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Nitric oxide synthase in dog urethra: a histochemical and pharmacological analysis

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1 To examine the presence of nitric oxide synthase (NOS) activity in female dog urethra, pharmacological experiments were performed using electrical field stimulation (EFS), guanethidine, atropine, N^G-nitro-L-arginine methyl ester and L-arginine, NOS immunohistochemistry using specific anti-NOS antibody, and reduced nicotinamide adenine dinucleotide phosphate (NADPH) diaphorase staining were also performed.

2 EFS caused frequency-dependent contractions in all urethral preparations, but in the presence of guanethidine and atropine, EFS caused significant relaxation in the proximal urethra and was without effect on the distal urethra.

3 In the presence of guanethidine, atropine, and N^G-nitro-L-arginine methyl ester, small contractions to EFS were re-established in the proximal urethra, but not in the distal urethra. N^G-nitro-D-arginine methyl ester had no such effect.

4 In the presence of guanethidine, atropine, and N^G-nitro-L-arginine methyl ester, the addition of L-arginine, restored the EFS-elicited relaxant responses previously seen with guanethidine and atropine alone in the proximal urethra (at 30 Hz; $12.89 \pm 5.27\%$ to $-2.44 \pm 4.43\%$, mean + s.e., $P < 0.05$). D-Arginine had no such effect.

5 In the distal urethra, the addition of N^G-nitro-L-arginine methyl ester and then L-arginine had no effect on responses to EFS in preparations treated with guanethidine and atropine.

6 Sodium nitroprusside caused relaxation in both the proximal and distal urethra. The relaxant responses per cm^2 cross sectional area in the proximal and distal urethra were 1.23 ± 0.29, and 2.02 ± 0.54 g cm^{-2} cross sectional area (mean $\pm$ s.e.), respectively: there was no significant difference between them.

7 Both NOS and NADPH diaphorase-positive neurones were present in dog urethra, the densities of both being higher in the proximal urethra than in the distal urethra.

8 These results show that female dog urethra possesses NOS nerves and that endogenous NO may play a role in relaxation in the proximal but not the distal urethra.

Keywords: Nitric oxide synthase; dog urethra; electrical stimulation; NADPH diaphorase

Introduction

Nitric oxide (NO) is a small, simple and highly toxic molecule. Recent data have shown that NO is synthesized from L-arginine by the enzyme NO synthase (NOS) (Palmer *et al.*, 1987; 1988), and to be a messenger in at least three systems; white blood cells, blood vessels, and neuronal structures (Moncada, 1992). NOS is regulated by calcium, calmodulin, and reduced nicotinamide adenine dinucleotide phosphate (NADPH) (Bredt & Snyder, 1989; 1990). Among neuronal constituents, NO not only plays a role in the central nervous system, but also in the peripheral nerves (Moore *et al.*, 1991). Evidence has accumulated to suggest that NO may be the transmitter in at least some non-adrenergic, non-cholinergic (NANC) nerves and may be a modulator in others. The L-arginine: NO pathway has been shown to have a functional role in the relaxation in several organs (Li & Rand, 1989; Bult *et al.*, 1990; Tottrup *et al.*, 1990). In urinogenital systems, the NANC relaxation of urethra (Pascual *et al.*, 1991; Andersson *et al.*, 1992; Rajfer *et al.*, 1992; Hashimoto *et al.*, 1993) and penile corpus cavernosum (Ignarro *et al.*, 1990; Holmquist *et al.*, 1991; Rajfer *et al.*, 1992) induced by electrical stimulation is mediated by NO. Furthermore, the presence of NOS in the rat (Burnett *et al.*, 1992) and human (Burnett *et al.*, 1993) penis has been confirmed by immunohistochemistry by use of a specific NOS antibody.

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Physiological evidence for the presence of NOS is provided by the observation that relaxation elicited by electrical nerve stimulation is reversed by a NOS inhibitor, and the fact that this reversal is inhibited by the NOS substrate, L-arginine. Histochemical analysis of the presence of NOS is made by immunohistochemical examination using anti-NOS antibody. In the early 1960s, Thomas & Pearse found that a subpopulation of neurones in the brain contained an enzyme termed nicotineamide adenine dinucleotide phosphate (NADPH) diaphorase, and NADPH diaphorase staining produces a Golgi-like staining of neuronal subpopulations in several brain regions (Thomas & Pearse, 1964). Until recently, the exact identity of the enzyme producing NADPH diaphorase activity was not known; however, biochemical studies have now demonstrated that the enzyme responsible for the histochemical reaction in neurones is actually NOS (Dawson *et al.*, 1991). In confirmation of this finding, it has been shown that all NADPH diaphorase positive neurones exhibit NOS immunoreactivity and contain NOS mRNA (Bredt *et al.*, 1991). Thus, NADPH diaphorase positive neurones are believed to be identical to NOS neurones.

