British Journal of Pharmacology (1996) 118, 392-399

Localization and activity of haem oxygenase and functional effects of carbon monoxide in the feline lower oesophageal sphincter

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1 In the feline lower oesophageal sphincter (LOS), the distribution of the carbon monoxide (CO) producing enzymes haem oxygenase (HO)-1 and -2 was studied by immunohistochemistry and confocal microscopy, the HO activity was measured and the possible role for CO as a mediator of relaxation was investigated.

2 HO-2 immunoreactivity was abundant in nerve cell bodies of the submucosal and myenteric plexus. Approximately 50% of the HO-2-containing myenteric cell bodies were also nitric oxide synthase- and vasoactive intestinal peptide (VIP)-immunoreactive. In addition, HO-2 immunoreactivity was seen in nerve fibres, in non-neuronal cells dispersed in the smooth muscle and in arterial endothelium. HO-1 immunoreactivity was confined to non-neuronal cells in the smooth muscle, similar to those positive for HO-2.

3 Activity of HO, measured as CO production, was observed in LOS homogenates at a rate of 1.00 ± 0.05 nmol mg^{-1} protein h^{-1}. This production was inhibited by the HO inhibitor, zinc protoporphyrin-IX (ZnPP).

4 In isolated circular smooth muscle strips of LOS, developing spontaneous tone, exogenously administered CO evoked a concentration-dependent relaxation reaching a maximum of $93 \pm 3\%$. This relaxation was accompanied by an increase in cyclic GMP, but not cyclic AMP levels. The relaxant response was attenuated by methylene blue, but unaffected by tetrodotoxin. Repeated exposure to CO resulted in a progressive reduction of the relaxant response.

5 ZnPP caused a rightward-shift of the concentration-response curves for the relaxant responses to VIP, peptide histidine isoleucine, and pituitary adenylate cyclase activating peptide 27.

6 ZnPP and tin protoporphyrin-IX (another inhibitor of HO) did not affect nonadrenergic, noncholinergic relaxations induced by electrical field stimulation. Nor did ZnPP affect relaxations induced by 3-morpholino-sydnonimine or forskolin.

7 The present findings, showing localization of HO immunoreactivity to both neuronal and non-neuronal cells of the feline LOS, ability of LOS to produce CO and a relaxant effect of CO in circular LOS muscle, suggest a role for CO as a peripheral messenger.

Keywords: Carbon monoxide; haem oxygenase; metalloporphyrins; nitric oxide synthase; nonadrenergic, noncholinergic nerves; oesophagogastric junction; peptide histidine isoleucine; pituitary adenylate cyclase activating peptide; smooth muscle; vasoactive intestinal peptide

Introduction

Nitric oxide (NO) has been established as a messenger molecule in signal transduction in both the central and peripheral nervous systems (Rand & Li, 1995; Zhang & Snyder, 1995). Recently, it was also suggested that carbon monoxide (CO) might be involved in neurotransmission (Maines, 1993; Dawson & Snyder, 1994). In the periphery, a role for CO has been proposed in vascular and gastrointestinal control (Farrugia *et al.*, 1993; Rattan & Chakder, 1993; Morita *et al.*, 1995; Ny *et al.*, 1995c; Prabhakar *et al.*, 1995).

The main source for endogenous CO in mammals is through the degradation of haem to biliverdin by the enzyme, haem oxygenase (HO; e.g. Maines, 1988; 1993). This enzyme exists in at least two different isoforms, the inducible HO-1, and the constitutive HO-2. HO-2 is believed to be associated with CO production in the brain, where HO-2 immunoreactivity and high levels of HO-2 mRNA have been detected in various neuronal structures (Ewing *et al.*, 1992; Verma *et al.*, 1993; Vincent *et al.*, 1994). HO activity and CO production have been demonstrated in various peripheral tissues, e.g. liver, spleen, and smooth muscle (Maines, 1988; Vreman & Stevenson, 1988; Morita *et al.*, 1995).

In the feline lower oesophageal sphincter (LOS), inhibitory control is exerted through nonadrenergic, noncholinergic (NANC) nerves. Some of these use NO as a transmitter, but other mediator(s), such as vasoactive intestinal peptide (VIP), may also be involved (Ny *et al.*, 1995a). Recent results suggest that CO may also be involved in feline LOS control (Ny *et al.*, 1995c).

In the present study, we investigated further the localization and activity of HO, and the possible role of CO as a mediator of mechanical activity in the feline LOS. The relation of HO/CO to VIP and the NO system was also studied. HO enzymes were localized by immunohistochemistry and confocal microscopy, the activity of the enzymes was quantified by measuring CO production and the functional effects of CO and HO inhibitors were investigated in strips from feline isolated LOS.

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Methods

Tissue preparation

Twenty-two adult male cats, with a weight of approximately 4.0 kg, were anaesthetized by i.v. administered α-chloralose (60 mg kg^{-1}) and killed by an i.v. injection of air or a saturated KCl solution. The distal two thirds of the oesophagus with adjacent parts of the stomach, were removed and opened longitudinally. The LOS was identified as a thickening of the circularly running, inner smooth muscle layer at the oesophagogastric junction, as described in detail previously (Ny *et al.*, 1995a). Whole wall specimens used for histological examination were put in formaldehyde. Tissue specimens used for functional studies and HO activity measurements were placed in an ice-cold Krebs solution (for composition, see below). Specimens were pinned flat with the mucosal side up and stretched to the *in vivo* length and the muscularis externa was dissected free. Transverse segments were prepared from the circular smooth muscle at the oesophagogastric junction. Tissue used for measurements of HO activity was stored in liquid nitrogen until used.

