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Hyperpolarization and inhibition of contraction mediated by nitric oxide released from enteric inhibitory neurones in guinea-pig taenia coli

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1 Inhibition of nitric oxide synthase by N^G-nitro-L-arginine (L-NNA) reduced the neurogenic relaxation of precontracted taenia coli only in the absence of atropine. The membrane hyperpolarization associated with the neurogenic relaxation was also reduced by inhibition of NOS only when atropine was absent.

2 The membrane hyperpolarization associated with the neurogenic relaxation of the taenia coli was inhibited by oxyhaemoglobin only in the absence of atropine. In the presence of atropine, oxyhaemoglobin did not reduce the i.j.p. or nerve-evoked relaxation.

3 Inhibition of NOS by L-NNA did not affect the overflow of [^{3}H]-ACh in response to electrical field stimulation (EFS), suggesting that, under the conditions of our experiments, endogenous NO did not modulate release of ACh. Sodium nitroprusside also had no effect on the neurogenic overflow of [^{3}H]-ACh; however, noradrenaline significantly reduced [^{3}H]-ACh overflow.

4 In summary, the postjunctional effects of neurally-released NO are not apparent in guinea-pig taenia coli when atropine is present. This implies muscarinic regulation of NO release or muscarinic regulation of another excitatory substance, such as tachykinin(s), that, when blocked, masks the postjunctional effects of NO. These data, together with previous studies, suggest a possible regulatory role for NO in enteric neurotransmission that may be more prominent in some species or tissues than others.

Keywords: Nitric oxide; non-adrenergic, non-cholinergic nerves; neuromodulation; taeni coli

Introduction

Nitric oxide (NO) or a related substance has been shown to be a mediator of non-adrenergic, non-cholinergic (NANC) inhibitory neurotransmission in many visceral smooth muscles, including longitudinal, circular and spincter smooth muscle of the gastrointestinal tract of many species (see Sanders & Ward, 1992; Rand & Li, 1995 for reviews). In the majority of these tissues NO appears to be synthesized in nerve terminals by a constitutive nitric oxide synthase (NOS; Bredt & Snyder, 1990) and reaches its postjunctional targets via diffusion. Evidence suggests that NO produces relaxation of smooth muscle via activation of guanylate cyclase, elevation of guanosine 3':5'-cyclic monophosphate (cyclic GMP) levels, activation of cyclic GMP-dependent protein kinase, and phosphorylation of a variety of cellular effectors (see Lincoln & Cornwell, 1993). Cyclic GMP-independent mechanisms may also contribute to the effects of NO in some smooth muscles (Bolitina *et al.*, 1994; Koh *et al.*, 1995).

This direct model of neurotransmission has been difficult to demonstrate in some muscles of the gastrointestinal tract. For example, inhibitors of NOS did not modify responses to inhibitory neurotransmission in the guinea-pig ileum (Wiklund *et al.*, 1993). Others found similar effects in the taenia coli (Knudsen & Tottrup, 1992) and trachea (Belvisi *et al.*, 1993) of the guinea-pig and suggested that the inhibitory effects of NO depend upon the integrity of cholinergic neurotransmission, because they could demonstrate effects of arginine analogues only if atropine was not present. Recent electrophysiological studies have supported this notion by showing that inhibitory junction potentials in taenia coli, initiated by electric field stimulation of intramural inhibitory neurones, are also unaffected by nitroarginine in the presence of atropine (Bridgewater *et al.*, 1995). These observations have led to the suggestion of a prejunctional effect of NO in regulating either acetylcholine (Knudsen & Tottrup, 1992) or tachykinin (Wiklund *et al.*, 1993) release. Thus, in some muscles, NO may have, in addition to a role as a primary neurotransmitter, a neuromodulatory role, regulating the release of other transmitter substances (see also Grider & Jin, 1993).

The present study examines further the role of NO in inhibitory neurotransmission in the guinea-pig taenia coli by looking at the mechanism of action of NOS inhibitors and oxyhaemoglobin on the electrical and mechanical responses of this tissue, and on the overflow of [^{3}H]-acetylcholine ([^{3}H]-ACh) from tissues preincubated with [^{3}H]-choline. A preliminary account of this work has been presented (Dalziel *et al.*, 1993).

Methods

General

Male albino guinea-pigs were killed by asphyxiation with CO_2 and exsanguination. The abdomen was opened and approximately 5 cm of the taenia coli removed. Lengths of muscle (approximately 1 cm) were cut and mounted in organ baths for measurement of mechanical or electrical activity or overflow of radiolabelled ACh (see below). Tissues were sustained in Krebs solution which was equilibrated with 95% O_2/5% CO_2 and maintained at 37.5±0.5°C and was of the following composition (mM): NaCl 110, KCl 4.6, $CaCl_2$ 2.5, $NaHCO_3$ 24.8, KH_2PO_4 1.2, $MgSO_4$ 1.2, glucose 5.6. Electrical field stimulation (EFS) induced mechanical and electrical responses and overflow of radiolabelled neurotransmitter were all abolished by tetrodotoxin (1 μM) indicating their neurogenic origin. The α-adrenoceptor antagonist, phentolamine (1 μM) and the β-

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adrenoceptor antagonist, propranolol (1 μM) were routinely added to the Krebs solution to negate the effects of neuronally released noradrenaline.

