Cyclic stretch activates p38 SAPK2-, ErbB2-, and AT1-dependent signaling in bladder smooth muscle cells

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Nguyen, Hiep T., Rosalyn M. Adam, Samuel H. Bride, John M. Park, Craig A. Peters, and Michael R. Freeman. Cyclic stretch activates p38 SAPK2-, ErbB2-, and AT1-dependent signaling in bladder smooth muscle cells. Am J Physiol Cell Physiol 279: C1155–C1167, 2000.—Cyclic mechanical stretch of bladder smooth muscle cells (SMC) increases rates of DNA synthesis and stimulates transcription of the gene for heparin-binding epidermal growth factor-like growth factor (HB-EGF), an ErbB1/EGF receptor ligand that has been linked to hypertrophic bladder growth. In this study we sought to clarify the signaling pathways responsible for mechanotransduction of the stretch stimulus. HB-EGF mRNA levels, DNA synthesis, and AP-1/Ets DNA binding activities were induced by repetitive stretch of primary culture rat bladder SMC. Inhibitors of the p38 SAPK2 pathway, the angiotensin receptor type 1 (AT1), and the ErbB2 tyrosine kinase reduced each of these activities, while an inhibitor of the extracellular signal-regulated kinase mitogen-activated protein kinase (Erk-MAPK) pathway had no effect. Stretch rapidly activated stress-activated protein kinase 2 (p38 SAPK2) and Jun NH2-terminal kinase (JNK)/SAPK pathways but not the Erk-MAPK pathway and induced ErbB2 but not ErbB1 phosphorylation. Angiotensin II (ANG II) a bladder SMC mitogen previously linked to the stretch response, did not activate ErbB2, and ErbB2 activation occurred in response to stretch in the presence of an ANG receptor inhibitor, indicating that activation of the AT1-mediated pathway and the ErbB2-dependent pathway occurs by independent mechanisms. p38 SAPK2 and JNK/SAPK signaling also appeared to be independent of the ErbB2 and AT1 pathways. These findings indicate that stretch-stimulated DNA synthesis and gene expression in normal bladder SMC occur via multiple independent receptor systems (e.g., AT1 and ErbB2) and at least one MAPK pathway (p38 SAPK2). Further, we show that the Erk-MAPK pathway, which in most systems is linked to receptor-dependent cell growth responses, is not involved in progression to DNA synthesis or in the response of the HB-EGF gene to mechanical forces.

MECHANICAL FORCES CAN AFFECT cellular structure and function, thereby altering rates and patterns of tissue growth (10). Strain, pressure, and shear stress can induce pathological remodeling of the muscle layers that form hollow organs, resulting in hypertrophic and/or hyperplastic growth. An experimental approach used by a number of groups to understand these processes has been to subject cells plated onto a deformable membrane to a quantifiable stretch-relaxation stimulus. Sadoshima and Izumo (45) demonstrated that repetitive stretch-relaxation (hereafter, stretch) of cardiac myocytes rapidly activates a number of signaling pathways, including Ras, mitogen-activated protein kinases (MAPKs), protein kinase C, s6 kinase (p90RSK), several phospholipases, and P450 pathways. This and subsequent reports demonstrate that activation of signaling pathways by stretch is not a degenerate event, since some pathways are activated by the stretch stimulus while others are not (24, 31, 36, 42, 45). This pathway selectivity is likely to reflect cell type-dependent signaling mechanisms underlying idioptypic cellular responses to mechanical stress (14).

In vitro stretch experiments were initially used to implicate autocrine activation of an angiotensin (ANG) receptor-dependent pathway as a mediator of muscle cell hypertrophy (47). The role of ANG-responsive signals, and other potential downstream signaling systems, in mecanoiinduction of cell growth has since been studied by several groups using heterologous cell types. ANG II has been implicated as a mediator of stretch-induced DNA synthesis in bladder smooth muscle cells (SMC) (37) and glomerular mesangial cells (24). Increases in steady-state levels or DNA binding activity of the AP-1 transcription factor have been observed in response to stretch of mesangial cells (2), cardiac myocytes (27), vascular SMC (23), and bladder SMC (36). Activation of the extracellular signal-regulated MAPKs (Erk-MAPKs) in response to stretch has been observed in vitro in cardiac myocytes and fibroblasts (27, 31, 45, 58), mesangial cells (24), and arterial SMC (39). These observations suggest that fibromuscular cells from diverse organs share common mechanisms of mechanochemoinal signal transduction leading to growth.

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Park et al. (36, 37) recently demonstrated that DNA synthesis and expression of the heparin-binding epidermal growth factor-like growth factor (HB-EGF) gene could be rapidly induced by repetitive stretch of normal diploid bladder SMC. HB-EGF is an activating ligand for the ErbB1/EGF receptor tyrosine kinase (RTK) and a potent SMC mitogen (reviewed in Ref. 41). It is normally expressed in some SMC in vivo, including in the genitourinary tract (18, 19). The observation that the HB-EGF gene and DNA synthesis rates are induced coordinately by stretch suggests that this mitogen plays a physiological role in mechanochemical signaling as an autocrine or paracrine growth factor. In bladder SMC, the HB-EGF gene expression response to stretch was inhibited by losartan, an antagonist of the angiotensin receptor type 1 (AT1), but not by PD-123319, an inhibitor of the angiotensin receptor type 2 (37). These results support a role for AT1 receptor activation as a critical aspect of mechanoregulation of the HB-EGF gene.

