PKC epsilon signaling effect on actin assembly is diminished in cardiomyocytes when challenged to additional work in a stiff microenvironment

Michael A. Mkrtschjan1 | Christopher Solís2 | Admasu Y. Wondmagegn2 | Janki Majithia2 | Brenda Russell2

1Department of Bioengineering, University of Illinois at Chicago, Chicago, Illinois
2Department of Physiology and Biophysics, University of Illinois at Chicago, Chicago, Illinois

Correspondence
Brenda Russell, Department of Physiology and Biophysics, University of Illinois at Chicago, 835 S. Wolcott Rm E202 (MC901), Chicago, IL 60612-7342.
Email: russell@uic.edu

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Abstract
The stiffness of the microenvironment surrounding a cell can result in cytoskeletal remodeling, leading to altered cell function and tissue macrostructure. In this study, we tuned the stiffness of the underlying substratum on which neonatal rat cardiomyocytes were grown in culture to mimic normal (10 kPa), pathological stiffness of fibrotic myocardium (100 kPa), and a nonphysiological extreme (glass). Cardiomyocytes were then challenged by beta adrenergic stimulation through isoproterenol treatment to investigate the response to acute work demand for cells grown on surfaces of varying stiffness. In particular, the PKCε signaling pathway and its role in actin assembly dynamics were examined. Significant changes in contractile metrics were seen on cardiomyocytes grown on different surfaces, but all cells responded to isoproterenol treatment, eventually reaching similar time to peak tension. In contrast, the assembly rate of actin was significantly higher on stiff surfaces, so that only cells grown on soft surfaces were able to respond to acute isoproterenol treatment. Förster Resonance Energy Transfer of immunofluorescence on the cytoskeletal fraction of cardiomyocytes confirmed that the molecular interaction of PKCε with the actin capping protein, CapZ, was very low on soft substrata but significantly increased with isoproterenol treatment, or on stiff substrata. Therefore, the stiffness of the culture surface chosen for in vitro experiments might mask the normal signaling and affect the ability to translate basic science more effectively into human therapy.

KEYWORDS
beta-adrenergic stimulation, CapZ, contractility, cytoskeleton, mechanobiology

1 | INTRODUCTION

In response to functional demands, muscle remodels at the macroscopic level by changing the shape, cytoskeletal content, and performance of individual cardiomyocytes (CMs). The mechanisms for CM shape and strength are not fully understood, but it is likely to involve multiple processes, such as gene transcription, protein translation, posttranslational modification, and the assembly of the sarcomeres in cell hypertrophy (Russell, Curtis, Koshman, & Samarel, 2010; Sanger, Wang, Fan, White, & Sanger, 2010). Exercise or chronic disease increases cell hypertrophy, which has been modeled by static or dynamic strain of CMs in culture to reveal mechanisms by which sarcomeres are added (Li & Russell, 2013; Lin, Li, Swanson, & Russell, 2013; Sharp, Simpson, Borg, Samarel, & Terracio, 1997; Torsoni, Marin, Veloso, & Franchini, 2005; Yang et al., 2016; Yu & Russell, 2005). Most studies are done either with acute or chronic loading models, but little is done to determine how cells respond to acute work when they are already in a chronically loaded pathological state.

It is well accepted that increased load leads to muscle bulking. A cell senses external forces impinging on it, which are balanced against forces generated internally by the sarcomere. Increased cell tension triggers mechanotransduction pathways, leading to thin filament assembly. Multiple mechanosensors detect increased mechanical loading to initiate actin filament assembly (Hoshijima, 2006; Skwarek-Maruszewska,
Hotulainen, Mattila, & Lappalainen, 2009). Forces are transmitted internally to the Z-disc, permitting an extensive amplification of filament assembly throughout the width and length of the CM. At the Z-disc, the thin actin filaments insert and reverse their polarity, making it the pivotal sarcomere assembling site in CMs (Gautel & Djuricin-Djuricin-Carugo, 2016). Upon mechanical stimulation of CMs, assembly may be controlled in an hour by an acute bout of activity through modification of the actin capping protein, CapZ, where load increases actin dynamics, filament assembly, and cell size (Lin et al., 2013). Mechanotransduction arising from stress or strain modifies the function of CapZ by phosphorylation via protein kinase C (PKC) (Disatnik, Buraggi, & Mochly-Rosen, 1994; Kim, 2014), and through acetylation (Lin, Warren, Li, McKinsey, & Russell, 2016). Here, we concentrate on phosphorylation, which has recently been thoroughly reviewed (Scruggs, Wang, & Ping, 2016). In particular, the PKCe isoform translocates to the Z-disc when CMs are activated (Disatnik et al., 1994), where it is anchored to the myofilaments (Hartman et al., 2009; Pyle, La Rotta, de Tombe, Sumandea, & Solaro, 2006; Robia, Ghatia, Robu, & Walker, 2001).

