



Pharmacological evidence that nitric oxide may be a retrograde messenger in the enteric nervous system

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- 1 The effects of inhibition of nitric oxide synthase on neuro-neuronal and neuromuscular transmission during motility reflexes in the small intestine of the guinea-pig were examined.
- 2 Isolated segments of intestine were secured in a three chambered organ bath so that different parts of the reflex pathways could be independently exposed to drug-containing solutions. Reflexes were evoked by distension or compression of the mucosa in two adjacent chambers and reflex responses were recorded from the circular muscle with intracellular microelectrodes in the third chamber. Thus, the actions of drugs at connections between sensory neurones and interneurons, between interneurons and other interneurons and at motor neurones could be distinguished.
- 3 N^G-monomethyl-L-arginine (L-NMMA; 100 μM), an inhibitor of nitric oxide synthase, did not affect the ascending excitatory reflex when added to either the central stimulation chamber or the recording chamber.
- 4 In contrast, L-NMMA (100 μM) enhanced the descending inhibitory reflex when added to the chamber in which stimuli were applied. This effect was prevented by prior exposure to L-arginine (100 μM), which had no effect by itself. Conduction of reflexes between the stimulus chamber and the recording chamber was unaffected by the presence of L-NMMA in an intervening chamber.
- 5 L-NMMA (100 μM) added to the recording chamber depressed the descending inhibitory reflex, an effect that was prevented by previous exposure to L-arginine.
- 6 The nitric oxide donor, sodium nitroprusside (100 μM), added to the stimulus chamber, depressed both ascending excitatory and descending inhibitory reflexes. When added to the middle chamber, sodium nitroprusside had no effect on conduction of reflexes through this chamber.
- 7 It is deduced that nitric oxide, released from the cell bodies of descending interneurons, suppresses transmission from synaptic connections made with them by enteric sensory neurones.

Keywords: Nitric oxide; intestinal motility; neurotransmitters; enteric nervous system; peristaltic reflex

Introduction

Over the last few years it has become clear that nitric oxide (NO) is almost certainly a transmitter of inhibitory motor neurones in the gastrointestinal tract (Stark & Szurszewski, 1992; Sanders & Ward, 1992). However, nitric oxide synthase (NOS) is not confined to neurones innervating muscle. It is found in many axons that terminate on the cell bodies and/or dendrites of neurones (Vincent & Kimura, 1992), although roles of NO in neuro-neuronal communication are not well defined by present experimental data.

In the guinea-pig gastrointestinal tract, immunohistochemical studies have shown that NOS is in inhibitory motor neurones innervating circular muscle, and in interneurons that run anally along the intestine and provide terminals to other myenteric ganglia (Costa *et al.*, 1992b; McConlogue & Furness, 1993). However, NOS-immunoreactivity is not found within neurones that have been identified electrophysiologically as sensory neurones, the circumferentially projecting AH/Dogiel type II neurones (unpublished observation). The NOS-containing interneurons are a subset of descending interneurons found within the guinea-pig small intestine and are also immunoreactive for several other bioactive substances, including vasoactive intestinal peptide, gastrin releasing peptide and dynorphin (Costa *et al.*, 1992a).

Mechanical stimulation of the intestinal wall evokes stereotyped reflexes consisting of excitation oral to the point of stimulation (ascending excitation) and relaxation anal to

the point of stimulation (descending inhibition) (Bayliss & Starling, 1899; Smith *et al.*, 1990; Yuan *et al.*, 1991). The neuronal pathways responsible for these reflexes include mechanosensitive enteric sensory neurones which excite either ascending or descending interneurons. The respective interneurons then excite either excitatory motor neurones or inhibitory motor neurones (Bornstein *et al.*, 1991a).

In this study, we have examined whether the descending interneurons that contain NOS, and presumably release NO, modulate neurotransmission in the reflex pathways that control intestinal motility. To distinguish the sites of action of NO, a three chambered organ bath was used. Each chamber was separately perfused and reflexes were evoked with mechanical stimuli (distension or compression of the mucosal villi (Yuan *et al.*, 1991; 1994)) in the central chamber and in one end chamber. The reflex responses were recorded intracellularly from the circular muscle in the chamber at the other end of the preparation. This arrangement allowed drugs to be applied selectively to the region of the active sensory neurones, to a region in which the only active synapses would be those of interneurons and to a region containing the motor neurones supplying the circular muscle (Yuan *et al.*, 1994).