In recent work in rabbit, sheep, and dog urethra, NO-dependent relaxation was only studied in either the proximal or the distal part of the urethra. The distribution of NOS throughout the urethra was not examined, and no comparison of pharmacological and histochemical studies was made. Thus, the objectives of the present study were to examine the difference in NOS activity between the proximal and distal

urethra of the female dog, and to compare the findings of pharmacological experiments using electrical field stimulation (EFS), the NOS inhibitor, N^G-nitro-L-arginine methyl ester (L-NAME), and the NOS substrate, L-arginine, and histochemical examinations using NOS immunohistochemistry using specific anti-NOS antibody and NADPH diaphorase staining.

Methods

Ten female mongrel dogs weighing 10 to 15 kg, were anaesthetized with sodium thiopentone (20 mg kg^{-1}, i.v.) and exanguinated from the common carotid arteries. A total urethral length of 50–60 mm from the bladder neck to just proximal from the entrance into the vestibule (Evans, 1980), was removed. The anterior wall of the vagina was removed with the urethra to avoid damaging the urethral structure. After removing surrounding connective tissues, the urethra was cut longitudinally in Krebs-Henseleit solution. Then, the urethra was cut into three pieces (proximal, middle, and distal) and longitudinal strips of proximal and distal urethra (approximately 2 mm wide, and 3 mm thick) were made and these were frozen in liquid nitrogen immediately after removal, for histochemical examinations. For functional experiments, urethral strips were mounted in Krebs-Henseleit solution of the following composition (mM): NaCl 130, KCl 5, $MgCl_2$ 1.2, $CaCl_2$ 2.5, $NaHCO_3$ 1.2 and glucose 11.4 at 4°C.

Pharmacological experiments

Just before pharmacological experiments, the length and weight of each tissue were measured to calculate the average cross sectional area. Transverse muscle strips (approximately 5–6 mm in width, 10 mm in length, and 2–3 mm in thickness) of proximal and distal urethra were mounted vertically in organ baths containing 5 ml Krebs-Henseleit solution.

The bathing medium was maintained at 37°C, pH 7.4, and was bubbled with a gas mixture consisting of 95% O_2 and 5% CO_2. Isometric tension was measured with FT03 force-displacement transducers (Grass Instruments, Quincy, MA) coupled to a general purpose amplifier (Stemtech Inc., Houston, Texas, U.S.A.) and an IBM PC computer with computer-based oscillograph and data acquisition system (DATAQ Instruments, Akron, OH, U.S.A.). The resting tension was set at 2.0 g, and all preparations were equilibrated for at least 60 min before the experiments were started. After the equilibration period, each experiment was started by exposing the preparation to a K^+ (135 mM) Krebs solution, which was prepared by replacing NaCl of Krebs solution with equivalent KCl, until two reproducible contractions (difference $<10\%$) were obtained.

EFS was provided by a Grass S88 stimulator (Grass Instruments, Quincy, MA, U.S.A.) and applied via two parallel platinum electrodes mounted at either end of the tissue strip. Parameters of EFS were as follows; 0.3 ms duration, frequencies of 0.5, 1, 2, 5, 10, 20, 30 and 50 Hz, 10 s train duration, and 2 min intervals at supramaximal voltage. Once a stable resting tension was achieved, the following investigations were performed.

(1) The control responses to EFS were recorded.

(2) Guanethidine (10^{-5} M) and atropine (2×10^{-6} M) were then added to the bathing medium in order to block both the adrenergic and cholinergic nerves. The responses to EFS were again recorded when the new level of tension had stabilized. To optimize the NANC conditions, a range of concentrations of guanethidine (10^{-6}–10^{-5} M) and atropine (10^{-7}–2×10^{-6} M) were tested, and guanethidine (10^{-5} M) and atropine (2×10^{-6} M) proved most effective in inhibiting contractile responses to EFS. The effect of treatment with tetrodotoxin (TTX; 10^{-5} M for 5 min) for a minimum of 15 min on the electrically-evoked response was also tested on some strips.

(3) After the inhibitory responses with guanethidine (10^{-5} M) and atropine (2×10^{-6} M) were obtained, the effect of L-NAME (10^{-4} M) on the responses to EFS was monitored after a 15 min preincubation. In some strips, N^G-nitro-D-arginine methyl ester (D-N(L-NAME) was used instead of L-NAME.

