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Le Feber, Joost, and Els van Asselt. Pudendal nerve stimulation induces urethral contraction and relaxation. *Am. J. Physiol.* 277 (*Regulatory Integrative Comp. Physiol.* 46): R1368–R1375, 1999.—In this study we measured urethral pressure changes in response to efferent pudendal nerve stimulation in rats. All other neural pathways to the urethra were transected, and the urethra was continuously perfused. We found fast twitch-like contractions, superimposed on a slow relaxation. The amplitude of the twitches was independent of the stimulation frequency below 26 Hz, whereas the relaxation depended highly on this frequency. The twitches were caused by striated urethral muscles, and the relaxation was caused by smooth muscles. Both were mediated by acetylcholine. We calculated the effective urethral relaxation as the absolute relaxation multiplied by the time fraction between the twitches. Maximum effective relaxation occurred at 8–10 Hz, exactly the frequency of spontaneous oscillations during bladder voiding in rats. Although the oscillatory sphincter contractions in rats during voiding may be needed in other mechanisms for efficient voiding, our data suggest that they may be a side effect of the actual purpose: urethral relaxation.

rat; urethral oscillations; nitric oxide; acetylcholine

DYNAMIC PROPERTIES of the lower urinary tract have been extensively studied over the years, and mechanical models have been developed (12, 42). More recently, attention has been paid to the innervation of the pelvic organs (1, 17, 26, 38), and the activity in the innervating nerves is being incorporated in these models (18, 19, 25, 29, 39). The extended models may enable differentiation between myogenic and neurogenic causes for dysfunction of the lower urinary tract or may improve the clinical use of neurostimulation as a treatment for lower urinary tract dysfunction in patients with spinal cord injury.

Most studies on lower urinary tract innervation have been done in animals, mostly rats. Like humans, rats have three major nerves that innervate the pelvic organs: the (predominantly sympathetic) hypogastric nerve, the (predominantly parasympathetic) pelvic nerve, and the (predominantly somatic) pudendal nerve. The sympathetic system is assumed to be active during urine storage, whereas the parasympathetic system controls bladder voiding. Despite the many similarities, there are some major differences between the human and the rat urinary system; one of the most striking is the oscillatory contractions of the external urethral sphincter (EUS) during voiding in rats (3, 6, 22, 24, 28, 40). The oscillations occur more often and are stronger in male rats than in female rats (22). The rat's normal pattern of bladder-EUS coordination has some superficial similarities to the dyssynergic pattern observed in humans with spinal cord injury. However, unlike the spinalized human, the rat is able to void quite successfully, and knowledge of the differences between the rat and human mechanism of bladder-EUS coordination has therefore been suggested to be of use in evaluating micturition problems in patients with spinal cord injuries (16).

The reason for the sphincter contractions in the rat, which occur at a rate of 6–10 per second (24, 28, 40), has been a subject of discussion for years. A pumplike function (22), a mechanism to sustain a high level of bladder afferents and therefore bladder contraction (16), and a mechanism necessary for territory marking (40) have been suggested. However, none of these explanations seems very satisfactory. Furthermore, it remains unclear why the oscillations occur at the specific frequency range of 6–10 Hz.

In this study, we investigated the relationship between efferent pudendal nerve activity and urethral resistance, as well as some of the major neurotransmitters involved. Urethral oscillations normally occurring during voiding contractions (6–10 Hz) were simulated by pudendal nerve stimulation at 2–70 Hz. We distinguished smooth muscle and striated muscle responses to pudendal stimulation. We will introduce a third explanation for the urethral oscillations, taking into account the frequency of the oscillations during spontaneous bladder voiding.

MATERIALS AND METHODS

Surgery

Sixteen male Wistar rats (444 ± 47 g) were anesthetized with urethan (1.2 g/kg ip) (23) and placed on a heated pad. A dorsal incision was made on the left side of the spine, just below S_3, to access the pudendal nerve. Two trimel-coated silver wires were twisted around the nerve (the undivided efferent and afferent branch) (26) at a distance of ~2 mm. The wound was then closed, and the animal was turned over on its back.

An abdominal midline incision was made to access the urethra. The right side pelvic and hypogastric nerves were transected. The left side pelvic and hypogastric nerves were freed from the underlying tissue and marked with sutures. Warm saline kept the abdomen moist during surgery. The animal was placed in a frame with a heated ground plate, and the abdominal wall was tied to the frame to create a basin. During the measurements, this basin was filled with warm paraffin oil.

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Experimental Setup

A 20-gauge angiocatheter was inserted through the bladder into the urethra. Through this needle, the pressure at the entrance of the urethra (P_{ura}) was measured with the use of a disposable pressure transducer and a Statham SP-1400 blood pressure monitor. Furthermore, the needle was used to pump saline through the urethra with a Harvard Apparatus (Millis, MA) infusion pump at a flow rate (Q) of 0.5 ml/min. The bladder was emptied and tied to prevent leakage from the urethra to the bladder and high bladder pressures.

A bipolar platinum-iridium electrode that consisted of two metal hooks at a distance of ~0.5 mm was lowered into the paraffin oil basin. Either the pelvic or the hypogastric nerve was guided over the electrode, which was used for stimulation. The silver wires around the pudendal nerve provided another stimulation electrode. A Hameg programmable function generator, HM-8130, generated the stimulation signals. The pressure signal was read into a personal computer at sample rates of 200 or 1,000 Hz, with the use of specially developed software driving a PCL 818 analog-to-digital converter. The stimulation signal was also sampled, at a rate of 25 kHz.

Measurement Conditions

Measurements were done under two conditions: *1*) with intact left pelvic and hypogastric nerves and *2*) with bilaterally transected pelvic and hypogastric nerves.

Intact left pelvic and hypogastric nerves. After transection of the right side pelvic and hypogastric nerves, in eight rats we electrically stimulated the left side pudendal nerve (frequency 2–70 Hz, amplitude 0.4–3.5 V, pulse width 50 μs) and measured the change in urethral pressure. Because the pudendal nerve electrode was very close to the sciatic nerve, pudendal stimulation also induced hindleg movement, which restricted the maximum stimulation amplitude. In four rats we measured the effect of hypogastric nerve stimulation on P_{ura}.

Bilaterally transected pelvic and hypogastric nerves. After exclusion of central reflexes by bilateral transection of the pelvic and hypogastric nerves, there were only pudendal efferent pathways to the urethra (see Fig. 1). The pudendal nerve was stimulated again (at maximum amplitude with only minor hindleg movement), and urethral pressure was measured. In seven rats, we determined the frequency dependence of the urethral pressure response to pudendal nerve stimulation by varying the stimulation frequency (f_{stim}) between 2 and 20 Hz (5 rats) or between 1 and 70 Hz (4 rats). In two of these rats both protocols were applied.