Mechanobiological cues read by a cell depend on the underlying material scaffold for microscale, cell-specific instruction (Engler et al., 2008; Wozniak & Chen, 2009). Stiffness of a 3D matrix significantly affects maturation and differentiation into myocytes (Jacot, Martin, & Hunt, 2010), as well as force generation (Bhana et al., 2010; Broughton & Russell, 2015; Curtis, Budyn, Desai, Samarel, & Russell, 2013; Hazeltine et al., 2012). The stiffness in the heart varies during development from embryonic/neonatal myocytes 5–10 kPa (Bhana et al., 2010) to the normal adult rat myocardium of 10–70 kPa (Yoshikawa, Yasuiki, Yagi, & Yamada, 1999). Infarct stiffness and collagen content increase dramatically with time, so that by 6-week post-infarct, it may rise to 400 kPa (Fomovsky & Holmes, 2010; Fomovsky, Thomopoulos, & Holmes, 2010). Traditionally, cell culture is done almost exclusively using flat, hard, plastic surfaces, which poorly mimic the external forces existing in normal living tissues. Therefore, most studies in culture actually mimic pathological conditions, which may obscure the more normal physiological signal transduction.

In this study, we use 10–100 kPa polyacrylamide substrata along with glass surfaces to mimic the softness of the healthy and pathological heart to study mechanotransduction. We explore the effects of the combination of acute and/or chronic loading on mechanosignaling by applying acute challenges through β-adrenergic receptor (βAR) stimulation of CMs cultured on substrata of varying stiffness. βAR activation by epinephrine or isoproterenol causes positive inotropy and chronotropy (Kaumann & Molenaar, 2008). We assess contractility, actin assembly, PKCe signaling and distribution, as well as the molecular interactions between PKCe and CapZ. The effect of local tension and acute demand on CM contractility is assessed by line scan kymographs (Broughton & Russell, 2015). Additionally, fluorescence recovery after photobleaching (FRAP) of actin-GFP is used to study actin dynamics (Hartman et al., 2009; Lin et al., 2013). Proteomics, colocalization, and Förster resonance energy transfer (FRET) are used to study the molecular interactions of Z-disc proteins and PKCe distribution in the sarcomere. By these approaches, we determine that the effect of PKCe signaling seen in a soft environment following βAR stimulation is diminished in CMs within a stiff environment, a phenomenon that could contribute to maladaptive responses in disease states.

## 2 | RESULTS

### 2.1 | Substrate stiffness and increased acute demand alter contractile time to peak tension

Following culture for 48 hr, contractile measurements of CMs were made on varying substrate stiffness with or without isoproterenol treatment. A representative image of a line scan readout on a 10 kPa substrate shows characteristic peaks from which time to peak tension was obtained (Figure 1a). Untreated cells grown on 10 kPa, 100 kPa, and glass substrata had time to peak tension values of 144 ms, 181 ms, and 206 ms, respectively (Figure 1b). Statistically significant differences were seen in untreated 100 kPa and glass groups when compared to untreated 10 kPa group (p < .01 and p < .0001, respectively), though there was no significance between 100 kPa and glass (Figure 1b). Time to peak tension in all CMs was significantly reduced by treatment with isoproterenol compared to untreated CMs grown on 10 kPa (Figure 1c), 100 kPa (Figure 1d), or glass (Figure 1e).

