

Restoration of Resting Sarcomere Length After Uniaxial Static Strain Is Regulated by Protein Kinase C ϵ and Focal Adhesion Kinase

Haytham Mansour, Pieter P. de Tombe, Allen M. Samarel, Brenda Russell

Abstract—Physiological or pathological stresses and strains produce longer or wider muscle cells, but resting sarcomere length remains constant. Our goal was to investigate the cellular mechanisms for controlling this optimal, resting sarcomere length. To do so, we cultured neonatal rat cardiomyocytes on microfabricated peg-and-groove, laminin-coated silicone surfaces and applied a uniaxial static strain of 10%. Sarcomere length was accurately measured by fast Fourier transform analysis of images before, within 5 minutes of, and 4 to 6 hours after imposition of the strain. Sarcomere length of aligned cardiomyocytes ($1.94 \pm 0.07 \mu\text{m}$) was lengthened acutely ($2.06 \pm 0.06 \mu\text{m}$), and recovered ($1.95 \pm 0.07 \mu\text{m}$) by 4 hours. Puromycin, an mRNA translational inhibitor, prevented recovery of resting sarcomere length by 4 hours, thus indicating a requirement for new protein synthesis in the recovery process. Furthermore, activation of protein kinase C ϵ (PKC ϵ) was necessary for length recovery, as nonselective PKC inhibitors [staurosporine ($5 \mu\text{mol/L}$) and chelerythrine chloride ($10 \mu\text{mol/L}$)], and a replication-defective adenovirus (Adv) encoding a dominant-negative mutant of PKC ϵ prevented the restoration of sarcomere length. To assess the importance of focal adhesion complexes, cardiomyocytes were infected with an Adv encoding a dominant-negative inhibitor of focal adhesion kinase (FAK) (Adv-GFP-FRNK). Adv-GFP-FRNK also prevented resting sarcomere length recovery, whereas a control Adv encoding only GFP did not. In conclusion, using our novel culture system, we provide evidence indicating that the length remodeling process requires new protein synthesis, PKC ϵ and FAK. (*Circ Res.* 2004;94:642-649.)

Key Words: microtopography ■ protein kinase C ■ signaling ■ mechanotransduction ■ remodeling

Cardiac hypertrophy is an adaptive response to increased mechanical load.^{1,2} Previous studies of loaded and unloaded cardiac tissue demonstrate a critical relationship between mechanical load and the regulation of myocyte remodeling.³ A constant sarcomere length is required for optimal tension development during altered loading conditions.⁴⁻⁹ Thus, an important outcome of cardiac remodeling is the maintenance of optimal resting sarcomere length despite changes in mechanical load leading to growth and lengthening of individual cardiomyocytes. Furthermore, the transmission of mechanical forces depends on the orientation of the cells and the function of specific cardiomyocyte cytoskeletal proteins involved in mechanochemical signal transduction. Consequently, numerous studies have examined the effect of mechanical forces on randomly oriented cardiomyocytes in cell culture.¹⁰⁻¹³ Using improved culture systems of aligned cardiomyocytes, investigators have now begun to examine the effects of the direction of strain, as well as its magnitude, on cell signaling and growth.^{12,14}

Recently, the focal adhesion complex has been shown to be a key mediator of cardiomyocyte mechanochemical signal

transduction. Focal adhesion kinase (FAK), a nonreceptor protein tyrosine kinase that localizes to focal adhesion complexes in response to cyclic stretch,^{15,16} has been shown to be intricately involved in converting mechanical signals into biochemical signals that regulate cardiomyocyte growth and survival. Other structural and functional proteins within the focal adhesion complex are also critically important for stretch-induced cellular remodeling.¹⁷ However, the cooperative association of integrins, the focal adhesion complex, and the actin cytoskeleton suggests an important role for FAK in the regulation of sarcomere length during periods of cardiomyocyte length remodeling.^{16,18,19}

Recent work demonstrates that the mechanical stretch sensor also involves proteins associated with the Z disc, such as protein kinase C (PKC).^{20,21} On activation, the PKC ϵ isoform translocates to the Z disc and regulates myofilament activity. PKC has also been widely studied in relation to cardiac hypertrophy.^{22,23} In addition, results indicating that PKC ϵ activation can lead to the tyrosine autophosphorylation and activation of FAK²⁴ have provided further evidence that PKC might be critical to the cardiomyocyte stretch response.

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From the Department of Physiology and Biophysics (H.M., P.P.d.T., B.R.), University of Illinois at Chicago, Chicago, Ill; the Cardiovascular Institute (A.M.S.), Loyola University Chicago Stritch School of Medicine, Maywood, Ill.

Correspondence to Brenda Russell, PhD, Professor, Department of Physiology and Biophysics, MC901, 835 S Wolcott Ave, Room E-202, University of Illinois at Chicago, Chicago, IL 60612. E-mail russell@uic.edu

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Therefore, we explored the mediation role of PKC ϵ between the Z-disc region and the focal adhesion complex in sarcomere length remodeling.

To explore the signaling mechanisms responsible for cardiac remodeling, our group has developed a 3-dimensional (3-D) culture system^{25–27} of aligned, well-attached cardiomyocytes. Culturing neonatal rat cardiomyocytes on this 3-D surface provides an environment for uniform application of mechanical strain to all cells. This system also permits cells to be suddenly stretched to study how myocyte length remodeling is regulated, and what intracellular processes are necessary for the recovery of resting sarcomere length. By using a combination of pharmacological and molecular approaches, we provide evidence for a critical role PKC ϵ and FAK in the restoration of resting sarcomere length after uniaxial static strain.

