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Nitrergic relaxation of the horse corpus cavernosum. Role of cGMP

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Abstract

The involvement of nitric oxide (NO) and the mechanisms mediating neurogenic relaxation were investigated in the horse corpus cavernosum. NADPH-diaphorase activity was expressed in nerve fibres around arteries and muscular bundles in the horse trabecular tissue. Relaxations in response to electrical field stimulation were tetrodotoxin (10^{-6} M)-sensitive, indicating their neurogenic origin. The NO synthase inhibitor, L-NO-arginine (L-NO-Arg, 3×10^{-5} M), abolished the electrically induced relaxations, which were significantly reversed by L-arginine (3×10^{-3} M). Exogenous NO (10^{-6}–10^{-3} M) evoked relaxations which were unaffected by L-NO-Arg. 1*H*-[1,2,4]oxadiazolo[4,3,-*a*]quinoxalin-1-one (ODQ, 5×10^{-6} M), an inhibitor of guanylate cyclase activation by NO, reduced the relaxations in response to electrical stimulation and exogenous NO. Iberiotoxin (3×10^{-8} M) or apamin (5×10^{-7} M), inhibitors of large and small conductance Ca^{2+}-activated K^+ channels, respectively, and glibenclamide (3×10^{-6} M), a blocker of ATP-sensitive K^+ channels, failed to modify the relaxations with NO. It is suggested that NO is present in nerve fibres of the horse corpus cavernosum and relaxes smooth muscle through a guanylate cyclase-dependent mechanism. Neither Ca^{2+}-activated nor ATP-sensitive K^+ channels seem to be involved in these relaxations.

Keywords: Corpus cavernosum (horse); NADPH-diaphorase; Nitric oxide (NO); Guanylate cyclase; K^+ channel

1. Introduction

Penile erection is initiated when, under parasympathetic influence, the sinusoids and the cavernosal and helicinal arteries dilate, with subsequent increase in blood flow to the lacunar spaces of the corpus cavernosum. Relaxation of the trabecular smooth muscle then follows, expanding and compressing the plexus of subtunical venules against the tunica albuginea, thus reducing venous outflow and increasing intracavernous pressure, leading to penis engorgement and erection (Andersson, 1993).

The peripheral nervous control of relaxation of trabecular smooth muscle is dependent on an interaction between adrenergic, cholinergic and nonadrenergic noncholinergic (NANC) mechanisms (Klinge and Sjöstrand, 1977; Hedlund and Andersson, 1985; Saenz de Tejada et al., 1988, 1989, 1991). The relaxation of trabecular tissue is achieved partly by cholinergic nerves, that suppress excitatory adrenergic neurotransmission, but also by the release of relaxing-NANC transmitter(s) from nerves and from the endothelium. Thus, NANC neurotransmission has been considered the most likely neural pathway involved in penile erection (Bowman and Gillespie, 1983; Andersson, 1993).

By means of radioimmunoassay and immunohistochemistry techniques, vasoactive intestinal peptide (VIP) has been localized in the corpus cavernosum (Shirai et al., 1990). Moreover, the observation that pelvic nerve stimulation caused an output of VIP both from the dog and cat penis (Willis et al., 1981; Andersson et al., 1987), led to the suggestion that VIP might be an important neurotransmitter in penile erection. However, VIP alone caused relatively poor erections even after direct injection into the corpus cavernosum (Juenemann et al., 1987; Wagner and Gerstenberg, 1987; Roy et al., 1990). Recently, Kim et al. (1995) suggested that VIP may contribute to NANC neurally mediated cavernosum relaxation, and that its mecha-

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nisms of relaxation involve the generation of prostanoids and nitric oxide (NO).

NO has been identified as an important inhibitory neurotransmitter involved in penile erection in man (Kim et al., 1991; Bush et al., 1992; Holmquist et al., 1992; Rajfer et al., 1992), rabbit (Ignarro et al., 1990; Kim et al., 1991; Bush et al., 1992; Holmquist et al., 1992) and dog (Hedlund et al., 1995). NO causes relaxation by activating soluble guanylate cyclase, resulting in accumulation of intracellular guanosine 3′:5′-cyclic monophosphate (cyclic GMP) (Ignarro et al., 1990). Cyclic GMP seems to elicit relaxation of smooth muscle by lowering the intracellular Ca^{2+} concentration through stimulation of sarcoplasmic Ca^{2+}-ATPase activity, or through opening of K^+ channels leading to hyperpolarization (Lincoln and Cornwell, 1991; Robertson et al., 1993). In addition, NO can directly stimulate Ca^{2+}-activated K^+ channels in both vascular (Bolotina et al., 1994) and colon (Koh et al., 1995) smooth muscle.

During erection, the local release of NO from endothelium and/or the nerves causes relaxation of corpus cavernosum smooth muscle. Evidence for a source of NO nerves supplying the trabecular tissue has been provided by the immunohistochemical localization of the enzyme that synthesizes NO, NO synthase, in penile erectile tissue from rat, man and dog (Burnett et al., 1992, 1993; Vizzard et al., 1994; Hayashida et al., 1996), as well as in the intracavernosum small penile arteries (Simonsen et al., 1995). Results of recent studies (Simonsen et al., 1995, 1997c; Recio et al., 1997), suggest that the reactivity of the erectile tissue of the horse is similar to that of the human (Hedlund and Andersson, 1985; Saenz de Tejada et al., 1989; Simonsen et al., 1997b). However, there are no data related to the presence and role of NO in the horse corpus cavernosum. The aim of the present study was to investigate both the possible involvement of the L-arginine/NO neural pathway in inhibitory neurotransmission, and the mechanism underlying relaxations in response to NO in the horse corpus cavernosum smooth muscle.

2. Material and methods

2.1. Tissue preparation

Corpus cavernosum tissue was obtained from adult horse penis in the local slaughterhouse, immediately after the animals were killed by stunning followed by exsanguination. The tissue was placed in cold physiological saline 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, $CaCl_2$ 1.5, 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.

2.2. NADPH-diaphorase histochemistry

Samples of isolated corpus cavernosum were immersed in 4% paraformaldehyde in 0.1 M sodium phosphate-buffered saline (PBS, pH 7.3) at 4°C for 24 h, and then placed in a cryoprotective PBS solution containing 30% sucrose for 24 h at 4°C. Sections (40 μm) were obtained using a freezing microtome and processed for NADPH-diaphorase histochemistry following the protocol of Vicent and Kimura (1992) with minor modifications. Briefly, the preparations were incubated in a medium containing β-NADPH 1 mg/ml, nitro-blue tetrazolium 0.25 mg/ml and 0.3% Triton X-100 in 0.1 M PBS (pH = 7.4), for 30–45 min at 37°C and protected from light. After incubation, the sections were rinsed in PBS, mounted, dehydrated in a graded ethanol series, cleared with xylene and covered with DePeX (neutral solution of polystyrene and plasticizers in xylene). The following controls of the histochemical reaction were carried out: (1) incubation without the substrate β-NADPH; (2) incubation without the chromogen nitro-blue tetrazolium in order to rule out possible nonspecific formation of reaction products; and (3) overfixation of the tissue (2 weeks in fixative medium). In all cases, no residual reaction was observed.

