Phosphorylation of extracellular signal-regulated kinases in urinary bladder in rats with cyclophosphamide-induced cystitis

Kimberly A. Corrow and Margaret A. Vizzard

University of Vermont College of Medicine, Department of Neurology and Anatomy and Neurobiology, Burlington, Vermont

Submitted 8 December 2006; accepted in final form 30 March 2007

Corrow KA, Vizzard MA. Phosphorylation of extracellular signal-regulated kinases in urinary bladder in rats with cyclophosphamide-induced cystitis. Am J Physiol Regul Integr Comp Physiol 293: R125–R134, 2007. First published April 4, 2007; doi:10.1152/ajpregu.00857.2006.—Phosphorylated ERK expression has been demonstrated in the central and peripheral nervous system after various stimuli, including visceral stimulation. Changes in the activation (i.e., phosphorylation) of extracellular signal-regulated kinases (pERK) were examined in the urinary bladder after 4 h (acute), 48 h (intermediate), or chronic (10 day) cyclophosphamide (CYP) treatment. CYP-induced cystitis significantly (P ≤ 0.01) increased pERK expression in the urinary bladder with intermediate (48 h) and chronic CYP treatment. Immunohistochemistry for pERK immunoreactivity revealed little pERK-IR in control or acute (4 h) CYP-treated rat urinary bladders. However, pERK expression was significantly (P ≤ 0.01) upregulated in the urothelium after 48 h or chronic CYP treatment. Whole mount preparations of urothelium/lamina propria or detrusor smooth muscle from control (noninflamed) rats showed no pERK-IR in PGP9.5-labeled nerve fibers in the suburothelial plexus. However, with CYP treatment (48 h, chronic), a few pERK-IR nerve fibers in the suburothelial plexus of whole mount preparations of bladder at the serosal edge of urinary bladder sections were observed. pERK-IR cells expressing the CD86 antigen were also observed in urinary bladder from CYP-treated rats (48 h, chronic). Treatment with the upstream inhibitor of ERK phosphorylation, U0126, significantly (P ≤ 0.01) increased bladder capacity in CYP-treated rats (48 h). These studies suggest that therapies targeted at pERK pathways may improve urinary bladder function in CYP-treated rats.

neurotrophins; mitogen-activated protein kinase; plasticity; inflammation; urothelium; U0126

THE P44 AND P42 MITOGEN-ACTIVATED PROTEIN KINASES (P44/42 MAPKS)/EXTRACELLULAR SIGNAL-REGULATED KINASES (ERK1 AND ERK2) ARE MEMBERS OF THE SERINE/THREONINE PROTEIN KINASES INVOLVED IN THE TRANSDUCTION OF NEUROTROPHIC AND NEUROCHEMICAL SIGNALS (18, 36, 38, 49). ERKs PLAY IMPORTANT ROLES IN CELL PROLIFERATION, DIFFERENTIATION, SURVIVAL, MEMORY FORMATION, AND NEURONAL PLASTICITY (36, 38). ERKs ARE ACTIVATED (I.E., PHOSPHORYLATED) IN THE DORSAL HORN OF THE SPINAL CORD AND IN BRAIN STEM NUCLEI AFTER PERIPHERAL SOMATIC OR VISCERAL (9, 15–17, 45) STIMULATION OR INFLAMMATION. THE CHEMICALLY CYCLOPHOSPHAMIDE (CYP)-INDUCED BLADDER INFLAMMATION MODEL IS ASSOCIATED WITH ALTERATIONS IN NEUROCHEMICAL (50, 54), ELECTROPHYSIOLOGICAL (57), ORGANIZATIONAL (55), AND FUNCTIONAL PROPERTIES (20) OF MICTURITION PATHWAYS. THESE CHANGES MAY BE MODULATED BY CHEMICAL MEDIATORS (E.G., NEUROTROPHINS, CYTOKINES, NEUROPEPTIDES) PRODUCED IN THE BLADDER, SPINAL CORD, OR DORSAL ROOT GANGLIA WITH CYSTITIS (6, 32, 50, 53, 54).