Interestingly, the observed increase in HB-EGF mRNA induced by stretch in bladder SMC was not inhibited by PD-98059 (3), a potent inhibitor of Mek1, a dual specificity kinase that activates the Erk1 and Erk2 MAPKs (37). The Erk-MAPKs are serine/threonine kinases involved in the control of cell cycle progression, which lie downstream of a variety of membrane-bound signal transducing molecules (16, 26, 28, 54). Most soluble inducers of cell growth, as well as solid-state inducers such as integrin-matrix interactions, activate the Erk-MAPK pathway (reviewed in Ref. 49). The finding that stretch-stimulated HB-EGF gene expression is insensitive to Erk-MAPK inhibition is surprising for a number of reasons. 1) The apparent dependence on ANG II signaling for stretch-induced HB-EGF expression, in the absence of dependence on signaling through Mek1, appears inconsistent with reports demonstrating that ANG II activates the Ras→Raf→Mek→Erk-MAPK pathway in a variety of cell types (24, 30, 35, 44, 57). 2) This finding also contrasts with a previous report identifying a Raf-1-activatable pathway as a mediator of HB-EGF gene expression in fibroblasts (32). Raf family kinases are the “classical” upstream activators of the Erk-MAPK pathway (25). 3) The effect of PD-98059 pretreatment of cells on the DNA synthesis response to stretch was not tested in our earlier report. However, if the Erk-MAPK pathway were not essential for the growth response to stretch, this would be in marked contrast to previous reports implicating signaling through Erk-MAPKs in stretch-stimulated (hypertrophic) growth of cardiac myocytes (27, 45), with reports of Erk-MAPK activation by mechanical loading in arterial smooth muscle (1), and possibly with other reports suggesting that the cell cycle G2/S phase transition is dependent on activation of this pathway (7, 9, 22, 28, 34, 56).

Bladder muscle hypertrophy arises in the urinary tract as an adaptive response to increased urethral resistance (38); however, the underlying mechanisms that promote tissue growth in this context are unknown. Pathological remodeling of bladder tissue may or may not be similar mechanistically to muscle hyperplasia and/or hypertrophy seen in the cardiovascular system in response to hemodynamic load or injury. In this study we sought to clarify the role of the Erk-MAPK pathway and other alternative signaling mechanisms in stretch stimulation of normal bladder SMC.

EXPERIMENTAL PROCEDURES

Bladder SMC Culture

Rat bladder SMC were isolated from 5-day-old Zucker rats (Charles River Laboratories, Willington, MA) using an enzymatic dispersion method described previously (37). Cells were cultured in medium 199 (GIBCO, Gaithersburg, MD) supplemented with 20% fetal bovine serum (FBS; Hyclone Laboratory, Logan, UT), penicillin (100 U/ml), and streptomycin (100 μg/ml; GIBCO), in a humidified 5% CO2-95% air atmosphere at 37°C. All experiments were performed on cells between passages 1 and 3.

Application of Cyclic Stretch-Relaxation

Approximately 1 × 10^5 SMC/well were plated onto six-well silicone elastomer bottomed culture plates coated with collagen type I (Bioflex; Flexcell, Hillsborough, NC). Cells were grown to 80% confluence and were rendered quiescent by 48 h incubation in medium 199 supplemented with 0.5% FBS. Two hours before stretch, the cells were incubated in serum-free medium 199 and were then subjected to continuous cycles of stretch-relaxation using the FX-3000 Flexercell Strain Unit (Flexcell). Each cycle consisted of 5 s of stretch and 5 s of relaxation (0.1 Hz), with 25% maximum radial stretch at the membrane periphery. It should be noted that the degree of stretch deformation is not uniform throughout different regions of the membrane; however, since samples are harvested from each well and pooled from several wells, variations in response due to variability in the degree of stretch deformation are averaged.

Inhibition Studies

Pharmacological inhibitors were added to the cell media 1 h before stretch in selected plates. All chemicals except losartan were purchased from Calbiochem (La Jolla, CA). Losartan was purchased from Merck (West Point, PA).

RT-PCR

Total RNA was extracted from nontreated and treated SMC using Tri-Reagent (Molecular Research Center, Cincinnati, OH) according to the manufacturer’s protocol. Reverse transcription was performed using 2 μg of total RNA, 100 units of Moloney’s murine leukemia virus RT (Ambion, Austin, TX), and 1 μg of oligo(dT)12–18 (GIBCO) as first-strand primer for 1 h at 42°C. cDNA was precipitated with polyacrylimide, resuspended in Tris (10 mM)-EDTA (0.1 mM) buffer. Primers were selected based on published gene sequences for rat HB-EGF (17), c-Jun (48), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (51) from the GenBank database. A 413-nt HB-EGF product was amplified using a sense 5′-TCC CAC TGG AAC CAC AAA CCA G-3′ (nt 157–178) and an antisense 5′-CCC ACG ATG ACA AGA AGA CAG AC-3′ (nt 570–548) primer pair. A 567-nt c-Jun product was amplified using a sense 5′-CAA CAT GCT CAG GGA ACA GGT G-3′ and an antisense 5′-GGA GTT CAT ACC GGA TCT AGC C-3′ primer pair. A 571-nt GAPDH

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product was amplified using a sense 5‘-TCA CCA TCT TCC AGG AGC G-3’ and an antisense 5‘-CTG TTT ACC ACC TTC TTG A-3’ primer pair. PCR reactions were performed using 22 μl of Platinum SuperMix (GIBCO), 0.5 μl of sense and antisense primer (20 pmol/μl), 0.1 μl of [32P]dCTP (3,000 Ci/mmol; Amersham, Arlington Heights, IL), and 2 μl of cDNA. PCR amplification was performed for 30 cycles at 94°C (denature), 58°C (anneal), and 72°C (extend) for 30 s each. The PCR products were visualized by polyacrylamide gel electrophoresis (5.1%, PAGE) and autoradiography. Normalization to GAPDH expression and a limiting dilution method were used to make semiquantitative comparisons between samples.