Functional experiments were performed on tissues from all animals. In separate groups of cats, tissues were used for histological investigations, measurement of HO activity, or measurement of cyclic nucleotide content.

Immunohistochemistry and confocal microscopy

Tissue specimens from 6 cats were fixed for 4 h in an ice-cold solution of 4% formaldehyde in phosphate-buffered saline (PBS; pH 7.4), and rinsed in 15% sucrose in PBS (at least 3 rinses during 3 days). The tissue pieces were then frozen at −40°C in isopenthane and stored at −70°C, before sectioning in a cryostat. Cryostat sections were cut at a thickness of 10 μm and thaw-mounted onto chrome-alum coated slides. For the simultaneous demonstration of two antigens (Wessendorf & Elde, 1985), sections were incubated at 25°C in the presence of two primary antisera, raised in two different species (rabbit anti-HO-2 and guinea-pig anti-VIP, or guinea-pig anti-nitric oxide synthase; NOS). The sections were first incubated overnight with HO-2-antiserum, rinsed in PBS, and then incubated overnight with VIP or NOS-antisera. After rinsing for 10 min in PBS (3 rinses), the sections were incubated for 90 min with Texas Red conjugated anti-rabbit immunoglobulins, rinsed, and incubated for 90 min with fluorescein isothiocyanate (FITC)-conjugated anti-guinea-pig immunoglobulins. Subsequently, the sections were mounted in glycerol/PBS with *p*-phenylenediamine to prevent fluorescence fading (Johnson & Araujo, 1981).

The sections were examined with an Olympus 3 × 50 system fluorescence microscope equipped with epi-illumination or with a confocal laser scanning microscope (Multiprobe 2001 CLSM; Molecular Dynamics, Inc.) equipped with an Ar/Kr laser and an inverted Nikon Diaphot TMD microscope. The confocal microscope analysis was performed as described previously (Ny *et al.*, 1995a). The confocal microscope allowed evaluation of 3-dimensional distribution, cellular distribution, and whether two immunoreactivities were localized within the same nerve structure. By making consecutive series, rather than simultaneous detection, signal/noise ratio was optimized and 'leakage' of FITC fluorescence into the Texas Red channel and *vice versa*, was eliminated.

The primary antisera used were: HO-1 and HO-2 rabbit antisera (Stressgen Biotech. Corp., Victoria, Canada; diluted 1:1000 in PBS), guinea-pig VIP-antiserum (Euro-Diagnostica, Malmö, Sweden; code no. B-GP-340-100; diluted 1:640 in PBS) and guinea-pig neuronal NOS antiserum (Euro-Diagnostica, Malmö, Sweden; code no. B225-1; diluted 1:500 in PBS). The secondary antibodies used were Texas Red-conjugated affinity-purified $F(ab')_2$ fragments of donkey anti-rabbit immunoglobulins (1:80; Jackson ImmunoResearch Inc, West Grove, PA, U.S.A., code no. 771-076-152) and FITC-conjugated goat anti-guinea-pig immunoglobulins (1:80 in PBS; Sigma, St Louis, MO, U.S.A., code no. F-6261). In control experiments, no immunoreactivity could be detected in sections incubated with primary antisera absorbed with excess of the respective antigens (100 μg ml^{-1}), or in the absence of primary antisera. The structures related are referred to as HO-1-, HO-2-, VIP- and NOS-immunoreactive (IR), as cross reactions with other antigens, sharing similar amino acid sequences, cannot be completely excluded.

Measurement of HO activity

LOS specimens of the muscularis externa, including the inner circular smooth muscle layer, the myenteric plexus, and the outer longitudinal smooth muscle layer, were put into 3–5 volumes (w/v) of KH_2PO_4 (0.1 mM, pH 7.4) and homogenized. The tissue homogenates were centrifuged at 18,000 *g* for 10 min at 4°C. An incubation mixture consisting of the supernatant of the tissue specimens, met-haemalbumin and β-NADPH, was made. The HO activity analysis was performed as described previously (Grundemar *et al.*, 1995). The incubation mixture was incubated at 37°C for 10 min in a water bath, protected from light. The incubation was terminated by placing the mixture on ice. In a control medium, met-haemalbumin and β-NADPH were replaced by phosphate buffer. In some experiments the HO inhibitor, zinc protoporphyrin-IX (ZnPP; 10 μM) was included in the incubation medium to which LOS tissue homogenates was added, and this mixture was subsequently incubated 10 min prior to HO analysis. A concentration of 10 μM ZnPP has in previous studies been shown to be effective in inhibiting HO activity (Vreman *et al.*, 1989). The incubation mixture and the control were then injected into a gas chromatograph (Varian 3400, U.S.A.) as described by Cavallin-Ståhl *et al.* (1978). The net production of CO was determined by subtracting the CO production in the tube containing substrates and tissue with that containing phosphate buffer and tissue only. A 99.9% CO gas was used as a standard, to which all calculations of CO production were related. The protein content in the supernatant was determined according to the method described by Bradford (1976), with bovine serum albumin used as a standard.

Functional studies

The tissue was pinned flat with the mucosal side up and stretched to its *in vivo* length. The mucosa was removed and circular smooth muscle preparations (1–2 mm wide and 20 mm long) were cut in the direction of the muscle fibres. Each preparation was then cut into 3–4 minor strips, and silk-ligatures were tied at both ends. There was a distance of 5 mm between the knots. The strips were transferred to thermostatically controlled (37°C) 5 ml tissue baths containing Krebs solution (for composition, see below) bubbled with 5% CO_2 and 95% O_2 (pH 7.4) and mounted between two L-formed hooks. One hook was attached to a force transducer (Grass FT03) for measurement of mechanical activity and the other was connected to a sledge, which allowed adjustment of the passive tension of the strip. The recordings were made on a Grass polygraph, 7D or E. The strips were stretched from resting length (L_R) until about 160% of L_R was reached (Ny *et al.*, 1995a), and thereafter allowed to equilibrate for 1 h. Only strips that developed a spontaneous, active tone during this period were used. The passive tension of the muscle strips was determined by exposure to a 'Ca^{2+}-free'-Krebs solution containing 0.1 mM ethylene glycol-*bis* (β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA; for composition, see below). The base-line obtained was used as the 0-level from which all calculations of relaxant responses were measured.

