British Journal of Pharmacology (1997) 122, 179–185

Evidence for a role for nitric oxide in relaxation of the frog oesophageal body to electrical field stimulation

[1]S.J. Williams & M.E. Parsons

Biosciences Division, University of Hertfordshire, College Lane, Hatfield, Hertfordshire, AL10 9AB

1 Electrical field stimulation (EFS) (1–10 Hz, 30 V, 2 ms) of frog oesophageal body strips resulted in frequency-dependent non-adrenergic, non-cholinergic (NANC) relaxations.

2 Tetrodotoxin (TTX) (10^{-6}–10^{-5} M) had no effect on EFS evoked relaxations with a 2 ms pulse width. At a pulse width of 0.5 ms only the responses to the highest frequency (10 Hz) were significantly inhibited by TTX at 10^{-5} M. Relaxations at the 2 ms pulse width were unaffected by ω-conotoxin (10^{-6} M), nifedipine (10^{-6} M) or cobalt (5×10^{-4} M).

3 N^G-nitro-L-arginine (L-NOARG) (10^{-6}–10^{-4} M), a nitric oxide synthase (NOS) inhibitor, caused a concentration-dependent inhibition of the EFS-induced NANC relaxant responses. The inhibitory effect of L-NOARG was both prevented and reversed by L-arginine but not D-arginine (5×10^{-3} M).

4 The phosphodiesterase type V inhibitor (PDE V), SK&F 96231 (10^{-7}–10^{-4} M), caused a concentration-dependent potentiation of both the percentage relaxation and the duration of the relaxant responses to EFS.

5 ODQ (10^{-7}–10^{-5} M), a guanylate cyclase inhibitor, produced a concentration-dependent inhibition of EFS-evoked NANC relaxations.

6 Oxyhaemoglobin (10^{-6} M), which binds nitric oxide (NO), inhibited NANC relaxations to EFS.

7 The NO donor sodium nitroprusside (SNP) (10^{-8}–10^{-4} M) produced a concentration-dependent inhibition of evoked tone. L-NOARG (10^{-4} M) had no effect on the SNP evoked relaxations. Preincubation with oxyhaemoglobin (10^{-6} M) caused a reduction in the SNP (10^{-6}–10^{-5} M) induced relaxations.

8 These results suggest NO is the relaxant transmitter of the frog oesophageal body and the source of NO may be non-neuronal.

Keywords: frog oesophagus; nitric oxide (NO); non-adrenergic, non-cholinergic (NANC); guanylate cyclase (GC); phosphodiesterase; guanosine 3′:5′-cyclic monophosphate (cyclic GMP); SK&F 96231; N^G-nitro-L-arginine (L-NOARG); tetrodotoxin (TTX)

Introduction

Non-adrenergic, non-cholinergic (NANC) neurones play an important role in the inhibitory innervation of the gastrointestinal tract (Burnstock & Costa, 1973). However, the nature of the inhibitory transmitter of these NANC nerves has been the subject of considerable debate. A number of studies have supported a role for vasoactive intestinal polypeptide (VIP) (Grider & Makhlouf, 1986; D'Amato *et al*., 1988; Grider & Rivier, 1990) while others have provided evidence that adenosine 5′ triphosphate (ATP) is the inhibitory transmitter in certain gastrointestinal tissues (Burnstock *et al*., 1978; Satchell, 1981). More recently there has been increasing evidence that nitric oxide (NO) is an important NANC inhibitory transmitter in the gastrointestinal tract (Boeckxstaens *et al*., 1990; Osthaus & Galligan, 1992; Williams & Parsons, 1995) including the oesophagus (Tøttrup *et al*., 1991; Knudsen *et al*., 1993).

NO has been shown to have a role in several oesophageal functions including relaxation of the lower oesophageal sphincter (LOS) (Knudsen *et al*., 1992), controlling the latency of the 'off' contraction (Murray *et al*., 1991; Yamato *et al*., 1992) and the timing of swallow induced oesophageal peristalsis (Yamato *et al*., 1992). Further support for the role of NO is provided by the presence of nitric oxide synthase (NOS) activity in the tissue (Ny *et al*., 1995; Singaram *et al*., 1995), the release of NO on NANC nerve stimulation (Murray *et al*., 1994) and an increase in the second messenger used by NO, guanosine 3′-5′-cyclic monophosphate (cyclic GMP) during stimulation (Torphy *et al*., 1986). There are also a number of oesophageal motor disorders in which disturbances in the NO inhibitory transmitter system have been implicated, for example diffuse oesophageal spasm (Konturek *et al*., 1995), achalasia (Wong *et al*., 1987) and congenital oesophageal stenosis (Singaram *et al*., 1995).

The majority of investigations into oesophageal motility have used the opossum as an animal model because of the similarity in the oesophageal muscle arrangement to man. The human oesophageal body proximally consists of striated muscle which is gradually replaced by smooth muscle so that the lower third to half is entirely smooth muscle (Goyal & Paterson, 1989). The striated muscle segment is under central control whereas the smooth muscle is under both central and intrinsic control. Electrical stimulation of the oesophagus when the vagal nerves have been severed leads to contractions in the striated muscle and peristalsis in the smooth muscle portion (Mukhopadhyay & Weisbrodt, 1975) demonstrating the importance of the smooth muscle and its intrinsic innervation in the co-ordination of oesophageal peristalsis.

In this study the possible role of NO in the oesophageal motility of the frog was investigated. The frog oesophagus, as in birds, reptiles and other amphibia, consists entirely of smooth muscle (Inglefinger, 1958) which may make it a good model for the human lower oesophagus because of the importance of the smooth muscle and its intrinsic innervation in the co-ordination of oesophageal peristalsis.

Methods

Frogs of either sex, weighing 15–25 g, were stunned, decapitated and had their spinal cord destroyed (pithed). The oesophagus was removed via a midline incision and opened

[1] Author for correspondence.

lengthwise. Strips of muscle measuring 10–15 mm in length and 2–3 mm wide were cut in the circumferential axis of the oesophagus. The strips were suspended in 15 ml organ baths between paired platinum ring electrodes. One end was attached to the bottom of the bath and the other to a force displacement transducer (Dynamometer UF1). The transducer was connected to a recorder (Lectromed 5041) via a preamplifier (Lectromed 3552) to record changes in isometric tension.