2.3. Experimental procedures

Corpus cavernosum strips (4 mm long and 2 mm wide) were suspended horizontally, placed parallel between two platinum electrodes, with one end connected to an isometric transducer (Grass FT O3C) and the other to a displacement unit, in 5 ml organ baths with PSS, 37°C, pH 7.4. The signal was recorded continuously on a polygraph (Grass 79E). A passive tension of 3 g was applied to corpus cavernosum strips and they were allowed to equilibrate for 60 min. The contractile capacity of the preparations was challenged by exposing the strips to 124 mM K^+-rich PSS (K^+-PSS) which was PSS with KCl replacing for NaCl on an equimolar basis. Corpus cavernosum strips were incubated with guanethidine (10^{-5} M) and atropine (10^{-7} M), during a period of 1 h with washing every 20 min. The drugs were present throughout both electrical field stimulation and exogenous NO experiments, to block adrenergic neurotransmission and muscarinic receptors, respectively. The preparations were contracted with phenylephrine (2×10^{-7} M), and when a stable tone was obtained electrical stimulation was performed with rectangular pulses (1 ms duration, 0.5–16 Hz, 20 s trains) at 3 min intervals from a Cibertec CS20 stimulator (Barcelona, Spain) with constant supramaximal current output adjusted to 75 mA. A first frequency–response curve was made with phenylephrine-contracted preparations. The horse corpus cavernosum strips were repeatedly washed and allowed to equilibrate for at least 1 h before they were incubated with either tetrodotoxin (10^{-6} M), N^G-nitro-L-arginine (L-NO-Arg, 3×10^{-5} M), L-arginine (L-Arg, $3 \times$

10^{-3} M), L-Arg (3×10^{-3} M) plus L-NO-Arg (3×10^{-5} M), N^G-nitro-D-arginine (D-NO-Arg, 3×10^{-5} M), apamin (5×10^{-7} M), glibenclamide (3×10^{-6} M), Iberiotoxin (3×10^{-8} M) or 1*H*-[1,2,4]oxadiazolo[4,3,-*a*]quinoxalin-1-one (ODQ, 5×10^{-6} M). After 20 to 30 min incubation with the corresponding drug, a second frequency–response curve was made. In each experiment, control curves were run in parallel. Cumulative concentration–response curves for exogenous NO (added as acidified $NaNO_2$ solution) and *S*-nitroso-L-cysteine were obtained with phenylephrine-precontracted corpus cavernosum strips by adding the drug directly to the bath. The preparations were incubated with the blocking agents for 30 min before the concentration–response curves were repeated. Relaxation experiments for both electrical field stimulation and exogenously added NO were performed in the same preparation.

At the end of the experiments, papaverine (10^{-4} M) was added to the organ bath with the aim of obtaining maximal relaxation of preparations.

2.4. Drugs and solutions

The following drugs were used: apamin, atropine sulphate, glibenclamide, guanethidine sulphate, iberiotoxin, L-arginine hydrochloride, β-nicotinamide adenine dinucleotide phosphate reduced form, nitro-blue tetrazolium, L-NO-Arg, D-NO-Arg, phenylephrine hydrochloride, papaverine hydrochloride, sodium nitrite, tetrodotoxin and Triton X-100 (Sigma, St. Louis, MO, USA), ODQ (Tocris Cookson, Bristol, UK). All drugs were dissolved in twice distilled water, except glibenclamide and ODQ which were dissolved in dimethyl sulphoxide, and papaverine, which was dissolved in 96% ethanol. Stock solutions were prepared and stored at -20°C and further fresh dilutions were prepared daily. The $NaNO_2$ solution of 1 M was prepared daily in distilled water with HCl (37%) obtaining a final pH of 2. This solution was placed on dry ice and protected from air. Further dilutions were made in this acidified solution. *S*-Nitroso-L-cysteine was prepared fresh just before use by reacting equimolar concentrations of L-cysteine and sodium nitrite under acidic conditions. Neither acid solvent nor $NaNO_2$ solution (pH = 7.4) had any effect on corpus cavernosum strips.

2.5. Calculations and statistics

For each frequency or concentration–response curve, the pulse frequency or drug concentration in the absence or presence of blocking agent required to give half-maximal relaxation (EF_{50} or EC_{50}, respectively) was determined with a computer programme (Graph Pad Inplot 4.1, San Diego, CA, USA), fitting the data to the Hill equation: $E/E_{max} = A(M)^n_H/(A(M) + EC_{50}(M)^n_H)$, where E/E_{max} is the relative response to the effective concentration of drug, $A(M)$, and $EC_{50}(M)$ are given in molar concentrations; n_H is a curve fitting parameter or Hill coefficient. The sensitivity and maximal relaxant responses of the exogenous NO and *S*-Nitroso-L-cysteine were expressed in terms of pD_2 and E_{max}, respectively. pD_2 was defined as the negative logarithm of EC_{50} ($-\log EC_{50}$). The results are expressed as mean values ± S.E.M. where *n* indicates the number of preparations studied in each set of experiments. Each parameter was determined from penis tissue of at least 4–5 different animals. Statistical significance of differences was calculated by one-way analysis of variance (ANOVA) and Student's *t*-test with a posteriori Bonferroni test (Wallenstein et al., 1980). A probability value less than 0.05 was considered significant.

3. Results

3.1. NADPH-diaphorase activity

Neural NO synthase-containing fibres were visualized by NADPH-diaphorase staining in cryostat cross-sections of the corpus cavernosum. NADPH-diaphorase activity was present along and between smooth muscle bundles of sinusoids in horse penis (Fig. 1A). These nerves present different sizes and coarse nerve trunks; wavy bundles of nerves usually oriented parallel to the muscle fascicles were found (Fig. 1B). The smooth muscle bundles show a clear network of varicose nerve terminals (Fig. 1C). The cavernosum tissue muscular arteries of different sizes were surrounded by a dense plexus of varicose nerves forming a plexus of the adventitia. NADPH-diaphorase activity was present in the arterial endothelium (Fig. 1D).

3.2. Responses to electrical field stimulation and exogenous NO

Horse corpus cavernosum strips were equilibrated to a passive tension of 2.7 ± 0.1 g ($n = 64$). Phenylephrine (2×10^{-7} M) induced sustained contractions of 4.7 ± 0.5 g ($n = 64$). In the presence of guanethidine (10^{-5} M) and atropine (10^{-7} M), electrical stimulation evoked frequency–response relaxations at 0.5–16 Hz, with an $EF_{50} = 3.1 \pm 0.2$ Hz and a maximum relaxation (E_{max}) obtained at 16 Hz which averaged $56.5 \pm 3.7\%$ ($n = 43$) of the tone induced by phenylephrine (2×10^{-7} M) (Fig. 2A). The electrically induced relaxations were reproducible, giving EF_{50} values and E_{max} of 3.0 ± 0.2 Hz and $60.4 \pm 4.1\%$ and 3.2 ± 0.3 Hz and $59.5 \pm 3.4\%$, in a first and second frequency–response curve, respectively, made with the same preparation ($n = 5$).

