Relaxation mechanisms induced by stimulation of nerves and by nitric oxide in sheep urethral muscle

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1. Isolated transverse and longitudinally oriented preparations of sheep urethra precontracted with noradrenaline responded to electrical field stimulation (EFS) with stimulus-dependent non-adrenergic, non-cholinergic (NANC) relaxations.

2. Exogenous nitric oxide (NO) (acidified $NaNO_2$), *S*-nitroso-L-cysteine (NC), sodium nitroprusside (SNP), 8-Br-cGMP, dibutyryl-cAMP, forskolin and isoprenaline each relaxed precontracted transverse urethral preparations in a concentration-dependent manner in order of potency: NC > forskolin > isoprenaline = SNP > NO > 8-Br-cGMP = dibutyryl-cAMP. Longitudinally oriented preparations responded to NO and NC with concentration-dependent relaxation, no different from that observed in transverse strips.

3. Methylene blue (MB) and oxyhaemoglobin (HbO_2) each shifted the concentration–response curve for NO to the right without affecting EFS-induced relaxation. Similarly, concentration-dependent responses to NC were not affected by MB. The inhibition of relaxation to NO by MB was prevented by superoxide dismutase, suggesting the inhibition was caused by extracellular generation of superoxide anions.

4. EFS-induced relaxation was accompanied by elevation of cGMP. However, for the same level of relaxation, exogenous NO and NC induced 15- and 23-times higher increases in cGMP values, respectively, than EFS. cAMP levels were not affected by EFS- or NO-induced relaxation, although a large increase accompanied relaxation induced by forskolin. Forskolin also increased cGMP content.

5. Pretreatment with MB reduced basal levels of cGMP and inhibited both relaxation and rise in cGMP levels induced by NO. SNP-elicited relaxant responses, in the presence of MB, were accompanied by an accumulation of cGMP; cAMP levels were unaffected. MB reduced cGMP levels induced by NC, while the relaxant response was unchanged.

6. In urethral preparations prelabelled with [^{3}H]myoinositol, exposure to NA caused an accumulation of [^{3}H]inositol phosphates, which was unaffected by pretreatment with 8-Br-cGMP or dibutyryl-cAMP.

7. EFS failed to induce a relaxant response in excess $[K^+]_o$-contracted preparations, while relaxation with exogenous NO was unaffected. Ouabain abolished EFS-induced relaxation and reduced responses to NO. Neither TEA nor glibenclamide affected relaxation to either EFS or NO.

8. Relaxation elicited by SNP was not accompanied by any change in cGMP or cAMP levels, and was unaffected by MB, HbO_2, K^+ channel blockers (TEA and glibenclamide), ouabain or high $[K^+]_o$ solution. This suggested that relaxation was caused by a mechanism independent of NO generation.

9. A dense network of NADPH diaphorase-positive fibres associated with both the circular and longitudinal smooth muscle layers of sheep urethra was found.

10. The possibility that a nitrosyl compound which releases NO in the target tissue is the NANC transmitter in sheep urethra is discussed. The data also suggest a role for increased cGMP levels in the relaxation mechanisms, whereas changes in cAMP levels, phosphoinositide hydrolysis or K^+ channel activation do not seem to be involved.

Urinary bladder voiding has been considered classically as a passive process arising from an increase in intravesical pressure. However, an initial drop in urethral pressure, followed by an increase in intravesical pressure, has been demonstrated in man during micturition (Tanagho & Miller, 1970), suggesting an active role for the urethra in this mechanism. A non-adrenergic, non-cholinergic (NANC) relaxation, unaffected by α- and β-adrenoceptor or muscarinic antagonists, but blocked by tetrodotoxin (TTX) and nitric oxide (NO)-synthesis inhibitors, has been described in isolated urethral preparations from various species in response to electrical field stimulation (EFS) (Andersson, García-Pascual, Forman & Tøttrupt, 1991; Dokita, Morgan, Wheeler, Yoshida, Latif-Pour & Weiss, 1991; García-Pascual, Costa, García-Sacristan & Andersson, 1991; Andersson, García-Pascual, Persson, Forman, & Tøttrupt, 1992; Persson & Andersson, 1992; Persson, Igawa, Mattiasson & Andersson, 1992; Thornbury, Hollywood & McHale, 1992). Furthermore, in conscious rats, blockade of the L-arginine–NO pathway induced bladder hyperactivity (Persson *et al.* 1992), an effect attributed to an inhibition of NO formation at the bladder outlet region, since a role for NO in the detrusor itself has not been established (Persson *et al.* 1992). These observations suggest that the L-arginine–NO pathway in the outlet region (bladder base, bladder neck and urethra) may be of pathophysiological importance in controlling the micturition reflex.

At present, it is not clear if NO is simply stored in and released from autonomic nerves or whether some intermediate compound, that generates NO on interacting with smooth muscle, is formed. *S*-Nitrosothiols have been considered recently as potential precursors of endothelium-derived NO (Myers, Minor, Guerra, Bates & Harrison, 1990). Furthermore, in sheep urethral smooth muscle, *S*-nitroso-L-cysteine (NC) induces a relaxant response similar to the effects of nerve stimulation (Thornbury *et al.* 1992), raising the possibility that a nitrosyl compound may be the proposed NO-derived transmitter.

It is generally accepted that NO and compounds such as sodium nitroprusside (SNP), which probably act through formation of NO, induce a smooth muscle relaxation associated with increased levels of cGMP (Ignarro, 1990). The role of changes in cGMP production in urethral relaxation induced by EFS of NANC nerves has not yet been determined. Furthermore, while it is assumed that NO increases cGMP levels, little is known about the transduction mechanisms within the smooth muscle which mediate the relaxant response, although cGMP is thought to reduce free intracellular calcium levels (Lincoln, 1989) and to inhibit phosphoinositide hydrolysis (Rapoport, 1986). In keeping with these observations, both a membrane hyperpolarization, through an increase in potassium conductance, and an activation of guanylate cyclase have been observed in gastrointestinal smooth muscle preceding relaxation induced by NO (Du, Murray, Bates & Conklin, 1991). On the other hand, NO-mediated smooth muscle relaxation concomitant with increases in level of cAMP has also been described (Ignarro, Harbison, Wood & Kadowitz, 1986; Brayden & Wellman, 1989; Heuzé-Joubert, Mennecier, Simonet, Laubie & Verbeuren, 1992).

The purpose of the present study was to clarify further whether or not NO is responsible for relaxation induced by EFS of NANC nerves in sheep urethra and to analyse the underlying mechanisms.

METHODS

Tissue preparation

Lower urinary tracts from female lambs (2–3 months old) were collected at the local slaughterhouse shortly after the animals 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; $NaHCO_3$, 24·9; KH_2PO_4, 1·4; ethylenediaminetetraacetic acid disodium salt (Na_2EDTA, Merck), 0·07; and glucose, 11. Specimens were transferred to Petri dishes containing Krebs solution at room temperature (20 °C). The urethra was taken out, opened longitudinally and the mucosa, periurethral fat and connective tissue removed by sharp dissection. Transverse or longitudinal strips (approximately 1 × 1 × 5 mm) were taken from the proximal urethra. Male Sprague–Dawley rats (250–350 g) were stunned and killed by cervical dislocation. The iliac arteries were removed and cut into rings of 2 mm length. All preparations were investigated on the day the animals were killed.

Recording of mechanical activity

The strips of urethral tissue 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 polygraph model 7D (Grass Instruments, Quincy, MA, USA). During the equilibration period (approximately 60 min) the resting tension was adjusted to 5–6 mN. The arterial rings were suspended on two intraluminal parallel wires (diameter 100 μm), introduced into organ baths containing Krebs solution, and connected to a Pioden strain gauge (Pioden UF1, UK) for isometric tension measurement. Rings were allowed to equilibrate for 90 min at a tension of 15 mN.

Electrical field stimulation

Preparations were stimulated with a Cibertec CS20 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 0·5–12 Hz were delivered at 2 min intervals. The voltage was supramaximal (current strength 75 mA) and the train duration was 5 s. Thus changes in the number of pulses also occurred at each frequency change.

Experimental procedure

Relaxant responses to EFS (supramaximal voltage, 0·5–12 Hz, 0·8 ms for 5 s, every 2 min), NO (present in acidified solutions of $NaNO_2$, 0·1–30 mM), NC (0·1–30 μM), SNP (0·1 μM to 1 mM), forskolin (0·1–100 μM), 8-Br-cGMP (1 μM to 1 mM), dibutyryl-

cAMP (1 μM to 1 mM) and isoprenaline (0·1–300 μM) were recorded in tissues precontracted with noradrenaline (NA, 50 μM). When the maximum contraction had been reached, stimulus–response, and cumulative concentration–relaxation curves were constructed. In some experiments, single responses to submaximal frequencies of EFS or to submaximal concentrations of NO and SNP were employed. When the effects of methylene blue (MB), oxyhaemoglobin (HbO_2), K^+ channel blockers or ouabain were studied, the drugs were added at the concentrations indicated 10–30 min before a second contraction–relaxation procedure was performed. From each animal, one preparation not receiving any treatment was always tested in parallel to study the reproducibility of the relaxation.

