Phosphatidylinositol 4,5-bisphosphate regulates CapZβ1 and actin dynamics in response to mechanical strain

Jieli Li and Brenda Russell
Department of Physiology and Biophysics, University of Illinois at Chicago, Chicago, Illinois

Submitted 12 June 2013; accepted in final form 10 September 2013

Li J, Russell B. Phosphatidylinositol 4,5-bisphosphate regulates CapZβ1 and actin dynamics in response to mechanical strain. Am J Physiol Heart Circ Physiol 305: H1614–H1623, 2013. First published September 16, 2013; doi:10.1152/ajpheart.00477.2013.—Mechanical stress causes filament remodeling leading to myocyte hypertrophy and heart failure. The actin capping protein Z (CapZ) tightly binds to the barbed end of actin filaments, thus regulating actin assembly. The hypothesis is that the binding between CapZ and the actin filament is modulated through phosphatidylinositol 4,5-bisphosphate (PIP2) and how the COOH-terminus of CapZβ1 regulates this binding. Primary neonatal rat ventricular myocytes (NRVMs) were strained at 10% amplitude and 1-Hz frequency. Dot blotting measured the PIP2 neonatal rat ventricular myocytes (NRVMs) were strained at 10% of green fluorescent protein-CapZ

Subcellular fractionation and antibody localization showed PIP2 decreased PIP2 level and its binding to CapZ. Therefore, the mechanistic responses to mechanical stress involve PIP2 signaling and CapZ (28). Hypertrophic remodeling to neurohumoral stimulation involves PIP2 signaling and CapZβ1 (15). Furthermore, according to the computational analysis, the COOH-terminus mutant of CapZβ1 has hydrophobic domains that might permit lipid binding to alter its interaction with actin (37). However, after mechanical stimulation, it is still not known whether PIP2 is directly involved in regulating CapZ, nor whether there is any subsequent control of actin assembly.

In strained muscle, the COOH-terminal extended ends of the α- and β-subunits of CapZ are the major actin-binding sites and act like two “tentacles” that are critical for actin capping (43). In vitro experiments show that phosphatidylinositol 4,5-bisphosphate (PIP2) regulates CapZ (16). Addition of PIP2 to actin filaments in a polymerization reaction leads to an increased assembly consistent with complete and rapid uncapping (20, 41). Myofilament tension generation is depressed by extraction of CapZ from cardiac myofilaments with PIP2, and tension can be restored to control level by addition of CapZ (28). Hypertrophic remodeling to neurohumoral stimulation involves PIP2 signaling and CapZβ1 (15). Furthermore, according to the computational analysis, the COOH-terminus subunit has hydrophobic domains that might permit lipid binding to alter its interaction with actin (37). However, after mechanical stimulation, it is still not known whether PIP2 is directly involved in regulating CapZ, nor whether there is any subsequent control of actin assembly.

In strained muscle, the COOH-terminal extended ends of the α- and β-subunits of CapZ are the major actin-binding sites and act like two “tentacles” that are critical for actin capping (43). In vitro experiments show that phosphatidylinositol 4,5-bisphosphate (PIP2) regulates CapZ (16). Addition of PIP2 to actin filaments in a polymerization reaction leads to an increased assembly consistent with complete and rapid uncapping (20, 41). Myofilament tension generation is depressed by extraction of CapZ from cardiac myofilaments with PIP2, and tension can be restored to control level by addition of CapZ (28). Hypertrophic remodeling to neurohumoral stimulation involves PIP2 signaling and CapZβ1 (15). Furthermore, according to the computational analysis, the COOH-terminus subunit has hydrophobic domains that might permit lipid binding to alter its interaction with actin (37). However, after mechanical stimulation, it is still not known whether PIP2 is directly involved in regulating CapZ, nor whether there is any subsequent control of actin assembly.

