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Effects of superoxide anion generators and thiol modulators on nitrergic transmission and relaxation to exogenous nitric oxide in the sheep urethra

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1 The effects of superoxide anion generators, the nitric oxide (NO) scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoine-1-oxyl 3-oxide (carboxy-PTIO), the specific guanylate cyclase inhibitor 1H-[1,2,4]-oxadiazole-[4,3-a]-quinoxalin-1-one (ODQ), and thiol modulating agents were investigated on relaxations induced by nitrergic stimulation and exogenous NO addition in the sheep urethra.

2 Methylene blue (MB, 10 μM), pyrogallol (0.1 mM) and xanthine (X, 0.1 mM)/xanthine oxidase (XO, 0.1 u ml^{-1}) inhibited NO-mediated relaxations, without affecting those induced by nitrergic stimulation. This resistance was not diminished following inhibition of endogenous Cu/Zn superoxide dismutase (Cu/Zn SOD) with diethyldithiocarbamic acid (DETCA, 3 mM), which almost abolished tissue SOD activity.

3 Carboxy-PTIO (0.1–0.5 mM) inhibited NO-mediated relaxations but had no effect on responses to nitrergic stimulation, which were not changed by treatment with ascorbate oxidase (2 u ml^{-1}).

4 Relaxations to NO were reduced, but not abolished, by ODQ (10 μM), while nitrergic responses were completely blocked.

5 The thiol modulators, ethacrynic acid (0.1 mM), diamide (1.5 mM), or 5,5′-dithio-bis (2-nitrobenzoic acid) (DTNB, 0.5 mM), and subsequent treatment with dithiothreitol (DTT, 2 mM) had no effect on responses to nitrergic stimulation or NO. In contrast, N-ethylmaleimide (NEM, 0.2 mM) markedly inhibited both relaxations.

6 L-cysteine (L-cys, 0.1 mM) had no effect on responses to NO, while it inhibited those to nitrergic stimulation, in a Cu/Zn SOD-independent manner.

7 Our results do not support the view that the urethral nitrergic transmitter is free NO, and the possibility that another compound is acting as mediator still remains open.

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Keywords: Nitrergic neurotransmitter; nitric oxide; urethra; superoxide anion; carboxy-PTIO; thiol modulator; ODQ; superoxide dismutase; diethyldithiocarbamic acid

Abbreviations: carboxy-PTIO, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoine-1-oxyl 3-oxide; Cu/Zn SOD, Cu/Zn superoxide dismutase; DETCA, diethyldithiocarbamic acid; DTNB, 5,5′-dithio-bis (2-nitrobenzoic acid); DTT, dithiothreitol; EFS, electrical field stimulation; L-cys, L-cysteine; MB, Methylene blue; NA, noradrenaline; NANC, non-adrenergic, non-cholinergic; NEM, N-ethylmaleimide; NO, nitric oxide; NOS, nitric oxide synthase; ODQ, 1H-[1,2,4]-oxadiazole-[4,3-a]-quinoxalin-1-one; X, xanthine; XO, xanthine oxidase

Introduction

The nitrergic nature of the non-adrenergic, non-cholinergic (NANC) relaxation of the urethral smooth muscle is now well established in several species, including man. NANC relaxation induced by electrical field stimulation (EFS) of nerves can be blocked by nitric oxide (NO)-synthesis inhibitors (Garcia-Pascual *et al*., 1991; Andersson *et al*., 1992) and is mediated by increases in cyclic GMP levels in smooth muscle (Garcia-Pascual & Triguero, 1994). Furthermore, the presence of constitutive NO synthase (NOS) activity has been shown in the urethra (Garcia-Pascual *et al*., 1996), and is localized in nerve fibres at the smooth muscle layer (Triguero *et al*., 1993). Nitrergic neurotransmission mediating relaxation has also been demonstrated in different smooth muscles from the gastrointestinal, respiratory and genital tracts (see Rand & Li, 1995a). However, controversy exists over the precise identity of the transmitter.

Free radical scavengers and superoxide anion generators have been shown to have a more pronounced effect on relaxations induced by authentic NO than on those induced by NANC nerve stimulation in different nitrergically-innervated gastrointestinal smooth muscles (see Gibson & Lilley, 1997). We have also observed, in the sheep urethral tissue, that both the NO scavenger oxyhaemoglobin and methylene blue (MB), which is acting in this tissue by generation of superoxide anions, inhibited responses to exogenous NO without affecting nitrergic relaxations (Garcia-Pascual & Triguero, 1994). These facts have led to the suggestion that a more stable precursor, instead of NO itself, is released by nerve terminals. In this sense, several relatively stable NO adducts, specially S-nitrosothiol compounds, have been investigated as possible nitrergic carriers (DeMan *et al*., 1995; 1998; Garcia-Pascual *et al*., 1999), but definitive experimental results supporting their role as nitrergic transmitters are still lacking.

It is already known that spontaneous release of NO does not account for the relaxant activity of several, chemically-different, NO donor compounds, while metabolic activation to NO at the target tissue or S-nitrosylation reactions more likely mediate their action (Kowaluk & Fung, 1990; Garcia-Pascual *et al*., 1999). Furthermore, while NO itself does not react with thiol groups under physiological conditions, alternative redox activated states of NO (NO^+), from endogenous species such

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as S-nitrosothiols or iron-nitrosyls, are likely to directly nitrosylate thiol groups of a wide variety of proteins of biological significance (Arnelle & Stamler, 1995). The involvement of S-nitrosylation reactions in the nitrergically-induced relaxation, which might suggest the existence of a NO carrier compound, has not been clearly elucidated yet. In the rat gastric fundus, DeMan *et al*. (1996a) found that different thiol modulators had no effect on relaxations induced by nitrergic stimulation or NO, while they inhibited those elicited by glyceryl trinitrate. However, in the same tissue, Barbier & Lefebvre (1994) observed that in preparations that were made tolerant to glyceryl trinitrate, which may be due to intracellular thiol depletion, they became less responsive to nitrergic stimulation and these responses were more sensitive to LY83583 inhibition, thus suggesting that the impairment of NO incorporation into a cellular thiol results in a larger output of the neurotransmitter being released as free NO.