Materials and Methods

Cell Culture

Animal experiments were performed according to Institutional Animal Care and Use Committee and NIH guidelines. Cardiomyocytes were isolated from neonatal Sprague-Dawley rats (Harlan, Indianapolis, Ind) as previously described.²⁸ Briefly, hearts were removed from 1- to 2-day-old rats. Isolated ventricular myocytes were plated on laminin (25 $\mu\text{g}/\text{mL}$)-coated silicone membranes (200 000 cells/ cm^2) and maintained in complete medium (Dulbecco's modified Eagle's medium/ Nutrient Mixture F-12 HAM without L-glutamine) (Sigma), standard amino acid concentrations plus palmitic (2.56 mg/L) and linoleic (0.84 mg/L) fatty acids, penicillin G/streptomycin solution (10 $\mu\text{L}/\text{mL}$), and gentamicin (50 mg/L) with 5% fetal bovine serum for 48 hours before strain experiments. Cytosine- β -D-arabino-furanoside (5 $\mu\text{g}/\text{mL}$) was added to prevent fibroblast proliferation.

Microfabrication of Textured Surface

Textured substrates for cell culture were fabricated as previously described.²⁶ Briefly, the parylene-textured surface, as optically imaged in Figure 1A, consisted of vertical pegs spaced 100 μm from center to center. Groove depth was 5 μm and spacing between grooves was 10 μm at the top and bottom surface. Peg height and diameter was 10 μm . The textured side of the parylene template was then embedded into unpolymerized silicone (Dupont) and a 40 g weight was applied for 15 seconds to equally distribute the silicone beneath the template. The silicone membrane was cured (48 hours, 25°C), then cleaned with 11 mol/L hydrochloric acid (1 hour), rinsed with distilled water, and dried at 37°C overnight. Membranes were then cut into a rectangle (6 \times 3 cm), loaded onto uniaxial static strain devices,¹⁴ and coated with laminin (25 $\mu\text{L}/\text{mL}$) dissolved in Dulbecco's Modified Eagle's Medium (Sigma) for 2 hours before cell plating.

Mechanical Strain

A strain was applied by manually turning two adjustable screws at the free end of the strain device (Figure 1B). A strain of approximately 10% was applied to longitudinally aligned cardiomyocytes in all experiments. The actual longitudinal strain applied to the membrane was determined using the micropegs on the textured surface as internal markers of length change of the membrane. When longitudinal strain was applied a small transverse compression occurred and was also measured. Uniformity of mechanical strain across the membrane was determined. Changes to substrate length were determined either parallel, or perpendicular, to the long axis of strain; each representing either longitudinal strain or transverse compression, respectively.

All myofibrillar images were captured after gluing (4 hours, 25°C) the silicone substrate to a glass slide with silicone glue (NuSil Med

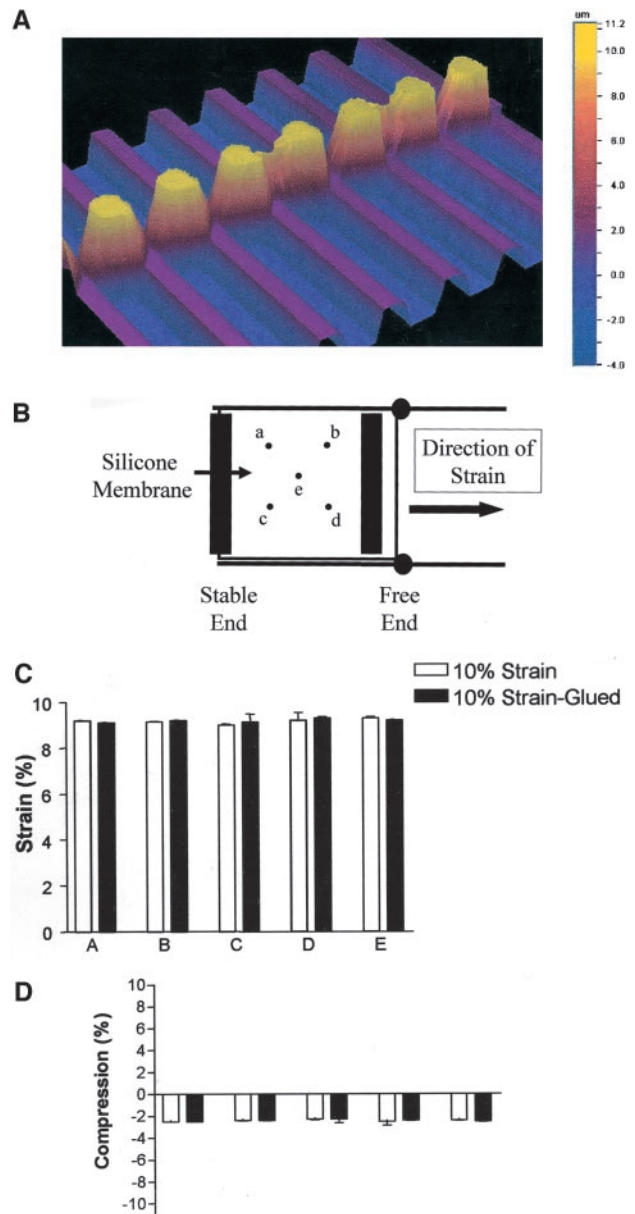


Figure 1. Strain determination of substrata. A, Computer-generated 3-D output of microtopography. B, Diagram illustrating static stretch device. Five representative regions (a, b, c, d, and e) were chosen to determine regional variation of strain and compression inherent in elastic material. C, Strain and compression were assessed immediately after 10% strain (■) and after attachment of strained membrane to glass slide (□) for sarcomere length measurement. Strain was determined as the change in length parallel to axis of strain. There were no significant differences among sarcomere length values from the 5 representative regions, before or after attachment to slide. D, Similarly, compression was determined as change in length perpendicular to axis of strain. Compression of membrane was uniform immediately after strain and after attachment to membrane (n=3 independent experiments).

