A spinal GABAergic mechanism is necessary for bladder inhibition by pudendal afferent stimulation

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McGee MJ, Danziger ZC, Bamford JA, Grill WM. A spinal GABAergic mechanism is necessary for bladder inhibition by pudendal afferent stimulation. Am J Physiol Renal Physiol 307: F921–F930, 2014. First published August 20, 2014; doi:10.1152/ajprenal.00330.2014.—Electrical stimulation of pudendal afferents can inhibit bladder contractions and increase bladder capacity. Recent results suggest that stimulation-evoked bladder inhibition is mediated by a mechanism other than activation of sympathetic bladder efferents in the hypogastric nerve, generating α-adrenergic receptor-mediated inhibition at the vesical ganglia and/or β-adrenergic receptor-mediated direct inhibition of the detrusor muscle. We investigated several inhibitory neurotransmitters that may instead be necessary for stimulation-evoked inhibition and found that intravenous picrotoxin, a noncompetitive GABA_A antagonist, significantly and reversibly blocked pudendal afferent stimulation-evoked inhibition of bladder contractions in a dose-dependent manner. Similarly, intrathecal administration of picrotoxin at the lumbosacral spinal cord also blocked bladder inhibition by pudendal afferent stimulation. On the other hand, glycineergic, adrenergic, or opioidergic mechanisms were not necessary for bladder inhibition evoked by pudendal afferent stimulation. These results identify a lumbosacral spinal GABAergic mechanism of bladder inhibition evoked by pudendal afferent stimulation.

METHODS

Acute nonsurvival experiments were conducted in 33 intact adult male cats (2.8–4.5 kg). All animal care and experimental procedures were approved by the Duke University Institutional Animal Care and Use Committee. Initial anesthesia was with ketamine-HCl (35 mg/kg im), and anesthesia was maintained with α-chloralose (65 mg/kg iv, supplemented at 15 mg/kg iv). End-tidal CO_2 was maintained at between 3 and 4% with artificial respiration. Blood pressure was monitored via a catheter placed in the carotid artery and a solid-state pressure transducer. Body temperature was maintained at 38°C using a heating pad, and 0.9% NaCl with 5% dextrose and 8.4 mg/l NaHCO_3 was administered intravenously (15 ml·kg⁻¹·h⁻¹).

The bladder was accessed through a midline abdominal incision, and a 3.5-Fr suprapubic catheter was inserted into the bladder dome and secured with a purse string suture. Bladder pressure was measured with a solid-state pressure transducer in series with the catheter and recorded. A 3.5- to 5-Fr catheter was used to occlude the urethra during bladder filling and isovolumetric conditions. The threshold volume for distension-evoked reflex contractions (DECs) was found by filling the bladder at 1–2 ml/min with either room temperature saline or 0.25% acetic acid (AA), used to irritate the bladder and mimic OAB symptoms (44). Electromyographic (EMG) activity was detected from the external anal sphincter (EAS) with paired wire...
electrodes, amplified (1,000), filtered (10 Hz–10 kHz), and recorded. The PN was exposed through an incision between the base of the tail and the ischial tuberosity and transection of the gluteofemoralis. After dissection of the ischiorectal fossa, the sensory (SN) and rectal perineal branches of the PN were visible. Further dissection of the distal portion of the SN branch revealed the dorsal nerve of the penis (DNP) (57, 58). For animals receiving intrathecal administration of picrotoxin, a partial laminectomy was performed from L6 to S3. A 20-G needle was used to puncture the dura and insert a PE-10 catheter for intrathecal infusion. The location of the intrathecal catheter and spread of injected volume were confirmed in a separate experiment using dye and postmortem dissection.

The PN and DNP were stimulated unilaterally with cuff electrodes around each branch (Fig. 1A). Electrodes were composed of single platinum contacts embedded within silicone cuffs, and pulse generators (Pulsar 6bp, FHC) were used to deliver stimulation (0.1-ms cathodic stimulus pulses, for 20–30 s) between the cuffs and subcutaneous 20-G needles. Both the PN and DNP were stimulated at 10 Hz, a frequency previously shown to produce strong bladder inhibition in cats (53). The stimulation amplitude was one (1T), two (2T), or three (3T) times the threshold (T) that produced a bulbocavernous reflex (BCR) EMG response in the EAS with 1-Hz stimulation.

