted with carbachol and NANC-mediated relaxations were elicited by electrical field stimulation through parallel platinum ring electrodes connected to a Grass S11 stimulator. Changes in isometric tension were recorded on a Lectromed 5041 recorder via a Lectromed 3552 preamplifier.

Guinea-pig taenia caeci

Dunken Hartley guinea-pigs of either sex weighing 650–900 g were fasted, with free access to water, for 24 h before they were killed by a blow to the head followed by exsanguination. The caecum was exposed via a midline incision and guinea-pig taenia caeci preparations 2 cm long were dissected from the tissue. The guinea-pig taenia caeci were mounted as described for rat fundal strips but placed under 0.5 g tension.

Guinea-pig isolated ileum longitudinal muscle with intact myenteric plexus

Dunken Hartley guinea-pigs of either sex weighing 400–900 g were fasted, with free access to water, for 24 h before they were killed by a blow to the head followed by exsanguination. The ileum was exposed via a midline incision and a 20 cm section of distal ileum was tied at the stomach end and removed. Sections of longitudinal muscle with intact myenteric plexus, 3 cm long, were prepared in a dissecting tray as described by Patel & Spraggs (1992). The sections of guinea-pig isolated ileum longitudinal muscle with intact myenteric plexus were mounted as described for rat fundal strips but were placed under 0.5 g tension and 10^{-6} M atropine was also present in the Krebs-Ringer solution.

Experimental design

Rat fundal strips In order to obtain NANC relaxations it was necessary to raise tone above basal. This was achieved by addition of carbachol which produced a sustained stable contractile response. In preliminary experiments a concentration-response curve to carbachol was established and from this a concentration producing maximal contraction (10^{-5} M) was used for all subsequent experiments. After carbachol administration a plateau was established before beginning the experimental procedure.

The first set of experiments involved the construction of random frequency-response curves to electrical field stimulation (1–16 Hz, 40 V, 0.5 ms pulse duration for 10 s every 5 min). Two experimental designs were used: (a) two random frequency-response curves separated by a 10 min interval and (b) one random frequency-response curve followed by washout, recontraction with carbachol and then a second curve.

Another series of experiments investigated the effect of a 10 min incubation (time obtained from preliminary experiments) with L-NOARG (10^{-4} M–5×10^{-3} M) between two random frequency-response curves. The experiments using 10^{-4} M were repeated in the presence of L-arginine (5×10^{-3} M), the physiological precursor of NO, administered 5 min prior to L-NOARG.

Because they reduced the evoked tone, the effect of SK&F 96231 or zaprinast (10^{-6}–10^{-4} M) on random frequency-response curves was examined by establishing a control curve, washing out the carbachol, equilibrating for 20 min with SK&F 96231 or zaprinast (times obtained from preliminary experiments), recontracting the tissue and constructing the test curve.

Guinea-pig taenia caeci As with rat fundal strips, tone was raised with carbachol (10^{-5} M) and random frequency-response curves (0.5–10 Hz, supramaximal voltage, 0.5 ms duration for 5 s every 2 min) were constructed using both designs.

The effects of L-NOARG (10^{-4} M) and SK&F 96231 (10^{-5} M) were studied with the same protocols as for rat fundal strips.

Guinea-pig isolated ileum longitudinal muscle with intact myenteric plexus As with the other tissues the tone needed to be raised, but in this tissue histamine produced the most stable contraction and 10^{-4} M was maximal.

Random frequency-response curves (1–20 Hz, 50 V, 0.3 ms duration for 1 s every 5 min) were constructed as described for the other tissues.

The effects L-NOARG (10^{-4} M), SK&F 96231 and zaprinast (10^{-6}–10^{-4} M) were studied with the same experimental designs as described previously.

In all tissues the relaxant effect of cumulative addition of SK&F 96231 or zaprinast alone on agonist-induced tone was established. For the rat fundal strips and guinea-pig isolated ileum longitudinal muscle with intact myenteric plexus the effect of SK&F 96231 on the duration of the responses to electrical field stimulation was also determined.