The organ baths were filled with Tyrode solution (composition mM: NaCl 137, KCl 2.7, $CaCl_2$ 1.8, $MgCl_2$ 0.88, NaH_2PO_4 0.36, $NaHCO_3$ 12.0 and glucose 5.5) containing indomethacin and guanethidine, both at 10^{-6} M. The tissues were placed under a tension of 1 g at room temperature, aerated with 95% O_2 and 5% CO_2 and allowed to equilibrate for at least 1 h with frequent washing. After the equilibration period the tissues were contracted with 10^{-5} M carbachol, a concentration which produced a maximal contraction. Once a plateau was established the preparations were stimulated via the platinum ring electrodes by a Grass S11 stimulator. This electrical field stimulation (EFS) ensured that all the neuronal elements within the tissue were stimulated.

Experimental design

The first set of experiments involved the construction of random frequency-response curves to electrical field stimulation (1–10 Hz, 30 V, 2 ms pulse width for 10 s every 5 min). Two experimental designs were used: (a) two random frequency-response curves separated by a 30 min interval and (b) one random frequency-response curve followed by washout and recontraction with carbachol 30 min later and then a second curve constructed.

Responses to a range of stimulus parameters (1–10 Hz, 30 V, 0.5–2 ms pulse width, 10 s every 5 min) were studied in the absence and presence of 10^{-6} M or 10^{-5} M tetrodotoxin (TTX) by use of a 10 min incubation between control and test random frequency-response curves. The same protocol was used to investigate the influence of ω-conotoxin (10^{-6} M), an N type calcium channel blocker, and cobalt (5×10^{-4} M) an inorganic calcium channel blocker. The L type calcium channel blocker, nifedipine (10^{-6} M), was studied in the same design but in tissues contracted with U46619 (10^{-7} M), because it relaxed carbachol-evoked tone.

Another series of experiments investigated the effect of the nitric oxide synthase inhibitor L-NOARG. Tissues were incubated with N^G-nitro-L-arginine (L-NOARG) (10^{-6}–10^{-4} M) for 25 min (the time was obtained from preliminary experiments) between two random frequency-response curves. These experiments were repeated with D-NOARG (10^{-4} M). Random frequency-response curves were constructed in the absence and presence of L-NOARG and L-arginine in two sets of experiments. The first series of experiments consisted of a control curve followed by incubation with L-arginine (5×10^{-3} M) for 15 min then a second curve followed by incubation with L-NOARG (10^{-4} M) (in the presence of L-arginine) for 25 min before a third curve. In the second series a control curve was established followed by incubation with L-NOARG. A second curve was established in the presence of L-NOARG followed by incubation with L-arginine (in the presence of L-NOARG) before a third curve. These experiments were repeated with D-arginine.

Because SK&F 96231 alone produced a reduction in carbachol-evoked tone it was examined by establishing a control curve to EFS, washing out the carbachol, equilibrating with SK&F 96231 (10^{-7}–10^{-4} M) for 25 min (the time was obtained from preliminary experiments), recontracting the tissue and constructing the test curve. In this SK&F 96231 design no significant reduction in carbachol evoked tone was observed.

The effects of the guanylate cyclase inhibitor, ODQ (30 min, 10^{-7}–10^{-5} M) and oxyhaemoglobin (10 min, 10^{-6} M), which binds NO, were studied with the same protocol as for L-NOARG.

Concentration-response curves to sodium nitroprusside (SNP, 10^{-8}–10^{-4} M) were constructed by contracting the tissues and applying a single concentration of SNP then washing out when the tissue had returned to the control contracted level. This was repeated for the other concentrations. The effect of L-NOARG (10^{-4} M) on SNP concentration-response curves was studied by incubating for 25 min before the SNP applications. The effect of oxyhaemoglobin (10^{-6} M) on relaxations evoked by SNP (10^{-6} or 10^{-5} M) was examined by use of a 10 min incubation before the addition of SNP to the organ bath.

Drugs

The following drugs were used: D-arginine, L-arginine, atropine sulphate, carbamylcholine chloride (carbachol), guanethidine sulphate, haemoglobin, indomethacin, nifedipine, D-NOARG, L-NOARG, SNP (sodium nitroprusside), TTX (tetrodotoxin) (Sigma, Poole, Dorset), cobalt (BDH, Lutterworth, Leicester), ODQ ([1,2,4] oxadiazolo[4,3-a] quinoxalin-1-one) (Tocris Cookson, Langford, Bristol), SK&F 96231 (2-(2-propoxyphenyl)-6-purinone) (synthesized and kindly donated by SmithKline and Beecham, Welwyn, Hertfordshire), U46619 (5Z,9a,11a,13E,15(S))-15-hydroxy-9(11)-methanoepoxyprosta-5,13-dien-1-oic acid) (Cascade Biochem Ltd, Reading, Berkshire).

PBS (phosphate buffered saline); 0.7 g $NaH_2PO_4H_2O$, 4.36 g $Na_2HPO_4H_2O$, 17.04 g NaCl, distilled water to 2 l and pH to 7.4.

All drugs were dissolved in distilled water with the exception of indomethacin, nifedipine, D-NOARG, L-NOARG, SK&F 96231, ODQ and U46619. Indomethacin (10^{-2} M) and U46619 (10^{-4} M) stock solutions were made up in ethanol, nifedipine (10^{-2} M) stock solution was made up in acetone, D-NOARG (10^{-2} M) and L-NOARG (10^{-2} M) were dissolved in 65 mM HCl, ODQ stock solution (10^{-2} M) was made up in DMSO (dimethyl sulphoxide), and SK&F 96231 (10^{-2} M) was dissolved in 1 M NaOH.

The oxyhaemoglobin stock solution was prepared according to Martin *et al.* (1986). The basic method was to make a stock solution of lyophilized haemoglobin, which is predominantly methaemoglobin, and then dialyse it at 4°C against PBS. PBS was used because of the low pH of the available distilled water. Spectrophotometry was used to ascertain whether the haemoglobin was now oxyhaemoglobin and to determine the concentration of the stock solution.

The volume added to the bath did not exceed 1% of the total volume and the concentrations presented are final bath concentrations. All appropriate time and solvent controls were performed (all $n=5$) and no significant changes were found.

