

Characterization and localization of nitric oxide synthase in non-adrenergic non-cholinergic nerves from bovine retractor penis muscles

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1 Partially purified soluble nitric oxide (NO) synthase was isolated from the bovine retractor penis muscle (BRP), a tissue in which the inhibitory response to non-adrenergic non-cholinergic nerve (NANC) stimulation appears to be mediated by NO or NO-like material.

2 NO synthase from BRP used L-arginine as a substrate, required NADPH, tetrahydrobiopterin, and FAD as co-factors and was Ca²⁺/calmodulin-dependent. The activity of NO synthase was inhibited by N^G-methyl-L-arginine and N^G-nitro-L-arginine, and haemoglobin blocked the effect of NO formed by the enzyme.

3 On reducing SDS polyacrylamide gel electrophoresis the apparent molecular mass of NO synthase from BRP was 160 ± 2 kDa, which is similar to that of the cerebellar NO synthase. Protein immunoblot and immunoprecipitation showed that NO synthase from BRP cross-reacted with the selective antiserum to neuronal NO synthase from rat cerebellum.

4 Immunohistochemistry using the same antiserum demonstrated that NO synthase in BRP was located exclusively within nerve fibres. Thus, autonomic nerves synthesizing the NANC neurotransmitter seem to contain an isoform of NO synthase which is similar to that from rat cerebellum.

Keywords: Calcium/calmodulin-dependent; calmodulin antagonist; cyclic GMP; L-arginine; nitric oxide; nitric oxide synthase; N^G-methyl-L-arginine; N^G-nitro-L-arginine; non-adrenergic non-cholinergic nerve; retractor penis

Introduction

Field stimulation of autonomic nerves in many organs produces inhibitory responses which are not regulated by the release of adrenergic or cholinergic neurotransmitters. In many tissues such as anococcygeus and retractor penis muscle, the relaxations caused by non-adrenergic non-cholinergic (NANC) nerve stimulation appear to be mediated by nitric acid or nitric oxide-like material (NO) (Gillespie *et al.*, 1989; Gibson *et al.*, 1990; Gillespie & Sheng, 1990; Sheng, 1991; Liu *et al.*, 1991). NO has also been shown to be released from NANC innervated canine ileocolonic junction upon field stimulation (Bult *et al.*, 1990). However, it is unclear whether the NANC neurotransmitter itself is NO or whether an unidentified neurotransmitter causes NO release from adjacent cells.

Various cell types have been found to synthesize NO. The enzyme responsible for NO synthesis (NO synthase) has been characterized and purified from cerebellum (Bredt & Snyder, 1990; Mayer *et al.*, 1990; Schmidt *et al.*, 1991), endothelial cells (Förstermann *et al.*, 1991; Pollock *et al.*, 1991), neutrophils (Yui *et al.*, 1991b), and cytotoxic macrophages (Yui *et al.*, 1991a). In all cases NO synthase requires NADPH as a co-factor and utilizes L-arginine as a substrate. The cerebellum contains a soluble, constitutive isoform of NO synthase (Bredt & Snyder, 1990; Mayer *et al.*, 1990; Schmidt *et al.*, 1991) whereas most of the constitutive NO synthase in endothelial cells is particulate (Förstermann *et al.*, 1991; Pollock *et al.*, 1991). Both isoforms are regulated by Ca²⁺ and calmodulin. In contrast, the NO synthase induced in macrophages by endotoxin and/or cytokines is Ca²⁺/calmodulin-independent (Yui *et al.*, 1991a).

In bovine retractor penis muscle (BRP) the inhibitory res-

ponse to nerve stimulation is mediated by the activation of soluble guanylyl cyclase and formation of guanosine 3':5'-cyclic monophosphate (cyclic GMP) (Bowman & Drummond, 1985). Nitric oxide mimics the response to NANC nerve stimulation and both effects can be inhibited by oxyhaemoglobin (Bowman *et al.*, 1982; Gillespie & Sheng, 1988; 1989). Further experiments showed that the endogenous formation of NO from L-arginine may be involved in NANC neurotransmission (Liu *et al.*, 1991; Sheng, 1991). Recently we showed that a Ca²⁺-dependent NO synthase was present in rat anococcygeus muscle (Mitchell *et al.*, 1991), a tissue which has an anatomical and pharmacological profile similar to BRP (Gillespie & Sheng, 1989). However, since smooth muscle preparations may contain more than one isoform of NO synthase, it is important to classify the isozyme responsible for the formation of NANC neurotransmitter. Here we show that NO synthase isolated from BRP is neuronal in origin and synthesizes the material which mediates NANC neurotransmission. Some of these observations have been reported in abstract from (Sheng *et al.*, 1991b).