Alternatively, Martin *et al*., (1994) suggested that free NO is, indeed, released by nerve terminals, but the presence of high levels of endogenous Cu/Zn superoxide dismutase (SOD) would be protecting NO from the action of superoxide anion generators. Since depletion of endogenous SOD with the copper chelator diethyldithiocarbamic acid (DETCA) led to effective inhibition by pyrogallol, LY83583 and hypoxanthine/xanthine oxidase (XO). In support of this hypothesis, Thomas *et al*. (1996) have demonstrated the localization of SOD and catalase in neural elements of the oesophageal myenteric and submucosal plexuses. In addition, other physiological antioxidants, such as ascorbic acid, which extracellular concentration ranged between 200–400 μM (Miele *et al*., 1994), have been shown to provide protection against NO inactivation by superoxide anions and by the direct NO scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoine-1-oxyl 3-oxide (carboxy-PTIO) (Lilley & Gibson, 1996). Ascorbic acid also appears to be released by 6-hydroxydopamine-resistant nerve terminals (Lilley & Gibson, 1997). However, the effect of extracellular ascorbic acid depletion on nitrergic responses has not been investigated yet.

The present study was designed to further investigate whether free NO or a NO-containing compound, accounts for the relaxant activity of the nitrergic transmitter in the sheep urethra. We have studied the effects of several superoxide anion-generating agents, the NO scavenger carboxy-PTIO, the selective guanylate cyclase inhibitor 1H-[1,2,4]-oxadiazole-[4,3-a]-quinoxalin-1-one (ODQ), and several thiol modulating agents on relaxant responses induced by nitrergic stimulation in comparison with those induced by exogenous NO. The influence of DETCA pretreatment on the effects of superoxide anion generators and the influence on the effect of carboxy-PTIO of extracellular ascorbate depletion by ascorbate oxidase treatment were also assessed.

Methods

Drugs

Atropine sulphate, ascorbate oxidase (from *cucurbita* species), L-cysteine hydrochloride monohydrate (L-cys), DETCA, diamide, 5,5′-dithio-bis (2-nitrobenzoic acid) (DTNB), DL-dithiothreitol (DTT), ethacrynic acid, guanethidine sulphate, MB, $\pm$-noradrenaline bitartrate (NA), N-ethylmaleimide (NEM), pyrogallol, Cu/Zn SOD (from bovine erythrocytes), X and XO (from buttermilk) were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). ODQ and carboxy-PTIO were purchased from Alexis Co. (Läufelfingen, Switzerland). Drugs were dissolved in distilled water except DTNB, which was dissolved in ethanol, and ethacrynic acid and ODQ in dimethyl sulphoxide. Solutions were stored at -20°C and working dilutions were made in 0.9% NaCl.

Preparation of NO solutions

A saturated NO aqueous solution was prepared daily in a sealed vial containing 20 ml of ice-cold distilled water (previously de-oxygenated with oxygen-free nitrogen for 60 min). The vial was exposed for 10 min to a stream of pure NO gas (L′Air liquide, Madrid, Spain) which had been previously bubbled through a KOH (10%) solution to remove nitrogen dioxide. Subsequent dilutions were prepared in sealed de-oxygenated vials by means of a gas-tight syringe. The NO concentrations in both the saturated and working dilutions were determined before use by means of a NO selective electrode (ISO-NO, World Precision Instruments, Hertfordshire, U.K.) which was calibrated daily by addition of sodium nitrite to a nitrogen-gassed solution of 0.1 mM KI in 0.1M H_2SO_4. The detector response was linear ($r>0.99$) over the concentration range tested (0–2.8 μM) with an average slope of 1.7 pA current/nM NO and the detection limit in our experimental conditions was 15 nM.

Tissue preparation and recording of mechanical activity

Lower urinary tracts from female lambs (2–3 months old) were collected at the local slaughterhouse shortly after sacrifice and transported to the laboratory in cold Krebs solution (composition in mM: NaCl 119, KCl 4.6, $CaCl_2$ 1.5, $MgCl_2$ 1.2, $NaHCO_3$ 15, KH_2PO_4 1.2, EDTA 0.01 and glucose 11). The urethra was removed, opened longitudinally and placed in a Petri dish filled with cold Krebs solution. The mucosa, most of the submucosa, fat and connective tissue were carefully eliminated by sharp dissection under a stereomicroscope. Then, transverse strips (approximately $1\times1\times5$ mm) were cut and mounted as previously described (García-Pascual *et al*., 1996) in 5 ml organ baths containing Krebs solution at 37°C and bubbled with a mixture of 95% O_2 and 5% CO_2 (pH 7.4). Tension was monitored with a Grass FT03C force displacement transducer and recorded on a Grass polygraph model 7D (Grass Instruments, Quincy, MA, U.S.A.). Strips were equilibrated at a resting tension of 15 mN for 60 min and exposed to guanethidine (50 μM) and atropine (1 μM) before experimentation.

Electrical field stimulation

Electrical field stimulation (EFS) was performed by means of two platinum electrodes placed parallel to the preparation, connected to a Grass S48 stimulator (Grass Instruments, Quincy, MA, U.S.A.) and coupled to a Med-lab stimulus splitter (Med-Lab Instruments, Loveland, CO, U.S.A.). Square-wave pulses of 0.8 ms duration at a frequency of 0.5–12 Hz were delivered at 2 min intervals. The voltage was supramaximal (current strength 200 mA) and the train duration was 5 s. This stimulation produces the release of the nitrergic transmitter from intrinsic urethral nerves (García-Pascual *et al*. 1991).

Experimental protocol

All experiments were performed on urethral preparations contracted with a submaximal concentration of noradrenaline (NA, 50 μM) and in the continuous presence of guanethidine

(10 μM) and atropine (1 μM). Relaxant responses to EFS and NO were analysed as follows: once the NA-induced contraction had reached a plateau, either frequency-response curves from 0.5 to 12 Hz were constructed or NO (1–300 μM) was added cumulatively in half-log increments of molar concentrations to perform a concentration-response curve. When the effects of drugs were studied, preparations were pre-exposed to the drug for the time indicated. Some substances (carboxy-PTIO, SOD or pyrogallol) were added on top of the NA-induced contraction 5 min before EFS or NO addition. Control preparations from the same animal, receiving only the drug solvent, were always run in parallel to serve as time control. When the effects of exogenous generation of superoxide anion by XO (0.1 u ml^{-1})/X (0.1 mM) were studied, XO was added 10 min before NA-induced contraction, thus allowing it to permeate into the tissue, followed by the addition of X, on top of the contraction, 5 min before EFS or NO addition. In other experiments, the possible interfering effect of endogenous ascorbate on the carboxy-PTIO action was studied by removing the extracellular ascorbate with ascorbate oxidase (2 u ml^{-1}) treatment for 30 min before carboxy-PTIO addition. In separate experiments, the effects of irreversible inhibition of endogenous Cu/Zn SOD with DETCA on the action of the superoxide generators (pyrogallol, methylene blue and XO/X), on EFS-induced relaxations were investigated. In these experiments, urethral preparations were incubated with DETCA (3 mM) for 2 h, followed by extensive washing before MB (10 μM), X (0.1 M) / XO (0.1 u ml^{-1}) or pyrogallol (0.1 M) addition. Results were compared with those obtained in the presence of MB, pyrogallol or XO/X without DETCA pre-treatment. The effect of DETCA treatment on EFS-induced relaxation in control conditions was also assessed.

