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A possible role for nitric oxide in the regulation of human ureteral smooth muscle tone in vitro

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Abstract There is ample evidence that nitric oxide (NO) is an important neurotransmitter in many tissues of the urogenital tract. The aim of the present study was to examine the possible role of NO in ureteral relaxation. Human ureteral rings were mounted in organ bath chambers and precontracted with KCl. Increasing doses of the NO donor linsidomine (SIN-1) were added with and without prior blockade of the NO/cGMP pathway by methylene blue and protein kinase (PK) inhibitors Rp-8-pCPT-cGMPS and Rp-8-CPT-cAMPS. Electrical field stimulation (EFS) was done before and after incubation with L-NOARG (N^G-nitro-L-arginine) and TTX (tetratodoxin). For detection of neuronal NO synthase (NOS), ureters were stained immunohistochemically. Ureteral strips were dose dependently relaxed by SIN-1; preincubation with methylene blue and protein kinase G inhibitor significantly reduced the SIN-1-induced relaxations. No effects of L-NOARG and TTX on EFS-induced tone alterations were found. NOS-positive neuronal axons and nerve-ending-like structures were found in the muscular layers. Our in vitro findings suggest that ureteral relaxation may involve the NO pathway.

Key words Ureter · Nitric oxide · Smooth muscle physiology

Introduction

In contrast to other urological tissues such as urethra, penis or bladder, little is known about the peripheral parasympathetic neurotransmission responsible for ureteral relaxation [1]. Recent research on ureteral neurotransmitters has focused on afferent innervation [12]. Studies on the efferent limb of the autonomic ureteral innervation were done over 2 decades ago when the concept of non-adrenergic/non-cholinergic (NANC) innervation had not been established. More recently, reports on the relaxing effect of vasoactive intestinal polypeptide (VIP) on the ureteral tissue in vitro were published [6], but no in vivo studies were carried out to further corroborate these findings. However, knowledge of the peripheral parasympathetic neurotransmitter(s) would not only be of academic but also of clinical interest since this may enable pharmacological ureteral relaxation in vivo. If selective enough, it may be used for treatment of ureteral and renal colics and the improvement of urinary stone or stone fragment passage.

NO is synthesized from L-arginine by two main classes of NO synthases (NOSs). The NOS occurring in peripheral nerves is a Ca^{2+}/calmodulin-dependent enzyme, which releases picomoles of NO in response to receptor stimulation [5, 14]. Data obtained by immunohistochemistry with rabbit antisera against –COOH and –NH_2 terminal fragments of cloned NOS from rat cerebellum [3] suggest that NOS is localized in nerve fibres of the porcine lower urinary tract, including detrusor, trigone and urethra [17].

The involvement of the L-arginine/NO pathway in the relaxation of isolated preparations from smooth muscle organs of the genitourinary tract has been demonstrated in various species by many investigators [10, 13]. NANC-induced NO release is considered to be of importance in the tone regulation of these tissues. This was based on experiments utilizing strip preparations from the detrusor, trigone, bladder neck, urethra and penile erectile tissue. Relaxation of urethral and bladder neck smooth muscle has been demonstrated in rat [19], pig [16, 18], dog [7] and humans [2]. NO induced concentration-dependent relaxation in preparations contracted by noradrenaline or carbachol. In all preparations, N^G-nitro-L-arginine (L-NOARG), an

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inhibitor of the synthesis of NO from L-arginine, almost diminished the relaxation elicited by electrical field stimulation. NANC-nerve-mediated relaxation of the isolated rabbit urethra could be inhibited by L-NOARG. The distribution of NOS-positive nerves in pig detrusor, trigone and urethra corresponds with tissue ability to response to NO-mediated relaxation. In the pig detrusor, the NO donor SIN-1 and NO, stimulating the activity of guanylate cyclase, relaxed carbachol and endothelin-1 (ET-1) contracted preparations by approximately 60% [17]. However, adenylate cyclase stimulating agent isoprenaline was more potent than SIN-1 and caused complete relaxation. These results agree with those of Morita et al., who found that, in rabbits, cGMP is related to urethral relaxation and cAMP is mainly related to urinary bladder relaxation [15]. There is also growing evidence that, during erection, release of NO produces relaxation of corpus cavernosum tissue. It was demonstrated in rabbit and human corpus cavernosum that NO or NO-related vasodilators such as nitroglycerin, sodium nitroprusside, *S*-nitroso-*N*-acetylpenicillamine and SIN-1 cause tissue relaxation through stimulation of soluble guanylate cyclase, thus increasing the tissue levels of cGMP [4, 8, 9, 11]. The clinical potential of the NO donor SIN-1 in the treatment of erectile dysfunction has been shown [22].