(4) Then, the effect of L-arginine (10^{-2} M) on preparations treated with L-NAME was tested. After ascertaining maximum stable inhibition by L-NAME (10^{-4} M), L-arginine was added to the bath in the continued presence of L-NAME, guanethidine, and atropine, and the response to EFS was monitored after the new levels of tension had stabilized. In some tissues, D-arginine was used instead of L-arginine.

(5) At the end of pharmacological experiment, 10^{-4} M sodium nitroprusside (a donor of NO) was added to the medium to measure the relaxant responses to exogenous NO. The change of tension was observed for 30 min.

Histochemical experiments

NADPH-diaphorase staining Urethral tissues were frozen and embedded in OCT compound (Miles Inc. Elkhat U.S.A.). Cryostat sections of 8 μm thickness were made, and mounted onto a glass slide and air dried. The slide-mounted tissues were fixed with 4% depolymerized paraformaldehyde in 0.1 M phosphate buffered saline at room temperature for 15 min. Fixed tissues were washed with 0.05 M Tris buffer (pH 7.4), and subsequently incubated with 1 mM βNADPH, 0.2 mM nitroblue tetrazolium, and 0.2% Triton X in 0.05 M Tris buffer (pH 7.4) at 37°C for 30 min. In control experiments, tissues were incubated in the absence of βNADPH.

NOS immunohistochemistry Urethral tissues were frozen and embedded in OCT compound (Miles Inc. Elkhat U.S.A.). Cryostat sections of urethral tissue were made, and fixed in the same way as for NADPH diaphorase staining. These tissues were permeabilized in 1% normal goat serum in 0.05 M Tris buffer at 4°C for 1 h. These tissues were then incubated with primary rabbit polyclonal antibody (Schmidt *et al.*, 1992) which had been raised against rat cerebellar NOS (100:1) in 1% bovine serum albumin and 0.05 M Tris buffer at 4°C overnight. Then, these tissues were stained with Avidin-Biotin Peroxidase using an LSAB kit (DAKO Corp., Carpenteria U.S.A.). 3-Amino-9-ethylcarbazole was used as chromogen, and Mayer's Haematoxylin was used for counter staining. In control experiments, tissues were incubated with non-immune rabbit serum instead of anti-NOS antibody or with anti-serum absorbed with excess NOS protein (Knowles *et al.*, 1989).

Parallel slide-mounted sections were fixed in 4% formaldehyde and stained with haematoxylin and eosin. All preparations were examined with an Olympus microscope.

Methods of analysis

Pharmacological experiments In each experiment, responses were calculated at % of maximum contraction, which was defined as the contractile response to EFS at 30 or 50 Hz, whichever produced the greater contraction, without any drug present. The differences between EFS-elicited contraction without any drug, and EFS-elicited contractions in the presence of the various drugs were examined.

Data are expressed as mean $\pm$ s.e.mean, and statistical analysis was performed by one-way ANOVA or unpaired t test. A P value <0.05 was taken to indicate statistical significance.

Histochemical experiments In NADPH-diaphorase staining, regions with a characteristic neurone form and with dark blue staining were identified as NADPH positive neurones. In NOS immunohistochemistry, regions with a characteristic neurone

form and red staining were identified as NOS positive neurones. For quantitative analysis, staining was assessed by counting the number of NOS and NADPH-positive nerve fibres present in 10 random fields (magnification × 200). Data are expressed as mean ± s.e.mean, and Student's t test for unpaired samples was used for statistical analysis, and the P value <0.05 was taken to indicate statistical significance.

Drugs

Atropine sulphate, guanethidine sulphate, L-arginine, D-arginine, sodium nitroprusside, N^G-nitro-L-arginine methyl ester, N^G-nitro-D-arginine methyl ester, tetrodotoxin, nitroblue tetrazolium, and Triton X-100 were all purchased from Sigma (St. Louis, MO, U.S.A.). βNADPH was purchased from Boehringer Mannheim (Germany). Stock solutions of atropine sulphate, guanethidine sulphate, L-arginine, D-arginine, sodium nitroprusside, L-N^G nitro-arginine methyl ester, D-N^G nitro-arginine methyl ester, and tetrodotoxin in distilled water were stored at −70°C. βNADPH, nitroblue tetrazolium, and Triton-X100 were dissolved just before use.

Results

Pharmacological experiments

The effect of EFS Both proximal and distal urethra showed frequency-dependent contractions. The highest contractile activity was achieved at 50 Hz in most preparations of both proximal and distal urethra, but in some tissues, the highest response was achieved at 30 Hz (Figures 1, 2 and Table 1).

The effect of guanethidine and atropine on EFS-elicited responses In the presence of guanethidine (10^{-5} M) and atropine (2×10^{-6} M), EFS-elicited contraction was completely inhibited, and proximal urethra showed typical relaxation (Figure 2), but distal urethra showed no contraction or relaxation (Figure 1).