Determination of cyclic GMP and AMP levels

Cyclic nucleotide determinations were made in urethral preparations equilibrated under tension and precontracted with NA (50 μM). Tissues were quickly frozen at the point of maximum relaxation induced by EFS, NC, NO, SNP or forskolin, by means of a stainless-steel clamp cooled in liquid nitrogen. Tone was monitored until the time of freezing. In some experiments MB (10 μM) was added and incubated for 30 min before exposure to the relaxing agents. The frozen segments were homogenized in trichloroacetic acid (1 ml TCA, 6 %) at 4 °C and centrifuged (2500 *g* for 15 min). Supernatants were collected and TCA extracted four times with 5 ml of water-saturated ether. The final aqueous phase was evaporated to dryness at 70–80 °C under a stream of nitrogen gas, and the residue was resuspended in 500 μl of 0·05 M sodium acetate buffer, pH 6·2. The concentration of cGMP and cAMP was determined in 100 μl aliquots by radioimmunoassay with commercial kits following the instructions of the manufacturer (New England Nuclear, Boston, MA, USA) for acetylated samples. The recovery of cGMP and cAMP was at least 90 %. The protein content of the TCA precipitate was quantified (Lowry, Rosebrough, Farr & Randall, 1951) using bovine serum albumin as standard and cyclic nucleotide levels were expressed as pmoles per milligram protein. Endothelium-intact vascular rings from rat iliac arteries, precontracted by prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$, 1 μM), were processed by the same method as described for the urethral preparations and cGMP levels in response to acetylcholine (10 μM) measured. Increases in cGMP levels induced by acetylcholine in this preparation were used as a positive control of the assay.

Measurement of inositol phosphates formation

[^{3}H]Inositol phosphates were measured as described by Emilsson & Sundler (1984) with minor modifications. Briefly, pieces of urethral tissue (15–20 mg) were preloaded with 15 μCi myo-2-[^{3}H]inositol (specific activity 20 Ci mmol^{-1}, New England Nuclear, Boston, MA,USA) in 3 ml of Krebs solution gassed with 95 % O_2 and 5 % CO_2 at 37 °C for 8 h. Unincorporated isotope was removed by washing for 30 min with fresh Krebs solution. In order to inhibit inositol-1-phosphatase, tissues were exposed to NA (50 μM) for 1 h, after pretreatment with LiCl (10 mM) for 30 min. In some experiments, 8-Br-cGMP (1 mM) or dibutyryl-cAMP (1 mM) was added 30 min before and during application of NA. The reaction was stopped by addition of 2 ml of a chloroform : methanol : 12 M HCl (1 : 1 : 0·02) mixture containing 0·05 % butyrated hydroxytoluene (Sigma, St Louis, MO, USA) as an antioxidant. Extraction and separation were performed as previously described (García-Pascual, Persson, Holmquist & Andersson, 1993). Total [^{3}H]inositol phosphates, comprising inositol mono-, di- and trisphosphate, were separated from the aqueous phase on propylene columns containing 1 ml anion exchange resin (formate form, AG1-X8, 200–400 mesh, Bio Rad Laboratories, Richmond, CA, USA) and aliquots were counted in an LKB model 1214 liquid scintillation spectrometer (Pharmacia, Wallac, Finland) at efficiency of about 50 %. Total ^{3}H content was determined from aliquots of the incubation medium, the tissue (dissolved in 1 ml Soluene-100, Packard, The Netherlands, at 60 °C for 30 min), and the different extracts. The measured accumulation of [^{3}H]inositol phosphates was related to the total ^{3}H content of the preparation.

Histochemistry for reduced nicotinamide–adenine dinucleotide phosphate (NADPH) diaphorase

Reduced nicotinamide–adenine dinucleotide phosphate (NADPH) diaphorase activity was localized histochemically (Dawson, Bredt, Fotuhi, Hwang & Snyder, 1991). The entire urethra was removed and immersed in 4 % paraformaldehyde in 0·1 M sodium phosphate-buffered saline (PBS, pH 7·3) at 4 °C, immediately after death and maintained in the fixative for 24 h. The tissue was subsequently placed in a cryoprotective PBS solution containing 15 % sucrose for 24 h at 4 °C. From a piece of the urethra frozen in dry ice, transverse sections (40 μm), comprising the whole urethral wall, were cut on a cryotome and extensively rinsed in PBS. The NADPH diaphorase reaction was performed by incubating in the dark at 37 °C for 30 min in 0·1 M PBS (pH 8·0) containing 1 mM β-NADPH, 0·5 mM Nitroblue Tetrazolium and 0·3 % Triton X-100. After incubation, sections were rinsed in PBS, mounted in slides pretreated with 10 % poly-L-lysine and viewed with bright field illumination.

Drugs

The following drugs were used: (±)-noradrenaline hydrochloride (Serva, Heidelberg, Germany), 8-Br-cyclic GMP, L-cysteine, dibutyryl-cyclic AMP, forskolin, glibenclamide, haemoglobin (bovine), (±)-isoprenaline sulphate, methylene blue, N^G-nitro-L-arginine, ouabain octahydrate, superoxide dismutase (bovine erythrocytes), tetraethylammonium chloride, tetrodotoxin, $NaNO_2$ (Sigma, St Louis, MO, USA); sodium nitroprusside (Merck, Darmstadt, Germany). Drugs were dissolved in distilled water except glibenclamide, which was dissolved in dimethyl sulphoxide. Stock solutions were prepared and then stored at −20 °C. Subsequent dilutions were made with 0·9 % NaCl containing 1 mM ascorbic acid. To study the effects of NO, an acidified solution of $NaNO_2$ was used (Furchgott, 1988). The $NaNO_2$ solution was freshly prepared by adding concentrated HCl until pH 2 was reached and the volume adjusted to a final concentration of 1 M. This stock solution was maintained cold and protected from air. Subsequent dilutions were made in diluted HCl (pH 2) immediately before use and added in volumes of 5–100 μl. Separate experiments showed that equivalent volumes of the acid solution, used as the vehicle for $NaNO_2$, did not affect the preparations. Non-acidified $NaNO_2$ (pH 7·4, i.e. NO_2^- above 3 mM) caused delayed (> 30 s), slow (time to peak > 3 min), persistent relaxations, whose amplitude was approximately 25 % of those elicited by acidified $NaNO_2$ at the same concentrations (not shown). Thus, relaxant responses to NO were measured no later than 30 s after addition, to avoid the direct effect of NO_2^-. Sodium nitroprusside was maintained in the dark to minimize light-induced degradation.

S-Nitroso-L-cysteine (NC) was prepared by reacting L-cysteine with sodium nitrite under acidic conditions (Field, Dilts, Ravichandran, Lenhert & Carnahan, 1978). To a reactant solution containing: 1 M HCl, methanol and concentrated H_2SO_4 (5:5:0·2), $NaNO_2$ (100 mM) and L-cysteine (50 mM) were added. Following reaction, the solution became red and exhibited a visible absorption maximum at 544 nm, characteristic of *S*-nitrosothiols. NC concentration was spectrophotometrically determined at this wavelength, assuming a molar absorptivity coefficient of 16·6 (Kowaluk & Fung, 1990). The HCl–MeOH–H_2SO_4 solution (pH close to zero) relaxed the urethra in volumes of 10 μl or more. Therefore, working dilutions were prepared in Krebs buffer immediately before use, kept on ice and added as 10–30 μl aliquots to 5 ml organ baths. A blank reactant solution, containing all the components except L-cysteine, was prepared and diluted in Krebs buffer in an identical manner. A relaxant response to the blank solution was observed when a dilution equivalent to 50 μM NC was reached. This relaxation could be ascribed to the release of NO from $NaNO_2$ in acidic medium. Thus, the maximum NC concentration tested was 30 μM, avoiding the effect of the remaining sodium nitrite in the NC solutions. Oxyhaemoglobin was prepared (Martin, Villani, Jothianandan & Furchgott, 1985) from a 1 mM solution of commercial haemoglobin (which contains a mixture of oxyhaemoglobin and the oxidized derivative methaemoglobin) by addition of a 10-fold molar excess of sodium dithionite ($Na_2S_2O_4$). The reducing agent was then removed by dialysis in 12 l of distilled water, and gassed with N_2 at 4 °C. The purity of the solutions of oxyhaemoglobin was determined spectrophotometrically.

