Substance P via NK1 receptor facilitates hyperactive bladder afferent signaling via action of ROS

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Chien, Chiang-Ting, Hong-Jeng Yu, Tser-Bin Lin, Ming-Kuen Lai, and Su-Ming Hsu. Substance P via NK1 receptor facilitates hyperactive bladder afferent signaling via action of ROS. Am J Physiol Renal Physiol 284: F840–F851, 2003; 10.1152/ajprenal.00187.2002.—We explored whether substance P (SP) via neurokinin (NK) receptor facilitates bladder afferent signaling and reactive oxygen species (ROS) formation in bladder in association with neurogenic inflammation. We evaluated ROS activity and cystometrograms as well as pelvic nervous activity in anesthetized rat bladder with SP stimulation. Our results showed that endogenous SP via NK1, not NK2, receptor mediated a micturition reflex. An increase in SP by electrical stimulation of the pelvic nerve or an increase in exogenous SP by intra-arterial or intrathecal administration can facilitate myogenic and neurogenic bladder contractions. Furthermore, exaggerated SP release increased ROS in the bladder and whole blood via increased mast cell degranulation, intercellular adhesion molecule expression, and leukocyte adhesion, a primary source of ROS in the inflamed bladder. Treatment with NK1-receptor antagonists or ROS scavengers reduced bladder intercellular adhesion molecule expression and ROS and ameliorated the hyperactive bladder response. Our study indicates that the mechanism by which SP participates in the neurogenic bladder may be complicated by its proinflammatory activity and its ability to stimulate ROS generation.

reactive oxygen species; micturition reflex; neurokinin receptor

THE TACHYKININS Substance P (SP) and neurokinins (NK) A and B belong to a family of neuropeptides that are widely distributed in the mammalian central and peripheral afferent nervous systems and produce their biological actions by activating three distinct receptor types, NK1, NK2, and NK3 (25). These afferents commonly innervate smooth muscle, submucosal layers, and blood vessels of visceral organs (4, 21, 34). On release from sensory afferents, SP and NKA, via NK1 and NK2 receptors, act on smooth muscle or blood vessels to regulate visceral motility and blood flow (21, 25). Recent evidence suggests that endogenous tachykinins may play a role in visceral inflammation, hyperreflexia, and hyperalgesia (4, 20). For example, in the rat urinary bladder, SP may be more relevant than NKA for the mediation of plasma protein extravasation and inflammatory response (19), whereas NKA is an important mediator of smooth muscle contraction (21). In addition, tachykinins may lead to expression of adhesion molecules by endothelial cells, chemotaxis and activation of immune cells, mucus secretion, and water absorption/secretion in the lungs, gastrointestinal tract, and genitourinary tract (1, 19, 32).

The mammalian bladder is richly innervated by capsaicin-sensitive, SP-containing afferent fibers (22, 37). Afferents signaling mechanical and chemical environments in the bladder evoke sacralombar reflexes in lumbar sympathetic neurons to increase bladder capacity or elicit parasympathetic bladder efferent excitation to trigger a normal micturition reflex (8, 9, 11). However, the bladder capacity and/or the micturition reflex may be altered in a number of pathophysiological conditions, such as interstitial cystitis, cyclophosphamide-induced cystitis, and irritant-induced hypersensitivity (14). The mechanisms responsible for bladder hyperactivity may vary from condition to condition and are likely complicated. Bladder biopsies in some patients diagnosed with interstitial cystitis have shown increased density of SP-containing fibers and NK1 receptors (15, 26, 29). Furthermore, administration of SP is known to cause bladder inflammation (1, 21, 39) and generation of reactive oxygen species (ROS) by inflammatory cells (3, 40). Thus SP may have a direct or an indirect role in neurogenic inflammation and regulation of bladder motility in various clinical conditions. Nonetheless, whether ROS generated by SP-reacted inflammatory cells contribute to bladder hyperactivity remains to be determined.

Our objective in the present study was to clarify the contribution and the possible mechanism of SP in bladder hyperreflexia. We showed that bladder hyperactivity caused by pelvic nerve stimulation is associated with an increased SP level in the bladder. We also used NK-receptor antagonists and free radical scavengers to test the role of SP- and ROS-induced hyperactivity, respectively. Our results clearly showed that SP via NK1-receptor activation enhances the micturition re-
flex and ROS release from the inflammatory cells and, consequently, leads to a hyperactive bladder.

MATERIALS AND METHODS

Drugs. The drugs/chemicals used in this study are listed in Table 1. The drugs were prepared and stored at −70°C, and subsequent dilutions of the drugs were made in saline on the day of the experiments.

Surgery. Adult female Wistar rats weighing 220–240 g were anesthetized with urethane (1.2 g/kg sc), which is well known to anesthetize the animals yet permit full reflex bladder contractions (8, 9). Maintenance of deep anesthesia was determined by the persistence of miotic pupils as judged from frequent inspection and by the lack of heart rate and arterial blood pressure (ABP) fluctuations in the absence of visceral stimuli (8). An experiment was terminated when the baseline mean ABP was <90 mmHg. Animal care and experimental protocol were in accordance with the guidelines of the National Science Council of the Republic of China (1997). All efforts were made to minimize animal suffering and the number of animals used throughout the experiment. At the end of each experiment, the animals were killed by an intravenous potassium chloride injection.

PE-50 catheters were placed in the left femoral artery for measurement of ABP and in the left femoral vein for administration of anesthetics. ABP was recorded on a polygraph (model RS3400, Gould) with a transducer (model P23 1D, Gould-Statham, Quincy, MA). A length of stretched PE-10 tubing inserted just above the bifurcation of the aorta from the right femoral artery was used for injection of various drugs. Body temperature was kept at 36.5–37°C by an infra-red light and was monitored with a rectal thermometer.