Exogenous NO (10^{-6}–10^{-3} M) induced relaxations with a pD_2 of 4.8 ± 0.1 and an E_{max} of $85.3 \pm 2.1\%$ ($n = 42$) of the phenylephrine-precontracted strips (Fig. 2A). These relaxations were reproducible in a second curve with a pD_2 of 4.7 ± 0.3 and an E_{max} of $83.2 \pm 2.4\%$ ($n = 5$). Papaverine (10^{-4} M) evoked a maximum relax-

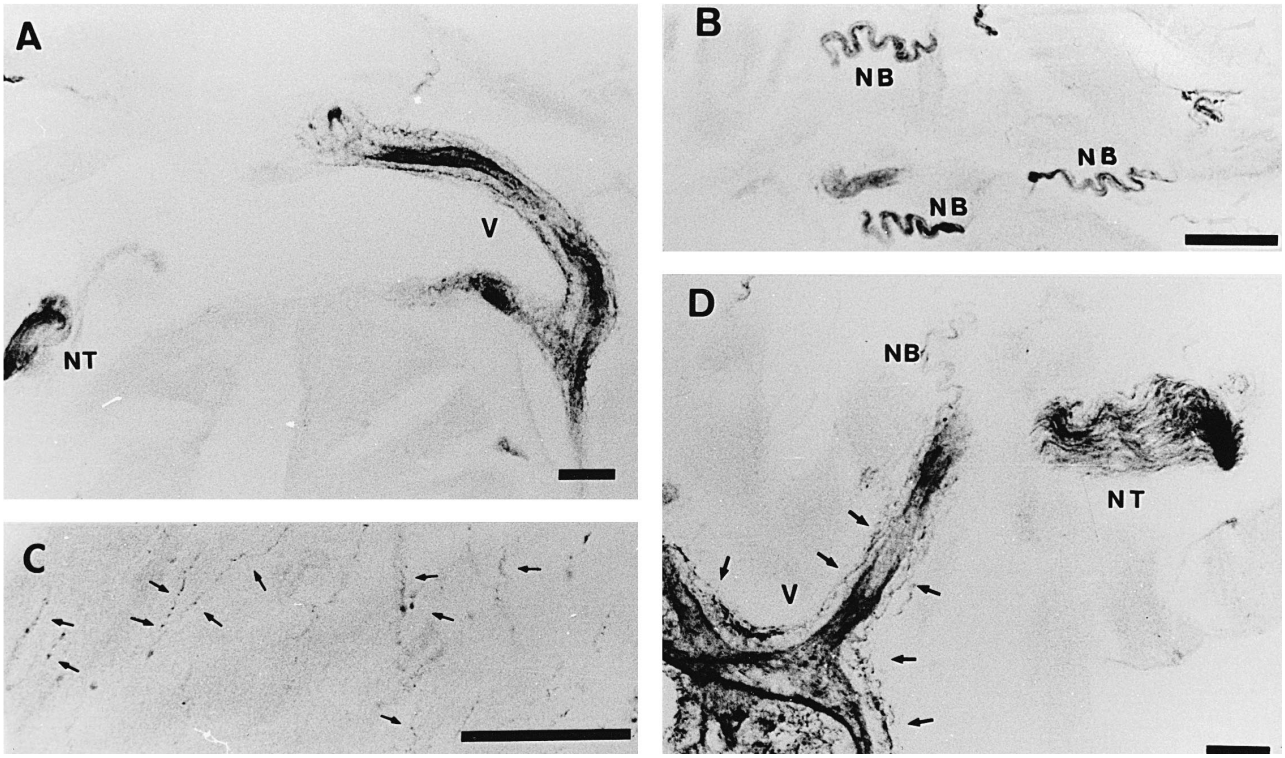

Fig. 1. Photomicrographs showing the NADPH-diaphorase activity in corpus cavernosum. (A) General view of smooth muscle bundles of sinusoids in the horse penis with a positive nerve trunk (NT) and blood vessels (V) surrounded by a dense plexus of positive varicose nerves. (B) Positive wavy nerve bundles (NB). (C) Network of positive varicose nerve terminals (arrows) in smooth muscle bundles. (D) Positive nerve bundle (NB) forming a dense plexus of varicose nerves (arrows) around the blood vessels (V) and a thick positive nerve trunk (NT) in horse corpus cavernosum. A positive NADPH-diaphorase reaction was present in the arterial endothelium. Scale bar = 100 μm.

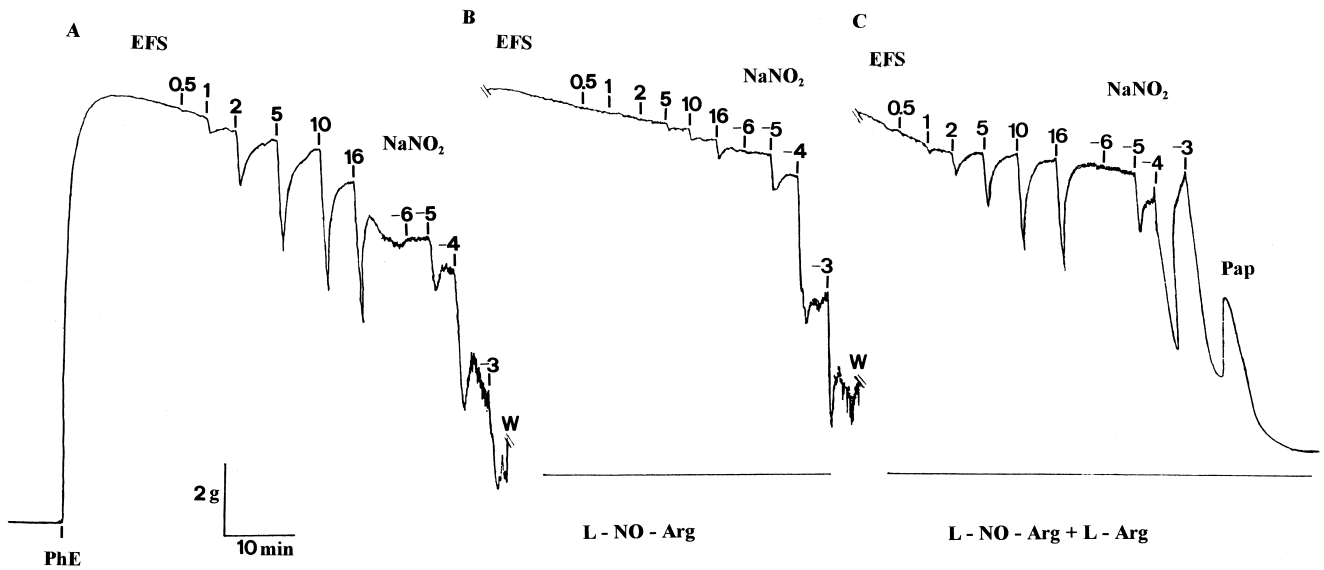

Fig. 2. Isometric force recordings showing the response of corpus cavernosum preparations of horse to electrical field stimulation (EFS, 0.5–16 Hz) and exogenous addition of NO (10^{-6}–10^{-3} M, added as an acidified solution of $NaNO_2$). Guanethidine (10^{-5} M) and atropine (10^{-7} M) were present throughout the experiment to block adrenergic and cholinergic responses, respectively. The corpus cavernosum strips were contracted with phenylephrine (PhE, 2×10^{-7} M) and frequency and concentration–response curves for EFS and NO were made in the absence (A) and the presence of L-NO-Arg (3×10^{-5} M) (B), and L-NO-Arg (3×10^{-5} M) plus L-Arg (3×10^{-3} M) (C). At the end of the experiment the preparations were relaxed with papaverine (Pap, 10^{-4} M). Numbers indicate frequency (Hz) or molar concentration in the bath, W: washout.

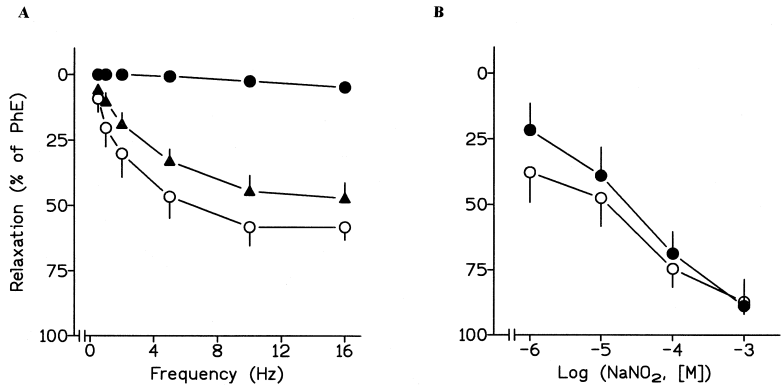

Fig. 3. Average relaxations in response to electrical field stimulation (A) and exogenous NO (in acidified sodium nitrite, $NaNO_2$) (B) in horse corpus cavernosum strips contracted with phenylephrine (2×10^{-7} M) in the presence of guanethidine and atropine (open circles, control), and the effect of L-NO-Arg (3×10^{-5} M) (closed circles) and L-NO-Arg (3×10^{-5} M) plus L-Arg (3×10^{-3} M) (closed triangles). Relaxations are expressed as percentages of the tension induced by phenylephrine and each point represents the mean ± S.E.M. for 8–10 preparations.

ation (100%) of 2×10^{-7} M phenylephrine-precontracted strips.

