Differential Effect of L-Cysteine in Isolated Whole-Bladder Preparations from Neonatal and Adult Rats

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ABSTRACT

The present study was undertaken to compare the effects of the thiol reagents L-cysteine and (diazene dicarboxylic acid bis 5*N,N*-dimethylamide) diamide on contractile activity of neonatal and adult rat bladders. In vitro whole-bladder preparations from Wistar rats were used to study the modulation of spontaneous bladder contractions by thiol reagents. After blocking cholinergic and adrenergic transmission with atropine and guanethidine, L-cysteine facilitated spontaneous bladder contractions in neonatal rat bladders. The effect of L-cysteine was suppressed by diamide. Diamide alone did not change basal activity of the neonatal rat bladder. The facilitatory effects of L-cysteine were reduced by the L-type Ca^{2+} channel-blocking agent nifedipine and the calcium-activated K^{+} channel opener NS1619 [1,3-dihydro-1-[2-hydroxy-5-(trifluoromethyl)phenyl]-5-(trifluoromethyl)-2*H*-benzimidazol-2-one]. ATP or suramin, a purinergic receptor antagonist, significantly inhibited the effect of L-cysteine in

neonatal bladders, whereas the nitric-oxide synthase inhibitor *N*^ω-nitro-L-arginine was ineffective. L-cysteine did not elicit any detectable effects in the adult rat bladder; whereas diamide caused a large-amplitude sustained tonic contraction. The contraction induced by diamide in adult bladder did not occur when the preparation was pretreated with L-cysteine. Also, L-Cysteine administered during the diamide-evoked contraction completely inhibited the contraction to diamide. In conclusion, our results suggest that L-cysteine has markedly different effects in isolated whole-bladder preparations from neonatal and adult rats. Thus thiol-sensitive mechanisms may modulate contractility by regulation of Ca^{2+} and K^{+} channels and/or purinergic transmission in the neonatal bladder. The effects of L-cysteine and diamide were reversed in adult bladders, indicating that the regulation of bladder contractility by thiols is markedly altered during postnatal development.

Intracellular thiols have been implicated in the regulation of smooth muscle contractile mechanisms (Reeve et al., 1995; Iesaki and Wolin, 2000; Schach et al., 2007). Diamide (diazene dicarboxylic acid bis 5*N,N*-dimethylamide), a thiol oxidant that is known to promote the reversible oxidation of glutathione and other protein thiols to their disulfide forms, causes vasodilation of precontracted pulmonary arteries (Reeve et al., 1995; Schach et al., 2007) and coronary arteries (Iesaki and Wolin, 2000). On the other hand, antioxidants such as coenzyme Q and duroquinone have the opposite effect, producing constriction of pulmonary arteries (Reeve et al., 1995). The diamide-induced relaxation in pulmonary ar-

teries has been attributed to the opening of K^{+} channels and suppression of store-operated Ca^{2+} channels (Schach et al., 2007), whereas in the coronary arteries the vasodilatory effect of diamide has been linked to a suppression of L-type Ca^{2+} channels and a reduction of extracellular Ca^{2+} influx (Iesaki and Wolin, 2000). Because reversible oxidative modification of proteins primarily involves the reactivity of the free thiol group of cysteine residues it is believed that the redox status of these thiols is important for regulating vascular smooth muscle function.

Previous studies have revealed that thiol reagents also influence the contractility of bladder smooth muscle (Resim et al., 2002). In bladder strips from adult rats, thiol-inactivating agents (ethacrynic acid and *N*-ethylmaleimide) induced contractions of the strips, whereas L-cysteine, glutathione, verapamil, or Ca^{2+} -free solutions reduced these contractions. However, L-cysteine and glutathione did not

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ABBREVIATIONS: NS1619, 1,3-dihydro-1-[2-hydroxy-5-(trifluoromethyl)phenyl]-5-(trifluoromethyl)-2*H*-benzimidazol-2-one; NO, nitric oxide; AUC, area under the curve; BK_{Ca} , large-conductance Ca^{2+} -activated K^{+} .

alter the contractions induced by electrical field stimulation or acetylcholine. It was concluded that thiol reagents may modulate contractile activity of the rat bladder by controlling the gating of Ca^{2+} channels.