In another set of experiments, we investigated the involvement of thiols in the responses elicited by nerve stimulation and exogenous NO, by using different thiol-modulating agents. N-ethylmaleimide (NEM, 0.2 mM), ethacrynic acid (0.1 mM)), diamide (1.5 mM) or 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB, 0.5 mM) were incubated for 30 min, followed by extensive washing. Then, preparations were again contracted with NA and frequency- or concentration-response curves performed. Since NEM-treatment depressed NA-contraction by 20–30%, lower concentrations of NA (1–30 μM) were applied to parallel controls to match the tone with that of treated strips. In preparations treated with DTNB, diamide or ethacrynic acid, a second concentration-relaxation procedure was performed after 30 min treatment with DTT (2 mM) (followed by washout), in an effort to overcome the action of these thiol depleting agents. The effect of excess thiol addition (L-cys 0.1 mM), both in the presence and absence of SOD (100 u ml^{-1}), was also evaluated. Furthermore, the effect of L-cys (1 mM) addition 10 min before incubation with NEM was also studied.

Assay of SOD activity

Total SOD activity in urethral supernatants was measured by the use of a SOD assay commercial kit (Calbiochem, San Diego, CA, U.S.A.). The experimental preparation, weighing 70–100 mg, consisted of four interconnecting strips in order to facilitate tissue rinsing. Two preparations were obtained from each animal and incubated in the organ baths under the same conditions as in tension recording experiments (Krebs solution at 37°C under a stream of 5% CO_2 and 95% O_2). After 1 h, preparations were exposed to DETCA (3 mM) for 2 h. Parallel preparations from the same animal, without treatment, served as controls. Tissues were subsequently blotted on filter paper, weighed, frozen in liquid nitrogen and pulverized in a liquid N_2 cooled stainless steel mortar. Tissues were homogenized in 0.25 M sucrose using a ground glass pestle homogenizer, and homogenates were then diluted in ice-cold 0.25 M sucrose to 10% w v^{-1} and centrifuged at 8500 × g for 10 min at 4°C. Supernatants were immediately assayed, following the kit manufacturer instructions. Before assay, 250 μl of supernatants were treated with 400 μl of ice-cold EtOH/$CHCl_3$ 62.5/37.5 v v^{-1} to remove interfering compounds (haemoglobin, albumin), centrifuged at 3000 × g for 10 min at 4°C and the upper aqueous layer collected for the assay.

SOD activity is expressed in SOD-525 units (as defined by Calbiochem) per mg protein. Protein concentration in tissue fractions was determined by the method of Lowry *et al.*, (1951).

Analysis of data

Relaxation responses were expressed as a percentage of the contraction induced by NA (50 μM). The negative logarithm of the NO concentration ($-\log EC_{30}$), or the logarithm of the frequency of EFS ($\log EF_{30}$) eliciting 30% relaxation, were determined by linear interpolation using the values immediately above and below that induced 30% reduction of the NA-induced tension. Results are expressed as mean ± s.e.mean and n denotes the number of animals used. Student's t-test (two tailed) was used for statistical comparisons. A value of $P < 0.05$ was considered significant.

Results

Effects of superoxide anion generators on urethral relaxations to nitrergic stimulation and NO

Urethral smooth muscle preparations were exposed to superoxide anions by the addition of pyrogallol (0.1 mM), XO (0.1 u ml^{-1}) plus X (0.1 mM) or MB (10 μM). As can be seen in Figure 1, concentration-dependent relaxations to exogenously added NO were significantly inhibited by these three agents. Prevention of the inhibitory effect of MB, X/XO and pyrogallol by the previous addition of SOD (200 u ml^{-1}) is also shown (Figure 1), demonstrating that these agents are acting by the extracellular generation of O_2^-. The fact that MB could be acting by chemical inactivation of NO, rather than by guanylate cyclase inhibition in the sheep urethra, has been previously reported (García-Pascual & Triguero, 1994; García-Pascual *et al.*, 1999), possibly due to its auto-oxidation in the presence of oxygen (McCord & Fridovich, 1970). In contrast, frequency-dependent nitrergic relaxation were not affected by any of these extracellular superoxide anion generators (Figure 2).

Effects of superoxide anion generators on nitrergic relaxation in DETCA-treated tissues

To investigate whether the presence of high endogenous levels of Cu/Zn SOD in the neuroeffector junction is protecting the nitrergic transmitter from inactivation by superoxide anions (Martin *et al.*, 1994), urethral preparations were pre-treated with the SOD inhibitor DETCA (3 mM, 2 h incubation followed by washout). This treatment did not significantly affect relaxant responses to NO (E_{max} were 67.9 ± 10.1 and 67.4 ± 10.3 and $-\log EC_{30}$ were 14.15 ± 0.29 and 4.43 ± 0.20, in

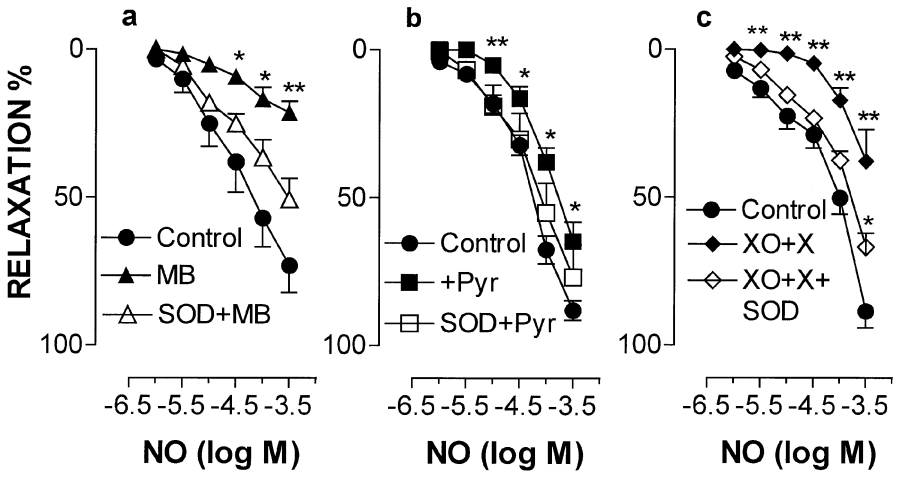

Figure 1 Effect of (a) methylene blue (MB, 10 μM), (b) pyrogallol (0.1 mM) and (c) xanthine (X, 0.1 mM) with xanthine oxidase (XO, 0.1 u ml^{-1}), in the absence and the presence of SOD (200 u ml^{-1}), on the concentration-response curve to nitric oxide (NO) in the sheep urethra. Results are expressed as percentage decrease of the noradrenaline (NA, 50 μM)-induced contraction and represent the mean$\pm$s.e.mean ($n=6-7$). *$P<0.05$; **$P<0.001$ significantly different from respective controls (Student's t-test for unpaired observations).