1-4013; NuSil Silicone Technology). The textured membrane was then cut free from the strain device to capture images.

Myofibrillar Orientation

Orientation was determined as previously described.²⁶ Briefly, cardiomyocytes grown on textured and nontextured surfaces were

photographed using a Nikon phase-contrast microscope ($\times 20$ objective). Parallel and perpendicular lined grids of known dimensions were superimposed over the images to determine cellular and myofibrillar orientation along the direction of strain. Orientation was determined both before and immediately after a 10% strain was applied to the textured and untextured (flat) surfaces.

Immunochemistry

At various time points (5 minutes to 48 hours) after the imposition of static strain, cells were washed in phosphate-buffered saline (PBS), fixed [4% paraformaldehyde (Fisher); 10 minutes], and washed (70% ethanol). Filamentous actin was stained with rhodamine-phalloidin (Molecular Probes) diluted in PBS (1:1000). Each membrane was incubated with 100 μ L of rhodamine-phalloidin for 50 minutes and then rinsed three times (5 minutes) in PBS. Before applying a cover slip, Vectashield mounting medium (Vector Laboratories) was applied to the surface of the silicone membrane to help preserve the stain. The mounting medium contained DAPI to stain nuclei. Adv-GFP- and Adv-GFP-FRNK-infected cells were viewed under a laser scanning confocal microscope (Zeiss LSM510).

Sarcomere Length Measurement

Cardiomyocyte images, stained for actin (rhodamine-phalloidin), were captured using a Nikon epifluorescent microscope ($\times 40$ objective) with a Spot RT Color camera (Diagnostics Instruments). Cells were selected based on sharpness of image and the number of continuous sarcomeric units in one myofibril. Images were converted to black and white and oriented in the horizontal direction. Sarcomere length measurements were acquired using computer-assisted software.²⁹ Briefly, a region of the image encompassing 10 to 20 sarcomeres along a myofibril was selected, and each longitudinal pixel line was transformed by fast Fourier transformation (FFT) into a spatial frequency domain. The power spectra were averaged, and the spatial frequency at peak power of the first-order harmonic in the spatial frequency domain was determined. This spatial frequency was then converted into median sarcomere length (SL) across the region. The system was calibrated with glass gratings of known spacing.

Protein Synthesis Determination

To determine the effect of inhibition of de novo protein synthesis on sarcomere length recovery, cardiomyocytes were cultured onto peg-and-groove textured membranes and treated with puromycin (10 μ mol/L, Sigma) for 30 minutes and 24 hours before application of strain. Myocytes remained in puromycin-containing medium after strain application. To ensure that this concentration of puromycin was sufficient to inhibit protein synthesis, protein synthetic rate was estimated by measuring the rate of [³H]leucine incorporation into total protein. Cardiomyocytes plated at a density of 500 000 cells/cm² were maintained in control medium, or medium containing puromycin (10 μ mol/L) or phorbol 12-myristate 13-acetate (PMA, 200 nmol/L; positive control) for 24 hours. [³H]leucine (2 μ Ci/dish) was added during the last 6 hours of treatment. After washing with cold PBS, macromolecules were precipitated with ice-cold 10% tricarboxylic acid (TCA), and scraped from the dishes. The insoluble protein was then washed twice with TCA, and then solubilized (60°C, 20 minutes) in 200 μ L of 0.2N NaOH. [³H]leucine incorporation was determined by liquid scintillation counting. Protein concentration was determined by Bradford assay. Protein synthesis was described as DPM/ μ g of protein.

Adenoviral Constructs

Replication-defective adenoviruses (Adv) used in this study were propagated in HEK293 cells, and purified by double CsCl centrifugation as previously described.²⁴ Adv were titrated by viral dilution in HEK293 cells grown in 96-well microtiter plates. PKC ϵ activity was inhibited using an Adv encoding a dominant-negative mutant of rabbit PKC ϵ (Adv-dnPKC ϵ), kindly provided by Dr Peipei Ping (University of California, Los Angeles).³⁰ An Adv encoding cytoplasmic β -galactosidase (Adv-c β gal) was used to control for non-

specific effects of Adv infection. Infection efficiency was assessed in preliminary studies by immunochemical staining with monoclonal anti- β gal antibody (Promega), as well as by immunochemical staining of the myc epitope tag (HA.11 polyclonal antibody PRB-101P, Covance Research Products) within the dnPKC ϵ transgene. A multiplicity of viral infection (MOI) of ≈ 50 was sufficient to infect $\approx 90\%$ of the cultured cells. Cardiomyocytes were infected 24 hours before uniaxial strain was applied.

FAK-dependent signal transduction was inhibited by Adv-mediated overexpression of GFP-tagged FRNK, the autonomously expressed, C-terminal region of FAK.³¹ An Adv expressing GFP alone was used to control for nonspecific effects of Adv infection. Preliminary studies indicated that an MOI of ≈ 10 was sufficient to infect $\approx 90\%$ of cells, based on GFP fluorescence. Cells were infected with either Adv-GFP or Adv-GFP-FRNK 24 hours before strain, and then sarcomere length changes were monitored in randomly selected cells over time. Both Adv-dnPKC ϵ and Adv-GFP-FRNK produced high levels of transgene overexpression, as previously analyzed in cardiomyocytes maintained in 2-D culture.^{31,32}

Data Analysis

All values are mean \pm SEM. Data were compared using one-way and two-way ANOVA or the Students unpaired *t* test. Differences among means were considered significant at $P < 0.05$. Data were analyzed using GraphPad statistical software.

