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Nitric oxide modulates bladder afferent nerve activity in the in vitro urinary bladder–pelvic nerve preparation from rats with cyclophosphamide induced cystitis

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Abstract

Effects of a nitric oxide (NO) donor (SNAP), NO substrate (L-arginine), and NO synthase inhibitor (L-NAME) on bladder afferent nerve (BAN) activity were studied in an in vitro bladder–pelvic nerve preparation from untreated or cyclophosphamide (CYP) treated rats. Distension of the bladder induced phasic bladder contractions (PBC) that were accompanied by multiunit afferent firing. Intravesical administration of SNAP (2 mM) which did not change the amplitude of PBC significantly decreased peak afferent firing from 79 ± 15 spikes/s to 44 ± 8 spikes/s in CYP pretreated but not untreated preparations. In CYP treated preparations SNAP also decreased by 33–55% BAN firing induced by isotonic distension of the bladder at 10–40 cm H_2O pressures. Electrical stimulation on the surface of the bladder elicited action potentials (AP) in BAN. SNAP significantly increased the voltage threshold by 75% ($p < 0.05$) and decreased by 45% ($p < 0.05$) the area of the AP evoked at submaximal stimulus intensity. Bath application of SNAP (2 mM) or L-arginine (50 mM) elicited similar inhibitory effects on the distension evoked BAN firing. The effects of L-arginine were blocked by bath application of L-NAME (20 mM). L-NAME alone did not alter BAN firing. In preparations from normal rats SNAP or L-arginine did not alter BAN activity. These results suggest that exogenous as well as endogenously generated NO depresses the excitability of sensitized but not normal BAN and that NO may have an antinociceptive function and modulate bladder hyperactivity induced by pathological conditions.

Keywords

Nitric oxide; Bladder sensation; Nitric oxide synthase; Bladder pain; Nociceptive afferent

1. Introduction

This study examined the effects of nitric oxide (NO) on mechano-sensitive bladder afferent nerves and the potential role of NO as an anti-nociceptive agent. Nitric oxide synthase (NOS) is expressed at various sites in the urinary bladder including afferent nerves (Vizzard et al., 1995, 1996; Vizzard, 1997), parasympathetic efferent nerves (Andersson and Persson, 1995; Vizzard et al., 1994), urothelial cells (Birder et al., 1998,2008; Birder, 2005); and smooth muscle (Andersson and Persson, 1993, 1995; Andersson and Wein, 2004; Birder et al., 1998, 2005,2008; Birder, 2005; de Groat, 2006; Moncada et al., 1991; Vizzard et al., 1994, 1997) suggesting that nitric oxide (NO) could have complex functions in the bladder. The smooth muscles of the bladder outlet and urethra are inhibited by NO, but detrusor

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muscle is relatively insensitive to these inhibitory effects (Andersson and Wein, 2004) except in neonatal bladders (Artim et al., 2009, 2011). However, in adult bladders intravesical administration of an NO donor suppresses bladder overactivity induced by chemical irritation with cyclophosphamide (CYP) (Ozawa et al., 1999); while intravesical administration of an NO scavenger (oxyhemoglobin) enhances reflex bladder activity (Pandita et al., 2000). These findings coupled with the demonstration that chemical and mechanical stimuli release NO from the urothelium (Birder et al., 1998, 2002) raise the possibility that NO functions as an inhibitory transmitter between the urothelium and the underlying afferent nerves.

The sensitivity of bladder afferent neurons to NO was revealed using patch clamp recording techniques in dissociated dorsal root ganglion cells (Yoshimura et al., 2001). Application of the NO donor, SNAP, suppressed voltage gated calcium channels via an intracellular signaling pathway involving cyclic GMP and protein kinase G. Other studies on sensory neurons from nodose ganglia showed that NO also suppresses voltage gated sodium channels (Bielefeldt et al., 1999; Li et al., 1998).

The identification of NO as a potential inhibitory neurotransmitter of afferent nerve activity in the bladder has focused attention on the role of NO in bladder pathology. In patients with painful bladder syndrome/interstitial cystitis the urine concentration of nitrite a metabolic product of NO is decreased suggesting that a reduction in NO release in the bladder might contribute to the disorder (Smith et al., 1996). Thus L-arginine, a substrate for NOS and the precursor of NO, was administered to increase NO levels (Smith et al., 1997). Patients treated with L-arginine exhibited reduced symptoms, raising the possibility that NO has an antinociceptive action (Smith et al., 1997).

L-arginine is also a substrate for arginase enzymes that convert L-arginine to ornithine and urea (Moncada et al., 1991). Arginase activity regulates the concentration of L-arginine available to NOS and in turn indirectly controls the synthesis of NO. Thus inhibition of arginase activity can enhance NO-dependent functions in isolated tissues (Baggio et al., 1999; Kim et al., 2001). Administration of an arginase inhibitor (N^{W}-hydroxy-nor-L-arginine, nor-NOHA) to chronic spinal cord injured rats with neurogenic bladder overactivity suppressed the amplitude and number of non-voiding contractions detected during cystometry. This effect was blocked by a NOS inhibitor (L-NAME) (Sasatomi et al., 2008) indicating that it was dependent on the synthesis and release of endogenous NO. Because the effect of nor-NOHA on non-voiding contractions was similar to the effect of capsaicin pretreatment that desensitizes C-fiber bladder afferent nerves (Cheng et al., 1995; de Groat, 2006), it was concluded that increased production of endogenous NO after arginase inhibition suppresses bladder overactivity by targeting the C-fiber afferent limb of the micturition reflex (Masuda et al., 2007).

In this study we obtained more direct evidence for an inhibitory action of NO on afferent activity using an in vitro whole bladder–pelvic nerve preparation. We examined the effect of an NO donor (SNAP), an NO substrate (L-arginine) and a NOS inhibitor (L-NAME) on bladder afferent nerve activity in normal bladders and in a cystitis model induced by pretreatment with CYP. Preliminary data have been presented in an abstract (Yu and de Groat, 2005).

