Nitric oxide-dependent and -independent hyperaemia due to calcitonin gene-related peptide in the rat stomach

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1 Calcitonin gene-related peptide (CGRP) potentially enhances mucosal blood flow in the rat stomach. The aim of this study was to examine whether CGRP also dilates extramural arteries supplying the stomach and whether the vasodilator action of CGRP involves nitric oxide (NO).

2 Rat CGRP- α (0.03–1 nmol kg⁻¹, i.v.) produced a dose-dependent increase in blood flow through the left gastric artery (LGA) as determined by an ultrasonic transit time technique in urethane-anaesthetized rats. Blockade of NO synthesis by N^G-nitro-L-arginine methyl ester (L-NAME, 20 and 60 μ mol kg⁻¹, i.v.) significantly reduced basal blood flow (BF) in the LGA and attenuated the hyperaemic activity of CGRP by a factor of 2.8–4. D-NAME tended to enhance basal BF in the LGA but had no influence on the dilator activity of CGRP. The ability of vasoactive intestinal polypeptide to increase left gastric arterial blood flow remained unaltered by L-NAME.

3 L-NAME (20 and 60 μ mol kg⁻¹, i.v.) evoked a prompt and sustained rise of mean arterial blood pressure (MAP) and caused a slight decrease in the hypotensive activity of CGRP. In contrast, D-NAME induced a delayed and moderate increase in MAP and did not influence the hypotensive activity of CGRP.

4 Rat CGRP- α dilated the isolated perfused bed of the rat LGA precontracted with methoxamine and was 3 times more potent in this respect than rat CGRP- β . The dilator action of rat CGRP- α in this preparation was not affected by L-NAME or D-NAME (40 μ M).

5 L-NAME (60 μ mol kg⁻¹, i.v.) reduced gastric mucosal blood flow as assessed by laser Doppler flowmetry and diminished the hyperaemic activity of rat CGRP- α in the gastric mucosa by a factor of 4.5, whereas D-NAME was without effect.

6 These data show that CGRP is a potent dilator of mucosal and extramural resistance vessels in the rat stomach. Its dilator action involves both NO-dependent and NO-independent mechanisms.

Keywords: Calcitonin gene-related peptide (CGRP); vasoactive intestinal polypeptide; blood flow; rat stomach; gastric mucosa; left gastric artery; vasodilatation; hypotension; nitric oxide; N^G-nitro-L-arginine methyl ester (L-NAME); D-NAME; inhibition of nitric oxide synthase

Introduction

Calcitonin gene-related peptide (CGRP) is a potent dilator of submucosal arterioles in the rat stomach (Chen *et al.*, 1992), an action by which it augments gastric mucosal blood flow (MBF) (Holzer & Guth, 1991). This and other findings suggest that CGRP plays a regulatory role in the gastric mucosal microcirculation. Arteries and arterioles in the rat stomach are surrounded by a dense plexus of primary afferent nerve fibres containing CGRP (Green & Dockray 1988; Sternini & Anderson, 1992). Activation of these neurones causes release of CGRP into the vascular bed of the stomach (Holzer *et al.*, 1990), and there is pharmacological evidence that CGRP participates in the gastric hyperaemic response to sensory nerve stimulation with capsaicin (Li *et al.*, 1991). The first objective of the present study was to investigate whether the vasodilator action of CGRP in the rat stomach is confined to the mucosal microvessels or whether CGRP also dilates extramural resistance vessels. This question was addressed by measuring blood flow in the gastric mucosa and in the left gastric artery (LGA) of anaesthetized rats and by use of the isolated perfused bed of the LGA.

The second objective was to inquire into the mechanism of the vasodilator action of CGRP in the rat stomach. In addition to a direct action on vascular smooth muscle, transmitter substances can regulate blood flow via release of vasodilator mediators such as nitric oxide (NO) (Moncada *et al.*, 1991). There is evidence that NO plays an important role in the regulation of gastric MBF (Walder *et al.*, 1990; Pique

et al., 1992; Lippe & Holzer, 1992; Tepperman & Whittle, 1992). CGRP-evoked dilatation of various extragastric vascular beds differs with regard to the involvement of NO and the endothelium, an important source of NO (Gardiner *et al.*, 1991; Abdelrahman *et al.*, 1992; Gray & Marshall, 1992), and there is a preliminary report suggesting that the hyperaemic action of CGRP in the rat gastric mucosa depends to some extent on NO (Whittle *et al.*, 1992). The question whether the vasodilator action of CGRP in the rat stomach involves NO was examined by the use of N^G-nitro-L-arginine methyl ester (L-NAME), a specific blocker of the constitutive NO synthase (Moore *et al.*, 1990; Rees *et al.*, 1990).

