British Journal of Pharmacology (1995) 116, 2582-2590 © 1995 Stockton Press All rights reserved 0007-1188/95 $12.00

Involvement of nitric oxide in the non-adrenergic non-cholinergic neurotransmission of horse deep penile arteries: role of charybdotoxin-sensitive K^+-channels

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1 The involvement of nitric oxide (NO) and the signal transduction mechanisms mediating neurogenic relaxations were investigated in deep intracavernous penile arteries with an internal lumen diameter of 600-900 μm, isolated from the corpus cavernosum of young horses.

2 The presence of nitric oxide synthase (NOS)-positive nerves was examined in cross and longitudinal sections of isolated penile arteries processed for NADPH-diaphorase (NADPH-d) histochemistry. NADPH-d-positive nerve fibres were observed in the adventitia-media junction of deep penile arteries and in relation to the trabecular smooth muscle.

3 Electrical field stimulation (EFS) evoked frequency-dependent relaxations of both endothelium-intact and denuded arterial preparations treated with guanethidine (10^{-5} M) and atropine (10^{-7} M), and contracted with 10^{-6} M phenylephrine. These EFS-induced relaxations were tetrodotoxin-sensitive indicating their non-adrenergic non-cholinergic (NANC) neurogenic origin.

4 EFS-evoked relaxations were abolished at the lowest frequency (0.5-2 Hz) and attenuated at higher frequencies (4-32 Hz) by the NOS inhibitor, N^G-nitro-L-arginine (L-NOARG, 3×10^{-5} M). This inhibitory effect was antagonized by the NO precursor, L-arginine (3×10^{-3} M). N^G-nitro-D-arginine (10^{-4} M) did not affect the relaxations to EFS.

5 Incubation with either the NO scavenger, oxyhaemoglobin (10^{-5} M), or methylene blue (10^{-5} M), an inhibitor of guanylate cyclase activation by NO, caused significant inhibitions of the EFS-evoked relaxations, and while oxyhaemoglobin abolished the relaxations to exogenously added NO (acidified sodium nitrite, 10^{-6}-10^{-3} M), there still persisted a relaxation to NO of 24.4±5.1% ($n=6$) in the presence of methylene blue.

6 Glibenclamide (3×10^{-6} M), an inhibitor of ATP-activated K^+-channels, did not alter the relaxations to either EFS-stimulation or NO, while the blocker of Ca^{2+}-activated K^+-channels, charybdotoxin (3×10^{-8} M), caused a significant inhibition of both the electrically-induced relaxations and the relaxations to exogenously added NO. Furthermore, charybdotoxin blocked relaxations induced by the cell permeable analogue of cyclic GMP, 8-bromo cyclic GMP (8 Br-cyclic GMP).

7 These results suggest that relaxations of horse deep penile arteries induced by NANC nerve stimulation involve mainly NO or a NO-like substance from nitrergic nerves. NO would stimulate the accumulation of cyclic GMP followed by increases in the open probability of Ca^{2+}-activated K^+-channels and hyperpolarization leading to relaxation of horse penile arteries.

Keywords: Penile arteries; electrical field stimulation; nitrergic nerves; nitric oxide; N^G-nitro-L-arginine; charybdotoxin; Ca^{2+}-activated K^+-channels; 8-Br-cyclic GMP

Introduction

Penile erection is initiated by activation of parasympathetic pelvic nerves leading to arterial dilatation. Relaxation of the erectile smooth muscle elements of the corpora cavernosa (trabecular smooth muscle) then follows, allowing blood filling of the sinoids and compression of the draining venules against the tunica albuginea, with entrapment of pressurized blood in the corpora cavernosa (Andersson *et al.*, 1984; Lue & Tanagho, 1987).

Despite the corpus cavernosum and penile vasculature being extensively innervated by cholinergic nerves (McConnell *et al.*, 1979; Stief *et al.*, 1989), penile erection has been shown to be atropine-resistant (Brindley, 1986; Stief *et al.*, 1989). Morphological demonstrations of penile nerves containing vasoactive intestinal peptide (VIP) (Larsson *et al.*, 1977; Polak *et al.*, 1981), and the observation that pelvic nerve stimulation caused an output of VIP from the canine penis, which was correlated to the haemodynamic events in penile erection (Willis *et al.*, 1981; Andersson *et al.*, 1984), led to the suggestion that VIP might be the neurotransmitter responsible for penile erection. However, VIP alone caused relatively poor erections even after direct injection into the corpus cavernosum (Juenemann *et al.*, 1987; Roy *et al.*, 1990).

Recent pharmacological and neurochemical studies have implicated nitric oxide (NO) as a transmitter in the neural vasodilator pathways to the penis. Evidence for a source of NO in nerves supplying the corpus cavernosum trabecular smooth muscle and penile vasculature has been provided by the immunocytochemical localization of the enzyme that synthesizes NO, NO synthase (NOS), in penile efferent nerves from the pelvis plexus of rat and man (Burnett *et al.*, 1992; Vizzard *et al.*, 1994). *In vivo* infusion of NOS inhibitors was shown to antagonize penile erection induced by electrical stimulation of the sacral part of the spinal cord in pithed rats (Finberg *et al.*, 1993), or pelvic nerve stimulation in dogs (Trigo-Rocha *et al.*, 1993) or rabbits (Holmquist *et al.*, 1991).

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A non-adrenergic non-cholinergic (NANC) neurotransmitter was observed to mediate the relaxation of the cavernous trabecular smooth muscle (Andersson *et al.*, 1987; Saenz de Tejada *et al.*, 1988) and was later suggested to be neuronally released NO (Ignarro *et al.*, 1990; Kim *et al.*, 1991; Pickard *et al.*, 1991; Holmquist *et al.*, 1992). In the bovine large dorsal penile arteries, an inhibitory neurotransmission was discovered by Klinge & Sjöstrand in 1974 and N^G- substituted analogues of L-arginine (Liu *et al.*, 1991) and haemolysate (Bowman & Gillespie, 1983) were shown to inhibit NANC relaxations. However, despite immunocytochemical studies providing evidence of nitrergic nerves around intracavernous deep penile arteries, the role of NO in these arteries, which control the blood flow between the systemic arterial circulation and the cavernous sinoids, has not been fully elucidated.

The purpose of the present study was to clarify whether the L-arginine/nitric oxide pathway is involved in the nerve-evoked relaxation of the deep penile arteries and which transduction mechanisms mediate these responses.

