Alterations in spinal cord Fos protein expression induced by bladder stimulation following cystitis

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Vizzard, Margaret A. Alterations in spinal cord Fos protein expression induced by bladder stimulation following cystitis, Am J Physiol Regulatory Integrative Comp Physiol 278: R1027–R1039, 2000.—These studies examined Fos protein expression in spinal cord neurons synaptically activated by stimulation of bladder afferent pathways after cyclophosphamide (CYP)-induced bladder inflammation. In urethan-anesthetized Wistar rats with cystitis, intravesical saline distension significantly (P < 0.0005) increased the number of Fos-immunoreactive (IR) cells observed in the rostral lumbar (L1, 35 cells/section; L2, 27 cells/section) and caudal lumbar-sacral (L6, 120 cells/section; S1, 96 cells/section) spinal cord compared with control animals, but Fos protein expression in the L5 segment was not altered. The topographical distribution of Fos-IR cells was also altered in the lumbosacral spinal cord. The majority of Fos-IR cells were distributed in the dorsal commissure (45%), with smaller percentages in the sacral parasympathetic nucleus (25%), medial dorsal horn (20%), and lateral dorsal horn (10%). These results demonstrate that urinary bladder distension produces increased numbers and an altered distribution pattern of Fos-IR cells after cystitis. This altered distribution pattern resembles that following noxious irritation of the bladder in control animals. Pretreatment with capsaicin significantly reduced the number of Fos-IR cells induced by bladder distension after cystitis. These data suggest that chronic cystitis can reveal a nociceptive Fos expression pattern in the spinal cord in response to a non-noxious bladder stimulus that is partially mediated by capsaicin-sensitive bladder afferents.

interstitial cystitis; allodynia; sacral parasympathetic nucleus; spinal cord; capsaicin; choline acetyltransferase

THE STORAGE AND PERIODIC ELIMINATION of urine are dependent upon the coordinated activity of various organs, including the urinary bladder, urethra, and external urethral sphincter (13). This coordination is mediated by reflex pathways organized in the lumbosacral spinal cord and also by voluntary and reflex mechanisms located in the brain. These pathways consist of the following three basic central elements: spinal preganglionic neurons, spinal interneurons, and extraspinal inputs from primary afferents and supraspinal neurons (13, 26).

Chronic pathological conditions inducing tissue irritation or inflammation can alter the properties of sensory pathways, leading to a reduction in pain threshold (allodynia) and an amplification of painful sensations (hyperalgesia) (9). Peripheral sensitization of primary afferents or changes in central synapses can contribute to the increased pain sensation (15, 18, 19). These changes have been linked with alterations in gene expression and synthesis of neurotransmitters (16, 22). It has been documented that tissue inflammation in visceral organs such as the urinary bladder can also increase afferent nerve sensitivity to noxious and non-noxious stimuli (21, 37). Changes in afferent excitability can elicit painful sensations as well as hyperactivity of the inflamed visceral organs. For example, patients receiving cyclophosphamide (CYP) for the treatment of neoplastic disorders often exhibit side effects of CYP therapy that include hemorrhagic cystitis, irritative voiding, and gross hematuria (35, 42, 50).

In addition, patients with interstitial cystitis, which is a painful, chronic urinary bladder inflammation syndrome, exhibit urinary frequency, urgency, and suprapubic and pelvic pain (23, 35).

Recent experiments involving a chemically (CYP)-induced urinary bladder inflammation in the rat have demonstrated alterations in neurochemical (17, 34, 48) and electrophysiological (24, 51) properties of bladder afferent neurons in the L6–S1 dorsal root ganglia (DRG). Animal studies have also indicated that CYP treatment in the rat induces cystitis that is characterized by histological changes in the urinary bladder as well as increased frequency of voiding in awake rats and urinary bladder hyperreflexia in anesthetized rats (28, 31, 45). These changes suggest considerable reorganization of reflex connections in the spinal cord and marked changes in the properties of micturition reflex pathways after CYP-induced cystitis. These changes may also be involved in the phenomenon of allodynia (e.g., pain at low to moderate urinary bladder distension) demonstrated by patients with interstitial cystitis (23, 35).

Previous studies (3, 4, 48) have used immediate early gene expression as a marker for postsynaptic activation of spinal cord neurons receiving afferent input from the lower urinary tract (LUT). Studies (3, 4, 48) have revealed that noxious and non-noxious stimulation of the rat LUT increased the number of Fos-immunoreactive (IR) neurons in discrete regions of the L6–S1 spinal cord, including the superficial lateral and medial dorsal horn (LDH and MDH, respectively), the dorsal commissure (DCM), and the region of the sacral para-
sympathetic nucleus (SPN). Noxious stimulation activated greater numbers of Fos-IR neurons in the DCM, whereas non-noxious stimulation elicited a greater response in the SPN (4). It was surprising that Fos protein expression was not detected in rostral lumbar (L1–L2) spinal segments after stimulation (noxious or non-noxious) of the LUT, although these segments receive afferent input from the urinary bladder (3).

The present studies examined Fos protein expression in spinal cord neurons synchronously activated by stimulation of bladder afferent pathways after CYP-induced bladder inflammation. It was hypothesized that after CYP-induced bladder inflammation increased numbers of Fos-IR cells induced by urinary bladder distension (non-noxious) would be observed in the spinal cord as a result of 1) central changes in synaptic plasticity and/or membrane excitability; 2) peripheral changes in afferent terminal excitability in the bladder; or 3) changes in tonic pain modulation systems. Our results demonstrate that urinary bladder distension produces increased numbers of Fos-IR cells as well as an altered distribution pattern of Fos-IR cells after CYP-induced bladder inflammation. This altered distribution pattern resembles that following noxious irritation (1% acetic acid) of the urinary bladder in control (non-inflamed) animals (3, 6). Pretreatment with capsaicin (CAP; C-fiber neurotoxin), significantly reduced the number of Fos-IR spinal cord cells induced by urinary bladder distension after CYP-induced inflammation. These data suggest that chronic bladder inflammation can reveal a nociceptive Fos expression pattern in the spinal cord in response to a non-noxious bladder stimulus that is partially mediated by CAP-sensitive (presumptive C-fibers) bladder afferents. Thus this situation is, in some ways, analogous to the altered visceral sensation of interstitial cystitis patients in which a normally non-noxious stimulus (bladder filling) is perceived as painful (23, 35).