2. Results

2.1. Effects of SNAP, an NO donor, administered by intravesical infusion on bladder afferent nerve activity under isovolumetric conditions

In rats pretreated with CYP (100 mg/kg, i.p.) 17 h prior to the experiments multiunit afferent activity in the pelvic nerve was induced by intravesical infusion of Krebs solution at the rate of 0.04 ml/min for 8 min (Fig. 1). Two components of afferent activity were identified: (1) phasic firing which occurred during bladder contractions and (2) tonic firing which occurred between bladder contractions. Phasic firing which was the focus of the present experiments reached a peak 5–7 min after the start of infusion and slowly declined after the infusion was stopped at 8 min. The average maximal intravesical pressure during bladder contractions was 11.5 ± 2.1 cm H_2O and the average maximal phasic afferent firing rate was 79 ± 15 spikes/s ($n = 14$) measured for a period of 10 min after the end of infusion. After emptying the bladder, the rhythmic bladder contractions and afferent firing returned to baseline. Repeated distensions with Krebs solution elicited similar responses; eg., during a second bladder filling the peak amplitude of bladder contractions was 10.5 ± 2.4 cm H_2O ($n = 10$) and the average peak firing was 81 ± 11 spikes/s ($n = 10$).

SNAP, an NO donor, administered by intravesical infusion in a concentration (2 mM) that suppresses reflex activity of CYP irritated bladders (Ozawa et al., 1999) did not significantly alter the average maximal intravesical pressure (9.1 ± 1.4 cm H_2O, $n = 14$, $p < 0.01$, Fig. 2A), but reduced by almost 50% ($p < 0.05$) the maximal phasic afferent firing (average 44 ± 8 spikes/s) during bladder contractions (Figs. 1 and 2B). The effects of SNAP were reversible within 30 min after washout and reproducible during a second application.

In normal, untreated preparations ($n = 6$) the average maximal intravesical pressure during bladder contractions was 16.1 ± 2.2 cm H_2O and the average peak firing rate was 33 ± 7 spikes/s. As reported previously (Yu and de Groat, 2008) this firing rate was significantly lower ($p < 0.05$) than the firing rate in preparations irritated by CYP. Intravesical administration of SNAP (2 mM) did not significantly change the average amplitude of the bladder contractions (12 ± 1.8 cm H_2O, Fig. 2C) or the average maximal afferent firing rate (40 ± 8 spikes/s) associated with the contractions ($n = 6$) (Fig. 2D).

2.2. Effects of intravesical infusion of SNAP on bladder afferent nerve activity evoked by isotonic distension of the bladder

In four CYP pretreated preparations afferent nerve activity was elicited by isotonic distension of the bladder with Krebs solution at pressures between 10 and 40 cm H_2O for 30 s at 30 s intervals to evaluate the pressure–response relationship of afferent nerve activity (Fig. 3; Table 1). The afferent firing reached a maximum within 5–10 s after isotonic distention of the bladder and then slowly declined indicating some adaptation at a constant intravesical pressure. Intravesical administration of SNAP (2 mM) significantly ($p < 0.05$) decreased by 33–55% the afferent firing induced by 10–40 cm H_2O pressure (Fig. 3;Table 1). The effects of SNAP were reversible after washout and repeatable. However intravesical application of SNAP (2 mM) in preparations from normal untreated rats did not significantly alter afferent firing induced by isotonic distension of the bladder at 10–40 cm H_2O pressures ($p > 0.05$, $n = 4$) (Fig. 4; Table 1).

In five additional CYP pretreated preparations afferent activity was elicited by isotonic distension of the bladder with Krebs solution at 10–40 cm H_2O pressures and SNAP (final concentration, 2 mM) was applied in the bath. SNAP significantly reduced by 40–50% the afferent firing (Fig. 5). The effects were reversible within 30 min after washout and repeatable during a second application.

2.3. Effect of NO substrate, L-arginine, on bladder afferent nerve activity

In seven CYP pretreated preparations L-arginine injected into bath (final concentration, 50 mM) significantly ($p < 0.05$) decreased the maximal afferent firing induced by isotonic bladder distension at 10–40 cm H_2O pressures by 15–52% (Figs. 6 and 7; Table 2). The inhibitory effect of L-arginine on afferent activity occurred within 5 min after injection into bath and persisted for at least 20 min. After washout with Krebs solution the bladder afferent nerve activity returned to control level. In six of these experiments when L-arginine was administered a second time at a 60 min interval after flushing the bath with Krebs solution it elicited a similar inhibitory effect on afferent firing (Fig. 6). This inhibitory effect of L-arginine on afferent nerve firing was blocked by administration of L-NAME (final concentration, 20 mM) in combination with 50 mM L-arginine (Fig. 7, $n = 7$; Table 2). However injection of L-NAME alone ($n = 7$) in the absence of L-arginine did not significantly alter afferent firing induced by isotonic distension bladder at 10–40 cm H_2O pressures. In four untreated control preparations the peak afferent nerve firing was not altered by bath application of L-arginine (final concentration, 50 mM) (Table 2).

2.4. Effects of intravesical infusion of SNAP on evoked compound action potentials in the pelvic afferent nerves

After filling the bladder with Krebs solution at the rate of 0.04 ml/min for 8 min electrical stimulation was applied with bipolar electrodes located on the serosal surface of the bladder close to the neck to activate afferent axons and evoke compound action potentials in the pelvic nerve (Fig. 8). Action potentials occurred at short and long latencies ranging 3–65 ms (Fig. 8) corresponding to axonal conduction velocities ranging from 6.6 to 0.2 m/s after correcting for the slower conduction velocity at the 27 °C bath temperature (Franz and Iggo, 1968). The largest amplitude action potentials in the recording occurred at latencies representing conduction velocities below 2 m/s. As reported previously (Yu and de Groat, 2008) the stimulus threshold for evoking action potentials in CYP pretreated preparations was significantly lower ($p < 0.05$) than in untreated preparations (Fig. 9).