The relaxations in response to electrical stimulation were completely inhibited in the presence of the neuronal sodium channels blocker, tetrodotoxin (10^{-6} M), with an $EF_{50} = 1.7 \pm 0.2$ Hz and $E_{max} = 72.7 \pm 7.4\%$ under control conditions and $E_{max} = 0\%$ in the presence of tetrodotoxin ($n = 8$). Incubation with the NO synthase blocker, L-NO-Arg (3×10^{-5} M), inhibited electrically elicited relaxations. Thus, the EF_{50}-values and E_{max} were 2.9 ± 0.6 Hz and $63.6 \pm 6.1\%$ in the absence, and 8.5 ± 1 Hz ($P < 0.001$, ANOVA followed by the Bonferroni test, $n = 10$) and $4.3 \pm 2.2\%$ ($P < 0.001$, ANOVA followed by the Bonferroni test, $n = 10$) in the presence of L-NO-Arg (3×10^{-5} M), respectively (Fig. 2B and Fig. 3A). The NO synthesis substrate, L-Arg (3×10^{-3} M), did not modify either the basal tension (2.7 ± 0.3 g and 2.7 ± 0.3 g, $n = 5$ in the absence and presence of L-Arg, respectively), or the electrically-induced relaxations ($EF_{50} = 1.9 \pm 0.3$ Hz and $E_{max} = 68.1 \pm 6.2\%$ and $EF_{50} = 1.8 \pm 0.5$ Hz and $E_{max} = 69.7 \pm 7.1\%$, $n = 5$ under control conditions and after treatment, respectively) of the horse corpus cavernosum strips. However, L-Arg (3×10^{-3} M) significantly reversed the inhibition caused by L-NO-Arg (3×10^{-5} M) with an $EF_{50} = 2.9 \pm 0.6$ Hz and $E_{max} = 63.6 \pm 6.1\%$ and $EF_{50} = 2.7 \pm 0.5$ Hz and $E_{max} = 47.2 \pm 6\%$, in controls and in the presence of L-NO-Arg (3×10^{-5} M) plus L-Arg (3×10^{-3} M), respectively ($P < 0.05$ vs. control, ANOVA followed by the Bonferroni test, $n = 10$) (Fig. 2C and Fig. 3A). D-NO-Arg (3×10^{-5} M), was without effect on electrically-induced relaxations ($EF_{50} = 3.5 \pm 1.2$ Hz and $E_{max} = 49.7 \pm 12.1\%$ in controls and $EF_{50} = 3.2 \pm 0.9$ Hz and $E_{max} = 45.6 \pm 8.4\%$) after D-NO-Arg treatment, $n = 4$).

Relaxations in response to exogenous NO were not changed in the presence of tetrodotoxin (10^{-6} M) with a $pD_2 = 4.8 \pm 0.2$ and $E_{max} = 91.4 \pm 14.2\%$ and $pD_2 = 4.6 \pm 0.4$ and $E_{max} = 81.8 \pm 14.3\%$ ($n = 6$) under control conditions and after treatment, respectively. The incubation with L-NO-Arg (3×10^{-5} M) did not alter the relaxations caused by exogenous NO. pD_2 and E_{max} being 5.4 ± 0.3 and $88.9 \pm 5.1\%$, and 4.9 ± 0.3 and $93.2 \pm 5.3\%$,

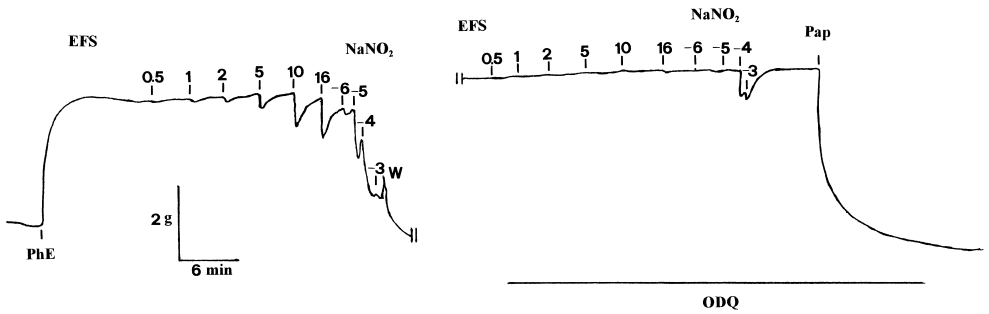

Fig. 4. Isometric force recordings showing the effect of ODQ (5×10^{-6} M) on the responses of horse corpus cavernosum strips to EFS (0.5–16 Hz) and exogenous addition of NO (10^{-6}–10^{-3} M, added as an acidified solution of $NaNO_2$). Guanethidine (10^{-5} M) and atropine (10^{-7} M) were present throughout the experiment, to block adrenergic neurotransmission and muscarinic receptors, respectively. The corpus cavernosum strips were contracted with phenylephrine (PhE 2×10^{-7} M) and when a sustained tone was obtained, frequency and concentration–response curves for EFS and NO were made in the absence and presence of ODQ (5×10^{-6} M). At the end of the experiment, papaverine (Pap, 10^{-4} M) was added to obtain the maximal relaxation of the horse corpus cavernosum preparations. Numbers indicate frequency (Hz) or molar concentration in the bath. W: washout.

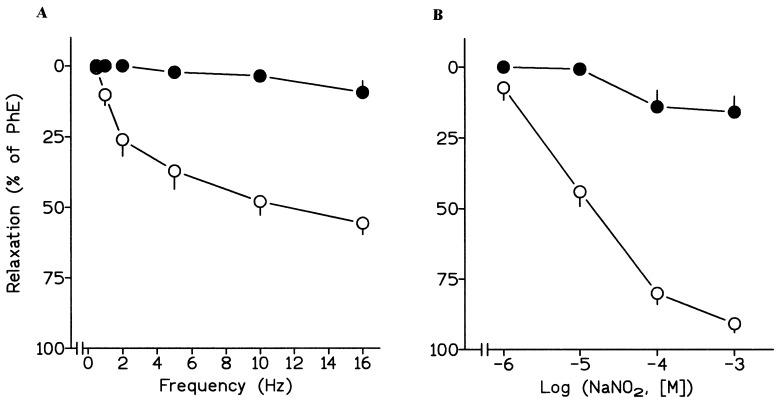

Fig. 5. Relaxations in response to electrical field stimulation (A) and exogenous NO (as acidified sodium nitrite, $NaNO_2$) (B) in horse corpus cavernosum strips contracted with phenylephrine (2×10^{-7} M) in absence (open circles) and presence (closed circles) of ODQ (5×10^{-6} M). Relaxations are expressed as percentages of the tension induced by phenylephrine, and each point represents the mean ± S.E.M. for 7–9 preparations.

