Urol Res (1997) 25:395–399

ORIGINAL PAPER

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Role of substance P in several models of bladder inflammation

Received: 5 November 1996 / Accepted: 17 June 1997

Abstract Substance P (SP) is a peptide found in the sensory nervous system which has multiple biologic effects including stimulation of muscle contraction, pain nociception, immune cell functions, plasma extravasation and a constellation of inflammatory effects. Here we investigate the role of SP in several animals models of bladder inflammation. Using the female Lewis rat, inflammation was induced using either xylene, lipopolysaccharide (LPS) or polyinosinic-polycytidylic acid (polyIC). Inflammation occurred rapidly (4 h) and was maintained in each model for at least 7 days. Each of these protocols decreased the bladder content of immunoreactive SP by approximately 50%, suggesting enhanced release. There was no change in the urinary frequency of these animals over 3 weeks, suggesting that urinary frequency changes are not mediated by acute inflammation. We also found that the SP receptor (NK_1) antagonist, (−)CP96345, was unable to block the inflammation produced by polyIC, suggesting that SP is not an obligatory mediator of immune cell stimulation in this model.

Key words Interstitial cystitis · Bladder inflammation · Substance P · Animal models

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Introduction

Substance P (SP), an 11 amino acid neuropeptide found in peripheral sensory fibers, is thought to play a role in nociception [6] and neurogenic inflammation [4]. Released by antidromic stimulation in skin, SP causes vasodilatation and plasma extravasation by both direct and indirect mechanisms involving vascular smooth muscle and interstitial mast cells [13]. Receptors for SP are also present on lymphocytes [11] and macrophages [3], and SP can act as both a chemoattractant [12] and an activator of immune cell function [7, 8]. The aim of the present study was to investigate the involvement of SP in several models of bladder inflammation

The xylene-induced model of Maggi et al. [10] has been suggested as a model with which to study neurogenic inflammation. Xylene is reported to specifically stimulate sensory (capsaicin-sensitive) nerves in the bladder which have been shown to lead to bladder hyperreflexia and histamine release from mast cells. Xylene is also reported to damage the urothelium in a histamine-independent, capsaicin-sensitive manner [4]. We have also investigated bladder inflammation produced by more classical immunologic means, i.e., that produced by lipopolysaccharide (LPS), a bacterial endotoxin, and polyinosinic-polycytidylic acid (polyIC), a synthetic ribonucleic acid (RNA) which is known to stimulate cytokine secretion (especially interferon secretion), in order to determine whether SP might play a role in immune-stimulated bladder inflammation as well. (These two models were chosen to mimic bacterial- and viral-induced inflammation respectively.)

Materials and methods

The experiments described were approved by the Universtiy of Massachusetts Medical Center animal use committee. The laboratory animal care and use program at the University of Massachusetts Medical Center at Worcester complies with all Federal and State laws regarding the care and use of experimental animals and with the Public Health Service Policy on Humane Care and Use of Laboratory Animals (NIH Guide for Grants and Contracts, Vol. 14, No. 8, June 25, 1985; as revised September 1986). The animal care and use program is also fully accredited by the American Association for Accreditation of Laboratory Animal Care (AAALAC).

Xylene instillation

Female Lewis rats weighing 180–220 g were used for all studies. These animals were anesthetized with ketamine/xylazine and a small abdominal incision (~1 cm) made to reveal the bladder. The bladders were emptied by gentle manual pressure and then 300 µl of 100% xylene was injected carefully into the bladder lumen using a 26 3/8G syringe needle. The bladders were tied off with a small piece of sterile string such that some leakage could occur but the majority of the xylene was maintained in the bladder lumen. (The pressure applied to the string did not appear sufficient to have blocked blood flow to the bladder, though the possibility of some ischemia does exist. We found, however, that tying off the bladder was necessary for 100% effectiveness of the xylene treatment.) The bladders remained tied for 5 min. The string was then removed, and the animals were sutured and stapled and permitted to recover. For control animals the bladder was poked with the needle but no xylene or other fluid was injected.

PolyIC and LPS instillation

For the polyIC and LPS instillation experiments we used 22 G intravenous catheters to instill polyIC (in various doses as described below) and LPS (1 mg/kg) (or diluent controls) directly into the bladder lumen of rats anesthetized as described above. Diluent used was 1 ml sterile, non-pyrogenic saline. The solutions were administered at a rate of 0.5 ml in 30 s followed by a 1 min delay in removing the catheters. Controls were instilled in identical fashion with sterile, pathogen-free saline.