Results

Membrane Strain

Silicone membranes were fabricated from a parylene template to generate a microtextured surface consisting of a combination of pegs and grooves (Figure 1A). To determine whether membrane strain was uniform, peg-to-peg distance was measured at representative positions across the membrane surface before and after 10% uniaxial stretch (Figure 1B). Actual strains at all five regions were similar after the applied strain ($9.17 \pm 0.11\%$, $n=3$) or after gluing the membrane to the slide ($9.19 \pm 0.07\%$, $n=3$) (Figure 1C). Slight transverse compression ($-2.40 \pm 0.08\%$, $n=3$) was noted immediately after applying a 10% strain. This compression remained constant after gluing the membrane to the slide ($-2.42 \pm 0.08\%$, $n=3$) (Figure 1D).

Growth of Cardiac Myocytes on Textured Surface

Cardiomyocytes grown on laminin-coated, flat surfaces were randomly oriented, as shown in the representative phase-contrast image depicted in Figure 2A. However, cardiomyocytes grown on microtextured surfaces were uniformly oriented along the long axis of the grooves (Figure 2B). In addition, sarcomeres (visualized by phalloidin staining) were randomly oriented in cardiomyocytes cultured on flat membranes (Figure 2C), as opposed to the aligned pattern on the microtextured surfaces (Figure 2D). Myofibrillar alignment was then quantified by image analysis. There was a high degree of alignment on textured surfaces, but not on flat surfaces before strain (flat, $6.0 \pm 2.6\%$, textured, $82 \pm 5.1\%$, $P < 0.001$, $n=3$) and immediately after strain (flat, $9.0 \pm 3.5\%$, textured, $75 \pm 3.7\%$, $P < 0.001$, $n=3$) (Figure 2E).

Regional Sarcomere Length Variation With Strain

We next examined the uniformity of sarcomere length within a myocyte at four different subcellular zones before and after strain (Figure 3). The four subcellular zones consisted of cells that (1) spanned the entire 100- μ m length of the groove and

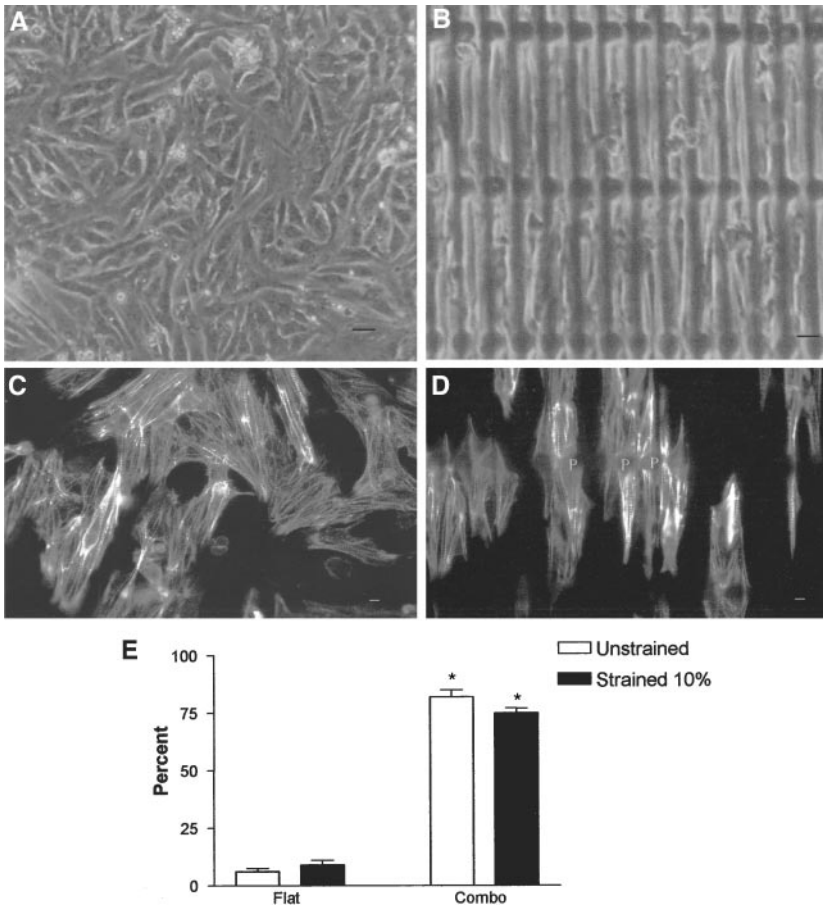


Figure 2. Alignment of cardiac myocytes on microtopography. Phase-contrast images of neonatal rat cardiomyocytes plated on laminin-coated flat surface (A) or textured peg-and-groove (B) (Combo) surface. Epifluorescence color images of cells stained for sarcomeric actin with rhodamine-phalloidin were converted to black and white for sarcomere length measurements. Sarcomeric organization on a flat surface with random myocyte orientation (C) or on the textured peg-and-groove combination with cells aligned along the long axis of the grooves (D). E, Alignment of individual myofibrils from randomly selected myocytes on flat or from textured surfaces was quantified before (□) and after (■) applying a strain of 10%. n=3 independent experiments. Scale bar=10 μm. P indicates location of peg. *P<0.01.

attached at both ends to pegs (Figure 3A), (2) attached to a peg at only one end (Figure 3B), (3) surrounded a peg (Figure 3C), or (4) were not attached to a peg at either end (Figure 3D). Cardiomyocytes were randomly selected and the sarco-

mere lengths were quantified and compared (Figure 3E). Sarcomere lengths accurately followed the 10% applied strain in every zone and there were no detectable inhomogeneities.