The effect of L-NAME Tissues were incubated with L-NAME for 15 min after the response to EFS in the presence of guanethidine (10^{-5} M) and atropine (2×10^{-6} M) had equilibrated. EFS-elicited contractions were significantly increased in the presence of L-NAME (at 30 Hz; −11.15 ± 3.18% to 12.89 ±

Table 1 % responses of maximum contraction of dog proximal urethra induced by electrical field stimulation

		(1) Control		*(2) Gu + Atr*		*(3) L-NAME*		*(4) L-Arg*	
Hz	*Number*	*Mean (%)*	*s.e.*	*Mean (%)*	*s.e.*	*Mean (%)*	*s.e.*	*Mean (%)*	*s.e.*
0.5	8	1.41	6.45	-13.29#	7.05	3.65	1.65	-3.98‡	1.80
1	10	1.95	6.09	-14.65#	7.83	1.87	2.15	-9.81‡	2.76
2	10	6.68	7.50	-27.2 ‡	9.55	5.61	3.91	-10.73‡	3.16
5	12	16.47	8.60	-20.40#	10.32	5.12	4.35	-12.58‡	3.77
10	12	29.57	5.86	-20.87*	11.94	4.91	4.32	-15.68‡	5.27
20	12	63.9	5.44	-20.49#	12.94	6.77	5.10	-4.35	3.68
30	12	89.76	3.40	-11.15‡	9.18	12.89	5.27	-2.44#	4.43
50	12	84.25	5.18	-19.5 ‡	7.19	15.24	5.50	-2.71#	3.91

Abbreviations: Gu: guanethidine, Atr: atropine, L-NAME: N^G-nitro-L-arginine methyl ester, L-Arg: L-arginine.
Control: responses to electrical field stimulation in the absence of drugs. Gu + Atr: responses to electrical field stimulation in the presence of guanethidine (10^{-5} M) and atropine (2×10^{-6} M). L-NAME: responses to electrical field stimulation in the presence of guanethidine (10^{-5} M), atropine (2×10^{-6} M), and N^G-nitro-L-arginine methyl ester (10^{-4} M). L-Arg: responses to electrical field stimulation in the presence of guanethidine (10^{-5} M), atropine (2×10^{-6} M), N^G-nitro-L-arginine methyl ester (10^{-4} M), and L-arginine 10^{-2} M.
#Significantly different from (3) ($P<0.05$); *significantly different from (3) ($P<0.01$); ‡significantly different from (3) ($P<0.005$).

