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Essential role for extracellular Ca^{2+} in JNK activation by mechanical stretch in bladder smooth muscle cells

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Kushida, Nobuhiro, Yukihito Kabuyama, Osamu Yamaguchi, and Yoshimi Homma. Essential role for extracellular Ca^{2+} in JNK activation by mechanical stretch in bladder smooth muscle cells. *Am J Physiol Cell Physiol* 281: C1165–C1172, 2001.—Mechanical stretch has been implicated in phenotypic changes as an adaptive response to stretch stress physically loaded in bladder smooth muscle cells (BSMCs). To investigate stretch-induced signaling, we examined the mitogen-activated protein kinase (MAPK) family using rat primary BSMCs. When BSMCs were subjected to sustained mechanical stretch using collagen-coated silicon membranes, activation of c-Jun NH_2-terminal kinase (JNK) was most relevant among three subsets of MAPK family members: the activity was elevated from 5 min after stretch and peaked at 10 min with an 11-fold increase. Activation of p38 was weak compared with that of JNK, and ERK was not activated at all. JNK activation by mechanical stretch was totally dependent on extracellular Ca^{2+} and inhibited by Gd^{3+}, a blocker of stretch-activated (SA) ion channels. Nifedipine and verapamil, inhibitors for voltage-dependent Ca^{2+} channels, had no effect on this JNK activation. Moreover, none of the inhibitors pertussis toxin, genistein, wortmannin, or calphostin C affected stretch-induced JNK activation, indicating that G protein-coupled and tyrosine kinase receptors are unlikely to be involved in this JNK activation. On the other hand, W-7, a calmodulin inhibitor, and cyclosporin A, a calcineurin inhibitor, prevented JNK activation by stretch. These results suggest a novel pathway for stretch-induced activation of JNK in BSMCs: mechanical stretch evokes Ca^{2+} influx via Gd^{3+}-sensitive SA Ca^{2+} channels, resulting in JNK activation under regulation in part by calmodulin and calcineurin.

stretch-activated ion channel; calmodulin; cyclosporin A; c-Jun NH_2-terminal kinase

BLADDER OUTLET OBSTRUCTION is a common disorder resulting from benign prostate hyperplasia, urethral stricture disease, and congenital anomaly. Although the etiology of bladder obstruction is still obscure, in most instances excessive mechanical overload might be involved in the pathogenesis. In human obstructed bladder, sustained stretch stress causes bladder wall remodeling, such as changes in both composition and amount of extracellular matrix and smooth muscle cells (7, 16, 37). An in vivo rat model with partial urethral ligature reveals smooth muscle hypertrophy and extracellular matrix deposition in the bladder wall, which are quite similar to bladder obstruction in humans (20, 21, 23, 35). However, little is known about the mechanisms by which mechanical stretch of the bladder smooth muscle produces intracellular signals leading to nuclear events, including expression of specific genes such as cyclooxygenase-2 (COX-2) and heparin-binding epidermal growth factor (HB-EGF) (27–30).

Recent studies have identified some of the intracellular signaling pathways that mediate the biological effects evoked by mechanical stimulation in vitro. The mitogen-activated protein kinases (MAPKs) constitute a family of serine/threonine kinases that mediate the transduction of external stimuli into intracellular signals that regulate cell growth and differentiation. These include the extracellular signal-regulated kinase (ERK) pathway that is stimulated in response to mechanical stress of many cell types (11, 12, 15, 17, 18, 22). Some reports indicated that another MAPK family member, c-Jun NH_2-terminal kinase (JNK), is activated by mechanical stretch in cardiac fibroblast cells (22), vascular smooth muscle cells (9), cardiac myocytes (17), and mesangial cells (12). JNK was shown to bind to a specific region within a transactivation domain of c-Jun and to phosphorylate serine residues 63 and 73 of c-Jun (4). In addition, JNK also phosphorylated transcription factors such as ATF-2 (8) and Elk-1 (36) and increased their transcription activating potential. p38 kinase (31), a member of the MAPK family, also plays an important role in cellular responses to various kinds of stress (32) such as ultraviolet stress (19). In the present study, we examined early signaling events provoked by sustained mechanical stretch using primary bladder smooth muscle cells (BSMCs) cultured on deformable silicon dishes and demonstrated a novel pathway to activate JNK in response to mechanical stretch in BSMCs.

MATERIALS AND METHODS

Cell culture. The primary cultures of rat BSMCs were isolated and maintained as described (29). In brief, bladders were obtained from Sprague-Dawley rats weighing 250–300

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g, and smooth muscle layers were dissected from epithelium and other extraneous tissues. Smooth muscle layer minced into 1- to 3-mm pieces was thoroughly washed with sterile phosphate-buffered saline (PBS) and incubated at 37°C for 4 h in medium 199 (GIBCO) containing 20% fetal bovine serum (FBS, Boehringer Mannheim), 0.125 mg/ml elastase (type III, Sigma), and 2.5 mg/ml collagenase (type I, Sigma). The resulting tissue suspension was triturated several times with a pipette and filtered through a 100-μm cell strainer (Falcon). Cells were recovered by centrifugation, and the pellet was resuspended in a growth medium consisting of medium 199, 20% FBS, 100 U/ml penicillin, and 0.1 mg/ml streptomycin (GIBCO). Cells were grown on 100-mm culture dishes (Falcon) and maintained in a humidified 5% CO_2-95% air atmosphere at 37°C with a change of medium every 3 days. All experiments were performed on cells between *passages 2* and *10*.

Application of mechanical stretch. BSMCs harvested from 100-mm dishes were seeded on six-well silicon elastomer-bottomed culture plates that had been coated with collagen type I (Bioflex, Flexcell, McKeesport, PA). After achieving 90% confluency, cells were subjected to sustained mechanical stretch using a controlled vacuum unit (Flexercell strain unit FX-3000, Flexcell) initially described by Banes et al. (2). The intensity of stretch was changed from 5 to 25% elongation by applying a vacuum at 15–20 kPa because BSMCs were detached from silicon membrane when strains over 25% were loaded. These procedures were carried out in a humidified incubator with 5% CO_2-95% air at 37°C.

