British Journal of Pharmacology (1996) 117, 717-723

Nitric oxide synthase activity and non-adrenergic non-cholinergic relaxation in the rat gastric fundus

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1 In the presence of atropine (1 μM) and guanethidine (5 μM), electrical field stimulation (EFS, 120 mA, 1 ms, 0.5-16.0 Hz, trains of 2 min) induced frequency-dependent relaxations of 5-hydroxytryptamine (3 μM)-precontracted longitudinal muscle strips from the rat gastric fundus.

2 L-Citrulline concentrations were measured in the incubation medium of precontracted strips before and after EFS to investigate nitric-oxide (NO) synthase activity and its possible relation to non-adrenergic non-cholinergic (NANC) relaxation.

3 Basal NO synthase activity was reflected by the finding of prestimulation levels of L-citrulline of $\approx$30 nM. These levels were unaffected by tetrodotoxin (3 μM) and N^G-nitro-D-arginine methyl ester (D-NAME, 100 μM), slightly reduced by a calcium-free medium and halved by N^G-nitro-L-arginine methyl ester (L-NAME, 100 μM).

4 EFS evoked significant, frequency-dependent increases in bath levels of L-citrulline at all frequencies tested. The increases evoked by 16-Hz EFS were abolished by tetrodotoxin (3 μM), a calcium-free medium and L-NAME (100 μM) but not by D-NAME (100 μM).

5 L-NAME (0.1 μM-1.0 mM) produced significant reduction of 4-Hz EFS-induced L-citrulline production (100% inhibition at 10 μM), but had less marked effects on basal production ($\approx$50% reduction at 100 μM) and 4-Hz EFS-induced NANC relaxation ($\approx$50% reduction at 1 mM).

6 L-Arginine (1 mM), but not D-arginine (1 mM), increased basal L-citrulline levels and reversed the inhibitory effect of L-NAME (10 μM).

7 These findings represent clear biochemical evidence of both basal and EFS-stimulated NO synthase activity in the rat gastric fundus.

Keywords: Nitric oxide (NO); L-citrulline; nitric oxide (NO) synthase; non-adrenergic non-cholinergic (NANC) relaxation; rat gastric fundus

Introduction

An awareness of the roles played by nitric oxide (NO) in neurotransmission and other physiological processes began to emerge after Palmer *et al.* (1987) demonstrated that the biological activity of endothelium-derived relaxing factor in blood vessels could be attributed to the release of this gas. NO was subsequently shown to be produced during a reaction catalyzed by a constitutive, calcium and calmodulin-dependent enzyme that converted L-arginine into equimolar amounts of NO and L-citrulline (Palmer & Moncada, 1989; Moncada & Palmer, 1990). This enzyme, termed NO synthase, was later isolated from the rat cerebellum (Bredt & Snyder, 1990) and a purified form was used to raise specific antisera. This development led to the immunohistochemical demonstration of NO in the vascular endothelium and in neurones of both the central and peripheral nervous systems (Bredt *et al.*, 1990), including those of the myenteric plexus (Llewellyn-Smith *et al.*, 1992).

NO is now known to be involved in non-adrenergic non-cholinergic (NANC) neurotransmission in the gastrointestinal, respiratory, genitourinary and vascular systems (for reviews, see Rand, 1992; Sanders & Ward, 1992). Its putative role in NANC relaxation of various types of smooth muscle is based primarily on observations that this response is diminished by L-arginine analogues such as N^G-monomethyl-L-arginine (L-NMMA), N^G-nitro-L-arginine (L-NOARG) and N^G-nitro-L-arginine methyl ester (L-NAME), all of which inhibit NO synthase. Direct demonstration of NO release is extremely difficult because in physiological solutions the gas is rapidly transformed into NO_2^- and NO_3^-. For this reason, NO synthesis and/or release are evaluated indirectly by use of bioassays or measurement of [^{3}H]-L-citrulline production from [^{3}H]-L-arginine or formation of NO_2^- and NO_3^- (Knowles & Moncada, 1994).

In the rat gastric fundus, L-NMMA, L-NOARG and L-NAME inhibit the relaxant response induced by electrical field stimulation (EFS) of intramural neurones, and this inhibition can be reversed in an enantiomeric manner by L-arginine (Li & Rand, 1990; D'Amato *et al.*, 1992a). In this tissue, the L-arginine analogues also reduce the EFS-evoked release of a vasorelaxant substance with biological properties similar to those of NO (Boeckxstaens *et al.*, 1991). The purpose of this study was to provide direct biochemical evidence of EFS-induced NO synthesis in rat gastric fundus strips by measuring L-citrulline levels in the bath medium and to examine the relationship between this enzymatic activity and the mechanical responses of the strips to EFS.