Experimental models and protocols. We used a transcystometer (via cannulation of the bladder dome) mimicking normal micturition of the bladder to evaluate the role of SP in the bladder micturition reflex and bladder hyperactivity (8, 18). Briefly, the urinary bladder was exposed through a midline incision of the abdomen, and urine was emptied by application of light pressure. A PE-50 T tube was inserted through the apex of the bladder dome. The bladders were filled by continuous infusion of 0.9% saline (0.15 ml/min) at room temperature and allowed to drain/micturate repeatedly via the urethra.

The change in bladder pelvic afferent nerve activity (PANA), bladder pelvic efferent nerve activity (PENA), ABP, and various parameters measured by the cystometrogram can be determined before and after intra-arterial or intrathecal administration of various chemicals. All chemicals were injected through the intra-arterial catheter in a volume of 1 ml/kg (0.20–0.25 ml) and were followed by 0.1 ml of heparinized saline. For intrathecal administration, a catheter (Portex, Hythe, Kent, UK) was inserted through the atlanto-occipital membrane for 8.5 cm, such that the tip of the catheter was placed just above the lumbosacral enlargement, as described previously (18). Chemicals were given intrathecally in a volume of 20 μl and were followed by 30 μl of saline.

In the first part of study, the animals were divided into three groups: group 1 was used to test the role of endogenous SP in the bladder micturition reflex by administration of NK-receptor antagonists; groups 2 and 3 were used to test the effect of exogenous SP and SP + NK-receptor antagonists on bladder hyperactivity. SP was given at 0.1–10 μg iv and 5 μg it. Plasma SP in the iliac vein was 35–1,200 ng/ml at 10 min after intra-arterial injection. To verify the SP activity via the NK1 receptor, we injected SP (group 2), SP + the NK1-receptor antagonist CP-96345 (group 3a), or the NK2-receptor antagonist SR-48968 (group 3b). The NK-receptor antagonists were given at 250–500 μg iv and at 5–250 μg it, doses that were lower than those used in previous studies (5–5,000 μg/kg iv and ≤250 μg/kg it) (17, 24, 27).

Cystometrogram for measurement of bladder response. The bladder catheter was connected via a T tube to a pressure transducer (model P23 1D, Gould-Statham), and the intravesical pressure (IVP) was recorded continuously on a polygraph (model RS3400, Gould, Cleveland, OH). The following parameters of bladder responsiveness were also measured: threshold volume (infused volume at the point preceding a micturition reflex), number of active contractions (>15 mmHg), micturition volume (volume of expelled urine collected in a preweighed tube), residual volume (amount of fluid remaining after a bladder contraction; infused volume = infusion rate – micturition volume), bladder capacity (residual volume + micturition volume), intercontraction interval (time lag between 2 micturition cycles), and basal pressures.

Recording of PANA and PENA. Multifiber PANA and PENA were measured simultaneously in eight rats. The two

Table 1. Drugs/chemicals

<table>
<thead>
<tr>
<th>Drugs/Chemicals</th>
<th>Name</th>
<th>Source</th>
<th>Function</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>SP</td>
<td>[Sar9, Met (O2)11]-substance P</td>
<td>Peninsula Laboratory (Belmont, CA)</td>
<td>Neuropeptide released from sensory nerves</td>
<td>27</td>
</tr>
<tr>
<td>CP-96345</td>
<td>(S)-N-methyl-N-[4-(4-acetylamino-4-phenylpiperidino)-2-(3,4-dichlorophenyl)-butyl]benzamide</td>
<td>Pfizer Central Research (Groton, CT)</td>
<td>NK1-receptor antagonist</td>
<td>33</td>
</tr>
<tr>
<td>SR-48968</td>
<td>(S)-N-methyl-N-[4-(4-acetylamino-4-phenylpiperidino)-2-(3,4-dichlorophenyl)-butyl]benzamide</td>
<td>Sanofi Recherche (Montpellier, France)</td>
<td>NK2-receptor antagonist</td>
<td>13</td>
</tr>
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<td>SOD</td>
<td>Superoxide dismutase</td>
<td>Sigma (St. Louis, MO)</td>
<td>Free radical scavenger</td>
<td>9</td>
</tr>
<tr>
<td>C3</td>
<td>Hexacarboxylic acid</td>
<td>Dr. T. Y. Luh (Dept. of Chemistry, National Taiwan University)</td>
<td>Free radical scavenger with C60 fullerene structure</td>
<td>12</td>
</tr>
<tr>
<td>FC3S</td>
<td>Hexa(sulfobutyl)fullerenes</td>
<td>Dr. L. Y. Chiang (Center for Condensed Matter Sciences, National Taiwan University)</td>
<td>Free radical scavenger with C60 fullerene structure</td>
<td>41</td>
</tr>
<tr>
<td>Lucigenin</td>
<td>N,N'-dimethylacridinium</td>
<td>Sigma (St. Louis, MO)</td>
<td>Chemiluminescence probe for O2</td>
<td>35</td>
</tr>
<tr>
<td>MCLA</td>
<td>2-Methyl-6-(4-methoxyphenyl)-3,7-dihydroimidazo[1,2-a]-pyrazin-3-one-hydrochloride</td>
<td>TCI-Ace, Tokyo Kasei Kogyo (Tokyo, Japan)</td>
<td>Chemiluminescence probe for O2</td>
<td>28</td>
</tr>
</tbody>
</table>
left pelvic nerve branches attached to the urinary bladder surface were isolated and simultaneously recorded by placement of the intact nerve fibers in parallel with two pairs of thin, bipolar stainless steel electrodes (8). The electrical signals were amplified 20,000-fold, filtered (high-frequency cutoff at 3000 Hz and low-frequency cutoff at 30 Hz) with an alternating-current preamplifier (model P511, Grass, Valley View, OH), continuously recorded on magnetic tape, and displayed on an oscilloscope (model 1604, Gould). The amplified signals (spikes) were transformed by a window discriminator (model 121, World Precision Instruments, Sarasota, FL) and analyzed with an impulse counter (Gould integrator amplifier 13-4615-70) that was set to count the total number of spikes per second (8, 9). The background activity, which could be caused by the nerve contact with electrodes, nerve damage during handling, and the equipment itself, was excluded from the window discriminator by adjustment of the threshold voltage (9).

