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Characterization of nitric oxide synthase activity in sheep urinary tract: functional implications

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1 To define further the role of nitric oxide (NO) in urinary tract function, we have measured the presence of nitric oxide synthase (NOS) activity, and its relationship with functional NO-mediated responses to electrical field stimulation (EFS) in the urethra, the detrusor and the ureter from sheep. NOS activity was assayed by the conversion of L-[^{14}C]-arginine to L-[^{14}C]-citrulline. Endogenous production of citrulline was confirmed by thin layer chromatography.

2 NOS enzymatic activity was detected in the cytosolic fraction from tissue homogenates with the following regional distribution (pmol citrulline mg^{-1} protein min^{-1}): urethra (33 ± 3.3), detrusor (13.1 ± 1.1) and ureter (1.5 ± 0.2). No activity was detected in the particulate fraction of any region.

3 NOS activity was dependent on Ca^{2+}-calmodulin and required exogenously added NADPH and tetrahydrobyopterin (BH_4) for maximal activity. Exclusion of calmodulin from the incubation mixture did not modify NOS activity, but it was significantly reduced in the presence of the calmodulin antagonist, calmidazolium, suggesting the presence of enough endogenous calmodulin to sustain the observed NOS activity.

4 NOS activity was inhibited to a greater extent by N^G-nitro-L-arginine (L-NOARG) and its methyl ester (L-NAME) than by N^G-monomethyl-L-arginine (L-NMMA), while 7-nitroindazole (7-NI) was a weak inhibitor and L-cannavine had no effect.

5 Citrulline formation could be inhibited by superoxide dismutase in an oxyhaemoglobin-sensitive manner, suggesting feedback inhibition of NOS by NO.

6 EFS induced prominent NO-mediated relaxations in the urethra while minor or no responses were observed in the detrusor and the ureter, respectively. Urethral relaxations to EFS were inhibited by NOS inhibitors with the rank order of potency: L-NOARG = L-NAME > 7-NI > L-NMMA.

7 In conclusion, we have demonstrated the presence of NO-synthesizing enzymatic activity in the sheep urinary tract which shows similar characteristics to the constitutive NOS isoform found in brain. We suggest that the enzymatic activity measured in the urethral muscle layer may account for the NO-mediated urethral relaxation during micturition whereas regulation of detrusor and ureteral motor function by NOS containing nerves is less likely.

Keywords: Nitric oxide; nitric oxide synthase; urethra; detrusor; ureter; nitrergic relaxation; urinary tract

Introduction

It has been suggested that nitric oxide (NO) has important regulatory functions in the urinary tract acting as a non-adrenergic, non-cholinergic (NANC)-relaxant transmitter. Specially relevant is the proposed role of NO in mediating the decrease in outlet resistance which accompanies micturition (Andersson, 1993). A rich supply of nitrergic nerves has been identified by NADPH-diaphorase histochemistry and nitric oxide synthase (NOS) immunohistochemistry in the outflow region (urethra, bladder neck and bladder base or trigone) of several species including rat (McNeill *et al.*, 1992), pig (Persson *et al.*, 1993), sheep (Triguero *et al.*, 1993) and human (Smet *et al.*, 1994a; Leone *et al.*, 1994). Furthermore, functional studies have demonstrated that isolated urethral and trigonal smooth muscles in all the species studied so far, respond to electrical field stimulation (EFS) with pronounced NANC relaxations, which can be blocked by NO synthesis inhibitors and mimicked by NO and NO donors (García-Pascual *et al.*, 1991; Andersson *et al.*, 1991; 1992; Persson & Andersson, 1992; Persson *et al.*, 1992; 1993; García-Pascual & Triguero, 1994). This suggests that NO, or a related compound, is the relaxant mediator, which acts by increasing guanosine 3':5'-cyclic monophosphate (cyclic GMP) levels in smooth muscle (García-Pascual & Triguero, 1994; Dokita *et al.*, 1994). Moreover, an increase in both nitrite and nitrate, the metabolites of NO, has been measured in human urethral preparations upon EFS, which was inhibited by N^G-nitro-L-arginine methyl ester (L-NAME) (Leone *et al.*, 1994). In addition, *in vivo* administration of N^G-nitro-L-arginine (L-NOARG) to foetal lambs resulted in incomplete bladder emptying, suggesting inadequate sphincter relaxation (Mevorach *et al.*, 1994).

In contrast to the outlet region, the functional relevance of NO in detrusor and ureteral physiology is questionable. It has been speculated that NO could have an inhibitory function, keeping the detrusor muscle relaxed during bladder filling (James *et al.*, 1991), and that it could modulate ureteral peristalsis (Hernández *et al.*, 1995). The presence of nerves containing the NO-synthesizing enzyme has been demonstrated, although in less density than in urethra, in the detrusor muscle of rat (McNeill *et al.*, 1992), pig (Persson *et al.*, 1993), sheep (Triguero *et al.*, 1993) and human (Smet *et al.*, 1994a) as well as in the muscular layer of the intravesical portion of the pig (Hernández *et al.*, 1995; Iselin *et al.*, 1995) and human (Goessl *et al.*, 1995) ureter, and in the human middle ureter (Smet *et al.*, 1994b), while they were sparse in the pelvic pig ureter (Iselin *et al.*, 1995) and absent in the distal ureter from sheep (Triguero *et al.*, 1993). However, attempts to find functional

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relaxations of neural origin sensitive to NOS inhibition have failed or only minor responses were obtained in both detrusor (Persson & Andersson, 1992; Persson *et al.*, 1992; 1993; Triguero *et al.*, 1993) and ureteral preparations (Triguero *et al.*, 1993; Obara *et al.*, 1995; Iselin *et al.*, 1995).

NO is synthesized by NOS, a cytochrome P450-like haem protein that catalyses the NADPH-dependent oxidation of L-arginine to form L-citrulline and NO. To date, three different NOS isoforms have been cloned and characterized which differ in their subcellular distribution, Ca^{2+}-calmodulin dependency and regulation mechanisms (Knowles & Moncada, 1994). Currently, an increasing number of NOS inhibitors, some of them exhibiting some preference for distinct NOS isozymes, are available which are useful tools for characterization of the NOS isoforms.

In the present study, we have characterized the enzymatic activity of NOS present in the muscular layer of the sheep urinary tract at three levels: urethra, detrusor and distal ureter. The dependence of enzyme activity on cofactors and differential inhibition by several NOS inhibitors were determined and relationships with functional reponses are discussed.

Methods

Tissue preparations

Lower urinary tracts from female lambs (2–3 months old, 18–22 kg bodyweight) were collected at the local slaughterhouse shortly after they had been killed. They were transported to the laboratory in cold Krebs solution of composition (mM): NaCl 119, KCl 4.6, $CaCl_2$ 1.5, $MgCl_2$ 1.2, $NaHCO_3$ 15, KH_2PO_4 1.2, ethylenediaminotetraacetic acid disodium salt (EDTA) 0.07 and glucose 11. The urethra and the bladder were opened longitudinally and the mucosa, most of the submucosa, fat and connective tissue were removed by sharp dissection. A segment, 5 cm in length, from the distal part of the ureter was cut and opened lengthwise. Transverse (urethra) or longitudinal (detrusor and distal ureter) strips (approximately $1 \times 1 \times 5$ mm) were cut and mounted in organ baths for recording of mechanical activity (see below). Detrusor preparations were obtained from the central part of the anterior wall of the bladder. Urethral and vesical smooth muscle pieces (1×1 cm), as well as ureteral segments, were pooled from 6–10 different animals and prepared for homogenization and NOS activity measurements (see below).