Intravesical administration of SNAP (2 mM) increased the stimulus threshold by 75% (from 4.9 ± 0.5 to 8.6 ± 1 V) (Fig. 9, $n = 13$, $p < 0.01$). In addition the area of evoked action potentials including the short and long latency potentials induced at a submaximal stimulus intensity (80 V, 0.15 ms duration) was significantly decreased ($p < 0.05$, $n = 13$) approximately 45% (from 4.9 ± 0.6 μV ms to 2.7 ± 0.5 μV ms) after administration of SNAP (Fig. 9A and B).

However in untreated preparations SNAP did not change ($p > 0.05$, $n = 6$, Fig. 9) either the threshold stimulus intensity for evoking action potentials (7.5 ± 2.2 V before and 9.8 ± 1.1 V after SNAP) or the area of evoked action potentials (3.1 ± 0.6 μV ms before and 2.5 ± 0.5 μV ms after SNAP) at a submaximal stimulus intensity (80 V, 0.15 ms duration).

3. Discussion

The present study revealed that an NO donor (SNAP) or endogenous NO produced by application of high concentrations of L-arginine suppresses the firing of mechano-sensitive bladder afferent nerves evoked by phasic bladder contractions or distension of the bladder in an in vitro bladder–pelvic nerve preparation. The NO mediated suppression was detected in bladders from rats with CYP induced cystitis but not in normal bladders. The inhibitory effect occurred after either intravesical or bath application of SNAP and was not associated with a decrease in low amplitude phasic bladder contractions. SNAP also increased the threshold and decreased the area of action potentials in the pelvic nerve evoked by electrical stimulation on the serosal surface of the bladder in CYP treated bladders but not in untreated

bladders. These results indicate that NO depresses the excitability of sensitized but not normal bladder afferent nerves and that depressant effects appear to be mediated by direct effects on afferent terminals and/or axons and not indirectly by a suppression of bladder smooth muscle. Thus NO may have antinociceptive actions and also suppresses bladder hyperactivity induced by pathological conditions.

The selective inhibitory effect of NO on afferents sensitized by CYP pretreatment is consistent with reports that intravesical administration of SNAP in vivo reduces reflex bladder hyperactivity in rats with CYP-cystitis but does not alter reflex bladder activity in normal animals (Ozawa et al., 1999). These observations suggest that the mechanisms underlying the NO inhibition of afferent activity are influenced by pathological conditions. Previous studies (Yoshimura et al., 2001) revealed that NO suppresses N-type Ca^{2+} channels in dissociated bladder dorsal root ganglion cells exhibiting TTX-sensitive or TTX-resistant action potentials but does not alter the resting membrane potential, spike threshold or frequency of firing induced by depolarizing current pulses. The inhibitory effect of NO is mediated by a cyclic GMP dependent intracellular signaling pathway because it is blocked by ODQ, an inhibitor of soluble guany-late cyclase and mimicked by application of 8-bromo-cyclic GMP.

Patch clamp studies on isolated baroreceptor neurons from rat nodose ganglia revealed that NO also inhibits TTX-sensitive and TTX-resistant Na^+ currents (Bielefeldt et al., 1999; Li et al., 1998; Matsuda et al., 1995). The inhibition was not mediated by cyclic GMP but by NO interaction with channel thiols leading to a 5–7 mV hyperpolarizing shift in the steady-state, voltage-dependent inactivation of the channels. Because the neurons express nNOS and application of NOS inhibitors or NO scavengers enhance Na^+ currents it was proposed that NO and/or NO related species act as autocrine regulators of Na^+ currents in baroreceptor sensory neurons (Li et al., 1998). This speculation was supported by in vivo pharmacological experiments which revealed that baroreceptor afferent activity was modulated by exogenous NO, NO scavengers or NOS inhibitors (Matsuda et al., 1995).

The studies of baroreceptor afferents (Matsuda et al., 1995) and isolated sensory neurons from DRG (Yoshimura et al., 2001) and nodose ganglia (Bielefeldt et al., 1999) raise the possibility that NO suppresses mechanosensitive afferent firing in CYP-treated bladders by several mechanisms including modulation of Na^+ and/or Ca^{2+} channels. Suppression of Ca^{2+} currents in afferent terminals could also indirectly suppress firing by reducing the release of excitatory afferent neurotransmitters, such as neurokinins that can act in an auto-feedback manner to enhance afferent excitability (Morrison et al., 1999; Sculptoreanu et al., 2009) or that can stimulate mast cells to amplify inflammatory conditions in the bladder and release substances that sensitize afferents (Ercan et al., 2006; Saban et al., 2002).

As reported previously (Yu and de Groat, 2008) and shown in the present experiments the peak bladder afferent firing elicited by isotonic distension of the bladder was greater in CYP irritated bladders than in normal bladders (Tables 1 and 2). Because this increase can be reversed by acute administration of a purinergic receptor antagonist in the in vitro bladder preparation (Yu and de Groat, 2008) it is likely that tonically released ATP and activation of P2X receptors on afferent nerves is responsible in part for the sensitization of the bladder afferents. CYP induced bladder irritation also increases the expression of P2X receptors in bladder afferent neurons and enhances the excitatory effects of ATP on these neurons (Dang et al., 2008). Thus the selective inhibitory effects of NO in CYP irritated bladders may be due to a suppression of purinergic excitatory mechanisms in bladder sensory pathways. ATP is released by urothelial cells in response to bladder distension or chemical stimulation (Birder et al., 2003; Ferguson et al., 1997; Kullmann et al., 2008) and is believed to act on adjacent suburothelial afferent nerves to enhance their excitability (Birder et al.,

2008;Cockayne et al., 2005; de Groat et al., 1999; Kullmann et al., 2008; Rong et al., 2002; Vlaskovska et al., 2001). Because exogenous NO is known to affect the properties of the urothelium (Birder et al., 2008), an action of NO in the present experiments on the urothelium to interrupt urothelial-afferent signaling might also contribute to the inhibition of afferent firing.