**METHODS**

**Chemical Cystitis**

As previously described (45, 47, 48), chemical cystitis was induced in Wistar rats of either sex (200–250 g) by CYP, which is metabolized to acrolein, an irritant eliminated in the urine (11). CYP (Sigma Chemical, St. Louis, MO; 75 mg/kg ip) was administered every 3rd day for 2 wk (n = 20) to elicit chronic irritation. All injections of CYP were performed under halothane (2%) anesthesia. At the end of the 2-wk CYP protocol, animals were anesthetized with urethan (0.9 mg/kg sc, 0.3 mg/kg ip), and the urinary bladder was exposed via an abdominal incision. Room temperature saline (0.9%) was infused (0.12 ml/min) via a needle (25 gauge) placed through the dome of the urinary bladder. Because the urethral outlet remained open, fluids were expelled during reflex bladder contractions. To confirm the previously demonstrated (3, 4) Fos expression pattern induced by infusion of a chemical irritant into the urinary bladder and to provide a direct comparison with the present results, room temperature 1% acetic acid was continuously infused (0.12 ml/min) into the urinary bladder of additional animals (n = 4, no CYP treatment) for a 2-h period, and the irritant was allowed to leak out the urethral outlet. Mineral oil was applied to the area around the urethral orifice to minimize irritation of the perineum.

**Capsaicin Treatment**

Birder and de Groat (4) have demonstrated that pretreatment of rats with CAP markedly suppressed Fos protein expression in the spinal cord induced by noxious stimulation (1% acetic acid) of the LUT; however, CAP pretreatment did not affect the distribution or numbers of Fos-IR cells in the spinal cord after distension of the urinary bladder by saline infusion (non-noxious). Thus it was concluded (4) that a specific population of nociceptors (C-fiber afferents) exists and responds to chemical irritation of the LUT. To determine whether Fos protein expression in the spinal cord induced by distension of the urinary bladder by saline in CYP-treated animals was mediated by a CAP-sensitive population of nerve fibers, some animals (n = 6) were pretreated 4–5 days before the experiment with the neurotoxin CAP to achieve desensitization of small-diameter primary afferents. CAP treatment was started 4–5 days before non-noxious stimulation of the LUT, at which point the animals were on day 9 or day 10 of the CYP protocol (2-wk duration).

As described previously (49), CAP solution containing 20 mg/ml CAP (Sigma) in 10% ethanol, 10% Tween 80, and 80% physiological saline was injected (125 mg/kg sc) in divided doses on 2 consecutive days: 25 and 50 mg/kg at a 12-h interval on the 1st day and 50 mg/kg on the 2nd day. Thirty-six to forty-eight hours after the last injection, the animals exhibited a negative eye-wipe test, which involved the application of a drop of dilute CAP solution (20 µg/ml) to the surface of the eye and counting the number of eye wiping movements. This test indicates the extent of desensitization to CAP (43).

**Euthanasia and Tissue Handling**

Two hours after infusion of saline or acetic acid into the urinary bladder, while still under urethan anesthesia, animals were killed by intracardiac perfusion first with oxygenated Kребs buffer (95% O2, 5% CO2) followed by 4% paraformaldehyde. Previous studies have determined that Fos protein expression in the spinal cord is maximal 2 h after intravesical infusion (3). After perfusion, the spinal cords were quickly removed and postfixed for 2–6 h. Tissue was then rinsed in PBS (0.1 M NaCl in phosphate buffer, pH 7.4) and placed in ascending concentrations of sucrose (10–30%) in 0.1 M PBS for cryoprotection. Spinal cord segments (L1, L2, L5–S1) were sectioned in the transverse plane at a thickness of 40 µm on a freezing microtome. These experimental protocols were approved by the University of Vermont Institutional Animal Care and Use Committee (99–059). Animal care was under the supervision of the University of Vermont's Office of Animal Care in accordance with the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) and National Institutes of Health guidelines. All efforts were made to minimize animal stress/distress and suffering and to use the minimum number of animals.

**Control Experiments**

Multiple control groups were used for these studies (Table 1).

Pan-Fos immunohistochemistry. As previously described (48), alternate spinal cord sections (L1, L2, L5–S1) were incubated for 72 h at 4°C with pan-Fos antisera (1:10,000; Genosys Biotechnologies, The Woodlands, TX) diluted in potassium phosphate-buffered saline (KPBS) plus 0.4% Triton X-100. This antibody recognizes Fos oncoproteins, and as
such detects Fos and Fos-related proteins. After the incubation, the antibody was visualized with an avidin-biotin horse-radish peroxidase complex using a Vectastain ABC kit (Vector Laboratories, Burlingame, CA). Tissue sections were then mounted on gelatin-coated slides, dehydrated in graded ethanol rinses, cleared in xylene, and coverslipped with Permount. All sections were examined with brightfield microscopy. Tissue from control (no treatment, sham, or vehicle treatment) and chronically irritated animals was processed at the same time. Control tests run without the primary or secondary antisera or with antisera preabsorbed with Fos protein eliminated Fos staining. Fos-IR was also detected by using an alternative c-Fos antisera (1:3,000; Santa Cruz Biotechnology, Santa Cruz, CA). Although the Fos-IR was comparable in number and distribution using either Fos antisera, visualization of Fos antigen by indirect immunofluorescence was only successful when using the pan-Fos antisera (see below). Thus the pan-Fos antisera is the preferred antisera and quantification of Fos-IR in this study is based on this antisera.