Methods

Tissue preparation

Penises from normal horses were obtained once a week at the local slaughterhouse, immediately after the animals were killed and placed in cold physiological salt solution (PSS). Throughout the subsequent dissection, the penis was bathed in cold PSS, 4°C, of the following composition (mM): NaCl 119, KCl 4.7, KH_2PO_4 1.18, $MgSO_4$ 1.17, $CaCl_2$ 2.5, ethylenediaminetetraacetic acid (EDTA) 0.027 and glucose 11. The solution was gassed with 5% CO_2 in 95% O_2 to maintain pH at 7.4.

NADPH diaphorase histochemistry

Deep penile arteries isolated by dissection from horse corpus cavernosum were immersed in ice-cold (4 °C) 4% paraformaldehyde with 0.1% glutaraldehyde in phosphate buffer (PB) 0.1 M, pH 7.2. After a 24 h fixation period, samples were kept in a cryoprotective solution of 15% sucrose in 0.1 M PB overnight. Cross and longitudinal sections (30 μm) were obtained with a cryostat and processed for NADPH diaphorase histochemistry following the protocol by Hope & Vincent (1989). The preparations were incubated in 0.1 M PB, pH = 8.0, containing 1 mM β-NADPH, 0.5 mM nitroblue tetrazolium and 0.3% Triton X-100 for 30–45 min at 37°C and protected from light. Controls for specificity of the staining were carried out by incubating some sections with either dicoumarol or L-canavanine, in order to prevent the activity of NADPH cytochrome P_{450} reductase activity and the inducible form of NO synthase, respectively (Schmidt, 1992). Sections were rinsed in PB, dehydrated and mounted on to poly-L-lysine covered slides for light microscopic examination.

Dissection and mounting

Branches of the dorsal penile arteries penetrating the tunica albuginea of the penis were localized, and its course was followed by dissection. The tunica albuginea and the corpus cavernosum of the penis were opened until the common profound artery, orientated parallel to the longitudinal axis of the penis and corresponding to the deep penile artery in man, was localized. Branches of deep penile arteries with an internal lumen diameter of 600–900 μm were dissected by carefully removing the adhering trabecular tissue and segments (3–4 mm long) of the arteries were subsequently mounted in 5 ml organ baths as ring preparations on two parallel stainless steel L-shaped legs. One of the hooks was connected to a transducer (Grass FT03) for isometric tension measurements, and the other to a length displacement device permitting the adjustment of the vascular diameter. In the case of endothelium-denuded preparations, the endothelium was mechanically removed by introducing a horse hair into the lumen of the arterial segments and rubbing back and forth 10–15 times before the segment was mounted.

The vessels were allowed to equilibrate in PSS, 37°C, pH 7.4 for about 30 min. The penile arterial segments were stretched applying increments of 0.5 g, and after each step contracted with 10^{-6} M phenylephrine to determine the optimal resting isometric tension for contraction. When the active developed contraction was within 10% of the previous contraction, that tension was considered optimal. The presence or absence of endothelium was assessed by exposure to the endothelium-dependent dilator, acetylcholine (10^{-5} M), in phenylephrine contracted vessels. The arteries were incubated with guanethidine (10^{-5} M) and atropine (10^{-7} M) and these drugs were kept present throughout the rest of the experiment to block adrenergic neurotransmission and muscarinic receptors, respectively.

Experimental procedure

Electrical field stimulation (EFS) was performed through two electrodes mounted parallel to the vessel segments using a Cibertec stimulator (Barcelona, Spain) with constant current output. Square pulses of 0.3 ms duration in 20 s trains with varying frequency (0.5–32 Hz, 10–640 pulses) were applied. Voltage of the stimulator was adjusted to deliver 75 mA. A first frequency-response curve in phenylephrine-contracted preparations was obtained. The preparations were repeatedly washed and allowed to equilibrate for at least 1 h before they were incubated with either N^G-nitro-L-arginine (L-NOARG, 3×10^{-5}–10^{-4} M), L-arginine (3×10^{-3} M), L-arginine (3×10^{-3} M), plus L-NOARG (3×10^{-5} M), N^G-nitro-D-arginine (D-NOARG, 10^{-4} M), indomethacin (10^{-5} M), tetrodotoxin (TTX, 10^{-6} M), L-NOARG (3×10^{-5} M) plus TTX (10^{-6} M), oxyhaemoglobin (10^{-5} M), methylene blue (10^{-5} M), charybdotoxin (3×10^{-8} M) or glibenclamide (3×10^{-6} M). After 10–30 min incubation with the corresponding drug, phenylephrine was added at a concentration adjusted to match the contraction during the first control curve, and a second frequency-response curve was then performed. In each experiment, frequency-response curves without any treatment were run in parallel.

Cumulative concentration-response curves to sodium nitrite ($NaNO_2$) were obtained in phenylephrine-contracted penile arteries by adding the agonist directly to the bathing medium. The bath solution was changed several times and the segments were incubated with the blocking agents for 30 min before the concentration-response curves were repeated.

At the end of each series of experiments, papaverine was added in a concentration of 10^{-4} M to attain maximum relaxation of the arteries.

Drugs

Acetylcholine hydrochloride, atropine sulphate, L-canavanine hydrochloride, charybdotoxin, dicoumarol, N^G-nitro-D-arginine, glibenclamide, guanethidine sulphate, indomethacin, L-arginine hydrochloride, N^G-nitro-L-arginine, methylene blue, phenylephrine hydrochloride, papaverine hydrochloride, sodium nitrite ($NaNO_2$) and tetrodotoxin (Sigma, St. Louis, MO, U.S.A.) were dissolved in twice distilled water, except indomethacin and glibenclamide, which were dissolved in 90% ethanol and dimethyl sulphoxide, respectively, and further diluted in water. Stock solutions were prepared and stored at −20 °C and further fresh dilutions were prepared daily. Stock solutions of guanethidine, atropine and phenylephrine were prepared for each experiment.

Oxyhaemoglobin was prepared from a 1 mM solution of commercial haemoglobin (bovine haemoglobin, Sigma) by addition of 10 mM sodium dithionite ($Na_2S_2O_4$, Sigma) (Martin *et al.*, 1985). The reducing agent converting methaemoglobin to oxyhaemoglobin was 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 giving a final concentration of $5-8 \times 10^{-4}$ M.

The $NaNO_2$ was freshly prepared as 1 M stock solutions by adjusting the pH to 2 by adding concentrated HCl (Cocks & Angus, 1990). This stock solution was kept cold and protected from air. Further dilutions were made in diluted HCl (pH 2) immediately before use and added in volumes of 5–10 μl. Neither equivalent volumes of the vehicle for $NaNO_2$ nor non-acidified $NaNO_2$ (pH = 7.4) up to 10^{-2} M caused relaxation of horse penile arteries.

