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Nitric oxide-induced cGMP accumulation in the mouse bladder is not related to smooth muscle relaxation

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Abstract

The functional role of nitric oxide (NO) and the guanylate cyclase/cGMP second messenger system was investigated in the mouse bladder. Electrical field stimulation and the NO-donor 3-morpholino-sydnonimin hydrochloride (SIN-1) did not induce relaxation of the carbachol-precontracted bladder. However, sodium nitroprusside (10^{-3} M) was found to enhance the contractile response to electrical field stimulation by $24 \pm 6\%$ ($n = 8$; $P < 0.05$) without affecting the contractile response to carbachol. The enhancement of bladder contractility evoked by sodium nitroprusside was inhibited by the guanylate cyclase inhibitor 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalime-1-one (ODQ; 10^{-6} M). Incubation of bladder strips with SIN-1 and sodium nitroprusside caused an increase in cGMP accumulation as measured by radioimmunoassay. Immunohistochemical studies showed cGMP-immunoreactivity in nerve fibres and in stromal cells, but not in smooth muscle bundles after exposure to NO-donors. The results show that NO-donors have no inhibitory effect on smooth muscle tone in the mouse bladder, but that NO may have a functional role as an excitatory neuromodulator. The targets of endogenous NO in the bladder may be the demonstrated cGMP-positive structures, i.e., nerves and stromal cells. © 2000 Published by Elsevier Science B.V.

Keywords: Urinary bladder; Urethra; Nitric oxide (NO); cGMP; Smooth muscle

1. Introduction

Endogenous nitric oxide (NO) is enzymatically synthesized from L-arginine by NO-synthase (NOS). The neuronal isoform of NOS (nNOS) has been demonstrated in nerves innervating the smooth muscle of the lower urinary tract in various species including man (Andersson and Persson, 1995; Burnett, 1995). Regional differences in the distribution of nNOS-positive nerves (Persson et al., 1993; Alm et al., 1995) and NOS-activity (Ehrén et al., 1994) have been reported, showing a more pronounced NOS-innervation/NOS-activity in the trigone and urethra than in the detrusor. This corresponds well with the ability of the smooth muscle to respond with NO-mediated relaxation in response to nerve stimulation, which was distinct in the trigone and the urethra, but practically absent in the detrusor (Persson and Andersson, 1992; Triguero et al., 1993). It is now well accepted that NO acts as an inhibitory transmittor in urethral smooth muscle, but the functional importance of NO in the regulation of detrusor smooth muscle tone is still unclear.

NO-mediated responses are linked to an increase in cGMP formation by activated guanylate cyclase (Murad, 1994). In urethral smooth muscle, NO-mediated relaxation has been reported to increase the tissue content of cGMP in several species (Dokita et al., 1994; Garcia-Pascual and Triguero, 1994; Persson and Andersson, 1994). On the other hand, for bladder relaxation cAMP formation linked with β-adrenoceptor stimulation seems to be more important than the NO/cGMP pathway (Morita et al., 1992; Persson and Andersson, 1994). Results obtained from animal bladders are in accordance with findings in the human bladder. Sodium nitroprusside or inhibition of cGMP hydrolysis by zaprinast has minor effect on human bladder smooth muscle tone (Truss et al., 1996), and cGMP formation in the human detrusor is less than in the urethra (Ehrén et al., 1994). In the human bladder, the cAMP pathway and the calcium/calmodulin-stimulated phosphodiesterase type 1 may be of functional importance in the regulation of muscle tone (Truss et al., 1996).

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Morphological studies to elucidate possible targets for NO in the lower urinary tract have been performed by Smet et al. (1996) by using immunohistochemical analysis of cGMP expression. The detrusor smooth muscle bundles did not show cGMP-immunoreactivity, but numerous stromal cells were found to express cGMP-immunoreactivity after stimulation by NO. It was suggested that these stromal cells, rather than the smooth muscle cells, are the cellular targets of NO in the bladder. Moreover, neuronal tissue may be the target of NO, since cGMP was also demonstrated in nerve fibres (Smet et al., 1996).

In the present study, we focused on the guanylate cyclase/cGMP second messenger system in the bladder in order to further elucidate the role of NO in this tissue. Urethral tissue was used as a control. Tissue bath experiments were performed for recording of muscle tension, a radioimmunoassay was used for measurement of cGMP levels, and cGMP immunohistochemistry for visualisation of cGMP-immunoreactive cells.

2. Material and methods

2.1. Tissue preparation

Female mice (10 weeks of age, BALB/c; Møllegaard, Denmark), were killed by CO_2 asphyxia, followed by exsanguination, and the bladder and urethra were dissected out. For functional studies, bladder strips ($1 \times 1 \times 5$ mm) were prepared from the middle part of the bladder. The urethra was opened longitudinally and one-strip preparation ($1 \times 1 \times 5$ mm) was prepared. The experimental procedures were approved by the Animal Ethics Committee of Lund University.

2.2. Functional studies

2.2.1. Recording of mechanical activity

For functional studies, the preparations were transferred to 5-ml organ baths containing Krebs solution (for composition, see below) at 37°C bubbled with a mixture of O_2 (95%) and CO_2 (5%), maintaining pH at 7.4. The strips were mounted between two L-shaped hooks by means of silk ligatures. One of the hooks was connected to a Grass Instruments FT03C force-displacement transducer for recording of isometric tension. The other was attached to a movable unit, permitting precise adjustment of preload tension. The transducer output was recorded using a Grass polygraph model 7D or E. During an equilibration period of 60 min, the tension of the bladder and urethral strips was adjusted to reach a final level of 2 mN. When subjected to electrical field stimulation, the preparations were mounted between two parallel platinum electrodes in the organ bath. Square wave pulses (duration 0.8 ms) were delivered at supramaximal voltage. The train duration was 5 s and the stimulation interval 120 s.