### 2.2 | Actin cytoskeletal assembly rate increases with isoproterenol treatment on soft substrata

Actin-GFP infected cells cultured for 48 hr were photobleached to observe the rate of fluorescence recovery of actin over a 7.5 min time period (Figure 2a). Increased substrate stiffness led to significantly increased kfrap on 100 kPa and glass substrata when compared to 10 kPa (p < .05), but no difference was observed between 100 kPa and glass groups (Figure 2b). kfrap values of untreated cells on 10 kPa, 100 kPa, and glass substrata were 2.8 × 10−4 s−1, 6.6 × 10−4 s−1, 6.7 × 10−4 s−1, respectively. Notably, isoproterenol treatment led to significantly increased kfrap only on 10 kPa (p < .05 for 15-min and 1 hr) (Figure 2c), but there was no significant difference with isoproterenol treatment for CMs grown on glass (Figure 2d).

### 2.3 | Time course of colocalization of phosphorylated PKCe and α-actinin with isoproterenol treatment of cardiomyocytes

CMs grown on 10 kPa, 100 kPa, or glass with membrane and cytosol extracted to retain the cytoskeleton were probed to study pPKCe distribution as a function of stiffness and for the time course of increased muscle beating caused by βAR stimulation. Representative fluorescence images of pPKCe and α-actinin are shown in 10 kPa (Figure 3a) or glass (Figure 3b). Untreated CMs on 10 kPa had little yellow compared to treated groups, indicating lower colocalization at the Z-disc. A redistribution of pPKCe was seen with isoproterenol treatment as evidenced by increased yellow at the Z-disc, with peak levels reached between 15 min and 1 hr for cells grown on the soft substrate (Figure 3a). However, cells grown on glass remained strongly striated throughout isoproterenol treatment (Figure 3b). Line scans shown for
FIGURE 1  Contractile time to peak tension decreases with increased stiffness and with isoproterenol treatment of CMs on soft substrata. (a) Representative line scan image acquisition showing how time to peak tension (TPT) was measured. Y-axis is line scan position; x-axis is time. (b) Time to peak tension measurements for cells grown on 10 kPa (n = 28 cells), 100 kPa (n = 27 cells), and glass (n = 21 cells). CMs grown with 1- or 24-hr isoproterenol treatment on (c) 10 kPa (n = 24 and 22 for treated cells, respectively), (d) 100 kPa (n = 27 and 17 for treated cells, respectively), or (e) glass (n = 26 and 24 for treated cells, respectively). Values are mean ± SE. **p < .01, ***p < .001, and ****p < .0001.

FIGURE 2  Actin dynamics increase with substrate stiffness but only respond to isoproterenol treatment on 10 kPa substrata. (a) Representative images showing FRAP experiment within region of interest (red box, ROI), pre-bleached (left panel), immediately following bleaching (middle panel), and after 7.5 min of recovery time (right panel). Scale bar, 10 μm. (b) k_{frap} values for cells grown 10 kPa, 100 kPa, or glass substrata (n = 13, 17, and 15, respectively), and with 15-min, 1-hr, or 24-hr isoproterenol treatment of CMs (c) on 10 kPa (n = 12, 11, and 10 for treated cells, respectively), or (d) on glass (n = 12, 12, and 14 for treated cells, respectively). Values are mean ± SE. *p < .05. [Color figure can be viewed at wileyonlinelibrary.com]
10 kPa and glass (left panels Figure 3a,b, respectively) were analyzed to acquire peak intensities and normalized to α-actinin. Histograms for ratios of pPKCε/α-actinin of fluorescence intensity (Figure 3c–e for 10 kPa, 100 kPa, and glass, respectively) confirm the visual impressions. A significant difference due to isoproterenol treatment was observed only on 10 kPa substrata (Figure 3c), with ratio changes at 15 min, 1 hr, and 24 hr of 1.35-, 1.51-, and 1.28-fold compared to untreated baseline (p < .01, p < .0001, p < .05, respectively). However, no changes in colocalization of pPKCε and α-actinin were observed on 100 kPa or glass.