Analysis of data

The mechanical responses of the vessels were measured as force and expressed as active wall tension, δT, which is the increase in measured force, δF, divided by twice the segment length. By use of a computer programme (GraphPad, Institute for Scientific Information, San Diego, California, U.S.A.), the concentration-response curves were fitted to the classical 'Hill equation': $R/R_{max} = A(M)^{n_H}/(A(M)^{n_H} + IC_{50}(M^{n_H}))$, where R/R_{max} is the relative response to the effective concentration of drug, A(M), and IC_{50}(M) is the concentration of agonist required to give half maximal vessel response (R_{max}), where A(M) and IC_{50}(M) are given in molar concentrations. n_H is a curve fitting parameter or 'Hill-coefficient'. Similarly, the frequency-response curves were fitted using the same programme and the EF_{50} values, which is the stimulus strength producing a 50% relaxation of the maximum relaxation reached, were determined.

The results are expressed as means ± s.e.mean, where *n* represents the number of animals studied in each experiment. The frequency or concentration-response curves before and after treatment were compared by analysis of variance (ANOVA) for repeated measures, and by paired test for comparisons of the individual concentrations or frequencies. Results comparing endothelium-intact and denuded preparations or experiments with 8-Br-cyclic GMP were evaluated by analysis of variance (ANOVA) followed by unpaired two-tailed *t* test. When multiple comparisons were made with a single control, values were analysed according to one-way analysis of variance (ANOVA) and Bonferroni method as an *a posterio* test (Wallenstein *et al.*, 1980). Probability levels under 5% were considered significant.

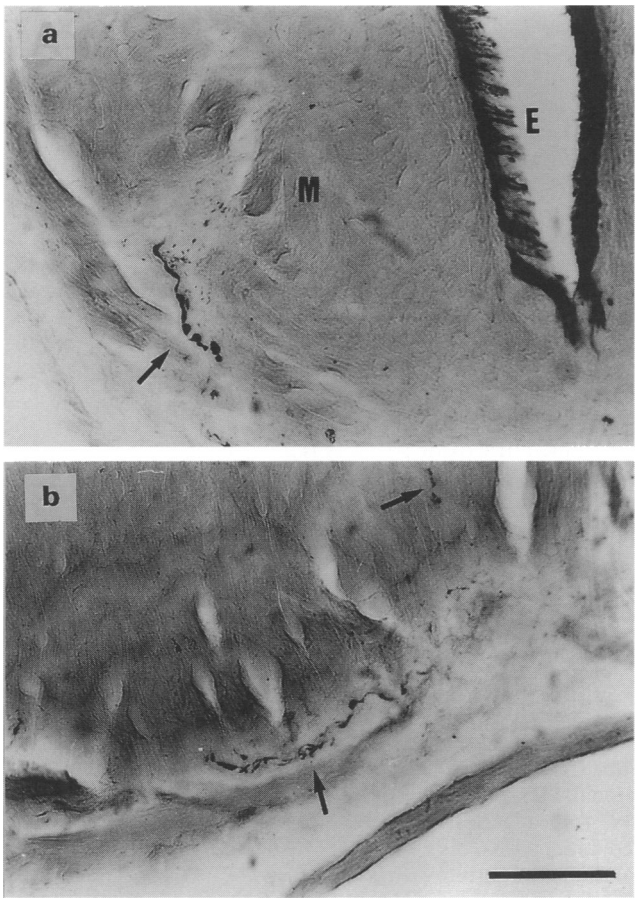

Figure 1 Histochemical distribution of NADPH-diaphorase activity in cross (a) and longitudinal (b) sections of horse deep penile arteries stained for NADPH-diaphorase histochemistry. (a) NADPH-diaphorase positive reaction is found in both endothelial cells (E) and nerve fibres (arrow) at the adventitia-media junction. Note the thick media (M) of these arteries. (b) Varicose NADPH-diaphorase positive nerves (arrows) running along the adventitia-media border and finer branching fibres in the outer part of the arterial media. Scale bar = 100 μm in (a) and (b).

Results

NADPH-diaphorase activity

NADPH-diaphorase (NADPH-d) activity was observed as a blue reaction product within both endothelial cells and nerve fibres of the thick-walled deep penile arteries (Figure 1a). A strong positive staining was found in the intima of all the arteries examined (Figure 1a), whereas varicose NADPH-d-positive nerve fibres were localized at the adventitia-media junction of deep penile arteries (Figure 1a,b). Fine positive fibres were observed in the outer part of the arterial media (Figure 1b).

Responses to electrical field stimulation (EFS)

The horse deep penile arteries were stretched to a passive tension of 3.9 ± 0.2 Nm^{-1} ($n = 121$), where phenylephrine (10^{-6} M) induced a stable contraction of 4.4 ± 0.2 Nm^{-1} ($n = 121$). Electrical field stimulation (EFS) evoked frequency-dependent relaxations at 0.5–2 Hz, while stimulation at 4–32 Hz induced a biphasic response consisting of contraction followed by relaxation (Figure 2a). After depletion of the adrenergic nerves with guanethidine, the contraction disappeared and only relaxation persisted in response to EFS (Figure 2b). In the presence of both guanethidine and atropine, EFS in a first frequency-response curve induced relaxations which peaked at 15 s with an $EF_{50} = 4.8 \pm 1.0$ Hz ($n = 11$) and a maximum relaxation which was obtained at 32 Hz and averaged 57.6 ± 4.2% ($n = 11$) of the tone induced by phenylephrine (10^{-6} M). The relaxations to EFS were reproducible with an $EF_{50} = 5.3 \pm 1.0$ Hz ($n = 11$) and maximum relaxation of 58.6 ± 4.2% ($n = 11$) in a second frequency-response curve constructed in the same vessel segments. In the presence of tetrodotoxin (TTX, 10^{-6} M), the EFS-induced relaxations were abolished at the lowest frequencies (0.5–16 Hz), while at 32 Hz small TTX-resistant relaxations (13.5 ± 5.4%, $n = 8$) persisted.