A preliminary study determined that 2–20 mg/kg of polyIC was required for consistent production of inflammation. The LPS dose was as previously described [16]. We did not pretreat the bladders with protamine sulfate [15] to destroy the glycosaminoglycan (GAG) layer; however, we did observe some blood in the urine (measured with Chemstrips) of all the rats after catheterization, indicating that some trauma may have occurred to the mucosa during treatment.

SP extraction and measurement

Bladders were removed at various times following treatment under anesthesia and the animals were killed. Bladders were cut in half longitudinally and each half was immediately weighed and placed in either 2 ml of 80% acid/acetone for peptide extraction, or in 2% paraformaldehyde for histologic preparation. Tissues for extraction were immediately minced with scissors and homogenized on ice using a Polytron homogenizer. The homogenates were kept at 4 °C overnight and centrifuged at 8000 *g* for 20 min. The supernatant fractions were isolated and mixed with an equal volume of petroleum ether. The lower, acetone-water layer was heated in a water bath to remove acetone and the aqueous residue was lyophilized.

Lyophilized extracts were resuspended in phosphate-buffered saline and brought to pH 7.4. SP content was measured by radioimmunoassay as previously described [5].

SP antagonist experiments

For the SP antagonist experiments the animals were maintained for 2 weeks prior to the experiments in a reverse dark/light environment (14 h light, 10 h dark) in order to facilitate measurement of urinary frequency in alert animals. The animals were anesthetized and injected with polyIC or saline as described above, in the dark (under red light). Experimental animals were pretreated with 7.5 mg/kg (−)CP96345 [17] 10 min prior to polyIC (or saline control) treatment. [The (−) CP96345 was a gracious gift from Pfizer, Groton, Conn.] This dose was shown previously to block the plasma extravasation due to capsaicin in the bladder [2] and to block the sialogogic effect of 1 µg/kg SP given intravenously. (0.175 g saliva produced with SP alone, 0.024 g produced with the antagonist present in addition to SP). After 24 h the animals were anesthetized, the bladders excised, and the animals killed. The bladders were immediately placed in 2% paraformaldehyde for histologic preparation.

Urinary frequency study

Prior to being killed, the rats in the SP antagonist experiment were assessed to see whether any changes in urinary frequency occurred due to the polyIC-induced inflammation and to determine whether the SP antagonist was able to block the potential change.

Urinary frequency was determined as previously described with slight modification [9]. Briefly, rat cages were lined with gel dryer filter paper (model 583, Bio-Rad, Calif.) Data were collected at 1000–1200 hours. The number of urinations was determined by counting the number of spots observed under UV light (λ = 244/356 nm). Spot counts were performed by an observer masked to the animal treatment.

Histology

Bladders were fixed in 2% paraformaldehyde for 24 h and were then transfered to buffered formalin and embedded in paraffin. Tissue sections thus prepared were stained with hematoxylin and eosin (H&E).

Statistical analyses

The effects of xylene and time on SP content were evaluated using analysis of variance for mixed models (ANOVA) by the method of

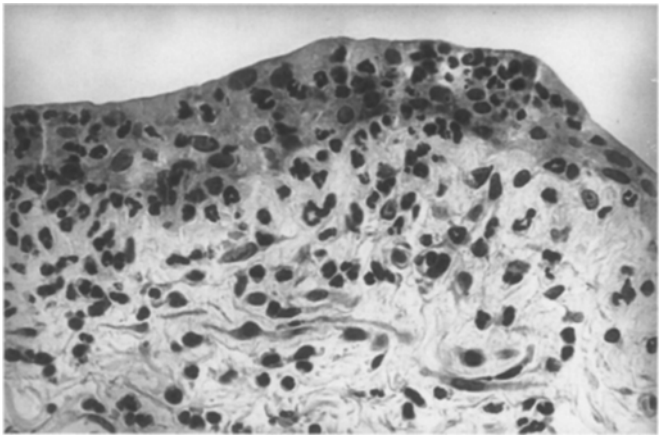


Fig. 1 Histology of bladder treated with polyinosinic-polycytidylic acid (polyIC). H & E stain of bladder tissue 24 h after exposure to polyIC. Tissue was prepared as described in the text. Note the epithelial and subepithelial inflammation. Magnification ×400

restricted estimation by maximum likelihood (REML). The reasonableness of the assumption of normality of the distribution of errors was evaluated graphically by inspection of frequency histograms on the residuals.