Presentation and statistical analysis of results

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

Results

Carbachol (10^{-5} M), in the presence of indomethacin and guanethidine (both at 10^{-6} M), produced a maximal and stable contraction of frog oesophageal body strips (0.80 g ± 0.05, $n=15$). EFS of these contracted tissues resulted in frequency-dependent NANC relaxations of the circular muscle. These relaxations were generally transient in nature and were often followed by small rebound contractions. Although the onset of relaxation and the time to peak effect was always rapid there was some variability in the duration of the response. The reproducibility of two random frequency-response curves, in

Table 1 The effect of tetrodotoxin on electrical field stimulation-induced relaxations

	Pulse width			
Frequency	*0.5 ms*		*2 ms*	
(Hz)	*Control relaxation* (%)	*Test relaxation* (%)	*Control relaxation* (%)	*Test relaxation* (%)
1	30.37±3.82	27.20±4.06	32.22±3.71	31.35±4.07
2	38.90±4.57	34.64±4.66	39.68±4.02	41.09±4.56
4	42.35±4.89	39.68±5.82	45.98±5.07	45.81±4.49
8	48.85±5.28	43.04±6.49	52.16±3.99	53.09±4.48
10	55.07±5.44	46.83±6.10*	57.72±4.44	55.56±4.33

Random frequency-response curves, 30 V for 10 s every 5 min at 0.5 ms ($n=7$) or 2 ms ($n=7$) pulse width, in the absence and presence of 10^{-5} M TTX (10 min). Values are mean with s.e.mean percentage relaxations of carbachol (10^{-5} M) evoked tone. *$P<0.05$ significantly different from controls (Student's t test for paired observations).

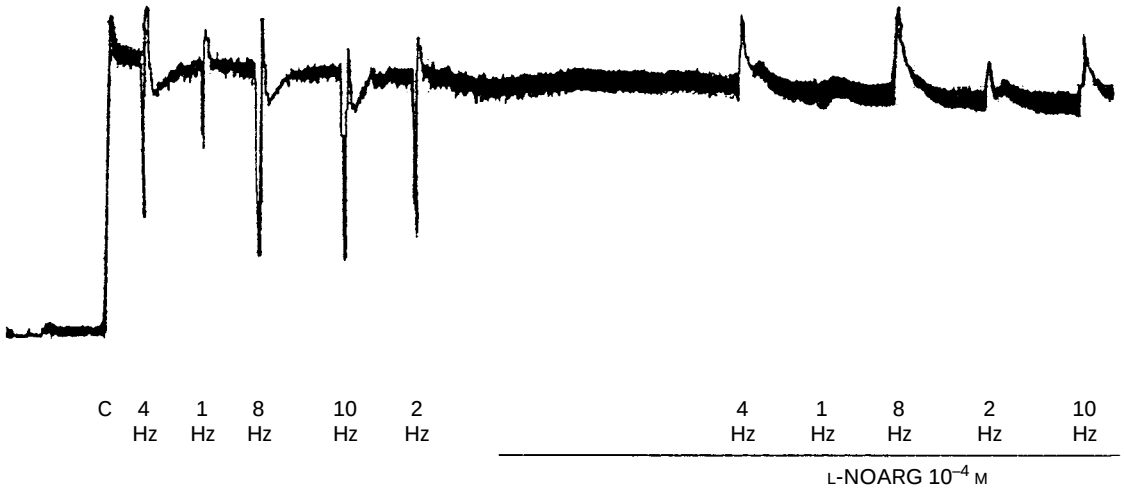

Figure 1 Original recording of frog oesophageal body circular strips contracted with 10^{-5} M carbachol (C) in the presence of indomethacin and guanethidine (both at 10^{-6} M) showing responses to electrical field stimulation (30 V, 2 ms, 1–10 Hz for 10 s every 5 min) in the absence and presence of L-NOARG (10^{-4} M, 25 min).

either of the experimental designs investigated (both $n=15$), demonstrated that they were suitable for examining the effect of compounds on these NANC relaxant responses.

At a concentration of 10^{-6} M, TTX did not effect the EFS-evoked relaxations at any frequency or pulse width studied ($n=6$). The higher concentration of TTX (10^{-5} M) had no effect on the NANC relaxant response at 2 ms pulse width ($n=7$) but at 0.5 ms the responses to the highest frequency (10 Hz) were slightly reduced but not abolished ($n=7$) (Table 1). Relaxations to EFS at the 2 ms pulse width were also unaffected by ω conotoxin (10^{-6} M, $n=5$), nifedipine (10^{-6} M, $n=5$) and cobalt (5×10^{-4} M, $n=5$) (data not shown).

L-NOARG caused a small, transient increase in carbachol-evoked tone for 5–10 min (0.56 g±0.03 to 0.64 g±0.03, $P<0.001$) in approximately half of the experiments. The effect of L-NOARG (10^{-4} M, 25 min) on random frequency-response curves was to abolish completely the EFS-evoked relaxations at all frequencies studied ($n=7$) and the relaxations were replaced by frequency-related contractions (Figure 1). When the experiments were repeated with other concentrations of L-NOARG (10^{-6} and 10^{-5} M), it was found that the inhibition was concentration-related with 10^{-6} M being the lowest concentration to cause a significant inhibition (Figure 2), for example at 8 Hz there was a 16.6%±1.44 inhibition. When these experiments were repeated with D-NOARG (10^{-4} M, 25 min) there was no significant difference between control and test random frequency-response curves ($n=6$) (data not shown).

L-Arginine alone had no effect on EFS-induced relaxations but it did cause a transient increase in carbachol-evoked tone of 0.15 g±0.02 for 5–10 min. Incubation with L-arginine

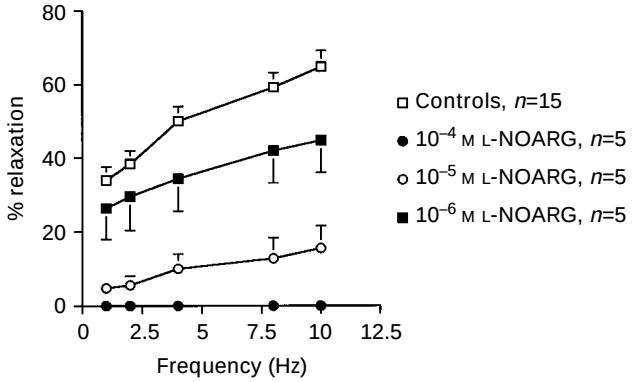

Figure 2 Random frequency-response curves (30 V, 2 ms, 1–10 Hz for 10 s every 5 min) for tissues contracted with carbachol (10^{-5} M) in the presence of indomethacin and guanethidine (both at 10^{-6} M). Curves constructed in the absence and presence of L-NOARG, 25 min. Values are mean with vertical lines showing s.e.mean.

(5×10^{-3} M, 15 min) was able both to prevent ($n=6$) and reverse ($n=6$) completely the inhibitory action of 10^{-4} M L-NOARG on random frequency-response curves. In contrast, D-arginine (5×10^{-3} M, 15 min) neither prevented ($n=5$) nor reversed ($n=5$), the L-NOARG inhibition of NANC relaxations (data not shown) but it did cause a transient increase in the evoked tone of 0.16 g±0.03 for 5–10 min.