Protein extraction. BSMCs treated by stretch were washed with ice-cold PBS and harvested with a cell scraper. Cells were recovered by centrifugation at 3,000 rpm for 1 min and solubilized in a lysis buffer consisting of 20 mM Tris·HCl (pH 7.5), 1% Nonidet P-40, 1 mM EDTA, 50 mM NaF, 50 mM sodium β-glycerophosphate, 0.05 mM Na_3VO_4, 10 μg/ml leupeptin, and 100 μM phenylmethylsulfonyl fluoride. After incubating on ice for 15 min, cell extracts were centrifuged at 15,000 rpm for 5 min. The resultant supernatant was collected and used as lysate after protein determination by Bradford assay (Bio-Rad).

In-gel kinase assay. For in-gel kinase assay of JNK, cell extracts (0.2 mg of protein) were resolved on 10% SDS-polyacrylamide gels containing 40 μg/ml of glutathione *S*-transferase (GST)-c-JUN (amino acids 1–79) copolymerized in separating gel (24). After electrophoresis, the gels were washed twice with 100 ml of 20% 2-propanol and 20 mM HEPES-NaOH, pH 7.5, and twice with *buffer A* (20 mM HEPES-NaOH, 5 mM β-mercaptoethanol) at room temperature. Gels were then incubated for 1 h in 100 ml of *buffer A* containing 6 M urea, which was followed by serial incubations in *buffer A* containing 0.05% Tween 20 and either 3, 1.5, or 0.75 M urea. Gels were washed several times with *buffer A* containing 0.05% Tween 20 at 4°C and incubated in a reaction buffer [20 mM HEPES-NaOH, pH 7.6, 20 mM $MgCl_2$, 20 mM β-glycerophosphate, 0.1 mM Na_3VO_4, 2 mM dithiothreitol (DTT), 50 μM ATP, and 5 μCi/ml of [γ-^{32}P]ATP (Amersham)] at 37°C for 1 h. The reaction was stopped by washing with 5% TCA and 1% sodium pyrophosphate. After several more washes, gels were dried, and the protein bands were detected by autoradiography.

For detection of ERK, cell extracts (0.2 mg of protein) were resolved on 10% SDS-polyacrylamide gels containing 0.5 mg/ml myelin basic protein (MBP, Sigma). After electrophoresis, SDS was removed from the gel by washing with *buffer B* (50 mM Tris·HCl, pH 8.0) containing 20% 2-propanol. Proteins in the gel were denatured in *buffer B* with 6 M guanidine-HCl for 1 h at room temperature and renatured in *buffer B* with 0.04% Tween 40 at 4°C for 16 h. The gel was incubated in *buffer C* (40 mM HEPES-NaOH, pH 7.5, 0.1 mM EGTA, 20 mM $MgCl_2$, 5 mM β-mercaptoethanol, 2 mM DTT) containing 25 μM ATP with 25 μCi/ml of [γ-^{32}P]ATP at 30°C for 1 h. After being washed extensively with 5% TCA and 1% sodium pyrophosphate, the gels were dried, and protein bands were detected by autoradiography.

In vitro kinase assay. For in vitro kinase assays, cell extracts containing 0.2 mg of protein were incubated with 1.0 μg of rabbit polyclonal antibody against JNK-1 or p38 (Santa Cruz Biotechnology) for 4 h at 4°C with rotation. Immune complex was recovered by incubating with protein G-Sepharose (Zymed) for 1 h at 4°C. The resultant immunoprecipitates were thoroughly washed and suspended in a reaction buffer (25 mM HEPES-NaOH, pH 7.5, 10 mM magnesium acetate, 50 μM ATP). Kinase reactions were carried out by incubating with [γ-^{32}P]ATP (50 μCi/ml) and with 1.0 μg of either GST-c-JUN (amino acids 1–79) or PHAS-1 (Stratagene) for 30 min at 30°C. The reaction products were resolved on 12.5% (for JNK-1) or 14% (for p38) SDS-polyacrylamide gels and visualized by autoradiography.

Western blotting. Cell lysates were resolved in a 10% SDS-polyacrylamide gel and electrotransferred to a nitrocellulose membrane. Immunodetection of JNK1, p38, and ERK1 was done using α-JNK1, α-p38, and α-ERK1 antibodies (Santa Cruz Biotechnology), respectively. Incubation with an anti-rabbit secondary antibody conjugated to horseradish peroxidase was followed by chemiluminescence detection (Amersham Pharmacia Biotech).

Other chemicals. Ionomycin, gadolinium chloride ($GdCl_3$), genistein, nifedipine, pertussis toxin (PTX), verapamil, and wortmannin were purchased from Sigma; KN-93 was from Calbiochem-Novabiochem; *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W-7) was from Seikagaku; and 1,2-bis-(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid tetrakis(acetoxymethyl ester) (BAPTA-AM), calphostin C, cyclosporin A (CsA), 12-myristate 13-acetate (PMA), and ryanodine were from Wako Chemicals.

RESULTS

Activation of MAPK family proteins in BSMCs exposed to mechanical stretch. Among MAPK family members, JNK and ERK activities were measured by in-gel kinase assay using GST-c-Jun and MBP as substrates, respectively. p38 activity was determined by in vitro kinase assay using PHAS-1 as a substrate. When BSMCs were exposed to mechanical stretch for various periods, the activities of both JNK1 and JNK2 were enhanced from 5 min after starting stretch and peaked at 10 min; an 11-fold increase was detected with stretch of 15% elongation for 10 min (Fig. 1*A*). The effect of intensity of stretch on the activity of JNK was also examined. Activation of JNK was observed from stretch with 10% elongation and reached a plateau at 15% elongation (Fig. 2). Similarly, the activity of p38 was enhanced by mechanical stretch in a time-dependent manner with a peak level at 5 min; the level was about fourfold that in nonstimulating control (Fig. 1*B*). Like JNK, activation of p38 was observed from stretch with 10% elongation and reached a plateau at 15% elongation (Fig. 2). On the other hand, ERK1 and ERK2 did not appear to be appreciably activated by mechanical stretch (Fig. 1*C*). The significant activation of ERK was not detected at any time points and at any