Contractile experiments

Lengths of taenia coli of approximately 1 cm were mounted in jacketed organ baths and allowed to equilibrate under a resting tension of 1 g for 60–90 min. Tissues were then precontracted with histamine (3 μM) and subjected to electric field stimulation (EFS) of the intrinsic nerves with square wave pulses (0.3 ms, supramaximal voltage at required frequency) from a stimulator (Grass S48) delivered via parallel platinum electrodes placed on either side of the tissue. Each tissue was subjected to 3 periods of precontraction, each lasting approximately 5 min and separated by 30 min, during which EFS at the various parameters of interest or drugs were added and the resulting mechanical responses observed. Control experiments showed that the responses during this protocol were reproducible. Mechanical responses were monitored with force transducers (Grass FT03) and displayed on chart paper (Grass 5DWCB). At the end of the experiment the tissues were lightly blotted on tissue paper and weighed to the nearest 0.1 mg. Mechanical responses were measured as g mg^{-1} of tissue wet weight and expressed as a percentage of the histamine-induced tone immediately prior to induced relaxation. In the presence of atropine, phentolamine and propranolol (all 1 μM), tetrodotoxin (1 μM) completely abolished relaxations to EFS indicating that these responses were mediated by NANC inhibitory nerves.

Electrophysiology experiments

Lengths of taenia coli (0.5–1.0 cm) were pinned to the Sylgard floor of an electrophysiological recording chamber which was continuously perfused with Krebs of the composition and temperature described above. In some experiments one end of the tissue was not pinned to the floor but was attached to a force transducer to allow measurement of mechanical responses of the whole tissue as well as electrical responses of individual cells. Muscles were allowed to equilibrate for 60–90 min before experiments were initiated. Single smooth muscle cells were impaled with glass microelectrodes filled with 3 M KCl with resistances ranging from 30–50 MOhms. Transmembrane potential was measured by a standard electrometer (WPI M-7000), and outputs displayed on an oscilloscope (Tektronix 5111). Signals were recorded on magnetic tape (Hewlett-Packard 3964A) and chart paper (Gould 2200). EFS of the intrinsic nerves was induced by square wave pulses (0.5 ms, supramaximal voltage at required frequency) via platinum electrodes using a Grass S44 stimulator. One second trains of pulses were used for electrophysiological experiments because longer duration trains of EFS caused dislodgement of the microelectrode. Where stated, nifedipine (1 μM) was added to the perfusion solution to suppress spiking activity and contraction of the preparation which led to the dislodgement of the microelectrode.

[^{3}H]-ACh labelling and overflow experiments

In each experiment three 1.5 cm lengths of taenia coli from the same guinea-pig were secured between parallel plate electrodes on a glass rod holder. This allowed easy transference of the preparation between different incubation tubes. The tissues were incubated for 2 h in a water bath at 37°C in Krebs solution of the above composition which also contained choline chloride (0.1 μM) and 9 μCi of [^{3}H]-choline chloride. Subsequent to this the tissues were washed for 2 h by sequentially passing them at 5 min intervals through tubes each containing 10 ml Krebs solution. Midway through this washing procedure the tissues were stimulated at 10 Hz for 5 s, since preliminary experiments had shown that this first stimulus produced very high overflow in comparison to subsequent stimuli. After the washing period the experiment was started and the tissue transferred at 1 min intervals through a series of tubes containing 3.5 ml of Krebs solution, all of which were retained for scintillation counting. After 10 min of collections to establish basal overflow, the tissue was stimulated twice, first at 2 Hz for 1 min and then, 20 min later, at 10 Hz for 30 s. After a period