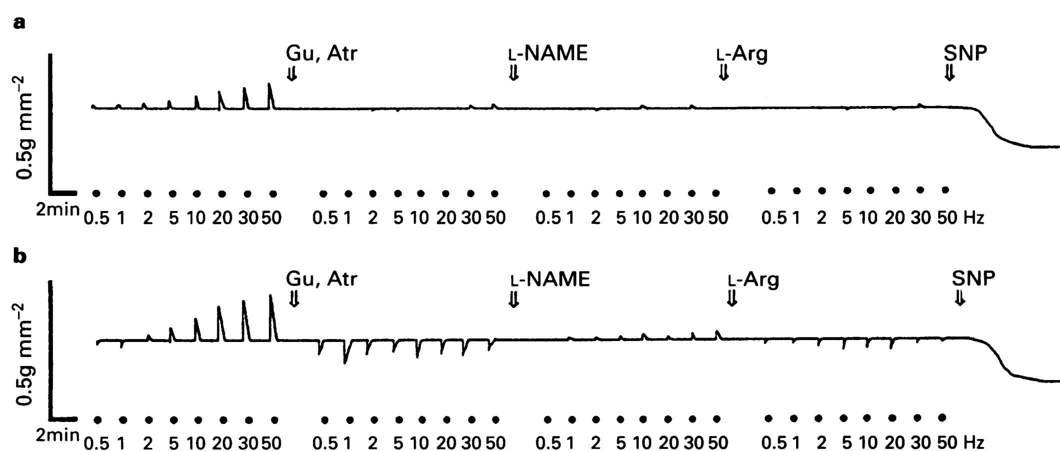

Figure 1 Representative responses of distal urethra (a) and proximal urethra (b) of female dog to electrical field stimulation (EFS) in the absence of drugs, and then in the presence of guanethidine (10^{-5} M), and atropine (2×10^{-6} M), then guanethidine (10^{-5} M), atropine (2×10^{-6} M), and N^G nitro-L-arginine methyl ester (10^{-4} M), and finally guanethidine (10^{-5} M), atropine (2×10^{-6} M), N^G nitro-L-arginine methyl ester (10^{-4} M) and L-arginine (10^{-2} M). After a series of experiments using EFS, sodium nitroprusside (10^{-4} M) was added to the medium. EFS comprises of 0.5, 1, 2, 5, 10, 20, 30 and 50 Hz, 0.3 ms pulse duration, and 10 ms train duration at supramaximum voltage. Dots mark the delivery of EFS. Abbreviations: Gu, guanethidine (10^{-5} M); Atr, atropine (2×10^{-6} M); L-NAME, N^G nitro-L-arginine methyl ester (10^{-4} M); L-Arg, L-arginine (10^{-2} M); SNP, sodium nitroprusside (10^{-4} M).

3.27%, $P<0.05$) in the proximal urethra (Figures 2, 3).

To optimize the effect of L-NAME, lower concentrations (10^{-5} M and 3×10^{-5} M) were also tested, and the highest inhibitory effect on NOS was obtained at 10^{-4}M. However, L-NAME had no effect on EFS-elicited responses in distal urethra.

D-NAME had no effect on EFS-elicited contractions in either proximal or distal urethra.

The effect of L-arginine After the response to EFS in the presence of guanethidine (10^{-5} M) and atropine (2×10^{-6} M), and L-NAME had equilibrated, and without washing the tissue, L-arginine (10^{-2} M) was added to the bathing solution and left in contact with the tissue for 15 min. In the presence of L-NAME, L-arginine (10^{-2} M) restored the EFS-elicited response (at 30 Hz; 12.89 ± 5.27% to −2.44 ± 4.43%, $P<0.05$) in the proximal urethra (Figures 2, 3, and Table 1). However, lower concentrations of L-arginine (10^{-3} M and 3×10^{-3} M) had no effect on EFS-elicited response in distal urethra. D-arginine had no effect on EFS-elicited responses in either the proximal or distal urethra.

The effect of sodium nitroprusside Sodium nitroprusside caused significant relaxation in both proximal and distal urethra (Figure 1). The relaxant responses per cm² cross sectional area were 1.23 ± 0.29 g cm² in the proximal urethra (n = 18), and 2.02 ± 0.54 g cm^{-2} in the distal area (n = 10), and there was no difference between them (Figure 4). In some tissues, 3×10^{-4} M, and 10^{-5} M sodium nitroprusside were tested. Although concentration-dependent relaxant effects were observed in both the proximal and distal portions of the urethra, there was no difference between responses of the proximal and the distal preparations at the same concentration (data not shown).

Histochemical examinations

Both NOS- and NADPH-positive nerve structures were seen in female dog urethra (Figure 5). In the proximal urethra, NADPH positive nerve trunks and nerve fibres were abundant in subadventitial and muscular layers (Figure 6), however, NADPH-positive neurones were hardly seen in distal urethra (Figure 7). These findings were almost identical in each dog.

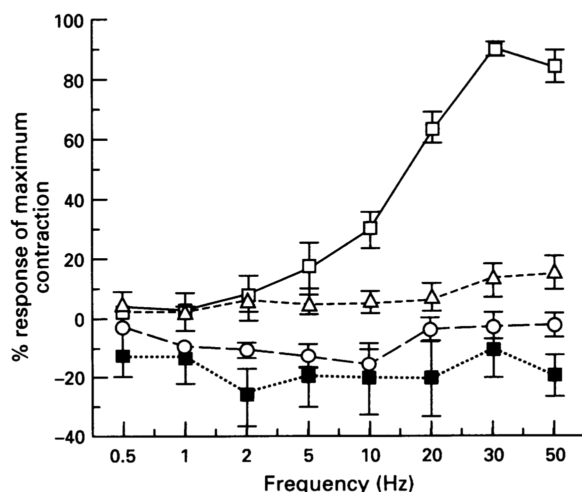

Figure 2 Frequency-response curves to electrical field stimulation, in the absence (□), and presence of guanethidine (10^{-5} M) and atropine (2×10^{-6} M) (■), guanethidine (10^{-5} M), atropine (2×10^{-6} M), and N^G nitro-L-arginine methyl ester (10^{-4} M) (△), and guanethidine (10^{-5} M), atropine (2×10^{-6} M), N^G nitro-L-arginine methyl ester (10^{-4} M), and L-arginine (10^{-2} M); (○), in the proximal urethra of female dog. Results are expressed as percentage of maximum control contraction before treatment, and are given as mean ± s.e.mean (n = 8–12).

