Intracellular signaling specificity in response to uniaxial vs. multiaxial stretch: implications for mechanotransduction

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The intrinsic ability of striated muscle cells to sense and respond to mechanical signals has been recognized for decades, and these responses have been implicated in a variety of biological processes ranging from organogenesis to regulation of tissue and cell size and, in cardiac tissue, the development of pathological disease (9, 15, 18, 22, 37). In addition to being able to sense mechanical signals, there is also evidence to suggest that muscle cells have the capacity to distinguish between different types of mechanical signals. For example, in skeletal muscle, chronic longitudinal stretch produces growth in length, defined by increased sarcomere number in series, whereas functional overload produces cross-sectional growth, as defined by increased sarcomere number in parallel (32). Growth in the heart also provides an example of different phenotypic responses to mechanical loading, with pressure overload resulting in increased ventricular wall thickness in contrast to volume overload resulting in increased ventricular volume (4). These examples highlight the evidence that in striated muscle tissue, different types of mechanical loading can produce distinct downstream adaptations. Furthermore, these examples suggest that specific cellular mechanisms may exist to sense and subsequently respond to the different mechanical forces delivered. However, because of the complexity of the mechanical environment within muscle tissue, the concept that different types of mechanical forces can elicit unique molecular events has not been well studied.

Defining the mechanical environment within a single muscle cell is technically very difficult, if not impossible, in vivo. This is because of numerous anatomical factors that include characteristics such as fiber orientation within the muscle (i.e., angle of pennation), interactions between the muscle fiber and its own extracellular matrix/basal lamina, and the mechanical impact that results from the complex recruitment of motor units so that an activated muscle fiber from one motor unit will be contracting next to an inactive fiber from a different, nonrecruited motor unit. All of these factors contribute to both the magnitude and the type of forces experienced by a single muscle fiber within the tissue in vivo. Thus, to study mechanotransduction-mechanosensory capacities of muscle cells, it is necessary to apply clearly defined mechanical stimuli, and for this we rely on the use of in vitro mechanical stimulators (stretch devices). These devices can be used within the confines of a cell culture environment, and the mechanical stimuli that are delivered to the culture membrane are easily controlled and can be readily defined.

In vitro stretch devices can generally be grouped into one of two classes, uniaxial or multiaxial. The major difference between a uniaxial and multiaxial stretch device is the type of mechanical deformation delivered to the elastic culture membrane. In this study, we utilized these two types of devices in an attempt to identify whether muscle cells have the fundamental ability to differentiate between different types of mechanical forces. Alterations in well-known signaling molecules were evaluated as a means to discern whether differential mechanotransduction-mechanosensory information was being processed. The molecules evaluated included the extracellular signal-regulated kinase (ERK), protein kinase B (PKB/Akt), and the 70-kDa S6 kinase (p70s6k). These signaling molecules were selected because previous reports have shown them to respond to mechanical stimuli and because they all have been...
implicated in the regulation of mechanical load-induced hypertrophy (1, 36).

The findings from these studies demonstrate that both uniaxial and multiaxial stretch induce an increase in PKB/Akt, and ERK phosphorylation but that only multiaxial stretch induces an increase in p70S6k phosphorylation. Evidence is provided suggesting that the unique activation of p70S6k is a result of myotubes sensing multiaxial vs. uniaxial tensile strains and is not due to differences in surface area deformation or shear stress. In addition, our findings demonstrate that disrupting the actin cytoskeleton inhibits the multiaxial stretch-induced phosphorylation of p70S6k, whereas pharmacological inhibitors that have been reported to block signaling to p70S6k after growth factor stimulation did not (6, 11, 12, 25). These findings suggest that a novel signaling pathway is used by multiaxial stretch to induce phosphorylation of p70S6k. It is also important to note that although disruption of the actin cytoskeleton blocked the multiaxial stretch-induced phosphorylation of p70S6k, it had no effect on the signaling to PKB/Akt, leading to the additional conclusion that mechanical stimuli can activate distinct mechanosensory-mechanotransduction pathways and that the activation of these pathways is specific to the types of mechanical forces applied.