A recent study from another lab (Aizawa et al., 2011) conducted in vivo in urethane anesthetized female rats indicated that intravenous administration of a large dose of L-arginine (300 mg/kg) suppressed the firing of Aδ and C-fiber bladder afferents evoked by intravesical infusion of saline or a chemical irritant, acrolein. The suppressant effects of L-arginine were blocked by pretreatment with L-NAME, a NOS inhibitor. It was concluded that endogenous NO can suppress normal as well as chemically sensitized myelinated and unmyelinated bladder afferents. Our data showing that SNAP increases the electrical threshold for evoking action potentials in myelinated Aδ pelvic afferent axons and suppresses the late component of evoked discharges (elicited by high intensity stimulation) mediated by C-fiber afferent axons is consistent with the report (Aizawa et al., 2011) that both types of bladder afferents are sensitive to NO. However our finding that afferent activity in non-irritated bladders is resistant to SNAP or L-arginine is not consistent with the in vivo data (Aizawa et al., 2011), raising the possibility that different experimental conditions (eg., anesthesia), or actions of L-arginine at other sites in vivo (eg., efferent nerves or spinal cord) that could indirectly alter afferent activity may have been involved in the action of NO on normal afferent nerves. A change in bladder compliance which was observed in the in vivo experiments after L-arginine administration (Aizawa et al., 2011) may be a reflection of this indirect action.

In the present study bath application of L-NAME did not alter peak afferent firing induced by isotonic distension of the bladder at a range of pressures (10–40 cm H_2O), indicating that the basal release of endogenous NO did not reach sufficient concentrations to modulate afferent nerve activity. This conclusion is consistent with an in vivo study (Masuda et al., 2007) showing that intravesical administration of L-NAME did not alter reflex activity of normal bladders. However in the same study L-NAME enhanced the bladder excitatory effect of intravesically administered capsaicin (Masuda et al., 2007), suggesting that endogenous NO, possibly released by capsaicin (Birder et al., 1998), inhibits the activation of capsaicin sensitive C-fiber afferent nerves. On the other hand, in the experiments of Aizawa et al., 2011 intravesical administration of L-NAME in urethane anesthetized rats enhanced the firing of Aδ and C-fiber bladder afferents during saline infusion suggesting that NO mediates a tonic inhibitory effect on afferents in the absence of bladder overactivity. The different results of the two in vivo experiments may be related to different recording conditions, i.e., reflex bladder activity versus afferent nerve recording. Experiments in other tissues (intestine, knee joint, carotid sinus) provide evidence that endogenous NO tonically modulates the activity of normal as well as sensitized afferent nerves (Aley et al., 1998; Beyak, 2010; Bielefeldt et al., 1999;Kelly et al., 2001; Matsuda et al., 1995; Page et al., 2009; Patil et al., 2004).

The suppression of electrically evoked afferent firing by intravesically administered SNAP occurred in CYP-treated preparations but not in normal preparations providing additional evidence that NO inhibition is unmasked in sensitized afferents. However the location of the sensitization is uncertain, because current from the stimulation electrodes on the serosal surface of the bladder, could excite axons on the bladder surface but also could pass through the bladder wall to excite intramural axons or even the afferent nerve terminals near the urothelium. CYP irritation reduced the electrical threshold for eliciting action potentials, a change that would be expected to occur near the lumenal surface because acrolein, the irritant metabolite of CYP, is carried in the urine (Lanteri-Minet et al., 1995). In addition

intravesical administration of SNAP which is likely to selectively target suburothelial afferent nerves was effective in increasing the electrical threshold and decreasing the area of the evoked action potentials also suggesting that the action potentials were generated near the lumenal surface. Mucosal afferents in the gastrointestinal tract are also tonically inhibited by endogenous NO, while afferents with receptors in the muscle layers are not inhibited (Page et al., 2009).

Although the physiological role of NO as a modulator of normal bladder sensory mechanisms is still unclear the evidence for such a role of NO in pathological conditions is stronger (Levy and Strassman, 2004; Lin et al., 2007; Lundberg et al., 1996; Sasatomi et al., 2008). As mentioned above, endogenous NO is released by urothelial cells; and this release could be increased in pathological conditions by upregulation of urothelial NOS expression (Birder et al., 2005). In addition NO may be released from bladder afferent nerves in pathological conditions, because nNOS which is not detectable in normal bladder afferent neurons (Vizzard et al., 1994, 1995, 1996; Vizzard, 1997) is markedly increased after CYP irritation of the bladder (Vizzard et al., 1996), or after spinal cord injury (Vizzard, 1997) or stimulation of TRPV1 receptors with capsaicin (Vizzard et al., 1995, 1996). Thus NO synthesized and released by afferent nerves as well as by adjacent urothelial cells may act in an autocrine and paracrine manner to provide an inhibitory modulation of sensitized bladder afferents that would reduce painful sensations as well as bladder overactivity in pathological conditions.