In strained muscle, the COOH-terminal extended ends of the α- and β-subunits of CapZ are the major actin-binding sites and act like two “tentacles” that are critical for actin capping (43). In vitro experiments show that phosphatidylinositol 4,5-bisphosphate (PIP2) regulates CapZ (16). Addition of PIP2 to actin filaments in a polymerization reaction leads to an increased assembly consistent with complete and rapid uncapping (20, 41). Myofilament tension generation is depressed by extraction of CapZ from cardiac myofilaments with PIP2, and tension can be restored to control level by addition of CapZ (28). Hypertrophic remodeling to neurohumoral stimulation involves PIP2 signaling and CapZβ1 (15). Furthermore, according to the computational analysis, the COOH-terminus subunit has hydrophobic domains that might permit lipid binding to alter its interaction with actin (37). However, after mechanical stimulation, it is still not known whether PIP2 is directly involved in regulating CapZ, nor whether there is any subsequent control of actin assembly.
treatment of neomycin, the increased kinetic rate of GFP-CapZ showed GFP-CapZ was significantly reduced. Means ± SE, n > 3. *P < 0.05. Bar = 10 μm.

Table 1. Recovery kinetics (K_frap) for CapZβ1 and actin under experimental conditions

<table>
<thead>
<tr>
<th>Experimental Conditions</th>
<th>CapZ K_frap (10^4 s⁻¹)</th>
<th>Actin K_frap (10⁻⁶ s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unstrained</td>
<td>1.97 ± 0.17</td>
<td>5.70 ± 0.71</td>
</tr>
<tr>
<td>Strained</td>
<td>3.96 ± 0.52*</td>
<td>9.66 ± 0.58*</td>
</tr>
<tr>
<td>Neomycin</td>
<td>1.90 ± 0.68</td>
<td>5.68 ± 1.86</td>
</tr>
<tr>
<td>Neomycin + strain</td>
<td>1.73 ± 0.60#</td>
<td>5.20 ± 0.498</td>
</tr>
<tr>
<td>Y27632</td>
<td>1.42 ± 0.17</td>
<td>5.72 ± 0.94</td>
</tr>
<tr>
<td>Y27632 + strain</td>
<td>1.10 ± 0.45#</td>
<td>3.76 ± 0.98#</td>
</tr>
<tr>
<td>C3 transferase</td>
<td>1.70 ± 0.65</td>
<td>6.08 ± 1.69</td>
</tr>
<tr>
<td>C3 transferase + strain</td>
<td>1.24 ± 0.40#</td>
<td>6.93 ± 0.84#</td>
</tr>
</tbody>
</table>

Values are means ± SE; CapZ, capping protein Z. *P < 0.05 vs. unstrained neonatal rat ventricular myocytes; #P < 0.05 vs. strained neonatal rat ventricular myocytes.

Continuous strain was blunted by intervention with PIP2 and RhoA, suggesting the importance of these molecules in regulation of physiological responses to strain.

MATERIALS AND METHODS

Ethical approval. All experiments were conducted following National Institutes of Health guidelines. All experiments were approved by the University Committee on the Use and Care of Animals at the University of Illinois at Chicago and the Biologic Resources Laboratory. All euthanasia was performed following the recommendations of the University Committee on the Use and Care of Animals and the Biologic Resources Laboratory and the Committee for the Human Use of Animals.

Cell culture. Hearts were removed and cells isolated from 1- to 2-day-old Sprague-Dawley rats with collagenase type II (Worthington, Lakewood, NJ) as previously described (6). NRVMs were resuspended, filtered through a metal sieve to remove large material, and plated at high density (1,000 cells/mm²) in PC-1 medium (Biowhitaker/Cambrex) on fibronectin-coated (25 μg/ml) FlexCell silicone membranes (200,000 cells/cm²). Cells were left undisturbed for 24 h in a 5% CO2 incubator. Unattached cells were removed by aspiration, and PC-1 media was replenished. Myocytes were incubated for another 24 h before the experiment.

Mechanical strain. Cyclic mechanical strain was generated with a Flexcell Strain Unit (Model FX-4000, Flexcell International, Hillsborough, NC). NRVMs in PC-1 medium were strained at 10% biaxially at 1 Hz for 1 (for signaling studies) or 48 h (for myocyte size) (36).

Adenovirus. Recombinant adenoviruses used were GFP, GFP-CapZβ1, GFP-CapZβ1-ΔC, and hemagglutinin-tagged dominant-negative RhoA (Asn19) (HA-RhoA-DN), as described in the original articles (4, 11, 17, 21). NRVMs were infected with CapZβ1, GFP-CapZβ1-ΔC (multiplicity of infection, 20), or HA-RhoA-DN (multiplicity of infection, 20) for 60 min at 37°C diluted in PC-1 medium. Viral medium was then replaced with virus-free PC-1 medium for 24 h as previously described (15, 21).

