British Journal of Pharmacology (1998) 123, 1055–1064

Myogenic nitric oxide synthase activity in canine lower oesophageal sphincter: morphological and functional evidence

[1]Anne Marie F. Salapatek, [2]Yu-Fang Wang, [2]Yu-Kang Mao, [2]Annette Lam & [2,3]Edwin E. Daniel

[1]Playfair Neurosciences Centre, The Toronto Hospital, 399 Bathurst St., Toronto, ON MST 2S8 and [2]Department of Biomedical Sciences, Division of Physiology and Pharmacology, Faculty of Health Sciences, McMaster University, 1200 Main St. W., Hamilton, ON L8N 3Z5, Canada

1 Studies on canine lower oesophageal sphincter (LOS) evaluated the existence and function of a myogenic, nitric oxide synthase (NOS) by use of immunocytochemistry for NOS isozymes, NADPH-d histochemistry, [^{3}H]-L-arginine to [^{3}H]-L-citrulline transformation. In addition, functional studies in the muscle bath were performed.

2 Smooth muscle bundles or freshly isolated smooth muscle cells of LOS were NADPH-d reactive but did not recognize some antibodies against neural, endothelial or inducible NOS. NADPH-d reactivity and immunoreactivity to a neural NOS antibody were colocalized in LOS enteric nerves. Muscle plasma membrane-enriched fractions from fresh and cultured LOS cells converted [^{3}H]-L-arginine to [^{3}H]-L-citrulline; activity was mostly Ca^{2+}/calmodulin-dependent.

3 N-Nitro-L-arginine (L-NOARG) persistently increased tone (blocked by L-arginine) in muscle strips despite blockade of nerve function. Nifedipine prevented or abolished L-NOARG-induced, but not carbachol-induced, contraction showing that tone increase by L-NOARG required functional L-Ca channels.

4 Membrane-bound, myogenic NOS in canine LOS may release NO continuously when Ca^{2+} entry through L-Ca channels occurs under physiological conditions and thereby modulate tone in LOS.

Keywords: Calcium; constitutive nitric oxide synthase; oesophageal sphincter; smooth muscle; tone

Introduction

The lower oesophageal sphincter (LOS) of many species has significant myogenic tone (reviewed by Daniel, 1992). Myogenic tone in the LOS can be defined as the ability of muscle to maintain a basal state of constriction due to direct activation of the smooth muscle by stretch (a modification of Johansson's definitions of myogenic reactivity and myogenic tone in vascular smooth muscle, 1989). Thus, investigators have noted that *in vitro* in the absence of neural activity, a significant amount of LOS tone remained (Daniel, 1992). Furthermore, canine LOS developed tetrodotoxin (TTX)-insensitive tone spontaneously when stretched *in vitro* (Allescher *et al*., 1988). This latter observation has proved to be a general phenomenon of many smooth muscle sphincters in other regions of the gastrointestinal tract and other parts of the body (reviewed by Watanabe, 1992).

However, the possibility that a spontaneous myogenic contraction mechanism left unchecked would result in spasm of sphincter musculature has not been addressed. When LOS muscle strips are adequately stretched, LOS tone develops rapidly but reaches a plateau. A relaxation mechanism may be present to counteract contractile forces and determine the set point at which basal active tone settles. The present study suggests that the height of this plateau may be determined by a mechanism of myogenic relaxation opposing myogenic contractile activities and dependent on activation of a constitutive nitric oxide synthase (cNOS) in the muscle.

Studies in the LOS of variety of different species have demonstrated that when muscle strips are studied *in vitro*, application of electrical field stimulation (EFS) causes a transient relaxation associated with an inhibitory junction potential (i.j.p.) (see Daniel, 1992). In the dog LOS, these i.j.ps have been shown at low frequencies and pulse durations to be due to release of a neurotransmitter since they are TTX-sensitive (Allescher *et al*., 1988). The transmitter appears to be nitric oxide (NO) released from non-adrenergic, non-cholinergic (NANC) nerve endings (DeMan *et al*., 1991; Jury *et al*., 1991). The latter conclusion was based on studies with authentic NO or NO-liberators, inhibitors of NOS (the enzyme which catalyzes the production NO from the substrate, L-arginine (L-Arg)), use of substrate stereoisomers and overcoming NOS inhibition with increasing substrate levels. However, in some studies of LOS in various species, authors have noted that inhibitors of NOS alone, in the absence of EFS, cause an increase in LOS tone (dog: Jury *et al*., 1991; human: Preiksaitas *et al*., 1994; Konturek *et al*., 1997; cat: Preiksaitis, H.G., personal communication). Furthermore, Murray *et al*. (1992) showed that putative inhibitors of guanylate cyclase (the enzyme proposed to be activated by NO) such as cystamine and methylene blue caused a prolonged increase in opossum LOS tone (>40 min).

We hypothesized that there was a constitutive, spontaneously active NOS, releasing NO in the LOS tissue preparation, but its cellular locus was uncertain. A baseline, ongoing release of NO from nerve endings could also have explained these results.

Evidence for the presence of NOS in peripheral tissues has been found not only in non-adrenergic, non-cholinergic (NANC) nerves (Bredt & Snyder, 1994; Berezin *et al*., 1994) but also in a variety of tissues such as endothelium and macrophages (reviewed by Moncada, 1992), kidney (Wilcox *et al*., 1992), uterine smooth muscle (Yallampalli *et al*., 1994) and myocardium (De Belder *et al*., 1993). NOS enzymes present in various tissues differ as to whether they are constitutive or induced and as to whether they are cytoplasmic or membrane-bound (reviewed by Förstermann *et al*., 1994). Grider & Jin (1993), Murthy *et al*. (1993) and Murthy & Makhlouf (1994)

[3] Author for correspondence.

demonstrated the existence of constitutive NOS in gastric and intestinal circular smooth muscles. Murthy & Makhlouf (1994) also showed that the gastric NOS is membrane-bound and activated by vasoactive intestinal peptide (VIP) involving a G-protein. They propose that VIP interacts with its receptor, in turn resulting in an increase in intracellular Ca^{2+} ($[Ca^{2+}]_i$). Ca^{2+}/calmodulin activates the NOS resulting in NO release, an increase in intracellular guanosine 3′ : 5′-cyclic monophosphate ($[cyclic\ GMP]_i$) and relaxation of the smooth muscle in part through the action of cyclic GMP-dependent protein kinase. However, earlier we found no evidence for the presence of cNOS or any NOS in circular muscle of canine intestine (Kostka *et al*., 1993). Therefore, if cNOS resides in canine LOS muscle, as well as in nerve, it may differ from other canine gastrointestinal muscles. Furthermore, the elevated steady state $[Ca^{2+}]_i$ levels in LOS smooth muscle which may account for its tone (reviewed by Daniel, 1992) may also be sufficient to activate such a myogenic cNOS. Indeed, the activation of cNOS by entering Ca^{2+} might provide a modulatory control of Ca^{2+} entrance and contraction by hyperpolarizing the membrane.

The aim of the present study was to evaluate the existence and distribution of NOS in intact LOS tissue preparations and isolated LOS cells, by use of NADPH-diaphorase (-d) histochemistry and immunofluorescence to antibodies for known NOS isoforms, neural (n), inducible (i) and endothelial (e). In addition, functional *in vitro* studies were performed in order to evaluate whether the presence of NADPH-d staining correlated with smooth muscle responses involving the L-arginine/NO pathway in the muscle bath or with NOS activity studied with a biochemical assay in isolated cells or various cellular membrane fractions. A complementary study found evidence for LOS cNOS at the single muscle cell level by use of whole cell patch clamp technologies (Salapatek *et al*., 1996). Preliminary accounts of this work were presented at Digestive Diseases Week, San Diego, U.S.A. (Salapatek & Daniel, 1995) and at a meeting of the American Motility Society, Wintergreen, U.S.A. (Mao *et al*., 1994).

Methods

Mongrel dogs of either sex were killed with an overdose of sodium pentobarbitone (100 mg kg^{-1}), according to a protocol approved by the McMaster Animal Care Committee and following the guidelines of the Canadian Council for Animal Care. The gastro-oesophageal (GO) region was excised and kept during dissection in cold Krebs-Ringer solution equilibrated with 5% CO_2 and 95% O_2 and having the following composition (in mM): NaCl 115.0, KCl 4.6, NaH_2PO_4 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 22.0, $CaCl_2$ 2.5 and glucose 11.0. The GO junction or LOS was opened on the gastric greater curvature side and the mucosae removed by sharp dissection. The muscular equivalent of the LOS was revealed as a thickened ring of muscle composed of clasp fibres with oblique gastric sling fibres on either side (Friedland *et al*., 1971). LOS muscle was consistently cut from the clasp region of the LOS since functional differences have been demonstrated between the different muscle bundles of the LOS in a number of species including man (Preiksaitas *et al*., 1994).