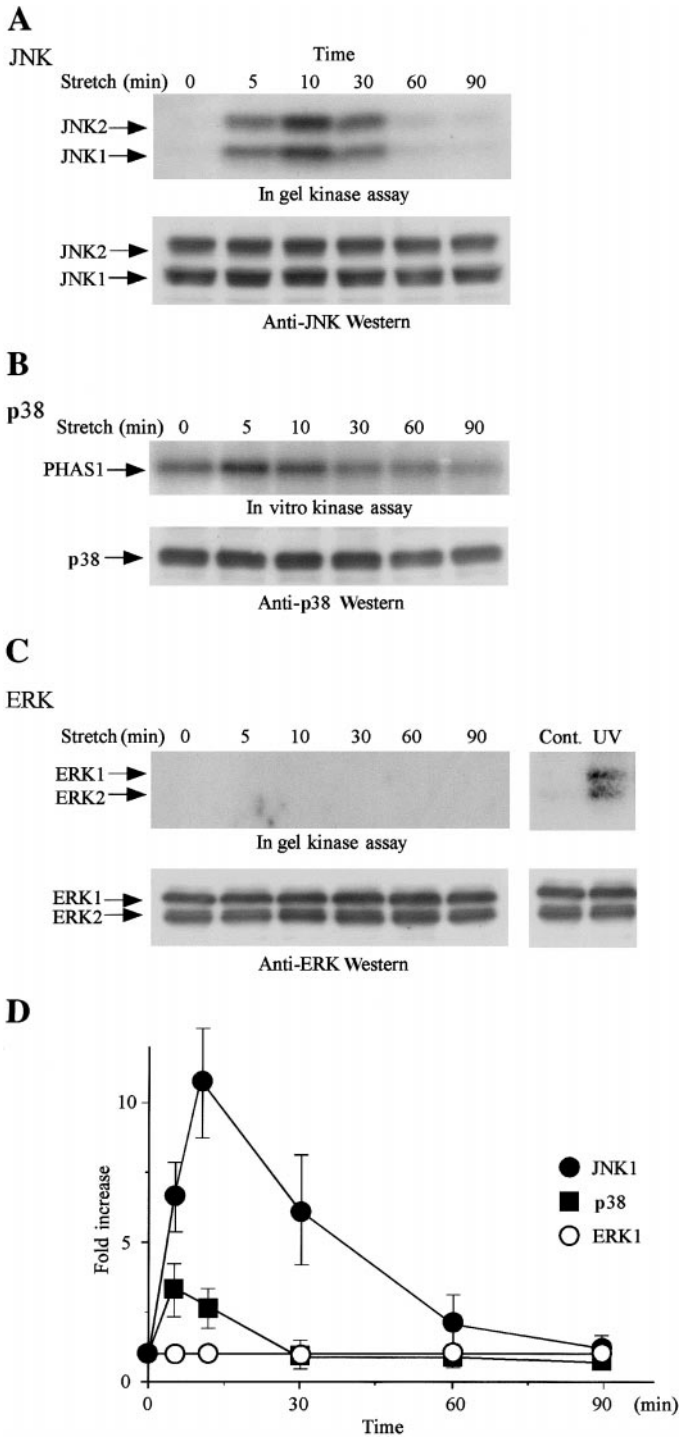

Fig. 1. Time-dependent mitogen-activated protein kinase (MAPK) activation by static mechanical stretch in bladder smooth muscle cells (BSMCs). *A–C*: BSMCs were exposed to static mechanical stretch (15% elongation) for periods indicated. Cell lysates were prepared and activities of c-Jun NH_2-terminal kinase (JNK; *A*), p38 (*B*), and extracellular signal-regulated kinase (ERK; *C*) were measured by in-gel kinase and in vitro kinase assays (*A–C*, *top*). The same filters were immunoblotted with each of the specific antibodies to demonstrate total amounts of JNK, p38, and ERK (A–C, *bottom*). UV, ultraviolet. *D*: activities of JNK, p38, and ERK at each time point were quantified by densitometry. The data are normalized by the protein amount, and the intensity at 0 min for each kinase was set at 1. Representative autoradiograms and immunoblottings and a summary of the results (means ± SE, $n = 6$) are shown.

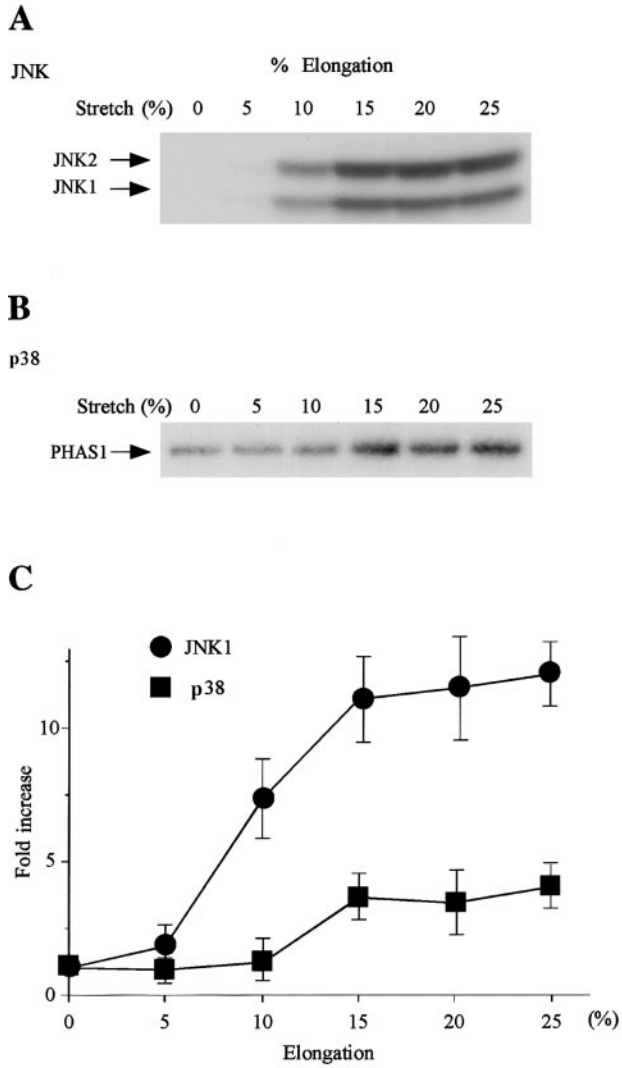

Fig. 2. Strength-dependent JNK and p38 activation in BSMCs. *A* and *B*: BSMCs were exposed to static mechanical stretch of various strengths for 10 min, and the activity of JNK was measured by in-gel kinase assay (*A*) and that of p38 by in vitro kinase assay (*B*). *C*: activities of JNK, p38, and ERK at each time point were quantified by densitometry. The data are normalized by the protein amount, and the intensity at 0 min for each kinase was taken as 1. Representative autoradiograms and a summary of the results (means ± SE, $n = 8$) are shown.

intensity from 10 to 25%, while the activity was enhanced by ultraviolet irradiation.

