

# Stimulus interval, rate and direction differentially regulate phosphorylation for mechanotransduction in neonatal cardiac myocytes

Samuel E. Senyo<sup>a,1</sup>, Yevgeniya E. Koshman<sup>a,1</sup>, Brenda Russell<sup>a,b,\*</sup>

<sup>a</sup> Department of Bioengineering, University of Illinois at Chicago, USA

<sup>b</sup> Department of Physiology and Biophysics (MIC 901), University of Illinois at Chicago, 835 S. Wolcott Avenue, Chicago, IL 60612-7342, USA

Received 21 July 2007; accepted 27 July 2007

Available online 8 August 2007

Edited by Veli-Pekka Lehto

**Abstract** The effect of interval, direction and rate of strain on mechanotransduction in neonatal rat cardiomyocytes is determined for focal adhesion kinase (Y397pFAK), extracellular signal-regulated kinase ERK1/2 (Thr<sup>183</sup>/Tyr<sup>185</sup>) and paxillin (pY31) and phosphorylation time courses to 10% strain assessed. Cells are non-responsive at 5 min but recover at 15 min ( $P < 0.03$ ) with FAK nuclear translocation by 30 min. Cyclic biaxial strain increased phosphorylation from slower to faster rates ( $P < 0.05$ ). Uniaxial strain to groove-aligned myocytes increased FAK and ERK1/2 phosphorylation transversely more than longitudinally ( $P < 0.05$ ). Mechanotransduction may have a refractory period of 5 min and differentiate directions and rates of strain.

© 2007 Federation of European Biochemical Societies. Published by Elsevier B.V. All rights reserved.

**Keywords:** Cardiomyocyte; Force direction; Mechanotransduction; Focal adhesion kinase; Refractory period; Nucleo-cytoplasmic transport

## 1. Introduction

A ventricular myocyte experiences changes in length and load with increased systolic wall stress due to pressure, or diastolic wall stress due to volume overload. These are transduced into biochemical signals that change the rate of protein synthesis, cell morphology, protein localization, phosphorylation and gene expression. The chemical agents that trigger signaling candidates are frequently studied but the equally important physical modes are less studied. Mechanical variables include the magnitude of load or strain, material stiffness, flow, shear stress and compression [1,2]. The direction of force affects responses of aligned tissue or cell culture [3–7].

Mechanotransduction begins at the focal adhesion complex as cells sense physical forces and transduce them to activate signaling cascades by phosphorylation of FAK, ERK, paxillin and others [8]. The nature and timing of the mechanical stimuli

greatly affects the ability of the myocyte to detect changes. Here, the interval between sudden strains is changed to test for presence of a refractory period by diminution of FAK and ERK phosphorylation. Changing the strain rate and strain direction simulates transverse stress in pressure overload and longitudinal strain in volume overload to test for rate and anisotropy of responses.

## 2. Materials and methods

### 2.1. Cell culture

Animal experiments were performed according to Institutional Animal Care and Use Committee and NIH guidelines. Neonatal rat ventricular myocytes (NRVM) were isolated from the hearts of 1–2-day-old Sprague–Dawley rats (Harlan, Indianapolis, IN) and plated on fibronectin-coated (12.5  $\mu\text{L}/\text{mL}$ ) silicone membranes (200000 cells/ $\text{cm}^2$ ). Standard methods were used for static [9], strain vector and rate studies [10]. Sigma antibiotic solution (5  $\mu\text{L}/\text{mL}$ ) was sometimes excluded for 48 h.

### 2.2. Mechanical strain

**2.2.1. Static strain.** Silicone membranes (Specialty Manufacturing Inc, Saginaw, MI) were mounted on strain devices [3] for 10% strain application [9] of either 5 or 15 min followed by a second strain of the same duration.

**2.2.2. Cyclic strain.** Mechanical deformation was varied for rate and vector (Model FX-4000<sup>TM</sup>, Flexercell International, McKeesport, PA). NRVM were strained 10% biaxially (BioFlex<sup>®</sup>) for 20 min in non-serum media at varied frequency to modulate the rate of strain expressed in %strain/time ( $\% \text{ s}^{-1}$ ).

**2.2.3. Strain vector.** Microfabricated grooved substrata (5  $\mu\text{m}$  deep), 10  $\mu\text{m}$  valley and ridge (Fig. 1) [11] were made in uniaxial dishes (Uniflex<sup>TM</sup>) by pressing the parylene mold against unpolymerized silicone (Dupont, Phoenix, AZ). NRVM were plated on grooves set perpendicular to the uniaxial stretch axis for transverse and parallel for the longitudinal 10% strain for 20 min at strain at about 1 Hz.