The PDE V inhibitor, SK&F 96231, caused a decrease in carbachol-induced tone and therefore it was studied with a

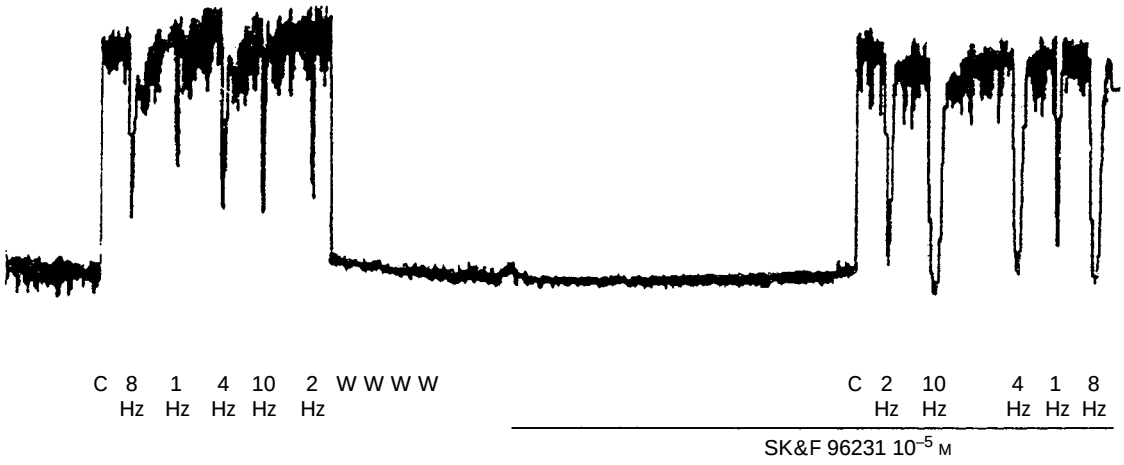

Figure 3 Original recording of frog oesophageal body circular strips contracted with 10^{-5} M carbachol (C) in the presence of indomethacin and guanethidine (both at 10^{-6} M) showing responses to electrical field stimulation (30 V, 2 ms, 1–10 Hz for 10 s every 5 min) in the absence and presence of SK&F 96231 (10^{-5} M, 25 min).

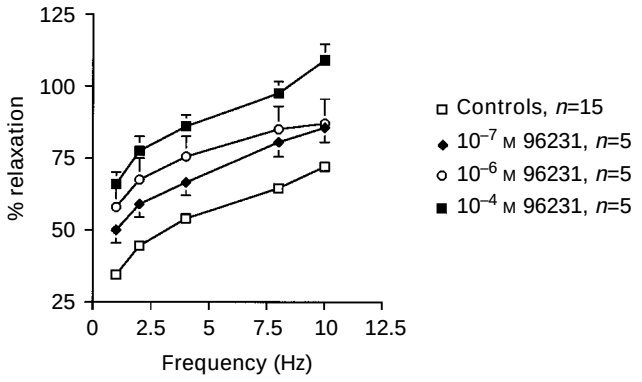

Figure 4 Random frequency-response curves (30 V, 2 ms, 1–10 Hz for 10 s every 5 min) in the absence and presence of SK&F 96231. Tissues were washed and preincubated for 25 min between the two curves. Values are mean with vertical lines showing s.e.mean.

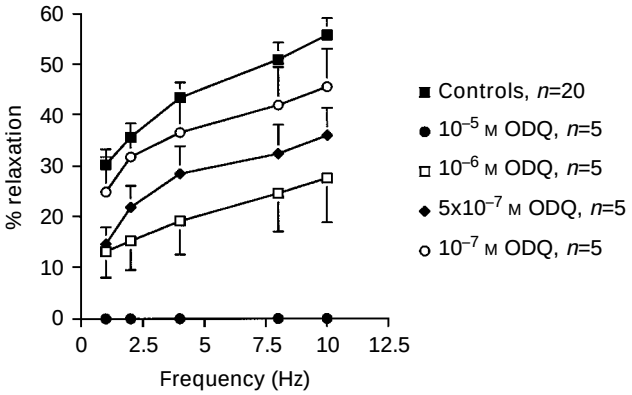

Figure 5 Random frequency-response curves (30 V, 2 ms, 1–10 Hz for 10 s every 5 min) in the absence and presence of ODQ (30 min). Values are mean with vertical lines showing s.e.mean.

wash and incubation period between random frequency-response curves. SK&F 96231 (10^{-5} M, 25 min) ($n=6$) caused an increase in both the percentage relaxation and the duration of the relaxant responses at all frequencies (Figure 3). For example the percentage relaxation to 10 Hz was increased by $40\pm8.34\%$ and the duration of the response was increased from 0.7 min $\pm$0.04 to 1.81 min $\pm$0.09 ($n=6$). Further experiments demonstrated that the effect of SK&F 96231 was concentration-dependent with 10^{-7} M being the lowest concentration to have a significant potentiating action (Figure 4). With the highest concentration of SK&F 96231 (10^{-4} M) used the relaxant response to stimulation at 10 Hz was over 100%, i.e. some relaxation of the basal tone was seen.

Investigations were carried out with the relatively selective inhibitor of NO sensitive guanylate cyclase (GC), ODQ. ODQ (30 min) had no effect on the carbachol-induced contraction and produced a concentration-related inhibition of EFS-evoked relaxations at all the frequencies tested with 10^{-7} M being threshold and 10^{-5} M causing complete abolition (Figure 5). When the EFS-evoked relaxant responses were abolished by ODQ they were replaced by frequency-dependent contractions.

The effect of haemoglobin (10^{-6} M, 10 min), which binds NO, on random frequency-response curves was a significant reduction at all the frequencies studied ($n=6$) (Figure 6), for example a reduction of $78.25\pm8.38\%$ at 10 Hz. On some occasions haemoglobin addition resulted in abolition of EFS-evoked relaxant responses at all frequencies and in these instances the relaxations were replaced by contractions.