Recovery of Resting Sarcomere Length With Uniaxial Static Strain

The recovery of resting sarcomere length was used as an indicator of the cardiac remodeling process. Initial analysis of sarcomere length over a period of 48 hours showed that recovery of resting sarcomere length occurred by 6 hours (data not shown). Subsequently, we analyzed sarcomere length changes from 0 to 6 hours more extensively from more than 100 cardiomyocytes from 7 independent cultures at each time point. Resting sarcomere lengths on textured and flat surfaces were similar before strain was applied ($1.94 \pm 0.07 \mu\text{m}$) (Figure 4). Sarcomere length increased to $2.06 \pm 0.06 \mu\text{m}$ immediately after applying a nominal 10% static strain to the textured surface. Sarcomere length recovered by 4 hours on the textured surface ($1.95 \pm 0.17 \mu\text{m}$). However, cardiomyocytes grown on flat surfaces never lengthened significantly after being strained 10% ($1.95 \pm 0.07 \mu\text{m}$), and consequently were still at rest length at the 4-hour time point ($1.98 \pm 0.02 \mu\text{m}$).

Effect of Translational Block on Sarcomere Length Recovery

To determine whether the restoration of resting sarcomere length required new protein synthesis, cells were treated with

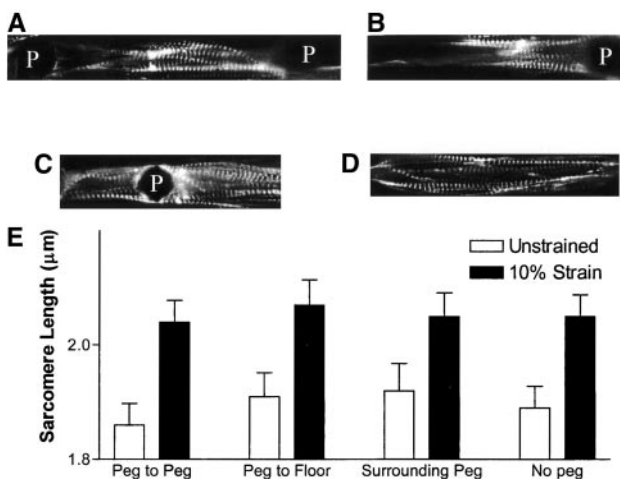


Figure 3. Sarcomere length measurements on microtextured surface. Variation of sarcomere length from different subcellular zones was assessed. Representative myocytes from regions where both ends are attached to pegs (A), only one end is attached to a peg (B), myocyte surrounds a single peg (C), and where no attachment to a peg is found (D). E, No significant differences in sarcomere lengths were found among the 4 zones either before (□) or after (■) strain.

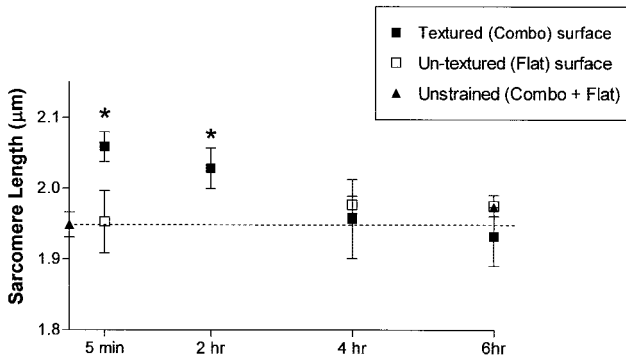


Figure 4. Resting sarcomere length recovery. Sarcomere lengths were measured over time from randomly selected myocytes before and after strain. Within 5 minutes of strain of the textured (■) silicone membrane by a nominal 10%, the sarcomere length increased from a resting value of 1.94 ± 0.07 to $2.06 \pm 0.06 \mu\text{m}$ ($*P < 0.001$). By 4 hours, the measured sarcomere length recovers on the textured (Combo) surface to $1.95 \pm 0.17 \mu\text{m}$. Myocytes cultured on a flat (□) membrane did not have a significant increase in sarcomere length when strained ($n > 100$ cells from 7 independent cultures).

puromycin ($10 \mu\text{mol/L}$) to inhibit mRNA translation. Preliminary studies were conducted to determine whether this puromycin concentration was sufficient to block protein synthesis, as estimated by [^3H]leucine incorporation into total protein (Figure 5A). Using PMA, a known hypertrophic agonist, as a positive control, we found a 50% decrease in protein synthetic rate in cardiomyocytes treated with puromycin for 24 hours, whereas PMA showed a 10% increase compared with unstimulated, control cells.