Methods

Surgical preparation of the animals for the in vivo experiments

All experiments were performed on female Sprague-Dawley rats, weighing 190–220 g. They were fasted for about 20 h before experimentation but allowed free access to water. After the induction of anaesthesia with urethane (1.5 g kg⁻¹, s.c.) the rats were fitted with a tracheal cannula to facilitate spontaneous respiration. The body temperature was kept at 36–37°C by means of a water-perfused heating pad. Mean arterial blood pressure (MAP) was recorded from a cannula in the right carotid artery. In many experiments the MAP signal was fed into a heart ratemeter and the heart rate (HR) and MAP were displayed on a chart recorder. For the i.v. administration of drugs a catheter was placed in a jugular

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vein through which saline (0.9% NaCl, wt/wt) was continuously infused at a rate of 1.5 ml h^{-1} to avoid dehydration. In some experiments the drugs were administered close arterially to the stomach, in which case the abdominal aorta was cannulated with a catheter (PE-20) in a retrograde direction so that the tip of the catheter lay just above the branching of the coeliac artery (Lippe *et al.*, 1989). After completion of surgery a period of at least 30 min was allowed for equilibration until the experiments were started.

Measurement of blood flow in the LGA

Blood flow in the LGA was determined by the ultrasonic transit time shift technique. This method involves a flow sensor, which contains two ultrasonic transducers passing a plane wave of ultrasound back and forth over the full width of the vessel alternately intersecting the flowing blood in upstream and downstream directions (Burton & Gorewit, 1984; Wang *et al.*, 1989). The shift in transit time which results from the flow of blood is integrated over the full width of the vessel and used to calculate the volume of blood flow ($\mu\text{l min}^{-1}$). After a midline laparotomy the LGA was separated from the surrounding tissue over a length of 4–5 mm under a binocular microscope. Care was taken to remove any fat tissue from the vessel. Blood flow was measured by an ultrasonic flow sensor (model 1RB, Transonic, Ithaca, New York, U.S.A.) which was placed around the LGA and connected to a small animal flowmeter (model T106, Transonic).

The experiments were started by priming the animals with i.v. injections of rat CGRP- α (100 pmol kg^{-1}) or vasoactive intestinal polypeptide (VIP, 300 pmol kg^{-1}) at 15 min intervals. Once the hyperaemic actions of these peptides were stable, saline (1 ml kg^{-1}), D-NAME (20 or $60 \text{ }\mu\text{mol kg}^{-1}$) or L-NAME (20 or $60 \text{ }\mu\text{mol kg}^{-1}$) was injected into the jugular vein. After a wait of 30 min, dose-response curves for the hyperaemic actions of CGRP and/or VIP were recorded. This was done by injecting increasing doses of CGRP (0.03 – 1 nmol kg^{-1}) and VIP (0.3 – 1 nmol kg^{-1}) at 15 min intervals. All peptide doses were injected at a volume of 1 ml kg^{-1} , the vehicle being saline.

Measurement of gastric mucosal blood flow with laser Doppler flowmetry

The stomach was exposed by a midline laparotomy, and a small bore (4 mm o.d.) plastic cannula was inserted via a small incision in the forestomach and tied in place, to allow free access to the gastric lumen (Tepperman & Whittle, 1992). Gastric MBF was recorded continuously with a laser Doppler flow monitor (model MBF3D, Moor Instruments, Devon) and an endoscopic laser optic probe (model P6B, 1.25 mm o.d.; Moor Instruments) which was inserted into the gastric lumen via the plastic cannula and allowed to rest gently on the gastric corpus mucosa (Tepperman & Whittle, 1992). Drug-induced changes in the laser Doppler flux readings were expressed as a percentage of the average flux recorded over a 3 min period immediately before administration of the drug. The experimental protocol was identical with that used for the measurement of blood flow in the LGA.

Measurement of gastric mucosal blood flow with the hydrogen gas clearance technique

The clearance of hydrogen from the gastric tissue was measured by a platinum needle electrode which, after a midline laparotomy, was inserted from the serosa into the basal portion of the gastric corpus mucosa and positioned at the submucosal border of the muscularis mucosae (Lippe & Holzer, 1992). MBF was estimated by computer-assisted analysis of the hydrogen clearance curves and expressed as $\mu\text{l min}^{-1} \text{ g}^{-1}$ (Lippe & Holzer, 1992). The experimental protocol involved alternating 15 min periods of saturation, and

desaturation, of the tissue with hydrogen gas. Measurements of MBF were taken during the periods of 45–60 min, 75–90 min, 105–120 min, 135–150 min, and 165–180 min after the start of the experiments. Since MBF measured by the hydrogen clearance technique represents flow averaged over a period of 15 min, only prolonged changes in blood flow can be measured. CGRP was infused, therefore, close arterially to the stomach via a catheter in the abdominal aorta. This catheter was continuously perfused with Krebs buffer pH 7.4 containing 0.2 mM acetic acid (i.e., the vehicle for CGRP) at a rate of 1.5 ml min^{-1} to keep the catheter patent and to avoid dehydration of the rat (no infusion of saline via a jugular vein was carried out in these experiments). At 60 min, D-NAME or L-NAME ($20 \text{ }\mu\text{mol kg}^{-1}$, 1 ml kg^{-1}) was administered to the stomach by slow injection into the aortic catheter. Rat CGRP- α (60 pmol min^{-1} , 0.03 ml min^{-1}) was infused via the same route during the period of 160–180 min.