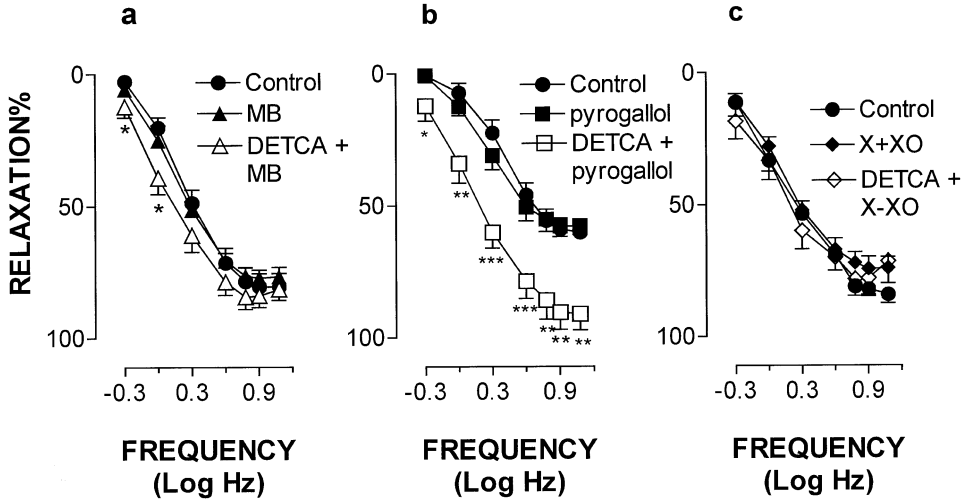

Figure 2 Effect of (a) methylene blue (MB, 10 μM), (b) pyrogallol (0.1 mM) and (c) xanthine (X, 0.1 mM) plus xanthine oxidase (XO, 0.1 u ml^{-1}) alone and after treatment with DETCA (3 mM, 2 h followed by washout) on the frequency-response curve to nitrergic stimulation in the sheep urethra. Results are expressed as percentage decrease of the noradrenaline (NA, 50 μM) -induced contraction and represent the mean$\pm$s.e.mean ($n=6-9$). *$P<0.05$; **$P<0.01$; ***$P<0.001$ significantly different from respective controls (Student's t-test).

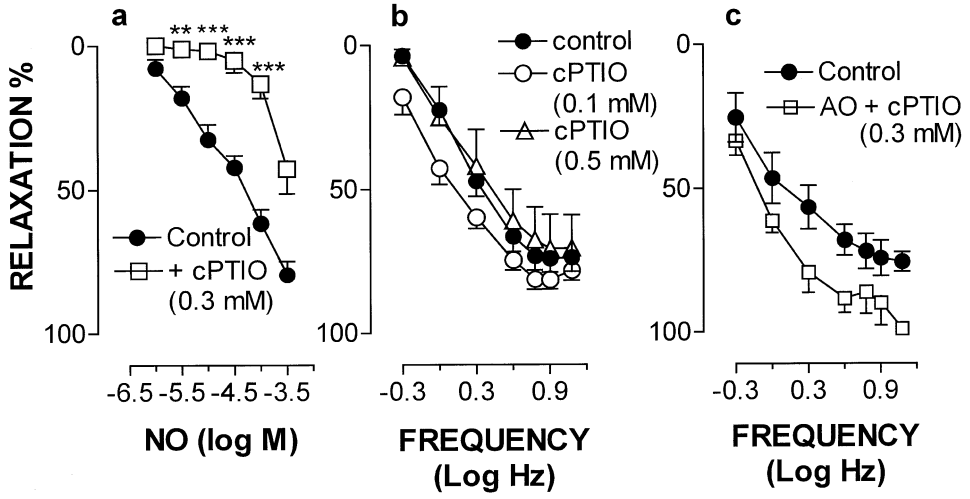

Figure 3 Comparison of carboxy-PTIO (0.1–0.5 mM) effects on the concentration-response curve to NO (a), and on the frequency-response curve to nitrergic stimulation, without (b) and after treatment with ascorbate oxidase (AO, 2 u ml^{-1} for 30 min) (c) in the sheep urethra. Carboxy-PTIO was added on top of the noradrenaline (NA, 50 μM) -induced contraction 5 min before electrical stimulation or NO addition. Results are expressed as percentage decrease of the NA-induced tone and represent the mean$\pm$s.e.mean ($n=5-6$). **$P<0.01$; ***$P<0.001$ significantly different from respective controls (Student's t-test for unpaired observations).

control and treated preparations, respectively, $n=6$), although it produced a slight increase in EFS-induced relaxations at low frequencies (E_{max} were 81.4 ± 3.0 and 84.1 ± 5.7, $P>0.05$, while log EF_{30} were -0.04 ± 0.06 and -0.33 ± 0.08, $P<0.05$, in control and treated preparations, respectively, $n=8$). However, the resistance of urethral nitrergic relaxation to inhibition by pyrogallol (0.1 mM), X (0.1 mM) plus XO (0.1 u ml^{-1}) and MB persisted in DETCA-treated tissues (Figure 2). A significant increase of relaxation was observed for the whole frequency-response curve in the presence of pyrogallol (Figure 2b), and at low frequencies in the presence of MB (Figure 2a), when compared with urethral preparations not exposed to DETCA. However, the effect of MB was not different to that observed in DETCA-treated strips in the absence of MB.

