Activation of the nitric oxide-cGMP pathway reduces phasic contractions in neonatal rat bladder strips via protein kinase G

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Am J Physiol Renal Physiol 297: F333–F340, 2009. First published June 3, 2009; doi:10.1152/ajprenal.00207.2009.—Nitric oxide (NO), a neurotransmitter in the lower urinary tract, stimulates soluble guanylyl cyclase (sGC) and in turn cGMP-dependent protein kinase G (PKG) to modulate a number of downstream targets. NO donors reduce bladder hyperactivity in some pathological models but do not affect normal bladder activity in the adult rat. In this study, the NO donor S-nitroso-N-acetyl-DL-penicillamine (SNAP; 100 μM) decreased the amplitude and frequency of spontaneous and carbachol-enhanced contractions in neonatal rat bladder strips, which are intrinsically hyperactive. This effect was blocked by inhibition of sGC and mimicked by application of a membrane-permeable cGMP analog (8-bromo-cGMP, 100 μM). Inhibition of PKG prevented or reversed the inhibitory effects of 8-bromo-cGMP. A portion of the SNAP-mediated inhibition was also dependent upon PKG; however, a short-lasting, sGC-dependent inhibitory effect of SNAP was still present after PKG inhibition. Inhibition of NO synthase with L-NAME (100 μM) did not change the amplitude or frequency of contractions. However, inhibition of endogenous phosphodiesterase (PDE)-5 with zaprinast (25 μM) reduced the amplitude and frequency of phasic contractions and increased the magnitude of inhibition produced by maximal concentrations of SNAP, suggesting that endogenous PDEs are constitutively active and regulate cGMP production. These results suggest that the NO-cGMP-PKG pathway may be involved in inhibitory control of the neonatal rat bladder.

NITRIC OXIDE (NO) has an important physiological role as a neurotransmitter in the peripheral and central nervous systems and as a vasodilator substance released from endothelial cells. NO, which is synthesized by nitric oxide synthase (NOS), activates soluble guanylyl cyclase (sGC) to produce cGMP. cGMP in turn activates cGMP-dependent protein kinase (PKG), which regulates many cellular processes including smooth muscle relaxation and ion channel function (20).

In the lower urinary tract, NO has the potential to function as a transmitter at various sites because NOS is expressed in afferent and efferent nerves, as well as in the urothelium, smooth muscle, and striated muscle (19). NO acts as an inhibitory neurotransmitter in the urethra, where it is released from parasympathetic nerves; however, its function in the urinary bladder is uncertain and seems to vary in different species. For example, NO donors abolish rhythmic activity induced by muscarinic stimulation in the mouse whole-bladder preparation (23), but increase the frequency of phasic activity in a guinea pig whole-bladder preparation (14). On the other hand, the contractile activity of the rat and rabbit bladder seems to be resistant to NO donors (1, 9, 25). Exposure to NO donors increases the levels of cGMP in serosal and intramuscular interstitial cells in the mouse bladder, and in interstitial cells in the guinea pig bladder located near the serosal surface, between muscle bundles and in the suburothelium (15–17, 22, 23).

In adult rats, where the detrusor muscle is thought to be comparatively insensitive to NO (32), several studies have demonstrated an effect of NO on reflex bladder activity in vivo. For example, in rats, intravesical administration of an NO donor suppresses cyclophosphamide-induced bladder hyperactivity (29), while the NO scavenger oxyhemoglobin induces bladder overactivity (30), likely to be due to a reduction of an inhibitory effect of NO generated in the urothelium (3, 4). In addition, the amplitude and number of nonvoiding contractions in chronic spinal cord-injured rats are decreased by treatment with an arginine inhibitor (35). This effect is prevented by treatment with a NOS inhibitor, demonstrating that the effect of the arginase inhibitor is due to increased NO production.