Trials to evaluate bladder responses to 10-Hz PN or DNP stimulation were conducted under isovolumetric conditions after saline infusion to 100% of the threshold volume for DECs under control conditions and following drug administration. In some animals, individual stimulation of both the PN and DNP was performed to compare the drugs’ effects on bladder inhibition at each stimulation location. For all drugs, a control trial was conducted before drug administration to confirm bladder inhibition with 10-Hz PN or DNP stimulation (Fig. 1B). In some animals where drug administration affected bladder inhibition, an additional trial was performed after a drug washout period of at least 2 h. To prevent interaction effects following the administration of multiple drugs, each animal received only one pharmacological treatment.

Picrotoxin, an antagonist for GABA_\_ receptors, was administered intravenously (iv; n = 11, 1.5 mg/kg iv) with saline (n = 8) or AA (n = 3) bladder infusion or intrathecalectly (n = 3, 5 mM, 0.2 ml, based on doses and volumes found to be effective in similar experiments) (21, 36) with saline bladder infusion. In three additional animals, picrotoxin (0.5 mg/kg iv) was administered to evaluate the effectiveness of picrotoxin at a lower dose. Phentolamine (2 mg/kg iv) and propranolol (1 mg/kg iv) (11, 18, 25, 55) were coadministered to block α- and β-adrenergic receptors (n = 7). Unilateral and bilateral hypogastric nerve transection was performed to eliminate sympathetic innervation of the bladder (n = 2). To block glycnergic receptors, increasing cumulative doses of strychnine (n = 4, 0.01–0.1 mg/kg iv) were administered, including to two animals who received cumulative doses up to 0.25 mg/kg iv (40). Increasing cumulative doses of naloxone (0.1–4.0 mg/kg iv), a competitive opioid antagonist, were administered to identify any opioidergic contribution to bladder inhibition (n = 3), as this was ambiguous from prior studies (7, 28).

Gallamine triethiodide (10 mg/kg iv initial dose with 5 mg/kg iv supplemented every 45 min), a paralytic and muscle relaxant, was administered throughout experiments with picrotoxin and strychnine to prevent convulsions caused by the pharmacological antagonists. Additional control trials were conducted after administration of gallamine to detect any change in stimulation-evoked inhibition of bladder contractions caused by administration of the paralytic.

Bladder inhibition produced by stimulation was quantified as normalized bladder pressure: the ratio of the mean bladder pressure during the entire time of stimulation to the mean bladder pressure during control DECs. Statistical significance was determined either by ANOVA or repeated-measures ANOVA with post hoc paired comparisons with Bonferroni correction (P < 0.05). Trials of stimulation of PN or DNP were pooled for analysis, as noted in RESULTS. Data are shown as means ± SE, unless otherwise stated.

RESULTS

Stimulation of the PN (10 Hz; n = 21 cats) or DNP (n = 12) inhibited distension-evoked bladder contractions in all animals in which each target nerve was stimulated. The DECs evoked by bladder filling were consistent over time, as reported in a previous study (5), and did not change following repeated stimulation of PN or DNP (Fig. 1B). The reduction in bladder pressure during PN and DNP stimulation was dependent on the amplitude of stimulation (P = 0.011, ANOVA) (Fig. 2), but there was no significant difference in the normalized bladder pressure during stimulation of PN or DNP (P = 0.337,
ANOVA). In the following results, we combined cases of PN and DNP stimulation for analysis because there was no significant interaction effect in the two-way ANOVA for bladder inhibition between the stimulation site and drug delivered ($P = 0.914$), indicating that there was no difference in drug effect on bladder inhibition by PN or DNP stimulation. Although the magnitude of bladder inhibition varied across animals, stimulation producing any decrease in normalized bladder pressure occurred in 100, 87.5, and 41.2% of trials at 3T, 2T, and 1T stimulation of the PN, respectively, and in 100, 83.3, and 50% of trials at 3T, 2T, and 1T stimulation of the DNP. Bladder inhibition evoked by PN or DNP stimulation was disrupted by the administration of picrotoxin, either intravenously or intrathecially, but was not blocked by phentolamine, propranolol, hypogastric nerve transection, strychnine, or naloxone.