It was also shown that thiol modification of cysteine residues within basic regions of the channel protein modulate the gating of large-conductance Ca^{2+} -activated K^+ (BK_{Ca}) channels, which are important in the postnatal down-regulation of bladder spontaneous contractions during maturation (Wu et al., 2005; Zhang and Horrigan, 2005). Also, it was suggested that the thiol-reactive compounds may modify the gating of the pore of P2X receptor channels (Holmgren et al., 1996; Liu et al., 1997; del Camino and Yellen, 2001; Li et al., 2008).

To further study the mechanisms underlying the effects of thiol reagents on bladder function, the present study was undertaken to compare the effects of diamide and L-cysteine on contractile activity of neonatal and adult rat bladders. Previous experiments (Sugaya and de Groat, 1994, 2000; Szell et al., 2003; Kanai et al., 2007; Ng et al., 2007) showed that neonatal and adult bladders have markedly different properties. Neonatal bladders exhibit large-amplitude, low-frequency rhythmic contractions, whereas adult bladders have low-amplitude, high-frequency contractions. The neonatal pattern converts to an adult pattern 4 to 5 weeks after birth but reappears in adults in pathological conditions such as chronic urethral outlet obstruction (Sugaya and de Groat, 2000) or spinal cord injury (Kanai et al., 2007). The present experiments explored the possibility that these differences might be related to differences in the role of thiols in regulating Ca^{2+} channels and BK_{Ca} channels on contractile mechanisms in neonatal and adult bladders. Also, we examined the possibility that L-cysteine might influence the purinergic mechanisms in the neonatal bladder.

Materials and Methods

Animals. The experimental procedures were approved by the animal care committee of the University of Çukurova (Tıbbi Bilimler Deneyisel Araştırma ve Uygulama Merkezi), and the studies were carried out in accordance with the *Principles of Laboratory Animal Care* (National Institutes of Health publication 86-23, revised 1984). A total of 56 neonatal (1–2 weeks old; 28 female and 28 male) and 12 adult (6 female and 6 male) Wistar rats were used in the experiments. All animals were kept under standard laboratory conditions (12-h light/12-h dark).

In Vitro Whole-Bladder Preparation. Rats were anesthetized with halothane and sacrificed by cervical dislocation. A whole-bladder preparation was prepared by using previously published techniques (Sugaya and de Groat, 2000; Ng et al., 2006). The bladder was exposed by a midline abdominal incision and removed from the abdomen by cutting at the bladder neck. A 26-gauge needle was inserted at the bladder neck and tied with 5-0 silk sutures. The needle was connected to an infusion pump and pressure transducer via polyethylene tubing and a three-way stopcock. The needle and tubing were filled with Krebs' solution (113 mM NaCl, 19.8 mM NaHCO_3 , 11.1 mM dextrose, 1.2 mM KH_2PO_4 , 4.7 mM KCl, 2.5 mM MgCl_2 , 1.7 mM CaCl_2). The bladder was placed between two platinum stimulating electrodes inside an organ bath filled with 37°C Krebs' solution and bubbled with 95% O_2 /5% CO_2 . Bladder pressure was recorded with data acquisition software (BIOPAC MP30 Systems, Inc., Goleta, CA). After a 30-min equilibration period, the bladder was filled slowly with Krebs' solution in 50- μl increments during intermittent electrical field stimulation (50 V, 1.5-ms pulse

