Video Article

Assessment of Myofilament Ca^{2+} Sensitivity Underlying Cardiac Excitation-contraction Coupling

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Abstract

Heart failure and cardiac arrhythmias are the leading causes of mortality and morbidity worldwide. However, the mechanism of pathogenesis and myocardial malfunction in the diseased heart remains to be fully clarified. Recent compelling evidence demonstrates that changes in the myofilament Ca^{2+} sensitivity affect intracellular Ca^{2+} homeostasis and ion channel activities in cardiac myocytes, the essential mechanisms responsible for the cardiac action potential and contraction in healthy and diseased hearts. Indeed, activities of ion channels and transporters underlying cardiac action potentials (*e.g.,* Na^+, Ca^{2+} and K^+ channels and the Na^+-Ca^{2+} exchanger) and intracellular Ca^{2+} handling proteins (*e.g.*, ryanodine receptors and Ca^{2+}-ATPase in sarcoplasmic reticulum (SERCA2a) or phospholamban and its phosphorylation) are conventionally measured to evaluate the fundamental mechanisms of cardiac excitation-contraction (E-C) coupling. Both electrical activities in the membrane and intracellular Ca^{2+} changes are the trigger signals of E-C coupling, whereas myofilament is the functional unit of contraction and relaxation, and myofilament Ca^{2+} sensitivity is imperative in the implementation of myofibril performance. Nevertheless, few studies incorporate myofilament Ca^{2+} sensitivity into the functional analysis of the myocardium unless it is the focus of the study. Here, we describe a protocol that measures sarcomere shortening/re-lengthening and the intracellular Ca^{2+} level using Fura-2 AM (ratiometric detection) and evaluate the changes of myofilament Ca^{2+} sensitivity in cardiac myocytes from rat hearts. The main aim is to emphasize that myofilament Ca^{2+} sensitivity should be taken into consideration in E-C coupling for mechanistic analysis. Comprehensive investigation of ion channels, ion transporters, intracellular Ca^{2+} handling, and myofilament Ca^{2+} sensitivity that underlie myocyte contractility in healthy and diseased hearts will provide valuable information for designing more effective strategies of translational and therapeutic value.

Video Link

The video component of this article can be found at https://www.jove.com/video/54057/

Introduction

Cardiac excitation-contraction (E-C) coupling is the fundamental scheme for analyzing mechanical properties of the myocardium, *i.e.,* the contractile function of the heart[1,2]. E-C coupling is initiated by membrane depolarization secondary to the activities of sarcolemmal ion channels (*e.g.,* the voltage-gated Na^+ channel, which can be measured *via* patch-clamp techniques). Subsequent activation of voltage-gated L-type Ca^{2+} channels (LTCCs) and Ca^{2+} influx *via* LTCCs trigger the bulk of Ca^{2+} release through ryanodine receptors (RyRs), increasing the cytosolic Ca^{2+} concentration from the nanomolar (nM) to micromolar (μM) level. Such an increase in cytosolic Ca^{2+} promotes Ca^{2+} binding to troponin C (TnC) in thin filaments and elicits conformational changes of the filament complex to facilitate the actin-myosin interaction and attains myocardial contraction[3]. Conversely, the cytosolic Ca^{2+} is re-uptaken back into the sarcoplasmic reticulum (SR) through the Ca^{2+}-ATPase in SR (SERCA2a) or is extruded out of the myocyte *via* the Na^+/Ca^{2+} exchanger and the plasmalemmal Ca^{2+} ATPase[1,2]. Consequently, the decline in cytosolic Ca^{2+} instigates conformational changes of thin filaments back to the original state, resulting in the dissociation of actin-myosin and myocyte relaxation[1-3]. In this scheme, the activity of SERCA2a is generally considered to determine the speed of myocardial relaxation because it accounts for 70 - 90% of cytosolic Ca^{2+} removal in most mammalian heart cells[1]. As such, abnormal Ca^{2+} handling by LTCC, RyR and SERCA2a, etc. has been considered the primary mechanisms for impaired contractility and relaxation in the diseased heart[1-4].

