CapZ and actin capping dynamics increase in myocytes after a bout of exercise and abates in hours after stimulation ends

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THE HEART MUSCLE REMODELS to the demands of exercise by exercise and abates in hours after stimulation ends. J Appl Physiol 114: 1603–1609, 2013. First published March 14, 2013; doi:10.1152/japplphysiol.01283.2012.—The time course of the response and recovery after acute activity seen in exercise is not well understood. The goal of this work is to address how proteins of the thin filament (actin and its capping protein CapZ) are changed by 1 h of mechanical stimulation and return to baseline over time. Neonatal rat ventricular myocytes in culture were subjected to cyclic 10% strain at 1 Hz for 1 h to mimic increased mechanical loading during exercise. CapZ and actin dynamics were analyzed by fluorescence recovery after photobleaching (FRAP) using CapZβ1-GFP, actin-GFP, or actin-RFP. After cyclic strain, CapZ dynamics increased above resting controls and abated 2–3 h after cessation of the cyclic strain. Similarly, actin dynamics initially increased and abated 1.5–2 h after the end of stimulation. Neurohormonal hypertrophic stimulation by phenylephrine or norepinephrine treatments also increased above resting controls and abated 2–3 h after cessation of the cyclic strain. Similarly, actin dynamics initially increased and abated 1.5–2 h after the end of stimulation. Neurohormonal hypertrophic stimulation by phenylephrine or norepinephrine treatments also increased above resting controls and abated 2–3 h after cessation of the cyclic strain. Similarly, actin dynamics initially increased and abated 1.5–2 h after the end of stimulation.

The goal of these experiments using neonatal rat ventricular myocyte (NRVM) in culture is to find how CapZ regulates sarcomeric actin filament remodeling with cyclic strain and return to baseline after stimulation ends. The hypothesis is that 1 h of mechanical stimulation is sufficient to regulate actin dynamics by CapZ in NRVM, which return to the control level over time. First, the dynamics of CapZ in NRVM are analyzed after 1 h of cyclic mechanical strain, and the time course is tracked for the next 3 h. Second, the dependence of actin dynamics on CapZ is measured. Finally, a CapZ β tentacle mutant determines its role in both CapZ and actin dynamics.

MATERIALS AND METHODS

Cell culture. Primary heart cultures were obtained from neonatal rats according to Institutional Animal Care and Use Committee and National Institutes of Health guidelines for the care and use of laboratory animals. Hearts were removed and cells were isolated from 1- to 2-day-old neonatal Sprague-Dawley rats with collagenase (Worthington), as previously described (6). The cells were re-suspended, filtered through a metal sieve to remove large material, and plated at high density (1,000 cells/mm²) in PC-1 medium (Biowhittaker/Cambridge, MA) with a 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 (16, 33). The flexibility of the β tentacle alters the mushroom structure, resulting in modified interactions with other proteins (9). Mutations or deletion of the β tentacle significantly affect capping properties (4, 17, 33). Also, the transgenic mouse with a β-tentacle mutation had impaired cardiac muscle structure (13). Thus the β tentacle is critical for CapZ-mediated actin filament assembly.

The actin cytoskeleton in mature muscle cells is a very stable structural component. Nonetheless, mature sarcomeric actin filaments in muscle cells incorporate G-actin within minutes, indicating their dynamic nature (11, 22). In recent studies, the dynamics of non-sarcomeric β-actin increased in adult cardiac myocytes under neurohormonal stimulation (3), but stimulation of α-sarcomeric actin has not been explored.

Pressure or volume overloading, such as aortic stenosis or increased peripheral resistance, initiates cardiac remodeling. When loads are encountered, cardiac myocytes integrate extracellular, intercellular, and intracellular forces into intracellular signals through the mechanotransduction pathways (21). However, destination for the signaling cascades and how filament assembly and sarcomere remodeling occur are less well understood. CapZ capping is dynamic, as assessed by fluorescence recovery after photobleaching (FRAP) of CapZβ1-GFP (14). That study also found the dynamics of CapZ were elevated by neurohormonal stimulation such as phenylephrine (PE) and endothelin-1 (ET1) through a PKC- and PIP2-dependent pathway. Also, although calcium signaling plays an important role in mechanical stimulation of myocyte, CapZ is not modified by intracellular calcium (15).

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brinectin 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. Cells were left in incubator for another 24 h before the experiment.

Mechanical strain. Seventy-two hours after cell isolation, with or without viral infection, cyclic mechanical strain was generated with a Flexcell Strain Unit (model FX-4000, Flexcell International, Hillsborough, NC). NRVMs were strained at 10% elongation biaxially at 1 Hz sinusoidally for 1 h in PC-1 medium. Strain magnitude, time, and waveform were user-assigned to the system that controls vacuum pressure to deliver calibrated strain values to available elastic substrates housed in the incubator.