Morphological studies

Histochemistry

Tissue preparation and sectioning Eight healthy dogs of either sex were killed and strips of LOS prepared as described above. LOS strips were then fixed in 4% paraformaldehyde in 0.1 M phosphate buffered saline (PBS pH 7.4) for 4 h at 4°C. The tissues to be used for cryostat sectioning were cut into small pieces and then stored in 15% sucrose containing PBS for cryoprotection at 4°C for 24 h and sectioned in 16 mm thickness in a cryostat (Leitz 1720 digital). The sections were collected on the slides coated with gelatin.

NADPH diaphorase (NADPH-d) histochemistry The sections were stained for NADPH-d activity by incubation in 0.1 M phosphate buffered saline (PBS) containing 1.2 mM β-NADPH, 0.24 mM nitroblue tetrazolium (NBT) (both from Sigma Chemical, St. Louis, MO) and 0.3% Triton X-100 at 37°C for 20 min. The reaction was stopped by rinsing the tissue in PBS. The control preparations were treated in the same way but without NADPH in the reaction solution. In addition, the specificity of staining for NADPH-d in whole mounts of various canine gastrointestinal tissues was examined by using pretreatment with heat, acid, alkaline denaturation or by substituting NADH for NADPH as described by Hope and Vincent (1989). Our results were very similar to theirs, supporting the assumption that our staining represented NADPH-d activity. None of these latter treatments resulted in positive staining in gastrointestinal musculature. The nature of the NADPH-d reactivity in LOS was further studied by comparing the time course for staining of nerves and muscle. After 10 min incubation, some nerves were lightly stained but muscle was not. After 20 min incubation, nerves were deeply stained and muscle was clearly stained. After 30 min incubation, both nerves and muscle were deeply stained. However, similar incubation (20 or 30 min) of canine antral, intestinal or colonic circular muscle did not result in staining except of nerves.

Localization of eNOS and iNOS immunoreactivity; localization of nNOS immunoreactivity and colocalization with NADPH-d Whole mount preparations and cryostat sections were incubated in 1 : 1000 dilution of rabbit anti-sera raised against nNOS purified from rat cerebellum (Euro-Diagnostica, Malmö, Sweden and in a 1 : 100 dilution of mouse anti-sera raised against eNOS purified from human endothelial cells (Transduction Laboratories, Lexington, KY, U.S.A.) in PBS at 4°C overnight. This antibody strongly stained blood vessel endothelium in canine gastro-intestinal tract as well as bovine cultured pulmonary artery endothelial cells. The antibody against iNOS (Transduction Laboratories) stained macrophages. At a dilution of 1 : 100 it failed to stain nerves or muscle on LOS. All antibodies were visualized with fluorescein isothiocyanate (FITC) labelled goat anti-rabbit goat anti-mouse antibodies (Jackson ImmunoResearch, PA, U.S.A.). Selected ganglia stained for nNOS were photographed and then the tissues were reprocessed for NADPH-d staining (see NADPH-d histochemistry section above).

Cell isolation and cell culture Isolated cells were prepared from canine LOS by a modification of a method used by Janssen and Sims (1992) for isolating viable canine smooth muscle cells. LOS smooth muscle strips were cut into squares approximately 2 mm^3. Smooth muscle squares were incubated in dissociation solution containing (in mM): NaCl 125.0, glucose 10.0, KCl 5.0, $CaCl_2$ 1.0, $MgCl_2$ 1.0, HEPES 10.0 and ethylenediamine tetraacetic acid disodium salt (EDTA, BDH Inc., U.S.A.) 2.5 to which was added collagenase (Blends F, H, or L, Sigma; 130.0 mg ml^{-1}), papain (Sigma; 130.0 mg ml^{-1}), (−)-1,4-dithio-L-threitol (L-DTT, Sigma; 15.4 mg ml^{-1}), and bovine serum albumin (Sigma; 100.0 mg ml^{-1}) and incubated

at 37°C. Tissues were washed with enzyme-free dissociation solution and then mechanically agitated with siliconized Pasteur pipettes to disperse tissue and isolate single smooth muscle cells. These cells also contracted to 60 mM KCl addition or to the muscarinic agonist carbachol (10^{-5} M), excluded the dye trypan blue (Sigma) but stained positively with smooth muscle a-actin antibody (Sigma). They also relaxed to their original shapes and lengths when contractile agents were washed out. Cells isolated by methods described above were transferred to culture medium (D-MEM, supplemented with 10% bovine serum albumin and 1.0% antibiotic-antimycotic (GIBCO BRL products, New York, U.S.A.). The cell suspension was transferred to Petri dishes and placed at 37°C in a humidified incubator, gassed with 95% air/5% CO_2. Once some cells had adhered to the dishes, the medium was changed every 3 days. Most studies were performed before confluence of cell cultures. When confluence was reached, some cultures were passaged once using 1.5 g l^{-1} trypsin (to dislodge cells in order to replate them).

A modification of a conventional histochemical technique was used to detect NADPH-d in the isolated cells (Hassall & Burnstock, 1986). Fixed cells were rinsed several times in PBS and then incubated in the same reaction solution as used in tissue sections. After the reaction had been stopped by dilution with PBS, the cells were suspended in 80% glycerol and 20% PBS, carefully mounted on slides and viewed under the microscope.

All tissue preparations were viewed on a Leitz microscope equipped with a fluorescence epi-illuminator with N2 filter for FITC for NOS and an illuminator for NADPH-d visualization. Kodak T-MAX 400 film was used for black and white photography.

Functional studies

Bioassay for NOS activity

Preparation of synaptosomal and smooth muscle plasma fractions Fractionation of smooth muscle and synaptosomal membranes from canine LOS followed the procedure described previously (Mao *et al*., 1993). Briefly, freshly dissected tissue was suspended in ice-cold isolation buffer (wet w/v ratio 1 : 10) containing 25 mM MOPS-NaOH, 10 mM $MgCl_2$, 8% (w:v) sucrose, pH 7.4 and thoroughly minced with scissors. The tissue suspension was homogenized by Polytron (15,000 r.p.m. for 10 s) followed by centrifugation at 750 *g* for 10 min to remove nuclei and cellular debris. The resulting postnuclear supernatant was centrifuged at 4,000 *g* for 10 min; then the supernatant was again centrifuged at 17,000 *g* for 10 min (the pellet was the P2 fraction, a synaptosome enriched fraction). The supernatant was subjected to high speed centrifugation at 120,000 *g* for 60 min. The pellet was resuspended in the buffer with a hand-held Teflon-coated homogenizer and was subjected to a centrifugation at 17,000 *g* for 10 min. The supernatant was MIC II (smooth muscle plasma membrane-enriched).

The binding of [^{3}H]-saxitoxin (Ahmad *et al*., 1988) was performed according to the methods already established in our laboratory. The protein content in each membrane fraction was determined by the Folin phenol method (Lowry *et al*., 1951).

Assay of NOS The assay of NOS activity was carried out in membrane vesicle preparations or isolated cells after pre-incubation for 5 min at 0°C with 0.1% Triton X-100 to permeabilize these membranes and allow access of exogenous compounds to the enzyme. The activity of NOS was determined as the rate of conversion of [^{3}H]-L-arginine to [^{3}H]-L-citrulline (Bredt & Snyder, 1989). The procedure used was as described previously (Kostka *et al*., 1993). Briefly, 150 μl aliquots of the cells treated by Triton X-100 or membrane fractions were supplemented with 0.4 mCi of [^{3}H]-L-arginine and 1 mM NADPH, into 200 μl. The reaction mixtures were incubated for 30 min at 37°C. The reaction was terminated by heating samples at 90°C for 5 min, followed by dilution with 1 ml of deionized water containing 1 mM and 1 mM L-citrulline. After being mixed, 1 ml of sample was applied to chromatographic columns containing 1 g of Dowex AG50WX-8 (Na^+ form). [^{3}H]-L-citrulline was eluted from the columns by 1 ml of water and was collected into scintillation vials. After the addition of 4 ml of scintillation fluid, the radioactivity of each sample was counted. In order to account for the residual radioactivity in the eluate, not derived from [^{3}H]-L-citrulline, heat-inactivated fractions or cells (boiled for 10 min) were used in the procedure described; the resultant radioactivity was considered background and subtracted from that obtained with only NADPH as supplement. Then any increase in radioactivity in fractions or cells not heat inactivated on addition of 0.1 mM Ca^{2+} and 1 mM calmodulin represents additional cNOS activity, while any decrease on addition of 0.1 mM EGTA represents loss of Ca^{2+}-dependent NOS. The EGTA-insensitive component in all preparations (membrane fractions, isolated or cultured cells) was reduced but not completely abolished by the NOS inhibitor N^G-nitro-L-arginine methylester (L-NAME, 1 mM) and therefore represented mostly non-specific activity. The activity insensitive to L-NAME was subtracted before the data were summarized. On the assumption that endogenous L-arginine was lost after permeabilization of cells and membrane isolation, NOS activity was expressed in terms of fmol transformed to L-citrulline and expressed as a percentage of basal NOS activity. The data sets were analysed by one-way ANOVA. The statistical significance of differences in the means was determined by Tukey-Kramer multiple comparisons test.