Cardiomyocytes cultured on microtextured surfaces were then treated with puromycin for 30 minutes or 24 hours before strain (Figure 5B). In cells treated with puromycin for 30 minutes, we found an initial increase in sarcomere length (0%, $1.94 \pm 0.2 \mu\text{m}$; 10%, $2.15 \pm 0.18 \mu\text{m}$, $P < 0.01$, $n = 6$). By 4 hours, the sarcomere length had returned to rest length ($1.93 \pm 0.12 \mu\text{m}$). Sarcomere length also acutely increased in cardiomyocytes treated with puromycin for 24 hours before the initial strain (0%, $1.93 \pm 0.15 \mu\text{m}$; 10%, $2.07 \pm 0.17 \mu\text{m}$, $n = 3$). However, sarcomere length did not recover by 4 hours ($2.00 \pm 0.15 \mu\text{m}$) or 6 hours ($2.06 \pm 0.18 \mu\text{m}$) after the initial strain ($P < 0.01$, $n = 3$). Thus, in cardiomyocytes treated with puromycin for 24 hours, resting sarcomere length recovery was partially inhibited, indicating a requirement for new protein synthesis during remodeling.

Effect of PKC Inhibition on Recovery of Resting Cardiac Sarcomere Length

We next used the nonisoform-specific PKC inhibitors, chelerythrine chloride and staurosporine, to evaluate the influence of PKC on the recovery of resting sarcomere length. As seen in Figure 6A, both inhibitors prevented the recovery of resting sarcomere length after a 10% static stretch, suggesting a role for one or more PKC isoenzymes in the remodeling process.

PKC ϵ is the major novel PKC isoenzyme expressed in cardiomyocytes. This isoenzyme has been implicated in both cytoskeletal remodeling, and the induction of specific aspects on cardiomyocyte hypertrophy. Therefore, we compared the

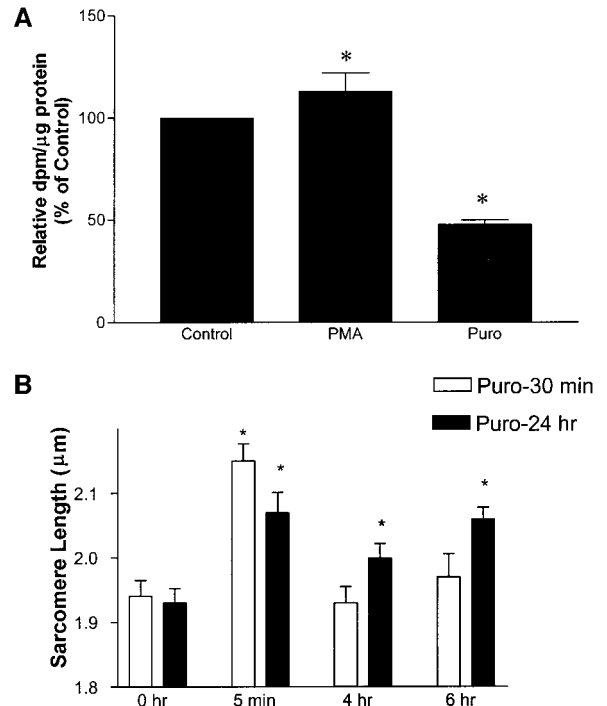


Figure 5. Inhibition of protein synthesis prevents sarcomere length recovery. A, [^3H]leucine incorporation was used to estimate protein synthesis rates of rat neonatal cardiomyocytes treated with puromycin ($10 \mu\text{mol/L}$) or PMA (200 nmol/L). PMA stimulation resulted in a significant increase in [^3H]leucine incorporation by approximately 10% ($112.97 \pm 15.79 \text{ dpm}/\mu\text{g}$ of protein; $P < 0.01$, $n = 3$), whereas puromycin treatment of myocytes resulted in a 50% decrease ($47.73 \pm 3.76 \text{ dpm}/\mu\text{g}$ of protein; $P < 0.01$, $n = 3$). B, Sarcomere length recovery was determined in myocytes cultured on textured peg-and-groove surfaces and treated with puromycin for 30 minutes and 24 hours before application of strain. Puromycin treatment inhibits recovery to resting values for cells treated for 24 hours ($2.00 \pm 0.15 \mu\text{m}$), but not after only 30 minutes ($1.93 \pm 0.12 \mu\text{m}$) ($n \geq 50$ cells from 4 independent cultures; $*P < 0.01$).

ability of cardiomyocytes overexpressing a dominant-negative mutant of PKC ϵ to undergo recovery of resting sarcomere length. Cells grown on microtextured surfaces were infected with Adv-dnPKC ϵ (50 MOI, 24 hours before stretch). Adv-c β gal (50 MOI) was used to control for non-specific effects of Adv infection. Sarcomere length acutely increased in cells infected with either Adv-c β gal or Adv-dnPKC ϵ (Figure 6B). However, only cells infected with Adv-c β gal were able to recover to their resting sarcomere lengths within the 4-hour recovery period ($P < 0.01$). These results indicate that PKC ϵ is a critical component of the signaling pathways responsible for resting sarcomere length recovery during cardiomyocyte remodeling.

FAK-Mediated Regulation of Resting Sarcomere Length Recovery Assessed With FRNK

We next examined whether GFP-FRNK overexpression affected the recovery of resting sarcomere length in response to static strain. The control GFP transgene (Figure 7A) was diffusely localized within the cardiomyocyte. However, by capturing optical sections ($0.1 \mu\text{m}$) throughout the thickness of the cell and at the cell-substratum interface, we verified