The neonatal rat bladder is intrinsically overactive during the first 3–4 wk of life (37). During this time, bladder activity is characterized by high-amplitude low-frequency spontaneous contractions that are likely to be myogenic, as they occur in the absence of nerve stimulation and are not blocked by inhibiting nerve activity with TTX (39). Later, during postnatal development, the spontaneous bladder activity is reduced to a low-amplitude high-frequency pattern characteristic of the normal adult rat. Spontaneous activity in the neonatal rat bladder is hypothesized to be initiated at the bladder dome (21) or the bladder neck region (39) and propagated throughout the detrusor muscle by a network of interstitial cells interconnected by gap junctions (21). Activity arising in the urothelium and/or lamina propria may contribute to the generation of the spontaneous contractions. These spontaneous contractions are thought to be under tonic inhibitory control by the central nervous system because in the neonatal rat spinal cord-bladder preparation, application of TTX or removal of the spinal cord increases spontaneous activity (38). This inhibition may be important for maintaining urinary continence in the pup and allowing the mother to control voiding via the perineal-to-bladder excitatory reflex. However, the neurotransmitter mediating the inhibition has yet to be identified.

This study investigated the effect of the NO-cGMP pathway on spontaneous bladder contractions in neonatal rat bladder strips to evaluate the possibility that NO has a role in the putative neural....

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inhibition of the neonatal bladder. The results demonstrate that activation of the NO-cGMP pathway inhibits spontaneous and carbachol-enhanced contractions in neonatal rat bladder strips, suggesting that NO may be an inhibitory neurotransmitter in the bladder during early postnatal development.

METHODS

Bladder strip preparation. All experimental procedures were approved and performed in accordance with the Institutional Animal Care and Use Committee of the University of Pittsburgh. Bladder strips from male and female neonatal (10–21 days old) Sprague-Dawley rats were prepared as described previously (39). Briefly, the bladder was removed from isolufurane (4% in O2)-anesthetized rats, placed in warm Krebs solution [composition (in mM): 118 NaCl, 4.7 KCl, 1.9 CaCl2, 1.2 MgSO4, 24.9 NaHCO3, 1.2 KH2PO4, and 11.7 dextrose, pH 7.4, bubbled with 95% O2-5% CO2], and cut into two to four longitudinal strips (~1.5 × 8–10 mm). Strips were tied with fine thread at each end and mounted in a vertical double-jacketed organ bath in oxygenated Krebs solution (15-ml volume) kept at 37°C via a circulating warm water bath. The tissue was allowed to equilibrate for 1–2 h before drug testing. Drugs from concentrated stock solutions were added directly to the organ bath. Because the amplitude and frequency of spontaneous contractions vary over several hours, in most experiments, 50–200 nM carbachol was applied to each strip to enhance spontaneous contractions without increasing baseline tension (28). This stabilized bladder strip activity so that contractions and the effects of drugs could be measured over several hours. After setting of baseline tension to 10 mN (1 g), spontaneous and carbachol-enhanced contractions were measured with a force displacement transducer (Grass, Astromed, West Warwick, RI). Data, including baseline tone and amplitude and frequency of spontaneous contractions, were recorded for offline analysis using Windaq software (DATAQ Instruments, Akron, OH) and analyzed using Excel (Microsoft, Redmond, WA) and Origin (version 7; Origin Lab, Northampton, MA).

Drugs used in this study include S-nitroso-N-acetyl-L-cysteine (SNAP), an NO donor; 8-bromo-cGMP, a cGMP analog; N\(^\text{-}\)nitro-L-arginine methyl ester hydrochloride (L-NAME), a NOS inhibitor; 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), an inhibitor of soluble guanylyl cyclase; Rp-8-bromo-PET-cGMPS hydrate (Rp-cGMPS), a cGMP-dependent protein kinase (PKG) inhibitor; zaprinast, a phosphodiesterase (PDE) 5 inhibitor; and carbac- chol, a cholinergic receptor agonist. Zaprinast, Rp-cCMPS and 8-bromo-cGMPS hydrate (Rp-cGMPS), a cGMP-dependent protein kinase (PKG) inhibitor; zaprinast, a phosphodiesterase (PDE) 5 inhibitor; and carba-