($n = 7$) in absence and in presence of the NO synthase inhibitor, L-NO-Arg (3×10^{-5} M), respectively (Fig. 2B and Fig. 3B). The NO donor, *S*-Nitroso-L-cysteine (10^{-7}–10^{-4} M) induced relaxations of phenylephrine-contracted corpus cavernosum strips, ($pD_2 = 6.1 \pm 0.2$ and an E_{max} of 95.4 ± 2.4%, $n = 7$).

3.3. Effect of the cyclic GMP inhibitor, ODQ

The inhibitor of soluble guanylate cyclase ODQ (5×10^{-6} M) had no effect on the resting tension of trabecular strips. ODQ (5×10^{-6} M) however, markedly reduced the electrically induced relaxations (Figs. 4 and 5A, Table 1). Similarly, incubation with ODQ (5×10^{-6} M) also inhibited the relaxations in response to exogenous NO, although a relaxation of 15.9 ± 5.6% ($n = 9$) still persisted at the highest concentration (10^{-3} M) (Figs. 4 and 5B, Table 1).

Table 1
The effect of the inhibitors of cGMP and K^+ channels, ODQ (5×10^{-6} M), iberiotoxin (IbTX, 3×10^{-8} M), apamin (Apa, 5×10^{-7} M), and glibenclamide (Glib, 3×10^{-6} M) on relaxations in response to EFS and exogenous NO in phenylephrine-precontracted (2×10^{-7} M) horse corpus cavernosum strips

	EFS			NO		
	n	EF_{50} (Hz)	E_{max} (%)	*n*	pD_2	E_{max} (%)
Control	7	3.2 ± 0.6	55.6 ± 4.3	9	4.7 ± 0.1	90.8 ± 3.2
ODQ	7	11.3 ± 2.7[a]	9.4 ± 4.3[b]	9	–	15.9 ± 5.6[b]
Control	5	4.7 ± 0.4	42.5 ± 5.6	5	4.5 ± 0.2	83.1 ± 5.5
IbTX	5	4.4 ± 0.6	37.9 ± 3.0	5	4.5 ± 0.4	78.0 ± 6.0
Control	5	2.6 ± 0.6	52.2 ± 8.1	8	4.5 ± 0.2	81.5 ± 5.9
Apa	5	3.0 ± 0.8	47.0 ± 6.4	8	4.8 ± 0.2	77.5 ± 5.3
Control	5	3.3 ± 0.4	47.1 ± 7.7	7	4.9 ± 0.1	73.9 ± 4.7
Glib	5	3.0 ± 0.5	51.4 ± 3.8	7	4.8 ± 0.1	77.8 ± 4.0

Values are means ± S.E.M.
n = Number of preparations from 4–5 different animals.
EF_{50} is the pulse frequency causing half-maximal relaxation and pD_2 is the negative logarithm of the EC_{50} ($-\log EC_{50}$).
E_{max} is the maximal relaxation expressed relative to the contraction induced by phenylephrine (2×10^{-7} M).
Significantly different values of parameters with respect to control values, as calculated by analysis of variance (ANOVA), followed by an a posteriori Bonferroni test.
[a] $P < 0.01$; [b] $P < 0.001$.

3.4. Effect of K^+ channel blockers

Iberiotoxin (3×10^{-8} M) and apamin (5×10^{-7} M), inhibitors of the large and small conductance Ca^{2+}-activated K^+ channels, respectively, had no effect on basal tension of the horse corpus cavernosum strips. Moreover, both the sensitivity and maximal relaxations in response to electrical stimulation and exogenous NO remained unchanged in the presence of either iberiotoxin or apamin (Table 1), respectively.

The blocker of ATP-activated K^+ channels, glibenclamide (3×10^{-6} M), did not change the basal tension of corpus cavernosum strips. Glibenclamide (3×10^{-6} M) had no effect on electrically induced relaxations or exogenously added NO (Table 1).

4. Discussion

The present study revealed that NADPH-diaphorase activity is expressed in nerves and that electrical field stimulation-released and exogenously added NO induces relaxations of the cavernosum trabecular smooth muscle from horse through a soluble guanylate cyclase-dependent mechanism. Iberiotoxin, apamin and glibenclamide-sensitive K^+ channels do not appear to contribute to these relaxations.

NADPH-diaphorase staining is considered as a marker for the enzyme NO synthase in brain and peripheral tissues

(Hope and Vincent, 1989; Dawson et al., 1991; Hope et al., 1991; Alm et al., 1993). During fixation with paraformaldehyde, only the NADPH-diaphorase activity associated with soluble NO synthase remains intact (Matsumoto et al., 1993). Thus, with the fixation procedure applied in the present study, the NADPH-diaphorase activity observed can be considered specific for NO synthase. NADPH-diaphorase-positive nerves were found in the horse corpus cavernosum as well as in human (Burnett et al., 1993), dog (Hedlund et al., 1995; Hayashida et al., 1996) and horse deep penile arteries (Simonsen et al., 1995). Moreover, the positive nerves of horse trabecular tissue have varions sizes. There are thick nerve trunks, wavy nerve bundles and a dense networks of varicose nerve terminals in smooth muscle bundles and muscular arteries in cavernosum tissue of horse penis. The localization of NO synthase suggests that NO may act as an inhibitory neurotransmitter in the horse corpus cavernosum.

The abolition by tetrodotoxin of electrically induced relaxations in the presence of guanethidine and atropine indicates that these responses in horse corpus cavernosum are mediated via a NANC neuronal pathway.

NO is synthesized by NO synthase, which catalyses the conversion of molecular oxygen (O_2) and the amino-acid L-arginine to form NO and L-citrulline. This reaction can be inhibited by various *N*-substituted analogues of L-arginine, such as *N*-methyl-, *N*-nitro- and *N*-amino-L-arginine, by competition with endogenous pools of substrate (Palmer et al., 1988). The inhibition of NO synthesis can be overcome by subsequent addition of the exogenous substrate, L-arginine. In the present study, the electrically induced relaxations of horse corpus cavernosum, were markedly inhibited by L-NO-Arg, but not by D-NO-Arg, and this inhibition was reversed by the addition of excess synthesis substrate, L-arginine. Addition of exogenous NO mimics the electrically induced relaxations but these are not affected by L-NO-Arg, which suggests that NO produces direct relaxation of the cavernosum trabecular smooth muscle. These results suggest that NO or a NO-related compound is involved in the NANC relaxations of the horse corpus cavernosum and confirm earlier observations in penile corpus cavernosum of human (Kimura et al., 1992; Rajfer et al., 1992), rabbit (Kim et al., 1991; Bush et al., 1992; Knispel et al., 1992; Holmquist et al., 1992), and dog (Hayashida et al., 1996). Moreover, the present data agree with results of recent studies showing the presence of a functional nitrergic innervation in both human (Simonsen et al., 1997b) and horse (Simonsen et al., 1995) penile resistance arteries. Cyclic GMP and cyclic adenosine 3′:5′-monophosphate (cAMP) are important intracellular messengers in smooth muscle cells, and NO usually causes relaxation by activating soluble guanylate cyclase by binding to the heme group on this enzyme, resulting in accumulation of intracellular cyclic GMP (Ignarro et al., 1990). Methylene blue has been applied as inhibitor of the NO-elicited soluble guanylate cyclase activation (Gruetter et al., 1981) and cyclic GMP accumulation (Martin et al., 1985). The relaxations induced by electrical field stimulation and exogenous NO were inhibited in the presence of methylene blue in several tissues of the urogenital tract such as trigone (Persson and Andersson, 1992) and intravesical ureter (Hernández et al., 1997) of the pig; urethra of several species (Andersson, 1993); dog (Hayashida et al., 1996), man and rabbit (Kim et al., 1991; Azadzoi et al., 1992) corpus cavernosum and horse deep penile arteries (Simonsen et al., 1995). However, methylene blue had shown nonspecific actions, i.e., inactivation of NO by generating superoxide anions which scavenge NO (Wollin et al., 1990) and inhibition of endothelial NO synthase (Mayer et al., 1993).