Recording of mechanical activity and electrical field stimulation

Strips were transferred to 5 ml thermostatically controlled (37°C) organ baths containing Krebs solution bubbled with a mixture of 95% O_2 and 5% CO_2, maintained at pH 7.4. The preparations were mounted between two L-shaped hooks by means of silk ligatures. One was connected to a Grass FT03C force-displacement transducer for the measurement of isometric tension. The other was fixed to a moveable unit to allow adjustment of tension. Isometric tension was recorded on a Grass poligraph model 7D (Grass Instruments, Quincy, MA, U.S.A.). During the equilibration period (approximately 60 min) the resting tension was adjusted to 8–10 mN.

Electrical field stimulation (EFS) was performed with a Cibertec CS 20 stimulator (Letica, Barcelona, Spain) connected to a pair of platinum electrodes placed parallel to the preparation. Square-wave pulses of 0.8 ms duration at a frequency of 0.5–20 Hz were delivered at 2 min intervals. The voltage was supramaximal (current strength 75 mA) and the train duration was 5 s.

Responses to EFS were studied in precontracted preparations and in the continuous presence of guanethidine (10 μM) and atropine (1 μM). Detrusor preparations were also pretreated with α-β methylene ATP (10 μM). Contractions of urethral preparations were evoked by noradrenaline (NA, 50 μM). Endothelin-1 (ET-1, 20 nM) was used to induce contractions in the detrusor and the ureter. These concentrations produced stable increases in tone (10.0 ± 1.1 mN, $n=20$, in the urethra; 10.3 ± 1.0 mN, $n=9$, in the detrusor; and 11.8 ± 1.9 mN, $n=9$, in the ureter) equivalent to 60–90% of the tension induced by a K^+ (120 mM) Krebs solution (prepared by replacing NaCl with equimolar amounts of KCl). When the effects of NOS inhibitors were studied, control responses to EFS (at 2 and 8 Hz) were recorded before incubating the urethral strip with the inhibitor for 30 min. Then the preparation was once again subjected to EFS. The tone of the preparation varied less than 15% during the experimental period. At the end of the experiment, the base line level was determined by changing the bath medium to a Ca^{2+}-free Krebs solution (prepared by omitting Ca^{2+} from the normal Krebs solution and adding 0.1 mM ethyleneglycol, *bis* (β-aminoethylether)-*N*-*N'*-tetraacetic acid (EGTA)). To test whether an antidromic invasion of primary sensory afferents was behind EFS-induced response in the ureter, EFS (10 Hz) was delivered before and after capsaicin desensitization to non-precontracted ureteral preparations. Desensitization was performed by treatment with capsaicin (10 mM) for 15 min followed by repeated washing for 1 h.

Measurement of nitric oxide synthase activity

NOS activity was determined by measuring the formation of L-[^{14}C]-citrulline from L-[^{14}C]-arginine according to the procedure described by Bredt & Snyder (1990) with slight modifications.

Tissues were homogenized in 4 volumes (w/v) of ice-cold homogenization buffer using a ground glass pestle homogenizer. Homogenization buffer contained: 20 mM HEPES, 320 mM sucrose, 0.5 mM EDTA, 1 mM dithithreitol (DTT), 1 mM phenylmethylsulphonyl fluoride (PMSF), 1 μM pepstatin A and 2 μM leupeptin (pH 7.2). Homogenates were centrifuged at 20,000 *g* for 40 min at 4°C and the resultant supernatant centrifuged again at 100,000 *g* for 60 min at 4°C to obtain the cytosolic fraction. The resultant pellet from the first centrifugation step was resuspended in ice-cold homogenization buffer containing 1 M KCl for 5 min, to remove loosely bound cytosolic proteins and after centrifugation (20,000 *g* for 40 min, 4°C) the supernatant was discarded and the particulate fraction resuspended in 6 volumes of homogenization buffer. NOS activity was determined in the cytosolic and KCl-washed particulate fractions. In some experiments, NOS activity was also determined in crude homogenates.

Cytosolic fractions were passed over a 1 ml column of AG50W-X8 (Na^+ form, prepared from the H^+ form) to remove endogenous arginine and the effluent was used for the enzyme and protein assays. Samples were stored in liquid nitrogen until use. L-[^{14}C]-arginine was purified before assay by anionic exchange cromatography on 1 ml columns of Dowex AG1-X8 (OH^- form, prepared from the acetate form).

Incubation mixtures contained 150 μl of cytosolic or particulate fractions and 50 μl of homogenization buffer containing (final concentration): 2 mM NADPH, 1 mM $CaCl_2$, 30 u ml^{-1} calmodulin, 3 μM tetrahydro-L-biopetrin (BH_4), 40 μM L-arginine and 0.5–0.7 μM L-[^{14}C]-arginine (0.2 μCi ml^{-1}). Samples were incubated for 60 min at 37°C in a shaking water bath. Preliminary experiments showed that the reaction was linear during this time. Incubation was terminated by the addition of 1.5 ml of ice-cold stop buffer (5 mM HEPES, 2 mM EDTA, pH 5.5). Samples were passed through a 1 ml column of AG50W-X8 (Na^+ form) and eluted with 2 ml of stop buffer. Radioactivity of the effluent was measured by liquid scintillation spectrometry. A parallel set of incubations was performed at 4°C and was considered as non-specific activity. In some experiments, incubations were performed in the absence of NADPH, BH_4, $CaCl_2$ (with 2 mM EGTA) or calmodulin; or in the presence of calmidazolium (without calmodulin), NOS inhibitors, superoxide dismutase (SOD) or oxyhaemoglobin.

Protein concentrations in tissue fractions were determined (Lowry *et al.*, 1951) with bovine serum albumin used as stan-

dard. The specific activity was expressed as pmol citrulline mg^{-1} protein min^{-1}. The detection limit of the assay in our experimental conditions was 0.20 pmol citrulline mg^{-1} protein min^{-1}.

Thin layer chromatography

Thin layer chromatography was performed: (1) on the eluate from the Dowex AG1-X8 to control the purity of the L-[^{14}C]-arginine used in the assay; and (2) on the reaction mixture after column separation to verify the formation of citrulline. For this purpose, the reaction mixture contained 2 μCi ml^{-1} of L-[^{14}C]-arginine and 34 μM L-arginine. The reaction was stopped with 100 μl stop buffer and eluted with 500 μl buffer on 0.5 ml of Dowex AG50W-X8.

Ten μl aliquots of the effluent and 0.5 μl of L-arginine, L-citrulline and L-ornithine (2 mg ml^{-1}) were each spotted onto silica gel plates and the plates were developed with a chloroform/methanol/amonium hydroxide (30%v)/water (0.5:4.5:3:1) solvent system. After location of amino acids of interest with ninhydrin (0.2% in ethanol) the silica gel was divided in 0.5 cm fractions, scraped and placed into scintillation vials for counting.

