Extracellular matrix-specific focal adhesions in vascular smooth muscle produce mechanically active adhesion sites

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Submitted 2 November 2007; accepted in final form 12 May 2008

Sun Z, Martinez-Lemus LA, Hill MA, Meininger GA. Extracellular matrix-specific focal adhesions in vascular smooth muscle produce mechanically active adhesion sites. Am J Physiol Cell Physiol 295: C268–C278, 2008; doi:10.1152/ajpcell.00516.2007.—Integrin-mediated mechanotransduction in vascular smooth muscle cells (VSMCs) plays an important role in the physiological control of tissue blood flow and vascular resistance. To test whether force applied to specific extracellular matrix (ECM)-integrin interactions could induce myogenic-like mechanical activity at focal adhesion sites, we used atomic force microscopy (AFM) to apply controlled forces to specific ECM adhesion sites on arteriolar VSMCs. The tip of AFM probes were fused with a borosilicate bead (2–5 μm) coated with fibronectin (FN), collagen type I (CNI), laminin (LN), or vitronectin (VN). ECM-coated beads induced clustering of α5- and β3-integrins and actin filaments at sites of bead-cell contact indicative of focal adhesion formation. Step increases of an upward (z-axis) pulling force (800–1,600 pN) applied to the bead-cell contact site for FN-specific focal adhesions induced a myogenic-like, force-generating response from the VSMC, resulting in a counteracting downward pull by the cell. This micromechanical event was blocked by cytochalasin D but was enhanced by jasplakinolide. Function-blocking antibodies to α5β1- and α6β4-integrins also blocked the micromechanical cell event in a concentration-dependent manner. Similar pulling experiments with CNI, VN, or LN failed to induce myogenic-like micromechanical events. Collectively, these results demonstrate that mechanical force applied to integrin-FN adhesion sites induces an actin-dependent, myogenic-like, micromechanical event. Focal adhesions formed by different ECM proteins exhibit different mechanical characteristics, and FN appears of particular relevance in its ability to strongly attach to VSMCs and to induce myogenic-like, force-generating reactions from sites of focal adhesion in response to externally applied forces.

integrins; myogenic mechanism; atomic force microscopy; mechanobiology; microcirculation

MECHANOTRANSDUCTION in vascular smooth muscle cells (VSMCs) is a fundamental mechanism underlying the vascular myogenic response (9), in which vascular smooth muscle contracts in response to increased intraluminal pressure or relaxes in response to decreased pressure (11, 21).1 This VSMC behavior is a key mechanism for the establishment of vascular tone and the autoregulation of blood flow (8). It is generally assumed that, in the myogenic response, a perturbation in intravascular pressure changes vascular wall tension/stress, which then triggers the contraction of the VSMC. The VSMC then exerts its contractile force on the extracellular matrix (ECM) it is embedded within. Evidence from isolated, intact arterioles indicates that inhibition of α5β1- and α6β4-integrins prevents myogenic constriction in response to an acute pressure elevation (23). It has also been demonstrated that ligation of α5β1-, α6β4-, and α6β4-integrins with ECM proteins modulates Ca2+ conductance through voltage-gated Ca2+ channels (32, 36). Thus, one plausible hypothesis for the myogenic mechanism involves force transmission to sites of integrin attachment with subsequent activation of the actomyosin contractile process (9, 35, 36).

Arising from their complex function in mediating cell attachment and signal transduction, integrins have been more widely recognized to be important for cellular mechanotransduction. Supporting evidence for integrin involvement in mechanotransduction comes from multiple cell types, for example, endothelial cells (30), fibroblasts (6), osteoblasts (26), neuronal cells (12), and VSMCs (14). Structurally, integrins are a family of transmembrane proteins interposed between ECM proteins and the cytoskeleton (29), thus providing a mechanical connection to the extracellular environment through which mechanical forces are envisioned to be bidirectionally transmitted through focal adhesion sites. Integrin association with the ECM leads to integrin clustering and subsequent association with a number of scaffolding proteins and signaling proteins that are linked as a series of connected elements with the cytoskeleton. Assembly of the scaffolding and signaling proteins and their hierarchical importance within an adhesion site are areas that are incompletely understood. Known processes include, but are not limited to, activation of FAK, recruitment of paxillin, vinculin, and Cas, activation of receptor tyrosine kinases and phosphatases, and cytoskeleton remodeling (see, e.g., Refs. 1, 5, 13, and 29). These processes of protein assembly continue as the focal contact matures into a dynamically regulated focal adhesion (4, 18, 20, 30). As focal adhesions mature, so, apparently, does the ability of the cell to transmit external physical forces and exert tensile forces to the surrounding ECM. Indeed, numerous pieces of evidence support the ability of cells to transmit forces through focal adhesions to their environment (4) and ultimately establish a mechanical balance (2, 20) that involves adaptation at the level of the focal adhesion site.