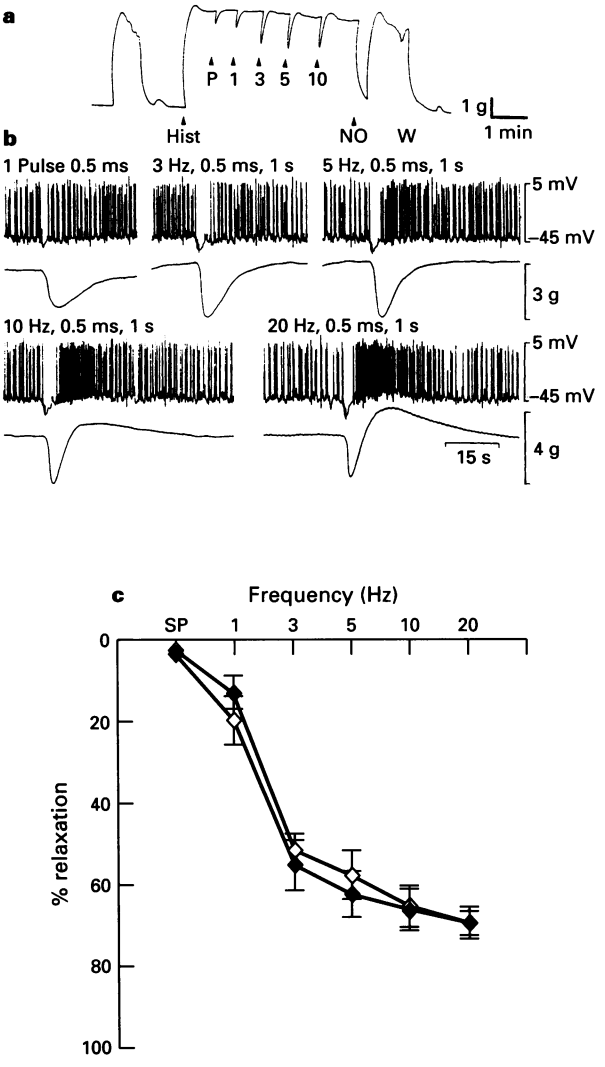

Figure 1 Responses to EFS of guinea-pig taenia coli pre-contracted with histamine (3 μM). (a) An example of relaxations to EFS delivered as a single pulse (P) or 5 s trains at 1 to 10 Hz and to addition of exogenous nitric oxide (NO, 1 μM). Note that the tissue displayed a frequency-dependent relaxation. At the higher frequencies and after application of NO the relaxation was followed by a 'rebound' contraction. The tracing also shows a spontaneous contraction preceding the histamine-induced contraction. (b) Illustrates simultaneous intracellular microelectrode and mechanical recordings in a preparation in response to EFS delivered as a single pulse or 1 s trains at 3 to 20 Hz. EFS produced a frequency-dependent hyperpolarization accompanied by an inhibition of spiking activity for the duration of the stimulus and a relaxation of the muscle. At higher frequencies (>5 Hz) an increase in spiking frequency and a rebound contraction occurred upon termination of the EFS. (c) Shows mean frequency-response curves obtained from the type of experiment illustrated in (a). Each of the two curves represents a separate period of histamine induced contraction, 30 min apart, during which the relaxation responses were obtained: (◇) initial response; (◆) 30 min later. The response is expressed as % relaxation from the tone induced by 3 μM histamine. SP refers to single pulse. In the absence of EFS the tone induced by histamine is well maintained for the duration of time required for the EFS protocol. Mean values ± s.e.mean are shown. $n=7$.

of 35 min the stimulation protocol was repeated. In experiments where the effect of the NOS inhibitor was examined the drug was included in the medium 30 min before the second pair of stimuli. In experiments where the effect of noradrenaline or SNP was examined, a more acute exposure was desirable and incubation with these substances was 4 min before and during the EFS. The 3.5 ml samples of Krebs solution were transferred to scintillation vials and made up to 20 ml with Ecolume scintillant (I.C.N. Biomedicals, Inc.). They were then counted in a Beckman LS100C scintillation counter twice for 2 min and averaged. The tissue was solubilized overnight in 1 ml of 10% NaOH, neutralized with HCl and buffered with HEPES prior to counting. Overflow of ^{3}H was calculated as fractional release from the tissue. The fractional release after incubation with drug (S_2) was expressed as a ratio of the release obtained by stimulation prior to exposure to the drug (S_1). It is generally well accepted that overflow of ^{3}H evoked by EFS from tissues preincubated with [^{3}H]-choline is a good measure of release of [^{3}H]-ACh (Richardson & Szerb, 1974). This technique has been employed to study the release of ACh from a variety of neuroeffector preparations including guinea-pig gastrointestinal tissues (e.g. Alberts *et al.*, 1982). Accordingly we refer to the release of ^{3}H as release of [^{3}H]-ACh.

Drugs

General N^G-nitro-L-arginine (L-NNA; Sigma), N^G-nitro-L-arginine, methyl ester (L-NAME; Sigma), N^G-monomethyl-L-arginine (L-NMMA; Calbiochem) and propranolol (Sigma) were used as the hydrochloride salt. Histamine (Sigma) was used as the dihydrochloride salt. Atropine (Sigma) was used as the sulphate salt, phentolamine (Ciba Geigy) as the mesylate salt and sodium nitroprusside (SNP; Sigma) as the dihydrate. Drugs were dissolved in distilled water as stock solutions of 10 or 100 mM and further dilutions made in Krebs solution. Tetrodotoxin (TTX; Sigma) was dissolved in distilled water to 300 μM and further dilutions made in Krebs solution. Nifedipine (Sigma) was dissolved in ethanol to 10 mM and subsequent dilutions made in Krebs solution. [^{3}H]-choline chloride was obtained from New England Nuclear.

Nitric oxide NO stock solution was prepared by bubbling ice-cold, deoxygenated (sonication under vacuum followed by purging with 100% N_2 gas) distilled water with 99% pure NO gas to give a saturated solution (1–1.5 mM, Ignarro *et al.*, 1987). In tension experiments, NO was delivered to the tissues by addition of the appropriate volume of stock solution directly to the organ chamber. In electrophysiology experiments, NO was delivered by adding appropriate volumes of stock solution to a 20 ml heated, bubbled reservoir of Krebs solution which fed the tissue chamber. The recording chamber could be rapidly perfused with the solution from the reservoir at a rate of 5 ml min^{-1}. The lag time for perfusion of the recording chamber from the reservoir was 1.5 s. The stated concentrations of NO have not been corrected for breakdown and therefore may be slightly overestimated. Addition of water alone instead of NO solution had no effect on electrical or mechanical activity.

Oxyhaemoglobin: Oxyhaemoglobin was prepared as a lysate of canine or human erythrocytes according to the method of Bowman & Gillespie (1982), with the exception that red cells were lysed by 1:1 addition of distilled water.

Data analysis

Statistical significance of differences between the means of data groups was determined by Student's *t* test for paired or unpaired data, as appropriate.

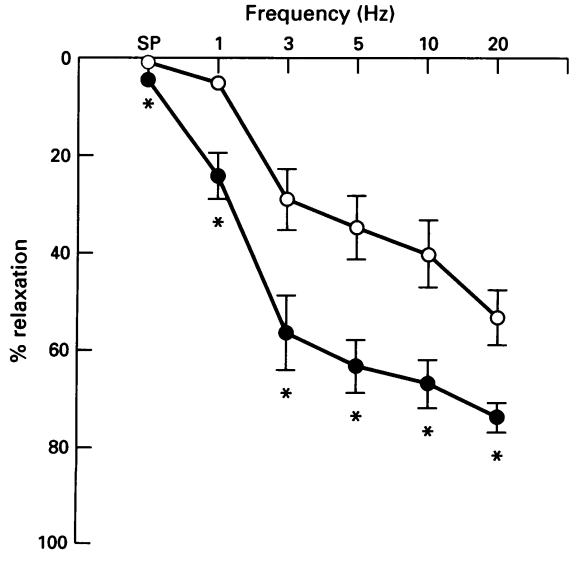

Figure 2 Mean frequency-response curves in guinea-pig taenia coli before (control, ○) and after pretreatment for 30 min with atropine (1 μM, ●). Experiments were conducted as described in the legend for Figure 1c. Note the significant enhancement of the EFS-evoked relaxation responses by atropine. Asterisks indicate a significant difference from control ($P<0.05$). $n=8$.