duration, 10 Hz for 15–30 s) to determine the bladder volume necessary to produce maximal bladder contractions. Field stimulation was delivered by a Grass S88 stimulator (Grass Instruments, Quincy, MA). The distended bladder was washed three times with 15 ml of fresh Krebs' solution and equilibrated for another 30 min, and then drug treatment was started. Amplitude and frequency of the spontaneous contractions before and after a drug were measured for 5 min within a 10-min observation period. The mean peak amplitude of the spontaneous contractions was normalized as a percentage of the maximal K^+ -evoked contraction amplitude. The K^+ -evoked contraction was induced at the end of the experiments by application of bath solution containing 80 mM KCl. Frequency of contractions was determined by counting the number of contractions over a 5-min interval. The adrenergic neuron-blocking agent guanethidine (2 μM) and the muscarinic cholinergic receptor antagonist atropine (2 μM) were always present in the bathing medium to block adrenergic and cholinergic transmission. In the experiments with neonatal rats, L-cysteine (0.1, 0.5, or 1 mM) was applied twice to the same tissue consecutively after a wash and recovery period ranging from 40 to 45 min. In our preliminary studies, we tested different concentrations of L-cysteine ranging from 0.1 to 2 mM and determined that 1 mM was an optimal concentration to obtain a reproducible facilitatory effect on basal spontaneous contractions of neonatal rat bladder. After the first application of the agent, the tissue was washed with fresh Krebs' solution. The exposure time of each L-cysteine application was 10 min. In some experiments, the effects of various agents, including the thiol-modifying agent diamide (200 μM), the L-type calcium channel blocker nifedipine (1 μM), the nitric-oxide (NO) synthase inhibitor N^{ω} -nitro-L-arginine (100 μM), the purinergic agonist ATP (50 μM), the purinergic antagonist suramin (200 μM), or the calcium-activated K^+ channel opener NS1619 (1,3-dihydro-1-[2-hydroxy-5-(trifluoromethyl)phenyl]-5-(trifluoromethyl)-2H-benzimidazol-2-one) (Edwards et al., 1994; Patel et al., 1998; Ng et al., 2006; Park et al., 2007; 30 μM), were examined during recording of spontaneous contractions in the presence or absence of L-cysteine (1 mM). In our preliminary studies, we tested different concentrations of diamide and determined that 200 μM was a suitable concentration to obtain a reproducible inhibitory effect on the responses to L-cysteine. We used only the highest concentration of L-cysteine to test the effect of the agents mentioned above because we determined that 1 mM was the optimal concentration to obtain reproducible potentiating effects on the basal spontaneous contractions of neonatal rat bladder. In adult rats, the effects of L-cysteine and/or diamide on the baseline spontaneous contractions were also examined to compare the effects with those elicited in neonatal rat bladders. In some adult rat bladders, we also tested the effects of diamide (200 μM) in the presence of ATP (50 μM) or suramin (200 μM).

Drugs. L-Cysteine, atropine, guanethidine, diamide, N^{ω} -nitro-L-arginine, ATP, and suramin were dissolved in distilled water. Nifedipine and NS1619 were dissolved in 75% ethyl alcohol (final concentration in the bath medium was 0.08%). Drugs were obtained from Sigma-Aldrich (St. Louis, MO).

Statistical Analysis. The spontaneous contractile activity was quantified by measuring the average maximal amplitude (cm H_2O), the frequency (contractions per min), and the area under the curve (AUC; cm H_2O per second). Amplitude and developed tension were expressed as a percentage of the KCl-induced contraction at the end of each experiment. All data are expressed as mean $\pm$ S.E.M. All of the data were evaluated with the Bonferroni corrected *t* test that was used in analysis of variance. A *p* value of less than 0.05 is considered significant. Statistical analysis was performed with GraphPad Prism software (San Diego, CA).