**Electrophoretic Mobility Shift Assay**

Bladder SMC were subjected to stretch for 2 h. In selected plates, cells were preincubated with SB-203580 (10 μM), PD-98059 (30 μM), AG-825 (3.5 μM), losartan (10 μM), or AG-825 with losartan for 1 h before stretch-induction. Non-stretched cells were incubated in parallel. Nuclear proteins were extracted using a method modified from Dignam et al. (15). Cells were scraped into cold PBS and centrifuged at 250 g for 5 min. The resultant pellet was resuspended and incubated in 0.4 ml of 10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 2 μg/ml aprotinin, 2 μg/ml leupeptin, 1 mM dithiothreitol (DTT), and 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF) for 15 min at 4°C. Non-ident P-40 (final concentration 3%, vol/vol) was added to the cell suspension and mixed vigorously; lysates were then centrifuged at 25,000 g for 30 s. The nuclear pellet was resuspended and incubated in 25 μl of 20 mM HEPES (pH 7.9), 0.42 M NaCl, 1 mM EDTA, 1 mM EGTA, 2 μg/ml aprotinin, 2 μg/ml leupeptin, 1 mM DTT, and 1 mM AEBSF for 30 min at 4°C. The suspensions were then centrifuged at 25,000 g for 5 min, and the supernatant was collected and stored at −80°C. Protein concentration was determined using the Bio-Rad DC protein assay (Bio-Rad Laboratories, Hercules, CA).

Electrophoretic mobility shift assays were performed by incubating 10 μg of the nuclear protein extract with 2 μg of poly(dI-dC) (Pharmacia, Piscataway, NJ) and 10^6 counts/min (cpm) of [32P]-end-labeled 21-mer double-stranded Ets consensus oligonucleotide (5‘-GAT CTC GAG CAG GAA GGT CCA-3’) or AP-1 consensus oligonucleotide (5‘-CGG TTG ATG ACT CCG GAA-3’) both from Santa Cruz Biotechnology, (Santa Cruz, CA) in 20 μl of 20 mM HEPES (pH 7.9), 50 mM KCl, 5 mM EDTA, 1 mM DTT, 3 mM MgCl2, and 5% glycerol for 20 min at 4°C. The double-stranded oligonucleotides were labeled with [32P] using T4 polynucleotide kinase (Roche, Indianapolis, IN). The DNA-protein complex was resolved on a 4% PAGE gel in 22 mM Tris-HCl, 22 mM boric acid, and 0.5 mM EDTA. To examine the specificity of binding, nuclear protein extracts were preincubated with 1-, 10-, 25-, and 50-fold molar excess of unlabeled competitor DNA fragment derived from the HB-EGF promoter region (−992 to −973, 5‘-TCT GAC TCA TCT TCC TTG TGT-3’) containing a composite AP-1/Ets binding site (underlined). To identify AP-1, nuclear protein extracts were preincubated with c-Fos polyclonal rabbit antibody (Santa Cruz Biotechnology) for 10 min before the addition of the radioactive DNA probe.

**Cell Growth Assays**

Tritiated thymidine incorporation. Bladder SMC were subjected to stretch for 48 h in the presence and absence of selected inhibitors. The control cells were incubated in parallel in the absence of stretch, and replicate plates were made to measure relative cell number. Eight hours before completion, 1 mCi/ml of [methyl-3H]thymidine (5 Ci:mmol; Amersham) was added to the culture medium of each well. After 48 h of stimulation, 3H incorporation into TCA-precipitable material was measured using an LKB Rackbeta liquid scintillation counter.

**Biomass assay.** A crystal violet assay was used to measure relative cell number. The culture medium was removed from replicate plates. Each well was treated with 500 μl of 1% glutaraldehyde, followed by 500 μl of 0.5% crystal violet, then washed with water and air dried; 500 μl of Sorenson’s solution (30 mM citric acid trisodium salt, 0.02 N hydrochloric acid, and 45% ethanol) were then added to elute the dye from the fixed cells. Absorbance at 570 nm was measured using a Bio-Rad 550 microplate reader. Results obtained from [3H]thymidine incorporation assay were adjusted based on the relative absorbance ratios between corresponding samples.

**Western Blot Analysis**

Cells were washed with cold PBS and harvested on ice using a solution containing 62.5 mM TrisCl (pH 6.8), 2% SDS, and 10% glycerol. Protein concentration was measured with the Bio-Rad DC protein assay. DT (50 mM) and 0.1% bromophenol blue were added to the lysates. Equivalent amounts of proteins were separated by electrophoresis using a 6%, 7.5%, or 12% SDS-polyacrylamide gel and transferred to polyvinylidene difluoride Immobilon-P membranes (Millipore, Bedford, MA). Membranes were probed with the following antibodies: polyclonal anti-human EGF receptor (Upstate Biotechnology, Lake Placid, NY); monoclonal phosphospecific anti-EGF receptor (Calbiochem); polyclonal anti-human ErbB2 (Upstate Biotechnology); polyvalent anti-human activated ErbB2 (Upstate Biotechnology); anti-Erk-MAPK, E10 monoclonal anti-phospho-Erk-MAPK (Thr-202/Tyr-204); anti-JNK/SAPK; anti-phospho-JNK/SAPK (Thr-183/Tyr-185); anti-c-Jun; and anti-phospho-c-Jun (Ser-63/Ser-73). All antibodies were purchased from New England BioLabs (Beverly, MA) unless otherwise indicated. To determine whether the dose of PD-98059 used was sufficient to block Erk-MAPK activation, quiescent bladder SMC were incubated with various concentrations of PD-98059 for 1 h and then treated with basic fibroblast growth factor (50 ng/ml) for 5 min and harvested for Western blot. Specific antigen-antibody complexes were detected with the Photo-100 Imaging Analysis System (Alpha Innotech, San Leandro, CA).