Recently, ODQ, a potent and selective inhibitor of NO-stimulated guanylate cyclase activity, has been synthesized (Garthwaite et al., 1995). The inhibition exerted by ODQ seems to be due to irreversible oxidation of the soluble guanylate cyclase prosthetic heme group (Schrammel et al., 1996). In our studies, ODQ markedly inhibited the relaxations induced by both electrical stimulation and exogenous NO in horse corpus cavernosum, which suggests that NO relaxes horse cavernosum trabecular smooth muscle mostly through activation of a soluble guanylate cyclase-dependent mechanism. The same has been observed in other tissues, such as the bovine pulmonary artery (Brunner et al., 1996), rat vascular smooth muscle (Moro et al., 1996) and lamb coronary small arteries (Simonsen et al., 1997a), where ODQ inhibited the relaxations in response to NO donors, *S*-nitroso-L-cysteine and acidified sodium nitrite. Recent studies showed that ODQ only caused a partial inhibition of the relaxant responses to exogenous NO in horse penile resistance arteries (Prieto et al., 1998) indicating that NO may relax this vascular bed through cGMP-independent mechanisms. This observation differs from the marked inhibition by ODQ of the NO-induced relaxations of the corpus cavernosum in the same species. These results suggest heterogeneity in the mechanisms by which NO relaxes the erectile tissues of the penis.

It is well established that NO may activate K^+ channels, either through cyclic GMP-dependent protein kinase (Robertson et al., 1993) or by direct opening of Ca^{2+}-activated K^+ channels without the requirement for cyclic GMP (Bolotina et al., 1994). Cyclic GMP has been suggested to cause relaxation of smooth muscle by lowering the intracellular Ca^{2+} concentration by either stimulating Ca^{2+}-ATPase activity or through opening of K^+ channels, leading to hyperpolarization and subsequent reduction of Ca^{2+} influx through voltage-operated Ca^{2+} channels (Lincoln and Cornwell, 1991; Robertson et al., 1993). The inhibitors of large conductance Ca^{2+}-activated K^+ channels, charybdotoxin and/or iberiotoxin have been reported to inhibit the relaxations induced by exogenous NO or NO donors in rabbit aorta (Bolotina et al., 1994) and in bovine

small bronchioles (Hernández et al., 1998), and those in response to both endogenous and exogenous NO in tracheal smooth muscle (Ellis and Conanan, 1994; Kannan and Johnson, 1995; Bialecki and Stinson-Fisher, 1995) and horse deep penile arteries (Simonsen et al., 1995). Iberiotoxin, a selective blocker of the large conductance Ca^{2+}-activated K^+ channels (Gálvez et al., 1990), which does not inhibit other types of voltage-dependent ion channels sensitive to charybdotoxin (Gálvez et al., 1990; Deutsh et al., 1991), did not affect the relaxations caused by either electrical stimulation or exogenous NO in the horse corpus cavernosum. These results suggest that large conductance Ca^{2+}-activated K^+ channels are not involved in the relaxant responses to NO in horse corpus cavernosum.

Small conductance Ca^{2+}-activated K^+ channels were shown to mediate the hyperpolarization and relaxations in response to electrical stimulation and the NO donor, *S*-nitroso-L-cysteine in rat gastric fundus (Kitamura et al., 1993), in guinea-pig ileal longitudinal muscle (Osthaus and Galligan, 1992), canine small intestine (Christinck et al., 1991), rat proximal duodenum (Martins et al., 1995) and lamb coronary small arteries (Simonsen et al., 1997a). Moreover, NO has been reported to activate both small and large conductance K^+ channels in colon smooth muscle from dog (Koh et al., 1995). In the present study, the selective inhibitor of the small conductance Ca^{2+}-activated K^+ channels, apamin (Cook, 1988), did not change the relaxations induced by either electrical stimulation or exogenous NO, which indicates that these types of channels do not appear to contribute to the NO-mediated NANC inhibitory neurotransmission of the horse corpus cavernosum.

ATP-sensitive K^+ channels (Cook and Quast, 1990) play an important role in the regulation of the genito-urinary tract smooth muscle (see the work of Andersson (1993)). Thus, ATP-sensitive K^+ channels were shown to mediate the NO-evoked hyperpolarization in rat and rabbit mesenteric arteries (Garland and McPherson, 1992; Murphy and Brayden, 1995) and the responses to both electrically released and exogenously added NO in pig intravesical ureter (Hernández et al., 1997). In horse corpus cavernosum, the inhibitor of ATP-sensitive K^+ channels, glibenclamide (Ashcroft and Ashcroft, 1990; Edwards et al., 1991), did not affect the relaxation in response to either electrical stimulation or exogenous NO, which indicates that ATP-sensitive K^+ channels do not seem to be involved in the NO-mediated relaxations in horse corpus cavernosum.

In summary, the present results suggest that the horse corpus cavernosum is innervated by nerve fibres containing NO. NO acts as an inhibitory neurotransmitter by relaxing trabecular smooth muscle through the activation of soluble guanylate cyclase. Neither large nor small conductance Ca^{2+}-activated, nor ATP-sensitive K^+ channels seem to be involved in the relaxations evoked by NO in the horse corpus cavernosum.

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References

Alm, P., Larsson, B., Ekblad, E., Sundler, F., Andersson, K.-E., 1993. Immunohistochemical localization of peripheral nitric oxide synthase-containing nerves using antibodies raised against synthesized L- and N-terminal fragments of a cloned enzyme from rat brain. Acta Physiol. Scand. 148, 421–429.

Andersson, K.-E., 1993. Pharmacology of lower urinary tract smooth muscles and penile erectile tissue. Pharmacol. Rev. 45, 253–308.

Andersson, P.O., Bjornberg, J., Bloom, S.S., Mellander, S., 1987. Vasoactive intestinal polypeptide in relation to penile erection in the cat evoked by pelvic and hypogastric nerve stimulation. J. Urol. 138, 419–426.

Ashcroft, S.J.H., Ashcroft, F.M., 1990. Properties and functions of ATP sensitive K channels. Cell. Signalling 2, 197–214.

Azadzoi, K.M., Kim, N., Brown, M.L., Goldstein, I., Cohen, R.A., Saenz de Tejada, I., 1992. Endothelium-derived nitric oxide and cyclooxigenase products modulate corpus cavernosum smooth muscle tone. J. Urol. 147, 220–225.

Bialecki, R.A., Stinson-Fisher, C., 1995. KCa channel antagonists reduce NO donor-mediated relaxation of vascular and tracheal smooth muscle. Am. J. Physiol. 268, L152–159.

Bolotina, V.M., Najibi, S., Palacino, J.J., Pagano, P.J., Cohen, R.A., 1994. Nitric oxide directly activates calcium-dependent potassium channels in vascular smooth muscle. Nature 368, 850–853.

Bowman, A., Gillespie, J.S., 1983. Neurogenic vasodilation in isolated bovine and canine penile arteries. J. Physiol. 341, 603–616.

Brunner, F., Schmidt, K., Nielsen, E.B., Mayer, B., 1996. Novel guanylyl cyclase inhibitor potently inhibits cyclic GMP accumulation in endothelial cells and relaxation of bovine pulmonary artery. J. Pharmacol. Exp. Ther. 277, 48–53.

Burnett, A.L., Lowenstein, C.J., Bredt, D.S., Chang, T.S.K., Snyder, S.M., 1992. Nitric oxide: a physiologic mediator of penile erection. Science 257, 401–403.