2.2.2. Experimental procedure

After the equilibration period, each experiment was started by exposing the preparations to K^+ (124 mM) Krebs solution (for composition, see below), until two reproducible contractions (difference $< 10\%$) had been obtained.

First, relaxant responses to the NO-donor 3-morpholino-sydnonimin hydrochloride (SIN-1), the cGMP analogue 8-bromo-cGMP (8-Br-cGMP), and electrical field stimulation were studied in precontracted bladder and urethral preparations. Contractions of bladder and urethral strips were evoked by carbachol (10^{-5} M) and by vasopressin (10^{-7} M), respectively. The effect of electrical field stimulation on bladder strips was studied in the presence and in the absence of L-arginine (10^{-3} M). In urethral preparations, frequency-dependent relaxations (2–32 Hz) were first studied in the absence of drug treatment. Thereafter N^G-nitro-L-arginine (L-NOARG) (10^{-4} M) or 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalime-1-one (ODQ) (10^{-6} M) was given 15 min before the preparations were once again subjected to electrical field stimulation. In addition, the effects of the novel phosphodiesterase type 5 inhibitors, E4021 (Kodama et al., 1994) and sildenafil (Boolell et al., 1996), were examined on the electrical field stimulation-induced responses (bladder 12 Hz; urethra 8 Hz).

The effect of the NO-donors, SIN-1 and sodium nitroprusside, were examined on bladder contractile activity evoked by electrical field stimulation and carbachol. After incubation with the phosphodiesterase inhibitors 3-isobutyl-1-methyl-xanthine (IBMX) (10^{-3} M) and zaprinast (10^{-4} M) for 15 min, bladder strips were stimulated with SIN-1 (10^{-4} M) or sodium nitroprusside (10^{-3} M) for 15 min before subjected to electrical field stimulation (2–60 Hz) or exposed to carbachol (10^{-8}–10^{-5} M). The control strip was incubated with IBMX (10^{-4} M) + zaprinast (10^{-4} M) for 30 min and then subjected to electrical field stimulation or carbachol.

The effects of L-arginine (10^{-3} M), L-NOARG (10^{-4} M), SIN-1 (10^{-4} M) and sodium nitroprusside (10^{-3} M) were tested on bladder contractions induced by electrical field stimulation (40 Hz). The effect of sodium nitroprusside (10^{-3} M) was further examined in the presence of IBMX (10^{-4} M) + zaprinast (10^{-4} M), sildenafil (10^{-5} M) or ODQ (10^{-6} M).

2.3. Determination of cGMP and cAMP levels

Bladder and urethral preparations were placed in organ baths containing Krebs solution at 37°C bubbled with a mixture of O_2 (95%) and CO_2 (5%). To prevent the breakdown of cyclic nucleotides, the solution contained the phosphodiesterase inhibitor zaprinast (10^{-4} M). After incubation with zaprinast for 30 min, the bladder tissues were stimulated by the NO-donors, SIN-1 (10^{-4} M) or sodium nitroprusside (10^{-3} M), for 20 min and then

frozen in liquid nitrogen. Frozen tissue was then homogenized at 4°C in 2 ml of 10% trichloroacetic acid using a hand glass homogenizer, and centrifuged at 2000 rpm (4°C) for 15 min. The pellet was reconstituted in 2 M NaOH and assayed for protein content using bovine serum albumin as a standard. The trichloroacetic acid in the supernatant was removed by five consecutive extractions with 5 ml of water-saturated diethyl ether. The aqueous phase was evaporated and the residue stored at −20°C. Residues were dissolved in 0.05 M sodium acetate and the amount of cGMP or cAMP was quantitated using [^{125}I] cGMP and [^{125}I] cAMP radioimmunoassay kits (DuPont, Wilmington, DEL, USA) according to kit instructions. All determinations of cyclic nucleotide levels and protein content were made in duplicate. Cyclic nucleotides were acetylated with acetic anhydride to increase the sensitivity of the assay. The final values of tissue cGMP were corrected for trace amounts of [^{3}H] cGMP, used to determine recovery (approximately 70%).

2.4. Immunohistochemistry

To stimulate cGMP formation, bladder tissue was placed in phosphate buffered saline (PBS, pH 7.4) at 37°C, and bubbled with a mixture of O_2 (95%) and CO_2 (5%) as previously described by Smet et al. (1996). The buffer contained the phosphodiesterase inhibitors IBMX (10^{-3} M) and zaprinast (10^{-4} M). After a 30-min incubation with IBMX and zaprinast, the tissues were stimulated by the NO-donors, SIN-1 (10^{-4} M) or sodium nitroprusside (10^{-3} M), for 20 min. Control tissues were not exposed to SIN-1 or sodium nitroprusside. The bladders were fixed in ice-cold 4% formaldehyde in PBS for 4 h. SIN-1 or sodium nitroprusside was also included in the fixative for stimulated tissue. The tissues were rinsed in PBS containing 15% sucrose for 2–3 days and then frozen in isopentane at −40°C and stored at −70°C. Cryostat sections were cut at a thickness of 8 μm.