Electrical field stimulation (EFS) was applied by means of platinum wire electrodes placed in parallel with the strip at a distance of 1 mm from the strip. The stimulation frequency was varied between 1–20 Hz. Five-second trains of square wave pulses (0.8 ms duration, supramaximal voltage) were

delivered every 2 min. In some experiments, continuous stimulation for 2 min was performed at 10 Hz. All experiments using EFS were carried out in the presence of scopolamine (1 μM).

Measurement of cyclic nucleotide concentrations

Guanosine 3':5'-cyclic monophosphate (cyclic GMP) and adenosine 3':5'-cyclic monophosphate (cyclic AMP) contents were analysed in LOS preparations at the spontaneously developed tension level (control), and after exposure to 24 μM CO. When a stable tension level was achieved, the strip was rapidly removed from the tissue bath and frozen in liquid nitrogen. The tissue was homogenized in 2 ml 10% trichloroacetic acid (TCA) in water with a glass-glass homogenizer, and centrifuged at 1500 *g* (4°C) for 10 min. The protein content was determined by the method described by Bradford (1976), with bovine serum albmunin used as a standard. The supernatants were extracted 5 times with 5 ml of water-saturated diethyl ether. The aqueous phase was evaporated and the residue stored at −20°C. Residues were dissolved in 0.05 M sodium acetate, and the amounts of cyclic GMP and cyclic AMP were quantified by using [^{125}I]-cyclic GMP and [^{125}I]-cyclic AMP radioimmunoassay kits (RIA-NEN, Du Pont Company, Boston, MA, U.S.A.). A [^{3}H]-cyclic AMP recovery marker was added to the TCA tissue homogenate in order to determine the recovery of cyclic GMP and cyclic AMP during ether extraction. The mean recovery was 71%.

Solutions

The Krebs solutions used had the following composition (in mM): Na^+-Krebs solution: NaCl 119, KCl 4.6, $NaHCO_3$ 15, $CaCl_2$ 1.5, $MgCl_2$ 1.2, NaH_2PO_4 1.2 and glucose 11, 'Ca^{2+}-free'-Krebs solution (in mM): NaCl 119, KCl 4.6, $NaHCO_3$ 15, $MgCl_2$ 1.2, NaH_2PO_4 1.2, glucose 11 and EGTA 0.1.

Drugs

The chemicals were obtained from the following sources: forskolin, haem (bovine), methylene blue, N^G-nitro-L-arginine (L-NOARG), β-NADPH, peptide histidine isoleucine (PHI), scopolamine, tetrodotoxin (TTX), vasoactive intestinal peptide (VIP) from Sigma Chemical Company (St Louis, MO, U.S.A.); pituitary adenylate cyclase activating peptide 27 (PACAP 27) from Peninsula Laboratories Inc (Belmont, CA, U.S.A.); tin protoporphyrin-IX (SnPP) and ZnPP from Porphyrin Products Inc (Logan, UT, U.S.A.), and 3-morpholino-sydnonimine (SIN-1) from Casella AG (Frankfurt, Germany).

ZnPP and SnPP were dissolved in 0.2 M NaOH. All other drugs were dissolved in saline. The preincubation time with ZnPP, SnPP and the combination of ZnPP and L-NOARG was 60 min. All experiments using ZnPP and SnPP were performed in darkness by covering the tissue bath with a black plastic film. The preincubation time with L-NOARG, methylene blue, and TTX was 30 min.

A saturated CO solution was prepared by bubbling deoxygenated saline with 99.9% CO gas (Ny *et al.*,1995c). Exposure to CO was achieved by adding increasing volumes of the saturated CO solution to the tissue bath.

Calculations and statistics

Statistical results are expressed as mean ± s.e.mean. When statistical differences between two means were determined, Student's unpaired *t* test was performed, whereas for multiple comparisons a one-way analysis of variance, ANOVA, followed by Dunnet's unpaired two-tailed *t* test was used. $P<0.05$ was regarded as significant. Outliers were checked for by Dixon's gap test. (*n*) is the number of strips tested, and, when not specifically indicated, refers to tissues from different animals. When statistical analyses between means were performed, all values refer to different animals. pIC_{50}, the negative logarithm of the concentration that evokes a 50% relaxation response compared to the 0-level (the Ca^{2+}-free Krebs, see above), was determined by regression analysis, using the values immediately above and below the half maximum response. VIP, PACAP 27, PHI, forskolin, and SIN-1 were added cumulatively and concentration-response curves were constructed.