Nerve growth factor (NGF) interactions with the high-affinity receptor, TrkA, may contribute to phosphorylated ERK expression in the urinary bladder after CYP-induced cystitis. TrkA can signal through at least six different pathways, including the MAPK/ERK pathway (36). Previous studies from several laboratories have suggested that neurotrophic factor expression in the urinary bladder may underlie the changes in neurochemical (50, 54) and electrical (57) phenotype of bladder-afferent neurons after urinary bladder dysfunction. Studies from our laboratory have demonstrated that CYP-induced cystitis alters the expression of NGF and receptor expression in the urinary bladder, dorsal root ganglia, and major pelvic ganglia (53). The NGF scavenging agent, REN1820, reduced bladder overactivity in CYP-induced cystitis in rats. Intrathecal (56), intramuscular (P. Zvara and M. A. Vizzard, unpublished data) delivery of NGF or adenovirus-mediated NGF overexpression (27) in the bladder induces bladder overactivity and bladder-afferent cell hyperexcitability (56) in control (noninflamed) rats.

Patients with interstitial cystitis, a painful, chronic urinary bladder inflammation syndrome, exhibit urinary frequency, urgency, and suprapubic and pelvic pain (41), and an involvement of C-fibers has been suggested (8). The overall hypothesis of this work is that neurotrophin-dependent changes in urinary bladder function with CYP-induced cystitis involve the ERK pathway. As initial steps in addressing this hypothesis, we determined: 1) pERK protein expression in the urinary bladder by Western blot analysis after CYP-induced cystitis of varying duration; 2) cellular location of pERK in urinary bladder of control rats or after CYP-induced cystitis using immunohistochemistry with an emphasis on urothelial cell, nerve fiber, and inflammatory cell infiltrate expression; 3) intensity of pERK immunoreactivity in the urothelium after CYP-induced cystitis using semiquantitative image analysis; and 4) the functional effect(s) of the upstream inhibitor of ERK phosphorylation, U0126, on bladder function 48 h after CYP treatment using conscious cystometry in rats.

MATERIALS AND METHODS

CYP-IJECTED CYSTITIS

Chemical cystitis was induced in adult female Wistar rats (175–250 g) by CYP treatment. CYP (Sigma ImmunoChemicals, St. Louis, MO) was administered: 1) 4 h (150 mg/kg ip) before euthanasia; 2) 48 h (150 mg/kg ip) before euthanasia or 3) administered every third day for 10 days to elicit chronic inflammation (75 mg/kg ip). Control

Address for reprint requests and other correspondence: M. A. Vizzard, Univ. of Vermont College of Medicine, Dept. of Neurology, D411 Given Bldg., Burlington, VT 05405 (e-mail: margaret.vizzard@uvm.edu).

http://www.ajpregu.org 0363-6119/07 $8.00 Copyright © 2007 the American Physiological Society
animals received no treatment. Rats were euthanized by isoflurane (4%) and thoracotomy, and bladders were harvested. The University of Vermont Institutional Animal Care and Use Committee approved all procedures.

Western Blot Analysis for pERK and total ERKs

The bladder (n = 6–10 for control and each CYP group) was harvested and homogenized, and aliquots were removed for protein assay. Samples (20 µg) were suspended in sample buffer for fractionation on Tris-Glycine gels (Invitrogen, Carlsbad, CA) and subjected to SDS-PAGE. Proteins were transferred to nitrocellulose membranes, and the efficiency of transfer was evaluated. Membranes were blocked for 1 h followed by rinsing in Tris-buffered saline Tween-20 (TBST). Membranes were then incubated in rabbit anti-p44/42 MAPK (1:1,000 in TBST; Cell Signaling Technology, Danvers, MA) or phospho-p44/42 (Thr202/Tyr204) MAPK (1:1,000 in TBST; Cell Signaling Technology) overnight at 4°C and rinsed in TBST the following day. Washed membranes were incubated in HRP-conjugated goat anti-rabbit IgG (1:5,000 in TBST; Jackson Immunoresearch, West Grove, PA) for 2 h at room temperature and rinsed in TBST. Membranes were then incubated with Western blot analysis detection reagents (General Electric Healthcare, Buckinghamshire, UK) for enhanced chemiluminescence. Blots were exposed to Biomax film (Kodak, Rochester, NY) and developed. Densitometric quantification of immunopositive bands was performed using Un-Scan-It Gel software (Silk Scientific, Orem, UT). Both bands (pERK1 and pERK2) were captured with the image analysis software, and the intensity of each band was determined separately and background intensities subtracted. The mean value of the two bands was calculated and normalized with the loading control (total ERK). To compare blots from urinary bladders of varying durations of CYP treatment, the mean for the normalized density values of control experiments was calculated and assigned the value of 100%. The normalized values of the CYP treatments were then expressed as percentages relative to the control experiments.