In the immunohistochemical procedure, sections were first incubated in PBS with 0.2% Triton X-100 for 2 h at room temperature. Some bladder sections were then incubated overnight at room temperature with a sheep antiserum against nNOS (1:4000; generous gift from Dr. P.C. Emson, The Babraham Institute, Cambridge, UK), rinsed, and incubated for 90 min with fluorescein isothiacyanate (FITC)-conjugated donkey anti-sheep immunoglobulins (IgG) (1:80; Sigma, St. Louis, MO, USA). For the simultaneous demonstration of two antigens, some bladder sections were incubated overnight at room temperature with a sheep antiserum against cGMP (1:2000; generous gift from Dr. J. De Vente, Rijksuniversiteit Limburg, Maastricht, The Netherlands), rinsed, and incubated overnight in a rabbit antiserum against protein gene product 9.5 (1:2000; UltraClone, Cambridge, England). The sections were then rinsed, and incubated for 90 min with FITC-conjugated donkey anti-sheep IgG (1:80; Sigma), rinsed, and incubated for 90 min with Texas Red-conjugated donkey anti-rabbit IgG (1:160; Sigma). After rinsing, the sections were mounted in glycerol/PBS with *p*-phenylenediamine (Merck, Darmstadt, Germany) to prevent fluorescence fading.

2.5. Drugs and solutions

The following substances were used: carbamylcholine chloride (carbachol), vasopressin, L-arginine hydrochloride, L-NOARG, 8-Br-cGMP, IBMX (Sigma), sodium nitroprusside (Nipride®, Roche, Switzerland), zaprinast (M and B 22948, May and Baker, Dagenham, UK), SIN-1 (Casella , Frankfurt am Main, Germany), ODQ (Tocris, Bristol, UK), sildenafil (Pfizer, Kent, UK), E4021 (Eisai, Tokyo, Japan). Stock solutions were prepared and then stored at −70 °C. Subsequent dilutions of the drugs were made with 0.9% NaCl. Sodium nitroprusside and SIN-1 were kept in dark vessels in order to minimise light-induced degradation. 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, glucose 5.5. A high K^+ solution containing 124 mM K^+ was obtained by Na^+ exchange for equimolar amounts of K^+ in the normal Krebs solution.

2.6. Analysis of data

Statistical analysis were performed by the use of Student's unpaired (two-tailed) *t*-test. A probability level

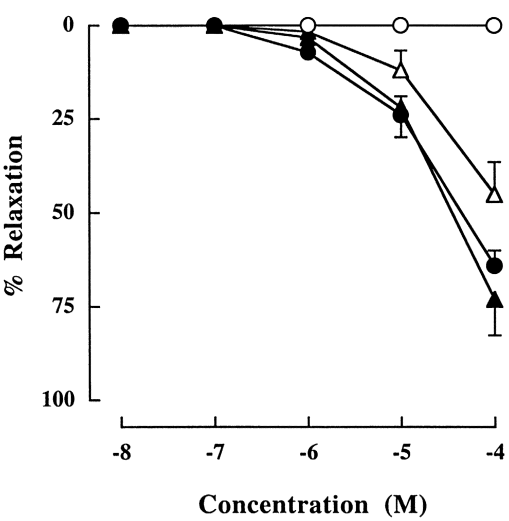

Fig. 1. Concentration–response curves for SIN-1 and 8-Br-cGMP in bladder preparations contracted by carbachol (SIN-1, ○; 8-Br-cGMP, △) and in urethral preparations contracted by vasopressin (SIN-1, ●; 8-Br-cGMP, ▲). Each point represents mean from six experiments with S.E.M. shown by vertical bars.

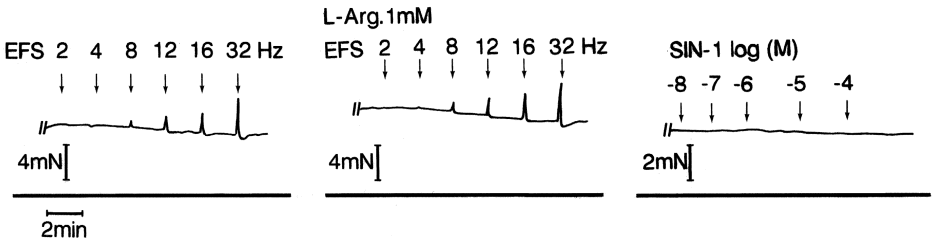

Fig. 2. The response to electrical field stimulation (EFS) in bladder preparations contracted above baseline (shown as a solid line) by carbachol. The experiment was performed in the presence and absence of L-arginine (L-Arg). SIN-1 was applied at the end of the experiment.

< 0.05 was accepted as significant. Results are given as mean values $\pm$ s.e.mean (S.E.M.).

