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Nitric oxide synthase in pig lower urinary tract: immunohistochemistry, NADPH diaphorase histochemistry and functional effects

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1 The distribution and colocalization of nitric oxide synthase (NOS)-like immunoreactivity and NADPH diaphorase activity in the pig lower urinary tract were investigated by immunohistochemical and histochemical staining techniques. Functional *in vitro* studies were performed to correlate the presence of NOS-immunoreactivity/NADPH diaphorase staining with smooth muscle responses involving the L-arginine/nitric oxide (NO) pathway.

2 NOS-immunoreactivity and NADPH diaphorase activity were expressed in nerve trunks and fine nerve fibres in and/or around muscular bundles in the detrusor, trigone and urethra. Thin nerve fibres that dispersed within the muscle bundles were mainly found in the urethral/trigonal area, whereas such fibres were less common in the detrusor.

3 Almost all neuronal structures that were NOS-immunolabelled were also stained for NADPH diaphorase. In contrast, the urothelium, which was intensively stained by the NADPH diaphorase technique, remained unstained by immunohistochemistry.

4 Electrical field stimulation of pig isolated trigonal and urethral preparations induced relaxations, which were inhibited by tetrodotoxin (1 μM) and N^G-nitro-L-arginine (L-NOARG, 10 μM).

5 L-Arginine (1 mM), but not D-arginine, inhibited (25–30%) electrically evoked detrusor contractions. This inhibition was reversed by L-NOARG (0.1 mM). L-Arginine did not inhibit detrusor contractions in the presence of scopolamine (1 μM) and had no direct smooth muscle effects *per se*.

6 Acetylcholine (1 nM–10 μM) caused concentration-dependent relaxations of noradrenaline-induced contractions in pig vesical arteries. Removal of the endothelium practically abolished the acetylcholine-induced relaxation. Pretreatment with L-NOARG (0.1 mM and 0.3 mM) caused a rightward shift of the concentration-response curves to acetylcholine, but the maximal relaxation obtained was significantly reduced (to 65 ± 12%; $n = 6$; $P < 0.05$) only at 0.3 mM L-NOARG.

7 In vessel segments contracted with K^+ (60 mM), acetylcholine induced concentration-dependent relaxations. When the vessels were incubated with 0.3 mM L-NOARG and then contracted with K^+ (60 mM) all relaxant responses to acetylcholine were abolished.

8 The presence of NO synthesizing enzyme in nerve fibres and the pharmacological evidence for NO-mediated relaxation of the trigone and urethra suggest that NO or a NO-related substance may have a role in inhibitory neurotransmission in these regions. In the detrusor, the presence of NO-synthesizing enzyme in nerves can be demonstrated, but its functional importance is unclear. NO, as well as other endothelium-derived factors seem to be involved in the endothelium-dependent acetylcholine-induced relaxation of pig vesical arteries.

Keywords: Nitric oxide; nitric oxide synthase; immunohistochemistry; NADPH diaphorase; urinary tract; N^G-nitro-L-arginine

Introduction

Much evidence has been produced showing that nitric oxide (NO) plays a major role in the non-adrenergic, non-cholinergic (NANC) inhibitory response in the urethra, bladder neck and trigone from various species (Garcia-Pascual *et al.*, 1991; Dokita *et al.*, 1991; Andersson *et al.*, 1991; 1992; Persson & Andersson, 1992; Persson *et al.*, 1992; Thornbury *et al.*, 1992). The relaxation induced by electrical stimulation is totally or partially abolished by enzymatic blockade of NO production by use of analogues of L-arginine, the natural substrate for NO synthase (NOS). Furthermore, in these studies NO and NO-donors (e.g. sodium nitroprusside and SIN-1) were shown to have postjunctional effects producing smooth muscle relaxations more effectively in the trigone and urethra than in the detrusor (Persson & Andersson, 1992; Persson *et al.*, 1992). Thus, while it seems that NO is released from nerves and acts like a neurotransmitter in the bladder outlet, the possible role of NO in the detrusor is still uncertain. A mechanism for detrusor relaxation mediated by release of NO, not from nerves, but from the smooth muscle itself, has been suggested (James *et al.*, 1991).

NO is synthesized from L-arginine by two main classes of synthase: a constitutive form that is present in the brain, endothelial cells and peripheral nerves, and an inducible form that can be induced in macrophages, endothelial cells and vascular smooth muscle cells by endotoxin and cytokines (e.g. Moncada, 1992). The constitutive NOS has been purified from rat cerebellum and antibodies raised against the enzyme have been used to determine the localization of NOS (Bredt *et al.*, 1990). By means of immunohistochemistry major populations of NOS positive nerves have been demonstrated throughout the peripheral nervous system in, e.g., the gastrointestinal tract (Bredt *et al.*, 1990; Ward *et al.*, 1992b; Young *et al.*, 1992; Saffrey *et al.*, 1992; Alm *et al.*, 1993), the urogenital tract (Burnett *et al.*, 1992; Sheng *et al.*, 1992; Alm *et al.*, 1993) and perivascular nerve fibres (Kummer *et al.*, 1992). The distribution of nerves staining for NOS does not seem to be associated with any particular neurotransmitter system, although some NOS immunolabelled nerves in the

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brain also express somatostatin, neuropeptide Y (NPY) and choline acetyltransferase (Dawson *et al.*, 1991), and in the intestine, VIP (Ward *et al.*, 1992b). However, extensive colocalization has recently been reported between NOS immunopositive nerves and nerves stained histochemically by the NADPH diaphorase method (Dawson *et al.*, 1991; Hope *et al.*, 1991; Alm *et al.*, 1993).

In an attempt to obtain highly specific antisera against neuronal NOS, we have immunized rabbits with fragments of the C- and N-terminal parts of the NOS sequence (Alm *et al.*, 1993), described from rat cerebellum (Bredt *et al.*, 1991). Antibodies raised against the two fragments visualized immunoreactive nerve structures in, e.g., the rat intestine, adrenal glands and penile erectile tissue. No immunoreactive staining was found in the endothelium, suggesting that these fragments of the NOS enzyme may be used for specific localization of neuronal NOS (Alm *et al.*, 1993).

The aim of the present study was to establish the distribution of NOS containing nerves, as studied by immunohistochemistry and NADPH diaphorase histochemistry, in the pig lower urinary tract. In addition, functional *in vitro* studies were performed in order to evaluate whether the presence of NOS immunoreactivity/NADPH diaphorase staining correlates with smooth muscle responses involving the L-arginine/NO pathway.

Methods

The bladder and urethra from female pigs were removed in a slaughterhouse shortly after the animals had been killed. The tissue was transported to the laboratory in cold Krebs solution (for composition, see below). The bladder and urethra were opened longitudinally and tissue pieces from the detrusor, trigone, urethra and vesical arteries were prepared. The specimens used for morphological studies were fixed for 3–4 h in an ice-cold freshly prepared solution of 4% formaldehyde in phosphate buffered saline (PBS, pH 7.4) and then rinsed in 15% sucrose in PBS (for 2–3 days). The tissue pieces were frozen at −40°C in isopentane and stored at −70°C.