In vitro *studies*

Recording of mechanical activity After the LOS region was exposed as described above, circular muscle strips approximately 20 mm × 2 mm were dissected free of underlying muscle layers so that the long axis of each strip was parallel to the circularly oriented clasp muscle fibres. Muscle strips were placed in 10 ml organ baths, bathed in Krebs-Ringer solution (a physiological salt solution, PSS, composition given above) at 37°C, and bubbled with 95% O_2 and 5% CO_2. Each strip was oriented vertically with the bottom end fixed to the electrode holder by silk ligature and the top end passed through a pair of concentric platinum electrodes, then fixed by a longer silk thread and ligature to a force diqplacement transducer (Grass FTOC3). Two grams of tension was applied to each strip. During an initial equilibration period of 30 min, all muscle strips rapidly contracted and spontaneously developed tone. Active tension was taken to be the difference between the observed tension and the minimum tension reached in Ca^{2+}-free PSS (made with no Ca^{2+} and 1 mM EGTA added). Muscle strip tension changes were displayed on a Beckman R611 Dynograph. Electric field stimulations were generated from a Grass S88 stimulator at 40 V, 5 pps, 10 s with 0.1, 0.2, and 0.3 ms pulse durations applied sequentially. These stimulation parameters were used since they were shown previously to produce TTX-sensitive relaxations (Allescher *et al*., 1988), a finding reconfirmed in these studies.

Experimental protocols and drugs used In all studies, muscle strips were equilibrated for 30 min over which time tone developed spontaneously. EFS relaxations were evoked in all strips with the parameters described above. Earlier studies (Allescher *et al*., 1988) revealed that cholinergic and adrenergic innervation did not contribute in a major way to responses to electrical field stimulation; e.g., addition of atropine resulted in a more prolonged relaxation. In additional studies not described here we found that 10^{-7} M atropine did not influence responses to L-NOARG. In initial experiments ($n=6$), after strips had relaxed to EFS, L-NOARG (10^{-4} M, a potent inhibitor of NOS, see Bredt & Snyder, 1994) was applied and EFS repeated. In all subsequent experiments (except for initial experimental control strips), neural toxins ω-conotoxin (GVIA) (ω-CTX, 10^{-7} M, a neuronal N-type Ca^{2+} channel blocker (McCleskey *et al*., 1987)) and TTX (10^{-6} M, a Na^{+} channel blocker) were added sequentially for a total of 30 min. EFS was then repeated on all strips. In all cases, there was no relaxation or contraction to EFS following exposure to nerve toxins, indicating that both inhibitory and excitatory nerve function were abolished. Neural toxins remained in the medium thereafter. After various experimental protocols (description to follow) a source of NO, sodium nitroprusside (NaNP, 10^{-4} M) or synondimine (SIN-1, 10^{-2} M) was then added to the same strips. Finally, 0 Ca^{2+} PSS was applied to all strips.

Experimental protocols The following experimental protocols ($n=6$ each) were performed after neural release of NO was blocked: (1) L-NOARG was applied for 30 min. (2) Muscle strips were pretreated with L-arginine (L-Arg, 10^{-3} M, a substrate for NOS production of NO and competitor for the same NOS site of action as L-NOARG) for 30 min (reviewed in reference Daniel, 1992). L-NOARG was subsequently added. (3) (a) Strips were pretreated with nifedipine (3×10^{-5} M, an L-type Ca^{2+} (L-Ca) channel blocker, $n=6$) for 15 min alone or (b) when carbachol (CCh, 10^{-5} M, a muscarinic receptor agonist, was added before and after nifedipine ($n=3$) or (c) nifedipine was added during the L-NOARG-induced contraction ($n=4$).

Drugs

All drugs for this portion of the study were purchased from Sigma Chemical Company (St. Louis, MO, U.S.A.) and were dissolved in PSS on the day of the experiment. Where required some drugs were first dissolved in 0.1 N HCl in high concentration and then diluted with PSS to concentrations required. The drugs were added to the bath in small volumes (ml) and diluents used to solubilize these reagents were used on control strips in similar volumes to exclude any action by them.

The peak contraction was measured, or the area under the tension trace was measured by use of a computerized microplanimeter (Laboratory Computer Systems, Cambridge, MA). Peak or area data were expressed as means $\pm$ s.e.mean and standardized by expressing means as percentages of baseline active tone or the rectangular area defined by the LOS tone level before drug addition and a set, arbitrary, period of time (6 min). The letter n indicates the number of experiments and is equivalent to the number of experimental animals. The data sets were analysed by one-way ANOVA or when appropriate a repeated measures ANOVA was used. The statistical significance of differences between means was determined by Tukey-Kramer multiple comparisons test, where P values less than 0.05 were considered significant ($^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$).

Results

Morphological studies: NADPH-diaphorase histochemistry and immunoreactivity

NADPH-d-positive nerve cell bodies and their processes were present within the myenteric plexus of LOS as shown in Figures 1a and 2b ($n=8$). Positively stained nerve cell bodies were found in every ganglion observed, appearing to occupy up to half the volume of each ganglion. As shown in Figure 2a and b, in whole mount preparations ($n=8$), these neurones appeared to be Dogiel type I (Dogiel, 1899) with some lamellae, dendrites and often a long axon. In the same preparation, all nerve cells which were reactive for NADPH-d were also immunoreactive for nNOS (see Figure 2a,b). However LOS muscle cells were not immunoreactive to nNOS. Many positive nerve fibres were observed to run parallel to the bundles of sphincter muscle (Figure 1a). NADPH-d reactivity

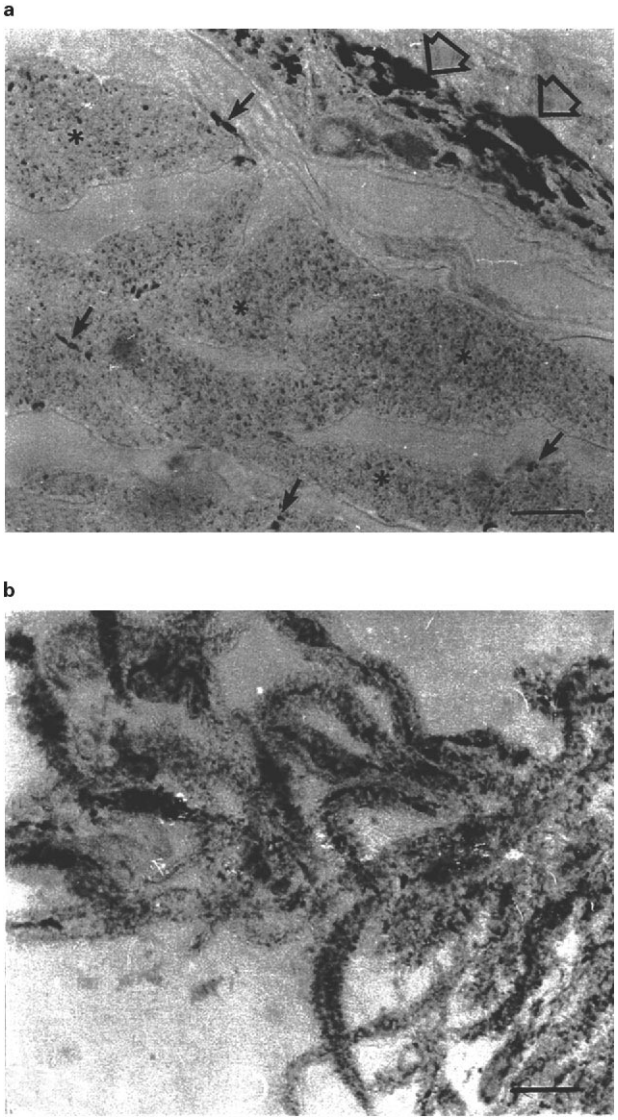

Figure 1 NADPH-d histochemistry. (a) Cryostat section from LOS of the canine showing NADPH-d positive reaction in neurones: both nerve cell bodies in myenteric plexus (empty arrows) and nerve fibres (arrows) and sphincter muscle cells (asterisks) ($n=8$). Bar: 25 mm. (b) Isolated circular smooth muscle cells from canine LOS showing high density, granular staining for NADPH-d. Bar: 25 mm ($n=4$ individual experiments).

was also found in the smooth muscle bundles in the tissue slice (Figure 1a) and in freshly isolated smooth muscle cells (Figure 1b, $n=4$). Under the same conditions, antral, small intestinal and colonic circular muscles did not stain. Isolated smooth muscle cells studied on four separate occasions were deeply stained with the staining characterized by densely stained granules for NADPH-d (Figure 1b). Endothelial cells but not nerves or muscle cells were recognized by eNOS antibody (not shown) and by the NADPH-d reaction (not shown). Neither nerves nor muscle were recognized by the iNOS antibody, which did recognize macrophages in lung tissue (unpublished data).

