Afferent Fibers of the Hypogastric Nerves Are Involved in the Facilitating Effects of Chemical Bladder Irritation in Rats

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Mitsui, Takahiko, Hidehiro Kakizaki, Shinobu Matsuura, Kaname Ameda, Mitsuhiro Yoshioka, and Tomohiko Koyanagi. Afferent fibers of the hypogastric nerves are involved in the facilitating effects of chemical bladder irritation in rats. J Neurophysiol 86: 2276–2284, 2001. To evaluate the role of bladder afferent fibers in the hypogastric nerves (HGN) in modulation of the micturition reflex induced by chemical bladder irritation, voiding behavior, continuous cystometry, and spinal c-fos expression following intravesical acetic acid instillation were investigated in rats with or without HGN transection. Voiding behavior and continuous cystometry were examined in unanesthetized conscious rats. Following chemical bladder irritation, a significant increase in urinary frequency associated with a marked decrease in the voided volume per micturition, was noted in control rats with the intact HGN, but not in HGN-transected rats. Continuous infusion of acetic acid in control rats elicited irritative bladder responses characterized by a marked decrease in the intercontraction interval and a marked increase in maximal vesical pressure, both of which were absent in capsaicin-desensitized rats. HGN transection prevented the decrease in the intercontraction interval but not an increase in maximal vesical pressure following chemical bladder irritation. Compared with saline infusion, acetic acid infusion caused a significant increase in c-fos expression at L1 and L6 of the spinal cord, and HGN transection significantly reduced c-fos expression in the dorsal horn of the spinal cord at L1 but not at L6. These results suggest that capsaicin-sensitive bladder afferent fibers in the HGN, which travel through the rostral lumbar spinal cord, have a role in urinary frequency caused by chemical bladder irritation.

INTRODUCTION

Autonomic nerves play a critical role in the control of lower urinary tract function (Kakizaki et al. 1997). Reflex contractions of the urinary bladder that occur in response to bladder distension are mediated via parasympathetic efferent fibers in the pelvic nerves (PLN). The PLN of the rat also include Aδ- and C-fiber bladder afferents responding to bladder distension (Sengupta and Gebhart 1994; Shea et al. 2000; Su et al. 1997). In the rat, parasympathetic preganglionic neurons originate in the intermediolateral gray matter in the lumbosacral spinal cord (L6–S2), while most afferent neurons that send axons to the bladder via the PLN are located in the dorsal root ganglia at the same level (de Groat 1986; Keast and de Groat 1992; Sharkey et al. 1983; Steers et al. 1991). Thus the L6–S1 spinal cord constitutes the spinal micturition center in the rat. On the other hand, the hypogastric nerves (HGN) convey sympathetic efferent fibers to provide inhibitory input to the bladder and excitatory input to the bladder neck (Hancock and Peveto 1979). In the rat, preganglionic sympathetic neurons that send fibers to the lower urinary tract originate in the rostral lumbar spinal cord, while afferent neurons that send axons to the lower urinary tract via the HGN are located in the dorsal root ganglia at the same level (de Groat and Saum 1972; Keast and de Groat 1992; Steers et al. 1991; Tabatabai et al. 1986).

A previous histological study showed that most afferent fibers innervating the musculature of the bladder body pass through the PLN, whereas the majority of afferent endings in the submucosal layer of the bladder are innervated by the HGN (Uemura et al. 1975), suggesting that afferent fibers in the PLN and HGN have different roles. It has been speculated that afferent fibers in the HGN are involved in the nociception or signaling inflammation of the pelvic viscera (Berkeley et al. 1993; Moss et al. 1997). However, the precise role of afferent fibers in the HGN in the control of bladder function has not been fully evaluated.

