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Modulation by nitric oxide of spontaneous motility of the rat isolated duodenum: role of tachykinins

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1 Incubation of proximal segments of the rat isolated duodenum with N^G-nitro-L-arginine (L-NOARG; 3-100 μM) produced a concentration-dependent increase in both resting tone and the amplitude of the spontaneous contractions. These effects were attenuated by concurrent incubation with L-arginine (1 mM) but not D-arginine (1 mM).

2 These changes in resting tone and motility induced by L-NOARG (30 μM) were substantially reduced by concurrent incubation with tetrodotoxin (1 μM) or hexamethonium (10 μM), implicating the involvement of a local neuronal response.

3 The L-NOARG-induced increase in duodenal motility was not, however, inhibited by atropine (1 μM), guanethidine (6.4 μM) phentolamine (1 μM), or indomethacin (10 μM), indicating a non-cholinergic, non-adrenergic and non-prostanoid-mediated contractile response.

4 The NK_1/NK_2 tachykinin receptor antagonist, (D-Pro^2, D-$Trp^{7,9}$ substance P, 1-10 μM), and the NK_2-receptor antagonists, MEN 10,207 and MEN 10,376 (1-5 μM), concentration-dependently reduced the effect of L-NOARG (30 μM) on spontaneous duodenal motility.

5 The resting tone and amplitude of the spontaneous contractions was likewise increased by incubation with N^G-monomethyl-L-arginine (L-NMMA; 100-1000 μM). However, incubation with L-NMMA (100 μM) attenuated the actions of more potent L-NOARG (30 μM) on resting motility.

6 Administration of *E.coli* endotoxin (3 mg kg^{-1}, i.v.) to the rat 5 h prior to tissue removal, at a time of known induction of NO synthase, reduced the amplitude of spontaneous contractions of the isolated duodenum, an effect inhibited by pretreatment of the rats with dexamethasone (1 mg kg^{-1}) 2 h prior to endotoxin challenge.

7 These findings indicate a role of endogenous NO in the modulation of spontaneous tone and motility in the rat duodenum. Induction of NO synthase may result in a reduction in spontaneous motility of the tissue. By contrast, inhibition of constitutive NO biosynthesis unmasks a contractile response that is neuronally mediated and involves tachykinin NK_2 receptors.

Keywords: Duodenal motility; nitric oxide synthase; NO; tachykinins; NK_2 receptors; NANC contractile response

Introduction

Nitric oxide (NO), synthesized from L-arginine by a calcium-dependent constitutive NO synthase located in neurones (see Moncada *et al.*, 1991), has been demonstrated to be a mediator of non-adrenergic, non-cholinergic (NANC) relaxation following neuronal stimulation in a wide range of intestinal smooth muscle preparations. These tissues include rat and guinea-pig stomach, duodenum or ileum, and the canine ileocecal junction or duodenum (Bult *et al.*, 1990; Toda *et al.*, 1990; Li & Rand, 1990; Desai *et al.*, 1991; Irie *et al.*, 1991; Boeckxstaens *et al.*, 1991a,b; Kanada *et al.*, 1992; Lefebvre *et al.*, 1992; Ward *et al.*, 1992; Wiklund *et al.*, 1993; Middleton *et al.*, 1993; Martinez-Cuesta *et al.*, 1995), as well as the human ileum and colon (Maggi *et al.*, 1991; Burleigh, 1992; Boeckxstaens *et al.*, 1993).

In other studies, incubation of strips of rat gastric forestomach and colon, canine ileocolonic junction and colon or human ileum and colon with NO synthase inhibitors such as N^G-monomethyl-L-arginine (L-NMMA), N^G-nitro-L-arginine (L-NOARG) or N^G-nitro-L-arginine methyl ester (L-NAME) increased basal tension of these preparations (Li & Rand, 1990; Boeckxstaens *et al.*, 1991a,b; 1993; Ward *et al.*, 1992; Niklasson *et al.*, 1992; Maggi *et al.*, 1992). In studies *in vivo*, intravenous administration of L-NAME did not influence resting intragastric pressure (Lefebvre *et al.*, 1992), yet caused a substantial increase in tone and spontaneous motility in the jejunum of the anaesthetized rat, an effect reversed by administration of L-arginine or atropine (Calignano *et al.*, 1992). These latter effects thus suggest a role for endogenous NO not only as a NANC neurotransmitter following stimulation, but in the regulation of resting motility in the small intestine, interacting with other local mediators.