Activation of JNK is dependent on extracellular Ca^{2+}. Because JNK was activated the most prominently among the three MAPK members in response to mechanical stretch, the activity of JNK was used as an indicator of intracellular events induced by mechanical stress. On the basis of the above results, we employed a condition of stretch with 15% elongation for 10 min for the following assessments. Previous reports have demonstrated that an increase of intracellular Ca^{2+} is important for activation of JNK (1, 33). Therefore, we examined the contribution of extracellular Ca^{2+} to the mechanical stress-induced activation of JNK using HEPES-buffered saline solution containing various

concentrations of $CaCl_2$ (Ca-HBS). When BSMCs suspended in this Ca-HBS were exposed to mechanical stretch, activation of JNK by mechanical stretch was totally dependent on extracellular Ca^{2+} concentrations (Fig. 3*A*). In addition, when BSMCs were suspended in HBS containing BAPTA-AM, a membrane-permeable Ca^{2+} chelator, mechanical stretch-induced JNK activation was abrogated in a concentration-dependent manner (Fig. 3*B*). On the other hand, ryanodine, an inhibitor of intracellular Ca^{2+} stores (Fig. 3*C*), had no effect on the JNK activation, suggesting that Ca^{2+} does not come from the release from intracellular stores.

Stretch-activated ion channels are required for JNK activation. It has been demonstrated by patch-clamp experiments that mechanical stretch-activated (SA) ion channels are involved in increasing of the intracellular Ca^{2+} concentration in some cell types (3, 25, 38) and that Gd^{3+} is a specific inhibitor for this SA ion channel (3). Thus we tested the effect of Gd^{3+} on the SA JNK activation. When BSMCs were treated with 50 μM $GdCl_3$, the stretch-induced activation of JNK was completely abrogated (Fig. 3*D*). In contrast, blockers of voltage-gated Ca^{2+} ion channels, nifedipine and verapamil, did not have any inhibitory effect on the stretch-induced JNK activation (Fig. 3*E*). These results indicate that an increase of intracellular Ca^{2+} via SA ion channels, rather than voltage-gated channels, is required for JNK activation.

Effects of signaling inhibitors on JNK activation. We tested a number of pharmacological agents in a search