In this study we examined the possible involvement of the L-arginine/NO pathway in ureteral relaxation in vitro using functional organ bath studies and immunohistochemistry.

Material and methods

Tissue source

Ureteral tissue from the mid ureter was taken from ten patients undergoing radical surgery for renal tumors without the involvement of the renal pelvis and ureter. Preoperative renal function in all patients was at least 100 ml/min. The tissue was immediately placed in a chilled organ protective solution (NaCl 15 mM, KCl 9 mM, $KHC_5H_6O_5$ 1 mM, $MgCl_2$ 4 mM, histidine 18 mM, tryptophan 2 mM, mannitol 30 mM, $CaCl_2$ 0.015 mM) and transported to the laboratory for further preparation.

Organ bath studies

Ureteral smooth muscle was carefully dissected free of surrounding tissue. Ureteral rings (5 mm) were cut and mounted in 10-ml organ bath chambers containing Ringer-Krebs solution (pH 7.2–7.4) of the following composition (mM): NaCl 119, $NaHCO_3$ 15, KCl 4.6, $CaCl_2$ 1.5, $NaHPO_4$ 1.2, $MgCl_2$ 1.2, Na [Ca^{2+}] ethylenediamine tetraacetate (EDTA) 0.1, glucose 11. The bath solution was continuously gassed with carbogen (95% O_2, 5% CO_2) and the temperature was maintained at 37 °C. The rings were attached to pressure transducers (Grass, Quincy, MA, USA) and a pretension of 10 mN was applied. The tissue was equilibrated for 60 min without further mechanical manipulation. Precontraction was done with 80 mM KCl, which ensured stable and reproducible contractions. The use of noradrenaline, carbachol, endothelin or $PGF_{2\alpha}$ failed to induce long-lasting contractions (data not shown).

When a stable contraction plateau was reached, increasing doses of SIN-1 (0.01–100 μM) were added to the bath chambers. Isotonic responses of the tissue were amplified and plotted with a thermowriter. The same approach was performed after preincubation of the tissue with methylene blue (50 μM).

Another series of functional experiments was conducted with electrically induced ureteral relaxation. For electrical field stimulation studies, an HSE stimulator type 215/I (Hugo Sachs Electronics, Freiburg, Germany) was used. The tissue was prepared and mounted as described above. Stimulation parameters were set as follows: stimulus amplitude 15 V, frequency 20 Hz, stimulus duration 5 s every 120 s, pulse duration 48 ms. When constant amplitudes were reached, L-NOARG (0.1–100 μM) was added to the bath chambers. Sensitivity of the electrical-evoked tissue responses towards TTX (0.2 and 2 μM) was also tested.

To further elucidate the role of the NO-guanylate cyclase-cGMP pathway in ureteral smooth muscle, another study was performed. The ureteral rings were preincubated 20 min prior to addition of SIN-1 (10 μM) with increasing doses of Rp-8-pCPT-cGMPS and Rp-8-CPT-cAMPS, membrane-permeable selective inhibitors of cGMP-activated protein kinase GI_α and cAMP-activated protein kinase, respectively.

Immunohistochemistry

For immunohistofluorescence the ureteral tissue was fixed in an immersion of 4% formaldehyde at 4 °C overnight. Small sections were cut with a microtome and incubated in a dilution (1:250) of a rabbit antiserum against neuronal NO synthase. Bound antibodies were visualized with fluorescin or (DAB)-conjugated pig anti-rabbit IgG diluted 1:40. Control stainings were done using the same approach but without the antiserum.