To determine the type of spinal neuron (interneuron vs. preganglionic neuron) expressing Fos-IR in the region of the intermediolateral cell column (L1-L6), dorsal commissural nucleus (DCN; L1), or SPN (L6), cholinergic preganglionic neurons (PGN) in the L1 (n = 6) or L6 (n = 6) segment were identified by an antibody against choline acetyltransferase (ChAT). For this analysis, Fos-IR was detected through the use of indirect immunofluorescence and this analysis was restricted to the L1 and L6 spinal segments because these segments exhibited the greatest number of Fos-IR cells. Sections were examined under a fluorescence photomicroscope with a multiband filter set for simultaneous visualization of Cy3 and Cy2 fluorophores. Cy2 was viewed by using a filter with an excitation range of 447–501 nm and an emission range from 510 to 540 nm; Cy3 was visualized with a filter with an excitation range of 506–596 nm and an emission range from 610–655 nm. In all cases, Fos-IR was visualized as red fluorescence and ChAT-IR was visualized as yellow-green fluorescence.

Assessment of Urinary Bladder Inflammatory Changes

Histology. Sections of the bladder wall (25 µm) from control situations (no treatment, sham, or vehicle treatment) and chronic CYP-treated animals were examined for inflammatory changes after hematoxylin/eosin (H/E) staining. Urinary bladders were harvested from animals killed by intracardiac perfusion but not subjected to any urinary bladder distension protocol. These bladders were blotted dry and the weights recorded. Subsequently, the tissue was postfixed in 4% paraformaldehyde, placed in ascending concentrations of sucrose (10–30%) in 0.1 M PBS for cryoprotection, and then sectioned and counterstained using the Mayer’s technique for hematoxylin and eosin (1a). Additional urinary bladders were harvested from animals receiving CYP vehicle, CYP vehicle, or CAP treatment. These urinary bladders were also blotted dry and the weights recorded.

ED-1 immunoreactivity. To assess the relative density of resident tissue macrophages in the urinary bladder after chronic CYP treatment compared with control situations, sections (25 µm) of urinary bladder were incubated with an ED-1 mononodal antibody (MAb) (1:100; Harlan Bioproducts for Science, Indianapolis, IN). The MAb ED-1 recognizes a cytoplasmic antigen (CD86) associated with phagolysosomes present in monocytes, most tissue macrophages, as well as some dendritic cell subpopulations (12).

Myeloperoxidase assay. Inflammation of the urinary bladder was also assessed with an assay for myeloperoxidase (MPO). Polymorphonuclear (PMN) cell infiltration is a characteristic of inflammation and MPO is a naturally occurring enzyme contained in the primary granules of the PMN cells. Greater MPO activity in a tissue represents increased PMN cell infiltration in inflamed tissue (8). Thus an MPO assay was performed on freshly harvested urinary bladder tissue obtained from pentobarbital sodium-anesthetized (120 mg/kg ip) animals. Euthanasia was confirmed with a bilateral thoracotomy. The MPO assay was performed as described by
Bradley et al. (8). Briefly, MPO was extracted from homogenized bladder tissue by suspending the material in 0.5% hexadecyltrimethylammonium bromide (Sigma) in 50 mM potassium phosphate buffer (pH 6.0) before sonication in an ice bath for 30 s. The specimens were freeze-thawed three times, and the sonication was repeated. Suspensions were assayed spectrophotometrically. Supernatant (0.1 ml) was mixed with 2.9 ml of potassium phosphate buffer containing 0.167 ml/ml of o-dianisidine dihydrochloride (Sigma) and 0.0005% hydrogen peroxide. The change in absorbency was measured at 460 nm. One unit of MPO activity was defined as that degrading one micromole of peroxide per minute at 25°C.

Quantification and statistical analysis. The goals of the present study were 1) to determine the relationship between the distribution of Fos-IR cells in specific spinal cord regions (see below) and 2) to determine the percentage of Fos-IR cells exhibiting ChAT-IR in the lateral horn of the L1 and L6 spinal segments. In this study, the absolute number of Fos-IR cells was not determined. Thus the number of cells exhibiting Fos-IR was estimated from 15–20 spinal cord sections from L1, L2, and L5–S1 segmental levels in four spinal cord regions for each animal: 1) MDH; 2) LDH; 3) DCM in L6–S1; and DCN in L1–L2; and 4) lateral laminae V–VII, including the SNp in L6–S1 or the IML in L1–L2. Fos-IR cells in the lateral horn region also exhibiting ChAT-IR were similarly counted. These spinal cord regions were the same as those examined in previous studies examining Fos distribution after LUT stimulation (3, 48). Spinal cord sections used for counts were separated by 120 µm to reduce the possibility of double counting. Counts of Fos-IR cells are presented as average numbers of cells per section or percentage change in numbers of cells per section. Comparisons between the number of Fos-IR spinal neurons in control or experimental situations were made using ANOVA. Animals processed and analyzed on the same day were tested as a block in the ANOVA. Thus day was treated as a blocking effect in the model. Two variables being tested in the analysis: 1) experimental manipulation versus control situation and 2) the effect of day (i.e., tissue from experimental and control groups of animals were processed on different days). When F ratios exceeded the critical value (p ≤ 0.05), the Newman-Keuls test was used for multiple comparisons among means. All values are expressed as means ± SE.