In the bilaterally transected rats, to investigate the neurotransmitters involved in urethral responses to the evoked pudendal nerve activity, we measured the effect of intravenous atropine [1 mg/ml, 0.2 ml (n = 1) or 0.4 ml (n = 3)] or N^{ω}-nitro-L-arginine methyl ester [L-NAME; 50 mg/ml (n = 2) or 100 mg/ml (n = 3); 0.2 ml] administration. These series of measurements were done at f_{stim} of 15 Hz. First, three control measurements were done, and then a drug was administered. If both drugs were given in the same animal, we allowed at least 1 h in between administrations.

Measurements

Under both conditions (bilaterally transected pelvic and hypogastric nerves), electrical stimulation of the pudendal nerve had a dual effect: fast twitch-like contractions were superimposed on a slow relaxation (see Fig. 2). The recorded urethral pressure was analyzed in blocks with a length equal to the stimulus period. The blocks were synchronized to the stimulation signal, which was recorded for this purpose. In each block, the maximum (P_{max}) and baseline pressures (P_0') during pudendal stimulation were determined. P_0' was defined as the mean pressure in the block after exclusion of the first twitch and the subsequent pressure undershoot (see Fig. 2). Before stimulation and at the end of the measurements, P_0' equaled the urethral pressure necessary to maintain a flow rate of 0.5 ml/min without nerve stimulation (P_0, see Fig. 2). P_0' described the relaxant response. An exponential function was fitted to P_0' to determine the time constant of relaxation, τ_{relax}.

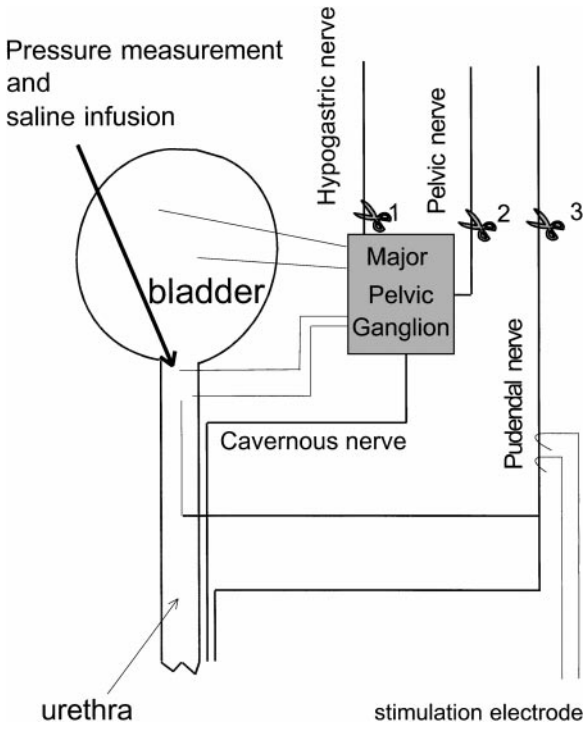

Fig. 1. Schematic drawing of left side innervation of rat lower urinary tract. The pudendal nerve was stimulated by an electrode that consisted of 2 silver wires twisted around the nerve. In our measurements a needle was inserted through the bladder into the urethra for saline infusion and pressure measurement. To determine the effect of only efferent pudendal stimulation, either the hypogastric and pelvic nerves were transected at *1* and *2* or the pudendal nerve was transected at *3*.

The pressure amplitude of the fast contraction (P_{twitch}) was calculated as the mean value of $P_{max} - P_0'$ during stimulation. The pressure amplitude of the slow relaxation (P_{relax}) was calculated as the difference between P_0 and the minimum value of P_0'.

In each rat P_{twitch} was expressed in decibels [= 20·log(amplitude)], and the responses were plotted on a logarithmic frequency axis (2). For each rat the horizontal part of the graph was shifted to 0 (normalization), and all graphs were averaged. A two-parameter model (cutoff frequency and slope) was fitted to the data. To evaluate the effect of the tested drugs, we had to distinguish between the decay with time that was always observed in the urethral pressure responses to pudendal nerve stimulation and the effect caused by the drug itself. We assumed that both drugs did not have any effect on P_{ura} after more than 30 min. The decay with time was estimated from the first three measurements (controls) and the measurements done more than 30 min after drug administration by fitting a straight line to these data. Urethral pressures measured within 30 min after drug administration were corrected using this line.

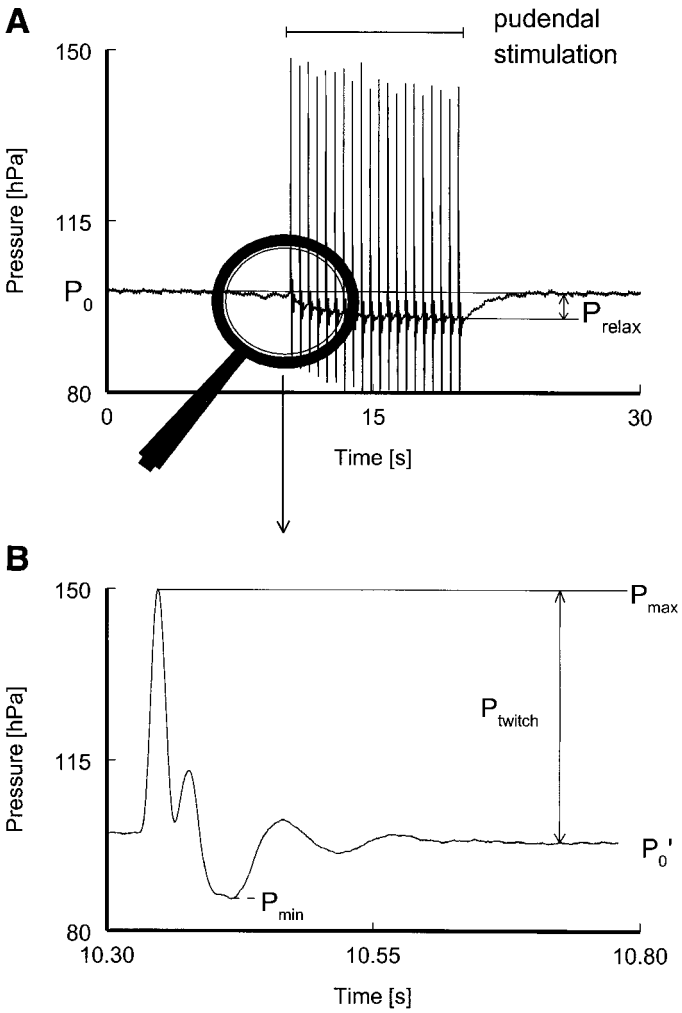

Fig. 2. *A*: electrical stimulation of the pudendal nerve (2 Hz in this example) had a dual effect: fast twitch-like contractions were superimposed on a slow relaxation. P_0, pressure necessary for urethral perfusion (after stimulation, urethral pressure slowly returned to baseline). P_{relax}, pressure amplitude of slow relaxation. *B*: magnification of portion of *A*. The oscillatory pressure waves after the first twitch are probably caused by the moving fluid that is suddenly stopped in a nonrigid chamber. P_{max} and P_{min}, maximum and minimum urethral pressure, respectively, during pudendal stimulation; P_{twitch}, pressure amplitude of fast contraction; P_0', baseline urethral pressure during pudendal stimulation.