Acetylcholine (10^{-5} M) caused a relaxation of 94.7 ± 1.0% ($n = 9$) of the phenylephrine-induced tone in endothelium-intact horse deep penile arteries, while no relaxant effect was observed in endothelium-denuded arteries ($n = 9$). However, neither the sensitivity nor maximal responses to EFS were significantly different in endothelium-intact and denuded arteries (Figure 3a). EF_{50} values and maximal relaxations were 3.7 ± 1.7 Hz and 73.5 ± 1.7% ($n = 9$) in endothelium-intact, and 2.4 ± 1.1 Hz and 70.6 ± 6.8% ($n = 9$) in endothelium-denuded arteries, respectively. Exogenously added NO, as acidified $NaNO_2$ (10^{-6}–10^{-3} M), also induced relaxations of similar magnitude and potency in endothelium-intact and denuded arteries (Figure 3b); the pIC_{50} and maximum relaxations were 4.75 ± 0.24 and 87.9 ± 4.3% ($n = 6$), and 4.59 ± 0.24 and 84.7 ± 4.4% ($n = 6$) in endothelium-intact and -denuded arteries, respectively. In endothelium-intact preparations the cyclo-oxygenase inhibitor, indomethacin (10^{-5} M), either had

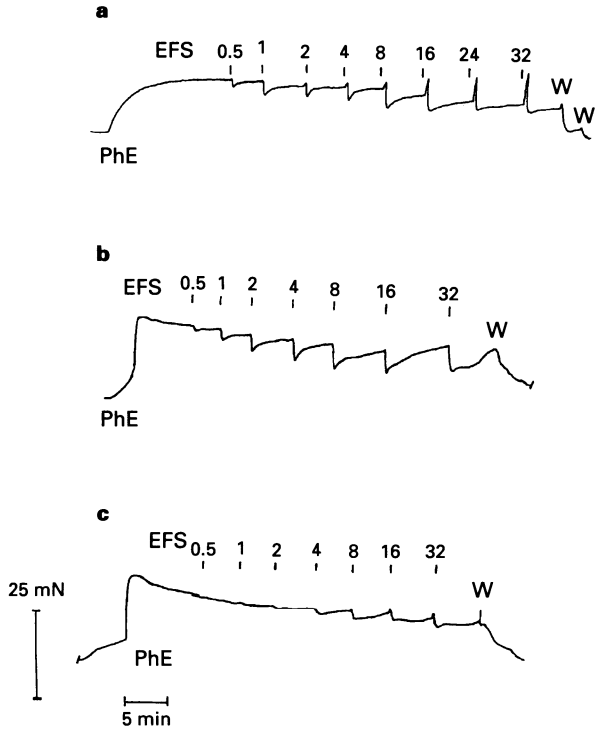

Figure 2 (a) Electrical field stimulation (EFS) with square pulses (0.3 ms, 20 s trains, supramaximal current) elicited only relaxations at low frequencies and a biphasic contraction/relaxation at higher frequencies in a horse deep penile artery precontracted with 10^{-6} M phenylephrine (PhE). (b) EFS-induced relaxations in an endothelium-intact deep penile artery treated with guanethidine and atropine to block adrenergic and cholinergic neurotransmission. (c) The same artery as in (b) after treatment with L-NOARG (3×10^{-5} M). Numbers indicate the frequency applied.

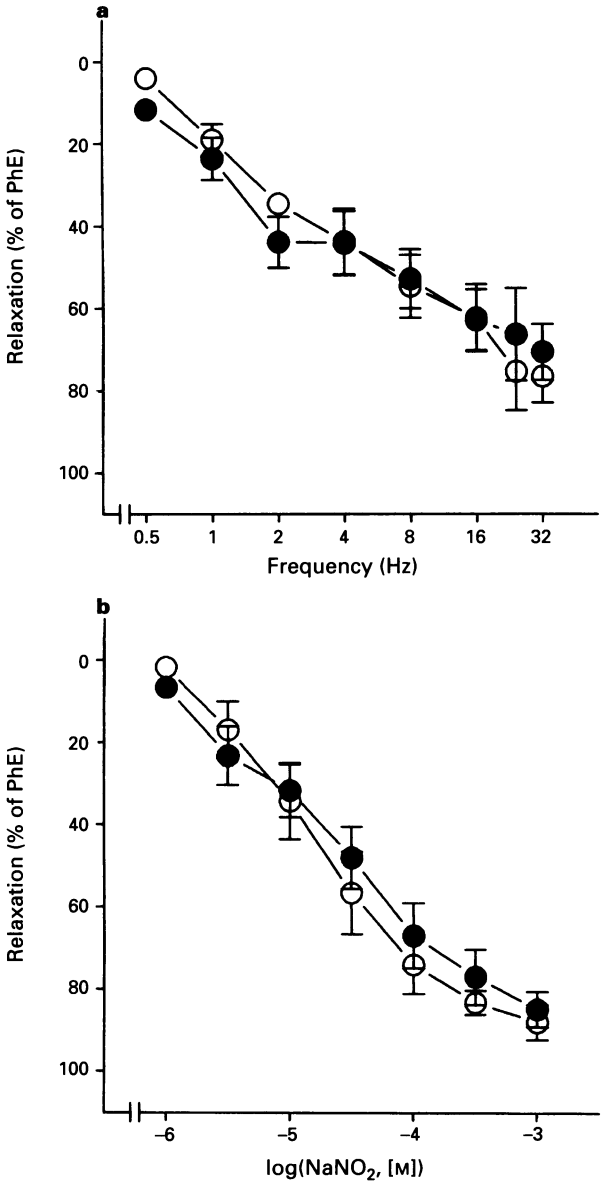

Figure 3 The effect of endothelial cell removal in horse deep penile arteries on the relaxations to (a) electrical field stimulation (0.3 ms, 20 s trains, supramaximal current) and (b) exogenous NO added in the form of acidified sodium nitrite ($NaNO_2$) in phenylephrine (PhE)-contracted endothelium-intact (○) and endothelium-denuded (●) arteries. Guanethidine and atropine were present throughout the experiments. Relaxations are expressed as percentages of the tension induced by PhE, and each point represents the mean ± s.e.mean of 5–9 preparations.

no effect on the inhibitory response to EFS ($n=6$) or increased the relaxations to EFS in preparations with responses of small magnitude in a first control curve ($n=4$) (data not shown).