Perfusion of the isolated bed of the LGA

Twenty minutes after the induction of anaesthesia with pentobarbitone (50 mg kg^{-1} , i.p.) the rats were laparotomized to expose the stomach. After cannulation of the LGA with a catheter (PE-20) the preparation was slowly flushed with 20 ml of Krebs buffer pH 7.4 containing heparin (20 iu ml^{-1}). The perfused stomach was excised and cut open along the greater curvature. The preparation thus was drained via the plexus of submucosal arterioles that had been cut during dissection. The preparation was transferred to a perfusion apparatus which consisted of an inclined pad maintained at 37°C . The stomach was placed on the pad with the mucosal side downwards and covered with parafilm to avoid dehydration. Oxygenated Krebs buffer pH 7.4 enriched with dextran F70 (3%, wt/wt) (Kwok *et al.*, 1988) was perfused through the preparation by means of a peristaltic pump at a rate of 1.35 ml min^{-1} . Proper perfusion of the preparation was checked visually by injecting 0.05 ml of Evans blue (10 mg ml^{-1}). The perfusion pressure was measured by way of a pressure transducer connected to the inflow cannula and displayed on a chart recorder.

After an initial equilibration period of 20 min the α -adrenoceptor agonist, methoxamine, was added to the perfusion solution to enhance perfusion pressure and thereby facilitate the study of dilator responses (Kawasaki *et al.*, 1990). The concentration of methoxamine (25 – $90 \text{ }\mu\text{M}$) was such that perfusion pressure rose by about 80–100 mmHg. After allowing a further 20 min for equilibration in the presence of methoxamine, two sets of experiments were carried out. In the first set of experiments the dilator responses to rat CGRP- α and CGRP- β were compared. To this end, increasing doses of the two peptides (3–100 pmol) were injected at volumes of 0.1 ml at 20 min intervals. The dilator response to 100 pmol CGRP- α was used to standardize the preparations at the end of each experiment and all other dilator responses were expressed as a percentage of that response. In the second set of experiments the effect of D-NAME and L-NAME on the dilator response to rat CGRP- α was investigated. For this purpose, a dose of CGRP (usually 10 pmol) causing approximately half-maximal relaxation of the methoxamine-contracted preparations was chosen as test dose and administered at 20 min intervals. After reproducible dilator responses to the test dose of CGRP had been obtained (usually after 3 dosings), D-NAME ($40 \text{ }\mu\text{M}$) was added to the perfusion medium. Following 3 applications of the test dose of CGRP, D-NAME was replaced with L-NAME ($40 \text{ }\mu\text{M}$), and CGRP was tested 3 times in the presence of L-NAME.

Substances and solutions

Rat CGRP- α , rat CGRP- β , and rat VIP (Peninsula, Heidelberg, Germany) were dissolved in 0.02 M acetic acid to give stock solutions of 0.1 mM. For intravascular administration,

the stock solutions were diluted with Krebs buffer pH 7.4 (i.a. infusion) or saline (i.v. injection). D-NAME (N^G-nitro-D-arginine methyl ester) and L-NAME (N^G-nitro-L-arginine methyl ester) came from Bachem (Bubendorf, Switzerland) and were dissolved (20 or 60 mM) in saline. Methoxamine (Sigma, Deisenhofen, Germany) was dissolved (0.7 M) in saline. Heparin was provided by Sandoz (Vienna, Austria) and dextran F70 was obtained from Serva (Heidelberg, Germany).

Calculation of data and statistics

Since D-NAME and L-NAME caused changes in gastric blood flow and MAP, all actions of CGRP and VIP on these parameters were expressed as percentage changes in order to account for the change in the baseline values (Abdelrahman *et al.*, 1992). All data are presented as mean $\pm$ s.e.mean. Statistical evaluation of the results was performed with the Mann-Whitney U test, Kruskal-Wallis H test or Wilcoxon test for pair differences as appropriate. Probability values of $P < 0.05$ were regarded as significant.

Results

Effect of L-NAME and D-NAME on blood flow in the LGA, MAP and HR

The basal levels of blood flow in the LGA and gastric mucosa, MAP and HR as measured before administration of saline, L-NAME or D-NAME are given in Table 1. There were no significant differences in these parameters between the different groups of rats used in the present study.