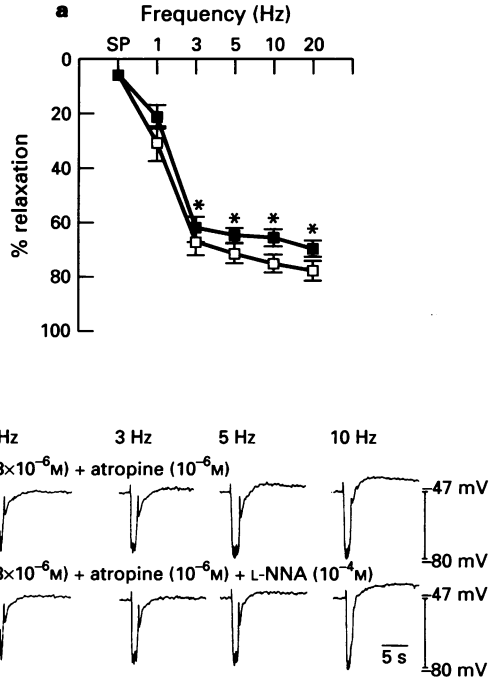

Figure 3 (a) Mean frequency-response curves in guinea-pig taenia coli in the presence of atropine (1 μM; control, □) and in the presence of atropine and after treatment with L-NNA (100 μM) for 30 min (■). There was no significant effect of L-NNA on responses to a single pulse or 1 Hz whereas there was a small attenuation of the relaxations evoked by EFS at 3 to 20 Hz. Asterisks indicate a significant difference from control. $n=7-8$. (b) Microelectrode recordings of membrane potential and the effect of EFS delivered as 1 s trains at 1 to 10 Hz. The upper panel shows hyperpolarizations obtained in the presence of histamine (3 μM) and atropine (1 μM) while the lower panel shows hyperpolarizations under similar circumstances but with pretreatment with L-NNA (100 μM). In the presence of atropine two distinct rates of recovery of the inhibitory junction potentials (i.j.ps) are revealed, a fast component that is followed by a much slower recovery to the prestimulus membrane potential. Note that L-NNA had no significant effect on the amplitude or duration of i.j.ps Mean data are shown in Table 1.

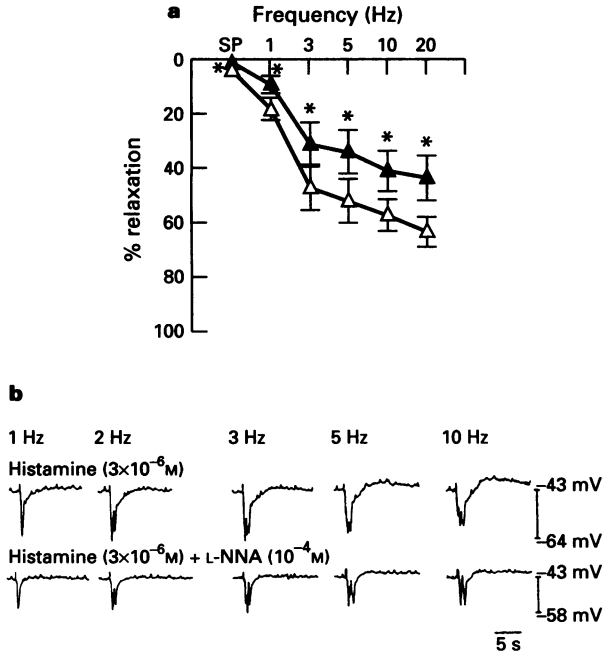

Figure 4 (a) Mean frequency-response curves in guinea-pig taenia coli before (△) and after treatment with L-NNA (100 μM) (▲) for 30 min. L-NNA produced a significant reduction in the size of the relaxation induced by a single pulse and by EFS at 1 to 20 Hz. The degree of attenuation ranged from 30 to 50%. Asterisks indicate a significant difference from control. $n=7-8$. These curves differ from those of Figure 3 in that atropine is absent. (b) Microelectrode recordings of membrane potential and the effect of EFS delivered at 1 s trains at 1 to 10 Hz. The upper panel shows inhibitory junction potentials (i.j.ps) in the presence of histamine (3 μM) while the lower panel shows i.j.ps in the presence of histamine but with pretreatment with L-NNA (100 μM). Note that L-NNA significantly reduced the amplitude of the i.j.ps. Mean data are shown in Table 1. These tracings differ from those of Figure 3 in that atropine is absent.

Results

Mechanical and electrical responses of the guinea-pig taenia coli

Under resting conditions, the guinea-pig taenia coli exhibited spontaneous contractures with a frequency of 1 per 2–3 min. Exposure to histamine (3 μM) produced a contraction (1.08 ± 0.09 g mg^{-1} tissue, $n=64$ preparations) which was well maintained and reproducible during the course of these experiments (each histamine challenge lasting approximately 5 min). A typical example of the mechanical response to EFS and exogenously applied NO in histamine precontracted muscles, is shown in Figure 1a. In the presence of histamine, periods of EFS produced a frequency-dependent relaxation followed by a 'rebound' contraction. The rebound contraction increased in amplitude at higher frequencies of stimulation. NO mimicked the effects of NANC nerve stimulation in that it also produced a transient relaxation followed by a rebound contraction. EFS also produced frequency-dependent hyperpolarization in membrane potential and inhibition of spiking activity. These events correlated with relaxation of the muscle (Figure 1b). Termination of EFS produced repolarization of membrane potential, recommencement of spiking activity and termination of the relaxation. The rate at which the relaxation response decayed was frequency-dependent and it was associated with a transient increased spiking activity. Rebound contraction occurred at higher frequencies (10 to 20 Hz). Figure 1c summarizes the effect of 5 s trains of pulses at various frequencies (1 pulse–10 Hz) of EFS. Each of the 2 curves represents a frequency-response curve carried out during separate histamine contractures 30 min apart.