Methods

In all experiments isolated segments of small intestine from adult guinea-pigs of 200–300 g, were used that were killed by a blow to the head and cutting the carotid arteries.

The dissection, superfusing solutions, recording procedures and methods for stimulation by distension or compression of

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the mucosa are described elsewhere (Yuan *et al.*, 1991; 1994). Opened segments were pinned, mucosa uppermost, in a three chambered organ bath in which it was possible to superfuse separately different regions of the intestine (Yuan *et al.*, 1994). The partitions in this bath were separated by 10 mm and each partition was 1 mm thick, the central chamber had a volume of 1 ml and the outer chambers volumes of 3 ml. Set into the base of the bath were 2 distending balloons (11 mm apart): one in the central chamber and the other at an end. Mechanical stimuli, distension and/or compression of the mucosal villi (Yuan *et al.*, 1991), were applied to the intestinal wall in two of the chambers, while intracellular recordings were made from the circular muscle in the third with the distance between the centre of the nearer stimulation balloon and the recording site being 11 mm (Figure 1). The sensory neurones typically project less than 1 mm along the intestine (Bornstein *et al.*, 1991b), so stimuli applied in one chamber would excite only sensory neurones in that chamber and conduction of reflex activity between chambers would be via interneurons. Intracellular recordings were made from either the oral end or the anal end of the intestinal segment, so that both the ascending excitatory and the descending inhibitory pathway were studied (Yuan *et al.*, 1991). Each chamber was perfused separately. Nicardipine (3 μM) was present throughout each experiment to reduce spontaneous and neurogenic contractions which might dislodge the intracellular recording electrodes (Smith *et al.*, 1990).

Drugs were made up fresh each day in physiological saline and added to either the central stimulation chamber or the recording chamber by altering the perfusate reservoir. Thus, drugs were applied to specific points within each reflex pathway. In a previous study using this organ bath arrangement, it was found that the responses to reflex stimulation (distensions and mucosal compressions each lasting 9 s) remained reproducible over at least 30 min following the first stimulus (Yuan *et al.*, 1994). Accordingly, drugs were added to the relevant chamber immediately following the initial control responses and their effects were tested 15 min later. The drugs were then washed out, and responses were again recorded after another 15 min. In all experiments, the durations of the stimuli were 9 s.

Data were analysed by measuring the maximum amplitudes of the excitatory junction potentials (i.j.ps) and inhibitory junction potentials (i.j.ps). Data are expressed as mean \pm standard error from at least 4 preparations. The significance of any differences was determined by a paired *t* test comparing the initial control with the response in the presence of a drug; differences were taken to be significant when $P < 0.05$. Washout results were used to determine whether effects were reversible.

Results

Compound e.j.ps were recorded in the circular muscle oral to the stimulus when the mucosa was distorted by compression or the intestine was distended. Compound i.j.ps were evoked anal to these stimuli. The different stimuli were delivered 2 min apart, so that any interactions between the reflexes were prevented (Yuan *et al.*, 1992).

Inhibition of NOS had markedly different effects on the ascending and descending reflex pathways. The NOS inhibitor L-NMMA (100 μM) (Moncada *et al.*, 1991) added to the chamber from which responses were evoked, to the chamber through which responses were conducted or to the chamber in which recordings were made, had no significant effect on compound e.j.ps recorded from the circular muscle oral to either stimulus (Figure 2). In contrast, L-NMMA altered the responses to activation of the descending inhibitory pathway, with its effect depending on the site of application.