Results

Immunohistochemistry and confocal microscopy

HO-2 immunoreactivity was observed in the cytoplasm of numerous nerve cell bodies in the submucosal and myenteric plexus (Figure 1a, c). Many of the HO-2 positive cell bodies were also NOS-IR (Figure 1b) and/or VIP-IR (Figure 1d). Approximately 50% of the HO-2-IR myenteric cell bodies were NOS-IR and/or VIP-IR. In general, all NOS and/or VIP containing cell bodies were also HO-2-IR. Confocal microscopy revealed that both HO-2 and NOS immunoreactivities were diffusely distributed in the cytoplasm of the cell bodies (Figure 1a-c), whereas VIP-immunoreactivity was observed in granular structures in close association to the cell nucleus, often localized to only one side of the cell (Figure 1d). HO-2 immunoreactivity occurred in a few nerve fibres and coarse nerve trunks, especially around the cell bodies in the myenteric and submucosal plexus, but only occasionally in the circular smooth muscle layer. Small cells with a non-neuronal appearance, staining positively for HO-2, were located around the myenteric plexus and dispersed in the smooth muscle layer. These cells were elongated and had an HO-2 positive cytoplasm surrounding the immunonegative nucleus. The endothelium of many arteries was also HO-2-IR (Figure 1e), whereas no HO-2 immunoreactivity was observed in veins.

With an antiserum against HO-1, immunoreactive non-neuronal cells, similar to those demonstrated by the HO-2 antiserum, were shown to be dispersed within the smooth muscle layer with a similar distribution pattern to the HO-2-IR non-neuronal cells, but, in addition, found also in the submucosal area (Figure 1f). No HO-1 immunoreactivity was observed in any nerve structures or in blood vessels.

HO activity

The rate of CO production in tissue homogenates from the LOS was 1.00 ± 0.05 nmol mg^{-1} protein h^{-1} ($n=10$; Figure 2). Inclusion of ZnPP (10 μM; $n=6$) in the incubation medium decreased the activity to 0.44 ± 0.07 nmol mg^{-1} protein h^{-1} ($P<0.001$). Inclusion of EGTA (0.1 mM; $n=6$) in the incubation medium resulted in a non-significant reduction of the HO activity to 0.84 ± 0.06 nmol mg^{-1} protein h^{-1}. The CO production was unaffected by preincubating the tissue strips with VIP (1 μM; $n=4$; 1.10 ± 0.19 nmol mg^{-1}protein h^{-1}) or SIN-1 (10 μM; $n=4$; 1.08 ± 0.24 nmol mg^{-1} protein h^{-1}) for 30 min before homogenisation of the tissue. No CO production was observed in LOS homogenates that were boiled before analysis.

Functional studies

Isolated circular smooth muscle strips of the LOS generated a spontaneous tension amounting to 11.8 ± 1.7 mN ($n=138$ from 22 animals). Exogenously administered CO induced a concentration-dependent relaxation reaching a maximal amplitude amounting to $93 \pm 3\%$ ($n=10$; Figures 3 and 4). Repeated exposure to CO, with intermittent washing periods, resulted in a progressive reduction and occasionally a final disappearance of the relaxant response (Figure 3). After such CO-induced desensitization, the relaxant responses to EFS remained unchanged compared to the control. Preincubation with methylene blue for 30 min inhibited the relaxant response

to CO ($n=10$; Figures 3 and 4). Methylene blue increased the muscle tone by $30\pm12\%$ ($n=10$). Pretreatment with tetrodotoxin (1 μM) for 30 min completely blocked relaxation induced by EFS, but did not affect the CO-induced relaxation ($n=6$; Figures 3 and 4).