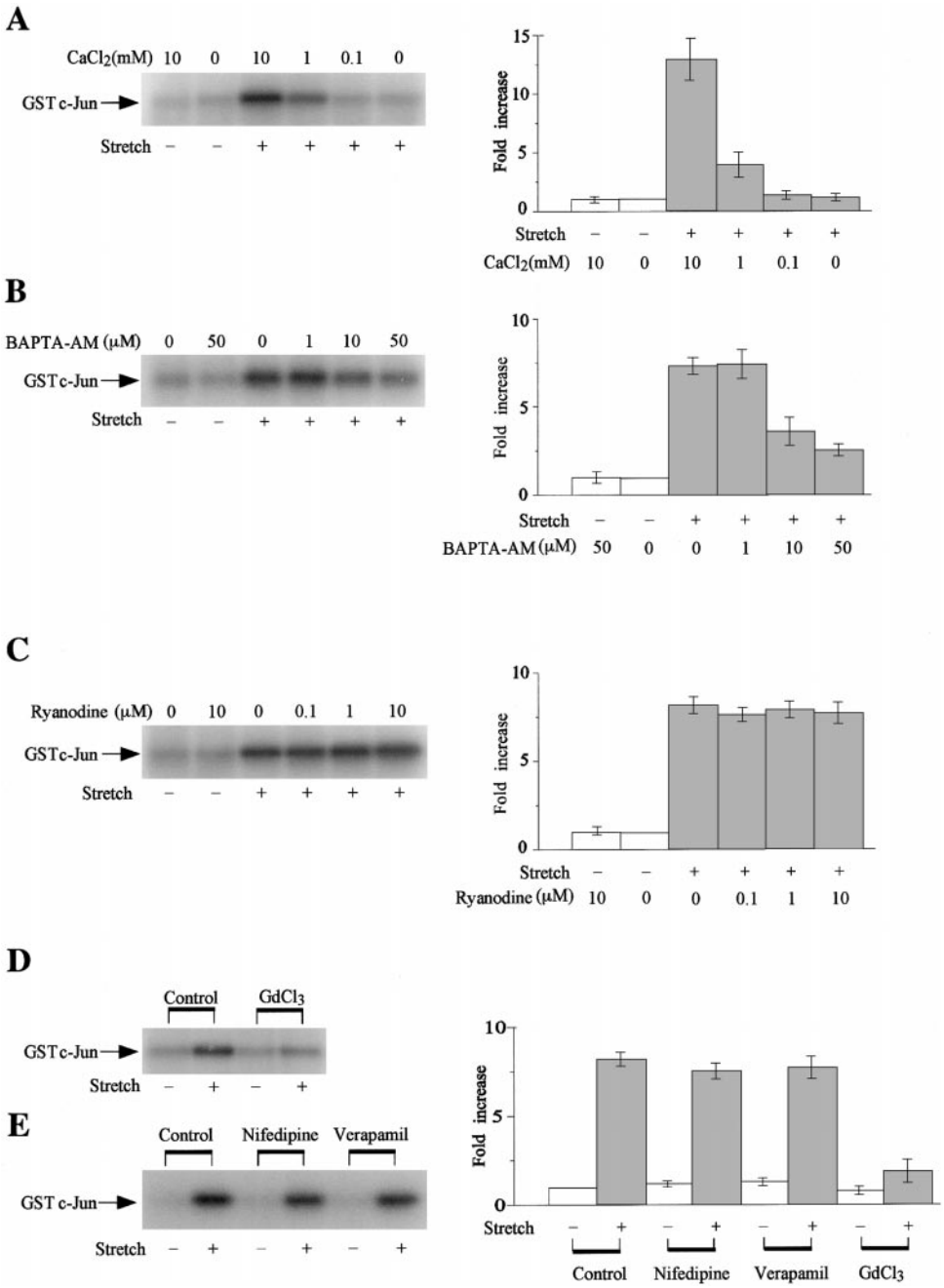

Fig. 3. Effect of various agents on stretch-induced JNK activation. *A*: BSMCs in HEPES-buffered saline solutions containing various concentrations of $CaCl_2$ (Ca-HBS) were subjected to static stretch with 15% elongation for 10 min. JNK activities were measured by in vitro kinase assay using glutathione *S*-transferase (GST)-c-Jun as substrate. *B* and *C*: BSMCs were incubated in HBS-Ca containing 1.0 mM $CaCl_2$ and various concentrations of 1,2-bis-(2-aminophenoxy) ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid tetrakis(acetoxymethyl ester) (BAPTA-AM) for 15 min (*B*) or ryanodine for 30 min (*C*), and cells were subjected to stretch. JNK activities were measured as described in Fig. 1. *D* and *E*: BSMCs were preincubated with 50 μM $GdCl_3$ (*D*), 10 μM nifedipine, or 10 μM verapamil (*E*), and cells were subjected to static stretch with 15% elongation for 10 min. JNK activities were measured as described in Fig. 1. The intensity of each band was quantified by densitometry. The data are normalized by JNK protein, and the intensity at 0 min was taken as 1. Representative autoradiograms and immunoblottings and summaries of the results (means ± SE, $n = 5$) are shown.

for the intracellular signaling pathways leading to activation of JNK in BSMCs by mechanical stretch. BSMCs were pretreated with genistein, a tyrosine kinase inhibitor, wortmannin, a phosphatidylinositol 3-kinase inhibitor, PTX, a G protein-coupled receptor inhibitor, or calphostin C, a protein kinase C (PKC) inhibitor, for 30 min and then exposed to stretch with 15% elongation for 10 min. None of these inhibitors could affect the stretch-induced JNK activation (Fig. 4).

We next examined the effects of W-7 and KN-93, which are specific inhibitors of calmodulin (CaM) and Ca^{2+}/CaM-dependent kinase II (CaM kinase II), respectively. As shown in Fig. 5*A*, pretreatment of BSMCs with W-7 for 30 min resulted in a loss of activation of JNK in a concentration-dependent manner, while that with KN-93 failed to affect the stretch-dependent JNK activation (Fig. 5*B*). W-7 at a low concentration (~1 μM) clearly abrogated the ionomycin-dependent JNK activation (Fig. 5*C*). However, W-7 even at a high concentration (~100 μM) had little effect on JNK activation induced by PMA (Fig. 5*D*). On the other hand, CsA, a calcineurin inhibitor, prevented the activation of JNK in a dose-dependent fashion; ~50% inhibition was observed at 1 μM CsA (Fig. 5*E*). These results indicate that CaM and calcineurin play a significant role in JNK activation by mechanical stretch in BSMCs.

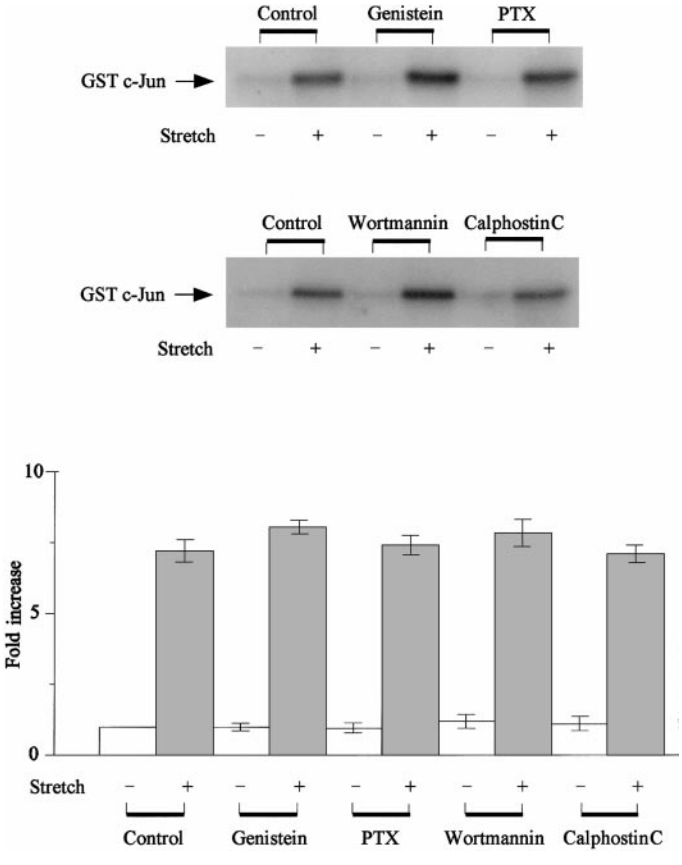

Fig. 4. Effect of signaling inhibitors on JNK activation. BSMCs were pretreated with 20 μM genistein, 50 μg/ml pertussis toxin (PTX), 100 nM wortmannin, or 0.5 μM calphostin C for 30 min, and cells were subjected to static stretch with 15% elongation for 10 min. JNK activities were measured by in vitro kinase assay. The intensity of each band was quantified by densitometory: the data are normalized by JNK protein, and the intensity at 0 min was taken as 1. Representative autoradiograms and summaries of the results (means ± SE, $n = 5$) are shown.