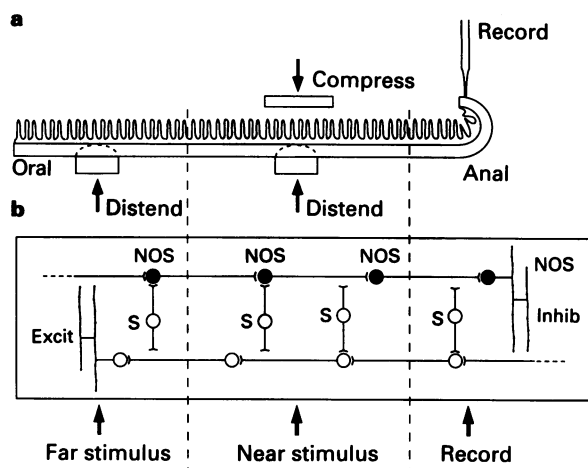


Figure 1 (a) Schematic diagram of the organ bath used in this study. Divisions between chambers are shown by dashed lines. (b) The neuronal circuit hypothesized to mediate motility reflexes in guinea-pig ileum (Bernstein *et al.*, 1991a). Chains of interneurons run orally (ascending interneurons) or anally (descending interneurons). At each level, interneurons receive input from local sensory neurones (S) and provide output to excitatory (ascending pathway) or inhibitory motor neurones supplying the circular muscle (descending pathway). Some descending interneurons and inhibitory motor neurones (filled cell bodies) contain NO synthase (NOS) (Costa *et al.*, 1992b). Drugs in the central chamber act on sensory neurones and interneurons in this chamber, but only on interneurons in pathways excited from the distant stimulation chamber. Drugs in the recording chamber act on transmission to motor neurones and on their transmission to the muscle.

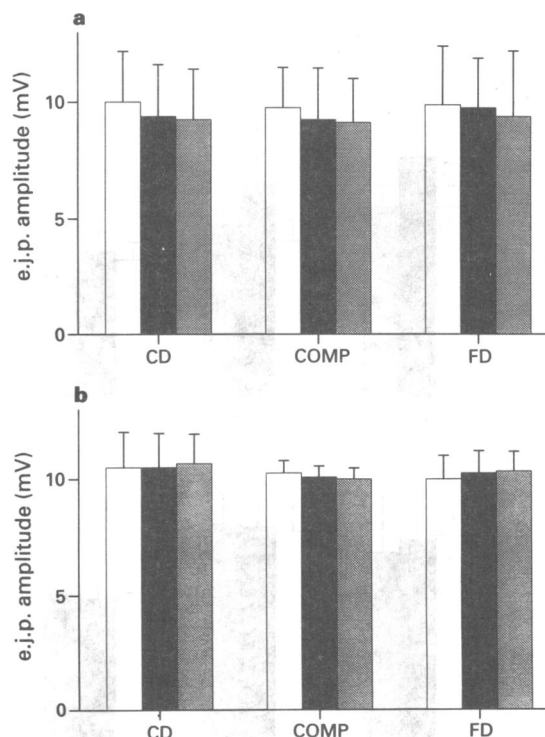


Figure 2 The NO synthase (NOS) inhibitor, N^G-monomethyl-L-arginine (L-NMMA; 100 μM) did not affect the ascending pathway when applied in the central (a) or the recording chamber (b). CD, distension in the central stimulation chamber; COMP, mucosal compression in the central chamber; FD, distension in the far chamber. Stimuli applied in the far chamber evoke reflexes which must propagate through the central chamber to be recorded, thus drugs in the central chamber would act on interneurons in the pathways excited from the far chamber. Open columns, controls; solid columns, responses in L-NMMA; and cross-hatched columns responses after washout.