VIP ($n=6$), PHI ($n=6$), and PACAP 27 ($n=6$) induced

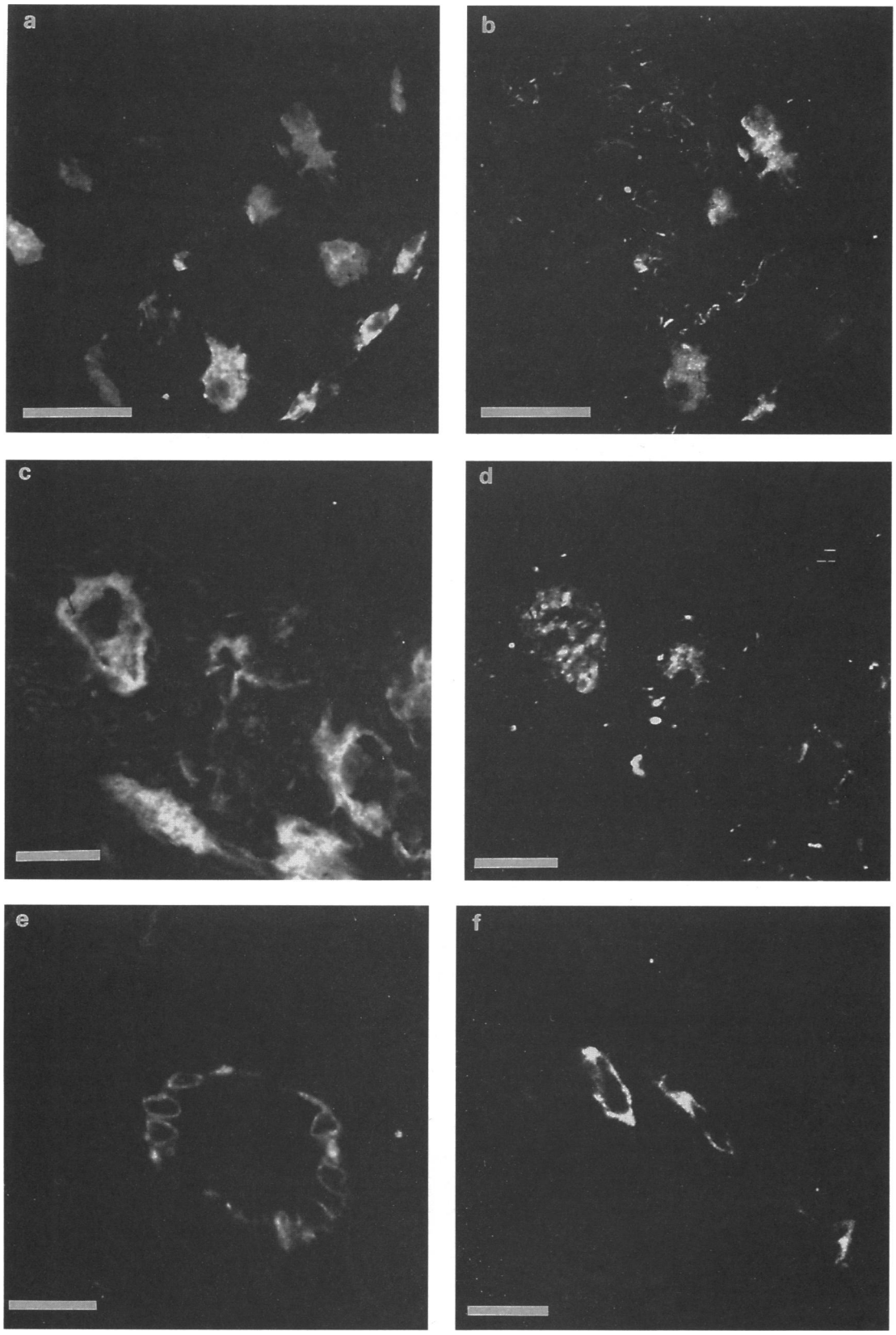

Figure 1 Haem oxygenase (HO) immunoreactivity as demonstrated by confocal microscopy in the feline lower oesophageal sphincter. (a) HO-2-IR myenteric nerve cell bodies (Texas Red-immunofluorescence). Bar 50 μm. (b) The same section as in (a) but immunoreactivity against nitric oxide synthase (FITC-immunofluorescence). Bar 50 μm. (c) HO-2-IR myenteric nerve cell bodies (Texas Red-immunofluorescence). Bar 20 μm. (d) Same section as in (c) but immunoreactivity against vasoactive intestinal peptide (FITC-immunofluorescence). Bar 20 μm. (e) HO-2 immunoreactivity in the endothelium of an artery (Texas Red-immunofluorescence). Bar 10 μm. (f) HO-1 immunoreactive cells, with a non-neuronal appearance, surrounding the smooth muscle bundles (Texas Red-immunofluorescence). Bar 10 μm.

concentration-dependent relaxations (1 nM – 1 μM) that were markedly attenuated by ZnPP (0.1 mM; Table 1 and Figure 5). SnPP (0.1 mM; $n=6$) had a slight inhibitory effect on VIP-induced relaxations (Table 1 and Figure 5). The relaxation induced by either SIN-1 ($n=6$) or forskolin ($n=6$) was unaffected by preincubation with ZnPP (0.1 mM; Table 1). ZnPP (0.1 mM; $n=6$) and SnPP (0.1 mM; $n=6$) did not affect relaxations induced by EFS (0.5 – 20 Hz in 5 s trains or continuous stimulation at 10 Hz for 2 min; Figure 6). ZnPP or SnPP did not affect muscle tone. L-NOARG (0.1 mM) abolished relaxations induced by low frequencies and markedly inhibited relaxations induced by high frequencies (Figure 6). An increase of tone was occasionally seen after L-NOARG treatment. This increase was 7 ± 3% ($n=12$), and tension returned to the initial level within 15 min. Treatment with a combination of ZnPP (0.1 mM) and L-NOARG (0.1 mM) had less inhibitory effect on electrically-induced relaxations than treatment with L-NOARG alone, particularly in the frequency range 4 – 12 Hz, where the difference was significant ($P<0.05$; Figure 6).

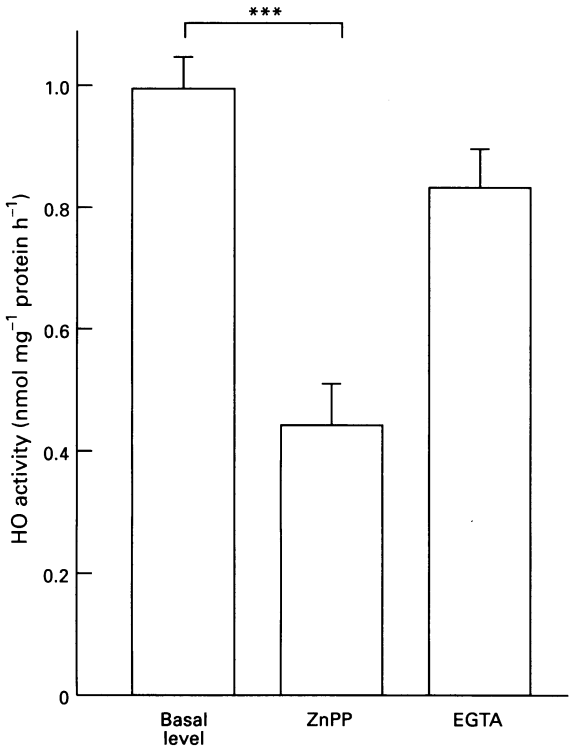

Figure 2 Haem oxygenase activity measured as carbon monoxide production in the feline lower oesophageal sphincter. Measurements were performed at basal level ($n=10$), and after including zinc protoporphyrin-IX (ZnPP; 10 μM; $n=6$), or EGTA (0.1 mM; $n=6$) in the incubation medium. Values are means ± s.e.mean. $^{***}P<0.001$.