4. Experimental procedures

4.1. Experimental preparation

Male Sprague Dawley rats (100–170 g, $n = 47$) were anesthetized with ketamine (50 mg/kg, i.m.); and the urinary bladder, urethra, prostate gland, seminal vesicles along with the attached pelvic nerves and the major pelvic ganglia were removed and placed in a 20 ml bath perfused with Krebs solution (1 ml/min) at a temperature of 27 °C and continuously bubbled with 95% O_2 and 5% CO_2. A low temperature was used to prolong the survival of the in vitro preparation. The Krebs solution had the following composition (mM/L): NaCl 128, KCl 1.8, $NaHCO_3$ 22, KH_2PO_4 1.5, $MgSO_4$ 1.3, glucose 10, $CaCl_2{\cdot}2H_2O$ 0.4, H_2O_2 0.4 at pH 7.4. A catheter (PE50) which was inserted through the urethra into the bladder was connected to an infusion pump to infuse solutions intravesically and to a pressure transducer to monitor bladder activity. The pelvic nerve on one side was placed in an adjacent chamber filled with paraffin oil and positioned on silver bipolar electrodes for recording multiunit afferent nerve activity. Standard electrophysiological methods (Yu and de Groat, 2008) were used to amplify and analyze the records.

4.2. Nerve recording

Afferent activity was elicited by intravesical infusion of Krebs solution at the rate of 0.04 ml/min for 8 min which also evoked rhythmic contractions of the bladder smooth muscle. After bladder filling multiunit afferent activity was measured (spikes/s) for 10 min using a pulse height discriminator-ratemeter and displayed on a rectilinear paper recorder and also recorded on a VCR for later off-line analysis. Afferent activity was also evoked by isotonic distension of the bladder with Krebs solution for 30 s periods at pressures of 10, 20, 30 and 40 cm H_2O. Chemicals were administered intravesically and/or into the bath.

Multiunit compound action potentials were also elicited by electrical stimulation (1 Hz, 0.15 ms pulse duration) using a pair of silver electrodes (diameter: 0.25 mm) positioned on the serosal surface close to the neck of the bladder (Yu and de Groat, 2010). Individual responses were averaged ($n = 5$) with a computer (Lab View program, National Instrument

Company). Based on the latencies of evoked action potentials in the pelvic nerve and the distance between stimulus and recording sites the conduction velocities of the afferent nerves were calculated. The distance between stimulating and recording electrodes ranged between 11 and 13 mm. Changes in the electrically evoked action potentials were studied after intravesical infusion (rate of 0.04 ml/min for 8 min) or following isotonic bladder distention with: (1) Krebs solution, (2) a single drug solution or (3) a solution containing a combination of drugs. In some experiments (n = 14) CYP 100 mg/kg was injected intraperitoneally 17 h prior to experiments to induce cystitis (Lanteri-Minet et al., 1995). All procedures utilized in this study were approved by University of Pittsburgh, Institutional Animal Care and Use Committee.

4.3. Analysis of data

Multiunit recordings of afferent activity are presented as peak firing frequency in spikes/s recorded under isovolumetric or isotonic conditions. The resting activity of afferent nerves was measured for 1 min before the start of bladder filling. The Lab View program (National Instrument Company) was used to analyze the area of evoked action potentials. All data are expressed as mean ± SE. Results were evaluated using two-way ANOVA followed by Bonferroni post tests using Prism 4 program (GraphPad software Inc, San Diego, CA).

4.4. Drugs

S-nitroso-N-acetyl-penicillamine (SNAP, Sigma) was dissolved in 0.5% DMSO solution at the concentration of 4 M and applied to the bath in a volume of 10 μl to produce a final bath concentration of 2 mM. For intravesical administration SNAP was dissolved in 0.5% DMSO and infused in a concentration of 2 mM. Application of the DMSO vehicle did not affect afferent firing or bladder activity. L-arginine (Sigma) and NG-Nitro-L-arginine methyl ester hydrochloride (L-NAME, Sigma) were dissolved in Krebs solution. Cyclophosphamide (Sigma) was dissolved in distilled water at a 40 mg/ml concentration for intraperitoneal injection.

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Abbreviations

NO	nitric oxide
NOS	nitric oxide synthase
SNAP	S-nitroso-N-acetyl-penicillamine
BAN	bladder afferent nerve
AP	action potential
DRG	dorsal root ganglia
CYP	cyclophosphamide
PBS	phasic bladder contractions
L-NAME	NG-Nitro-L-arginine methyl ester hydrochloride

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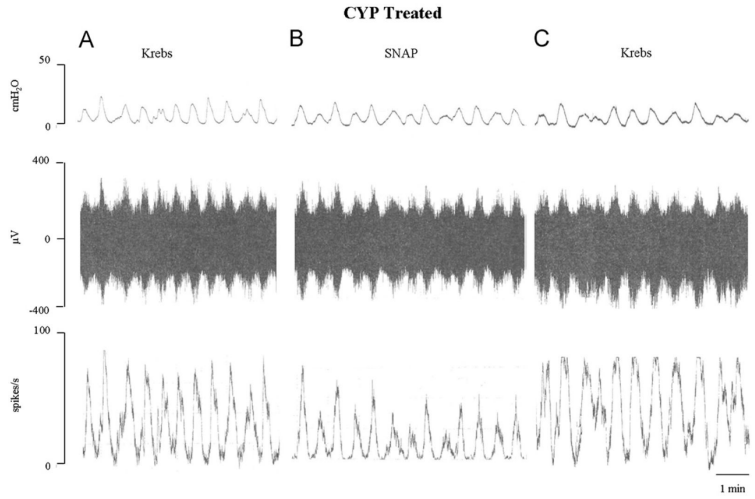

Fig. 1.

Inhibitory effects of intravesical administration of SNAP (2 mM) on bladder afferent nerve firing induced by intravesical infusion of Krebs solution at the rate of 0.04 ml/min for 8 min in a CYP pretreated preparation. The top traces represent bladder contractile activity measured as intravesical pressure in cm H_2O, the middle traces represent afferent nerve firing (μV) and the bottom traces represent ratemeter recording of afferent firing (spikes/s). All records were obtained in the same preparation with 30–60 min between recordings. (A) Control recording 10 min after filling the bladder with Krebs solution. (B) Intravesical infusion of SNAP induced a slow decline in afferent firing. (C) Afferent firing recovered after washout of SNAP with Krebs solution. Horizontal calibration represents 1 min.