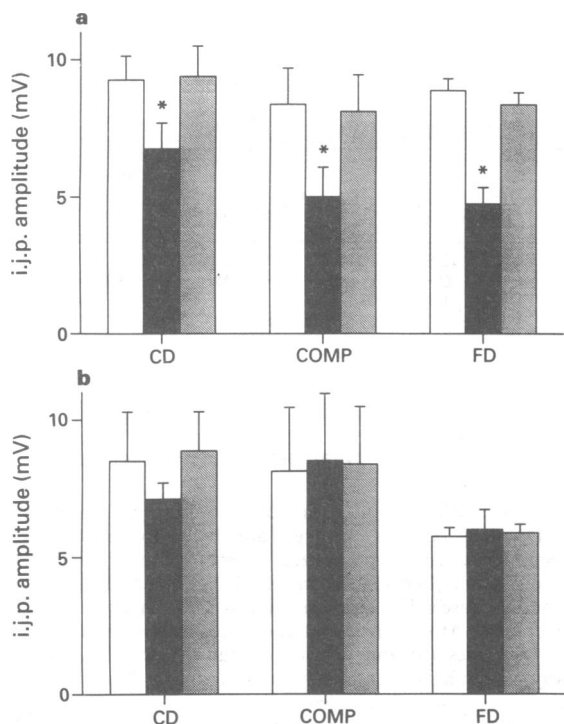


Figure 3 The effects of N^G-monomethyl-L-arginine (L-NMMA 100 μ M) in the recording chamber on descending inhibitory reflexes in control solutions (a) and when L-arginine (100 μ M) was present throughout the experiment (b). CD, distension in the central stimulation chamber; COMP mucosal compression in the central chamber; and FD distension in the far chamber. Open columns, controls; solid columns, responses in L-NMMA and cross-hatched columns, responses after washout. *Significantly different from control ($P < 0.05$; paired t test).

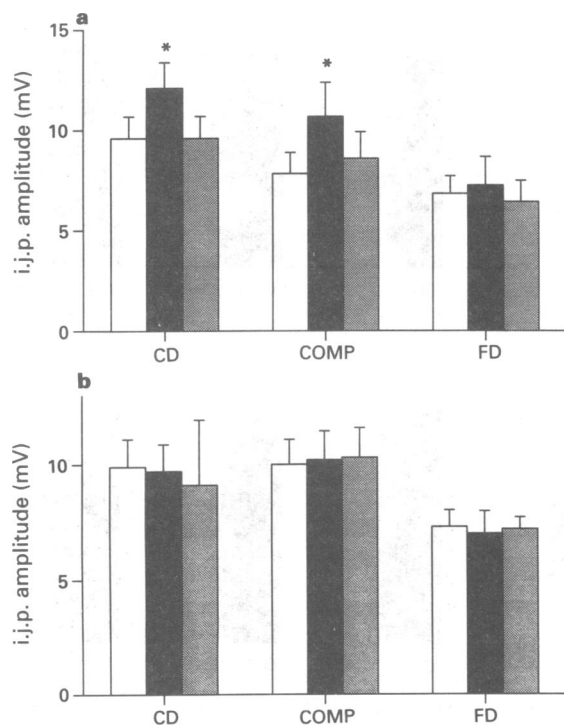


Figure 4 The effects of N^G-monomethyl-L-arginine (L-NMMA 100 μ M) in the central stimulation chamber on descending inhibitory reflexes in control solutions (a) and when L-arginine (100 μ M) was present throughout the experiment (b). CD, distension in the central stimulation chamber; COMP mucosal compression in the central chamber; and FD distension in the far chamber. Open columns, controls; solid columns, responses in L-NMMA and cross-hatched columns, responses after washout. *Significantly different from control ($P < 0.05$; paired t test).

When added to the recording chamber, L-NMMA (100 μ M) depressed compound i.j.ps evoked by either distension or compression of the mucosal villi (Figure 3a). When L-arginine (100 μ M), the normal substrate for NOS, was added to this chamber it produced no change in the reflex (not illustrated). The depression produced by L-NMMA, however, was completely abolished when L-arginine, which opposes the effect of synthase inhibition (Moncada *et al.*, 1991), was present throughout the experiments (Figure 3b).

Addition of L-NMMA (100 μ M) to the central chamber enhanced descending reflexes evoked by stimuli applied in this chamber (Figure 4a). L-Arginine (100 μ M) had no effect by itself, but prevented the enhancement produced by L-NMMA (Figure 4b).

Conduction of reflexes through the central chamber was unaffected by L-NMMA (Figure 4a). In these experiments, reflexes were initiated from the oral end chamber, recordings were made in the anal end chamber and the L-NMMA was placed in the central chamber.

While the effects of inhibition of endogenous NO synthesis were confined to the descending pathway, the effects of the NO donor sodium nitroprusside (SNP) were not (Figure 5). When reflexes were elicited from the central chamber and recordings were made in the adjacent chamber, SNP (100 μ M) added to the central chamber depressed both ascending and descending reflexes evoked by mucosal compression or by distension (Figure 5). There was no effect of SNP on conduction of either ascending or descending reflexes along the intestine in experiments in which the end chambers were used for stimuli and recording and SNP was added to the chamber in between (Figure 5).