Effects of N^G-nitro-L-arginine, L-arginine and N^G-nitro-D-arginine on the responses to EFS

D-NOARG (10^{-4} M) had no significant effect on the relaxations to EFS of horse deep penile arteries, while 10^{-4} M L-NOARG caused a significant rightward shift in the frequency-response curve (Figure 4a). In endothelium-intact horse deep penile arteries, L-NOARG (3×10^{-5} M) increased the basal tension by 0.7 ± 0.2 Nm^{-1} ($n=9$), while no contractile effect was observed in endothelium-denuded arteries ($n=4$). In endothelium-intact preparations, the phenylephrine-induced contractions were also increased in the presence of L-NOARG (3×10^{-5} M), and therefore, the concentration of phenylephrine was reduced to 5×10^{-7} M to obtain contractions (7.2 ± 1.5 Nm^{-1}, $n=9$) comparable to those in control curves (7.0 ± 1.0 Nm^{-1}, $n=9$). Pretreatment with L-NOARG (3×10^{-5} M) inhibited the relaxations to EFS at the lowest frequencies, while slowly developing relaxations peaking after 1 min were still observed at 16, 24 and 32 Hz (Figure 2c), and $53.6 \pm 4.8\%$ ($n=9$) relaxation still remained at 32 Hz after L-NOARG incubation (Figure 4b). These persisting relaxations were blocked in the presence of tetrodotoxin (Figure 4b). L-Arg (3×10^{-3} M) alone ($n=4$) had no effect on the relaxations to EFS, but significantly reversed the inhibition induced by L-NOARG (Figure 4b). The relaxations to acidified $NaNO_2$ were unchanged in the presence of L-NOARG (3×10^{-5} M); the pIC_{50} and maximum relaxations were 4.50 ± 0.29 ($n=6$) and $78.6 \pm 8.6\%$ ($n=6$), respectively, in the absence, and 4.52 ± 0.17 ($n=6$) and $91.5 \pm 5.6\%$ ($n=6$), respectively, in the presence of 3×10^{-5} M L-NOARG. In the presence of 3×10^{-5} M L-NOARG, papaverine (10^{-4} M) relaxed the phenylephrine-contracted arteries by $99.8 \pm 0.1\%$ ($n=9$).

Effect of oxyhaemoglobin and methylene blue

In endothelium-intact penile arteries, oxyhaemoglobin (10^{-5} M) and methylene blue (10^{-5} M) induced an increase in basal tension of 0.6 ± 0.3 Nm^{-1} ($n=10$) and 0.8 ± 0.3 Nm^{-1} ($n=12$), respectively. The contractions to phenylephrine were also increased in the presence of these two inhibitors and the concentration of phenylephrine was reduced to 5×10^{-7} M to obtain contractions comparable to those in control curves: the contractions to phenylephrine were 7.0 ± 0.8 Nm^{-1} and 7.0 ± 0.8 Nm^{-1} ($n=10$) in the absence and the presence of oxyhaemoglobin (10^{-5} M), respectively, while in the absence

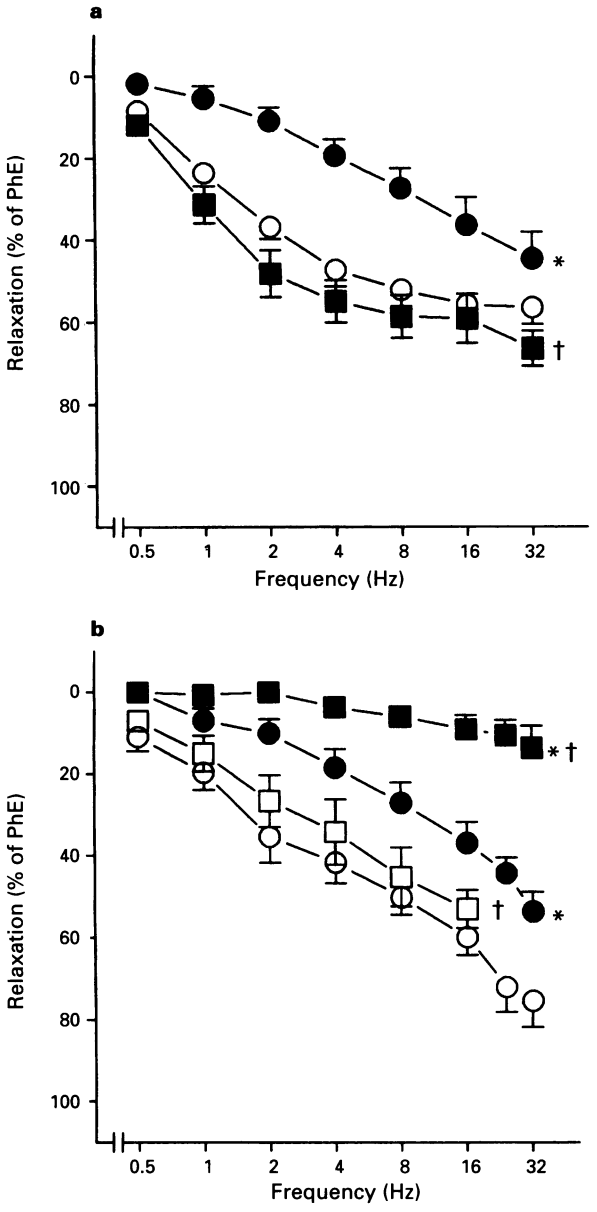

Figure 4 Average relaxations to electrical field stimulation (0.3 ms, 20 s trains, supramaximal current) in horse deep penile arteries contracted with phenylephrine in the presence of guanethidine and atropine (○, control), and (a) the effect of L-NOARG (10^{-4} M) (●) or D-NOARG (10^{-4} M) (■); or (b) the effect of L-NOARG (3×10^{-5} M) (●), L-NOARG and L-ARG (3×10^{-3} M) (□), or L-NOARG (3×10^{-5} M) and tetrodotoxin (10^{-6} M) (■). Relaxations are expressed as percentages of the tension induced by phenylephrine (PhE), and each point represents the mean±s.e.mean of 5–10 endothelium-intact arteries (one per animal). Significantly different curve, tested by analysis of variance followed by Bonferroni method: $^{*}P<0.05$, versus control curve; $^{\dagger}P<0.05$ versus curve performed in the presence of L-NOARG.

and the presence of 10^{-5} M methylene blue phenylephrine-induced contractions were 5.4±0.6 Nm^{-1} and 5.1±0.8 Nm^{-1} ($n=12$), respectively.

The scavenger of NO, oxyhaemoglobin (10^{-5} M), reduced the relaxations to EFS in endothelium-intact arteries although a relaxation of 33.8±7.9% ($n=5$) was still observed at 32 Hz. In contrast to L-NOARG, oxyhaemoglobin completely abolished the relaxations to exogenously added NO (present in acidified solutions of $NaNO_2$) (Figure 5a–b). Methylene blue (10^{-5} M) inhibited the relaxant response to EFS in phenylephrine-contracted preparations, and antagonized the relaxations to acidified $NaNO_2$. However, a relaxation of 28.6±9.8% ($n=6$) and 24.4±5.1% ($n=6$) still persisted at the highest frequency of EFS (32 Hz) and concentration of acidified $NaNO_2$ (10^{-3} M), respectively (Figure 5c–d).