The tissues responded to SNP, the NO donor, with concentration-dependent relaxations. Preincubation with L-NOARG (10^{-4} M) had no inhibitory effect on the SNP-induced relaxations ($n=5$). Experiments to investigate the effect of haemoglobin (10^{-6} M) on SNP-induced relaxations found that there was a significant reduction in the percentage relaxation but there was no effect on the duration of the relaxant response ($n=6$). Haemoglobin produced a mean reduction in the response to 10^{-6} M SNP of $52.8\pm5.1\%$ ($n=6$) and to 10^{-5} M SNP of $32.4\pm5.5\%$ ($n=6$). For comparison, the response to EFS at 2 Hz, which produced a similar percentage relaxation to that evoked by 10^{-6} M SNP, was inhibited by $85.13\pm6.31\%$.

Discussion

Strips of frog oesophageal body circular muscle, contracted with carbachol, responded to EFS with frequency-dependent NANC relaxations of a transient nature. Few studies have looked at the effect of EFS on contracted oesophageal body preparations, but when this has been investigated, for example, in the cat (Behar *et al.*, 1989) and human oesophagus (Tøttrup

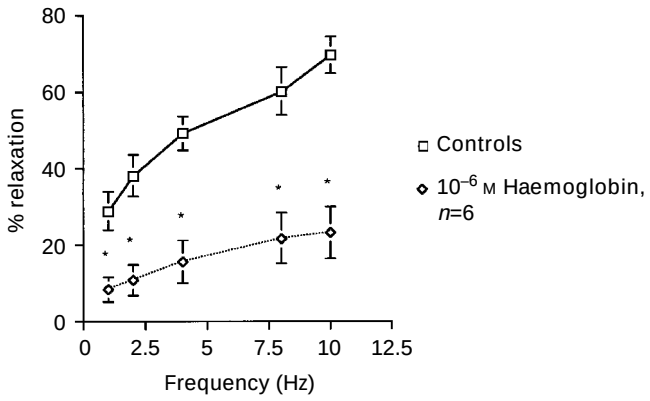

Figure 6 Random frequency-response curves (30 V, 2 ms, 1–10 Hz for 10 s every 5 min) in the absence and presence of haemoglobin (10^{-6} M, 10 min) ($n=4$). Values are mean with vertical lines showing s.e.mean. *$P<0.01$ significantly different from controls (Student's t test for paired observations).

et al., 1990), it was found that EFS resulted in relaxations. These findings are in agreement with those presented here for the frog oesophagus.

The failure of even a high concentration (10^{-5} M) of TTX to abolish these relaxant responses suggests in the main that the responses do not involve the opening of Na^+ channels. However, there is evidence that some Na^+ channels are resistant to TTX (Yoshida, 1994) but these can be blocked by inorganic calcium channel blockers such as cadmium and cobalt. However, in this study cobalt did not inhibit the EFS-evoked relaxations. These NANC relaxations of the frog oesophageal body may involve other ion channels. ω Conotoxin, an N type Ca^{2+} channel blocker and nifedipine, an L type Ca^{2+} channel blocker did not inhibit the relaxant responses which suggests Ca^{2+} channels are not involved.

Investigations by Daniel *et al*. (1979) in the opossum LOS found that EFS, in the presence of TTX, at pulse widths greater than 3 ms and up to 20 ms, resulted in relaxations. EFS at longer pulse widths can directly stimulate muscle and cause contraction, which was the anticipated result, but not the TTX-insensitive relaxations observed. Studies by these workers with black widow spider venom suggested these relaxant responses were not myogenic. Daniel *et al*. (1979) suggested that at the range of pulse widths which produced relaxations, a structure with a time constant longer than that required to stimulate axons was stimulated to release an inhibitory substance. These structures were later identified as interstitial cells (Daniel & Daniel-Posey, 1984). The pulse duration time employed for frog oesophageal body in the present study was 2 ms and, although this is not quite as long as that used by Daniel *et al*. (1979), it may be long enough to stimulate interstitial cells if they are present in the frog oesophagus.

The NOS inhibitor L-NOARG produced a small, transient increase in the induced tone in approximately half the tissues. An L-NOARG-induced increase in basal or induced tone has been observed in a number of other gastrointestinal smooth muscle tissues, for example in the rat gastric fundus (Boeckxstaens *et al*., 1991a; D'Amato *et al*., 1992a) and canine ileocolonic junction (Boeckxstaens *et al*., 1991b). This increase in tone in the presence of L-NOARG could be interpreted as uncovering a tonic inhibitory influence of endogenous NO or a direct action by L-NOARG on smooth muscle.

L-NOARG produced a concentration-dependent inhibition of EFS-evoked relaxations with a complete abolition at all frequencies with 10^{-4} M. In contrast, the inactive isomer D-NOARG had no effect on tone or relaxations to EFS. There have been other studies showing a complete abolition of NANC relaxant responses with L-NOARG, for example Tøttrup *et al*. (1991) with the opossum LOS and Huizinga *et al*. (1992) in the canine colon. This total inhibition of EFS-induced NANC relaxations could indicate that NO is the sole transmitter in the frog oesophagus and the other tissues. Most investigators have found only partial inhibition of NANC relaxant responses of smooth muscle of the gastrointestinal tract by L-NOARG, for example the canine ileocolonic junction (Boeckxstaens *et al*., 1990), rat gastric fundus and guinea-pig ileal longitudinal muscle (Williams & Parsons, 1995). These studies suggested that NO was not the sole transmitter mediating these NANC relaxant responses.

The inhibitory effect of L-NOARG was completely reversed or prevented by an excess of the substrate of NO biosynthesis, L-arginine, but not D-arginine. Some investigators have found only a partial antagonism by L-arginine of inhibition produced by L-arginine substituted analogue NOS inhibitors (Osthaus & Galligan, 1992; Williams & Parsons, 1995), whereas others have found complete antagonism (Gibson *et al*., 1990; Boeckxstaens *et al*., 1991b). In the present studies the complete reversal and prevention by L-arginine of the inhibitory effect of L-NOARG and the ineffectiveness of D-arginine supports a selective NOS inhibition in this study. Further support is provided by the use of SNP, an NO donor, which caused concentration-dependent relaxations which were unaffected by L-NOARG. This demonstrates that the ability of the smooth muscle to relax is not altered by L-NOARG. Taken together these findings suggest L-NOARG is acting specifically on the transmitter system, i.e. NO production from L-arginine by NOS, and not producing a non-specific postjunctional effect. In the current studies L-arginine caused a small transient increase in the agonist-evoked tone, but this would appear to be a non-specific effect because D-arginine also caused an increase in contracted tone. Haemoglobin, in the oxyhaemoglobin form, is frequently employed as a tool to assess the involvement of NO in NANC transmission. Haemoglobin inhibits NO activity because of the high affinity the haem group has for NO (Gibson & Roughton, 1957). In the studies described here, haemoglobin caused, for example, an 84% inhibition of EFS-induced NANC relaxations at 10 Hz, and on some occasions a complete abolition. These findings support a role for NO or an NO related compound being the transmitter involved in these relaxations. The results presented here compare well with those obtained by a number of other investigators, for example with the mouse anococcygeus Gibson *et al*. (1992) found an 87% inhibition of NANC relaxations and Knudsen *et al*. (1992) around a 70% reduction with the opossum LOS. Incomplete inhibition could reflect some lack of tissue penetration by haemoglobin because of its large molecular size or the involvement of another transmitter. The results obtained with NOS inhibitors and haemoglobin strongly support NO being the NANC inhibitory transmitter in the frog oesophageal body.