Effects of atropine and NOS inhibitors, alone and in combination, on neurogenic relaxations and membrane potential

Incubation with atropine (10^{-6}M) for 30 min produced a large increase in the relaxation response to EFS at all frequencies

Table 1 Summary of the effects of L-NNA on inhibitory junction potentials in the absence and presence of atropine

Experimental manipulation (n = 5)	*Freq.*	*RMP* (mV)	*i.j.p. amplitude* (mV)	*i.j.p. duration* (s)
Control (Histamine 3×10^{-6}M)		-39.1 ± 2.6		
	1 Hz		23.8±2.4	2.2±0.34
	2 Hz		20.25±1.5	3.3±0.5
	3 Hz		21.75±0.5	3.5±0.6
	5 Hz		18.5±0.7	3.7±0.2
	10 Hz		14.5±1.0	3.6±0.23
L-NNA (10^{-4}M)		-34.5 ± 3.6		
	1 Hz		16.4±1.7*	2.0±0.2
	2 Hz		15.8±1.4*	2.5±0.4
	3 Hz		15.5±1.3*	2.6±0.4
	5 Hz		12.0±3.1*	2.6±0.4
	10 Hz		14.5±1.5	2.9±0.5
Atropine (10^{-6} M)		-41.2 ± 3.3		
	1 Hz		26.8±0.3*	2.6±0.6
	2 Hz		26.7±0.9*	4.0±0.5
	3 Hz		27.7±0.3*	4.7±1.0
	5 Hz		28.3±1.7*	4.9±1.1
	10 Hz		28.3±1.7**	3.5±1.9
Atropine (10^{-6}M) + L-NNA (10^{-4} M)		-35.6 ± 3.5		
	1 Hz		27.2±1.7	2.5±0.5
	2 Hz		31.4±1.5*	3.4±0.1
	3 Hz		31.6±1.3*	3.2±0.2
	5 Hz		31.7±1.2**	3.1±0.1
	10 Hz		31.3±0.5**	2.4±0.5

Control experiments were performed in the presence of histamine (3×10^{-6}M) to mimic the experimental conditions of mechanical experiments. Other drugs were all added in the presence of histamine.
*The amplitude of the i.j.p. was statistically different ($P<0.05$) from the control.
**The amplitude of the i.j.p. was statistically different ($P<0.005$) from the control.
Note: L-NNA in the presence of atropine did not significantly reduce the i.j.p. compared with that in atropine alone.

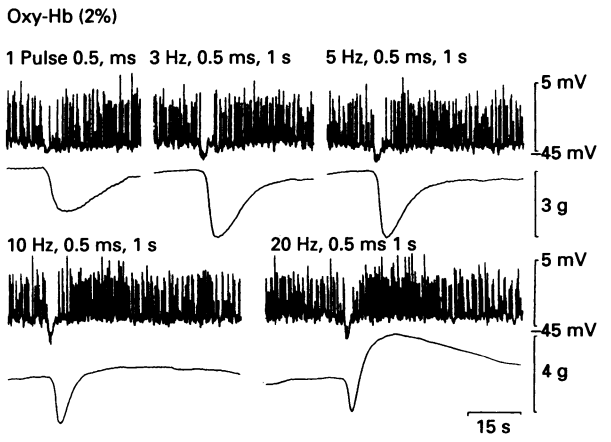

Figure 5 Simultaneous intracellular microelectrode and mechanical recordings of a preparation after treatment with oxyhaemoglobin (OxyHb; as a 2% haeme lysate solution) for 20 min. EFS was delivered as a single pulse of 1 s trains at 3 to 20 Hz as indicated. The intracellular recording was performed from the same cell shown in Figure 1b. Note that the EFS-induced hyperpolarizations and relaxations persist after oxyhaemoglobin treatment and do not differ from control (Figure 1b).