Drugs

The following drugs were used: L-arginine, atropine sulphate, carbamylcholine chloride (carbachol), guanethidine sulphate, indomethacin, L-NOARG (Sigma, Poole, Dorset), histamine acid phosphate (Merck, Lutterworth, Leicester), SK&F 96231 (2- (2-propoxyphenyl) -6-purinone) and zaprinast (synthesized and kindly donated by SmithKline Beecham, Welwyn, Hertfordshire). All drugs were dissolved in distilled water with the exception of indomethacin, L-NOARG, SK&F 96231 and zaprinast. Indomethacin was dissolved in ethanol, L-NOARG was dissolved in 65 mM HCl and both SK&F 96231 and zaprinast were dissolved in 1 M NaOH. The volume added to the bath did not exceed 1% of the total volume and the concentrations reported are final bath concentrations.

Presentation and statistical analysis of results

Results are expressed as a percentage relaxation of the agonist-induced contraction for each individual response curve and are quoted as mean ± s.e.mean for the number of animals stated. The statistical analysis performed were either Student's one tailed *t* test for unpaired data or Student's two tailed *t* test for paired data. *P* values of less than 0.05 were considered statistically significant.

Results

Rat fundal strips

In the presence of carbachol (10^{-5} M), indomethacin (10^{-6} M) and guanethidine (10^{-6} M), electrical stimulation induced relaxations of rapid onset that were frequency-dependent. The responses were of a transient nature but demonstrated a more sustained duration at the 16 Hz frequency. Random frequency-response curves showed no significant difference at any frequency tested in either experimental design.

L-NOARG (10^{-4} M) caused a partial inhibition of the NANC relaxations (Figure 1 and Table 1). Increasing the concentration to 10^{-3} M did not increase the degree of inhibition obtained. However, at 5×10^{-3} M the response to 1 and 2 Hz was abolished, and that to 4 Hz almost abolished (Table 1). At the two highest frequencies responses to EFS were still obtained in the presence of 5×10^{-3} M L-NOARG but the pattern of relaxation was dramatically altered. At 8 Hz the majority, and at 16 Hz the whole, of the response occurred after stimulation had ceased. At the lower frequencies the inhibitory effect of L-NOARG was partially reversed by preincubation with L-arginine (5×10^{-3} M) ($n = 5$); for example at 1 Hz the percentage inhibition produced by L-NOARG (10^{-4} M) was reduced from 80% ± 18 to 58% ± 9.

Since both SK&F 96231 and zaprinast when applied to the contracted tissued caused a reduction in the evoked tone, the

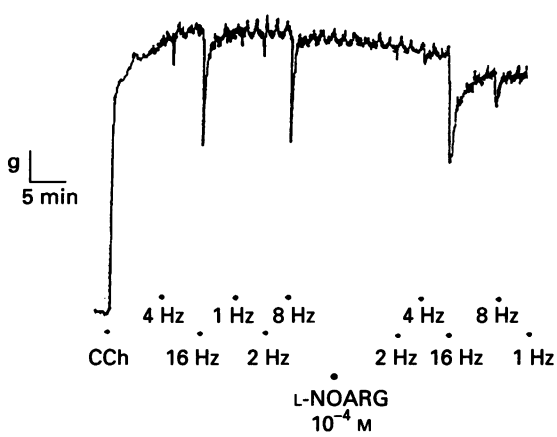

Figure 1 Original recording of rat fundal strip contracted with carbachol 10^{-5} M (CCh) in the presence of indomethacin (10^{-6} M) and guanethidine (10^{-6} M), showing the responses to electrical stimulation (40 V, 0.5 ms for 10 s every 5 min) in the absence and presence of 10^{-4} M N^G-nitro-L-arginine (L-NOARG).

Table 1 The effect of N^G-nitro-L-arginine (L-NOARG) on the electrical field stimulated relaxations of the rat fundal strip

	% inhibition of EFS		
Frequency (Hz)	*L-NOARG (10^{-4} M)*	*L-NOARG (10^{-3} M)*	*L-NOARG (5×10^{-3} M)*
1	80% ± 9	76% ± 13	100% ± 0
2	70% ± 9	72% ± 13	100% ± 9
4	61% ± 8	65% ± 3	97% ± 5
8	51% ± 6	62% ± 7	74% ± 5
16	0% ± 3	5% ± 3	12% ± 3

The experiments were performed during a carbachol-induced contraction (10^{-5}M) in the presence of guanethidine (10^{-6}M) and indomethacin (10^{-6}M). The 10^{-4}M L-NOARG experiments were carried out during the contraction ($n = 10$). The 10^{-3}M ($n = 4$) and 5×10^{-3}M ($n = 4$) experiments were carried out in the preincubation design because of the effect the L-NOARG had on the induced tone. Results are expressed as percentage inhibition compared with controls.