**Antagonism of GABA$_A$ Reversibly Blocked Bladder Inhibition**

Intravenous picrotoxin (1.5 mg/kg) blocked bladder inhibition by PN and DNP stimulation at 1T, 2T, and 3T amplitudes. The normalized bladder pressure during pudendal afferent stimulation was significantly higher following administration of picrotoxin ($P < 0.001$, ANOVA, $n = 11$: 9 PN, 2 DNP) (Figs. 2 and 3). Administration of 1.5 mg/kg picrotoxin blocked bladder inhibition and produced significantly higher normalized bladder pressures than controls and paralyzed control trials ($P < 0.001$, post hoc with Bonferroni correction). Although the average normalized bladder pressure produced by stimulation was dependent on stimulation amplitude, picrotoxin blocked the decrease in normalized bladder pressure for all amplitudes (Fig. 2). The loss of inhibition following intravenous picrotoxin was reversible and stimulation-evoked inhibition returned following a washout period of 2 h ($n = 4$), indicating that the loss of bladder inhibition was not due to a loss of stimulation effectiveness (Figs. 3 and 4). In three animals, a lower dose of picrotoxin (0.5 mg/kg iv) was administered and did not block inhibition of bladder contractions by pudendal afferent stimulation (Fig. 4A). There was no significant difference between normalized bladder pressures during control and lower dose picrotoxin trials ($P = 0.908$, post hoc with Bonferroni correction). Normalized bladder pressure during stimulation-evoked inhibition following administration of the paralytic but before administration of picrotoxin was not significantly different from that of control trials ($P = 0.569$, post hoc with Bonferroni correction), demonstrating that the loss of inhibition was not a result of coadministration of the paralytic (Figs. 3 and 4). In one cat, 33-Hz stimulation, previously demonstrated to evoke reflex contraction of the bladder (53), continued to evoke robust bladder contractions after the administration of picrotoxin, even though 10-Hz stimulation-evoked inhibition was blocked.

**Fig. 2.** Effect of stimulation amplitude on bladder inhibition generated by PN and DNP stimulation. Results are shown of average normalized bladder pressure under control conditions and after administration of picrotoxin for 1T, 2T, and 3T stimulation of PN ($n = 8$) and DNP ($n = 5$). Picrotoxin significantly blocked the reduction in normalized bladder pressure for all amplitudes of both PN and DNP stimulation ($P < 0.001$, ANOVA). There was a significant effect of stimulation amplitude on normalized bladder pressure ($P = 0.011$, ANOVA); normalized bladder pressure was significantly higher with 1T stimulation than 3T stimulation ($P = 0.004$, post hoc paired comparisons with Bonferroni correction).

**Fig. 3.** Intravenous picrotoxin (1.5 mg/kg) reversibly blocked inhibition of reflex bladder contractions by pudendal afferent stimulation. Representative bladder pressure traces show effect of 3T stimulation of the PN or DNP on bladder contractions before and after administration of high-dose picrotoxin. Control and paralytic trials were performed before administration of picrotoxin. Washout trials occurred at least 2 h after picrotoxin administration. Bar indicates when stimulation was on.
Inhibition and the dissection demonstrated successful placement of the intrathecal picrotoxin (*P < 0.001, ANOVA, n = 3: 2 PN, 1 DNP; P < 0.001 post hoc paired comparisons with Bonferroni correction) (Fig. 6). Similar to intravenous picrotoxin administration with saline bladder infusion, bladder inhibition by pudendal afferent stimulation with AA bladder infusion returned after >2 h of washout.

**Administration of Other Pharmacological Antagonists**

We tested a number of other interventions to investigate other mechanisms that might contribute to pudendal afferent stimulation-evoked bladder inhibition. Table 1 summarizes the experiments performed where we did not observe effects of the interventions on pudendal afferent stimulation-evoked inhibition of DECs.