Functional studies:

NOS activity in LOS Figure 3 summarizes the results from the assay of NOS activity from 4 independent membrane fractions or isolated LOS cell batches (freshly isolated or cultured smooth muscle cells). Ca^{2+}-calmodulin enhanced and EGTA partially inhibited the activities of the NOS enzyme in synaptosome-enriched preparations (P2) and smooth muscle plasma membrane-enriched preparations (Mic II). Inhibition by EGTA was incomplete, amounting to 42, 70, 39 and 82% in the P2, Mic II, isolated cell-derived and cultured cell-derived membranes, respectively. This finding is similar to that made by others in previous studies using this methodology on a similar gastrointestinal preparation (Kostka *et al*., 1993). The origin of the EGTA-insensitive NOS activity is unknown, but it should be noted that among membranes from isolated cells were included membranes from all cell types in the LOS, including nerve bundles, interstitial cells of Cajal, macrophages and other immune cells. There was a high level of saxitoxin binding in the synaptosomal fraction, indicating its origin. There was also a smaller but significant amount of saxitoxin binding in the Mic II fraction. However, the Ca^{2+}-calmodulin dependent activities of the two fractions expressed mg^{-1} protein were inconsistent with the explanation that NOS activity in the Mic II fraction was a result of its neural contamination; the values were 95 and 165 fmol mg^{-1} protein in the P2 and Mic II fractions, respectively. In order to rule out further that the NOS activity found in Mic II was derived from synaptosomal contamination, we examined the pattern of NOS enzyme activity in permeabilized freshly isolated and in cultured smooth muscle cells. Basal NOS activities in all preparations (membranes or cells) were high but the Ca^{2+}-calmodulin dependent components were similar, 195 and 230 fmol/10^6 cells for cultured and isolated cells, respectively. Moreover, saxitoxin binding and hence neural contamination was neglible in the fractions from cultured cells. Thus, the NOS activity from these fractions cannot be ascribed to neural contamination. Contamination by interstitial cells of Cajal is also an unlikely explanation. Previous studies (Berezin *et al*., 1987; Allescher *et al*., 1988) showed that there were few such cells, always associated with inhibitory nerve bundles and within or near muscle bundles. Figure 1a indicates the paucity of such arrays, labelled by small arrows. We also never noticed cells with the configuration of interstitial cells of Cajal stained by NADPH-d in cryostat sections of cells isolated from LOS.

In vitro *studies* As shown in Figure 4a, EFS relaxations at short pulse durations caused relaxations which were abolished

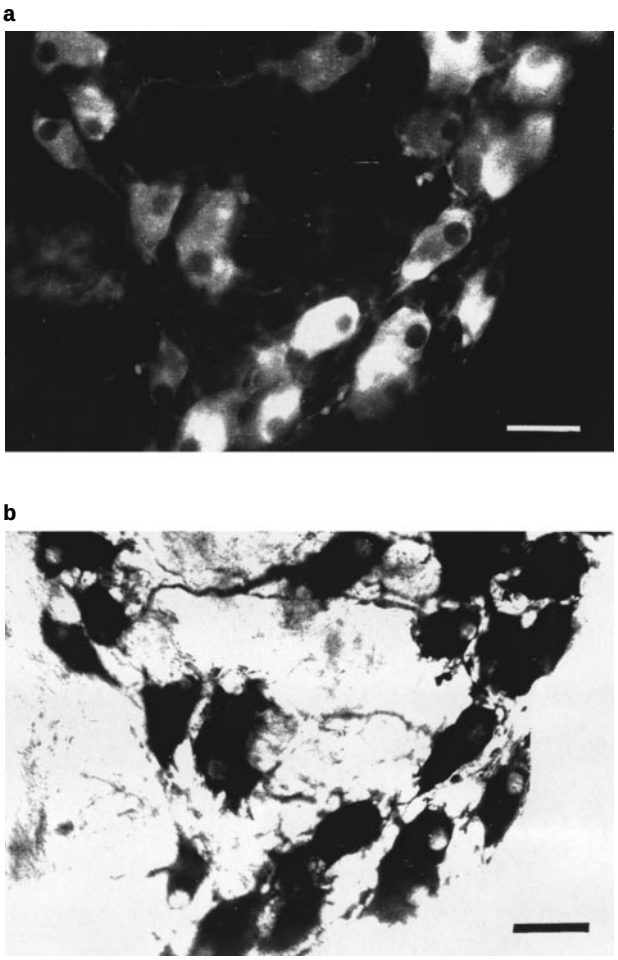

Figure 2 Colocalization of nNOS immunoreactivity and NADPH-d activity. Micrographs showing the colocalization of nNOS and NADPH-d in the myenteric plexus of whole mount preparations of the canine LOS. All nerve cell bodies containing NOS (a) also stained for NADPH-d (b). The calibration bar is 50 μm.

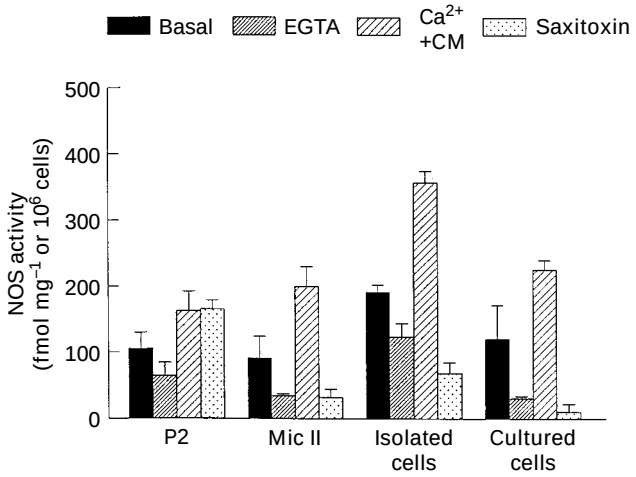

Figure 3 NOS activity in the LOS. The NOS activity in fractions or smooth muscle cells isolated or cultured (primary cultures for 2–3 days) from the canine LOS. This activity was assessed by the conversion of [^{3}H]-L-arginine to [^{3}H]-L-citrulline and expressed as a mean percentage of basal NOS activity; L-NOARG-insensitive activity was subtracted. Addition of 0.1 μM EGTA reduced basal NOS activity significantly in all 4 cell and membrane preparations. Addition of 0.1 mM Ca^{2+} and 0.1 μM calmodulin (CM) significantly increased NOS activity over basal levels. To clarify the extent of contamination of these fractions of cells by nerve membranes, binding of [^{3}H]-saxitoxin was determined (Ahmad *et al*., 1988). Saxitoxin binding was high in the P2 fraction but also present in MIC II and freshly isolated cells. Saxitoxin binding was negligible (not different from zero) in cultured LOS smooth muscle cells.

without substitution of contraction after application of the neural toxins, ω-CTX (10^{-7} M) and TTX (10^{-6} M). In six separate experiments (not shown), L-NOARG (10^{-4} M), a NOS inhibitor, when added alone, also abolished EFS relaxations at the same pulse durations and increased LOS tone significantly over pre-drug levels. Neural toxins alone did not significantly increase LOS tone (Figures 4a and 5). Subsequent to abolition of EFS relaxations, and with neural toxins still present in the bath, addition of L-NOARG (10^{-4} M) caused a prolonged significant increase in LOS tone (>30 min) over control levels (see Figures 4b and 5). (Note, that the increase in LOS tone with L-NOARG alone was not significantly different from that observed with L-NOARG plus toxins). LOS tone observed with time after L-NOARG did not decrease significantly (see Figures 5 and 6). In six experiments, sodium nitroprusside (NaNP, 10^{-4} M), an NO liberator, was added last in the presence of L-NOARG (10^{-4} M) and neural toxins. NaNP completely relaxed LOS smooth muscle strips as judged by the absence of further additional relaxation when 0 Ca^{2+} PSS was applied (Figures 4c and 5).

In subsequent studies, TTX and ω-CTX were added and shown to abolish EFS-induced relaxation before the experiment proceeded. Figure 6 shows the results of six separate experiments in which the effect of pretreatment with L-Arg (10^{-3} M), a NOS substrate, were studied. After L-Arg addition, LOS tone was significantly reduced at 1.5 h compared to that at 1.0 h when L-Arg was added, but this change was not significantly different from the change in control strips over the same time interval. However, L-Arg pretreatment of strips (see Figure 6) prevented the previously observed (see Figure 5) marked increase in LOS tone with L-NOARG, consistent with the conclusion that the actions of L-NOARG resulted from inhibition of NOS.