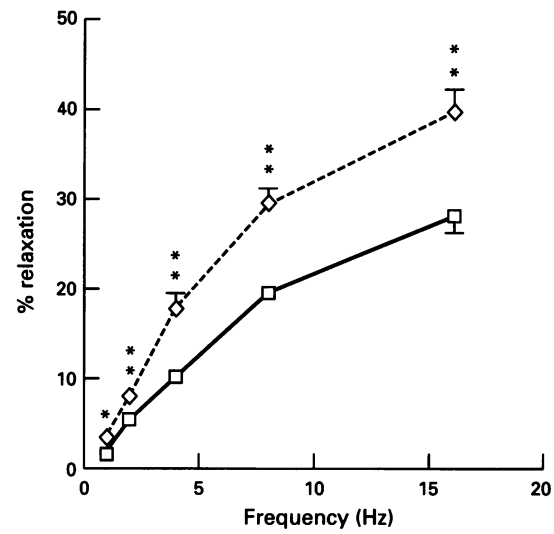

Figure 2 Relaxation responses of rat fundal strips to electrical stimulation (40 V, 0.5 ms for 10 s every 5 min). Tissues contracted with carbachol (10^{-5} M) in the presence of indomethacin (10^{-6} M) and guanethidine (10^{-6} M). Random frequency-response curves in the absence (□) and in the presence (◇) of 10^{-5} M SK&F 96231; tissues were washed and preincubated for 20 min between the two curves ($n = 5$). Values are mean ± s.e.mean. *$P < 0.01$, **$P < 0.001$ significantly different from first curve (Student's *t* test for paired observations).

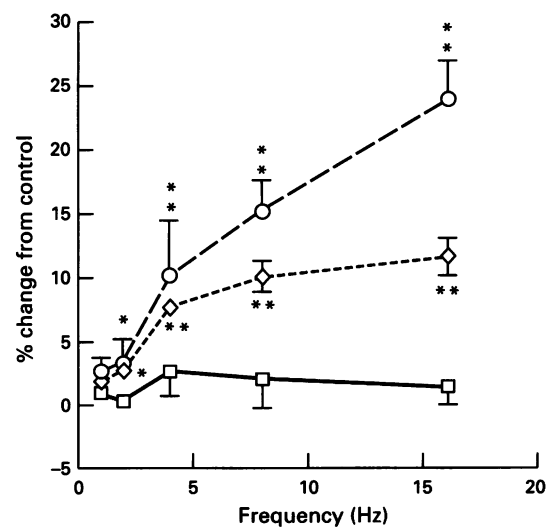

Figure 3 The percentage change from control responses in rat fundal strips contracted with carbachol (10^{-5} M) in the presence of indomethacin (10^{-6} M) and guanethidine (10^{-6} M). Random frequency-response curves in the presence of SK&F 96231 10^{-6} M (□) ($n = 7$), 10^{-5} M (◇) ($n = 19$) and 10^{-4} M (○) ($n = 5$). Values are mean with s.e.mean. *$P < 0.05$, **$P < 0.01$ significantly different from first curve (Student's *t* test for unpaired observations).

preincubation design was employed. SK&F 96231 (10^{-6}–10^{-4} M) produced a concentration- and frequency-dependent increase in the EFS-induced relaxations (Figures 2 and 3). At the lowest concentration, 10^{-6} M ($n=4$), there was a small but statistically insignificant increase at all frequencies tested. SK&F 96231 10^{-5} M and 10^{-4} M produced a concentration-dependent potentiation at all frequencies ($n=5$). The potentiation at 10^{-4} M may not be maximal, but concentrations greater than this could not be tested because the compound precipitated out of solution in the organ bath. Although SK&F 96231 produced a slight reduction in the plateau response achieved to carbachol, before constructing the second EFS relaxant-response curve, this was minimal compared to the degree of potentiation of the response. For example, there was a 12% reduction in tone with SK&F 96231 10^{-5} M but the minimum increase in the relaxant response was 40% at 16 Hz ($n=5$).