Stimulation (10 Hz) of either PN or DNP at 3T continued to evoke consistent bladder inhibition, and there was no significant difference between the reduction in normalized bladder pressure during stimulation after any of the following interventions (Table 1): 1) naloxone administered in cumulative doses (0.1–4.0 mg/kg iv, P = 0.568, ANOVA, n = 3: 2 DNP, 1 PN); 2) strychnine, administered in cumulative doses (0.025–0.25 mg/kg iv, P = 0.541, ANOVA, n = 4; 3 DNP, 1 PN); and 3) neither unilateral nor bilateral hypogastric nerve transection suppressed or eliminated bladder inhibition caused by PN stimulation ranging from 2T to 10T (P = 0.909, ANOVA, n = 2). Although 3T PN and DNP stimulation continued to evoke consistent bladder inhibition, there was a main effect of coadministration of α- and β-adrenergic antagonists (phentolamine, 2 mg/kg iv) and (propranolol, 1 mg/kg iv) with repeated-measures ANOVA (P = 0.039, n = 7: 4 PN, 3 DNP). Post hoc paired comparisons with Bonferroni correction revealed that normalized bladder pressure was significantly higher following phentolamine (P = 0.015), but not propranolol (P = 0.065). However, normalized bladder pressure during stimulation remained low following phentolamine, indicating that robust bladder inhibition was still intact (Table 1).

Although a smaller number of animals was used in some of the non-GABAergic antagonist experiments, the risks of a type II error for strychnine (β = 0.04), naloxone (β = 0.17), and hypogastric nerve transection (β = 0.02), where a statistically significant difference in normalized bladder pressure may not have been detected, were still quite low.

**DISCUSSION**

This study of the mechanisms of bladder inhibition by pudendal afferent stimulation was prompted by studies suggesting that stimulation-evoked bladder inhibition is not mediated by the activation of sympathetic outflow (28, 55), as previously asserted, and by previous reports of differing...
contributions of opioidergic mechanisms to stimulation-evoked bladder inhibition (7, 28). The present results reveal that a spinal GABAergic mechanism mediates the inhibitory pudendovesical pathway. Picrotoxin, a GABA<sub>A</sub> receptor antagonist, blocked pudendal afferent stimulation-evoked bladder inhibition and was effective when administered in a sufficient dose either systemically or locally to the lumbo-sacral spinal cord. Consistent with our results, other studies have reported that GABA and muscimol (GABA<sub>A</sub> receptor agonist) when administered intravenously or intrathecally inhibit the micturition reflex in rats (27). Furthermore, a recent report also found that lower doses of picrotoxin did not disrupt pudendal afferent stimulation-mediated inhibition of bladder contractions during cystometry with saline.

Fig. 5. Intrathecal (i.t.) picrotoxin reversibly blocked inhibition of reflex bladder contractions by pudendal afferent stimulation. A: representative bladder pressure traces from control, paralytic, and i.t. picrotoxin trials of 3T PN stimulation. Bar indicates when stimulation was on. The low bladder pressure seen in the picrotoxin trial example is a result of periodic DECs. After the onset of the next DEC, inhibition of the contractions was blocked by i.t. picrotoxin. B: bladder pressure during PN or DNP stimulation, normalized to pressure during control DECs, following i.t. picrotoxin and washout (P = 0.005, ANOVA, n = 3: 2 PN, 1 DNP). *P < 0.05, post hoc paired comparisons with Bonferroni correction.

Fig. 6. Intravenous picrotoxin reversibly blocked inhibition of acetic acid (AA)-induced bladder contractions by pudendal afferent stimulation. A: representative bladder pressure traces from 1 animal with 3T PN stimulation for control fill with AA following intravenous picrotoxin and following picrotoxin washout. Bar indicates when stimulation was on. B: bladder pressure during 3T stimulation, normalized to pressure during control DECs, was significantly different following administration of picrotoxin (P < 0.001, ANOVA, n = 3: 2 PN, 1 DNP). *P < 0.001, post hoc paired comparisons with Bonferroni correction.
were given to 0.25 mg/kg. Loss of bladder inhibition with picrotoxin. In 2 of 4 animals receiving strychnine, the maximum dose was 0.1 mg/kg; in the remaining 2 animals, additional doses (0.05) were performed only when there was a general drug effect on stimulation effectiveness. Washout trials were performed in a subset of animals that showed restoration of bladder control.