Materials and drugs

L-[U-^{14}C]-arginine (specific activity 330 mCi $mmol^{-1}$) was obtained from New England Nuclear (Boston, MA, U.S.A.). Dowex AG1-X8 (acetate form, 100–200 mesh) and AG50W-X8 (H^+ form, 100–200 mesh) were purchased from Bio Rad Laboratories (Richmond, CA, U.S.A.). Ready Safe scintillation liquid was from Beckman Instruments (Fullerton, CA, U.S.A.). Z 122688 silica gel 20 × 5 cm thin layer chromatography plates were obtained from Aldrich Chemicals. (6**R**)-5,6,7,8-tetrahydro-L-bipterin (BH_4) and 7-nitroindazole were from RBI (Natick, MA, U.S.A.). All other chemicals were purchased from Sigma Chemicals (St Louis, MO, U.S.A.). Drugs were dissolved in distilled water except 7-nitroindazole and endothelin-1 which were dissolved in methanol and in bovine serum albumin (0.05%), respectively. Oxygen-free water was used to dissolve BH_4. Stock solutions were prepared and then stored at −20°C. Subsequent dilutions were made with 0.9% NaCl containing 1 mM ascorbic acid or with homogenization buffer (for NOS assay). Capsaicin (8-methyl-N-vanillyl-6-nonenamide, Sigma Chemicals) was dissolved in a mixture (2:1:7) of absolute ethanol: Tween 80 (Sigma): 0.9% NaCl and then diluted in saline prior to use. Solvent controls were used when needed. Oxyhaemoglobin was prepared from a 1 mM solution of commercial haemoglobin (bovine) by addition of a 10 fold molar excess of sodium dithionite followed by gel filtration with a Sephadex G-25 column as described by Murphy & Noack (1994). The concentration of oxyhaemoglobin was determined by the cyanomethemoglobin method (Drabkin & Austin, 1935). The stock solution was divided in aliquots and stored in liquid nitrogen.

Analysis of data

Relaxation responses were normalized by expressing them as a fraction of the reduction in tone following exposure to Ca^{2+}-free Krebs solution. Normalized results are expressed as a percentage of the maximum relaxation obtained during the first response of each preparation. IC_{50} values (drug concentrations producing 50% inhibition of relaxation or NOS activity) were determined for each individual experiment by linear interpolation. The K_m and V_{max} values were estimated by non-linear analysis of the model. $V = (V_{max} \times [S])/(K_m + [S])$ in which V is the initial velocity (pmol citrulline mg^{-1} protein min^{-1}) and [S] is the L-arginine concentration (μM). K_i values were obtained by linear regression analysis according to the method of Dixon for competitive inhibitors, assuming a K_m value of 10 μM.

$$\frac{1}{v} = \frac{K_m}{V_{max} \cdot K_1 \cdot S} \cdot I + \frac{1}{V_{max}} \cdot \left(1 + \frac{K_m}{S}\right)$$

IC_{50} values and kinetic constants are expressed as geometric means with 95% confidence intervals. All other data are given as means ± s.e.mean. n denotes number of experiments (all with different animals). Statistical analysis was performed by one-way analysis of variance and Student's t test (two-tailed) for unpaired data with a probability value <0.05 being regarded as significant.

Results

Nitric oxide-mediated responses to electrical field stimulation in different regions of the sheep urinary tract

Responses to EFS (0.5–20 Hz) were elicited in precontracted preparations taken from the proximal urethra, the central portion of the detrusor and the distal third of the ureter. Experiments were carried out in the presence of guanethidine (10 μM), atropine (1 μM) and α,β-methylene ATP (10 μM, in the detrusor) in order to reduce contractions induced by potential excitatory mediators. Urethral preparations were contracted with noradrenaline (NA, 50 μM), whereas strips of detrusor and ureter were stimulated with endothelin-1 (ET-1, 20 nM), obtaining stable levels of tension (about 10 mN) in all the regions. In the urethra, EFS caused pronounced and frequency-dependent relaxations that were completely blocked by L-NOARG, (0.1 mM); however, only 4 out of 9 detrusor preparations showed small (10 times lower) but L-NOARG (0.1 mM)-sensitive relaxations (Figure 1, Table 1). Strips of ureter developed spontaneous phasic contractile activity (mean frequency 7.2 ± 0.7 contractions min^{-1}, $n = 15$), which persisted on top of the ET-1-induced contraction. Responses to EFS in ureter segments consisted of a transient suppression of the spontaneous rhythmic activity, which was not affected by L-NOARG treatment (Figure 1, Table 1). The inhibitory response to EFS was, however, suppressed in 10 out of 30 preparations of ureter examined after acute desensitization to capsaicin (10 μM) *in vitro*. In the remaining preparations the inhibition was slight or was not observed. Capsaicin also induced inhibition of the spontaneous motility that was reserved by washing (Figure 2).

Distribution of nitric oxide synthase activity in different regions of the sheep urinary tract

Initial studies demonstrated that NOS activity, as measured by the L-[^{14}C]-citrulline formation assay, was exclusively located in the cytosolic fraction of urinary tract tissues, while negligible activity remained in the KCl-washed particulate fraction of all regions. To exclude the possibility that necessary cofactors might have been lost during the centrifugation steps, total NOS activity in the cytosolic fraction (without removing endogenous arginine) was compared with that found in crude homogenates, but no change in activity could be detected (not shown). All other results refer to the cytosolic fraction unless noted.

Thin layer chromatography was used to confirm that radioactivity measured corresponded to citrulline. The R_F values of arginine, ornithine and citrulline standards were 0.51 ± 0.01; 0.61 ± 0.006 and 0.82 ± 0.006 ($n = 5$), respectively. Thin layer chromatography of the Dowex effluent showed that 84–95% of the radioactivity comigrated with citrulline standard.

NOS activity was measured in samples taken from the urethra, detrusor and the distal third of the ureter. Significant differences in NOS activity were found amongst the three regions. As shown in Table 1, the highest NOS specific activity was present in the urethra and the lowest in the ureter. These