Chemicals and drugs

All laboratory chemicals, unless otherwise indicated, were obtained from either Sigma (St. Louis, MO, USA) or E. Merck Chemicals (Darmstadt, Germany). SIN-1 (linsidomine) was generously provided by Hoechst Germany. Rp-8-pCPT-cGMPS and Rp-CPT-cAMPS were purchased from Biolog (Bremen, Germany). Stock solutions of drugs were made up in saline. NO synthase antiserum for immunohistochemistry was a gift from Dr. Mayer, Graz University, Austria; DAB-conjugated pig anti-rabbit IgG was purchased from Daco-Patts (Hamburg, Germany).

Analysis of data

All functional experiments were repeated 6–8 times. Relaxant responses of tissue preparations are expressed as the percentage of maximal contraction induced by KCl (80 mM) or electrical field stimulation (EFS). All data are given as means ± standard deviations of the mean. Statistical analysis was conducted by Student's *t*-test. A probability (*P*) value of less than 0.05 was accepted as significant.

Results

SIN-1

During organ bath studies none of the ureteral rings showed spontaneous contractile activity. After

precontraction with 80 mM KCl, SIN-1 induced dose-dependent relaxations in the ureteral rings from 2.3 ± 4.0% at 0.1 μM to 78.2 ± 5.5% ($n = 8$) at 100 μM (Fig. 1).

Influence of methylene blue, Rp-8-pCPT-cGMPS and Rp-8-CPT-cAMPS on SIN-1-induced relaxations

In the ureteral preparations preincubated with methylene blue for 20 min, relaxations induced by 100 μM SIN-1 were markedly reduced to 50.2±4.4% ($n = 8$) compared to non-preincubated rings (Fig. 2). In the presence of increasing doses of Rp-8-pCPT-cGMPS (0.01–100 μM), the relaxing effect of 10 μM SIN-1 was slightly but significantly reduced from 35.0 ± 3.6% to 22.5±2.3% ($n = 6$). In contrast, SIN-1-induced relaxations were not altered after preincubation of the ureteral rings with the protein kinas A (PKA)-inhibitor Rp-8-CPT-cAMPS ($n = 6$) (Fig. 3).

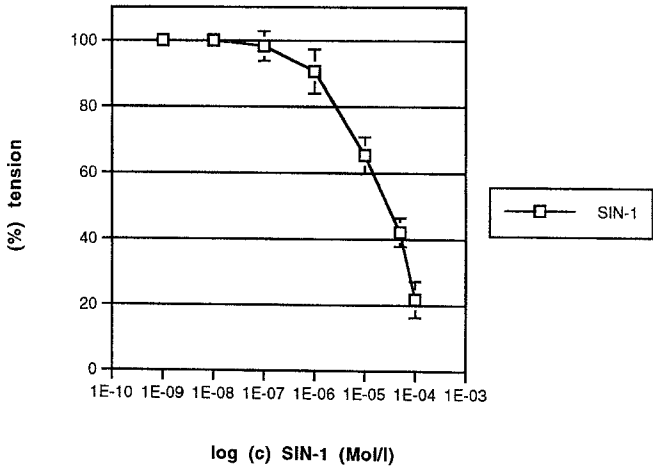

Fig. 1 Relaxing effect of SIN-1 on human ureteral rings precontracted with 80 mM KCl. Results are given as percent of the maximum tension induced by KCl and are the means ± SD of eight experiments

EFS experiments

In all preparations studied, EFS induced frequency-dependent transient relaxations. The relaxant effect of EFS remained unaltered even after several stimulations. Relaxation was occasionally followed by contraction amplitude. L-NOARG in concentrations from 1 to 10 μM did not diminish EFS-induced relaxations, even an increase in concentration to 100 μM failed to reveal any blocking of the relaxant effects ($n = 6$). After TTX treatment, EFS-induced responses of the tissue were unchanged compared to EFS-evoked relaxations without TTX pretreatment, which proved the non-neurogenic origin of the EFS effects (Fig. 4). Therefore, blockade studies of the NO pathway with EFS-induced ureteral relaxations were abolished.

Immunohistochemistry

In immunohistochemistry, ureteral tissue showed rich staining for NO-containing nerves and NO-containing nerve endings within the smooth musculature (Fig. 5). Their density was semiquantitatively classified as ++ [7]. The NO-immunoreactive nerves were observed extending transversely through all layers of the mid-ureter, forming dense plexi. They could be seen running parallel with the transverse axis of smooth muscle bundles.

