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Mediators and mechanisms of relaxation in rabbit urethral smooth muscle

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1 Electrophysiological and mechanical experiments were performed to investigate whether the nitric oxide (NO)-mediated relaxation of rabbit urethral smooth muscle is associated with a hyperpolarization of the membrane potential. In addition, a possible role for vasoactive intestinal peptide (VIP) and carbon monoxide (CO) as relaxant agents in rabbit urethra was investigated.

2 Immunohistochemical experiments were performed to characterize the NO-synthase (NOS) and VIP innervation. Possible target cells for NO were studied by using antisera against cyclic GMP. The cyclic GMP-immunoreactivity was investigated on tissues pretreated with 1 mM IBMX, 0.1 mM zaprinast and 1 mM sodium nitroprusside.

3 Intracellular recordings of the membrane potential in the circular smooth muscle layer revealed two types of spontaneous depolarizations, slow waves with a duration of 3–4 s and an amplitude of 30–40 mV, and faster (0.5–1 s), more irregular depolarizations with an amplitude of 5–15 mV. The resting membrane potential was 39 ± 1 mV ($n=12$). Application of NO (30 μM), CO (30 μM) or VIP (1 μM) did not change the resting membrane potential.

4 Both NO (1–100 μM) and VIP (1 nM–1 μM) produced concentration-dependent relaxations amounting to $87 \pm 4\%$ and $97 \pm 2\%$ ($n=6$), respectively. The relaxant effect of CO (1–30 μM) amounted to $27 \pm 4\%$ ($n=5$) at the highest concentration used.

5 Immunohistochemical experiments revealed a rich supply of NOS-immunoreactive nerve fibres in the smooth muscle layers. Numerous spinous cyclic GMP-immunoreactive cells were found interspersed between the smooth muscle bundles, mainly localized in the outer layer. These cells had long processes forming a network surrounding the smooth muscle bundles. VIP-immunoreactivity was sparse in comparison to NOS-immunoreactive nerves.

6 The rich supply of NOS-immunoreactive nerve fibres supports the view that NO is an important NANC-mediator in the rabbit urethra. In contrast to several other tissues, the relaxant effect of NO in the rabbit urethra does not seem to be mediated by hyperpolarization. The network of cyclic GMP-immunoreactive cells may constitute target cells for NO, but their function remains to be established.

Keywords: Carbon monoxide; cyclic GMP; electrophysiology; lower urinary tract; nitric oxide; vasoactive intestinal peptide

Introduction

Nitric oxide (NO) has been demonstrated to be an important inhibitory neurotransmitter in the lower urinary tract (Andersson & Persson, 1993; Burnett, 1995). Although the mechanism of relaxation is not fully understood, a general observation is that NO-mediated responses in smooth muscle preparations are linked to an increase in guanosine 3′,5′-cyclic monophosphate (cyclic GMP) formation (Sneddon & Graham, 1992; Zhang & Snyder, 1995). This has also been demonstrated in the rabbit urethra (Morita *et al*., 1992; Dokita *et al*., 1994; Persson & Andersson, 1994). Subsequent activation of a cyclic GMP-dependent protein kinase has been suggested to hyperpolarize the cell membrane, probably by causing a leftward shift of the activation curve for the K^+-channels, thus increasing their open probability (Robertson *et al*., 1993; Peng *et al*., 1996). There have also been studies suggesting that NO might act directly on the K^+-channels (Bolotina *et al*., 1994; Koh *et al*., 1995). Other mechanisms for NO-induced relaxations, mediated by cyclic GMP, might involve reduced intracellular Ca^{2+} levels by intracellular sequestration, or reduced sensitivity to Ca^{2+} (Warner *et al*., 1994), both acting without changing the membrane potential.

Electrophysiological recordings from urethral smooth muscle are scarce, probably due to technical difficulties caused by large amounts of connective tissue. Thus, a possible NO-mediated hyperpolarization, associated with the NO-mediated relaxation, has not been investigated in the urethra. However, Ito & Kimoto (1985) demonstrated a hyperpolarization following NANC-stimulation in some preparations of urethral smooth muscle from male rabbits. Furthermore, KRN 2391, a combined NO-donor and K^+-channel opener (Ogawa, 1994), had a pronounced relaxant effect accompanied by hyperpolarization in the female rabbit urethra (Waldeck *et al*., 1995). These effects were suggested to be mediated predominantly through NO-dependent mechanisms, since the relaxant effect was less sensitive to K^+-channel blockade. However, the possibility that the hyperpolarization was a pure K^+-channel opening effect, not mediated by NO, cannot be excluded. Thus, further investigations are needed to clarify whether NO acts by hyperpolarizing the membrane potential in the rabbit urethra.

Additional inhibitory systems with as yet unknown mediators have been observed in the urethra from pig (Bridgewater & Brading, 1993; Werkström *et al*., 1995) and dog (Hashimoto *et al*., 1993). Vasoactive intestinal peptide (VIP) and carbon monoxide (CO) are two potential candidates for these inhibitory effects, since VIP- and haem oxygenase-immunoreactivities have been demonstrated in the pig urethra (Persson *et al*., 1995; Werkström *et al*., 1997). Furthermore, like NO, the relaxant effect of CO is thought to be mediated by increased levels of cyclic GMP.