Chemical irritation has been used to produce acute inflammation of the bladder. Intravesical instillation of acetic acid as a chemical irritant of the bladder is known to facilitate micturition reflex in the rat. Continuous intravesical instillation of acetic acid is accompanied by an increase in the frequency of bladder contractions as well as an increase in the frequency of external urethral sphincter bursting during micturition (Kakizaki and de Groat 1996). In addition, spinal expression of an immediate early gene, c-fos, increases dramatically following continuous intravesical infusion of acetic acid compared with nonnoxious stimulation of the bladder (saline infusion) (Birder and de Groat 1992; Kakizaki et al. 1996; Menetry et al. 1989).

The present study was carried out to evaluate the role of bladder afferent fibers in the HGN in response to bladder irritation with acetic acid. Changes in bladder function were detected by monitoring voiding behavior and by cystometry while presumed nociceptive neural activity was examined by an immunohistochemical study on spinal c-fos expression.

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the 30th annual meeting of International Continence Society (Mitsui et al. 2000).

METHODS

Adult female Wistar rats weighing between 160 and 220 g were used in the present study. All experimental procedures were performed in accordance with the Guidelines for the Care and Use of Laboratory Animals by the Animal Research Committee of Hokkaido University School of Medicine.

HGN transection

After anesthesia with intraperitoneal injection (ip) of pentobarbital sodium (40 mg/kg), bilateral HGN were exposed through a midline abdominal incision and sectioned completely at the level of the inferior mesenteric ganglion. Experiments were performed 1 wk after HGN transection.

Voiding behavior

Rats were placed in metabolic cages (Nalgene Metabolic Cage, Nalg, New York) to examine voiding behavior. The voided urine was collected on an electronic scale (Libror EB-340HW, Shimadzu, Kyoto, Japan) connected to a microcomputer for recording micturition frequency and volume (Chancellor et al. 1994; Ozawa et al. 1999). Data were recorded and stored using data acquisition software (Balancewin, Shimadzu, Kyoto, Japan). Micturition parameters evaluated were as follows: 1) total urine output for 3 h, 2) number of micturitions, and 3) voided volume per micturition.

Voiding behavior was recorded before and after intravesical acetic acid instillation in control rats (n = 7) and HGN-transected rats (n = 7). Three days after the first observation of voiding behavior for 3 h at night (10:00 P.M to 1:00 A.M.), rats were anesthetized with ether inhalation, which was started at about 8:30 P.M. A urethral catheter (PE-50, Clay-Adams, Parsippany, NJ) was introduced, and 0.5 ml of 0.5% acetic acid in saline (a physiological solution consisting of 0.9% NaCl) was instilled into the bladder and retained for 20 min. Then the acetic acid solution was withdrawn, and the bladder was irrigated with saline. Ether inhalation was discontinued, and complete recovery from the anesthesia was confirmed by normal locomotion about 1 h following the cessation of ether inhalation. Next the second observation of voiding behavior was performed for 3 h at night (10:00 P.M to 1:00 A.M.). Through preliminary experiments, 0.5% acetic acid was determined appropriate for studying voiding behavior.

Results are expressed as means ± SE. For comparisons, the Wilcoxon signed-ranked test and Student’s t-test were used, and P < 0.01 was considered significant.

Continuous cystometry

Following anesthesia with pentobarbital (40 mg/kg, ip), a polyethylene catheter (PE-60, Clay-Adams, NJ) was implanted into the bladder through the dome, as described previously (Yaksh et al. 1986).

