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

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Received 18 October 2000; accepted in final form 7 June 2001

Kushida, Nobuhiro, Yukihiro 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 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 activator protein-1 (AP-1) pathway that is activated in response to mechanical stretch in cardiac myocytes (17), and mesangial cells (12). JNK was activated 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 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 g. Essential role for extracellular Ca$^{2+}$ in JNK activation by mechanical stretch in bladder smooth muscle cells.
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% CO2-95% 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 (Flexcell; Springfield, VA). Following 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 stretch was performed at 37°C for 4 h at 4°C with rotation. Immune complex was revealed by incubating with protein G-Sepharose (Zymed) at 1°C for 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-32P]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 (GdCl3), 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 tetraakis(acetoxyethyl) 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. 1A). 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. 1B). 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. 1C). The significant activation of ERK was not detected at any time points and at any

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

Activation of JNK is dependent on extracellular Ca\textsuperscript{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\textsuperscript{2+} is important for activation of JNK (1, 33). Therefore, we examined the contribution of extracellular Ca\textsuperscript{2+} to the mechanical stress-induced activation of JNK using HEPES-buffered saline solution containing various

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\textsubscript{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.
concentrations of CaCl₂ (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 Ca²⁺ concentrations (Fig. 3A). In addition, when BSMCs were suspended in HBS containing BAPTA-AM, a membrane-permeable Ca²⁺ chelator, mechanical stretch-induced JNK activation was abrogated in a concentration-dependent manner (Fig. 3B). On the other hand, ryanodine, an inhibitor of intracellular Ca²⁺ stores (Fig. 3C), had no effect on the JNK activation, suggesting that Ca²⁺ 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 Ca²⁺ concentration in some cell types (3, 25, 38) and that Gd³⁺ is a specific inhibitor for this SA ion channel (3). Thus we tested the effect of Gd³⁺ on the SA JNK activation. When BSMCs were treated with 50 μM GdCl₃, the stretch-induced activation of JNK was completely abrogated (Fig. 3D). In contrast, blockers of voltage-gated Ca²⁺ ion channels, nifedipine and verapamil, did not have any inhibitory effect on the stretch-induced JNK activation (Fig. 3E). These results indicate that an increase of intracellular Ca²⁺ 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 for inhibitors of JNK activation. We used a number of pharmacological agents in a search for inhibitors of JNK activation. We used a number of pharmacological agents in a search for inhibitors of JNK activation.

**Fig. 3.** Effect of various agents on stretch-induced JNK activation. A: BSMCs in HEPES-buffered saline solutions containing various concentrations of CaCl₂ (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 CaCl₂ and various concentrations of 1,2-bis-(2-aminophenoxy)ethane-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 GdCl₃ (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. 5A, 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. 5B). W-7 at a low concentration (~1 μM) clearly abrogated the ionomycin-dependent JNK activation (Fig. 5C). However, W-7 even at a high concentration (~100 μM) had little effect on JNK activation induced by mechanical stretch. W-7 at a low concentration (~1 μM) clearly abrogated the ionomycin-dependent JNK activation (Fig. 5C). However, W-7 even at a high concentration (~100 μM) had little effect on JNK activation induced by mechanical stretch (Fig. 4).

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, myocytes, and vascular endothelial cells. 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. 3A); 2) Gd\(^{3+}\) (Fig. 3D) and BAPTA-AM (Fig. 3B), 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. Alternatively, the involvement of Ca\(^{2+}\) in JNK activation has been reported in some experiments. 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.
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\(^{2+}/\text{CaM}\) complex binds to and modulates the activities of multiple key signal transducing enzymes, including CaM kinase and Ca\(^{2+}/\text{CaM}\)-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\(^{2+}/\text{CaM}\)-dependent signaling pathway associated with JNK activation. On the other hand, KN-93 had no effect on the activation (Fig. 5B). 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 (15) 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 calcium channels in the bladder wall. The effect of CsA is quite interesting. It is well known that CsA functions as an inhibitor of calcium channels in the bladder wall. The effect of CsA is quite interesting.

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