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In the present study we have focused on the mechanism of action for NO in the rabbit urethral smooth muscle. Thus, we have performed isometric tension recordings and electrophysiological experiments in order to elucidate whether the NO-mediated relaxation is associated with a hyperpolarization. In addition, we have used antibodies against NO-synthase (NOS) and cyclic GMP in order to characterize the NOS innervation and the potential target cells for NO. Furthermore, a possible role for CO and VIP in the rabbit urethral smooth muscle was investigated.

Methods

Tissue preparation for isometric tension recordings

Female New Zealand white rabbits (3 kg) were used after approval from the Animal Ethics Committee, Lund University. The rabbits were killed by a blow on the head, followed by exsanguination, before the urinary bladder and urethra were dissected out and placed in cold Krebs solution.

The bladder and urethra were opened longitudinally and the mucosa was removed from the proximal part of the urethra. Circular smooth muscle strips ($1 \times 1 \times 5$ mm) from this part of the tissue were prepared and mounted by means of silk ligatures between two hooks in 5 ml tissue baths, allowing recording of isometric tension. The temperature of the tissue bath was maintained at 37°C and the Krebs solution was bubbled with 95% O_2 and 5% CO_2 yielding a pH of 7.4.

After an equilibration period (60 min), each experiment was started by adding 124 mM K^+ Krebs solution (see below) to examine the contractile capacity of the preparations. Initial experiments were performed to determine a concentration of noradrenaline (NA) that produced a contraction corresponding to 75% of maximal NA-induced contraction. Based on these experiments we studied the relaxant effects on tissues precontracted by 3 μM NA. When the concentration-response relationship was investigated, the relaxant drugs were administered in a cumulative manner. To investigate whether a relaxant effect was dependent on K^+-channel opening, the relaxant ability was assessed on preparations precontracted by 80 mM K^+-Krebs (see below).

Tissue preparation for electrophysiological experiments

After the mucosa had been removed from the proximal urethra, as described above, the outer longitudinal smooth muscle layer was removed, and the remaining circular smooth muscle layer was pinned on a sylgard bottom in a 2 ml tissue bath. The preparation was superfused (1 ml min^{-1}) with Krebs solution and the preparation was allowed to equilibrate for 1 h. The headstage, where the electrode was attached, was mounted on a Burleigh Inchworm motor (Burleigh Instruments, U.S.A.) used to penetrate the tissue. The electrodes were made in a horizontal puller, Flaming/Brown micropipette puller, model P-87 (Sutter Instruments Co, U.S.A.) and filled with 3 M KCl, yielding an electrode resistance of 50–75 MΩ. The amplifier was an Axoprobe-1A from Axon Instruments, U.S.A. The membrane potential was simultaneously displayed on an oscilloscope and registered on a pen recorder. The electrode was advanced through the tissue from the serosal side with 0.5 μm steps and very fast acceleration. An acceptable impalement was characterized by a sharp decrease in potential and a stable recording of the membrane potential.

Immunohistochemistry

Transversal sections of the rabbit proximal urethra were used for immunohistochemical investigations of neuronal NOS-, VIP- and cyclic GMP-immunoreactivities. When cyclic GMP-immunoreactivity was investigated, the tissue was pretreated with 1 mM isobutyl methylxanthine (IBMX) and 0.1 mM zaprinast. Subsequent stimulation of guanylate cyclase was performed by adding 1 mM sodium nitroprusside (SNP) for 10 min (Smet *et al.*, 1996). The tissue was fixed in 4% formaldehyde in ice-cold phosphate buffered saline (PBS, pH 7.4). After 4 h the tissue was rinsed repeatedly in PBS with 15% sucrose (4°C). The general procedure for immunohistochemistry was performed as described by Ny *et al.* (1995). Briefly, the cryostat sections, cut at a thickness of 7–12 μm, were air-dried and then incubated in 0.2% Triton X-100 and 0.1% BSA in PBS, followed by rinsing in PBS before incubation with the antibodies. The antiserum used were NOS antisera raised in sheep (1:2000, Dr P.C. Emson, The Babraham Institute, Cambridge, U.K.), previously described by Herbison *et al.* (1996), VIP antisera raised in guinea-pig (1:640, Euro-Diagnostica, Malmö, Sweden) and antisera against cyclic GMP raised in sheep (1:500; Dr J. DeVente, Section of Neuropsychology, Limburg University, Maastricht, Netherlands). Immunoreactivities were visualized with fluorescein isothiocyanate conjugated (FITC) donkey anti-sheep immunoglobulins (1:80, Sigma, St. Louis, MO, U.S.A.) or Texas Red $F(ab)_2$ donkey-anti guinea-pig immunoglobulins (1:80, Jackson ImmunoResearch Inc., West Grove, PA, U.S.A.). The sections were examined in an Olympus BX60 fluorescence microscope. In control experiments, no immunoreactivity could be detected in the absence of primary antisera. The related structures are referred to as NOS, VIP and cyclic GMP-immunoreactive (IR), as cross-reactions with other antigens, sharing similar chemical structures could not be completely excluded.