Methods

General

Male and female Wistar rats (150-250 g) were fasted with free access to water for $\approx$24 h before they were killed by decapitation and exsanguinated. The gastric fundus was removed and one or two longitudinal muscle strips (3 x 20 mm) were prepared as described by Vane (1957). The strips were immediately transferred to organ baths or glass tubes (see below)

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containing Krebs solution, 37°C, bubbled with a 95:5 O_2/CO_2 mixture, of the following composition (mM): NaCl 118.5, KCl 4.8, $CaCl_2$ 1.9, KH_2PO_4 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 25 and glucose 10.1 (pH 7.4). The Krebs solution contained atropine 1 μM, guanethidine 5 μM and 5-hydroxytryptamine (5-HT) 3 μM (to raise strip tone and allow recording of NANC relaxations). Different strips were used for functional experiments and for measurement of L-citrulline production (see below).

Functional experiments

The strips were mounted between parallel platinum electrodes (20 mm long, 4 mm wide, 5 mm apart) and placed inside 5-ml organ baths under a 1-g load. Auxotonic changes in length, magnified 5–10 times, were recorded by Harvard smooth muscle transducers attached to Rikadenki R-01 single-pen recorders. After a 60-min equilibration period, during which the bath solution was changed every 10 min, the strips were subjected to isolated EFS with a Palmer Bioscience 6012 Stimulator linked in series with a Basile Biological Research Apparatus constant-current unit. EFS consisted of rectangular, bipolar pulses of constant duration (1 ms), amplitude (120 mA) and pulse train duration (2 min); frequencies ranged from 0.5–16.0 Hz. Between trains of EFS, the bath solution was changed every 5 min.

Experiments for L-citrulline measurements

The strips were suspended under a 1-g load between parallel platinum electrodes (20 mm long, 3.5 mm wide, 3 mm apart) inside 4-ml silanized glass tubes containing 2 ml of Krebs solution. During the initial 60-min equilibration period, the strips were transferred every 10 min to a different tube containing fresh bath solution. This phase was followed by two or more incubation series, each consisting of 3 consecutive 2-min incubations (in different tubes) before, during and after EFS, as described above. After each 2-min incubation, the bath solution was collected on ice and stored at −20°C. In the interval between two incubation series, the medium was changed every 5 min (as in functional experiments). At the end of each experiment, the strip was blotted and weighed to evaluate the relationship between strip weight and the amount of L-citrulline recovered from the organ bath. No correlation could be demonstrated within the weight range of 88.1–196.1 mg (121.8 ± 2.9 mg, mean ± s.e.mean, $n = 56$) and the results were thus expressed as pmol ml^{-1}. Total evoked L-citrulline production was calculated for each strip by subtracting prestimulation levels from those observed during the EFS (both calculated on a total volume of 2 ml) and expressed as absolute values (pmol).

L-Citrulline measurement

L-Citrulline was measured in the incubation medium by *o*-phthaldialdehyde precolumn derivatization, reversed-phase high-performance liquid chromatography (h.p.l.c.) and fluorimetric detection (excitation at 330 nm, emission at 450 nm) as described by Jones & Gilligan (1983) with the following modifications.

The L-citrulline derivative was analysed with a LiChrosphere 100 RP-18 column (5 μm, 5 mm i.d. × 125 mm) (Merck, Darmstadt, Germany). The derivative was resolved using a linear gradient (flow rate of 1.3 ml min^{-1}) starting with 0.05 M aqueous potassium dihydrogen-phosphate, pH 6.3, and ending after 15 min with acetonitrile:H_2O:methanol (4:2:4). The latter solvent was continued for 5 min, and the column was then re-equilibrated for 5 min with the initial buffer before the injection of the next sample. The h.p.l.c. system (Jasco, Tokyo, Japan) consisted of a 880-PU pump, a 880-02 controller for gradient programming, a Rheodyne injection valve 7125i with a 100-μl filling loop and a 821-FP spectrofluorometer. Quantification of the h.p.l.c. results was obtained with a CR6A Chromatopak (Shimadzu, Kyoto, Japan). The chemicals utilized were of analytical grade and the solvents were of chromatographic grade.

Experimental protocols

EFS frequency-response curves The strips were subjected to isolated EFS at increasing frequencies (0.5–16.0 Hz) to study the effects on strip tone and L-citrulline production. In functional experiments, strips were allowed to recover basal tone before subsequent EFS. In the experiments performed for L-citrulline measurements, the interval between stimulations was increased progressively from 10 to 30 min as higher frequencies were used. All relaxations were expressed as peak amplitudes and the results expressed as percentages of the maximal response observed with 16-Hz EFS in each strip.