MATERIAL AND METHODS

Materials. Peroxidase-conjugated anti-rabbit was purchased from Vector Laboratories (Burlingame, CA). FITC-conjugated anti-rabbit was purchased from Jackson Laboratories (Bar Harbor, ME). Phosphospecific (threonine-202/tyrosine-204) ERK1/2, (serine-473) PKB/Akt, and (threonine/serine-421/424) p70S6k were purchased from Cellular Signaling (Lake Placid, NY). Polyvinylidene difluoride (PVDF) membranes were purchased from Millipore (Bedford, MA). Enhanced chemiluminescence (ECL) detection reagent was purchased from Amersham Pharmacia Biotech (Amersham, UK). The DC protein assay kit was purchased from Bio-Rad (Hercules, CA). Dulbecco’s modified Eagle’s medium (DMEM), fetal bovine serum (FBS), and horse serum (HS) were purchased from Invitrogen (Carlsbad, CA). Cytochalasin D, gadolinium, genistein, and anti-α-actinin antibody were purchased from Sigma (St. Louis, MO). PD-098059, bisindolylmaleimide I (BIM), and LY-294002 were purchased from Calbiochem (San Diego, CA).

Cell culture. C2C12 skeletal myotube cultures were grown by plating C2C12 myoblasts on type I collagen-coated Bioflex membranes (Flexcell International, Hillsborough, NC) at a density of ~1.5 × 10⁴ cells/cm² as previously described (17). Myoblasts were maintained in low-glucose DMEM supplemented with antibiotics (100 μg/mL streptomycin and 100 U/mL penicillin; Sigma) and 10% FBS. Upon confluence (~48 h), cells were switched to DMEM supplemented with antibiotics and 10% HS to promote differentiation. Cells were maintained in this medium for 24 h and then switched back to the 10% FBS medium for an additional 5–6 days, after which distinct multinucleated myotubes were present. Media were changed every 48 h, and fresh medium was added 18 h before the initiation of mechanical stretch. Three hours before the initiation of stretch, culture plates were removed from the incubator, rapidly placed on the uniaxial or multiaxial stretch device, and returned to the incubator.

Mechanical stretch. Two separate devices were used to stretch the C2C12 myotube cultures. The uniaxial stretch device consisted of a slight modification of the device described by Clark et al. (5). This is a motor-driven system that applies uniaxial stretch to a rectangle-shaped membrane. The sides of the membrane are unconstrained, so there is minor compression transverse to the direction of applied stretch. This is a feature of incompressibility and is very similar to what would happen if an isolated cell were stretched on a single axis.

The multiaxial device (FX-3000; Flexcell International, Hillsborough NC) is a vacuum-operated system that produces nonuniform multiaxial (radial and circumferential) tensile strains with very little compression on a circle-shaped membrane (7). The airflow through the stretch plates is unrestricted, which diminishes the potential for internal pressure gradients in the cell culture plates. This is important because it allows the rate of deformation of the membrane to be a true reflection and a result of the rate of pressure change via the vacuum system.

The stretch programs employed in both devices were designed to produce cyclic 15% membrane stretch, cyclic 11% increases in membrane surface area (ΔSA) or cyclic 24% ΔSA. These changes were produced at a frequency of 1 Hz in a triangular wave form. Stretch cycles were sustained for 45 s followed by 15 s of rest. This pattern was repeated for a total of 10 min.

The ΔSA produced by the uniaxial stretch device was calculated from previously generated finite analysis (5). These data indicate that a 15% and 32% stretch along the major axis of the membrane produces an 11% and 24% ΔSA, respectively. The calculations for ΔSA produced by the multiaxial stretch device were based on spherical deflection geometry in which ΔSA is represented by a spherical cap (described below) (2). These calculations indicate that 7% and 15% membrane stretch produce an 11% and 24% ΔSA, respectively.

Application of fluid shear stress. Skeletal myotubes grown on type I collagen-coated Bioflex membranes were subjected to 10 min of fluid shear with the use of an orbital shaker in the incubator (Armalab, Bethesda, MD) as described by Kraiss et al. (23). This technique produces nonuniform laminar shear with the majority of cells being exposed to near-maximal (peak) shear stress (τₘₚ), which can be calculated as

$$\tau_{max} = a \left( \frac{\eta f}{2\pi} \right)^{\frac{1}{3}}$$

where a is the radius of orbital rotation (1.27 cm), p is the density of the culture medium (1.0 g/cm³), η is the viscosity of the medium (0.01 g·cm⁻¹·s⁻¹) (3), and f is the frequency of rotation (rotations/s) (23). At a frequency of 1 Hz (1 rotation/s), τₘₚ = 2.0 dyn/cm².