Solutions

A Krebs solution of the following composition was used (mM): NaCl 119, KCl 4.6, $CaCl_2$ 1.5, $MgCl_2$ 1.2, $NaHCO_3$ 15, NaH_2PO_4 1.2 and glucose 5.5. High K^+ solutions containing 80 mM K^+ and 124 mM K^+ were obtained by exchanging Na^+ for equimolar amounts of K^+ in the normal Krebs solutions.

Drugs

The following drugs were used: (−)-noradrenaline hydrochloride (NA), vasoactive intestinal peptide (VIP), 8-Br-cyclic GMP, IBMX (Sigma Chemical Company, St. Louis, MO, U.S.A.), levcromakalim (SmithKlein Beecham, U.K.), sodium nitroprusside (SNP) Nipride, Roche, Basel, Switzerland), zaprinast (M&B 22948, May & Baker, Dagenham, U.K.). Stock solutions were prepared and then stored in −70°C. Subsequent dilutions were made with 0.9% NaCl. Stock solutions of NO and CO were prepared by bubbling deoxygenated saline for 10 min with NO or CO gas (AGA Gas AB Stockholm, Sweden).

Analysis of data

The effects of the relaxant drugs are expressed as % relaxation of the agonist-induced contraction. Results are given as mean values ± s.e.mean. E_{max} denotes the relaxation obtained at the highest concentration used of the relaxant

agent. The pEC_{50} values denote the negative logarithm of the concentration which relaxes the preparation by 50% of the agonist induced contraction; *n* denotes the number of animals.

Results

Effects on the membrane potential

Intracellular recordings of the membrane potential in the circular smooth muscle layer revealed spontaneous depolarizations in 9 out of 12 preparations. Two types of spontaneous depolarization were observed, slow waves with a duration of approximately 3–4 s and amplitude of 30–40 mV (Figure 1a), and faster (0.5–1 s), more irregular depolarizations with an amplitude of 5–15 mV (Figure 1b). In 3 preparations, occasional spontaneous hyperpolarizations were observed. The resting membrane potential was 39 ± 1 mV ($n=12$). Exogenous application of NO (30 μM), CO (30 μM) or VIP (1 μM) did not change the membrane potential (Figure 2a). Transmural nerve stimulation was performed during simultaneous recording of the membrane potential to record a possible effect on the membrane potential of the endogenously released transmitter(s). However, these experiments did not indicate any changes in the membrane potential associated with the inhibitory neurotransmission. To verify the electrophysiological technique and the viability of the tissue, some preparations were exposed to levcromakalim (100 μM). This resulted in a pronounced hyperpolarization from -38 ± 4 mV to -64 ± 8 mV ($n=4$; Figure 2b).

Isometric tension recordings

Rabbit urethral strip preparations do not develop a spontaneous tone. Thus, the resting tension is passive and is not sensitive to relaxant agents such as NO, CO or VIP. The tone induced by 3 μM NA amounted to 5.3 ± 0.5 mN ($n=23$), which was $70\pm8\%$ of the K^+ (124 mM)-induced contractions. Application of NO (1–100 μM) to the precontracted tissue induced a short-lasting concentration-dependent relaxation which amounted to $87\pm4\%$ ($n=6$) at the highest concentration used (Figure 3a). In contrast, CO

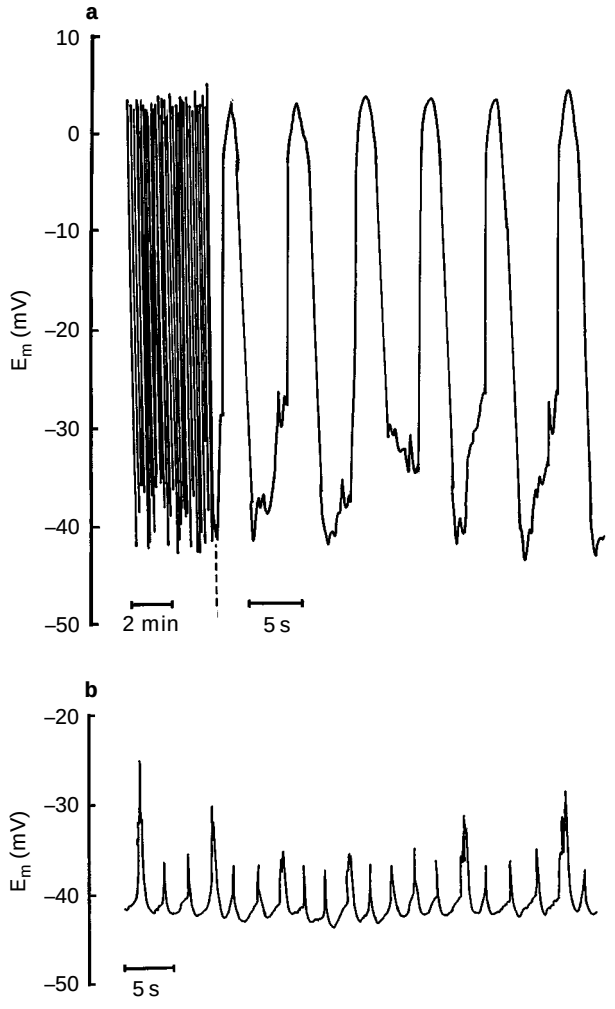

Figure 1 Original tracings showing (a) slow spontaneous depolarizations, and (b) fast irregular spontaneous depolarizations in rabbit urethral smooth muscle, registered by an intracellular microelectrode. Spontaneous depolarizations occurred in 9 out of 12 preparations.

