Nitrergic and cholinergic innervation of the rat lower urinary tract after pelvic ganglionectomy

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Persson, Katarina, Per Alm, Bengt Uvelius, and Karl-Erik Andersson. Nitrergic and cholinergic innervation of the rat lower urinary tract after pelvic ganglionectomy. Am. J. Physiol. 274 (Regulatory Integrative Comp. Physiol. 43): R389–R397, 1998.—The possible coexistence of nitric oxide (NO) and acetylcholine in the rat major pelvic ganglion (MPG) was examined by double immunohistochemistry using antisera raised against NO synthase (NOS) and choline acetyltransferase (ChAT). The smooth muscle responses of the isolated bladder and urethra were recorded after bilateral cryoganglionectomy of the MPG, focusing on the possible development of denervation supersensitivity. In the MPG, NOS immunoreactivity (ir) was seen in a large number of cell bodies, but it was not as abundant as ChAT-ir cell bodies. Double immunolabeling showed that all NOS-ir cell bodies also displayed ChAT-ir. In ganglionectomized bladders, the electrical field stimulation (EFS)-evoked contractile response was markedly reduced. When compared with control bladders, detrusor strips from ganglionectomized rats were more sensitive to carbachol as revealed by a lower negative logarithm of the drug concentration eliciting 50% relaxation (6.5 ± 0.04 vs. 5.9 ± 0.07). In the urethra, the NO-mediated relaxant response to EFS was practically abolished by ganglionectomy, whereas no difference was found in sensitivity to 3-morpholinosydnonimine hydrochloride (SIN-1). SIN-1 produced an equal increase in tissue levels of guanosine 3',5'-cyclic monophosphate in urethral preparations from control and ganglionectomized rats. The results suggest that the NOS-ir nerves that mediate inhibition of rat urethral smooth muscle tone originate from the MPG and contain ChAT. No denervation supersensitivity to nitrergic stimulation was observed in the urethra after ganglionectomy.

ChAT in cell bodies in the MPG; and

the existence, and possible coexistence, of NOS and cholinergic, adrenergic, purinergic, and peptidergic neurons (8), and recently nitric oxide synthase (NOS)-containing cells have also been demonstrated (2, 22, 36).

Specific markers for cholinergic neurons in the peripheral nervous system have been lacking. Although the histochemical technique for detection of acetylcholinesterase has been used in the past, this enzyme does not appear to be a specific marker for cholinergic nerves. Recently, antibodies raised against choline acetyltransferase (ChAT), the enzyme that catalyzes the biosynthesis of acetylcholine, have been developed for visualization of peripheral cholinergic nerves (18, 32). Many investigators have suggested that NOS is located primarily in parasympathetic cholinergic as opposed to sympathetic adrenergic pathways (2, 22), but the evidence in the lower urinary tract is circumstantial. Nerves immunolabeled for NOS, but not for tyrosine hydroxylase (TH), have been assumed to represent parasympathetic cholinergic fibers (2, 36). Furthermore, chemical denervation studies have shown that both the distribution of NOS-containing nerves and NO-mediated urethral relaxations are unaffected by 6-hydroxydopamine treatment (30). Whether NOS and ChAT are coexpressed in the rat MPG has, to the best of our knowledge, not been investigated.

Retrograde axonal tracing studies in the rat suggest that NOS-containing fibers in the MPG project almost exclusively to the urethra (36). In agreement with this, NOS-containing (nitrergic) nerves in the smooth muscle are more frequent in the urethra than in the bladder (5). Transmural stimulation of urethral tissue of several species evokes relaxations that are sensitive to inhibitors of NOS (5). However, no functional denervation studies seem to have been performed to verify that the MPG is the source for the nitrergic nerves and neurotransmission in the urethral smooth muscle. In rats, the MPG is a well-defined ganglion, which can be removed surgically or, as in this study, destroyed by a cryoprobe (13).