RESULTS

Activation of the NO-cGMP pathway inhibits carbachol-enhanced spontaneous contractions in neonatal rat bladder strips. Bladder strips from neonatal rats (10–21 days of age) exhibited high-amplitude, low-frequency spontaneous contrac-

Fig. 1. S-nitroso-N-acetyl-l-cysteine (SNAP) and 8-bromo-cGMP reduce the amplitude and frequency of carbachol-enhanced spontaneous contractions (SC\(_{\text{carb}}\)). A: carbachol-induced enhancement of spontaneous activity in a bladder strip from neonatal rat. Arrow indicates time of carbachol (100 nM) application. B: concentration dependence of SNAP-mediated inhibition of SC\(_{\text{carb}}\) amplitude (n = 7 for each concentration). C and E: inhibition of SC\(_{\text{carb}}\) by 100 μM SNAP (C) and 100 μM 8-bromo-cGMP (E). D and F: summary data showing average effect of 100 μM SNAP (n = 15) and 100 μM 8-bromo-cGMP (n = 13) on SC\(_{\text{carb}}\) amplitude (D) and frequency (F). G: summary of inhibition of SC\(_{\text{carb}}\) amplitude by 2 consecutive applications of 100 μM SNAP followed by 1 application of 100 μM 8-bromo-cGMP (n = 12). *P < 0.05 compared with control. #P < 0.05 between groups. **P < 0.05 compared with maximum SNAP inhibition by 2-tailed t-test with layered Bonferroni correction.
Table 1. Effects of SNAP and 8-bromo-cGMP on carbachol-enhanced neonatal bladder strip activity

<table>
<thead>
<tr>
<th></th>
<th>Amplitude of SC&lt;sub&gt;carb&lt;/sub&gt;</th>
<th>Frequency of SC&lt;sub&gt;carb&lt;/sub&gt;</th>
<th>Inhibition of Amplitude</th>
<th>Inhibition of Frequency</th>
</tr>
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<tbody>
<tr>
<td>100 µM SNAP (n = 15)</td>
<td>33.2±4.3%*</td>
<td>10.25±6.2%*</td>
<td>14.9±4.7%*</td>
<td>3.6±10.8%</td>
</tr>
<tr>
<td>100 µM 8-bromo-cGMP (n = 13)</td>
<td>55.2±2.5%*</td>
<td>28.2±3.8%*</td>
<td>49.2±3.2%*</td>
<td>28.8±3.9%*</td>
</tr>
</tbody>
</table>

Values are means ± SE expressed as percentage of drug-induced reduction measured as percentage of control; n = no. of strips. SNAP, S-nitroso-N-acetyl-L-penicillamine; SC<sub>carb</sub>, carbachol-enhanced spontaneous contractions. *P < 0.05 vs. control by Student’s t-test with layered Bonferroni corrections when multiple comparisons were made.

Table 2. Effects of SNAP and 8-bromo-cGMP on neonatal bladder strip activity

<table>
<thead>
<tr>
<th></th>
<th>Amplitude of SC&lt;sub&gt;carb&lt;/sub&gt;, 2nd Drug Application</th>
<th>Amplitude of Spontaneous Contractions</th>
<th>Frequency of Spontaneous Contractions</th>
</tr>
</thead>
<tbody>
<tr>
<td>100 µM SNAP</td>
<td>38.6±7.6%* (n = 13)</td>
<td>54.5±10.5%* (n = 6)</td>
<td>38.7±13.6%* (n = 6)</td>
</tr>
<tr>
<td>100 µM 8-bromo-cGMP</td>
<td>53.3±9.15%* (n = 12)</td>
<td>39.2±7.4%* (n = 5)</td>
<td>39.9±11.1%* (n = 5)</td>
</tr>
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</table>