Zaprinast (10^{-6}–10^{-4} M) also produced a concentration-dependent potentiation of the EFS-induced relaxations (Figure 4). In contrast to 10^{-5} M SK&F 96231, zaprinast at the same concentration only produced significant potentiation at the higher frequencies (4, 8 and 16 Hz). Concentrations of zaprinast higher than 10^{-4} M were not investigated because they produced a marked reduction in the evoked tone even in the preincubation design.

The duration of NANC nerve-stimulated relaxant responses were increased after incubation with SK&F 96231. For example at 10^{-5} M SK&F 96231, the total duration of the response to 4 Hz was increased by 67 % from 0.55± 0.04 to 0.92±0.08 min ($n=4$).

Guinea-pig taenia caeci

Electrical field stimulation, in the presence of carbachol (10^{-5} M) produced frequency-dependent, rapid onset and transient relaxations which were followed by rebound contractions. Random frequency-response curves performed showed no significant differences at any of the frequencies investigated in either design.

Incubation with 10^{-4} M L-NOARG ($n=6$) between random frequency-response curves produced no significant differences between the control and test curves.

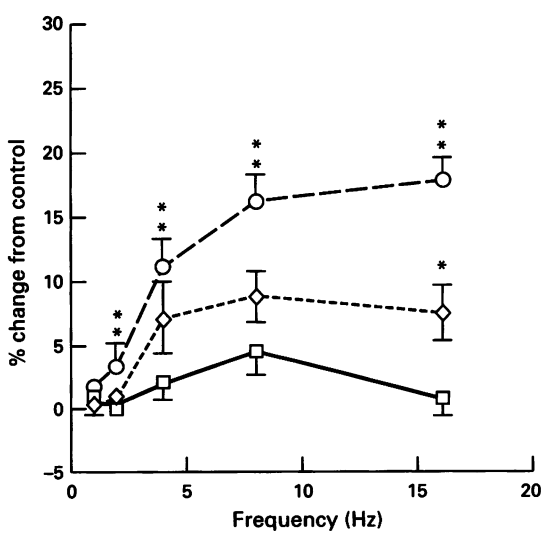

Figure 4 The percentage change from control responses in rat fundal strips contracted with carbachol (10^{-5}M) in the presence of indomethacin (10^{-6}M) and guanethidine (10^{-6}M). Random frequency-response curves in the presence of zaprinast 10^{-6}M (□) ($n=7$), 10^{-5}M (◇) ($n=8$) and 10^{-4}M (○) ($n=7$). Values are mean with s.e.mean. *$P<0.05$, **$P<0.01$ significantly different from first curve (Student's t test for unpaired observations).

Preincubation with SK&F 96231 (10^{-5} M) between frequency-response curves ($n=5$) had no significant potentiating effect on the relaxant response at any of the frequencies but did produce a small but significant reduction at the 1 Hz frequency (control; 13.2% ±3.8, test; 7.2%±1.8, $P<0.05$, Student's t test for paired observations).

Guinea-pig isolated ileum longitudinal muscle with intact myenteric plexus

In the presence of histamine (10^{-4} M), electrical field stimulation resulted in rapid onset, transient and frequency-dependent relaxations. Random frequency-response curves performed in either design showed no significant difference between them except a small, but significant, difference at the 20 Hz frequency when a wash period occurred between curves (first curve 44.8%±1.9, second curve 48.4%±2.5, $P<0.05$, Student's t test for paired observations, $n=4$).

Incubation with L-NOARG (10^{-4} M) produced a frequency-dependent inhibition of the relaxant responses except at the lowest frequency, 1 Hz ($n=4$). Increasing the concentration of L-NOARG to 10^{-3} M and 5×10^{-3} M (which did not significantly affect the tone in this tissue) did not produce a significant further reduction in the responses to EFS ($n=7$). In this tissue preincubation with L-arginine (5×10^{-3} M) caused a complete reversal of the L-NOARG (10^{-4} M) inhibitions at 3 and 5 Hz and a significant reversal at 10 and 20 Hz ($n=3$) (Table 2).