Analysis of data

Results were normalized by expressing relaxation as a fraction of tension induced by the contractile agonist. Normalized results were expressed as a percentage of the maximum relaxation obtained during the first response of each preparation and given as means ± S.E.M. EC_{50} and EF_{50} values (concentration or stimulus strengths producing a 50 % relaxation of the maximum contraction) were determined graphically and expressed as geometric means with their 95 % confidence intervals. 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. *n* denotes the number of preparations (all from different animals).

RESULTS

Relaxations in response to electrical field stimulation, nitric oxide, sodium nitroprusside, *S*-nitroso-L-cysteine and cyclic nucleotide-elevating agents

Transverse urethral preparations were precontracted with NA (50 μM). This concentration of NA produced a stable increase in tone (mean 20·9 ± 2·3 mN, $n = 64$) equivalent to 55–60 % of the maximum response to this agonist. EFS (5 s duration, 0·5–12 Hz) of NA-contracted strips produced stimulus-dependent, transient relaxations (time to peak of 10–12 s). A maximum response of 65·7 ± 3·5 % ($n = 16$) was obtained at 8 Hz ($EF_{50} = 2·68 \pm 0·86$ Hz). Longitudinal preparations contracted by NA (50 μM) (19·8 ± 1·2 mN) also responded with reproducible stimulus-dependent relaxations to EFS. The maximum relaxant response comprised 81·2 ± 7·3 % ($n = 6$) of the NA-induced increase in tension ($EF_{50} = 1·4 \pm 0·2$ Hz). No differences were found in stimuli dependence between circularly and longitudinally oriented preparations and all responses were abolished by tetrodotoxin (1 μM) and by N^G-nitro-L-arginine (0·1 mM) (not shown).

Exogenously added NO (present in acidified solutions of $NaNO_2$), NC, SNP, 8-Br-cGMP, dibutyryl-cAMP, forskolin and isoprenaline each concentration-dependently relaxed transverse urethral preparations precontracted with NA. The rank order of potency was: NC > forskolin > isoprenaline = SNP > NO > 8-Br-cGMP = dibutyryl-cAMP (Table 1). Responses to NO and NC were rapid in onset, transient (time to peak of 20–30 s) and rapidly returned to the preapplication level (Figs 1 and 3), while

Table 1. Relaxant effects of nitric oxide (NO, acidified $NaNO_2$) *S*-nitroso-L-cysteine (NC), 8-Br-cGMP, dibutyryl-cAMP (d-cAMP), sodium nitroprusside (SNP), isoprenaline (Iso) and forskolin (Forsk) on noradrenaline (50 μM)-contracted urethral preparations

	n	$EC_{50} \times 0·1$ mM [a]	E_{max} [b]
NO	10	125 (79–199)[efg]	68·4 ± 4·8 (30 mM)
NC	14	0·12 (0·07–0·20)[def]	74·6 ± 5·6 (30 μM)
8-Br-cGMP	8	[c]	36·6 ± 8·1 (1 mM)
d-cAMP	9	[c]	48·8 ± 6·7 (1 mM)
SNP	14	1·5 (0·6–3·6)[dg]	78·5 ± 4·5 (3 mM)
Isoprenaline	7	0·97 (0·5–1·9)[dg]	83·2 ± 7·8 (0·3 mM)
Forskolin	7	0·18 (0·07–0·4)[def]	81·7 ± 3·9 (0·1 mM)

[a] EC_{50}, concentrations causing 50 % of the maximum relaxation of induced contraction. Means with associated 95 % confidence intervals. [b] E_{max}, maximum relaxation obtained at the highest drug concentration used (indicated between brackets). Means ± S.E.M. [c] Agents failed to produce 50 % relaxation. *n* denotes number of animals tested. [d] Significantly different ($P < 0·001$) when compared with NO. [e] Significantly different ($P < 0·05$) when compared with SNP. [f] Significantly different ($P < 0·01$) when compared with isoprenaline. [g] Significantly different ($P < 0·05$) when compared with forskolin and NC.

relaxations to the other agents were slower and sustained (SNP had a time to peak of 2–3 min, forskolin and isoprenaline of 4–6 min and the cyclic nucleotide analogues 8-Br-cGMP and dibutyryl-cAMP needed 5–15 min to gain a stable level of tension). NO and NC each induced transient and concentration-dependent relaxation in longitudinally oriented preparations, which did not differ significantly from those observed in transverse strips. The maximum effect was $53{\cdot}0 \pm 13{\cdot}8$ % at 10 mM and $76{\cdot}0 \pm 9{\cdot}4$ % at 30 μM, of the NA-induced tone, for NO and NC, respectively (EC_{50} for NO = 10·5 (3·5–31·6) mM; EC_{50} for NC = 10·2 (4·1–25·1) μM; $n = 6$). Unless otherwise indicated, the rest of the experiments were made on transverse urethral preparations since no differences were found between the responses of circularly and longitudinally oriented strips.

Effects of methylene blue and oxyhaemoglobin on relaxations induced by electrical field stimulation, nitric oxide, *S*-nitroso-L-cysteine or sodium nitroprusside

Pre-incubation (30 min) with methylene blue (MB) (10 μM) did not affect either the resting tension of the urethral preparations or the contractile response to NA (50 μM), but significantly inhibited dose-dependent relaxations induced by exogenous NO, reducing its maximum response to 29 ± 3 % of control values ($n = 6$, $P < 0{\cdot}001$). In contrast, at the same concentration, MB failed to affect significantly stimulus- and dose-dependent relaxant responses to EFS, NC and SNP (Figs 1, 2 and 3).

Oxyhaemoglobin (HbO_2, 3–10 μM for 10 min) did not change resting tension but reduced (by 10 % at 3 μM, $P < 0{\cdot}05$, and by 24 % at 10 μM, $P < 0{\cdot}05$) the level of tension produced by NA (50 μM), and when added on top of contraction caused a progressive fall in tension. Short periods of pre-incubation were used (10 min) and relaxant responses were evaluated in relation to the degree of muscle tension in each moment to exclude interferences of the spontaneous decrease in tone with the measured drug-induced responses (García-Pascual *et al.* 1991).

Oxyhaemoglobin produced a concentration-dependent rightward shift of concentration–relaxation curves to NO (Fig. 4). The inhibition was overcome with higher concentrations of NO with no change in maximum relaxation. However, stimulus-dependent relaxation induced by EFS was not significantly inhibited by HbO_2. Relaxant responses induced by a submaximal concentration of SNP (0·1 mM) were also unaffected by pretreatment with HbO_2 (10 μM). SNP-induced relaxation was $95{\cdot}1 \pm 6{\cdot}3$ % ($n = 6$) and $93{\cdot}4 \pm 2{\cdot}9$ % ($n = 6$) of control values, in the absence and presence of HbO_2, respectively. Furthermore,

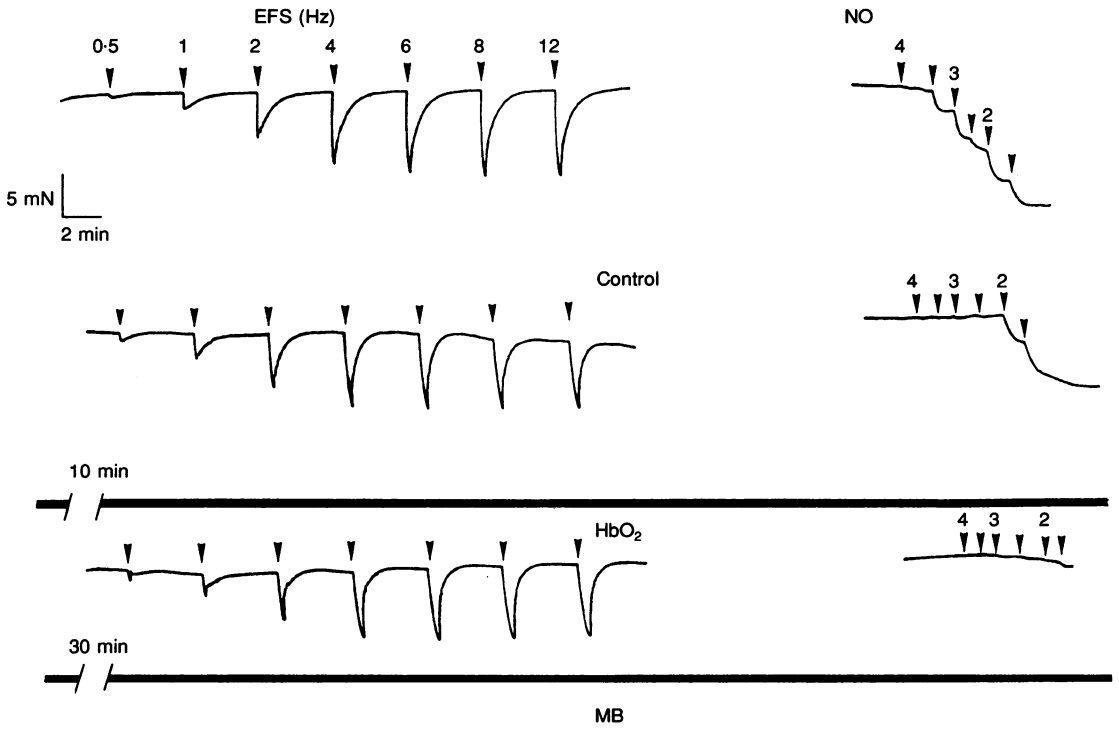

Figure 1. EFS- and NO-induced relaxations are dissimilarly affected by MB and HbO_2
Differential effects of oxyhaemoglobin (HbO_2, 10^{-5} M) and methylene blue (MB, 10^{-5} M) on responses to electrical field stimulation (EFS) and to exogenously added nitric oxide (NO, present in acidified solutions of $NaNO_2$) (−log M) in sheep urethral muscle. Preparations were precontracted by noradrenaline (50 μM).