Burnett, A.L., Tillman, S.L., Chang, T.S.K., Epstein, J.I., Lowenstein, C.J., Bredt, D.S., Snyder, S.H., Walsh, P.C., 1993. Immunohistochemical localization of nitric oxide synthase in the autonomic innervation of the human penis. J. Urol. 150, 73–76.

Bush, P.A., Aronson, W.J., Buga, G.M., Rajfer, J., Ignarro, L.J., 1992. Nitric oxide is a potent relaxant of human and rabbit corpus cavernosum. J. Urol. 147, 1650–1655.

Christinck, F., Jury, J., Cayabyab, F., Daniel, E.E., 1991. Nitric oxide may be the final mediator of nonadrenergic, noncholinergic inhibitory function potentials in the gut. Can. J. Physiol. Pharmacol. 69, 1448–1458.

Cook, N.S., 1988. The pharmacology of potassium channels and their therapeutic potential. Trends Pharmacol. Sci. 9, 21–28.

Cook, N.S., Quast, U., 1990. Potassium channel pharmacology. In: Cook, N.S. (Ed.), Potassium Channels. Chichester, pp. 181–258.

Dawson, T.M., Bredt, D.S., Fotuhi, M., Hwang, P.M., Snyder, S.H., 1991. Nitric oxide synthase and neuronal NADPH-diaphorase are identical in brain and peripheral tissues. Proc. Natl. Acad. Sci. USA 88, 7797–7801.

Deutsh, C., Price, M., Lee, S., King, V.F., García, M.L., 1991. Characterization of high affinity binding sites for charybdotoxin in human T-lymphocytes. J. Biol. Chem. 266, 3668–3674.

Edwards, G., Henshaw, W.M., Miller, M., Weston, A.H., 1991. Compari-

son of the effects of several potassium-channel openers on rat bladder and rat portal vein in vitro. Br. J. Pharmacol. 102, 679–686.

Ellis, J.L., Conanan, N.D., 1994. Effect of potassium channel blockers on relaxations to a nitric oxide donor and to nonadrenergic nerve stimulation in guinea pig trachea. J. Pharmacol. Exp. Ther. 271, 782–785.

Gálvez, A., Giménez-Gallego, G., Reuben, J.P., Roy-Contancin, L., Feigenbaum, P., Kaczorowski, G.J., García, M.L., 1990. Purification and characterization of an unique, potent, peptidyl probe for the high conductance calcium-activated potassium channel from venom of the scorpion *Buthus tamulus*. J. Biol. Chem. 265, 11083–11090.

Garland, C.J., McPherson, G.A., 1992. Evidence that nitric oxide does not mediate the hyperpolarization and relaxation to acetylcholine in the rat small mesenteric artery. Br. J. Pharmacol. 105, 427–435.

Garthwaite, J., Southam, E., Boulton, C.L., Nielsen, E.B., Schmidt, K., Mayer, B., 1995. Potent and selective inhibition of nitric-oxide-sensitive guanylylcyclase by 1*H*-[1,2,4] oxadiazolo [4,3,-*a*] quinoxalin-1-one. Mol. Pharmacol. 48, 184–188.

Gruetter, C.A., Kadowitz, P.J., Ignarro, L.J., 1981. Methylene blue inhibits coronary arterial relaxation and guanylate cyclase activation by nitroglycerine, sodium nitrite and anyl nitrite. Can. J. Physiol. Pharmacol. 59, 150–156.

Hayashida, M., Okamura, T., Tomoyoshi, T., Toda, N., 1996. Neurogenic nitric oxide mediates relaxation of canine corpus cavernosum. J. Urol. 155, 1122–1127.

Hedlund, H., Andersson, K.-E., 1985. Comparison of the responses to drugs on adrenoceptors and muscarinic receptors in human isolated corpus cavernosum and cavernous artery. J. Auton. Pharmacol. 5, 81–88.

Hedlund, P., Larsson, B., Alm, P., Andersson, K.-E., 1995. Distribution and function of nitric oxide containing nerves in canine corpus cavernosum and spongiosum. Acta Physiol. Scand. 155, 445–455.

Hernández, M., Prieto, D., Orensanz, L.M., Barahona, M.V., Jiménez-Cidre, M., Rivera, L., García-Sacristán, A., Simonsen, U., 1997. Involvement of a glibenclamide-sensitive mechanism in the nitrergic neurotransmission of the pig intravesical ureter. Br. J. Pharmacol. 120, 609–616.

Hernández, M., Elmedal, B., Mulvany, M.J., Simonsen, U., 1998. Mechanisms of relaxations of bovine isolated bronchioles by the nitric oxide donor, GEA 3175. Br. J. Pharmacol. 123, 895–905.

Holmquist, T.F., Andersson, K.-E., Hedlund, H., 1992. Characterization of the inhibitory neurotransmission in the isolated corpus cavernosum from rabbit and man. J. Physiol. London 449, 295–311.

Hope, B.T., Vincent, S.R., 1989. Histochemical characterization of neuronal NADPH-diaphorase. J. Histochem. Cytochem. 37, 653–661.

Hope, B.T., Michael, G.J., Knigge, K.M., Vincent, S.R., 1991. Neuronal NADPH-diaphorase is a nitric oxide synthase. Proc. Natl. Acad. Sci. USA 88, 2811–2814.

Ignarro, L.J., Bush, P.A., Buga, A., Wood, K.S., Fukoto, J.M., Rajfer, J., 1990. Nitric oxide and cyclic GMP formation upon electrical field stimulation cause relaxation of corpus cavernosum smooth muscle. Biochem. Biophys. Res. Commun. 170, 843–850.

Juenemann, K.-P., Lue, T.F., Luo, J.-A., Jadallah, S.A., Nunes, L.L., Tanagho, E.A., 1987. The role of vasoactive intestinal polypeptide as a neurotransmitter in canine penile erection: a combined in vivo and immunohistochemical study. J. Urol. 138, 871–877.

Kannan, M.S., Johnson, D.E., 1995. Modulation of nitric oxide-dependent relaxation of pig tracheal smooth muscle by inhibitors of guanylyl cyclase and calcium activated potassium channels. Life Sci. 56, 2229–2238.

Kim, N., Azadzoi, K.M., Goldstein, I., Saenz de Tejada, I., 1991. A nitric oxide-like factor mediates nonadrenergic-noncholinergic neurogenic relaxation of penile corpus cavernosum smooth muscle. J. Clin. Invest. 88, 112–118.

Kim, Y.C., Kim, J.H., Davies, M.G., Hagen, P.O., Carson, C.C., 1995. Modulation of vasoactive intestinal polypeptide (VIP)-mediated relaxation by nitric oxide and prostanoids in the rabbit corpus cavernosum. J. Urol. 153, 807–810.

Kimura, K., Tamura, M., Kawanishi, Y., Kagawa, S., 1992. Nitric oxide (NO) as non-adrenergic non-cholinergic neurotransmitter in human corpus cavernosum. Jpn. J. Pharmacol. 58, 385.

Kitamura, K., Lian, Q., Carl, A., Kuriyama, H., 1993. *S*-Nitrocysteine, but not sodium nitroprusside, produces apamin-sensitive hyperpolarization in rat gastric fundus. Br. J. Pharmacol. 109, 415–423.

Klinge, E., Sjöstrand, N.O., 1977. Comparative study of some isolated mammalian smooth muscle effectors of penile erection. Acta Physiol. Scand. 100, 354–367.

Knispelz, M.M., Goessl, C., Beckmann, R., 1992. Nitric oxide mediates relaxation in rabbit and human corpus cavernosum smooth muscle. Urol. Res. 20, 253–257.