Figure Preparation

Tissue sections were examined and photographed with Ektachrome Elite II film with an Olympus photomicroscope. Photographic slides were subsequently scanned into Photoshop 3.0 with the aid of a Polaroid SprintScan 35. Images were minimally adjusted for brightness and contrast and appropriately cropped. Images were imported into Canvas 6.0, where groups of images were assembled and labeled. Composite figures were printed on a Codonics NP-1600 Photographic Network printer (Middleburg Heights, OH).

RESULTS

Control Experiments

Urethan-anesthetized control animals not subjected to any experimental procedures (control group 1) or urethan-anesthetized control animals receiving CYP vehicle, CAP vehicle, or CAP treatment without any further experimental procedure (control groups 4, 7, 10) exhibited low numbers of Fos-IR cells (<2 cells/section) in the spinal segments examined (L1, L2, L5–S1). As previously demonstrated (48), urethan-anesthetized animals receiving CYP every 3rd day for 2 wk also exhibited low numbers of Fos-IR cells comparable to those observed in control animals not subjected to any experimental procedures (control group 13). Sham-operated control animals in which the urinary bladder was exposed through a midline abdominal incision and a needle placed through the dome of the urinary bladder (control group 2) or sham-operated animals receiving CYP vehicle, CAP vehicle, or CAP (control groups 5, 8, 11) exhibited low numbers of Fos-IR cells (<3 cells/section) in the L5–S1 spinal segments examined. However, as previously noted (4, 7), greater numbers of Fos-IR cells (5–10 cells/section) were observed after sham surgery in the rostral lumbar (L1–L2) spinal segments that receive afferent input from the abdominal wall. There was no effect of day on the numbers of Fos-IR cells observed.

Fos Protein Expression Induced by Non-noxious Stimulation of the LUT

Urethan-anesthetized animals with a constant infusion (0.12 ml/min) of saline through a needle inserted into the bladder dome (control group 3) or urethan-anesthetized animals receiving CYP vehicle, CAP vehicle, or CAP (control groups 6, 9, 12) with a constant intravesical infusion of saline exhibited significantly (P ≤ 0.005) greater numbers of Fos-IR cells in the lumbar (L6–S1) spinal cord. No differences in the numbers of Fos-IR cells in the L6–S1 spinal cord were observed among groups 3, 6, 9, and 12. There was no effect of day on the numbers of Fos-IR cells observed.

Thus data from these animals are presented together as control distension data. Infusion of saline induced repeated micturition reflexes at 3- to 5-min intervals during the duration (2 h) of the experiment. In agreement with previous studies (4), intravesical saline infusion induced the expression of Fos-IR cells in the L6 (56.0 ± 8.5 Fos-IR cells/section) and S1 (44.0 ± 5.2 Fos-IR cells/section) spinal cord, whereas few Fos-IR cells were observed in the L1, L2, or L5 spinal segments (range 5–10 cells/section) (Fig. 1). Fos-IR cells were observed in specific regions of the L6–S1 spinal cord. The largest percentage (68 ± 4.5% in L6, 65 ± 3.2% in S1) of Fos-IR cells were observed in the region of the SPN (Figs. 2 and 3). Smaller percentages of Fos-IR cells were present in the DCM (22 ± 7.2% in L6, 20 ± 5.6% in S1), MDH (7 ± 2.2% in L6, 10 ± 2% in S1), and LDH (3 ± 1.2% in L6, 5 ± 1.7% in S1) (Figs. 2 and 3). The majority of Fos-IR cells in the L1 spinal cord were distributed in the MDH (90 ± 3.5%) with significantly smaller percentages being fairly equally distributed in the region of the IML (2 ± 1.2%), DCN (5 ± 2.5%), and LDH (3 ± 1.7%) (Figs. 4 and 5). The distribution of Fos-IR cells in the L2 spinal cord after intravesical saline infusion was similar to that observed in the L1 segment.

Inflammation of the Urinary Bladder Induced by CYP Treatment

Histological examination and an assay of MPO activity established inflammation of the urinary bladder.
After chronic CYP-induced urinary bladder inflammation but not after CYP vehicle, CAP vehicle, or CAP treatment, urinary bladder weight significantly increased (141 ± 12.1 mg) compared with control animals (81.14 ± 1.5 mg) (Fig. 6). As previously demonstrated (45–48), gross microscopic analysis of urinary bladders from animals treated chronically with CYP resulted in extensive regions of mucosal erosion, ulcerations, edema, and, in some instances, petechial hemorrhages. Histological changes evident after chronic CYP treatment included edema of the lamina propria and plasma cell infiltrates in the lamina propria, submucosa, and perivascular tissue. Some of these cellular infiltrates included macrophages, as detected with an ED1 monoclonal antibody that recognizes a cytoplasmic antigen (CD86) associated with phagolysosomes present in monocytes, most tissue macrophages, as well as some dendritic cell subpopulations (12) (Fig. 7). MPO activity in control animals not subjected to any experimental procedure or in animals receiving CYP vehicle, CAP vehicle, or CAP treatment was similar. Thus MPO activity among these groups is presented together as control MPO activity (0.51 ± 0.05 U·min⁻¹·µg⁻¹ of bladder tissue). After CYP treatment for 2 wk, MPO activity significantly (P ≤ 0.005) increased (2.32 ± 0.42 U·min⁻¹·µg⁻¹ of bladder tissue) (Fig. 6).