L-NAME (20 and 60 $\mu\text{mol kg}^{-1}$, i.v.) led to an immediate and sustained increase in MAP, which was accompanied by a significant decrease in HR and blood flow through the LGA (Table 2). The dose of 20 $\mu\text{mol kg}^{-1}$ L-NAME appeared to be maximally effective, because the effects of a 3 times higher dose of the drug (60 $\mu\text{mol kg}^{-1}$) were not different from those of 20 $\mu\text{mol kg}^{-1}$ L-NAME (Table 2). The vehicle (saline,

1 ml kg^{-1}) had no influence on MAP, HR and blood flow through the LGA (Table 2).

D-NAME (20 and 60 $\mu\text{mol kg}^{-1}$, i.v.) caused a delayed and moderate increase in MAP but failed to change HR (Table 2). Whilst the hypertensive effect of L-NAME was fully developed within 5–10 min after injection of the drug, it took more than 15 min until the hypertensive effect of D-NAME reached the level of statistical significance. The increase in MAP caused by 20 $\mu\text{mol kg}^{-1}$ D-NAME did not differ from that caused by 60 $\mu\text{mol kg}^{-1}$ D-NAME (Table 2). Unlike L-NAME, D-NAME tended to increase blood flow through the LGA, but this effect reached statistical significance only in the experiments involving 20 $\mu\text{mol kg}^{-1}$ D-NAME (Table 2).

Effect of L-NAME and D-NAME on the hyperaemic action of CGRP in the LGA

Intravenous injection of rat CGRP- α (0.03 to 1 nmol kg^{-1}), but not of the vehicle (saline, 1 ml kg^{-1}), evoked an immediate and transient increase in blood flow through the LGA (Figure 1a). This action of CGRP was dose-dependent (Figure 2).

L-NAME (20 and 60 $\mu\text{mol kg}^{-1}$, i.v.) reduced the potency of CGRP in augmenting blood flow through the LGA when compared with the activity of CGRP in rats treated with saline (1 ml kg^{-1}), whereas D-NAME (20 and 60 $\mu\text{mol kg}^{-1}$) had no effect (Figure 2). Following injection of L-NAME the CGRP dose-response curve was shifted to the right in a parallel manner, with no depression of the maximal hyperaemic response to CGRP (Figure 2). The doses of 20 and 60 $\mu\text{mol kg}^{-1}$ L-NAME did not differ in their ability to attenuate the hyperaemic activity of CGRP (Figure 2). Graphical extrapolation at the level of 140% increase in LGA blood flow showed that 20 $\mu\text{mol kg}^{-1}$ L-NAME shifted the CGRP dose-response curve by a factor of 4 (Figure 2a) compared with a shift of 2.8 caused by 60 $\mu\text{mol kg}^{-1}$ L-NAME (Figure 2b). This observation indicates that the dose of 20 $\mu\text{mol kg}^{-1}$ L-NAME is maximally effective in antagonizing the dilator action of CGRP in the LGA.

Effect of L-NAME and D-NAME on the hypotensive action of CGRP

Intravenous injection of rat CGRP- α (0.03 to 1 nmol kg^{-1}) induced an immediate and transient fall in MAP which was accompanied by a transient increase in the heart rate (HR) (Figure 1a). The hypotensive (Figure 2) and tachycardiac (data not shown) actions of CGRP were dose-dependent; the vehicle (saline, 1 ml kg^{-1}) was devoid of any effect (data not shown).

L-NAME (20 and 60 $\mu\text{mol kg}^{-1}$, i.v.) diminished the hypotensive potency of CGRP when compared with the activity of CGRP in rats treated with saline (1 ml kg^{-1}) (Figure 2). The effect of L-NAME consisted of a parallel rightward shift of the CGRP dose-response curve, the magnitude of the shift (1.8–2 fold) being independent of whether 20 or 60 μmol

Table 1 Basal values of mean arterial blood pressure (MAP), heart rate (HR), blood flow in the left gastric artery (BF/LGA), gastric MBF measured by laser Doppler flowmetry (LDF Flux/GM) and gastric MBF measured by the hydrogen gas clearance technique (Gastric MBF) as measured before administration of N^G-nitro-L-arginine methyl ester (L-NAME), D-NAME or their vehicle (saline)

Parameter	Unit	Value	n
MAP	mmHg	90 $\pm$ 1.1	81
HR	min ⁻¹	431 $\pm$ 5.7	58
BF/LGA	$\mu\text{l min}^{-1}$	530 $\pm$ 30	58
LDF Flux/GM	arbitrary units	98 $\pm$ 7.1	21
Gastric MBF	$\mu\text{l min}^{-1} \text{g}^{-1}$	546 $\pm$ 35	14

The values shown are mean $\pm$ s.e.mean.