Western blot analysis. Skeletal myotubes were collected in lysis buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Nonidet NP-40, 1 mM β-glycerol phosphate, 1 mM Na₃VO₄, 1 μg/mL leupeptin, and 1 mM PMSF) and measured for protein concentration with the DC protein assay kit. From the myotube homogenate, samples containing 10–60 μg of protein were dissolved in Laemmli buffer and subjected to electrophoretic separation by SDS-PAGE on 7.5% acrylamide gels as previously described (16). After electrophoretic separation, proteins were transferred to a PVDF membrane, blocked in 5% blotto [5% powdered milk in TBST (Tris-buffered saline, 1% Tween 20)] for 3 h followed by an overnight
incubation at 4°C with primary antibody. After overnight incubation, membranes were washed for 30 min in TBST and then probed with anti-rabbit antibody for 45 min at room temperature. After another 30 min of washing in TBST, the blots were developed with ECL. Once the appropriate image was captured, membranes were stained with Coomassie blue to verify equal loading in all lanes. Densitometric measurements were carried out using the FluorSmax Imager with QuantityOne software (Bio-Rad). It should be noted that PKB/Akt and p70S6k contain multiple phosphorylation sites and that the phos- phosphospecific antibodies against these proteins often reveal a doublet or triplet banding pattern (see Figs. 1–7). The doublet and triplet banding pattern likely reflects changes in mobility that result from different phosphorylation states of the protein; thus, when images were quantified, all of the doublet and triplet bands were counted as a single entity. The phosphospecific ERK antibody reveals two distinct bands that represent ERK1 (44 kDa) and ERK2 (42 kDa). For quantification, both ERK1 and ERK2 were each counted as a single entity.

**Immunohistochemistry.** C2C12 myotubes were subjected to 10 min of cyclic 15% multiaxial stretch and immediately washed two times with ice-cold PBS, fixed in 4% paraformaldehyde for 10 min, and permeabilized with PBS containing 0.5% Triton X-100 for 10 min. Permeabilized cultures were blocked with PBS containing 1% bovine serum albumin (BSA) and 10% HS for 60 min, followed by an overnight incubation with primary antibody in 1% BSA at 4°C. The following dilutions of primary antibody were used: anti-α-actinin (1:2,000) and anti-p70S6k 421/424 (1:50). After incubation, the myotubes were washed four times with 1% BSA-PBS and then incubated with FITC-conjugated secondary antibody (1:100) for 1 h at room temperature. After another series of washes in 1% BSA-PBS, cultures were covered with Vectashield mounting medium (Vector Laboratories, Burlingame, CA), and a continuous series of images was constructed from individual images taken with identical exposure times. After an individual image was captured, the culture plate was moved with a micromanipulator, with care taken to keep a portion of the previous field in view. The overlapping fields from each exposure were used to reconstruct the continuous series of images. In other experiments, C2C12 myotubes were grown on six-well plastic dishes (Corning, NY), fixed and permeabilized as described above, and incubated with fluorescein-conjugated phalloidin from Molecular Probes (Eugene, OR) in PBS (1:40). All immunofluorescent images were captured on a Kodak DC290 camera taken through a Nikon Diaphot 200 microscope and imported into Adobe Photoshop for analysis.

**Pharmacological inhibitors.** Before initiation of stretch, culture medium was replaced with DMEM containing 10% FBS and vehicle (DMSO) or 10 μM cytochalasin D, 500 μM gadolinium(III) chloride, 250 μM genistein, 50 μM PD-98059, 20 μM BIM, or 100 μM LY-294002. The concentration for each of the inhibitors employed was based on previously published work and testing that preceded the stretch experiments (8, 21, 24, 29). After a 45-min preincubation, myotubes were subjected to 10-min cyclic 15% multiaxial stretch as described above and then collected for Western blot analysis.
Groups were considered significant if Student-Newman-Keuls post hoc analysis. Differences between groups were considered significant if \( P \leq 0.05 \).