In this study, we used atomic force microscopy (AFM) to directly measure and apply nanoscale force to ECM-induced focal adhesion sites. The goal of our study was to test the hypothesis that mechanically pulling at a site of integrin-ECM adhesion would induce myogenic-like, micromechanical events...
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F-12 supplemented with 10% FBS, 10 mM HEPES (Sigma, St. Louis, MO), 2 mM L-glutamine, 1 mM sodium pyruvate, 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B. Cells were maintained in 60-mm tissue culture dishes (Falcon, BD Laboratory, Lincoln, NJ) in a humidified incubator (Heraeus Instruments, Newtown, CT) with 5% CO2 at 37°C. For AFM experiments, cells were cultured in 35-mm tissue culture dishes with a no. 1 glass coverslip bottom (World Precision Instruments, Sarasota, FL). Low-passage cells (passages 3–10) were used in all experiments. Except for HEPES, all reagents were purchased from Invitrogen (Carlsbad, CA).

### Isolated vessel experiments.
Arterioles were isolated from the cremaster skeletal muscle of pentobarbital-anesthetized rats (60 mg/kg) as we have previously described (11). The cremaster muscle was isolated, and a distal segment of the external spermatic artery (first-order arteriole 1A; 120–190 μm inner diameter, passive) was excised and placed in a chamber containing Krebs buffer [composed of (in mM) 112 NaCl, 12 glucose, 25.5 NaHCO3, 9.1 HEPES, 1.2 MgSO4, 2.5 CaCl2, 1.2 KH2PO4, and 4.7 KCl]. Two glass micropipettes (tip diameter: 40–60 μm) were filled with Krebs buffer plus 0.5% BSA were used to cannulate the arteriole. The arteriole was set to a length such that lateral bowing did not occur up to intraluminal pressures of 120 mmHg and then pressurized to an in vivo level of 70 mmHg. Vessels typically developed spontaneous tone, resulting in constriction to 50–60% of their passive diameter. Vessel dimensions were measured using a closed-circuit videomicroscopy system coupled to a video caliper (Cardiovascular Research Institute, Texas A&M University Health Science Center). The video caliper allowed lumen diameter to be stored and recorded online as a continuous trace using Powerlab software (AD Instrument). After a spontaneous tone developed in the vessel, the intraluminal pressure was lowered to 30 mmHg, and the vessel was allowed to stabilize to reach a steady state. The intraluminal pressure was then elevated by 15 mmHg in a stepwise manner, and the whole system was thermoequilibrated for 1 h. This equilibration time was longer than the suggested by the manufacturer and was determined to effectively reduce drift experimentally. After thermal equilibration, the protein-

### MATERIALS AND METHODS

#### Cell isolation and cell culture.
Microvascular smooth muscle cells (mVSMCs) were isolated from the first-order feed arteriole (100–150 μm diameter) of Sprague-Dawley rat cremaster skeletal muscles using previously described methods (36). Cells were cultured in DMEM/H9262 (mVSMCs) were isolated from the first-order feed arteriole (100 –150 μm) of Sprague-Dawley rat cremaster skeletal muscles using previously described methods (36). Cells were cultured in DMEM-