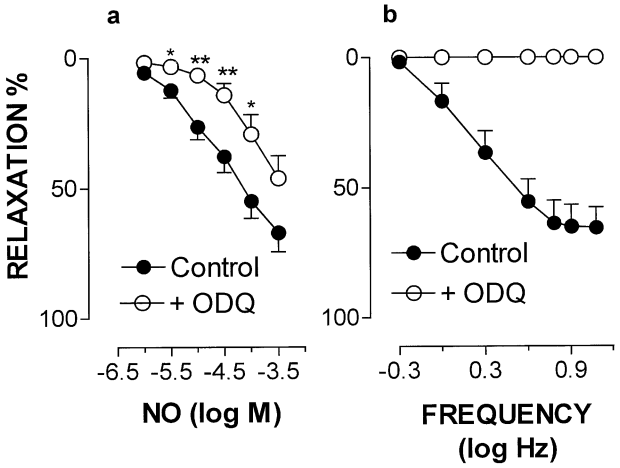

Figure 4 Effects of ODQ (10 μM) on the concentration-response curve to NO (a) and on the frequency-response curve to nitrergic stimulation (b) in the sheep urethra. ODQ was added 30 min before noradrenaline (NA). Results are expressed as percentage decrease of the NA (50 μM) -induced contraction and represent the mean$\pm$ e.s.mean ($n=6-7$). *$P<0.05$; **$P<0.01$ significantly different from controls (Student's t-test for unpaired observations).

Effects of DETCA treatment on SOD activity of urethral muscle

Homogenates of urethral smooth muscle were analysed for total SOD activity in both control conditions and after 2 h of treatment with DETCA (3 mM) under the same conditions as in organ bath studies. The SOD activity was 9.3 ± 2.29 units mg^{-1} protein ($n=4$) in control preparations and 0.8 ± 0.56 units mg^{-1} protein ($n=4$) ($P<0.001$) in DETCA-treated strips.

Effects of carboxy-PTIO on urethral relaxation to nitrergic stimulation and NO

The NO scavenging agent carboxy-PTIO (0.3 mM) induced a significant inhibition of the concentration-response curve to NO (Figure 3a) while at 0.1 and 0.5 mM it did not significantly affect the frequency-response curve to nitrergic stimulation (Figure 3b). Pre-treatment with ascorbate oxidase (2 u ml^{-1} for 30 min) did not modify the ineffectiveness of carboxy-PTIO (Figure 3c).

Effects of ODQ on the urethral relaxation to nitrergic stimulation and NO

The guanylate cyclase inhibitor ODQ (10 μM) completely abolished relaxation elicited by nitrergic stimulation (Figure 4b) and significantly inhibited, but did not abolish, that induced by exogenous NO (Figure 4a). ($-\log EC_{30}$ for NO was 4.8 ± 0.16 in the absence and 3.9 ± 0.26 in the presence of ODQ, $n=7$, $P<0.05$).

Effects of thiol modulators on urethral relaxation to nitrergic stimulation and NO

We have investigated the involvement of thiol groups in the mechanisms of release and/or action of the nitrergic transmitter and in the effect of NO by the use of several types of thiol-modulating agents: NEM (non-specific alkylating agent of both protein and non protein thiols) (Murphy *et al*., 1991), diamide (alkylating agent that oxidizes protein sulphydryl groups and depletes intracellular glutathione)

Table 1 Effects of thiol modulating agents on EFS- and NO-induced relaxations of the sheep urethra

		NO			*EFS*	
Treatment	E_{max} (%)	$-log\ EC_{30}$	n	E_{max} (%)	$log\ EF_{30}$	n
Control	77 ± 7.2	4.7 ± 0.14	5	75 ± 6.9	-0.11 ± 0.09	6
+DTNB	79 ± 8.9	4.7 ± 0.16	5	80 ± 6.9	0.002 ± 0.07	6
Control	78 ± 5.9	5.0 ± 0.22	5	80 ± 7.2	-0.01 ± 0.12	6
+DTNB+DTT	71 ± 2.6	4.7 ± 0.14	5	79 ± 5.4	0.003 ± 0.07	6
Control	53 ± 6.5	4.1 ± 0.21	4	75 ± 4.8	-0.09 ± 0.09	6
+Diamide	64 ± 14.5	4.0 ± 0.18	4	68 ± 5.4	0.02 ± 0.09	6
Control	68 ± 9.2	4.4 ± 0.21	4	79 ± 3.3	-0.25 ± 0.09	6
+Diamide+DTT	64 ± 11.5	4.6 ± 0.57	4	70 ± 3.6	-0.02 ± 0.07	6
Control	65 ± 10.4	1.5 ± 0.31	6	74 ± 8.5	0.16 ± 0.10	9
+Ethacrynic ac.	71 ± 12.1	4.4 ± 0.27	6	64 ± 4.7	0.22 ± 0.06	9
Control	62 ± 6.7	4.6 ± 0.22	6	66 ± 5.8	0.15 ± 0.09	9
+Ethacrynic ac.+DTT	69 ± 8.3	4.4 ± 0.20	6	64 ± 4.9	0.19 ± 0.07	9

Responses to electrical field stimulation (EFS) or NO were performed on the top of a NA (50 μM)-induced contraction. E_{max} is the maximum relaxant response and is expressed as a percentage of the contraction to NA. $-\log EC_{30}$ and $\log EF_{30}$ are the concentrations of NO (negative logarithms) of the frequency of EFS (positive logarithms) eliciting 30% of relaxation. DTNB (0.5 mM) diamide (1.5 nM) or ethacrynic acid (0.1 mM) were applied for 30 min, followed by extensive washing, before addition of NA. A second concentration-relaxation procedure was performed after 30 min treatment with DTT (2 mM, followed by washout). Parallel preparations not receiving any treatment served as controls. Values are mean$\pm$s.e.mean, n indicates number of experiments. No significant differences were observed between means (students t-test).

(Murphy *et al*., 1991), ethacrynic acid (glutathione-S-transferase inhibitor, which induces depletion of the glutathione pool) (Lau & Benet, 1992), DTNB (relatively impermeant thiol-specific oxidizing agent) and DTT (thiol-specific reducing agent) (Campbell *et al*., 1996). The results obtained show that the relaxation to either nitrergic stimulation or to NO, were not significantly affected by the 30 min treatment (followed by washout) with diamide (1.5 mM), DTNB (0.5 mM) or ethacrynic acid (0.1 mM). Furthermore, subsequent treatment with DTT (2 mM, 30 min followed by washout) was also without effect (Table 1). None of these thiol modulators affected the basal tension or the NA-induced contraction.