Methods

Crude soluble fraction

BRP muscles from the local slaughter house were frozen in liquid nitrogen immediately after tissue dissection. The following experiments were performed at 4°C except when indicated otherwise. Muscles were homogenized (1:5) in buffer 1 (50 mM Tris/HCl, pH 7.4 containing 0.1 mM EDTA, 0.1 mM EGTA, 12 mM 2-mercaptoethanol, 1 mM phenylmethylsulphonyl fluoride, 3 μM leupeptin, and 1 μM pepstatin A) with a commercial blender. The homogenate was filtered through muslin, centrifuged (100,000 g for 1 h) and the supernatant (crude soluble fraction) collected.

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DEAE Sephacel chromatography

The crude soluble fraction (500 ml) was loaded into a DEAE Sephacel anion exchange column (1 ml) by recirculating it 3–4 times through the column. The column was then washed with 10 column volumes of buffer 2 (buffer 1 containing 10% glycerol) and partially purified NO synthase was eluted with 3 column volumes of buffer 2 containing 0.3 M NaCl.

2', 5'-ADP-Sepharose chromatography

The crude soluble fraction (500–1000 ml) was loaded onto a 2', 5'-ADP-Sepharose column (0.5 ml) by recirculating it 3–5 times through the column. The column was washed with 10 column volumes of buffer 2, then 3 column volumes of buffer 2 containing 0.5 M NaCl, followed by 10 column volumes of buffer 2. Partially purified soluble NO synthase was eluted with 5 column volumes of buffer 2 containing NADPH (10 mM).

Assay of activation of guanylyl cyclase in RFL-6 cells

The increase of cyclic GMP in rat foetal lung fibroblasts (RFL-6 cells) was used as a measure of NO activity as described by Ishii *et al.* (1991). Briefly, RFL-6 cells cultured in 12-well plates (5×10^5 cells per well) were washed twice with Locke solution (composition mM: NaCl 154, KCl 5.6, CaCl₂ 2, MgCl₂ 1, NaHCO₃ 3.6, glucose 5.6 and HEPES 10; pH 7.4) containing 3-isobutyl-1-methylxanthine (IBMX, 0.3 mM). Cells were then equilibrated at 37°C for 20 min in the same solution. Superoxide dismutase (SOD, 20 units ml⁻¹) was added 3 min before addition of L-arginine (100 μM), NADPH (100 μM), calmodulin (30 units ml⁻¹), tetrahydrobiopterin (3 μM), and FAD (1 μM). The reaction was started by adding partially purified NO synthase from BRP. In some experiments, some of the co-factors were omitted. The incubation was continued for another 3 min. The reaction was stopped by aspirating the medium, adding 0.5 ml of ice-cold sodium acetate (50 mM, pH 4.0) to the RFL-6 cells, and rapidly freezing the samples with liquid nitrogen. The content of cyclic GMP in each sample was determined by radioimmunoassay. Experiments with defined Ca²⁺ concentrations were performed in Ca²⁺-free Locke solution (pH 7.4) containing IBMX (0.3 mM), SOD (20 units ml⁻¹), and EGTA (1 mM). Various amounts of CaCl₂ were added and free Ca²⁺ concentrations were calculated (Segal, 1986). In some experiments the calmodulin antagonists, calmidazolium or trifluoperazine were added to the RFL-6 cells in the absence of exogenous calmodulin. In another series of experiments, the L-arginine analogues N^G-methyl-L-arginine (L-NMA) or N^G-nitro-L-arginine (L-NNA) were added to the cells in the presence of 10 μM L-arginine.

Western blot analysis of NO synthase from BRP

Polyclonal antisera against NO synthase from rat cerebellum were raised in three rabbits (Schmidt *et al.*, 1992). NO synthase from BRP, rat cerebellum and endothelial cells was partially purified by 2', 5'-ADP affinity chromatography and separated on a 7.5% SDS polyacrylamide gel, then transferred to a nitrocellulose membrane. All subsequent procedures were performed at room temperature. The transferring buffer contained 40 mM Tris/HCl pH 7.55, 0.3 M NaCl and 0.3% Tween 20. The membrane was blocked with 7% fat-free dried milk and then incubated with antiserum at a 1:1000 dilution for 1 h. The NO synthase band were identified with a horseradish peroxidase-conjugated secondary antibody against rabbit IgG (1:5000) and chemiluminescence (Amersham).