Effect of 5-HT on L-citrulline production In separate experiments, each strip was subjected to 4-Hz EFS before and 30 min after the addition of 5-HT 3 μM to the bath medium and levels of L-citrulline were measured in the bath solution. Forty minutes elapsed between the two stimulations.

Effects of tetrodotoxin, calcium-free Krebs solution and L-NAME on L-citrulline production Each strip was subjected to a control stimulation at 16 Hz and L-citrulline levels in the bath medium were measured. The same procedure was later repeated after 20-min incubations with tetrodotoxin (TTX) 3 μM, L-NAME and its enantiomer D-NAME (both 100 μM) and after a 30-min incubation in a calcium-free bath medium. Sixty minutes elapsed between control and post-treatment stimulations.

The calcium-free medium was prepared by substituting the Ca^{2+} normally present in the Krebs solution with an equimolar concentration of Mg^{2+}. The medium was renewed every 5 min during the 30-min incubation so that any calcium diffusing into the medium from the tissue was removed. In previous mechanical studies, this protocol produced a total loss of tone in rat gastric fundus strips that had been precontracted with 5-HT (Currò *et al.*, 1994). Nonetheless, to verify the effects observed in these experiments, tests were repeated with the same calcium-free medium plus the addition of the calcium chelator, ethylene glycol-O,O′-*bis*(2-aminoethyl)-N,N,N′,N′-tetraacetic acid (EGTA), 1 mM.

Concentration-dependent effects of L-NAME. Experiments were conducted to evaluate the effects of L-NAME on basal production of L-citrulline and on strip relaxation and L-citrulline production induced by 4-Hz EFS. In the mechanical experiments, each strip was exposed to consecutive incubations with increasing concentrations of L-NAME (0.1 μM–1 mM). In those conducted for measurement of L-citrulline production, a single concentration within the 0.1–10 μM range was used for each strip. In both cases, EFS was delivered 20 min after the addition of L-NAME.

In experiments conducted for L-citrulline assays, the strips tested with L-NAME 10 μM were subjected to a third EFS after a 20-min incubation with L-NAME 10 μM + L-arginine (L-Arg) 1 mM or L-NAME 10 μM + D-arginine (D-Arg) 1 mM. Basal and EFS (4 Hz)-evoked L-citrulline production by other strips were also measured after 20-min incubations with L-Arg or D-Arg 1 mM alone.

Drugs used

The following were used: atropine sulphate, guanethidine sulphate, 5-HT creatinine sulphate, tetrodotoxin (Sigma, St Louis, MO, U.S.A.); L-NAME, D-NAME (Bachem, Bubendorf, Switzerland); L-arginine, D-arginine (Novabiochem, Laufelfingen, Switzerland); L-citrulline, EGTA, *o*-phthaldialdehyde (Fluka, Buchs, Switzerland).

Statistical analysis

The results were evaluated by means of Student's paired and unpaired t test. All values are reported as means ± s.e.mean. $P<0.05$ was considered statistically significant.

Results

EFS frequency-response curves

At frequencies ranging from 0.5–16.0 Hz, EFS caused frequency-dependent NANC relaxation of the gastric fundus strips as well as statistically significant increases in bath-medium levels of L-citrulline (Figure 1). Relaxations began immediately and increased rapidly to near-maximal levels. After stimulation at frequencies of ≤4 Hz, basal strip tone was restored almost immediately but progressively longer intervals were required for recovery after EFS at higher frequencies (Figure 1).

Basal concentrations of L-citrulline measured before each EFS showed no significant variations and averaged 35.8 ± 0.5 pmol ml^{-1} (range 32.1–41.0 pmol ml^{-1}, $n=36$). EFS-induced increases ranged from 22.5 ± 1.3% at 0.5 Hz ($n=6$) to 174.9 ± 6.3% at 16 Hz ($n=6$). A return to basal levels was observed in the first 2 min after termination of EFS, regardless of the frequency used.