Koh, S.A., Campbell, J.A., Carl, A., Sanders, K.M., 1995. Nitric oxide activates multiple potassium channels in canine colonic smooth muscle. J. Physiol. 489, 743–753.

Lincoln, T.M., Cornwell, T.L., 1991. Towards and understanding of the mechanism of action of cyclic AMP and cyclic GMP in smooth muscle relaxation. Blood Vessels 28, 129–137.

Martin, W., Villani, G.M., Jothianandan, D., Furchgott, R.F., 1985. Selective blockade of endothelium-dependent and glyceryl trinitrate-induced relaxation by haemoglobin and by methylene blue in the rabbit aorta. J. Pharmacol. Exp. Ther. 232, 708–716.

Martins, S.L.R., De Oliveira, R.B., Ballejo, G., 1995. Rat duodenum nitrergic-induced relaxations are cGMP-independent and apamin-sensitive. Eur. J. Pharmacol. 284, 265–270.

Matsumoto, T., Nakane, M., Pollock, J.S., Ku, J.E., Förstemann, U., 1993. A correlation between soluble brain nitric oxide synthase and NADPH-diaphorase activity is only seen after exposure of the tissue to fixative. Neurosci. Lett. 155, 61–64.

Mayer, B., Brunner, F., Schmidt, K., 1993. Inhibition of nitric oxide synthesis by methylene blue. Biochem. Pharmacol. 45, 367–374.

Moro, M.A., Russel, R.J., Cellek, S., Lizasoain, I., Su, Y., Darley-Usmar, V.M., Radomski, M.W., Moncada, S., 1996. cGMP mediates the vascular and platelet actions of nitric oxide: confirmation using an inhibitor of the soluble guanylyl cyclase. Proc. Natl. Acad. USA 93, 1480–1485.

Murphy, M.E., Brayden, J.E., 1995. Nitric oxide hyperpolarizes rabbit mesenteric arteries via ATP-sensitive potassium channels. J. Physiol. 486, 47–58.

Osthaus, L.E., Galligan, J.J., 1992. Antagonists of nitric oxide synthesis inhibit nerve-mediated relaxations of longitudinal muscle in guinea-pig ileum. J. Pharmacol. Exp. Ther. 260, 140–145.

Palmer, R.M.J., Ashton, D.S., Moncada, S., 1988. Vascular endothelial cells synthesize nitric oxide form L-arginine. Nature 333, 664–666.

Persson, K., Andersson, K.-E., 1992. Nitric oxide and relaxation of pig lower urinary tract. Br. J. Pharmacol. 106, 416–422.

Prieto, D., Simonsen, U., Hernández, M., García Sacristán, A., 1998. Contribution of K^+ channels and ouabain-sensitive mechanisms to the endothelium-dependent relaxations of horse penile small arteries. Br. J. Pharmacol. 123, (In press).

Rajfer, J., Aronson, W.J., Bush, P.A., Dorey, F.J., Ignarro, L.J., 1992. Nitric oxide as a mediator of relaxation of the corpus cavernosum in response to nonadrenergic, noncholinergic neurotransmission. New Engl. J. Med. 326, 90–94.

Recio, P., López, P.G., Fernández, J.L.G., García Sacristán, A., 1997. Pharmacological characterization of adrenoceptors in horse corpus cavernosum penis. J. Auton. Pharmacol. 17, 191–198.

Robertson, B.E., Schubert, R., Hescheler, J., Nelson, M.T., 1993. cGMP-dependent protein kinase activates Ca-activated K channels in cerebral artery smooth muscle cells. Am. J. Physiol. 265, C299–C303.

Roy, J.B., Petrone, R.L., Saia, S.I., 1990. A clinical trial of intracavernous vasoactive peptide to induce penile erection. J. Urol. 143, 302–304.

Saenz de Tejada, I., Blanco, R., Goldstein, I., Azadzoi, K., De las Morenas, A., Krane, R.J., Cohen, R.A., 1988. Cholinergic neurotransmission in human corpus cavernosum: I. Responses of isolated tissue. Am. J. Physiol. 254, H468–472.

Saenz de Tejada, I., Kim, N., Laga, I., Krane, R.J., Goldstein, I., 1989. Regulation of adrenergic activity in penile corpus cavernosum. J. Urol. 142, 1117–1121.

Saenz de Tejada, I., Moroukian, P., Tessier, J., Kim, J.J., Goldstein, I., Frohrib, D., 1991. The trabecular smooth muscle modulates the capacitor function of the penis. Studies on a rabbit model. Am. J. Physiol. 260, H1590–H1595.

Schrammel, A., Behrends, S., Schmidt, K., Koesling, D., Mayer, B., 1996. Characterization of 1*H*-[1,2,4] oxadiazolo [4,3,-*a*] quinoxalin-1-one as a heme-site inhibitor of nitric oxide-sensitive guanylyl cyclase. Mol. Pharmacol. 50, 1–5.

Shirai, M., Maki, A., Takanami, M., Ando, K., Nakamura, K., Yanaihara, N., Yanaihara, C., Iguchi, K., Fujita, T., Iwanaga, T., 1990. Content and distribution of vasoactive intestinal polypeptide (VIP) in cavernous tissue of human penis. Urology 35, 360–363.

Simonsen, U., Prieto, D., Saenz de Tejada, I., García-Sacristán, A., 1995. Involvement of nitric oxide in the non-adrenergic non-cholinergic neurotransmission of horse deep penile arteries: Role of charybdotoxin-sensitive K^+-channels. Br. J. Pharmacol. 333, 664–666.

Simonsen, U., García-Sacristán, A., Prieto, D., 1997a. Apamin-sensitive K^+ channels involved in the inhibition of acetylcholine contractions in lamb coronary small arteries. Eur. J. Pharmacol. 329, 153–163.

Simonsen, U., Prieto, D., Delgado, J.A., Hernández, M., Resel, L., Saenz de Tejada, I., García-Sacristán, A., 1997b. Nitric oxide is involved in the inhibitory neurotransmission and endothelium-dependent relaxations of human small penile arteries. Clin. Sci. 92, 269–275.

Simonsen, U., Prieto, D., Hernández, M., Saenz de Tejada, I., García-Sacristán, A., 1997c. Adrenoceptor-mediated regulation of the contractility in horse penile resistance arteries. J. Vasc. Res. 34, 90–102.

Vicent, E.R., Kimura, H., 1992. Histochemical mapping of nitric oxide synthase in the rat brain. Neuroscience 46, 755–784.

Vizzard, M.A., Erdman, S.L., Forstermann, U., Croat, W.C., 1994. Differential distribution of nitric oxide in neural pathways to the urogenital organs (urethra, penis, urinary bladder) of the rat. Brain Res. 646, 279–291.

Wagner, G., Gerstenberg, T., 1987. Intracavernosal injection of vasoactive intestinal polypeptide (VIP) does not induce erection in man per se. World J. Urol. 5, 171–182.

Wallenstein, S., Zucker, G.L., Fleiss, J.L., 1980. Some statistical methods useful in circulation research. Circ. Res. 47, 1–9.

Willis, E., Ottesen, B., Wagner, G., Sundler, F., Fahrenkrug, J., 1981. Vasoactive intestinal polypeptide (VIP) as a possible neurotransmitter involved in penile erection. Acta Physiol. Scand. 113, 545–547.

Wollin, M.S., Cherry, P.D., Rodenburg, J.M., Messina, E.J., Kaley, G., 1990. Methylene blue inhibits vasodilatation of skeletal muscle arterioles to acetylcholine and nitric oxide via extracellular generation of superoxide anion. J. Pharmacol. Exp. Ther. 254, 872–876.