## 3. Western blots and immunocytochemistry

Proteins were probed with antibodies: FAK (BD transduction, Lexington, KY); pY397 FAK (Biosource, Camarillo, CA); Thr<sup>183</sup>Tyr<sup>185</sup> of p42/p44 (ERK1/2) (Promega, Madison, WI); and pY31 paxillin (Invitrogen, Carlsbad, CA) visualized by enhanced chemiluminescence (ECL, Amersham, Arlington Heights, IL) and quantified by laser densitometry. Immunostained images (Nikon Microphot-FXA) were digitally captured with a Spot RT CCD (Diagnostic Instruments) [11].

\*Corresponding author. Address: Department of Physiology and Biophysics (M/C 901), University of Illinois at Chicago, 835 S. Wolcott Avenue, Chicago, IL 60612-7342, USA. Fax: +1 312 996 6312. E-mail address: russell@uic.edu (B. Russell).

<sup>1</sup> Authors contributed equally to this work.

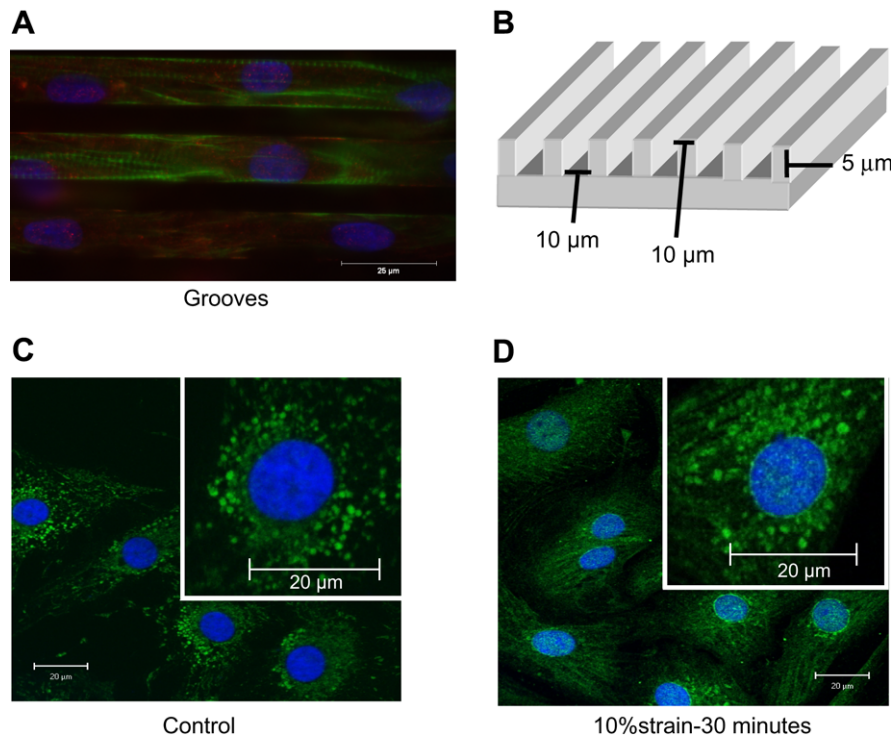


Fig. 1. Aligned NRVM and nuclear translocation. (A) NRVM on microtextured grooves. FAKpY397 (red), actin (green), nucleus (DAPI, blue). (B) Substrata diagram with dimensions. (C, D) NRVM on flat membrane. Total FAK (green).

#### 4. Data analysis

All values are means  $\pm$  S.E.M., with  $n = 4$  or more. Data were analyzed using two-way ANOVA. Differences among means were considered significant at  $P < 0.05$ . Data were analyzed using GraphPad statistical software.

#### 5. Results and discussion

##### 5.1. FAK nuclear translocation

The subcellular localization in control NRVM of FAK seen with anti-FAK antibody had a punctuate pattern representing sites of focal adhesions but was entirely absent from the nuclei (Fig. 1C). However, after 30 min of 10% static strain FAK was redistributed to spots within and around the nuclei (Fig. 1D). Strained myofibrils were stained but not in control (Fig. 1).

Nuclear-cytoplasmic shuttling may be a mechanism by which the rapid translocation of FAK from the cytoplasm to the nucleus and perinuclear region occurs. FAK and ERK1/2 phosphorylation initiate the Ras/MAPK pathway that dissociates a cytoplasmic complex enabling entry to the nucleus for hypertrophic gene expression [12,13]. Many other focal adhesion-associated proteins shuttle to the nucleus, including zyxin, paxillin and muscle LIM protein [14,15] where they regulate chromatin structure, transcription, mRNA processing and export. Interestingly, FAK accumulates in myocytes of failing hearts of spontaneously hypertensive rats [14].

##### 5.2. Stretch-induced FAK, ERK and paxillin activation in myocytes

The time course of FAK phosphorylation in response to 10% static stretch (Fig. 2A) reached a peak of 47% at

5 min and remained elevated for 30 min (Fig. 2B). The time course of ERK1/2 phosphorylation in response to 10% static stretch peaked at 1 min at 50% and decreased by 30 min (Fig. 2C,D). A single 10% static strain resulted in a 40% increase in the level of pY31-paxillin, which peaked at 15 min and decreased by 30 min ( $P < 0.05$ ) (Fig. 2E and F). These time courses are similar but the magnitude was lower than seen after brief endothelin and other stimulation [16]. Changes in FAK, ERK and paxillin protein expression levels were neither expected nor found since times are too brief for any significant increase in translation to occur.