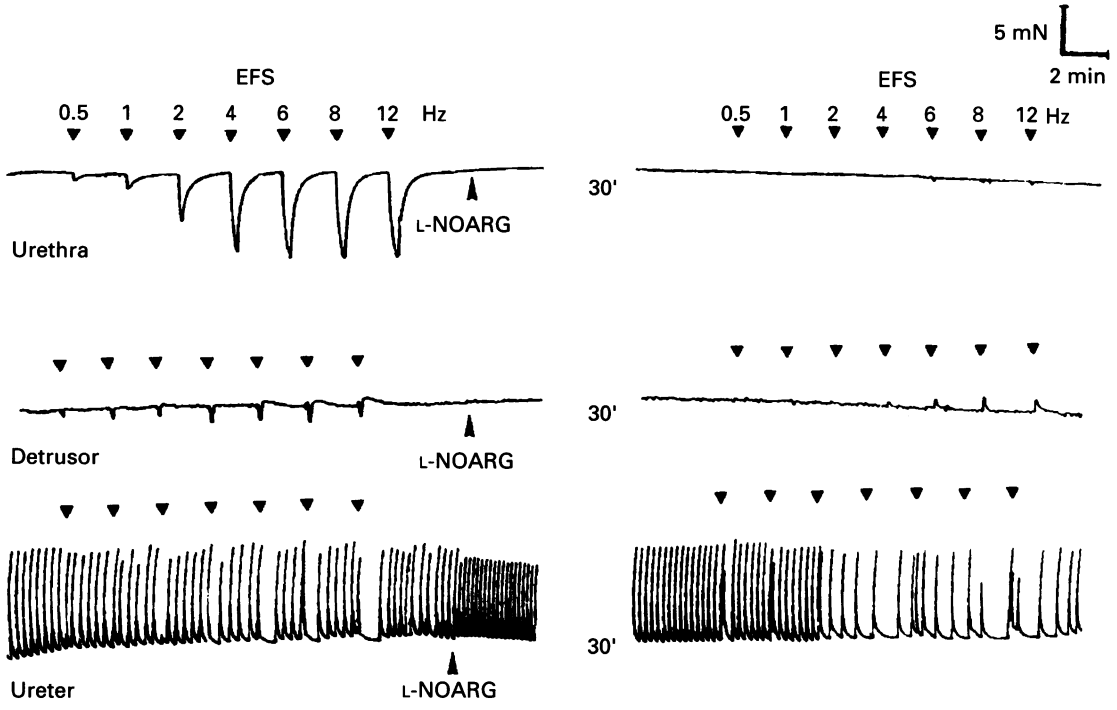

Figure 1 Effects of electrical field stimulation (EFS, 0.5–12 Hz, 0.8 ms for 5 s, supramaximum voltage) on preparations from different regions of the sheep urinary tract before and after pretreatment with N^G-nitro-L-arginine (L-NOARG, 0.1 mM). Preparations were pretreated for 30 min with guanethidine (10 μM) and atropine (1 μM) (10 μM α,β-methylene ATP was also present in the detrusor) and then contracted with noradrenaline (NA, 50 μM) (urethra) or endothelin-1 (ET-1, 20 nM) (detrusor and ureter).

Table 1 NOS specific activity and amplitude of relaxation induced by electrical field stimulation (EFS) at 8 Hz in different regions of the sheep urinary tract

Region	*NOS Specific activity* (pmol citrulline mg^{-1} protein min^{-1})	n	*Relaxation* (%)	n
Uretha	33.0 ± 3.3	21	49.0 ± 3.6	20
Detrusor	13.1 ± 1.1	17	5.6 ± 2.9	9
Ureter	1.5 ± 0.2	10	0	9

NOS activity was measured in the cytosolic fraction by the conversion of L-[^{14}C]-arginine to L-[^{14}C]-citrulline in the presence of 2 mM NADPH, 1 mM $CaCl_2$, 30 u ml^{-1} calmodulin, 3 μM BH_4 and 40 μM L-arginine. Relaxations are expressed as % decrease in tone from a contraction to noradrenaline (50 μM) in the urethra, or to endothelin-1 (20 nM) in the detrusor and the ureter. Preparations were pretreated with guanethidine (10 μM) and atropine (1 μM) before contraction and EFS (8 Hz). α,β-methylene ATP (10 μM) was also present in the detrusor. Means ± s.e. mean. *n* denotes number of experiments.

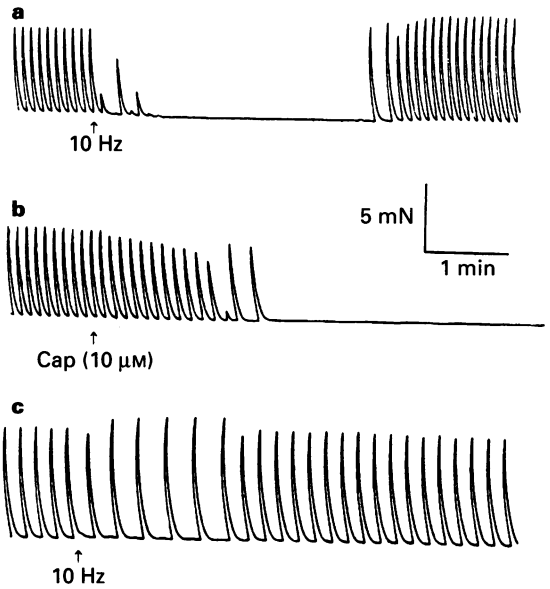

Figure 2 Typical tracing illustrating the inhibitory effect of capsaicin (Cap, 10 μM) on the transient suppression of peristalsis of the ureter induced by electrical field stimulation (EFS, 10 Hz, 0.8 ms for 5 s, supramaximum voltage). Responses to EFS are shown before (a) and after (c) capsaicin treatment for 15 min followed by 1 h period of washing. Capsaicin application also had an inhibitory effect on ureteral motility (b).

differences in NOS activity correlate with the NO-dependent functional responses to EFS in the three regions: higher in the urethra, lower in the bladder and absent in the ureter (Table 1). It is noteworthy that the NOS activity measured in the bladder and ureter is proportionally higher than their ability to develop a NO-mediated relaxation response. Thus, the NOS specific activity was 2.5 fold lower in the detrusor than in the urethra, while the relaxant responses to EFS, when present, were only 11% of the urethral ones (Table 1).

Characteristics of nitric oxide synthase in sheep urinary tract tissues

In experiments conducted at different L-arginine concentrations (ranging from 0 to 100 μM), formation of citrulline followed Michaelis-Menten kinetics (Figure 3). The apparent K_m and the apparent V_{max} of NOS were estimated to be 9.9 ± 1.2 μM and 56.7 ± 14.7 pmol citrulline mg^{-1} protein min^{-1} ($n = 3$), respectively, in the urethra; and 9.1 ± 1.0 μM and 24.5 ± 10.2 pmol citrulline mg^{-1} protein min^{-1} ($n = 3$), respectively, in the detrusor.

The cofactor requirements of the urinary tract NOS are shown in Figure 4 and Table 2. The enzyme, in all three re-

gions, was Ca^{2+}-dependent and partially dependent on NADPH and BH_4 (Figure 4). No significant Ca^{2+}-independent activity was detected, as reflected by reduction of activity by more than 90% in the absence of Ca^{2+} and in the presence of 2 mM EGTA, except in the ureter. In this tissue, 30% activity persisted, which was, however, very close to the minimally detectable NOS activity and not significantly different from activity values measured in the presence of L-NOARG (0.1 mM) ($P>0.05$). In the absence of NADPH, NOS activity was decreased by 48%, 53% and 70% in the urethra, detrusor and ureter, respectively, while in the absence of BH_4, it was inhibited by 42%, 27% and 46% in the urethra, detrusor and ureter, respectively. In addition, the enzymatic activity was virtually abolished in the three regions by L-NOARG (0.1 mM)-treatment, while L-canavanine (0.1 mM had no effect, demonstrating the specificity of NOS activity determinations (Figure 4). Suppression of exogenous calmodulin in the reaction mixture had no effect on NOS activity, but calmodulin-dependency was demonstrated by the pronounced inhibitory effect of the calmodulin antagonist, calmidazolium at 100 μM (Table 2), suggesting endogenous staturating calmodulin concentration are present in crude enzyme preparations.

To investigate whether enzymatically formed NO (together with citrulline) in the assay medium might be able to inhibit NOS through a feedback mechanism, NOS activity in urethral cytosolic fraction was assayed in the absence and presence of SOD (a scavenger of superoxide and protector of NO inactivation, 5000 u ml^{-1}), oxyhaemoglobin (a scavenger of NO, 10 μM) or their combination. As shown in Figure 5, the enzyme was inhibited to 55% of controls in the presence of SOD, and this effect was prevented by oxyhaemoglobin. Nevertheless, oxyhaemoglobin alone was not by itself able to increase NOS activity.