**Signal Transduction Pathway Reporting Systems Analysis**

Pathway-specific transactivator plasmids and reporter plasmids (Stratagene, La Jolla, CA) were transfected into 1 × 10^6 bladder SMC utilizing the LipO TAXI transfection reagent (Stratagene) per the manufacturer’s protocol. The transactivator plasmid expresses a fusion protein consisting of the activation domain of either c-Jun (residues 1–223) for the
JNK/SAPK pathway or Elk1 (residues 307–427) for the Erk-MAPK pathway, joined to the yeast GAL4 (residues 1–147) DNA binding domain. Constitutive expression of the transactivator plasmids is driven by the cytomegalovirus immediate early promoter. The reporter plasmid contains a synthetic promoter (TATA box) with five tandem repeats of GAL4 binding elements, controlling the expression of the firefly luciferase gene. Direct or indirect phosphorylation of the transcription activation domain of the fusion protein by stretch allows the fusion protein to activate the GAL4 promoter. As a negative control, SMC were transfected with plasmids containing only the GAL4 DNA binding domain sequence and the reporter plasmids. As a positive control, SMC were transfected with plasmids that constitutively expressed either MAPK kinase (MEKK; residues 380–672, for the JNK/SAPK pathway) or Mek1 (S218/222E,D32–51, for the Erk-MAPK pathway) protein kinase along with the transactivator and reporter plasmids. The transfected cells were incubated for 12 h in medium 199 with 20% FBS and then for 24 h in medium 199 with 0.5% FBS. The transfected cells were then subjected to stretch for 12 h as described. Luciferase activity was measured using Promega (Madison, WI) reagents according to the manufacturer’s instructions.

**Statistical Analysis**

Means and SD were calculated. Paired Student’s t-test was used to determine statistical significance (P < 0.05).

**RESULTS**

*Stretch-Stimulated HB-EGF Gene Expression Is Inhibited by AT1 Receptor, ErbB2 Receptor, and p38 SAPK2 Antagonists*

To determine whether bladder SMC respond to stretch by activating discrete signaling pathways, we initially evaluated the response of the HB-EGF gene to stretch in cells treated with a series of pharmacological inhibitors that antagonize signaling through a number of pathways. Cells were made quiescent by culture in low serum for 48 h, and the inhibitors were added 1 h before initiation of the stretch stimulus (0.1 Hz, 25% maximum radial stretch). HB-EGF mRNA levels were determined after 4 h of stretch, a time point we previously reported to be the peak of the HB-EGF mRNA stretch response (37). Seven drugs were tested: PD-98059 (an inhibitor of Erk-MAPK activation), SB-203580 (p38 SAPK2 inhibitor), losartan (AT1 inhibitor), AG-1478 (ErbB1 inhibitor), PD-153035 (ErbB1 inhibitor), AG-825 (ErbB2 inhibitor), and SU-4984 [FGF, platelet-derived growth factor (PDGF), and insulin-like growth factor (IGF) receptor inhibitor].

Consistent with our previous findings (37), HB-EGF mRNA levels in stretched cells were markedly decreased in the presence of losartan compared with control (Fig. 1). The ratio of HB-EGF mRNA levels in stretched and nonstretched cells preincubated with losartan was 17.9 under vehicle conditions and 1.3 and 1.0 in the presence of 10 and 100 μM losartan, respectively (P < 0.005 compared with control). SB-203580 and AG-825 were also effective inhibitors. The ratio of HB-EGF mRNA levels in stretched and nonstretched cells preincubated with 5 and 10 μM SB-203580 was 9.2 ± 2.7 and 3.9 ± 1.2, respectively (P = 0.064 and P = 0.011 compared with control). In the presence of AG-825, HB-EGF mRNA induction with stretch was significantly decreased (stretched/nonstretched: 0.8 ± 0.7 at 0.35 μM and 1.3 ± 1.1 at 3.5 μM, P < 0.005 compared with control). In agreement with our previ...
ous results, PD-98059, an inhibitor of Erk-MAPK pathway activation, did not inhibit HB-EGF gene induction by stretch at any dose (at 30 μM, P = 0.46 compared with control). These findings suggest that the stretch signal is transmitted through AT1- and p38 SAPK2-dependent pathways. Surprisingly, the specific inhibitor of ErbB2, a member of the EGF receptor family, was also a potent inhibitor of the stretch response.

To determine whether pharmacological inhibition of the ErbB2-related receptor, ErbB1, resulted in a similar inhibition of HB-EGF mRNA induced by stretch, quiescent bladder SMC were stretched in the presence of two ErbB1 inhibitors, PD-153035 and AG-1478. HB-EGF mRNA levels in cells stretched in the presence of PD-153035 (0.25 nM) were not significantly different from those of control (stretched/nonstretched: 10.9 ± 3.5 vs. 9.8 ± 2.9 seen in the control, P > 0.05). In cells stretched in the presence of AG-1478, a statistically significant reduction in HB-EGF mRNA levels in stretched cells was noted only at 100 μM (P = 0.015 for 100 μM, a concentration 400 times greater than the IC50 for ErbB1 inhibition (29)). The FGF-, PDGF-, and IGF receptor inhibitor SU-4984 did not inhibit the increase in HB-EGF mRNA levels with stretch at 4 and 40 μM (stretched/nonstretched: 9.2 ± 1.8 and 10.2 ± 1.0, P > 0.05 compared with control).

Stretch Increases AP-1 and Ets DNA Binding Activity

The above results led us to hypothesize that the response of the HB-EGF gene to stretch is mediated by AT1-, ErbB2-, and p38 SAPK2-dependent pathways, which are independent of signaling through the Erk-MAPK pathway. To test this hypothesis using an independent assay system, we carried out electromophoretic mobility shift analysis to assess the DNA binding activity of the AP-1 transcription factor in response to stretch. We and others have demonstrated increases in AP-1 activity in stretched cells (23, 27, 36). We also recently reported that an AP-1 site at position −988 to −982 (relative to the translation start site) in the HB-EGF promoter is a "stretch-responsive element" (36). This AP-1 site is a composite transcription factor binding site, with a functional Ets binding site lying immediately adjacent (32). We reported that inactivating mutations in both the AP-1 and Ets components of this site block HB-EGF promoter activation by stretch (36). To determine whether AP-1 or Ets DNA binding activities are affected by the same antagonists shown to be active when HB-EGF mRNA levels were used as an experimental end point, we performed mobility shift analysis using AP-1 and Ets consensus oligonucleotides.