Figure 7 shows the results of 6 experiments in which nifedipine, an L-type Ca^{2+} channel blocker (3×10^{-5} M), was added 1.25 h for 15 min before L-NOARG addition. Nifedipine caused a profound decrease in LOS tone and prevented the L-NOARG-induced contraction observed in the control strips. The tone after nifedipine was similar to that

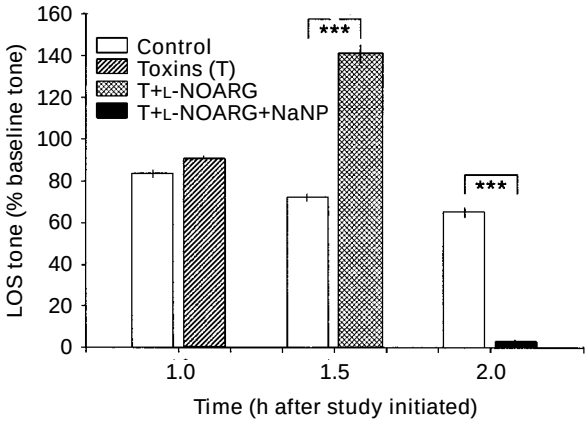

Figure 5 Effect of L-NOARG and NaNP on LOS tone after neural blockade. A graphical summary of the results presented in Figure 4. The control strips were not treated with drugs. All data were standardized by expressing LOS tone resulting as a percentage of the original or baseline tone developed in each strip. Tone was measured three times during the experiment, 1, 1.5 and 2 h after the study was initiated. LOS tone did not significantly decrease with time alone or on addition of neural toxins, ω-CTX (10^{-7} M) and TTX (10^{-6} M). However, neural toxins did abolish EFS relaxations in toxin-treated strips as shown in Figure 4 ($n=12$). L-NOARG (10^{-4} M), a NOS inhibitor, added in the presence of toxins increased LOS tone for more than 30 min significantly over the time matched control level at 1.5 h, $141.3\pm8.1\%$ compared to $72.3\pm4.4\%$, $P<0.001$, $n=6$, respectively. NaNP (10^{-4} M), a NO liberator, in addition to toxins and L-NOARG, significantly decreased LOS tone over the time matched control level at 2 h, to $2.8\pm0.4\%$ compared to the control value of $65.4\pm4.8\%$, $P<0.001$, $n=6$, respectively.

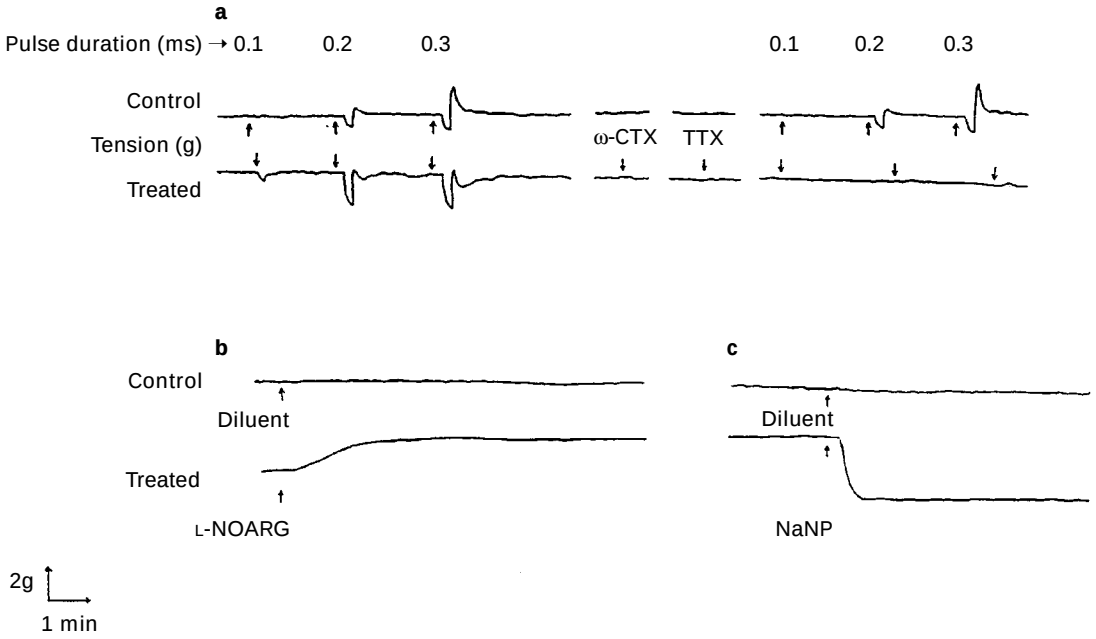

Figure 4 Effect of L-NOARG and NaNP on LOS tone after neural blockade. A typical trace of tension recordings from two LOS strips: control strip and treated strip. Control strips were stimulated with EFS and exposed to diluent rather than active agents. Treated strips were stimulated with EFS and exposed to TTX and ω-CTX. (a) EFS relaxations are shown for the three pulse durations applied at the arrows. There was no significant change in LOS tone after the neural toxins, ω-CTX (10^{-7} M) and TTX (10^{-6} M), were applied to the treated strip. However, EFS relaxations were abolished in the toxin treated strip but not in the control strip. (b) With neural toxins still in the bath, L-NOARG (10^{-4} M), a NOS inhibitor, caused a prolonged increase in LOS tone. (c) Subsequent addition of NaNP (10^{-4} M), a NO liberator, to the treated strip greatly reduced LOS tone ($n=6$).

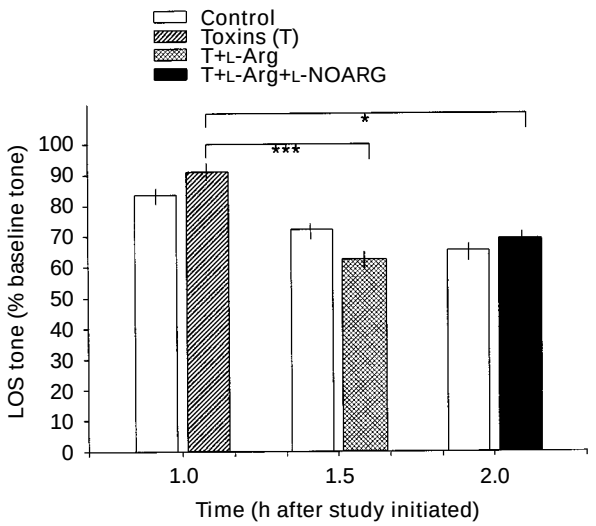

Figure 6 Effect of L-arginine (L-Arg) pretreatment on L-NOARG response. Data for this figure were standardized by expressing the LOS tone developed in each strip as a percentage of the basal tone developed in each strip. Tone was measured at 1, 1.5 and 2 h after the study was initiated. L-Arg addition did not significantly reduce LOS tone compared to the time matched control at 1.5 h and 2 h ($n=6$), although tone was significantly decreased at both 1.5 and 2 h when compared to previous tone levels measured in the same strips at 1 h, $62.5\pm4.8\%$ compared to $90.8\pm4.3\%$, $P<0.001$, and $68.9\pm5.1\%$ versus $90.8\pm4.3\%$, $P<0.05$, respectively. There was no significant increase in LOS tone with L-NOARG (10^{-4} M) when strips were pretreated with L-Arg ($n=6$) as shown at 2 h when drug-treated strips were compared to time matched control or to the pre-L-NOARG values at 2.0 h.

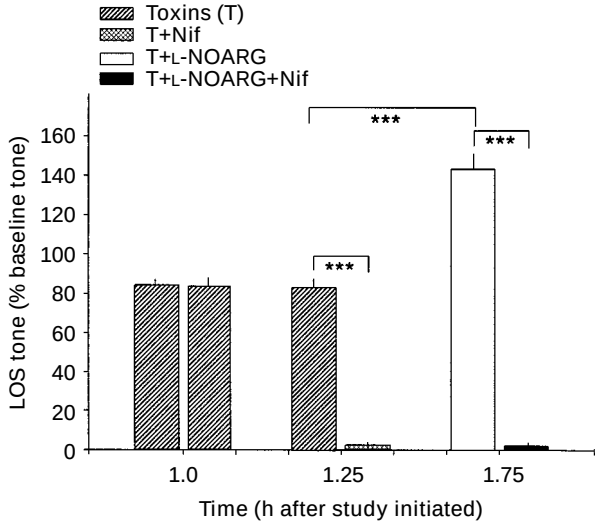

Figure 7 The effect of nifedipine, an L-type Ca^{2+} channel blocker, on the L-NOARG response after neural blockade ($n=6$). In this study, all LOS strips were treated initially with nerve toxins. The left hand columns in each pair of columns represent muscle strips to which neural toxins were present and tone was measured at 1 h and again at 1.25 h; then L-NOARG was added and tone was measured a third time at 1.75 h when the maximum contraction occurred. The right hand columns in each pair represent tone measured in muscle strips treated with toxins in the same way as the other strips, and measured at 1 h and 15 min after pretreatment with nifedipine (Nif; 3×10^{-5} M) at 1.25 h, before L-NOARG addition. Tone after L-NOARG was measured at 1.75 h. Data were standardized by expressing LOS tone resulting as a percentage of the original or baseline tone developed in each strip. After strips were treated with neural toxins, ω-CTX (10^{-7} M) and TTX (10^{-6} M), there were no significant changes in LOS strips tone over baseline (not shown). Toxin action was verified by the failure of muscle strips to relax electrical field stimulation (see Figure 4a). Nifedipine addition (3×10^{-5} M) significantly reduced LOS tone over the time matched control at 1.25 h, $2.4\pm0.3\%$ versus $82.5\pm3.5\%$, respectively. As observed in Figures 4b and 5, there was a significant increase in LOS tone with L-NOARG (10^{-4} M) in strips not exposed to nifedipine but not in strips pretreated with nifedipine, as shown at 1.75 h, $142.9\pm8.5\%$ (compared to 82.5%) and $2.0\pm0.3\%$ (compared to 2.4%).