Immunoprecipitation of NO synthase from BRP

Polyclonal IgG against NO synthase from rat cerebellum was purified by protein A Superose chromatography (Pharmacia).

Partially purified soluble NO synthase from BRP (by 2', 5'-ADP chromatography, 18 μg per aliquot) was incubated overnight with different concentrations of purified antibody. The control experiment was performed by using normal rabbit IgG. After adding protein A Sepharose beads (18 μg per aliquot) to each aliquot, samples were centrifuged (20,000 g) for 1 h. The supernatant from each aliquot was assayed for NO synthase activity by activation of guanylyl cyclase in RFL-6 cells.

Histochemical determination of NO synthase in BRP

Frozen BRP was mounted onto microtome chucks. Oblique or longitudinal sections (8 μm thick) were cut and thaw-mounted onto microscope slides, fixed by immersion in acetone for 5 min and then air-dried. For NO synthase staining, the sections were incubated with polyclonal antiserum to rat cerebellar NO synthase (1:100) or the pre-immune serum (1:100) diluted in phosphate buffered saline (PBS) containing 1% bovine serum albumin (BSA) at 37°C for 30 min and then, in peroxidase conjugate (1:50, in 1% BSA/PBS) for 30 min. The peroxidase label was developed by using 3', 3'-diaminobenzidine dissolved in imidazole buffer for 6–10 min, washed with Tris buffer (50 mM, pH 7.6), dehydrated and mounted. It has been reported that NO synthase from rat cerebellum has NADPH diaphorase activity (Hope *et al.*, 1991). For labelling NADPH diaphorase, the tissue sections were incubated for 20–30 min at 37°C with nitro blue tetrazolium (0.5 mM) and NADPH (1 mM) in Tris (50 mM, pH 7.6) containing Triton X-100 (2%), counterstained with eosin, and then dehydrated by use of a graded series of ethyl alcohol concentrations.

Protein determination

Protein concentrations were determined as described by Bradford (1976) with bovine serum albumin used as the standard.

Drugs

Compound R24571 (calmidazolium), calmodulin, 3', 3'-diaminobenzidine, ethylenediaminetetraacetic acid (EDTA), ethylene glycol-bis (β-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA), flavin adenine dinucleotide (FAD), haemoglobin, 3-isobutyl-1-methylxanthine (IBMX), leupeptin, L-arginine hydrochloride, 2-mercaptoethanol, nitro blue tetrazolium, N^G-nitro-L-arginine (L-NNA), pepstatin A, phenylmethylsulphonyl fluoride, superoxide dismutase, trifluoperazine dihydrochloride were purchased from Sigma (U.S.A.); NADPH tetrasodium salt was obtained from Boehringer Mannheim (Germany); Tetrahydrobiopterin from Schircks (Switzerland); N^G-methyl-L-arginine (L-NMA) was synthesized as described (Patthy *et al.*, 1977).

Results

Soluble NO synthase isolated from BRP produced NO as deduced by stimulation of cyclic GMP accumulation and conversion of L-arginine to L-citrulline (data not shown). The results mentioned below were obtained by measuring the cyclic GMP content in RFL-6 cells as this is a sensitive bioassay technique for the detection of NO (Ishii *et al.*, 1991). The specific activities of partially purified NO synthase from BRP (detected in 5×10^5 RFL-6 cells) were 28.08 ± 8.69 nmol cyclic GMP mg⁻¹ protein by 2',5'-ADP affinity chromatography ($n = 9$) and 11.82 ± 0.32 pmol cyclic GMP mg⁻¹ protein by DEAE anion exchange chromatography ($n = 3$). Figure 1a shows that NO synthase from BRP used L-arginine as a substrate. Superoxide dismutase (20 units ml⁻¹) was required for the detection of NO synthase activity, while oxyhaemoglobin (10^{-5} M) completely inhibited