Fos Protein Expression Induced by Non-noxious Stimulation of the LUT

In urethan-anesthetized animals with CYP-induced urinary bladder inflammation, a constant infusion of saline through a needle inserted into the bladder dome significantly (P ≤ 0.0005) increased the number of Fos-IR cells observed in both the rostral lumbar (L1, 35 ± 3.5 cells/section; L2, 27 ± 2.6 cells/section) and caudal lumbosacral (L6, 120 ± 10.5 cells/section; S1, 96 ± 8.2 cells/section) but Fos protein expression in L5 (12 ± 1.5 cells/section) was not altered (Fig. 1). In addition to an increase in the magnitude of Fos protein expression after intravesical saline infusion, the topographical distribution of Fos-IR cells was also altered in animals with CYP-induced bladder inflammation (Figs. 1–5). In the rostral lumbar (L1) spinal cord, the majority of Fos-IR cells were equally distributed in the IML (35 ± 4.5%) and DCN (35 ± 3.8%), with smaller percentages being distributed in the MDH (25 ± 2.5%) and LDH (5 ± 1.9%; Fig. 5). The topographical distribution of Fos-IR cells in the L2 spinal cord was similar to that observed in the L1 spinal segment. In the L6 spinal segment, the majority of Fos-IR cells were distributed in the DCM (45 ± 4.3%) with smaller percentages in the SPN (25 ± 5.2%), MDH (20 ± 3.1%), and LDH (10 ± 2.2%; Fig. 3A). Similarly, in the S1 spinal segment, the majority of Fos-IR cells were distributed in the DCM (43 ± 3.8%), with smaller

![Fig. 1. Histogram showing segmental distribution of Fos-immunoreactive (IR) cells/section (s) in rat spinal cord (L1–S1) after intravesical saline distension in control animals, in animals treated chronically with cyclophosphamide (CYP), and in animals pretreated with the C-fiber neurotoxin, capsaicin (CAP), before chronic CYP treatment. #P ≤ 0.05 vs. CYP-treated animals; **P ≤ 0.005 vs. CYP-treated animals; *P ≤ 0.0005 vs. control.](http://ajpregu.physiology.org/)

![Fig. 2. Brightfield photographs from sections (40 µm) of L6 spinal cord showing distribution of Fos-IR cells after intravesical saline distension in control animals (A), animals treated chronically with CYP (B), and animals pretreated with neurotoxin, CAP, before chronic CYP treatment (C). D: distribution of Fos-IR cells in L6 spinal segment after lower urinary tract irritation with 1% acetic acid (AA) in control animals. MDH, medial dorsal horn; LDH, lateral dorsal horn; DCM, dorsal commissure; CC, central canal; SPN, sacral parasympathetic nucleus. Calibration bar = 110 µm.](http://ajpregu.physiology.org/)
percentages in the SPN (27 ± 4.8%), MDH (18 ± 2.5%), and LDH (12 ± 1.8%) (Fig. 3B). There was no effect of day on the numbers of Fos-IR cells observed.

Non-noxious Stimulation of LUT in CYP-Treated Animals Compared with Noxious Stimulation of LUT in Control Animals

The magnitude of Fos-IR cells and the change in the topographical distribution of Fos-IR cells after intravesical saline infusion in CYP-treated animals was similar to that previously reported for control animals with a constant intravesical irritant infusion (3, 4). To confirm these previous studies and to provide a direct comparison for the present study, control animals were continuously infused with 1% acetic acid for 2 h through a needle placed through the dome of the urinary bladder (Fig. 2). In confirmation of previous results (3, 4), the majority of Fos-IR cells in the L6 spinal cord were distributed in the DCM (52.4 ± 3.7%), with smaller percentages in the SPN (25.1 ± 6.5%), MDH (17.2 ± 5.6%), and LDH (5.3 ± 3.6%; Fig. 2). Smaller numbers of Fos-IR cells (10–15 Fos-IR cells/section), comparable to that observed with intravesical saline infusion, were observed in the rostral lumbar (L1–L2) or L5 spinal segments. Thus the topographical distribution of Fos-IR cells in the lumbosacral spinal cord after either noxious stimulation of the LUT in control animals or non-noxious stimulation of the LUT in CYP-treated animals was similar. In contrast, the large number of Fos-IR cells in the rostral lumbar (L1–L2) spinal segments induced by noxious stimulation of the LUT in CYP-treated (Figs. 1, 4, and 5) animals does not parallel that observed for noxious stimulation of the LUT in control animals, where few Fos-IR cells are observed (4). There was no effect of day on the numbers of Fos-IR cells observed.

Preganglionic Neurons (ChAT-IR) Exhibiting Fos Protein After Intravesical Saline Distension After CYP-Induced Cystitis

We determined whether Fos-IR cells in autonomic centers in the L1 (IML and DCN) and L6 (SPN) spinal cord also exhibit ChAT-IR and represent either sympathetic (L1) or parasympathetic (L6) preganglionic neurons. In control animals with constant saline infusion through a needle placed in the urinary bladder, no Fos-IR cells in the region of either the IML or DCN in the L1 segment also exhibiting ChAT-IR (Table 2). Similarly, no Fos-IR cells in the region of the SPN in the L6 spinal segment exhibited ChAT-IR (Table 2). In animals treated chronically with CYP and subsequently with constant saline infusion, a significant percentage of Fos-IR cells in three regions examined (IML, 47.5 ± 5.8%; DCN, 28.2 ± 6.9%; SPN, 32.2 ± 5.2%) exhibited ChAT-IR (Table 2). In CYP-treated animals, 54.7 ± 6.9% of PGNs (ChAT-IR) in the L6 SPN were Fos-IR. Fos-IR cells expressing ChAT-IR (presumptive PGNs) were located ventral to cells exhibiting only Fos-IR (presumptive interneurons; Table 2, Fig. 8). Thus in the region of the SPN, interneurons are located dorsal to the more ventrally located PGNs (Fig. 8). There was no effect of day on the numbers of Fos-IR, ChAT-IR, or Fos + ChAT-IR cells observed.