It is well known that when neuromuscular transmission is interrupted, e.g., after nerve section, the target organ becomes supersensitive to the transmitter substance released by the nerve terminal (37). A presynaptic type of supersensitivity occurs when a greater fraction of the transmitter released or administered exogenously reaches the receptor because of loss of transmitter removal. Postjunctional supersensitivity occurs in the target organ and involves an increase in receptor numbers or nonspecific hyperexcitability (37). In both the rat detrusor (9) and urethral (10) smooth muscle, supersensitivity to muscarinic receptor stimulation was reported to develop after bilateral ganglionectomy. Removal of basal NO-mediated vasodilator tone in cardiovascular systems in vivo leads to a specific supersensitivity to nitrovasodilators (23). It is not known whether the smooth muscle of the lower urinary tract responds with supersensitivity to NO after bilateral ganglionectomy.

In the present study, we wanted to investigate 1) the effect on NOS-containing nerves in the rat detrusor and urethra after bilateral pelvic cryoganglionecomy; 2) the existence, and possible coexistence, of NOS and ChAT in cell bodies in the MPG; and 3) whether or not supersensitivity to NO develops after ganglionectomy.
Muscle responses to electrical field stimulation (EFS) and agonists were examined, and single- and double-labeling immunohistochemistry were performed. A preliminary report including some of these findings has been presented previously (31).

MATERIALS AND METHODS

Twenty-five female Sprague-Dawley rats (body weight 200–250 g) were used in this study. Ten rats underwent ganglionectomy, and fifteen rats were used as controls (8 for functional studies and 7 for immunohistochemistry). The experimental protocol was approved by the Animal Ethics Committee, Lund University.

Ganglionectomy. The rats were anesthetized with ketamine (100 mg/kg im, Ketalar; Parke-Davis, Barcelona, Spain) and xylazine (15 mg/kg im, Rompun; Bayer, Leverkusen, Germany). The lower abdomen was opened, and the area of the pelvic ganglia was exposed. A 5 × 7 mm area between the proximal urethra, the ureter, and the vaginal-rectal border was frozen several times with a metal probe containing liquid nitrogen, providing complete destruction of the pelvic ganglion (13). Care was taken to avoid injury to adjacent tissues. The procedure was repeated on the other pelvic ganglion, and the abdomen was closed. The bladders were emptied daily by means of external compression, and the animals were killed 3 wk postoperatively. This time was chosen on the basis of previous studies on the ganglionectomized rat urinary tract (9, 10).

Tissue handling. The rats were killed by CO2 asphyxia, and the bladders together with the urethras were removed en bloc. Fat and connective tissue were carefully removed, and the bladders and urethras were opened by longitudinal incisions.

Urethral and bladder specimens aimed for tissue bath studies and measurement of cyclic nucleotide levels were processed as described below.

For immunohistochemical evaluation of the effect of ganglionectomy on the urethral and bladder innervation, specimens of bladder and urethra were immersion-fixed for 4 h in an ice-cold, freshly prepared solution of 4% formaldehyde in phosphate-buffered saline (PBS; 0.1 M, pH 7.4) and then rinsed in ice-cold 15% sucrose in PBS for 2–3 days. The tissue specimens were subsequently frozen in isopentane at −40°C and stored at −70°C.

For dissection of the MPG in the controls, the rats were anesthetized with ketamine (100 mg/kg im) and xylazine (15 mg/kg im) and perfused through the ascending aorta via a cannula through the left ventricle of the heart, first with 100 ml of ice-cold, calcium-free Krebs solution (containing 1 g/l sodium nitrate and 10,000 IU/l of heparin), and then with 300 ml of a freshly prepared solution of 2% formaldehyde and 0.2% picric acid in 0.1 M phosphate buffer (pH 7.2). The ganglia were removed and then immersion-fixed at room temperature for ~5 h, rinsed overnight in ice-cold 15% sucrose in PBS, and then frozen and stored as previously described.

Immunohistochemistry. Cryostat sections were cut at a thickness of 10 μm and thaw-mounted onto chrome-alum-coated slides and then preincubated in PBS with 0.2% Triton X-100 for 2 h.

Sections of bladders and urethras were incubated overnight in the presence of antisera raised in rabbits against neuronal NOS (1:1,500; Euro-Diagnostica, Malmö, Sweden) or protein gene product 9.5 (PGP; 1:2,000; UltraClone, Cambridge, UK). The sections were rinsed in PBS (three rinses during 10 min) and then incubated for 90 min with fluorescein isothiocyanate (FITC)-conjugated swine anti-rabbit immunoglobulins (lg) (1:80; Dakopatts, Stockholm, Sweden), rinsed, and mounted in PBS-glycerol with p-phenylenediamine to prevent fluorescence fading.