In order to confirm the findings of PKCε phosphorylation, an independent biochemical approach was used with Western blot analysis.
on the cytoskeletal fraction of CMs (Figure 3f,g). PKCε phosphorylation on 10 kPa substrata following isoproterenol treatment began to increase by 15 min (not significant) and reached a maximum at 1 hr ($p < .01$), which was sustained but not significant at 24 hr. From untreated group, changes of 1.19-, 1.63-, and 1.32-fold are seen at 15-min, 1-hr, and 24-hr treatments, respectively. Changes were not observed with isoproterenol treatment for cells grown on stiff substrata (data not shown).

### 2.4 Molecular interactions of CapZ-GFP and pPKCε with stiffness and isoproterenol

Direct molecular interactions between CapZ-GFP and pPKCε were assessed by FRET (Figure 4a). Under glass and isoproterenol treatment for 1 hr, a typical ROI shown (Figure 4b left panel) was fully bleached after 20 scans (right panel), while the reference ROI was unchanged. Increasing donor fluorescence intensity (CapZ, green) was accompanied by concomitant acceptor intensity decay of pPKCε (red) (Figure 4c). The regression line between the acceptor intensity and transfer efficiency for this example (Figure 4d) showed a FRET response. Varying substrate stiffness resulted in a significant change in transfer efficiency in cells grown on 100 kPa substrata compared to 10 kPa ($p < .01$). Transfer efficiency was higher on glass substrata but not significant ($p = .07$) (Figure 4e). Low transfer efficiency was seen for untreated cells on 10 kPa substrata, but this was significantly increased with 1-hr isoproterenol treatment ($p < .05$) (Figure 4f). Isoproterenol treatment did not lead to significant changes on stiff substrata (Figure 4g,h). These data suggest an increase in the molecular interactions between CapZ and pPKCε under stiff conditions or when challenged to additional work under soft conditions.

**FIGURE 4** FRET analysis of PKCε interaction with CapZ at the Z-disc. CMs grown on 10 kPa and 100 kPa substrata or glass were infected by CapZ-GFP. Untreated and 1-hr isoproterenol treated CMs were compared following component extraction, fixation, and immunolabeling with pPKCε antibody (S729) and red secondary antibody for confocal imaging and FRET analysis. (a) Diagram showing how molecular range interactions of CapZ with the GFP (green) as donor and a secondary antibody to pPKCε (red) as acceptor. (b) A typical scan for a striated CM on glass with 1-hr isoproterenol treatment before scanning a region of interest (ROI) and the unbleached reference panel (ref.). Right panel shows after 20 scans the red acceptor is bleached leaving only the green donor Scale bar: 5 μm fluorescence. (c) Photobleaching trace of pPKCε acceptor (solid, red circles) over 20 scans showing steady bleaching over time while the acceptor in the reference region is unchanged (open, red circles). The GFP donor (solid, green circles) increased slightly in bleached ROI (expanded in upper area for clarity) relative to the donor in the reference region (open, green circles). (d) Linear fit of acceptor intensity and transfer efficiency, where the intercept represents the recovered transfer efficiency (intercept = 2.56%, $R^2 = 0.69$). (e) Transfer efficiency significantly increased in 100 kPa compared to 10 kPa but was not significantly different on glass ($p = .07$). (f), (g), (h) changes in transfer efficiency when treated with isoproterenol on different stiffness conditions. Only 10 kPa exhibited a significant change following treatment ($n = 19$ cells for 10 kPa and glass; $n = 10$ cells for 100 kPa). Values are mean ± SE. *$p < .05$ and **$p < .01$ [Color figure can be viewed at wileyonlinelibrary.com]
The objective of this study was to investigate the response to acute work demand for cells grown on surfaces of varying stiffness. In particular, the PKCε signaling pathway and its role in actin assembly dynamics were examined. Interestingly, novel findings suggest that pathological stiffness diminishes the effect of PKCε signaling on actin assembly in neonatal rat heart cells. Immunofluorescence studies found that substrate stiffness similar to the normal neonatal heart leads to significantly increased PKCε activation in the CM cytoskeleton upon isoproterenol treatment, whereas this response is not seen on pathological and nonphysiological substrate stiffness. The FRAP and FRET data further support the notion that at the pathological stiffness, CapZ is already phosphorylated, loosening the capping conformation, which results in increased actin assembly rate. A proposed mechanism is depicted (Figure 5), where upon translocation of PKCε to the sarcomere, CapZ is phosphorylated, leading to a conformational change and decreased association with the actin barbed end. In that scenario, isoproterenol treatment could not lead to additional phosphorylation of CapZ, and the actin assembly rate would remain the same. Conversely, CMs grown on physiological stiffnesses are free to undergo CapZ phosphorylation by PKCε and increases assembly rate accordingly.