Results

Neonatal Rat Bladder Basal Spontaneous Contractions. We first determined the profile of spontaneous con-

tractions of isolated whole bladders obtained from neonatal rats. Spontaneous contractions appeared in 1- to 2-week-old bladders after they were filled to 150 to 200 μ l, which was the volume required for maximal field stimulation-evoked contractions. The mean amplitude of the spontaneous contractions was $2.91 \pm 0.24\%$ of K^+ -evoked contraction ($n = 48$; Figs. 1 and 2). The mean frequency was 4.41 ± 0.27 contraction/min ($n = 48$). Most of the neonatal rats exhibited very small amplitude spontaneous contractions. Some of the prep-

arations (19%) did not have detectable spontaneous contractions. Those bladders were not evaluated.

Effects of L-Cysteine on Spontaneous Contractions in the Neonatal Rat Bladder. After blocking muscarinic cholinergic and adrenergic responses with atropine (2 μ M) and guanethidine (2 μ M), which did not alter basal spontaneous activity or baseline pressure, L-cysteine (0.1–1 mM) enhanced spontaneous contractions in a concentration-dependent manner (Fig. 1). L-Cysteine (0.1, 0.5, and 1

Fig. 1. a, the facilitatory effects of L-cysteine (0.1, 0.5, and 1 mM) on the spontaneous contractions in neonatal rat bladder. Low concentrations of L-cysteine enhance spontaneous contractions without producing a tonic contraction; higher concentrations produce tonic contractions. w represents washout. b, amplitude is presented as percentage of K^+ -evoked contractions, AUC is presented as cmH₂O per second, and frequency is presented as contractions per min. Results are expressed as mean $\pm$ S.E. ($n = 6-8$). *, $p < 0.05$, significant difference from control basal spontaneous contractions.

Fig. 2. a, the inhibitory effect of diamide on the responses to L-cysteine (1 mM) in neonatal rat bladder. w represents washout. b, amplitude is presented as percentage of K^+ -evoked contractions, AUC is presented as cmH₂O per second, and frequency is presented as contractions per min. Bars indicate control (A), 1 mM L-cysteine (B), 1 mM L-cysteine + 200 μ M diamide (C), washout (D), and the second application of 1 mM L-cysteine (E). Results are expressed as mean $\pm$ S.E. ($n = 6-8$). *, $p < 0.05$, significant difference from control. +, $p < 0.05$, significant difference from 1 mM L-cysteine.

mM) significantly increased the amplitude and AUC of the spontaneous contractions (Fig. 1b; amplitude increased to $1652.7 \pm 84.6\%$ of the basal spontaneous contractions; $p < 0.001$). Also, this agent was ineffective on the frequency at 0.1 and 0.5 mM and caused a significant decrease at 1 mM. A low concentration of L-cysteine (0.1 mM) increased spontaneous contractions without causing a tonic contraction, whereas 0.5 and 1 mM concentrations of L-cysteine evoked initial large-amplitude transient contractions (4.2 ± 1.3 and 13.4 ± 1.5 cmH₂O, respectively) followed by large-amplitude and low-frequency phasic contractions (Fig. 1a). In 80% of the preparations, these phasic contractions were superimposed on a low-amplitude, transient (3–5 min) elevation of basal tone (2.3 ± 0.5 cmH₂O). The facilitating effect of L-cysteine on the spontaneous contractions persisted for at least 15 to 20 min and was reversible in 40 to 45 min after washout of the drug. The responses to the highest concentration (1 mM) of L-cysteine were more stable and reproducible compared with the lower concentrations (0.1 and 0.5 mM) (Fig. 1).

Effects of Diamide or Nifedipine on the Facilitatory Effect of L-Cysteine in Neonatal Rat Bladders. In the presence of 200 μ M diamide, which did not alter spontaneous contractions or baseline pressure, the initial large increase in baseline pressure evoked by 1 mM L-cysteine and the secondary facilitatory effect on the amplitude and frequency of spontaneous contractions was completely inhibited and the effect on AUC was markedly reduced (Fig. 2). Within 30 to 40 min after washout the inhibitory effect of diamide was completely reversible (Fig. 2). Pretreatment with the L-type calcium channel blocker nifedipine (1 μ M) completely abolished basal spontaneous contractions and the responses evoked by L-cysteine (Table 1). The solvent of nifedipine did not affect the responses (data not shown).