NADPH diaphorase histochemistry

Tissue demonstration of NADPH diaphorase activity was performed by incubating tissue sections with 1 mM β-NADPH and 0.5 mM nitro blue tetrazolium dissolved in 50 mM Tris/HCl buffer (pH 8.0) containing 0.2% Triton X-100 for 30–120 min at 37°C. After rinsing in PBS the sections were mounted in Kaiser's glycerol gelatin. In some sections β-NADPH was excluded, or exchanged for 1 mM α-NADPH or 1 mM β-NADP. Various other methods were also used to study the specificity of the β-NADPH diaphorase reaction. The heat-stability was checked by preincubating some sections at 77°C for 5 min. Other pretreatments included incubation with 0.1 mM N^G-nitro-L-arginine (L-NOARG) at room temperature for 40 min, 0.1 mM dicoumarol, or 1 and 5 mM L-canavanine for 60 min, or incubation with 1 mM 5,5'-dithio-*bis*(2-nitrobenzoic acid) (DTNB) for 90 min. All substances used for preincubation were also present during the NADPH diaphorase activity reaction, except DTNB, which was present only at the preincubation.

The NADPH diaphorase activity obtained by the various procedures was judged subjectively.

NOS immunohistochemistry

Tissue sections, cut at a thickness of 9 μm, were air-dried for 15 min and then incubated in PBS containing 0.2% Triton X-100 for 2 h. Incubation took place overnight at room temperature in an antiserum, raised in rabbits against a 15-amino acid C-terminal fragment of NOS, diluted 1:1280 (Alm *et al.*, 1993) in PBS containing 0.2% Triton X-100. After rinsing in PBS the sections were incubated with FITC, (diluted 1:80 in PBS) for 90 min, followed by rinsing in PBS. The sections were then mounted in buffered PBS/glycerol containing *p*-phenylenediamine to prevent fluorescence fading (Johnson & Araujo, 1981) and investigated in a fluorescence microscope. The immunoreactivity was documented by microphotography, after which the cover-slips were carefully detached from the slides and subsequently rinsed overnight in PBS. Tissue demonstration of NADPH diaphorase activity was then undertaken in the same section. As cross-reaction to other proteins sharing amino acid sequences with the synthesized products cannot be excluded, the immunoreactive products were referred to as NOS-like immunoreactive (NOS-IR). Details and characterization of the antiserum are described elsewhere (Alm *et al.*, 1993).

In control experiments, sections were incubated in the absence of the primary antiserum or with antiserum absorbed with the soluble, purified homogenate from rat cerebellum (Knowles *et al.*, 1989).

Functional studies

Recording of mechanical activity The preparations were transferred to 2.5 or 5 ml organ baths containing Krebs solution maintained at 37°C by a thermoregulated water circuit. The Krebs solution was bubbled with a mixture of 95% O_2 and 5% CO_2, maintaining pH at 7.4. The strips were mounted between two L-shaped hooks by means of silk ligatures. One of the hooks was connected to a Grass Instrument FT03C force-displacement transducer for registration of isometric tension and the other was attached to a movable unit. By varying the distance between the hooks the tension could be adjusted. The transducer output was recorded on a Grass Polygraph model 7D or E. During an equilibration period of 45–60 min, the preparations were stretched until a stable tension was obtained.

When subjected to electrical field stimulation (EFS), the preparations were mounted between two parallel platinum electrodes in the organ baths. Transmural stimulation of nerves was performed using a Grass S48 or S88 stimulator delivering single square wave pulses (duration 0.8 ms) at supramaximum voltage. The polarity of the electrodes was reversed after each pulse by means of a polarity-changing unit. The train duration was 5 s and the stimulation interval 120 s.

Tissue preparations and experimental procedures The preparations were investigated on the same day as the tissue was obtained, or stored for 24 h at 4°C in Krebs solution before investigation. There was no difference in the response to drugs between preparations investigated on the first day and those studied the day after.

Detrusor: Preparations (1 × 2 × 5 mm), stripped of mucosa, were prepared from the anterior wall of the detrusor. The tension of the preparations was adjusted during the equilibration period to a final level of 4–6 mN. After the equilibration period, each experiment was started by exposing the detrusor preparations to a K^+ (124 mM) Krebs solution (for composition, see below), until two reproducible contractions (difference $<10\%$) had been obtained. The following investigations were performed: (1) The supramaximum voltage was determined individually for the detrusor preparations at 20 Hz, then electrically-induced contractions were recorded at 10, 20 or 40 Hz. At least three consecutive reproducible contractions (difference $<10\%$) were required before the different drugs (L-arginine, D-arginine, L-NOARG, SIN-1, propranolol, isoprenaline or scopolamine) were added to the baths. (2) Concentration-response relations for carbachol (10 nM–0.1 mM) with or without preincubation with L-arginine (1 mM), L-NOARG (0.1 mM) or SIN-1 (0.1 mM). (3) Responses of L-arginine (1 mM) and SIN-1 (0.1 mM) in precontracted (carbachol 10 μM) muscle strips.

Urethra and trigone: Smooth muscle strips (1 × 2 × 5 mm), stripped of mucosa, were prepared from the trigone and urethra. The urethral strips were cut transversely from the proximal part of the urethra. The trigone strips were taken in an oblique direction from the internal urethral orifice towards one of the ureteric orifices. The tension of the urethral and trigonal strips was adjusted during the equilibration period to 4–8 mN. Trigonal strips were initially exposed to a K^+ (124 mM)-Krebs according to the procedure previously described for the detrusor. Responses to EFS in urethral and trigonal preparations were studied after precontraction of the strips, to a stable level, with noradrenaline (10 μM). First, the supramaximum voltage was determined individually for each strip at 8 Hz. This frequency has previously been shown to cause maximal relaxation of the pig urethra and trigone (Persson & Andersson, 1992). Thereafter, relaxant responses at 8 Hz were studied in the presence or absence of L-NOARG (10 μM) and TTX (1 μM). SIN-1 (0.1 mM) was added to the baths at the end of the experiment followed by determination of baseline level by changing the bath medium to a Ca^{2+} free solution.