Among the 10 samples from the 10 female dogs, an average of 183 ± 45.6, and 45 ± 19.6 positively stained fibres were recorded in 10 random fields, in the proximal and distal part of the urethra, respectively. The former value was significantly higher than that of the latter ($P<0.05$).

No neuronal structure was stained without βNADPH. No neuronal structure was stained with non-immune rabbit serum instead of anti-NOS antibody or with anti-serum absorbed with excess NOS protein.

Discussion

The present results from pharmacological and histochemical experiments demonstrate that the proximal urethra from female dogs has NOS activity, but that the distal urethra does not. In the proximal urethra, in the presence of guanethidine and atropine to block adrenergic and cholinergic nerves, respectively, EFS caused relaxation. Subsequent addition of L-NAME abolished the EFS-induced relaxation, which was then

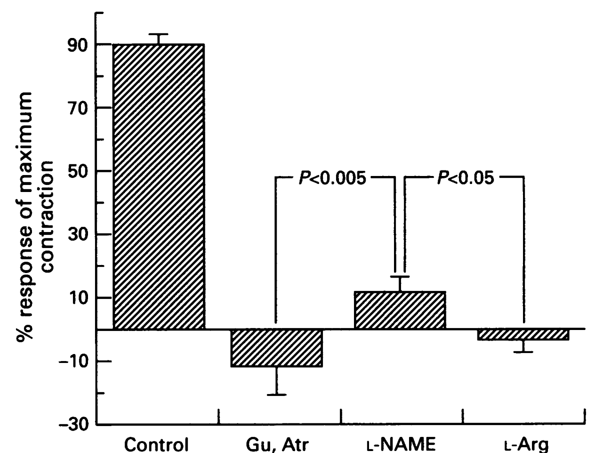

Figure 3 Responses of dog proximal urethra induced by electrical field stimulation at 30 Hz, in the absence (Control) and presence of guanethidine (10^{-5} M) and atropine (2×10^{-6} M) (Gu, Atr); guanethidine (10^{-5} M), atropine (2×10^{-6} M) and N^G nitro-L-arginine methyl ester (10^{-4} M) (L-NAME); and guanethidine (10^{-5} M), atropine (2×10^{-6} M), N^G nitro-L-arginine methyl ester (10^{-4} M), and L-arginine (10^{-2} M); (L-Arg). Results are expressed as percentage of maximum control contraction in the absence of drug, and are given as mean ± s.e.mean (n = 12).

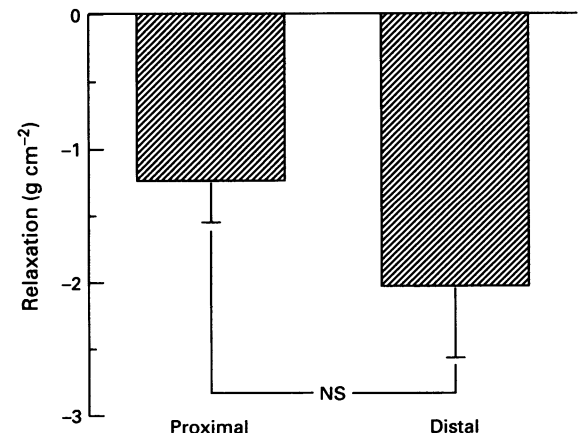

Figure 4 Relaxant responses of proximal and distal urethra of female dogs induced by sodium nitroprusside (10^{-4} M). Results are expressed as g cm^{-2} cross sectional area, and given as mean ± s.e.mean (n = 18; proximal, n = 10; distal).