Preincubation with SK&F 96231 (10^{-6} M) had no significant effect on the relaxant responses to EFS ($n=4$). Higher concentrations of SK&F 96231 (10^{-5}–10^{-4} M) produced a concentration-dependent potentiation of the responses at all frequencies except 20 Hz (Figure 5a).

Incubation with SK&F 96231 increased the total duration of the response to EFS particularly at the higher frequencies as illustrated in Figure 5b. For example incubation with 10^{-4} M SK&F 96231 increased the duration of response to 10 Hz by 130% from 0.54±0.01 to 1.24±0.11 min ($n=4$) and the response to 20 Hz increased by 220% from 0.64±0.06 to 2.04±0.20 min ($n=4$).

SK&F 96231 or zaprinast alone had an effect on the three smooth muscle preparations. Cumulative administration of either SK&F 96321 or zaprinast during agonist-induced tone in all the tissues resulted in a concentration-dependent and tissue-dependent relaxation as illustrated in Table 3. Both compounds had a significantly greater effect on the guinea-pig taenia caeci than isolated ileum longitudinal muscle. The effects on the rat fundal strip were significantly less than on the guinea-pig ileal preparation. In all three tissue preparations

Table 2 Inhibitory effect of N^G-nitro-L-arginine (L-NOARG, 10^{-4}M) on EFS induced relaxations of guinea-pig isolated ileum longitudinal muscle with intact myenteric plexus, in the absence and presence of L-arginine (5×10^{-3}M)

	%inhibition of EFS	
Frequency (Hz)	*L-NOARG*	*Preincubation with L-arginine L-NOARG*
1	0±2	0±1
3	14±5	2±1*
5	22±6	0±3**
10	41±5	21±5**
20	58±5	42±3*

Experiments were performed during a histamine-induced contraction (10^{-6}M) in the presence of guanethidine, indomethacin (10^{-6}M) and atropine (10^{-6}M), ($n=4$ for both protocols). *$P<0.05$, **$P<0.01$ significant difference when pretreated with L-arginine. Results are expressed as % inhibition of control.

zaprinast caused larger relaxant responses than SK&F 96231 which were statistically significant at all concentrations tested except on the rat fundal strip at 10^{-4} M.

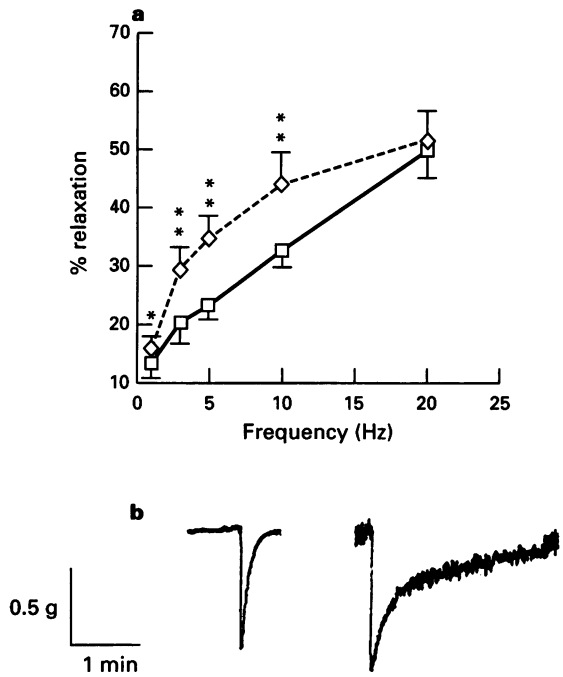

Figure 5 (a) Percentage relaxations of longitudinal muscle with intact myenteric plexus contracted with histamine (10^{-4}M) in the presence of atropine (10^{-6}M), showing responses to electrical stimulation (50V, 0.3ms for 1s every 5min). Random frequency-response curves in the absence (□) and in the presence (◇) of 10^{-4}M SK&F 96231, with washing between ($n=4$). Values are mean with s.e.mean. *$P<0.05$, **$P<0.01$ significantly different from first curve (Student's t test for paired observations). (b) Original trace of longitudinal muscle with intact myenteric plexus contracted with histamine (10^{-4}M), the effect of a 20min incubation with 10^{-4}M SK&F 96231 on the duration time of responses to electrical field stimulation of 10Hz at 50V, 0.3ms for 1s every 5min.