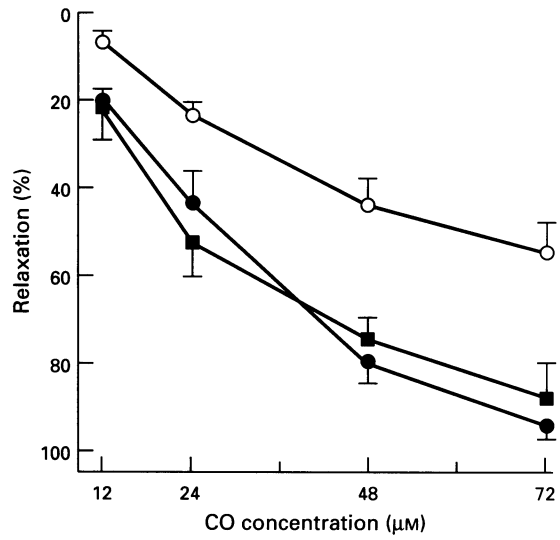

Figure 4 Effect of exogenously administered carbon monoxide on resting tension in circular smooth muscle strips from the feline lower oesophageal sphincter ($n=10$; ●), after pretreatment with methylene blue (30 μM; $n=10$; ○), and tetrodotoxin (1 μM; $n=6$; ■). Values are means ± s.e.mean.

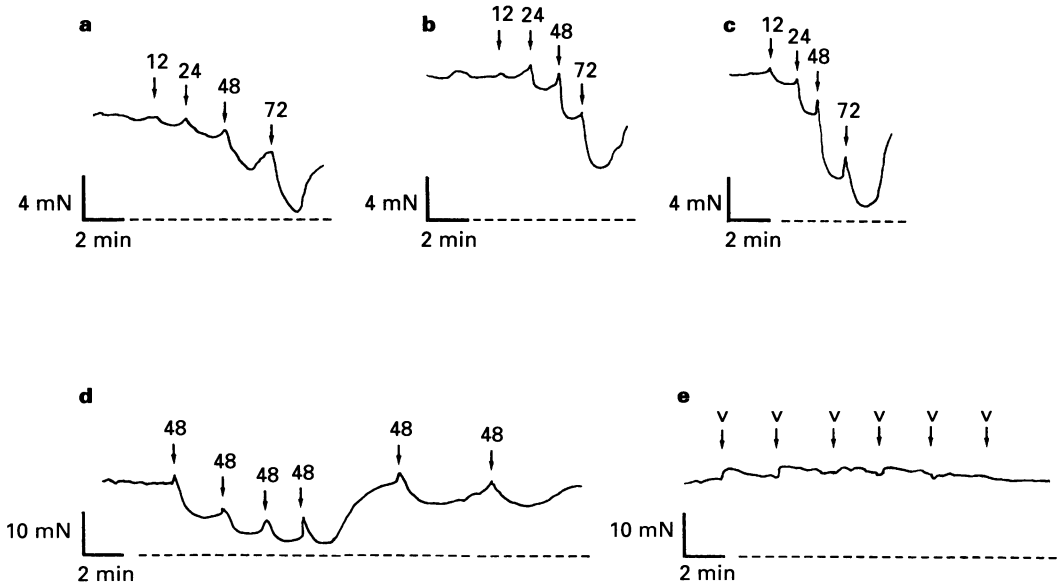

Figure 3 Original tracing illustrating the effect of exogenously administered carbon monoxide (in μM) on resting tension in circular smooth muscle strips from the feline lower oesophageal sphincter. (a) without pretreatment, (b) after pretreatment with methylene blue (30 μM) for 30 min, and (c) after pretreatment with tetrodotoxin (1 μM) for 30 min. (d) after repeated exposure to 48 μM carbon monoxide resulting in a marked reduction of the response, (e) after repeated exposure to vehicle (v).

Table 1 Inhibitory effects of metalloporphyrins (ZnPP and SnPP; 0.1 mM) on relaxation evoked by various agents in circular smooth muscle preparations from the feline lower oesophageal sphincter

	pIC_{50} (M)	P	*Relaxation (%) at highest concentration*	P	n
VIP	7.98±0.12	NA	94±3	$P<0.05$	6
VIP + ZnPP	–		57±14		6
VIP	7.98±0.12	$P<0.05$	94±3	NS	6
VIP + SnPP	7.40±0.27		92±5		6
PHI	7.14±0.14	NA	86±6	$P<0.01$	6
PHI + ZnPP	–		57±6		6
PACAP 27	6.85±0.15	NA	84±14	$P<0.01$	6
PACAP 27 + ZnPP	–		35±11		6
Forskolin	6.52±0.14	NS	98±2	NS	6
Forskolin + ZnPP	6.12±0.14		85±13		6
SIN-1	6.24±0.26	NS	93±5	NS	5
SIN-1 + ZnPP	5.68±0.18		92±5		5

For abbreviations, see text.
NS, not statistically significant; NA, not applicable (no pIC_{50} value was calculated since the relaxation in some preparations was below 50%).
*The highest concentration for VIP, PHI and PACAP 27 was 1 μM, and for forskolin and SIN-1 10 μM.

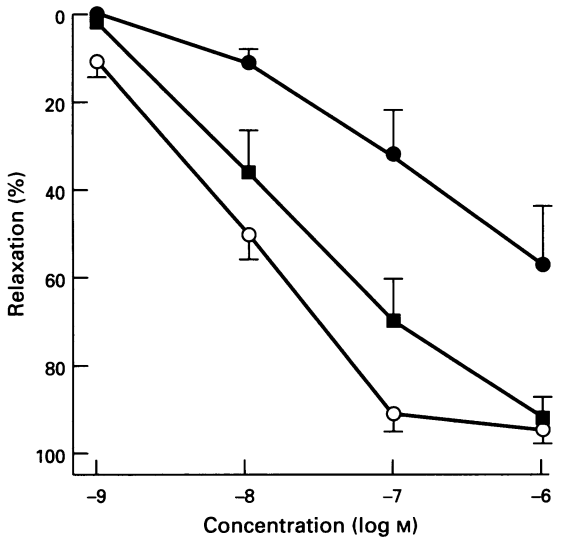

Figure 5 Effects of vasoactive intestinal peptide on resting tension in circular smooth muscle strips from the feline lower oesophageal sphincter after pretreatment with zinc protoporphyrin-IX (ZnPP; $n=6$; ●), tin protoporphrin-IX (SnPP; $n=6$; ■), and control ($n=6$;○). Values are means±s.e.mean.