Chemical stimulation may activate different signaling pathways than a single mechanical strain. Vascular endothelial cells are continuously exposed to both mechanical and chemical stimuli. Mechanical (shear) and chemical (VEGF) stimuli diverge at the VEGF receptor 2 (Flk-1) and employ different components of the complex signaling network in regulating downstream molecules, such as ERK [17]. This may explain the difference in the peak activation levels of phosphorylation for FAK and ERK1/2 between mechanical or chemical stimuli.

##### 5.3. Refractory period in FAK and ERK activation

Phosphorylation levels were assessed for different intervals between two 10% strains 5 or 15 min apart (Fig. 3A and B). There was no further response of Y397pFAK phosphorylation ( $P > 0.07$ ) for the second 5 min strain (Fig. 3C) but the second 15 min strain was significantly increased by 30% ( $P < 0.03$ ) (Fig. 3D). There was no further response of pERK1/2 phosphorylation ( $P > 0.07$ ) for the second 5 min strain but the second 15 min strain significantly increased the level of ERK1/2 phosphorylation by 50% ( $P < 0.03$ ) (Fig. 3E and F).

Refractory periods are common in physiology and describe the period of time during which an organ or cell is incapable

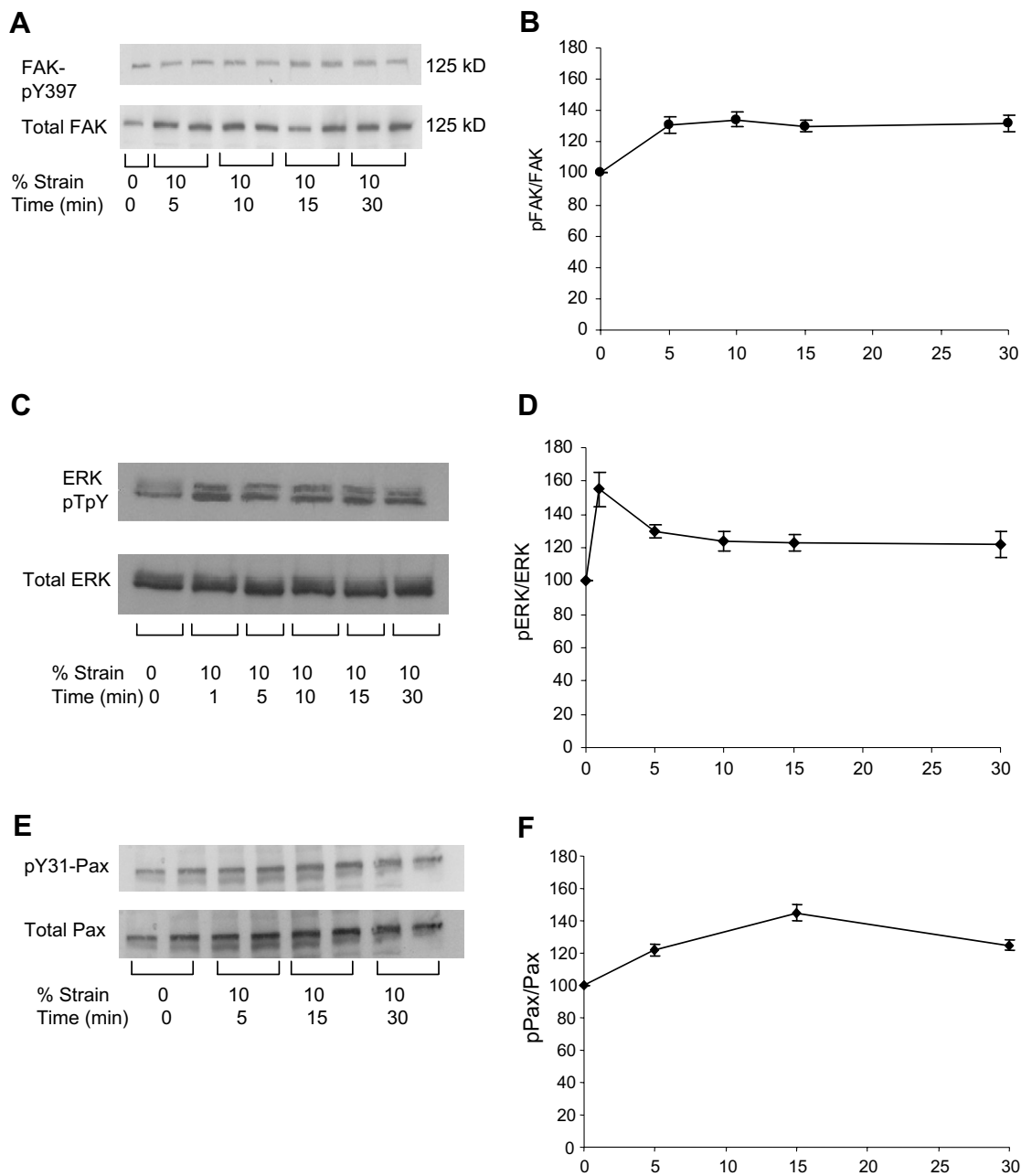