Vesical arteries: Extramural arteries (inner diameter = 250–400 μm) supplying the detrusor were taken from the fascia adjacent to the lateral surface of the bladder, 2–4 cm before entering the bladder wall. The vessels were then dissected free from surrounding tissue under a microscope and cut into 1–2 mm long ring segments. After mounting, the vessels were stretched stepwise during the equilibration period to a stable tension of 2–4 mN. Each experiment was started by exposing the vessels to a K^+ (124 mM)-Krebs solution. At least two reproducible contractions (difference $<10\%$) were required before the experiments were begun: (1) The vessel segments were precontracted with a submaximal concentration of noradrenaline (50–80% of the initial K^+ (124 mM)-induced contraction). The noradrenaline concentration (0.5–6 μM) was chosen individually for each segment since this ensured that the precontracted level was the same, relative to K^+, in all experiments. Separate experiments, with different levels of noradrenaline-induced tension, showed that when the tension was higher than 80% of K^+, the relaxation to acetylcholine was impaired. (2) The presence of an intact endothelium was confirmed in each preparation by addition of acetylcholine (1 μM), and only vessels exhibiting $>80\%$ relaxation of the noradrenaline-induced tension were accepted for relaxation studies. (3) To study the endothelium-dependent relaxation, the endothelium was removed by allowing carbogen gas to flow through the lumen for 2–3 min. Loss of relaxant response to acetylcholine (1 μM) was considered to indicate endothelium removal. (4) The effects of L-NOARG (0.1 mM–0.3 mM) and indomethacin (1 μM) on acetylcholine-induced relaxations were examined by adding the drugs either during a noradrenaline-induced contraction or before the contraction. In all cases the drugs were in contact with the vessels over a period of at least 15 min before concentration-response curves for acetylcholine were constructed. (5) In some experiments the vessels were pretreated with phentolamine (1 μM) and indomethacin (1 μM) and contracted with 60 mM K^+ in the presence or absence of 0.3 mM L-NOARG. Concentration-response curves to acetylcholine or SIN-1 were then obtained. (6) Relaxant responses to exogenous NO (acidified $NaNO_2$ (pH 2), as described by Furchgott *et al.*, 1988) and SIN-1 were recorded in vessels without endothelium. Separate experiments showed that the vehicle (H_2O, pH 2), in equivalent volumes, had no relaxing effect *per se*.

Drugs and solutions

The following drugs were used: (−)-noradrenaline hydrochloride, carbamylcholine chloride, acetylcholine chloride, isoprenaline hydrochloride, nitro blue tetrazolium, propranolol hydrochloride, tetrodotoxin, scopolamine hydrochloride, N^G-nitro-L-arginine (L-NOARG), L-arginine hydrochloride, D-arginine hydrochloride, β-NADPH, tris(hydroxymethyl) aminomethane (Tris), 5,5′-dithio-*bis*-(2-nitrobenzoic acid) (DTNB), L-canavanine, dicoumarol, (Sigma Chemical Company, St Louis, MO, U.S.A.), indomethacin (Confortid, Dumex, Copenhagen, Denmark), phentolamine methane sulphonate (Ciba-Geigy, Basel, Switzerland), Triton X-100, *p*-phenylenediamine, Kaiser's glycerol gelatin, ethyleneglycol *bis* (β-aminoethylether)-N,N′-tetraacetic acid (EGTA) (Merck, Darmstadt, Germany), FITC (fluorescein isothiocyanate)-conjugated swine antirabbit immunoglobulins (Dakopatts, Stockholm, Sweden), SIN-1 (3-morpholino-sydnonimine hydrochloride) was a gift from Dr Kunstmann, Cassella AG, Germany. Stock solutions were prepared and then stored at − 70°C. Subsequent dilutions of the drugs were made with 0.9% NaCl, and when appropriate, 1 μM ascorbic acid was added as an antioxidant. The reported concentrations are the calculated final concentrations in the bath solution.

The Krebs solution used had the following composition (mM): NaCl 119, KCl 4.6, $CaCl_2$ 1.5, $MgCl_2$ 1.2, $NaHCO_3$ 15, NaH_2PO_4 1.2, glucose 11. K^+-Krebs solutions (60 and 124 mM) were prepared by replacing NaCl with equimolar amounts of KCl. Ca^{2+}-free medium was prepared by omitting Ca^{2+} from the normal Krebs solution and adding 0.1 mM EGTA.

Analysis of data

The relaxant effects of EFS and drugs were normalized and have been expressed as percentage reduction in tension. In urethral and trigonal strips, baseline level was defined as the tension reached after exposing the strips to Ca^{2+}-free medium. The effect of drugs on electrically induced detrusor contractions are expressed as percentage change of control contractions obtained before drug administration. The − log IC_{50} values (the negative logarithm of the drug concentration producing 50% of the maximum relaxation obtained) were determined graphically for each curve by linear interpolation. *P* values were determined by an unpaired Student's *t* test (two-tailed) corrected for multiple comparisons by Bonferroni's method, and considered significant if lower than 0.05. *n* denotes the number of preparations and *N* the number of animals. When the number of preparations and animals is the same, only *n* is given. Results are given as mean values ± s.e.mean.

Results

NADPH diaphorase histochemistry

NADPH diaphorase positive varicose nerve fibres of various thickness, and coarse nerve branches, were found in and/or around muscular bundles in the detrusor, trigone and urethra (Figure 1). In general, the nerve fibres in the trigone and urethra were much thinner and more dispersed within the muscular tissue than in the detrusor. In the outer parts of the smooth musculature and the surrounding connective tissue, some NADPH diaphorase positive nerve cells in ganglionic structures could be seen. In the urothelium of all tissues, the NADPH diaphorase reaction was found to be particularly strong. Frequently, NADPH diaphorase positive nerve fibres could be seen around arteries, but only rarely around veins. A positive NADPH diaphorase reaction was found in the endothelium of some arteries. Arteries that displayed a positive endothelial and/or perivascular NADPH diaphorase activity were often seen in the lamina propria and in the outer parts of the smooth musculature. No NADPH diaphorase activity was seen in the endothelium of veins. In segments of the extramural vesical artery, located in the fascia adjacent to the lateral surface of the detrusor, NADPH diaphorase positive staining was found in thin nerve fibres surrounding the vessels and in the endothelium.