found after NaNP or Krebs solution with zero Ca^{2+}. When nifedipine was added during the L-NOARG-induced contraction, LOS tone was reduced significantly and nearly abolished, as shown in Figure 8. Carbachol (10^{-5} M) added before nifedipine increased tone by a mean of 7.72 ± 0.62 g; after nifedipine, carbachol still evoked a mean increase in strip tension of 4.90 ± 0.78 g (in 10 muscle strips, $n=4$) (not shown).

Discussion

In this study, NADPH-d reactivity was demonstrated in LOS tissue slice preparations in nerves of the myenteric plexus in both cell bodies and processes. The staining in the myenteric plexus was like that observed by others in cat (Ny *et al*., 1995) and human (Singaram *et al*., 1994) oesophagus. However, unlike other studies which did not comment on NADPH-d staining in muscle or evaluate it in isolated muscle cells, positive staining was also demonstrated in whole LOS tissue slices (in muscle) and in single freshly isolated LOS smooth muscle cells. This staining found was localized in morphologically-identified, isolated, LOS smooth muscle cells and was clustered in a dense particulate manner.

However, as clear as the NADPH-d reactivity appeared in freshly isolated LOS smooth muscle cells, there remained a possibility of nerve membrane fragment contamination found in freshly isolated cells (later documented by the small but significant [^{3}H]-saxitoxin binding present in the smooth muscle membrane fraction Mic II). When the activities of these two fractions were compared in terms of Ca^{2+}-calmodulin dependent [^{3}H]-L-citrulline production mg^{-1} protein, it was clear that the activity in the Mic II fraction could not be explained as a result of contaminating nerve particles. LOS circular smooth muscle cells were also cultured. The possibility of nerve cell contamination was dismissed since cultured LOS smooth muscle cells also demonstrated high levels of NOS activity and did not have any nerve cell contamination as demonstrated by the extremely low level of [^{3}H]-saxitoxin binding (which was not significantly different from zero). In some fractions, there was a Ca^{2+}-calmodulin insensitive production of [^{3}H]-L-citrulline. Its cellular locus was not determined, but it was very small in membranes from cultured cells and cannot account for our findings.

The specificity of our NADPH-d procedures for NOS activity was studied further. We found colocalization of immunoreactivity for nNOS and reactivity for NADPH-d in myenteric plexus neurones and processes. Endothelial cells were recognized by antibodies to eNOS and were also reactive for NADPH-d. However, muscle cells were not immunoreactive to the antibodies tested against either n- or e-NOS, and an antibody against iNOS was also ineffective. Thus, this NADPH-d reactivity in LOS muscle appears to represent a NOS which differs from other constitutive enzymes. The fact that the myogenic NOS differed from nNOS was supported by the finding that it was membrane bound (see below). Also, that this NADPH-d reactivity represented an activity related to NOS was supported by the finding that the time course of staining was similar to that for nNOS, positive after 20 min

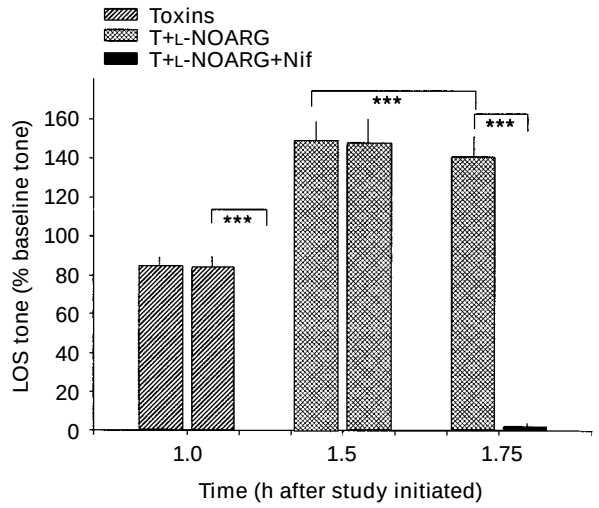

Figure 8 The effect of nifedipine added after L-NOARG. In this study, all LOS strips were treated initially with nerve toxins. The first column of each grouping of two columns represents the control strips which were treated with neural toxins then with L-NOARG and nothing else. The second column in each grouping of two columns represents muscle strips treated in the same way as control strips except that nifedipine was added before the final tone measurement, after nerve toxins and L-NOARG. All data were standardized by expressing LOS tone resulting as a percentage of the original or baseline tone developed in each strip. Tone was measured three times during the experiment, 1, 1.5 and 1.75 h after the study was initiated. LOS tone did not significantly change with the addition of neural toxins (not shown). However, neural toxins, ω-CTX (10^{-7} M) and TTX (10^{-6} M), did abolish EFS relaxations in toxin treated strips as shown in Figure 4. L-NOARG (10^{-4} M), a NOS inhibitor, added in the presence of toxins at 1.5 h increased LOS tone significantly over previous levels at 1.0 h, for control columns $148.4 \pm 10.7\%$ compared to $84.1 \pm 3.8\%$, $P < 0.001$, respectively; for the second column in each pair $147.6 \pm 10.3\%$ versus $83.6 \pm 3.7\%$, $P < 0.001$, respectively. Nifedipine (Nif, 3×10^{-7} M), an L-type Ca^{2+} channel blocker, when added after toxins and L-NOARG, significantly decreased LOS tone over the time matched control level at 1.75 h, to $2.1 \pm 0.2\%$ compared to $140.7 \pm 10.8\%$, $P < 0.001$, $n = 4$, respectively.

incubation and maximal after 30 min incubation. NADPH-d reactivity was absent in other non-sphincteric canine gastrointestinal muscles tested (Daniel, unpublished).

Myogenic NOS in canine LOS appeared to be membrane-bound. To date, the endothelial NOS isoform is the only isoform known to be membrane-bound (Pollack *et al*., 1993) suggesting that there may be other similarities between the LOS myogenic cNOS and endothelial cNOS, such as shared epitopes. Murthy *et al*. (1993) have described a NOS in gastric and intestinal smooth muscles sharing some of the characteristics of the LOS NOS we describe here, including being membrane-bound. However, the NOS activity in the gastric and intestinal muscle membranes required G-protein activation initiated by VIP. Furthermore, immunostaining of this smooth muscle NOS was not demonstrated with antibodies raised against neural NOS. Therefore, Murthy *et al*. (1993) suggested that their NOS may be unlike either neural or endothelial NOS. In our study, antibodies for neural and endothelial isoforms of NOS did not stain isolated LOS smooth muscle cells, but did stain neurones and endothelial cells, respectively. We conclude that the canine LOS cNOS isoform must have some structural differences to the epitopes recognised by these known, available antibodies. Mechanistically, a plasma membrane location for LOS myogenic NOS could make it subject to regulation and possibly ongoing activation by high Ca^{2+} concentrations near the membrane, as proposed by Van Breemen *et al*. (1995), or generally high steady state $[Ca^{2+}]_i$, as may occur in tonically contracting muscles such as sphincters (see Daniel, 1992).

The presence of NADPH-d histochemistry and NOS activity in isolated smooth muscle cells and their membrane fractions was correlated with smooth muscle responses involving the L-arginine/NO pathway in the muscle bath. The functional studies provided evidence for a non-neural NOS since L-NOARG, the most potent inhibitor of both neural and endothelial NOS (reviewed in Bredt & Snyder, 1994), caused an increase in tone (implying that an active NOS had been blocked) in LOS smooth muscle strips. A neural release of NO was ruled out because EFS-induced relaxations were also blocked by neural toxins acting to inhibit action potential transmission in axons and Ca^{2+} entrance into nerve endings. In LOS strips, there was no change in tone on nerve blockade, demonstrating that tonic NO release from nerves was not occurring in LOS strips. Subsequent addition of NaNP to LOS strips, in the presence of L-NOARG and neural toxins, resulted in almost complete relaxation. This showed that the tissue was still susceptible to NO-induced relaxation. The specificity of the effect of L-NOARG was confirmed by the ability of excess L-Arg to compete for the active NOS site and inhibit L-NOARG actions (reviewed in Bredt & Snyder, 1994). L-Arg alone did not reduce LOS tone significantly compared to time matched controls. This finding may indicate that endogenous L-Arg was adequate to saturate the already active NOS.