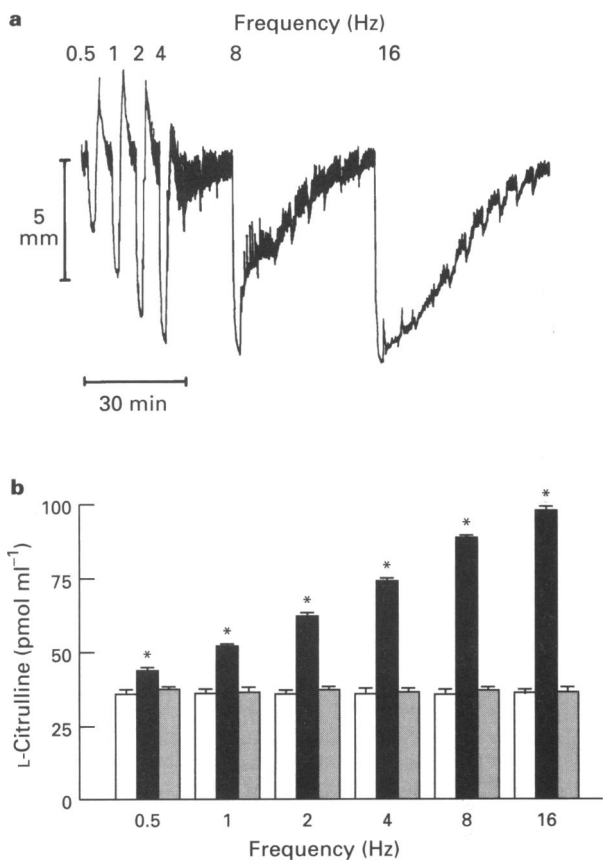

Figure 1 Mechanical and biochemical effects of electrical field stimulation (EFS, 0.5–16.0 Hz, 120 mA, 1 ms, pulse trains of 2 min) on 5-HT (3 μM)-precontracted longitudinal muscle strips from the rat gastric fundus under non-adrenergic non-cholinergic (NANC) conditions. (a) Representative tracing of frequency-dependent relaxations induced in one muscle strip. (b) L-Citrulline concentrations measured in the incubation medium before (open columns), during (solid columns) and after (hatched columns) EFS. Each column represents the mean (± s.e. mean) concentration per 2-min collection fraction produced by 6 strips. Significant differences with respect to prestimulation levels: $^*P<0.001$.

Figure 2 shows mean frequency-response curves for EFS-induced strip relaxation and total evoked L-citrulline formation, which were closely related (Figure 3).

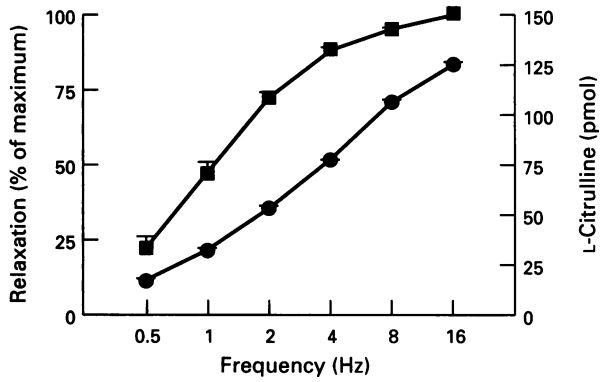

Figure 2 Mean frequency-response curves for both relaxation (■) and total L-citrulline production (●) induced by EFS (0.5–16.0 Hz, 120 mA, 1 ms, pulse trains of 2 min). Each point represents the mean ± s.e. mean of responses observed in 6 (●) or 8 (■) strips. Relaxations, measured as peak amplitudes, are expressed as percentages of the maximal relaxation induced in the same strip by 16-Hz EFS. Total evoked L-citrulline production was calculated by subtracting the prestimulation concentration from that observed during stimulation.

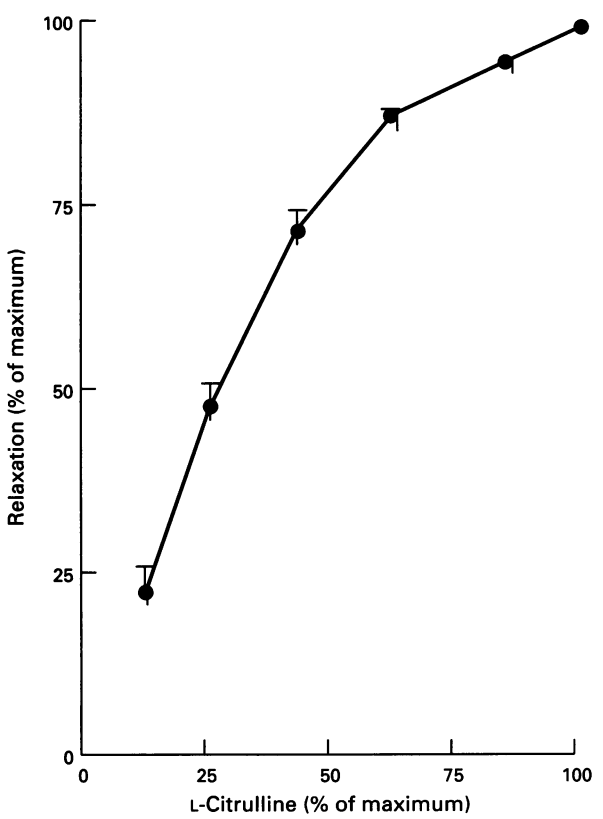

Figure 3 Correlation between EFS (0.5–16.0 Hz, 120 mA, 1 ms, pulse trains of 2 min)-induced NANC relaxation and total evoked L-citrulline production (calculated by subtracting the prestimulation concentration from that observed during stimulation) by rat gastric fundus strips. Data are expressed as percentages of maximal values and are shown here as means ± s.e.mean of responses observed in 6 (L-citrulline production) or 8 (relaxation) strips.