At the tissue level, translocation of PKCε to the sarcomere has been associated with cardiac hypertrophy (Mochly-Rosen et al., 2000), a phenomenon correlated with increased actin assembly rate. PKCε translocation has also been shown in vitro following CM stretch (Vincent et al., 2006). Our laboratory has demonstrated that mechanical stimulation through increased substrate stiffness or cyclic mechanical stretch leads to greater actin dynamics, which is mediated through a variety of signaling molecules, including PKCε, PIP2, and FAK (Li, Mkrtschjan, Lin, & Russell, 2016; Lin, Swanson, Li, Mkrtschjan, & Russell, 2015). It has been shown that isoproterenol treatment leads to rapid translocation of PKCε to the cell particulate fraction (Li, Cai, Liu, & Guo, 2015), making it a strong candidate here for implication in increased actin assembly rate. Indeed, this is supported by the increased FRAP kinetics that were seen 15 min after isoproterenol treatment.

We demonstrate that although the physiological metric of time to peak tension is altered by substrate stiffness, they each respond rapidly to βAR stimulation through isoproterenol stimulation, reaching common peak measurements. It is well known that changes in the mechanical environment lead to aberrations in CM contractility. In particular, a substrate tuned in stiffness to the natural myocardium yields embryonic CMs that work optimally (Engler et al., 2008). Although the stiffness of the underlying substrata affected contractility, βAR stimulation still led to increases in contractile velocity, which is most easily explained by changes in calcium handling. Phosphorylation of phospholamban can be mediated by a PKCε-dependent mechanism (Okumura et al., 2014) or by other signaling molecules.

CMs here were grown on substrata of varying stiffness for 2 days before the acute isoproterenol treatment to provide time for the initial and subsequent response to occur. Lack of response under high stiffness could be part of a more persistent desensitization mechanism to β agonists. It is known that catecholamine response is depressed in the failing heart (El-Armouche & Eschenhagen, 2009). Our results suggest a desensitization mechanism in single myocytes with 2 days of chronic high stiffness that caused a blunted response of PKCε to isoproterenol. This time might be sufficient for activation of beta signaling desensitization mechanisms like upregulation of G-coupled receptor kinase 5 (Islam & Koch, 2012; Zelarayan et al., 2009). Results here suggest that the chronic pathological state can be replicated by manipulating substrate stiffness levels.

It seems likely that filaments are built to serve the functional work being demanded by the myocyte, and that local mechanical conditions ultimately regulate filament assembly and muscle mass. Findings demonstrate that the CM responds to acute changes in demand differentially based on the underlying matrix stiffness, a condition which would be altered with cardiac fibrosis. This could ultimately affect the response an individual might have to particular drug therapies. Results also suggest that the matrix stiffness on which cells are cultured has implications for drug screening studies. Since cells become nonresponsive to treatment when grown for several days on a pathologically stiff surface, investigators might fail to detect results that are necessary to translate in vitro studies more effectively to human therapy.