In the detrusor and trigone, the specificity of the NADPH

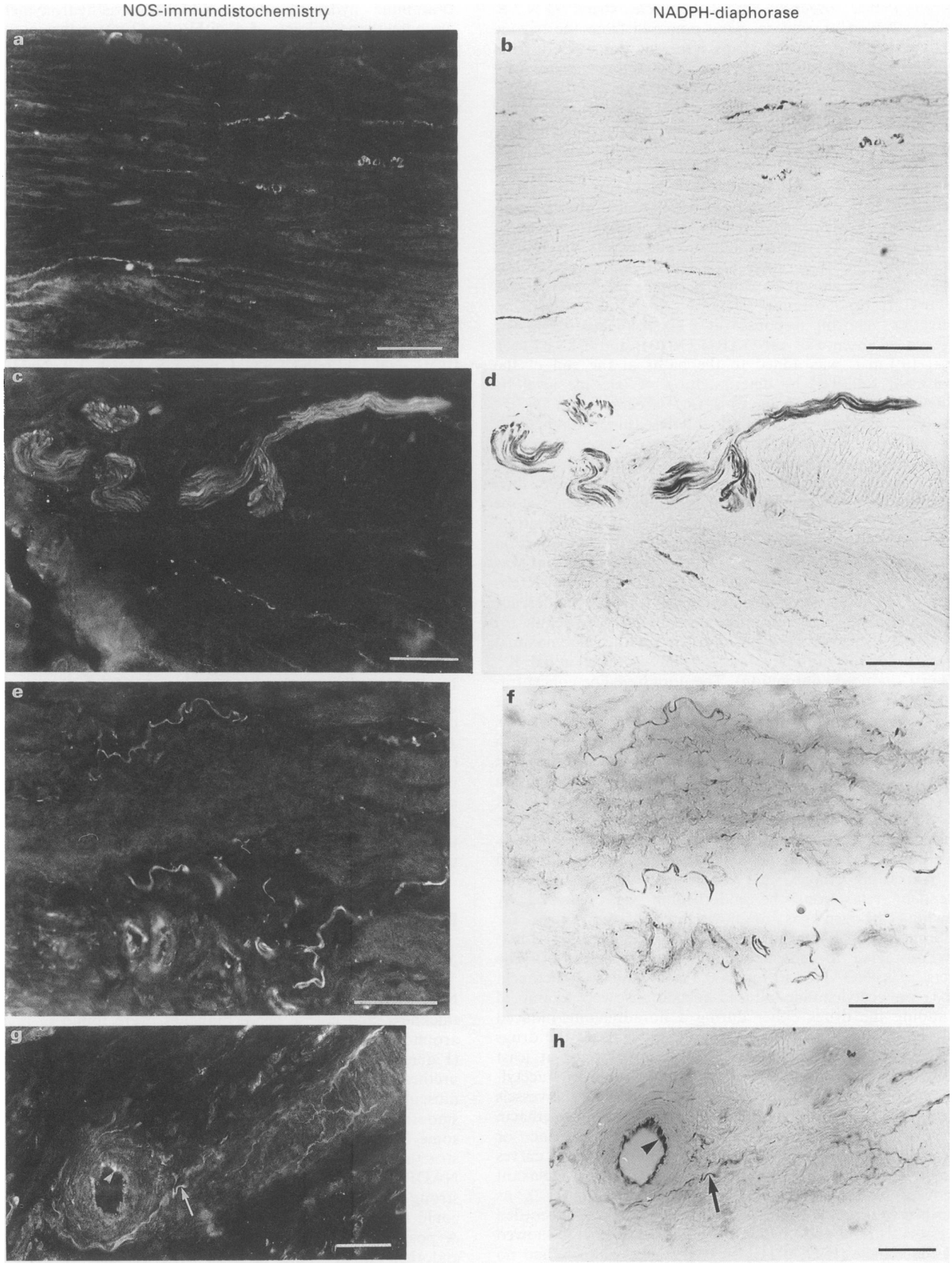

Figure 1 Microphotographs showing the distribution and colocalization of nitric oxide synthase (NOS)-like immunoreactivity and NADPH diaphorase activity in tissue sections of the pig lower urinary tract. The sections were processed for NOS immunohistochemistry (a, c, e, g) and subsequently for NADPH diaphorase (b, d, f, h). (a–d) Immunohistochemical localization of NOS, showing thin nerve fibres (a) and coarse nerve trunks (c) in the smooth muscle layer of the detrusor. Almost identical staining was observed using the NADPH diaphorase technique (b, d). (e, f) Sections of urethral tissue showing thin NOS-immunolabelled nerve fibres dispersed within the smooth muscle layer (e). Almost all NOS-immunolabelled nerve fibres were also stained for NADPH diaphorase (f). (g, h) Intramural vessel in the trigone area surrounded by numerous NOS-immunoreactive (g) and NADPH diaphorase positive (h) nerve fibres (arrows). The endothelium of the vessel (arrowheads) is strongly positive for NADPH diaphorase activity, while inconsistent staining is demonstrated by immunohistochemistry. (Scale bar = 50 μm).

diaphorase reaction was studied by various procedures. The neuronal and endothelial NADPH diaphorase reaction was abolished, or virtually abolished, by heat and DTNB, or if β-NADPH was excluded or substituted with α-NADPH. When β-NADP was used instead of β-NADPH, nearly all structures in the tissue reacted strongly and nerves could not be separated from other structures in the tissue. L-NOARG, dicoumarol, and L-canavanine were essentially without effect on the NADPH diaphorase reaction. A NADPH diaphorase reaction in the urothelium could still be observed if β-NADPH was substituted with α-NADPH or in the presence of L-canavanine, dicoumarol or L-NOARG, or if the sections were pretreated with DTNB.

NOS immunohistochemistry

NOS-IR nerve fibres in the pig lower urinary tract were mainly distributed in the smooth muscle layers of the detrusor, trigone and urethra regions (Figure 1). A gradient in the density of immunolabelled fibres was discernible, showing the highest density of NOS-IR fibres in the urethra and the lowest in the detrusor. The NOS antiserum labelled different types of neuronal structures, ranging from coarse trunks to delicate fibres, throughout all regions. Coarse nerve trunks with NOS-IR fibres were observed mainly in the adventitia but also in the muscle layers. Finer nerve trunks consisting of NOS-IR fibres were observed in the connective tissue between the smooth muscle bundles. In addition, delicate immunolabelled fibres coursed along and dispersed within the smooth muscle bundles. Thin NOS-IR fibres could be found in the lamina propria of all regions, mostly in the vicinity of vessels. However, no immunolabelling could be observed in the urothelium.

The NOS antiserum labelled vessels localized in the lamina propria, intramurally and close to the adventitia in all regions. Labelling occurred in the endothelial lining of vessels in the lamina propria, and in perivascular varicose fibres innervating them. Likewise, intramural vessels in all regions were surrounded by thin NOS-IR nerves, that formed plexi around the vessels. NOS-IR labelling also seemed to be confined to the endothelial lining of some intramural vessels, but vessels without both NOS-IR perivascular labelling and endothelium staining were observed. Perivascular fibres around the extramural vesical artery displayed NOS-IR labelling. Thin, delicate fibres as well as nerve trunks were present in the tunica adventitia and occasionally the former penetrated superficially in the tunica media. The endothelium was also stained.

In sections where the antiserum was absorbed with antigen extracted from rat cerebellum, immunoreactivity of neuronal structures was abolished. However, immunoreactivity was still observed in the endothelium of vessels. No staining of neuronal structures or endothelium was found when the primary antiserum was omitted.

Correlation between NADPH diaphorase staining and NOS immunoreactivity

NADPH diaphorase staining in the lower urinary tract showed both similarities and differences with the NOS immunohistochemistry. Thus, the distribution of fibres in the smooth musculature obtained with the NADPH-diaphorase technique was almost identical to that revealed with NOS immunohistochemistry (Figure 1). However, the fibres were sometimes better visualized with the NOS antiserum than after NADPH diaphorase staining. In addition, the gradient with highest density of stained fibres in the urethra was also revealed with the NADPH-diaphorase staining. In contrast to the NADPH diaphorase technique, no labelling was observed in the urothelium with NOS immunohistochemistry. Moreover, the endothelium of vessels was more frequently stained using the NADPH diaphorase technique than immunohistochemistry (Figure 1g, h).

Functional studies

Detrusor EFS of pig detrusor strips at 10, 20 and 40 Hz produced phasic contractions. Separate experiments showed that these frequencies corresponded to approximately 30, 70 and 100% of the maximal response induced by EFS. Detrusor preparations were treated with L-NOARG (1 μM–0.1 mM), added cumulatively to the bath, and the contractile response to EFS at 10, 20 and 40 Hz studied. L-NOARG (10 μM) increased the contractions by a maximum of 12 ± 2% at 10 Hz, 12 ± 5% at 20 Hz, and 3 ± 4% at 40 Hz ($n = 6$ for all; data not shown). Compared to time-matched control preparations, running in parallel, these increases were not statistically significant.