Neurohormonal hypertrophic stimulation. Control cells were either stimulated or left unstimulated before photobleaching. The neurohormonal treatment times chosen were sufficient to induce hypertrophy after photobleaching (FRAP) of CapZβ1-GFP as a percentage of prebleach intensity with unstrained control (dashed line, Non-S) and within 1 h after cyclic strain ended [solid line, S (0–1 h)]. D: $K_{frap}$ values for CapZβ1 normalized to unstrained cells; 0 to −1 h ($n = 5$), 1 to −2 h ($n = 4$), and 2 to −3 h ($n = 4$). Values are means ± SE. *Significant difference ($P < 0.01$).

Fig. 1. Time course of CapZβ1 dynamics after cyclic mechanical strain. Neonatal rat ventricular myocytes (NRVMs) infected by CapZβ1-GFP were either unstrained or subjected to 1-Hz cyclic 10% strain for 1 h. A and B: confocal images of CapZβ1-GFP with FRAP shown before bleach (0 s), after bleach (2 s), and 15 min later in cells in the following conditions: A, unstrained with resting spontaneous beating or B, 1 h after cyclic strain has ended. Region of interest (ROI) shown as dashed white boxes (3.75 μm × 3.75 μm). Insert: higher magnification images of area delineated by the solid white boxes in the lower magnification image. C: fluorescence recovery after photobleaching (FRAP) of CapZβ1-GFP as a percentage of prebleach intensity with unstrained control (dashed line, Non-S) and within 1 h after cyclic strain ended [solid line, S (0–1 h)]. D: $K_{frap}$ values for CapZβ1 normalized to unstrained cells; 0 to −1 h ($n = 5$), 1 to −2 h ($n = 4$), and 2 to −3 h ($n = 4$). Values are means ± SE. *Significant difference ($P < 0.01$).
Immunostaining and confocal microscopy: NRVMs were washed with PBS, fixed with 4% paraformaldehyde (Sigma Aldrich) for 10 min, placed in cold 70% ethanol, and stored at −20°C until immunostaining. Cells were rehydrated in PBS (Sigma Aldrich) for 10 min. After rehydration, cells were incubated on a shaker table in 1% BSA in PBS for 1 h at 25°C. Primary α-actinin (AbCam) antibody was diluted (1:250) in 1% BSA in PBS. The antibody was allowed to incubate on a shaker table at 4°C overnight. Cells were then rinsed in PBS at 25°C and blocked in 1% BSA in PBS for 1 h at 25°C. Secondary antibody (Molecular Probes) was diluted at a ratio of 1:500 in 1% BSA in PBS and incubated for 1 h at 25°C. Cells were washed in PBS. Anti-fade reagent with DAPI (Molecular Probes) was added, and cover slips were mounted on glass slides.

Analysis of fluorescence recovery after photobleaching. Fluorescence recovery after photobleaching (FRAP) analysis was done as in previous publications (14, 18, 29). For FRAP of CapZβ1-GFP, up to 10 beating and well striated cells (as evidenced by CapZβ1-GFP) were randomly selected for each FRAP. FRAP images were obtained by a Zeiss LSM 710 confocal microscope. The intensity (I_{frap}) of the region of interest (ROI; area = 3.75 × 3.75 μm) was observed both before (t₀) and immediately after (t₁) bleaching at full power. Data were collected every 2 min because of the slow dynamics of CapZ (24) with lower excitation power (1 to ~3%) and 800- to ~1,000-ms duration until the end of the 15 min. Images were analyzed using the Zeiss Imaging Browser. Plotted intensity values are given as a percentage of the difference between I_{frap} (t₀) and I_{frap} (t₁). Binding of CapZ to the actin filament has two binding states (30), so FRAP curves of CapZ were fit using nonlinear regression in OriginPro (OriginLab, Northampton, MA):

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

(1)

The average kinetic constant (K_{frap}) for dynamics was calculated using the following formula:

Fig. 2. Time course for actin dynamics after cyclic mechanical strain. NRVMs infected by actin-GFP were either unstrained or subjected to 1-Hz cyclic 10% strain for 1 h. A and B: confocal images of actin-GFP with FRAP shown before bleach (0 s), after bleach (2 s), and 8 min later in cells in the following conditions: A, unstrained with resting spontaneous beating B, 1 h after cyclic strain has ended. Regions of interest (ROIs) are shown as dashed white boxes (3.75 × 3.75 μm). Inset: higher magnification images of area delineated by the solid white boxes in the lower-magnification image. C: FRAP of actin-GFP as a percentage of prebleach intensity with unstrained control (dashed line, Non-S) and within 1 h after cyclic strain ended [solid line, S (0–1h)]. D: K_{frap} values for actin-GFP normalized to unstrained cells; 0 to −0.5 h (n = 6), 0.5 to −1 h (n = 5), 1 to −1.5 h (n = 4), 1.5 to −2 h (n = 8), and 2 to −3 h (n = 7) after strain were compared. Values are means ± SE. Significant difference: *P < 0.01; #P < 0.05.