4 | MATERIALS AND METHODS

4.1 | Isolation of neonatal rat ventricular myocytes

Primary heart cultures were obtained from 1–2 day old neonatal rats according to the Institutional Animal Care and Use Committee at the University of Illinois at Chicago. Hearts were removed, and CMs were isolated from 1–2 day old Sprague-Dawley rats with collagenase type II (Worthington, Lakewood, NJ) as previously described (Li et al., 2016). Neonatal rat ventricular CMs were re-suspended, filtered
through a 70 μm nylon sieve to remove large material, and plated in PC-1 medium (Lonza Group, Basel, Switzerland). Dishes were coated with fibronectin for at least 2 hr prior to plating. Polyacrylamide (10 kPa and 100 kPa) substrates were functionalized according to previous studies prior to incubation with fibronectin (Li et al., 2016).

4.2 | Line scans of live cardiomyocytes to measure contraction

Following 48-hr culture and drug treatment, CMs were placed into a temperature/CO₂ chamber connected to a Zeiss 710 or 880 (Zeiss, Oberkochen, Germany) confocal microscope system. Polarized, isolated individual CMs were selected in an unbiased manner, and a single line was scanned repeatedly at high speed along the contractile axis of the CM using transmitted light. Output kymographs were then analyzed using Zen software to measure the time to peak tension. At least 17 cells over three separate cultures were analyzed for each condition.

4.3 | Actin fluorescence recovery after photobleaching

CMs were grown in culture 24 hr before infecting cells with actin-GFP virus (ThermoFisher Scientific, Waltham, MA). Cells were cultured an additional 24 hr before FRAP studies were carried out on adequately expressing striated cells within a temperature/CO₂ chamber. A region of actin was photobleached to at least 50% of its original fluorescence intensity and re-imaged every 5 s for 450 s to monitor the rate of fluorescence recovery. The rate constant, kfrap, was determined using a single parameter, nonlinear regression fit in OriginLab Pro (OriginLab Corp., Northampton, MA). At least 10 cells were measured for each condition, coming from 3 or more separate cultures. The actin-GFP construct used is specific for β-actin, which has been shown to localize to the Z-disc (Benz et al., 2013). It has previously been used effectively as a reporter of actin dynamics (Lin et al., 2015).

4.4 | Immunofluorescence

CMs were fixed using a 10% formalin solution, incubated overnight at 4 °C in a 1:200 primary antibody, 1% BSA, and 0.1% Tween-20 solution. Primary antibodies used for IF were anti-phospho-PKCe (S729) (pPKCe) (ab88241, abcam, Cambridge, MA), and anti-α-actinin (ab9465, abcam). Dishes were then washed and incubated at room temperature for 1 hr in a secondary antibody (Goat Anti-Rabbit IgG H&L Alexa Fluor 488 or Goat anti-Mouse IgG H + L Alexa Fluor 568, Cat. # A-11034 and A-11004, ThermoFisher Scientific) in PBS. Secondary antibodies were diluted at 1:400 for α-actinin and 1:200 for pPKCe. Cells were counterstained using Vectashield Antifade mounting medium with DAPI (Vector Laboratories, Burlingame, CA). No changes were observed with antibodies to total-PKCe (ab124806, abcam) under any condition, and so no data are reported.

4.5 | Analysis of PKCe localization and phosphorylation using immunofluorescence

CMs grown in all conditions were fractionated to remove the membrane and cytosol but retain the cytoskeletal and nuclear fractions using the ProteoExtract Subcellular Proteome Extraction kit (MilliporeSigma, Billerica, MA) as described previously (Boateng et al., 2007; Lin et al., 2016; Ryba et al., 2017). CMs were then probed with anti-α-actinin and pPKCe. Phosphatase inhibitor (not standard with kit, #539131, MilliporeSigma) was included to preserve sites of phosphorylation. CMs were then probed with secondary antibodies and mounted. For each condition, intensity levels were normalized on a week-by-week basis to account for small variations in antibody dilutions or probing efficiency. Due to differences in optical properties, analysis was only conducted between cells grown on the same material/stiffness with the same antibody combination. For normalization, a maximum laser intensity and gain were found in untreated dishes and scaled back to account for potential experimental changes in levels, thus preventing peaking of fluorophore signal in subsequent dishes where changes might occur. This process was done for α-actinin and pPKCe. Following the setting of levels, all dishes within that stiffness and antibody combination were imaged at the same settings. Polarized, isolated CMs were chosen in an unbiased manner. Following acquisition, ImageJ (NIH, Bethesda, MD) and a custom MATLAB (MathWorks, Natick, MA) script were used for analysis. Five line scans of at least 5 sarcomeres within a single cell were taken of α-actinin and pPKCe. Peak intensities of each Z-disc localization were determined, averaged, and the ratio of pPKCe/α-actinin was calculated and recorded. At least 5 cells were analyzed for each condition per culture, coming from at least 3 separate cultures.