### Protein coating of beads.
AFM probes with biotin-labeled borosilicate beads were purchased from Novascan (Ames, IA) with a spring constant of 0.01 N/m. ECM proteins [fibronectin (FN), collagen type I (CNI), laminin (LN), and vitronectin (VN)] were first biotinylated using EZ-Link Sulfo-NHS-LC-Biotin ( Pierce, Rockford, IL) following instructions provided by the manufacturer. Briefly, 200 μl of protein (0.5 or 1 mg/ml) were mixed with 6–12 μl of Sulfo-NHS-LC-Biotin (10 mg/ml) in a microcentrifuge tube (Corning, Corning, NY). The mixture was incubated on ice for 2 h and filtered through a microcentrifuge tube (Millipore, Bedford, MA). Filtered proteins were dissolved in Dulbecco’s PBS (DBPS) with 0.1% NaN3 (Sigma-Aldrich) to a concentration of 0.5 mg/ml. AFM probes with biotin-labeled borosilicate beads were incubated with avidin (1 mg/ml, Sigma-Aldrich) for 5 min at room temperature. Probes were then washed five times with DBPS and incubated with biotinylated protein for 6 min at room temperature, followed by another five times of DBPS washing. Polystyrene fluorescent beads (5.46 μm diameter) were purchased from Bangs Laboratory (Fishers, IN) with dragon green (480/520-nm excitation/emission) and streptavidin coating. Beads were spun down and washed three times with DBPS. After being washed, beads were incubated with biotin-conjugated ECM proteins at room temperature for 30 min on a rolling plate. Beads were then washed three times with DBPS and resuspended in 80 μl DBPS.

### AFM force application and measurement.
To apply pulling forces to the VSMC surface, the AFM was operated in contact mode with the scan size set to 0.1 μm. The experiment was performed at room temperature, and VSMCs were incubated in HBSS. To minimize drift, after the probe was submerged in cell bath, the whole system was thermoequilibrated for 1 h. This equilibration time was longer than that suggested by the manufacturer and was determined to effectively reduce drift experimentally. After thermal equilibration, the protein-

#### Isolated vessel experiments.
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#### Instruments.
A Bioscope AFM System (model IIIa or IVa, Digital Instruments, Santa Barbara, MA) was mounted on an Axiovert 100 TV inverted microscope (Carl Zeiss, Thornwood, NY). AFM data were collected and analyzed using NanoScope (Digital Instruments) and MatLab (MathWorks, Natick, MA) software. Immunofluorescently labeled cells were visualized using either a Meridian ULTIMA Z-Laser Confocal Microscope System or a SP2 Confocal/Multiphoton Microscope System (Leica, Bannockburn, IL).

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Borosilicate glass beads (5 μm diameter) were purchased from SPI. Beads (1 mg) were mixed with CNI or FN and incubated at room temperature for 3 h. Beads were then spun down, washed three times with DBPS, and resuspended in 80 μl HBSS.

AFM force application and measurement. To apply pulling forces to the VSMC surface, the AFM was operated in contact mode with the scan size set to 0.1 μm. The experiment was performed at room temperature, and VSMCs were incubated in HBSS. To minimize drift, after the probe was submerged in cell bath, the whole system was thermoequilibrated for 1 h. This equilibration time was longer than that suggested by the manufacturer and was determined to effectively reduce drift experimentally. After thermal equilibration, the protein-

![AFM data analysis](http://ajpcell.physiology.org/)
coated beads were brought into contact with the cell surface and were kept in a static force neutral position on the cell surface for 25–30 min. The deflection set point was then manually adjusted to apply step increases of pulling force (800–1,600 pN) to the VSMC at the site of bead contact. The pulling force was generated by the bending of the AFM cantilever and was calculated according to Hooke’s law as follows:

\[ F = d \times k \]

where \( F \) is force (in pN), \( d \) is cantilever deflection (in nm), and \( k \) is the cantilever spring constant (in pN/nm). Bead displacements were recorded for 4 min in the contact mode. Height data were recorded continuously and analyzed to obtain quantifiable changes in the magnitude of the micromechanical event of the VSMC following the upward pull by AFM.

To examine the role of the actin cytoskeleton, the VSMC micromechanical event was evaluated in the presence of cytochalasin D (13.3 μM, 10 min of incubation), a F-actin depolymerizing agent, or jasplakinolide (0.2 μM, 10 min of incubation), a F-actin stabilizing agent. A paired experimental design was used for these experiments. A control response to pulling with the AFM (800 pN) was obtained before treatment, and a second response was then recorded after the addition of cytochalasin D or jasplakinolide to the cell bath. Vehicle control experiments were also performed.

To evaluate the involvement of α5- and β3-integrins, specific function-blocking antibodies to α5- and β3-integrins were added into the cell bath, and AFM force application and measurements were then performed as described above. Parallel control experiments were performed without antibodies.