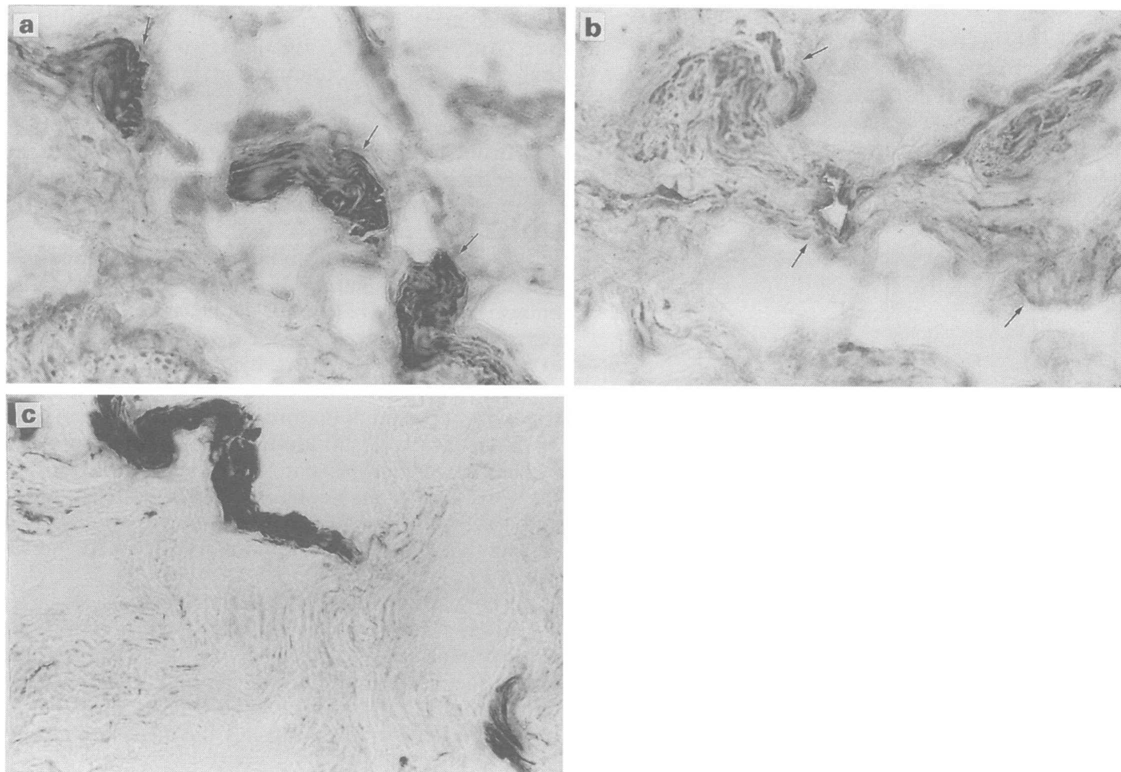

Figure 5 Nitric oxide synthase (NOS) immunohistochemistry and reduced nicotinamide adenine dinucleotide phosphate (NADPH) diaphorase staining in the proximal urethra of female dog. (a) NOS immunohistochemistry using specific anti-NOS antiserum (Schmidt *et al.*, 1992); a large nerve trunk (arrow) is positively stained. (b) NOS immunohistochemistry with preimmune serum (negative control); a nerve trunk (arrow) is negatively stained. (c) NADPH diaphorase staining; a NADPH diaphorase positive nerve trunk is present. Reduced from $\times 400$.

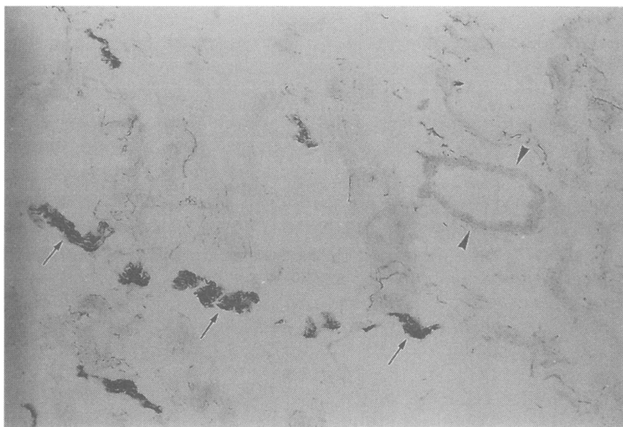

Figure 6 Reduced nicotinamide adenine dinucleotide phosphate (NADPH) diaphorase staining in the proximal urethra of female dog. Many NADPH diaphorase positive nerve trunks (arrow), and thin nerve fibres are seen. Vessel (arrowheads) is also positively stained. Reduced from $\times 200$.

restored by addition of L-arginine. Such a profile of activity is consistent with the presence of NOS activity. On the other hand, in the presence of guanethidine and atropine, the distal urethra did not show such a relaxant response to EFS. Furthermore, subsequent addition of L-NAME, and then L-NAME and L-arginine were without effect. These results suggest that the distal urethra does not possess NOS activity.

In all parts of the nervous system studied so far, NOS distribution is identical to that of NADPH diaphorase, and NOS immunohistochemistry and NADPH diaphorase staining are regarded as identifying the same substance within neurones (Dawson *et al.*, 1991). In previous studies relating to urethra and NOS, the involvement of NO as an inhibitory NANC-transmitter has been based mainly on the findings that neuronally-mediated relaxant responses in the urethra, bladder neck, and trigone were prevented by NOS-inhibitors, and exogenous NO mimicked the responses to NANC-nerve stimulation (Pascual *et al.*, 1991; Andersson *et al.*, 1992). However, morphological evidence that NO may act as a neurotransmitter in the urethra is necessary to strengthen the hypothesis of NOS distribution in the urethra. Recently, a study involving both histochemistry (NOS immunohistochemistry and NADPH diaphorase staining), and function in pig lower urinary tract has been reported (Persson *et al.*, 1993). In this study, NOS and NADPH diaphorase positive nerve trunks and fine nerve fibres were found mainly in the proximal urethra and trigone, whereas such fibres were less common in the detrusor. However, the distribution of NOS nerves in the distal urethra was not examined. In the present study, it is confirmed that both NOS and NADPH positive neurones are predominantly present in the proximal urethra of the female dog, but are scarcely found in distal urethra. These findings are consistent with the functional study.