Effects of 5-HT on bath levels of L-citrulline

5-HT (3 μM) had no effect on basal L-citrulline production (27.3±2.6 pmol ml^{-1} vs 27.8±2.5 pmol ml^{-1} without 5-HT, $n=4$) or that evoked by EFS at 4 Hz (64.8±5.8 pmol ml^{-1} vs 64.9±5.9 pmol ml^{-1} without 5-HT, $n=4$).

Effects of TTX, calcium-free Krebs solution and L-NAME

TTX, 3 μM, calcium-free Krebs solution and L-NAME, 100 μM, all abolished the increase in L-citrulline synthesis evoked by 16-Hz EFS (Figure 4) but had different effects on basal levels. TTX did not affect basal concentrations of L-citrulline (100.6±1.3% of control levels, $n=4$), but significant reductions were observed with the other two treatments. The effect of L-NAME, 100 μM, on prestimulation L-citrulline levels was more marked (50.0±0.5% of controls, $n=4$, $P<0.001$) than that of the calcium-free medium (89.1±2.9% of controls, $n=6$, $P<0.05$). There were no significant differences between the effects of the calcium-free medium with and without EGTA. Prior to EFS the concentration of L-citrulline found in the medium containing the chelator was significantly lower than that found when normal Krebs solution was used (29.1±3.0 vs 33.1±3.3 pmol ml^{-1}, $n=4$, $P<0.01$), but this reduction (to 88.1±0.3% of controls) was not significantly different from that produced by the calcium-free medium without EGTA ($P=0.79$). Strip incubation with D-NAME, 100 μM, had no effect on basal (33.4±3.3 vs 33.0±3.2 pmol ml^{-1}, respectively before and after D-NAME, $n=4$) or total EFS-evoked production of L-citrulline (126.3±1.8 vs 125.8±1.8 pmol, respectively before and after D-NAME, $n=4$).

Concentration-dependent effects of L-NAME

L-NAME dose-dependently inhibited basal L-citrulline production, as well as EFS-induced strip relaxation, but its most marked effects were observed on the formation of L-citrulline provoked by EFS (Figure 5). The addition of 10 μM of L-NAME to the bath medium, which completely abolished the EFS-induced increase in L-citrulline synthesis, produced only mild inhibition of basal production (22.6±1.9% of controls, $n=4$) and strip relaxation (7.9±2.3% of controls, $n=10$). At the maximal concentration used in the mechanical experiments (1 mM), L-NAME reduced the relaxant response by 47.0±3.9% with respect to controls ($n=10$). Strips tested with L-NAME (10 μM) were subsequently subjected to a 20-min incubation with L-NAME 10 μM + L-Arg 1 mM. In these experiments, the inhibitory effects of L-NAME on both basal and EFS-induced L-citrulline formation were reversed and both parameters were increased with respect to values observed prior to incubation with L-NAME alone (basal formation: 125.1±0.1% of controls, $n=4$, $P<0.01$; total EFS-evoked formation: 135.3±7.9% of controls, $n=4$, $P<0.05$) (Figure 6a). When these same experiments were performed using D-Arg instead of L-Arg, there was no reversal of the inhibitory