Urinary Bladder Preparation

Sections. Whole bladders from control (n = 6) and CYP treatment groups (n = 6 each) were postfixed in 4% paraformaldehyde, placed in ascending concentrations of sucrose (10–30%), frozen, sectioned (7 µm), and mounted on gelled (0.5%) microscope slides.

Whole mount bladder preparation. The urinary bladder from control (n = 3) and CYP treatment groups (n = 3) was dissected and placed in Krebs solution. The bladder was cut open along the midline and pinned out with maximum stretch to a Sylgard-coated dish. The bladder was incubated for 1.5 h at room temperature in cold fixative (2% paraformaldehyde + 0.2% picric acid), and urothelium was removed as previously described (60). The urothelium has suburothelial structures associated with it, but we use the term urothelium in this paper to refer to both urothelium and suburothelial structures.

ERK-Immunoreactivity in Bladder

Urinary bladder sections or whole mounts were incubated with phospho (p)Erk1/2 (pERK1:1:1,000; Cell Signaling Technology) in PBS, Tween-20 with goat serum (PBGT) overnight at room temperature then washed (3 × 10 min) in 0.1 M PBS. Tissue was then incubated in Cy3-conjugated goat anti-rabbit antibody (1:500 in PBGT; Jackson) for 2 h at room temperature followed by washing (3 × 10 min in PBS) and cover slipping with Citifluor (Citifluor, London, UK). Sections of the bladder wall and whole mounts from control and experimental treatments (acute, intermediate, chronic CYP-induced cystitis) were examined for pERK-immunoreactivity (IR). Groups of experimental animals were processed simultaneously to decrease the incidence of variation in staining and background that can occur between animals and on different processing days. To demonstrate pERK-IR in neuronal fibers in the urinary bladder, urinary bladder whole mounts were incubated with a cocktail of primary and secondary antibodies against pERK and the pan-neuronal marker, protein gene product 9.5 (PGP9.5; 1:15; Abcam, Cambridge, MA). Following washing (3 × 10 min with PBS), the slides were coverslipped with Citifluor. Control sections incubated in the absence of primary or secondary antibody were also processed and evaluated for specificity or background staining levels. In the absence of primary antibody, no positive immunostaining was observed. In the present study and other studies using the same pERK antisera (9, 15, 24, 28, 36, 38, 45, 47), control tissues exhibited little pERK-IR.

To determine the cellular identity of pERK in inflammatory cell infiltrates, some bladder sections were also immunostained with antibodies against ED-1 or histamine. The monoclonal antibody ED-1 recognizes a cytoplasmic antigen (CD86) associated with phagocytes in monocytes, most tissue macrophages, and some dendritic cell populations (10). Increased urinary levels of histamine and tryptase have been demonstrated in interstitial cystitis (40), suggesting a role for mast cells in this syndrome. Thus, histamine immunoreactivity was used to identify mast cells. Urinary bladder sections were incubated in a cocktail of primary and secondary antibodies against pERK and either ED1 (1:500, Serotec, Raleigh, NC) or histamine (1:400; Accurate, Westbury, NY) and processed as described above.

Assessment of Positively Stained Urinary Bladder Regions

Staining observed in experimental tissue was compared with that observed from experiment-matched negative controls. Urinary bladders exhibiting immunoreactivity that was greater than the background level observed in experiment-matched negative controls were considered positively stained. In this study, we have focused on pERK-IR in the urothelium, bladder nerves in the detrusor and suburothelial plexus and detrusor smooth muscle. All sections of urinary bladder or entire whole mounts of urothelium + suburothelium or detrusor muscle from each animal for each group (control, 4 h, chronic, 48 h) were examined with fluorescence microscopy for evidence of pERK-IR as described below.