Nerve fiber with PANA was confirmed by its ability to show increased activity in response to small increments in IVP by saline infusion via T tube. Nerve fiber with PENa had minimal activity until a threshold volume/pressure in the bladder produced a bursting discharge causing a micturition contraction (Fig. 1) (8, 9, 36).

Electrical stimulation of the pelvic nerve. The next study was intended to determine whether nerve activity causing bladder hyperactivity was accompanied by release or/and elevation of SP in bladder. An electric current of square-wave pulses with pulse duration of 0.05 ms was applied from a stimulator (model S88, Grass, Quincy, MA) through a stimulus isolation unit (model SIU5B, Grass) and a constant-current unit (model CCU1A, Grass).

Six rats were given a solution of atropine (1 mg·ml⁻¹·kg⁻¹) to effectively inhibit the muscarinic (parasympathetic) effect. One branch of the pelvic nerve was dissected from the left major pelvic ganglion to the urinary bladder and was identified by a bladder contraction elicited by electrical stimulation (8). The urinary bladder was electrically stimulated (1–10 Hz) for 5 min, and bladder response, including IVP and the number of active contractions, was recorded. Blood samples from the bladder outflow (iliac vein) were obtained for determination of SP level before and during each period of stimulation.

Measurement of SP. Plasma levels of SP in the iliac vein were measured as described previously (5). The study was intended to determine whether pelvic nerve activity results in elevation and release of SP in the bladder. Briefly, supernatant from plasma samples was diluted with the same volume of buffer A (RIK-BA-1, Peninsula Laboratory). Then each sample was passed slowly through a C18 Sep-Pak column (RIK-SEPCOL-1, Peninsula Laboratory). The column was washed with 9 ml of buffer A and eluted with 3 ml of buffer B (RIK-BB-1, Peninsula Laboratory). The eluted samples were dried by vacuum centrifugation and stored at −70°C for later analysis. An SP enzyme immunoassay kit (Cayman Chemical, Ann Arbor, MI) was used to detect the SP level. Each sample was dissolved in 1% HCl, diluted to a suitable concentration with enzyme immunoassay buffer, and assayed in duplicate. The SP, which was linked to acetylcholinesterase as a tracer, and rabbit SP antiserum were added to the sample and incubated in the assay plate at 4°C for 18 h. Then, the wells were rinsed five times with washing buffer. Ellman’s reagent was added for development of the plates in each well. After development, the plates were read at 410 nm, and SP levels were calculated.

Detection of ROS production in urinary bladder after exogenous SP administration. SP is capable of causing intracellular ROS generation and release by inflammatory cells and, perhaps, other types of cells (3, 40). Thus we want to examine whether ROS generated after exogenous SP administration contributes to bladder hyperactivity. The ROS generation in response to SP stimulation was measured in whole blood (obtained from the femoral artery) and bladder by a chemiluminescence (CL) detection method as described previously (7). In addition, the possible cellular source of ROS in the urinary bladder was examined by the fluorescence emitted after dichlorofluorescein (DCFH) diacetate infusion (38).

A change in the bladder surface during the filling and micturition states of the transcystometric model may affect the measurement of ROS. Thus an isovolumetric model allowing minimal bladder surface change was adopted for measurement of ROS generation from the bladder in vivo.

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**Fig. 1.** Saline infusion (0.15 ml/min) into the urinary bladder evoking normal micturition demonstrated with a transcystometric bladder model. A: simultaneous recordings of pelvic afferent nervous activity (PANA) and pelvic efferent nervous activity (PENA), intravesical pressure (IVP), and arterial blood pressure (ABP) at a slow chart speed. B: at a fast chart speed, accumulated urine in the urinary bladder gradually enhances mechanoreceptor-dependent PANA. Up to a threshold volume, the enhanced PANA evokes a bursting type of PENA and triggers a series of fluctuating bladder contractions to expel the urine. A mild increase in ABP, defined as a vesicovascular reflex, is associated with a micturition reflex. N, neurogenic bladder contraction.
Eighteen rats were used and grouped (see below) in this part of the experiment. To establish an isovolumetric condition, we inserted one PE-50 tube into the bladder through the urethra and tied it in place with a ligature around the urethral orifice (23). The catheter was connected to a separate pressure transducer and an infusion pump via a T tube connector. Transurethral filling (0.15 ml/min) of 0.9% saline into the urinary bladder via the urethral catheter was done until rhythmic bladder contractions occurred. The infusion was stopped, and the bladder was maintained under constant-volume conditions by ligation of the ureter bilaterally.