Differences in SP content between shams and LPS- or polyIC-treated animals were evaluated using Student's *t*-test. The reasonableness of the assumption of normality of the distribution of errors was evaluated graphically by inspection of frequency histograms on the residuals.

Results

Inflammatory effects

Sections from sham-treated animals appeared normal at all times except for some slight edema at 4 h and 24 h.

Within 4 h of polyIC treatment (Fig. 1), a marked inflammatory response was initiated in the bladders as determined by H&E stain. Similar findings were observed 4 h after xylene and LPS treatment, with the exception that the epithelial tissue layer was damaged in the case of xylene treatment (data not shown) whereas it was intact in the LPS and polyIC models. In all cases the epithelium and lamina propria were infiltrated with abundant neutrophils and the vasculature was congested. At 24 h the infiltrate was even more apparent. The infiltrate was almost gone at 7 days while the congestion and edema remained. In the xylene model, the epithelium appeared to be repairing itself by 7 days in that more of the tissue had a complete transitional epithelium.

Effects on bladder SP levels

Xylene treatment increased the bladder weight slightly but not significantly over control values (Fig. 2). Xylene also induced a rapid (within 4 h) 50% decrease in bladder SP content as compared with sham-treated animals when defined as picomoles per gram of tissue wet weight (Fig. 3). This decrease was still significant when the SP content was defined as picomoles per whole bladder (data not shown). The decrease was maintained up to at least 7 days after treatment.

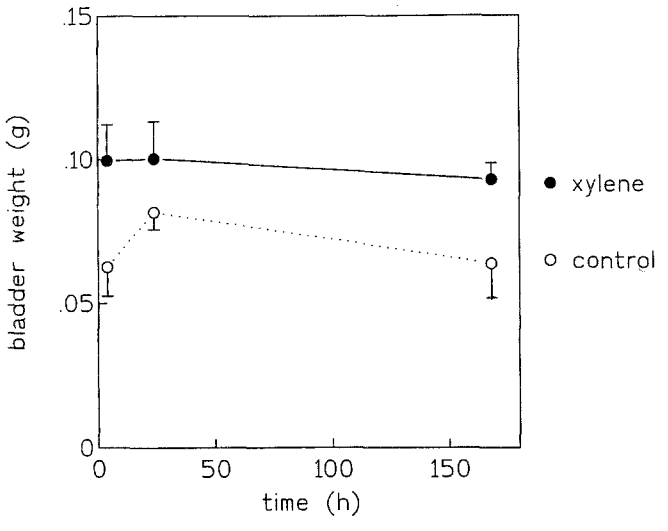


Fig. 2 Change in bladder weights in xylene-treated animals as compared with sham-injected controls. Bladders weights shown are wet weights. No significant difference in weights was found ($P > 0.05$). Error bars show the SE

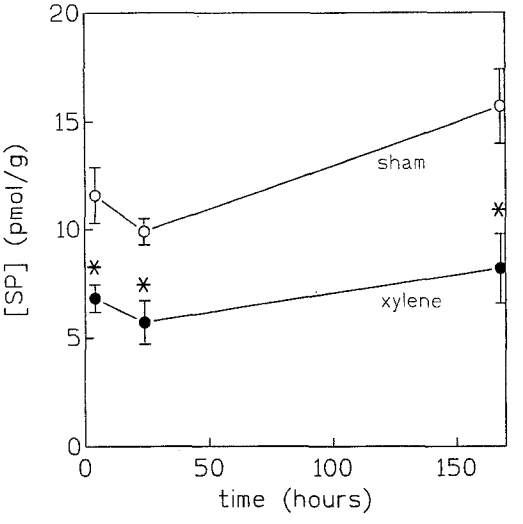
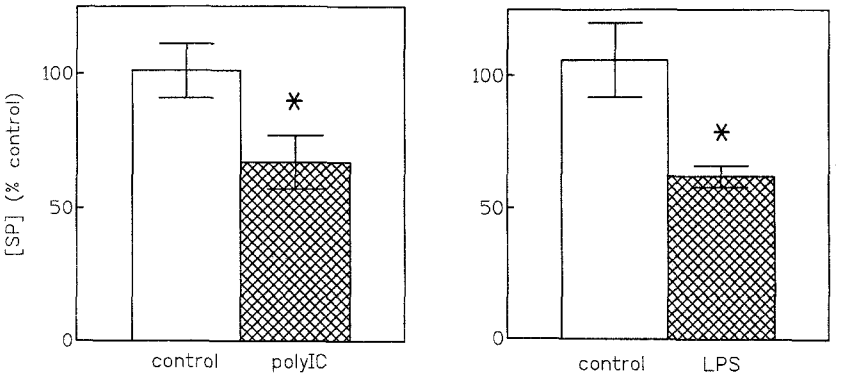