In contrast, pre-treatment with NEM (0.2 mM, 30 min followed by washout) markedly inhibited relaxation induced by both nitrergic stimulation and NO (Figure 5a,c). This inhibitory effect on nitrergic relaxation was prevented by the previous addition (5 min before NEM) of an excess of thiol (L-cys, 1 mM) (Figure 5c). It has to be noted that contractile responses to NA were also reduced after treatment with NEM by 20–30%, which points to the involvement of non-specific effects in the action of NEM. Addition of L-cys (0.1 mM) had no detectable effect on the relaxation-response relationship for NO (Figure 5b), while the frequency-response curves to nitrergic stimulation were significantly depressed (Figure 5d). The inhibitory effect of L-cys on the nitrergic relaxation was not affected by previous addition (5 min before) of SOD (100 u ml^{-1}) and the presence of SOD alone had also no significant effect on nitrergic responses (Figure 5d).

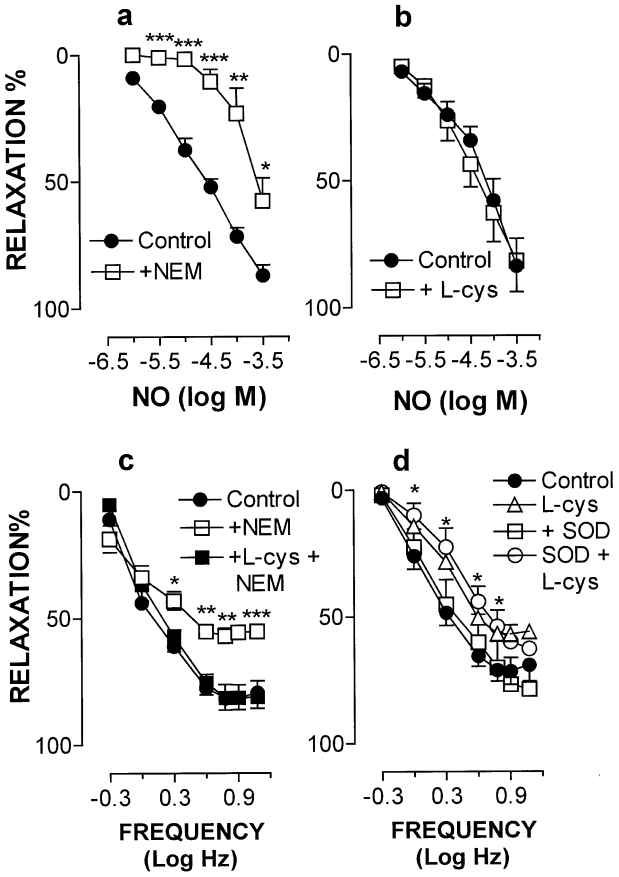

Figure 5 Concentration-response curves to NO (a and b) and frequency-response curves to nitrergic stimulation (c and d) of the sheep urethra, in the absence and after treatment with NEM (0.2 mM), 30 min followed by washout (a and c), with and without previous addition of L-cysteine (L-cys, 1 mM) (c), or in the absence and presence of L-cys (0.1 mM) (b and d), superoxide dismutase (SOD, 100 u ml^{-1}) or both (c). Results are expressed as percentage decrease of the noradrenaline (NA, 50 μM) -induced contraction and represent the mean ± s.e.mean ($n=6-7$). *$P<0.05$; **$P<0.01$; ***$P<0.001$ significantly different from controls (Student's *t*-test for unpaired observations).

Discussion

In the urinary tract, NANC relaxant neurotransmission controls the decrease in bladder outlet resistance that precedes micturition. In contrast to the urethra of some species, such as pig (Werkström *et al*., 1995) and dog (Hashimoto *et al*., 1993), which have both NO-dependent and -independent components, no doubts exist about the sole nitrergic origin of the inhibitory neurotransmission in the sheep urethra (García-Pascual *et al*., 1991; 1996), but there is no evidence that the NO produced by nerves is, in fact, the ultimate mediator of transmission. The results of the present study show that relaxant responses elicited by nitrergic stimulation of the sheep urethra are resistant to the action of the superoxide anion generators pyrogallol, MB and X/XO, at concentrations that effectively inhibited responses induced by exogenously applied NO. Dissimilar effects of different superoxide anion generating-agents on both nitrergic relaxations and responses to authentic NO are common features in practically all the nitrergically-innervated smooth muscles: pyrogallol, hypoxanthine/XO and LY83583 in the bovine retractor penis muscle (Martin *et al*., 1994); duroquinone and X/XO in the mouse anococcygeus (Lilley & Gibson, 1996); pyrogallol and duroquinone in the rat anococcygeus (La & Rand, 1999); LY83583, hypoxanthine/XO, pyrogallol and duroquinone in the rat gastric fundus (DeMan *et al*., 1996a; Lefebvre, 1996); pyrogallol in the canine ileocolonic junction (Boeckxstaens *et al*., 1994) and LY83583 and pyrogallol in the mouse corpus cavernosum (Göçmen *et al*., 1998). To explain these discrepancies, Martin *et al*. (1994) proposed that high levels of endogenous Cu/Zn SOD, which may be produced and released by nerve terminals (Thomas *et al*., 1996), are protecting NO at the neuroeffector junction from the inactivating action of superoxide anions, while they can easily inactivate exogenous NO before it reaches the tissue. This hypothesis is supported by experiments showing that pre-treatment with the copper chelator DETCA, which inhibits both intra- and extracellular Cu/Zn SOD, made responses to nitrergic stimulation sensitive to inhibition by pyrogallol, LY83583 and hypoxanthine /XO in the bovine retractor penis, effects that were reversed by exogenous Cu/Zn SOD (Martin *et al*., 1994). However, contradictory results also exist in the literature. In some tissues, such as the rat gastric fundus (Lefebvre, 1996), the rat (La & Rand, 1999) and mouse anococcygeus (Lilley & Gibson, 1996) or the mouse corpus cavernosum (Göçmen *et al*., 1998), DETCA pre-treatment only increased the inhibitory effect on nitrergic responses of intracellular generators of superoxides (LY83583 or duroquinone), but not those acting by generation of superoxide anions at the extracellular milieu (X/XO or pyrogallol). Furthermore, the action of DETCA was not reversed by exogenous SOD, which cannot enter the cell, thus suggesting that the interaction of the endogenous nitrergic transmitter with superoxides occur mainly at the intracellular level. In the present study, the resistance of the nitrergic urethral relaxations to inhibition by the extracellular superoxide anion generators pyrogallol, MB and X/XO was not diminished by pre-treatment with DETCA, although it was very effective in

reducing total SOD activity in the supernatants of homogenated urethral smooth muscle. From these results, it can be argued that the presence of CU/Zn SOD in the tissue does not account for the differential effects of superoxide anion generators on nitrergic and NO-mediated responses.