In reality, free cytosolic Ca^{2+} that functions as the messenger in E-C coupling accounts for around 1% of total intracellular Ca^{2+} and the majority of Ca^{2+} is bound to intracellular Ca^{2+} buffers[5,6]. This is due to the fact that various Ca^{2+} buffers are abundant in cardiac myocytes, *e.g.,* membrane phospholipids, ATP, phosphocreatine, calmodulin, parvalbumin, myofibril TnC, myosin, SERCA2a, and calsequestrin in the SR.[5,6,7]. Among them, SERCA2a and TnC are the predominant Ca^{2+} buffers[5,6,7]. Furthermore, Ca^{2+} binding to its buffers is a dynamic process during twitch (*e.g.,* 30-50% of Ca^{2+} binds to TnC and dissociate from it during Ca^{2+} transients[7]) and the change in Ca^{2+} binding cause additional "release" of free Ca^{2+} to the cytosol, results in the alterations of the intracellular Ca^{2+} concentration. Consequently, perturbation of the intracellular Ca^{2+} level induces abnormal myofilament movements, which are the precursors of contractile dysfunction and arrhythmias[8,9]. Many

factors (both physiological and pathological) can be the sources of post-transcriptional modifications of myofilament proteins, which influence myofilament Ca^{2+} buffering and myofilament Ca^{2+} sensitivity[8-10]. Recently, it was reported that mutations in myofilament proteins increase the Ca^{2+} binding affinity and intracellular Ca^{2+} handling, triggering pause-dependent potentiation of Ca^{2+} transients, abnormal Ca^{2+} release, and arrhythmias[8]. In line with this concept, we have also shown that myofilament Ca^{2+} desensitization in hypertensive rat hearts secondary to the up-regulation of neuronal nitric oxide synthase is associated with elevated diastolic and systolic Ca^{2+} levels[11], which in turn, increases the vulnerability of the LTCC to Ca^{2+}-dependent inactivation[12]. Hence, myofilament Ca^{2+} sensitivity is an "active" regulator of intracellular Ca^{2+} homeostasis and myocyte contractile function. It has become necessary to analyze interactions between myofilament and Ca^{2+} handling proteins for thorough investigation of myocyte E-C coupling and cardiac function.

Here, we describe a protocol that assesses the changes of myofilament Ca^{2+} sensitivity in isolated cardiac myocytes. Comprehensive analysis of intracellular Ca^{2+} profile, myofilament Ca^{2+} sensitivity and contraction will unearth novel mechanisms underlying myocardial mechanics.

Protocol

The protocol is in accordance with the Guide for the Care and Use of Laboratory Animals published by the UN National Institutes of Health (NIH Publication No. 85-23, revised 1996). It was approved by the Institutional Animal Care and Use Committee (IACUC) of Seoul National University (IACUC approval no.: SNU-101213-1).