Fig. 2. Stretch-induced FAK, ERK and paxillin. NRVM 10% static strain for 1–30 min. (A, C, E) Western blots: anti-FAKpY397, anti-ERKp42/44, anti-pY31; (B, C, E) time courses normalized to controls: pFAK/FAK ( $n = 4$ ,  $*P < 0.04$ ); pERK1/2/ERK1/2 ( $n = 4$ ,  $*P < 0.04$ ); pPax/Pax ( $n = 4$ ,  $*P < 0.05$ ).

of response, such as action potential initiation of nerves. Here, a second strain at the 5-min interval yielded no further response although the 15-min interval significantly increased phosphorylation levels for both FAK and ERK1/2. Thus, a second 10% strain yields 20% total strain that induces FAK and ERK higher phosphorylation (Fig. 3), that is not realized when two steps are given separately at the 5-min interval of non-responsiveness but are detectable by 15 min. This time scale fits with the time course of peak phosphorylation followed by the subsequent dephosphorylation of the different proteins (Fig. 2B and D) [16]. Results suggest that mechano-transduction in cardiomyocytes has a refractory period of at

least 5 min during which a new dynamic intervention might not be detected in a normal manner. Note that NRVM beat spontaneously throughout static stretch correlating to a sudden change of chamber volume in the heart that continues to beat as it adapts to a sudden extension of myocyte length.

## 6. Strain rate discrimination of frequency-dependent FAK and ERK1/2 phosphorylation

Increased phosphorylation of FAK397 and ERK1/2pTEpY were found with increased rate of strain produced by higher