Table 2 Effect of i.v. injected saline (1 ml kg^{-1}), N^G-nitro-L-arginine methyl ester (L-NAME) and D-NAME (20 and 60 $\mu\text{mol kg}^{-1}$) on mean arterial blood pressure (MAP), heart rate (HR), blood flow in the left gastric artery (BF/LGA), and laser Doppler flux readings in the gastric mucosa (LDF Flux/GM) as measured 30 min post-drug injection

Parameter	Saline (1 ml kg^{-1})	L-NAME (20 $\mu\text{mol kg}^{-1}$)	L-NAME (60 $\mu\text{mol kg}^{-1}$)	D-NAME (20 $\mu\text{mol kg}^{-1}$)	D-NAME (60 $\mu\text{mol kg}^{-1}$)
MAP	102 $\pm$ 1% (21)	160 $\pm$ 4% (13)**	169 $\pm$ 5% (17)**	116 $\pm$ 3% (13)**	120 $\pm$ 3% (16)**
HR	103 $\pm$ 1% (14)	93 $\pm$ 3% (13)**	94 $\pm$ 3% (8)*	102 $\pm$ 1% (13)	100 $\pm$ 1% (9)
BF/LGA	100 $\pm$ 4% (15)	67 $\pm$ 5% (13)**	65 $\pm$ 7% (8)**	128 $\pm$ 12% (13)*	120 $\pm$ 13% (9)
LDF Flux/GM	104 $\pm$ 7% (7)	ND	76 $\pm$ 7% (7)**	ND	106 $\pm$ 4% (7)

The values shown are expressed as a percentage of the values recorded immediately before administration of saline, L-NAME or D-NAME and are mean $\pm$ s.e.mean. The number of observations (animals) is given in parentheses.

* $P < 0.05$, ** $P < 0.01$ versus saline. ND, not determined.

Figure 1 (a) Recordings of the actions of rat calcitonin gene-related peptide- α (CGRP) and vasoactive intestinal polypeptide (VIP), injected i.v., on heart rate (HR), mean arterial blood pressure (MAP) and blood flow in the left gastric artery (BF/LGA). (b) Recording of the action of rat CGRP, injected i.v., on blood flow in the gastric mucosa as determined by laser Doppler flowmetry (LDF flux/GM).

Figure 2 Dose-response relationship for the ability of i.v. injected rat calcitonin gene-related peptide- α (CGRP) to increase blood flow in the left gastric artery (BF/LGA) and to decrease mean arterial blood pressure (MAP) in rats pretreated with (a) saline (NaCl , 1 ml kg^{-1} ○), N^G -nitro-D-arginine methyl ester (D-NAME, $20 \mu\text{mol kg}^{-1}$ ●) or L-NAME $20 \mu\text{mol kg}^{-1}$ ◆) or (b) with saline (NaCl , 1 ml kg^{-1} ○), D-NAME ($60 \mu\text{mol kg}^{-1}$ ●) or L-NAME ($60 \mu\text{mol kg}^{-1}$ ◆). These drugs were injected i.v. 30 min before the recording of the dose-response curve to CGRP was begun. The ordinate scale shows the percentage changes in BF/LGA and MAP. Results are mean with s.e.mean shown by vertical bars; $n = 7-9$. * $P < 0.05$, ** $P < 0.01$ versus respective values recorded in rats treated with saline.

kg^{-1} L-NAME had been administered (Figure 2). The tachycardiac action of CGRP was not altered by L-NAME in any consistent manner (data not shown).

D-NAME (20 and $60 \mu\text{mol kg}^{-1}$, i.v.) failed to influence the hypotensive potency of CGRP (Figure 2), with the only exception that the fall in MAP evoked by 0.1 nmol kg^{-1} CGRP was significantly inhibited by $20 \mu\text{mol kg}^{-1}$ D-NAME (Figure 2a). The tachycardiac response to CGRP was unchanged by D-NAME (data not shown).

Effect of L-NAME on the hyperaemic action of VIP in the LGA

Experiments involving VIP were carried out to test whether or not the action of a vasodilator peptide other than CGRP is changed by L-NAME as compared with the action of the peptide in the presence of D-NAME. Like CGRP, i.v. injection of VIP (0.3 to 1 nmol kg^{-1}) caused an immediate fall in MAP associated with an increase in HR and blood flow through the LGA (Figure 1a). The potency of VIP in dilating the LGA was lower than that of CGRP (compare Figure 2 with Figure 3), and the duration of the actions of VIP on the cardiovascular system was shorter than that of the actions of CGRP (Figure 1a). The ability of VIP to augment blood flow through the LGA did not differ between rats treated with D-NAME or L-NAME ($20 \mu\text{mol kg}^{-1}$, i.v.) as was the case for the tachycardiac action of VIP (data not shown). However, L-NAME tended to reduce the hypotensive action of VIP when compared with the hypotensive responses to VIP in rats treated with D-NAME, although this effect of L-NAME did not consistently reach the level of statistical significance (Figure 3).