Effect of nitric oxide synthase inhibitors on enzymatic activity and relaxation responses to electrical field stimulation

Figure 6 compares NOS inhibition in cytosolic fractions from both urethra and detrusor. Citrulline formation was measured in standard incubation mixtures containing 40 μM L-arginine, in the absence or in the presence of increasing concentrations of L-NOARG, L-NAME, N^G-monomethyl-L-arginine (L-NMMA) or 7-nitroindazole. All inhibitors displayed concentration-dependent inhibition in both tissues. The calculated K_i values for the inhibitors are listed in Table 3. As can be seen, no differences in the inhibiton constants were observed between the urethra and the detrusor. The rank order of potency was found to be L-NOARG = L-NAME > L-NMMA > 7-nitroindazole.

The dose-dependency inhibitory effect of NOS inhibitors were also studied on the relaxation responses induced by EFS at 2 and 8 Hz in precontracted urethral preparations (Figure 7). The rank order of potency for inhibition of relaxation was L-NOARG = L-NAME > 7-nitroindazole > L-NMMA at both frequencies. The magnitude of the inhibition was not different at low or high frequencies except for L-NMMA. Thus relaxation responses at 2 and 8 Hz were inhibited by L-NMMA

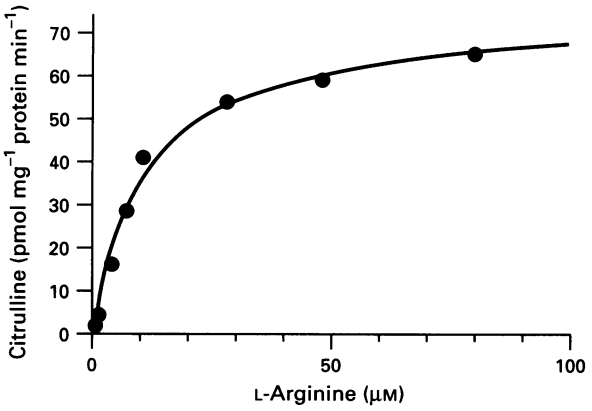

Figure 3 Representative experiment showing the plot of velocity for NOS activity (pmol citrulline mg^{-1} protein min^{-1}) vs concentration of L-arginine in the sheep urethra. NOS activity was measured in the presence of 1 mM $CaCl_2$, 2 mM NADPH, 30 u ml^{-1} calmodulin, 3 μM BH_4 at different L-arginine concentrations (0–100 μM). $K_m = 11.43 \pm 1.59$ μM; $V_{max} = 74.8 \pm 3.3$ pmol mg^{-1} protein min^{-1}: The K_m and V_{max} values were estimated by non-linear regression, fitting the data to a rectangular hyperbola ($r = 0.996$).

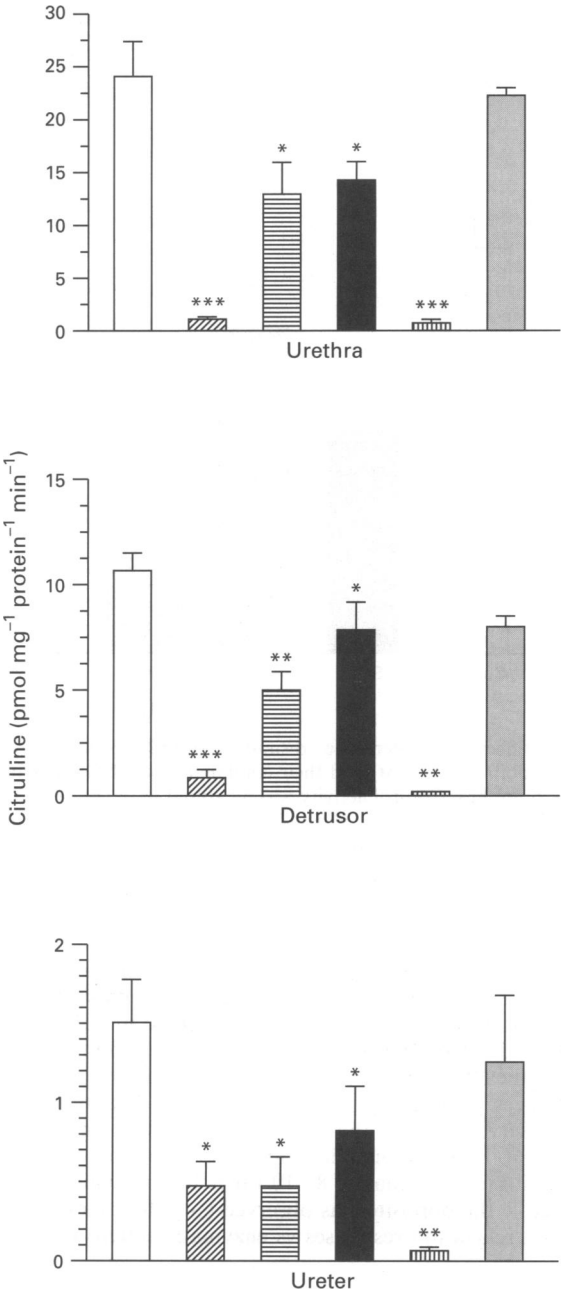

Figure 4 Effect of cofactor removal or inhibitor addition on NOS activity in different regions of the sheep urinary tract. NOS activity was measured in the presence of 1 mM $CaCl_2$ (except $-Ca^{2+}$, together with 2 mM EGTA, diagonally hatched columns), 2 mM NADPH (except −NADPH, horizontally hatched columns), 30 u ml^{-1} calmodulin, 3 μM BH_4 (except $-BH_4$, solid columns) and 40 μM L-arginine. Some reaction mixtures contained N^G-nitro-L-arginine (0.1 mM, vertically hatched columns) or L-canavanine (0.1 mM, stippled columns). Open columns show control values in the presence of all cofactors and the absence of inhibitors. Data are means ± s.e.mean ($n = 3-5$). $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ vs control.

Table 2 Calmodulin-dependency of NOS activity in different regions of the sheep urinary tract

Treatment	NOS activity (pmol citrulline mg^{-1} protein min^{-1}) Urethra	Detrusor	Ureter
Control	45.9 ± 5.4	15.2 ± 1.4	1.4 ± 0.2
−Calmodulin	48.7 ± 7.7	12.3 ± 1.6	0.8 ± 0.1
+CMZ (10 μM)	33.4 ± 11.4	17.6 ± 2.4	0.6 ± 0.4
+CMZ (100 μM)	3.5 ± 0.9**	1.3 ± 0.6**	0.5 ± 0.3*

Control NOS activity was measured in the presence of 2 mM NADPH, 1 mM $CaCl_2$, 40 μM L-arginine, 3 μM BH_4 and 30 u ml^{-1} calmodulin (except −calmodulin and CMZ). Some reaction mixtures contained calmidazolium (CMZ, 10 or 100 μM, also without calmodulin) as indicated. Data are means ± s.e.mean. (n = 3-4). *P<0.05, **P<0.01 vs control.