In the present study, the mechanisms by which endogenous NO modulates spontaneous tone and motility in the rat isolated proximal duodenum, have been investigated. Thus, the nature of the contractile responses to L-NOARG has been explored by use of a variety of antagonists, including those affecting the neurokinin NK_1 and NK_2 receptors. In addition, to evaluate the actions of endogenous high concentrations of NO produced as a consequence of expression of inducible NO synthase (iNOS) in gastro-intestinal tissue (Salter *et al.*, 1991; Boughton-Smith *et al.*, 1993), the actions of endotoxin challenge *in vivo* on the subsequent motility *in vitro* of the duodenal tissue removed 5 h later, have also been studied.

Methods

Tissue preparation and isometric tension recording

Segments (2.5 cm) of proximal duodenum were removed from male Wistar rats (250-300 g) after exsanguination. Two tis-

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sues from each rat were rinsed and were set up in organ baths filled with 25 ml of modified Krebs' solution (composition in mM: NaCl 118, KCl 4.8, $MgSO_4$ 1.2, KH_2PO_4 1.2, $CaCl_2$ 1.25, $NaHCO_3$ 24 and glucose 11). The solution was maintained at 32°C (a temperature known to produce a more consistent basal motility) and aerated with a mixture of 95% O_2 and 5% CO_2. Mechanical activity of the duodenal segments, under a resting tension of 1 g, was measured isometrically with a Grass FT-03 transducer connected to a Lectromed MT 8P coupler and Rikadenki R-64 recorder.

Experimental protocols

After 1 h equilibration period, the effect of L-NOARG (3–100 μM), was studied on the spontaneous activity exhibited by the duodenal segments over a 15 min period. In studies on the actions of L-arginine (1 mM) or its enantiomer D-arginine (1 mM), these were added to the organ bath 5 min prior to L-NOARG administration. Each tissue was exposed to only a single concentration of L-NOARG.

For the evaluation of the possible mediator involved in the contractile effect of L-NOARG, experiments were performed in the presence of atropine (1 μM), guanethidine (6.4 μM), indomethacin (10 μM), tetrodotoxin (1 μM), hexamethonium (10 μM), phentolamine (1 μM) and propranolol (1 μM) as well as the tachykinin NK_1/NK_2 receptor antagonist (D-Pro^2, D-$Trp^{7,9}$ substance P; 1–10 μM) and the NK_2 receptor antagonists MEN 10,376 (0.05–5 μM) and MEN 10,207 (1–5 μM). These drugs were added to the organ bath, 15 min prior to the addition of L-NOARG.

The amplitude of spontaneous motility was also measured immediately after the 1 h *in vitro* equilibration period, following the administration of *E.coli* endotoxin (3 mg kg^{-1}, i.v.) to rats, 5 h before removal of the duodenal tissue. The effect of pretreatment of the rats with dexamethasone (1 mg kg^{-1}, s.c.), 2 h before endotoxin, was also investigated. In control studies, the vehicle for endotoxin, isotonic saline, was administered to the animals.

Drugs

The drugs used were L- and D-arginine hydrochloride, atropine sulphate, atenolol, guanethidine monosulphate, hexamethonium bromide, indomethacin, lipopolysaccharide from *E. coli* (LPS, serotype 0111:B4), N^G-nitro-L-arginine (L-NOARG), phentolamine hydrochloride, propranolol hydrochloride, substance P, (D-Pro^2, D-$Trp^{7,9}$) substance P, tetrodotoxin and the other reagents (Sigma Chemical Company, Poole, Dorset); dexamethasone sodium phosphate (Merck), N^G-monomethyl-L-arginine acetate (Wellcome Foundation Ltd, Beckenham, Kent), [Tyr^5, D-$Try^{6,8,9}$, Arg^{10}] neurokinin A4-10 (MEN 10,207) and [Tyr^5, D-$Trp^{6,8,9}$, Lys^{10}] neuokinin 4–10 (MEN 10,376) (Peninsula Labs Europe, Merseyside). All drugs were dissolved in isotonic saline except indomethacin, which was dissolved in 5% w/v sodium bicarbonate solution, and LPS which was dissolved in 0.1% bovine serum albumin in saline.