Seven days after the catheter implantation, continuous cystometry was performed without anesthesia. Three different experimental animal groups were compared: 1) control rats (n = 7), 2) capsaicin-desensitized rats (n = 6), and 3) HGN-transected rats (n = 8). In capsaicin-desensitized rats, capsaicin (75 mg/kg) was administered subcutaneously 4 days before experiments in a solution containing 20 mg/ml capsaicin (Sigma) in 10% ethanol, 10% Tween 80, and 80% saline. In preliminary studies, subcutaneous administration of 75 mg/kg capsaicin could achieve desensitization that was confirmed by a negative eye wipe test and a marked increase in latency to withdrawal in the hot plate test. Rats were placed in a restraining cage (KN-326, Natsume, Tokyo, Japan) sufficiently large enough to permit them to adjust themselves in a normal crouched posture and narrow enough to prevent front-to-back rotation. The bladder catheter was connected to a pressure transducer (AP-641G, Nihon Koden, Tokyo, Japan) and a microinjection pump (STC-523, Terumo, Tokyo, Japan). Room-temperature saline was infused constantly at a rate of 0.1 ml/min, and intravesical pressure was recorded continuously. Following saline infusion, 0.1% acetic acid was infused constantly at the same infusion rate. Since acetic acid was continuously infused into the bladder, we used a lower concentration (0.1%) of acetic acid than that (0.5%) used for experiments on voiding behavior. Continuous intravesical infusion of 0.1% acetic acid induced irritative bladder responses even in urethral-anesthetized rats (Kakizaki and de Groat 1996). The frequency of bladder contractions stabilized and became fairly regular after about 30 min of infusion of either saline or 0.1% acetic acid. Three micturition cycles were collected after a 60-min stabilization period following saline infusion. Then the infusion was switched to acetic acid. After another 60-min stabilization period, three micturition cycles were again collected. The means of the intercontraction interval (ICI) and maximal voiding pressure (MVP) in these micturition cycles were compared in each group.

Results are expressed as means ± SE. For comparisons between values obtained during saline and acetic acid infusion, paired Student’s t-tests were used, and P < 0.01 was considered significant.

Spinal c-fos expression

After urethan anesthesia (1.2 g/kg, ip), the urinary bladder was exposed through a lower midline abdominal incision, and fluid (either saline or 1% acetic acid) was infused (0.12 ml/min) for 2 h via a needle (23 gauge) inserted through the bladder dome just before the start of infusion. Spinal c-fos expression was examined in the following three groups: 1) saline infusion with the intact HGN (n = 3), 2) 1% acetic acid infusion with the intact HGN (n = 3), and 3) 1% acetic acid infusion with the HGN transection in advance (n = 3). A 2-h infusion time and 1% acetic acid were adopted in the present study according to previous studies (Birder and de Groat 1992, 1993; Kakizaki et al. 1996). Since the urethra remained open, fluid infused into the bladder could be expelled or leak out during the continuous infusion. If 1% acetic acid was infused into the bladder, mineral oil was applied to the area around the urethral meatus to reduce the possibility of irritation of the perineal skin and vaginal mucosa near this region.

Two hours after infusion of either saline or acetic acid, the animals were killed via intracardiac perfusion of 0.1 M, pH 7.4, phosphate-buffer (PB) followed by 4% paraformaldehyde fixative in PB (0.1 M, pH 7.4). The spinal cord was then removed and postfixed for 24 h in the same fixative at 4°C before cryoprotection in 0.1 M, pH 7.4. The spinal cord was then cleaved in xylene, and placed under a coverslip with toluene-based synthetic resin mounting medium. All sections were examined with bright-field microscopy.

Since previous studies analyzed the distribution of Fos-positive cells within the spinal cord following bladder stimulation (Birder and de Groat 1992; Cruz et al. 1994), analysis in the present study was restricted to the L1 and L6 spinal segment, where the majority of the HGN and the PLN afferent terminals project, respectively. Cells exhibiting Fos immunoreactivity were counted in three different spinal cord regions: the dorsal horn (DH), dorsal commissure (DCM),
and intermediolateral gray matter (ILG; Fig. 1, A and B). In the L₁ spinal segment, since the numbers of Fos-positive cells in the DCM and ILG regions were significantly smaller than in the DH, the Fos-positive cells in the DCM and ILG were counted together (Fig. 1B). Counts of Fos-positive cells were performed on 20 sections and presented as average numbers of cells per section. All values are expressed as means ± SE. To evaluate changes in the number of Fos-positive cells in each group, ANOVA followed by the Mann-Whitney U test was used for analyzing differences in the distribution of Fos-positive cells in specific areas of the spinal cord, and \( P < 0.01 \) was considered significant.