Actin-GFP expression. Actin-GFP expression was induced by CellLight-Rightegens BacMam 2.0 Actin-GFP (Invitrogen). As previously described (21), NRVMs were transfected according to the manufacturer’s instructions. Infected NRVMs were returned to the culture incubator for at least 24 h. Transfected NRVMs exhibited an actin organization with a striated structure in myocytes. Furthermore, colocalization of actin-GFP and phalloidin indicated that the actin-GFP was successfully incorporated into all the F-actin structures within NRVMs (21). Thus actin-GFP can be considered to reflect the structure and behavior of F-actin accurately in transfected myocytes.

Inhibitors. Neomycin has a high affinity for PIP2 and readily sequesters any available PIP2 in the cellular milieu, rendering it unavailable for its normal physiological roles (2). C3 transferase...
inhibits Rho proteins by ADP-ribosylation on asparagine 41 in the effector binding domain of the GTPase, thereby blocking their action. NRVMs were pretreated with C3 transferase (0.25 μg/ml) or ROCK inhibitor Y27632 (10 μmol/l) for 1 h, or neomycin (500 μM) for 4 h, and then strained for 1 h.

**Dot blots.** Whole cell lysates were extracted from NRVMs in each experimental condition, spotted onto nitrocellulose membrane, probed with PIP2 antibody (1:500, mouse IgG, Abcam, Cambridge, MA), GAPDH (1:1,000, Sigma), and detected using a horseradish peroxidase-conjugated secondary antibody (anti-mouse, horseradish peroxidase, Cell Signaling Technology, Boston, MA) and enhanced chemiluminescence.

**Subcellular fractionation and localization of PIP2.** For subcellular fractionation of myocytes, the Calbiochem ProteoExtract Subcellular Proteome Extraction Kit was used (EMD Millipore, Billerica, MA). This method uses a previously described detergent-based protocol (5). Cellular proteins were sequentially extracted into four compartments: cytosolic, membrane/organelles, nuclei, and cytoskeleton. Briefly, NRVMs (~1 × 10^6/well) were seeded in Flexcell plates. After 1 h cyclic strain, NRVMs were permeabilized with Digitonin-EDTA in buffer I and kept at 4°C for 10 min (releasing cytosolic proteins without solubilizing membrane proteins). The supernatant was collected as the cytosolic fraction. Subsequently, the NRVMs were extracted with Triton-EDTA in buffer II and kept at 4°C for 30 min. The supernatant contained the membrane fraction. The NRVMs were next recovered with Tween 20-deoxycholate-benzonase in buffer III and kept at 4°C for 10 min. The supernatant contained the nuclear fraction. Finally, the cytoskeleton remaining was immunostained with a PIP2 antibody (1:200, mouse IgG, Abcam) and α-actinin antibody (1:200, Cell Signaling Technology, Danvers, MA). Cells were briefly washed three times in PBS between each extraction fraction to prevent cross contamination. After each fraction, cells were observed by microscopy to ensure that they were still attached to the dish. Cell integrity is maintained throughout the fractionation process. The accuracy of the fractionation method was verified with antibodies to well-documented subcellular distribution markers (heat shock protein 70 for cytosol, 1:200; β-integrin for membrane, 1:1,000; and Histone H2B for nuclei, 1:500) (anti-heat shock protein 70 from Santa Cruz Biotechnology, Dallas, TX; anti-β-integrin from EMD Millipore; and anti-Histone H2B from Imgenex, San Diego, CA).