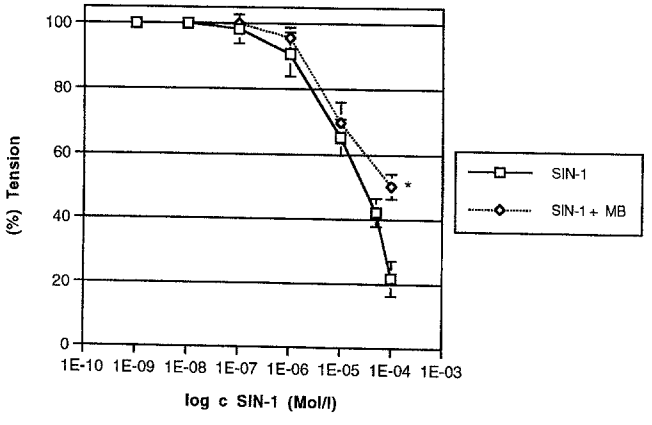

Fig. 2 Effect of 50 μM methylene blue (*MB*) on the relaxant response of KCl-precontracted ureteral rings to SIN-1. * indicates statistical significance ($P < 0.05$)

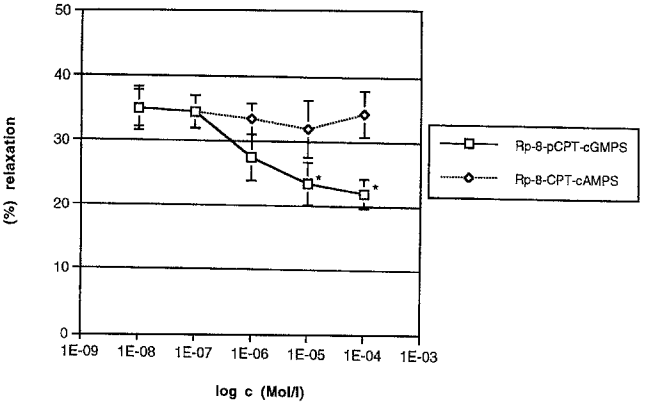

Fig. 3 Effect of increasing concentrations of protein kinase inhibitors Rp-8-pCPT-cGMPS and Rp-8-CPT-cAMPS on the relaxant response of KCl-contracted ureteral rings to 10 μM SIN-1. * indicates statistical significance ($P < 0.05$)

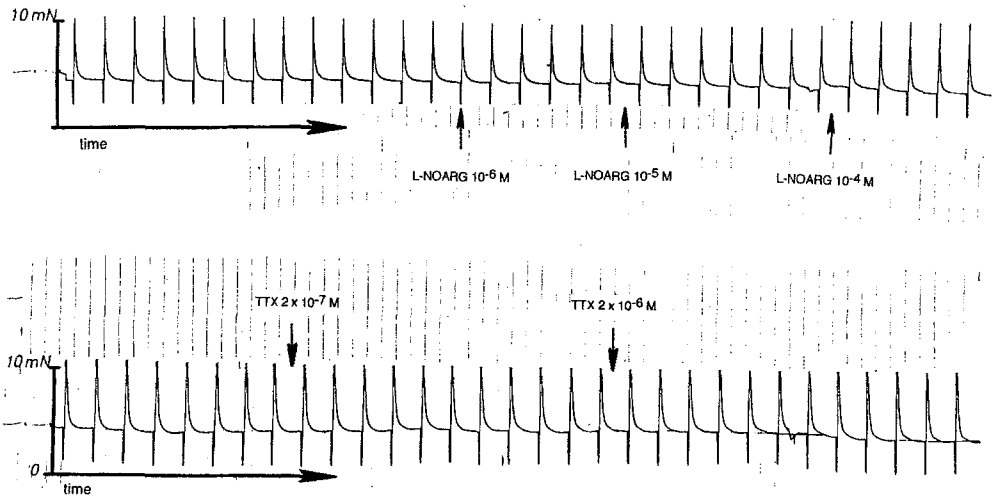

Fig. 4 Original recordings of an EFS experiment. The upward amplitudes represent contractions of the ureteral rings; the downward amplitudes represent relaxations. No effect of the increasing concentrations of L-NOARG and TTX on EFS-evoked tone alterations of human ureteral rings was found