1. Buffer Preparation (Table Materials and Equipment)

1. Prepare 300 ml of fresh isolation solution on the day of the experiment (in mM: NaCl, 135; KCl, 5.4; $MgCl_2$, 3.5; glucose, 5; HEPES, 5; Na_2HPO_4, 0.4; taurine, 20; pH 7.4 with NaOH).
2. Prepare 100 ml of fresh storage solution on the day of the experiment (in mM: NaCl 120; KCl 5.4; $MgSO_4$ 5; $CaCl_2$ 0.2; sodium-pyruvate 5; glucose 5.5; taurine 20; HEPES 10; mannitol 29; pH 7.4 with NaOH).
3. Prepare 1L of perfusion solution (in mM: NaCl, 141.4; KCl, 4; NaH_2PO_4, 0.33; $MgCl_2$, 1; HEPES, 10; glucose, 5.5; $CaCl_2$, 1.8; mannitol, 14.5; pH 7.4 with NaOH).
4. Prepare 30 ml of collagenase solution 1 by adding collagenase (1 mg/ml), protease (0.1 mg/ml), bovine serum albumin (BSA, 1.67mg/ml), and Ca^{2+} (0.05 mM) to 25 ml of isolation solution.
5. Prepare 20 ml of collagenase solution 2 by adding collagenase (1 mg/ml), BSA (1.67 mg/ml), and Ca^{2+} (0.05 mM) to 16.7 ml of isolation solution.
6. Prepare 40 ml of BSA solution by adding 0.4 g of BSA to 40 ml of isolation solution. Separate 10 ml and 30 ml into two beakers. Add Ca^{2+} to 30 ml of BSA solution so that the final Ca^{2+} concentration in the BSA solution is 1 mM.

2. Preparation for the Isolation of Left Ventricular (LV) Myocytes

1. Transfer 8-12 week-old, male Sprague-Dawley (SD) rats in clean transport cages from the animal facility to the preparation and isolation room.
2. Heat two water baths to 37 ^{o} C.
   Note: Use one water bath for the water - jacketed reservoir and the perfusion tubes of the Langendorff perfusion system. Use the other water bath to agitate myocardial tissue trunks in order to separate single myocytes.
3. Add 5 ml and 3.3 ml of BSA solution (without Ca^{2+}) to 25 ml of collagenase solution 1 and 16.7 ml collagenase solution 2 to make up the volume to 30 ml and 20 ml, respectively.
4. Add isolation solution (100 ml) and collagenase solution 1 (30 ml) to column 1 (Col. 1) and column 2 (Col. 2) in the Langendorff perfusion system, respectively (**Figure 1A**).
5. Oxygenate the isolation solution and collagenase solution 1 in Col. 1 and Col.2 *via* the oxygen connection tube in the Langendorff perfusion system (**Figure 1A**). Similarly, oxygenate collagenase solution 2 and the BSA solution in the shaking water bath with 100% O_2 *via* the oxygen connection tube.

3. Isolation of LV Myocytes

1. Anesthetize a SD rat *via* intraperitoneal injection of pentobarbital sodium (30 mg/kg) and confirm the anesthetic status by toe pinching and lack of withdrawal reflection.
2. Move the rat to a dissection tray. In the supine position, secure the four legs to the sides of the body with tape.
3. Apply thoracic mid-axial incisions with surgical scissors to open the chest, ensuring not to damage the heart. Use another pair of clean scissors to dissect the heart from the connecting vessels (*e.g.,* superior and inferior vena cava, pulmonary vessels, and aorta) and pericardial membrane[13].
4. Leave a portion of the aorta of a sufficient length (5 - 8 mm), clamp the aorta with fine forceps, and rapidly mount the cannula of the Langendorff perfusion system within 1 min (**Figure 1A**). Tie suture thread 4/0 tightly over the aorta.
5. Turn on the valve on Col. 1 and perfuse the isolated heart with pre-warmed and oxygenated isolation solution for 10 min (perfusion rate: 12-14 ml/min). Turn off the valve on Col. 1, turn on the valve on Col. 2 and perfuse the heart with collagenase solution 1 for 8 - 10 min.
6. Dismount the digested heart by cutting the aorta and transfer it by holding the aorta with forceps into the flask containing fresh isolation solution (**Figure 1Bi**). Use fine scissors and forceps to cut most of the LV free wall (including the septum) into smaller pieces (~22 mm, **Figure 1Bii**).
7. Transfer the pieces into a flask containing pre-warmed and oxygenated fresh collagenase solution 2 (**Figure 1Biii**). Shake for 10 min. Keep delivering oxygen to the myocyte-containing collagenase solution 2.