Effect of *N*^ω-Nitro-L-Arginine on the Facilitatory Effect of L-Cysteine in Neonatal Rat Bladders. Pretreatment with the *N*^ω-nitro-L-arginine (100 μ M), a NO synthase inhibitor, did not affect the amplitude, frequency, or AUC of the phasic contractions induced by 1 mM L-cysteine (Table 1). *N*^ω-nitro-L-arginine alone did not alter the basal activity of the neonatal rat bladder (not shown).

Effects of ATP, Suramin, or NS1619 on the Facilitatory Effect of L-Cysteine in Neonatal Bladders. Ten minutes after pretreatment with ATP (50 μ M), which initially evoked a transient contraction (1–3 min in duration; 16.5 ± 1.33 cmH₂O), the initial bladder contraction induced by L-cysteine was decreased by $82.0 \pm 4.7\%$ and the facilitatory effect of L-cysteine (1 mM) on spontaneous contractions was significantly inhibited (Fig. 3b). Suramin (200 μ M), a nonselective purinergic receptor antagonist, also signifi-

cantly decreased the initial contraction ($35.0 \pm 5.3\%$ decrease) and the enhancement of the spontaneous contractions evoked by L-cysteine (1 mM) (Fig. 3). However, the inhibitory effect of suramin was less than the effect of ATP. NS1619 (30 μ M), a potassium channel opener, significantly inhibited both the initial contraction evoked by L-cysteine (1 mM) ($77.0 \pm 6.1\%$ suppression) and the facilitation of spontaneous contractions (Fig. 4). The effect of NS1619 was similar in magnitude to the effect of ATP. Suramin or NS1619 alone did not change basal activity of the neonatal rat bladder (not shown). The solvent of NS1619 did not affect the responses (data not shown).

Effect of L-Cysteine and Diamide on Activity of the Adult Rat Bladder. Spontaneous contractions in bladders from adult rats were lower in amplitude (1.52% of K⁺-evoked contractions) and higher in the frequency (6.43 ± 0.24 contractions/min, $n = 6$) than in neonatal bladders. L-Cysteine (1 mM) did not elicit any detectable effects in the adult rat bladder (Fig. 5a; $n = 6$), whereas diamide (200 μ M) caused a large-amplitude sustained tonic contraction followed by low-amplitude, high-frequency spontaneous contractions superimposed on the sustained contraction (Fig. 5b; $n = 6$). The amplitude of the contraction (9.57 ± 1.34 cm H₂O) elicited by diamide was 22.7% of the K⁺-evoked contraction. The amplitude, AUC, and frequency of the spontaneous contractions superimposed on the sustained contraction evoked by diamide were not significantly different from those in the control period before drug administration (not shown). The contraction induced by diamide did not occur when the preparation was pretreated with 1 mM L-cysteine (Fig. 5a and Table 2). Also, 1 mM L-cysteine administered during the diamide-evoked contraction completely inhibited the contraction to diamide ($97.0 \pm 5.1\%$ inhibition; Fig. 5b). ATP (50 μ M) or suramin (200 μ M) caused a decrease that was not significant on the contraction induced by 200 μ M diamide (Table 2).