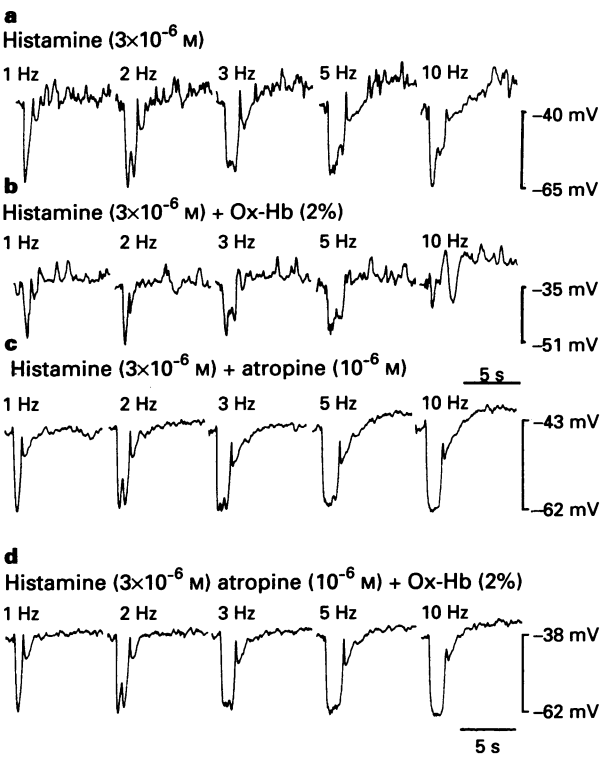

Figure 6 The effect of oxyhaemoglobin (2%) on inhibitory junction potentials (i.j.ps) recorded in the absence and presence of atropine. (a) Microelectrode recordings of membrane potential and the effect of EFS (0.5 ms duration) delivered as 1 s trains at 1 to 10 Hz under control conditions (histamine, 3×10^{-6} M). (b) The effect of oxyhaemoglobin (OxyHb; 2% for 20 min.) OxyHb reduced the amplitude of i.j.ps at all frequencies tested compared to control. (c) In the presence of atropine (10^{-6} M) the irregular oscillations in membrane potential were reduced and inhibitory responses to EFS appeared more robust. (d) In the continued presence of atropine, oxyhaemoglobin had no effect on i.j.ps in response to EFS.

($P<0.05$; Figure 2). In the presence of atropine, L-NNA (100 μM, 30 min) produced a small degree of attenuation of EFS-induced NANC relaxations at 3–20 Hz ($P<0.05$) and no significant effect at a single pulse of 1 Hz (Figure 3a; compare with Figure 4a). L-NNA (100 μM, 30 min) had no effect on the amplitude of membrane hyperpolarizations ($P>0.05$; Figure 3b); however, the duration of inhibitory junction potentials was reduced at all frequencies tested, although values did not reach stastical significance. L-NMMA (100 μM) and L-NAME (100 μM) had a similar effect to L-NNA on mechanical responses ($n=5$ and 6 respectively, data not shown). None of the NOS inhibitors had any effect on the mechanical or electrical responses to exogenously applied NO (data not shown).

In the absence of atropine, L-NNA (100 μM) reduced the magnitude of NANC relaxations (Figure 4a) and membrane hyperpolarizations (Figure 4b) at all frequencies tested (single pulse–20 Hz). This attenuation in relaxation was significantly greater than the relaxation observed with L-NNA (100 μM) in the presence of atropine ($P<0.05$ at all frequencies). A summary of the effects of atropine and L-NNA on the EFS-evoked membrane hyperpolarizations is presented in Table 1.

Effect of oxyhaemoglobin on neurogenic relaxations and membrane potential

In the presence of atropine (1 μM), oxyhaemoglobin (2%) had no effect on the EFS-induced relaxations or membrane hyperpolarizations at any of the frequencies tested, whereas in all of the tissues it abolished responses to exogenously applied NO (1 μM). In 4 experiments L-NAME (100–200 μM) was added in the presence of oxyhaemoglobin; however, the relaxations to EFS were not different from those in oxyhaemoglobin alone. Figure 5 shows the simultaneous electrical and mechanical recordings in response to EFS in the presence of oxyhaemoglobin (2% for 20 min). The recording is from the same cell shown in Figure 1b. In the absence of atropine, oxyhaemoglobin caused a significant reduction in the amplitude of EFS-induced membrane hyperpolarizations at all the frequencies tested (Figure 6). A summary of the effects of oxyhaemoglobin in the presence and absence of atropine, on EFS evoked membrane hyperpolarizations is presented in Table 2.

The time course for the reduction in the amplitude produced by oxyhaemoglobin was rapid. Within 2–3 min of its addition, oxyhaemoglobin had produced maximum inhibition (Figure 7a). The reversal of the inhibition of the i.j.p. by oxyhaemoglobin was slower, taking approximately 15–20 min for complete recovery of its effect. In comparison, the time course of the block of i.j.ps by TTX (10^{-6} M) is illustrated in Figure 7b. The i.j.p. was almost completely inhibited by TTX. Both substances showed a similar time period for recovery.

[^{3}H]-ACh overflow experiments

The experimental protocol necessary for the detection of [^{3}H]-ACh, required the stimulation of the enteric nerves within muscles by EFS for 1 min at 2 Hz and 30 s at 10 Hz (see methods). Due to the short half-life of NO (see Figure 1), the more stable NO donor, SNP, was used in these experiments. Incubation with L-NNA (100 μM) or SNP (10 μM) had no effect on overflow of [^{3}H]-ACh from the taenia coli stimulated at 2 Hz whereas noradrenaline (10 μM) decreased overflow by approximately 70% (Figure 8). However at 10 Hz stimulation, L-NNA, SNP or noradrenaline did not significantly affect overflow of [^{3}H]-ACh. Overflow of [^{3}H]-ACh was abolished by incubation with TTX (1.0 μM) for 5 min (not shown).

Discussion

The guinea-pig taenia coli muscle exhibits NANC inhibitory responses consisting of hyperpolarization, cessation of spiking

Table 2 Summary of the effects of oxyhaemoglobin on inhibitory junction potentials in the absence and presence of atropine