Visualization and semiquantitative analysis of pERK-IR in urinary bladder sections. Five urinary bladder sections from each animal in the control (n = 6) and experimental groups (n = 6) were examined under an Olympus fluorescence photomicroscope (Optical Analysis, Nashua, NH) with a multiband filter set for visualization of the Cy3 fluorophore. Cy3 was visualized with a filter with an excitation range of 560–596 nm and an emission range from 610–655 nm. Grayscale images acquired in tiff format here imported into MetaMorph image analysis software (version 4.5r4; Universal Imaging, Downingtown, PA) as previously described (26, 58). Images were calibrated for pixel size by applying a previously created calibration file to convert all measurements from pixels to square micrometers. The free-hand drawing tool was selected and the urothelium was drawn and measured in total pixels per area (Fig. 1, B and D). A threshold within an intensity range of 100–250 grayscale values was applied to the region of interest in the least brightly stained condition first. The same intensity range of 100–250 grayscale values was applied to the region of interest in the least brightly stained condition first. The same threshold was subsequently used for all images. Average intensity was calculated within the outlined area (Fig. 1, B and D). The average intensity represents the average value of all of the pixels above the threshold value (Fig. 1, B and D). Percent pERK expression above threshold in the total area selected was then calculated and averaged for all samples from control (n = 6) and CYP-treated rats (n = 6 each). Percentage pERK expression in CYP-treated rats was therefore expressed as a percentage of control.

Intravesical Catheter Placement

A lower midline abdominal incision was performed during animal anesthesia (n = 8). Polyethylene (PE) tubing (PE-50) with the end...
flared by heat was inserted into the dome of the bladder and secured in place with a 6–0 nylon purse string suture (20, 21, 59). The distal end of the tubing was sealed, tunneled subcutaneously and externalized at the back of the neck. Abdominal and neck incisions were closed with 4–0 nylon sutures. Animals received the analgesic, buprenorphine (0.05 mg/kg sc), every 12 h for 48 h after surgery; the animals were maintained for 72 h after surgery to ensure complete recovery. For intravesical administration of an inhibitor of ERK phosphorylation, rats were anesthetized with 2% isoflurane, and 1.0 ml U0126 was injected through the bladder catheter; the animals were maintained under anesthesia to prevent expulsion of U0126 through a voiding reflex. In this procedure, U0126 remained in the bladder for 30 min at which time, U0126 was drained, the bladder washed with saline and animals recovered from anesthesia for 20 min before experimentation.

Cystometry in Conscious Animals

Control rats (n = 6) and CYP-treated animals (48 h; n = 6) were treated with an inhibitor of ERK phosphorylation (U0126; Promega, Madison, WI) for cystometry. The effectiveness of intravesical U0126 (500 μg/kg) administration was evaluated in rats 48 h after a single injection of CYP (150 mg/kg ip). These experiments were performed in the same CYP-treated rats before and after treatment with U0126. A control group of CYP-treated rats receiving intravesical administration of vehicle (DMSO; Sigma, St. Louis, MO) (n = 6) or no treatment (n = 6) were also evaluated. For cystometry in conscious rats, an unrestrained animal was placed in a Plexiglas cage with a wire bottom. Before the start of the recording, the bladder was emptied and the catheter was connected via a T-tube to a pressure transducer (Grass Model PT300, West Warwick, RI) and microinjection pump (Harvard Apparatus 22, South Natick, MA). For intravesical drug administration studies, a Small Animal Cystometry Lab Station (MED Associates, St. Albans, VT) was used for urodynamics. Saline solution was infused at room temperature into the bladder at a rate of 10 ml/h to elicit repetitive bladder contractions. At least four reproducible micturition cycles were recorded after the initial stabilization period of 25–30 min. The following cystometric parameters were recorded in each animal: filling pressure (pressure at the beginning of the bladder filling), threshold pressure (bladder pressure immediately prior to micturition), micturition pressure, micturition interval (time between micturition events), bladder capacity, void volume, presence and amplitude of NVCs. In these rats, residual volume was less than 10 μl; therefore, voided volume and bladder capacity were similar. For the present study, NVCs were summed over five micturition cycles with a cycle being defined as a single voiding event. At the conclusion of the experiment, the animal was euthanized (4% isoflurane plus thoracotomy). The concentration of U0126 used in these studies was based upon previous studies (15, 37, 46).