DISCUSSION

Mechanical overload is possibly involved in remodeling of bladder wall, and such stretch stress may cause phenotypic changes in BSMCs. However, little is known about signaling mechanisms induced by static stress in smooth muscle cells. To understand intracellular events activated by mechanical stretch, we examined MAPK family members using primary cultures of BSMCs maintained on collagen-coated silicon membranes with a vacuum-operated stretch-inducing device. The results demonstrate the following. *1*) Mechanical stretch mainly activates JNK in BSMCs. p38 was moderately activated, but ERK was not activated at all. *2*) Mechanical stretch evokes an influx of extracellular Ca^{2+}, which is required for this JNK activation. *3*) Gd^{3+}-sensitive SA ion channels and CaM are possibly involved in the regulation of this JNK pathway. Although the molecular basis and activation mechanism of nonselective types of SA ion channels are still obscure, an important role for these channels has been proposed in *Xenopus* oocytes (38), myocytes (3), and vascular endothelial cells (25). However, there is no direct evidence to support a pathway by which an increase in intracellular Ca^{2+} induced by mechanical stretch could activate JNK. We observed that *1*) elimination of extracellular Ca^{2+} abrogated stretch-induced JNK activation (Fig. 3*A*); *2*) Gd^{3+} (Fig. 3*D*) and BAPTA-AM (Fig. 3*B*), but not ryanodine, nifedipine, and verapamil (Fig. 3, *C* and *E*), inhibited this JNK activation; and *3*) Ca^{2+} influx was induced by G protein- and tyrosine kinase-free mechanisms (Fig. 4). These results suggest a hypothesis, i.e., stretch stress induces BSMCs to open Gd^{3+}-sensitive SA ion channels by unknown mechanisms, resulting in Ca^{2+} influx, which leads to an increase in the intracellular Ca^{2+} concentration. The sustained or oscillating high Ca^{2+} levels activate JNK. It has been demonstrated that in endothelial cells, mechanical stretch caused a transient Ca^{2+} increase within a few seconds via SA ion channels that declined to the initial basal Ca^{2+} concentration in 100 s (25). Alternatively, the involvement of Ca^{2+} in JNK activation has been reported in some experiments (1, 33, 39). However, these two events have never merged in one pathway because it requires some minutes for stretch-induced JNK activation to occur, while the intracellular Ca^{2+} event occurs in seconds. In fact, in our BSMCs system, a single cycle of stretch-relax stimulation, as well as static stretch up to 1 min, did not cause JNK activation (data not shown). These findings suggest that a sustained or oscillating intracellular Ca^{2+} concentration may be required for stretch-induced JNK activation.

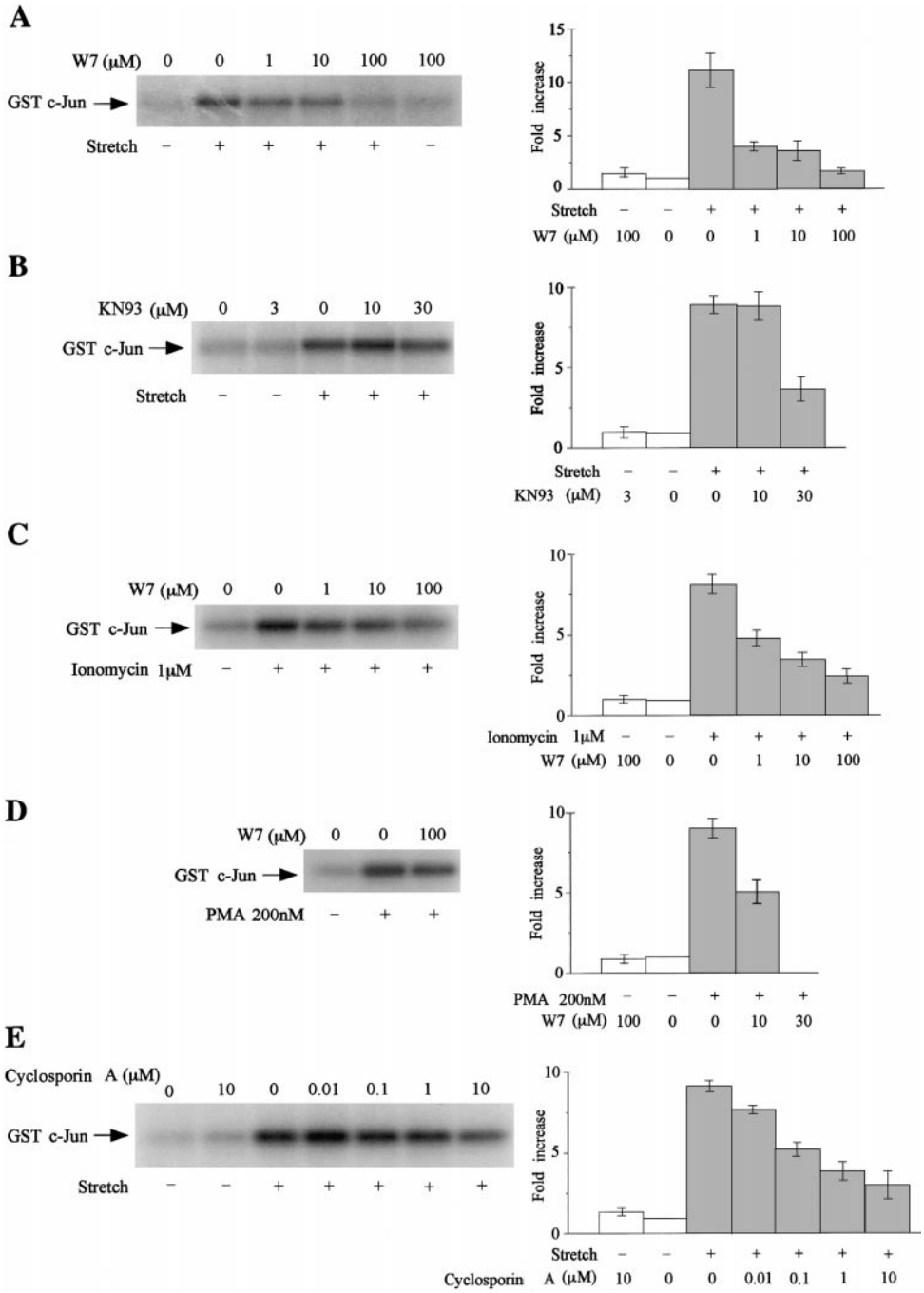

Fig. 5. Effect of inhibitors downstream of Ca^{2+}. *A* and *B*: BSMCs were incubated with various concentrations of calmodulin (CaM) inhibitor, *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W-7; *A*), or CaM kinase inhibitor, KN-93 (*B*), for 30 min and subjected to static stretch with 15% elongation for 10 min. JNK activities were measured by in vitro kinase assay. *C* and *D*: BSMCs were pretreated with various concentrations of W-7 for 30 min and then stimulated by 1 μM ionomycin (*C*) or 200 nM phorbol 12-myristate 13-acetate (PMA) (*D*) for 10 min. JNK activities were measured as described. *E*: BSMCs were preincubated with various concentrations of cyclosporin A for 30 min and subjected to static stretch with 15% elongation for 10 min. JNK activities were measured as described. The intensity of each band was quantified by densitometory: the data are normalized by JNK protein, and the intensity at 0 min was taken as 1. Representative autoradiograms and summaries of the results (means ± SE, $n = 5$) are shown.