Materials

Drugs used were atropine (Pharmachemie BV) and L-NAME (Sigma), both dissolved in saline (0.9% NaCl), and urethan (Sigma), which was dissolved in water.

Experiments were carried out as outlined in the Erasmus University of Rotterdam Guidelines for the Care and Use of Laboratory Animals, which in general follow the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*. All data are presented as means ± SD.

RESULTS

In 16 rats, we measured urethral responses to electrical stimulation of the pudendal nerve. At a perfusion rate of 0.5 ml/min we measured a pressure loss of ~1 hPa in the connecting tubing. Pudendal nerve stimulation had a dual effect: fast twitch-like contractions on each stimulus pulse were superimposed on a slow relaxation (Fig. 2). The amplitude of the fast contractions (P_{twitch}), the amplitude of the relaxation (P_{relax}), and the pressure necessary for urethral perfusion (P_0) were measured. Stimulation of the hypogastric nerve (15–30 V) induced slow urethral contraction without twitches ($\Delta P = 6 \pm 3$ hPa) in three of four rats. In one rat we measured no effect.

To exclude the possible effects of afferent pudendal stimulation, either the stimulated pudendal nerve was transected centrally to the stimulation electrode (Fig. 1, *transection 3*; 1 rat), or the bilateral pelvic and hypogastric nerves were transected (Fig. 1, *transections 1* and *2*; 12 rats). After transection, again twitches superimposed on slow relaxation were observed. Figure 3 shows an example of the development of P_{max}, P_{min}, and P_0' before, during, and after pudendal nerve stimulation. P_0, P_{relax}, and P_{twitch} were determined. In all rats, P_{relax} and P_{twitch} increased with the stimulation amplitude. In two rats, pudendal nerve stimulation hardly had any effect, probably because of insufficient stimula-

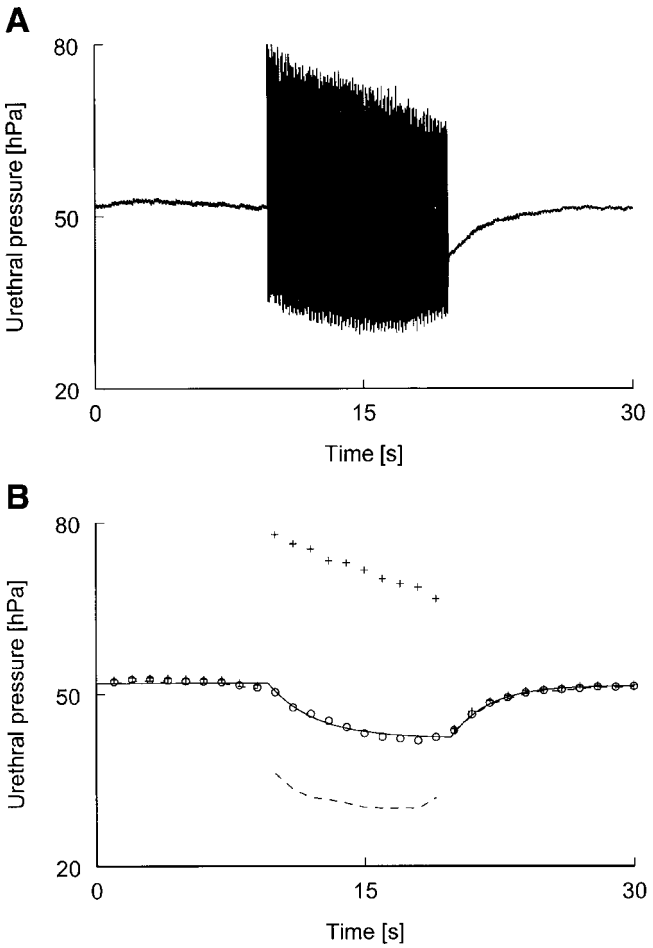

Fig. 3. Measured urethral pressure in response to pudendal nerve stimulation after bilateral transection of the hypogastric and pelvic nerves (*A*). The results were analyzed in blocks that had the same length as the stimulus period. An example of such a block is shown in Fig. 2*B*. *B*: in each block the maximum (+; P_{max}) and minimum pressures (−; P_{min}) were determined. Furthermore, the steady-state pressure in each block (P_0') was calculated (○). Change over time of P_{max}, P_{min}, and P_0' are shown in *B* [stimulation frequency (f_{stim}) = 15 Hz; for clarity, only one +, −, and ○ are shown per second]. An exponential function was fitted to P_0', and a time constant of 3 ± 1 s was found.

tion amplitude. In these rats, the amplitude could not be increased further because of violent hindleg movement; therefore, they were excluded from further analysis. P_0 did not change after pelvic nerve transection ($\Delta P_0 = 0 \pm 10\%$, -0.9 ± 5.4 hPa) and was slightly but significantly reduced after hypogastric nerve transection ($\Delta P_0 = -6 \pm 4\%$, -3.2 ± 2.1 hPa; paired *t*-test: $P = 0.02$). Transection of both nerves had no significant effect on P_0 ($\Delta P_0 = -4 \pm 11\%$, -1.7 ± 5.2 hPa; paired *t*-test: $P > 0.2$). Statistical tests were applied to absolute pressure changes. The average P_0 in all rats was 51 ± 18 hPa. Transection of the hypogastric and pelvic nerves had no influence on P_{twitch}.