To determine the involvement of Src family kinases, cells were incubated with 5 μM PP2 or PP3 (EMD Biosciences, San Diego, CA) for 30 min at room temperature, and AFM force application and measurements were then performed as described above.

Cell staining for immunocytochemistry and confocal microscopy.

Cells were allowed to grow until 50% confluent on glass-bottom tissue culture dishes. FN-coated fluorescent beads were added to the culture dish, and cells were incubated with the beads for 2 h at 37°C. Cells were then washed with DPBS and fixed with 2% paraformaldehyde, followed by the addition of glycine buffer (0.1 mM glycine) for paraformaldehyde quenching. After being washed with PBS, cells were incubated with a primary antibody (1:200 dilution in labeling buffer composed of 150 mM NaCl, 15 mM Na3C6H5O7, 0.05% Triton X-100) overnight at 4°C. The secondary antibody was added, and cells were incubated for 1 h at room temperature. Nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI) for visualization.

Fig. 2. A: trans-illumination image a bead-fused atomic force microscopy (AFM) probe applied to surface of a VSMC. B: force required to detach the bead from the cell surface plotted as a function of contact time. C: displacement of a fibronectin (FN)-coated bead on the VSMC surface in response to step increases of pulling force applied by AFM. The FN-coated bead fused to the AFM cantilever was brought into contact with the VSMC surface to form molecular connections. Top, step increases of pulling force (z-direction) applied onto the bead-cell connection site using AFM. Bottom, corresponding z-direction movements of the FN-coated bead. D: schematics of the VSMC response to a step increase of pulling force. E: myogenic response of an intact isolated rat cremaster skeletal muscle arteriole induced by a stepwise increase of the intraluminal pressure. Arrows depict time points of application of 15 mmHg pressure steps; vessel diameters were normalized by the vessel diameter at 70 mmHg (D/D70), and are shown in a percentage scale. F: vessel responses to a step pressure change from 45 to 60 mmHg. Data represent means ± SE.
X-100, and 2% BSA) at 4°C overnight. Cells were then washed six times with cold buffer (composed of 150 mM NaCl, 15 mM Na$_3$C$_6$H$_5$O$_7$, and 0.05% Triton X-100), followed by an incubation with Cy5-conjugated secondary antibody (1:100 dilution in labeling buffer) or Alexa 568-conjugated phalloidin for 1 h at room temperature in a dark environment. Labeled cells were then washed six times with cold buffer and imaged on a confocal microscope using excitation wavelengths of 488 nm and 647 nm sequentially. A through-focus image set was collected for each cell with a $z$-step interval of 0.2 μm. Images were analyzed using ImagePro Plus software (Media Cybernetics, Carlsbad, CA) and Matlab (MathWorks).

Reagents. Human plasma FN was purchased from Invitrogen, rat plasma FN was purchased from EMD Biosciences, and human natural VN was purchased from BD Bioscience. Mouse LN and cytochalasin D were purchased from Sigma. CNI was isolated from the rat tail as previously reported (28). For confocal microscopy, rabbit anti-α$_5$-integrin polyclonal antibody, rabbit anti-rat β$_3$-integrin polyclonal antibody, mouse anti-FAK monoclonal antibody, and rabbit anti-paxillin monoclonal antibody (Millipore) were used as primary antibodies, and Cy5-conjugated goat anti-rabbit IgG antibody (Jackson ImmunoResearch) was used as the secondary antibody. Jasplakinolide, Alexa 647-phalloidin, and Alexa 568-phalloidin were purchased from Molecular Probes. For integrin-blocking experiments, antibodies HMα5-1 and F11 were purchased from Pharmingen.

Viscoelasticity analysis. To analyze the mechanical properties of the collective cellular structures associated with ECM-labeled beads,
a mechanical model of viscoelasticity was adopted from Bausch et al. (3) to describe the viscoelastic behavior of cells. The model was modified to fit the experimental condition where the cantilever-attached bead was pulled by an upward vertical force rather than by a tangential force. Briefly, the cellular structure was modeled by a mechanical circuit, known as a Kelvin body, in which the springs represent the elastic elements and the dashpot represents the viscous elements in the cell structure (Fig. 1, A and B).