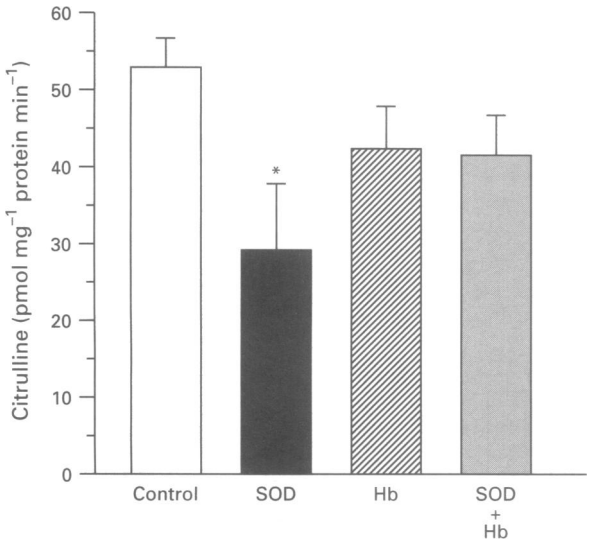

Figure 5 Effect of superoxide dismutase (SOD, 5000 u ml^{-1}), oxyhaemoglobin (Hb, 10 μM) and their combination on NOS activity in the sheep urethra. Control activity was measured in the presence of 2 mM NADPH, 1 mM $CaCl_2$, 30 u ml^{-1} calmodulin, 3 μM BH_4 and 40 μM L-arginine. Some reaction mixtures contained SOD, Hb or both as indicated. Data are means ± s.e.mean (n = 3–4). *P<0.05 vs control.

by 53% and 21%, respectively (P<0.05). A comparison of IC_{50} values for NOS inhibitors in enzymatic activity studies vs functional studies in urethral tissues is presented in Table 4. L-NOARG and L-NAME were about 3 fold more potent in NOS activity studies than in relaxation ones, but with both methods no differences in potency exist between them. The most surprising observation was the drastic reduction in inhibitory potency of L-NMMA on relaxation responses compared to NOS activity (approximately 8–10 times lower), while for 7-nitroindazole the opposite was observed (~ 3 fold increase in potency on relaxation responses vs enzymatic activity).

Discussion

Our results show the presence of the enzymatic system for the synthesis of NO within the muscular layer of urethra, detrusor and ureter from sheep, supporting previous morphological and functional data which suggest the involvement of NO in the NANC transmission of the urinary tract (see Introduction). L-[^{14}C]-arginine was converted by cytosolic fractions of urinary tract tissues to a radiolabelled molecule with the cation exchange and thin layer chromatography profile of citrulline. Formation of citrulline by alternative

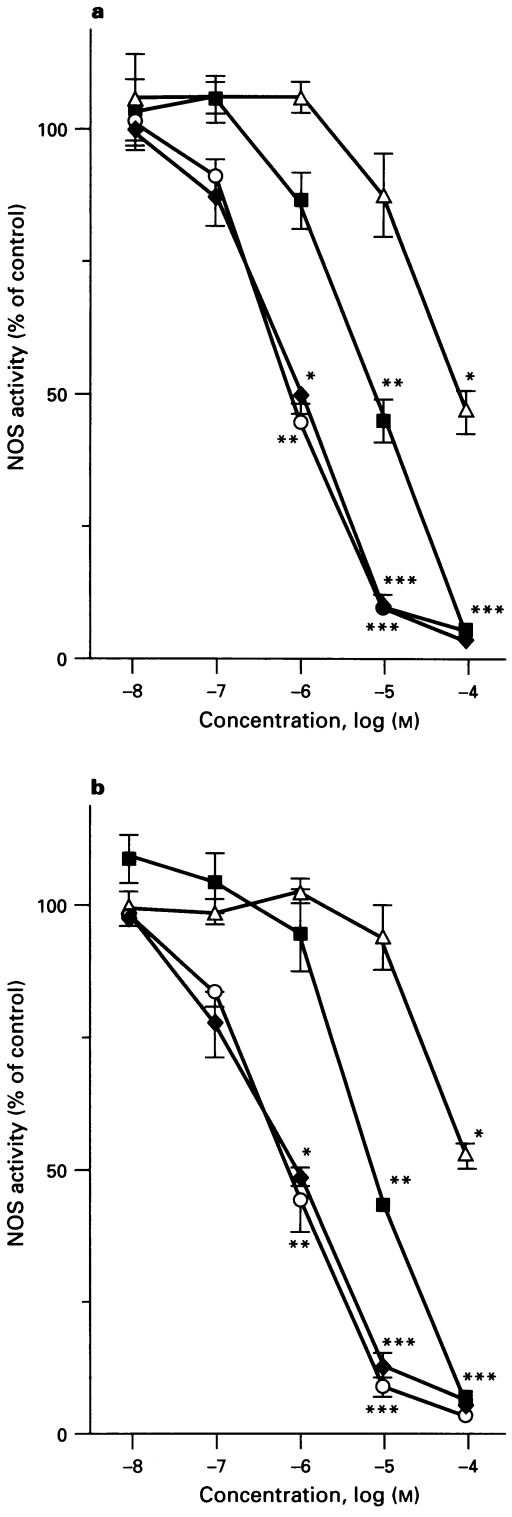

Figure 6 Effect of N^G-nitro-L-arginine (o), N^G-nitro-L-arginine methyl ester (◆), N^G-monomethyl-L-arginine (■) and 7-nitroindazole (△) on NOS activity in the sheep urethra (a) and detrusor (b). Values are expressed as percentage of control NOS activity for each data set (49.9 ± 4.1 and 16.4 ± 1.8 pmol citrulline mg^{-1} protein min^{-1} in the urethra and the detrusor, respectively). Data are means ± s.e.mean (n = 3–4). *P<0.05, **P<0.01, ***P<0.001 vs control.

pathways such as arginine conversion to ornithine by arginase, and subsequent formation of citrulline by ornithine transcarbamoylase, can be discarded in our assay because: (1) The ornithine intermediate was not seen on thin layer chromatography and (2) enzymatic activity was specifically inhibited by L-arginine analogues while L-canavanine, a strong arginase inhibitor (Hrabák *et al.*, 1994), had no effect.

Table 3 Inhibition constants (K_i) of NOS inhibitors in the sheep urethra and detrusor

	K_i (μM)			
Inhibitor	*Urethra*		*Detrusor*	
L-NOARG	0.25	(0.17–0.32)	0.21	(0.04–0.38)
L-NAME	0.21	(0.14–0.27)	0.28	(0.05–0.50)
L-NMMA	1.01	(0.09–1.93)	1.04	(0.39–1.68)
7-NI	22.6	(2.0–42.7)	26.8	(19.3–34.2)

K_i values were estimated from the plots of 1/velocity versus inhibitor concentration according to the method of Dixon, assuming competitive inhibition and for a K_m value of 10 μM. Data are mean values of 4–5 separate experiments with 95% confidence limits in parentheses. L-NOARG = N^G-nitro-L-arginine; L-NAME = N^G-nitro-L-arginine-methyl ester; L-NMMA = N^G-monomethyl-L-arginine and 7-NI = 7-nitroindazole.

In the present study, NOS activity measured in the sheep urethra, detrusor and ureter was exclusively located in the cytosolic fraction, was Ca^{2+} and calmodulin-dependent and required the addition of NADPH and BH_4 for maximal activity. The K_m for the cytosolic activity was similar in both urethral and detrusor preparations (9.9 μM and 9.1 μM), and is in accordance with values described for the constitutive enzyme found in brain (8.4 μM, Knowles *et al.*, 1990; 10 μM, Klatt *et al.*, 1993). Taken together, these findings suggest that the enzymatic activity measured actually reflects the activity of a neural NOS isoform (nNOS) (Knowles & Moncada, 1994) from NANC nerves, whereas the endothelial and membrane-associated isoform (eNOS) (Knowles & Moncada, 1994) is not significantly present in our homogenates. However the possibilty cannot be exlcuded that part of the eNOS could have been released into the cytosol upon cell disruption during the homogenization procedure.