GABAergic Picrotoxin

Inhibition by Pudendal Afferent Stimulation

GABAergic Mechanisms are Necessary for Bladder Inhibition by Pudendal Afferent Stimulation

Picrotoxin, administered intravenously (1.5 mg/kg) to block GABA<sub>A</sub> receptors, reversibly blocked inhibition of reflex bladder contractions by pudendal afferent stimulation at all stimulation amplitudes. PN and DNP stimulation-evoked bladder inhibition was blocked by picrotoxin similarly across multiple amplitudes of stimulation, suggesting that they both employ the same neurotransmitter mechanisms. The loss of pudendal afferent stimulation-evoked inhibition was not due to a loss of the effect of stimulation at the electrode because inhibition returned after a washout period of at least 2 h. Because the pharmacokinetics of picrotoxin are not well documented, we chose a washout period of 2 h that corresponded to the time when the effects on blood pressure diminished. Low-dose picrotoxin (0.5 mg/kg) failed to block inhibition by pudendal afferent stimulation, indicating that

<table>
<thead>
<tr>
<th>Mechanism Investigated</th>
<th>Treatment or Pharmacological Antagonist</th>
<th>Dose and Route</th>
<th>Average NBP Before Treatment</th>
<th>Average NBP After Treatment</th>
<th>Average Within-Animal ΔNBP</th>
<th>Post Hoc Tests</th>
<th>Notes</th>
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<tbody>
<tr>
<td>Adrenergic</td>
<td>Phenolamine (α-adrenergic antagonist)</td>
<td>2 mg/kg iv</td>
<td>n = 7</td>
<td>0.383 ± 0.03</td>
<td>0.454 ± 0.03*</td>
<td>NBP compared with control was significantly higher for phenolamine, but not propranolol.</td>
<td>Bladder inhibition remained for both.</td>
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<td>Propranolol (β-adrenergic antagonist)</td>
<td>1 mg/kg iv</td>
<td>n = 7</td>
<td>0.383 ± 0.03</td>
<td>0.433 ± 0.03</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Sympathetic</td>
<td>Hypogastric nerve transection</td>
<td>Unilateral, bilateral</td>
<td>n = 2</td>
<td>0.332 ± 0.09</td>
<td>0.351 ± 0.04</td>
<td>0.019 ± 0.13</td>
<td>N/A</td>
</tr>
<tr>
<td>Glycinergic</td>
<td>Strychnine (glycine antagonist)</td>
<td>0.1 mg/kg iv</td>
<td>n = 4</td>
<td>0.525 ± 0.11</td>
<td>0.544 ± 0.13</td>
<td>0.019 ± 0.08</td>
<td>N/A</td>
</tr>
<tr>
<td>Opioidergic</td>
<td>Naloxone (opioid antagonist)</td>
<td>0.1–4.0 mg/kg iv</td>
<td>n = 3</td>
<td>0.452 ± 0.09</td>
<td>0.388 ± 0.09</td>
<td>−0.064 ± 0.16</td>
<td>N/A</td>
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<tr>
<td>GABAergic</td>
<td>Picrotoxin (GABA&lt;sub&gt;A&lt;/sub&gt; antagonist)</td>
<td>0.5 mg/kg iv</td>
<td>n = 3</td>
<td>0.599 ± 0.11</td>
<td>0.646 ± 0.06</td>
<td>0.046 ± 0.08</td>
<td>N/A</td>
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<tr>
<td></td>
<td>Picrotoxin (GABA&lt;sub&gt;A&lt;/sub&gt; antagonist)</td>
<td>1.5 mg/kg iv</td>
<td>n = 8</td>
<td>0.625 ± 0.06</td>
<td>1.098 ± 0.02†</td>
<td>0.473 ± 0.06</td>
<td>Inhibition returned following washout period (n = 4).</td>
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<td></td>
<td>Picrotoxin (GABA&lt;sub&gt;A&lt;/sub&gt; antagonist)</td>
<td>5 mmol i.t.</td>
<td>n = 3</td>
<td>0.595 ± 0.07</td>
<td>1.115 ± 0.04†</td>
<td>0.519 ± 0.11</td>
<td>Inhibition returned following washout period (n = 2).</td>
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Values are means ± SE. Experiments evaluating bladder inhibition following the administration of phenolamine and propranolol, hypogastric nerve transection, strychnine, naloxone, and picrotoxin were performed independently in each animal. The dose and route of treatment or drug administration and number of experiments performed (n) are each listed. The average normalized bladder pressure (NBP) is listed before and after treatment and represents the normalized fraction of bladder inhibition with stimulation across animals at the highest dose point. Average NBP values >1 indicate that pudendal afferent stimulation did not inhibit bladder contractions. The average within-animal change (Δ) in NBP is also shown to illustrate that trends in NBP before and after treatment were consistent across animals. There was a significant main effect of coadministration of adrenergic antagonists on NBP (P = 0.039, ANOVA), although robust bladder inhibition was preserved. Post hoc paired comparisons with Bonferroni correction showed that NBP was significantly higher than control following phenolamine (†P = 0.015), but not propranolol (P = 0.065). There was a significant effect of treatment on stimulation-evoked inhibition with high-dose intravenous (iv) or intrathecal (i.t.) picrotoxin administration (†P < 0.001, ANOVA). Post hoc paired comparisons with Bonferroni correction (P < 0.05) were performed only when there was a general drug effect on stimulation effectiveness. Washout trials were performed in a subset of animals that showed loss of bladder inhibition with picrotoxin. In 2 of 4 animals receiving strychnine, the maximum dose was 0.1 mg/kg; in the remaining 2 animals, additional doses were given to 0.25 mg/kg.