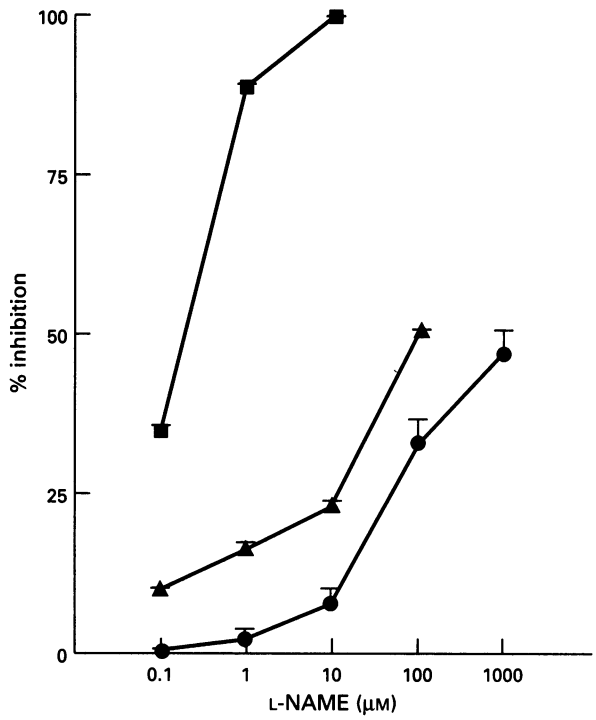

Figure 5 Concentration-dependent effects of L-NAME on basal production of L-citrulline (▲) and on NANC relaxation (●) and total L-citrulline production (■) induced by EFS (4.0 Hz, 120 mA, 1 ms, pulse trains of 2 min) of rat longitudinal gastric fundus strips. Each point represents the mean±s.e.mean of responses observed in 4 (▲,■) or 10 (●) strips. The point representing the effect of L-NAME 100 μM on basal production of L-citrulline has been drawn from the experiments shown in Figure 4.

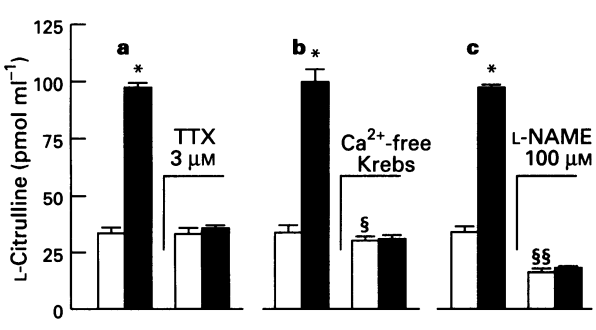

Figure 4 Effects of tetrodotoxin (TTX; 3 μM) (a), a calcium-free medium (b) and L-NAME 100 μM (c) on L-citrulline levels in the incubation medium of rat gastric fundus strips before (open columns) and during (solid columns) EFS (16.0 Hz, 120 mA, 1 ms, pulse trains of 2 min). Each column represents the mean (±s.e.mean) concentration per 2-min collection fraction produced by 4 (a,c) or 6 (b) strips. Significant differences between corresponding prestimulation and stimulation values: *$P<0.001$; significant differences with respect to control prestimulation levels: §$P<0.05$, §§$P<0.001$.

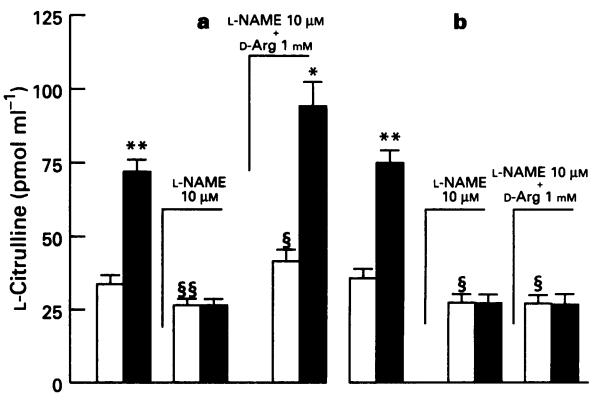

Figure 6 Effects of L-arginine (L-Arg) (a) and D-arginine (D-Arg) (b) (both 1 mM) on 10 μM L-NAME-induced inhibition of basal (open columns) and EFS (4.0 Hz, 120 mA, 1 ms, pulse trains of 2 min)-evoked production of L-citrulline (solid columns) by rat gastric fundus strips. Each column represents the mean±s.e.mean concentration per 2-min collection fraction produced by 4 strips. Significant differences between corresponding prestimulation and stimulation values: *$P<0.01$, **$P<0.001$; significant differences with respect to control prestimulation levels: §$P<0.01$, §§$P<0.001$.

effects of L-NAME on either basal or EFS-induced production (Figure 6b).

The addition of L-Arg (1 mM) alone significantly increased basal concentrations of L-citrulline (141.6±1.3% of controls, $n=4$, $P<0.01$) (Figure 7a) but had no effect on the total production evoked by 4-Hz EFS (76.0±7.9 vs 71.6±3.5 pmol, $n=4$, NS). D-Arg (1 mM) had no effect on either basal or stimulated production (Figure 7b).

Discussion

Various groups have provided evidence of NO involvement in NANC relaxation of gastrointestinal smooth muscle (for review, see Sanders & Ward, 1992). The earliest reports of this type of neurotransmitter activity in the rat gastric fundus were published by Li & Rand (1990), and these findings have been strengthened and expanded by those of a number of other investigators. In this tissue, NO appears to be the principal mediator of NANC relaxation induced by low-frequency or short-duration EFS, while vasoactive intestinal polypeptide and peptide histidine valine seem to be responsible for the response observed with high-frequency or sustained stimulation (Li & Rand, 1990; D'Amato *et al.*, 1992a,b; Boeckxstaens *et al.*, 1992; Currò *et al.*, 1994).