Discussion

The use of nitric oxide synthase inhibitors to identify nitrergic transmission has been employed by a number of investigators but the use of phosphodiesterase V inhibitors has been very limited. The present study has employed these two complementary approaches to investigate the role of NO in NANC relaxations of three gastrointestinal tissues.

The results obtained demonstrate that the nitric oxide synthase inhibitor, L-NOARG, is able to reduce the NANC relaxant responses of the rat fundal strip. The inhibition was frequency-dependent and the responses at the lower frequencies were completely abolished suggesting that NO was the sole transmitter. These findings are consistent with those of other workers for example Boeckxstaens *et al.* (1992), investigating the rat gastric fundus, demonstrated inhibition with L-NOARG of 85% at 2 Hz and 50% at 4 Hz and clearly indicated a role for NO in the relaxant response of this tissue. However, when the rat fundal strips were stimulated at 16 Hz, 10^{-4} M L-NOARG did not significantly reduce the relaxant response. This could be because the amount of NO released at this frequency is large enough to overcome the competitive inhibition produced by L-NOARG or the predominant transmitter released at this frequency may not be NO, as suggested by Li & Rand (1989), or that nitric oxide synthase is not completely inhibited by these concentrations. Increasing the concentration of L-NOARG to 5×10^{-3} M did cause a significant reduction in the responses to 16 Hz, but the responses at the higher frequencies were still not completely abolished, suggesting that NO was not the sole transmitter released, although a non specific effect at this high concentration of L-NOARG cannot be precluded. The fact that the highest concentration of L-NOARG dramatically altered the pattern such that the relaxation occurred after cessation of the stimulus supports the concept that another transmitter was involved. Several studies have suggested that VIP is the inhibitory transmitter of gastric relaxation in the guinea-pig (Grider *et al.*, 1985; Grider & Rivier, 1990) and cat (D'Amato *et al.*, 1988). More recent evidence suggests there is co-transmission of NO and VIP, such that at the lower frequencies NANC relaxations are mediated mainly by NO and at the higher frequencies both NO and VIP are involved (Li & Rand, 1990; Boeckxstaens *et al.*, 1992; D'Amato *et al.*, 1992; Barbier & Lefebvre, 1993) and the present results are compatible with this evidence. L-Arginine, the biological precursor of NO, has been reported to cause reduction of L-NOARG inhibition of the

Table 3 Effect of cumulative addition of SK&F 96231 or zaprinast on agonist-induced tone

Concentration (M)	% relaxation of tone: Fundal strips	Longitudinal muscle	Taenia
SK&F 96231			
10^{-6}	0	0	1.27±0.46●●
5×10^{-6}	0.83±0.53	3.36±1.07*	7.33±1.29●
10^{-5}	3.0±0.68	7.65±1.52**	14.54±1.89●
5×10^{-5}	8.25±1.24	19.77±3.48**	33.0±3.16●●
10^{-4}	20.11±3.0	28.65±4.93	52.62±4.10●●
Zaprinast			
10^{-6}	0.73±0.36	3.51±1.03*	4.30±1.14
5×10^{-6}	6.47±0.88	16.4±3.29*	21.98±4.75
10^{-5}	7.05±1.39	27.26±3.53**	50.89±5.36●●
5×10^{-5}	14.59±1.81	46.02±5.40**	79.99±4.27●●
10^{-4}	19.03±1.92	52.74±6.29**	83.62±3.63●●

Rat fundal strip ($n=4$) and guinea-pig taenia caeci ($n=4$) experiments were performed during a carbachol (10^{-5}M)-induced contraction in the presence of guanethidine (10^{-6}M) and indomethacin (10^{-6}M). The guinea-pig isolated ileum longitudinal muscle with intact myenteric plexus experiments ($n=4$) were performed during a histamine (10^{-4}M)-induced contraction in the presence of atropine (10^{-6}M), guanethidine (10^{-6}M) and indomethacin (10^{-6}M). The statistical analysis performed was 1 tailed Student's t test for unpaired data. *$P<0.05$ and **$P<0.01$ significant difference between fundal strip and isolated ileum longitudinal muscle with intact myenteric plexus. ●$P<0.05$ and ●●$P<0.01$ significant difference between isolated ileum longitudinal muscle with intact myenteric plexus and taenia caeci. Results expressed as mean percentage relaxations ± s.e.mean.