Effect of glibenclamide and charybdotoxin

The blocker of ATP-activated potassium-channels, glibenclamide, had no effect on either basal tension, contractions to phenylephrine, or relaxations to EFS or exogenously added NO as acidified $NaNO_2$ (Figure 6), while the inhibition of Ca^{2+}-activated potassium-channels with 3×10^{-8} M charybdotoxin induced an increase in basal tension of endothelium-intact segments of 1.4±0.2 Nm^{-1} ($n=8$) and the concentration of phenylephrine had to be reduced to 5×10^{-7} M to obtain precontracting levels comparable (6.4±0.6 Nm^{-1}) to those observed in the absence (5.4±0.8 Nm^{-1}, $n=8$) of the antagonist. Charybdotoxin significantly inhibited responses to EFS at all frequencies applied (1–32 Hz) (Figure 6a) and also to acidified $NaNO_2$ in phenylephrine-contracted horse deep penile arteries (Figure 6b).

In the presence of 3×10^{-5} M L-NOARG, the cell permeable analogue of cyclic GMP, 8-Br-cyclic GMP (1 mM) relaxed phenylephrine-contracted arteries by 81.7±8.1% ($n=5$). In the presence of charybdotoxin the relaxations induced by 8-Br-cyclic GMP were significantly reduced to 10.1±5.2% ($n=5$, $P<0.001$, Students t test). In the presence of L-NOARG (3×10^{-5} M), papaverine (10^{-4} M) relaxed contracted arteries by 99.3±0.7% in the absence, and by 84.7±5.2% ($n=8$, $P<0.05$, Students t test) in the presence of 3×10^{-8} M charybdotoxin.

Discussion

Both the finding of NADPH-d positive nerves and the endothelium-independent neurogenic relaxations to EFS, which are markedly reduced by the inhibitor of NOS, L-NOARG, provide evidence for the presence of a functional nitrergic innervation in the horse deep penile arteries.

NADPH-d staining is considered as a specific marker for NOS (Hope & Vincent, 1989), although NADPH-diaphorase activity is associated not only with the constitutive form of NOS, but also with the inducible isoenzyme and microsomal NADPH cytochrome P450 reductase (Schmidt, 1992). Using an appropriate fixation with paraformaldehyde, other forms of NADPH-d activity are inactivated and only the NADPH-d activity associated with soluble NOS remains intact (Matsumoto *et al.*, 1993). Thus, with the fixation procedure applied in the present study, the NADPH-d activity observed in the deep penile arteries can be considered specific for NOS. The presence of NADPH-d positive nerve fibres at the media-adventitia junction and distribution of finer fibres in the outer part of the media of horse deep penile arteries is consistent with the NOS-neuronal staining of deep penile arteries reported for rat (Burnett *et al.*, 1992; Vizzard *et al.*, 1994) and man (Burnett *et al.*, 1993). Thus, the nitrergic innervation of the penile arterial circulation seems to be preserved across various mammalian species.

The persistence of relaxations to EFS after inhibition of the adrenergic and cholinergic neurotransmission and its abolition in the presence of tetrodotoxin provide evidence for a NANC neurogenic relaxation in horse deep penile arteries.

Recently, endothelium-dependent, NO-mediated tetrodotoxin-sensitive relaxations have been observed in rabbit aortic rings (Persico *et al.*, 1993) and hindlimb (Gustafsson *et al.* 1994). However, in the present study although a strong NADPH-d positive reaction was found in the endothelial cell layer, mechanical endothelial cell removal did not alter the relaxations to EFS suggesting that the NANC transmitter is released from stimulated nerves and acts directly on the smooth muscle cells, as reported for bovine cerebral arteries (Ayajiki *et al.*, 1993). This observation is further supported by