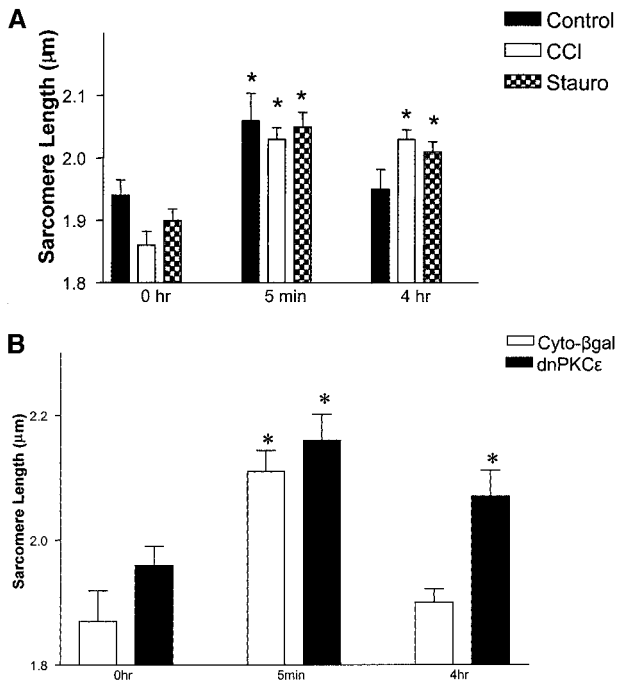


Figure 6. Effect of PKC inhibition on sarcomere length recovery. A, Time course for resting sarcomere length recovery was determined using the nonselective PKC inhibitors chelerythrine chloride or staurosporine. Fresh media was applied with inhibitors 30 minutes before strain and remained for the duration of the experiment. All treated and untreated (control) myocytes exhibited an increase in sarcomere length when strained. However, myocytes treated with PKC inhibitors did not recover resting sarcomere length, whereas control cells did recover. Sarcomere length measurements 4 hours after strain were significantly different than rest length ($n \geq 30$ cells from 4 chelerythrine chloride- and 3 staurosporine-treated cultures; $*P < 0.01$). B, Sarcomere length recovery was then determined for Adv-dnPKC ϵ - and Adv-Cyto β gal-infected cardiac myocytes. Sarcomere length recovery was inhibited in myocytes infected with Adv-dnPKC ϵ , but control Cyto β gal-infected cardiac myocytes recovered well. $n \geq 30$ cells from 4 independent cultures; $*P < 0.01$.

that GFP-FRNK localized to costameric structures and focal adhesions, as previously described (Figure 7B).^{26,31} Exchange of endogenous FAK with its truncated form, FRNK, subsequently prevented the recovery of resting sarcomere length following 10% strain (Figure 7C). The failure to recover resting sarcomere length in Adv-GFP-FRNK-infected myocytes was not due to nonspecific effects of adenoviral infection, as length recovery proceeded normally in cells infected with Adv-GFP.

Discussion

Our first novel finding indicates that the neonatal rat cardiomyocyte can add approximately one sarcomere per hour and effectively compensate for an increase in resting sarcomere length after an applied strain of 10%. We calculate this rate by knowing the following facts: a cardiomyocyte spans the 100 μm from one peg to another; the strain lengthens a cell 10% or 10 μm ; the sarcomere length is about 2 μm ; and it takes 4 hours to return to the original, optimal sarcomere length. Our second novel finding illustrates that PKC, specifically PKC ϵ , is necessary for the recovery of resting

sarcomere length. A PKC ϵ -dependent signaling pathway appears critical in linking external mechanical stimulation to the internal environment. Our third finding is that the focal adhesion complex, through FAK, is also necessary for the cardiac remodeling process.

It has been difficult to design experiments to determine the cellular basis of length regulation in vivo. Cardiac atrophy and subsequent hypertrophy in vivo was previously demonstrated by severing and then reattaching the chordae tendinea of a single right ventricular cat papillary muscle to alter the load on the myocytes.³ Cell shape changed creating a longer cell but sarcomere length was not measured. Cardiomyocyte dimensions also change, cross-sectionally and longitudinally, in response to increases in pressure or volume, respectively.^{2,33} Left ventricular myocytes isolated from patients with ischemic cardiomyopathy were shown to have longer and thinner cellular dimensions than cells from control tissue, but had normal resting sarcomere lengths,³³ again suggesting that rest length is a tightly controlled process.

Although cyclic stretching of cardiomyocytes on a flat surface results in changes in overall cell size,^{14,34} this in vitro model system provides only limited information about sarcomere length changes, and thus limited insight regarding the regulatory mechanisms involved in load-induced structural remodeling. Our findings suggest that the average sarcomere length of cardiomyocytes grown on flat, laminin-coated surfaces does not change when strained. Because these cells were randomly oriented, only a small fraction of the cardiomyocytes was precisely oriented in the direction of the applied vector. In addition, earlier work by our group²⁶ using similarly fabricated, textured surfaces demonstrated that focal adhesion proteins, such as paxillin and vinculin, were expressed at levels similar to those observed in the intact, neonatal heart. In particular, we suspect that the vertical topography of the 3-D microtextured surface provides a better geometry for anchorage, and thus an increase in the number of focal adhesion complexes necessary for cell attachment. However, anchorage to the vertical pegs was not required for the transmission of length changes from the membrane to the sarcomeres, as sarcomeric extension was similar in cells lying in the grooves that were not attached to the pegs. We suspect that the vertical costameres on the sidewalls of the grooves are also important topographical features for efficient mechanotransduction. Thus, our microtextured surface consisting of both pegs and grooves has unique advantages for mechanochemical signaling studies, especially with respect to the role of the focal adhesion complex.