In four rats we determined the frequency dependence of P_{twitch}. We stimulated the pudendal nerve at frequencies between 1 and 70 Hz and plotted the frequency responses as in a Bode diagram. The amplitude of the twitches (P_{twitch}) was found to be independent of the stimulation frequency up to 26 Hz (Fig. 4). Beyond this frequency, the amplitude of P_{twitch} decreased because there was no longer complete relaxation between two consecutive twitches. In all rats, the relaxation (P_{relax}) depended highly on the stimulation frequency. In five rats we quantified this frequency dependence (Fig. 5). To avoid interference of the contractions with P_{relax}, these measurements were done up to 20 Hz only. For analysis each measurement was divided into blocks with a length equal to the stimulus period. P_0' described the relaxant response during stimulation (see MATERIALS AND METHODS and Fig. 3). Fitting an exponential function to the relaxation showed a time constant of 3 ± 1 s (Fig. 3). The absolute values of P_{relax} were stimulation amplitude dependent, and the hindleg movement limited the maximum stimulation amplitude. Still, considerable urethral pressure decreases of ~30 hPa could be observed. On average, P_{relax} increased by 70% after bilateral transection of the pelvic and hypogastric nerves. However, this increase was not significant ($P = 0.3$, paired *t*-test).

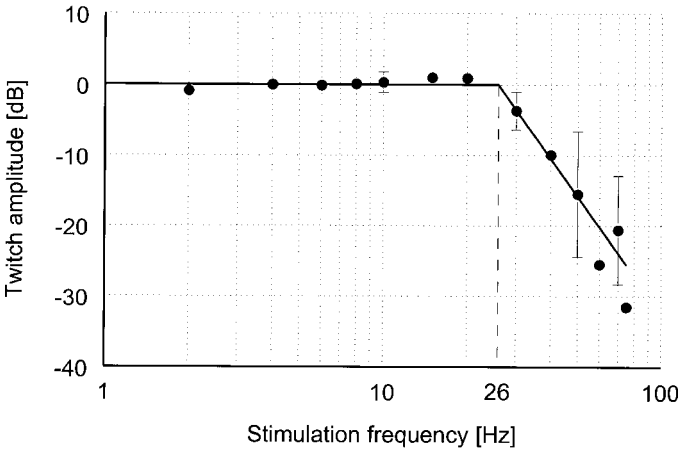

Fig. 4. Amplitude of twitches as a function of stimulation frequency, measured after bilateral transection of the hypogastric and pelvic nerves. As in a Bode diagram, P_{twitch} is expressed in dB [$= 20 \cdot \log$(amplitude)], and the frequency was plotted on a logarithmic scale. In each rat the horizontal part of the graph was shifted to 0 dB for normalization, and all graphs ($n = 4$) were averaged. ●, Mean P_{twitch} (±SD). Fitted line had 2 degrees of freedom: cutoff frequency and slope.

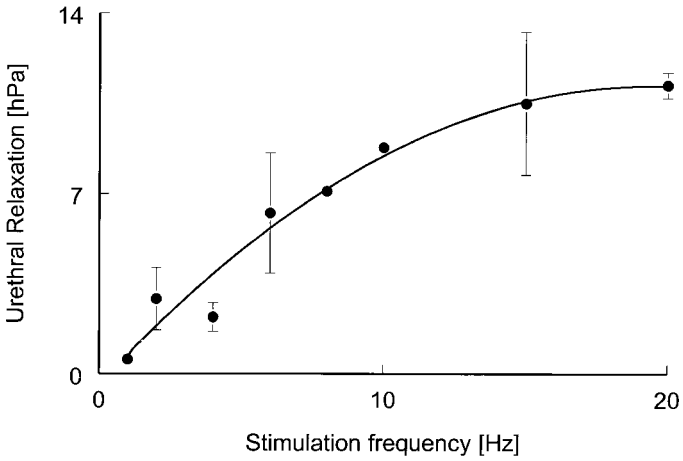

Fig. 5. To assess urethral relaxation as a function of the stimulation frequency, we normalized the relaxation at each frequency in all rats to the relaxation at 8 Hz in that rat. All normalized values were averaged and multiplied by the average relaxation at 8 Hz [P_{relax}(8 Hz) ~ 7.1 ± 5.6 hPa]. Measurements were done after bilateral transection of the pelvic and hypogastric nerves. ●, Measured P_{relax}; SDs refer to differences between rats. Fitted line: $P_{relax} = -0.42 + 1.1 \cdot f_{stim} - 0.031 \cdot f_{stim}^2$.

On average a twitch-like contraction lasted 31 ± 4 ms and had an amplitude of 23 ± 15 hPa. The twitch amplitude (P_{twitch}) depended highly on the stimulation voltage. In contrast to P_{twitch}, the twitch width did not depend on the stimulation amplitude. In three rats we varied the stimulation amplitude and determined the ratio, P_{twitch}/P_{relax}. At very low stimulation intensities only slight twitches were observed; at higher voltages P_{relax} appeared to increase linearly with P_{twitch} (see Fig. 6). We found that $P_{twitch}/P_{relax} \sim 3$.

We investigated the neurotransmitters involved in the pudendus-induced urethral relaxation by administration of the nitric oxide synthase (NOS) inhibitor L-NAME and the muscarinic receptor antagonist atropine.

Over the course of the experiments (56 ± 9 min), the effects of pudendal nerve stimulation (P_{relax} and P_{twitch})

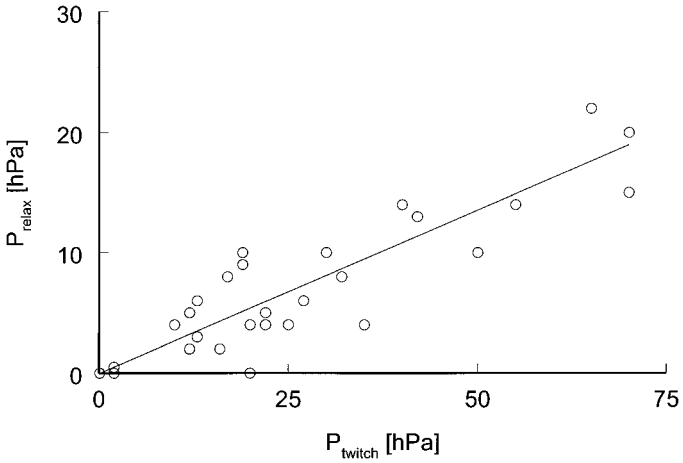

Fig. 6. In 3 rats, amplitude of pudendal nerve stimulation was varied. Amplitude of evoked twitches (P_{twitch}) and slow relaxation (P_{relax}) were measured. Measurements were done after bilateral transection of the pelvic and hypogastric nerves. Data of all rats were pooled (○). Fitted line: $P_{relax} = 0.3 \cdot P_{twitch} - 0.04$.

generally decreased. A straight line was fitted to the first three (control) values of P_{relax} and the values measured more than 30 min after drug administration. The slope of this line was used as an estimate for the decrease of P_{relax} with time and was used to correct for this time effect (mean slope, 0.14 ± 0.10 hPa/min).