(56). These results identify a clear and compelling mechanism for pudendal afferent stimulation-evoked inhibition of the bladder and may inspire the development of therapies for restoration of bladder control.

GABAergic Mechanisms are Necessary for Bladder Inhibition by Pudendal Afferent Stimulation

Picrotoxin, administered intravenously (1.5 mg/kg) to block GABA<sub>A</sub> receptors, reversibly blocked inhibition of reflex bladder contractions by pudendal afferent stimulation at all stimulation amplitudes. PN and DNP stimulation-evoked bladder inhibition was blocked by picrotoxin similarly across multiple amplitudes of stimulation, suggesting that they both employ the same neurotransmitter mechanisms. The loss of pudendal afferent stimulation-evoked inhibition was not due to a loss of the effect of stimulation at the electrode because inhibition returned after a washout period of at least 2 h. Because the pharmacokinetics of picrotoxin are not well documented, we chose a washout period of 2 h that corresponded to the time when the effects on blood pressure diminished. Low-dose picrotoxin (0.5 mg/kg) failed to block inhibition by pudendal afferent stimulation, indicating that
the effect on inhibition was dose dependent and required sufficient blockade of GABA_A receptor activity.

Picrotoxin was administered intrathecally in some animals to identify the location of picrotoxin-mediated blockade of bladder inhibition. Intrathecal picrotoxin produced results similar to those following administration of intravenous picrotoxin and blocked bladder inhibition by pudendal afferent stimulation. Inhibition and the consequent stimulation-evoked reduction in bladder pressure returned after 2 h of washout. Therefore, although the pharmacodynamics of intrathecal administration and the concentration of drug delivered were different from intravenous administration, picrotoxin’s effects were still reversible.

The key finding that spinal GABAergic mechanisms mediate bladder inhibition by pudendal afferent stimulation does not rule out contributions of other mechanisms, although our data suggest that adrenergic, glycnergic, and opioidergic mechanisms may not be necessary for robust inhibition. Parallel pathways at the spinal and supraspinal levels may both be involved in bladder control and activated by pudendal afferent stimulation, as bladder excitation with pudendal afferent stimulation involves both spinobulbospinal and spinal reflexes (54).

Neural circuits in the periaqueductal gray (PAG) and pontine micturition center (PMC) are known to employ GABA (16) but are unlikely to be primarily responsible for pudendal stimulation-evoked reflex bladder inhibition, as bladder inhibition by pudendal afferent stimulation remains intact following chronic spinal cord injury (45, 47). Any central GABAergic pathways that are involved would have also been antagonized after administration of intravenous picrotoxin. Intrathecal blockade of GABA was important because central GABAergic mechanisms are known to exist in the PAG (42), and these results demonstrated that the GABAergic neurons participating in pudendal afferent stimulation-evoked inhibition are located in the lumbosacral spinal cord.

The GABA_A pathway identified here may control sacral parasympathetic output via inhibition of preganglionic cells in the sacral parasymathetic nucleus (SN) by sacral interneurons or recurrent inhibition from the SN (39). GABA_A receptors are located throughout the dorsal horn and in the dorsal gray commissure (DGC) (2). GABA_A receptors also occur on neurons in the SN (3), whose dendrites extend to the DGC (13, 30), a site of pudendal afferent innervation (49). There may be multiple inhibitory pathways to the SN (17), including both presynaptic and postsynaptic GABAergic inhibition from other areas of the sacral spinal cord (2, 35). Inhibitory feedback neurons from the SN are known to produce recurrent inhibition of the bladder circuit (12) and may be GABAergic. Presynaptic inhibition of pelvic afferents by primary afferent depolarization may also be GABAergic (6, 38).