the effect of NO formed by NO synthase from BRP (Figure 1a). The L-arginine analogues, L-NMA and L-NNA showed a concentration-dependent inhibition of the enzyme activity (Figure 1b). The activities of NO synthase partially purified by DEAE anion exchange chromatography were reduced in the absence of exogenous NADPH ($36.62 \pm 6.13\%$ of control activity, $n = 3$) or tetrahydrobiopterin ($19.9 \pm 3.9\%$ of control activity, $n = 3$). The material eluted from 2',5'-ADP Sepharose showed very little dependence on tetrahydrobiopterin ($87.97 \pm 12.89\%$ of control activity, $n = 3$). We have found that NO synthase from other tissues partially purified by 2', 5'-ADP chromatography still contained tetrahydrobiopterin (Schmidt *et al.*, unpublished observations). The enzyme activity was partially reduced in the absence of FAD ($46.44 \pm 3.13\%$ of control activity, $n = 3$) or calmodulin ($64.31 \pm 1.87\%$ of control activity, $n = 3$). NO synthase from BRP was found to be Ca^{2+} -dependent. There was no detectable enzyme activity at a Ca^{2+} concentration ≤ 1 nM, and enzyme activity reached a maximum at 500 nM Ca^{2+} (Figure 1c). Partially purified NO synthase showed only a partial dependence on exogenous calmodulin, probably because the sample still contained significant amounts of endogenous calmodulin as we reported previously for partially purified preparations from endothelial cells (Förstermann *et al.*, 1991). To test whether NO synthase from BRP is calmodulin-dependent, we also investigated the effects of two calmodulin antagonists on the enzyme activity. Figure 1d shows that both calmidazolium (0.1 or 0.3 μM) and trifluoperazine (1 or 3 μM) markedly inhibited the activity of NO synthase with calmidazolium being approximately 10 fold more potent than trifluoperazine.

Protein immunoblot analysis showed that soluble NO synthase from BRP partially purified by 2', 5'-ADP affinity chromatography cross-reacted with a polyclonal antiserum generated against NO synthase from rat cerebellum (Figure 2a). The denatured molecular mass of NO synthase from BRP was 160 ± 2 kDa. The antiserum was selective in that it did not cross-react with soluble (data not shown) and particulate NO synthase from endothelial cells (Figure 2a). Identical results were obtained by using similar antisera raised in two other rabbits (data not shown). The partially purified NO synthase from BRP (by 2', 5'-ADP chromatography) contained many other proteins, as SDS polyacrylamide gel electrophoresis revealed more than 20 protein bands when stained with Coomassie blue (data not shown). To ensure the single band shown in Figure 2a was NO synthase, we performed immunoprecipitation experiments. NO synthase from BRP bound to purified IgG to rat cerebellar NO synthase and was precipitated by adding protein A Sepharose beads. Figure 2b shows that the activity of NO synthase was reduced by higher concentration of purified IgG to cerebellar NO synthase but not by normal rabbit IgG.

Immunohistochemistry demonstrated that antiserum raised against rat cerebellar NO synthase stained only nerve fibres in BRP from both longitudinal (Figure 3a) and oblique sections (Figure 3b). Similar results were obtained with nitro blue tetrazolium used to demonstrate the NADPH diaphorase activity (Figure 3c). In contrast, Figure 3d shows no staining in a longitudinal section treated with rabbit pre-immune serum.

Discussion

It has been known for several years that some smooth muscle preparations contain an inhibitory neurotransmitter, the response of which cannot be blocked by any known receptor antagonists, but can be inhibited by oxyhaemoglobin (Bowman *et al.*, 1982; Gillespie & Sheng, 1989). Recently, as L-arginine analogues such as L-NMA and L-NNA became established as inhibitors to the synthetic pathway of endothelium-derived relaxing factor, these compounds were found to inhibit the relaxation caused by stimulation of NANC