Intravenous administration of L-NAME reduced P_{relax} in four of five rats by 30% on average and had no significant influence on P_{twitch} (ΔP_{twitch} was 0.7 ± 3.9 hPa; paired *t*-test: $P = 0.74$). Intravenous administration of atropine reduced P_{relax} in all rats by 40% ($n = 4$) and did not affect P_{twitch}. An example of the effect of both drugs is shown in Fig. 7.

DISCUSSION

In this study we measured urethral pressure (P_{ura}) in response to pudendal nerve stimulation. To allow urethral pressure measurement, the urethra was perfused at a constant flow rate (0.5 ml/min). At this flow rate the urethral pressure was 51 ± 18 hPa, which is in agreement with an earlier study ($P_{ura} \sim 56 \pm 22$ hPa) (19).

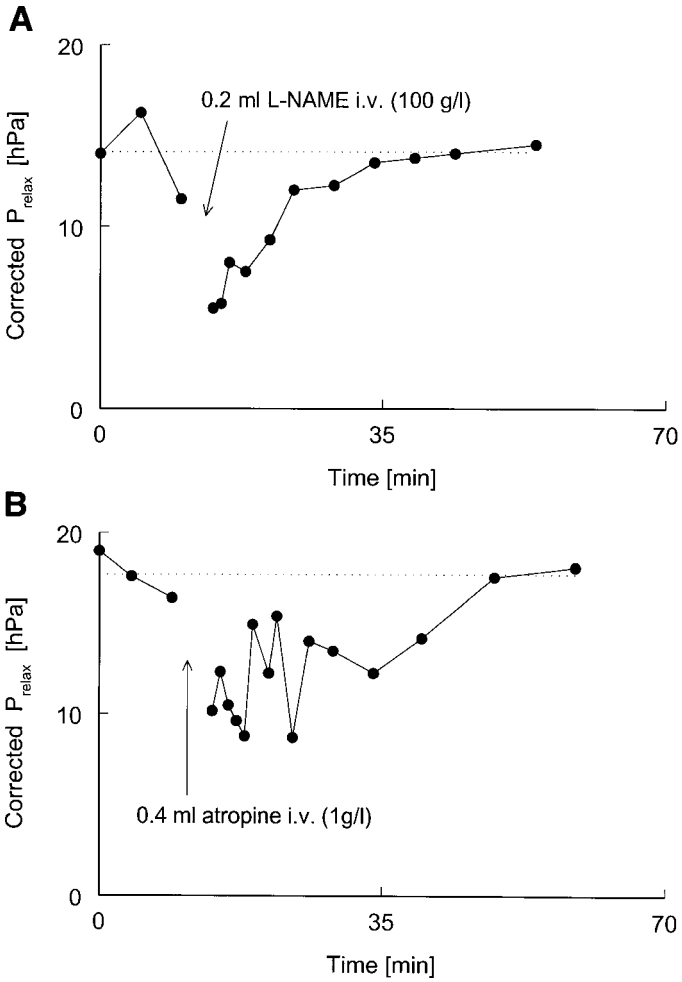

Fig. 7. Effect of N^{ω}-nitro-L-arginine methyl ester (L-NAME; *A*) and atropine (*B*) on P_{relax}. After bilateral transection of the pelvic and hypogastric nerves, 3 control measurements were done in which the pudendal nerve was stimulated with an amplitude high enough to induce clearly detectable urethral relaxation (f_{stim} = 15 Hz). Either drug was then administered, and pudendal nerve stimulation was repeated at the same frequency and amplitude. In both experiments a decrease of P_{relax} with time interfered with the effect of the drugs. It was assumed that the effect of both drugs had vanished after 30 min, which allowed estimation of the time effect from the first 3 (controls) and last 2 measurements. All responses were corrected for this estimated decrease with time (ΔP_{relax} = 0.14 ± 0.10 hPa/min on average).

The rat urinary sphincter has been shown to receive both autonomic and somatic input (43). The somatic input is conducted through the pudendal nerve (26), whereas the autonomic input is generally assumed to be provided via the major pelvic ganglion (7). The pudendal nerve anatomy allows a clear distinction between an efferent and an afferent trunk (26), the afferent trunk contributing ~84% to the total number of fibers (13). Although the pudendal nerve is generally considered somatic, the efferent trunk appears to contain ~25% sympathetic fibers. The other 75% are parasympathetic or somatic motor fibers. Only 12% of the efferent fibers are myelinated and are suggested to be somatic motor fibers (13).

After the initial transection of the right pelvic and hypogastric nerve, there were four possible neural pathways to control urethral pressure: left pelvicus, left hypogastricus, and left or right pudendus. As in the experiments done by Kihara and de Groat (15), electrical stimulation of the hypogastric nerve increased P_{ura}. In their experiments, the central pathway was transected. Therefore, the increased urethral pressure had to be caused by efferent hypogastric nerve stimulation. It is thus probable that the hypogastric nerve provides an excitatory (adrenergic) input to the smooth muscle of the proximal urethra. Fraser et al. (11) showed that electrical stimulation of parasympathetic pathways in the anesthetized rat produces urethral smooth muscle relaxation sensitive to NOS inhibition.

After subsequent transection of the left pelvic and hypogastric nerves only pudendal efferents controlled urethral resistance.

The first (rostral) half of the proximal urethra consists of three layers of smooth muscle, which receive mainly adrenergic innervation. In the innermost layer, cholinergic innervation has been described as well (43). In the second half, a single layer of smooth muscle cells is surrounded by a layer of mixed smooth and striated muscle cells and a layer of striated muscle. Both adrenergic and cholinergic nerves have been found in these layers. Ganglion cells have been described between the striated muscle layer and smooth muscular coat (43).

In our study, after bilateral transection of the pelvic and hypogastric nerves, electrical stimulation of the pudendus induced fast twitch-like contractions upon each stimulus pulse superimposed on a slow relaxation. At low stimulation frequencies (≤20 Hz) the pressure reached a stable level P_0' before the next twitch contraction. The fast pressure undershoot (P_{min}, Fig. 2). was considered to be an artefact caused by the sudden stop of moving fluid in a flexible chamber. This point of view is supported by the fact that P_0' at the end of stimulation (and not P_{min}) equals the urethral pressure immediately after stimulation (Fig. 2). Thus P_0' described the relaxant response. We determined the time constant of this relaxation (τ_{relax} = 3 ± 1 s). Other studies have

shown that bladder smooth muscle contracts with a time constant of ~3.1 ± 1.1 s (41) or ~3.4 s (18). We therefore concluded that the slow decrease in urethral pressure was caused by smooth muscle relaxation.