relaxant response to electrical field stimulation in a variety of gastrointestinal tissues; rat gastric fundus (Boeckxstaens *et al.*, 1991a), guinea-pig gastric fundus (Lefebvre *et al.*, 1992), canine ileocolonic injection (Boeckxstaens *et al.*, 1990) and opossum lower oesophageal sphincter (Murray, J. *et al.*, 1991; Tøttrup *et al.*, 1991). In the present studies the inhibitory effect of L-NOARG was partially reversed by L-arginine indicating that L-NOARG is acting, at least in part, by NO synthase inhibition. This also provides further evidence to support a role for NO in the relaxations of this tissue.

It is known that NO activates guanylyl cyclase and increases cyclic GMP levels (Arnold *et al.*, 1977) and there is evidence that increased cyclic GMP levels are associated with relaxation of tissues induced by stimulation of enteric nerves in a number of gastrointestinal tissues e.g. human lower oesophageal sphincter (Barnette *et al.*, 1991), canine anal sphincter (Grous *et al.*, 1990) and opossum lower oesophageal sphincter (Torphy *et al.*, 1986; Barnette *et al.*, 1989). Cyclic nucleotides are metabolized by phosphodiesterase isoenzymes and cyclic GMP breakdown is primarily through phosphodiesterase V. Inhibitors of phosphodiesterase V would be expected to increase the availabilty of cyclic GMP and so potentiate the size and/or duration of any relaxant responses evoked by electrical field stimulation which involved a transmitter, e.g. NO, that used this transduction pathway. In the present study the relaxations to electrical stimulation of the rat fundal strip were increased in both size and duration after treatment with SK&F 96231, a relatively selective phosphodiesterase V inhibitor (Murray, 1993). Another phosphodiesterase V inhibitor, zaprinast, also demonstrated a potentiation of NANC relaxant responses. The degree of potentiation achieved with both these compounds was very similar which would be expected from their similar inhibitory IC_{50} values on the phosphodiesterase V isoenzyme (Murray, K.J. *et al.*, 1991). However, zaprinast had a significantly greater effect on the induced tone either during a contraction or compared to controls. This difference may reflect the lower selectivity of zaprinast for the phosphodiesterase V isoenzyme since it has significant inhibitory action on the phosphodiesterase I isoenzyme (Murray, K.J. *et al.*, 1991). The magnitude of the potentiation caused by phosphodiesterase V inhibition on the rat fundal strips in absolute terms is not large, with a maximum of 75% at 10^{-5} M SK&F 96231, but it is consistent with other reports in the literature. For example Gibson & Mirzazadeh (1989) found that zaprinast caused a 50% increase in the NANC nerve-induced relaxations of the anococcygeus muscle. Measurement of tissue levels of cyclic GMP after phosphodiesterase inhibitor administration have also only shown a maximum increase of approximately 110% when tested on the guinea-pig taenia coli *in vitro* (Hills *et al.*, 1988). Other published studies using zaprinast have produced conflicting results with some demonstrating potentiation for example in the opossum lower oesophageal sphincter (Rattan & Moummi, 1989) and the mouse anococcygeus (Gibson & Mirzazadeh, 1989) while others have demonstrated an increase in cyclic GMP levels but no potentiation of the relaxant response for example in the canine colon (Barnette *et al.*, 1993) and the opossum lower oesophageal sphincter (Barnette *et al.*, 1989). The latter authors suggested this could possibly be due to there being two forms of phosphodiesterase able to metabolize cyclic GMP with different sensitivities to zaprinast. As mentioned previously, there is evidence that zaprinast also has an inhibitory effect on phosphodiesterase I as well as phosphodiesterase V (Murray, K.J. *et al.*, 1991; Murray, 1993) and this lack of selectivity could underlie some of these conflicting data. SK&F 96231 exhibits a greater degree of selectivity and therefore may be a more appropriate drug.