NO activates guanylyl cyclase (GC) and increases cyclic GMP levels (Arnold *et al*., 1977) and there is evidence that an increase in cyclic GMP levels is associated with relaxation of gastrointestinal tissues when enteric nerves are stimulated. This has been demonstrated in, for example, human LOS (Barnette *et al*., 1991), canine internal anal sphincter (Grous *et al*., 1991) and opossum LOS (Torphy *et al*., 1986; Barnette *et al*., 1989). The metabolism of cyclic nucleotides is mediated by the phosphodiesterase (PDE) family of isoenzymes with cyclic GMP metabolism mainly due to the PDE V isoenzyme. If cyclic GMP breakdown was prevented by PDE V inhibition, increased availability of cyclic GMP would be expected to result in a potentiation of the size and/or duration of relaxant responses which involved a transmitter, e.g. NO that used this transduction pathway. In this study the relatively selective PDE V inhibitor SK&F 96231 (Murray, 1993) was investigated. SK&F 96231 alone caused a decrease in carbachol-induced tone, which may reflect an ongoing tonic turnover of cyclic GMP. A decrease in tone of vascular and non-vascular tissues caused by PDE V inhibitors has been observed by other investigators, for example Barbier & Lefebvre (1992), Martin *et al*. (1986), Gibson & Mirzazadeh (1989) and Williams & Parsons (1995). SK&F 96231 increased both the size and the duration of electrically-induced relaxations. The magnitude of the potentiation caused by SK&F 96231 was larger than that

observed in other studies. For example previous studies by Williams & Parsons (1995) found a 75% increase in NANC relaxant response of the rat fundal strip and Gibson & Mirzazadeh (1989) found that zaprinast, another PDE V inhibitor, produced a 58% increase in NANC nerve-induced relaxations of the mouse anococcygeus.

With the possible exception of atrial natriuretic peptide (ANP) and carbon monoxide, NO is the only transmitter in the gatrointestinal tract which uses cyclic GMP as its second messenger system. ANP can also induce smooth muscle relaxation which is associated with a rise in cyclic GMP (Fiscus *et al*., 1985) but ANP activates particulate GC (Waldman *et al*., 1984) and NO activates soluble GC (Waldman & Murad, 1987). Investigations here used the potent and relatively selective inhibitor of NO sensitive GC, ODQ (Garthwaite *et al*., 1995). This compound has been shown to have no effect on cyclic GMP-independent relaxations produced for example by isoprenaline. ODQ produced a concentration-dependent inhibition of electrically-induced NANC relaxations with a complete abolition at 10^{-5} M. The results obtained with SK&F 92631 and ODQ support the involvement of GC activation of cyclic GMP in the frog oesophageal body in NANC relaxations.

The data presented provide substantial evidence to support NO being the NANC transmitter of the frog oesophagus and this is in agreement with other studies in different species. However, the source of NO in this tissue has not been identified. The lack of effect of TTX and the calcium channel blockers could suggest that the NO is from a non-neuronal location. NO release from non-neuronal sources has been previously demonstrated. Grider *et al*. (1992) in isolated gastric smooth muscle cells found VIP caused relaxation and stimulated NO production in the target cells. They suggested VIP stimulates NO production in the muscle cells and that this NO can both relax the smooth muscle cells directly and diffuse from them to enhance VIP release from neurones presynaptically. The results of the present study in the frog oesophagus do not support the involvement of VIP in the relaxant responses to EFS because they were completely abolished by L-NOARG but they do not exclude the possibility of synergistic co-release. In the Grider *et al*. (1992) study the VIP was neurally released so if this was occurring in the frog then TTX should have blocked the responses.

Another possible non-neuronal source of NO could be the ICC (interstitial cells of Cajal). ICC have been demonstrated in the circular muscle of the oesophagus in opossum (Daniel & Posey-Daniel, 1984), dog (Berezin *et al*., 1994) and man (Faussone-Pellegrinin & Cortesini, 1985). Daniel *et al*. (1979) and Daniel & Posey-Daniel (1984) suggested that they may be the structures responsible for the TTX-insensitive relaxation of the opossum oesophagus to EFS of longer pulse duration, NOS immunoreactivity has been found in ICC (Berezin *et al*., 1994) and isolated ICC have been demonstrated to release NO (Publicover *et al*., 1993). It is possible that in the frog oesophageal body, TTX-resistant relaxations may be due to stimulation of ICC and release of NO from them which results in smooth muscle relaxation.

The data provided by the present study strongly support NO as the NANC inhibitory transmitter of the frog oesophageal body, although the source of NO is not known and may be non-neuronal in origin. The data presented do not exclude the possibility of co-release of other transmitters.

References

ARNOLD, W.P., MITTAL, C., KATSUKI, S. & MURAD, F. (1977). Nitric oxide activates guanylate cyclase and increases guanosine 3′,5′-cyclic monophosphate levels in various tissue preparations. *Proc. Natl. Acad. Sci. U.S.A*., **76,** 3203 – 3207.

BARBIER, A.J. & LEFEBVRE, R.A. (1992). Effect of 3-isobutyl-1-methylxanthine and zaprinast on non-adrenergic, non-cholinergic relaxation in the rat gastric fundus. *Eur. J. Pharmacol*., **210,** 315 – 323.

BARNETTE, M.S., BARONE, F.C., FOWLER, P.J., GROUS, M., PRICE, W.J. & ORMSBEE, H.S. (1991). Human lower esophageal sphincter relaxation is associated with raised cyclic nucleotide content. *Gut*, **32,** 4 – 9.

BARNETTE, M.S., TORPHY, T.J., GROUS, M., FINE, C.F. & ORMSBEE, H.S. (1989). Cyclic GMP: A potential mediator of neurally- and drug induced relaxation of the opossum lower esophageal sphincter. *J. Pharmacol. Exp. Ther*., **249,** 524 – 528.