The method for detection of ROS from the organ surface after 2-methyl-6-(4-m ethoxyphenyl)-3,7-dihydroimidazo(1,2-a)-pyrazin-3-one hydrochloride administration (0.2 mg·ml⁻¹·h⁻¹ ia) was adapted from the technique described by Chien et al (7) for demonstration of ROS production in the hyperactive bladder. The rat was maintained on a respirator (1.0–1.5 ml tidal volume, 80–90 cycles/min, 20–30 cmH₂O inspiratory pressure) and a circulating water pad at 37°C until photon detection. For exclusion of photon emission from sources other than the urinary bladder, the animal was housed in a light shield box with a sham bladder. Only the bladder window was left unshielded and was positioned under a reflector, which reflected the photons from the exposed bladder surface onto the detector area. The 2-methyl-6-(4-m ethoxyphenyl)-3,7-dihydroimidazo(1,2-a)-pyrazin-3-one hydrochloride-enhanced CL signal from the bladder surface was measured continuously before and during SP (10 μg) administration by use of a CL analyzing system (CLD-110, Tohoku Electronic Industrial, Sendai, Japan).

**Cellular origin of ROS in the bladder after SP administration.** We conducted DCFH diacetate tissue staining to determine the cellular origin of ROS in the bladder after SP administration. DCFH diacetate is a stable nonfluorescent compound that can diffuse into cells, is hydrolyzed to DCFH, and is thereby trapped within the cells (38). DCFH is oxidized by ROS to yield dichlorofluorescein (DCF), a fluorescent molecule (38).

At 10 min after SP (10 μg ia) administration, the bladder was filled transurethrally with 5 μM DCFH diacetate (Sigma, St. Louis, MO) and 1 μM propidium iodide (PI; Sigma) in a volume of 0.5 ml of saline for an additional 30 min and then washed out with saline. The bladder was removed, cut, and embedded in Tissue-Tek optimal cutting temperature compound (Sakura Finetek, Torrance, CA). Frozen sections (10 μm thick) were obtained, and cellular fluorescence intensity in the tissue sections was examined by fluorescence microscopy (model DMRD, Leica Microsystems Wetzlar, Wetzlar, Germany).

In addition, a portion of the urinary bladder was cut and fixed in 10% neutral buffered formalin solution, dehydrated in graded ethanol, and embedded in paraffin. Sections (5 μm) of bladders were stained with hematoxylin and eosin and Giemsa and evaluated for the extent of inflammatory cell accumulation and number of mast cells (31).

**Effects of SP-induced ROS formation on bladder hyperactivity.** We detected formation of ROS in the bladder of SP-treated rats. Next, we performed studies to determine the role of ROS in bladder hyperactivity. Before SP stimulation, baseline ABP, IVP, and ROS amounts were recorded for 30 min as a control value. After 10 min of SP stimulation, the rats (n = 18) were divided into groups and treated with saline (n = 2), CP-96345 (500 μg, n = 4), SR-48968 (500 μg, n = 2), superoxide dismutase (SOD, 500 U, n = 4), FC₃S (250 μg, n = 3), or C₅ (250 μg, n = 3). The three antioxidants (SOD and the fullerenes FC₃S and C₅) were used to test whether the SP-induced bladder hyperactivity can be ameliorated by free radical scavengers. The ABP and cystometrogram were monitored simultaneously for 60 min. After measurement, the bladder tissues and leukocytes isolated from 4 ml of whole blood were collected and stored at −70°C for immunoblotting analysis.

**Immunoblot analysis for ICAM and β-actin.** The immunoblotting method was described previously (7). We measured the amounts of ICAM and β-actin in bladder tissues and leukocytes of SP-treated rats. For protein analysis, bladder samples and leukocytes were homogenized with a prechilled mortar and pestle in extraction buffer, which consisted of 10 mM Tris-HCl (pH 7.6), 140 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 1% NP-40, 0.5% deoxycholate, 2% β-mercaptoethanol, 10 μg/ml pepstatin A, and 10 μg/ml aprotinin. The mixtures were homogenized completely by vortexing and kept at 4°C for 30 min. The homogenate was centrifuged at 12,000 g for 12 min at 4°C, the supernatant was collected, and protein concentration was determined by Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA). Antibodies raised against ICAM (catalog no. AF583, R & D Systems, Minneapolis, MN) and β-actin (catalog no. A5316, clone AC-74, Sigma) were used. Both of these antibodies cross-react with respective rat antigens.

SDS-PAGE was performed on 12.5% separation gels in the absence of urea, and the gels were stained with Coomassie brilliant blue. Proteins on the SDS-PAGE gels, each lane containing 30 μg of total protein, were transferred to nitrocellulose filters. The immunoreactive bands were detected by incubation with the antibody described above, the secondary antibody-alkaline phosphatase, and, finally, nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate, toluidine salt (Roche Diagnostic, Mannheim, Germany) stock solution for 30 min at room temperature.

**Data acquisition and statistical analysis.** All nervous activity (PANA and PEN A) was expressed as the number of spikes per second. We analyzed the PANA and the frequency of bursting PENA before and after drug treatment. Values are means ± SE. Data were subjected to analysis of variance, followed by Duncan’s multiple-range test for assessment of the differences among groups. Student’s paired t-test was used to detect differences between control and drug treatment. P < 0.05 was considered to indicate statistical significance.