Effect of L-NAME and D-NAME on the hyperaemic action of CGRP in the gastric mucosa

Intravenous injection of rat CGRP- α (0.1 to 1 nmol kg^{-1}) caused an immediate and transient increase in blood flow through the gastric mucosa as measured by laser Doppler flowmetry (LDF) (Figure 1b). The hyperaemic action of CGRP in the gastric mucosa was dose-dependent (Figure 4) but the amplitude of the CGRP-evoked hyperaemia in the gastric mucosa was considerably smaller than that in the LGA (Figure 2). Injection of the vehicle (saline, 1 ml kg^{-1} , i.v.) was devoid of any effect (data not shown).

L-NAME ($60 \mu\text{mol kg}^{-1}$, i.v.) led to a sustained decrease in blood flow through the gastric mucosa as measured by LDF whereas the same dose of D-NAME or the vehicle (saline, 1 ml kg^{-1}) did not alter the LDF signal (Table 2). The

Figure 3 (a) Dose-dependent increase in blood flow in the left gastric artery (BF/LGA) and (b) decrease in mean arterial blood pressure (MAP) in response to i.v. injection of vasoactive intestinal polypeptide (VIP) in rats treated with N^G -nitro-D-arginine methyl ester (D-NAME, $20 \mu\text{mol kg}^{-1}$, open columns) or L-NAME ($20 \mu\text{mol kg}^{-1}$, solid columns). These drugs were injected i.v. 30 min before the recording of the actions of VIP was begun. The ordinate scale shows the percentage changes in BF/LGA and MAP. Results are mean with s.e.mean shown by vertical bars; $n = 8$. * $P < 0.05$ versus respective values recorded in rats treated with D-NAME.

Figure 4 Dose-response relationship for the ability of i.v. injected rat calcitonin gene-related peptide- α (CGRP) to increase blood flow in the gastric mucosa as measured by laser Doppler flowmetry (LDF/GM) in rats pretreated with saline (NaCl, 1 ml kg⁻¹ ○), N^G-nitro-D-arginine methyl ester (D-NAME, 60 μ mol kg⁻¹ ●) or L-NAME (60 μ mol kg⁻¹ ◆). These drugs were injected i.v. 30 min before the recording of the dose-response curve to CGRP was begun. The ordinate scale shows the percentage changes in BF/LGA. Results are mean with s.e.mean; $n = 7$. ** $P < 0.01$ versus respective values recorded in rats treated with saline.

potency of CGRP in augmenting blood flow through the gastric mucosa was reduced by L-NAME when compared with the activity of CGRP in rats treated with saline (Figure 4). Graphical extrapolation at the level of 40% increase in the LDF signal showed that L-NAME caused a 4.5 fold shift of the CGRP dose-response curve to the right, with no apparent depression of the maximal action of the peptide (Figure 4). In contrast, D-NAME failed to affect the hyperaemic activity of CGRP in the gastric mucosa (Figure 4).

The inhibitory effect of L-NAME on the CGRP-evoked hyperaemia in the gastric mucosa was confirmed in experiments in which gastric MBF was measured by the hydrogen gas clearance technique (Table 3). Close arterial injection of L-NAME (20 μ mol kg⁻¹) to the stomach reduced gastric MBF by about 40%, whereas the same dose of D-NAME had no influence on MBF (Table 3). L-NAME prevented the gastric hyperaemia evoked by close arterial infusion of CGRP (60 pmol min⁻¹) to the stomach, which resulted in a 70% increase in gastric MBF in rats treated with D-NAME (Table 3). Saline-treated rats were not used in these experiments which were conducted solely to confirm that L-NAME, relative to D-NAME, inhibits the vasodilator action of CGRP in the gastric mucosa.

Table 3 Effect of N^G-nitro-D-arginine methyl ester (D-NAME) and L-NAME (20 μ mol kg⁻¹, i.a.) on basal blood flow (Basal MBF) and calcitonin gene-related peptide (CGRP)-evoked hyperaemia (CGRP hyperaemia) in the gastric mucosa as measured by the hydrogen gas clearance technique

Parameter	D-NAME	L-NAME
Basal GMBF	94 $\pm$ 14% (7)	59 $\pm$ 4% (7)*
CGRP hyperaemia	173 $\pm$ 25% (7)	103 $\pm$ 11% (7)*

D-NAME and L-NAME (20 μ mol kg⁻¹) were injected close arterially to the stomach 100 min before close arterial infusion of CGRP (60 pmol min⁻¹) to the stomach. The values shown are expressed as a percentage of the values recorded immediately before administration of L-NAME or D-NAME (Basal MBF) or immediately before administration of CGRP (CGRP hyperaemia) and are mean $\pm$ s.e.mean. The number of observations (animals) is given in parentheses.