Statistical analysis

The amplitude of the spontaneous motility of the duodenum was determined by averaging the maximal contractions taken over the 15 min experimental period, prior to and following drug administration. Changes in resting tone were determined from the mid-point of the pre- and post-treatment amplitude values, averaged over the 15 min experimental periods. The effect of L-NOARG on the motility of duodenal segments was expressed as changes (mg) in tone and the mean amplitude of spontaneous contractions. The values are shown as mean ± s.e.mean for the numbers of rats indicated (n). Student's t test for unpaired observations was used as appropriate, where P values of less than 0.05 were considered to be significant.

Results

Effects of L-NOARG on resting tone and motility

After a 1 h equilibration period, the duodenal tissue exhibited spontaneous motility, the amplitude of which ranged from 400 to 1100 mg, with a mean value of 894 ± 62 mg (n = 36). L-NOARG (3–100 μM) induced an increase in resting tone and in the amplitude of spontaneous motility of duodenal segments (Figure 1), effects that were sustained for at least 15 min and which were concentration-dependent (Figure 2a and b). Preincubation with L-arginine (1 mM) but not D-arginine (1 mM) prevented this effect of L-NOARG, as shown in Figure 2a and b. L-Arginine (1 mM) alone had no effect on the spontaneous activity of duodenum (n = 5).

The increase in resting tone and amplitude in the duodenal segments caused by L-NOARG (30 μM) was substantially attenuated by concurrent incubation with the neurotoxin, tetrodotoxin (1 μM) or with the ganglion blocker, hexamethonium (10 μM), as shown in Table 1, in concentrations that had no consistent or significant action on resting tone (Δ1 ± 1 and 30 ± 23 mg, n = 13 and 17 respectively) or amplitude (Δ2 ± 1 and 1 ± 1 mg respectively). However, these effects of L-NOARG were not significantly altered by incubation with guanethidine (6.4 μM), phentolamine (1 μM) or indomethacin (10 μM) as shown in Table 1, in concentrations that also had no consistent or significant effect on resting tone (Δ30 ± 30, 1 ± 2, 2 ± 1 mg n = 6, 9 and 8 respectively) or amplitude (Δ1 ± 1, 2 ± 1 and 1 ± mg respectively).

Incubation with atropine (1 μM), which significantly reduced resting tone and amplitude (by 223 ± 45 and 310 ± 102 mg, n = 8; P < 0.05), had no significant effect on the responses to L-NOARG (30 μM), as shown in Table 1.

Incubation of the duodenal segments with D-Pro^2, D-$Trp^{7,9}$ substance P (1–10 μM) substantially inhibited the contractile responses to L-NOARG (30 μM), the increase in tone being near-maximally reduced and the increase in amplitude being abolished (Figure 3a and b). Likewise, the contractile response to L-NOARG was dose-dependently reduced by incubation with MEN 10,207 (1–5 μM) or MEN 10,376 (0.05–5 μM), with both the increase in tone and amplitude being abolished with the highest concentrations of each antagonist (Figure 3a and b).

These tachykinin antagonists produced inconsistent and transient actions on duodenal resting motility. Thus, D-Pro^2, D-$Trp^{7,9}$ substance P (10 μM) produced a transient increase in tone (475 ± 142 mg, n = 6), with irregular changes in amplitude, which returned to basal levels 2–5 min after addition to the organ bath, prior to the addition of L-NOARG. Incubation with MEN 10,376 (5 μM) caused a transient decrease in tone (−663 ± 101 mg, n = 6), which returned to resting levels within 5 min. By contrast MEN 10,207 (5 μM) produced an initial fall in tone (−167 ± 45 mg, n = 6), which rapidly reversed to an increase of tone (400 ± 76 mg), but returned to resting levels within 5 min.

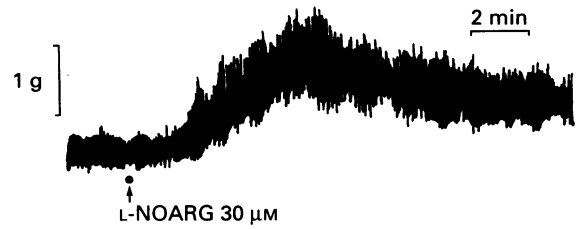

Figure 1 Effects of L-NOARG (30 μM) on resting tone (mg) and spontaneous contractions of the rat isolated duodenum.