Upon the application of a step increase of force, the bead displacement could be described by the function below:

$$\frac{x(t)}{F} = \frac{1}{k_0} \left[ 1 - \frac{k_1}{k_0 + k_1} \times \exp(-t/\tau) \right]$$

where the relaxation time $\tau$ is given by

$$\tau = \frac{\gamma_0 (k_0 + k_1)}{k_0 k_1}$$

where $F$ represents the step increase of pulling force, $t$ is time, and $x$ is the vertical bead displacement. $k_0$ and $k_1$ represent the elasticity of the elastic elements of the cell structure (in pN/nm) and are measures of the ability of the solid cellular structure (i.e., the cytoskeleton) to resist elastic deformation caused by extensional stress. $\gamma_0$ is the viscosity of the viscous elements (pN·nm·s) and is a measure of the ability of the amorphous cellular materials to resist deformation caused by extensional stress. The equations were used to numerically fit the experimental measurements (data acquired within the first 2 s after the application of AFM pulling force, Fig. 1C), and $k_1 + k_0$ and $\gamma_0$ were calculated to represent the pulling elasticity and viscosity of the cell structure under the beads. The pulling elasticity and viscosity reflected the strength of the cell attachment to the bead. High values of elasticity and viscosity indicate a tight (or rigid) cell-bead attachment, and vice versa.

Statistical analysis. Results were compared using paired or unpaired Student’s $t$-tests. Significance was assumed at a P value of ≤0.05.

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Fig. 4. A and B: effect of function-blocking antibodies for $\alpha_5\beta_1$ and $\alpha_5\beta_3$ integrins (HMα5-1 and F11, respectively) on the VSMC mechanoresponse. For control, $n = 7$; for HMα5-1 (20 $\mu$g/ml), $n = 7$; for HMα5-1 (50 $\mu$g/ml), $n = 6$; for F11 (20 $\mu$g/ml), $n = 4$; and for F11 (50 $\mu$g/ml), $n = 8$. C and D: pulling elasticity and viscosity of control VSMCs and cells treated with HMα5-1 or F11. Arrows indicate the time points of force application. Data represent means ± SE.
examine if integrins and actin filaments were associated with FN-coated beads after placement on the cell surface. FN-coated beads were placed on VSMCs and allowed to form adhesions on the cell surface. As shown in Fig. 3, A and B, both \( \alpha_5 \) and \( \beta_3 \)-integrins formed ring-shaped clusters around the FN-coated bead, indicating the presence of these integrins and supporting the involvement of both \( \alpha_5 \beta_1 \) and \( \alpha_6 \beta_3 \)-integrins. Actin filaments were also observed to cluster around the FN-coated bead (Fig. 3C), demonstrating the close association of cytoskeletal elements beneath the FN-coated bead.

The involvement of integrins in the micromyogenic event was further tested by blocking \( \alpha_5 \) and \( \beta_3 \)-integrins with specific function-blocking antibodies (HM\( \alpha_5 \)-1 and F11, respectively). The presence of either antibody did not abolish the ability of the VSMC to establish an adhesion with the FN-coated bead, but the presence of either antibody significantly inhibited the micromyogenic event (Fig. 4, A and B). The inhibitory effect of the antibodies was concentration dependent (20 or 50 \( \mu \)g/ml). A concentration-dependent increase in initial bead displacement was observed with both inhibitory antibodies, indicating that the pulling elasticity and viscosity of adhesion site and underlying cytoskeletal attachments were reduced in the presence of either antibody (Fig. 4, C and D).

To evaluate the role of the actin cytoskeleton, the micromyogenic event was studied in the presence of cytochalasin D (13.3 \( \mu \)M), an actin filament depolymerizing agent, or jasplakinolide (0.2 \( \mu \)M), an actin filament stabilizing agent. Cytochalasin D totally abolished the VSMC force response, with the FN-coated bead being quickly pulled away from the cell surface, indicating that the adhesive strength of the focal adhesion with the FN bead was significantly reduced (Fig. 5, A and B). In contrast, after the application of jasplakinolide (0.2 \( \mu \)M), the micromyogenic event was augmented, resulting in more rapidly developing tension (0.59 \pm 0.11 vs. 0.34 \pm 0.18 nm/s; Fig. 5, C and D). DMSO was applied as a vehicle control for jasplakinolide and cytochalasin D and had no effect on micromyogenic event (data not shown). As anticipated, cytochalasin D decreased the cell elasticity compared with jasplakinolide and the DMSO control, suggesting an effect on the intracellular cytoskeletal elements associated with the FN bead (Fig. 5E). Collectively, these results provide evidence for the involve-
ment of the actin cytoskeleton in the micromyogenic event that occurs with the FN-coated bead.