**PIP2 bead affinity precipitation.** NRVMs were transfected with adeno-GFP, adeno-GFP-CapZβ1, or GFP-CapZβ1-ΔC. The myocytes were washed in PBS and lysed and sonicated in 500 μl of wash/bind buffer containing 10 mM HEPES (pH 7.4), 0.25% Nonidet P-40, and 150 mM NaCl. After sonication and centrifugation at 10,000 g for 10 min in 4°C, 50 μl of 50% PIP2-conjugated agarose beads (Echelon Bioscience, Salt Lake City, UT) in slurry were added to the supernatant. After overnight incubation at 4°C, beads were removed by centrifugation, washed three times with wash/bind buffer to remove unbound proteins, and then washed with lysis buffer (1% Triton X-100, 1% Nonidet P-40, 50 mM NaCl, 0.1 M KCl, 50 mM HEPES (pH 7.4), 0.5 mM PMSF, 0.5 mM sodium vanadate, and 0.5 mM sodium orthovanadate) to remove lysate proteins. The PIP2 beads were then eluted with 20 μl of 150 mM NaCl, 50 mM HEPES (pH 7.4), 0.5% Nonidet P-40, and 0.5% Triton X-100. The eluate was analyzed by Western blotting with anti-CapZ and anti-α-actinin antibodies. The gel image was captured using a phosphor imager, and the band intensities were quantified using ImageJ software.

---

**Fig. 2.** Increased dynamics of CapZβ1 in NRVMs after 1 h of cyclic strain depends on the RhoA/ROCK pathway. *A:* microscopic images of living NRVMs infected with GFP-CapZβ1, RhoA-inhibited (C3 transferase), ROCK inhibited (Y27632) in no-strain or strained groups. FRAP of ROI for GFP-CapZβ1 before, immediately after, and 30 min after photobleaching showed that both C3 transferase and Y27632 attenuated responses in the strained groups. **B:** **K**<sub>FRAP</sub> with all groups normalized to no-strain group showed GFP-CapZβ1 in the strained group had increased kinetic rates. With the treatment of C3 transferase or Y27632, the increased kinetic rate of GFP-CapZβ1 in strained myocytes was significantly reduced. Means ± SE; n > 3. *P < 0.05. Bar = 10 μm.
washed three times in wash/bind buffer. The proteins were eluted from the PIP$_2$ beads by heating at 50°C for 10 min in 2× SDS-PAGE buffer. CapZβ1 or CapZβ1-ΔC was detected by anti-GFP (mouse, 1:1,000, Enzo Life Sciences). The bands of Western blot analysis are detected with an imager (Bio-Rad, Hercules, CA).

**Analysis of fluorescence recovery after photobleaching.** Recently, several microscopic techniques, such as analysis of fluorescence recovery after photobleaching (FRAP) (29, 15), have begun to yield important qualitative and quantitative information on the processes that promote and regulate actin assembly in living myocytes. For FRAP of GFP-CapZβ1, at least five well-behaved and strained myocytes (as evidenced by GFP-CapZ tag) were randomly selected for each experiment. The GFP fusion protein was irreversibly bleached by laser excitation (488 μm) at full power in a uniform square region of interest (ROI) lying midway between the myocyte nucleus and periphery. The intensity of the ROI was observed both before ($t_0$) and immediately after ($t_1$) bleaching and intermittently every 2 min until the end of the 30 min. Images were analyzed using Zeiss Imaging Browser. Plotted intensity values are given as a percentage of the difference between ROI ($t_0$) and ROI ($t_1$). Curves were fitted using nonlinear regression for two binding states in OriginPro (OriginLab, Northampton, MA):

$$ROI(t) = 1 - C_1 e^{-K_{off1}t} - C_2 e^{-K_{off2}t}$$  \[(1)\]

Average kinetic constant (K$_{frap}$) for dynamics was calculated using the following formula:

$$K_{frap} = C_1K_{off1} + C_2K_{off2}$$  \[(2)\]

For FRAP of actin-GFP, the ROI was observed both before ($t_0$) and immediately after ($t_1$) bleaching, and intermittently every 20 s until the end of the 8-min study, referred to the recovery time of actin-GFP in previous study (3). Also, actin has one binding state. Thus the equation for curve fitting using nonlinear regression in OriginPro was

$$ROI(t) = 1 - C_1 e^{-K_{off1}t}$$  \[(3)\]

And K$_{frap}$ was calculated using the following formula:

$$K_{frap} = C_1K_{off1}$$  \[(4)\]

**Statistics.** Data are presented as means ± SE. Statistical significance was determined by one-way ANOVA. Significance was taken at $P < 0.05$.