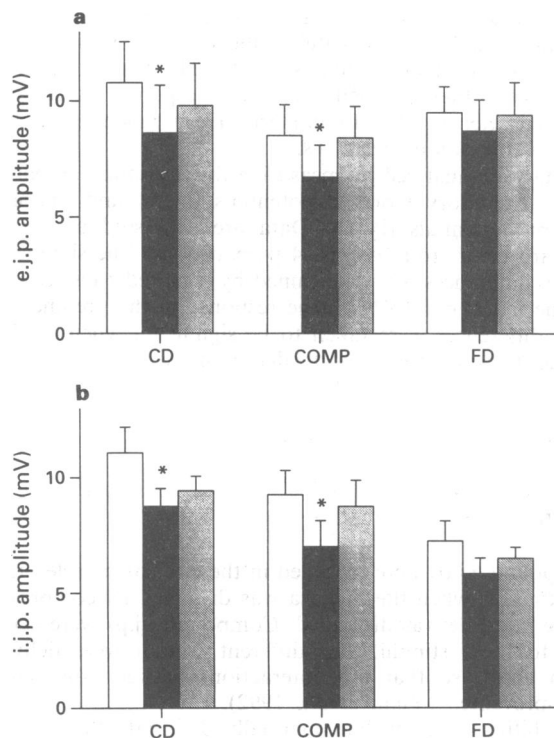


Figure 5 The effect of a NO donor, sodium nitroprusside (100 μ M), added to the central stimulation chamber on (a) the ascending reflex ($n = 5$) and (b) the descending reflex ($n = 6$). CD, distension in the central stimulation chamber; COMP mucosal compression in the central chamber; and FD distension in the far chamber. Open columns, controls; solid columns, responses in L-NMMA; and cross-hatched columns, responses after washout. In each case, the NO donor depressed reflexes evoked from the central chamber, but had no significant effect on transmission of reflexes through this chamber. *Significantly different from control ($P < 0.05$; paired t test).

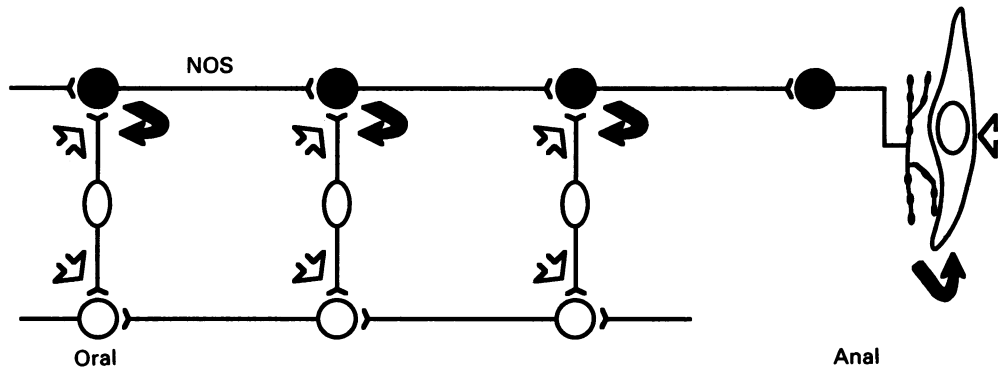


Figure 6 Potential sites of action of endogenous (solid arrow) and exogenous (open arrow) NO in the reflex pathways. Neurons that contain NOS have filled cell bodies, those lacking NOS have open cell bodies. Blockade of the synthesis of NO appears to depress inhibitory transmission from inhibitory motor neurones to the circular muscle, but has no effect on excitatory transmission. It also appears to enhance transmission from sensory neurones to interneurons in the reflex pathways. NO supplied by the donor, SNP, depressed transmission from sensory neurones to both ascending and descending pathways. However, the only neuronal sources of endogenous NO that could alter sensory to interneurone transmission are the cell bodies of NOS-containing descending interneurons.