**RESULTS**

The PANA and PEN A, IVP, and ABP in rats with normal micturition reflex were recorded simultaneously. The IVP was gradually increased on accumulation of saline in the urinary bladder, causing activation of PANA. The frequency of PANA over time was progressively increased by the increase in bladder filling volume. When a threshold volume (~0.42–0.62 ml, mean 0.5 ml) was reached to evoke a micturition reflex, PANA quickly reached its peak (i.e., enhanced PANA), and then a bursting efferent discharge (PENA) that lasted for 7–15 s was detected (Fig. 1). The bursting PENA can simultaneously trigger an active neural reflex-mediated bladder contraction, as shown by a quick rise in IVP. ABP was slightly and transiently elevated (for ~15–35 s) in accordance with the abrupt rise in IVP and the neurogenic bladder contraction (Fig. 1).
Endogenous SP via NK₁ receptor participates in a normal micturition reflex. Intra-arterial administration of CP-96345 (250 μg) significantly decreased the frequency of PANA over time, the frequency of bursting PENA, and active contractions (P < 0.05) and increased bladder capacity (Fig. 2, Table 2; P < 0.05). This result suggested that intra-arterial CP-96345 exerted a partial inhibition on afferent neurotransmission of the micturition reflex to increase the bladder capacity. A more significant effect of CP-96345 was detected when it was administered intrathecaly. The mean frequency of PANA over time increased after intrathecal CP-96345 administration, while the peak frequency of PENA dropped to zero, indicating that lumbosacral spinal NK₁-receptor blockade completely inhibited the afferent and efferent neural transmission of the micturition reflex. Inhibition in micturition parameters is displayed as a function of dose (Fig. 3, Table 2). The abrupt and dose-dependent hypotensive response to intrathecal administration of the NK₁ antagonist indicates CP-96345 leakage into the systemic circulation. In contrast, intra-arterial or intrathecal administration of SR-48968 had no influence on the micturition reflex (Table 2), suggesting that SP via mainly the NK₁, not the NK₂, receptor me-

Table 2. Effects of intra-arterial or intrathecal administration of SP, CP-96345, and SR-48968 on micturition parameters in urethane-anesthetized rats

<table>
<thead>
<tr>
<th>Drugs</th>
<th>n</th>
<th>Frequency</th>
<th>IVP, mmHg</th>
<th>BC, ml</th>
<th>MV, ml</th>
<th>RV, ml</th>
<th>BP, mmHg</th>
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<tbody>
<tr>
<td>SP</td>
<td>6</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>10 μg ia</td>
<td></td>
<td>Before</td>
<td>3.5 ± 0.4</td>
<td>26.0 ± 2.7</td>
<td>0.61 ± 0.10</td>
<td>0.50 ± 0.07</td>
<td>0.11 ± 0.02</td>
</tr>
<tr>
<td></td>
<td></td>
<td>After</td>
<td>8.6 ± 1.5*</td>
<td>31.1 ± 3.2*</td>
<td>0.33 ± 0.08*</td>
<td>0.21 ± 0.06*</td>
<td>0.12 ± 0.02</td>
</tr>
<tr>
<td>5 μg it</td>
<td>6</td>
<td>Before</td>
<td>3.9 ± 0.6</td>
<td>28.1 ± 2.0</td>
<td>0.56 ± 0.11</td>
<td>0.56 ± 0.11</td>
<td>0.14 ± 0.02</td>
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<tr>
<td></td>
<td></td>
<td>After</td>
<td>7.3 ± 1.8*</td>
<td>29.2 ± 3.5</td>
<td>0.30 ± 0.06*</td>
<td>0.30 ± 0.04*</td>
<td>0.10 ± 0.02</td>
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<tr>
<td>CP-96345</td>
<td>6</td>
<td>Before</td>
<td>4.2 ± 0.9</td>
<td>23.4 ± 2.0</td>
<td>0.62 ± 0.11</td>
<td>0.50 ± 0.07</td>
<td>0.12 ± 0.03</td>
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<tr>
<td>250 μg ia</td>
<td></td>
<td>After</td>
<td>2.4 ± 0.4*</td>
<td>24.0 ± 2.3</td>
<td>0.89 ± 0.16*</td>
<td>0.75 ± 0.10*</td>
<td>0.14 ± 0.02</td>
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<td>5 μg it</td>
<td>6</td>
<td>Before</td>
<td>3.7 ± 0.5</td>
<td>24.0 ± 3.0</td>
<td>0.60 ± 0.11</td>
<td>0.50 ± 0.09</td>
<td>0.12 ± 0.03</td>
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<td></td>
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<td>After</td>
<td>1.9 ± 0.2*</td>
<td>25.1 ± 2.9</td>
<td>DI</td>
<td>DI</td>
<td>DI</td>
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<tr>
<td>250 μg it</td>
<td>6</td>
<td>Before</td>
<td>4.0 ± 0.6</td>
<td>23.9 ± 3.0</td>
<td>0.62 ± 0.10</td>
<td>0.48 ± 0.09</td>
<td>0.13 ± 0.03</td>
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<td>After</td>
<td>0.9 ± 0.1*</td>
<td>24.5 ± 3.0</td>
<td>DI</td>
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<td>DI</td>
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<tr>
<td>SR-48968</td>
<td>6</td>
<td>Before</td>
<td>4.2 ± 0.5</td>
<td>23.6 ± 2.4</td>
<td>0.62 ± 0.10</td>
<td>0.44 ± 0.07</td>
<td>0.17 ± 0.03</td>
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<td>250 μg ia</td>
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<td>After</td>
<td>4.5 ± 0.7</td>
<td>23.9 ± 2.5</td>
<td>0.64 ± 0.10</td>
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<tr>
<td>250 μg it</td>
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<td>Before</td>
<td>4.0 ± 0.6</td>
<td>24.6 ± 2.6</td>
<td>0.59 ± 0.10</td>
<td>0.40 ± 0.08</td>
<td>0.18 ± 0.03</td>
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<td></td>
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<td>After</td>
<td>4.4 ± 0.7</td>
<td>25.0 ± 2.8</td>
<td>0.61 ± 0.11</td>
<td>0.43 ± 0.09</td>
<td>0.17 ± 0.04</td>
</tr>
</tbody>
</table>