Previous studies have indicated that increased intracellular Ca^{2+} mediates protein tyrosine phosphorylation, such as focal adhesion kinase $p125^{FAK}$ and $p130^{CAS}$ in vascular endothelial cells (26), and Ca^{2+}-dependent activation of receptor tyrosine kinase, such as EGF receptor in vascular smooth muscle cells (15). However, these signaling pathways should not contribute to stretch-induced JNK activation in BSMCs because tyrosine kinase inhibitor and PKC inhibitor did not affect JNK activation (Fig. 4). Thus we next tested CaM-dependent pathways. The Ca^{2+}/CaM complex binds to and modulates the activities of multiple key signal transducing enzymes, including CaM kinase and Ca^{2+}/CaM-dependent Ca^{2+} phosphatase, calcineurin. As a calmodulin inhibitor, W-7 attenuated JNK activation evoked by mechanical stretch or ionomycin (Fig. 5, *A* and *C*), a Ca^{2+}/CaM-dependent signaling pathway associated with JNK activation. On the other hand, KN-93 had no effect on the activation (Fig. 5*B*). These results suggest that other CaM kinases (e.g.. CaM kinase IV), rather than CaM kinase II, are associated with stretch-induced activation of JNK.

We demonstrated here that Ca^{2+} influx is essential for stretch-induced JNK activation and that ERK is not activated by mechanical stretch. It is of interest that these observations are quite different from previous

findings obtained from cardiomyocytes (17). Komuro and co-workers (17) observed in cardiomyocytes that EGTA has small effects on the increase in stretch-induced JNK activation. Furthermore, ERK is activated by mechanical stretch but at a faster time course than the activation of JNK. Tissue differences explain this discrepancy. That is, upstream signaling pathways for JNK and ERK activation would be different between BSMCs and cardiomyocytes.

There have been reports that activation of JNK by mechanical stretch induces expression of immediate early genes in cardiomyocytes (17) and vascular smooth muscle cells (15). JNK activation leads to phosphorylation of c-Jun (4), which is a component of the transcription factor AP-1. Thus it is thought that JNK activation by mechanical stretch regulates gene expression through AP-1 activation (12, 17). In this context, specific gene products including HB-EGF are critical; they act as a mitogen for smooth muscle cells (6, 10), and AP-1 is an essential element in their transcriptional activation in response to a mechanical stretch stimulus (27). Moreover, these changes in the gene expression system might induce phenotypic changes in BSMCs, possibly resulting in remodeling of the bladder wall. The effect of CsA is quite interesting. It is well known that CsA functions as an inhibitor of calcineurin, a ubiquitously expressed phosphatase that is activated by Ca^{2+} and Ca^{2+}/CaM. However, recent findings have indicated that CsA inhibits the Ca^{2+}-dependent activation of JNK in lymphocytes (13, 14, 34) and an in vivo model of mouse heart (5), which are similar to our results (Fig. 3*E*). These observations suggest the idea that CsA acts as a blocker of Ca^{2+} ion channels rather than an inhibitor of calcineurin.

REFERENCES

1. **Araki S, Haneda M, Togawa M, and Kikkawa R.** Endothelin-1 activates c-Jun NH_2-terminal kinase in mesangial cells. *Kidney Int* 51: 631–639, 1997.
2. **Banes AJ, Gilbert J, Taylor D, and Monbureau O.** A new vacuum-operated stress-providing instrument that applies static or variable duration cyclic tension or compression to cells in vitro. *J Cell Sci* 75: 35–42, 1985.
3. **Davis MJ, Meininger GA, and Zawieja DC.** Stretch-induced increases in intracellular calcium of isolated vascular smooth muscle cells. *Am J Physiol Heart Circ Physiol* 263: H1292–H1299, 1992.
4. **Derijard B, Hibi M, Wu IH, Barrett T, Su B, Deng T, Karin M, and Davis RJ.** JNK1: a protein kinase stimulated by UV light and Ha-Ras that binds and phosphorylates the c-Jun activation domain. *Cell* 76: 1025–1037, 1994.
5. **De Windt LJ, Lim HW, Haq S, Force T, and Molkentin JD.** Calcineurin promotes protein kinase C and c-Jun NH_2-terminal kinase activation in the heart. Cross-talk between cardiac hypertrophic signaling pathways. *J Biol Chem* 275: 13571–13579, 2000.
6. **Dluz SM, Higashiyama S, Damm D, Abraham JA, and Klagsbrun M.** Heparin-binding epidermal growth factor-like growth factor expression in cultured fetal human vascular smooth muscle cells. Induction of mRNA levels and secretion of active mitogen. *J Biol Chem* 268: 18330–18334, 1993.
7. **Gilpin SA, Gosling JA, and Barnard RJ.** Morphological and morphometric studies of the human obstructed, trabeculated urinary bladder. *Br J Urol* 57: 525–529, 1985.
8. **Gupta S, Campbell D, Derijard B, and Davis RJ.** Transcription factor ATF2 regulation by the JNK signal transduction pathway. *Science* 267: 389–393, 1995.
9. **Hamada K, Takuwa N, Yokoyama K, and Takuwa Y.** Stretch activates Jun N-terminal kinase/stress-activated protein kinase in vascular smooth muscle cells through mechanisms involving autocrine ATP stimulation of purinoceptors. *J Biol Chem* 273: 6334–6340, 1998.
10. **Higashiyama S, Abraham JA, Miller J, Fiddes JC, and Klagsbrun M.** A heparin-binding growth factor secreted by macrophage-like cells that is related to EGF. *Science* 251: 936–939, 1991.
11. **Hu Y, Bock G, Wick G, and Xu Q.** Activation of PDGF receptor alpha in vascular smooth muscle cells by mechanical stress. *FASEB J* 12: 1135–1142, 1998.
12. **Ishida T, Haneda M, Maeda S, Koya D, and Kikkawa R.** Stretch-induced overproduction of fibronectin in mesangial cells is mediated by the activation of mitogen-activated protein kinase. *Diabetes* 48: 595–602, 1999.
13. **Ishizuka T, Chayama K, Takeda K, Hamelmann E, Terada N, Keller GM, Johnson GL, and Gelfand EW.** Mitogen-activated protein kinase activation through Fc ϵ receptor I and stem cell factor receptor is differentially regulated by phosphatidylinositol 3-kinase and calcineurin in mouse bone marrow-derived mast cells. *J Immunol* 162: 2087–2094, 1999.
14. **Ishizuka T, Kawasome H, Terada N, Takeda K, Gerwins P, Keller GM, Johnson GL, and Gelfand EW.** Stem cell factor augments Fc ϵ RI-mediated TNF-α production and stimulates MAP kinases via a different pathway in MC/9 mast cells. *J Immunol* 161: 3624–3630, 1998.
15. **Iwasaki H, Eguchi S, Ueno H, Marumo F, and Hirata Y.** Mechanical stretch stimulates growth of vascular smooth muscle cells via epidermal growth factor receptor. *Am J Physiol Heart Circ Physiol* 278: H521–H529, 2000.
16. **Kim KM, Kogan BA, Massad CA, and Huang YC.** Collagen and elastin in the obstructed fetal bladder. *J Urol* 146: 528–531, 1991.
17. **Komuro I, Kudo S, Yamazaki T, Zou Y, Shiojima I, and Yazaki Y.** Mechanical stretch activates the stress-activated protein kinases in cardiac myocytes. *FASEB J* 10: 631–636, 1996.
18. **Kudoh S, Komuro I, Hiroi Y, Zou Y, Harada K, Sugaya T, Takekoshi N, Murakami K, Kadowaki T, and Yazaki Y.** Mechanical stretch induces hypertrophic responses in cardiac myocytes of angiotensin II type 1a receptor knockout mice. *J Biol Chem* 273: 24037–24043, 1998.
19. **Lin A, Minden A, Martinetto H, Claret FX, Lange-Carter C, Mercurio F, Johnson GL, and Karin M.** Independent human MAP-kinase signal transduction pathways defined by MEK and MKK isoforms. *Science* 267: 682–685, 1995.
20. **Lindner P, Mattiasson A, Persson L, and Uvelius B.** Reversibility of detrusor hypertrophy and hyperplasia after removal of infravesical outflow obstruction in the rat. *J Urol* 140: 642–646, 1988.
21. **Lluel P, Duquenne C, and Martin D.** Experimental bladder instability following bladder outlet obstruction in the female rat. *J Urol* 160: 2253–2257, 1998.
22. **MacKenna DA, Dolfi F, Vuori K, and Ruoslahti E.** Extracellular signal-regulated kinase and c-Jun NH_2-terminal kinase activation by mechanical stretch is integrin-dependent and matrix-specific in rat cardiac fibroblasts. *J Clin Invest* 101: 301–310, 1998.
23. **Monson FC, McKenna BA, Wein AJ, and Levin RM.** Effect of outlet obstruction on ^{3}H-thymidine uptake: a biochemical and radioautographic study. *J Urol* 148: 158–162, 1992.
24. **Murakami M, Sonobe MH, Ui M, Kabuyama Y, Watanabe H, Wada T, Handa H, and Iba H.** Phosphorylation and high level expression of Fra-2 in v-src transformed cells: a pathway of activation of endogenous AP-1. *Oncogene*: 142435–142444, 1997.
25. **Naruse K and Sokabe M.** Involvement of stretch-activated ion channels in Ca^{2+} mobilization to mechanical stretch in endothelial cells. *Am J Physiol Cell Physiol* 264: C1037–C1044, 1993.
26. **Naruse K, Yamada T, Sai XR, Hamaguchi M, and Sokabe M.** $p125^{FAK}$ is required for stretch dependent morphological response of endothelial cells. *Oncogene* 17: 455–463, 1998.