* $P < 0.05$ versus D-NAME.

Effect of L-NAME and D-NAME on the dilator action of CGRP in the isolated bed of the LGA

Injection of rat CGRP- α reduced the perfusion pressure in the left gastric arterial bed precontracted with methoxamine (Figure 5a). The CGRP-induced decline in the perfusion

Figure 5 (a) Recording of the action of rat calcitonin gene-related peptide- α (CGRP) on the perfusion pressure in the isolated bed of the rat left gastric artery in the presence of N^G-nitro-D-arginine methyl ester (D-NAME, 40 μ M; first injection of CGRP) and L-NAME (40 μ M; second injection of CGRP). Ordinate scale: perfusion pressure generated by the addition of methoxamine (50 μ M). (b) Dose-dependent actions of rat CGRP- α (●) and rat CGRP- β (○), given as bolus injections, on the perfusion pressure in the isolated bed of the left gastric artery precontracted with methoxamine (25–90 μ M). Ordinate scale: reduction of the perfusion pressure, expressed as a percentage of the perfusion pressure generated by methoxamine. Results are mean $\pm$ s.e.mean; $n = 8$.

Table 4 Effect of saline, N^G-nitro-D-arginine methyl ester (D-NAME) and L-NAME on the perfusion pressure and the relaxant action of calcitonin gene-related peptide (CGRP) in the isolated bed of the rat left gastric artery precontracted with methoxamine

Treatment	Perfusion pressure (mmHg)	Hypotension induced by CGRP (%)
Saline	92 $\pm$ 9	47 $\pm$ 3
D-NAME	99 $\pm$ 11	47 $\pm$ 4
L-NAME	115 $\pm$ 11	43 $\pm$ 6

Results are mean $\pm$ s.e.mean; $n = 7$. No significant differences. The preparations were continuously perfused with saline containing methoxamine. D-NAME and L-NAME (40 μ M) were administered by perfusion, the perfusion pressure being measured 20 min after their infusion was started and immediately before a bolus injection of rat CGRP- α (10–30 pmol) was performed. The relaxant action of CGRP is expressed relative to the perfusion pressure generated by methoxamine.

pressure, i.e. vasodilatation, was reproducible for up to 5 h when successive injections of rat CGRP- α (10–30 pmol) were carried out at 20 min intervals. The dilator responses to rat CGRP- α and rat CGRP- β were dose-dependent, rat CGRP- α being at least 3 times more potent than rat CGRP- β (Figure 5b). Perfusion of D-NAME (40 μ M) did not significantly alter the perfusion pressure and failed to influence the vasodilator action of rat CGRP- α (Table 4). Perfusion of L-NAME (40 μ M) increased the perfusion pressure (Figure 5a), an effect that slowly waned with time (Table 4). The vasodilator action of CGRP was not significantly changed in the presence of L-NAME (Table 4).

Discussion

The present data confirm that pmol doses of CGRP increase blood flow in the rat gastric mucosa (Holzer & Guth, 1991) and, in addition, show that CGRP is at least as potent in dilating extramural arterial vessels of the rat stomach as it is in dilating mucosal microvessels. The gastric hyperaemic action of CGRP was demonstrated both *in vivo* and in the isolated perfused bed of the LGA *in vitro*. This isolated preparation, which is drained via submucosal arterioles that have been cut during dissection, was used to measure the resistance of extramucosal arterial vessels in the stomach. Rat CGRP- α was chosen as the molecular form of CGRP to be tested in the *in vivo* experiments, since this variant of CGRP prevails in the primary afferent nerve fibres that supply the splanchnic arterial bed (Mulder et al., 1988; Sternini & Anderson, 1992). Additional experiments revealed that rat CGRP- α is about 3 times more potent than rat CGRP- β in reducing the resistance of the left gastric arterial bed *in vitro*, which is consistent with the finding that human CGRP- α is more active in increasing blood flow in the rabbit stomach than human CGRP- β (Bauerfeind et al., 1989).

L-NAME and D-NAME were employed to examine whether the CGRP-evoked hyperaemia in the LGA and gastric mucosa of the rat involves NO as a secondary vasodilator mediator. As shown previously, inhibition of NO synthesis by L-NAME resulted in hypertension and bradycardia (Gardiner et al., 1990; Rees et al., 1990) and in a decline of gastric blood flow (Lippe & Holzer, 1992; Tepperman & Whittle, 1992; Whittle et al., 1992). In contrast, D-NAME was without influence on HR and tended to increase blood flow in the LGA rather than to decrease it. D-NAME, though, enhanced MAP albeit this effect of D-NAME was delayed and less pronounced than that of L-NAME. The present finding of a hypertensive effect of D-NAME corroborates the study of Abdelrahman et al. (1992) but is at odds with the negative findings of Gardiner et al. (1990) and Rees et al. (1990).