In the presence of L-arginine (1 mM), contractions induced by EFS at 10, 20 and 40 Hz were reduced by 28 ± 3%, 30 ± 2%, and 25 ± 4% ($n = 6$ for all), respectively (Figure 2). This action of L-arginine was inhibited ($P < 0.01$) by 0.1 mM L-NOARG (Figure 2). D-Arginine (1 mM; $n = 4$) was without effect on EFS-induced detrusor contractions (Figure 3). Propranolol (1 μM) had no effect *per se*, and did not affect the L-arginine-induced decrease in contractile response (26 ± 2% at 20 Hz; $n = 6$ after propranolol pretreatment; Figure 3). On the other hand, in the presence of scopolamine (1 μM), L-arginine had no effect ($n = 6$) on electrically evoked (20 Hz) detrusor contractions (Figure 3). The scopolamine- and L-arginine resistant part of the contraction amounted to 12 ± 4% ($n = 6$). The NO-donor SIN-1 (1 μM–0.3 mM) had only minor effects on electrically evoked (20 Hz) detrusor contractions (8 ± 3% reduction at 0.1 mM, $n = 5$). Isoprenaline (1 nM–10 μM) caused concentration-dependent inhibition of the EFS-induced detrusor contractions (20 Hz), that amounted to 52 ± 11% ($n = 5$) at 10 μM.

Concentration-response curves to carbachol (10 nM–0.1 mM) were unaffected by pretreatment with L-arginine (1 mM, $n = 5$), L-NOARG (0.1 mM; $n = 4$) or SIN-1 (0.1 mM; $n = 5$). L-Arginine (1 mM) had no influence on tension when applied to preparations precontracted by carbachol (10 μM; $n = 6$), whereas SIN-1 (0.1 mM) in the same protocol reduced the tension by 40 ± 5% ($n = 6$; data not shown).

Urethra and trigone EFS of urethral and trigonal strips at 8 Hz produced pure relaxant responses. All responses were practically abolished by TTX (1 μM), verifying that they were nerve-mediated (Figure 4a). The relaxations amounted to 47 ± 5% ($n = 7$, $N = 4$) and 49 ± 6% ($n = 8$, $N = 5$), for urethral and trigonal preparations, respectively. Addition of L-NOARG (10 μM) to urethral and trigonal preparations

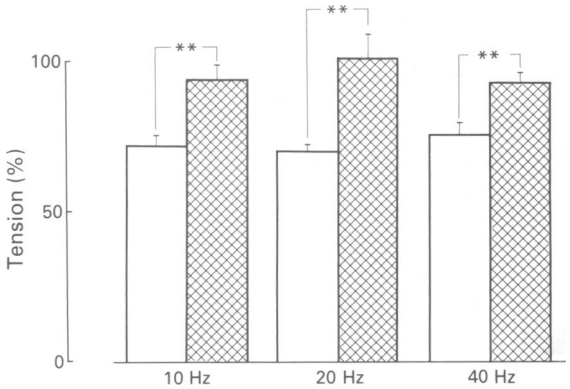

Figure 2 Effect of L-arginine on contractions induced by electrical field stimulation at 10, 20 and 40 Hz in pig isolated detrusor smooth muscle preparations. Open columns indicate the response to L-arginine (1 mM) and cross-hatched columns the response to L-arginine after pretreatment with N^G-nitro-L-arginine (L-NOARG, 0.1 mM). Results are expressed as a percentage of the contractions before drug treatment and given as mean ± s.e.mean ($n = 6$). $^{**}P < 0.01$.

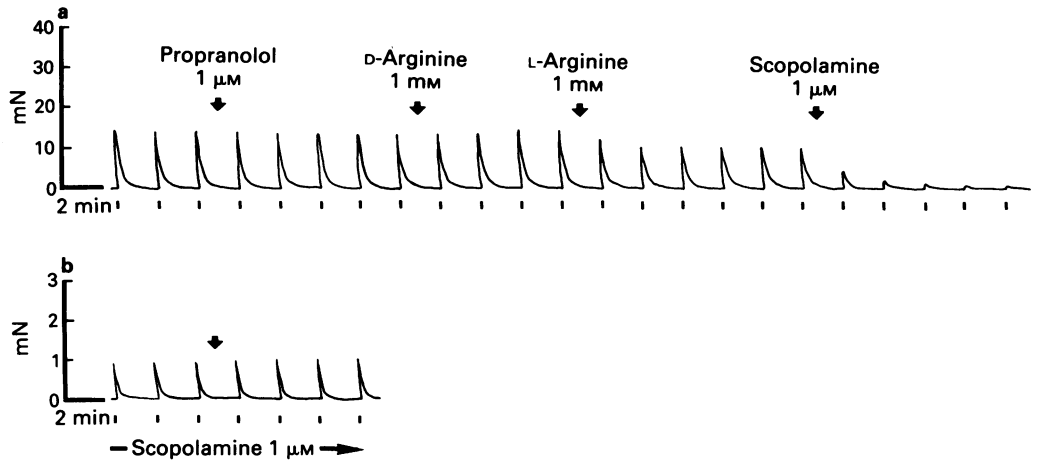

Figure 3 Recordings of contractions evoked by electrical field stimulation at 20 Hz in pig isolated detrusor smooth muscle preparations. In (a) is shown the effect of L-arginine (1 mM) and in (b) the lack of effect in the presence of scopolamine (1 μM). Bars indicate time of stimulation (supramaximum voltage, 0.8 ms pulses, 5 s train duration).

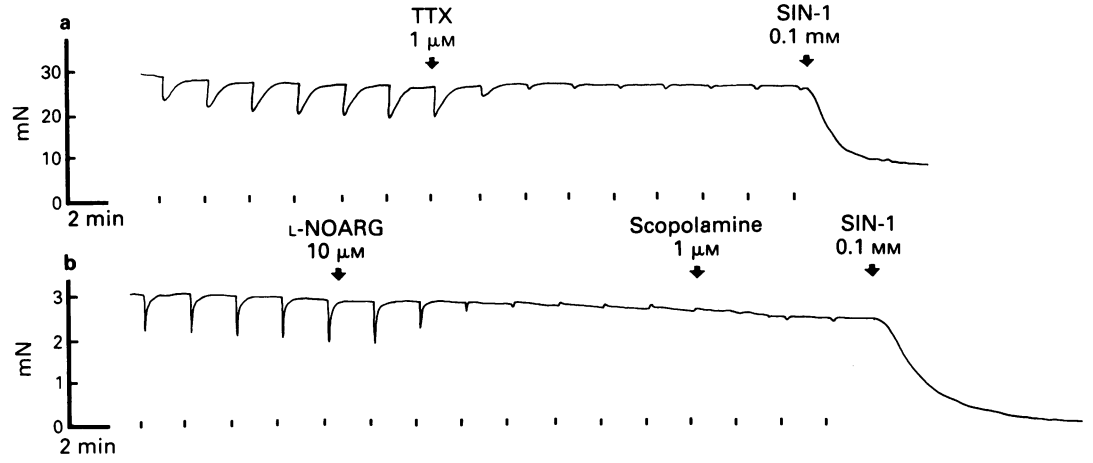

Figure 4 Recordings of relaxations evoked by electrical field stimulation at 8 Hz in (a) the pig isolated trigone and (b) the pig isolated urethra demonstrating the response to tetrodotoxin (TTX; 1 μM) and N^G-nitro-L-arginine (L-NOARG, 10 μM) in noradrenaline-contracted preparations. The NO-donor 3-morpholino-sydnonimine (SIN-1, 0.1 mM) was applied at the end of the experiment. Baseline level was defined as the level reached after exposing the strips to Ca^{2+}-free solution. Bars indicate time of stimulation (supramaximum voltage, 0.8 ms pulses, 5 s train duration).