**RESULTS**

Uniaxial and multiaxial stretch induce distinct signaling events. Uniaxial and multiaxial stretch devices deliver different types of mechanical forces to the cell culture membrane. The uniaxial stretch device employed in this study produced a single axis of uniform tensile strain associated with a small magnitude of compression (5), whereas the multiaxial device produced nonuniform tensile strain through two axes (radial and circumferential) (7). In an effort to determine whether C2C12 myotubes could distinguish between the different forces produced by the uniaxial and multiaxial stretch devices, alterations in the phosphorylation of signaling molecules PKB/Akt, ERK, and p70S6K were assessed after 10 min of 15% cyclic (1 Hz) stretch. At 10 min, both types of stretch induced a similar increase in PKB/Akt serine-473 (Fig. 1A) and threonine-308 phosphorylation (data not shown). Stretch on both devices also produced an increase in ERK phosphorylation, but the increase in ERK phosphorylation induced by multiaxial stretch was significantly greater in magnitude than the increase produced by uniaxial stretch (Fig. 1B) \( (P \leq 0.05) \). Only multiaxial stretch induced an increase in p70S6K phosphorylation (Fig. 1C). The multiaxial stretch-specific increase in p70S6K phosphorylation was evident with p70S6K threonine-serine-421/424 (Fig. 1C) and p70S6K threonine-389 phosphospecific antibodies, as well as with changes in gel mobility of total p70S6K (data not shown). It should be noted that multiaxial stretch-specific increase in glycogen synthase kinase-3\( \beta \) (GSK-3\( \beta \)) serine-9 phosphorylation was also observed (data not shown).

To determine whether the differences in signaling events between uniaxial and multiaxial stretch were the result of differences in the time course of activation, we assessed alterations in the phosphorylation of PKB/Akt, ERK, and p70S6K after 60 min of 15% cyclic (1 Hz) stretch. The results from these experiments indicated that PKB/Akt serine-473 phosphorylation was not elevated after 60 min or either uniaxial or multiaxial stretch (Fig. 1D). Furthermore, at 60 min, both uniaxial and multiaxial stretch produced a similar change in ERK phosphorylation (Fig. 1E), whereas only multiaxial stretch produced a significant increase in p70S6K threonine-serine-421/424 phosphorylation (Fig. 1F).

Stretch-sensitive signaling events are not responsive to fluid shear stress. The initial results of this study suggested that the multiaxial stretch-specific signaling events were the result of differences in the mechanical forces delivered through the elastic culture membrane. However, cyclic stretch produces motions in the overlying culture medium that, in turn, exert reactive forces (fluid shear stress) on the myocyte culture. A variety of cell types are sensitive to fluid shear stress, and signaling events characteristic of this response include increases in ERK and p70S6K phosphorylation (19, 23). This effect is cell-type specific and dependent on the magnitude of fluid shear stress applied; for example, an increase in the magnitude of fluid shear stress produces an increase in the magnitude of phosphorylation (19, 23). In an effort to establish whether any of the signaling events induced by cyclic stretch were a result of fluid shear stress, myocyte cultures were subjected to 10 min of 2 dyn/cm\(^2\) peak fluid shear stress on an orbital rotary shaker at 1 Hz frequency. This magnitude of fluid shear stress is up to fourfold that produced during cyclic 1 Hz, 7% stretch (11% \( \Delta SA \)) on the multiaxial device (3). Subjecting the myocytes to fluid shear stress did not alter PKB/Akt, ERK, or p70S6K phosphorylation (Fig. 2, A–C). These results suggest that the signaling events induced by cyclic stretch evolved independently of fluid shear stress and therefore were the result of the mechanical forces delivered through the elastic culture membrane. However, it should be noted that the cellular response to shear stress depends on both the magnitude of shear stress and the type of shear stress (static vs. pulsatile).
The orbital rotary shaker used in this study produces static flow with a constant rate of fluid shear stress, whereas cyclic stretch produces pulsatile shear stress, and therefore we cannot fully rule out the possibility that shear stress may have contributed to the signaling events produced by cyclic stretch.

Different signaling events induced by uniaxial and multiaxial stretch are not due to differences in the magnitude of surface area deformation. One of the major differences in the mechanical forces produced by stretch on the uniaxial and multiaxial devices is the magnitude or dose of surface area deformation (ΔSA) produced by a given amount of stretch. As a result of transverse compression, uniaxial 15% stretch produces an 11% ΔSA, whereas 15% stretch on the multiaxial device produces a 24% ΔSA. To determine whether the differences in signaling events after 15% stretch on the uniaxial and multiaxial devices resulted from differences in the magnitude of ΔSA, the magnitude of uniaxial stretch was increased to 32%, which produced a 24% ΔSA, and the magnitude of multiaxial stretch was decreased to 7%, which produced an 11% ΔSA (see MATERIALS AND METHODS for calculations). These alterations allowed for the comparison of uniaxial and multiaxial stretch-induced signaling events at both 11% and 24% ΔSA.