**RESULTS**

**Increased CapZβ1 dynamics induced by mechanical strain depend on the PIP$_2$ pathway.** The GFP-CapZβ1 had strong striations in NRVMs (Fig. 1A), and signals were detected in ROI (Fig. 1B). The FRAP experiments revealed differences after 1 h of 10% 1-Hz cyclic strain (Fig. 1B, and Table 1). The GFP-CapZβ1 had faster dynamics in strained myocytes compared with the unstrained group (3.96 ± 0.52 vs. 1.97 ± 0.17, $\times10^{-3}$s$^{-1}$, $P < 0.05$), meaning that a faster protein exchange was occurring in strained myocytes. Notably, strained myocytes treated with neomycin (a known PIP$_2$ scavenger), had dynamics of GFP-CapZβ1 that were significantly slower than strained myocytes (1.73 ± 0.60 vs. 3.96 ± 0.52, $\times10^{-3}$s$^{-1}$, $P < 0.05$), but no significance was found in unstrained myocytes treated with neomycin alone (1.73 ± 0.60 vs. 1.90 ± 0.68, $\times10^{-3}$s$^{-1}$) (Fig. 1B). Thus PIP$_2$ inhibition abrogated strain-induced alterations in CapZβ1 kinetics, suggesting the rate of CapZβ1 exchange after mechanical stimulation was dependent on the PIP$_2$ level and signaling.

**Increased CapZβ1 dynamics induced by mechanical strain depend on the RhoA/ROCK pathway.** The effect of C3 transferase (RhoA inhibitor) or Y27632 (ROCK inhibitor) on CapZβ1 dynamics induced by mechanical strain in NRVMs was examined using FRAP of CapZβ1 transfected myocytes (Fig. 2A). C3 transferase markedly inhibited the strain-induced increases of CapZβ1 dynamics (1.24 ± 0.40 vs. 3.96 ± 0.52, $\times10^{-3}$s$^{-1}$, $P < 0.05$) (Fig. 2B). Signaling by ROCK was assessed. A RhoA downstream serine kinase involved in the actin filament organization, mediated strain-induced increased CapZβ1 dynamics in NRVMs. The increased CapZβ1 dynamics induced by strain were markedly reduced after Y27632 treatment (1.10 ± 0.45 vs. 3.96 ± 0.52, $\times10^{-3}$s$^{-1}$, $P < 0.05$), but no change was observed in Y27632 treated myocytes that were not strained (1.10 ± 0.45 vs. 1.42 ± 0.17, $\times10^{-3}$s$^{-1}$).

**Increased actin dynamics induced by mechanical strain depend on both PIP$_2$ and RhoA/ROCK pathways.** The actin-GFP had strong striations in NRVMs, and signals were detected in ROI (Fig. 3, A and B). After 1 h of 10% 1-Hz cyclic strain, the actin-GFP had a faster dynamic protein exchange in strained compared with the unstrained myocytes (9.66 ± 0.58 vs. 5.70 ± 0.71, $\times10^{-4}$s$^{-1}$, $P < 0.05$) (Fig. 3C). The increased dynamics of actin-GFP had increased CapZ dynamics induced by mechanical strain in NRVMs calculated using FRAP of CapZβ1 transfected myocytes (Fig. 2A). C3 transferase markedly inhibited the strain-induced increases of CapZβ1 dynamics (1.24 ± 0.40 vs. 3.96 ± 0.52, $\times10^{-3}$s$^{-1}$, $P < 0.05$) (Fig. 2B). Signaling by ROCK was assessed. A RhoA downstream serine kinase involved in the actin filament organization, mediated strain-induced increased CapZβ1 dynamics in NRVMs. The increased CapZβ1 dynamics induced by strain were markedly reduced after Y27632 treatment (1.10 ± 0.45 vs. 3.96 ± 0.52, $\times10^{-3}$s$^{-1}$, $P < 0.05$), but no change was observed in Y27632 treated myocytes that were not strained (1.10 ± 0.45 vs. 1.42 ± 0.17, $\times10^{-3}$s$^{-1}$).
actin-GFP seen with neomycin treatment of strained myocytes was markedly reduced compared with untreated, strained myocytes (5.20 ± 0.49 vs. 9.66 ± 0.58, ×10⁻⁴ s⁻¹, P < 0.05). With the inhibition of RhoA or ROCK pathway (treated with C3 transferase or Y27632), the dynamics of actin-GFP were significantly slower than strained myocytes (6.93 ± 0.84 or 3.76 ± 0.98 vs. 9.66 ± 0.58, ×10⁻⁴ s⁻¹, P < 0.05). The FRAP experiments demonstrated that the dynamic exchange of actin-GFP depended on PIP2 and the RhoA/ROCK pathways after cyclic strain.