NO has been shown to act prejunctionally to enhance or inhibit release of neurotransmitters (Daniel *et al*., 1994; Hryhorenko & Fox-Threlkeld, 1994; Jing *et al*., 1995), but this is unlikely to be occurring in our experiment since ω-CTX, an N-type Ca^{2+} channel blocker, should prevent Ca^{2+} influx and release of transmitters from the prejunctional terminal. In other studies in canine intestine, we have found that ω-CTX alone abolished NANC relaxations and i.j.ps (Daniel *et al*., 1994; Cayabyab *et al*., 1997). Instead, NO of muscle origin is probably acting directly on a soluble guanylate cyclase in LOS smooth muscle resulting in an increase in cyclic GMP (Torphy *et al*., 1986). As described by Murthy *et al*. (1993) and Murthy & Makhlouf (1994), activation of a cyclic GMP-dependent protein kinase may then lead to relaxation.

Previously we observed that the membrane potentials of these cells were about -45 mV (Jury *et al*., 1991), at which membrane potential most L-Ca channels are expected to be closed (Catterall, 1995). However, the tone in canine LOS muscle clearly involves Ca^{2+} entry through these channels since the tone disappeared when nifedipine was added. Thus, L-Ca channels are open in this issue. Unlike the cases described by Murthy *et al*. (1993) and Murthy & Makhlouf (1994), in which NOS was activated by a VIP-induced release of Ca^{2+} from stores, the NOS of canine LOS appears to be activated by the continuous entry of Ca^{2+} through L-Ca channels unless blocked by L-NOARG.

These results demonstrate the existence of a constitutive, membrane-bound form of NOS in the circular smooth muscle of the canine LOS which releases NO in an ongoing basis without neural input or mediator activation. This NOS activity may constitute a myogenic relaxation mechanism acting to limit the contractility or stabilize the tone developed in this sphincter. Tone development in this tissue, unlike in the feline LOS (Biancani *et al*., 1994) in which spontaneous phospholipase C activity produces some IP_3 and Ca^{2+} release from stores, requires continuous Ca^{2+} entry since nifedipine, an L-type Ca^{2+} channel blocker, abolished tone. After nifedipine, L-NOARG-induced LOS contraction was also absent. Ongoing entrance of Ca^{2+} through L-Ca channels in the unstimulated LOS may

contribute both to tone initiation by activation of myosin light chain kinase (reviewed in Ebashi, 1991) and to its limitation by activation of myogenic NOS to release NO and limit Ca^{2+} entry.

If NO limits contraction by hyperpolarizing cells to limit Ca^{2+}-channel opening (Catterall, 1995) and/or by enhancing uptake of Ca^{2+} into the sarcoplasmic reticulum (SR) (Cayabab & Daniel, 1995), the two mechanisms may be related in that increased Ca^{2+} uptake into the SR may enhance functioning of the SR, as postulated in the 'buffer-barrier' hypothesis of Van Breemen *et al.* (1995), and increase $[Ca^{2+}]_i$ near the plasmalemma. This could enhance activation of Ca^{2+}-sensitive K^+ channels and activate myogenic NOS localized in that region. In patch clamp studies, when endogenous NO production is blocked with L-NOARG, K^+ channels close resulting in depolarization and possibly enhanced opening of voltage-sensitive L-type Ca^{2+} channels, leading to Ca^{2+} influx and further contraction. These findings suggest that the endogenously active, constitutive, myogenic NOS acts to release NO and hyperpolarize the membrane by activating K^+ channels (Salapatek *et al.*, 1996). VIP does not appear to be involved in this mechanism, since similar effects of L-NOARG were obtained in isolated cells or in tissues after neural blockade, and secondly VIP is known to cause relaxation of this smooth muscle by pharmacomechanical mechanisms which do not involve membrane potential changes or K_{Ca} channel opening (Jury *et al.*, 1991; Jury & Daniel, 1997).

The findings of the present study suggest that canine LOS tone is under tonic myogenic inhibition which is important for the functioning of the LOS. If this occurs in the human LOS, it could be therapeutically relevant to pathologies of the LOS where neural controls are deranged (either diminished or augmented), thereby providing another myogenic site with the potential to be manipulated pharmacologically.

A.M.S. was a recipient of a Research Award for an abstract of this work which was supported by the AGA Foundation at Digestive Diseases Week (San Diego, 1995). The authors wish to thank Michelle Brazas, Herman Fernandes and Scott Roberts, students of the McMaster University Honours Biopharmacology Program who performed the initial baseline experiments for this study. This study was also supported by the Medical Research Council of Canada.

References

AHMAD, S., ALLESCHER, H.D., MANAKA, H., MANAKA, Y. & DANIEL, E.E. (1988). [^{3}H]saxitoxin as a marker for canine deep muscular plexus neurons. *Am. J. Physiol.,* **255,** G462 – 469.

ALLESCHER, H.D., BEREZIN, I., JURY, J. & DANIEL, E.E. (1988). Characteristics of canine lower esophageal sphincter: a new electrophysiological tool. *Am. J. Physiol.,* **255,** G441 – 453.

BEREZIN, I., ALLESCHER, H.-D. & DANIEL, E.E. (1987). Ultrastructural localization of VIP-immunoreactivity in canine distal oesophagus. *J. Neurocytology,* **16,** 749 – 757.

BEREZIN, I., SNYDER, H., BREDT, D.S. & DANIEL, E.E. (1994). Ultrastructural localization of nitric oxide synthase in canine small intestine and colon. *Am. J. Physiol.,* **266,** C981 – C989.

BIANCANI, P., HARNETT, K.M., SOHN, U.D., RHIM, B.Y., BEHAR, J., HILLEMAN, C. & BITAR, K.N. (1994). Differential signal transduction pathways in LES tone and response to ACh. *Am. J. Physiol.,* **266,** G767 – 774.

BREDT, D.S. & SNYDER, S.H. (1989). Nitric oxide mediates glutamate-linked enhancement of cGMP levels in the cerebellum. *Proc. Natl. Acad. Sci. U.S.A.,* **86,** 9030 – 9033.

BREDT, D.S. & SNYDER, S.H. (1994). Nitric oxide: A physiologic messenger molecule. *Ann. Rev. Biochem.,* **63,** 175 – 195.

CATTERALL, W.A. (1995). Structure and function of voltage-gated ion channels. *Ann. Rev. Biochem.,* **64,** 493 – 531.

CAYABYAB, F.S. & DANIEL, E.E. (1995). K^+ channel opening mediates hyperpolarization by NO donors and inhibitory junction potentials in opossum esophagus. *Am. J. Physiol.,* **268,** G831 – 842.

CAYABYAB, F.S., JIMENEZ, M., VERGARA, P., DE BRUIN, H. & DANIEL, E.E. (1997). Influence of nitric oxide and vasoactive intestinal peptide on the spontaneous and triggered electrical and mechanical activities of the canine ileum. *Can. J. Physiol. Pharmacol.,* (in press).

DANIEL, E.E. (1992). Lower esophagus: structure and function. In *Sphincters: Normal Function – Changes in Diseases*. eds Daniel, E.E., Tomita, T., Tsuchida, S. & Watanabe, M. pp. 49 – 66. Boca Raton, Florida: Raven, CRC Press.

DANIEL, E.E., HAUGH, C., WOSKOWSKA, Z., CIPRIS, S., JURY, J. & FOX-THRELKELD, J.E.T. (1994). Role of nitric oxide-related inhibition in intestinal function: relation to vasoactive intestinal polypeptide. *Am. J. Physiol.,* **266,** G31 – G39.

DE BELDER, A.J., RADOMSKE, M.W., WHY, H.F.J., RICHARDSON, P.J., BUCKNALL, C.A., SALAS, E., MARTIN, J.F. & MONCADA, S. (1993). Nitric oxide synthase activities in human myocardium. *Lancet,* **341,** 84 – 85.

DE MAN, J.G., PELCKMANS, P.A., BOEKXSTAENS, G.E., BULT, H., OOSTERBOSCH, L., HERMAN, A.G. & VAN MAERCKE, Y.M. (1991). The role of nitric oxide in inhibitory non-adrenergic non-cholinergic neurotransmission in the canine lower oesophageal sphincter. *Br. J. Pharmacol.,* **103,** 1092 – 1096.