Values are means ± SE expressed as percentage of drug-induced reduction measured as percentage of control; n = no. of strips. *P < 0.05 vs. control by Student’s t-test with layered Bonferroni corrections when multiple comparisons were made.
In addition, zaprinast increased the duration of SNAP-mediated inhibition. In the presence of zaprinast, contraction amplitude was inhibited by 41.5 ± 5.6% 60 min after SNAP application, while in the absence of zaprinast contraction amplitude had completely recovered at this time (Fig. 5D, P < 0.05). Zaprinast pretreatment did not alter the 8-bromo-cGMP-mediated inhibition of SCcarb amplitude (50.8 ± 6.7% inhibition alone vs. 44.7 ± 8.7% inhibition after zaprinast, P > 0.05). The maximal inhibition elicited by SNAP after zaprinast was similar to that induced by 8-bromo-cGMP (Fig. 5F). Zaprinast (25 μM) alone elicited a small but significant reduction in SCcarb frequency (11.8 ± 2.3%, P < 0.05) but did not change the effect of SNAP on SCcarb frequency (Fig. 5E).

**DISCUSSION**

The present results indicate that the frequency and amplitude of rhythmic smooth muscle activity in bladder strips of neonatal rats are sensitive to the inhibitory effects of an intracellular sGC-cGMP-PKG signaling pathway that can be activated by an exogenously administered agent that generates NO. The inhibitory pathway is modulated by PDE-5 activity. Zaprinast, a PDE-5 inhibitor, enhanced the inhibitory effect of SNAP and produced a small inhibitory effect when applied alone, suggesting that low levels of cGMP are present in the tissue. However, the full inhibitory pathway does not seem to be tonically active under the conditions of our experiments, in which bladder nerves are quiescent. Still, bladder nerves that express neuronal NOS are presumably able to synthesize and release NO when activated. Thus the present experiments raise the possibility that NO might play a role in the previously identified neural-inhibitory pathway that regulates spontaneous contractions in the in vitro spinal cord-urinary bladder preparation of the neonatal rat (38).

**Signaling pathway.** In the present study, an NO donor, SNAP, reduced the amplitude and frequency of spontaneous contractions as well as carbachol-enhanced contractions in neonatal rat bladder strips. ODQ completely blocked the effects of SNAP, indicating that the effects of NO were mediated via activation of sGC. The cGMP analog 8-bromo-cGMP mimicked the inhibitory effect of SNAP but elicited a slower-onset and longer-lasting response, consistent with the slow...
passage of the large molecule across the plasma membrane, and its resistance to metabolism by endogenous phosphodiesterases. Treatment with zaprinast, a PDE-5 inhibitor, increased the efficacy and duration of action of SNAP such that it produced effects similar to those of 8-bromo-cGMP. This suggests a role for endogenous PDE-5 in regulating the NO signaling pathway in the neonatal bladder.

Pretreatment of bladder strips with a PKG inhibitor reduced SNAP inhibition and completely prevented or reversed 8-bromo-cGMP inhibition of SCAmb amplitude, suggesting that the effects of cGMP are dependent upon PKG. An early component of SNAP-induced inhibition that was resistant to PKG inhibition was short-lived, lasting only 10 min. Taken together, these data suggest that the classic NO-cGMP-PKG pathway can regulate bladder activity in the neonatal rat and provide evidence for a second inhibitory pathway initiated by NO that is sGC dependent but PKG independent.

The inhibitory effect of NO on neonatal bladder strips contrasts with the lack of effect of NO on in vitro bladder preparations from adult rats and other species including human, pig, and rabbit (2, 9, 25). Thus the sensitivity to NO-mediated inhibition may be lost during postnatal development. This suggests that either the sGC-PKG signaling pathway is downregulated or that the smooth muscle contractile mechanisms become resistant to this pathway. The data in this paper are insufficient to distinguish between these two possibilities.

Therefore, further studies of the mechanisms underlying NO-mediated inhibition in the neonatal rat during development and adult bladder are needed to address this question.