Multiaxial stretch-induced phosphorylation of p70S6k is not localized to regions of large tensile strain. Another important difference in the mechanical forces produced by stretch on the uniaxial and multiaxial devices involved the uniformity of strains. On the uniaxial device, stretch of the culture membrane...
occurs in a uniform distribution, whereas on the multiaxial device, stretch of the culture membrane occurs in a nonuniform distribution (5, 7). The nonuniform distribution in the multiaxial device is most prominent at the outer edge of the membrane, where a rapid increase in the radial axes and a gradual decrease in the circumferential axes of tensile strain are present (Fig. 4A). In this region of the membrane, the magnitude of the radial tensile strain reaches more than twofold the average radial tensile strain produced across the remainder of the membrane (7). This region of large tensile strain encompasses ~10% of the membrane surface area. If the stretch-induced increase in p70S6k phosphorylation (85%) were localized to the area of large tensile strain, the magnitude of phosphorylation in this region would have to be 8.5-fold greater compared with the remainder of the culture membrane, a magnitude of difference that should be detectable with immunohistochemistry.

To determine whether the increase in p70S6k phosphorylation was localized to the region of large tensile strain, we reconstructed a composite of immunohistochemical images, extending from the periphery to the center of the well, from >50 individual images. As a positive control for the uniform distribution of staining, myotube cultures were stained with an anti-α-actinin antibody (Fig. 4, B and D). Images generated with the same phosphospecific p70S6k (Fig. 4, C and E) antibodies used in the Western blot analysis suggest that p70S6k phosphorylation was evenly distributed across the membrane as opposed to being localized to the region of large tensile strain. These data, in conjunction with the observation that p70S6k phosphorylation did not increase after 32% tensile strain (24% ΔSA) on the uniaxial device, support the conclusion that large magnitudes of tensile strain, resulting from the nonuniform distribution of stretch on the multiaxial device, are not responsible for the different signaling events induced by the uniaxial and multiaxial stretch devices.

**Multiaxial stretch-induced phosphorylation of p70S6k and PKB/Akt is stimulated by distinct mechanosensory-mechanotransduction pathways.** Our data indicate that multiaxial stretch-specific signaling events were induced by mechanical forces delivered through the elastic culture membrane and that these mechanical forces were not due to differences in ΔSA or localized regions of large tensile strain. To determine which mechanisms might be involved in the multiaxial stretch-specific signaling, we evaluated changes in p70S6k phosphorylation in myotube cultures that had been incubated with a well-known compound that disrupts the actin cytoskeleton, cytochalasin D. Disruption of the actin cytoskeleton (Fig. 5, A and B) completely blocked the multiaxial stretch-induced increase in p70S6k phosphorylation (Fig. 5C). However, disruption of the cytoskeleton had no effect on the multiaxial stretch-induced phosphorylation of PKB/Akt (Fig. 5D) or ERK (data not shown). These data demonstrate that signaling to p70S6k and PKB/Akt occurs through stimulation of distinct mechanosensory-mechanotransduction pathways and that the actin cytoskeleton is a vital component of the pathway that imparts multiaxial stretch-specific signaling to p70S6k.

**Multiaxial stretch-induced phosphorylation of p70S6k is not inhibited by genistein, LY-294002, PD-98059, BIM, or gado- linium.** In an effort to more clearly define the signaling pathways leading to the multiaxial stretch-induced phosphorylation of p70S6k, we incubated C2C12 myotubes with pharmacological inhibitors. As noted in MATERIALS AND METHODS, the concentra-
tion employed for each of the inhibitors was based on several previously published reports (8, 21, 24, 29). The inhibitors tested include genistein, LY-294002, PD-98059, BIM, and gadolinium. With the exception of BIM, all of these agents altered the baseline phosphorylation of p70S6k; however, none of these agents were able to prevent the multiaxial stretch-induced increase in p70S6k phosphorylation, indicating that the mechanosensory-mechanotransduction pathway leading to p70S6k is not fully dependent on mechanisms sensitive to these inhibitors (Fig. 6).