P_{twitch} was not frequency dependent up to 26 Hz, which corresponds to a time constant of ~40 ms, 80 times faster than the smooth muscle time constant. Skeletal muscles have been shown to follow frequencies up to 2 Hz (32). However, this latter study concerned load-moving skeletal muscle, which decreased the maximum frequency. Isometric twitch contraction in the soleus muscle of mice was shown to have a contraction time of 21 ms (21), even less than the 31 ms measured in our study. We concluded that the twitches in our study were caused by striated muscle contractions. Because all other neural pathways to the urethra had been transected, both the smooth muscle relaxation and the striated muscle contraction must have been induced by efferent pudendal nerve firing [caused either directly by efferent stimulation or indirectly via the pudendo-pudendal reflex (27)].

Transection of the left hypogastric and pelvic nerve had no influence on the tonic urethral pressure. Similar results were obtained by Kakizaki et al. (14) after ganglionic blockade. Tonic urethral resistance is probably not neurally regulated by the pelvic or hypogastric nerve.

In our study, the effect of hypogastric and pelvic nerve transection on P_{relax} was probably underestimated because there was always a spontaneous decay with time, which was not compensated for. Thus a possible increase is masked by a decrease with time. Yet, we still measured a P_{relax} that tended to increase after hypogastric and pelvic nerve transection, although not significantly.

If P_{relax} increased after bilateral transection of the hypogastric and pelvic nerves, what may have caused this increase?

Stimulation of pudendal afferent fibers (originating in sex organs) has been shown to have an inhibitory effect on the bladder (9, 20). Inhibition of the micturition reflex may include an excitatory reflex (sympathetic adrenergic or parasympathetic cholinergic) to the urethral smooth muscle. The most probable pathway would be the sympathetic system because this is generally accepted to be active during the urine storage phase (8). Furthermore, far more adrenergic nerves than cholinergic nerves were found in the proximal urethra (43). Stimulation of the hypogastric nerve induced increased urethral resistance (15). Thus, before transection of the pelvic and hypogastric nerves, the relaxant effect of pudendal nerve stimulation may have been masked by a central reflex via the hypogastric nerve that induced contraction of urethral smooth muscle.

In in vitro experiments done by others, electrical stimulation of urethral tissue also induced both relaxation and contraction of smooth muscle (14, 31, 34). The contraction was no longer observed after supramaximal α-adrenergic stimulation (31), which indicates that the smooth muscle contraction upon electrical stimulation was mediated by adrenergic pathways and therefore sympathetic.

Nonadrenergic, noncholinergic-mediated relaxation of urethral smooth muscle has been described (3, 31), whereas norepinephrine has been found to be the major excitatory transmitter (5). Parlani et al. (31) reported atropine-sensitive relaxation of urethral muscle strips on acetylcholine stimulation.

In our study, atropine reduced the smooth muscle relaxation by ~40% and had no influence on the striated muscle contractions. Thus the smooth muscle relaxation was at least partially mediated by cholinergic activation of muscarinic receptors. Acetylcholine had an excitatory effect on the urethral striated musculature, but this effect was not blocked by atropine because the contraction is mediated by nicotinic receptors (14, 22).

The smooth muscle relaxation in our study was also reduced (by ~30 %) after L-NAME administration. We therefore concluded that the relaxation was also (partially) mediated by nitric oxide (NO). Persson et al. (33) described a dense innervation of NOS-containing nerves in the pig urethra and suggested a possible corelease of NO and acetylcholine because a considerable overlapping of NOS immunoreactivity and acetylcholinesterase (AChE) staining was found. In a more recent paper, they (34) suggested that the function of cholinergic nerves in the rat urethra is mainly to release inhibitory NO and to evoke urethral relaxation.

NO might also be released on cholinergic stimulation from endothelium cells (30). In the urethra, this may seem unlikely because cholinergic fibers were mainly found in the outer layers (43), whereas endothelium cells are located on the inner side. However, Persson et al. (35) found AChE-positive terminals that ran in close association with the urothelium, with branches arborizing into it. NOS-immunoreactive nerves were also observed in the suburothelial stroma (35).

NO may also be released from ganglion cells that are found in the urethra (43). In the major pelvic ganglion coexistence of cholinergic and nitrergic nerves has been demonstrated (34). This mechanism may play a role, but if so, it is not the single pathway because it does not explain the reduced relaxation after atropine administration that we found. Atropine has no effect on the nicotinic receptors in ganglion cells. Possibly, acetylcholine (also) has a relaxant effect on urethral smooth muscle, which is not mediated by NO. This is emphasized by our finding that a muscarinic receptor blocking agent induces stronger reduction of the urethral relaxation than an NO synthase inhibitor. Moreover, it was shown that L-NAME may block muscarinic receptors, and thus even the urethral relaxation that was reduced after L-NAME administration may have been mediated by acetylcholine instead of NO (4).

However, either of these mechanisms implies an important role of acetylcholine in pudendal nerve-controlled urethral relaxation.

If acetylcholine has an inhibitory effect on urethral smooth muscle and an excitatory effect on urethral striated muscle, and these types of muscle cells intermingle rather freely in the rat urethra (43), the ques-

tion arises: what is the predominant effect of acetylcholine release?

Our results show that the urethral relaxation is highly dependent on the frequency of pudendal nerve stimulation. The amplitude and the duration (~31 ms) of the striated muscle contractions, however, are independent of this stimulation frequency, up to 26 Hz. On average the amplitude of the contractions was three times larger than the amplitude of the relaxation. We concluded that during contraction of the striated muscles, the urethra was always closed. The relaxation was therefore only effective in the fraction of time when the striated muscles did not contract.

We calculated the effective urethral relaxation (EUR) as the urethral smooth muscle relaxation multiplied by this fraction ($1 - 0.031 \cdot f_{stim}$). The result is shown in Fig. 8. From other studies it is known that rats normally void at bladder pressures of ~40 hPa (40). P_{ura} necessary for perfusion at a flow rate as low as 0.5 ml/min generally exceeded this pressure (~51 ± 18 hPa), and thus urethral relaxation is needed to enable bladder emptying. Figure 8 shows that the EUR is maximum at frequencies between ~7 and ~15 Hz, the frequency range of natural urethral oscillations during voiding. In our measurements the absolute value of the maximum EUR was ~7 hPa, but this depended highly on the stimulation amplitude and may be much larger if all nerves fire at maximum frequency.

Thus the dominant effect of acetylcholine on urethral resistance depends on the frequency of release. An oscillatory release at 7–15 Hz induces maximum EUR. The frequency of sphincter oscillations during evoked voiding contractions in anesthetized animals is in the lower half of this frequency range with maximum EUR. This may be so because the lower frequencies require less metabolic activity, but it has also been described that the use of anesthetics lowers the oscillation frequency (28). Thus the oscillations during voiding occur at the frequency that maximizes urethral relaxation. Moreover, the oscillations are observed more frequently in male rats than in female rats. This may be explained by their higher need for urethral relaxation during voiding; male rat urethras are approximately four times longer than female rat urethras.