Table 2. Effect of methylene blue (MB) on the cyclic nucleotide accumulation induced by electrical field stimulation (EFS), nitric oxide (NO, acidified $NaNO_2$), *S*-nitroso-L-cysteine (NC), sodium nitroprusside (SNP) and forskolin (Forsk) in urethral preparations precontracted with noradrenaline (NA, 50 μM)

Treatment	*n*	cGMP (pmol (mg protein)$^{-1}$)	cAMP (pmol (mg protein)$^{-1}$)	Relaxation (%)
Control (NA 50 μM)	7	2·8 ± 0·52	56 ± 9·2	—
MB (10 μM)	7	1·3 ± 0·23*	—	—
EFS (2 Hz, 5 s)	8	2·2 ± 0·6	60 ± 3·7	28 ± 2·9
EFS (4 Hz, 5 s)	8	5·1 ± 0·53*	64 ± 13·4	40 ± 2·0
EFS (8 Hz, 5 s)	8	5·7 ± 1·06**	58 ± 7·2	66 ± 4·5
EFS (2 Hz, 60 s)	8	6·8 ± 1·22**	70 ± 19·8	66 ± 6·5
NO (6 mM)	8	101·3 ± 33***	69 ± 7·1	65 ± 4·8
NO + MB (10 μM)	8	27·3 ± 8·1***	68 ± 7·7	22 ± 3·5
NC (30 μM)	7	154·2 ± 52***	—	77 ± 3·3
NC + MB (10 μM)	7	7·4 ± 2·5**	—	65 ± 7·0
SNP (0·1 mM)	8	3·9 ± 0·8	69 ± 6·3	57 ± 2·0
SNP + MB (10 μM)	8	25·2 ± 8·1***	82 ± 10·1	67 ± 5·6
Forsk (0·1 mM)	8	8·3 ± 1·1***	1503 ± 158***	78 ± 5·9

Values are means ± S.E.M.; *n*, number of preparations taken from different animals. Tissues were incubated with MB for 30 min. Relaxation is expressed as the percentage decrease of the maximal contraction with NA (50 μM). Equiactive concentrations of NO, NC, SNP and Forsk, giving a relaxation of 60–70 %, were chosen. Tissues were frozen at the point of maximum relaxation (8 ± 1 s for EFS; 25 ± 5 s for NO; 33 ± 2 s for NC; 2·0 ± 0·2 min for SNP and 4·5 ± 0·5 min for Forsk). $*P < 0·05$, $**P < 0·01$, $***P < 0·001$ (significantly different from control values).

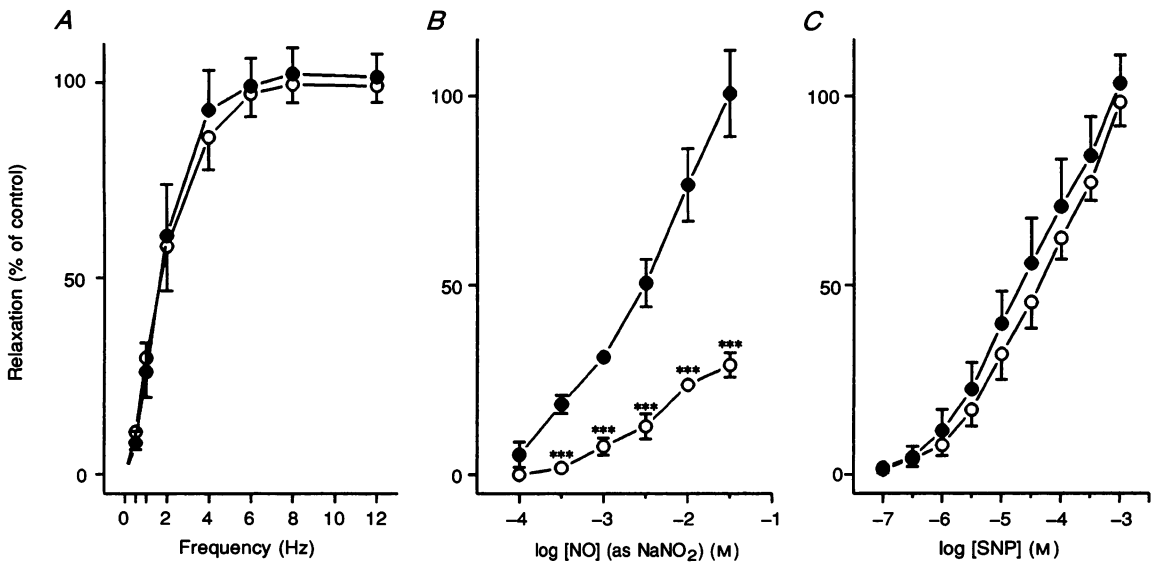

Figure 2. MB inhibits relaxant responses to NO without affecting those to either EFS or SNP
The effect of methylene blue (MB) on the relaxation induced by electrical field stimulation (5 s at different frequencies) (*A*), nitric oxide (acidified solutions of $NaNO_2$) (*B*) and, sodium nitroprusside (*C*), in sheep urethral preparations precontracted by noradrenaline (50 μM). The preparations were pretreated with MB (10 μM) for 30 min. Results are expressed as a percentage of the maximum relaxation before treatment and given as means ± S.E.M. ($n = 6$–8). ●, control experiments; ○, in the presence of 10 μM MB. $***P < 0·001$.

when added to preparations relaxed by SNP, HbO_2 (10 μM) did not reverse relaxation and even caused a further decrease in tension (not shown).

Effect of superoxide dismutase on the response to methylene blue

To investigate the possible role of superoxide anion (O_2^-) in mediating the inhibitory action of methylene blue (MB) on NO-elicited relaxation in sheep urethra, superoxide dismutase (SOD, 200 U ml^{-1}) was added 5 min before MB (10 μM). SOD prevented the inhibitory effects of MB on dose-dependent relaxation to NO at low concentrations (Fig. 5). At the highest NO concentration, the control responses and the relaxation in the presence of MB plus SOD were not significantly different ($P > 0.05$, $n = 6$–8). This indicates that part of the effect elicited by MB may be related to the formation of O_2^-. In the absence of MB, SOD did not modify the response to NO (not shown).

Cyclic nucleotide levels and relaxation to electrical field stimulation, nitric oxide, *S*-nitroso-L-cysteine and sodium nitroprusside; effects of methylene blue

Table 2 shows the levels of cGMP and cAMP measured in sheep urethral preparations precontracted with NA (50 μM), at the point of maximum relaxation induced by EFS (2–8 Hz, 8 ± 1 s, $n = 24$), NO (6 mM, 25 ± 5 s, $n = 16$), NC (30 μM, 33 ± 2 s, $n = 14$), SNP (0·1 mM, 2·0 ± 0·2 min, $n = 16$) and forskolin (0·1 mM, 4·5 ± 0·5 min, $n = 8$). The drug concentrations used were those inducing 60–70 % relaxation of NA-elicited tone.

Pretreatment, for 30 min, with MB (10 μM) significantly reduced basal levels of cGMP to approximately 50 % of control values (Table 2). This reduction was not accompanied by variations in the level of tension induced by NA (50 μM).