Values are means ± SE. Frequency, no. of active contractions (>15 mmHg) during 10-min period; IVP, intravesical pressure during micturition; BC, bladder capacity; MV, micturition volume; RV, residual volume; BP, baseline pressure; DI, dribbling incontinence. *P < 0.05 vs. Before.
mediates the afferent micturition pathway in the lumbosacral spinal cord (Fig. 2, right, and Fig. 3). Pelvic nerve stimulation enhances bladder hyperactivity and SP release. In rats, an increase in frequency (1–10 Hz) of pelvic nerve stimulation significantly enhanced the duration of bladder contraction, decreased intercontraction intervals (1.6 ± 0.4, 0.6 ± 0.2, 0.4 ± 0.1, and 0.3 ± 0.1 min at 0, 1, 5, and 10 Hz, respectively), and, at the same time, increased the release of SP to the bladder outflow (59 ± 8, 94 ± 14, 190 ± 30, and 254 ± 36 ng/ml at 0, 1, 5, and 10 Hz, respectively; Fig. 4). The data clearly showed that bladder hyperactivity caused by pelvic nerve stimulation is associated with a dose-dependent increase in SP in the bladder. Furthermore, we suggest that, in addition to SP, NKA and other substances are released from sensory pelvic afferents during electrical stimulation and, consequently, lead to a prolonged bladder contraction.

Exogenous SP facilitates the micturition reflex in bladder hyperreflexia. At residual volume immediately after micturition, no significant PANA and PENA were observed, and the IVP remained at the basal level when saline infusion was stopped (Fig. 5A). At ~5–10 s after intra-arterial administration of SP (0.1–5 μg), the bladder revealed an elevated IVP and dose-dependently enhanced PANA and PENA (Fig. 5B, fast trace).

During filling, the bladder revealed increased PANA by mechanical distension of bladder afferents. Shortly after intra-arterial administration of SP (10 μg), a bursting PENA was followed by a prolonged (~10-min) tonic bladder contraction (Fig. 5C). At 10 min after SP stimulation, the bladder remained hyperactive, as shown by the increase in the frequency of active contractions (number of active contractions; P < 0.05) and the decrease in bladder capacity (P < 0.05; Table 2). Our results further indicated that intra-arterial administration of SP can initiate the micturition reflex response in bladders containing a subthreshold amount (residual volume) of fluid, e.g., by activating NK receptors in smooth muscle and sensory neurons and by increasing the afferent discharge (PANA) to the central nervous system to result in a hyperactive bladder. A similar result was reported previously (23).

Similarly, intrathecal SP administration also induced bladder hyperactivity (Fig. 6), as evidenced by an increase in the number of active contractions, a
SP-induced ROS in hyperactive bladder

Fig. 5. Excitatory effects of intra-arterial SP on micturition parameters at residual volume. A: at residual volume without saline infusion, SP increased PANA, PENA, and IVP and lowered ABP in a dose-dependent manner. B: SP triggered myogenic (M) bladder contraction and PANA- and PENA-mediated neural (N) bladder contraction. C: at residual volume with continuous saline infusion, SP produced a tonic contraction followed, during its relaxation phase, by a series of rhythmic contractions (micturition reflex), which persisted even when the tonic contraction had returned to resting values. Note increased frequency of micturition (or shortened intercontraction interval) after SP stimulation.

increase in bladder capacity, and an elevation of baseline pressure (Table 2). The increased baseline pressure may be due to an increased release of tachykinins and other substances by continuous motor impulses to the smooth muscle through the pelvic parasympathetic nerves, which is brought about by micturition reflex afferents. These data suggest that increased SP in the rat lumbosacral spinal cord also plays a role in inducing bladder hypersensitivity via an afferent pathway.

Increased ROS formation from whole blood and bladder surface after exogenous SP administration. The SP-treated whole blood samples showed a dose-dependent increase in ROS formation: 78 ± 20, 336 ± 67, 560 ± 105, 1,035 ± 213, 1,201 ± 196, and 1,108 ± 230 counts/10 s at 0, 0.1, 1, 10, 50, and 100 μg of SP, respectively (Fig. 7A). The source of whole blood ROS was leukocytes, not erythrocytes and plasma (Fig. 7C). Coincubation with the NK1-receptor antagonist CP-96345 (50 μg) prevented SP-induced ROS generation from whole blood, which was reduced from 1,065 ± 210 to 97 ± 18 counts/10 s in response to treatment with 50 μg of SP (Fig. 7B). In contrast, SR-48968 had no significant inhibitory effect on SP-induced ROS generation: 1,065 ± 210 vs. 899 ± 177 counts/10 s (P > 0.05). Coincubation with the free radical scavengers SOD, C3, and FC4S also significantly reduced the SP-induced ROS generation: 220 ± 51, 430 ± 89, and 190 ± 56 counts/10 s, respectively (P < 0.05). The magnitude of the reduction exhibited by free radical scavengers was slightly lower than that exhibited by CP-96345, suggesting that directly blocking upstream NK1-receptor activation by a long-lasting effect of the nonpeptide CP-96345 is more efficient than scavenging downstream ROS activity by free radical scavengers.

Blood samples from rats subjected to intra-arterial SP treatment were examined for ROS generation. ROS in whole blood increased significantly from 101 ± 18 to 2,198 ± 365 counts/10 s after SP stimulation (Fig. 7D). This increase was reduced somewhat by simultaneous treatment with CP-96345 (756 ± 134 counts/10 s), SOD (897 ± 168 counts/10 s), C3 (1,312 ± 260 counts/10 s), or FC4S (1,032 ± 185 counts/10 s), but not by SR-48968 (2,250 ± 368 counts/10 s).