Discussion

The present experiments revealed that thiol reagents have markedly different effects in isolated whole-bladder preparations from neonatal and adult rats. L-Cysteine, a thiol-containing amino acid, induced an initial large-amplitude contraction and facilitated spontaneous activity in a concentration-dependent manner in neonatal rat bladders. The effect of L-cysteine was suppressed by diamide, a thiol-oxidizing agent, indicating that contractile activity in the neonatal bladder can be stimulated by high concentrations of reduced thiols. The facilitatory effects of L-cysteine were also reduced by an L-type Ca²⁺ channel-blocking agent and a K⁺

TABLE 1

The effects of nifedipine or *N*^ω-nitro-L-arginine on phasic contractions of isolated whole-bladder preparation induced by 1 mM L-cysteine in neonatal rats

Nifedipine completely blocked the responses to L-cysteine. Frequency is shown in contractions per minute. The results are presented as the percentage of the maximal response to 80 mmol/l KCl-induced contraction at the end of each experiment (mean $\pm$ S.E., $n = 6$).

	Amplitude	AUC	Frequency
	%		
Control	2.64 ± 0.25	2.41 ± 0.5	1.73 ± 0.5
L-Cysteine (1 mM)	$24.8 \pm 1.65^*$	$24.2 \pm 1.33^*$	$5.48 \pm 0.25^*$
<i>N</i> ^ω -Nitro-L-arginine (100 μ M)	$27.1 \pm 2.12^*$	$24.6 \pm 2.17^*$	$4.00 \pm 0.17^*$
Nifedipine (1 μ M)	0.0^*	0.0^*	0.0^*

*, $p < 0.05$, significant difference from control responses before drug application.

Fig. 3. a, the inhibitory effect of suramin (200 μ M) or ATP (50 μ M) on the responses to L-cysteine (1 mM) in neonatal rat bladder. w represents washout. b, amplitude is presented as percentage of K⁺-evoked contractions, AUC is presented as cm H₂O per s, and frequency is presented as contractions per min. Bars indicate control (A), 1 mM L-cysteine (B), 50 μ M ATP + 1 mM L-cysteine (C), 200 μ M suramin + 1 mM L-cysteine (D), washout (E), and the second application of 1 mM L-cysteine (F). Results are expressed as mean $\pm$ S.E. ($n = 6-8$). *, $p < 0.05$, significant difference from control. +, $p < 0.05$, significant difference from 1 mM L-cysteine.

Fig. 4. a, the inhibitory effect of NS1619 on the responses to L-cysteine in neonatal rat. w represents washout. b, amplitude is presented as percentage of K⁺-evoked contractions, AUC is presented as cm H₂O per s, and frequency is presented as contractions per min. Bars indicate control (A), 1 mM L-cysteine (B), 1 mM L-cysteine + 30 μ M NS1619 (C), washout (D), and the second application of 1 mM L-cysteine (E). Results are expressed as mean $\pm$ S.E. ($n = 6-8$). *, $p < 0.05$, significant difference from control. +, $p < 0.05$, significant difference from 1 mM L-cysteine.

channel opener consistent with the data obtained in vascular smooth muscle that thiols can modulate contractility by regulating Ca²⁺ and K⁺ channels. The facilitatory effect of L-cysteine occurred in the presence of a cholinergic muscarinic

receptor antagonist and an adrenergic neuron-blocking agent, indicating that the effect did not depend on an interaction with cholinergic or adrenergic excitatory mechanisms. However, ATP or suramin, a purinergic receptor antagonist, significantly

Fig. 5. Representative tracings showing the differential effects of L-cysteine and diamide in adult rat bladder function. **a**, the preparation was pretreated with 1 mM L-cysteine. **b**, the relaxant effect of L-cysteine on the contractile effect of diamide is shown. **w** represents washout.

TABLE 2

The contractile effect of 200 μ M diamide in the presence of L-cysteine (1 mM), ATP (50 μ M), or suramin (200 μ M) in the isolated adult rat whole-bladder preparation

The results are presented as the percentage of the maximal response to 80 mmol/L KCl-induced contraction at the end of each experiment (mean $\pm$ S.E., $n = 6$).