Multiaxial stretch-induced release of paracrine factors is not sufficient for phosphorylation of p70S6k. To determine whether the multiaxial stretch-induced phosphorylation of p70S6k was due to the release of paracrine factors, we incubated static C2C12 myotubes with conditioned media from stretched myotubes. The results of these experiments indicated that a stretch-induced release of paracrine factors was not sufficient for the induction of p70S6k phosphorylation (Fig. 7). Furthermore, the stretch-induced release of paracrine factors also was not sufficient for the induction of PKB/Akt or ERK phosphorylation (data not shown).

DISCUSSION

The initial aim of this study was to determine whether skeletal muscle cells have the fundamental ability to differentiate between different types of mechanical forces. To address this aim, we subjected skeletal myotube cultures to uniaxial or multiaxial cyclic stretch. The initial findings demonstrated that skeletal muscle cells can differentiate between uniaxial and multiaxial cyclic stretch as determined by unique increases in the phosphorylation of p70S6k in response to multiaxial stretch.

The mechanical forces produced during cyclic stretch include those delivered through the elastic culture membrane as well as fluid shear stress. Fluid shear results from motions in the overlying cell culture medium that, in turn, impose reactive forces (i.e., fluid shear stress) on the cells in culture. Endothelial cells are a well-known example of a cell type that is very sensitive to fluid shear forces. In particular, application of fluid shear forces on endothelial cells promotes signaling to p70S6k (23). In contrast, the results with C2C12 myotubes demonstrated that application of fluid shear did not result in signaling changes in any of the molecules studied. This lack of a signaling response indicates that 1) the signaling responses produced by either uniaxial or multiaxial stretch resulted primarily from changes in the mechanical forces delivered through the elastic culture membrane, and 2) the unique signaling responses in the multiaxial vs. the uniaxial stretch system likely resulted from the different mechanical forces associated with the multiaxial stretch. In addition, the observation that C2C12 myotubes did not exhibit signaling responses similar to those of endothelial cells when exposed to fluid shear stress demonstrated that mechanosensory-mechanotransduction systems exhibit cell-type specificity, and this may be associated with the unique functional demands of the cells in their tissue environment.

Further experiments were conducted to discern whether the unique response of p70S6k to multiaxial stretch was the result of differences in the magnitude of ΔSA (Fig. 3) or differences in localized regions of large strain (Fig. 4). These experiments demonstrated that the multiaxial stretch-specific signaling events were not a result of differences in ΔSA or localized regions of large tensile strain, and thus we propose that the multiaxial stretch-specific signaling events resulted from an intrinsic capacity of myotubes to distinguish between uniaxial and multiaxial tensile strains.

Fig. 5. Multiaxial stretch-induced phosphorylation of p70S6k and PKB/Akt are stimulated by distinct mechanosensory-mechanotransduction pathways. C2C12 myotubes were subjected to 10 min of 15% cyclic (1 Hz) multiaxial stretch. Before (45 min) initiation of stretch (STR), culture medium was replaced with medium containing a vehicle or 10 μM cytochalasin D (CytoD). Myotubes incubated in the presence of vehicle (A) or CytoD (B) were stained with phalloidin. In the presence of vehicle, the myotubes show well-defined actin stress fibers that are abolished by CytoD (×3,000 magnification). Cell homogenates were separated by SDS-PAGE, and Western blotting was performed with P-p70S6k (C) or P-PKB (D) antibodies. Values are presented as means ± SE and expressed as a percentage of a static vehicle-treated sample (control) (n = 7–12/group) from 3 independent experiments. Static samples are represented as light shaded bars, and stretched samples are represented as dark shaded bars. STR, stretched samples. *Significantly different from static samples of similar treatment (P ≤ 0.05).
To understand how myotubes might distinguish between the different types of stretch, it is important to have a conceptual sense of the cellular architecture associated with mechanotransduction systems. Recent reports have stated that much of the cell’s signal transduction machinery is physically immobilized to the cytoskeleton and is spatially integrated within the focal adhesion complex (FAC) (18, 30). Force-dependent alterations in the spatial organization of FAC signaling proteins or force-dependent changes in protein conformation could expose new binding sites and, in turn, activate signaling cascades. The cytochalasin D experiments performed in this study demonstrated that disruption of the cytoskeleton blocked the multiaxial stretch-induced signaling to p70S6k but had no effect on signaling to PKB/Akt, a molecule activated by both uniaxial and multiaxial stretch. These results are significant because they indicate that p70S6k and PKB/Akt are activated by distinct mechanosensory-mechanotransduction pathways and that the activation of these pathways is specific to the types of mechanical forces applied. In addition, they indicate that the actin cytoskeleton is a vital component of the mechanotransduction system that imparts the multiaxial stretch-specific signaling to p70S6k. A conceptual model of an actin cytoskeleton-linked, multiaxial stretch-specific mechanosensor is described in Fig. 8.