The fact that *in vitro* EFS-induced NANC relaxation of this tissue is attenuated in a concentration-dependent, stereo-specific manner by NO-synthase inhibitors (L-NMMA, L-NOARG, L-NAME) is considered to be the most convincing evidence of NO involvement in this response (Li & Rand, 1990; Boeckxstaens *et al.*, 1991; D'Amato *et al.*, 1992a). Additional support has been provided by observations that L-NOARG also inhibits the EFS-induced release of a labile factor with vasodilator properties that resemble those of NO (Boeckxstaens *et al.*, 1991). L-NAME, L-NOARG and L-NMMA are the L-arginine analogues that have been most widely used in studies of this type. L-NAME was chosen for use in the present study because, like L-NOARG, it is significantly more potent than L-NMMA and has the added practical advantage of being somewhat more water-soluble than L-NOARG.

Constitutive neuronal NO synthase catalyzes the reaction between L-arginine and oxygen, which yields equimolar quantities of NO and L-citrulline. Because of the extreme instability of NO, concentrations of L-citrulline were used in this study as an index of NO synthase activity. The levels (≈30 nM) of L-citrulline found in the bath medium prior to EFS indicate that this substance is synthesized by rat gastric fundus tissue under basal conditions. The fact that unstimulated production was reduced by ≈50% by L-NAME (100 μM) indicates that at least half of the L-citrulline recovered under basal conditions is the result of NO synthase activity, although it is possible that further reductions might have been achieved with higher doses of L-NAME. In any case, it is likely that a certain percentage of the L-citrulline we recovered was produced by enzymes other than NO synthase. To our knowledge no other enzymatic pathways have been described for L-citrulline synthesis in the rat gastric fundus, but we cannot exclude the possibility that enzymes of the urea cycle are present in some of these cells. It is impossible to tell what portion of total intracellular L-citrulline production is represented by the concentrations measured in the bath medium. The fate of intracellular L-citrulline resulting from NO synthase activity in the rat gastric fundus is still obscure. Extracellular transfer may depend on a non-selective neutral carrier (which is distinct from the cationic amino-acid y^+ carrier that is responsible for transporting L-arginine into the cell) that has been shown to transfer exogenous L-citrulline into murine J774 macrophages (Baydoun *et al.*, 1994). Reconversion of L-citrulline to L-arginine via an arginosuccinate synthase/lyase pathway has also been described in bovine aortic endothelial cells (Hecker *et al.*, 1990) and J774 macrophages (Baydoun *et al.*, 1994), but this recycling process cannot produce enough substrate to sustain more than ≈20% of normal NO synthesis in J774 cells (Baydoun *et al.*, 1994). It is therefore probable that the L-citrulline recovered from the bath medium in our study represents almost all of that produced by NO synthase.

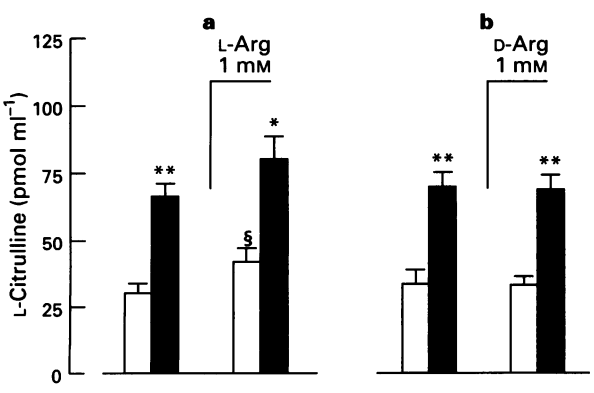

Figure 7 Effects of L-arginine (L-Arg) (a) or D-arginine (D-Arg) (b) (both 1 mM) on basal (open columns) and EFS (4.0 Hz, 120 mA, 1 ms, pulse trains of 2 min)-evoked production of L-citrulline (solid columns) by rat gastric fundus strips. Each column represents the mean (±s.e.mean) concentration per 2-min collection fraction produced by 4 strips. Significant differences between corresponding prestimulation and stimulation values: $*P<0.01$, $**P<0.001$; significant differences with respect to control prestimulation levels: $§P<0.01$.