SMC exposed to mechanical stretch displayed increased AP-1 DNA binding activity compared with controls (Fig. 2A), consistent with our previous report (36). The increased AP-1 binding activity in stretched SMC was inhibited in a dose-dependent fashion by preincubation of the nuclear extracts with an unlabeled competitor oligonucleotide corresponding to positions −993 to −973 of the HB-EGF promoter, which includes both the stretch-responsive AP-1 and Ets sites.

In mobility shift experiments with an Ets consensus oligonucleotide, cells exposed to stretch also demonstrated an increase in Ets DNA binding activity compared with nonstretched cells (Fig. 2B), consistent with our previous demonstration that an inactivating mutation within the Ets component of the composite AP-1/Ets site abolished the HB-EGF promoter's stretch response (36). Ets binding activity was inhibited in a
dose-dependent fashion by preincubation of the nuclear protein extracts with unlabeled competitor oligonucleotides corresponding to positions −993 to −973 of the HB-EGF promoter (the Ets site is at position −981 to −974). McCarthy et al. (32) previously demonstrated that Ets-2 binds to and activates transcription from this site; however, we were unable to confirm Ets-2 binding in electrophoretic mobility shift assays using a commercially available Ets-2 antibody (see EXPERIMENTAL PROCEDURES). Ets DNA binding in response to stretch was also significantly inhibited by treatment of cells with losartan and AG-825 before stretch. Because of our findings on the response of stretched cells to p38 SAPK2 and Erk-MAPK pathway inhibitors, we also assessed whether inhibition of either pathway affected stretch-stimulated Ets binding activity. Ets proteins have been reported to be activated by both the p38 SAPK2 and the Erk-MAPK pathways (55). Incubation with SB-203580 before stretch ablated stretch-stimulated Ets binding activity; however, prior incubation with PD-98059 had no inhibitory effect on the stretch response.

Collectively, these data provide independent evidence that signaling from the stretch stimulus is mediated by p38 SAPK2-, ErbB2-, and AT1-dependent mechanisms. Neither AP-1 DNA binding nor Ets DNA binding stimulated by stretch was inhibited appreciably by the Mek inhibitor, PD-98059, strongly suggesting that Erk-MAPK signaling is a dispensable pathway. Interestingly, while AT1- and ErbB2-dependent signals converge on both the AP-1 and Ets components of the stretch-responsive element in the HB-EGF promoter, the p38 SAPK2-dependent signal appears to activate only the Ets component of this site.

**Stretch-Stimulated Growth Is Inhibited by p38 SAPK2 and ErbB2 Antagonists**

To determine whether the same pathway-specific antagonists, identified as inhibitors of the HB-EGF gene expression response and AP-1 and Ets activation in response to stretch, also inhibited the growth response to stretch, we assessed relative DNA synthesis rates in bladder SMC treated with the p38 SAPK2, Erk-MAPK, ErbB1, and ErbB2 antagonists. The p38 SAPK2 antagonist, SB-203580 (10 μM), significantly inhibited the stretch response (cpm × 10³/cell number = 32 ± 4.9 compared with 117 ± 23 in cells stretched in the presence of vehicle alone, P < 0.05), whereas PD-98059 (30 μM) had no inhibitory effect compared with control (Fig. 3A).

![Fig. 3. [3H]thymidine incorporation assay of stretched SMC in presence of: DMSO (vehicle control), PD-98059 (PD, 30 μM; an inhibitor of Erk-MAPK) activation, and SB-203580 (SB, 10 μM; p38 SAPK2 inhibitor) (A); AG-1478 (ErbB1 inhibitor) and PD-153035 (ErbB1 inhibitor) (B); AG-825 (ErbB2 inhibitor) and SU-4984 (FGF, PDGF, and IGF receptor inhibitor) (C). Stretch-stimulated growth was inhibited by SB-203580, high-dose AG-1478, and AG-825 but not by PD-98059, PD-153035, or SU-4984 (except at high doses where toxicity was evident in cultures). cpm, Counts/min.](http://ajpcell.physiology.org/issue/)

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C1160 STRETCH STIMULATION OF BLADDER SMOOTH MUSCLE CELLS
AG-1478, the selective ErbB1 inhibitor, inhibited DNA synthesis at concentrations $\geq 1 \mu M$. However, only at high doses (100 $\mu M$) was the inhibition of stretch-stimulated growth complete (Fig. 3B). A second ErbB1 inhibitor, PD-153035 (0.025–250 nM), had no demonstrable inhibitory effect on stretch-stimulated growth (Fig. 3B). AG-1478 at high doses has been reported to inhibit ErbB2 indirectly by sequestering it in inactive ErbB1-ErbB2 heterodimers (4); therefore, one possible reason for the discrepant observations seen with two ErbB1 inhibitors is the inactivation of ErbB2 by AG-1478. Bladder SMC stretched in the presence of the specific ErbB2 inhibitor, AG-825, demonstrated a highly attenuated growth response to stretch, in a dose range (0.35–3.5 $\mu M$) thought to be specific for ErbB2 inhibition (Fig. 3C) (8, 20). In contrast, SU-4984, the PDGF-, FGF-, and IGF-receptor inhibitor demonstrated growth inhibition only at doses at which toxicity was evident in the cultures (>40 $\mu M$, Fig. 3C), suggesting that signaling through these receptor systems is dispensable for a robust growth response to stretch.  