Fig. 3 Change in bladder substance P (SP) content in response to xylene as compared with sham-injected animals. Same animals as in Fig. 2. Error bars show the SE. All points were significantly different from sham controls and independent of time ($n = 16$, $^*P < 0.00001$, ANOVA: see Materials and methods)

Fig. 4 Effect of lipopolysaccharide (LPS) or polyIC on bladder SP content. Average of two experiments each. *Significantly different from sham levels. Error bars show the SE. (PolyIC: $n = 7$, $P = 0.03$; LPS: $n = 5$, $P = 0.05$, Student's *t*-test)



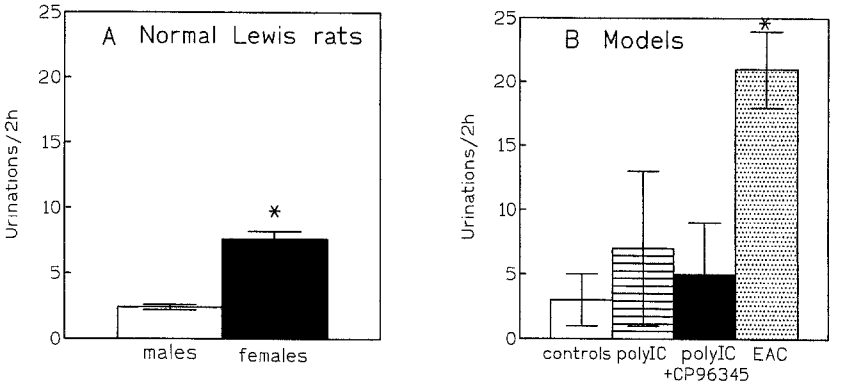


Fig. 5A,B Effect on urinary frequency of polyIC treatment with or without (−)CP96345. **A** Normal male Lewis rats versus normal female Lewis rats ($P < 0.0001$; $n = 14$ males, 8 females; 8 weekly measurements). **B** Effects of treatment with saline ($n = 4$), polyIC ($n = 7$) or polyIC plus (−)CP96345 ($n = 7$). Results for animals with experimental autoimmune cystitis (EAC) are shown for comparison; data are reported elsewhere [9]