27. **Park JM, Adam RM, Peters CA, Guthrie PD, Sun Z, Klagsbrun M, and Freeman MR.** AP-1 mediates stretch-induced expression of HB-EGF in bladder smooth muscle cells. *Am J Physiol Cell Physiol* 277: C294–C301, 1999.
28. **Park JM, Borer JG, Freeman MR, and Peters CA.** Stretch activates heparin-binding EGF-like growth factor expression in bladder smooth muscle cells. *Am J Physiol Cell Physiol* 275: C1247–C1254, 1998.
29. **Park JM, Yang T, Arend LJ, Schnermann JB, Peters CA, Freeman MR, and Briggs JP.** Obstruction stimulates COX-2 expression in bladder smooth muscle cells via increased mechanical stretch. *Am J Physiol Renal Physiol* 276: F129–F136, 1999.
30. **Park JM, Yang T, Arend LJ, Smart AM, Schnermann JB, and Briggs JP.** Cyclooxygenase-2 is expressed in bladder during fetal development and stimulated by outlet obstruction. *Am J Physiol Renal Physiol* 273: F538–F544, 1997.
31. **Raingeaud J, Gupta S, Rogers JS, Dickens M, Han J, Ulevitch RJ, and Davis RJ.** Pro-inflammatory cytokines and environmental stress cause p38 mitogen-activated protein kinase activation by dual phosphorylation on tyrosine and threonine. *J Biol Chem* 270: 7420–7426, 1995.
32. **Schaeffer HJ and Weber MJ.** Mitogen-activated protein kinases: specific messages from ubiquitous messengers. *Mol Cell Biol* 19: 2435–2444, 1999.
33. **Schmitz U, Ishida T, Ishida M, Surapisitchat J, Hasham MI, Pelech S, and Berk BC.** Angiotensin II stimulates p21-activated kinase in vascular smooth muscle cells: role in activation of JNK. *Circ Res* 82: 1272–1278, 1998.
34. **Su B, Jacinto E, Hibi M, Kallunki T, Karin M, and Ben-Neriah Y.** JNK is involved in signal integration during costimulation of T lymphocytes. *Cell* 77: 727–736, 1994.
35. **Uvelius B, Persson L, and Mattiasson A.** Smooth muscle cell hypertrophy and hyperplasia in the rat detrusor after short-time infravesical outflow obstruction. *J Urol* 131: 173–176, 1984.
36. **Whitmarsh AJ, Shore P, Sharrocks AD, and Davis RJ.** Integration of MAP kinase signal transduction pathways at the serum response element. *Science* 269: 403–437, 1995.
37. **Workman SJ and Kogan BA.** Fetal bladder histology in posterior urethral valves and the prune belly syndrome. *J Urol* 144: 337–339, 1990.
38. **Yang XC and Sachs F.** Characterization of stretch-activated ion channels in *Xenopus* oocytes. *J Physiol (Lond)* 431: 103–122, 1990.
39. **Zohn IE, Yu H, Li X, Cox AD, and Earp HS.** Angiotensin II stimulates calcium-dependent activation of c-Jun N-terminal kinase. *Mol Cell Biol* 15: 6160–6168, 1995.