8. Move the myocyte - suspension to a 10 ml centrifuge tube using droppers with a suction bulb and add Ca^{2+} - containing BSA solution (1:1 in volume). Centrifuge at 30 g for 2 min and discard the supernatant. Re-suspend the myocyte pellet in 5 ml of BSA solution. Centrifuge and discard the supernatant.
9. Disperse the myocyte pellets and keep myocytes in 10 ml of pre-oxygenated storage solution at RT (**Figure 1Biv-vi**).
10. Repeat the procedures (steps 3.7 - 3.9) with the remaining LV tissue in the flask.
    Note: Keep repeating the procedures (steps 3.7 - 3.9) again until most of the LV tissue disappears to obtain a good yield of myocytes.
11. Keep the myocytes in storage solution for 6-8 hr at RT until the end of experiments.

4. Simultaneous Measurements of Intracellular Ca^{2+} Transients and Myocyte Contraction

1. Load LV myocytes with the acetoxymethyl ester Fura-2 AM (2 µM).
   Note: Perform all loading procedures and experiments with loaded myocytes in a dark tube (**Figure 1Bvii**).
   1. Centrifuge the myocyte suspension (1 ml) at 2,000 x g for 10 sec. Discard the supernatant and re-suspend the myocyte pellet in 1 ml of Tyrode solution with a low Ca^{2+} concentration (250 µM, Table Materials and Equipment).
   2. Add Fura-2AM and poloxamer 407 (2 µl), gently disperse the myocyte suspension, and keep the mixture at RT (20 - 24^{o} C) for 15 min (**Figure 1Bvii**).
   3. Centrifuge the mixture for 5 sec, discard the supernatant and disperse the myocyte pellet in 1 ml of perfusion solution containing 500 µM Ca^{2+}. After 10 min, centrifuge the mixture for 5 sec and discard the supernatant.
   4. Add fresh perfusion solution (500 µM Ca^{2+}, 1 ml) and keep the Fura-2AM - loaded myocyte pellet in this solution for recordings.

2. Measurement of LV myocyte contraction and intracellular Ca^{2+}
   1. Before recording, fill the perfusion tube that runs through a water jacket with the Tyrode solution, which is pre-warmed to 36 ^{o} C.
   2. Place a few drops of the Fura 2 AM - loaded LV myocyte suspension on the chamber of an inverted fluorescence microscope for 5 - 8 min. Slowly perfuse the Tyrode solution (2.2 ml/min).
   3. Press the "start" button on the front panel of the digital stimulator to start field stimulation (2 Hz).
      Note: The output voltage (10 V, 5 msec duration) is applied to the myocytes in the chamber through platinum wires positioned on either side of the chamber.
      1. Select myocytes that contract stably (not those displaying hyper- or hypo-contraction) for recording.
   4. Adjust the myocyte of choice in the horizontal position of the video-based sarcomere detection system and adjust the focus of the microscope to obtain optimal images of sarcomeres. Position the purple rectangular box on the area in which clear sarcomeres are clearly observed until the average of sarcomere lengths (red peak) displays a singular sharp peak (**Figure 2A**, lower image).
   5. Record the changes of sarcomere length in response to field stimulation (**Figure 2A and B**).
      Note: Use the loaded myocytes within 1 - 2 hr.
   6. Adjust the aperture of the camera so that the field in the video-based sarcomere detection system is the size of the myocyte (**Figure 2A**). Stimulate the myocyte with excitation at 360 nm/380 nm and emission at 510 nm (acquisition frequency 1,000 Hz). Record sarcomere shortening and the Fura2 AM ratio with field stimulation (**Figure 2B**).

5. Assessment of Myofilament Ca^{2+} Sensitivity

1. Average the sarcomere lengths and Ca^{2+} transients at steady-state (10 - 20 traces) and plot the phase-plane loop of the Fura-2 ratio *vs.* sarcomere length of the same myocyte (both with measured values and delta changes; **Figure 2C**).
2. In each plot, define Fura -2 ratio at 50% relaxation (EC_{50}, **Figure 2C**). Compare both the loop and EC_{50} of each intervention.