Sections of MPG were double immunolabeled (for the simultaneous demonstration of two antigens) by first being incubated with a rabbit antisem to ChAT (1:2,000; BioMerieux, Göttingen, Germany) rinsed in PBS (three rinses during 10 min), and then incubated overnight with an antisem raised in guinea pig to neuronal NOS (1:500; Euro-Diagnostica) or in guinea pig to vasoactive intestinal polypeptide (VIP; 1:1,280; Euro-Diagnostica) or a mouse monoclonal antisem to TH (1:250–1:500; Incstar, Stillwater, MN). After rinsing, the sections were incubated for 90 min with Cyanine 3 (Cy3)-conjugated affinity-purified anti-rabbit lg (1:300; Jackson ImmunoResearch, West Grove, PA), rinsed, and then incubated with FITC-conjugated goat anti-guinea pig lg (1:80) or FITC-goat anti-mouse lg (1:80) (both purchased from Sigma, St. Louis, MO). After rinsing, the sections were mounted as previously described. Approximately 25 sections from each ganglion were examined.

The sections were examined in an Olympus epifluorescence microscope equipped with appropriate filter settings for Cy3 and FITC-immunofluorescence. No specific ChAT-ir or TH-ir could be observed in sections incubated in the presence of PBS or in normal rabbit or mouse lg (diluted as for the corresponding antiserum). Because cross reactions with antigens sharing similar sequences cannot be excluded, the structures demonstrated are referred to as ChAT-ir, NOS-ir, PGP-ir, VIP-ir, and TH-ir.

Recording of mechanical activity. The bladder and urethra were separated at the level of the bladder base, and circular strips (1 × 1 × 5 mm) were prepared from the middle third of the detrusor. The proximal part of the urethra was divided into two strips taken in the longitudinal direction. Thereafter, the strips were transferred to 5-ml tissue baths containing Krebs solution (in mM: 119 NaCl, 4.6 KCl, 1.5 CaCl2, 1.2 MgCl2, 15 NaHCO3, 1.2 NaH2PO4, and 11 glucose) maintained at 37°C by a thermoregulated circuit. The Krebs solution was continuously bubbled with a mixture of 95% O2 and 5% CO2, resulting in a pH of 7.4. Muscle strips were suspended between two L-shaped hooks by means of silk ligatures. One hook was connected to a movable unit allowing adjustment of passive tension, and the other to a Grass FT03C force transducer. Isometric tension was recorded using a Grass polygraph (7D). After they were mounted, the strips were stretched to a passive tension of 4 mN and allowed to equilibrate for 45–60 min before further experiments were performed.

EFS. Transmural stimulation of nerves was accomplished by means of two platinum electrodes placed on either side of the preparations. EFS was performed using a Grass S48 or S88 stimulator delivering single square-wave pulses (duration 0.8 ms) at selected frequencies. The voltage giving maximal response was determined individually for each strip. Train duration was 5 s and the stimulation interval 2 min. The polarity of the electrodes was shifted after each pulse by means of a polarity-changing unit. Frequency-response curves were constructed, and frequency producing 50% relaxation (EFS0) values were calculated. The EFS0 values may give information on the nature of released transmitters.

Experimental protocol. Each experiment was started by exposing the preparations to a K+ (124 mM) Krebs solution until two reproducible contractions had been obtained. In urethral preparations, the relaxant effect of EFS (0.5–30 Hz) and 3-morpholinosydnonimine hydrochloride (SIN-1; 10 mM) was studied after precontraction by a submaximal concentration (50–80% of the response to K+ of arginine
vasopressin (AVP; 5–100 nM). Because the response to K⁺ was larger in ganglionectomized rats, a higher concentration of AVP had to be given to urethral strips from ganglionectomized rats to attain this tension level. The contractile response to carbachol (10 nM–30 µM) added cumulatively was also studied. In detrusor preparations, frequency-response relationships for EFS (1–60 Hz) and concentration-response curves for carbachol (10 nM–30 µM) were constructed. In detrusor preparations, the relaxant effect of SIN-1 (10 nM–0.1 mM) was studied after precontraction by carbachol (10µM).