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 *et al.*, 1977). Several histological studies (Gu *et al.*, 1984; Crowne & Burnstock, 1989) have demonstrated a rich occurrence of NANC neurones in the bladder outlet region, in support of activation of NANC-inhibitory nerves as a possible mechanism for urethral relaxation. Thus, NOS positive nerves might be identical to the NANC-inhibitory nerve in the urethra, and NO might have some important role in relaxation of urethra during micturition. However, the reasons for the different distribution of NOS nerves in proximal and distal urethra are unknown. Nerve-mediated relaxation of the distal urethra may be produced by cholinergic nerve stimulation or other relaxa-

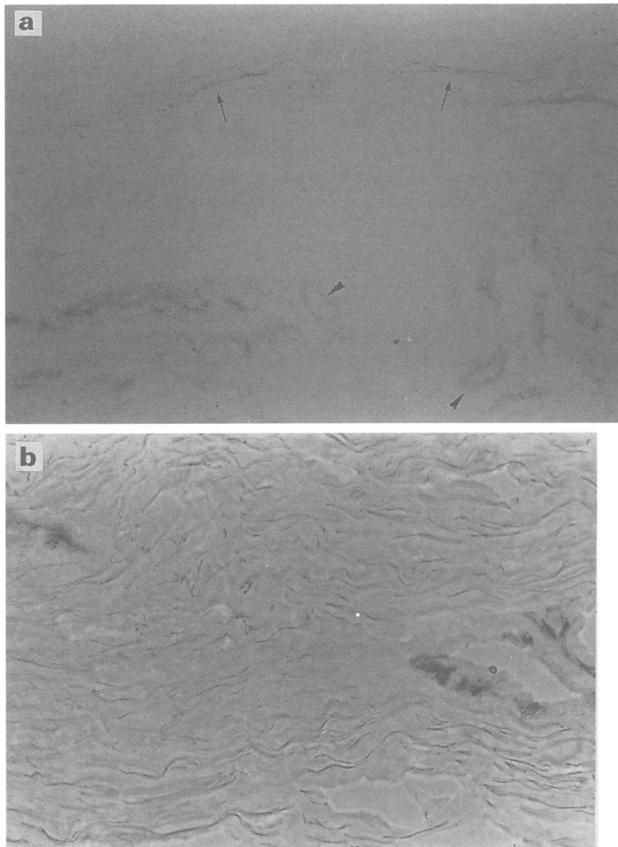

Figure 7 Reduced nicotinamide adenine dinucleotide phosphate (NADPH) diaphorase staining in the distal urethra of female dog (a),(b). Thin nerve fibres (arrows) are seen in the submucosal layer but NADPH positive nerve trunk is not seen in the muscular layer. Walls of vessels (arrowheads) are faintly stained.

tion-mediating factors, such as vasoactive intestinal polypeptide (Andersson *et al.*, 1983). However, our results showing that EFS with guanethidine and atropine did not elicit relaxation or contraction indicate the lack of any NANC relaxant innervation. The continence mechanism of proximal and distal urethra of female dog are twofold, comprising a purely passive component (transmission of bladder pressure to the urethra) and a purely active stress component (Heidler *et al.*, 1987; Thüroff *et al.*, 1987). According to our results and those of these previous studies on the continence mechanism, the following speculation related to urethral relaxation during micturition can be proposed; the proximal urethra and trigone contract by a passive mechanism, and both distal urethra and external sphincter contract by an active mechanism during storage of urine. On the other hand, during micturition, the trigone and proximal urethra relax by an active mechanism (by NOS nerve), and distal urethral relaxation occurs by a passive mechanism (by suspension of active contraction). Although the distal urethra may not possess NOS activity, both proximal and distal urethra showed relaxation by exogenous NO, suggesting that NO may be a modulator of NANC nerve in distal urethra of female dog.

In conclusion, NOS immunohistochemistry and NADPH diaphorase staining showed that NOS nerves are localized predominantly in the proximal region of the urethra, but are rare in the distal urethra of female dog. This is consistent with the functional data showing NANC nerve-mediated relaxation of the proximal urethra, but not the distal urethra.

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