3. Results

3.1. Functional studies

3.1.1. The effect of SIN-1, 8-Br-cGMP and electrical field stimulation on precontracted bladder and urethral preparations

In bladder preparations contracted by carbachol, SIN-1 did not induce any relaxation (Figs. 1 and 2), whereas 8-Br-cGMP induced a concentration-dependent relaxation amounting to $45 \pm 8\%$ ($n = 6$) at 10^{-4} M (Fig. 1). The effect of electrical field stimulation on precontracted bladder strips was studied in the presence and absence of L-arginine (10^{-3} M). In both cases, no relaxation but a contractile response was obtained (Fig. 2). In urethral preparations contracted by vasopressin, both SIN-1 and 8-Br-cGMP induced a concentration-dependent relaxation (Figs. 1 and 3). At 10^{-4} M, the response amounted to $64 \pm 3\%$ ($n = 6$) and $73 \pm 10\%$ ($n = 6$), respectively. Electrical field stimulation of urethral preparations induced frequency-dependent relaxations (Fig. 3), with a response amounting to $25 \pm 2\%$ at 32 Hz ($n = 6$). The NOS-inhibitor L-NOARG (10^{-4} M) abolished the relaxation (Fig. 3), whereas the guanylate cyclase inhibitor ODQ (10^{-6} M) inhibited the relaxation in three strips out of six. In three strips, a small relaxation was observed at high frequencies of stimulation (Fig. 3).

3.1.2. The effect of sildenafil and E4021 on the response to electrical field stimulation in precontracted bladder and urethral preparations

The phosphodiesterase type 5 inhibitors sildenafil and E4021 increased the amplitude of the electrical field stimulation-induced relaxations in the precontracted urethra (Fig. 4a,b). The duration of the relaxations was prolonged in response to high concentrations of sildenafil (Fig. 4b). In the bladder, electrical field stimulation did not induce relaxation in the presence of sildenafil or E4021 (data not shown).

3.1.3. The effect of NO-donors, L-arginine and L-NOARG on bladder contraction

The effect of the NO-donors, SIN-1 (10^{-4} M) or sodium nitroprusside (10^{-3} M), on bladder contractions induced by electrical field stimulation or carbachol was examined in the presence of IBMX (10^{-4} M) and zapri-

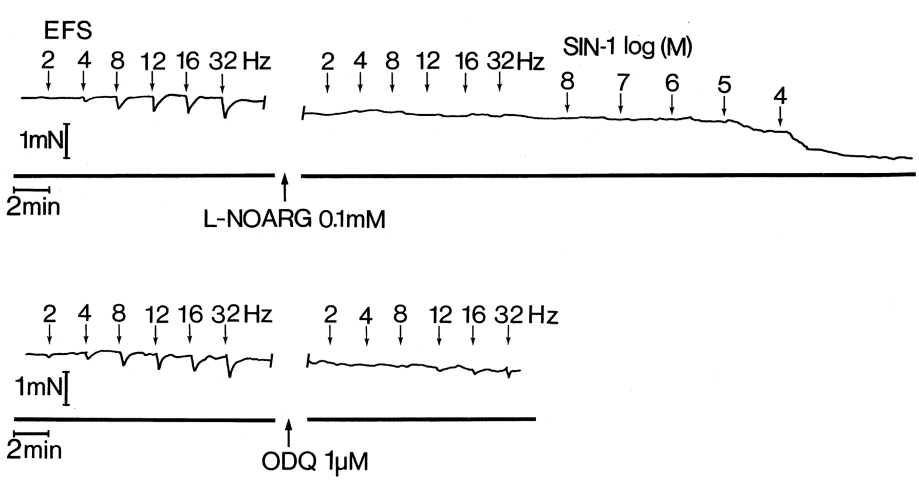

Fig. 3. The effect of L-NOARG (upper panel) and ODQ (lower panel) on relaxation induced by EFS in the urethra. Precontraction above baseline (shown as a solid line) was induced by vasopressin. SIN-1 was applied at the end of the experiment.

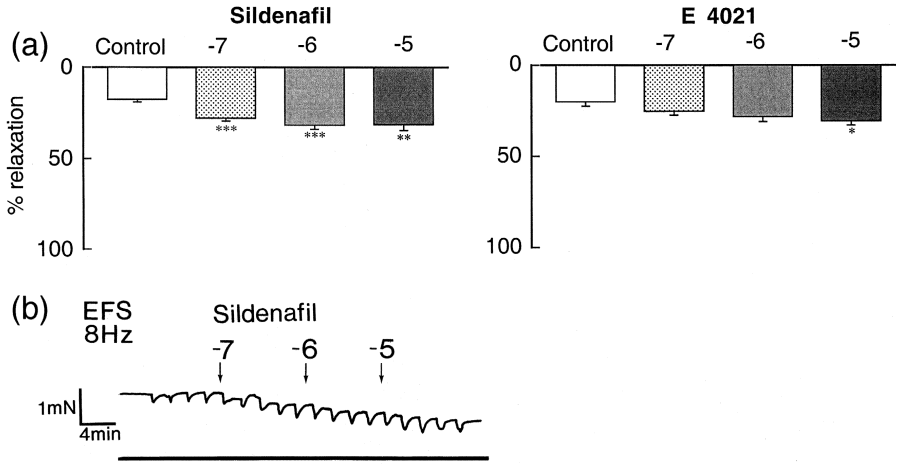

Fig. 4. (a) The effect of the phosphodiesterase inhibitors, sildenafil and E4021, on relaxation induced by electrical field stimulation at 8 Hz in urethral preparations contracted by vasopressin. Results are expressed as percentage relaxation of vasopressin-induced tension, and each column represents mean from six experiments with S.E.M. shown by vertical bars. (b) Tracing illustrating the effect of sildenafil on EFS-induced relaxation. Precontraction above baseline (shown as a solid line) was induced by vasopressin.

nast (10^{-4} M). Neither SIN-1 ($n = 6$) nor sodium nitroprusside ($n = 6$) affected the contractile response to carbachol (Fig. 5a). Sodium nitroprusside ($n = 8$), but not SIN-1 ($n = 6$), increased the contractile response to electrical field stimulation at high frequencies of stimulation ($P < 0.05$) (Fig. 5b).