Measurement of cyclic nucleotide levels. Urethral preparations used for determination of cyclic nucleotide levels were precontracted with AVP and exposed to SIN-1 (10 nM–0.1 mM). At the maximal relaxation level, the strips were frozen in liquid nitrogen. Control experiments (without application of SIN-1) were performed in the same way. The frozen tissue was homogenized on ice in 10% trichloroacetic acid using a hand glass homogenizer. Following centrifugation, the supernatant was first subjected to ether extraction, then evaporated at 50°C. The pellet was reconstituted in 2 M NaOH and analyzed for protein content according to Bradford using the Bio-Rad protein assay (Kemiila, Stockholm, Sweden). Residues were dissolved in 0.05 M sodium acetate, and the amount of guanosine 3',5'-cyclic monophosphate (cGMP) and adenosine 3',5'-cyclic monophosphate (cAMP) was quantitated using 125I-labeled cGMP and 125I-labeled cAMP radioimmunoassay kits (DuPont, Wilmington, DE) according to manufacturers’ instructions. The samples were acetylated to increase the sensitivity of the assay. [3H]cAMP was added to the homogenate to determine recovery. Recovery was estimated as 70%.

Drugs and solution. The following drugs were used in functional studies: scopolamine hydrochloride, carbamylcholine chloride, tetrodotoxin, N-nitro-L-arginine (Sigma), and AVP acetate (Peninsula Laboratories, Belmont, CA). SIN-1 was a gift from Dr. Kunstman, Cassella, Germany. Stock solutions were prepared and then stored at -70°C. All drugs were dissolved in saline or distilled water. K⁺-Krebs solution (124 mM) was prepared by replacing NaCl with equimolar amounts of KCl.

Data analysis. Relaxant responses are expressed as percent reduction of the AVP (urethra)- or carbachol (detrusor)-induced tension. Contractile responses are expressed as percentage of the initial contraction induced by K⁺ (124 mM). The negative logarithm of the drug concentration eliciting 50% relaxation (pEC₅₀) or EF₅₀ was determined by linear regression, using the values immediately above and below half-maximal relaxation. For contractile agonists, the pEC₅₀ value refers to the negative logarithm of the drug concentration producing 50% of the maximal contraction obtained, and the EF₅₀ value refers to the frequency producing 50% of the maximal response. Results are expressed as mean values ± SE, and n denotes the number of experiments (animals) performed. Student’s unpaired t-test (two tailed) was used for statistical analysis. Statistical significance was accepted when P < 0.05.

**RESULTS**

Effects of ganglionectomy on the detrusor and urethra. Bilateral pelvic ganglionectomy caused a marked increase in tissue weight from 60 ± 1.4 mg (n = 6) to 268 ± 15 mg (n = 8) in the detrusor (P < 0.001) and from 36 ± 2 mg to 98 ± 3 mg in the urethra (n = 8, P < 0.001).

Immunoreactivities to NOS and the general nerve marker, PGP, were used to evaluate the result of the denervation procedure. PGP-ir and NOS-ir nerve fibers were distributed in the rat lower urinary tract as previously described in detail (3). Briefly, in control rats, PGP labeled numerous nerve fibers in the smooth muscle of the detrusor (Fig. 1A) and urethra, ranging from coarse nerve trunks to varicose fibers. In contrast, NOS-ir nerves were scarce in the detrusor region, whereas a dense supply was found in the smooth muscle of the urethra (Fig. 1C). ChAT-ir nerve fibers could not be detected in the detrusor or urethral muscle.

In the bladder and urethra of ganglionectomized animals, nerve fibers immunoactive to PGP and NOS were practically absent (Fig. 1, B and D). However, some PGP-ir or NOS-ir nerve fibers were occasionally found in the striated muscle that surrounds the middle and distal part of the rat urethra.