**VSMC response to force applied through CNI-, VN-, or LN-coated beads.** To determine whether other ECM proteins induced a similar micromyogenic event, beads coated with CNI, VN, or LN were applied to the cell surface. Beads coated with BSA alone were applied to the cell surface as a control for the ECM proteins. As shown in Fig. 6a, CNI, VN, or LN did not induce the micromyogenic event from VSMCs. Force applied to sites of adhesion with CNI-coated beads exhibited strong and stable attachment that resisted the direction of pull by AFM. In comparison, beads coated with VN or LN were gradually but continuously pulled away from the cell surface using similar pulling force to that used for FN and CNI. This indicates a much weaker form of adhesion with these ECM proteins under the conditions of our experiments. In contrast to BSA-coated beads, CNI-coated beads (Fig. 8, E and F). PP2 significantly impaired the ability of FN to elicit the micromyogenic event. However, both FAK and paxillin were found clustered near the FN- and CNI-coated beads (Fig. 8, E and F). To further evaluate a possible mechanism, we examined whether tyrosine phosphorylation events mediated by Src were involved. Cells were treated with PP2, a specific inhibitor, to block Src family kinases. PP2 significantly reduced the elasticity and viscosity of cell attachment to both FN- and CNI-coated beads compared with the PP3 control group. Although the FN-coated bead continued to maintain its attachment to the cell, PP2 abolished the micromyogenic-like response to pulling through the FN-coated bead. PP2 significantly impaired the ability of the CNI-coated bead to withstand the pulling force, and the bead was gradually pulled away from the cell under constant force (Fig. 8, A–D). These results suggested that in mVSMCs, the Src-mediated signaling pathway plays an important regulatory role in the mechanisms that strengthen adhesive attachment for FN and CNI, and it is important for the development of the micromyogenic event.

**DISCUSSION**

The goal of this study was to determine if nanoNewton scale forces applied through specific ECM-integrin focal adhesion sites could induce myogenic-like micromechanical events from single mVSMCs. Our results indicate that forces applied to an FN-induced focal adhesion site induced mVSMCs to respond with a micromyogenic event. The micromyogenic event was dependent on interactions with α5β1-integrin, α6β3-integrin, the actin cytoskeleton, and cSrc activity. To our knowledge, this is the first study using AFM technology to quantitatively measure the mechanical responses to applied force at single focal adhesion sites formed with specific ECM proteins. In contrast to FN, force applied to focal adhesion sites induced with CNI, VN, or LN were not able to elicit the micromyogenic event, suggesting that FN could be of particular relevance to the mechanosensing and transducing pathways in mVSMCs.

In this study, we estimated the scales of time and pulling force to mimic those approximated to occur at the single cell level in an intact arteriole and that result in a myogenic response. This was done to strengthen our ability to correlate observations at the single cell level with the myogenic behavior.
of intact arteriole. As shown in Fig. 2E, during the myogenic response, the isolated arteriole constricted over a 3- to 5-min period following an initial distention. Similarly, the micromyogenic event in mVSMCs occurred over a similar 3- to 5-min interval. In the intact arteriole, the micromyogenic response to a step increase of pressure of 15 mmHg converts to an extensional stress of 2,000 pN/μm² on the vessel wall. Assuming that mVSMCs are the major force-bearing components in the vessel wall, we estimated that mVSMC-ECM interactions could experience forces close to that magnitude. For our experiments, we selected values for pulling from 800 to 1,600 pN, with most experiments conducted at 800 pN. With the use of 2- to 5-μm-diameter beads, the applied forces experienced at a single focal adhesion site would be ~30–200 pN/μm². The absolute rate of tension development by the single mVSMC is ~0.34 nm/s (rate of downward bead displacement on cell), lower than the predicted rate of cell length change in the intact vessel wall (~41.6 nm/s). However, the estimated rates of cell deformation, obtained by normalization with their respective dimension scale, fall into the same magnitude (1.4 vs. 4.2 times 10⁻⁵·s⁻¹). These quantitative estimates suggest that the micromyogenic behavior that we observed at single focal adhesion sites in mVSMCs closely parallel the estimated mechanical events envisioned to occur in the intact arteriole.