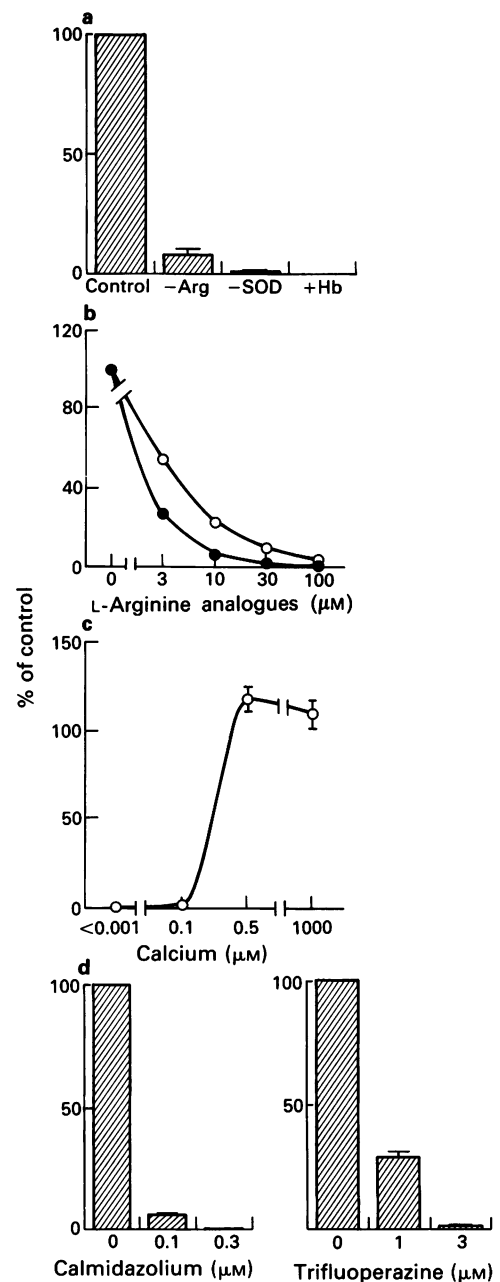


Figure 1 Characterization of NO synthase from bovine retractor penis muscle. Activity of NO synthase partially purified by 2', 5'-ADP affinity chromatography was detected by the cyclic GMP accumulation in 5×10^5 RFL-6 cells in the presence of L-arginine (100 μM), superoxide dismutase (20 units ml^{-1}), NADPH (100 μM), tetrahydrobiopterin (3 μM), and calmodulin (CaM, 30 units ml^{-1}) (control). (a) The activity of NO synthase was reduced in the absence of L-arginine (- Arg) or superoxide dismutase (- SOD) and in the presence of oxyhaemoglobin (+ Hb). Control activity was 18.66 ± 1.20 nmol cyclic GMP mg^{-1} protein. (b) NO synthase activity was inhibited in a concentration-dependent fashion by N^G -methyl-L-arginine (O) or N^G -nitro-L-arginine (●) in the presence of 10 μM L-arginine. Control activity was 45.43 ± 3.65 nmol cyclic GMP mg^{-1} protein. (c) The activity of NO synthase was tested at different concentrations of Ca^{2+} buffered with EGTA (1 mM). Control activity (20.25 ± 1.68 nmol cyclic GMP mg^{-1} protein) was measured in normal Locke buffer containing 2 mM Ca^{2+} . (d) Calmidazolium (0.1 or 0.3 μM) and trifluoperazine (1 or 3 μM) inhibited the activity of NO synthase. Control activity was 12 ± 0.77 nmol cyclic GMP mg^{-1} protein in the absence of exogenous calmodulin. Columns or points represent means with s.e. shown by vertical bars except where the error lies within the symbol, $n = 3$.