EFS-induced relaxations of the guinea-pig isolated ileum longitudinal muscle with intact myenteric plexus were found to be inhibited by L-NOARG but unlike the rat fundal strip the degree of inhibition increased with increasing frequency. There have been few studies on the identity of the inhibitory transmitter or transmitters in this tissue, but inhibition of NANC relaxations by NO synthase inhibitors has also been demonstrated by Osthaus & Galligan (1992). Also an investigation into the identity of the NANC inhibitory neurotransmitter of the rat ileum suggested that it was unlikely to be VIP or ATP since desensitization to these putative transmitters did not affect relaxations to transmural field stimulation (Yagasaki *et al.*, 1983). In the present studies the phosphodiesterase V inhibitor, SK&F 96231, increased the magnitude of the relaxant response to electrical field stimulation except at the highest frequency and increased the duration of the response at all frequencies. These data are consistent with nerve stimulation releasing NO which then acts via cyclic GMP to cause smooth musle relaxation, although it is unlikely that NO is the only relaxant transmitter. The increased duration of the NANC responses seen in both the stomach and small intestine in the presence of a phosphodiesterase V inhibitor has not been reported before but would be predicted if NO, which has a very short half life, was activating guanylyl cyclase to produce cyclic GMP which itself is rapidly metabolised.

In contrast the NANC relaxations of the guinea-pig taenia caeci were totally refractory to inhibition by L-NOARG. Similar results were reported by Rand & Li (1990), but there have been some reports of a small L-NOARG sensitive component in the relaxant response; for example Piotrowski *et al.* (1993) who demonstrated a maximum inhibition of 18%. Although the discrepancies between the studies remain unexplained the lack of potentiation with SK&F 96231 taken together with the inactivity of L-NOARG strongly argues against a role for NO and supports the concept that another transmitter, probably ATP, is involved (Satchell, 1981).

SK&F 96231 and zaprinast alone caused a concentration- and tissue-dependent relaxation of the agonist-induced tone in all three tissues. Both the sensitivity to zaprinast and SK&F 96231 and the maximum degree of relaxation, were found to be in the order stomach < small intestine < caecum. This is the reverse order for the ability of SK&F 96231 to potentiate NANC-induced relaxant responses. These tissue differences presumably reflect differences in basal guanylyl cyclase/cyclic GMP levels which could be established by measuring tissue content.

There have been few *in vivo* gastrointestinal studies relevant to the present investigation. A study by Gustafsson *et al.* (1993) in the anaesthetized cat showed that basal jejunal tone and phasic activity were increased by L-NOARG and the effect was partially reversed by L-arginine suggesting that there is a tonic inhibition of small intestinal motility by endogenous neuronal NO. Scott *et al.* (1994) found that phosphodiesterase V inhibitors decreased ileal and colonic motility with no effect on the lower oesophageal sphincter in the anaesthetized dog. Elevation of cyclic GMP levels can therefore have a relaxant effect on the small and large bowel.

Whether or not manipulation of nitric oxide synthase or phosphodiesterase V enzymes will have any therapeutic applications in gastrointestinal motility disorders remains to be established. In patients suffering from achylasia, Wong *et al.* (1987) found that administration of nitroglycerin, a NO donor, improved early oesophageal emptying. There is also evidence that nitric oxide synthase is absent in the innervation of the affected musculature in infantile hypertrophic pyloric stenosis (Vanderwinden *et al.*, 1993) and Hirschsprung's disease (Vanderwinden *et al.*, 1993). Clearly the phosphodiesterase V inhibitors relax gastrointestinal smooth muscle in their own right as well as potentiating NANC nerve stimulations. The fact that there are tissue differences in the effects of these compounds indicates that some selectivity for different regions of the gut can be achieved and therefore they may be of value in motility disorders such as irritable bowel syndrome.

In conclusion, this study has demonstrated the value of employing two complementary approaches in investigating the role of NO as a NANC inhibitory transmitter in the gut. The results presented suggest that NO is involved in the NANC

nerve-mediated relaxations of the gastric fundus and guinea-pig isolated ileum longitudinal muscle with intact myenteric plexus but not the guinea-pig taenia caeci. The data do not exclude the possiblity that there is co-release of other relaxant transmitters.

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