stimulated by EFS resulted in a marked decrease in the relaxant response with time. Maximal inhibition was reached within 10–15 min and in some preparations the response changed into a contraction (Figure 4b). Scopolamine (1 μM) reversed these contractions to a weak relaxation (Figure 4b). SIN-1 (0.1 mM) produced pronounced relaxations of both urethral (88 ± 2%, $n = 7$, $N = 4$) and trigonal strips (84 ± 3%, $n = 8$, $N = 5$) (Figure 4).

Vesical arteries Acetylcholine (1 nM–10 μM) caused concentration-dependent relaxations of noradrenaline-induced contractions in pig vesical arteries. A maximum relaxation of 97 ± 1% ($n = 16$) was obtained with acetylcholine 10 μM, and the $-\log IC_{50}$ value was 7.1 ± 0.1. The maximum relaxation (93 ± 3%, $n = 6$) and the $-\log IC_{50}$ value (7.1 ± 0.1) were not affected by preincubation with indomethacin (1 μM) (Figure 5). Removal of the endothelium practically abolished the acetylcholine-induced relaxation, since maximum relaxation was reduced to 4.7 ± 1.9% ($n = 8$, $P < 0.001$) (Figure 5). Pretreatment with L-NOARG (0.1 mM and 0.3 mM) caused a rightward shift of the concentration-response curves, but $-\log IC_{50}$ values were not calculated since no defined maxima were obtained. The maximal relaxations obtained amounted to 88 ± 6% ($n = 6$) and 65 ± 12% ($n = 6$, $P <$ 0.05) of the noradrenaline-induced contraction after pretreatment with L-NOARG, 0.1 mM and 0.3 mM, respectively (Figure 5).

In vessel segments contracted with 60 mM K^+ (contraction level: 54 ± 4%, $n = 10$, $N = 5$, of the initial K^+ 124 mM response), acetylcholine induced concentration-dependent relaxations (Figure 6). The relaxation obtained at 10 μM amounted to 65 ± 9% in K^+ (60 mM)-contracted vessels (compared to 97 ± 1% in noradrenaline-contracted vessels). When the vessels were incubated with 0.3 mM L-NOARG and then contracted with K^+ 60 mM (contraction level: 100 ± 2%, $n = 10$, $N = 5$ of the initial K^+ 124 mM response) all relaxant responses to acetylcholine were abolished (Figure 6). In contrast, SIN-1 produced relaxations of K^+ 60 mM contracted vessels both in the absence and presence of L-NOARG (data not shown). In arteries where the tension was raised with K^+ (124 mM), acetylcholine (1 μM) reduced the tension by 24 ± 2% ($n = 6$).

Addition of L-NOARG (0.1 mM) to vessel segments at baseline caused a small, approximately 3–4%, increase in tension. On the other hand, if L-NOARG (0.1 mM) was applied on top of a noradrenaline-induced contraction, it resulted in a pronounced increase in tension (26 ± 4%, $n = 12$). No increase in tension was seen when L-NOARG

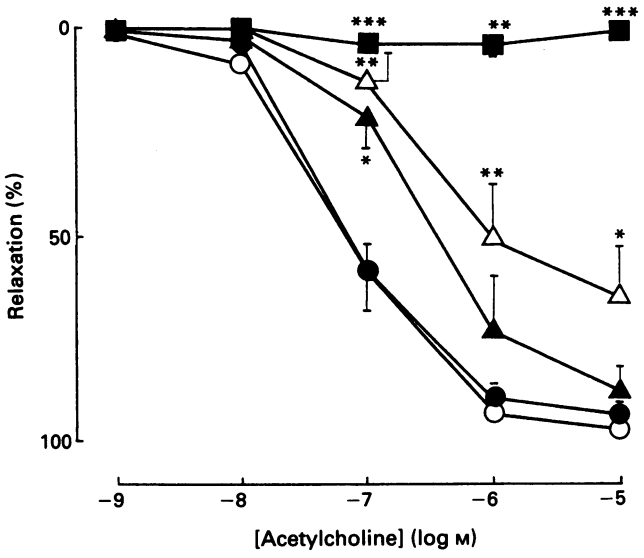

Figure 5 Relaxation of pig isolated vesical arteries induced by acetylcholine in controls (○) or in the presence of 1 μM indomethacin (●), 0.1 mM N^G-nitro-L-arginine (L-NOARG, ▲), 0.3 mM L-NOARG (△) or in vessels without endothelium (■). Results are expressed as percentage relaxation of the noradrenaline-induced tension and given as mean ± s.e.mean ($n = 6-16$). $*P<0.05$; $**P<0.01$; $***P<0.001$.

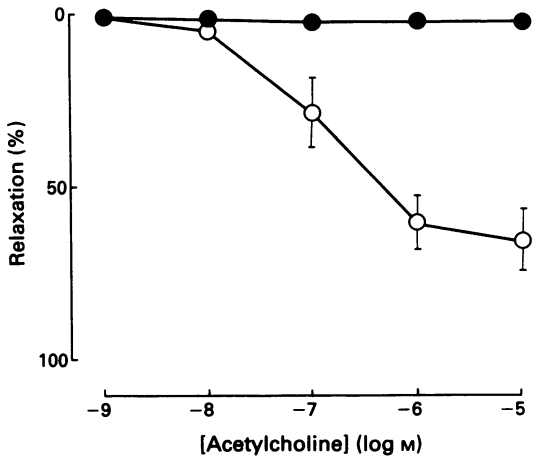

Figure 6 Relaxation of pig isolated vesical arteries induced by acetylcholine in K^+ (60 mM)-contracted preparations in the absence (○) or presence of N^G-nitro-L-arginine (L-NOARG, 0.3 mM) (●). The vessels were pretreated with phentolamine (1 μM) and indomethacin (1 μM) for 30 min. Results are expressed as percentage relaxation of the K^+ (60 mM)-induced tension and given as mean ± s.e.mean ($n = 5$).

(0.1 mM) was administered to vessels without endothelium ($n = 7$).

Noradrenaline-contracted pig vesical arteries without endothelium responded to increasing concentrations of SIN-1 and NO (present in acidified solution of $NaNO_2$) with concentration-dependent relaxations. SIN-1 (10 μM) relaxed the vessels by 96 ± 3% ($n = 6$) and NO (1 mM) by 97 ± 2% ($n = 6$; data not shown).