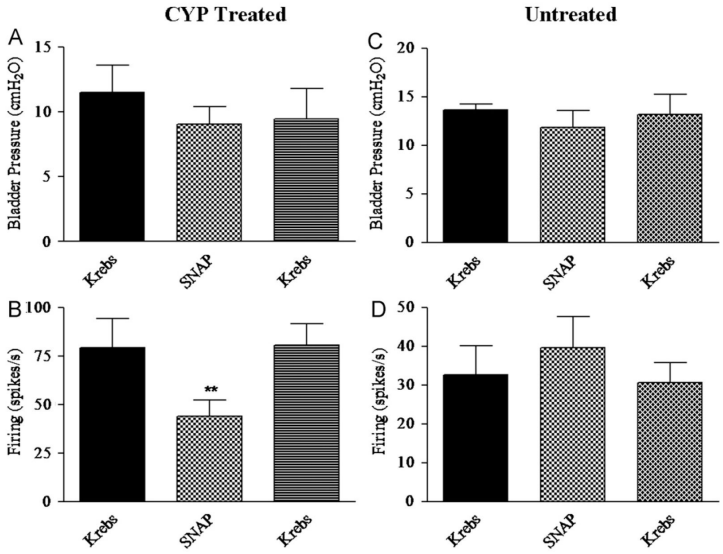

Fig. 2.

Effects of SNAP (2 mM) administered by intravesical infusion (0.04 ml/min for 8 min) on the peak amplitude (cm H_2O) of phasic bladder activity (A and C) and bladder afferent firing (spikes/s) (B and D) from CYP pretreated (A and B) and untreated rats (C and D). SNAP did not change bladder pressures in either CYP pretreated and untreated preparations. However SNAP significantly (** $p < 0.05$, $n = 14$) decreased pelvic nerve afferent firing in CYP pretreated preparations (B) but not in untreated preparations (D).

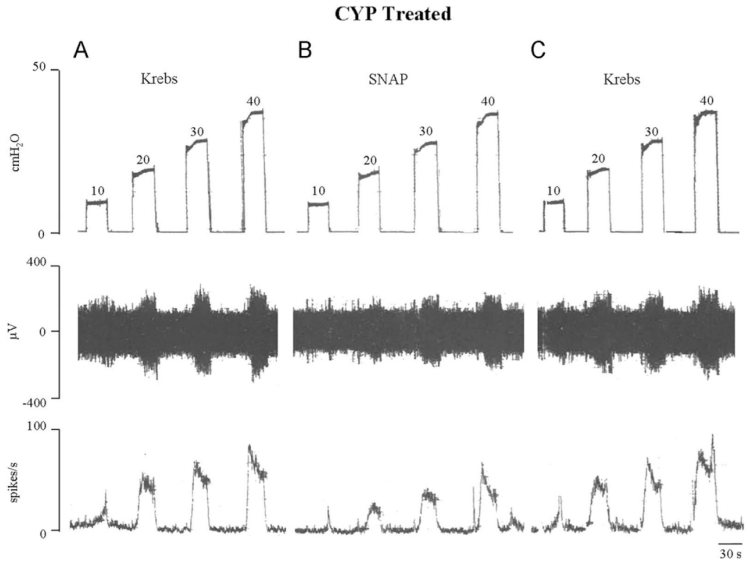

Fig. 3.

Effects of SNAP (2 mM) on bladder afferent nerve firing induced by isotonic distention of the bladder at 10, 20, 30, and 40 cm H_2O in a CYP pretreated preparation. All records were obtained in the same preparation with 30–60 min between recordings. Top traces are intravesical pressure in cm H_2O; middle traces show afferent nerve firing and bottom traces represent ratemeter recording of afferent firing. (A) Bladder distention with Krebs solution. (B) Decreased distention evoked afferent firing after intravesical infusion of SNAP (2 mM). (C) Afferent firing recovered after washout of SNAP. Vertical calibrations are the same as in Fig. 1. Horizontal calibration is 30 s.

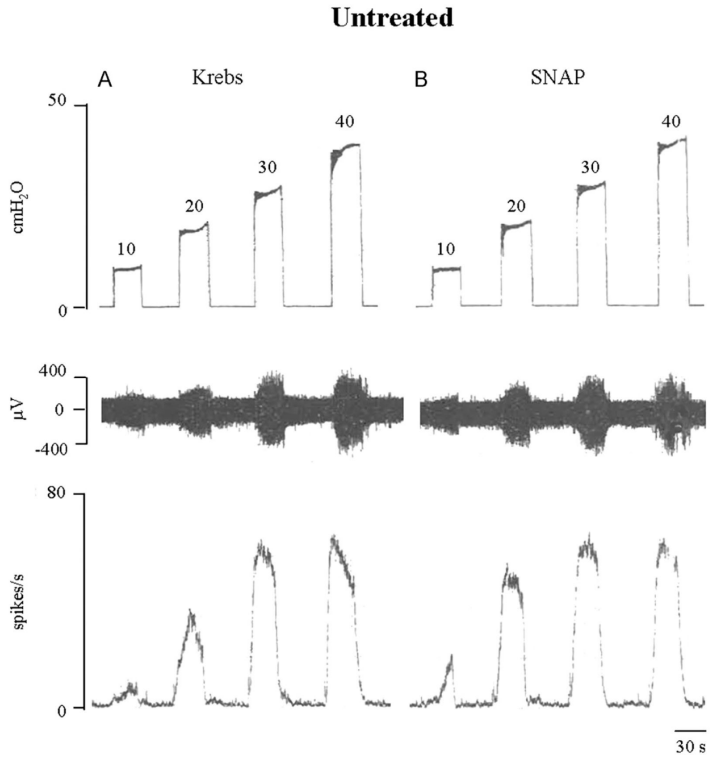

Fig. 4.