Interactions between L-NMMA and L-NOARG

L-NMMA (100 and 1000 μM), caused a concentration-dependent increase in the spontaneous activity of duodenal segments, but this effect was substantially smaller than that observed in response to L-NOARG (30 μM) as shown in Figure 4a and b. Moreover, in the presence of L-NMMA (100 μM), the increase in the resting tone and amplitude induced by L-NOARG (30 μM) was inhibited by $75 \pm 9\%$ and $53 \pm 9\%$ respectively ($P<0.01$ and $P<0.05$, $n=5$).

Effect of endotoxin

Administration of LPS (3 mg kg^{-1}, i.v.) 5 h prior to tissue removal significantly reduced the amplitude of spontaneous duodenal contractions (from 854 ± 62 to 552 ± 22 mg respectively, $n=6$ for each, $P<0.01$). This effect was inhibited by

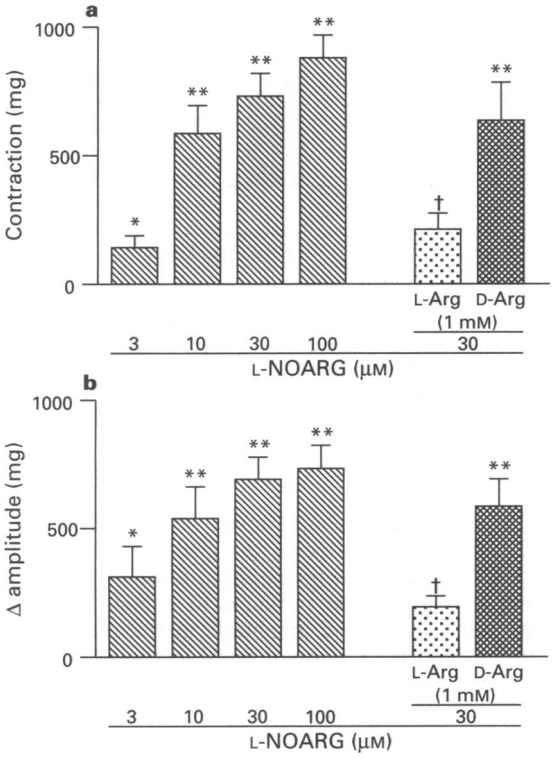

Figure 2 Effect of N^G-nitro-L-arginine (L-NOARG, 3–100 μM) on (**a**) the resting tone and (**b**) the amplitude of the spontaneous contractions of the rat isolated duodenum observed for 15 min after a 1 h equilibration period. A significant increase in tone was observed with each concentration of L-NOARG. L-Arginine (L-Arg) or D-arginine (D-Arg) in a concentration of 1 mM, was added 5 min prior to L-NOARG (30 μM) administration. The results, shown as the increase in tone (mg), and the increase in amplitude (Δmg) are the mean $\pm$ s.e.mean of 10–12 studies for each group, where significant increase from basal is shown as $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ and significant inhibition by L-arginine as $^{\dagger}P<0.001$.