**RESULTS**

**Voiding behavior**

Total micturition volume for 3 h increased slightly without statistical significance after acetic acid instillation in both control (from 1.26 ± 0.01 to 1.69 ± 0.28 ml, mean ± SE) and HGN-transected rats (from 1.03 ± 0.19 to 1.60 ± 0.22 ml; Fig. 2A). There was no statistically significant difference between control and HGN-transected rats. Before acetic acid instillation, the number of micturitions for 3 h in control rats was significantly greater than in HGN-transected rats (Fig. 2B). After acetic acid instillation, the number of micturitions for 3 h increased significantly both in control (from 7.4 ± 0.2 to 24.1 ± 2.4, \( P < 0.001 \)) and HGN-transected rats (5.1 ± 0.5 to 9.0 ± 1.0, \( P < 0.01 \)). The increase in the number of micturitions was significantly greater in control rats than in HGN-transected rats (\( P < 0.001 \); Fig. 2B). Before acetic acid instillation, the voided volume per micturition was not different between control and HGN-transected rats (Fig. 2C). After acetic acid instillation, the voided volume per micturition decreased significantly in control rats (from 0.17 ± 0.01 to 0.07 ± 0.01 ml, \( P < 0.001 \)), whereas there was no significant decrease in HGN-transected rats (from 0.21 ± 0.03 to 0.17 ± 0.02 ml; Fig. 2C). Thus the voided volume per micturition after acetic acid instillation was significantly smaller in control rats than in HGN-transected rats (\( P < 0.001 \)).

**Continuous cystometry**

Histological evaluation of the bladder after intravesical instillation of 0.1% acetic acid revealed extensive, severe mucosal degeneration accompanied by submucosal edema and infiltration of inflammatory cells (data not shown). However, intravesical instillation of 0.1% acetic acid was well tolerated in conscious rats, and they behaved normally except for occasional licking of the lower abdomen.

In control rats, acetic acid infusion elicited irritative bladder responses characterized by a marked decrease in ICI (from 10.8 ± 1.6 min during saline infusion to 6.1 ± 1.9 min after acetic acid infusion, \( P < 0.0002 \)) and a marked increase in MVP (from 19.3 ± 4.1 mmHg during saline infusion to 29.1 ± 4.5 mmHg after acetic acid infusion, \( P < 0.002 \); Figs. 3A and 4). In capsaicin-desensitized rats, there were no irritative bladder responses; ICI: 10.6 ± 1.2 min with saline and 10.7 ± 2.2 min with acetic acid; MVP: 17.8 ± 1.4 mmHg with saline and 18.3 ± 1.7 mmHg with acetic acid (Figs. 3B and 4). In HGN-transected rats, the volume-evoked micturition reflex was preserved, and there was no significant difference in ICI and MVP during saline infusion compared with control rats. After acetic acid infusion, although a marked increase in MVP was elicited (from 18.4 ± 1.8 mmHg with saline to 29.3 ± 3.1 mmHg with acetic acid, \( P < 0.001 \)), ICI did not decrease significantly in HGN-transected rats (12.2 ± 1.6 min with saline and 11.8 ± 3.1 min with acetic acid; Figs. 3C and 4).

**C-fos expression**

L₁ LEVEL. Compared with saline infusion (DH: 14.4 ± 1.8 cells/section; DCM: 19.7 ± 2.7 cells/section; ILG: 19.6 ± 2.2 cells/section), acetic acid infusion with or without HGN transection caused a significant increase in the number of Fos-positive cells in all three regions, and there was no difference between HGN-intact and HGN-transected rats (DH: 30.7 ± 2.3 cells/section; DCM: 50.5 ± 2.9 cells/section; ILG: 39.9 ± 2.4 cells/section in HGN-intact rats; DH: 31.0 ± 3.0 cells/section; DCM: 50.5 ± 2.6 cells/section; ILG: 40.5 ± 2.8 cells/section in HGN-transected rats; Figs. 5 and 6).