Effects of SNAP (2 mM) on bladder afferent nerve firing induced by isotonic distention of the bladder at 10, 20, 30, and 40 cm H_2O in an untreated preparation. Top traces are intravesical pressure in cm H_2O; middle traces show afferent nerve firing and bottom traces represent ratemeter recording of afferent firing. (A) Distention of the bladder with Krebs solution. (B) Intravesical infusion of SNAP did not alter afferent firing. Calibrations are the same as in Fig. 3.

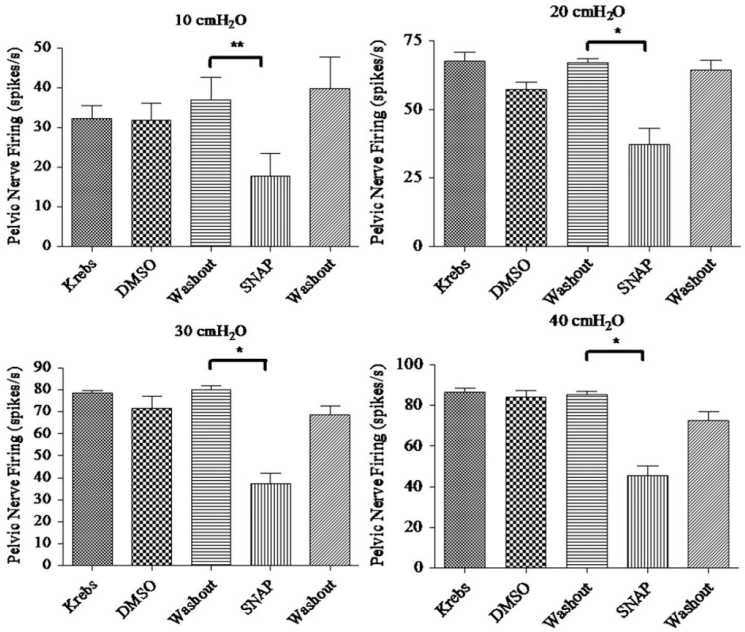

Fig. 5.

Effects of bath application of SNAP (2 mM final concentration) and vehicle (0.5% DMSO) on bladder afferent firing induced by isotonic distention of the bladder with Krebs solution at pressures of 10–40 cm H_2O in CYP pretreated preparations. SNAP decreased pelvic nerve firing (n=5, **$p < 0.01$, *$p < 0.05$) but DMSO had no effect. The depressant effect of SNAP was reversed 30 min after washout.

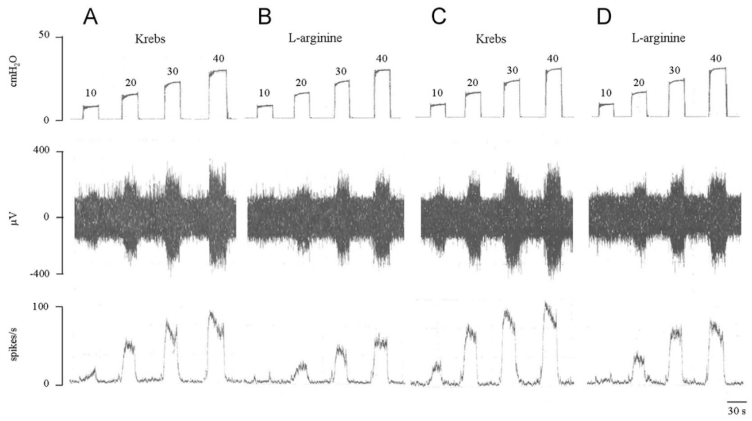

Fig. 6.

Effects of repeated administration of L-arginine (50 mM) on bladder afferent nerve firing induced by isotonic distention of the bladder at 10, 20, 30 and 40 cm H_2O with Krebs solution. All records were obtained in the same CYP pretreated preparation with 30–60 min between recordings. Top traces are intravesical pressure in cm H_2O; middle traces show afferent nerve firing and bottom traces represent ratemeter recording of afferent firing. (A) Control responses induced by bladder distention when the bath was perfused with Krebs solution prior to L-arginine administration. (B) Decreased afferent nerve firing after administration of L-arginine into the bath (50 mM, final concentration). (C) After washout of L-arginine. (D) After second administration of L-arginine. Calibrations are the same as in Fig. 3.

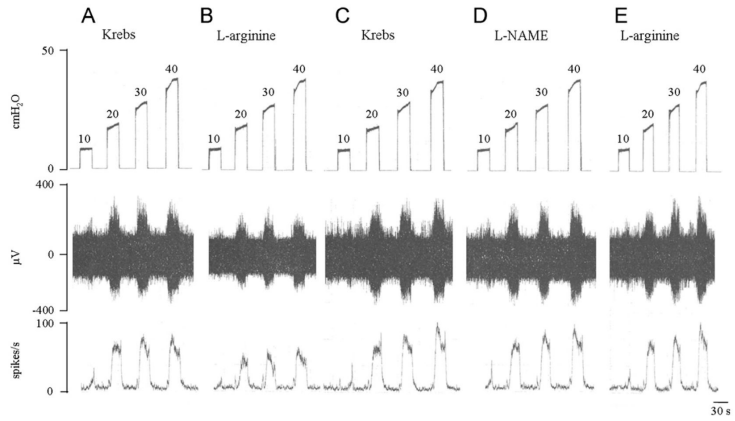

Fig. 7.

L-NAME administered in the bath (20 mM, final concentration) blocks the depression of bladder afferent firing elicited by L-arginine administered in the bath (50 mM, final concentration). All records were obtained in the same CYP pretreated preparation with 30–60 min between recordings. Afferent firing in all recordings was induced by isotonic distention of the bladder with Krebs solution. Top traces are intravesical pressure in cm H_2O; middle traces are afferent nerve firing and bottom traces are ratemeter recording of afferent firing. (A) Control responses induced by bladder distention when the bath was perfused with Krebs solution prior to L-arginine administration. (B) Decreased afferent nerve firing after administration of L-arginine into the bath (50 mM, final concentration). (C) After washout of L-arginine. (D) After administration of L-NAME afferent firing was not changed. (E) The second administration of L-arginine in the presence of L-NAME did not suppress afferent nerve firing. Calibrations are the same as in Fig. 3.