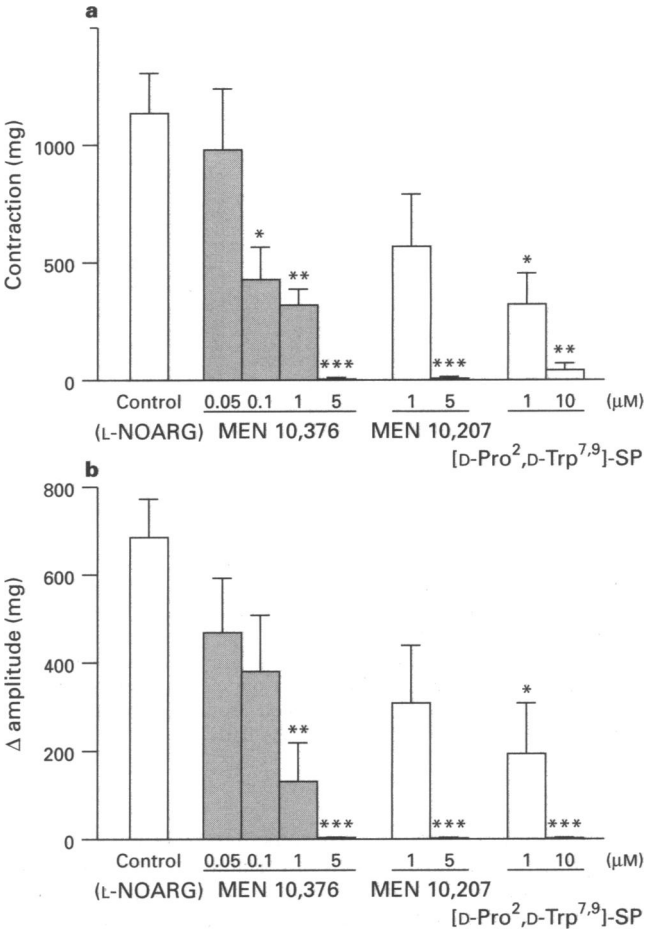

Figure 3 Effect of the NK$_1$/NK$_2$-receptor antagonist D-Pro^2, D-Trp^{7,9} substance P (1–10 μM) and the NK$_2$-receptor antagonists, MEN 10,376 (0.05–5 μM) and MEN 10,207 (1–5 μM) on (**a**) the tone and (**b**) the amplitude of spontaneous contractions in the rat isolated duodenum provoked by N^G-nitro-L-arginine (L-NOARG, 30 μM). Results, shown as the change in tone (mg) and in amplitude (Δmg) are the mean $\pm$ s.e.mean of 6 observations in each group, where significant inhibition of the control response to L-NOARG is given as $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$.

Table 1 Effect of N^G-nitro-L-arginine (L-NOARG; 30 μM) on the tone and the amplitude on spontaneous activity of rat isolated duodenum in presence of different pharmacological agents

Agent	(μM)	(n)	*Contraction* (mg)	Δ *amplitude* (mg)
L-NOARG	(30)	26	1030 ± 102	621 ± 63
+ Atropine	(1)	8	1012 ± 188	785 ± 217
+ Guanethidine	(6.4)	6	1083 ± 202	566 ± 149
+ Phentolamine	(1)	9	1033 ± 228	455 ± 44
+ Indomethacin	(10)	8	900 ± 256	585 ± 98
+ Tetrodotoxin	(1)	13	$123 \pm 54^{***}$	$71 \pm 49^{***}$
+ Hexamethonium	(10)	7	$178 \pm 114^{***}$	$228 \pm 121^{**}$

Results, shown as the contraction (mg) and increase in amplitude (mg) following incubation with L-NOARG (30 μM) and the various agents, are expressed as the mean $\pm$ s.e.mean of (n) studies, where significant change from the control responses to L-NOARG alone is given as $^{**}P<0.01$, $^{***}P<0.001$.

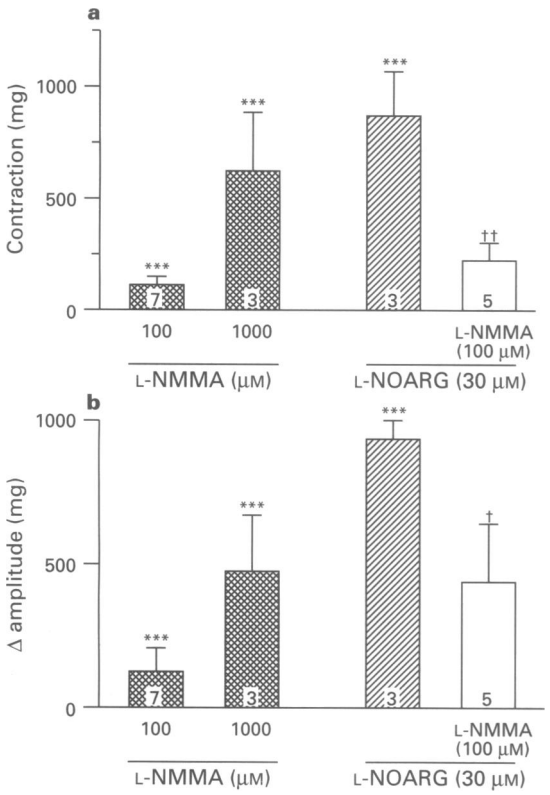

Figure 4 Effect of N^G-mono-methyl-L-arginine (L-NMMA; 100–1000 μM) on (**a**) the resting tone and (**b**) amplitude of contractions in the rat isolated duodenum, on the contractile responses to N^G-nitro-L-arginine (L-NOARG; 30 μM). Results, shown as the contraction (mg) and the increase in amplitude (Δmg) are the mean $\pm$ s.e.mean of (*n*) studies, where significant increases from resting levels are given as $^{***}P<0.001$, and significant reduction from the responses to L-NOARG alone, as $^{\dagger}P<0.05$, and $^{\dagger\dagger}P<0.01$.