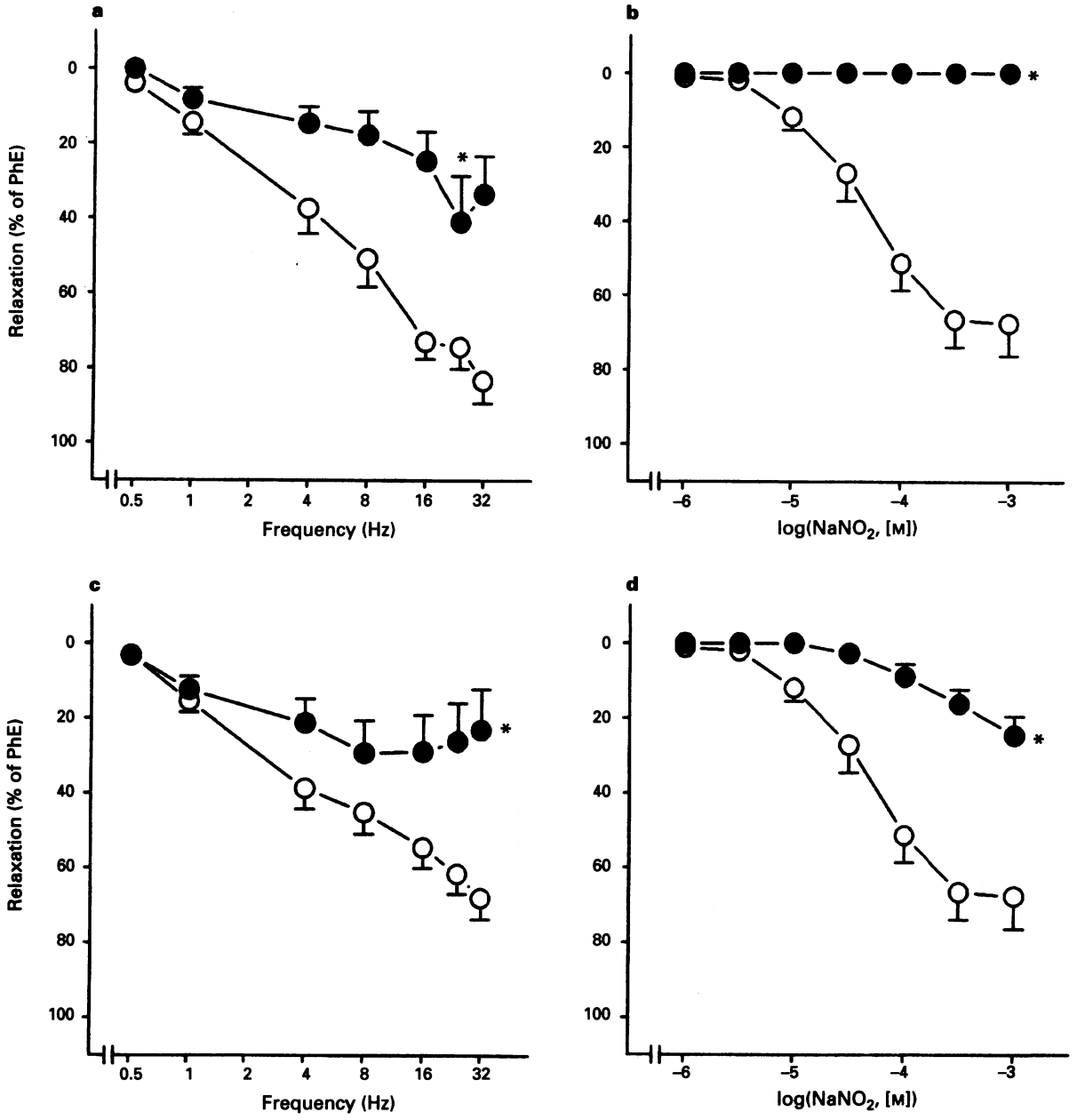

Figure 5 Relaxations to electrical field stimulation (EFS, 0.3 ms, 20 s trains, supramaximal current) (a,c) and exogenous NO (in acidified sodium nitrite, $NaNO_2$) (b,d) in endothelium-intact horse deep penile arteries contracted with phenylephrine (PhE), and effects of 10^{-5} M oxyhaemoglobin (a,b) and 10^{-5} M methylene blue (c,d). Control responses are represented by (○) and those in the presence of oxyhaemoglobin or methylene blue by (●). Relaxations are expressed as percentages of the tension induced by PhE, and each point represents the mean ± s.e.mean of 5–6 arteries (one per animal). Significantly different responses compared to control curve by analysis of variance for repeated measures: $^*P<0.05$.

the obvious large distance between the adventitia-media junction and the endothelium in the very thick-walled deep penile arteries (Figure 1).

NOS utilizes L-arginine and molecular oxygen as substrates with the generation of NO and citrulline. The inhibition of NOS by L-NOARG was competitive and stereospecific, since L-arginine reversed the inhibitory action of L-NOARG, and D-NOARG was ineffective on the EFS-induced relaxations of horse deep penile arteries. Similar observations have been reported earlier for the bovine dorsal penile artery (Liu *et al.*, 1991) and the corpora cavernosa of man and rabbit (Holmquist *et al.*, 1992). The present results provide the first evidence for an endogenous NO-containing compound, synthesized from L-arginine, as a major component of the NANC inhibitory transmission in horse intracavernous deep penile arteries.

Nitrovasodilators stimulate the soluble guanylate cyclase in smooth muscle when NO binds to a haeme group linked to the enzyme (Craven & de Rubertis, 1978). Haemoglobin, which can scavenge NO through its ferrous group, abolished the relaxations to exogenous NO (acidified $NaNO_2$) and markedly inhibited the relaxations to EFS, while the inhibitor of guanylate cyclase, methylene blue (Gruetter *et al.*, 1981), did not cause total inhibition of the relaxations to either EFS or NO (acidified $NaNO_2$) in horse deep penile arteries. These results suggest that NO released by electrical stimulation of dilator nerves causes arterial relaxation via a cyclic GMP-dependent mechanism. However, these results require some caution, since methylene blue has been shown to cause inactivation of NO through either superoxide anion generation (Wolin *et al.*, 1990) or inhibition of NOS at higher concentrations (Mayer *et al.*, 1993).

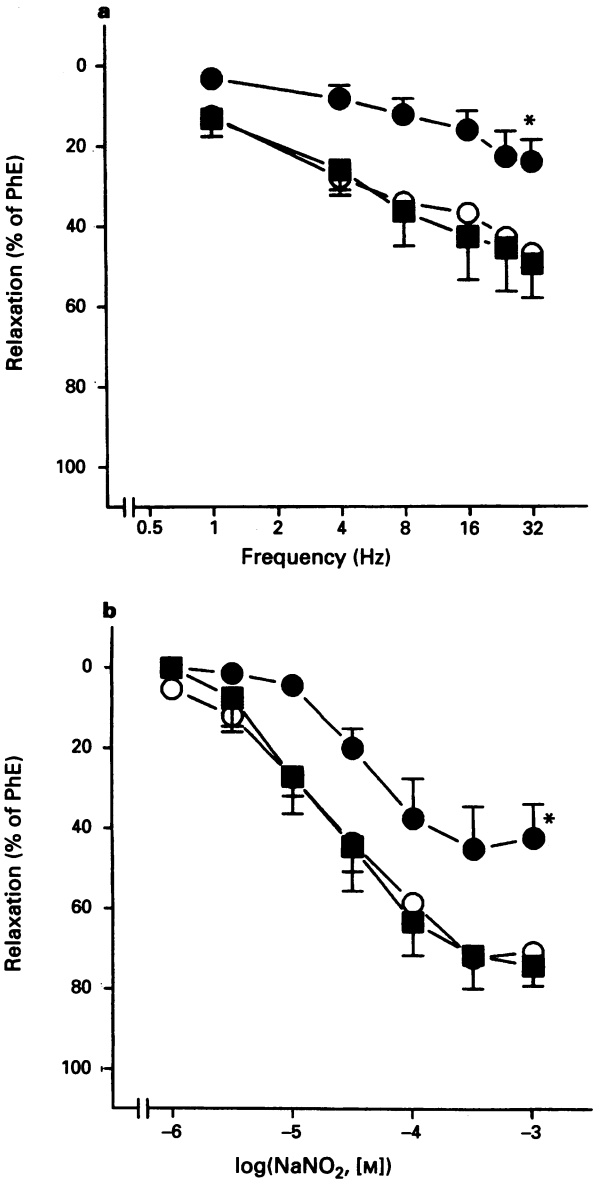

Figure 6 Relaxations to electrical field stimulation (0.3 ms, 20 s trains, supramaximal current) (a) and exogenous NO (in acidified sodium nitrite, $NaNO_2$) (b) in horse deep penile arteries contracted with phenylephrine (PhE). Control responses are represented by (○), those in the presence of 3×10^{-8} M charybdotoxin by (●) and those in the presence of 3×10^{-6} M glibenclamide by (■). Relaxations are expressed as percentages of the tension induced by PhE, and each point represents the mean ± s.e.mean of 4–8 endothelium-intact arteries (one per animal). Significantly different responses compared to control curve by analysis of variance for repeated measures: $^*P < 0.05$.