Although D-NAME, the enantiomer believed to be inactive on the NO synthase (Gardiner et al., 1990; Rees et al., 1990; Moncada et al., 1991), changed MAP and blood flow in the LGA to some extent, it did not alter the action of CGRP in inducing hypotension and increasing gastric blood flow. In contrast, L-NAME caused a small reduction of the CGRP-evoked fall in MAP, as has been reported previously (Abdelrahman et al., 1992), and a more pronounced inhibition of the CGRP-induced hyperaemia in the LGA and gastric mucosa. The enantiomer specificity of the effect of L-NAME in inhibiting the vasodilator activity of CGRP indicates that L-NAME interfered with CGRP-evoked hyperaemia by way of inhibition of NO formation and not by way of its vasoconstrictor effect. The validity of this conclusion is supported by a number of other findings. (i) The hypotensive action of CGRP is not blunted when the α -adrenoceptor agonist phenylephrine is used to augment MAP (Abdelrahman et al., 1992). (ii) The antagonistic effect of L-NAME on the CGRP-induced gastric hyperaemia *in vivo* was overcome by increasing the dose of CGRP. (iii) The dilator action of VIP on the LGA *in vivo* was left unaltered by L-NAME. VIP

is present in nerve fibres around splanchnic blood vessels (Della et al., 1983), and the present results show that, as in other vascular beds (Burnstock, 1990), VIP dilates the LGA in a NO-independent manner. (iv) Hyperaemia in the gastric mucosa evoked by glyceryl trinitrate remains unaltered by blockade of NO formation (Walder et al., 1990). Thus, inhibition of the L-arginine:NO system does not cause general suppression of the dilator capacity of gastric resistance vessels but counteracts the hyperaemic responses to CGRP (this study) and pentagastrin (Walder et al., 1990; Pique et al., 1992) in a selective manner.

The data obtained with L-NAME signify, therefore, that the dilator action of CGRP on the gastric circulation *in vivo* involves two mechanisms, a NO-dependent process that is activated by low doses of the peptide and a NO-independent process that becomes apparent with higher doses of CGRP. This inference is affirmed by the fact that the doses of L-NAME used here (20 and 60 μ mol kg⁻¹) appeared to be maximally effective. The contribution of NO to the hyperaemic action of CGRP was similar in the LGA and gastric mucosa, as in both tissues L-NAME caused a parallel rightward shift of the CGRP dose-response curves by a factor of 2.8–4.5, abolishing the dilator action of low doses of CGRP. This means that, if the low levels of circulating CGRP (Diez Guerra et al., 1988) were to regulate blood flow through the stomach, they would do so in a primarily NO-dependent manner. Further evidence for a contribution of NO to the hyperaemic action of CGRP in the gastric mucosa comes from a study in which a low dose of L-NAME (8 μ mol kg⁻¹) was found to attenuate the vasodilator action of CGRP (Whittle et al., 1992) and from the present experiments in which gastric MBF was measured by the hydrogen gas clearance technique.

Unlike *in vivo*, L-NAME failed to inhibit significantly the dilator action of CGRP in the isolated bed of the LGA *in vitro*, an observation that is in line with a report that the relaxant action of the peptide on LGA strips does not require the presence of the endothelium, an important source of NO (Bråtevit et al., 1991). Although the discrepancy between the *in vivo* and *in vitro* observations cannot yet be explained conclusively, it is conceivable that the system which forms NO in response to CGRP either is no longer present or is functionally damaged in the *in vitro* preparations. Whatever the reason, our findings point to a potential limitation in the use of isolated perfused vascular preparations for the study of the L-arginine:NO system.

In conclusion, the hyperaemic action of CGRP in the rat gastric circulation involves both NO-dependent and NO-independent mechanisms. This dual mode of action is in keeping with the dual distribution of CGRP receptors to both endothelium and smooth muscle of gastrointestinal arteries and arterioles in the rat (Sternini et al., 1991). CGRP released from sensory nerve endings participates in the rise of MBF caused by capsaicin-induced sensory nerve stimulation (Li et al., 1991), hyperaemia strengthening the resistance of the gastric mucosa to experimental injury (Lippe et al., 1989; Holzer et al., 1991). The mucosal hyperaemic responses to CGRP (this study) and sensory nerve stimulation by capsaicin (Whittle et al., 1992) or acid back-diffusion (Lippe & Holzer, 1992) as well as the gastric mucosal protective actions of CGRP (Lambrecht & Peskar, 1992) and capsaicin-induced sensory nerve stimulation (Peskar et al., 1991) all depend on NO. These findings indicate that NO is a major secondary mediator of the hyperaemic and protective actions of CGRP released from sensory nerve endings in the stomach.

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