The observation that mVSMCs could generate a micromyogenic force in response to force applied to the adhesion with FN is consistent with similar mechanical observations in other cell types. For example, in fibroblasts, Choquet et al. (6) reported that cells generated sufficient force to pull a bead coated with FN7-10 (a major integrin-binding motif) against a trapping force (5–60 pN) applied through laser tweezers. This suggested that the response involved integrin-mediated mechanosensing and cytoskeleton strengthening. In another study, Heidemann et al. (15) attached a LN-coated microglass needle to fibroblasts and demonstrated that the cell could contract against a force of 10–30 nN. In contrast to these observations, mVSMCs did not respond to force applied through LN. This could be related to differences in cell type. Integrin-mediated cellular force has also been demonstrated in processes such as bending of collagen fibrils by chondrocytes (22), contraction of fibrin clots by smooth muscle (37), and contraction of collagen gels by embryo cells (17). Our results also provide further quantitative biomechanical evidence suggesting that integrins are important sites for mechanosensing and force transmission in mVSMCs. In addition, our results point to FN as an important ECM protein that is linked to micromyogenic behavior in mVSMCs.

Fluorescent imaging of mVSMCs immunolabeled for α₅β₁-integrin, α₅β₃-integrin, and actin filaments showed accumulation of these proteins beneath the FN-coated beads (Fig. 4). In addition, FAK and paxillin were also found around the FN-coated bead (Fig. 8). The association of these proteins at the point of contact with the FN-coated bead indicated that mVSMCs formed a focal adhesion-like structure around the FN-coated bead. It was also observed in our experiments that the force required to completely detach the FN-coated bead from the VSMC increased with increasing duration of contact time. This indicated that a progressive biomechanical process was contributing to the formation of the adhesion complex between FN and the cell.

Disruption of the actin cytoskeleton by cytochalasin D abolished the cellular force response and reduced the mechanical elasticity of the focal adhesion site in response to pulling. This is consistent with the notion that the actin cytoskeleton is the major force-bearing elastic structure in the cell (19, 27, 33). Interestingly, blockade of actin depolymerization with jas-
plakinolide acutely enhanced the micromyogenic response. Supportive observations have been recently described by Zhang et al. (38). They showed that jasplakinolide enhanced the mechanical gating of nonselective stretch-inhibited cation channels during osmosensory transduction in neuron cells, whereas cytochalasin-D reduced it (38). In addition, Cipolla et al. (7) have shown that actin polymerization is strongly enhanced during the myogenic response in intact isolated
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were performed. We observed that bead adhesions to the same extent but eliminated the micro-mechanocommunications through a tyrosine phosphorylation process. PP2 treatment reduced the viscosity and elasticity of CNI adhesions and also attenuated the VSMC myogenic-like response to FN-coated beads, suggesting that EGFR-related signaling pathways may also be involved in VSMC mechanotransduction. Future studies will be necessary to address the cross-talk between these signaling pathways in VSMCs and identify the relevant phosphorylation targets. Work by Wu et al. (36) has clearly demonstrated that FN-induced cSrc activation is linked to phosphorylation of the L-type Ca\(^{2+}\) channel, providing one possibly important link to the micromyogenic event observed in our study.

In summary, our results indicate that mechanical force applied to a focal adhesion induced with FN can elicit a micromyogenic event in mVSMCs in response to the application of a constant force to the focal adhesion site. This myogenic-like response is not observed with CNI, VN, or LN, suggesting ECM specificity. The qualitative presence of \(\alpha_5\beta_1\)-integrin, \(\alpha_5\beta_3\)-integrin, actin, paxillin, and FAK at the adhesion sites with these ECM proteins is not sufficient to explain the FN-induced micromyogenic effect. However, a role for cSrc appears to be important. Further work will be necessary to delineate the specific cell signaling pathways and critical focal adhesion proteins to understand the mechanism of this myogenic-like response. Understanding this micromyogenic event may provide significant new insights into the mechanism of the vascular myogenic response.

GRANTS

This work was supported by National Heart, Lung, and Blood Institute Grants HL-58960 and HL-062863 (to G. A. Meininger).

REFERENCES

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