Representative Results

LV myocytes are isolated from normal and hypertensive rat hearts. Rod-shaped myocytes with clear striations (representing sarcomeres) and stable contractions in response to field stimulation are considered to be the optimal myocytes and are selected for recordings (**Figure 2A**). In the example shown in F**igure 2A**, a Fura 2 AM -loaded LV myocyte is positioned horizontally and the aperture of the camera is adjusted so that the myocyte occupies most of the recording field and minimal background area is included. In the recording field, adjust the dimensions of the purple box by clicking and dragging this box on the computer screen (through the recording program). When the average sarcomere length shows one sharp red peak, start recording (**Figure 2A**, lower image).

Both sarcomere length and intracellular Ca^{2+} transients (Fura-2 ratios) are recorded simultaneously from the same myocytes (**Figure 3A**). The average traces of sarcomere length and Ca^{2+} transients are shown in **Figure 3B**. Individually, diastolic/systolic sarcomere lengths, time to peak (PT), and sarcomere shortening are measured to investigate the amplitude and dynamics of myocyte contractility. Time to 50% relaxation (TR_{50}) is analyzed to assess the relaxation of the myocyte. Similarly, the diastolic and systolic Fura-2 ratios (Ca^{2+} transients), time to peak (PT) of Ca^{2+} transients and time constant of Ca^{2+} transient decay (tau) are analyzed to assess myocyte contraction and relaxation (**Table 1**). In this example, the amplitudes of Ca^{2+} transients are moderately increased in LV myocytes from a hypertensive rat and contraction is moderately reduced (**Figure 3B** and **Table 1**).

Furthermore, the relationships between the Fura - 2 ratio and sarcomere length (indicating the myofilament Ca^{2+} sensitivity of LV myocytes) are plotted in both sham and hypertensive rats (**Figure 3C**). The Fura 2 - sarcomere length trajectory during the relaxation phase of the myocyte defines a quasi-equilibrium of cytosol Ca^{2+}, myofilament Ca^{2+} binding, and sarcomere length[14]; therefore, the relaxation phase is compared between the two groups. The rightward shift of the trajectory in myocytes from the hypertensive group indicates a reduced myofilament response to Ca^{2+} (**Figure 3C**). Accordingly, the intracellular Ca^{2+} concentration required for half relaxation (EC_{50}) is increased (**Figure 3C**), referring to myofilament Ca^{2+}-desensitization in hypertension.