Inhibition of bladder hyperactivity by free radical scavengers. In the isovolumetric condition without SP stimulation, the frequency of bladder contractions was 3.4 ± 0.5 active contractions/10 min, and the basal level of bladder ROS was maintained at 140 ± 25 counts/10 s. Intra-arterial SP stimulation significantly reduced ABP (from 124 ± 5 to 78 ± 6 mmHg, P < 0.05) and increased the frequency of active contractions (7.3 ± 1.7 active contractions/10 min, P < 0.05) and the bladder ROS generation (Fig. 8A; from 140 ± 25 counts/10 s before SP stimulation to 2,560 ± 345 counts/10 s after 10 min of SP, P < 0.05). At 10 min after SP stimulation, intra-arterial saline had no effect on the SP-induced response. The response of the hyperactive bladder and the increase in ROS generation could be maintained for >30 min.

Pretreatment of NK-receptor antagonist is well known to reduce tachykinin-induced hyperactivity (18, 19, 21, 22); therefore, for evaluation of the possibly pharmacotherapeutic potential of agents on SP-induced hyperactivity, we administered the test agents 10 min after SP stimulation. At 10 min after SP stimulation, intra-arterial administration of CP-96345 significantly decreased the frequency of bladder contractions (to 3.4 ± 0.8 active contractions/10 min, P < 0.05) and reduced, in part, ROS generation from the bladder surface (Fig. 8C, right; from 2,705 ± 453 to 1,266 ± 320 counts/10 s, P < 0.05). Intra-arterial application of SOD (Fig. 8D, right), C3, and FC4S also significantly decreased the frequency of bladder contractions (to 4.5 ± 1.1, 4.9 ± 1.2, and 4.2 ± 1.0 active contractions/10 min with SOD, C3, and FC4S, respectively).
and ROS generation (to 1,556 ± 345, 1,956 ± 455, and 1,705 ± 345 counts/10 s with SOD, C3, and FC4S, respectively). However, SR-48968 had no effects on SP-induced hyperactivity and ROS production (Fig. 8B). These data showed that ROS plays a role in SP-induced bladder hyperactivity.

**Cellular origin of ROS in SP-induced bladder inflammation.** Sections from the bladder mucosa and smooth muscle obtained 40–45 min after SP administration revealed infiltration of leukocytes and the presence of several degranulated mast cells (Fig. 9). Sections of urinary bladders from control animals exhibited no signs of inflammation at the end of the experiments.

We used DCFH diacetate and PI under a fluorescence microscope for localization of ROS production and cell viability in bladder tissue, respectively. We found green fluorescence of DCF mainly in neutrophils in and around vessels (Fig. 9F) and in the submucosal layer (Fig. 9, G and H) of the bladder. In a similar location of the green fluorescence of DCF, red fluorescence of PI was observed (Fig. 9, F–H), indicating the presence of ROS-induced cellular damage. Neither green DCF fluorescence nor red PI fluorescence was observed in the control bladders (Fig. 9E).

**Effect of NK-receptor antagonist or SOD on ICAM expression in SP-treated bladder and leukocytes.** Expression of ICAM and β-actin in the bladder and leukocytes after SP treatment was assessed by immunoblotting with antibodies against ICAM and β-actin (Fig. 10). ICAM expression was detected in control bladder tissues was dose dependently increased by SP stimulation: 1.0 ± 0.3-, 2.0 ± 0.3-, and 3.5 ± 0.5-fold for control, 1 µg of SP, and 10 µg of SP, respectively. However, the enhanced ICAM expression was significantly inhibited by CP-96345 (from 3.5 ± 0.5- to 1.3 ± 0.3-fold) or SOD (from 3.5 ± 0.5- to 1.9 ± 0.3-fold). SR-48968 had no effect on enhanced ICAM expression (3.5 ± 0.5- vs. 3.1 ± 0.6-fold). ICAM expression in leukocytes was unaffected by SP stimulation.

**DISCUSSION**

The present study demonstrates for the first time the involvement of ROS generation in SP-mediated blad-
der hyperreflexia. SP is a sensory neuropeptide present in small myelinated Aδ-fibers and in small-diameter unmyelinated C fibers (22). Once released from bladder afferent nerves and the sacral spinal cord, SP is involved in the mechanoreceptor-mediated micturition reflex. In rats, systemic administration of capsaicin for depletion of SP resulted in urine retention or an increased volume/pressure threshold for micturition, implicating an excitatory role of SP in the afferent micturition pathway (6, 22). We demonstrated that intraarterial administration of exogenous SP could initiate the micturition reflex response in bladders containing a subthreshold amount (residual volume) of fluid. The SP-mediated micturition reflex or bladder hyperactivity is mainly through NK1 receptors. We further showed that exogenous or excessive SP stimulation also resulted in increased ROS generation. NK1-receptor blockade or free radical scavengers could inhibit micturition pathway facilitation and SP-mediated ROS generation and, subsequently, ameliorate SP-induced bladder hyperactivity.

Fig. 8. In vivo response of SP-induced bladder hyperactivity and bladder ROS generation in an isovolumetric model. A: intra-arterial SP injection induced significant vasodilation and bladder hyperactivity, as well as bladder ROS production. A and B: saline or SR-48968 had no effect on SP-induced bladder hyperactivity and ROS production. C: treatment with CP-96345 reduced the frequency of micturition and decreased, in part, ROS generation in the bladder (right). D: superoxide dismutase (SOD) injection decreased ROS generation in the bladder and reduced the frequency of micturition (right).