Immunoreactivity of cell bodies in the MPG. In the MPG, most cell bodies displayed ChAT-ir that covered the cytoplasm around the dark and unlabeled nuclei. Many of the ChAT-ir cell bodies were surrounded by ChAT-ir varicose terminals (Figs. 2A and 3A). NOS-ir was seen in a large number of cell bodies, but it was less abundant compared with ChAT-ir cell bodies (Fig. 2B). Double immunolabeling showed that all NOS-ir cell bodies also displayed ChAT-ir (Fig. 2, A and B). TH-ir was also observed in cell bodies, covering the cytoplasm around dark and unlabeled nuclei (Fig. 3B). In comparison to ChAT-ir and NOS-ir cell bodies, the number of TH-ir cell bodies was less. Double immunolabeling showed that ChAT-ir and TH-ir were localized in separate cell bodies (Fig. 3, A and B). In addition, all of the VIP-ir cell bodies in the MPG appeared to be ChAT-ir (data not shown).

Effects of ganglionectomy on functional responses in the detrusor. EFS (1–60 Hz) of the control rat detrusor muscle produced frequency-dependent, tetrodotoxin (1 µM)-sensitive contractions (Fig. 4A). The contractions were partly reduced by scopolamine (1 µM), suggesting a cholinergic origin. However, a scopolamine-resistant component of 40% was evident (Fig. 4A). EF₅₀ was 6.7 ± 0.3 Hz (n = 6; Table 1). In ganglionectomized bladders, the EFS-evoked response was markedly reduced, although a contraction of 36 ± 4% (n = 8) remained at 60 Hz (Fig. 4B). However, the major part of this contraction (75%) was resistant to tetrodotoxin (1 µM).

When compared with control bladders, detrusor strips from ganglionectomized rats were more sensitive to carbachol (P < 0.001) as revealed by a lower pEC₅₀ value (6.5 ± 0.04 vs. 5.9 ± 0.07) (Fig. 4C, Table 1). Also, the maximal tension developed in response to carbachol was higher in the ganglionectomized group. The NOS inhibitor N-nitro-L-arginine (L-NNA) (0.1 mM) did not affect the concentration-response relationships for carbachol (n = 3, data not shown). The relaxant effect of the NO donor SIN-1 (10 nM–0.1 mM) was minor in detrusor strips from both control (20 ± 3%; n = 6) and ganglionectomized (19 ± 2%; n = 8) rats (Table 1). The contractile response to K+ (124 mM) in ganglionectomized bladders did not differ from that of controls (Table 1).
Effects of ganglionectomy on functional responses in the urethra. Transmural nerve stimulation of the control rat urethra, precontracted with AVP, produced tetrodotoxin (1 µM)-sensitive relaxations. The relaxations were abolished by L-NNA (0.1 mM), suggesting involvement of NO. In all preparations, the response changed into a contraction after addition of L-NNA (Figure 5A). In control rats, EFS (0.5–30 Hz) caused frequency-dependent relaxations and a maximal relaxant response of 58 ± 6% (n = 8) was obtained at 16 Hz (Fig. 5B, Table 1). The relaxant response to EFS was markedly affected by ganglionectomy (10 ± 5%; P < 0.001) (Fig. 5B), and in six of eight rats the relaxation was abolished.

SIN-1 caused concentration-dependent, long-lasting relaxations (70 ± 5%, n = 6) of urethral strips precontracted with AVP. No difference was found between urethral strips from control and ganglionectomized rats with regard to magnitude or sensitivity to SIN-1-induced relaxations (Fig. 5C, Table 1). The contractile response to exogenously applied carbachol (10 nM–0.1 mM) was also examined in the urethra. These data showed that the sensitivity (pEC50 value) to carbachol was similar in urethral strips from control and ganglionectomized rats (Table 1). However, the maximal contractile response to carbachol was lower (P < 0.05) in ganglionectomized animals (Table 1). The tension developed in response to K+ (124 mM) was higher in ganglionectomized rats (Table 1). The difference remained after correction for strip weight, being 0.5 ± 0.05 mN/mg tissue (n = 8) in the control group and 0.8 ± 0.05 mN/mg tissue (n = 8; P < 0.001) in the ganglionectomized group.