Statistics

All values are means ± SE. Comparisons of pERK protein concentration, pERK-IR or urodynamic parameters in urinary bladder samples after CYP-induced cystitis were made using ANOVA. Percentage data from image analysis were arcsin transformed to meet the requirements of this statistical test. Animals, processed and analyzed on the same day, were tested as a block in the ANOVA. When F ratios exceeded the critical value (P ≤ 0.05), the Newman-Keul’s post hoc test was used to compare experimental means among groups.

Figure Preparation

Digital images were obtained using a CCD camera (MagnaFire SP; Optronics; Optical Analysis, Nashua, NH) and LG-3 frame grabber attached to an Olympus microscope (Optical Analysis). Exposure times were held constant when acquiring images from control and experimental animals processed and analyzed on the same day. Images were imported into Adobe Photoshop 9.0 (Adobe Systems, San Jose, CA), where groups of images were assembled and labeled.

Fig. 1. Semiquantitative analysis of pERK-immunoreactivity in the urothelium after cyclophosphamide (CYP)-induced cystitis. The urothelium (U) was outlined (green) and measured in total pixels per area (A–D). A threshold encompassing an intensity range of 100–250 grayscale values was applied to the region of interest (A–D). The same threshold was subsequently used for all images. Percentage data from image analysis were arcsin transformed to meet the requirements of this statistical test. Animals, processed and analyzed on the same day, were tested as a block in the ANOVA. When F ratios exceeded the critical value (P ≤ 0.05), the Newman-Keul’s post hoc test was used to compare experimental means among groups.

Digital images were obtained using a CCD camera (MagnaFire SP; Optronics; Optical Analysis, Nashua, NH) and LG-3 frame grabber attached to an Olympus microscope (Optical Analysis). Exposure times were held constant when acquiring images from control and experimental animals processed and analyzed on the same day. Images were imported into Adobe Photoshop 9.0 (Adobe Systems, San Jose, CA), where groups of images were assembled and labeled.
RESULTS

Upregulation of pERK in Whole Urinary Bladder From CYP-Treated Rats

Western blot analysis of whole urinary bladder from control and CYP-treated rats demonstrated significant (P ≤ 0.01) upregulation of pERK expression with CYP treatment (48 h or chronic) (Fig. 2). No increase in pERK expression in urinary bladder was detected 4 h after CYP treatment.

pERK-IR in Urothelium with CYP-Induced Cystitis

The expression of pERK-IR was extremely low or absent in urothelium from urinary bladder sections from control (Fig. 3A) and acute (4 h; Fig. 3B) CYP treatment. Little if any pERK-IR was present in suburothelial structures (Fig. 3A) or detrusor smooth muscle of control rats. Intermediate (48 h; Fig. 3C) CYP-induced cystitis increased (4.0-fold; P ≤ 0.01) pERK-IR (Figs. 3E, 4A) in the urothelium and diffuse pERK-IR was also observed in suburothelial structures (Figs. 3C, 4A). Similar to that observed for control rats, no pERK staining was observed in the detrusor smooth muscle with CYP treatment (Fig. 4B). pERK-IR was also increased (P ≤ 0.05; Fig. 3D) in the urothelium of rats treated chronically (Fig. 3D) with CYP, but this increase was significantly reduced (Fig. 3E) compared with the 48-h time point and had a patchy distribution in the urothelium (Fig. 3D).