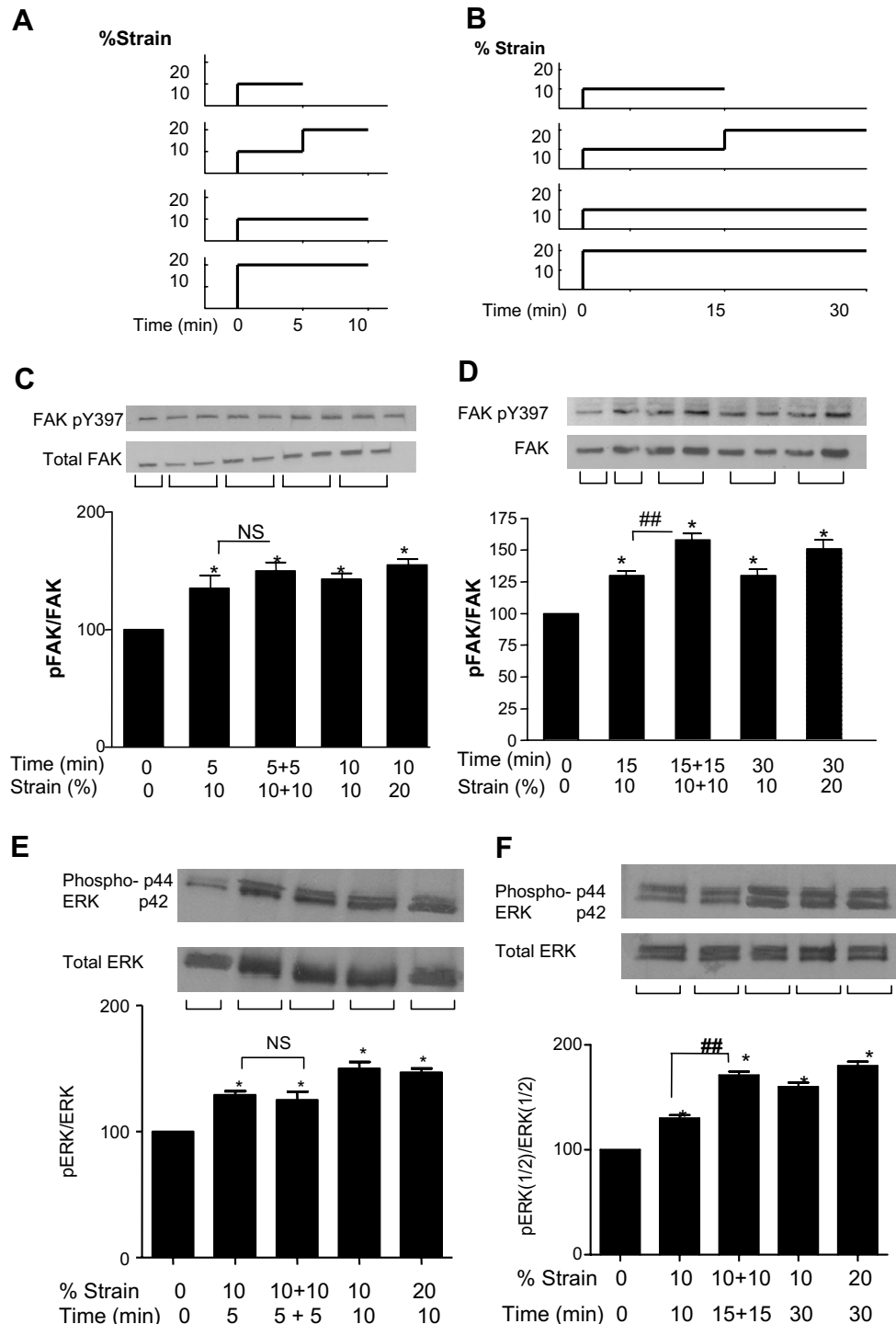


Fig. 3. Refractory period in FAK and ERK. (A, B) Protocols for 10% strain delivery and maintenance. (C, D) Western blots and histograms: Y397pFAK/FAK (5 min), (5 + 5 min); (15 + 15 min). (E, F) Western blots and histograms: ERK1/2 (5 min); (5 + 5 min); (15 + 15 min).  $N = 4$ , \* $P < 0.05$  normalized to control, \*\* $P < 0.05$  between the first and second strain.

cyclic frequency for biaxial perturbation of 10% strain for 20 min (Fig. 4). Western blotting for FAK after strain rates of  $\sim 10\% \text{ s}^{-1}$ ,  $\sim 20\% \text{ s}^{-1}$ , and  $\sim 40\% \text{ s}^{-1}$  induced 75%, 114%, and 125% of baseline, respectively, with statistical significance ( $P < 0.05$ ), (Fig. 4A). The drop in phosphorylation at the lower frequency was surprising (Fig. 4B and C). Similar strain rates

correlated with a rapid increase in ERK1/2pTEpY of 87%, 290%, and 355%, respectively, significantly greater than baseline ( $P < 0.05$ ) (Fig. 4D and E).

ERK1/2 phosphorylation was detectable at 2 min for 40%  $\text{s}^{-1}$  (not shown), as expected given response for static strain (Fig. 2D). The rate of loading regulates the transmittance of