Effects of ganglionectomy on SIN-1-induced cGMP accumulation. In control rats, SIN-1 caused a significant (P < 0.001) increase in tissue levels of cGMP in urethral preparations from 2.2 ± 0.1 to 4.4 ± 0.2 pmol/mg protein (n = 7, Fig. 6). Also in the ganglionectomized group, SIN-1 produced an increase in cGMP levels from 2.0 ± 0.2 to 4.3 ± 0.5 pmol/mg protein (n = 7, P < 0.01). Corresponding cAMP levels were not changed by application of SIN-1 (data not shown).

DISCUSSION

Most investigators (1, 17), with some exception (22), have found that the adult rat bladder lacks intramural neurons and that the pelvic ganglia provide the auto-
nomic nerve supply to the bladder. Although some accessory ganglia, located around the distal part of the ureter, may contribute to bladder innervation (8), pelvic ganglionectomy in rats is considered a good experimental model for bladder denervation studies. It seems that some ganglion neurons appear in the bladder wall subsequent to pelvic ganglionectomy (35), but nerve sprouting is not believed to be of significance after bilateral postganglionic denervation of the rat bladder (11). The parasympathetic innervation of the proximal urethra in rats, as in the case of the bladder, is mainly of extrinsic origin (10, 20). However, part of the sympathetic innervation of the urethra may emanate from intramural neurons (20). In the present study, PGP-ir and NOS-ir nerve fibers were practically abolished in urethral and bladder preparations from ganglionectomized animals, verifying that the denervation procedure had been successful. Accordingly, the nitricergic innervation of the rat lower urinary tract smooth muscle appears to originate from the MPG. In our study, some NOS-ir fibers were distributed in the striated muscle that surrounds the middle and distal part of the urethra. NOS-ir fibers within the striated muscle layer could also be detected, although less frequently, after ganglionectomy. This supports previous studies (28) that found that NOS-containing fibers in the rat external urethra are conveyed both via the MPG and the somatic pudendal nerve.

Recently, immunoreactivities for NOS, and, as a marker for cholinergic neurons, ChAT, were found to frequently coexist in many preganglionic fibers in the rat (24). In this study, we investigated the possible coexistence of NOS-ir and ChAT-ir in postganglionic cell bodies in the MPG. Retrograde axonal tracing investigations in combination with NOS immunohistochemistry of postganglionic neurons innervating the urethra and bladder (36) suggest that the NOS-ir neurons detected in the MPG in this study project almost exclusively to the urethra. The number of ChAT-ir cells in the MPG clearly exceeded the number of NOS-ir cells. Double-labeling immunohistochemistry revealed that all NOS-ir cell bodies in the MPG exhibited ChAT-ir. In addition, the VIP-ir population of neurons was also ChAT-ir. It was also confirmed, as recently described (18), that ChAT-ir cells were distinct from TH-ir cells in pelvic neurons. This supports previous studies showing that NOS is not present in TH-ir cells in the rat MPG (2, 36) and that NOS-ir as well as NO-mediated urethral relaxations are unaffected by treatment with the sympathetic neurotoxin 6-hydroxypamin (30). From the present study, it is concluded

![Fig. 2. Major pelvic ganglion. Localization of choline acetyltransferase (ChAT) immunoreactivity (A) and NOS immunoreactivity (B) in nerve cell bodies of the same section is shown. Scale bar = 100 µm.](image1)

![Fig. 3. Major pelvic ganglion. Localization of ChAT immunoreactivity (A) and tyrosine hydroxylase (TH) immunoreactivity (B) in nerve cell bodies of the same section is shown. Arrows indicate cell bodies immunoreactive for TH but not for ChAT. Scale bar = 100 µm.](image2)
that, in the rat MPG, ChAT/NOS-ir neurons constitute one population of neurons that is separate from sympathetic TH-ir neurons. However, there appear to be some exceptions in, for example, the male pelvic neurons of humans (16), where coexpression of NOS and TH has been demonstrated.