Discussion

The present study demonstrates that NOS-IR and NADPH diaphorase activity are expressed in nerve trunks and fine nerve fibres throughout the pig lower urinary tract. NOS-IR and NADPH diaphorase staining were present in neuronal structures both within and/or around smooth muscle bundles in the detrusor, trigone and urethra. Thin nerve fibres that dispersed within the muscle bundles were mainly found in the urethral/trigonal area, whereas such fibres were less common in the detrusor area. In previous studies, the involvement of NO as an inhibitory NANC-transmitter has been based mainly on the fact that relaxant responses to EFS of nerves in the urethra, bladder neck and trigone were inhibited by NOS-inhibitors and that exogenous NO mimicked the response to NANC-nerve stimulation (Garcia-Pascual *et al.*, 1991; Dokita *et al.*, 1991; Andersson *et al.*, 1991; 1992; Persson & Andersson, 1992; Persson *et al.*, 1992; Thornbury *et al.*, 1992). Furthermore, relaxations of the rabbit urethra in response to sodium nitroprusside (Morita *et al.*, 1992) and electrical stimulation (Persson & Andersson, 1993) seem to be associated with increases in smooth muscle content of cyclic GMP. Measurement of NOS activity in the rat urogenital tract, by monitoring the conversion of [^{3}H]-arginine to [^{3}H]-citrulline, revealed a high catalytic activity in the bladder neck and urethra (Burnett *et al.*, 1992). By localization of the enzyme involved in the synthesis of NO, morphological evidence for the assumption that NO may act as a neurotransmitter in the lower urinary tract now exists, strengthening the hypothesis of a role for NO in this tissue. The morphological findings in the present study, demonstrating that the detrusor region expresses a low density of thin NOS-IR/NADPH diaphorase stained fibres compared to the outlet region, are consistent with previous functional findings that NO may serve as a NANC inhibitory neurotransmitter mainly in the outlet region (Persson & Andersson, 1992; Persson *et al.*, 1992).

The observation that NADPH diaphorase histochemistry can be used to identify neuronal NOS (Hope *et al.*, 1991; Dawson *et al.*, 1991) has resulted in several reports about the distribution of NADPH diaphorase positive nerves in peripheral tissues, including the lower urinary tract (Larsson *et al.*, 1992; Keast, 1992; McNeill *et al.*, 1992; Grozdanovic *et al.*, 1992). In the rat urinary tract, NADPH diaphorase positive staining was observed in the urethra, close to the urothelium (Keast, 1992) and in the bladder base (McNeill *et al.*, 1992). Conflicting results have been reported concerning the NADPH diaphorase staining of the detrusor of different species. No staining could be identified in the rat detrusor by Keast (1992), but McNeill *et al.* (1992) and Grozdanovic *et al.* (1992) found staining in the rat and mouse detrusor, respectively.

In this and a previous study (Larsson *et al.*, 1992), the specificity of the NADPH diaphorase staining in the pig lower urinary tract was examined. L-NOARG, in a concentration sufficient to inhibit the enzyme functionally, caused no reduction in NADPH diaphorase staining. This suggests that the portion of the NOS enzyme that yields the NADPH diaphorase staining is not identical to, and does not interact with, the site of NO production. The marked increase in staining using β-NADP most probably reflects non-specific staining by other enzymes with diaphorase activity (Hope & Vincent, 1989). Dicoumarol, reported to inhibit some non-specific diaphorase staining (Ernster *et al.*, 1962), and L-canavanine, known to inhibit the inducible form of NOS (Iyengar *et al.*, 1987), were without effect on the NADPH diaphorase reaction found in this study. However, DTNB and α-NADPH abolished the NADPH diaphorase activity in nerves and in the endothelium, but not in the urothelium. Thus, the neuronal and endothelial NADPH diaphorase activity, suggested to indicate NOS (Dawson *et al.*, 1991; Hope *et al.*, 1991), exhibited characteristics different from the activity found in the urothelium.

Immunohistochemistry and NADPH-diaphorase histochemistry performed on the same preparations in the pig lower urinary tract, revealed an extensive colocalization of the two markers in all regions. However, the urothelium of all regions was intensively stained by the NADPH dia-

phorase technique, whereas no immunolabelling of the urothelium could be observed. The reason for this may be, as discussed above, that the NADPH diaphorase technique stains additional enzymes besides NOS, or that NADPH diaphorase staining of the urothelium represents an isoform of NOS not readily detectable with our antiserum The antiserum used in this study, raised against a fragment of the rat cerebellar NOS, has been shown to be selective for neuronal compared to endothelial NOS in rat tissue (Alm *et al.*, 1993). This was not the case with antisera raised against the whole enzyme (Bredt *et al.*, 1990; Schmidt *et al.*, 1992). In tissues from pig lower urinary tract, the present antiserum stained besides neuronal structures, also the endothelium of some vessels. Thus, it seems that our rat neuronal NOS antiserum recognizes a protein in the pig endothelium that is similar, but not identical to the neuronal one; possibly endothelial NOS.

Activation of inhibitory nerves to the detrusor might be one factor, behind the largely unknown mechanism, by which the detrusor muscle relaxes to promote urine storage (e.g. De Groat & Kawatani, 1985). In the stomach, NO has been suggested to have a function as a mediator of adaptive relaxation to accommodate food and fluid (Desai *et al.*, 1991). Likewise, a neurogenic inhibition involving NO release would theoretically be a possible factor for bladder relaxation during filling. However, a role for NO in detrusor relaxation has been questioned (Persson & Andersson, 1992; Persson *et al.*, 1992), due to difficulties in demonstrating relaxant responses upon nerve stimulation, and because the potency of NO-related agonists was low in the detrusor. In the present study, NOS-IR nerves were demonstrated in the pig detrusor muscle, although not as pronounced as in the outlet region, which indeed suggests that NO is present in neuronal structures in the detrusor. Even if NO does not seem to act as a neurotransmitter causing direct smooth muscle relaxation of the detrusor smooth muscle, it is possible that the L-arginine/NO system modulates the bladder tone by other mechanisms. L-Arginine was found to be without postjunctional effects on the detrusor muscle, but caused a small inhibition of the electrically evoked detrusor contractions that was reversed by L-NOARG. This may be explained by L-arginine stimulating neuronal NOS to yield an increased NO production. NO production, in turn, may be initiated by contractile activation. However, the NO production in the absence of exogenous L-arginine was apparently not sufficient to depress significantly the electrically induced contractions.

L-Arginine had no effect after muscarinic receptor blockade, indicating that the effect of L-arginine was restricted to cholinergic neurotransmission. Thus, available information suggests that the effect of L-arginine on the cholinergic neurotransmission in the pig detrusor may be due to either functional antagonism at the smooth muscle level, or to prejunctional inhibition of acetylcholine release. It has been suggested that the L-arginine/NO pathway affects cholinergic neurotransmission in guinea-pig trachea at the level of the smooth muscle or via a pre-junctional inhibition of acetylcholine release (Belvisi *et al.*, 1991). However, recent studies found no evidence for an affect on acetylcholine release either in guinea-pig trachea or human airway smooth muscle (Brave *et al.*, 1991; Ward *et al.*, 1992a), suggesting that functional antagonism accounts for the inhibitory action of NO rather than attenuation of transmitter release. However, it cannot be excluded that L-arginine *per se* and/or NO caused a release of an unknown substance, which in turn modulated the cholinergic response.