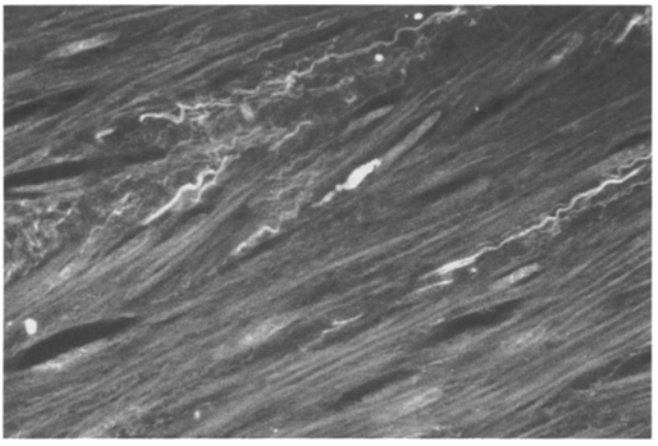

Fig. 5 Microphotographs of transverse sections of the human ureteral wall labelled with fluorescin-conjugated pig anti-rabbit IgG for visualization of bound antibodies against human neuronal NO-synthase. Nerve fibre-like structures stained positively for NOS are found between smooth muscle fibres, ×290

Discussion

Ureteral physiology has become a source of interest in the field of urological research. However, ureteral relaxation could not be achieved by neurostimulation in vitro and, with the exception of VIP, no putative neurotransmitters have yet been found in vitro. Only recently, our own studies [23] as well as those of others [21] found a possible involvement of NO in the regulation of ureteral smooth muscle tone. As expected in analogy to other urological smooth muscle tissues, our results, as described above, strongly suggest the role of NO as a peripheral neurotransmitter responsible for ureteral relaxation.

Our in vitro results demonstrated a relaxation of precontracted ureteral tissue by SIN-1, the active metabolite of molsidomine, which was used as NO donor, because it represents a substance with a well-established pharmacological profile: SIN-1 releases NO non-enzymatically [20]. These observations are supported by the second series of experiments showing a decrease of the relaxant activity of SIN-1 on precontracted ureteral rings after preincubation with methylene blue, which inhibits the soluble guanylate cyclase, thus specifically interfering with the NO pathway. The slight effect of blockade by methylene blue on the relaxing effect of SIN-1 may be explained by an insufficent incubation time, an insufficent dosage of methylene blue or competitive mechanisms at the enzyme level. Nevertheless, our results obtained with preincubation of the ureteral preparations with selective inhibitors of protein kinase G I prior to the addition of SIN-1 seem to support the role of the NO/guanylate cyclase/cGMP pathway in the regulation of ureteral smooth muscle tone.

To further delineate a possible involvement of the NO pathway in the physiological process of ureteral relaxation, a series of experiments was performed on the relaxant effect of EFS on ureteral tissue. It is generally accepted that EFS induces changes of the smooth muscle tone by release of neurotransmitter substances from their storage sides within the tissue. With EFS, frequency-dependent ureteral relaxation could be achieved. However, since addition of TTX was not followed by a significant decrease of the EFS-induced relaxations compared to EFS without TTX pretreatment, the effect of EFS must be considered as a direct effect on the ureteral smooth muscle and not as a stimulus to release cellular neurotransmitters.

These results suggest that, in the isolated ureter preparations, relaxations involving NO or an NO-containing compound can be elicited. The physiological significance remains to be fully established; however, it may be speculated that NO or a related compound acts as a physiological transmitter mediating ureteral

relaxation in vitro. Our functional results are furthermore corroborated by the immunological findings with dense staining of NOS. We observed nerve-fibre-like staining as well as synapse-type staining. These morphological observations are supported by similar studies that also showed dense NOS staining within the ureteral wall [16]. Further in vivo studies should be carried out to confirm our in vitro hypothesis of a possible role of NO as neurotransmitter for ureteral relaxation. Given positive results, a new pharmacological approach for the treatment of urinary colics and facilitation of urinary stone passage may arise since different mechanisms involved in the NO pathway can be influenced pharmacologically.

Conclusions

Our in vitro findings suggest that ureteral relaxation may involve the L-arginine/NO pathway. These functional results are supported by our morphological findings, suggesting a physiological NO-specific relaxation. These results may contribute to a new therapeutic approach in the treatment of ureteral colics.

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