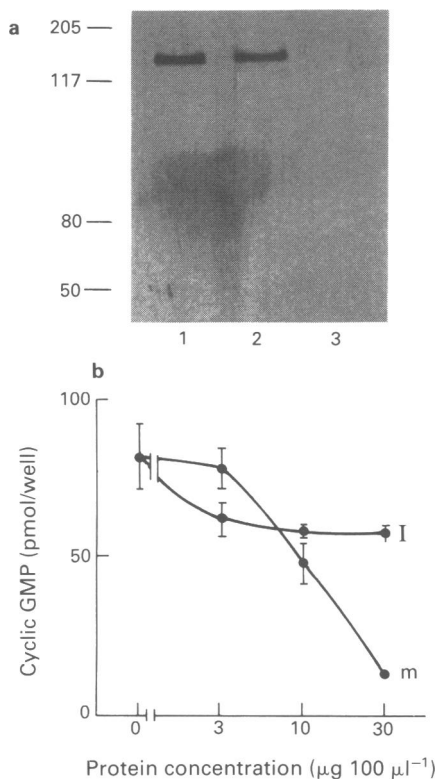


Figure 2 Western blot and immunoprecipitation of NO synthase from bovine retractor penis (BRP) muscle. (a) Soluble NO synthase from rat cerebellum (lane 1, 2 μg) and from BRP (lane 2, 18 μg) but not solubilized particulate NO synthase from bovine aortic endothelial cells (lane 3, 15 μg) cross-reacted with the antiserum against NO synthase from rat cerebellum. All NO synthase preparations were partially purified by 2', 5'-ADP affinity chromatography. Positions of molecular mass (M_r) markers are shown ($M_r \times 10^3$). (b) NO synthase from BRP bound to purified rabbit IgG raised against NO synthase from rat cerebellum (m) was precipitated by protein A Sepharose beads. The enzyme which did not bind to normal rabbit IgG (I) was not precipitated. The activity of NO synthase in the supernatant fraction was determined by cyclic GMP accumulation in 5×10^5 RFL-6 cells. Points represent means with s.e. shown by vertical bars, $n = 3$.

nerves in these tissues (Gillespie *et al.*, 1989; Gibson *et al.*, 1990; Liu *et al.*, 1991). In a bioassay cascade, stimulation of NANC-innervated canine ileocolonic junction leads to relaxation of the subjacent detector tissues (Bult *et al.*, 1990). It is still unclear, however, whether NO is released directly from nerve endings or from other cells within the donor tissue. The results of the present study clearly demonstrate that NO synthase in the NANC-innervated BRP is located exclusively in nerves. Therefore, NO, which causes relaxation following electrical stimulation of BRP and other NANC-innervated tissues, is likely to be synthesized within nerves and act as a NANC neurotransmitter.

The co-factor requirement of the BRP enzyme and its regulation by Ca^{2+} and calmodulin was very similar to the constitutive NO synthase from rat cerebellum and from endothelial cells (Bredt & Snyder, 1990; Schmidt *et al.*, 1991; Pollock *et al.*, 1991). The antiserum raised against NO synthase from rat cerebellum was selective in that it did not cross-react with NO synthase from endothelial cells either from immunoblot or from immunohistochemical staining (Figure 2a; Schmidt *et al.*, unpublished observations). Therefore, the soluble NO synthase isolated from BRP is clearly

not from vascular endothelial cells in this preparation. In addition, staining for NADPH diaphorase activity showed a distribution similar to NO synthase activity in BRP. This co-localization has previously been shown for cerebellar NO synthase and NADPH diaphorase (Hope *et al.*, 1991). According to protein immunoblot analysis, the denatured molecular mass of NO synthase in BRP was 160 ± 2 kDa, which is very similar to that of the enzyme isolated from cerebellum (Bredt & Snyder, 1990; Mayer *et al.*, 1990; Schmidt *et al.*, 1991). Also, on FPLC Superose 6 gel permeation chromatography (Pharmacia) the NO synthase from BRP eluted in the same fraction as NO synthase isolated from rat cerebellum (data not shown; Schmidt *et al.*, 1991). Thus, BRP contains an isoform of NO synthase which is similar to that from rat cerebellum. Since immunohistochemical staining of BRP with the antiserum to rat cerebellar NO synthase was only detected in nerve fibres, the soluble NO synthase in BRP is neuronal in origin and is responsible for NANC neurotransmission.

Some NO synthase activity was also detected in the particulate fraction of BRP showing similar characteristics to the soluble enzyme (data not shown). However, the small amount of particulate material we obtained was not enough for a detailed study of particulate NO synthase from BRP. Whether the particulate NO synthase from BRP is derived from microvascular endothelial cells (which contain predominantly particulate enzyme) or from nerves warrants further investigation.

It has been reported that L-NNA but not L-NMA inhibited the inhibitory response to NANC nerve stimulation in BRP (Liu *et al.*, 1991). In our experiment using RFL-6 cells to detect NO-like activity, both L-NMA and L-NNA inhibited the activity of NO synthase in either the soluble or the particulate fraction. The difference between our results and the previous report (Liu *et al.*, 1991) may be due to differences in uptake and/or metabolism of NMA in intact versus broken tissue (Hecker *et al.*, 1990).

The amount of NO synthesized within NANC nerves during electrical stimulation was enough to increase cyclic GMP content in BRP (Bowman & Drummond, 1985). We have previously reported that the increase of cyclic GMP in the bovine trachea is due to the generation of NO following addition of carbachol (Sheng *et al.*, 1991a). However, it was not clear whether NO was generated within the smooth muscle or other cells. Possibly in this latter tissue carbachol may stimulate the release of NO from NANC nerves within the trachea, rather than from the smooth muscle cells.