Pharmacological Evaluation of the Fos Protein Response to Non-noxious Stimuli of the LUT in CYP-Treated Animals

CAP. Previous studies (4) have demonstrated that pretreatment with CAP (100 mg/kg sc) significantly suppressed the expression of Fos protein induced by noxious (acetate acid) stimulation of the LUT. In contrast, CAP treatment did not affect the Fos protein...
induced by non-noxious (saline) stimulation of the LUT. To determine whether CAP-sensitive (presumptive C-fibers) fibers are contributing to the expression of Fos protein after non-noxious (saline) stimulation of the LUT after CYP-induced cystitis, animals pretreated with CAP (125 mg/kg sc) were used in the present studies. In these studies, pretreatment with CAP in a dose that produced complete desensitization to the eye-wipe test significantly decreased, but did not elmi-

Fig. 4. Brightfield photographs from sections (40 µm) of L1 spinal cord showing distribution of Fos-IR cells after intravesical saline distension in control animals (A), animals treated chronically with CYP (B), and animals pretreated with the neurotoxin, CAP, before chronic CYP treatment (C). D: distribution of Fos-IR cells in L1 spinal segment after lower urinary tract irritation with 1% AA in control animals. DCN, dorsal commissural nucleus; IML, intermediolateral cell column. Calibration bar = 100 µm.

Fig. 5. Histogram showing distribution of Fos-IR cells in 4 regions of L1 spinal cord after intravesical saline distension in control animals, in animals treated chronically with CYP, and in animals pretreated with the C-fiber neurotoxin, CAP, before chronic CYP treatment. Values represent percentage of total population of Fos-IR cells induced in each experimental paradigm. The 4 regions analyzed include IML, DCN, MDH, and LDH. Inset: drawing of a hemisection of L1 spinal cord depicting these 4 regions. *P < 0.0005. Comparisons were made 1) between control and CYP-treated animals and 2) between CYP + CAP-treated animals and CYP-treated animals.

Fig. 6. Histograms showing increase in bladder weight (A) and increase in myeloperoxidase (MPO) activity (B) after chronic CYP treatment. *P < 0.0005.
DISCUSSION

The present study demonstrates that urinary bladder distension produces increased numbers of Fos-IR cells as well as an altered distribution pattern of Fos-IR cells after the chronic administration of CYP to induce bladder inflammation (cystitis). This chronic, CYP-induced cystitis is associated with a significant increase in urinary bladder weight, MPO activity, and infiltration of tissue macrophages. Gross microscopic analysis of urinary bladders from animals treated with CYP revealed extensive regions of mucosal erosion, ulcerations, edema, and petechial hemorrhages. The altered distribution pattern of Fos protein resembles that following noxious irritation (1% acetic acid) of the urinary bladder in control (noninflamed) animals (3, 6). A significant percentage (55%) of parasympathetic PGNs in the SPN exhibited Fos-IR after saline distension of the urinary bladder after CYP-induced cystitis, whereas no parasympathetic PGNs exhibited Fos-IR in control (noninflamed) animals after intravesical distension. Pretreatment with CAP (C-fiber neurotoxin) significantly reduced the number of Fos-IR spinal cord cells induced by urinary bladder distension after CYP-induced inflammation. These data suggest that chronic bladder inflammation can reveal a nociceptive Fos expression pattern in the spinal cord in response to a non-noxious bladder stimulus that is partially mediated by CAP-sensitive (presumptive C-fibers) bladder afferents. Thus this situation, in some ways, is analogous to the altered visceral sensation of interstitial cystitis patients in which a normally non-noxious stimulus (bladder filling) is perceived as painful (23, 35).

Table 2. Fos-IR and Fos + ChAT-IR cells in autonomic nuclei in control and CYP-treated animals after intravesical saline distension

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>L1</th>
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<tr>
<td>Profiles/s</td>
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<td>(% colabel)</td>
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<td>8.5 ± 2.8</td>
</tr>
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<td>ChAT-IR</td>
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<td>11.5 ± 2.8</td>
</tr>
<tr>
<td>Profiles/s</td>
<td>7.2 ± 4.5</td>
<td>8.7 ± 3.2</td>
</tr>
<tr>
<td>(% colabel)</td>
<td>0</td>
<td>28.2 ± 6.9</td>
</tr>
<tr>
<td>Fos + ChAT</td>
<td>0</td>
<td>25.2 ± 4.2</td>
</tr>
<tr>
<td>(% colabel)</td>
<td>0</td>
<td>15.7 ± 3.7</td>
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Control distension

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<th>L6</th>
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<td></td>
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<td>27.8 ± 6.2</td>
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<td>6.8 ± 2.9</td>
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<td>ChAT-IR</td>
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<tr>
<td>Profiles/s</td>
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<td>(% colabel)</td>
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CYP + distension