	Amplitude
	%
Diamide	22.7 $\pm$ 2.67
L-Cysteine + diamide	0.0*
ATP + diamide	18.2 $\pm$ 2.6
Suramin + diamide	20.4 $\pm$ 4.1

*, $p < 0.05$, significant difference from control responses to diamide used alone.

inhibited the effect of L-cysteine in neonatal bladders, suggesting that this agent may enhance the spontaneous contractions by enhancing ATP release from nerves and/or facilitating purinergic excitatory responses. Thus thiol-sensitive mechanisms may modulate purinergic transmission in the neonatal bladder. The effects of L-cysteine and diamide were reversed in adult bladders, indicating that the regulation of bladder contractility by thiols is markedly altered during postnatal development.

Although the mechanism underlying the excitatory effect of L-cysteine in the neonatal bladder was not examined in the present experiments, studies conducted in other laboratories that focused on the actions of thiol reagents on neurons and various smooth muscle raise the possibility that several actions may be involved in the effects of thiols on the bladder. For example, in sensory neurons L-cysteine enhances T-type Ca^{2+} channel activity and increases neuronal excitability (Nelson et al., 2005). In bladder smooth muscle T-type Ca^{2+} channels have been implicated in the generation of phasic contractile activity (Chow et al., 2003; Sui et al., 2003; Yanai et al., 2006). Thus, if L-cysteine enhances T-type Ca^{2+} channels in bladder smooth muscle as it does in sensory neurons this could contribute to the excitatory effect of this agent in the neonatal bladder. However, we need further studies to examine a possible relationship between L-cysteine and T-type Ca^{2+} channels.

The effects of L-cysteine on L-type Ca^{2+} channels may also be important. Diamide, which blocked the effect of L-cysteine in the neonatal bladder, suppresses L-type Ca^{2+} channels

and relaxes various types of smooth muscle, whereas antioxidants, such as coenzyme Q, which would be expected to have actions similar to those of cysteine, have the opposite effect (Reeve et al., 1995). The complete suppression of the L-cysteine excitatory effects in neonatal bladder by nifedepine, an L-type Ca^{2+} channel blocker, indicates that L-type channels are essential for the excitatory effects.

The dramatic reversal in the effects of the thiol reagents in adult bladders suggests that the functions of the molecular targets for these reagents are altered during postnatal development. The results of previous experiments with different thiol reagents in adult rat bladder strips supports this conclusion. In adult bladders sulfhydryl-inactivating agents (*N*-ethylmaleimide and ethacrynic acid) induced contractions that were suppressed by L-cysteine, glutathione, verapamil, and Ca^{2+} -free bathing solution (Resim et al., 2002). On the other hand, L-cysteine and glutathione did not affect the contractions induced by electrical stimulation of intramural nerves that were suppressed by atropine or a Ca^{2+} channel blocker. It was concluded that 1) neurally mediated bladder contractions in adult rats are mediated by different mechanisms than the contractions induced by SH alkylating agents and 2) the alkylating action of the SH reagents on SH groups in Ca^{2+} channel protein leads to an opening of the channels. If this is correct then the Ca^{2+} channel-gating mechanisms or the types of Ca^{2+} channels controlling bladder activity are clearly different in adult and neonatal bladders. This difference may be correlated with the down-regulation of spontaneous bladder activity that occurs during postnatal development and reflect the emergence of mature bladder functions that promote more efficient urine storage and dependence on centrally generated neural activity for voiding.