The regulation of p70S6k phosphorylation is complex and involves the integration of signals from numerous pathways (31). These major pathways have been shown to include tyrosine kinases (for example, FAK), PKC, MEK, and phosphatidylinositol 3-kinase (PI3K) (6, 11, 12, 25). Thus it was surprising that the application of pharmacological inhibitors that have been reported to target these pathways did not block the multiaxial stretch-induced increase in p70S6k phosphorylation. Another mechanism that has been implicated in the regulation of p70S6k is an alteration in cellular calcium concentrations, and myotubes have been shown to contain mechanically sensitive ion channels that, when stretched, induce a calcium influx (14, 29). However, gadolinium, which has been reported to block these mechanically activated ion channels, did not inhibit the multiaxial stretch-induced increase in p70S6k phosphorylation, indicating that gadolinium-sensitive pathways are not required for the multiaxial stretch-induced signaling to p70S6k. The pharmacological inhibitor studies suggest that the multiaxial stretch-induced increase in p70S6k phosphorylation is independent of tyrosine kinases, PKC, MEK, PI3K, and mechanically activated ion channels. These findings are consistent with the concept that multiaxial stretch induces p70S6k phosphorylation through a novel pathway that is distinct from the well-defined growth factor pathways (17). The results from the conditioned media experiments also indicate that the release of paracrine growth factors is not sufficient for the activation of p70S6k phosphorylation with multiaxial stretch. Thus the multiaxial stretch-induced signaling to p70S6k appears to occur through a direct mechanical mechanism. Because signaling through p70S6k has been implicated in the regulation of load-induced cardiac and skeletal muscle hypertrophy, a better understanding of the mechanosensory-mechanotransduction pathway leading to p70S6k could provide new insights into the mechanisms involved in load-induced hypertrophy (1, 33, 35).

The results of this study highlight the concept of specificity in mechanotransduction. Although this concept is only begin-
A recent study by Kumar et al. (24) also provides evidence that distinct mechanosensors may be contributing to results in which different types of mechanical forces activate different mechanosensory-mechanotransduction pathways. In this study, strain was applied either along the longitudinal axis of the muscle fibers or transverse to the fibers, and the result was an induction of ERK phosphorylation through different upstream pathways. This work deserves particular attention, because uniaxial stretch has been used previously to induce alignment of fusing myotubes with the longitudinal axis of the membrane (38). One consideration with the results from this study may be that uniaxial stretch, applied along the longitudinal axis, activates the common ERK cascade, as described in the study by Kumar et al. (24), whereas multiaxial stretch, being simultaneously longitudinal and transverse, represents activation of both the longitudinal and the transverse ERK cascade. In this study, the myotubes subjected to uniaxial stretch were randomly aligned, resulting in cells being subjected to a distribution of longitudinal and transverse stretch, and this feature discounts the possibility that orientation of stretch contributed to the observed differences between uniaxial and multiaxial stretch. Thus these results provide further evidence that different mechanical forces activate distinct mechanosensory-mechanotransduction pathways; whether the specificity arises through the activation of unique mechanosensors remains to be determined.

To date, the complexity of the in vivo cellular environment has limited our ability to observe specificity in mechanotransduction. However, the results of this study first clearly show that muscle cells can differentiate between uniaxial and multiaxial cyclic stretch and, second, demonstrate that specificity is a fundamental property of the mechanotransduction machinery. With the development of new analytical techniques, the ability to characterize and/or model the mechanical environment in vivo is rapidly advancing. These models are providing...
new insights into how the mechanical environment is altered during the onset of pathological adaptations such as cardiac hypertrophy following a myocardial infarct and atherosclerosis (10, 26). Combining this information with the use of well-defined in vitro studies will greatly accelerate our understanding of how mechanical information is converted in specific physiological and pathological adaptations.

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