<table>
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<td>Profiles/s</td>
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<td>(% colabel)</td>
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Number of Fos-immunoreactive (IR) cells and/or choline acetyltransferase (ChAT)-IR cell profiles/section (s) induced by intravesical saline distension in control or cyclophosphamide (CYP)-treated animals in the intermediolateral cell column (IML), dorsal commissural nucleus (DCN), or sacral parasympathetic nucleus (SPN) regions of the L1 and L6 spinal cord, respectively. After chronic CYP treatment, a significant increase in the percentage of Fos-IR cells exhibiting ChAT-IR was observed (Fos + ChAT). In addition, there was a significant increase in the percentage of sympathetic or parasympathetic preganglionic neurons exhibiting Fos-IR (ChAT + Fos). *P < 0.005.
Chronic pathological conditions such as tissue inflammation or irritation can induce changes in the properties of somatic sensory pathways, leading to hyperalgesia and allodynia. Peripheral sensitization of primary afferents or changes in central synapses can contribute to the increased pain sensation (15, 18). It has been documented that tissue inflammation in visceral organs such as the urinary bladder can also increase afferent nerve sensitivity to noxious and non-noxious stimuli (20, 37). Changes in afferent excitability can elicit painful sensations as well as hyperactivity of the inflamed visceral organs. For example, patients receiving CYP for the treatment of neoplastic disorders often exhibit side effects of CYP therapy that include: hemorrhagic cystitis, irritative voiding, and gross hematuria (35, 42, 50). In addition, patients with interstitial cystitis, which is a painful, chronic urinary bladder inflammation syndrome, exhibit urinary frequency, urgency, and suprapubic and pelvic pain (23, 35). When CYP is injected systemically for the treatment of cancer, the urinary bladder is the organ most affected by the toxic actions of acrolein, a metabolite of CYP excreted in the urine (11, 29). Histological analysis of the urinary bladder in interstitial cystitis patients shows edema, vasodilation, and proliferation of nerve fibers with chronic infiltration of inflammatory cells such as mast cells (25). Animal studies have also indicated that CYP treatment in the rat induces cystitis, which is characterized by histological changes in the urinary bladder as well as increased frequency of voiding in awake rats and urinary bladder hyperreflexia in anesthetized rats (27, 28, 31, 45, 46, 48).

Recent experiments involving CYP-induced urinary bladder inflammation have demonstrated alterations in neurochemical (34, 47, 48) and electrophysiological (24, 51) properties of bladder afferent neurons in the L6–S1 dorsal root ganglia. Previous studies using additional animal models of chemically induced urinary bladder inflammation (cystitis) have indicated that cystitis initiates urinary bladder hyperactivity by sensitizing mechanosensitive afferents and/or recruitment of silent afferents, which are normally unresponsive to mechanical stimuli such as urinary bladder distension (14, 15, 21, 37). These changes suggest considerable reorganization of reflex connections in the spinal cord and marked changes in the properties of bladder afferents after cystitis.

The present study has demonstrated that after CYP-induced urinary bladder inflammation, urinary bladder distension results in an increased expression of Fos-IR in spinal neurons as well as an altered expression pattern of Fos-IR in specific regions of the rostral lumbar and caudal lumbosacral spinal cord, which play a role in micturition reflexes. The spinal cord regions (LDH, SPN, DCM, IML) that exhibit Fos protein induced by intravesical saline distension after CYP-induced cystitis correspond to those regions previously shown to exhibit increased growth-associated protein...
induced inflammation. These data suggest that chronic
induced by urinary bladder distension after CYP-
significantly reduced the number of Fos-IR spinal cord cells
(i.e., urinary bladder) pain.

May provide a neuroanatomical substrate for visceral
bladder in CYP-treated animals or after irritation of
previously found to exhibit a significant reduction in urinary bladder capac-
ity and a slightly reduced in the amplitude (mmHg) of
micturition contractions. Thus it is unlikely that signifi-
cant increases in urinary bladder pressure contribute
to the increased expression of Fos protein observed in
the present studies, given that bladder pressure in
CYP-treated animals are comparable or somewhat
reduced compared with controls (31).

The altered Fos distribution pattern in CYP-treated
animals after intravesical saline distension resembles
that following noxious irritation (1% acetic acid) of the
urinary bladder in control (noninflamed) animals (3, 6).
Recent studies by Al-Chaer et al. (1) have demonstrated
the presence of cells adjacent to the central canal that
respond selectively to visceral (colonic) stimulation,
including mustard oil-induced inflammation, but not to
cutaneous stimulation. Al-Chaer et al. (1) have sug-
gested that these postynaptic dorsal column neurons
may provide the physiological basis for primary vis-
ceral hyperalgesia. It is interesting that in the present
study, larger numbers of cells in the DCM, a region
complementary to that described by Al-Chaer (1), ex-
press Fos protein after saline distension of the urinary
bladder in CYP-treated animals or after irritation of
the lower urinary tract (4, 48). Thus some of these cells
may provide a neuroanatomical substrate for visceral
(i.e., urinary bladder) pain.

Pretreatment with CAP (C-fiber neurotoxin) signifi-
cantly reduced the number of Fos-IR spinal cord cells
induced by urinary bladder distension after CYP-
induced inflammation. These data suggest that chronic
bladder inflammation can reveal a nociceptive Fos
expression pattern in the spinal cord in response to a
non-noxious bladder stimulus that is partially medi-
ated by CAP-sensitive (presumptive C-fibers) bladder
afferents. Thus this situation, in some ways, is analo-
gous to the altered visceral sensation of interstitial
cystitis patients in which a normally non-noxious stimu-
lus (bladder filling) is perceived as painful (23, 35). In
addition, the present results demonstrate a prominent
sensitization of rostral lumbar spinal cord pathways
after CYP-induced cystitis. In control animals, neither
noxious nor non-noxious stimulation of the LUT in-
duces Fos protein expression in rostral lumbar spinal
neurons. However, after CYP-induced cystitis, saline
bladder distension does induce Fos protein expression
in spinal neurons in the L1–L2 spinal segments. Clin-
cal application of intravesical CAP for voiding dysfunc-
tion was first reported by Maggi et al. (30) and the use
of CAP and resiniferatoxin therapy for overactive blad-
ter treatment has recently been reviewed (10). To our
knowledge the present results are among the first to
demonstrate the involvement of CAP-sensitive bladder
afferents in mucituation reflexes and in the expression
of Fos protein induced by these reflexes after CYP-
induced cystitis.