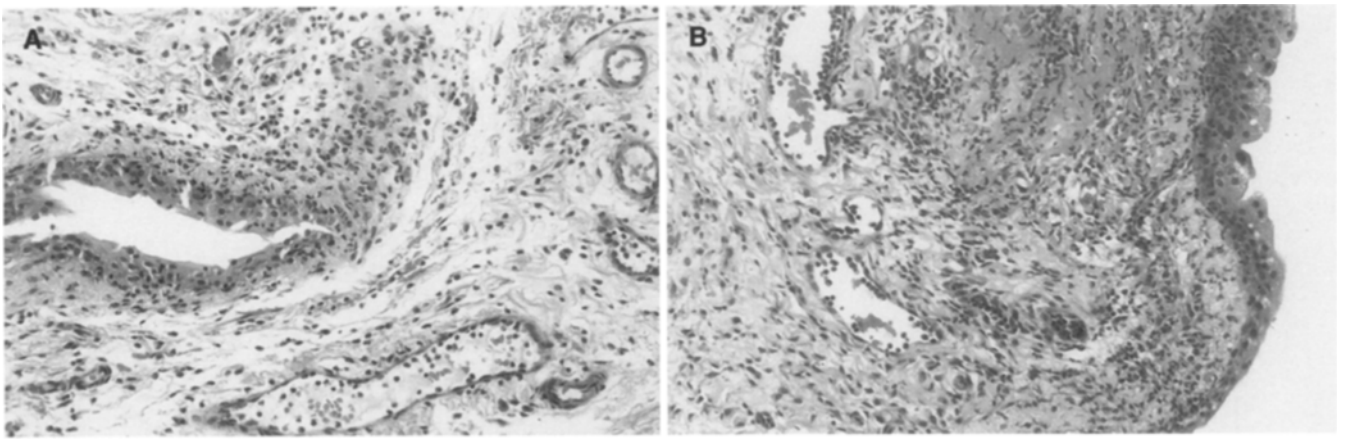


Fig. 6A,B Lack of effect of the SP antagonist (−)CP96345 on the inflammation induced by polyIC. **A** Section from the bladder of a female Lewis rat treated intravesically with 20 mg/kg polyIC. **B** Section from the bladder of a female Lewis rat treated intravesically with 20 mg/kg polyIC 10 min after pretreatment with 7.5 mg/kg intraperitonial (−)CP96345. Note that, if anything, the inflammation is enhanced in the (−)CP96345-treated animal

Both LPS and polyIC also caused a decrease (~50%) in bladder SP content 24 h after treatment when compared with saline-instilled controls (Fig. 4).

Effects on urinary frequency

While normal male Lewis rats exhibited a fairly constant urinary frequency from rat to rat and week to week of 2.4 ± 0.2 urinations per 2 h (range 1–4 urinations per 2 h), the urinary frequency of females varied more (range 1–16 urinations per 2 h) and was significantly elevated (7.6 ± 0.6 urinations per 2 h) over that of males (Fig. 5A). The urinary frequency of polyIC- and LPS-treated rats was found not to differ significantly from that of normal female rats (Fig. 5B). In contrast, rats with experimental autoimmune cystitis (EAC), an autoimmune rat model of human interstitial cystitis [9], exhibited a significantly elevated urinary frequency (Fig. 5B).

Effects of SP-receptor antagonist (−)CP96345

We found that pretreatment of animals with-CP96345 did not diminish polyIC-induced bladder inflammation as assessed by leukocyte infiltration and vascular congestion (Fig. 6).

Discussion

We have confirmed the finding of Maggi et al. [10] that xylene causes a marked inflammatory response in the rat bladder and that bladder levels of SP decrease concomitantly regardless of whether one measures bladder levels with respect to weight or total bladder content. Likewise we have found that LPS and polyIC both cause a marked inflammatory response in the rat bladder and that bladder SP levels are decreased by these treatments as well. Thus SP may exacerbate or maintain an inflammatory response in this tissue. We could not, however, block the initial, 24 h inflammatory response to polyIC with an antagonist to SP (NK1) receptors, (−)CP96345. Thus the SP-mediated response is not the only stimulus responsible for priming an inflammatory response to polyIC in the bladder and, in fact, the release of SP may be secondary to the inflammation.

This finding is in contrast to recent findings which show that immune complex mediated inflammation is dependent on SP and SP (NK_1) receptors [1]. These

findings also appear in direct contrast to those of Maggi et al. [10] for capsaicin-induced inflammation, which should be analogous to our xylene model. They found that pretreatment with (±)CP96345 did inhibit the SP-mediated plasma extravasation in the bladder, indicating a direct role for SP in this model of inflammation. They did not, however, get complete inhibition of plasma extravasation in the presence of (±)CP96345. Thus had they looked at the tissue histology, there might have been some inflammatory response which went undetected, which at 24 h might not appear any different than our response. The polyIC-induced inflammatory response may also initially depend more on cytokine production than the capsaicin/xylene response or the immune complex response. Thus although any SP enhancement of cytokine production [9] should have been blocked, the basal response to polyIC may have been enough to cause inflammation, or there may be other compensatory stimuli. Thus different modes of *initiating* inflammation may be more or less dependent on the presence of SP despite the release of SP from the bladder.

These findings do not, of course, rule out a role for SP in the pathogenesis of all bladder inflammation. Rather they indicate that SP is not the single initiator of inflammation in the bladder and that blockade of SP is not sufficient to protect the bladder against all inflammatory responses. What these findings do suggest is that once inflammation is initiated, SP will be released and this release may help to maintain or augment the inflammatory response. More importantly, the release of SP may still cause other symptoms associated with bladder inflammation such as hyperreflexia, and pain. Urinary frequency, up to 3 weeks after inflammatory insult, is apparently not affected by inflammation.

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