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The average kinetic constant \( K_{\text{frap}} \) was calculated using the following formula:

\[
K_{\text{frap}} = C_1 K_{\text{off1}} + C_2 K_{\text{off2}}
\]  

For FRAP of actin-GFP and actin-RFP, data were collected every 10 s (actin-GFP) or 20 s (actin-RFP) because of the higher dynamics of the actin (20) until the end of the 8 min (28). Since actin binding has only one binding state (23), the equation for curve fitting using nonlinear regression in OriginPro was:

\[
I_{\text{frap}}(t) = 1 - C_1 e^{-K_{\text{off1}}t}
\]  

Statistics. Data are presented as means ± SE. For FRAP assays, the sample number was defined as individual cells, of which one to five cells were analyzed per culture in at least three separate cultures analyzed per experimental condition. Statistical significance was determined for FRAP curves by Student’s t-test. Significance was taken at \( P < 0.01 \) or \( P < 0.05 \) as indicated.

RESULTS

Effects of cyclic mechanical strain on sarcomeric CapZ dynamics. To test the potential of mechanical stimulation to induce thin-filament remodeling, the dynamics of the CapZ was measured by FRAP in NRVMs with or without 1 h of 1-Hz, 10% cyclic strain (Fig. 1, A and B). Within 1 h after cyclic strain, the FRAP profile was altered significantly (Fig. 1C). The kinetic constant \( K_{\text{frap}} \) gained from the results of curve fitting (Eq. 2) was significantly increased by \( \approx 80\% \) within 1 h after cyclic strain (Fig. 1D and supplemental Table 1 available online at the Journal of Applied Physiology website) compared with nonstrained cells. The increased CapZ1 dynamics abated after cessation of cyclic strain and returned to the control level by 2 h after the end of strain (Fig. 1D).

Effects of cyclic mechanical strain on sarcomeric actin dynamics. Because of the role of CapZ in the regulation of actin filament assembly, the dynamics of the sarcomeric actin may also be altered. NRVMs infected with actin-GFP fusion protein showed the striated pattern, permitting dynamics of actin-GFP gained from the results of curve-fitting (Eq. 2) was significantly increased by \( \approx 50\% \) within 1 h after cyclic strain (Fig. 2D and supplemental Table 2 available online at the Journal of Applied Physiology website). The elevated sarcomeric actin dynamics returned to the control level 1.5 to \( \approx 2 \) h after the cessation of cyclic strain (Fig. 2D).

Effects of neurohormonal hypertrophic stimulation on sarcomeric actin dynamics. To test whether the sarcomeric actin dynamics are also elevated by neurohormonal stimulation as shown for CapZ (14), NRVMs were treated with PE (10 \( \mu \text{M} \)) or norepinephrine (NE; 20 \( \mu \text{M} \)), and FRAP of actin-GFP was measured (Fig. 3). FRAP results demonstrated that actin dynamics increased by \( \approx 65\% \) 24 h after PE treatment and by \( \approx 90\% \) 48 h after NE treatment. Importantly, the effective treatment time of 24 or 48 h for neurohormonal stimulation is much longer than the 1 h of cyclic strain.

Localization and dynamics of mutant CapZ1-GFP fusion proteins in vitro. Since both CapZ and sarcomeric actin dynamics have similar time courses in response to cyclic strain and the previous studies are all from in vitro experiments, a possible interaction between CapZ and the actin filaments was addressed. It is known that the CapZ1 mutant with its tentacle (COOH terminus) deleted (CapZ1ΔC) is critical in CapZ-actin interaction (4). In our study, the CapZ1ΔC-GFP showed a Z-disc localization (Fig. 4, A and B), but the recovery profile of CapZ1ΔC-GFP in NRVMs showed a significant difference from wild-type CapZ1-GFP (Fig. 4C). Results of curve-fitting by two binding-state model (Eq. 2) demonstrated that CapZ1ΔC had elevated \( K_{\text{frap}} \) by \( \approx 95\% \), confirming the importance of the \( \beta \) tentacle for CapZ binding (Fig. 4D).