Our results contrast with those found by Mitchell *et al.* (1991) in the rat anococcygeous muscle, another NANC innervated tissue, where Ca^{2+}-dependent NOS activity was found in both soluble and particulate fractions. Also, Dokita *et al.* (1994), in the rabbit urethra, described higher NOS activity present in the particulate fraction than in the soluble one, which was Ca^{2+}-independent and inhibited by L-canavanine > L-NMMA > L-NOARG. A similar Ca^{2+} -independant enzyme (also inhibited more by L-canavanine than by L-NMMA or L-NOARG) was measured in infected urine pellets reflecting the activity of an inducible isoform (iNOS) in inflammatory cells (Smith *et al.*, 1994). Although species differences cannot be excluded, the use of tissues pre-exposed to inflammatory or infectious conditions may explain these discrepancies. On the other hand, in accordance with our study, no Ca^{2+}-independent NOS activity has been found in the guinea-pig bladder (Ehrén *et al.*, 1995) or in the human urogenital tract (Ehrén *et al.*, 1994).

Besides cofactor requirements, NOS isoforms can also be identified by their differential sensitivity to inhibition by different compounds. The most widely used NOS inhibitors are N^G substituted analogues of the substrate L-arginine, such as L-NMMA, L-NOARG and its methyl ester (L-NAME). It appears that constitutive enzymes (nNOS and eNOS) are more sensitive to inhibition by L-NOARG and L-NAME than the inducible enzyme (iNOS), which, in turn, seems to be effectively inhibited by L-NMMA rather than by L-NOARG (Komori *et al.*, 1994). Another structural analgoue of L-arginine, L-canavanine, shows some selectivity towards iNOS (McCall *et al.*, 1989), while the fused heterocyclic 7-nitroindazole has been described as a selective inhibitor for nNOS (Moore *et al.*, 1993). Our results show that NOS activity in both urethra and detrusor is strongly inhibited by L-NOARG and L-NAME, less inhibited by L-NMMA, while 7-NI was a weak inhibitor and L-canavanine had no effect. Similar K_i values were observed in both tissues further supporting the existence of only one type of

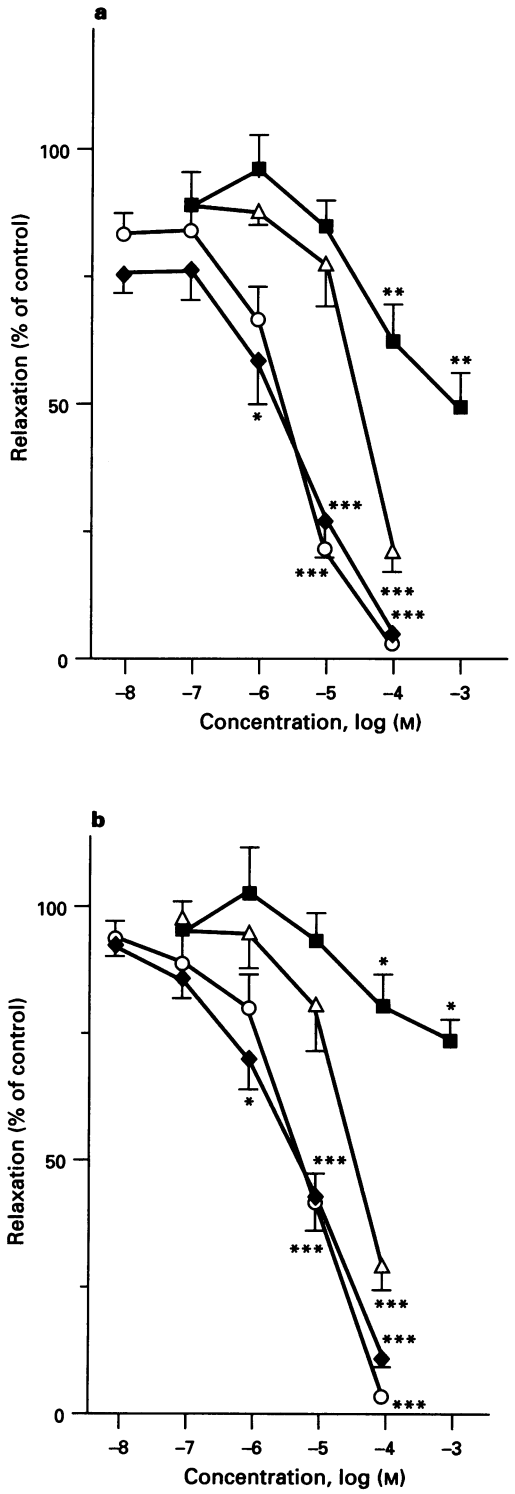

Figure 7 Effect of N^G-nitro-L-arginine (o) N^G-nitro-L-arginine methyl ester (◆), N^G-monomethyl-L-arginine (■) and 7-nitroindazole (△) on the relaxation induced by electrical field stimulation at 2 (a) and 8 Hz (b) in sheep and urethral preparations precontracted by noradrenaline (50 μM). The preparations were pretreated with NOS inhibitors for 60 min. Guanethidine (10 μM) and atropine (1 μM) were present throughout the experiment. Results are expressed as a percentage of the control relaxation before treatment (4.4 ± 0.1 and 6.6 ± 0.6 mN at 2 and 8 Hz, respectively). $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.01$ vs control.

NOS isozyme in the urethra and the detrusor (and probably also in the ureter). Since the kinetic constants reported here are stimated at a single long time point (60 min of incubation), our results will not account for the possibility of a slow inhibition process such as that described for L-NOARG and L-NMMA

Table 4 Effect of NOS inhibitors on enzymatic activity versus relaxation induced by electrical field stimulation in the sheep urethral smooth muscle

	IC_{50} (μM)		
		Relaxation	
Inhibitor	*Nos activity*	*2 Hz*	*8 Hz*
L-NOARG	0.67	2.45	5.81
	(0.47–0.95)	(0.95–6.32)	(2.35–14.4)
L-NAME	0.66	1.51	4.92
	(0.26–1.65)	(0.23–9.50)	(1.62–14.7)
L-NMMA	7.24	57.5	a
	(2.81–18.6)	(36.3–91.2)	
7-NI	85.1	26.3	33.8
	(25.8–251)	(12.5–54.9)	(14.1–81.2)

IC_{50} values (concentration of inhibitor giving 50% inhibition of control response) were obtained by linear interpolation. NOS activity was measured in the cytosolic fraction by the conversion of L-[^{14}C]-arginine to L-[^{14}C]-citrulline in the presence of 2 mM NADPH, 1 mM $CaCl_2$, 30 u ml^{-1} calmodulin, 3 μM BH_4 and 40 μM L-arginine. Relaxation responses to EFS (2 and 8 Hz) were studied in urethral preparations pretreated with guanethidine (10 μM) and atropine (1 μM) and contracted with noradrenaline (50 μM). Drugs were present for 60 min. [a] Agents failed to produce 50% inhibition. Data are mean values of 3–5 experiments with 95% confidence intervals in parentheses. Differences between IC_{50} values at 2 Hz and at 8 Hz were not significant (unpaired *t* test).