*Increased PIP2 production with mechanical strain depend on the RhoA/ROCK pathway.* The PIP2 production was significantly increased in NRVMs after a 1-h period of cyclic strain (2.22 ± 0.26 normalized to control of untreated, unstrained cells) (Fig. 4, A and B). The strain-induced increase in PIP2 (2.22 ± 0.26) was markedly reduced by preventing RhoA/ROCK signaling through pharmacological inhibition with C3 exoenzyme (1.33 ± 0.03) or the ROCK-specific inhibitor Y-27632 (0.98 ± 0.01). However, no change was observed in basal PIP2 expression in neomycin-treated, unstrained myocytes (Fig. 4, A and B).

The RhoA inhibitor C3 exoenzyme inhibits not only RhoA but also RhoB and RhoC. To further evaluate the involvement of RhoA in strain-induced PIP2 production, NRVMs were transiently transfected with adenovirus of HA-RhoA-DN. Myocytes expressing RhoA were identified by positive anti-HA-tag staining (data not shown). The control group of NRVM was transiently transfected with the GFP adenovirus. Most transfected myocytes showed clear striations when stained for actin, and only a few nonstriated fibroblasts were transfected (data not shown). Thus the majority of transfected cells were myocytes in both unstrained and experimental groups. Expression of RhoA-DN significantly attenuated (1.09 ± 0.18) the amount of PIP2 induced by mechanical strain (2.85 ± 0.32, P < 0.05) (Fig. 4, C and D). Thus these data strongly suggested a role for RhoA in regulating strain-induced PIP2 production in cardiac myocytes.

**Sarcomeric distribution of PIP2 in cultured neonatal myocytes.** The fractions were collected in the order of cytosolic, membranes/organelles, and nuclei. For a biochemical analysis, subcellular fractions of cardiac myocytes were analyzed by Western blot analysis using proteins with known subcellular localization for verification (Fig. 5A). Detergent-based subcellular fractionation could be successfully used to isolate the main compartments of cultured myocytes while maintaining cell attachment and integrity.

Redistribution of PIP2 was determined under the experimental conditions of cyclic strain. The cytoskeleton remained after...
removal of membranes, cytosol, and nuclei and was observed by microscopy to determine the subcellular location of PIP2. Indeed, PIP2 was distributed in a striated pattern colocalized with \( \beta \)-actinin at the Z disc (Fig. 5B).

**CapZ binding to PIP2.** Strong interaction between PIP2 and CapZ was found using PIP2 beads, (Fig. 6A), whereas GFP alone did not interact with PIP2 beads. Significantly more GFP-CapZ bound to PIP2 in strained (1.47 ± 0.17) than in unstrained cardiac myocytes (\( P < 0.05 \)) (Fig. 6B). Thus CapZ was a PIP2-interacting protein, which depended on mechanical strain.

To test the effect of the \( \beta \)-tentacle on PIP2 binding, GFP-CapZ-ΔC was transfected in NRVMs and proteins were affinity precipitated with PIP2-conjugated beads. Over threefold more GFP-CapZ-ΔC bound to PIP2, compared with GFP-CapZ (Fig. 7, A and B).

**Attenuation of myocyte hypertrophy induced by mechanical strain with inhibition of RhoA or PIP2.** To determine if the effects of PIP2 on cardiac hypertrophy could be attenuated, NRVMs were pretreated with HA-RhoA-DN, GFP, or neomycin and subjected to cyclic strain for an additional 48 h. HA-RhoA-DN or neomycin alone had no effects on myocardial size or phenotype (data not shown), indicating that the effect of HA-RhoA-DN or neomycin was not secondary to a toxic cellular effect. Cyclic strain (48 h) induced approximately a 38% increase in myocyte size, which was inhibited by neomycin pretreatment (Fig. 8). Similarly, the marked increase in myocyte size induced by cyclic strain was also significantly inhibited by HA-RhoA-DN (Fig. 8). These results suggested that HA-RhoA-DN or neomycin attenuated the cardiac myocyte hypertrophy induced by cyclic strain.