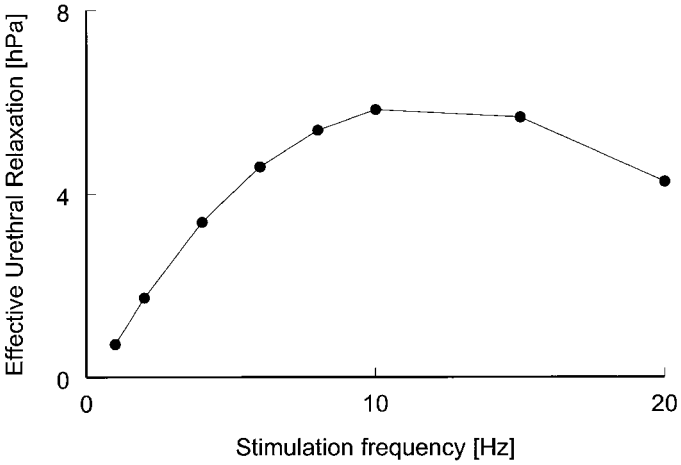

Fig. 8. Effective urethral relaxation was calculated by multiplying the urethral relaxation (Fig. 5, fitted line) with the time fraction in which the urethra was not closed because of a twitch contraction. Mean duration of twitches was 31 ms, and therefore this time fraction was calculated as $1 - (0.031 \cdot f_{stim})$.

It is generally assumed that the striated urethral muscles are controlled by somatic motor fibers that are presumably myelinated (13) and that the smooth muscles receive unmyelinated parasympathetic innervation. Because myelinated nerve fibers show a lower stimulation threshold (37), it might be expected that only twitches at low stimulation amplitude would be found. From our measurements we could not determine such a threshold amplitude (Fig. 6). However, other studies showed that the stimulation threshold for unmyelinated fibers is one order of magnitude higher than the threshold for myelinated fibers (36). Thus we probably stimulated only myelinated fibers, and only the number of recruited myelinated fibers increased with increasing stimulation amplitude. Both the twitch amplitude and the relaxant amplitude increased. Inasmuch as it is unlikely that all muscle cells in the urethral wall receive direct innervation (10), there may be myelinated axons that have influence on both striated and smooth muscle cells.

The major argument for the theory that the oscillations are needed to pump the urine through the urethra is the observation that blocking the oscillations by either pudendal nerve transection (6) or administration of *d*-tubocurarine (22) increases the residual urine in the bladder or completely abolishes effective voiding. However, pudendal nerve transection obviously abolishes both the twitches and the slow relaxation, whereas *d*-tubocurarine also blocks ganglionic transmission, and ganglionic blockade has been shown to abolish the oscillatory reflex (22). In both situations there is no acetylcholine release and thus no effective urethral relaxation. Kakizaki et al. (14) showed that the striated muscle contractions could be blocked by α-bungarotoxin while there still was smooth muscle relaxation (although masked by adrenergic smooth muscle contraction).

In conclusion, urethral oscillation during bladder emptying in rats may have a function in territory marking, or it may pump urine through the urethra. In this study, we showed that the pudendal nerve induces urethral relaxation, which is, at least partially, mediated by acetylcholine. Acetylcholine also induces contraction of striated urethral muscle, and the overall effect on urethral resistance depended on the frequency of acetylcholine release. We therefore introduced the (frequency dependent) effective urethral relaxation that was maximum at the frequency of the spontaneous oscillations. Thus, although the oscillatory sphincter contractions in rats during voiding may be needed in other mechanisms for efficient voiding, they may very well be a side effect of the actual purpose of pudendal acetylcholine release: urethral relaxation.