BEHAR, J., GUENARD, V., WALSH, J.H. & BIANCANI, P. (1989). VIP and acetylcholine: neurotransmitters in esophageal circular smooth muscle. *Am. J. Physiol*., **257,** G380 – G385.

BEREZIN, I., SNYDER, S.H., BREDT, D.S. & DANIEL, E.E. (1994). Ultrastructural localization of nitric oxide synthase in canine small intestine and colon. *Am. J. Physiol*., **266,** C981 – C989.

BOECKXSTAENS, G.E., PELKMANS, P.A., BOGERS, J.J., BULT, H., DEMAN, J.G., OOSTERBOSCH, L., HERMAN, A.G. & VAN MAERCKE, Y.M. (1991a). Release of nitric oxide upon stimulation of nonadrenergic, noncholinergic nerves in the rat gastric fundus. *J. Pharmacol. Exp. Ther*., **256,** 441 – 447.

BOECKXSTAENS, G.E., PELKMANS, P.A., BULT, H., DEMAN, L., HERMAN, A.G. & VAN MAERCKE, Y.M. (1990). Nonadrenergic, noncholinergic relaxation mediated by nitric oxide in the ileocolonic junction. *Eur. J. Pharmacol*., **190,** 239 – 246.

BOECKXSTAENS, G.E., PELKMANS, P.A., BULT, H., DEMAN, L., HERMAN, A.G. & VAN MAERCKE, Y.M. (1991b). Evidence for nitric oxide as mediator of non-adrenergic, non-cholinergic relaxations induced by ATP and GABA in the canine gut. *Br. J. Pharmacol*., **102,** 434 – 438.

BURNSTOCK, G. & COSTA, M. (1973). Inhibitory innervation of the gut. *Gastroenterology*, **64,** 141 – 144.

BURNSTOCK, G., COCKS, T., KASAKOV, L. & WONG, H. (1978). Direct evidence for ATP release from non-adrenergic, non-cholinergic ('purinergic') nerves in the guinea-pig taenia coli and bladder. *Eur. J. Pharmacol*., **31,** 360 – 362.

D'AMATO, M., CURRÒ, D., MONTUSCHI, P., CIABATTONI, G., RAGAZZONI, E. & LEFEBVRE, R.A. (1992a). Release of vasoactive intestinal polypeptide from the rat gastric fundus. *Br. J. Pharmacol*., **105,** 691 – 695.

D'AMATO, M., CURRÒ, D., MONTUSCHI, P., CIABATTONI, G., RAGAZZONI, E. & LEFEBVRE, R.A. (1992b). Evidence for dual components in the non-adrenergic, non-cholinergic relaxation in the rat gastric fundus: role of endogenous nitric oxide and vasoactive intestinal polypeptide. *J. Autonom. Nerv Syst*., **37,** 175 – 186.

D'AMATO, M., DEBEURME, F.A. & LEFEBVRE, R.A. (1988). Comparison of the effect of vasoactive intestinal polypeptide and inhibitory non-adrenergic, non-cholinergic neurone stimulation in the cat gastric fundus. *Eur. J. Pharmacol*., **152,** 71 – 82.

DANIEL, E.E., CRANKSHAW, J. & SARNA, S. (1979). Prostaglandins and tetrodotoxin-insensitive relaxation of opossum lower esophageal sphincter. *Am. J. Physiol*., **236,** E153 – 172.

DANIEL, E.E. & POSEY-DANIEL, V. (1984). Effects of scorpion venom on structure and function of esophageal lower sphincter (LES) and body circular muscle (BCM) from opossum. *Can. J. Physiol. Pharmacol*., **62,** 360 – 373.

FAUSSONE-PELLEGRINI, M.S. & CORTESINI, C. (1985). Ultrastructural features and localization of the interstitial cells of cajal in the smooth muscle coat of human esophagus. *J. Submicrosc. Cytol*., **17,** 187 – 197.

FISCUS, R.R., RAPOPORT, R.M., WALDMAN, S.A. & MURAD, F. (1985). Atriopeptin II elevates cyclic GMP, activates cyclic GMP-dependent protein kinase and causes relaxation in rat thoracic aorta. *Biochem. Biophys. Acta*, **846,** 179 – 184.

GARTHWAITE, J., SOUTHAM, E., BOULTON, C.L., NIELSON, E.B., SCHMIDT, K. & MAYER, B. (1995). Potent and selective inhibition of nitric oxide-sensitive guanylyl cyclase by 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one. *Mol. Pharmacol*., **48,** 184 – 188.

GIBSON, A., BABBEDGE, R., BRAVE, S.R., HART, S.L., HOBBS, A.J., TUCKER, J.F., WALLACE, P. & MOORE, P.K. (1992). An investigation of some S-nitrosothiols, and of hydroxy-arginine, on the mouse anococcygeus. *Br. J. Pharmacol.*, **107,** 715–721.

GIBSON, A. & MIRZAZADEH, S. (1989). N-methylhydroxylamine inhibits and M&B 22948 potentiates relaxations of the mouse anococcygeus to non-adrenergic, non-cholinergic field stimulation and to nitrovasodilator drugs. *Br. J. Pharmacol.*, **96,** 637–644.

GIBSON, A., MIRZAZADEH, S., HOBBS, A.J. & MOORE, P.K. (1990). L-N^G-monomethyl arginine and L-N^G-nitro arginine inhibit non-adrenergic, noncholinergic relaxation of the mouse anococcygeus muscle. *Br. J. Pharmacol.*, **99,** 602–606.

GIBSON, Q.H. & ROUGHTON, F.J.W. (1957). The kinetics and equilibria of the reactions of nitric oxide with sheep haemoglobin. *J. Physiol.*, **136,** 507–526.

GOYAL, R.K. & PATERSON, W.G. (1989). Esophageal motility. In *Handbook of Physiology. The Gastrointestinal System. Motility and Circulation*. Vol. 1, ed. Shultz, S.G., Wood, J.D. & Rauner, B.B., Bethesda, MD: Am. Physiol. Soc.

GRIDER, J.R. & MAKHLOUF, G.M. (1986). Colonic peristaltic reflex: identification of vasoactive intestinal peptide as mediator of descending relaxation. *Am. J. Physiol.*, **251,** G40–G45.

GRIDER, J.R., MURTHY, K.S., JIN, J.-G. & MAKHLOUF, G.M. (1992). Stimulation of nitric oxide from muscle cells by VIP: prejunctional enhancement of VIP release. *Am. J. Physiol.*, **262,** G774–G778.