**DISCUSSION**

The present study demonstrates that 1 h of cyclic strain increases the dynamics of CapZ and actin in NRVMs and that these changes depend on the PIP2 pathway. Furthermore, PIP2 production increases after cyclic strain and is dependent on the RhoA/ROCK pathway. PIP2 redistributes to the sarcomeric cytoskeleton, and more PIP2 is bound to CapZ after cyclic strain. With deletion of COOH-terminal of CapZ, more CapZ-ΔC binds to PIP2, which shows that the tentacle is not the major binding site of PIP2. However, its removal appears to release an inhibition enabling higher affinity PIP2 binding to the remainder of the CapZ molecule, suggesting the interaction of PIP2 and CapZ affects the COOH-terminal binding affinity to CapZ, thereby affecting actin assembly. Cardiac hypertrophy caused by 48 h cyclic strain is blunted by inhibition of PIP2 pathways. A potential scheme for how PIP2 regulates actin assembly under mechanical strain is shown diagrammatically in Fig. 9. After a 1-h period of cyclic strain, focal adhesion kinase (FAK) activates...
the RhoA/ROCK pathway, which activates phosphatidylinositol 4-phosphate 5-kinase (PIP5K) to produce more PIP2. Some of the extra PIP2 binds to CapZ increasing the CapZ dynamics, and resulting in a more rapid actin exchange. This suggests that after many hours of cyclic strain, a possible mechanism for cell hypertrophy is the accumulation of thin filament assembly triggered partially by the increased PIP2 level and its binding to CapZ.

The mechanical signals are transmitted from the extracellular matrix via integrins to the cytoskeleton. PIP2 has been implicated in the regulation of focal adhesions during changes in cell attachment to the extracellular matrix. PIP2 activates several key focal adhesion components including vinculin and talin (7, 13, 32). These signaling pathways are activated by forces delivered from the outside of the cell or to those generated internally with both directions leading to actin filament assembly at the focal adhesions (18).

Fig. 6. PIP2 binding to CapZβ1 increases after 1 h of cyclic strain. A: PIP2-conjugated beads were used to affinity precipitate GFP-CapZβ1. The bound CapZβ1 was detected with anti-GFP antibody by Western blot analysis. Total CapZβ1, as well as GFP, in whole lysate were detected with anti-GFP antibody in no-strain and strained NRVMs. After 1 h cyclic strain (10%, 1 Hz), more CapZβ1 bound to PIP2. B: Western blot analysis normalized to the no-strain NRVMs. Means ± SE; n = 3. *P < 0.05.

Fig. 7. PIP2 binding increases with the CapZ terminal deletion (CapZβ1ΔC). A: PIP2-conjugated beads were used to affinity precipitate GFP-CapZβ1 or GFP-CapZβ1ΔC. The bound CapZβ1 or CapZβ1ΔC was detected with anti-GFP antibody by Western blot analysis. Total CapZβ1, CapZβ1ΔC, or GFP in whole lysate were detected with anti-GFP antibody in NRVMs. More CapZβ1ΔC bound to PIP2, compared with CapZβ1. B: Western blot analysis normalized to the CapZβ1 group. Means ± SE; n = 3. *P < 0.05.
which PIP2 regulates CapZ and actin kinetics. However, the removal of the tentacle enhances PIP2 binding to the capping portion of CapZβ1. The COOH-terminus inhibits the interaction of PIP2 and CapZβ1. Thus the interaction of PIP2 and CapZβ1 affects the actin binding affinity, which may be partially responsible for changes in the dynamics of CapZ and actin measured by FRAP. The mechanism of structure of CapZβ1 binding with PIP2 needs to be further studied.

PIP2 has a direct role in regulating actin assembly by interaction with partnering proteins in many cells. In platelets, the half-life for a capped filament is 28 min, whereas the half-life to remain uncapped is only 0.2 s. Interestingly, the addition of PIP2 in these platelets reduces the half-life of the capped filament to 46 s, suggesting PIP2 regulation of actin filament capping dynamics (34). Gelsolins (calcium-dependent actin-binding proteins) exposed to high lipid concentrations in vitro can bind PIP2 molecules (40). Also in vitro, PIP2 affects actin dynamics by direct association with the actin-binding protein villin, resulting in unstable F-actin (38). PIP2 also binds to α-actinin and inhibits α-actinin bundling activity (10, 12). More PIP2 colocalizes with α-actinin at Z disc after mechanical strain (Fig. 5). Thus PIP2 is critical to actin filament regulation by interacting with actin binding proteins in many situations.