Unlike the induced NO synthase from macrophages or smooth muscle cells (Busse & Mülsch, 1990; Rees *et al.*, 1990; Yui *et al.*, 1991a) the enzyme isolated from BRP is Ca^{2+} /calmodulin-dependent. Therefore, the synthesis of NO in NANC nerves is likely to be regulated by the intracellular Ca^{2+} concentration. Depolarization of NANC nerves can lead to Ca^{2+} influx which, in turn, can activate NO synthase. The synthesized NO will be released from the NANC nerves and directly activates guanylyl cyclase in adjacent smooth muscle cells. Thus, unlike classical neurotransmitters, the NANC neurotransmitter does not act on a membrane receptor, but the haeme-moiety of soluble guanylyl cyclase functions as its 'receptor'. As NANC nerves are present in many tissues in the gastrointestinal, urino-genital, and vascular systems (Toda *et al.*, 1990a,b; Boeckstaens *et al.*, 1990; 1991; Tøttrup *et al.*, 1991; Dokita *et al.*, 1991; Kim *et al.*, 1991; Liu *et al.*, 1991). NO, acting as a neurotransmitter, may play an important physiological role in the regulation of smooth muscle tone in these organs.

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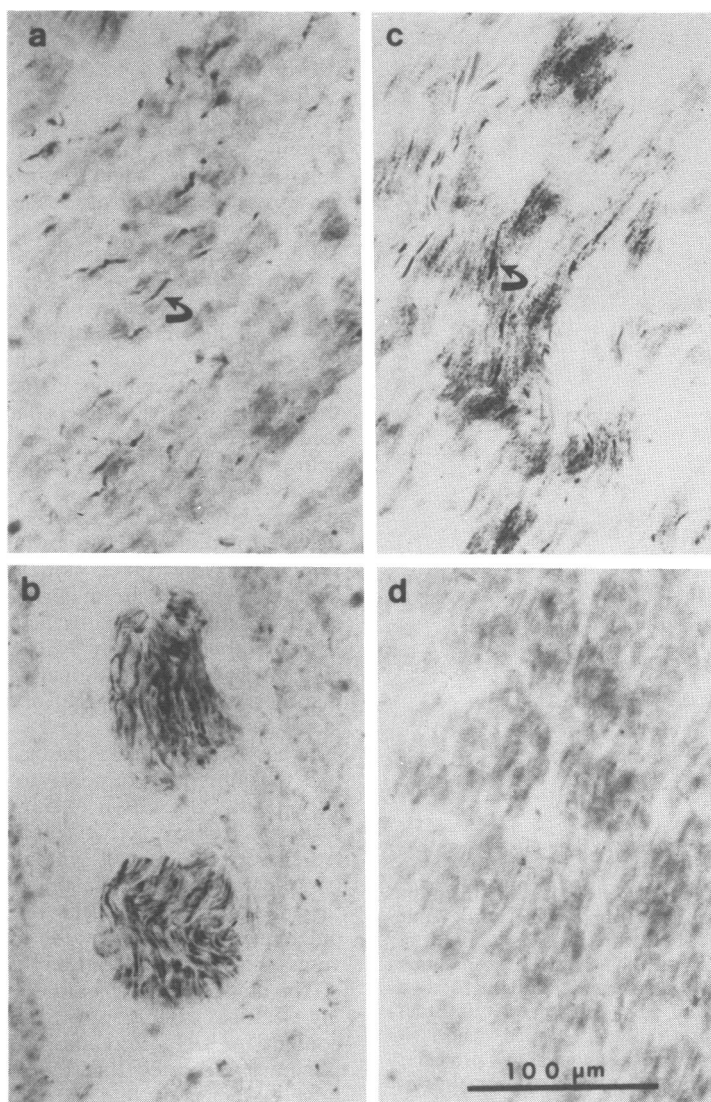


Figure 3 Localization of NO synthase in bovine retractor penis muscle (BRP). NO synthase stained with antiserum against NO synthase from rat cerebellum in (a), an oblique section and (b), longitudinal section was located in BRP in the nerve fibres traversing the smooth muscle. NADPH diaphorase, the localization of which is similar to NO synthase in brain, was also localized to nerves in the longitudinal section (c). The control (d) shows no staining in muscle longitudinal section treated with pre-immune rabbit serum. The arrows indicate the nerve fibres in BRP.

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