L₆ LEVEL. Acetic acid infusion in HGN-intact rats caused a marked increase in the number of Fos-positive cells in the DH (51.2 ± 2.2 cells/section) compared with saline infusion (19.9 ± 2.5 cells/section; Figs. 7 and 8). In HGN-transected rats, however, the number of Fos-positive cells in the DH following acetic acid infusion was significantly smaller (31.1 ± 4.2 cells/section) than in HGN-intact rats (Fig. 8). The decrease in the number of Fos-positive cells in HGN-transected rats was primarily noted in lamina I (Fig. 7). On the other hand, in the DCM and ILG, acetic acid infusion did not increase the number of Fos-positive cells either in HGN-intact rats (8.6 ± 2.1 cells/section) or HGN-transected rats (6.6 ± 1.6 cells/section).
DISCUSSION

The present study revealed that transient or continuous intravesical instillation of acetic acid in conscious rats provoked hyperactive bladder responses, which were characterized by a decrease in the voided volume per micturition and an increase in micturition frequency. Since capsaicin desensitization prevented the occurrence of hyperactive bladder responses following acetic acid infusion, these responses were mediated via capsaicin-sensitive afferent fibers. HGN transection prevented a decrease in the voided volume per micturition (voiding section), compared with saline infusion (6.4 ± 1.5 cells/section; Fig. 8).

FIG. 2. Voiding behavior before and after intravesical instillation of acetic acid in control (n = 7) and hypogastric nerve (HGN)-transected rats (n = 7). A: total micturition volume for 3 h. B: the number of micturitions for 3 h. * P < 0.01, ** P < 0.001. C: the voided volume per micturition. ** P < 0.001.
A  Control

(1) saline

(2) acetic acid

B  Capsaicin desensitization

(1) saline

(2) acetic acid

C  HGN-transection

(1) saline

(2) acetic acid

behavior) as well as an increase in micturition frequency (continuous cystometry) following acetic acid instillation, suggesting that capsaicin-sensitive bladder afferent fibers in the HGN have a role in urinary frequency caused by chemical bladder irritation. Since coordinated micturition is mediated by a spinobulbospinal pathway passing through a relay center in the rostral brain stem, it seems reasonable to conclude that the HGN bladder afferent fibers convey an excitatory input induced by chemical bladder irritation to the higher center for micturition, thus causing the facilitation of micturition frequency.

In the study of voiding behavior, the number of micturitions before acetic acid instillation was smaller in HGN-transected rats than in control rats. This finding may indicate a facilitative role of the HGN in the frequency of micturition. However, the voided volume per micturition before acetic acid instillation was not different between control and HGN-transected rats. Furthermore, in HGN-transected rats, the volume-evoked micturition reflex was preserved, and there was no significant difference in ICI and MVP during continuous infusion of saline compared with control rats. Thus it seems unlikely that the HGN have a significant role in micturition under circumstances without acute inflammation of the bladder or in micturition induced by saline infusion.

Interestingly, urine output increased following acetic acid instillation in both control and HGN-transected rats, although there was no statistical difference between before and after acetic acid instillation in either group. Similar findings were reported in a previous study (Ozawa et al. 1999). It may be speculated that a self-protection mechanism against bladder irritation induces an increase in fluid intake, and, as a result, total micturition volume (urine output) increases after intravesical acetic acid instillation. Or, it may be an effect of recent anesthesia (ether inhalation in this study).

Continuous infusion of acetic acid in control rats elicited an increase in MVP compared with that obtained during saline infusion. Theoretically, an increase in voiding pressure is caused by alterations of bladder contractility and/or urethral resistance. Acetic acid infusion could activate bladder afferents as well as urethral afferents, because infused acetic acid was expelled through the urethra. Activation of bladder afferents by acetic acid may facilitate the excitatory bladder reflex (vesico-vesical contraction reflex), while activation of urethral afferents may facilitate bladder contractions (urethro-vesical contraction reflex) or may alter urethral activity (urethro-urethral contraction reflex). Acetic acid infusion is known to alter urethral activity during micturition (Kakizaki and de Groat 1996). These changes in urethral activity caused by acetic acid may alter the urethral resistance during micturition, thus contributing to an increase in voiding pressure. Bladder and urethral afferents that mediate the increase in voiding pressure following acetic acid infusion are capsaicin-sensitive afferents that are not included in the HGN but possibly in the PLN, since capsaicin desensitization, but not HGN transection, prevented the increase in voiding pressure following acetic acid.