Cyclic strain of cardiomyocytes evokes many other intracellular signals that work in parallel to PIP signaling that lead to cardiomyocyte hypertrophy (1). One key component of the integrin complex is FAK, which is also part of the mechano-transduction signaling pathway (30). In mechanically strained cardiac myocytes, FAK phosphorylation at Y397 rapidly activates RhoA (39). PIP2 is generated by PIP5K which phosphor-ylates phosphatidylinositol 4-phosphate at the D-5 position of PI(4)P to produce PIP2 and increases the PIP2 level. Some of the increased PIP2 colocalizes with α-actinin in the Z disc. More PIP2 binds to CapZ, resulting in an increase in the kinetics of CapZ and also increase in the kinetics of actin. This suggests that after many hours of cyclic strain a possible mechanism for cell hypertrophy is the accumulation of thin filament assembly triggered partially by the increased PIP2 level and its binding to CapZ.
the inositol ring. PIP5K is also directly or indirectly activated by RhoA and its effector ROCK (26, 44). Cellular and molecular biology studies have indicated a pivotal role of RhoA/ROCK signaling in cardiac hypertrophy. RhoA and ROCK are rapidly activated by mechanical strain in cardiac myocytes (39). The RhoA/ROCK pathway is important for setting the PIP2 level (Fig. 4). However, the direct mechanism of RhoA on regulating PIP2 may be very complex and need to be further studied. To date, at least 15 RhoA effector proteins have been identified. Some of these are putative protein kinases and lipid kinases, such as 1,2-diacylglycerol kinase, phosphatidylinositol 3-kinase, and PIP5Ks (22). Nonetheless, after mechanical strain, activated RhoA/ROCK produces PIP2, which interacts with CapZ and subsequently alters cardiomyocyte cell size by mechanisms that are blocked by RhoC inhibition.

A complication in understanding the response to strain is that it is known to increase the intracellular Ca2+ concentration through strain-activated membrane channel opening (31). Ca2+ is an essential cofactor for both MAPK and protein kinase C signaling pathways that induce cardiac hypertrophy (24). However, CapZ is insensitive to Ca2+ (25); therefore, changes in dynamics cannot be directly due to altered calcium.

It is clear that PIP2 plays a major role in hypertrophic responses, since in our present study, removal of PIP2 blocked the increase in cell size normally found by two days of cyclic strain. A brief, 1-h period of cyclic strain is sufficient to initiate PIP2 binding to CapZ, modulate actin dynamics, and thereby regulate actin cytoskeleton assembly. While the Z disc is not a lipid membrane, it does have the capacity to bind PIP2 since it is known to increase the intracellular Ca2+ concentration by modulating actin dynamics, and thereby regulating PIP2, which interacts with CapZ and subsequently alters cardiomyocyte cell size by mechanisms that are blocked by RhoC inhibition.

ACKNOWLEDGMENTS

We thank Dr. Allen M. Samarel (Loyola University Chicago Stritch School of Medicine, Maywood, IL) for the gift of the CapZ DNA constructs and Dr. Viswanathan Natarajan (Department of Pharmacology College of Medicine, University of Illinois at Chicago) for the gift of Rho A-DN constructs. We thank Erik Swanson and Dr. Ke Ma for expert assistance with the FRAP experiments.

GRANTS

This work was supported by National Heart, Lung, and Blood Institute Grant HL-62426.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

J.L. conception and design of research; J.L. performed experiments; J.L. analyzed data; J.L. and B.R. interpreted results of experiments; J.L. prepared figures; J.L. drafted manuscript; B.R. edited and revised manuscript; B.R. approved final version of manuscript.

REFERENCES


5. Boateng SY, Belin R, Deenen DL, de Ridder KM, Arigoni P, Boelsterli UA. Increased in cell size normally found by two days of cyclic strain, activated RhoA/ROCK produces PIP2, which interacts with CapZ and subsequently alters cardiomyocyte cell size by mechanisms that are blocked by RhoC inhibition.