DOGIEL, A.S. (1899). Über den Bau der Ganglien in den Geflechten des Darmes und der Gallenblase des Menschen und der Säugetiere. *Arch. Anat. Physiol.,* **1899,** 130 – 158.

EBASHI, S. (1991). Excitation-contraction coupling and the mechanism of muscle contraction. *Ann. Rev. Physiol.,* **53,** 1 – 16.

FÖRSTERMANN, U., CLESS, E.I., POLLOCK, J.S., NAKANE, M., SCHWARZ, P., GATH, I. & KLEINERT, H. (1994). Nitric oxide synthase isozymes. *Hypertension,* **23,** 1121 – 1131.

FRIEDLAND, G.W., KOHATSU, S. & LEWIN, K. (1971). Comparative anatomy of feline and canine gastric sling fibers. Analogy to human anatomy. *Am. J. Dig. Dis.,* **16,** 495 – 507.

GRIDER, J.R. & JIN, J-G. (1993). VIP-induced nitric oxide (NO) production and relaxation in isolated muscle cells of the gut in human and other mammalian species. (Abstract). *Gastroenterology,* **104,** A515.

HASSALL, C.J.S. & BURNSTOCK, G. (1986). Intrinsic neurones and associated cells of the guinea-pig heart in culture. *Brain Res.,* **364,** 102 – 113.

HOPE, B.T. & VINCENT, S.R. (1989). Histochemical characterization of neuronal NADPH-diaphorase. *J. Histochem. Cytochem.,* **37,** 653 – 661.

HRYHORENKO, L.M. & FOX-THRELKELD, J.E.T. (1994). Tonic nitric oxide (NO) release inhibits field stimulated (FS) acetylcholine (ACH) release from canine ileal myenteric plexus free circular muscle. *J. Pharmacol. Exp. Ther.,* **271,** 918 – 926.

JANSSEN, L.J. & SIMS, S.M. (1992). Acetylcholine activates non-selective cation and chloride conductances in canine and guinea-pig tracheal myocytes. *J. Physiol.,* **453,** 197 – 218.

JING, L., INOUE, R., TAHIRO, K., TAKAHASHI, S. & ITO, Y. (1995). Role of nitric oxide in non-adrenergic, non-cholinergic relaxation and modulation of excitatory neuroeffector transmission in the cat airway. *J. Physiol.,* **483,** 225.

JOHANSSON, B. (1989). Myogenic tone and reactivity: definitions based on muscle physiology. *J. Hypertension,* **7,** S5 – S8.

JURY, J., AHMEDZADEH, N. & DANIEL, E.E. (1991). A mediator derived from arginine mediates inhibitory junction potentials and relaxations in lower esophageal sphincter: an independent role from vasoactive intestinal peptide. *Can. J. Physiol. Pharmacol.,* **70,** 1182 – 1189.

JURY, J. & DANIEL, E.E. (1997). Lack of electrophysiological effect of VIP on canine LES opossum LES and opossum BCM. *Gastroenterology,* **112,** A757.

KONTUREK, J.W., THOR, P., LUKASZYK, A., GABRYELEWICZ, A., KONTUREK, S.J. & DOMSCHKE, W. (1997). Endogenous nitric oxide in the control of esophageal motility in humans. *J. Physiol. Pharmacol. (Poland),* **48,** 201 – 209.

KOSTKA, P., JANG, E., WATSON, E.G., STEWART, J. & DANIEL, E.E. (1993). Nitric oxide synthase in the autonomic nervous system of canine ileum. *J. Pharmacol. Exp. Ther.,* **264,** 234 – 239.

LOWRY, O.H., ROSEBROUGH, N.J., FARR, A.L. & RANDALL, R.J. (1951). Protein measurement with the folin phenol reagent. *J. Biol. Chem.,* **193,** 265–275.

MAO, Y.-K., WANG, Y.-F. & DANIEL, E.E. (1993). Distribution and characterization of VIP binding in canine lower esophageal sphincter. *Gastroenterology,* **105,** 1370–1377.

MAO, Y.-K., WANG, Y.-F. & DANIEL, E.E. (1994). Canine LES cells and perhaps interstitial cells of cajal (ICC) contain cNOS; it is not nNOS. (Abstract). *Gastroenterology,* **107,** 1231.

MCCLESKEY, E.W., FOX, A.P., FELDMAN, D.H., CRUZ, L.J., OLIVERA, B.M., TSIEN, R.W. & YOSHIKAMI, D. (1987). ω-Conotoxin: direct and persistent blockade of specific types of calcium channels in neurons but not muscle. *Proc. Natl. Acad. Sci. U.S.A.,* **84,** 4327–4331.

MONCADA, S. (1992). The L-arginine:nitric oxide pathway. *Acta Physiol. Scand.,* **145,** 201–227.

MURRAY, J.A., DU, C., LEDLOW, A., MANTERNACH, P.L. & CONKLIN, J.L. (1992). Guanylate cyclase inhibitors: effect on tone, relaxation, and cGMP content of lower esophageal sphincter. *Am. J. Physiol.,* **263,** G97–101.

MURTHY, K.S. & MAKHLOUF, G.M. (1994). Vasoactive intestinal peptide/pituitary adenylate cyclase-activating peptide-dependent activation of membrane-bound NO synthase in smooth muscle mediated by pertussis toxin-sensitive G_{i1-2}. *J. Biol. Chem.,* **269,** 15977–15980.

MURTHY, K.S., ZHANG, K.-M., JIN, J.-G., GRIDER, J.R. & MAKHLOUF, G.M. (1993). VIP-mediated G-protein-coupled Ca^{2+} influx activates a constitutive NOS in dispersed gastric muscle cells. *Am. J. Physiol.,* **265,** G660–G671.

NY, L., ALM, P., LARSSON, B., EKSTRÖM, P. & ANDERSSON, K.-E. (1995). Nitric oxide pathway in cat esophagus: localization of nitric oxide synthase and functional effects. *Am. J. Physiol.,* **268,** G59–G70.

POLLOCK, J.S., JAKANE, M., BUTTERY, L.D., MARTINEZ, A., SPRINGALL, D., PLAK, J.A., FÖRSTERMANN, U. & MURAD, F. (1993). Characterization and localization of endothelial nitric oxide synthase using specific monoclonal antibodies. *Am. J. Physiol.,* **265,** C1379–C1387.

PREIKSAITIS, H.G., TREMBLAY, L. & DIAMANT, N.E. (1994). Nitric oxide mediates inhibitory nerve effects in human esophagus and lower esophageal sphincter. *Dig. Dis. Sci.,* **39,** 770–775.

SALAPATEK, A.M. & DANIEL, E.E. (1994). Novel calcium handling in canine lower esophageal sphincter smooth muscle. (Abstract). *Gastroenterology,* **106,** A1049.

SALAPATEK, A.M.F. & DANIEL, E.E. (1995). Modulation of canine lower esophageal sphincter tone by nitric oxide synthase in muscle. (Abstract). *Gastroenterology,* **108,** A681.

SALAPATEK, A.M.F., FOX-THRELKELD, J.E.T. & DANIEL, E.E. (1996). Mechanism of nitric oxide-induced LES relaxation involves intracellular calcium modulation. (Abstract). *Gastroenterology,* **110,** A750.

SINGARAM, C., SENGUPTA, A., SWEET, D.J. & GOYAL, R.K. (1994). Nitrinergic and peptidergic innervation of the human oesophagus. *Gut,* **35,** 1690–1696.

TORPHY, T.J., FINE, C.F., BURMAN, M., BARNETTE, M.S. & ORMSBEE, III, H.S. (1986). Lower esophageal sphincter relaxation is associated with increased cyclic nucleotide content. *Am. J. Physiol.,* **251,** G786–G793.

VAN BREEMEN, C., CHEN, Q. & LAHER, I. (1995). Superficial buffer barrier function of smooth muscle sarcoplasmic reticulum. *Trends Pharmacol. Sci.,* **16,** 98–104.

WATANABE, M. (1992). General properties of sphincters. In *Sphincters: Normal function – Changes in Diseases.* ed. Daniel, E.E., Tomita, T., Tsuchida, S. & Watanabe, M. pp. 3–5, Boca Raton, Florida: Raven, CRC Press.

WILCOX, C.S., WELCH, W.J., MURAD, F., GROSS, S.S., TAYLOR, G., LEVI, R. & SCHMIDT, H.H. (1992). Nitric oxide synthase in macula densa regulates glomerular capillary pressure. *Proc. Natl. Acad. Sci. U.S.A.,* **89,** 11993–11997.

YALLAMPALLI, C., IZUMI, H., BYAM-SMITH, M. & GARFIELD, R.E. (1994). An L-arginine-nitric oxide-cyclic guanosine monophosphate system exists in the uterus and inhibits contractility during pregnancy. *Am. J. Obstetrics Gynecology,* **170,** 175–185.

(Received September 2, 1997
Revised November 24, 1997
Accepted December 2, 1997)