Although CAP pretreatment significantly reduced
the number of Fos-IR cells induced by saline distension
of the urinary bladder in CYP-treated animals, these
effects were incomplete. Recent experiments in the rat
have demonstrated a complex organization of pelvic
nerve afferent fibers innervating the urinary bladder
(37). In the rat, Aδ and C-fiber bladder afferent fibers
show considerable overlap in the response threshold as
well as in the pressure/volume characteristics to which
each fiber subfraction responds (37). The following
types of pelvic afferent fibers have been identified in the
rat (37): 1) low-threshold mechanoreceptors with small
myelinated axons (A-δ) and unmyelinated axons (C
fibers); 2) high-threshold mechanoreceptors with small
myelinated axons (A-δ) and unmyelinated axons (C
fibers); and 3) silent receptors with small myelinated
axons (A-δ) and unmyelinated axons (C fibers) that are
mechanically unresponsive but may be chemosensitive.
In the present studies, the CAP data suggest the
possibility that both A-δ and C-fiber bladder afferents
are activated by urinary bladder distension in the
CYP-treated animals. Incomplete effects of CAP treat-
ment would be due to the CAP insensitivity of the A-δ
bladder afferents. In light of the above characteristics
of pelvic visceral afferents (37), it is also possible that
in the rat, some A-δ and C-fiber bladder afferents are
normally silent or inactive. After CYP-induced inflam-
lation, these fiber types may become sensitized or
awakened and contribute to the altered Fos protein
expression. Previous studies (4) have suggested that a
specific population of nociceptors (C-fiber afferents)
exists and responds to chemical irritation of the LUT in
rats without an inflamed urinary bladder. The present
results suggest that two populations of nociceptors (A
and C afferents) may contribute to the altered Fos
pattern induced by saline distension of the urinary
bladder in the presence of chronic urinary bladder inflammation. In both control animals and in animals treated chronically with CYP, saline bladder distension induced Fos protein expression in the region of the SPN. Because the SPN contains interneurons and projection neurons as well as PGNs (5, 6, 33), the question arises as to which cell populations in the region of the SPN are expressing Fos-IR. Whereas the IML region has traditionally been viewed as a closed nucleus containing only sympathetic PGNs, recent studies using transneuronal tracing with pseudorabies virus have revealed groups of interneurons intermingled with PGNs (2). Thus the possibility exists that the IML also contains interneurons and projection neurons in addition to PGNs. In the present experiments, no Fos-IR cells in the region of the IML or SPN in control animals after saline bladder distension were identified as PGNs based upon neurochemical phenotype (ChAT-IR). In contrast, after CYP-induced cystitis, a significant percentage of sympathetic PGNs (25% in L1 IML, 16% in L1 DCN) expressed Fos-IR after saline bladder distension. Similarly, a large percentage (55%) of parasympathetic PGNs in the L6 SPN of CYP-treated animals exhibited Fos-IR after saline bladder distension. This percentage (55%) is similar to that observed after noxious distension of the proximal colon in a rat model (32). Colon distension induced the expression of Fos protein in 40% of parasympathetic PGNs (identified on the basis of NADPH-d histochemistry) in the S1 spinal segment (32). In contrast, acetic acid irritation of the urinary bladder in control animals induced the expression of Fos protein in 20% of parasympathetic PGN, identified on the basis of retrograde labeling from the major pelvic ganglion (6). Thus, despite a similar distribution of Fos-IR cells and a similar magnitude of Fos protein expression after acetic acid infusion into the urinary bladder and saline distension of the urinary bladder in CYP-treated animals, these two stimuli may, in fact, be quite different in terms of the chemical phenotype of the Fos-IR cells activated.

A possible mechanism underlying the increased expression of Fos-IR in spinal neurons in LUT pathways after CYP-induced cystitis may involve neurotrophic factors (NTFs) (19, 36) or neural activity arising in the bladder. According to this hypothesis (19, 36), cystitis could change organ function that in turn can change afferent pathways from the organs. It is now widely accepted that target organs can influence the neurons that innervate them (38–40, 44). Previous experiments have demonstrated the influence of target organ-neuron interactions in the adult animal (38–40, 44). Nerve growth factor is significantly increased in the urinary bladder after partial urethral obstruction (39). Partial urethral obstruction leads to increased resistance to urine flow and in turn to increased bladder work and ultimately to bladder hypertrophy (38, 40, 41). This is also accompanied by hypertrophy of afferent neurons in the L6–S1 DRG and postganglionic efferent neurons in the major pelvic ganglia (MPG) (38) as well as increased expression of GAP-43 in bladder afferent projections to the spinal cord (lateral collateral pathway of Lissauer) (39, 41). The hypertrophied bladder exhibits markedly increased levels of nerve growth factor, and autoimmunization against nerve growth factor reduces but does not completely abolish the MPG neuronal hypertrophy (44) but does eliminate the increase in expression of GAP-43 in the lateral collateral pathway (39). This suggests that neurotrophic factors released in the hypertrophied bladder are partly responsible for the change in neuronal morphology and neurochemistry of bladder afferent projections. Similarly, alterations in Fos protein expression and the efficacy of the miturition reflexes may also be modulated by neurotrophic factors in the inflamed urinary bladder (45, 46).

Recent studies have demonstrated sudden and dramatic alterations in urinary bladder neurotrophic factor mRNA after CYP-induced cystitis (45, 46). These changes in neurotrophic factor mRNA include significant increases in nerve growth factor mRNA but also indicate that other neurotrophic factors (brain derived neurotrophic factor, glial derived neurotrophic factor, ciliary neurotrophic factor, neurotrophin 3, and neurotrophin 4) may also contribute to changes in lower urinary tract function after CYP-induced cystitis. These studies (45, 46) suggest that neurotrophic factors other than nerve growth factor may contribute to changes in the neurochemical, electrophysiological, and organizational properties of the lower urinary tract after CYP-induced cystitis. The contribution of nerve growth factor or other neurotrophic factors to the alterations in Fos-IR observed in the present study is not known but is a focus of future studies.

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