Relaxation induced by EFS was accompanied by stimulation-related elevations in cGMP; cAMP levels were

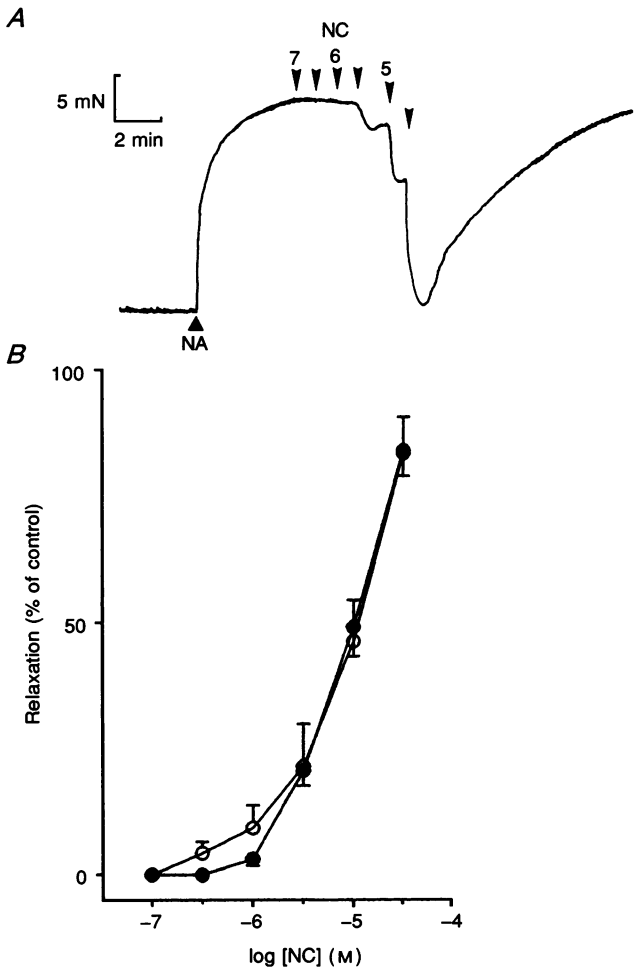

Figure 3. NC induces transient, concentration-dependent, relaxations that are unaffected by MB
A, relaxant response induced by cumulative addition of concentrations of *S*-nitroso-L-cysteine (NC, −log M) on sheep urethra precontracted by noradrenaline (NA, 50 μM). *B*, dose–response curves showing the effect of methylene blue (MB) on the relaxation induced by *S*-nitroso-L-cysteine in sheep urethral preparations contracted by noradrenaline (50 μM). The preparations were pretreated with MB (10 μM) for 30 min. Results are expressed as a percentage of the maximum relaxation before treatment and given as means ± S.E.M. ($n = 7$). ●, control experiments; ○, in the presence of 10 μM MB.

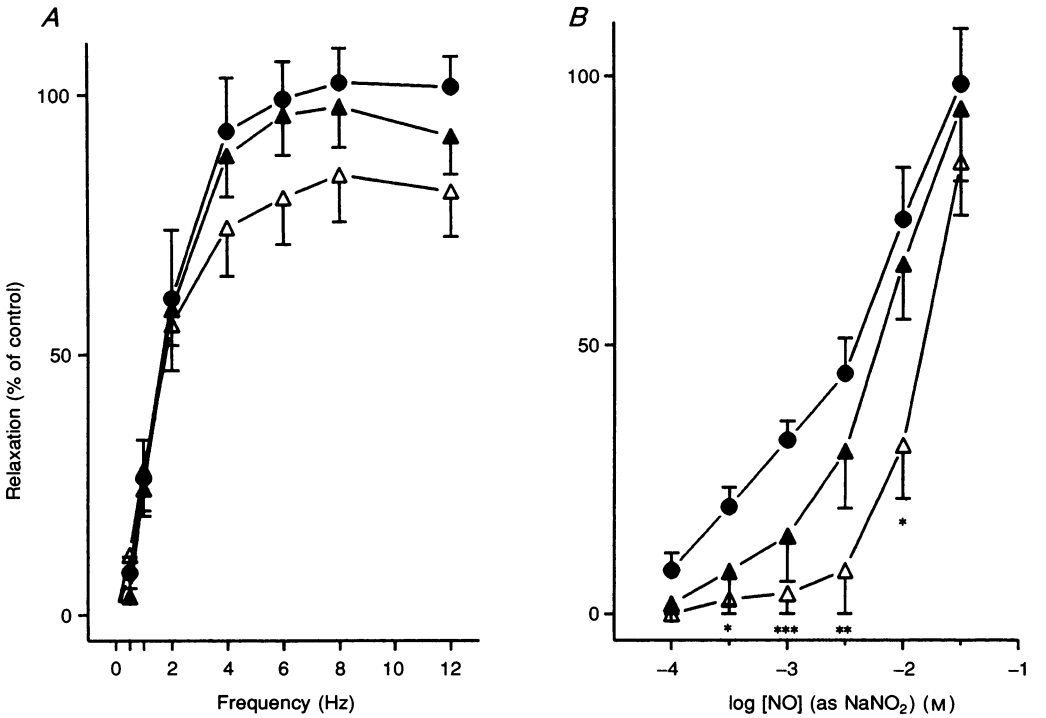

Figure 4. Relaxation, induced by NO but not by EFS, is inhibited by HbO_2

Stimulus–response and concentration–response curves showing the effect of oxyhaemoglobin (HbO_2) on the relaxation induced by electrical field stimulation (5 s at frequencies shown) (*A*) and nitric oxide (acidified solutions of $NaNO_2$) (*B*), in sheep urethral preparations precontracted by noradrenaline (50 μM). The preparations were pretreated with HbO_2 (5 or 10 μM) for 10 min. Results are expressed as a percentage of the maximum relaxation before treatment and given as means ± S.E.M. ($n = 4$–8). ●, control experiments; ▲, in the presence of 5 μM HbO_2; △, in the presence of 10 μM HbO_2. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

unaltered (Table 2). Increases of cGMP were slight but significant at frequencies above 2 Hz (a 2-fold increase from basal levels was observed at 8 Hz). Neither the maximum relaxation nor the cGMP accumulation elicited by EFS was significantly elevated by increasing the stimulation time to 60 s (2 Hz), although the relaxant response was maintained throughout the stimulation period (Table 2).

A very large (36-fold) increase in cGMP levels was induced by NO (6 mM). Pretreatment for 30 min with MB (10 μM) significantly reduced both the relaxant response and the concomitant cGMP accumulation evoked by NO, but a large increase in cGMP levels (9·7-fold from basal values) persisted. cAMP levels were not affected by any of these treatments (Table 2).

A large accumulation of cGMP (55-fold increase from basal levels) was induced by *S*-nitroso-L-cysteine (30 μM) which was reduced but not abolished by MB (10 μM), and a significantly higher value of cGMP compared with basal levels (2·6-fold) persisted. Relaxation with NC was, however, completely unaffected by MB (10 μM) (Table 2).

Relaxation induced by SNP (0·1 mM) was not accompanied by significant changes in either cGMP or cAMP levels. In contrast, in the presence of MB (10 μM), a 9-fold increase in cGMP values was observed without modification of cAMP levels (Table 2).

Forskolin (0·1 mM) induced 27- and 9-fold increases in cAMP and cGMP values, respectively (Table 2).

As a positive control for the cGMP assay, levels of cGMP were measured in unrubbed iliac arteries from rats following relaxation by acetylcholine. This relaxation is caused by NO released from endothelial cells. Arterial rings under tension were precontracted with $PGF_{2\alpha}$ (1 μM) and relaxed by acetylcholine (50 μM). Basal values of cGMP in precontracted arterial rings were 9·1 ± 0·6 pmol (mg protein)$^{-1}$ ($n = 4$). Acetylcholine (50 μM) completely relaxed arterial preparations and induced a 14-fold increase in cGMP values (129·5 ± 7·5 pmol (mg protein)$^{-1}$, $n = 3$, $P < 0.001$) after 60 s.