Wood & Garthwaite (1994) suggested that NO can cross the neuroeffector junction in less than 1 ms, due to its extremely rapid diffusion rate. Therefore, inactivating agents must be able to reduce the half life of NO to the sub-msec range to be effective. Given the high rate of reaction of NO with superoxides to form peroxynitrite (6.7×10^9 M^{-1} s^{-}) (Huie & Padmaja, 1993), such a reduction could be achieved, especially when high concentrations of superoxides are used, such as in the present study. On the other hand, in the hypothesis of Wood & Garthwaite, 1 nM NO was considered to be the minimal effective concentration, using the rat aorta as a model for smooth muscle tissues, an assumption that cannot be applied to the urethral smooth muscle. Alternatively, under conditions of SOD inhibition by DETCA, if the nitrergic transmitter is released as free NO, it should be inhibited by extracellular superoxide anions present at the neuroeffector junction. So, inhibition by superoxides only at an intracellular site may reflect the rapid inactivation of NO as it is being formed from the neuronal NOS, thus blocking its incorporation into a carrier molecule. Also, DETCA, as a copper chelator, might have other effects on free copper ions, known to produce S-nitrosothiol decomposition (McAninly *et al*., 1993), or on some other copper-dependent enzymes which are involved in metabolizing NO-dependent compounds (Gordge *et al*., 1996), and in this way it might stabilize the nitrergic transmitter. Indeed, we have observed that treatment with DETCA produced a slight potentiation of the urethral nitrergic relaxation at low frequencies of stimulation, an effect that was even larger and extended to the whole frecuency-response curve in the presence of pyrogallol. La & Rand (1999) also found, in the rat anococcygeus, a slight increase in nitrergic relaxations after DETCA treatment and in the presence of pyrogallol. We do not have a clear explanation for this effect, although a stabilizing effect of DETCA on a NO-carrier molecule, acting as the nitrergic neurotransmitter, might lead to a reduction in the release of free NO at the neuroeffector junction and its subsequent inactivation by superoxide anions, thus increasing the postsynaptic action of the transmitter. We have previously described that the spontaneous release of free NO from different S-nitrosothiols and sodium nitroprusside was decreased in the presence of sheep urethral homogenates (Garcia-Pascual *et al*., 1999). However, in conditions favouring their homolytic cleavage to NO (addition of excess thiol and Cu^{2+}), this 'sequestering' effect of the tissue was considerably reduced, suggesting a direct transfer of NO from these compounds to tissue constituents, possibly by trans-nitrosylation reactions. On the other hand, treatment of tissues with DETCA may lead to the formation of both ferric and ferrous complexes of DETCA, which act as NO scavengers (Mulsch *et al*., 1995). Thus, another possibility is that inactivation of free NO by these DETCA complexes or by superoxide anions, following treatment with DETCA, might reduce the NO concentration in the vicinity of NOS and thus counteract the feedback inhibition of the enzyme (Garcia-Pascual *et al*., 1996), leading to an increase in the formation and release of the nitrergic transmitter.

Similarly to superoxide anion generators, NO scavengers have been proved to be more effective on responses induced by exogenous NO than on nitrergic relaxations, but discrepancies between tissues and species have also been observed. Nitrergic responses in some tissues, such as the bovine retractor penis muscle (Bowman *et al*., 1982) or the rat anococcygeus (Li & Rand, 1993), had been shown to be inhibited by oxyhaemoglobin, while in the rat gastric fundus (Jenkinson *et al*., 1995), the rat anococcygeus (La *et al*., 1996) or the sheep urethra (Garcia-Pascual & Triguero, 1994), oxyhaemoglobin differentially affected responses to exogenous NO and nitrergic stimulation. Furthermore, the NO scavenger carboxy-PTIO did not modify responses to nitrergic nerve stimulation (Li & Rand, 1999; Rand & Li, 1995b; La *et al*., 1996), with the exception of the bovine retractor penis muscle (Paisley & Martin, 1996). In the present study we show the ineffectiveness of carboxy-PTIO on urethral relaxations induced by nitrergic stimulation, while those induced by exogenous NO were significantly reduced. It is well known that oxyhaemoglobin and carboxy-PTIO, which has a molecular weight 2000 times lower, are equally effective in blocking vascular relaxations mediated by EDRF release or by exogenous addition of NO (La *et al*., 1996), indicating that both compounds are able to penetrate between the endothelium and the adjacent smooth muscle cells (50 – 100 nm). So, it would be difficult to argue that they are not able to reach the wider gap (260 nm approximately) that exists between nerve varicosities and smooth muscle cells. Furthermore, as indicated by Rand & Li (1995b), the rate of reaction of carboxy-PTIO with NO (1.01×10^4 M^{-1} s^{-1}) and the high concentration of the scavenger applied, make sure that it could reduce the effective concentration of NO at the neuroeffector junction, in spite of its extremely rapid diffusion (Wood & Garthwaite, 1994). In fact, the effective inhibition of both oxyhaemoglobin and carboxy-PTIO on nitrergic relaxation in the retractor penis muscle (Paisley & Martin, 1996), would indicate that there are not such spatial or temporal constraints.

It has been suggested that other natural antioxidants, such as ascorbic acid, present in the redox environment of the tissue or released by nerve terminals (Lilley & Gibson, 1997), may also protect NO against attack by superoxide anions and carboxy-PTIO (Lilley & Gibson, 1996). Furthermore, treatment with ascorbate led to recovery from the impairment of NO-mediated vasodilatation after oxidant stress (Dudgeon *et al*., 1998), and improved vasodilatation in essentially hypertensive patients (Taddei *et al*., 1998). It is known that ascorbic acid may reduce carboxy-PTIO to N-hydroxy-carboxy-PTIO, which cannot react with NO (Tsunoda *et al*., 1994). In fact, we have observed that carboxy-PTIO (0.1 mM) was effective in inhibiting the spontaneous release of NO from a solution of S-nitroso-L-cysteine, as measured by a selective NO-electrode (Garcia-Pascual *et al*., 1999), but the addition of ascorbic acid (1 mM) greatly reduced the inhibitory action of carboxy-PTIO (unpublished observations). In the present study, we have tried to remove extracellular ascorbate by treatment with ascorbate oxidase (Scorza *et al*., 1997). However, this treatment was ineffective in making the urethral nitrergic relaxation sensitive to the carboxy-PTIO action, suggesting that the presence of high levels of extracellular ascorbate does not account for the lack of carboxy-PTIO inhibition on nitrergic responses. These experiments must be interpreted with caution, since ascorbate and other antioxidants, like GSH or α-tocopherol, are interlinked metabolically, so changes in one can lead to compensatory changes in the others (Meister, 1994). Thus, actual measurements of ascorbate levels after depletion experiments, as well as the changes in other antioxidants, needs to be made to confirm this suggestion. On the other hand, carboxy-PTIO is also ineffective on urethral relaxations induced by several S-nitrosothiols, sodium nitroprusside and glyceryl trinitrate,

supporting the view that these 'NO donors' do not act by extracellular generation of NO (Garcia-Pascual *et al*., 1999).