(Klatt *et al.*, 1994), which could lead to a slight underestimation of K_i. However, the observed K_i values for L-NOARG (0.2 μM), L-NAME (0.2 μM) and L-NMMA (1 μM) are not substantially different from those reported for the nNOS from brain in similar conditions (Komori *et al.*, 1994). Unexpectedly, 7-nitroindazole, reported to be a selective inhibitor of nNOS (Moore *et al.*, 1993), showed a weak effect on NOS activity in the urethra and detrusor with K_i values around 25 μM. However, it has been shown that inhibition of nNOS by 7-nitroindazole was competitive vs L-arginine and vs BH_4. Thus, the presence of high concentrations of BH_4 in the assay medium would diminish its sensitivity to inhibition (Wolff *et al.*, 1994). In fact, K_i values of 35 μM for 7-nitroindazole have been reported at saturating concentrations of BH_4 (Wolff *et al.*, 1994).

When the effects of these NOS inhibitors were evaluated on the relaxation induced by EFS in urethral preparations *in vitro*, the rank order of potency was L-NOARG = L-NAME > 7-nitroindazole > L-NMMA. Like NOS enzymatic activity, relaxation responses were more potently inhibited by L-NOARG and L-NAME, although IC_{50} values were about 3 fold higher than in citrulline formation studies, probably indicating that actual concentrations inside cells are much lower because of restricted uptake by the tissue. In contrast to enzymatic assays, L-NMMA had a weak inhibitory effect on nitrergic urethral relaxations, suggesting that this L-arginine analogue enters NOS-containing neurones very poorly or that non specific effects are counteracting its inhibitory action. In fact, it has been shown that L-NMMA is slowly metabolized to NO and citrulline during long time periods of incubation (Klatt *et al.*, 1994). This effect could also account for an overestimation of the inhibitory potency of L-NMMA in enzymatic assays, as production of citrulline from L-NMMA would reduce specific activity of the assay medium. In contrast to L-NMMA, 7-nitroindazole was a more potent inhibitor of EFS-induced relaxation than of NOS activity. Non-specific effects of 7-NI has also been described, suggesting that its use as selective inhibitor of NOS must be carefully considered. Thus, 7-nitroindazole induced smooth muscle relaxations (Medhurst *et al.*, 1994) and inhibited the purinergic component of the EFS-induced response in the vas deferens (Allawi *et al.*, 1994), through NOS-independent mechanisms.

To address the question of whether NO, generated in equimolar amounts with citrulline in the assay medium, may inhibit NOS activity through feedback mechanisms, we examined the effects of compounds that either prolong (SOD) or diminish (oxyhaemoglobin) the biological half life of NO. It has been suggested that in the presence of BH_4, large amounts of superoxide are generated by BH_4 autoxidation leading to NO inactivation to peroxynitrite, explaining why, in some cases, the addition of high concentrations of NO had no inhibitory effect on citrulline formation (Mayer *et al.*, 1995). This can also explain why, in the present study, NOS activity was not increased in the presence of oxyhaemoglobin. However, NOS-catalyzed citrulline formation was effectively inhibited by SOD in an oxyhaemoglobin-sensitive manner. Since SOD acts by protecting NO from superoxide inactivation and then raising NO concentration, our results suggest that enzymatically formed NO could mediate feedback inhibition of NOS in the urinary tract.

In an attempt to show that NOS activity measured by citrulline formation assay accounts for the relaxation induced by EFS in urinary tract tissues, we compared NOS specific activity of urethral, detrusor and ureteral preparations with their ability to develop NO-mediated EFS-induced relaxations. Prominent differences in NOS activity were found in the urethra, detrusor and ureter, which correlate with the density of nitrergic nerves present in the three regions (Triguero *et al.*, 1993; Persson *et al.*, 1993). In the urethra, a close correlation was found between density of NO-contained nerves (Triguero *et al.*, 1993), NOS enzymatic activity and functional responses elicited by EFS, suggesting that the measured enzymatic activity actually reflects the activity of NOS present in nitrergic nerves which assists NANC urethral inhibitory neurotransmission. In contrast, functional NO-mediated responses in the detrusor were much lower than those expected for its level of both NOS specific activity and density of nitrergic nerves. In accordance with our results, in the pig (Persson *et al.*, 1993) and rat detrusor (Persson *et al.*, 1992), no relaxant responses could be elicited in precontracted preparations. There is much controversy about whether inhibitory innervation plays a role in the detrusor. Although Vizzard *et al.* (1994) proposed a sensory function for NO as a constituent of bladder primary sensory afferents, NOS containing nerves in the bladder submucosa are not sensitive to systemic capsaicin treatment, nor does L-NOARG affect capsaicin-induced relaxation, making the NO contribution to sensory system questionable (Persson *et al.*, 1995). Recent investigations have shown that only after chemical irritation of the bladder, but not in control conditions, NO facilitates the micturition reflex (Kakizaki & DeGroat, 1995) and bladder afferent neurones exhibit NOS immunoreactivity (Vizzard *et al.*, 1995). Therefore, NO does not seem to be present in primary afferent neurones under physiological conditions, but might be induced by inflammation. Alternatively, a role of NO as neuromodulator of the cholinergic transmission has been suggested in the pig detrusor (Persson *et al.*, 1993), where L-arginine inhibited the atropine-sensitive detrusor contraction evoked by EFS.

In the sheep ureter, responses to EFS consisted of transient suppression of the ongoing spontaneous activity which could be inhibited by *in vitro* capsaicin-desensitization, suggesting the involvement of the capsaicin-sensitive sensory innervation of the ureter. Similarly, in the middle ureter from rats and guinea-pigs, both capsaicin and EFS had an inhibitory effect which was ascribed to the release of calcitonin-gene related peptide from sensory nerve terminals (Maggi & Giuliani, 1991). However, no relaxant responses could be elicited in the sheep ureter by EFS, and L-NOARG treatment had no effect either on responses to EFS or on the spontaneous motility. These results correlate with the absence of NADPH-diaphorase positive nerves in the sheep ureteral muscular layer. It should be noted that the whole wall of the ureter, including the submucosa, was used for NOS activity measurements. Thus, the small, but significant, specific activity observed may reflect the presence of submucosal and perivascular NOS containing neurones (Triguero *et al.*, 1993), but not motor innervation of

the muscle layer. In contrast to the sheep, nitrergic innervation of the ureter muscle layer has been described in pig (Hernández *et al.*, 1995; Iselin *et al.*, 1995) and human subjects (Smet *et al.*, 1994b; Obara *et al.*, 1995; Goessl *et al.*, 1995), but NO-mediated functional responses were observed only in the pig intravesical ureter (Hernández *et al.*, 1995).

In conclusion, the NO-synthesizing enzymatic system is present in the sheep urinary tract with a profile of cofactor-dependency and inhibition by NOS inhibitors that is compatible with the suggestion that nNOS, present in NANC nerves, is the isozymic form responsible for enzymatic citrulline production. Correlation between biochemical and functional data was found only in the urethra, where NOS present in nerves may assist urethral relaxation which accompanies micturition, whereas control of the motor function of the detrusor and ureter by nitrergic innervation seems less likely. The possibility that NO has a sensory function in physiological or pathological conditions or that it might participate as a neuromodulator merits further attention.

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