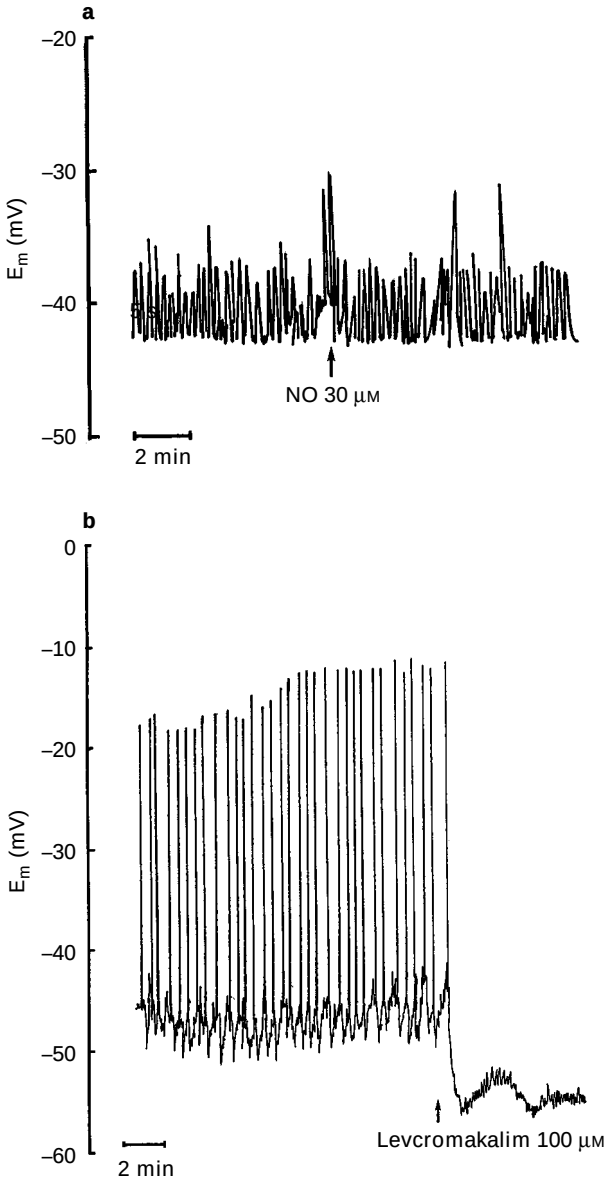

Figure 2 Intracellular recording of the resting membrane potential in the rabbit urethra during application of (a) NO (30 μM) and (b) levcromakalim (100 μM).

(1–30 μM) was less effective and produced slow relaxations amounting to 27±4% (n=5; Figure 3b). Higher concentrations were excluded due to vehicle effects. An analogue to the proposed second messenger for NO and CO, 8-Br-cyclic GMP (10 nM–100 μM), slowly and concentration-dependently relaxed the precontracted tissue to a maximal level of 74±5% (n=6). Another potential NANC-transmitter in the rabbit urethral smooth muscle, VIP (1 nM–1 μM), produced relaxations which amounted to 97±2% (n=6) at the highest concentration used (Figure 3c). For comparative purposes, we also studied the relaxant effect of the K^+-channel opener, levcromakalim (10 nM–10 μM). This drug produced relaxations amounting to 55±8% (n=6). The relaxant effects of NO (1–100 μM), CO (1–30 μM), VIP (1 nM–1 μM) and levcromakalim (10 nM–100 μM) are illustrated in Figure 4.

To study further the possible involvement of the K^+-channels in the relaxant mechanisms in the rabbit urethra, we investigated the relaxant effect of NO, VIP and levcromakalim on strips precontracted by 80 mM K^+-Krebs. Contractions induced by K^+ (80 mM) amounted to 3.1±0.4 mN (n=22), 29±3% of the K^+ (124 mM)-induced contractions. Application of NO (30 μM) and VIP (100 nM) resulted in relaxations amounting to 38±7% and 55±6% (n=4), respectively, while the relaxant effect of levcromakalim (10 μM) was negligible (6±3%; n=4) (Figure 5).