Basal spontaneous contractions are also sensitive to BK_{Ca} channel regulation, and these channels act as a brake on muscle activity (Wu et al., 2005). Elevated intracellular Ca^{2+} levels after smooth muscle contractions open BK_{Ca} channels, repolarize the membrane potential, and inactivate L-type Ca^{2+} channels. It was showed that BK_{Ca} channels are important in the postnatal down-regulation of bladder spontaneous contractions during maturation (Wu et al., 2005). Spontaneous contractions in both normal adult and diabetic bladders can be augmented by blocking BK_{Ca} channels with iberiotoxin (Herrera et al., 2000, 2001; Imai et al., 2001; Buckner et al., 2002; Hashitani et al., 2004; Nakahara et al., 2004). Our findings that opening BK channels after NS1619 treatment results in a decrease in facilitatory effects of L-cysteine on spontaneous contractions suggests that L-cysteine can modulate contractility by regulating K^{+} channels. Previous articles showed that thiol modification of cysteine residues within basic regions of the channel protein modulate the gating of BK_{Ca} channels (Zhang and Horrigan, 2005). In addition, Lang and Harvey, 2002 suggested that the NO donor *S*-nitroso-L-cysteine might modify thiols on these channels. These findings support the speculation that L-cysteine might have an important role in the modification of thiols on BK_{Ca} channels in neonatal rat bladder. On the other hand, the ineffectiveness of L-cysteine in adult bladder may be caused by BK_{Ca} channels becoming less important in controlling the activity of the bladder in adult rats (Ng et al., 2007).

The present experiments also examined the possibility that L-cysteine might influence the purinergic mechanisms in

the neonatal bladder. It was suggested that the thiol-reactive compounds may modify the gating of the pore of P2X receptor channels (Holmgren et al., 1996; Liu et al., 1997; del Camino and Yellen, 2001; Li et al., 2008). It was shown that conserved cysteine residues in the extracellular loop of the human P2X(1) receptor form disulfide bonds and are involved in receptor trafficking to the cell surface (Ennion and Evans, 2002). Also, a recent study showed that conserved extracellular cysteines and disulfide bonds may differentially regulate the inhibitory effect of ethanol in rat P2X4 receptors (Yi et al., 2009). In the present study, ATP, a purinergic receptor agonist, or suramin, a purinergic receptor antagonist, significantly inhibited the effect of L-cysteine in neonatal bladders. The inhibitor effect of ATP on the facilitating effect of L-cysteine may be caused by a possible desensitization of the purinergic receptors. These results suggest that this agent may enhance the spontaneous contractions by enhancing ATP release from nerves and/or facilitating purinergic excitatory responses. Thus, thiol-sensitive mechanisms may modulate purinergic transmission in the neonatal bladder.

In the present study, we also examined the role of NO in the mechanism of the facilitatory effect elicited by L-cysteine in the neonatal bladder. It has been suggested that NO can be released from urothelium and affect the excitability of adjacent afferent nerves (Andersson and Persson, 1995; Ozawa et al., 1999; Yoshimura et al., 2001; Birder et al., 2002). Neonatal bladders are sensitive to the inhibitory effects of NO, whereas adult bladders are relatively insensitive to NO (Artim et al., 2009). However, the NO synthase inhibitor *N*^ω-nitro-L-arginine did not affect the responses elicited by L-cysteine on neonatal bladder. This finding indicates that an NO pathway does not play a role in the facilitating effect of L-cysteine.

In conclusion, our experiments with thiol reagents suggest that L-cysteine has markedly different effects in isolated whole-bladder preparations from neonatal and adult rats. L-Cysteine facilitated spontaneous activity in a concentration-dependent manner in neonatal rat bladders, presumably by modulation of contractility by regulation of Ca²⁺ and K⁺ channels and/or purinergic transmission in the neonatal bladder. NO does not mediate the effect of L-cysteine in the neonatal bladder. The similarity between spontaneous bladder activity in neonatal rats and the spontaneous bladder activity in rats with chronic partial bladder outlet obstruction or spinal cord injury has prompted the speculation that primitive intrinsic bladder activity, which is present in the early postnatal period and declines during maturation, can reappear in adults in response to pathological conditions. Thus a more complete understanding of the actions of thiol reagents on bladder activity and the physiological mechanisms that underlie the developmental changes in the responses to these agents may provide new insights into the pathophysiology of the overactive bladder.

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