The effects of L-arginine (10^{-3} M), L-NOARG (10^{-4} M), SIN-1 (10^{-4} M) and sodium nitroprusside (10^{-3} M) were further investigated on bladder contractions induced by electrical field stimulation (40 Hz). L-arginine or L-NOARG had no effect on bladder contractions induced by electrical field stimulation (data not shown). SIN-1 or sodium nitroprusside did not affect bladder contractions in the absence of phosphodiesterase inhibitors (Fig. 6). However, in the presence of IBMX and zaprinast, SIN-1 and sodium nitroprusside increased the contractions induced by electrical field stimulation by $8.7 \pm 2.0\%$ ($n = 6$) and $24 \pm 6\%$ ($n = 6$), respectively (Fig. 6). The enhancement of bladder contractions induced by sodium nitroprusside was abolished by ODQ (10^{-6} M; Fig. 6). Sodium nitroprusside did not enhance electrical field stimulation-induced bladder contractions in the presence of sildenafil (data not shown).

3.2. cGMP / cAMP levels

The NO-donors SIN-1 and sodium nitroprusside evoked an increase in cGMP production in bladder tissue, but

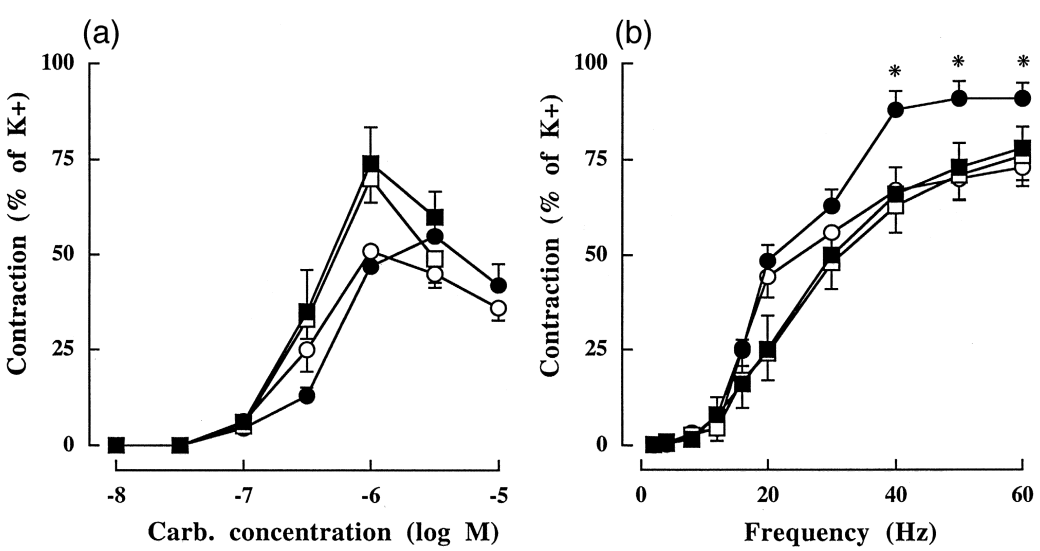

Fig. 5. Concentration–response relations for (a) carbachol and frequency–response relations for (b) electrical field stimulation-induced contraction shown in bladder preparations. Responses were recorded after pretreatment with SIN-1 (■) or its control (□), and after pretreatment with sodium nitroprusside (SNP) (●) or its control (○). Each point represents mean ($n = 6$–8) with S.E.M. shown by vertical bars; $^{*}P < 0.05$.

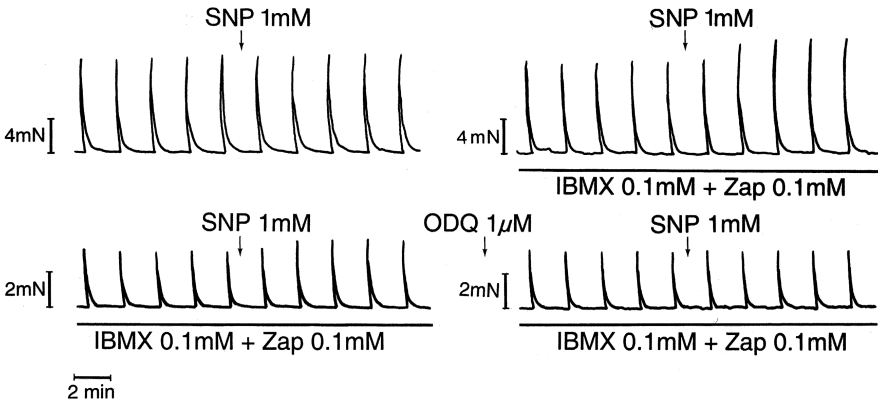

Fig. 6. The effect of SNP on electrical field stimulation-induced contractions in bladder preparations. The upper panel shows the effect of SNP in the absence and presence of IBMX + zaprinast (Zap). The lower panel shows the inhibitory effect of ODQ.

particularly in urethral tissues (Fig. 7). SIN-1 increased the cGMP level from 0.33 ± 0.04 to 1.6 ± 0.3 pmol/mg protein in bladder tissue ($P < 0.01$; $n = 6$) and from 2.6 ± 0.4 to 13 ± 2 pmol/mg protein in urethral tissue ($P < 0.001$; $n = 6$). Sodium nitroprusside increased cGMP levels from 0.52 ± 0.07 to 0.89 ± 0.10 pmol/mg protein in bladder tissue ($P < 0.05$; $n = 6$), and from 3.0 ± 0.4 to 6.3 ± 0.3 pmol/mg protein in urethral tissue ($P < 0.001$; $n = 6$) (Fig. 7). Neither SIN-1 nor sodium nitroprusside increased cAMP levels in bladder tisssue. In urethral tissue, sodium nitroprusside significantly increased cAMP levels ($P < 0.01$, $n = 6$; Fig. 7).