Experimental manipulation (n = 5)	*Freq.*	*RMP* (mV)	*i.j.p. amplitude* (mV)	*i.j.p. duration* (s)
Control (Histamine 3×10^{-6} M)		-42.7 ± 3.7		
	1 Hz		23.2 ± 1.3	2.0 ± 0.49
	2 Hz		22.2 ± 1.1	1.7 ± 0.39
	3 Hz		24.7 ± 0.99	1.8 ± 0.37
	5 Hz		18.7 ± 1.4	2.0 ± 0.24
	10 Hz		15.8 ± 2.1	2.5 ± 0.78
Oxyhaemaglobin (2%)		-36.9 ± 2.3		
	1 Hz		15.2 ± 1.98**	1.03 ± 0.17
	2 Hz		17.8 ± 0.86*	1.2 ± 0.04
	3 Hz		16.7 ± 1.5*	1.3 ± 0.08
	5 Hz		14.0 ± 2.0*	1.4 ± 0.12
	10 Hz		8.8 ± 0.49**	1.6 ± 0.33
Atropine (10^{-6} M)		-36.0 ± 3.5		
	1 Hz		26.7 ± 2.9	2.2 ± 0.52
	2 Hz		25.0 ± 1.1	2.3 ± 0.9
	3 Hz		24.0 ± 1.0	2.4 ± 0.85
	5 Hz		23.5 ± 1.5*	2.7 ± 0.9
	10 Hz		24.0 ± 1.0*	2.3 ± 0.7
Atropine (10^{-6}M) + oxyhaemaglobin (2%)		-36.8 ± 3.7		
	1 Hz		26.5 ± 2.6	2.2 ± 0.8
	2 Hz		22.0 ± 1.0	1.8 ± 0.6
	3 Hz		22.0 ± 1.2	2.0 ± 0.6
	5 Hz		22.0 ± 1.0*	2.1 ± 0.4
	10 Hz		24.0 ± 1.0*	1.9 ± 0.5

Control experiments were performed in the presence of histamine (3×10^{-6}M) to mimic the experimental conditions of mechanical experiments. Other drugs were all added in the presence of histamine.
*The amplitude of the i.j.p. was statistically different ($P<0.05$) from control.
**The amplitude of the i.j.p. was statistically different ($P<0.005$) from control.
Note: Oxyhaemaglobin in the presence of atropine did not significantly reduce the i.j.p. compared with that in atropine alone.

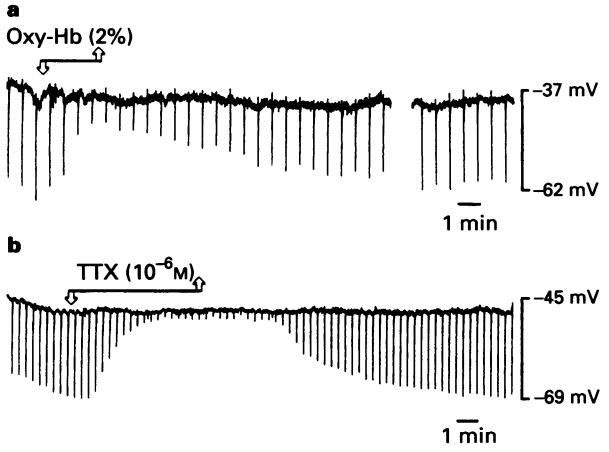

Figure 7 (a) Time course of the inhibition of oxyhaemoglobin on inhibitory junction potentials (i.j.ps) to a steady state within 5 min. Reversal of this inhibition took approximately 15–20 min. Break in the record is for a time period of 5 min. In comparison the time course of the effect of TTX (10^{-6}M) is illustrated in (b). Almost complete block of i.j.ps was achieved within 5 min and reversal of this block within 15 min.

activity, and relaxation if muscles are precontracted (Burnstock *et al.*, 1964). Despite many studies, the identity of the inhibitory neurotransmitter(s) in this tissue has been elusive. Among candidates that have been considered are ATP (Burnstock *et al.*, 1970) and vasoactive intestinal polypeptide (VIP) (Fahrenkrug, 1979; Furness *et al.*, 1981; Iselin *et al.*, 1988) but contradictory evidence concerning these agents has also been presented (Mackenzie & Burnstock, 1980; Westfall *et al.*, 1982). Motoneurones of the guinea-pig taenia coli contain NOS-like immunoreactivity, and these neurones co-express VIP-like immunoreactivity (Furness *et al.*, 1992). Therefore, it appears that the apparatus for NO production is located in inhibitory neurones. The possibility that NO may be the inhibitory transmitter has been examined in the guinea-pig taenia coli (Knudsen & Tottrup, 1992). Inhibitors of NO synthesis caused a reduction in neurogenic relaxation, but this effect of NO-synthase (NOS) inhibitors was dependent on an intact cholinergic mechanism. It was suggested that endogenous NO may have a prejunctional modulatory role on the cholinergic nerves and inhibitors of NOS reduce NANC relaxations by removing NO-mediated inhibition of acetylcholine release (Knudsen & Tottrup, 1992).

We have confirmed the findings of Knudsen & Tottrup (1992), in that inhibition of NOS by L-NNA reduced the neurogenic relaxation of precontracted taenia coli only in the absence of atropine. We have further shown that the membrane hyperpolarization associated with neurogenic relaxation is also reduced by inhibition of NOS when atropine is absent. These observations are important because atropine, a cholinoceptor antagonist, is often included in experiments designed to study NANC transmission. If atropine is included as part of the experimental paradigm, an effect of NOS inhibitors could go undetected and a false conclusion could be reached about the involvement of NO in NANC transmission. For example, Bridgewater *et al.* (1995) reported recently that L-NNA had no effect on inhibitory junction potentials in guinea-pig taenia coli treated with atropine and these authors concluded that the relaxant effect of nitrergic stimulation in the taenia coli is independent of changes in membrane potential. Our observations are entirely consistent with those of Bridgewater *et al.* (1995) when our experiments were performed in the presence of atropine, but when atropine was omitted, inhibition of NOS or addition or oxyhaemoglobin caused attenuation in membrane hyperpolarizations of the taenia coli to EFS.