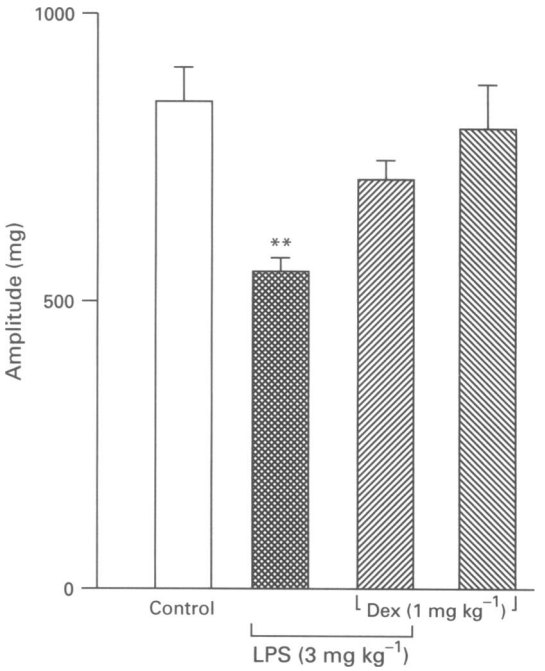

Figure 5 Effects of pretreatment (2 h) with dexamethasone (Dex, 1 mg kg^{-1}, s.c.) on the changes in the amplitude of the spontaneous contractions of the rat isolated duodenum observed 5 h following challenge *in vivo* with endotoxin (LPS, 3 mg kg^{-1}, i.v). Results shown as the amplitude (mg), and the mean $\pm$ s.e.mean of 4–6 observations in each group, where significant reduction is shown as $^{**}P<0.01$.

pretreatment of the rats with dexamethasone (1 mg kg^{-1}, s.c.), 2 h prior to challenge with LPS, the amplitude being not significantly different from the control value (Figure 5). Dexamethasone pretreatment alone had no significant effect on the amplitude of the contractions of the resting tissue (Figure 5).

Discussion

The present study indicates that under resting conditions *in vitro*, the NO synthase inhibitor L-NOARG substantially increases the spontaneous tone and contractions of the rat isolated duodenum, an action reversed by L-arginine but not D-arginine. Thus, NO not only mediates the NANC relaxation (Irie *et al.*, 1991; Martinez-Cuesta *et al.*, 1995), but also modulates the spontaneous motility in the rat isolated duodenum.

The contractile response of the duodenum to L-NOARG was greatly inhibited by tetrodotoxin and by hexamethonium, indicating a neuronal involvement. However, this response was not affected by adrenoceptor blockade, nor by cholinergic blockade with atropine nor by the cyclo-oxygenase inhibitor, indomethacin. These latter findings thus contrast with those in segments of the canine ileum, where the increase in spontaneous tonic and phasic contractile activity provoked by L-NAME was substantially inhibited not only by hexamethonium, but by atropine, indicating a cholinergic component (Daniel *et al.*, 1994). Moreover, in studies in the anaesthetized rat, the increase in resting intraluminal pressure and phasic contractions in the rat small intestine following intravenous administration of L-NAME was inhibited by treatment with atropine (Calignano *et al.*, 1992). However, such inhibitory actions of atropine in the latter study may reflect its observed effect in substantially reducing the resting tone and spontaneous contractions of the preparation, therefore indirectly attenuating the contractile response to L-NOARG. Despite reducing resting tone and amplitude in the present *in vitro* study in the duodenum, atropine failed to abolish the response to L-NOARG. The current findings thus suggest that inhibition of NO synthesis exposes a non-prostanoid, NANC contractile response under these resting conditions in the rat duodenum.

The atropine-resistant contractions of the canine isolated colon to electrical field stimulation have previously been shown to be enhanced in the presence of L-NMMA. This stimulated NANC contractile response was inhibited by the non-selective NK_1/NK_2 tachykinin receptor antagonist, D-Pro^2, D-Trp^{7,9} substance P, as well as by the more-selective NK_2 receptor antagonist, MEN 10,376 (Shuttleworth *et al.*, 1993). In the present study, D-Pro^2, D-Trp^{7,9} substance P, likewise inhibited the increased basal tone and spontaneous contractions of the duodenum brought about by incubation with L-NOARG. Furthermore, both the NK_2-receptor antagonists, MEN 10,207 and MEN 10,376, abolished the increase in duodenal tone and amplitude caused by L-NOARG, implicating the involvement of NK_2-receptor activation.