Immunohistochemistry

A rich supply of NOS-IR nerve fibres were found, predominantly in the circular smooth muscle layer, although substantial NOS-immunoreactivity was observed in the longitudinal layer as well. These nerves were lined along the smooth muscle bundles (Figure 6a), and occasionally, multiple NOS-IR nerve fibres were gathered in nerve trunks

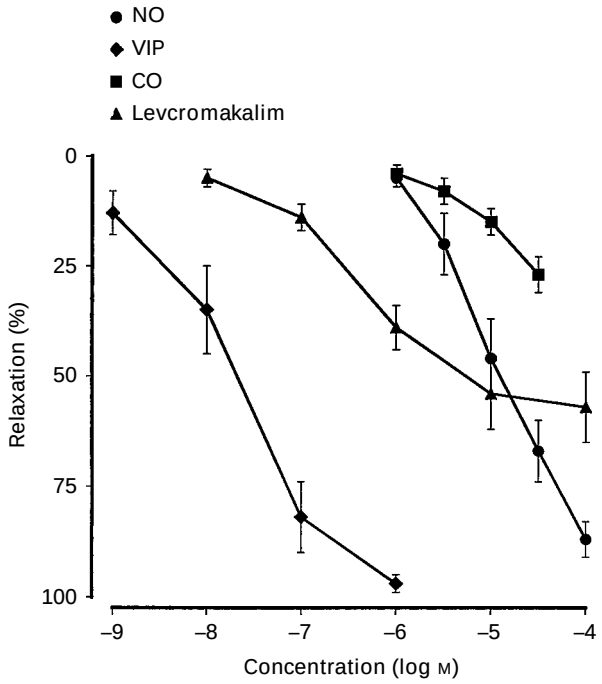

Figure 4 The concentration-response relationship for NO (1–100 μM), VIP (1 nM–1 μM), CO (1–30 μM) and levcromakalim (10 nM–100 μM) in the NA (3 μM)-contracted rabbit urethra. Values represent mean (n=5–6) and vertical lines show s.e.mean.

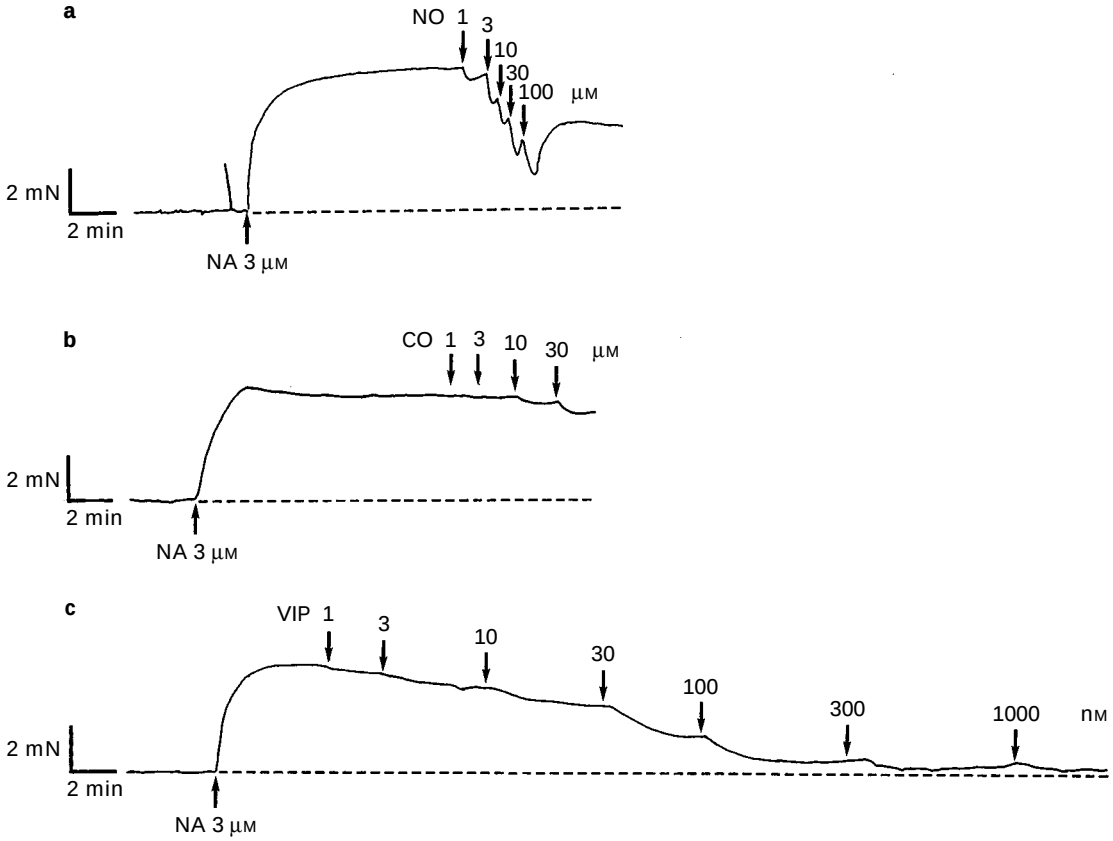

Figure 3 Original tracings showing the relaxant effect of (a) NO, (b) CO, (c) VIP on rabbit urethral smooth muscle precontracted by 3 μM NA.