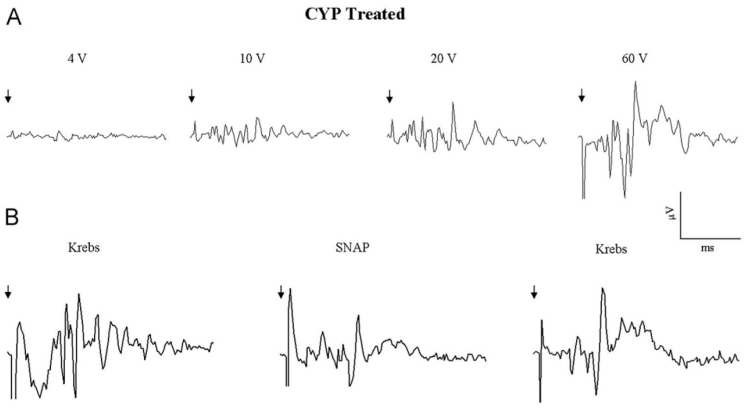

Fig. 8.

(A) Electrical stimulation (indicated by arrows) on the serosal surface of the urinary bladder with a range of stimulus intensities (4–60 V) elicits action potentials on the pelvic nerve in a CYP pretreated preparation. Evoked potentials represent computer averages of five individual records. (B) Action potentials elicited on the pelvic nerve by submaximal (1 Hz, 80 V, 0.15 ms) electrical stimulation of the urinary bladder are suppressed (middle tracing) 10 min after intravesical administration of SNAP (2 mM). Control responses (left and right tracings) were obtained during intravesical infusion of Krebs solution before and after administration of SNAP. Records in (A and B) were obtained in different preparations. Vertical and horizontal calibrations represent 400 μV and 25 ms, respectively, in (A) and 400 μV and 20 ms in (B).

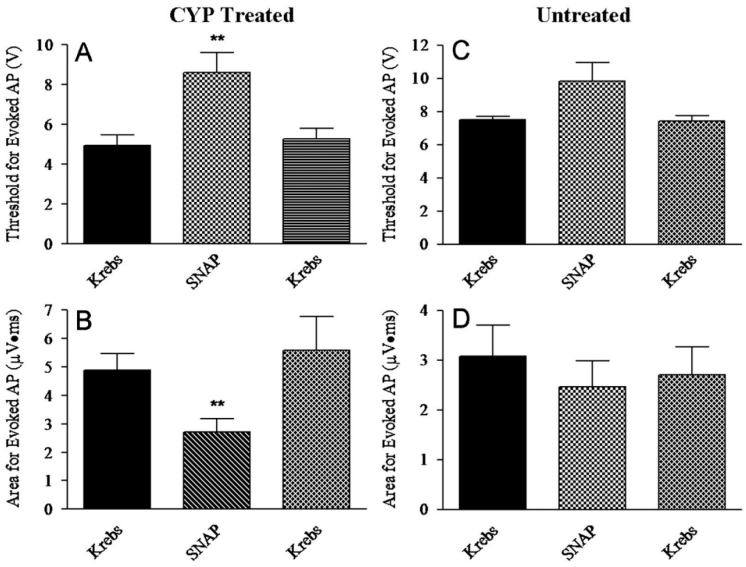

Fig. 9.

Effects of intravesical infusion of SNAP (2 mM) on the stimulus threshold (A and C) and the area (B and D) of the electrically evoked action potentials recorded on the pelvic nerve in CYP pretreated preparations (A & B) and in untreated preparations (C and D). All measurements were obtained from computer averages of five individual evoked responses. SNAP increased threshold for evoked action potentials (A) and decreased area for evoked action potentials (B) in CYP pretreated preparations ($n = 14$, $p < 0.01$), but did not change these parameters in untreated preparations ($n = 6$, $p > 0.05$).

Table 1

Effects of SNAP (2 mM) administered by intravesical infusion on bladder afferent firing (spikes/s) induced by isotonic distention of the bladder in untreated and CYP treated preparations.

Bladder pressure (cm H_2O)	Untreated (n = 4)			CYP treated (n = 4)		
	Krebs	SNAP	Washout	Krebs	SNAP	Washout
10	25±7	24±6	27±7	29±4	13±2 **	27±7
20	51±11	50±10	52±9	67±1	35±5 *	70±6
30	57±10	54±11	62±10	84±3	46±10 *	82±8
40	67±2	63±3	64±5	89±4	59±12 *	88±8

Note: The numbers of experiments is shown in parentheses. Data are mean±SE of peak afferent firing.

* Afferent activity is significantly less than firing in Krebs solution $p < 0.05$.

** Afferent activity is significantly less than firing in Krebs solution $p < 0.01$.

Table 2

Effects of L-arginine (50 mM) on bladder afferent firing (spikes/s) induced by isotonic distention of the bladder in untreated and CYP treated preparations.

Bladder pressure (cm H_2O)	Untreated (n = 4)		CYP treated (n = 7)		
	Krebs	L-arginine	Krebs	L-arginine	L-arginine+L-NAME
10	24±8	18±7	27±3	13±3**	25±3
20	49±11	44±11	70±9	44±8**	74±10
30	67±8	64±9	80±5	54±6**	84±7
40	70±8	68±10	92±6	69±5*	94±8

Note: The number of experiments is shown in parentheses.

Data are mean±SE of peak afferent firing.

*Afferent activity is significantly different than firing in Krebs solution $p < 0.05$.

**Afferent activity is significantly different than firing in Krebs solution $p < 0.01$.