ErbB2 Is Activated in Response to Stretch

Next we assessed whether ErbB1 and ErbB2 were activated in response to mechanical stretch. Total cell lysates were harvested at 10, 30, and 60 min following initiation of the stretch stimulus, and proteins were subjected to Western blot analysis using antibodies specific for the phosphorylated forms of both receptors. ErbB2 was activated within 10 min following stretch, and by 60 min ErbB2 phosphorylation was no longer evident (Fig. 4A). In contrast, increases in ErbB1 phosphorylation were not evident in response to stretch.

Because autocrine signaling through AT1 receptors was previously linked to the growth response to stretch in bladder SMC, we determined the effect of ANG II on ErbB2 activation. Quiescent bladder SMC were incubated with ANG II (1 $\mu M$) without stretch stimulation or were preincubated with losartan (10 $\mu M$) before stretch stimulation. Western blot analysis demonstrated that ErbB2 was not phosphorylated by ANG II stimulation (Fig. 4B). Stretch-induced phosphorylation of ErbB2 was also not inhibited by the AT1 receptor inhibitor losartan or by the p38 SAPK2 inhibitor, SB-203580 (Fig. 4C), indicating that ErbB2 activation by stretch occurs independently of AT1- or p38 SAPK2-dependent pathways.

Stretch Activates p38 SAPK2 and JNK/SAPK but not Erk-MAPK Signaling

The above results implicate signaling through the p38 SAPK2 pathway in stretch-induced gene expression, transcription factor binding activity, and DNA synthesis. To verify that the p38 SAPK2 pathway is activated by stretch, we performed Western blot analysis with a phosphospecific p38 SAPK2 antibody. An increase in p38 phosphorylation was detected within 30 min of stretch, with a peak at 1.5 h (% p38 SAPK2, $P = 220 \pm 37$, compared with 20 $\pm 12$ at time 0, $P < 0.01$) and decreased after 2 h of stretch (Fig. 5A).

In contrast to the observed increase in p38 SAPK2 signaling, Erk-MAPK phosphorylation was not detected in response to stretch (Fig. 5B). To confirm the lack of Erk-MAPK activation with stretch, we used a functional Erk-MAPK activation assay. A transactivator plasmid expressing a fusion protein containing the Erk-MAPK activation domain of Elk-1 (53) joined to the yeast GAL4 DNA binding domain was cotransfected with a reporter plasmid containing a series of GAL4 DNA binding elements upstream from the luciferase gene. In transfected cells subjected to stretch, there was no significant increase in luciferase reporter activity in stretched cells (1.7 $\pm 0.2$ compared with 1.8 $\pm 0.5$ for nonstretched cells, Fig. 5C), confirming that stretch did not detectably activate Erk-MAPK
signaling. In contrast, the Erk-MAPK pathway was demonstrably activated by FGF-2 (Fig. 5D), indicating that this pathway is functional in bladder SMC. To confirm that the dose of PD-98059 (30 μM) used in the HB-EGF mRNA, AP-1, and Ets transcription factor activation and cell growth assays was sufficient to block Erk-MAPK signaling, Erk-MAPK phosphorylation in response to FGF-2 was examined in the presence of PD-98059. Western blot analysis indicated that there was a marked inhibition of Erk-MAPK activation at 30 μM (Fig. 5D).

To assess whether the p38 SAPK2 pathway was the only MAPK pathway activated by stretch, we also determined whether stretch activated the JNK/SAPK pathway. Like the p38 SAPK2 pathway, the JNK/SAPK pathway is frequently activated in response to cell stress. Western blot analysis demonstrated that stretch induced phosphorylation of the JNK/SAPK protein within 30 min following stimulation (% JNK phosphorylation = 30 ± 12 compared with 5 ± 3 for time 0, P = 0.025, Fig. 5E). As an independent assay to assess JNK/SAPK activation, transfection experiments with a c-Jun transactivator plasmid demonstrated a significant increase in reporter activity in the transfected SMC following stretch (64 ± 19 vs. 11 ± 4, P = 0.002, compared with nonstretched cells, Fig. 5F). As an additional test, we also examined whether increases in phosphorylated c-Jun were detectable following stretch. Western blot analysis of stretched cells dem-
onstrated a time-dependent increase in the amount of both c-Jun and phosphorylated c-Jun protein (Fig. 5G), which peaked between 1 and 4 h and then decreased after 8 h. Semiquantitative RT-PCR analysis also demonstrated an increase in the amount of c-Jun mRNA following stretch (~15-fold increase at 1 h compared with time 0, data not shown).

Western blot analysis of nonstretched cells stimulated with ANG II demonstrated time-dependent phosphorylation of p38 SAPK2 but not JNK/SAPK (Fig. 6A). Furthermore, stretch-stimulated phosphorylation of both p38 SAPK2 and JNK/SAPK was not inhibited by losartan (Fig. 6B). Together, these findings provide additional evidence that signaling downstream from stretch is functionally distinct from signaling mediated by AT1 activation. Because stretch-stimulated DNA synthesis was inhibited by ErbB2 or p38 SAPK2 MAPK inhibitors, we also assessed whether stretch-induced activation of p38 SAPK2 and JNK/SAPK pathways was dependent on ErbB2 activation. Western blot analysis demonstrated that phosphorylation of p38 SAPK2 and JNK/SAPK increased after stretch in the presence of AG-825 (0.35 μM), indicating that ErbB2 signaling is not essential for p38 and JNK/SAPK activation by stretch (Fig. 6C).

