Pudendal nerve stimulation induces urethral contraction and relaxation

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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.

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 parasym pathetic) pelvic nerve, and the (predominantly somatic) pudendal nerve. The sympathetic system is assumed to be active 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 S3, 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 approximately 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.
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 efferent pathways to the urethra (see Fig. 1). The pudendal nerve was stimulated again (at maximum amplitude with 10.22±0.33.5 on July 5, 2017 http://ajpregu.physiology.org/ Downloaded from
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_{\text{twitch}}), the amplitude of the relaxation (P_{\text{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 (ΔP = 6 ± 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_{\text{max}}, P_{\text{min}}, and P_0 before, during, and after pudendal nerve stimulation. P_0, P_{\text{relax}}, and P_{\text{twitch}} were determined. In all rats, P_{\text{relax}} and P_{\text{twitch}} increased with the stimulation amplitude. In two rats, pudendal nerve stimulation hardly had any effect, probably because of insufficient stimula-

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_{\text{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_{\text{max}} and P_{\text{min}}, maximum and minimum urethral pressure, respectively, during pudendal stimulation; P_{\text{twitch}}, pressure amplitude of fast contraction; P_0, baseline urethral pressure during pudendal stimulation.

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. 2B. B: in each block the maximum (+; P_{\text{max}}) and minimum pressures (−; P_{\text{min}}) were determined. Furthermore, the steady-state pressure in each block (P_{\circ}) was calculated (○). Change over time of P_{\text{max}}, P_{\text{min}}, and P_0 are shown in B (stimulation frequency (f_{\text{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 (\( \Delta P_0 = 0 \pm 10\% \), \( -0.9 \pm 5.4 \text{ hPa} \)) and was slightly but significantly reduced after hypogastric nerve transection (\( \Delta P_0 = -6 \pm 4\% \), \( -3.2 \pm 2.1 \text{ hPa} \); paired t-test: \( P = 0.02 \)). Transection of both nerves had no significant effect on \( P_0 \) (\( \Delta P_0 = -4 \pm 11\% \), \( -1.7 \pm 5.2 \text{ hPa} \); paired t-test: \( P > 0.2 \)). Statistical tests were applied to absolute pressure changes. The average \( P_0 \) in all rats was \( 51 \pm 18 \text{ hPa} \). Transection of the hypogastric and pelvic nerves had no influence on \( P_{\text{twitch}} \).

In four rats we determined the frequency dependence of \( P_{\text{twitch}} \). 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 (\( P_{\text{twitch}} \)) was found to be independent of the stimulation frequency up to 26 Hz (Fig. 4). Beyond this frequency, the amplitude of \( P_{\text{twitch}} \) decreased because there was no longer complete relaxation between two consecutive twitches. In all rats, the relaxation (\( P_{\text{relax}} \)) depended highly on the stimulation frequency. In five rats we quantified this frequency dependence (Fig. 5). To avoid interference of the contractions with \( P_{\text{relax}} \), 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. \( P_0 \) describes the relaxant response during stimulation (see MATERIALS AND METHODS and Fig. 3). Fitting an exponential function to the relaxation showed a time constant of \( 3 \pm 1 \text{ s} \) (Fig. 3). The absolute values of \( P_{\text{relax}} \) were stimulation amplitude dependent, and the hindleg movement limited the maximum stimulation amplitude. Still, considerable urethral pressure decreases of \( -30 \text{ hPa} \) could be observed. On average, \( P_{\text{relax}} \) 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](http://ajpregu.physiology.org/)

**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, \( P_{\text{twitch}} \) is expressed in dB [\( = 20 \log(\text{amplitude}) \)], 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. \( ullet \), Mean \( P_{\text{twitch}} \) (\( \pm \text{SD} \)). Fitted line had 2 degrees of freedom: cutoff frequency and slope.

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

We investigated the neurotransmitters involved in the pudendal-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 \( (56 \pm 9 \text{ min}) \), the effects of pudendal nerve stimulation (\( P_{\text{relax}} \) and \( P_{\text{twitch}} \))...
generally decreased. A straight line was fitted to the first three (control) values of $P_{\text{relax}}$ 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 $P_{\text{relax}}$ 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 $P_{\text{relax}}$ in four of five rats by 30% on average and had no significant influence on $P_{\text{twitch}}$ ($\Delta P_{\text{twitch}}$ was 0.7 ± 3.9 hPa; paired t-test: $P = 0.74$). Intravenous administration of atropine reduced $P_{\text{relax}}$ in all rats by 40% ($n = 4$) and did not affect $P_{\text{twitch}}$. An example of the effect of both drugs is shown in Fig. 7.

**DISCUSSION**

In this study we measured urethral pressure ($P_{\text{ura}}$) 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 ($P_{\text{ura}}$ = 56 ± 22 hPa) (19).

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 $P_{\text{ura}}$.

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 ($P_{\text{min}}$, 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 $P_{\text{min}}$) equals the urethral pressure immediately after stimulation (Fig. 2). Thus $P_0$ described the relaxant response. We determined the time constant of this relaxation ($t_{\text{relax}} = 3 ± 1$ s). Other studies have
shown that bladder smooth muscle contracts with a time constant of $\sim 3.1 \pm 1.1$ s (41) or $\sim 3.4$ s (18). We therefore concluded that the slow decrease in urethral pressure was caused by smooth muscle relaxation.

$P_{\text{twitch}}$ was not frequency dependent up to 26 Hz, which corresponds to a time constant of $\sim 40$ 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 effective pudendal nerve firing (caused either directly by efferent stimulation or indirectly via the pudendo-pudendal reflex (27)).

Transsection 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 $P_{\text{relax}}$ 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 $P_{\text{relax}}$ that tended to increase after hypogastric and pelvic nerve transection, although not significantly.

If $P_{\text{relax}}$ 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 $\alpha$-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 $\sim 40\%$ 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 $\sim 30\%$) 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 nitricergic 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-
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 contraction 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·fstim). The result is shown in Fig. 8. From other studies it is known that rats normally void at bladder pressures of ~40 hPa (40), Pura 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 sphencter 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.

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. It is much 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.
REFERENCES