pERK Expression in Urinary Bladder Nerve Fibers After CYP Treatment

To determine whether pERK-IR was present in the suburothelial nerve plexus, whole mount preparations of urinary bladder were prepared to aid in the visualization of the nerve plexus. No pERK-IR nerve fibers were observed in the suburothelial plexus from control rats. In contrast, on rare occasions, pERK-IR nerve fibers were observed in the suburothelial plexus in CYP-treated rats (48 h; Fig. 4, D–F) or chronic CYP-treated rats (not shown). pERK-IR nerve fibers in the detrusor or urothelium whole mounts exhibited colocalization with the pan-neuronal marker, PGP9.5. These pERK-IR nerve fibers were observed in all CYP-treated (48 h, chronic) rats, but the number of pERK-IR nerve fibers in each animal was very small (range 1–5 nerve fibers/animal). The vast majority of PGP-IR nerve fibers in the suburothelial plexus did not exhibit pERK-IR. Some pERK-IR nerve fibers were observed at the serosal edge of bladder sections after CYP treatment and could be traced for short distances into the detrusor muscle (Fig. 4C). In whole mount and sections of the urinary bladder after CYP treatment (48 h, chronic), small, rounded cells without dendritic processes or with short dendritic processes were observed to exhibit pERK-IR (Fig. 4, A and B). These pERK-IR cells were abundant within the suburothelial region (Fig. 4A) and scattered throughout the detrusor smooth muscle (Fig. 4B). These cells show a similar morphology to mast cells and macrophages previously described in the urinary bladder after CYP-induced cystitis (20, 26). Double-labeling experiments demonstrated that these pERK-IR cells expressed the CD86 antigen (e.g., macrophages, dendritic cells) (Fig. 4G). In contrast to robust expression of CD86 antigen on numerous pERK-IR cells in the bladder, only weak histamine-IR was observed in some pERK-IR cells (Fig. 4H). pERK-IR cells expressing the CD86 antigen were also observed in the urinary bladder from rats treated chronically with CYP (Fig. 4I).

U0126, an Inhibitor of ERK Phosphorylation, Reduces Bladder Overactivity Associated with CYP-Induced Cystitis

Consistent with previous studies, CYP-induced cystitis (48 h) decreased (P ≤ 0.01) bladder capacity (75%) and the intercontraction interval between voiding events (Figs. 5, A and B, and 6A). CYP-induced cystitis also increased (P ≤ 0.05) bladder tension not associated with urine release (nonvoiding bladder contractions; Fig. 6B) and reduced threshold pressure without affecting either filling or micturition pressure (Table 1). To evaluate whether increased pERK expression and signaling participated in CYP-induced cystitis-associated bladder overactivity, U0126, an upstream inhibitor of ERK phosphorylation, was administered intravesically. Compared with cystitis animals presented with vehicle, intravesical administration of U0126 (500 μg/kg) significantly increased bladder capacity in CYP-treated rats 2.25-fold (Fig. 6A; P ≤ 0.01) and reduced the number and amplitude of nonvoiding bladder contractions, although this change was not significant (Fig. 6B). Intravesical administration of U0126 did not affect filling or micturition pressure, but threshold pressure was significantly increased in the CYP-treated animals with U0126 (Table 1). Changes in bladder capacity and threshold pressure observed in CYP-treated rats receiving U0126 treatment lasted ~60 min. CYP-treated rats receiving intravesical administration of vehicle (DMSO) showed no changes in bladder capacity, filling, threshold, or micturition pressure compared with CYP-treated groups. Consistent with a previous study (9) evaluating an ERK inhibitor on bladder function, intravesical
administration of U0126 in control (noninflamed) rats had no effect on bladder function.

**DISCUSSION**

Changes in the activation (i.e., phosphorylation) of pERK were examined in the urinary bladder after (4 h; acute), 48 h (intermediate), or chronic (10 day) CYP treatment. These studies demonstrate several novel findings: 1) CYP-induced cystitis significantly increased pERK expression in the urinary bladder with acute (48 h) and chronic CYP-treatment; 2) weak pERK-IR was present in control or acute (4 h) CYP-treated rat urinary bladders, but expression was significantly upregulated in the urothelium after 48 h or chronic CYP-treatment; 3) CYP treatment (48 h) resulted in the appearance of a few pERK-IR nerve fibers in the suburothelial plexus of the bladder and at the serosal edge of urinary bladder sections compared with control. After CYP treatment, pERK-IR cells expressing the CD86 antigen were observed throughout the bladder. Treatment with the upstream inhibitor of ERK phosphorylation, U0126, significantly increased bladder capacity in CYP-treated rats (48 h). These studies suggest that therapies targeted at pERK pathways may improve urinary bladder function in CYP-treated rats.