Effects of cyclic nucleotide analogues on [^{3}H]inositol phosphate accumulation induced by noradrenaline

In control urethral preparations prelabelled with [^{3}H]myo-inositol, [^{3}H]inositol phosphates, at 60 min, comprised 2·98 ± 0·21 % ($n = 6$) of the total ^{3}H content. Exposure to

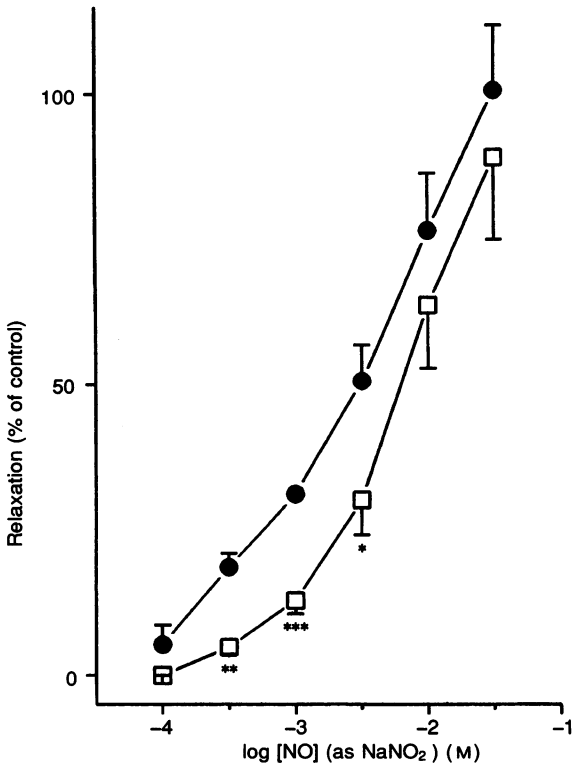

Figure 5. SOD prevents the inhibitory effect of MB on NO-induced relaxation

Effects of methylene blue (MB) on the relaxation induced by nitric oxide (acidified solutions of $NaNO_2$) in the presence of superoxide dismutase (SOD) in sheep urethral preparations precontracted by noradrenaline (50 μM). The preparations were pretreated with MB (10 μM) for 30 min. SOD (200 U ml^{-1}) was added 5 min before MB and was present throughout the experiment. Results are expressed as a percentage of the maximum relaxation before treatment and given as means ± S.E.M. ($n = 6$–8). ●, control experiments; □, in the presence of 10 μM MB plus 200 U ml^{-1} SOD. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

NA (50 μM) for 60 min caused a significant accumulation of [^{3}H]inositol phosphates amounting to 274 ± 19 % of time-matched controls ($n = 6$, $P < 0.001$) (Fig. 6). Pretreatment for 30 min, with 8-Br-cGMP (1 mM) or dibutyryl-cAMP (1 mM) had no significant effect on the accumulation of [^{3}H]inositol phosphates produced by 60 min exposure to NA (50 μM) ([^{3}H]inositol phosphates increased by 296 ± 30 %, $n = 6$, and by 263 ± 21 %, $n = 6$ of untreated controls, in the presence of 8-Br-cGMP and dibutyryl-cAMP, respectively) (Fig. 6).

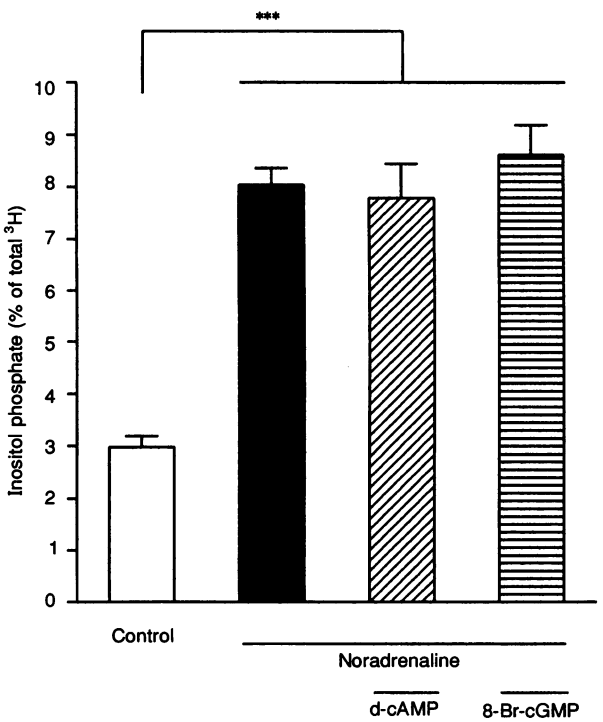

Figure 6. Cyclic nucleotide permeant analogues do not affect accumulation of [^{3}H]inositol phosphates induced by NA

The effect of 8-Br-cGMP and dibutyryl-cAMP on the accumulation of inositol phosphates induced by noradrenaline (NA). Preparations, prelabelled with myo-[^{3}H]inositol for 8 h, were exposed to NA (50 μM) for 60 min in the absence (■), presence of 8-Br-cGMP (1 mM) (▤), or dibutyryl-cAMP (1 mM) (▨). When included, cyclic nucleotide analogues were added 30 min before the preparations were exposed to NA. Untreated controls were run in parallel (□). Inositol phosphate formation is expressed as a percentage of total ^{3}H content. Each column gives the mean ± S.E.M. of six experiments. *** Significantly greater than tissues unexposed to NA ($P < 0.001$).

Effects of high $[K^+]_o$, ouabain and K^+ channel blockers on the relaxation induced by electrical field stimulation, nitric oxide and sodium nitroprusside

Urethral preparations exposed to 120 mM K^+-depolarizing Krebs solution (prepared by replacing NaCl with equimolar amounts of KCl in the normal Krebs solution) developed a stable contraction amounting to $14{\cdot}0 \pm 1{\cdot}1$ mN ($n = 15$). A second contraction induced by the additional application of NA (50 μM) was not significantly different from that elicited by $[K^+]_o$ (120 mM) ($17{\cdot}2 \pm 1{\cdot}0$ mN, $n = 15$). In 120 mM $[K^+]_o$-contracted preparations, EFS (supramaximal voltage, 0·8 ms, 8 Hz, for 5 s) failed to induce a relaxant response, whereas relaxations to NO (6 mM) and SNP (0·1 mM) were not significantly different from those elicited in NA-contracted strips (Fig. 7).

Pretreatment for 15 min with ouabain (0·5 mM), after a period of approximately 10 min, caused a gradual increase in tension amounting to $9{\cdot}7 \pm 2{\cdot}4$ mN in twelve out of eighteen preparations. Addition of NA (50 μM) induced a further increase in tension to a level not significantly different from that elicited prior to ouabain treatment ($102{\cdot}7 \pm 5{\cdot}1$ % of control response to NA before treatment, $n = 8$). In the presence of ouabain, EFS-induced (8 Hz) relaxation was abolished and responses to NO (6 mM) were reduced to $39{\cdot}4 \pm 5{\cdot}6$ % ($n = 7$, $P < 0{\cdot}001$), whereas SNP-elicited relaxation was not affected significantly (Fig. 7).

In order to ascertain the contribution of K^+ channels to relaxation, the effects of the K^+ channel blockers tetraethylammonium (TEA, 1 mM) and glibenclamide (3 μM) were examined. Neither affected either basal tone or the contractile response to NA (50 μM) ($97{\cdot}1 \pm 3{\cdot}4$ %, $n = 11$, and $94{\cdot}0 \pm 5{\cdot}5$ %, $n = 9$, in the presence of TEA and glibenclamide, respectively). Relaxation with EFS (supramaximum voltage, 0·8 ms, 8 Hz, for 5 s), NO (6 mM) or SNP (0·1 mM) in tissues exposed to TEA or glibenclamide were not significantly different from those of the controls (Fig. 7).

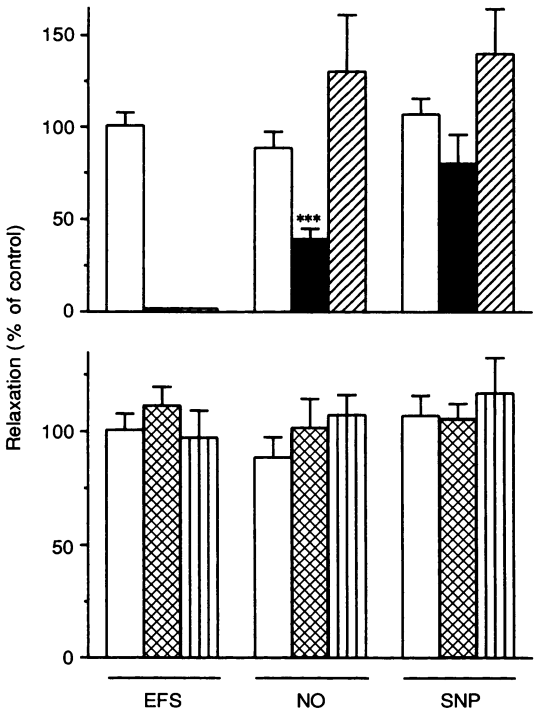

Figure 7. K^+ channel blockers do not affect relaxation, but high $[K^+]_o$ or ouabain impair EFS-induced responses

Effect of ouabain (0·5 mM, ■), $[K^+]_o$ (120 mM, ▨), tetraethylammonium (TEA, 1 mM, ▩) and glibenclamide (3 μM, ▥) each on the relaxation induced by electrical field stimulation (EFS, supramaximal voltage, 0·8 ms, 8 Hz for 5 s), nitric oxide (NO, acidified $NaNO_2$, 6 mM) and sodium nitroprusside (SNP, 0·1 mM) on sheep urethra. The preparations were precontracted by noradrenaline (50 μM) and relaxed by EFS, NO or SNP before and after 30 min pretreatment with ouabain, TEA or glibenclamide. Relaxation in $[K^+]_o$ (120 mM)-contracted preparations was compared with those in which contractions were induced by noradrenaline (50 μM). Results are expressed as a percentage of the maximum control relaxation before treatment and given as means ± S.E.M. ($n = 5$–8). ***$P < 0{\cdot}001$ when compared with control experiments run in parallel (□).