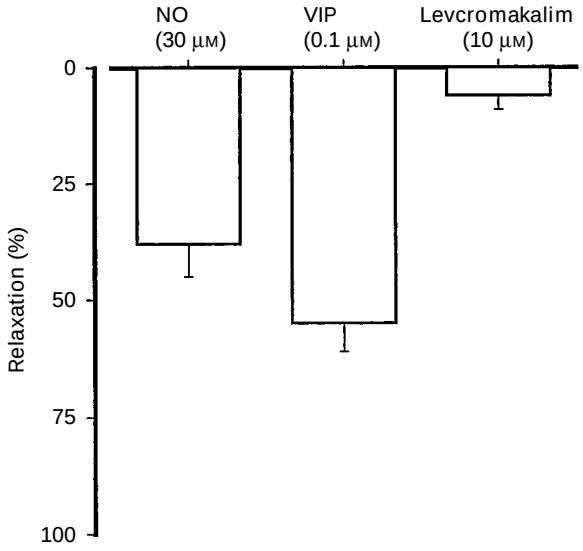

Figure 5 Relaxant effects of NO (30 μM), VIP (0.1 μM) and levcromakalim (10 μM) on the rabbit urethral smooth muscle precontracted by 80 mM K^+-Krebs. Values represent mean ± s.e.mean ($n=4$).

(Figure 6b). No NOS-immunoreactivity was observed in vascular endothelium or in the mucosal layer. Possible target cells for NO were localized by using antisera raised against cyclic GMP. Numerous spinous-formed cyclic GMP-IR cells were found interspersed between the smooth muscle bundles in the longitudinal smooth muscle layer. Similar cells were also observed, but much less frequently, in the circular smooth muscle layer. The cyclic GMP-IR cells had long processes forming a network surrounding the smooth muscle bundles (Figure 7a). Cyclic GMP-IR nerves were detected in several preparations. Occasionally, these nerves run in parallel, forming trunks (Figure 7b). Intense cyclic GMP-immunoreactivity was also observed in the endothelial layer of small vessels. No cyclic GMP-immunoreactivity was observed in either non-vascular or vascular smooth muscle cells. Control preparations exposed to IBMX and zaprinast only, did not show any cyclic GMP-immunoreactivity. VIP-IR nerve fibres were sparse in comparison to the NOS innervation and no nerve trunks were observed. The VIP-immunoreactivity was not restricted to any particular layer, but was observed in both the longitudinal and circular smooth muscle layers, as well as in the submucosal layer (Figure 8).

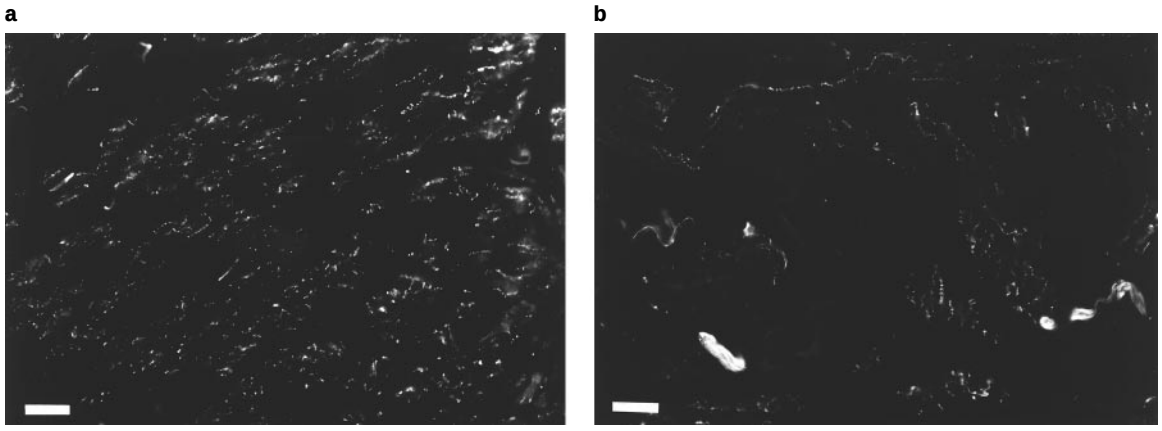

Figure 6 NOS-IR structures in the rabbit proximal urethra, visualized by NOS-antisera raised in sheep, and FITC-immunofluorescence. (a) NOS-IR nerve fibres lined along the smooth muscle bundles. (b) NOS-IR nerve fibres gathered in nerve trunks. Bars 50 μm.