3.3. Immunohistochemistry

In bladders stimulated by NO-donors, cGMP-immunoreactivity was found in vascular endothelium, in neuronal

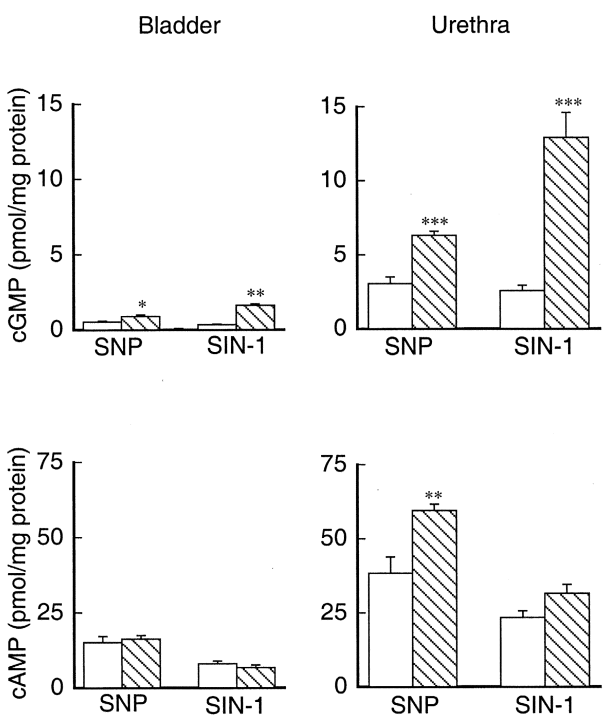

Fig. 7. Effect of SIN-1 and SNP on cGMP (upper panel) and cAMP (lower panel) accumulation in the bladder and urethra. Control strips (open bars) or strips stimulated by the NO-donors (hatched bars) were frozen in liquid nitrogen and cGMP and cAMP content were determined by radioimmunoassay. Data are given as mean ± S.E.M. ($n = 6$); $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

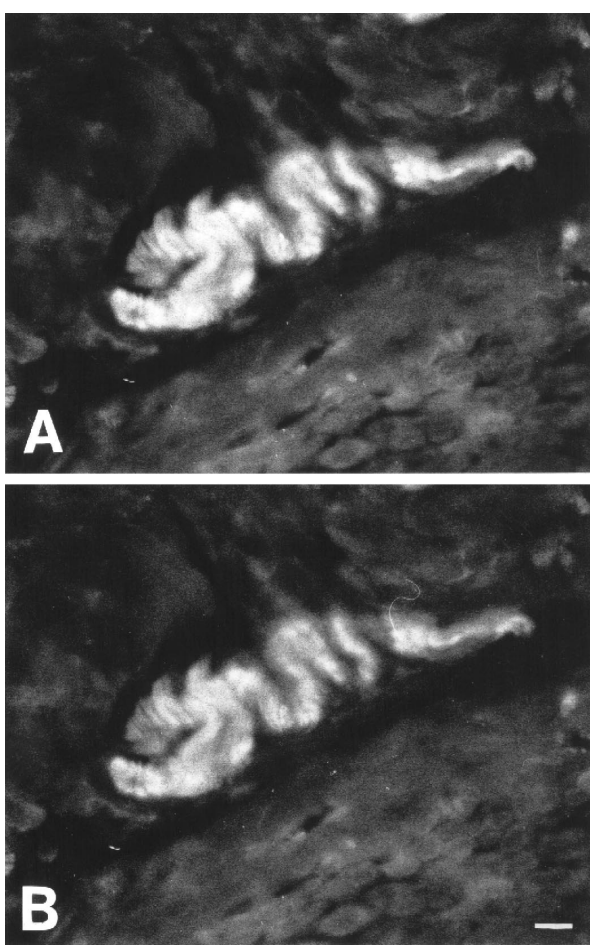

Fig. 8. Double immunolabelling of cGMP (A) and protein gene product 9.5 (B) in a nerve trunk within the bladder. Scale bar = 12 μM.

structures (Fig. 8) and in stromal cells located in the lamina propria and between smooth muscle bundles (Fig. 9B,C). Unstimulated preparations did not show any cGMP-immunoreactivity (Fig. 9A). The stromal cells had a dendritic appearance and were negative for the neuronal marker protein gene product 9.5, suggesting a non-neuronal origin. The smooth muscle bundles did not show any immunoreactivity for cGMP. Neuronal NOS-immunoreactivity was found in nerve trunks and in nerve cell bodies mainly at the serosal side of the bladder. Thin nerve fibres within the smooth muscle layer also showed nNOS-immunoreactivity (data not shown).