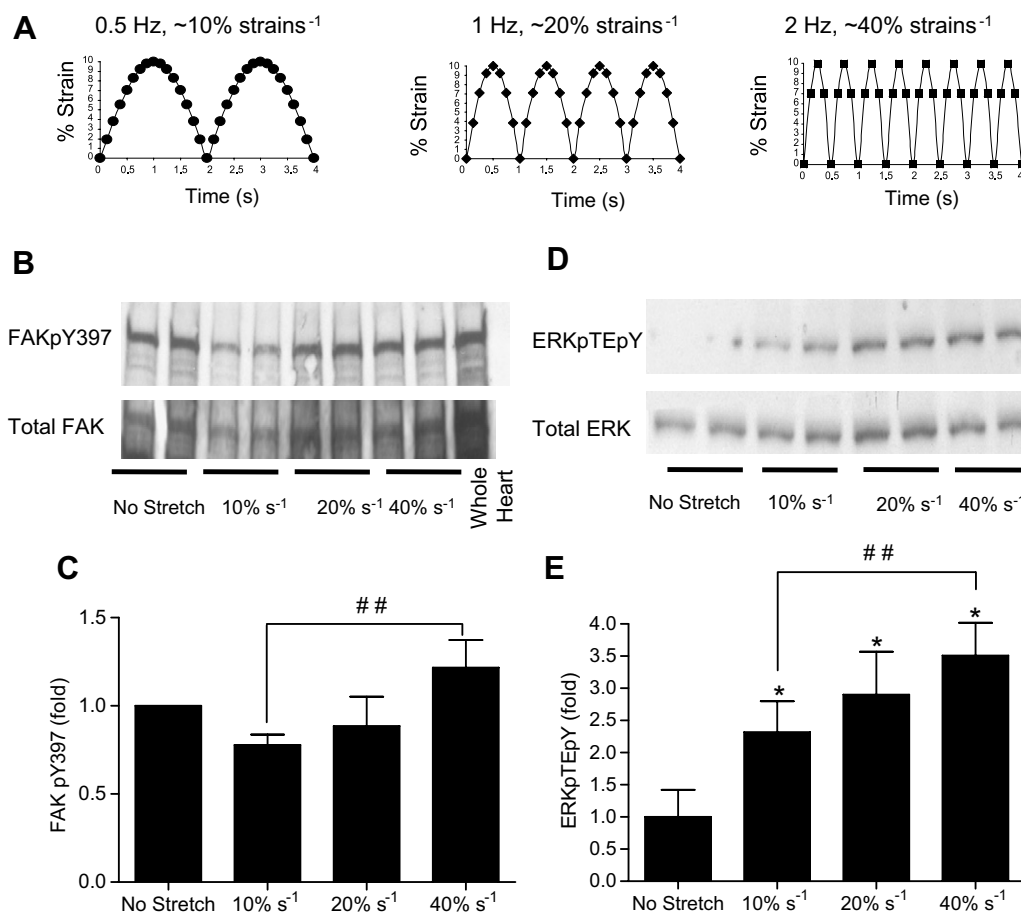


Fig. 4. Frequency-dependent phosphorylation. (A) Cyclic biaxial strain rate for 20 min; slow 0.5 Hz (~10% s<sup>-1</sup>), medium 1.0 Hz (~20% s<sup>-1</sup>), and fast 2 Hz (~40% s<sup>-1</sup>). (B, C) Western blot and histogram; FAK pY397 (10% s<sup>-1</sup> vs. 40% s<sup>-1</sup>, ## *P* < 0.05, *n* = 7). (D, E) ERK pTEpY/ERK (*n* = 4; \* *P* < 0.05).

force and overall activation of proteins relative to inherent relaxation constants of the cellular architecture. Strain rate sensitivity in a biaxial system may resolve from viscoelastic properties and relaxation time constants of the cellular membrane, focal adhesive complex, and the cytoskeleton [18]. These time constants could reflect growth of focal adhesive complexes that is unlikely to occur in minutes, so that signaling pathways are a more likely explanation.

The magnitude of the FAK phosphorylation here was two- and three-fold lower for static and cyclic strain, respectively, than other reports [19]. Removal of antibiotic cocktail from culture media for 48 h prior to strain increased levels of Y397pFAK to 124% ± 12% (*n* = 4). This is not surprising since streptomycin and its analogs (gentamicin and netilmicin) are common aminoglycosidic antibiotics that have been reported to block L-type Ca<sup>2+</sup> channels and stretch-activated channels in cardiac, skeletal, and vascular smooth muscle. In addition streptomycin can block [Ca<sup>2+</sup>]<sub>i</sub> transients and contraction in unstretched preparations [20]. Additionally, stretch may elicit entry from calcium channel populations in a rate dependent manner. The density of myocytes in culture also affects FAK expression and basal phosphorylation and the high density NRVM (2 × 10<sup>3</sup> cells/mm<sup>2</sup>) used here yields a lower concentration of FAK protein and a higher level of basal FAK phosphorylation.

## 7. Strain vector discrimination by FAK and ERK1/2 phosphorylation

NRVM align within grooved substrates as shown with FAKpY397 stain in culture (Fig. 1B). NRVMs stretched 20 min with 10% transverse strain had 25% higher FAKpY397 phosphorylation to total FAK (*P* < 0.05) than unstretched controls (Fig. 5). However, FAK phosphorylation level for longitudinal strain resembled the unstretched rest group. NRVMs stretched at 10% strain had pTEpY to total ERK (Fig. 5D and E) of 360% for transverse strain and 290% for longitudinal strain, both significantly higher than the unstretched NRVM (*P* < 0.05), though not differ from each other (transverse vs. longitudinal, *P* < 0.10).

The anisotropic geometry of the cardiac myocyte may allow for distinct pathways of force recognition and transmittance. Mechanical forces induce protein conformation altering the dynamic state of focal adhesive proteins [21] perhaps contributing to vector dependent phosphorylation of FAK or a FAK activating protein. Separate directional pathways are implicated by static transverse and longitudinal loading to activate stress induced-MAP kinase. Potentially longitudinal perturbation activates a separate pathway that also runs into ERK activation as shown in similar studies with elongation of tissue sections from murine diaphragm [22]. Interestingly,