DISCUSSION

Mechanosensing mechanisms mediate hypertrophic and hyperplastic growth of muscle cells in response to pathogenic stimuli in the cardiovascular system, kidney glomeruli, and the lower urinary tract. In this report, we have identified several features of the mechanism of mechanically induced stimulation of DNA synthesis and growth factor gene expression in normal bladder SMC. Bladder muscle can undergo hypertrophy, which includes both proliferative and hypertrophic cell growth, in response to a number of urological conditions that constrict urine outflow. Because bladder muscle has not been studied extensively, the signaling mechanisms downstream from bladder muscle cell activation are largely undefined. It is not known how mechanotransduction in bladder muscle cells might resemble (or differ) from other muscle types, such as cardiac muscle, which also undergo pathological growth in response to mechanical stress. This question relates ultimately to the design of more effective pharmacotherapies that target key intracellular signaling systems in a tissue-specific manner with minimal adverse sequelae. In this study we identified three aspects of the mechanosensing signaling mechanism in bladder SMC that have not been described in studies of mechanical stimulation of other cell types and thus are particularly novel observations: 1) a lack of involvement of the Erk-MAPK pathway in progression to DNA synthesis and an apparent requirement for signaling through both 2) the p38 SAPK2 pathway, and 3) the receptor tyrosine kinase ErbB2.

The Erk-MAPK pathway is typically activated downstream from growth factor receptor, G protein-coupled receptor (GPCR), and integrin receptor activation and is believed to be critically important in mitogenic signaling in a variety of cell types (3, 7, 16, 22, 24, 25, 49, 57). Activation of the losartan-sensitive angiotensin receptor type 1 (AT1), a member of the GPCR superfamily, has been shown to activate the Erk-MAPKs in several types of muscle cells (30, 35, 44). An earlier study of bladder SMC from our group (37) identified an autocrine loop, involving ANG II secretion and AT1 receptor activation, as one mediator of stretch-induced increases in DNA synthesis and as an essential mediator of stretch stimulation of HB-EGF gene expression. Stimulation of DNA synthesis in response to stretch was significantly, but not completely, inhibited by losartan. Signaling through the AT1 receptor has been shown previously to result in Erk-MAPK pathway activation (24, 35, 44, 57). Therefore, we anticipated at

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*Fig. 5.* A: Western blot analysis of p38 SAPK2 phosphorylation (p38 SAPK2-P) in bladder SMC following stretch. Band intensities were quantified, and the percentage of phosphorylated to nonphosphorylated p38 SAPK2 was determined. B: Western blot analysis of Erk-MAPK phosphorylation (Erk-MAPK-P) in bladder SMC following stretch. Band intensities were quantified, and the percentage of phosphorylated to nonphosphorylated Erk-MAPK was determined. C: Erk-MAPK reporter assay. To confirm that Erk-MAPK signaling was not activated in response to stretch, a functional Erk-MAPK activation assay was used. A plasmid encoding an Erk-MAPK target fusion protein (Erk1 fused to a GAL4 DNA binding domain sequence) and a luciferase reporter plasmid activatable by the fusion protein were transiently transfected into bladder SMC. These cells were then subjected to stretch for 12 h, and luciferase activity was determined. As a negative control, SMC were transfected with plasmids containing only the GAL4 DNA binding domain sequence and the reporter plasmids. As a positive control, SMC were transfected with plasmids that constitutively expressed Mek1 protein kinase along with the transactivator and reporter plasmids. Luciferase activity in nonstretched and stretched bladder SMC was expressed as a percentage of their respective positive controls. D: Western blot analysis of Erk-MAPK phosphorylation in bladder SMC 5 min following FGF (50 ng/ml) stimulation in the presence of various concentrations of PD-98059. E: Western blot analysis of JNK/SAPK phosphorylation (JNK/SAPK-P) in bladder SMC following stretch. Band intensities were quantified, and the percentage of phosphorylated to nonphosphorylated JNK/SAPK was determined. F: JNK/SAPK reporter assay. To confirm that JNK/SAPKs were phosphorylated in response to stretch, a functional JNK/SAPK activation assay was used. The JNK/SAPK specific fusion protein (c-Jun fused to a GAL4 DNA binding domain sequence) plasmid and luciferase reporter plasmid were transiently transfected into bladder SMC. As a positive control, SMC were transfected with plasmids that constitutively expressed mitogen-activated protein kinase kinase (MEKK) protein kinase along with the transactivator and reporter plasmids. Luciferase activity in nonstretched and stretched bladder SMC was expressed as a percentage of the positive controls. G: Western blot analysis of c-Jun phosphorylation (c-Jun-P) in bladder SMC following stretch. Band intensities were quantified, and the percentage of phosphorylated to nonphosphorylated c-Jun were determined. All panels show means and SD for 2 or 3 independent trials.
the outset of this study that signaling through the Erk-MAPKs would be observed, and possibly shown to be important, in the response of bladder SMC to stretch. However, in this same report we also showed that PD-98059, a specific inhibitor of the Erk-MAPK activator Mek1, did not significantly suppress the induction of the HB-EGF gene by stretch, suggesting that the Erk-MAPK pathway might be dispensable for some stretch responses in this cell type. We used this observation as a point of departure for the present series of experiments.

Our results indicate that signaling through the Erk-MAPK pathway is not required for any of the stretch responses we evaluated here; thus we conclude that the stretch-responsive mechanism that stimulates cell growth in bladder SMC acts independently of this pathway. This conclusion is based on a number of independent lines of evidence. Stretch did not detectably activate Erk-MAPK signaling up to 12 h following initiation of the stretch stimulus; in contrast, activation of both the p38 SAPK2 and JNK/SAPK pathways was observed within 30 min after the cells were subjected to stretch. PD-98059, an inhibitor of Erk-MAPK activation, did not show inhibitory activity in several assays related to stretch-stimulated gene expression, including increases in steady-state HB-EGF mRNA levels or increases in AP-1 and Ets transcription factor binding activity. Finally, PD-98059 did not inhibit stretch-stimulated increases in DNA synthesis rate.