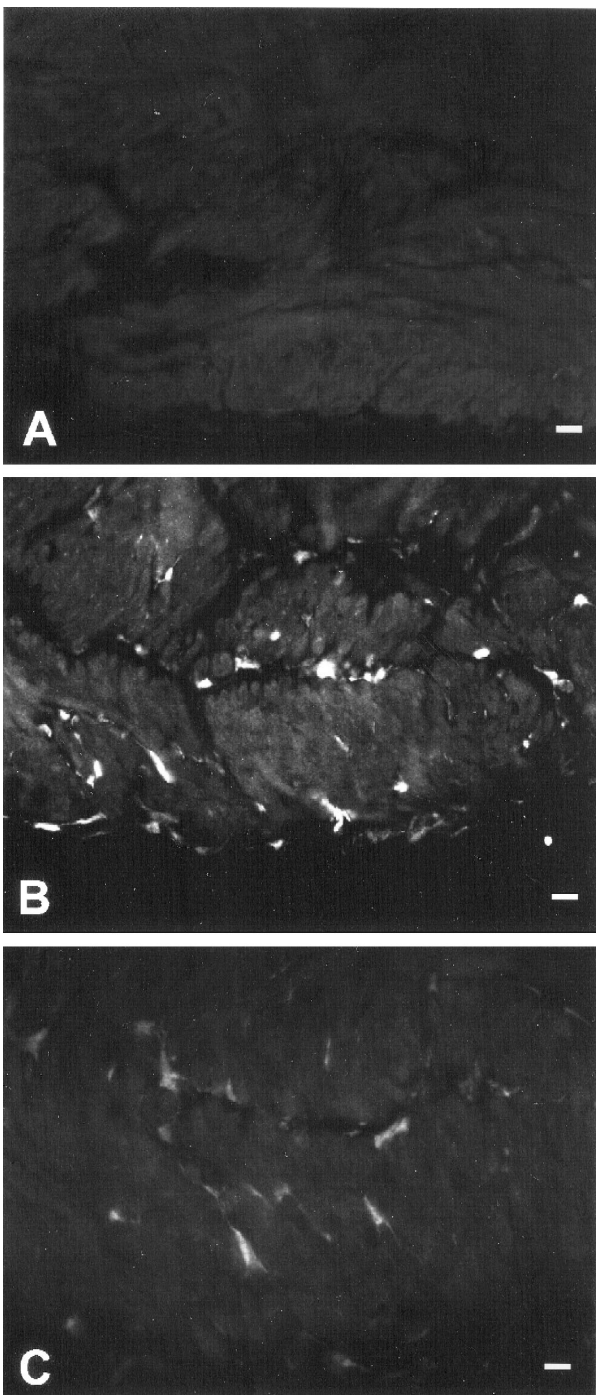

Fig. 9. Demonstration of cGMP-immunoreactive stromal cells in the bladder. (A) Unstimulated control bladders show no cGMP-immunoreactivity. (B, C) cGMP-positive cells are seen in between muscle bundles in SNP-stimulated bladders. Scale bars A, B = 15 μM; C = 10 μM.

4. Discussion

A species difference regarding the involvement of NO in the urethral relaxation has been noted. In the rabbit and rat, the relaxation is NO-dependent (Andersson et al., 1992; Persson et al., 1992), while the urethral relaxation in the pig and dog seems to be mediated by NO and a non-nitrergic, unknown component (Bridgewater et al., 1993; Hashimoto et al., 1993; Werkström et al., 1995). In the guinea pig urethra, the non-nitrergic component seems to dominate (Werkström et al., 1998). In the present study, the NOS inhibitor L-NOARG completely inhibited the electrical field stimulation-induced urethral relaxation, suggesting that NO is the main, if not sole, inhibitory transmitter in the mouse urethra. The type of phosphodiesterase that is involved in cGMP degradation in the mouse urethra has not previously been investigated. We showed that two specific phosphodiesterase type 5 inhibitors, E4021 and sildenafil, increased the relaxant response by electrical field stimulation in the precontracted mouse urethra. Thus, sildenafil, a novel oral agent for the treatment of penile erectile dysfunction, appears to enhance NO-mediated responses in the mouse urethra similar to its effect in human corpus cavernosum (Ballard et al., 1998).

To gain insight into the possible role of NO in the bladder, various experiments were performed using different methods. Although a rich number of nNOS immunoreactive nerves was observed in the mouse bladder, several observations in the present study argue against a direct functional role of nerve-derived NO in mediating detrusor relaxation. First, electrical field stimulation and the NO-donor SIN-1 failed to induce relaxation of precontracted preparations. Second, SIN-1 and sodium nitroprusside were found to increase cGMP levels in the bladder tissue, but the cGMP accumulation was not correlated to smooth muscle relaxation. These findings are in disagreement with those reported by Chung et al. (1996), who showed that rat bladder preparations were capable of relaxing to NO with a simultaneous elevation of cGMP. The source of NO used in the study by Chung et al. (1996) was derived from a molecule that was photoactivated to release NO, and thus, different from the NO-donor used in our study. The fact that 8-Br-cGMP, but not SIN-1, evoked relaxation of the mouse bladder suggests that the bladder is potentially able to relax in response to accumulation of cGMP. NO/cGMP-mediated smooth muscle relaxation is proposed to involve activation of cGMP-dependent protein kinase I (cGKI) (Lincoln et al., 1995). In bladder strips from

cGKI-deficient mice, the 8-Br-cGMP-induced relaxation is practically abolished (Persson et al., 1999). These experiments show that the bladder relaxation evoked by 8-Br-cGMP is mediated through cGKI. It is possible that the NO-donor used in our study did not generate sufficient NO/cGMP for full activation of cGKI or, alternatively, that the activation pathway of cGKI used by 8-Br-cGMP is not normally activated by endogenous cGMP.