Smooth muscle cells have been suggested as a possible source of NO formation in the human detrusor muscle (James *et al.*, 1991) as well as in the gastrointestinal tract (Grider *et al.*, 1992). An inducible form of NOS has been demonstrated in vascular smooth muscle cells (Rees *et al.*, 1990) but the physiological relevance of an inducible enzyme, involved in immunological responses, for normal detrusor relaxation seems limited. On the other hand, if smooth muscle cells express a constitutive form of NOS it must be different from the neuronal and endothelial NOS since in this study no labelling of the musculature could be detected with markers for the constitutive enzyme.

Interestingly, retrograde axonal tracing has recently been performed to examine the origin of NADPH diaphorase positive nerves in the rat bladder (McNeill *et al.*, 1992). Numerous NADPH diaphorase positive nerves were present in the major pelvic ganglia and the (T_{13}–L_2, L_6 and S_1) dorsal root ganglia, but not in the inferior mesenteric ganglia. This indicates that if NO contributes to the function in the detrusor, it is most likely through the parasympathetic and/or sensory nervous system. Colocalization studies of NOS, choline acetyltransferase and various peptides are currently being carried out, and may help to elucidate whether some portion of the peripheral nervous system in the lower urinary tract uses NO as a cotransmitter.

Urodynamic studies have revealed a decrease in urethral pressure, in normal micturition, 5–15 s before the detrusor contracts (Scott *et al.*, 1964; Tanagho & Miller, 1970; Low, 1977). Whether this reflects an involvement of a NANC-transmitter reducing the urethral tone is not known. Several histological studies (e.g. Gu *et al.*, 1984; Crowe & Burnstock, 1989) have demonstrated a rich occurrence of NANC-nerves in the bladder outlet region, in support of activation of NANC-inhibitory nerves as a possible mechanism for urethral relaxation. In the present study, histological evidence was provided that nerves coursing through the smooth muscle bundles in the pig urethra and trigone contained the enzyme producing the inhibitory substance NO. In addition, the fact that transient relaxant responses induced by electrical stimulation of pig urethral and trigonal preparations were practically abolished by inhibition of NOS, suggests that NO might have a functional role in urethral relaxation. However, NANC-relaxations evoked at high frequencies in the dog urethra do not seem to be mediated by NO (Hashimoto *et al.*, 1993), implying that at least two neurogenic responses are involved in dog urethral relaxation.

In the unanaesthetized rat, intra-arterial administration of a NOS-inhibitor caused bladder hyperactivity and decreased bladder capacity (Persson *et al.*, 1992). The mechanisms behind bladder hyperactivity are not known, but changes in afferent and/or efferent activity due to lack of inhibitory substances in either the detrusor or the outlet region might be one possible mechanism (Gu *et al.*, 1983; Chapple *et al.*, 1992). Since the NO innervation was found to be particularly well developed in the outlet region, it seems reasonable to assume that the observed bladder hyperactivity evoked by inhibition of NOS, was caused by functional disturbance in this region. In agreement with this, it has been suggested that bladder hyperactivity may be initiated from the bladder outlet region, rather than from the detrusor muscle itself (Hindmarsh *et al.*, 1983; Low *et al.*, 1989). In the present study, L-arginine was found to have an inhibitory effect on the cholinergic neurotransmission in the pig detrusor. This was not found in the rat detrusor (Persson *et al.*, 1992). Despite this, it cannot be excluded that the bladder hyperactivity observed in the rat was due to a lack of inhibitory mechanisms modulating the efferent neurotransmission in the detrusor.

The majority of the vessels throughout the pig detrusor, trigone and urethra were supplied by fine plexus of NOS-IR fibres. Moreover, labelling of the endothelium was seen in some, although not all vessels. It is known that acetylcholine produces relaxations of vessels entirely dependent on the presence of endothelium (Furchgott & Zawadzki, 1980) by release of an endothelium-derived relaxing factor (EDRF) identified as NO (Palmer *et al.*, 1987). The endothelium-dependent acetylcholine-induced relaxation of the pig vesical artery was only partially inhibited by the NOS-inhibitor, L-NOARG. This indicates that NO, provided that the NO formation is completely blocked by L-NOARG, is not the only endothelium-derived factor activated in response to

acetylcholine. Indomethacin had no effect on the relaxation, excluding contribution of a cyclo-oxygenase product. Vascular smooth muscle cells are able to relax upon hyperpolarization and an endothelium-derived hyperpolarizing factor (EDHF) has been postulated (e.g. Taylor & Weston, 1988). If the L-NOARG-resistant, acetylcholine-induced relaxation of the pig vesical artery is mediated by EDHF, an inhibition would be expected if the vessels are pretreated with L-NOARG and contracted with a potassium solution high enough to counteract the effect of EDHF (Nagao & Vanhoutte, 1992). This was also found and indicates that the overall relaxant response to acetylcholine in the pig vesical artery is determined by a combined effect of NO release and possible activation of a hyperpolarizing factor. It was further shown that L-NOARG, in endothelium-intact preparations, caused a marked increase in tension of preconstricted vessels. This was not found in vessels at resting level, suggesting that in vessel segments maintained under active tone, NO generation occurs continuously.

Neuronally-mediated vasorelaxations in e.g. bovine penile artery (Liu *et al.*, 1991) and dog, bovine and monkey cerebral arteries (Toda & Okamura, 1990; Gonzalez & Estrada, 1991) have been reported to be blocked by NOS-inhibitors. In preliminary experiments, long-lasting relaxations evoked by EFS were recorded in the pig vesical artery. However, the responses were unaffected by tetrodotoxin (TTX) and L-NOARG, which raises questions about the origin of the relaxations.

The physiological role of neuronal and endothelial NO-mediated vasodilatation in the bladder can only be speculated upon. The urinary bladder wall frequently undergoes changes in wall tension during filling, which may be sufficient to affect the blood flow. If the blood flow decreases during filling of the bladder (Dunn, 1974; Siroky *et al.*, 1990) an activation of vasodilator mechanisms has to take place in order to supply oxygen to the detrusor muscle. There is evidence from e.g. the guinea-pig coronary vasculature (Park *et al.*, 1992) and rabbit pulmonary circulation (Persson *et al.*, 1990) that hypoxia stimulates the release of NO. This may be the case also in bladder vessels.

In conclusion, the data obtained by immunohistochemistry and NADPH diaphorase staining suggest that NOS is localized in nerve fibres throughout the pig lower urinary tract and predominantly in the outflow region. This is consistent with functional data showing NANC nerve-mediated relaxation, involving the L-arginine/NO pathway, in trigonal and urethral smooth muscle preparations. In the detrusor, the presence of NO-synthesizing enzymes in nerves can be demonstrated, but its functional importance is unclear. NO, as well as other endothelium-derived factors seems to be involved in the endothelium-dependent acetylcholine-induced relaxation of pig vesical arteries.

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