4.6 | Immunoblotting

The extraction kit was used per manufacturer protocol with the addition of phosphatase inhibitors as above. Samples were then prepared for western blot by adding 4X Laemmli sample buffer (BioRad, Inc., Hercules, CA). Protein extracts from sarcomeric subcellular fractions were resolved by SDS/PAGE and transferred to polyvinyl difluoride. Blots were blocked for 1 hr in 5% nonfat skim milk at room temperature and washed three times for 10 min each in tris-buffered saline, pH 7.5, containing 0.1% Tween-20. Blots were then probed in primary antibody overnight at 4 °C. Antibodies were diluted in 2.5% bovine serum albumin and used at the following dilutions: 1:1000 anti-pPKCe [S729] (#06–821, MilliporeSigma) and anti-sarcomeric α-actinin.

4.7 | Förster resonance energy transfer for molecular interactions of phosphorylated PKCe and CapZ

FRET interactions between CapZ-GFP and pPKCe by the acceptor photobleaching method (König et al., 2006) were used to determine nano-range interaction with higher resolution than can be determined with IF confocal microscopy of the Z-disc. CMs were infected with a mouse CapZp1-GFP adeno-associated virus (MOI 20), incubated for 1 hr, washed with media, and left overnight as described (Hartman...
et al., 2009). CMs were incubated with 2.5 μM isoproterenol for 1 hr. Dishes were treated with a cytosolic and membrane extraction buffer as described above. After gentle washing, CMs were fixed, incubated with a 1:100 dilution of anti-pPKCε antibody, a secondary antibody chosen because of its smaller size (F(ab′)2 anti-rabbit Alexa Fluor 555, A21430, ThermoFisher Scientific), a second formalin treatment to stabilize the acceptor probe, and mounted.

FRET response was determined by the acceptor photobleaching method. Serial scans of donor CapZ-GFP (exc: 488, em: 509) and acceptor (pPKCε secondary antibody, ex: 568, em: 603) signals were captured with alternating acceptor photobleaching steps in designated regions of interest (ROIs). Twenty scans taken over a 2- to 3-min time period were necessary to attain complete bleaching of the GFP donor necessary for the FRET analysis and curve fitting. Preliminary testing showing the optimal result was with an ROI of 100 × 500 μm². Average donor fluorescence intensities in the acceptor photobleached ROI (D), a reference ROI (Dᵣ), and a background ROI (Dᵦ) are used to compute the FRET efficiency Eᵢ in step i:

\[
E(i) = \left[1 - \frac{D(0) - Dᵣ}{D(i) - Dᵦ} \right]^2 \left(\frac{D(0)}{D(i)}\right) \times 100\%
\]

where D(0) and Dᵣ are the fluorescence intensities before the photobleaching sequence begins. The recovered transfer efficiency is estimated by extrapolation of E as a function of the acceptor intensity to the origin, in order to determine the intercept with the y-axis that represents the recovered transfer efficiency.

4.8 | Statistics

Statistical analysis of experiments involving substrata of varying stiffness was done using one-way ANOVA followed by Tukey’s multiple comparisons test. For experiments testing the response of cells grown on a single stiffness following isoproterenol treatment (3 or more groups), the untreated group was considered the control, and one-way ANOVA followed by Dunnett’s multiple comparisons test was used. Student’s t test was used in experiments comparing two groups.

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ORCID

Michael A. Mkrtschjan @ http://orcid.org/0000-0001-6185-1210
Christopher Solís @ http://orcid.org/0000-0003-4584-7363

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