From studies on the NANC contraction of the guinea-pig duodenum following electrical stimulation, it has been proposed that there may be a co-operativity between NK_1 and NK_2 receptor-mediated responses, (Zagorodynyuk *et al.*, 1995). However, previous pharmacological studies have suggested a predominance of NK_2 over NK_1 receptors in the rat duodenum and that activation of NK_1 receptors is only of minor significance for the contractile responses of this tissue to tachykinins (Rahman *et al.*, 1994). Although it is therefore likely that only stimulation of NK_2 receptors predominates in the current investigation, additional studies will be required to characterize more fully the tachykinin receptors involved, as well as to confirm the identity of the endogenous ligand and its source in the contractile response of the duodenum exposed by L-NOARG. In the circular muscle of the guinea-pig stomach,

NK_2-receptor agonists elicited a contractile response, while inhibiting the relaxation, the production of NO and the release of vasoactive intestinal peptide provoked by NK_1 stimulation (Jin *et al.*, 1993). Such differential actions following tachykinin receptor activation, on the overall functional responses of intestinal tissue will be both species- and tissue-specific. The NK_2-receptor antagonists themselves had no consistent effect on resting motility, with MEN 10,376 causing only a very transient fall in resting tone and MEN 10,207 having a short-lived biphasic action. This makes difficult the identification of a role of endogenous tachykinins in the modulation of duodenal motility. Thus, the possibility that inhibition of neuronal NO synthesis leads to or enhances tachykinin release in the rat duodenum warrants evaluation.

To determine whether the responses to L-NOARG were shared with other NO synthase inhibitors, the effects of L-NMMA were investigated. By comparison with L-NOARG, only high concentrations of L-NMMA elicited a contractile response of the duodenal tissue. Furthermore, a similar interaction between of L-NMMA and L-NOARG have been observed in the bovine retractor penis and rat anococcygeus muscle, where L-NMMA alone had no effect in concentrations up to 1 mM (Martin *et al.*, 1993). It was proposed that L-NMMA could act as a partial inhibitor of neuronal NO synthase, producing a variable blockade but inhibiting the subsequent inhibition by L-NOARG (Martin, 1993). Such a mechanism requires further analysis, but along with the present study, these findings indicate that the use of L-NMMA in studies on nitrergic neuronal responses should be regarded with some caution.

Exogenous NO, released from the NO donor, nitroprusside relaxes the rat isolated duodenum (Irie *et al.*, 1991; Martinez-Cuesta *et al.*, 1995). To evaluate the effects of an excess of endogenous NO, *E. coli* lipopolysaccharide was administered *in vivo* in a dose shown to induce NO synthase in gastrointestinal tissue (Boughton-Smith *et al.*, 1993). Segments of duodenal tissue removed at a time of known expression of iNOS tissue following endotoxin challenge (Salter *et al.*, 1991; Boughton-Smith *et al.*, 1993), showed a significant reduction in the amplitude of the spontaneous contractions. This reduction in basal motility was prevented by pretreatment with dexamethasone before endotoxin challenge, a treatment previously shown to prevent the expression of iNOS in gastrointestinal tissue (Salter *et al.*, 1991; Boughton-Smith *et al.*, 1993). Confirmation of a role for iNOS awaits availability of highly selective iNOS inhibitors devoid of actions on constitutive NOS. These present findings do however suggest that events involving iNOS contribute to the motility dysfunction associated with endotoxaemia.

The cellular source of the NO released under such conditions is not known. The expression of iNOS has been detected in rat intestinal epithelial cells (Tepperman *et al.*, 1993) and may also occur in the intestinal smooth muscle cells. By contrast, NO involved in the physiological modulation of NANC responses in many tissues is considered to be of neuronal origin. In the rat duodenum, NO synthase has been identified in the myenteric plexus by use of immunohistochemical techniques (Bredt *et al.*, 1991). Furthermore, cold storage of duodenal tissue which destroys fine neurones, abolished the nitrergic NANC relaxation to nicotine and substantially reduced the total NO synthase activity in that tissue, indicating a predominantly neuronal location (Martinez-Cuesta *et al.*, 1995). The present observations thus indicate a complex interaction between constitutive NO, presumably of neuronal origin, and the actions of endogenous tachykinins acting predominantly on NK_2 receptors in the modulation of spontaneous motility in the rat isolated duodenum.

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