GRIDER, J.R. & RIVIER, J.R. (1990). Vasoactive intestinal polypeptide (VIP) as a transmitter of inhibitory motor neurones of the gut: evidence from the use of selective VIP antagonists and VIP antisera. *Am. J. Physiol.*, **253,** G774–778.

GROUS, M., JOSLYN, A.F., THOMPSON, W. & BARNETTE, M.S. (1991). Change in intracellular cyclic nucleotide content accompanies relaxation of the isolated canine internal and sphincter. *J. Gastrointest. Motil.* **3,** 46–52.

HUIZINGA, J.S., TOMLINSON, J. & PINTIN-QUEZADA. (1992). Involvement of nitric oxide in nerve mediated inhibition and action of vasoactive intestinal peptide in colonic smooth muscle. *J. Pharmacol. Exp. Ther.*, **260,** 803–808.

INGLEFINGER, F.J. (1958). Esophageal motility. *Physiol. Rev.*, **38,** 533–584.

KNUDSEN, M.A., FRØBERT, O. & TØTTRUP, A. (1993). The role of the L-arginine-nitric oxide pathway for peristalsis in the opossum oesophageal body. *Scand. J. Gastroenterol.*, **29,** 1083–1087.

KNUDSEN, M.A., SVANE, D. & TØTTRUP, A. (1992). Action profiles of nitric oxide, S-nitroso-L-cysteine, SNP, and NANC responses in opossum lower esophageal sphincter. *Am. J. Physiol.*, **262,** G840–G846.

KONTUREK, J.W., GILLESSEN, A. & DOMSCHKE, W. (1995). Diffuse esophageal spasm: a malfunction that involves nitric oxide? *Scand. J. Gastroenterol.*, **30,** 1041–1045.

MARTIN, W., MORGAN, R.O., SMITH, J.A. & WHITE, D.G. (1986). Atriopeptin II-induced relaxation of rabbit aorta is potentiated by M&B 22,948 but not blocked by haemoglobin. *Br. J. Pharmacol.*, **89,** 557–561.

MUKHOPADHYAY, A.K. & WEISBRODT, N.W. (1975). Neural organization of esophageal peristalsis: role of the vagus nerve. *Gastroenterology*, **94,** 444–447.

MURRAY, J., BATES, J.N. & CONKLIN, J.L. (1994). Nerve-mediated nitric oxide production by opossum lower esophageal sphincter. *Dig. Dis. Sci.*, **39,** 1872–1876.

MURRAY, J., DU, C., LEDLOW, A., BATES, J.N. & CONKLIN, J.L. (1991). Nitric oxide: mediator of nonadrenergic noncholinergic responses of opossum esophageal muscle. *Am. J. Physiol.*, **261,** G401–G406.

MURRAY, K.J. (1993). Phosphodiesterase V_A inhibitors. *Drug News Prospectives*, **6,** 150–156.

NY, L., ALM, P., LARSSON, B., EKSTROM, P. & ANDERSSON, K.-E. (1995). Nitric oxide pathway in cat esophagus: localization of nitric oxide synthase and functional effects. *Am. J. Physiol.*, **268,** G59–G70.

OSTHAUS, L.E. & GALLIGAN, J.J. (1992). Antagonists of nitric oxide synthesis inhibit nerve mediated relaxations of longitudinal muscle in guinea-pig ileum. *J. Pharmacol. Exp. Ther.*, **260,** 140–145.

PUBLICOVER, N.G., HAMMOND, E.M. & SANDERS, K.M. (1993). Amplification of nitric oxide signalling by interstitial cells isolated from canine colon. *Natl. Acad. Sci. U.S.A.*, **90,** 2087–2091.

SATCHELL, D.G. (1981). Nucleotide pyrophosphatase antagonises responses to adenosine 5′-triphosphate and non-adrenergic, non-cholinergic inhibitory nerve stimulation in the guinea-pig isolated taenia coli. *Br. J. Pharmacol.*, **74,** 319–321.

SINGARAM, C., SWEET, M.A., GAUMNITZ, E.A., CAMERON, A.J. & CAMILLERI, M. (1995). Peptidergic and nitrinergic denervation in congenital esophageal stenosis. *Gastroenterology*, **109,** 275–281.

TORPHY, T.J., FINE, C.F., BURMAN, M., BARNETTE, M.S. & ORMSBEE, H.S. (1986). Lower esophageal sphincter relaxation is associated with increased cyclic nucleotide content. *Am. J. Physiol.*, **251,** G786–G793.

TØTTRUP, A., FORMAN, A., FUNCH-JENSEN, P., RAUNDAHL, U. & ANDERSSON, K.-E. (1990). Effects of transmural field stimulation in isolated muscle strips from human esophagus. *Am. J. Physiol.*, **258,** G344–G351.

TØTTRUP, A., SVANE, D. & FORMAN, A. (1991). Nitric oxide mediating NANC inhibition in opossum lower esophageal sphincter. *Am. J. Physiol.*, **260,** G385–G389.

WALDMAN, S.A. & MURAD, F. (1987). Cyclic GMP synthesis and function. *Pharmacol. Rev.*, **39,** 163–196.

WALDMAN, S.A., RAPOPORT, R.M. & MURAD, F. (1984). Atrial natriuretic factor elicits an endothelium-independent relaxation and activates particulate guanylate cyclase in vascular smooth muscle. *Proc. Natl. Acad. Sci. U.S.A.*, **81,** 7661–7664.

WILLIAMS, S.J. & PARSONS, M.E. (1995). Nitric oxide, an enteric nonadrenergic-noncholinergic relaxant transmitter: evidence using phosphodiesterase V and nitric oxide synthase inhibition. *Br. J. Pharmacol.*, **116,** 1789–1796.

WONG, R.H.K., MAYDONOVITCH, C., GARCIA, J.E., JOHNSON, L.F. & CASTELL, D.O. (1987). The effect of terbutaline sulphate, nitroglycerin, and aminophylline on lower esophageal sphincter pressure and radionuclide esophageal emptying in patients with achalasia. *J. Clin. Gastroenterol.*, **9,** 386–389.

YAMATO, S., SAHA, J.K. & GOYAL, R.K. (1992). Role of nitric oxide in lower esophageal sphincter relaxation to swallowing. *Life Sci.*, **50,** 1263–1272.

YOSHIDA, S. (1994). Tetrodotoxin-resistant sodium channels. *Cell. Mol. Neurobiol.*, **14,** 227–244.

(Received December 9, 1996
Revised April 24, 1997
Accepted May 7, 1997)