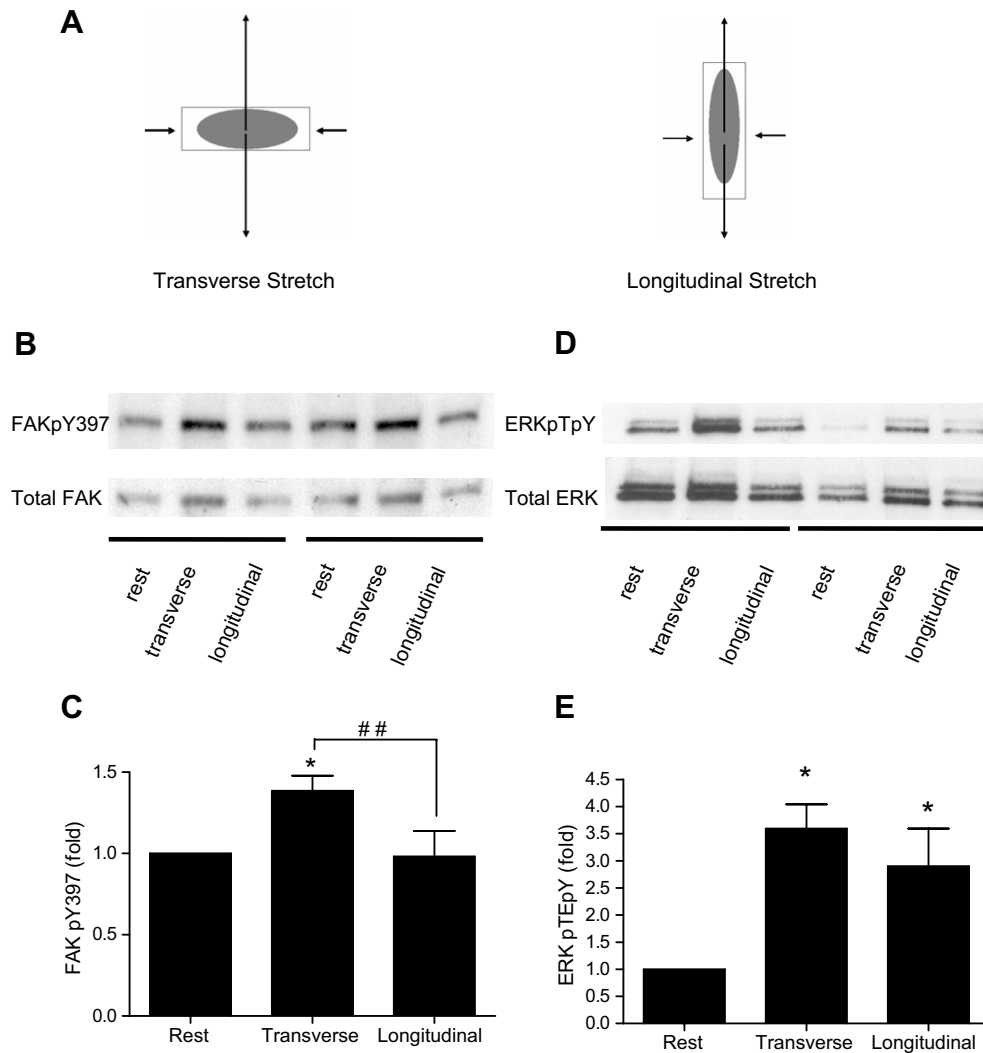


Fig. 5. Direction dependent phosphorylation by Western blot and histogram. (A) 10% cyclic strain transverse or longitudinal. (B, C) Y397pFAK/FAK (\* $P < 0.05$  vs. control \*\* $P < 0.05$  transverse vs. longitudinal,  $n = 4$ ). (D, E) ERKpTEpY/ERK: trend for transverse strain greater than longitudinal ( $P < 0.10$ ). Both greater than control (\* $P < 0.05$ ,  $n = 4$ ).

microtubule density is implicated in direction and frequency-dependent changes in viscosity over 1–10 Hz perturbation of single adult cardiac myocytes [18]. Subsequent conformational changes may rely in part on the protein orientation; thus force vectors aligned with mobile protein components have a greater probability to modulate protein structural arrangement. NRVM used here are spontaneously beating, thereby adding a dynamic factor potentially complicating the experiments since cross-bridge formation affects longitudinal tensile stiffness [18]. Nonetheless, results detected an anisotropy even though the spontaneous beating was present for strains delivered along both vectors suggesting that the intrinsic beating does not override the externally applied stimuli.

## 8. Conclusion

Results show a refractory period of at least 5 min for FAK phosphorylation and more sensitivity in the transverse direction. Results for asymmetric and temporal detection add another dimension of complexity to whole heart mechanical

studies in addition to the well known chemical and the simple mechanical perturbation studies. Thus, myocytes detect both temporal and anisotropic force changes that may be a critical for adaptation and maintenance of cardiac function when the mechanical demands on the heart are undergoing dynamic transformations due to disease.

*Acknowledgments:* We thank Tejal Desai, Allen Samarel and Samuel Boateng for advice and encouragement. The work was supported by NIH (HL 64956, HL 077995, HL62426) and American Heart Association Fellowship 558225.

## References

- [1] Holmes, J.W. (2004) Candidate mechanical stimuli for hypertrophy during volume overload. *J. Appl. Physiol.* 97 (4), 1453–1460.
- [2] Lorenzen-Schmidt, I., Schmid-Schonbein, G.W., Giles, W.R., McCulloch, A.D., Chien, S. and Omens, J.H. (2006) Chronotropic response of cultured neonatal rat ventricular myocytes to short-term fluid shear. *Cell Biochem. Biophys.* 46 (2), 113–122.
- [3] Simpson, D.G., Majeski, M., Borg, T.K. and Terracio, L. (1999) Regulation of cardiac myocytes protein turnover and myofibrillar