**FIG. 4.** Results of continuous cystometry in conscious control rats (n = 7), capsaicin-desensitized rats (n = 6), and HGN-transected rats (n = 8). A: intercontraction interval (ICI). ***P < 0.0002. B: maximal vesical pressure (MVP). * P < 0.002, ** P < 0.001.
infusion. It seems likely that urethral afferents in the pudendal nerves are also involved.

Several studies have shown that capsaicin-sensitive afferents in the lower urinary tract facilitate micturition and that desensitization of these afferents by systemic administration of capsaicin can increase bladder capacity in urethan-anesthetized rats (Holzer-Petsche and Lembeck 1984; Maggi et al. 1986; Santicioli et al. 1985). However, in another study, pretreatment with capsaicin did not cause significant changes in voiding parameters in urethan-anesthetized rats, including the volume threshold for micturition and the amplitude, duration, and interval of reflex bladder contractions (Cheng et al. 1993). In our study, capsaicin desensitization did not alter voiding parameters during saline infusion in conscious rats. On the other hand, capsaicin desensitization completely prevented changes in ICI and MVP following acetic acid infusion. Thus hyperac-

FIG. 5. Photographs of the L6 spinal cord. Fos-positive cells were identified by dark spots within the nuclei. A: HGN-intact control rat after acetic acid infusion. B: HGN-transected rat after acetic acid infusion.

FIG. 6. The number of Fos-positive cells at the dorsal horn (DH), dorsal commissure (DCM), and intermediolateral gray matter (ILG) of the L6 spinal cord following saline infusion in HGN-intact control rats (n = 3) or following acetic acid infusion in HGN-intact (n = 3) or HGN-transected rats (n = 3). * P < 0.0001.
tive bladder responses following acetic acid infusion are mediated via capsaicin-sensitive fibers.

The present study revealed that, compared with saline infusion, chemical irritation of the bladder in urethan-anesthetized rats caused a significant increase in the number of Fos-positive cells at the L1 and L6 spinal cord levels. The HGN transection significantly reduced the number of Fos-positive cells in the DH but not in the DCM and ILG of the L1 spinal cord, or in the L6 spinal cord. Since the HGN transection did not abolish c-fos expression at the L1 spinal cord, neural pathways other than the HGN are also involved in c-fos expression at the L1 spinal cord. It is speculated that an intersegmental pathway throughout the spinal cord (e.g., spinal tract neurons) contributes to c-fos expression at the L1 spinal cord. A previous study has demonstrated that an increase in c-fos expression at the Th12–L2 spinal cord following bladder irritation was mainly noted in lamina I (Cruz et al. 1994). Lamina I contains a majority of nociception-specific neurons (Menetrey et al. 1977), and there are massive projections from lamina I of the spinal cord to the thalamus (Lima and Coimbra 1988). Taking into account the results of voiding behavior and continuous cystometry, it is speculated that HGN bladder afferents, via the L1 spinal level, convey an excitatory input induced by chemical bladder irritation to the higher center for micturition.
resulting in the facilitative modulation of micturition frequency. The supraspinal micturition center, which is involved in the acetic acid–induced facilitation of micturition frequency, may be the thalamus, periaqueductal gray, or pontine micturition center itself, although there is no definite evidence. It will be of interest to investigate the higher center involved in the modulation of micturition frequency by HGN bladder afferents. Such studies might provide insights into the development of a new strategy for pharmacological treatment of urinary frequency that will target the CNS.

REFERENCES