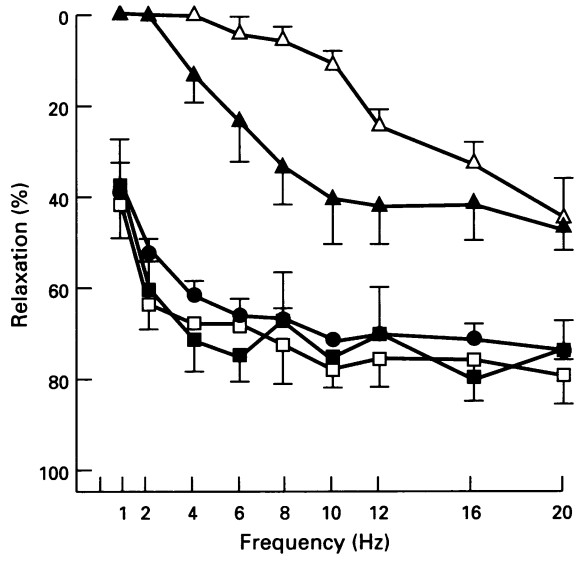

Figure 6 Effects of electrical field stimulation on resting tension in circular smooth muscle strips from the feline lower oesophageal sphincter following pretreatment with 0.1 mM zinc protoporphyrin-IX (ZnPP; $n=6$; □), 0.1 mM tin protoporphyrin-IX (SnPP; $n=6$; ■), 0.1 mM N^G-nitro-L-arginine (L-NOARG; $n=6$; △), 0.1 mM ZnPP+0.1 mM L-NOARG ($n=6$; ▲), and control ($n=6$; ●). Values are means±s.e.mean.

Effects of CO on cyclic nucleotide concentrations

The relaxation evoked by exogenously administered CO (24 μM) was accompanied by a 79% ($P<0.05$) increase of tissue cyclic GMP levels (2.8±0.3 before and 5.0±0.7 pmol mg^{-1} protein h^{-1} after CO stimulation; $n=10$), but cyclic AMP levels remained unchanged (from 38.9±2.4 to 41.6±3.4 pmol mg^{-1} protein h^{-1}; $n=10$; Figure 7).

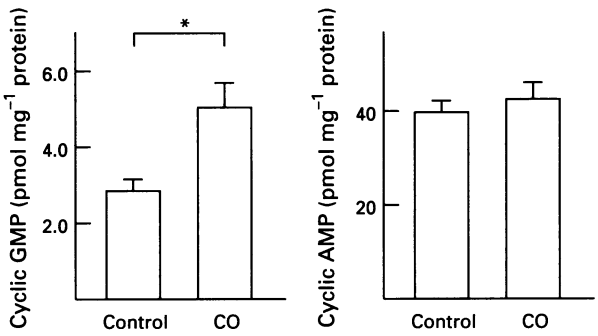

Figure 7 Concentrations of cyclic GMP ($n=10$) and cyclic AMP ($n=10$) in circular smooth muscle strips from the feline lower oesophageal sphincter after treatment with 24 μM carbon monoxide. Values are means±s.e.mean. *$P<0.05$.

Discussion

Recent studies have suggested that CO may have a role as a mediator in cardiovascular control (Ewing *et al.*, 1994; Grundemar *et al.*, 1995; Prabhakar *et al.*, 1995) and in neurotransmission in the brain (Dawson & Snyder, 1994). Furthermore, a role for CO has been proposed in NANC nerve regulation of gastrointestinal motility (Rattan & Chakder,

1993). This study shows that CO can be produced in the feline LOS and that the CO production may have different sources, since HO could be demonstrated in nerve structures, arterial endothelium, and interstitial cells with a non-neuronal appearance, dispersed in the smooth muscle layer. HO-2, the constitutive form, had, as expected, the widest distribution of the isoenzymes, and was the only type observed in neuronal structures. An abundance of HO-2- immunoreactivity was seen in neuronal cell bodies, but HO-2- immunoreactivity was only rarely observed in nerve fibres in the smooth muscle layer. HO-2 immunoreactivity was present in approximately twice as many cell bodies in the myenteric plexus as NOS and/or VIP immunoreactivity. HO-2 immunoreactivity and HO-2 mRNA have previously been detected in certain regions in the central nervous system (Ewing & Maines, 1992; Maines *et al.*, 1993; Verma *et al.*, 1993; Vincent *et al.*, 1994; Dwyer *et al.*, 1995), often localized within the same nerve structures as guanylate cyclase, but only occasionally within the same neurones as NOS. In the feline LOS, we observed HO-2 immunoreactivity also in non-neuronal tissue, such as in the endothelium of many arteries. These findings are in agreement with previous studies demonstrating HO-2 immunoreactivity and HO activity in blood vessels (Ewing *et al.*, 1994; Cook *et al.*, 1995; Grundemar *et al.*, 1995). Small HO-2 immunoreactive cells, with a non-neuronal appearance, were also seen around the smooth muscle bundles near the myenteric plexus. Similar small cells were found also to be HO-1 immunoreactive. The size, form and localization of these HO-1/HO-2 positive cells are similar to those of the interstitial cells of Cajal (Daniel & Berezin, 1992). However, it remains to be established if the population of HO-1/HO-2 immunoreactive cells is related to the interstitial cells of Cajal and/or has a role in the control of smooth muscle motility.