EFS-induced L-citrulline synthesis differed in several respects from that observed prior to stimulation. The rise in medium levels of L-citrulline provoked by EFS was, for example, eliminated by TTX, which had no effect on basal production, indicating that propagated neuronal action potentials are not involved in the latter phenomenon. The relatively mild reduction in basal levels seen when Ca^{2+} was removed from the bath medium suggests that unstimulated L-citrulline synthesis in these cells is mediated primarily by a constitutive Ca^{2+}-independent isoform of NO synthase. Although constitutive neuronal NO synthase is generally believed to be a Ca^{2+}-dependent enzyme, Ca^{2+}-independent isoforms appear to be present in the canine ileum (Kostka *et al.*, 1993) and in the aortic endothelium of pigs (Mülsch *et al.*, 1989) and rats (Rees *et al.*, 1990). In contrast, the increased production provoked by EFS, which was completely abolished by calcium withdrawal and L-NAME, appears to involve a Ca^{2+}-dependent form of NO synthase.

The effects of L-NAME on both basal and EFS-stimulated L-citrulline synthesis are competitive phenomena that can be reversed by substrate excess, i.e. L-arginine but not D-arginine, but these experiments also suggested the presence of two distinct isoforms of NO synthase. While exogenous L-arginine increased basal levels of L-citrulline in the bath medium, it had no effect on the total production observed after delivery of EFS. This finding indicates that, while basal NO synthase activity can be upregulated by increased substrate availability, the activated NO synthase system is saturated at lower (i.e. endogenous) substrate levels. The effects of L-arginine on NANC relaxation of the rat gastric fundus are similar to those exerted on L-citrulline production; exposure to L-arginine causes a decrease in the tone of precontracted strips (Li & Rand, 1990; D'Amato *et al.*, 1992a) but has no effect on EFS-induced NANC relaxation (Li & Rand, 1990). The two isoforms of NO also appear to be differentiated by their sensitivities to L-NAME, the Ca^{2+}-independent form involved in basal L-citrulline production being less susceptible than the Ca^{2+}-dependent form that mediates stimulated production. These characteristics are identical to those described by Rees *et al.* (1990) for the two forms of NO synthase that seem to be present in the endothelium of the porcine aorta.

The most surprising results of our study were the differential effects of L-NAME on L-citrulline formation and strip relaxation induced by EFS. This L-arginine analogue produced

concentration-dependent inhibition of both phenomena, but its effects on L-citrulline production were much more marked. At a concentration of 10 μM, L-NAME completely abolished the EFS-induced increase in L-citrulline bath levels, but its effects on strip relaxation were much less significant (i.e. reduction of $\approx$8%), and the highest concentration of L-NAME used in this study (1 mM) attenuated the relaxant response by only $\approx$50%. The possibility that neurotransmitter(s) other than NO might be involved in NANC relaxation of the rat gastric fundus is not sufficient to explain the increasingly effective inhibition of relaxation achieved with L-NAME concentrations greater than 10 μM. In addition to its effects on intracellular NO synthase activity, L-NAME might also affect the transport of L-citrulline into the extracellular space. The neutral carrier responsible for the transmembrane passage of L-citrulline is also known to transport L-NOARG and L-NAME (Baydoun & Mann, 1994). Thus, concentrations of L-NAME that have relatively limited repercussions on NO synthase activity within the cell (reflected by mild inhibition of the relaxant response) could markedly reduce the quantity of L-citrulline transferred into the bath medium by occupying this neutral carrier. It is also possible, however, that L-NAME reduces NANC relaxation by unidentified mechanisms unrelated to its inhibition of NO synthase. This hypothesis implies the absence of a direct relationship between the activation of this enzyme and NANC relaxation. A number of studies have, in fact, questioned the role of NO *per se* as a NANC neurotransmitter and suggested that the substances released by NANC nerves in various peripheral tissues (Knudsen *et al.*, 1992; Li & Rand, 1993; Rajanayagam *et al.*, 1993; Rand & Li, 1993; Liu *et al.*, 1994), including the rat gastric fundus (Hobbs *et al.*, 1991; Barbier & Lefebvre, 1992; 1994; Kitamura *et al.*, 1993; Jenkinson *et al.*, 1995) might actually be nitrosothiols.

In conclusion, this study provides biochemical evidence of electrically induced, Ca^{2+}-dependent, tetrodotoxin-sensitive activation of NO synthase in the rat gastric fundus. Our findings also support previous reports indicating that there are actually two distinct isoforms of this enzyme in the gastrointestinal tract.

Supported by MURST 60% and CNR Grant No. 93.0406. CT04. The authors are grateful to Prof. G. Ciabattoni for his invaluable advice on the preparation of this paper and to Ms M.E. Kent for editorial assistance.

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(Received July 25, 1995
Revised September 29, 1995
Accepted October 4, 1995)