In addition, we previously showed that adenovirally mediated overexpression of GFP-FRNK in nonaligned cultures of neonatal rat ventricular myocytes caused the displacement of endogenous FAK from focal adhesions within 24 hours.³¹ FRNK overexpression also markedly inhibited cell spreading and sarcomeric assembly in response to endothelin-1 added to the culture medium, and prevented the PKC ϵ -dependent autophosphorylation of FAK.³⁵ Furthermore, we recently demonstrated that focal adhesion proteins accumulate near each peg in aligned myocyte cultures,²⁶ suggesting that focal adhesion complexes may be important sites for the both structural remodeling and mechanochemical signaling.

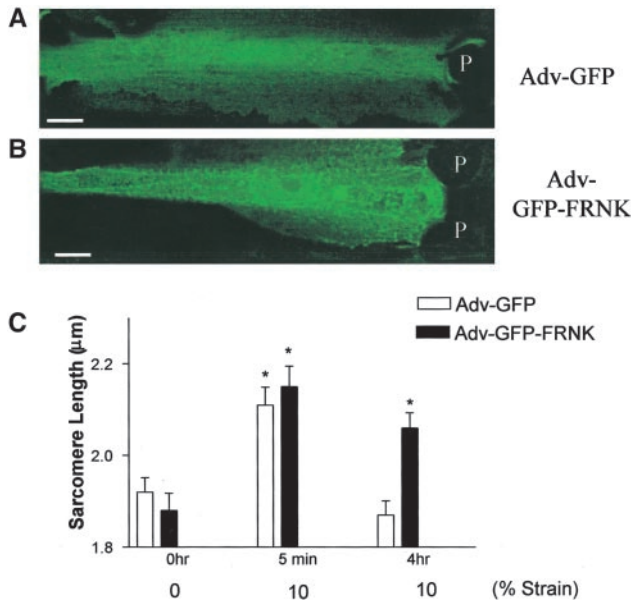


Figure 7. Effect of FRNK expression on resting sarcomere length recovery. Cardiomyocytes were infected for 24 hours with either Adv-GFP (A) or Adv-GFP-FRNK (B) on textured surfaces before strain. Confocal microscopy near the surface of the membrane illustrates the diffuse localization of GFP in the control cells, but the striated appearance of GFP fluorescence in Adv-GFP-FRNK-infected cells. C, Sarcomere lengths in myocytes infected with GFP-FRNK were unable to recover by 4 hours after strain, but recovery was normal in myocytes infected with GFP alone. $n > 25$ cells from 3 independent cultures. Adv-GFP, Unstrained $1.92 \pm 0.13 \mu\text{m}$, Strained 10% 4 hours later $1.87 \pm 0.12 \mu\text{m}$; Adv-GFP-FRNK, Unstrained $1.88 \pm 0.10 \mu\text{m}$, Strained 10% 4 hours later $2.06 \pm 0.12 \mu\text{m}$; $*P < 0.001$.

Our work also contributes to the growing body of evidence implicating PKC isoenzymes as mediators of cardiac remodeling. The present study does not indicate a direct association between PKC ϵ and FAK, nor does it indicate whether the two signaling kinases reside in the same, or parallel stretch-activated signaling pathways. However, previous studies from our group have demonstrated that PKC ϵ lies upstream of FAK in a signaling pathway activated in response to endothelin-1 receptor stimulation in neonatal rat cardiomyocytes.^{24,35} Thus, it is conceivable that PKC ϵ and FAK reside in a similar pathway activated in response to mechanical load. FAK, in turn, activates a number of crucial downstream signaling cascades that regulate cell spreading, focal adhesion formation, cytoskeletal reorganization, and gene expression changes.^{18,19} Inhibition of PKC ϵ - and FAK-dependent signaling may have interfered with the earliest steps in mechanochemical signaling, and therefore prevented resting sarcomere length recovery.

It is also conceivable that PKC ϵ and FAK are involved more directly in structural remodeling. Focal adhesion complexes are found both at the periphery and along costameric structures in the heart.³⁶ The localization of FAK at the end of a cell contributes to mRNA accumulation near the site of the integrin/FAK complex.³⁷ This evidence supports the hypothesis that control of sarcomeric protein addition in cardiac muscle, as similarly shown in skeletal muscle models with mechanical strain, is tightly regulated within cells.^{38–39} The

lack of increased sarcomere length in our strained unaligned flat cultures illustrates the importance of the costameric orientation. The longitudinal orientation of cells within grooves further supports the differential regulation of gene expression seen in longitudinal and transverse aligned cells associated with mechanotransduction signaling cascades.

The close relationship of the integrin receptor and the focal adhesion complex at the costameres and intercalated disc of cardiomyocytes suggests that it could be a site of the mechanical sensor. FAK is closely associated with a number of other key cytoskeletal proteins, such as paxillin, and critical kinases such as Rho kinase and Src.²⁴ More work needs to be done to determine the effect FAK has on these other supporting proteins during cardiac remodeling. However, with the evidence we have provided, we can conclude that FAK plays a critical role in the cellular response to mechanical stimulation. It is likely that a mechanical signal from the extracellular environment is transduced through the focal adhesion complex during mechanical deformation triggering a signaling cascade that could travel to Z discs for mechanical coordination of cytoskeletal remodeling. Intracellular cytoskeleton organization has been shown to depend on mechanical stimulation.³⁷

In conclusion, our study investigated the remodeling of resting sarcomere length in cardiac myocytes as seen in in vivo situations of volume overload. An important and novel finding in our study is the addition of about one sarcomere per hour when myocytes are strained 10% to compensate for the increase in resting sarcomere length. These additional sarcomeres are sufficient to return the cell to an optimal sarcomere rest length. In addition, our study suggests that the regulation of the resting sarcomere length recovery process is mediated by PKC ϵ and FAK.

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