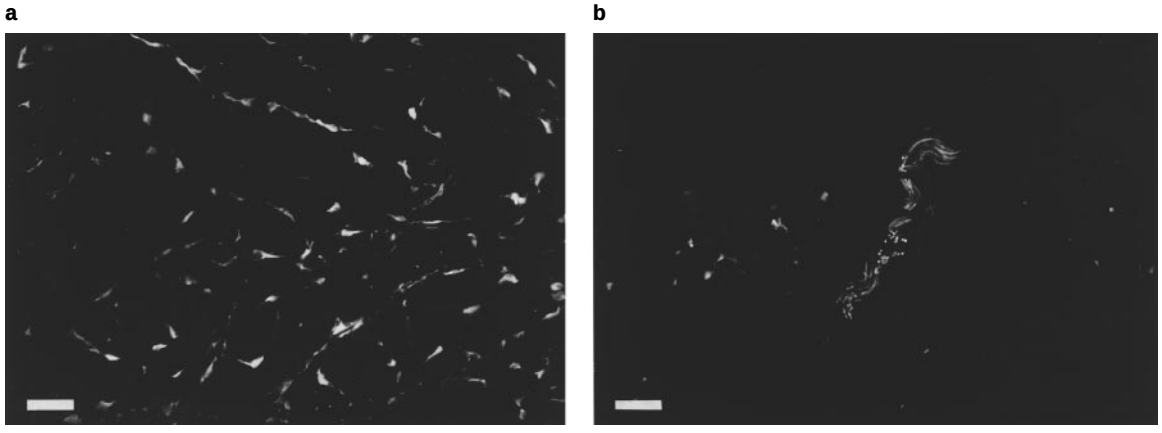

Figure 7 Cyclic GMP-IR structures in the rabbit proximal urethra visualized by cyclic GMP-antisera raised in sheep, and FITC-immunofluorescence. (a) Spinous-formed cyclic GMP-IR cells forming a network surrounding the smooth muscle cells mainly in the outer longitudinal smooth muscle layer. (b) Cyclic GMP-IR nerve fibres gathered in nerve trunk. Bars 50 μm.

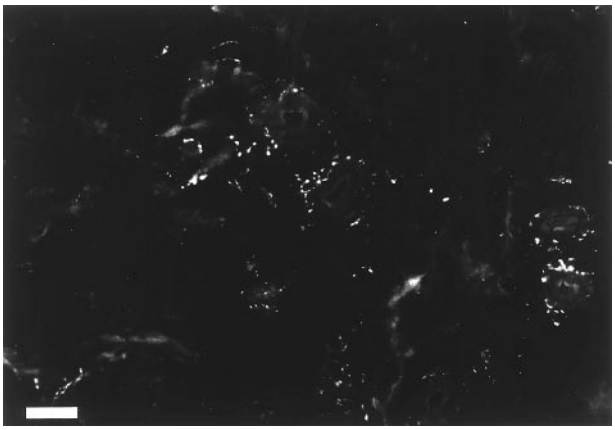

Figure 8 VIP-immunoreactivity in the rabbit proximal urethra visualized by VIP-antisera raised in guinea-pig, and Texas Red-immunofluorescence. VIP-immunoreactive nerve fibres were observed in both the longitudinal and circular layer, as well as in the submucosal layer. However, the occurrence was sparse in comparison to the NOS innervation, and no nerve trunks were observed. Bar 50 μm.

Table 1 The pEC_{50} and E_{max} values for NO, CO, 8-Br-cyclic GMP and VIP in the rabbit urethra precontracted by NA (3 μM)

Drug	*pEC_{50}*	*E_{max}* (%)	(n)
NO	4.8±0.1	87±4	(6)
CO	–	27±5	(5)
VIP	7.7±0.2	97±2	(6)
8-Br-cyclic GMP	4.8±0.1	74±5	(6)
Levcromakalim	5.8±0.2	58±8	(6)

The values represent the mean±s.e.mean ($n=5-6$). E_{max} denotes the relaxation obtained at the highest concentration used of the relaxant agent and is expressed as % relaxation of the agonist-induced contraction. The pEC_{50} values represent the negative logarithm of the concentration which relax the preparation to 50% of the agonist-induced contraction.

Discussion

Spontaneous electrical activity occurred in the majority of the preparations from the rabbit urethra. The depolarizations were of two types, slow waves with high amplitude, and short irregular spikes with low amplitude. Similar findings have previously been obtained by Hashitani *et al.* (1996). They suggested a myogenic origin, caused by spontaneous 'quantal-like' release of Ca^{2+} from intracellular stores, acting on Ca^{2+}-activated Cl^- channels to produce the short irregular spikes. Possibly, the slow waves are generated by summation of these irregular spikes. Since it is established that chloride is concentrated inside the smooth muscle cells (Aickin, 1990), Ca^{2+} activated Cl^- channels may represent a potentially important depolarizing mechanism (Hogg *et al.*, 1994).

In contrast to several other smooth muscle tissues in which NO acts as an inhibitory NANC-transmitter (Dalziel *et al.*, 1991; Thornbury *et al.*, 1991; Ward *et al.*, 1992), our results suggest that the NO-mediated inhibitory neurotransmission in the rabbit urethral smooth muscle is mediated through mechanisms which do not involve membrane hyperpolarization. This contradicts results obtained by Ito & Kimoto (1985). They observed inhibitory junction potentials in some recordings from rabbit urethral smooth muscle. However, these experiments were performed before the discovery of NO as an NANC-mediator, and thus, an NO-mediated origin was not demonstrated. Furthermore, in contrast to our study these experiments were performed on male rabbits, and a sex difference cannot be excluded. Thus, an alternative mechanism of action for NO, which does not affect the membrane potential, seems to exist in the female rabbit urethra. An NO-mediated relaxant effect, without an effect on the resting membrane potential, has also been demonstrated in feline tracheal smooth muscle (Jing *et al.*, 1995).