GABAergic Mechanisms Participate in Inhibition of Both Nociceptive and Nonnociceptive Bladder Reflexes

Intravenous picrotoxin also reversibly blocked pudendal afferent stimulation-evoked inhibition of reflex bladder contractions induced by AA installation. Although previous reports suggested that separate pathways may govern inhibition of nociceptive and nonnociceptive bladder contractions (44, 56), GABAergic mechanisms appear critical for inhibition of both types of contractions. A recent study of low-dose picrotoxin found that picrotoxin blocked inhibition of nociceptive, but not nonnociceptive, bladder contractions (56). This finding, when combined with our results, suggests that the nociceptive bladder state produced by infusion of AA may shift the response to picrotoxin to lower doses since blockade of pudendal afferent inhibition of nociceptive bladder contractions occurred at a lower dose than nonnociceptive bladder contractions.

Because AA infusion is frequently used as a model of OAB in animal models (28), this finding has important implications for research for the treatment of OAB. Following spinal cord injury, an alternate reflex pathway for bladder control emerges, driven by nociceptive C-fiber afferent activity from the bladder (20). Therefore, pudendal afferent inhibition of nonnociceptive bladder contractions that result from either OAB or spinal cord injury is likely to be mediated by GABA_A.

Other Mechanisms May Not Be Necessary for Bladder Inhibition by Pudendal Afferent Stimulation

We investigated a number of potential contributors to bladder inhibition and found little to no effect on bladder inhibition by pudendal afferent stimulation of the blockade of adrenergic, sympathetic, glycnergic, or opioidergic mechanisms. Although it is not clear that 3T stimulation can be considered supramaximal (Fig. 2), subtle effects of non-GABAergic antagonists may have been obscured by the use of 3T as opposed to lower amplitudes of stimulation.

Bladder inhibition by low-frequency PN or DNP stimulation was not blocked by coadministration of α- and β-adrenergic antagonists, indicating that adrenergic mechanisms, which were assumed to be a primary mechanism of bladder inhibition evoked by afferent stimulation (15), may not be necessary for this type of inhibition. Administration of phentolamine resulted in a statistically significant increase in normalized bladder pressure during pudendal afferent stimulation; however, the small effect size indicates robust bladder inhibition persisted under this treatment. In contrast to the large effect of picrotoxin (change in normalized bladder pressure = 0.473), the change in normalized bladder pressure following phentolamine was quite small (change in normalized bladder pressure = 0.071), indicating that α-adrenergic signaling may play a modulatory role.

Inhibition was preserved following unilateral and bilateral hypogastric nerve transection, replicating prior results (55, 59), and indicating that activity in the hypogastric nerve is not required for pudendal afferent-mediated bladder inhibition of DECs. Previous studies have suggested that sympathetic mechanisms mediate bladder inhibition-evaluated stimulation of pelvic afferents (15) or intravaginal stimulation (26), which might employ different mechanisms than stimulation of somatic afferents in the PN. Furthermore, mechanisms of inhibition may be different at low bladder pressures (<5 cmH_2O), where hypogastric transmission is thought to be required, and high bladder pressures (>15 cmH_2O), where inhibition is preserved following hypogastric transection (19).

Administration of strychnine to block glycine receptors also failed to block bladder inhibition, consistent with a previous study where strychnine failed to block bladder inhibition produced by rectal distension or perianal stimulation (17). In two animals, stimulation continued to produce strong inhibition of
reflex bladder contractions after the dose of strychnine was increased to 0.25 mg/kg iv, confirming that the absence of effect was not caused by too low a dose of strychnine. This dose approaches the lethal dose (0.33 mg/kg iv) (31) and was shown to adequately block glycine receptors in other reflex pathways (40).