In addition to increase cGMP levels in the urethra, sodium nitroprusside was also found to increase urethral cAMP levels. This may be explained by sodium nitroprusside causing a cGMP-mediated inhibition of phosphodiesterase type 3 and a subsequent accumulation of cAMP. If the rise in cAMP evoked by sodium nitroprusside participates in sodium nitroprusside-induced urethral relaxations is not known. However, the adenylate cyclase/cAMP activator forskolin is able to produce relaxation of the mouse urethra (unpublished observations), demonstrating that the mouse urethra may relax in response to cAMP accumulation.

The dissociation between functional responses and accumulation of cGMP may be explained by the existence of targets for NO/cGMP that are unrelated to regulation of smooth muscle tone. Immunohistochemical studies revealed that the smooth muscle bundles of the bladder did not show any positive staining for cGMP after exposure to NO-donors. In agreement with previous studies in the human and guinea-pig bladder (Smet et al., 1996; Werkström et al., 1998), cGMP-immunoreactivity was found in nerve fibres and in stromal cells located primarily in the lamina propria. These structures may act as targets for NO in the bladder and account for the accumulation of cGMP as measured by radioimmunoassay. The role of the stromal cells for bladder function, as well as the significance of the found cGMP accumulation, is at present unclear.

A role for NO as a neurotransmitter causing direct relaxation of the bladder smooth muscle is thus not supported by the present study, but it is possible that NO acts as a neuromodulator of the efferent or afferent neurotransmission in the bladder. It has been found that inhibition of NOS in rats causes bladder overactivity (Persson et al., 1992) and that mice lacking neuronal NOS show increased micturition frequency (Burnett et al., 1997). The mechanism behind this overactivity is not known but may result from a lack of inhibitory NO in either the detrusor or outlet region. One possibility is that NO may be involved in the regulation of the threshold for bladder afferent firing. In the present study, we investigated the effect of NO on the excitatory neurotransmission in the mouse bladder. It was demonstrated that sodium nitroprusside and SIN-1 increased the contractile response induced by electrical field stimulation, but not by carbachol, suggesting that NO acts prejunctionally to enhance the release of excitatory transmitter. This is in agreement with the report by Liu and Lin-Shiau (1997), showing an enhancement of the electrical field stimulation-induced contraction by NO in the mouse bladder. On the other hand, it has been reported that NO may act prejunctionally to inhibit acetylcholine release in the pig bladder (Persson et al., 1993).

The morphological findings that some nerves in the mouse bladder expressed cGMP-immunoreactivity support a neuromodulatory function of NO in the bladder. NO has been reported to modulate neurotransmitter release in several tissues. A stimulatory effect of NO on neurotransmitter release in autonomic ganglia (Scott and Bennett, 1993), vascular tissue (Modin et al., 1994) and vas deferens (Vladimirova et al., 1994) has been reported. An inhibitory action of NO on neurotransmission was found in the gastrointestinal tract (Hebeiss and Kilbinger, 1998). In the majority of the above studies, the modulatory effects of NO on neurotransmission could be mimicked by 8-Br-cGMP and/or inhibited by guanylate cyclase inhibitors suggesting involvement of cGMP. In our study, the enhancement of bladder contractions evoked by sodium nitroprusside involved cGMP, as shown by the inhibitory effect of ODQ. The mechanism by which the NO/cGMP pathway modulates the transmitter release in the mouse bladder is not clear. An increased calcium concentration in the nerve terminal can lead to an increase in secretion of neurotransmitters (Kuba and Kumamoto, 1990). As reflecting the complex effect of NO on neurotransmitter release, cGMP may modulate $[Ca^{2+}]_i$ levels in both directions, increasing it (Schaad et al., 1995) or decreasing it (Ruth et al., 1993; Meriney et al., 1994). In addition, NO-mediated, cGMP-independent $[Ca^{2+}]_i$ modulation in the nerve terminal was also reported by several investigators (Hoyt et al., 1992; Ohkuma et al., 1998).

The facilitatory effect of SIN-1 and sodium nitroprusside on excitatory neurotransmission in the mouse bladder was only found in the presence of the phosphodiesterase inhibitors, IBMX and zaprinast. Thus, a prolonged cGMP accumulation seems to be necessary for demonstration of NO-induced enhancement of bladder neurotransmission. The facilitatory effect on neurotransmission evoked by NO was only seen at high frequencies of stimulation. At high frequencies, electrical field stimulation-induced contractions in the mouse bladder are mainly mediated by the cholinergic component, while the non-adrenergic, non-cholinergic component dominates at low frequencies of stimulation (unpublished observations). Therefore, NO seems to facilitate mainly acetylcholine release in the mouse bladder. This view is different from that reported by Liu and Lin-Shiau (1997), who suggested that NO mainly enhanced transmitter release from non-cholinergic bladder nerves. However, in the presence of IBMX and zaprinast, scopolamine was found to abolish all electrical field stimulation-induced contractions, which excluded studies of NO on the non-cholinergic component in our system.

In conclusion, NO-donors cause accumulation of cGMP in mouse bladder strips, but do not induce smooth muscle relaxation. NO was found to cause a cGMP-dependent facilitation of excitatory neurotransmission in the bladder.

Nerves and stromal cells, but not smooth muscle cells, expressed cGMP-immunoreactivity and may represent the cellular targets of endogenous NO in the bladder.

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