NADPH diaphorase activity

Dense networks of NADPH diaphorase-positive varicose nerve fibres were consistently found running in parallel with both the circular (Fig. 8) and longitudinal smooth muscle layers of sheep urethra. NADPH diaphorase-positive nerve trunks and ganglionic cell bodies were also seen (not shown).

DISCUSSION

Non-adrenergic, non-cholinergic relaxation evoked by EFS in the urethra of sheep (García-Pascual *et al.* 1991; Thornbury *et al.* 1992) and other species including man (Andersson *et al.* 1991, 1992; Dokita *et al.* 1991; Persson & Andersson, 1992; Persson *et al.* 1992) are blocked by the NO synthesis inhibitors N^G-monomethyl-L-arginine and N^G-nitro-L-arginine, but not by the D-enantiomers. The inhibitory effect of these L-arginine analogues was reversed by L- but not by D-arginine. NO, or a NO-containing compound, derived from L-arginine, may thus be involved in inhibitory neurotransmission in urethral smooth muscle. In the present paper, NADPH diaphorase-positive fibres have been demonstrated in dense nerve networks coursing parallel to smooth muscle cells. Assuming that NADPH diaphorase activity reflects neurons containing NO synthase (Dawson *et al.* 1991), the localization of NO synthetic activity in neurons of sheep urethra has been shown. This nervous network is independent of adrenergic innervation; chemical sympathectomy with 6-hydroxydopamine does not affect relaxation of sheep urethral muscle induced by EFS (García-Pascual *et al.* 1991).

However, differences between the relaxation induced by EFS of nerves and by exogenously added NO have emerged in the present study and these question the identity of the transmitter as authentic NO. The neuronal release of a nitrosyl compound (rather than free NO), which would in turn release NO in the target tissue, could account for the differences observed.

Methylene blue, a proposed specific inhibitor of soluble guanylate cyclase (Martin *et al.* 1985), inhibited NO-induced relaxations and the accumulation of cGMP without affecting the relaxant responses to EFS in the present study and both rabbit urethra (Andersson *et al.* 1992) and pig trigone (Persson & Andersson, 1992). The inhibitory effect of MB was reversed by SOD, a scavenger of superoxide, suggesting that MB acted by the extracellular generation of O_2^-. Since a reduced form of MB can generate O_2^- by auto-oxidation in the presence of oxygen (McCord & Fridovich, 1970), and since NO is known to be inactivated by O_2^- (Palmer, Ferrige & Moncada, 1987), our results suggest that MB in sheep urethra could be acting by chemical inactivation of NO, rather than by guanylate cyclase inhibition. Indeed, the extracellular generation of O_2^- by MB is believed to mediate the inhibition of NO-dependent vasodilator responses in rat cremaster arterioles (Wolin, Cherry, Rodenburg, Messina & Kaley, 1990) and in cat cerebral arteries (Marshall, Wei & Kontos, 1988).

Oxyhaemoglobin, like MB, showed dissimilar effects on EFS- and NO-induced relaxation of sheep urethra. At concentrations which induced a parallel rightward shift in the concentration–response curves to NO, stimulation-dependent nerve-mediated relaxation was not affected significantly by HbO_2. HbO_2 elicits its inhibitory action by binding NO directly to form a nitrosyl–haemoprotein complex which prevents the passage of NO to smooth

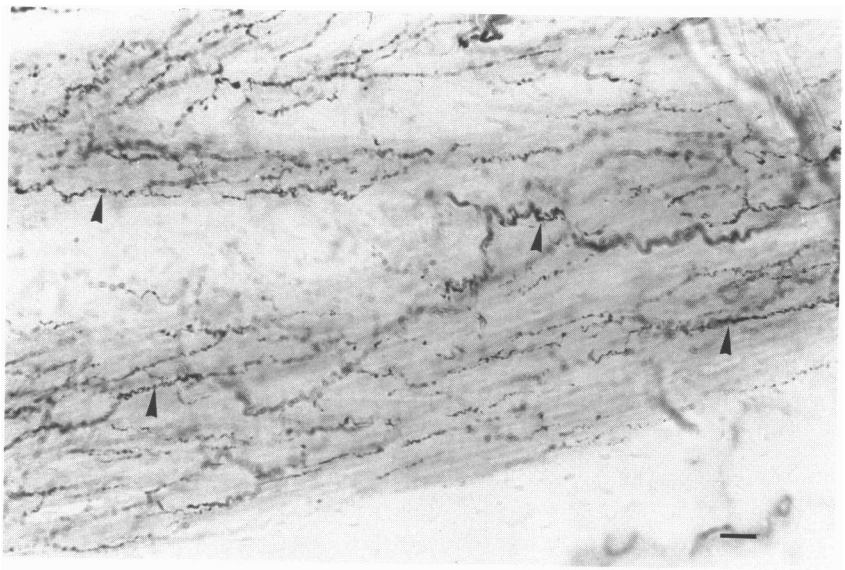

Figure 8. Smooth muscle bundles have a rich NADPH diaphorase-positive innervation
Histochemical localization of NADPH diaphorase-positive nerve fibres (▲) in the circular muscle layer from a transverse section of sheep urethra. Calibration bar = 25 μm.

muscle cells (Martin *et al.* 1985). In view of the molecular size of HbO_2, only a small proportion may have reached the neuromuscular junction; this may explain the discrepancy in efficacy. Together, the results obtained with MB and HbO_2 show that NANC relaxations of sheep urethra are resistant to both extracellular inhibitors (O_2^- and HbO_2). Two hypotheses could explain these findings. First, a short diffusion path for the released NO, from nerve terminals to smooth muscle cells, may account for the lack of effect of O_2^- and HbO_2 on NANC relaxation. Since NO-mediated NANC relaxation can be inhibited by O_2^- and HbO_2 and potentiated by O_2^- scavengers (Sneddon & Graham, 1992), NO, present in the synaptic cleft, may be susceptible to these extracellular inhibitors. Alternatively, a more stable precursor of NO could be released by nerve terminals and converted to NO in the target tissue. Vascular endothelium-derived relaxing factor (EDRF) may not be identical to NO (Greenberg, Wilcox & Rubany, 1990), as before release, NO is bound to a carrier molecule such as cysteine, to form *S*-nitrosothiol compounds (Myers, Minor, Guerra, Bates & Harrison, 1990). These are less sensitive than NO to inactivation by O_2^- and to inhibition by HbO_2 (Furchgott, Jothianandan & Khan, 1992), and following denitrosation at the external smooth muscle membranes, *S*-nitrosothiols can yield NO to the cytoplasm (Kowaluk & Fung, 1990). In support of this view, in the present study both NC and EFS induced potent and reversible relaxation, that was not affected by MB. Moreover, *S*-nitroso-L-cysteine and nerve stimulation produced a similar relaxation of sheep urethra (Thornbury *et al.* 1992).

In both neurons and smooth muscle, NO is believed to act via stimulation of soluble guanylate cyclase, with a subsequent increase in intracellular cGMP (Ignarro, 1990). In sheep urethra, EFS produced stimulation-dependent relaxation and rises in cGMP. 8-Br-cGMP, a permeant analogue of cGMP, also elicited concentration-dependent relaxation. However, the ability of EFS, NO and NC to each increase cGMP levels differed. cGMP levels in response to exogenously added NO and NC, were 15 and 23 times higher, respectively, than those produced by EFS for the same level of relaxation. This difference did not seem to be due to a time-dependent accumulation of cGMP in preparations relaxed by NO or NC, because no further elevation in cGMP was observed when the duration of EFS was increased from 5 to 60 s, emphasizing a clear dissociation between cGMP levels and relaxation. Smooth muscle relaxation can occur with cGMP increases of 2- to 10-fold, although its capacity to accumulate cGMP can reach 200-fold (Kukovetz & Holzmann, 1989). The physiological significance of such high concentrations of cGMP is unknown. In the present study, complete relaxation of rat iliac arteries by acetylcholine was obtained with a 14-fold increase in cGMP values. In other visceral smooth muscles, in which NO-mediated relaxation occurs, increases in cGMP following EFS, similar to that found in sheep urethra (1·6- to 3-fold), have been reported (Grous, Ormsbee & Barnette, 1990; Kanada *et al.* 1992). Stimulation of nerves is likely to induce a much more localized cGMP increase than when the whole tissue is exposed to exogenous NO or NC; this makes quantitative comparison difficult.