Bladder inhibition by pudendal afferent stimulation remained intact following intravenous administration of naloxone, a competitive antagonist of μ-opioid receptors, that also blocks, albeit with lower affinity, κ- and δ-opioid receptors (22). Prior studies reported variable effects of blocking opioid receptors on pudendal afferent stimulation-evoked bladder inhibition. Naloxone blocked bladder inhibition by low-amplitude pudendal afferent stimulation in cats (7) and mechanical skin stimulation in rats (23). Inhibition of bladder contractions evoked by the infusion of AA, but not saline, with titrial nerve stimulation was blocked by naloxone (44). Later experiments demonstrated that naloxone had no effect on pudendal stimulation-evoked inhibition of bladder overactivity in cats (28). We found that inhibition by either PN or DNP stimulation remained intact following administration of naloxone across a range of doses and stimulation amplitudes. The maximum dose (4.0 mg/kg iv) was much higher than in previous studies, and pudendal afferent stimulation continued to produce robust inhibition of DECs. Although opioids did not provide a major contribution to reflex bladder inhibition evoked by stimulation of pudendal afferents, it is possible that they modulate neurotransmitter release or modify parasympathetic outflow (37).

Effectiveness of Stimulation-Evoked Inhibition

Our results demonstrate that 3T PN or DNP stimulation at 10 Hz robustly produced bladder inhibition in control trials. Stimulation at 1T and 2T produced significant bladder inhibition, but was less robust, consistent with results of other studies (53). The nomenclature used here for threshold and stimulation amplitude (1T = BCR reflex EMG threshold) should be distinguished from other studies, where 1T is determined as the threshold to evoke inhibition and would, in general, represent a higher amplitude.

There were no significant differences in bladder inhibition or the effect of pharmacological antagonists on stimulation of the compound PN or DNP. Different mechanisms of inhibition could be employed by stimulation of different nerve pathways (50); however, bladder inhibition was consistent across DNP and PN stimulation. Additionally, because stimulation amplitude was normalized to the BCR threshold for each nerve, differences in neural activation between the nerves likely did not affect the results. Stimulation of the sacral roots, which activates both pelvic and pudendal afferents, could engage neural network mechanisms other than those activated with PN or DNP stimulation (50).

The administration of the paralytic gallamine served two purposes in these experiments: first, preventing convulsions due to systemic intravenous administration of the pharmacological antagonists, and second, eliminating any contribution of re-afference to bladder activity. Re-afference, the activation of sensory (afferent) mechanisms by muscle contraction evoked by electrical stimulation of efferent axons, can produce reflex bladder contractions (48, 57), and it was important to eliminate the secondary effects of stimulation of PN efferents to study clearly the effect of low-frequency pudendal afferent stimulation-evoked bladder inhibition.

In these experiments, we found that picrotoxin increased excitability of the bladder, and DECs occurred at lower bladder volumes than control trials. This is consistent with previous studies where administration of GABA_A antagonists excited the bladder (24, 27), although a recent study reported that picrotoxin increased bladder capacity in cats (56).

Perspectives and Significance

Pudendal afferent stimulation-evoked inhibition was previously thought to be mediated through bladder relaxation mechanisms used in normal control of the lower urinary tract via the hypogastric nerve and adrenergic receptors. The results presented here indicate that bladder inhibition evoked by pudendal afferent stimulation relies on inhibition of the excitatory control of the bladder, rather than activation of a bladder relaxation pathway. Bladder inhibition by stimulation of pudendal afferents may be via synaptic inhibition of the SPN via GABA_A from local interneurons or presynaptic inhibition of parasympathetic afferents. Although additional inhibitory mechanisms exist at the pelvic ganglia and bladder muscle (14, 26), these are not necessary for pudendal afferent-evoked bladder inhibition as systemic adrenergic blockade failed to block inhibition by pudendal afferent stimulation.

These experiments demonstrated that GABA_A in the lumbo sacral spinal cord is required for bladder inhibition by pudendal afferent stimulation and that glycineric, adrenergic, and opioidergic mechanisms may not be necessary. These results have important implications for neuromodulation via PN stimulation and may impact the understanding of the mechanisms of sacral neuromodulation, which may activate afferents from both the pelvic nerve and PN. Bladder inhibition by sacral neuromodulation, particularly in cases after spinal cord injury, likely utilizes a sacral spinal network that is mediated by GABA. These results also indicate that pharmacological interventions, such as GABA agonists for OAB, should be investigated to improve the efficacy of existing neuromodulation techniques.

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DISCLOSURES

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AUTHOR CONTRIBUTIONS


REFERENCES