Fig. 9. Mast cells (arrows) and leukocytes (pluses) in control (A) and SP-induced inflamed (B) urinary bladder. Magnification ×400. Mast cells and leukocytes primarily occurred in the vicinity of the blood vessels in the SP-treated bladder, but not in the control bladder. C and D: some leukocytes adhered to the endothelium of one vessel, where mast cell degranulation was found. Dichlorofluorescein diacetate and propidium iodide under a fluorescence microscope were used for localization of ROS production and cell viability in bladder tissue. Green fluorescence of dichlorofluorescein was apparent in vessels (F) and submucosal layer (G and H), where numerous cells with red fluorescence (propidium iodide staining) were found (G and H), suggesting oxidative damage. Neither green nor red fluorescence was observed in the control bladder (E).
aptic potential in the neurons of the spinal cord and neurotransmitter producing a slow excitatory postsynaptic response in the bladder. SP can also be a centrally excitatory neurotransmitter via smooth muscle contraction at the peripheral site of action via SP treatment.

Fig. 10. Top: Western blot analysis of homogenates of rat bladders and leukocytes subjected to SP stimulation with specific antibodies to intercellular adhesion molecule (ICAM) and β-actin. Note dose-dependent increase in ICAM (85 kDa) expression after SP (1 and 10 μg) stimulation. ICAM expression appeared to be decreased after treatment with CP-96345 (CP, 500 μg) and SOD (500 μg), but not SR-48968 (SR, 500 μg). In leukocytes, SP had no effect on ICAM expression. Equal protein loading was displayed by β-actin. Bottom: mean densitometric data. *P < 0.05 compared with bladders without SP treatment. #P < 0.05 compared with bladders with 10-μg SP treatment.

In our study, the cystometrogram and electrophysiological recording techniques in urethane-anesthetized rats are valuable tools for analyzing the neural control of the urinary bladder (6, 9, 36). Efficient bladder voiding is triggered primarily by the cumulative afferent activities from the bladder mechanoreceptors during bladder distension, which subsequently elicits parasympathetic bladder efferent excitation to evoke a micturition reflex response when a threshold volume/pressure is reached (8, 9). A bladder hyperactivity or hyperactivity indicates initiation of a micturition reflex response, even in bladders containing a subthreshold amount (residual volume) of fluid. A normal bladder contraction is coordinated by efferent purinergic, cholinergic, muscarinic, and nicotinic (somatic) elements (9). In addition, several neuropeptides (e.g., SP and NKA) have been demonstrated in the rat urinary bladder, yet their roles in normal micturition and bladder hyperactivity remain to be further elucidated (21).

Stimulation by exogenous SP or NK-receptor agonists has been shown to induce bladder hyperactivity (18, 21, 22). Maggi (22) demonstrated facilitation of reflex micturition by intravesical administration of [β-Ala³]-NKA (an NK₂-receptor agonist), suggesting a peripheral site of action via smooth muscle contraction in the bladder. SP can also be a centrally excitatory neurotransmitter producing a slow excitatory postsynaptic potential in the neurons of the spinal cord and mediating a dorsal root C fiber reflex to the dorsal horn neurons, leading to bladder hyperreflexia (Fig. 3, Table 2). Thus SP exerts effects on the peripheral bladder and the central spinal cord to facilitate the micturition pathway and, consequently, lead to bladder hyperactivity. The SP-mediated hyperactivity can be inhibited by intra-arterial or intrathecal administration of CP-96345 (an NK₁-receptor antagonist), but not SR-48968 (an NK₂-receptor antagonist).

It is also known that a peripheral release of tachykinins determines a set of responses (loosely defined as neurogenic inflammation) that includes vasodilatation, plasma protein extravasation, smooth muscle contraction, stimulation of afferents, and inflammation (19). To further complicate matters, sensory neurons and immune cells can express and release tachykinins, which may also contribute to neurogenic inflammation in the bladder. SP (0.3–1 μM) is able to induce, in a dose-dependent manner, secretion of various cytokines (e.g., interleukins-1 and -6 and tumor necrosis factor-α) from cultured lymphocyte-enriched mononuclear cells isolated from human peripheral blood (10). In addition, SP has been shown to cause a proinflammatory change in tissues, such as degradation of mast cells and leukocyte adhesion to the venular endothelium (39), by a mechanism of SP-enhanced ICAM expression in the bladder tissue (Fig. 10). The possible involvement of SP and its NK₁ receptor in pathophysiological changes of bladder inflammation has been underscored by a recent study showing a dramatic reduction in antigen-induced cystitis in NK₁-receptor-deficient mice (31).

As a consequence of SP-immune and -inflammatory cell interaction, a variety of substances, such as histamine, cytokines, and ROS, are released (3, 10, 32, 39). In whole blood and leukocytes incubated with SP, ROS activity was displayed in a dose-dependent manner and SP-induced ROS release and ICAM expression are inhibited by CP-96345, but not SR-48968, confirming a mediating role of the NK₁ receptor in triggering ROS generation in leukocytes. We further showed that SP-induced ROS generation in the bladder as well as bladder hyperreflexia can be partly ameliorated by CP-96345 or, noticeably, by free radical scavengers. It is not totally unexpected that SP-induced ROS formation may contribute to the myogenic and neural hyperactivity. ROS are known to be involved in changes in muscle tone, vascular smooth muscle strip contraction (2, 30), and increased neural activity/conduction velocity in vitro by mechanisms such as alterations in membrane conductance, calcium homeostasis, calcium-dependent processes, and eicosanoid and nitric oxide metabolism (2, 16, 42).

In summary, our studies provided direct evidence that SP participates in the micturition reflex response in bladders by activating NK receptors to facilitate the afferent pathway. Increased SP stimulation may enhance the afferent discharge (PANA) to the central nervous system to result in a hyperactive bladder. On the other hand, increased nerve activity by other means may result in increased release of SP, further complicating bladder hyperactivity. Our study indi-
cates that the mechanism by which SP participates in the neurogenic bladder may be complicated by its proinflammatory activity and its ability to stimulate ROS generation.

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