Knudsen & Tottrup (1992) proposed that NO may act by

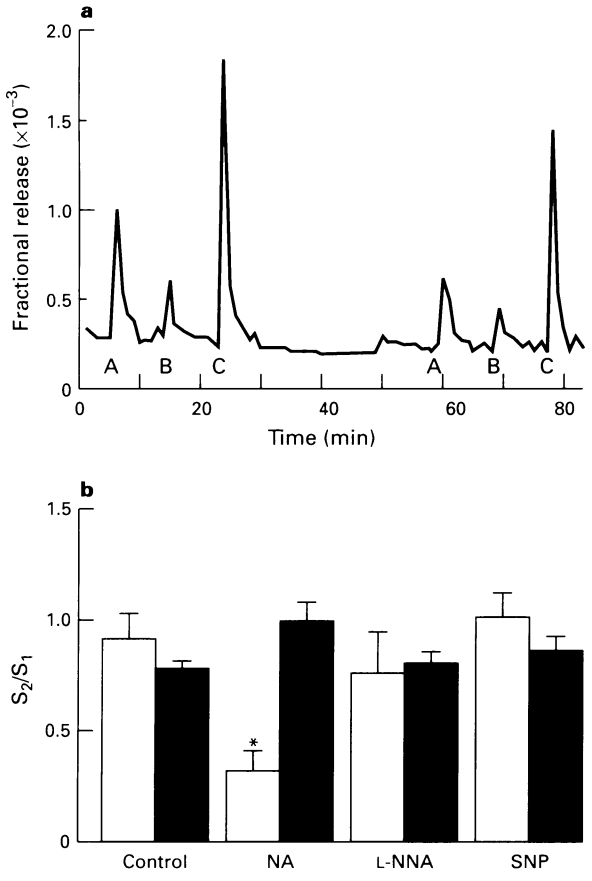

Figure 8 EFS-evoked overflow of [^{3}H]-ACh from the guinea-pig taenia coli. (a) A representative experiment showing ^{3}H overflow (expressed as fractional release from the tissue) with time. EFS (A, 2 Hz for 1 min; B, 10 Hz for 5 s; C, 10 Hz for 30 s) produced a significant increase in ^{3}H-overflow. The EFS evoked release was blocked by tetrodotoxin (1 μM). (b) Summary of the effects of EFS (2 Hz for 1 min, open columns, and 10 Hz for 30 s, solid columns) on [^{3}H]-ACh overflow expressed as the ratio of a second stimulation period (S_2) to the first (S_1). Pretreatment with L-NNA (100 μM) or SNP (10 μM) between S_1 and S_2 had no effect on [^{3}H]-overflow whereas noradrenaline (NA, 10 μM) decreased [^{3}H]-overflow evoked by 2 Hz EFS by 70%. At 10 Hz EFS, L-NNA, SNP or NA did not significantly affect overflow of [^{3}H]-ACh. $n = 5$.

inhibiting the release of acetylcholine from cholinergic nerves. Such an effect would be obscured by atropine. We found that inhibitions of NOS by L-NNA did not affect the overflow of [^{3}H]-ACh in response to EFS, suggesting that, under the conditions of our experiments, endogenous NO did not modulate release of ACh. This observation was supported by the observation that SNP also had no effect on the neurogenic overflow of [^{3}H]-ACh. We found that noradrenaline reduced [^{3}H]-ACh overflow, suggesting that the assay was sensitive enough to register the effects of prejunctional inhibition. Others have also reported that inhibition of NOS did not affect [^{3}H]-ACh overflow from the guinea-pig ileum (Wiklund *et al.*, 1993), although these authors were able to show that exogenous NO reduced neurogenic overflow of [^{3}H]-ACh. It would appear from these studies that excess NO may have some effect on ACh release, but the endogenous levels, produced by enteric inhibitory neurones are not sufficient to produce inhibition of ACh release.

At present it is unclear how atropine masks NO-dependent responses. In studies of the guinea-pig ileum others reported that NOS inhibitors enhanced contractile responses to electrical field stimulation, and these effects were blocked by the substance P antagonist, spantide (Wiklund *et al.*, 1993). These authors suggested that NO inhibits neurotransmission mediated by substance P, possibly by prejunctional neuromodulation. Such a mechanism could also explain our results, and therefore, we suggest the following scheme: muscarinic receptors exist on prejunctional membranes of excitatory neurones that release ACh and tachykinins (see Brookes *et al.*, 1991). ACh normally restricts the amount of tachykinins released via prejunctional muscarinic receptors. Blockade of these receptors by atropine increases tachykinin release, and this excitatory substance masks the inhibitory effects of neurally-released NO. Of course full testing of this hypothesis will require sensitive measurements of substance P release and the effects of muscarinic stimulation on this pathway. It is also possible that muscarinic receptors might be located on prejunctional membranes of inhibitory neurones. To explain our results, activation of these receptors would have to be facilitatory (if not obligatory) to NO release, and their blockade would have to reduce significantly the amount of NO produced by stimulation of nerves. Actually, prejunctional muscarinic receptors on inhibitory nerves have been reported (Li & Rand, 1989), but these receptors appear to be coupled to the inhibition of release of NANC inhibitory transmitters. Blocking these receptors would not be expected to reduce responses due to NO.

In summary, the postjunctional effects of neurally-released NO are not apparent in guinea-pig taenia coli when atropine is present. This implies muscarinic regulation of NO release or muscarinic regulation of another excitatory substance, such as tachykinins, that, when blocked, masks the postjunctional effects of NO. These data, together with previous studies, suggest a possible regulatory role for NO in enteric neurotransmission that may be more prominent in some species or tissues than others, e.g. canine colon versus guinea-pig taenia coli.

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