Fig. 6. Western blot analysis of p38 SAPK2 and JNK/SAPK phosphorylation (p38 SAPK2-P and JNK/SAPK-P) following stimulation with 1 μM ANG II without stretch stimulation (A); preincubation with 10 μM losartan (AT1 inhibitor) 1 h before stretch stimulation (B); and preincubation with 0.35 μM AG-825 (ErbB2 inhibitor) 1 h before stretch stimulation (C). Time above each panel indicates time following ANG II or stretch stimulation. Ultraviolet-treated cells served as positive control (+) for JNK/SAPK phosphorylation.
MAPK and p38 SAPK2 pathways in mesangial cells under conditions where the JNK/SAPK pathway was not activated (24). Taken together with our results, these observations suggest that MAPK pathway activation by mechanical forces is highly cell type dependent. Importantly, in the present study a selective pharmacological inhibitor of the p38 SAPK2 pathway was able to completely suppress DNA synthesis induced by stretch, indicating that p38 SAPK2 pathway activation is likely to be an important mediator of growth stimulation by mechanical forces in this cell type. Consistent with this finding, we also found that pharmacological suppression of p38 SAPK2 pathway signaling inhibited stretch-induced increases in steady-state HB-EGF mRNA and Ets transcription factor binding activity but, interestingly, not AP-1 transcription factor binding activity. AP-1 activation by stretch has been observed in bladder SMC (23) and other cell types (23, 27). Our data indicate that the p38 SAPK2 pathway appears to selectively regulate Ets transcription factor activity but not AP-1 activity, providing further evidence for selectivity in mechanochemical signaling leading to cell growth. Although stretch activates DNA synthesis in mesangial cells, similarly to the findings in bladder SMC, Ingram et al. (24) did not determine whether inhibition of p38 SAPK2 activity resulted in inhibition or attenuation of stretch-induced DNA synthesis. We are not aware of other studies using either pharmacological or genetic methods to directly implicate signaling through the p38 SAPK2 pathway in a proliferative response to mechanical forces, although this pathway has been implicated in cell proliferation activated by other stimuli (11, 12).

We also found that stretch-activated signaling is dependent on a losartan-inhibitable mechanism. These results are consistent with our own published work on bladder SMC and on published studies of cardiomyocytes (47), although stretch does not increase DNA synthesis in cardiomyocytes, a result thought to reflect the largely hypertrophic response of the heart muscle to increased hemodynamic load. ANG II is a hypertrophic stimulator of heart muscle growth and is involved in cardiac remodeling seen in hypertension and myocardial infarction (43). Several studies have suggested that ANG II may be an important autocrine mediator of stretch-induced gene expression, DNA synthesis, and/or protein synthesis in a variety of cells (5, 46, 47). The HB-EGF gene was shown previously to be positively regulated by ANG II in aortic SMC (50). These results suggest that HB-EGF may be a downstream activator of muscle growth in contexts where AT1 receptor activation occurs in response to pathologic stimuli.

The ErbB2 RTK, a member of the EGF receptor family and a promiscuous “coreceptor” for other ErbB family members and for other cytokines and growth factor receptors (13, 21, 40), was also activated rapidly (within 10 min) in response to the stretch stimulus. ErbB2 activation is likely to be a selective event, because activation of ErbB1/EGF receptor in response to stretch was not observed within 60 min of the stretch stimulus. Consistent with this interpretation, the specific ErbB2 inhibitor AG-825 was a potent antagonist of stretch-stimulated DNA synthesis, HB-EGF mRNA induction, and AP-1/Ets transcription factor DNA binding activity, whereas two selective ErbB1 inhibitors exhibited significantly less inhibitory potency. These experiments, for the first time, implicate ErbB2 as a direct mediator of signaling downstream from a mechanical stimulus. ErbB2 has been the subject of extensive study as an oncoprotein that is overexpressed in some human cancers (52). Its role in muscle cell types is still poorly explored. We recently identified expression of ErbB2 mRNA and protein in human and rodent bladder SMC in vivo and in vitro (6), suggesting the possibility that ErbB2 activation might occur in the urinary tract in response to overdistension of the bladder wall. This possibility remains to be investigated. RTKs can be activated in a ligand-independent manner in response to integrin receptor ligation and ligand-dependent activation of GPCRs. Ligand-independent activation of the PDGF receptor α was recently reported in response to stretch (23). GPCR-dependent activation of both ErbB1 and ErbB2 have been reported (13, 30); however, our results appear to exclude downstream activation of ErbB2 by the AT1 receptor in the context of stretch stimulation, because losartan did not inhibit ErbB2 activation by stretch and because direct AT1 activation by ANG II did not activate ErbB2. Consequently, ErbB2 activation must occur through another, unknown route. Consistent with this conclusion, both p38 SAPK2 and JNK pathway activation were not inhibited by either losartan or AG-825, the ErbB2 inhibitor, indicating that p38 SAPK2 and JNK/SAPK pathway activation occurs independently of ErbB2- or AT1-mediated pathways.

In summary, our results indicate that stretch-stimulated activation of bladder SMC leading to growth is mediated by a conventional signaling pathway, involving the ANG II receptor system, a conserved pathway, involving the p38 SAPK2 system that is activated by mechanical signals in some cells and not others, and a novel pathway, involving the ErbB2 tyrosine kinase. The relevance of ErbB2 activation to mechanochemical signaling in other cell types awaits further study. Significantly, at least one drug directed specifically against ErbB2 is now in use clinically to treat breast cancer (33), indicating that ErbB2-dependent signaling is potentially a feasible target for therapeutic intervention. Finally, our results have shown that the classical Erk-MAPK pathway, which has been proposed as a general mediator of cell cycle progression, is not involved in the stretch responses we evaluated here, including cell cycle entry and mitogenic stimulation. Because these studies were carried out with normal, diploid cells at early passage, they may be relevant to mechanisms of mechanosignaling in bladder tissue in vivo.

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