Sodium nitroprusside, which is thought to generate NO at or in the smooth muscle (Ignarro *et al.* 1981), should mimic the relaxation of NO or a NO-like substance released by inhibitory nerves. This was not found in the present study. Relaxation with SNP was not accompanied by measurable changes in total cGMP content nor were these relaxant responses affected by MB or HbO_2, suggesting that SNP does not release NO spontaneously in the extracellular medium. SNP may be metabolised by a plasma membrane-associated enzyme (Kowaluk, Seth & Fung, 1992), distinct from those that generate NO from nitroglycerin, organic nitrite esters and *S*-nitrosothiols. A differential distribution of the enzymatic systems needed for biotransformation of different nitrocompounds may account for the heterogeneous sensitivity to these agents in some tissues. Indeed, a rise in tissue cGMP content may not completely explain smooth muscle relaxation induced by SNP (Vidal, Vanhoutte & Miller, 1991; Hu, Honda, Murad & Hoffman, 1992) and the release of NO from nitrocompounds may not be necessary for relaxation. Mechanisms other than cGMP accumulation, such as the uncoupling of oxidative phosphorylation, the stimulation of prostaglandin synthesis and membrane hyperpolarization, have been proposed to explain smooth muscle relaxation induced by such nitrovasodilators as SNP (Ahlner & Axelsson, 1987). Relaxant responses to SNP were unaffected by K^+ depolarizing solution, ouabain or K^+ channel blockers, making membrane hyperpolarization involvement unlikely. The present results suggest that SNP may not be a suitable model for studying NO-mediated responses in this tissue.

Striking differences were seen between the changes in cGMP content induced by different nitrocompounds in the presence of MB, and the relationship with the observed relaxation. Relaxant responses to NC were not inhibited by MB significantly, while the cGMP levels were greatly reduced to values similar to those induced by EFS. Increases in cGMP induced by NO in the presence of MB remained very high, at a time when relaxant responses were reduced to 20%. SNP did not modify basal cGMP levels, but in the presence of MB, a large accumulaton of cGMP was measured, although relaxation was not modified. These effects of MB do not seem to be due to a direct action of the dye on urethral smooth muscle, since MB decreased, rather than increased, the basal levels of cGMP. Gryglewski, Zembowicz, Salvemini, Taylor & Vane (1992) showed that, in rabbit aorta, the rises in cGMP content induced by SNP and by sodium nitrite were enhanced by MB, while those elicited by nitroglycerine and *S*-nitroso-*N*-acetylpenicillamine were unaffected. They suggested that MB may act as an intracellular carrier for

NO from SNP to soluble guanylate cyclase. On the other hand, Kowaluk *et al.* (1992) observed that reducing agents increase the enzymatic degradation of SNP to yield NO, and this could also apply for a redox compound such as MB. The pharmacology of MB is complex; besides the generation of O_2^- and the inhibition of cGMP formation, other effects have been proposed (Mayer, Brunner & Schmidt, 1993). It would appear that methylene blue is not as potent nor as selective an inhibitor of soluble guanylate cyclase as was previously assumed. The present results show that relaxation with EFS and NC is accompanied by small increases in cGMP, which are not affected by MB, while changes in cGMP values in the presence of MB are not related to relaxation. These observations could be explained by the existence of multiple pools of cGMP in the cell, and so relaxation may be controlled by changes in the cGMP content of compartments that represent a small fraction of the total cellular nucleotide content. Activation of particulate forms of guanylate cyclase, which are not inhibited by MB, could mediate the relaxation and a particulate form of guanylate cyclase has been purified from retinal rod outer segments that is activated by SNP and NO (Horio & Murad, 1991). Additional mechanisms, independent of cGMP, contributing to EFS-induced relaxation of sheep urethra, cannot, however, be discarded.

Although NO activates cGMP production, an endothelium-dependent elevation of cAMP has also been observed in cerebral (Brayden & Wellman, 1989) and pulmonary arteries (Ignarro *et al.* 1986) and in isolated perfused rat kidney (Heuzé-Joubert *et al.* 1992); NO could thus also activate smooth muscle adenylate cyclase. Sheep urethral strips were dose-dependently relaxed by agents which increase cAMP levels (forskolin, isoprenaline and dibutyryl-cAMP). Forskolin, which activates the catalytic subunit of adenylate cyclase, significantly increased the concentration of cAMP in our preparation. These findings suggest a second messenger function for cAMP in mediating sheep urethral relaxation. Whether or not relaxation with forskolin is mediated exclusively by cAMP remains unclear; relaxant responses to EFS, exogenous NO and SNP were not, however, accompanied by increases in levels of cAMP.

The mechanism of smooth muscle relaxation by cGMP-elevating agents, like NO, is controversial. cGMP, via cGMP-dependent protein kinase, seems to be able to lower free intracellular calcium concentration by one or more independent mechanisms (Lincoln, 1989). However, changes in calcium fluxes may be secondary to changes in the intracellular levels of inositol phosphates. Cyclic nucleotides may inhibit smooth muscle contraction through inhibition of phosphoinositide hydrolysis (Rapoport, 1986, 1991). Thus, inhibition of agonist-dependent phospholipase C activation could result in a decreased formation of both inositol 1,4,5-trisphosphate and 1,2-diacylglycerol as well as a decreased activation of protein kinase C. Urethral smooth muscle may use hydrolysis of membrane phospholipids for the long-term regulation of agonist-induced tone (García-Pascual *et al.* 1993). In the present study, significant increases in inositol phosphate levels were observed after exposure to noradrenaline. However, pretreatment with high concentrations of cyclic nucleotide permeant analogues did not reduce inositol phosphate formation. Consequently, the relaxant effect of agents which raise cyclic nucleotide levels in sheep urethra, including NO, are unlikely to be due to an inhibition of phosphoinositide hydrolysis.

The relaxation in response to EFS varied with the method of inducing contraction. In urethral segments contracted by high $[K^+]_o$, EFS was ineffective. Perhaps, the different mechanisms of increasing intracellular calcium by NA and K^+ could affect the ability of NO to relax urethral smooth muscle. Thus, if the primary effect of elevated cGMP is to lower intracellular calcium, the membrane depolarization by K^+, which promotes calcium influx through voltage-operated calcium channels, should antagonize the actions of cGMP. This agrees with previous observations that an increase in calcium influx by Bay K 8644 inhibited EFS-induced relaxation in sheep urethra (García-Pascual *et al.* 1991). On the other hand, relaxant responses to NO in K^+- and NA-contracted preparations were not significantly different. In some vascular smooth muscle, endothelium-dependent relaxation to some agents is produced by membrane hyperpolarization through an increase in K^+ conductance. Since, in these tissues, exogenous NO does not elicit hyperpolarization another substance, termed endothelium-dependent hyperpolarizing factor (EDHF), released from the endothelium in addition to NO (Nagao & Vanhoutte, 1992), could be responsible. In contrast, in gastrointestinal smooth muscles, NO mimics inhibitory junction potentials evoked by stimulation of NANC nerves whose effects are mediated by an increase in K^+ conductance (Du *et al.* 1991). In sheep urethral preparations, relaxation induced by either EFS or NO was unaffected by the presence of two K^+ channel blockers, TEA, which blocks several types of K^+ channel, and the more specific blocker of the ATP-sensitive K^+ channel, glibenclamide. Thus, it seems unlikely that an enhancement of K^+ conductance and the associated hyperpolarization are involved in the relaxation. On the other hand, the Na^+–K^+-ATPase inhibitor ouabain inhibited EFS-induced relaxation suggesting that the hyperpolarization resulting from Na^+–K^+ pump activation might participate in the relaxation. However, relaxation by NO was inhibited by ouabain but not by high K^+ concentrations making this view unlikely. A high $[K^+]_o$, by attenuating the K^+ gradient across the plasmalemma, depolarizing the tissue and inactivating the hyperpolarization mechanisms which produce relaxation, would have been expected to inhibit NO relaxation. Alternatively, inhibition of the Na^+–K^+-ATPase activity by ouabain results in membrane depolarization and calcium entry through both voltage-operated calcium channels and the

Na^+–Ca^{2+} exchange mechanism (Ozaki & Urakawa, 1978). Thus, the inhibitory action of ouabain on NO-induced responses could arise from an increase in intracellular calcium availability, which would compete with relaxation mechanisms. Whether or not a hyperpolarizing factor other than NO is released by NANC nerve terminals which contributes to the relaxation is unknown. A presynaptic inhibitory depolarizing effect on NO release cannot be ruled out.

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Acknowledgements

The authors wish to thank Dr Carmen Estrada and Dr Carmen Gonzalez for providing vascular preparations for cGMP determinations. This work was supported by a Grant from the Complutense University (G. Precomp. 90–1).

Received 26 March 1993; revised 24 August 1993; accepted 15 September 1993.