In the periphery, relaxant effects of exogenously applied CO have been demonstrated in blood vessels from various species (Furchgott & Jothianadan, 1991; Lefer *et al.*, 1993; Zygmunt *et al.*, 1994), and in gastrointestinal tissue (Utz & Ullrich, 1991; Rattan & Chakder, 1993). The present results demonstrate relaxant effects of CO on feline LOS smooth muscle. Exogenous CO is considered to produce effects on smooth muscle by an activation of guanylate cyclase, resulting in an increase in cyclic GMP levels, and relaxation. This seems to be the case also in the feline LOS, where an increase of cyclic GMP concentrations after CO exposure, and an inhibiting effect of methylene blue on the CO-induced relaxation could be demonstrated. However, the effects of methylene blue may not be attributed to inhibition of guanylate cyclase alone, since methylene blue is known also to generate superoxide anions (Rand & Li, 1995). This may contribute to its ability to increase resting tone. CO has also been found to have a stimulating effect on potassium currents in the intestine (Farrugia *et al.*, 1993). A similar mechanism contributing to relaxation of the LOS cannot be excluded. NO has previously been shown to be an important relaxant mediator of feline LOS (Ny *et al.*, 1995a). NO seems to be more potent than CO in producing relaxation of the feline LOS, and has the ability to increase cyclic GMP concentrations 10–20 fold (Ny *et al.*, 1995a), whereas CO in the present study produced only an approximately two fold increase in the cyclic GMP concentration. This is in agreement with the general view that CO is a less effective stimulator of soluble guanylate cyclase than NO (Mayer *et al.*, 1994; Stone & Marletta, 1994). In the brain, CO has been suggested as a regulator of NOS activity and/or soluble guanylate cyclase (Maines *et al.*, 1993; Verma *et al.*, 1993). In this study, we were unable to demonstrate such a regulatory function for CO in NO-mediated functions, since EFS-induced relaxations were unaffected by exposure to CO, even at a time when the CO-induced response had practically disappeared after frequent exposure to CO.

A possible way of establishing a role for CO as a messenger molecule in the nervous system would be the use of HO inhibitors. Certain metalloporphyrins are inhibitors of HO; ZnPP and SnPP being the most widely used (Maines, 1993). In functional experiments, ZnPP has been demonstrated to affect neurotransmission and these effects have been coupled to inhibition of CO production (Rattan & Chakder, 1993; Stevens & Wang, 1993; Verma *et al.*, 1993; Zhuo *et al.*, 1993; Shinomura *et al.*, 1994). In the present study, HO activity, measured as CO production in LOS homogenates, was markedly inhibited when ZnPP was present in the incubation medium. However, in the intact LOS tissue, ZnPP and SnPP did not affect electrically induced NANC relaxations, or relaxations induced by forskolin and SIN-1, respectively. In contrast, ZnPP inhibited the relaxations induced by VIP, PHI, and PACAP 27. These observations are not in agreement with those made by Rattan & Chakder (1993) on the opossum inner anal sphincter. They found inhibitory effects of ZnPP on electrically-induced NANC relaxations and on the VIP-induced relaxation, but PHI-induced relaxation was unaffected. Recently, we have shown that receptor-mediated relaxation by VIP and atrial natriuretic peptide of the rat aorta was markedly attenuated not only by ZnPP but also by other porphyrins. It was suggested that porphyrins may interfere with mechanisms taking place in the plasma membrane instead of affecting HO (Ny *et al.*, 1995b). Effects of ZnPP, unrelated to inhibition of HO, have recently also been demonstrated in other tissues (Linden *et al.*, 1993; Meffert *et al.*, 1994; Tøttrup *et al.*, 1995). In previous investigations, ZnPP was shown to inhibit guanylate cyclase in tissue homogenates (Ignarro *et al.*, 1984) and *in vivo* (Luo & Vincent, 1994). However, we found no effect of ZnPP on the relaxation induced by SIN-1, which is believed to activate soluble guanylate cyclase via release of NO. This might indicate that the ZnPP molecule does not reach the intracellularly located HO, or that the guanylate cyclase activated by SIN-1/NO is not blocked by ZnPP. It is known that ZnPP is photoreactive and may exhibit unspecific effects if exposed to light (Zygmunt *et al.*, 1994). Hence, all experiments in this study were carried out in darkness, which should exclude inactivation of ZnPP as an explanation for the results obtained. A combination of ZnPP and L-NOARG seems to have less inhibitory effect on EFS-induced relaxations than L-NOARG alone. The reasons for this are unknown.

In summary, this study has demonstrated HO activity in the feline LOS, which may come from different sources. HO-2 immunoreactivity observed in nerve cell bodies often coexisted with NOS and/or VIP. Furthermore, exogenously applied CO produced relaxation associated with an increase in cyclic GMP levels. Event if the role of CO in peripheral neurotransmission and other functions has to be established, the present experiments have demonstrated the presence of haem oxygenase isoforms and suggest that CO may be a messenger molecule in peripheral tissues.

This work was supported by the Swedish Medical Research Council (grants no 6837 and 11205), the Swedish Natural Science Research Council (grant no 8554-311), the Medical Faculty, Lund University, the Åke Wiberg Foundation, the Crafoord Foundation, the Royal Physiographic Society, Lund, the Swedish Society for Medical Research and the Thelma Zoegas' Foundation. The authors are grateful to the Circulatory Research Unit, Department of Physiology and Biophysics, Lund University, for supply of tissue, and to Mrs Maj-Britt Johansson for technical help.

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(Received August 8, 1995
Revised January 18, 1996
Accepted January 23, 1996)