It seems reasonable to believe that the relaxant effect of NO in the rabbit urethra is mediated by increased levels of cyclic GMP. Evidence for this has been demonstrated by several investigators (Morita *et al.*, 1992; Dokita *et al.*, 1994; Persson & Andersson, 1994). Accordingly, the cyclic GMP-analogue, 8-Br-cyclic GMP, was able to induce relaxation, further supporting the view of cyclic GMP as a mediator of relaxation in rabbit urethra. The mechanism by which cyclic GMP induces relaxation are not fully understood, but it is suggested that elevated levels of cyclic GMP stimulate a cyclic GMP-dependent protein kinase, which phosphorylates K_{Ca} channels, and thus increases their open probability, leading to a hyperpolarization (Robertson *et al.*, 1993). Furthermore, cyclic GMP might affect sequestration of intracellular Ca^{2+}, affect Ca^{2+} extrusion pumps and/or decrease the sensitivity for Ca^{2+} (Warner *et al.*, 1994). The latter may occur without a change in the membrane potential. If this is the mechanism for the NO-induced relaxation in rabbit urethra it needs to be confirmed.

The widely occurring NOS-IR nerve fibres revealed in this study support the view of NO as the main inhibitory NANC-mediator in rabbit urethra (Andersson & Persson, 1993). To localize target cells for NO, we used antisera raised against cyclic GMP. The spindle-shaped cyclic GMP-IR cells formed a network around and between the smooth muscle bundles. Cells with similar shape have previously been demonstrated under the same conditions (inhibition of phosphodiesterases and stimulation by SNP) in the canine proximal colon (Shuttleworth *et al.*, 1993) and in the guinea-pig intestinal wall (Young *et al.*, 1993). In addition, a recent study (Smet *et al.*, 1996) demonstrated similar cells in guinea-pig and human bladder/urethra. In contrast to our results, Smet *et al.* (1996) also found smooth muscle cells exhibiting cyclic GMP-immunoreactivity in the urethra. The occurrence of cyclic GMP-immunoreactivity in smooth muscle cells seems logical, since NO is believed to stimulate guanylate cyclase with subsequent cyclic GMP-formation in these cells. However, a variable occurrence of cyclic GMP-IR smooth muscle cells has been demonstrated previously by Shuttleworth *et al.* (1993) in canine colon, and Young *et al.* (1993) found cyclic GMP-IR smooth muscle cells in only 3 out of 16 preparations from guinea-pig intestine. We found a similar variation in occurrence of cyclic GMP-IR smooth muscle cells in mice large intestine (unpublished observations). The variable cyclic GMP-immunoreactivity in smooth muscle cells might be due to the levels of cyclic GMP being under the detection limit of the immunohistochemical technique. Alternatively, it may be speculated that the cyclic GMP-IR cells demonstrated in this study are the major target cells for NO and mediate smooth muscle relaxation through, for example, gap junctions. However, this hypothesis may be questioned by the fact that inhibitory NANC relaxations are most marked in the circular layer (Mattiasson *et al.*, 1989), while the cyclic GMP-IR cells were observed mainly in the outer longitudinal smooth muscle layer. Thus, the cyclic GMP-IR cells may represent target cells for NO with an as yet unknown function.

Another inhibitory neurotransmitter candidate in the lower urinary tract is VIP. VIP-immunoreactivity has been demonstrated in nerves of the pig urethra, where it was partly co-

localized with NOS-immunoreactivity (Persson *et al*., 1995). In the present study VIP-IR nerve fibres occurred throughout the smooth muscle layers, although the distribution was not as extensive as NOS-IR structures. Exogenous administration of VIP induced a pronounced relaxation of the urethral smooth muscle and like NO, the relaxant mechanism for VIP in the urethral smooth muscle seems to be independent of changes in the membrane potential. Again, this is in contrast to observations in the gastrointestinal tract, e.g., the feline oesophageal smooth muscle (Ny *et al*., 1997), where exogenously applied VIP resulted in a relaxation and a distinct hyperpolarization.

The ability of NO and VIP to relax K^+-contracted preparations further strengthens the hypothesis that the relaxant mechanisms for these compounds (NO and VIP) are independent of hyperpolarization. In contrast, the K^+-channel opener levcromakalim, which relaxed the NA-contracted preparations, was almost unable to relax the K^+-contracted preparations.

With regard to the differences observed between the urethral smooth muscle and the smooth muscle tissues from the digestive tract, it may be speculated that a major difference is apparent between the tissues regarding the action of NO and VIP. The peristalsis occurring in the gastrointestinal tract is initiated by membrane potential variations, resulting in slow waves. Thus, it may be argued that inhibitory neurotransmission through hyperpolarization (inhibitory junction potentials) is favoured in smooth muscle tissues exerting a contractile pattern controlled by electrical signalling.

CO-forming enzymes (haem oxygenase isoenzymes) have been demonstrated in the pig urethra, and exogenously applied CO produced a pronounced relaxation of the pig urethral smooth muscle (Werkström *et al*., 1997). Since available antiserum for haem oxygenase isoenzymes are produced in rabbits, no immunohistochemical investigations of CO-forming enzymes could be performed in this study. However, the relaxant effect of exogenously applied CO in the rabbit urethra was weak, implicating that CO is not an important mediator of relaxation in this tissue.

In conclusion, the present results support the view that NO is the predominant inhibitory neurotransmitter in the rabbit urethra. However, in contrast to several other smooth muscle preparations where NO acts as an inhibitory transmitter, no effect of NO on the membrane potential was found. In addition, possible target cells for NO were demonstrated by using antisera raised against cyclic GMP. A physiological role for these cells remains to be established.

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