Although the classical signal transduction pathway ascribed to NO is the activation of soluble guanylate cyclase and a subsequent increase of the cyclic GMP levels in the smooth muscle cells, recent studies have demonstrated that endothelium-derived NO is capable of inducing hyperpolarization of vascular smooth muscle through activation of K^+-channels (Cowan *et al.*, 1993; Bolotina *et al.*, 1994; Vanheel *et al.*, 1994). In addition, nicorandil, which is believed to relax smooth muscle through activation of ATP-sensitive K^+-channels and release of NO, was recently demonstrated to induce relaxation of human isolated cavernous arteries. These relaxations were blocked by the antagonist, glibenclamide, suggesting the presence of ATP-activated K^+-channels in these arteries (Hedlund *et al.*, 1994). However, glibenclamide did not affect the relaxations to either EFS or acidified $NaNO_2$ in the horse deep penile arteries, in a concentration earlier shown to be effective in rabbit mesenteric arteries (Khan *et al.*, 1993). These results do not exclude the presence of this type of K^+-channel in horse penile arteries, but indicate that ATP-activated K^+-channels are not involved in the NO-mediated relaxations in these arteries.

In contrast, the antagonist of Ca^{2+}-activated K^+-channels, charybdotoxin, significantly inhibited the relaxations to EFS. This inhibitory effect appears to be postjunctional since charybdotoxin also effectively inhibited the relaxations to exogenous NO added as acidified $NaNO_2$. Charybdotoxin has recently been shown to augment the NO-mediated relaxations to EFS in the canine ileocolonic junction, but through a presynaptic mechanism (De Man *et al.*, 1993). In horse deep penile arteries, NO or a NO-related compound released from intramural nerve endings causes relaxation through increases in Ca^{2+}-activated K^+-channel conductance of the arterial smooth muscle cells.

Three different mechanisms have been proposed for hyperpolarization through activation of Ca^{2+}-activated K^+-channels by endothelium-derived NO in vascular smooth muscle. Novel studies have demonstrated that endothelium-derived NO opens Ca^{2+}-activated K^+-channels through a cyclic GMP-independent mechanism (Bolotina *et al.*, 1994; Vanheel *et al.*, 1994). However, opening of these channels can also occur through cyclic GMP-dependent mechanisms either via cyclic GMP-dependent protein kinase (Tare *et al.*, 1990; Khan *et al.*, 1993; Robertson *et al.*, 1993) or, as recently shown, directly by cyclic GMP (Goulding *et al.*, 1994).

In the present study, the marked inhibitory effect of charybdotoxin on the relaxations to the cyclic GMP analogue, 8-Br-cyclic GMP, strongly supports a role for Ca^{2+}-activated K^+-channels in the cyclic GMP-mediated relaxations of horse penile arteries, and therefore, in the relaxations induced by NO released from nitrergic nerves in the arterial wall. Interestingly, charybdotoxin caused greater inhibition of the relaxations to 8-Br-cyclic GMP than of a similarly sized relaxation to EFS or exogenous NO. This apparent discrepancy could be ascribed to the different concentration and access to the Ca^{2+}-activated K^+-channels in the cell membrane of the exogenously added 8-Br-cyclic GMP compared to those of the endogenous cyclic GMP formed after activation of guanylate cyclase by NO or EFS. This is also supported by the observation of much slower relaxation of contracted penile arteries to 8-Br-cyclic GMP (2 min to reach maximum relaxation) compared to the relaxations of similar magnitude to either NO (10–15 s) or EFS (15 s). The differential effect of charybdotoxin is unlikely to be related to the method used to generate NO, since neither the acid vehicle applied to dissolve sodium nitrite nor sodium nitrite itself did have any effect on penile arteries. Moreover, both methylene blue and charybdotoxin inhibited to a similar extent the relaxation to both EFS and exogenous NO, thus suggesting that NO might partly relax the smooth muscle by non-cyclic GMP mechanisms insensitive to charybdotoxin.

Intracavernous injection of papaverine has been widely used in the treatment of impotence (Lue & Tanagho, 1987). Papaverine induces relaxation of vascular smooth muscle mainly through the inhibition of the cyclic nucleotide phosphodiesterases followed by increases in levels of intracellular cyclic AMP and to a lesser degree of cyclic GMP (Rüegg, 1992). The latter effect might explain why the relaxations to papaverine, in contrast to those to 8-Br-cyclic GMP, are only slightly inhibited in the presence of charybdotoxin.

After treatment with high concentrations of L-NOARG, oxyhaemoglobin or methylene blue, which all are inhibitors of the L-arginine/NO pathway, there still persist significant relaxations to EFS in horse deep penile arteries, which are slow in onset, development and recovery, but sensitive to TTX, suggesting a neurogenic origin. Thus, we cannot exclude the presence of another inhibitory transmitter that is released concomitantly with NO. Several neurotransmitters such as VIP (Polak *et al.*, 1981; Willis *et al.*, 1981; Andersson *et al.*,

1984) and calcitonin gene-related peptide (CGRP) (Stief *et al.*, 1991) have been proposed as mediators of the vasodilatation associated with penile erection.

In summary, the present study of horse deep penile arteries, which control the blood flow between the systemic arterial circulation and the cavernous sinoids, indicates that the main inhibitory neurotransmitter causing vasodilatation in erection in this tissue is NO or a NO-related compound. NO probably acts through the activation of guanylate cyclase with accumulation of cyclic GMP that increases the open probability of Ca^{2+}-activated K^+-channels causing hyperpolarization and relaxation of the penile arteries. Thus, one might speculate whether in some cases, application of agonists that open Ca^{2+}-activated K^+-channels would provide a more physiological treatment for impotence.

We thank the Madrid Municipal Slaughterhouse for kindly supplying fresh tissue. This work was supported by project No. PM 92-0031 of DGICYT and PR 188/92-4103 of UCM. U.S. was supported by a grant from Universidad Complutense de Madrid. I.S.D.T. was supported by grants from the National Institute of Health (Rol-DK-39080 and DK-40487). The Spanish laboratory is participating in the European Working Party on Resistance Artery Disease (EURAD).

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(Received January 10, 1995
Revised July 21, 1995
Accepted July 27, 1995)