- structure in vitro by specific directions of stretch. *Circ. Res.* 85, 59–69.
- [4] Kurpinski, K., Chu, J., Hashi, C. and Li, S. (2006) Anisotropic mechanosensing by mesenchymal stem cells. *Proc. Natl. Acad. Sci. USA* 103 (44), 16095–16100.
- [5] Gopalan, S.M., Flaim, C., Bhatia, S.N., Hoshijima, M., Knoell, R., Chien, K.R., Omens, J.H. and McCulloch, A.D. (2003) Anisotropic stretch-induced hypertrophy in neonatal ventricular myocytes micropatterned on deformable elastomers. *Biotechnol. Bioeng.* 81 (5), 578–587.
- [6] Camelliti, P., Gallagher, J.O., Kohl, P. and McCulloch, A.D. (2006) Micropatterned cell cultures on elastic membrane as an in vitro model of myocardium. *Nat. Protoc.* 1 (3), 1379–1391.
- [7] Bullard, T.A., Hastings, J.L., Davis, J.M., Borg, T.K. and Price, R.L. (2007) Altered PKC expression and phosphorylation in response to the nature, direction, and magnitude of mechanical stretch. *Can. J. Physiol. Pharmacol.* 85 (2), 243–250.
- [8] Samarel, A.M. (2005) Costameres, focal adhesions, and cardiomyocytes mechanotransduction. *Am. J. Physiol. Heart Circ. Physiol.* 289, H2291–H2301.
- [9] Mansour, H., de Tombe, P.P., Samarel, A.M. and Russell, B. (2004) Restoration of resting sarcomere length after uniaxial static strain is regulated by PKC $\epsilon$  and FAK. *Circ. Res.* 94, 642–649.
- [10] Boateng, S.Y., Hartman, T.J., Aluwalia, N., Vidula, H., Desai, T.A. and Russell, B. (2003) Inhibition of fibroblasts proliferation in cardiac myocytes by surface microtopography. *Am. J. Physiol. Cell Physiol.* 285, C171–C182.
- [11] Motlagh, D., Hartman, T.J., Desai, T.A. and Russell, B. (2003) Microfabricated grooves recapitulate neonatal myocyte connexin43 and N-cadherin expression and localization. *J. Biomed. Mater. Res. A* 67 (1), 148–157.
- [12] Sadoshima, S. and Izumo, S. (1997) The cellular and molecular response of cardiac myocytes to mechanical stress. *Annu. Rev. Physiol.* 59, 551–571.
- [13] Wang, J.G., Miyazu, M., Xiang, P., Li, S.N., Sokabe, M. and Naruse, K. (2005) Stretch-induced cell proliferation is mediated by FAK-MAPK pathway. *Life Sci.* 76 (24), 2817–2825.
- [14] Yi, X.P., Zhou, J., Huber, L., Qu, J., Wang, X., Gerdes, A.M. and Li, F. (2006) Nuclear compartmentalization of FAK and FRNK in cardiac myocytes. *Am. J. Physiol. Heart Circ. Physiol.* 290, H2509–H2515.
- [15] Boateng, S.Y., Belin, R.J., Geenen, D.L., Margulies, K.B., Martin, J.L., Hoshijima, M., de Tombe, P.P. and Russell, B. (2007) Cardiac dysfunction and heart failure are associated with abnormalities in the subcellular distribution and amounts of oligomeric muscle LIM protein. *Am. J. Physiol. Heart Circ. Physiol.* 292, H259–H269.
- [16] Heidkamp, M.C., Bayer, A.L., Scully, B.T., Eble, D.M. and Samarel, A.M. (2003) Activation of focal adhesion kinase by protein kinase C $\epsilon$  in neonatal rat ventricular myocytes. *Am. J. Physiol. Heart Circ. Physiol.* 285, H1684–H1696.
- [17] Wang, Y., J, Chang, Chen, K.D., Li, S., Li, J.Y., Wu, C. and Chien, S. (2007) Selective adapter recruitment and differential signaling networks by VEGF vs. shear stress. *Proc. Natl. Acad. Sci. USA* 104 (21), 8875–8879.
- [18] Nishimura, S., Nagai, S., Katoh, M., Yamashita, H., Saeki, Y., Okada, J., Hisada, T., Nagai, R. and Sugiura, S. (2006) Microtubules modulate the stiffness of cardiomyocytes against shear stress. *Circ. Res.* 98 (1), 81–87.
- [19] Torsoni, A.S., Constancio, S.S., Nadruz, W., Hanks, S.K. and Franchini, K.G. (2003) Focal adhesion kinase is activated and mediates the early hypertrophic response to stretch in cardiac myocytes. *Circ. Res.* 93, 140–147.
- [20] Belus, A. and White, E. (2003) Streptomycin and intracellular calcium modulate the response of single guinea-pig ventricular myocytes to axial stretch. *J. Physiol.* 546 (Pt 2), 501–509.
- [21] Vogel, V. (2006) Mechanotransduction involving multimodular proteins: converting force into biochemical signals. *Annu. Rev. Biophys. Biomol. Struct.* 35, 459–488.
- [22] Kumar, A., Chaudhry, I., Reid, M.B. and Boriek, A.M. (2002) Distinct signaling pathways are activated in response to mechanical stress applied axially and transversely to skeletal muscle fibers. *J. Biol. Chem.* 277 (48), 46493–46503.