Site of action of NO. There are several possible targets of NO-cGMP signaling in the neonatal rat bladder preparation. Experiments in other species (e.g., guinea pig and mouse) in which bladder activity is sensitive to NO might provide insights into the mechanism of action of NO. For example, in guinea pig bladder, an NO donor increases the levels of cGMP in interstitial cells/myofibroblasts (15) and 8-bromo-cGMP reduces contractile activity (40). Interstitial cells, which communicate via gap junctions, are thought to underlie the propagation of rhythmic contractile activity through the bladder wall. Since NO can cause uncoupling of neuronal gap junctions (31), it is possible that NO suppresses phasic contractions by inhibiting the propagation of signals through the interstitial cell network in the bladder. Our data are consistent with an effect on propagation as the frequency as well as the amplitude of spontaneous activity were reduced by NO.

NO might also act directly on the smooth muscle. One possible NO target within smooth muscle is Rho kinase. PKG phosphorylates and inactivates RhoA, a G protein that activates Rho kinase (36). Rho kinase inhibition reduces the peak and sustained components of carbachol-induced contractions in mouse bladder (8) and decreases bladder tone (34). Alternatively, NO may target various types of potassium channels. For
example, in guinea pig bladder myocytes, an NO donor opens ATP-sensitive K channels in a cGMP-dependent manner (7). Large-conductance Ca$^{2+}$/H$^{+}$-activated K channels, which are known to regulate spontaneous activity in the neonatal rat bladder (18, 28) and which are activated by NO in vascular smooth muscle (24), are also potential targets for NO in bladder smooth muscle.

NO could also alter phasic activity in the bladder indirectly by acting on intramural nerves. Although phasic activity in the neonatal rat bladder is myogenic and occurs in the presence of TTX, which blocks nerve action potentials, spontaneous release of a neurotransmitter from nerves can, under certain conditions, modulate phasic contractile activity in the neonatal rat bladder (28). NO is known to inhibit transmitter release from efferent autonomic nerves (41) and to inhibit calcium channels in bladder afferent neurons that are important for release of afferent neurotransmitters (42). Thus NO could modulate phasic contractile activity by suppressing spontaneous release of excitatory neurotransmitters.

Species differences in effects of NO. Effects of NO in the neonatal rat bladder might be different from effects in the guinea pig and mouse bladder because the latter contain a more complex intramural nervous system composed of autonomic ganglion cells and local reflex pathways (10) that are not present in the rat bladder (11). This might account for some of the divergent findings reported in these species. For example, in guinea pig detrusor strips, SNAP increased the amplitude and frequency of spontaneous bladder activity and baseline tone, but in a sGC-independent manner, while in the same strips, 8-bromo-cGMP reduced contractile activity (40). In the mouse bladder, the peak amplitude and plateau of carbachol-induced contractions were suppressed by 8-bromo-cGMP, but the phasic contractions were not suppressed (8). Another study of the mouse bladder found no effect of NO donors, but 8-bromo-cGMP relaxed carbachol-precontracted bladder strips (9). Thus differences in the species, age of animal, types of contractile activity, and pharmacological agents should all be considered when the effects of NO on bladder activity are evaluated.
Physiological role of NO-sGC-cGMP signaling pathway. In neonatal rat bladder strips, inhibition of endogenous PDE-5 with zaprinast reduced the amplitude and frequency of SCcarb, indicating that sufficient cGMP is generated under the conditions of our experiments to inhibit bladder contractions. Furthermore, zaprinast increased the maximal effect of an exogenous NO donor, suggesting that there is sufficient endogenous PDE to limit NO efficacy. PDE-5 inhibitors such as sildenafil, which are used clinically to treat erectile dysfunction by increasing cGMP levels in penile smooth muscle, seem to also improve lower urinary tract symptoms associated with benign prostatic hyperplasia (26, 27). This suggests that the NO-cGMP-inhibitory pathway may be active under conditions of bladder dysfunction. In some pathological conditions, such as spinal cord injury or bladder outlet obstruction, the normal pattern of low-amplitude spontaneous activity is converted to a large-amplitude neonatal-like pattern of activity (39). This raises the possibility that pathology may cause relatively quiescent adult bladders to revert to a spontaneous neonatal pattern and initiate the reemergence of NO-mediated inhibitory effects. Therefore, the neonatal rat bladder may be a useful model for detrusor overactivity in adults.

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