Voiding involves firing in parasympathetic pathways causing bladder contraction by activation of muscarinic receptors and urethral relaxation (15). Acetylcholine constricts the smooth muscle of the urethra in several species in vitro (4), but the role of the rich cholinergic innervation of the urethra for micturition has not been fully understood. One possibility is that acetylcholine acts prejunctionally in the urethra to suppress norepinephrine release (21). No labeling of postganglionic, intramural nerve fibers was found with the ChAT antiserum in the present investigation. This may indicate that the concentration of ChAT in the postganglionic terminals, as opposed to ChAT levels in cell bodies and preganglionic terminals, is below the detection limit. However, assuming that coexistence of NOS and ChAT in the MPG also implies that NO and acetylcholine coexist in intramural nerve fibers, it may be suggested that the function of cholinergic nerves in the urethra is mainly to release inhibitory NO and to evoke urethral relaxation. Indeed, stimulation of parasympathetic pathways in the anesthetized rat produces urethral smooth muscle relaxation sensitive to NOS inhibition (12). The interplay between acetylcholine and NO in regulation of urethral motility is not known, but in several studies (for review, see Ref. 19), modulatory effects of NO on parasympathetic nerve activity have been reported.

Although species differences exist, acetylcholine seems to be the main mediator of bladder contraction (4). In line with previous studies (9, 20), bilateral ganglionectomy of the rat urinary bladder resulted in loss of reflex-mediated voiding, bladder growth, reduced response to EFS, and supersensitivity to muscarinic agonists. Supersensitivity to muscarinic receptor

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<tr>
<th>Stimulus</th>
<th>Control</th>
<th>Ganglionectomy</th>
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<tr>
<td>Detrusor</td>
<td></td>
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<tr>
<td>K⁺ (124 mM), mN</td>
<td>23 ± 4</td>
<td>22 ± 3</td>
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<tr>
<td>EFS</td>
<td>6.7 ± 0.3</td>
<td>13 ± 1.2†</td>
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<tr>
<td>EF₅₀, Hz</td>
<td>112 ± 4</td>
<td>36 ± 4†</td>
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<tr>
<td>Carbachol</td>
<td>5.9 ± 0.07</td>
<td>6.5 ± 0.04‡</td>
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<tr>
<td>pEC₅₀</td>
<td>111 ± 6</td>
<td>142 ± 8†</td>
</tr>
<tr>
<td>E₅₀, %</td>
<td>20 ± 3</td>
<td>19 ± 2</td>
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<tr>
<td>Urethra</td>
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<tr>
<td>K⁺ (124 mM), mN</td>
<td>3.4 ± 0.3</td>
<td>7.3 ± 0.6‡</td>
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<tr>
<td>EFS</td>
<td>8.5 ± 2.4</td>
<td>ND</td>
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<tr>
<td>EF₅₀, Hz</td>
<td>58 ± 6</td>
<td>10 ± 5‡</td>
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<tr>
<td>Carbachol</td>
<td>5.7 ± 0.09</td>
<td>5.7 ± 0.08</td>
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<tr>
<td>pEC₅₀</td>
<td>84 ± 11</td>
<td>54 ± 6*</td>
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<tr>
<td>E₅₀, %</td>
<td>4.6 ± 0.1</td>
<td>4.4 ± 0.2</td>
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<tr>
<td>SIN-1</td>
<td>70 ± 5</td>
<td>63 ± 5</td>
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Results are expressed as means ± SE from 6 to 8 animals. EFS, electrical field stimulation; SIN-1, 3-morpholinosydnonimine hydrochloride; EF₅₀, frequency producing 50% relaxation or contraction; E₅₀, maximal relaxant or contractile effect; pEC₅₀, negative logarithm of the drug concentration producing 50% relaxation or contraction; ND, not determined. Statistical analysis by Student's unpaired t-test, control vs. ganglionectomy: *P < 0.05, †P < 0.01, ‡P < 0.001.
stimulation in the rat bladder does not appear to be associated with increased density or affinity of muscarinic receptors (26). It was speculated that bladder supersensitivity to muscarinic receptor stimulation was attributable to the general loss of micturition reflex activity. The NOS inhibitor L-NNA did not influence the bladder sensitivity to carbachol. The simultaneous disappearance of inhibitory NOS-containing nerves is thus unlikely to explain the found supersensitivity to carbachol. In contrast to findings on circular strips from the male rat urethra (10), no denervation supersensitivity to carbachol was found after ganglionectomy in our study, using longitudinal strips from the female rat. The influence of the cholinergic activity in the regulation of urethral motility may differ between sexes. Also, in longitudinal rat urethra preparation, the excitatory innervation is less pronounced than in circular preparations (6).