Taking together, our results do not suggest that NO *per se* is released by nerve terminals in the sheep urethra. It is noteworthy that relaxations of the sheep urethra elicited by nitrergic nerve stimulation were well matched by concentrations of exogenous NO higher than 10 μM. This sensitivity is much lower than that described in other nitrergically-innervated tissues, such as the anococcygeus muscle and gastric fundus (Rand & Li, 1995a). Other differences found between the relaxation to exogenous NO and to nitrergic stimulation was that the latter was significantly inhibited by addition of L-cys, while responses to exogenous NO were unaffected. The inhibitory action of L-cys does not seem to be due to generation of superoxide anions (Jia & Furchgott, 1993), because previous addition of SOD did not prevent its action. It has been reported that L-cys may have a more complex effect given its ability to complex metal ions present in the buffer (Feelisch *et al*., 1994), leading to S-nitrosothiols stabilization in solution, or it may also, in combination with a NO adduct, promote trans-nytrosylation reactions and the subsequent formation of the less stable S-nitroso-L-cysteine (Keshive *et al*., 1996). This latter action of L-cys may convert a large proportion of the transmitter to NO, therefore reducing its post-synaptic relaxant activity.

Involvement of thiols in the urethral nitrergic relaxation has been studied by the use of different thiol modulators. However, none of these agents, with the exception of NEM, had any effect on nitrergic responses or NO-dependent relaxations. Furthermore, the effect of NEM seems to reflect unspecific actions of this compound on intracellular kinases or phosphatases, since it also reduced the contraction to NA and inhibited, in a similar degree, the relaxations induced by several S-nitrosothiols, sodium nitroprusside and glyceryl trinitrate in the sheep urethra (García-Pascual *et al*., 1999). The fact that L-cys abolished the inhibitory effect of NEM could be interpreted by prevention of the interaction of NEM with thiols. Thus, our results do not favour the view that thiols are involved in mediating urethral nitrergic neurotransmission. However, since cellular thiol pools are tied to each other and to other metabolic pathways, together with the fact that not all sulphydryl groups are equally accessible to oxidant agents, it can not be excluded that the thiol modulators used, and in the concentration tested, would not be able to interact with the specific thiol group involved in the synthesis and/or post-synaptic effect of the nitrergic transmitter. In accord with our results, DeMan *et al*., (1996a) have found, in the rat gastric fundus, that the thiol modulators buthionine sulphoximine, ethacrynic acid, sulphobromophtaleine and diamide did not affect responses to NANC nerve stimulation or NO. Moreover, the addition of Cu^{2+}, which enhanced relaxation to S-nitrosothiols, did not affect responses to nitrergic stimulation, suggesting that the nitrergic neurotransmitter is not a S-nitrosothiol (DeMan *et al*., 1996b). Other studies, however, propose the involvement of some NO adduct as the nitrergic mediator (Barbier & Lefebvre, 1994; Göçmen *et al*., 1998).

It is conceivable that the nitrergic mediator may not be the same in all nitrergically-innervated tissues, which could account for the subtle variations observed in different species and tissues. Alternatively, NO produced in nitrergic terminals might not act as a true transmitter, but as a neuromodulator, promoting the release of another NANC neurotransmitter, which could be resistant to extracellular chemical inactivators and scavengers of NO. In this sense, presynaptic NO-induced release of VIP, has been shown in rat and rabbit stomach (Jin *et al*, 1996). This type of 'in cascade' neurotransmission might produce a nitrergically-induced response, being susceptible of NOS inhibition, but that would not be mediated by NO movements across the neuroeffector junction. In this sense, it is noteworthy that NO does not induce hyperpolarization of urethral smooth muscle cells from the rabbit urethra (Waldeck *et al*, 1998), while it is a common feature to NO and NANC stimulation in gastrointestinal smooth muscles (Sneddon & Graham, 1992). Furthermore, a recent report in rabbit urethral smooth muscle has showed that, under phosphodiesterase inhibition and stimulation by sodium nitroprusside, cyclic GMP-immunoreactivity was evident in a spindle-shaped cell population, which form[5] a network around and between the smooth muscle bundles, but was absent in the smooth muscles cells (Waldeck *et al*., 1998). These spindle cells may represent the target for NO with a yet unknown function. We have found, in the present study, another difference between relaxant responses to nitrergic stimulation and NO. Thus, while the former were abolished by the specific guanylate cyclase inhibitor ODQ, responses to NO were much less affected. Indeed, we have previously reported that, for the same level of relaxation, NO induced a 15 times higher increase in cyclic GMP values than NANC stimulation (García-Pascual & Triguero, 1994). These results may be explained by considering that relaxation is brought about by small increases in cyclic GMP, and that higher levels of the nucleotide are not able to induce further relaxation; thus exogenous NO, which has access to the whole tissue, produce such a high level of cyclic GMP that could overcome the inhibition by ODQ, reaching enough cyclic GMP accumulation to trigger relaxation. Alternatively, a contribution of additional mechanisms, independent of cyclic GMP in the NO actionn cannot be discarded (Weisbrod *et al*., 1998). In contrast, nitrergic stimulation might produce a more localized rise in cyclic GMP, which could be completely inhibited by ODQ. The above mentioned spindle-shaped cells described by Waldeck *et al*. (1998) in the rabbit urethra could be a likely candidate for such localized cyclic GMP increases, although this possibility should be further studied.

In summary, our results show that nitrergically-induced relaxations of the sheep urethra, in contrast to those elicited by exogenous NO, are resistant to extracellular superoxide anion generators (even after Cu/Zn SOD depletion by DETCA treatment) as well as to the direct NO scavenger carboxy-PTIO (even after treatment with ascorbate oxidase). These results do not favour the view that endogenous antioxidants are protecting neuronally released NO from inactivation. Furthermore, nitrergic responses, but not those induced by NO, were inhibited by L-cys and completely abolished by ODQ, although both relaxations were not similarly affected by different thiol modulators. Our results do not support the view that NO *per se* is released by nerve terminals in the sheep urethra; whether some NO adduct, or another neurotransmitter is acting as the nitrergic mediator remains an open question.

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