Fig. 3. Actin dynamics after neurohormonal stimulation. \( K_{\text{frap}} \) values for actin-GFP FRAP in cells 4 h \((n = 5)\) or 24 h \((n = 4)\) after phenylephrine (PE) treatment and 24 h \((n = 6)\) or 48 h \((n = 4)\) after norepinephrine (NE) treatment, normalized to unstrained cells (Non-strain) or 0–1 h after strain. S(0–1h), 0–1 h after strain. Values are means ± SE. *Significant difference (\( P < 0.01 \)).

DISCUSSION

In this study, three novel findings are reported. First, in the NRVM primary culture system, the dynamics of CapZ, one of the major regulators of actin-filament assembly, was found to be elevated by mechanical strain at the end of a brief period of stimulation but to abate over the next 2 h. Second, the dynamics of sarcomeric actin in NRVMs share a similar time course to CapZ for both the increase and abatement. Third, the \( \beta \)1-tentacle COOH terminus of CapZ1 may play an important role in filament assembly since sarcomeric actin dynamics are significantly enhanced when the tentacle is deleted. Thus it
seems possible that muscle hypertrophy stimulated by mechanical forces 1) begins to remodel the actin filament within an hour, 2) abates over 2 h after cessation of stimulation, and 3) involves the tentacle of the β1 subunit of CapZ.

**Time course of the response of NRVM to mechanical stimulation.** Mechanotransduction rapidly reacts to stimulation through signaling and posttranslational modifications, such as phosphorylation of existing proteins, thereby initiating sarco-
Fig. 5. Model of interactions between CapZ and sarcomeric actin. A: in unstrained conditions when NRVMs beat spontaneously but are not growing, the CapZ tentacle tightly caps the barbed end of the actin filament [on rate (light gray arrow) > off rate (dark gray arrow)]. This is possibly due to structural change of the β tentacle (light gray arrow) through posttranslational modification. The β tentacle of CapZ is released from its actin-binding state, which leads to an elevated actin off rate (arrows with two directions).

Comparison of the effects of mechanical strain and neurohormonal hypertrophic stimulation. Both mechanical strain and neurohormonal stimulation result in the increase of CapZ and sarcomeric actin dynamics, suggesting that both alter actin filament assembly during hypertrophy. PE and NE elicit hypertrophy and cardiac remodeling via small G proteins and ions, such as calcium, are also involved in control of actin-filament assembly in response to different functional demands of the heart.

In conclusion, the interaction of CapZ and actin in the thin filament is altered after strain in NRVM in culture. A model is proposed to explain the possible role of the β tentacle of CapZ in NRVM after mechanical stimulation. One hour of cyclic strain is sufficient to increase the dynamics of sarcomeric actin and neurohormonal stimulation result in the increase of CapZ and sarcomeric actin dynamics, suggesting that both alter actin filament assembly during hypertrophy. PE and NE elicit hypertrophy and cardiac remodeling via small G proteins and share many signaling pathways with mechanical stimulation, such as Ras, Rac, Rho, and MAPKs (19). Noticeably, although only 1 h of cyclic mechanical strain alters actin capping and actin dynamics, it takes days for neurohormonal stimulation to produce similar changes (1 day for PE, 2 days for NE).

Role of the β1 tentacle of CapZ in regulation of capping properties in NRVM. A model is proposed for the mechanism by which the interaction between CapZ and actin regulates actin assembly after a brief period of mechanical stimulation (Fig. 5). This model is based on results in NRVM of CapZ and actin dynamics after cyclic strain or with the CapZβ1 terminal deletion mutant. Under normal conditions when adaptation is not occurring, the two tentacles of CapZ tightly cap the barbed end of the actin filament, resulting in a low actin mobility and a low actin off rate (Fig. 5A). The deletion of the β tentacle significantly weakens the actin capping and increases CapZ dynamics, thus elevating the off rate of actin (Fig. 5B). After brief mechanical stimulation, the capping of CapZ is also decreased, and the off rate of actin is elevated (Fig. 5C).

The binding of CapZ to the actin filament, however, is not controlled only by the CapZβ1 tentacle. The CapZα tentacle is the target of CapZ inhibitors (30), but the regulatory role of the α tentacle in mechanical strain-induced sarcromeric actin remodeling has not yet been investigated. Undoubtedly, other partnering proteins, posttranslational modifications, and ions, such as calcium, are also involved in control of actin-filament assembly in response to different functional demands of the heart.

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

AUTHOR CONTRIBUTIONS

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