In the present study, we removed NO-producing urethral nerves by cryoganglionectomy and found that nerve-evoked relaxations were markedly reduced in the absence of neuronal NOS. These data complement previous pharmacological investigations in the rat (7, 29) and provide further support for the notion that NOS-ir fibers in the urethra play a pivotal role in the regulation of urethral relaxation. It may be argued that if the nitricergic system is the predominating inhibitory system in the rat urethra, a denervation supersensitivity to NO may be expected after ganglionectomy. Altered reactivity of vascular smooth muscle to nitrovasodilators has been reported after changes in basal NO tone. A supersensitivity to nitrovasodilators occurs after removal of the endothelium (33) or after pharmacological inhibition of NOS in vivo (23). In contrast, a vascular hyporesponsiveness to nitrovasodilators occurs after incubation with endotoxins (34) known to induce NOS. In view of these observations, we speculated that a "subnormal" supply of nerve-derived NO after denervation might increase the sensitivity of the urethral smooth muscle to exogenous NO. However, no change in the relaxant response to the NO donor SIN-1 was found in urethral preparations from ganglionectomized rats compared with control rats. Thus vascular smooth muscle cells that are tonically and continuously regulated by endothelium-derived NO appear to be more sensitive to alterations in NO supply than smooth muscle cells in the neuromuscular junction. A reason for this discrepancy may be that muscle cells in the neuromuscular junction are exposed to nerve-derived NO only during short, intermittent periods.

Smooth muscle relaxant effects of NO in various tissues relate to cGMP synthesis (25). The sensitivity of

![Fig. 5. Functional response of urethral strips to ganglionectomy. A: tracing is shown of the N\(^{\text{\text{-}}\)nitro-L-arginine (L-NNA) sensitivity of electrically induced relaxations. Relaxant responses to electrical field stimulation (B) and the NO donor 3-morpholinosydnonimine hydrochloride (SIN-1) (C) of urethral strips from control (○) and ganglionectomized (●) rats are shown. Results are expressed as mean ± SE (n = 6–8).](http://ajpregu.physiology.org/)

![Fig. 6. Accumulation of cGMP. cGMP accumulation in response to the NO donor SIN-1 (0.1 mM) in urethral preparations obtained from control rats and from rats 3 wk after ganglionectomy is shown. Open bars, unstimulated; hatched bars, stimulated with SIN-1 (0.1 mM). Results are expressed as means ± SE (n = 7). **P < 0.01; ***P < 0.001 compared with unstimulated preparations.](http://ajpregu.physiology.org/)

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the cGMP system may partly be dependent on the degree of previous NO exposure, and extensive NO production after treatment with endotoxin and interleukin-1β causes downregulation of cGMP accumulation in aortic rings (27). Although we were unable to demonstrate supersensitivity to NO when studying the muscle relaxant response, ganglionectomy may lead to supersensitivity to NO at the level of the guanylate cyclase. Indeed, the NO-mediated relaxation of the urethra is known not to be well correlated with the degree of intracellular increase in cGMP (14). In the present study, the cGMP accumulation in response to the NO donor SIN-1 was not different before and after ganglionectomy. It may be speculated that because NO acts via a general intracellular messenger, guanylate cyclase, and not on a specific membrane receptor, NO-mediated responses may be less susceptible to sensitivity changes than classical transmitters. Furthermore, it cannot be excluded that the urethral smooth muscle cells in ganglionectomized rats are exposed to NO from nonneuronal sources in amounts sufficient to provide a basal stimulation of the NO/cGMP pathway.

In conclusion, both morphological and functional studies suggest that the NOS-ir nerves that mediate stimulation of the NO/cGMP pathway are less susceptible to sensitivity changes than classical transmitters. Furthermore, it cannot be excluded that the urethral smooth muscle cells in ganglionectomized rats are exposed to NO from nonneuronal sources in amounts sufficient to provide a basal stimulation of the NO/cGMP pathway.

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