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REFERENCES

1. **Baljet, B., and J. Drukker.** The extrinsic innervation of the pelvic organs in the female rat. *Acta Anat. (Basel)* 107: 241–267, 1980.
2. **Barrata, R., and M. Solomonow.** The dynamic response model of nine different skeletal muscles. *IEEE Trans. Biomed. Eng.* 37: 243–251, 1990.
3. **Bennett, B. C., M. N. Kruse, J. R. Roppolo, H. D. Flood, M. Fraser, and W. C. de Groat.** Neural control of urethral outlet activity in vivo: role of nitric oxide. *J. Urol.* 153: 2004–2009, 1995.
4. **Buxton, I. L., D. J. Cheek, D. Eckman, D. P. Westfall, K. M. Sanders, and K. D. Keef.** N^G-nitro-L-arginine methyl ester and other alkyl esters of arginine are muscarinic receptor antagonists. *Circ. Res.* 72: 387–395, 1993.
5. **Chen, H. I., and A. F. Brading.** The contribution of α-adrenoceptors to neurally mediated contractions of the rabbit urethral smooth muscle. *Br. J. Pharmacol.* 106: 302–306, 1992.
6. **Conte, B., C. A. Maggi, M. Parlani, G. Lopez, S. Manzini, and A. Giachetti.** Simultaneous recording of vesical and urethral pressure in urethane-anesthetized rats: effect of neuromuscular blocking agents on the activity of the external urethral sphincter. *J. Pharmacol. Methods* 26: 161–171, 1991.
7. **Creed, K. E.** Innervation of the smooth muscle of the lower urinary tract. *J. Smooth Muscle Res.* 31: 1–4, 1995.
8. **De Groat, W. C.** Anatomy of the central neural pathways controlling the lower urinary tract. *Eur. Urol.* 34: 2–5, 1998.
9. **De Groat, W. C., and W. D. Steers.** Neuroanatomy and neurophysiology of penile erection. In: *Contemporary Management of Impotence and Infertility*, edited by E. A. Tanagho, T. F. Lue, and R. D. McClure. Baltimore, MD: Williams and Wilkins, 1988, p. 3–27.
10. **Elbadawi, A.** Ultrastructure of vesicourethral innervation. I. Neuroeffector and cell junctions in the male internal sphincter. *J. Urol.* 128: 180–188, 1982.
11. **Fraser, M. O., H. D. Flood, and W. C. de Groat.** Urethral smooth muscle relaxation is mediated by nitric oxide (NO) released from parasympathetic postganglionic neurons (Abstract). *J. Urol.* 153: 461A, 1995.
12. **Griffiths, D. J.** Hydrodynamics and mechanics of the bladder and urethra. In: *Urodynamics: Principles, Practice, and Application* (2nd ed.), edited by A. R. Mundy, T. P. Stephenson, and A. J. Wein. New York: Churchill Livingstone, 1994, p. 71–81.
13. **Hulsebosch, C. E., and R. E. Coggeshall.** An analysis of the axon population in the nerves to the pelvic viscera in the rat. *J. Comp. Neurol.* 211: 1–10, 1982.
14. **Kakizaki, H., M. O. Fraser, and W. C. de Groat.** Reflex pathways controlling urethral striated and smooth muscle function in the male rat. *Am. J. Physiol.* 272 (*Regulatory Integrative Comp. Physiol.* 41): R1647–R1656, 1997.
15. **Kihara, K., and W. C. de Groat.** Sympathetic efferent pathways projecting to the bladder neck and proximal urethra in the rat. *J. Auton. Nerv. Syst.* 62: 134–142, 1997.
16. **Kruse, M. N., H. Noto, J. R. Roppolo, and W. C. de Groat.** Pontine control of the urinary bladder and external urethral sphincter in the rat. *Brain Res.* 532: 182–190, 1990.
17. **Langworthy, O. R.** Innervation of the pelvic organs of the rat. *Investig. Urol. (Berl.)* 2: 491–511, 1965.
18. **Le Feber, J., E. van Asselt, and R. van Mastrigt.** Neurophysiological modeling of voiding in rats: bladder pressure and postganglionic bladder nerve activity. *Am. J. Physiol.* 272 (*Regulatory Integrative Comp. Physiol.* 41): R413–R421, 1997.
19. **Le Feber, J., E. van Asselt, and R. van Mastrigt.** Neurophysiological modeling of voiding in rats: urethral nerve response to urethral pressure and flow. *Am. J. Physiol.* 274 (*Regulatory Integrative Comp. Physiol.* 43): R1473–R1481, 1998.
20. **Lindström, S., M. Fall, C.-A. Carlsson, and B.-E. Erlandson.** The neurophysiological basis of bladder inhibition in response to intravaginal electrical stimulation. *J. Urol.* 129: 405–410, 1983.
21. **Luff, A. R.** Dynamic properties of the inferior rectus, extensor digitorum longus, diaphragm and soleus muscles of the mouse. *J. Physiol. (Lond.)* 313: 161–171, 1981.
22. **Maggi, C. A., S. Giuliani, P. Santicioli, and A. Meli.** Analysis of factors involved in determining urinary bladder voiding cycle in urethan-anesthetized rats. *Am. J. Physiol.* 251 (*Regulatory Integrative Comp. Physiol.* 20): R250–R257, 1986.
23. **Maggi, C. A., and A. Meli.** Suitability of urethane anesthesia for physiopharmacological investigations. III. Other systems and conclusions. *Experientia* 15: 531–537, 1986.
24. **Maggi, C. A., P. Santicioli, and A. Meli.** The nonstop transvesical cystometrogram in urethane-anesthetized rats: a simple procedure for quantitative studies on the various phases of urinary bladder voiding cycle. *J. Pharmacol. Methods* 15: 157–167, 1986.
25. **Mallory, B., W. D. Steers, and W. C. de Groat.** Electrophysiological study of micturition reflexes in rats. *Am. J. Physiol.* 257 (*Regulatory Integrative Comp. Physiol.* 26): R410–R421, 1989.
26. **McKenna, K. E., and I. Nadelhaft.** The organization of the pudendal nerve in the male and female rat. *J. Comp. Neurol.* 248: 532–549, 1986.
27. **McKenna, K. E., and I. Nadelhaft.** The pudendo-pudendal reflex in male and female rats. *J. Auton. Nerv. Syst.* 27: 67–77, 1989.
28. **Mersdorf, A., R. A. Schmidt, and E. A. Tanagho.** Urodynamic evaluation and electrical and pharmacologic neurostimulation. The rat model. *Urol. Res.* 21: 199–209, 1993.
29. **Noto, H., J. R. Roppolo, W. D. Steers, and W. C. de Groat.** Electrophysiological analysis of the ascending and descending components of the micturition reflex pathway in the rat. *Brain Res.* 549: 95–105, 1991.
30. **Palmer, R. M. J., A. G. Ferrige, and S. Moncada.** Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. *Nature* 327: 524–526, 1987.
31. **Parlani, M., B. Conte, and S. Manzini.** Nonadrenergic, noncholinergic inhibitory control of the rat external urethral sphincter: involvement of nitric oxide. *J. Pharmacol. Exp. Ther.* 265: 713–719, 1993.
32. **Partridge, L. D.** Signal-handling characteristics of load-moving skeletal muscle. *Am. J. Physiol.* 210: 1178–1191, 1966.
33. **Persson, K., P. Alm, K. Johansson, B. Larsson, and K.-E. Andersson.** Coexistence of nitrergic, peptidergic and acetylcholine esterase-positive nerves in the pig lower urinary tract. *J. Auton. Nerv. Syst.* 52: 225–236, 1995.
34. **Persson, K., P. Alm, B. Uvelius, and K.-E. 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.
35. **Persson, K., K. Johansson, P. Alm, B. Larsson, and K. E. Andersson.** Morphological and functional evidence against a sensory and sympathetic origin of nitric oxide synthase-containing nerves in the rat lower urinary tract. *Neuroscience* 77: 271–281, 1997.
36. **Petruska, J. C., C. H. Hubscher, and R. D. Johnson.** Anodally focused polarization of peripheral nerve allows discrimination of myelinated and unmyelinated fiber input to brainstem nuclei. *Exp. Brain Res.* 121: 379–390, 1998.
37. **Rattay, F.** The basic mechanism for the electrical stimulation of the nervous system. *Neuroscience* 89: 335–346, 1999.
38. **Steers, W. D.** Rat: overview and innervation. *Neurourol. Urodyn.* 13: 97–118, 1994.
39. **Steers, W. D., and W. C. de Groat.** Effect of bladder outlet obstruction on micturition reflex pathways in the rat. *J. Urol.* 140: 864–871, 1988.
40. **Van Asselt, E., J. Groen, and R. van Mastrigt.** A comparative study of voiding in rat and guinea pig: simultaneous measurement of flow rate and pressure. *Am. J. Physiol.* 269 (*Regulatory Integrative Comp. Physiol.* 38): R98–R103, 1995.
41. **Van Koeveringe, G. A., and R. van Mastrigt.** Excitatory pathways in smooth muscle investigated by phase-plot analysis of isometric force development. *Am. J. Physiol.* 261 (*Regulatory Integrative Comp. Physiol.* 30): R138–R144, 1991.
42. **Van Mastrigt, R.** Application of animal and physical models to human cystometry. In: *Micturition*, edited by J. O. Drife, P. Hilton, and S. L. Stanton. London: Springer Verlag, 1990, p. 59–79.
43. **Watanabe, H., and T. Y. Yamamoto.** Autonomic innervation of the muscles in the wall of the bladder and proximal urethra of male rats. *J. Anat.* 128: 873–886, 1979.