The ERK/MAPK pathway is believed to play important roles in cellular proliferation and development but additional roles, including contributing to neuronal plasticity after nerve injury or inflammation, have been demonstrated (18, 23, 31). Although pERK expression has been demonstrated in human corneal epithelial cells in response to hyperosmolar stress (28), we believe the present studies are the first to demonstrate...
PERK expression in the urothelial cells of the urinary bladder in response to CYP-induced cystitis. A recent study (42) has demonstrated increases in pERK1/2 in whole urinary bladder after CYP treatment (48 h). pERK-IR was also observed in the Sub U (A, arrows). This staining was patchy and probably represented staining of inflammatory infiltrates (A, arrows). pERK-IR inflammatory infiltrates were also observed in the detrusor smooth muscle (M, arrows) after CYP treatment (48 h). Protein gene product 9.5 (PGP9.5) staining of nerve fibers in the U/Sub U whole mount preparation (D). Fluorescence photographs of pERK-IR nerve fibers in the detrusor (C) or nerve fibers in the Sub U plexus in whole-mount preparations of the U/Sub U (E) in CYP-treated rats. pERK-IR nerve fibers in the Sub U plexus (E) were rarely observed in contrast to nerve fibers in bladder sections (C). The rarely observed pERK-IR nerve fibers in the Sub U plexus colocalized with the pan-neuronal marker, protein gene product 9.5 (arrows; F). Merged image that demonstrates overlap between pERK and PGP immunostaining in the Sub U plexus (F, arrows). The vast majority of PGP9.5 immunostained nerve fibers do not exhibit pERK-IR (D, F). pERK-IR inflammatory cell infiltrates were present in the urinary bladder of rats with CYP treatment (48 h (G, H), chronic (I)). Merged images of double-labeled immunostaining revealed that pERK-IR (red) was present in cells that expressed the cytoplasmic antigen, CD86 (e.g., macrophages, dendritic cells) (green, arrows). Weak histamine-IR was present in some pERK-IR cells (histamine-IR, green, arrows). Calibration bar represents 80 μm in A–D, 120 μm in E–I.

pERK expression in the urothelial cells of the urinary bladder in response to CYP-induced cystitis. A recent study (42) has demonstrated increases in pERK1/2 in whole urinary bladder after CYP-induced cystitis. In contrast to the present study, which demonstrates significant upregulation of pERK1/2 in the urinary bladder of female rats treated for 48 h or chronically with CYP, the study by Qiao and Gulick (42) demonstrates upregulation of pERK1/2 in bladder of female rats treated 8 h with CYP and no change in pERK1/2 at 48 h. The reasons for the differences in the time course of the upregulation of pERK1/2 in urinary bladder after CYP treatment are not known but the slower upregulation of pERK1/2 in the present study may reflect the time required for metabolism of CYP to acrolein, the agent inducing bladder irritation, and for upregulation of inflammatory mediators, including NGF, to sufficient concentrations that may activate ERK1/2 (see below). A faster first course of upregulation of pERK1/2 in bladder would be expected for direct, receptor-activated mechanisms. In addition, the slower time course of pERK1/2 upregulation in bladder may also reflect slow migration of inflammatory cells expressing the CD86 antigen into the urinary bladder. Alternative reasons for these differences may include differences in the strain of rats used, suppliers or estrous cycle that was not determined in either study.

Previous studies have shown pERK-IR in nerve fibers and in inflammatory cells following peripheral nerve inflammation (1,
34). In the present study, pERK-IR was occasionally demonstrated in nerve fibers in the suburothelial plexus of bladder whole mounts and at the edge of the serosal border and penetrating into the detrusor smooth muscle in bladder sections. It has been previously demonstrated that afferent nerve fibers contribute to both the suburothelial plexus (2, 3) and to the dense innervation of the detrusor muscle (3). The pERK-IR may represent immunoreactivity in Schwann cells, as previously described (1, 34). In the present study, pERK-IR was also observed in macrophages, and weak pERK-IR was present in mast cells in the urinary bladder after CYP-induced cystitis, consistent with previous studies (1, 34). We conclude that the majority of inflammatory cells expressing pERK-IR in the urinary bladder express the CD86 antigen and include macrophages and dendritic cell populations, whereas there is minimal representation from mast cells.