Discussion

The present observations indicate that NO can modulate intestinal reflexes via an action on neurones, in addition to its role in inhibitory neuromuscular transmission (Figure 6). Immunohistochemical studies indicate that NOS is found within descending interneurons and inhibitory motor neurones, but not in enteric sensory neurones (Costa *et al.*, 1992b). The failure, however, of either L-NMMA or SNP to alter transmission of the reflexes along the intestine suggests that, despite the presence of this enzyme in descending interneurons, NO is not involved in orthograde transmission from such neurones during descending inhibitory reflexes. Nevertheless, the enhancement of descending, but not ascending, reflexes by L-NMMA suggests that NO released from the descending interneurons inhibits transmission at some point in the descending reflex pathway. As this effect is observed when L-NMMA is in the stimulation chamber, the most likely sites of action are the synapses between sensory neurones and interneurons. If NO acts at these synapses, it would be as a retrograde transmitter, released by the interneurone cell body, or dendrites, and acting back on the synaptic endings of the sensory neurone to inhibit transmission (Figure 6). Interestingly, NO from the donor, SNP, inhibits both ascending and descending reflexes, when added to the stimulation chamber. This suggests that responsiveness to exogenous NO occurs at sensory neurone to interneurone synapses, but not interneurone to interneurone synapses of both ascending and descending reflexes.

The conclusion that SNP acts at sensory to interneurone connections is consistent with the results of Kunze *et al.* (1993). They found that transmission from sensory neurones to descending interneurons is largely via slow excitatory synaptic potentials (e.p.s.ps), and it has been reported that NO (from SNP) selectively depresses non-cholinergic slow e.p.s.ps in myenteric neurones (Tamura *et al.*, 1993). Neither the present results nor those of Tamura *et al.* (1993) can exclude the possibility that the effect of NO is on the intracellular mechanism responsible for generation of the slow e.p.s.ps within the NOS-immunoreactive interneurons, rather than being on the release of neurotransmitter from sensory neurones. Such an intracellular site of action would account for the specificity of the NOS inhibitor for the descending pathway.

The reduction of the amplitudes of the compound i.j.ps seen when L-NMMA was added to the recording chamber provides further evidence that NO plays a significant role in transmission from inhibitory motor neurones to the circular muscle. The simplest explanation for this observation is that

L-NMMA interferes with the synthesis and release of NO from the inhibitory motor neurones, thereby reducing its effects on the circular muscle. This is consistent with the results of a variety of other workers who have come to similar conclusions as a result of experiments employing electrical stimulation to excite the inhibitory motor neurones (for reviews see Sanders & Ward, 1992; Stark & Szurszewski, 1992). However, this interpretation conflicts with the observations of Smith and colleagues, who found that the compound i.j.ps evoked in descending reflexes in the guinea-pig small intestine were abolished by apamin (Smith & Furness, 1988; Smith *et al.*, 1990). Electrically evoked i.j.ps in this preparation have two components: an initial fast component, which is blocked by apamin and a later, slower component, which is insensitive to apamin, but is blocked by inhibitors of NOS (Niel *et al.*, 1983; Lyster *et al.*, 1992). The apamin-sensitive component is, if anything, enhanced by inhibition of NOS (Lyster *et al.*, 1992). The compound i.j.ps evoked by distension reach their peak amplitude within 0.5 s of the beginning of the response (Smith *et al.*, 1990); accordingly, the amplitudes measured in the present experiments almost certainly correspond to the fast component of the electrically evoked i.j.ps. Thus, there is a discrepancy between the effects of inhibition of NOS on the electrically evoked fast i.j.ps and reflexly evoked i.j.ps, which might suggest that, although NO does not play a role in transmission between interneurons, it may play a role in transmission between interneurons and the inhibitory motor neurones.

The difficulty of interpreting the results of NOS inhibition in the recording chamber highlights a general problem when drugs which may act at several sites to have opposing effects are applied to all classes of neurones simultaneously. A knowledge of effects of NOS inhibitors at specific sites does not allow the final outcome for behaviour of the whole organ to be readily predicted when inhibition of NOS facilitates one arm of the reflex arc, and depresses, but does not abolish, the other. Nor does application of a NOS inhibitor to the whole organ allow the sites of action to be clearly deduced. The final outcome in each case will depend on the relative roles of the different interacting parts of the system.

This work was supported by a grant from SmithKline Beecham Australia and a grant from the National Health and Medical Research Council of Australia. We thank Mr Jim Pringle for his help in constructing the organ bath and Dr Tom Cocks for helpful comments on the manuscript.

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(Received June 28, 1994

Revised September 5, 1994

Accepted September 28, 1994).