Downstream target genes in the central nervous system that are modulated by the ERK/MAPK pathway are not clearly understood but a number of possibilities have been suggested (31, 36, 49). One possible target is cAMP response element-binding protein (CREB), phosphorylated (pCREB) by pERK upon translocation from the cytoplasm to the nucleus (31, 36). pCREB is involved in the regulation of several nociceptive-related target genes (31, 36) including c-Fos, substance P, dynorphin, CGRP, cyclooxygenase-2, NGF, and brain-derived neurotrophic factor. Most of these target genes are upregulated in the urinary bladder, spinal cord, and/or DRG after CYP-induced cystitis (20, 51–54). In addition, we have previously shown pCREB expression in lumbosacral DRG after CYP-induced cystitis (43). Other downstream targets of pERK include a number of proinflammatory cytokines (28), including tumor necrosis factor-α, IL-1β, and IL-6, each of which is also upregulated in the urinary bladder after CYP-induced cystitis (32). Thus, neurotrophins, neuropeptides and/or proinflammatory cytokines produced in the inflamed urinary bladder may contribute to urinary bladder dysfunction (i.e., overactivity) with CYP-induced cystitis. This suggestion is consistent with previous studies that demonstrate NGF blockade (21) and an antagonist pituitary adenylate cyclase activating polypeptide (PACAP)6-38 to the neuropeptide PACAP (6, 7) reduce CYP-induced bladder overactivity.

Intravesical administration of U0126, a potent and selective MEK inhibitor (24), significantly increased bladder capacity and tended to decrease nonvoiding bladder contractions in CYP-treated rats in the present study. The lack of a significant decrease in the number of nonvoiding contractions may be related to dosage of U0126, and/or lack of penetration to the detrusor smooth muscle with intravesical instillation. Studies by Cruz et al. (9) have also demonstrated that intrathecal administration of the ERK inhibitor, PD98059, also decreased

Fig. 5. Intravesical administration of U0126, an inhibitor of ERK phosphorylation (500 μg/kg), increased bladder capacity (reduced voiding frequency) after cyclophosphamide (CYP)-induced cystitis. Continuous cystometrogram recordings in control (A), 48-h CYP-treated rats + vehicle (B) (DMSO) and 48-h CYP-treated rats + U0126 (C). Arrows point to some nonvoiding bladder contractions. As expected, CYP treatment decreased bladder capacity and increased voiding frequency (B). In CYP-treated rats further treated with intravesical instillation of U0126, bladder capacity was increased and voiding frequency reduced (C). The x-axis represents the time (s), and the y-axis represents the intravesical pressure (cm H₂O). The amount of saline infused (μl) is also illustrated.
Table 1. Intravesical pressures during continuous filling cystometry in control, CYP-treated, CYP-treated + U0126 and CYP + vehicle-treated rats

<table>
<thead>
<tr>
<th></th>
<th>Filling Pressure, cmH2O</th>
<th>Threshold Pressure, cmH2O</th>
<th>Micturition Pressure, cmH2O</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>9.2 ± 3.0</td>
<td>13.0 ± 2.0</td>
<td>16.1 ± 2.0</td>
</tr>
<tr>
<td>CYP (48 h)</td>
<td>9.7 ± 2.0</td>
<td>8.2 ± 1.2*</td>
<td>17.2 ± 2.0</td>
</tr>
<tr>
<td>CYP (48 h) + U0126</td>
<td>10.2 ± 1.6</td>
<td>12.0 ± 1.0†</td>
<td>15.4 ± 1.0</td>
</tr>
<tr>
<td>CYP (48 h) + vehicle</td>
<td>10.3 ± 1.0</td>
<td>9.5 ± 1.1</td>
<td>16.2 ± 1.0</td>
</tr>
</tbody>
</table>

Threshold pressure was significantly reduced in CYP-treated rats. Intravesical instillation of U0126 (500 μg/kg iv) subsequently increased the threshold pressure to control values. *P ≤ 0.05 compared to control; †P ≤ 0.05 compared to CYP; n = 6 for control and CYP-treated groups (48 h).
PERK IN BLADDER PATHWAYS WITH CYSTITIS

R133

REFERENCES