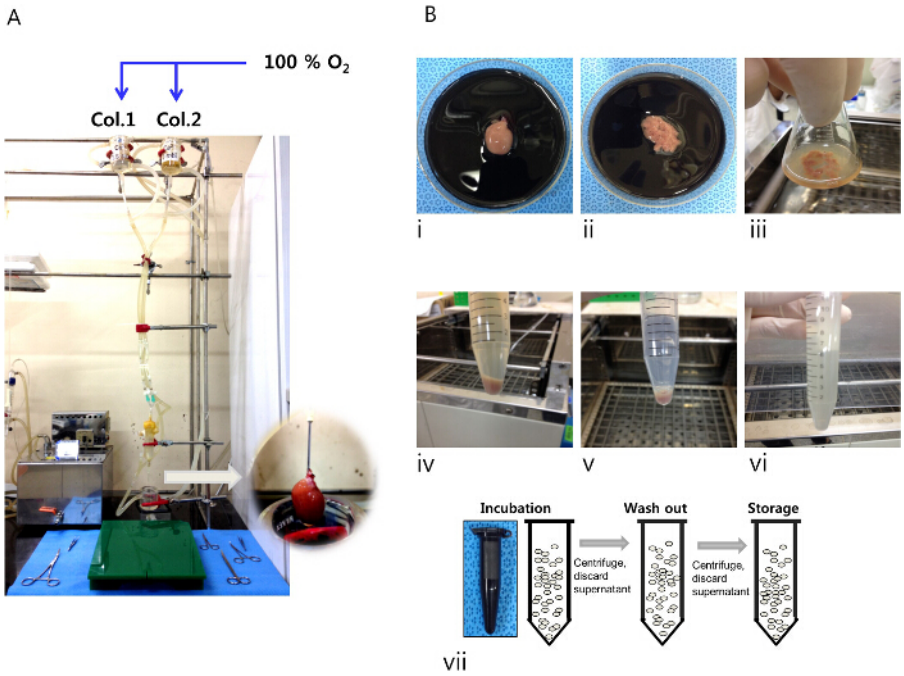

Figure 1. Procedures for isolating LV myocytes from rat heart. (A) The Langendorff perfusion system used to perfuse the isolation solution into the heart cannulated *via* the aorta (inset, magnified image of the mounted heart on the perfusion system). **(B)** i - iii: The heart after digestion with collagenase solution 1 and dissected LV tissue in a dish and flask. **(B)** iv - vi: Myocyte suspension after addition of collagenase solution 2, myocyte pellet after centrifugation, and re-suspended myocyte pellet in storage solution. **(B)** vii: Incubation, incubate LV myocytes in Fura 2AM - containing isolation solution (2 µM Fura 2 AM, 250 µM Ca^{2+} and with 2 µl poloxamer 407); washout, wash LV myocytes with isolation solution with 500 µM Ca^{2+}; storage, keep Fura-2 AM - loaded LV myocytes in fresh isolation solution containing 500 µM Ca^{2+}. Please click here to view a larger version of this figure.

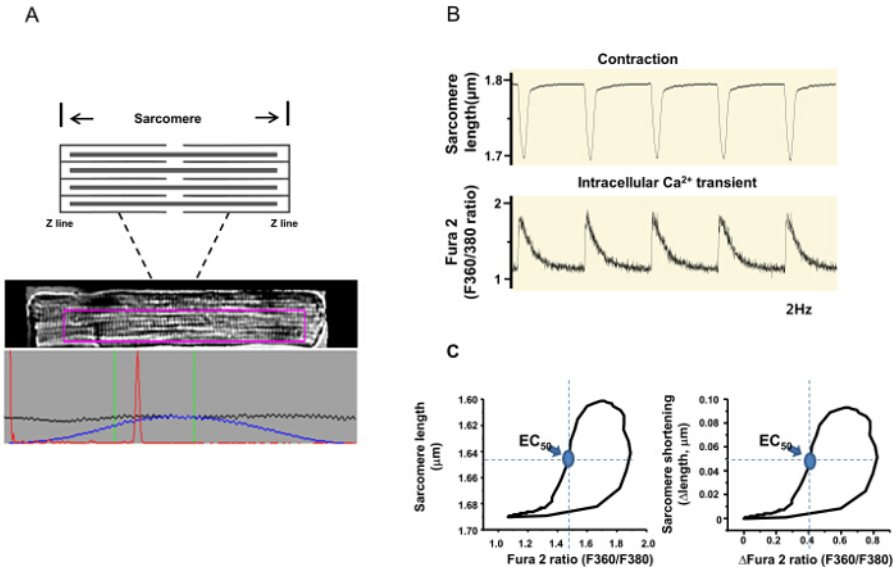

Figure 2. Measurement of sarcomere shortening and the Fura-2 ratio (indicative of the intracellular Ca^{2+} level). (A) A diagram of sarcomere, an image of a Fura-2 -loaded LV myocyte and the averaged sarcomere length (the red peak) are displayed on the computer. In the lower panel, the black line is the average of each horizontal pixel line within the purple region of interest. The blue line is the same data zeroed at each end. The red line is the fast Fourier transform (FFT) power spectrum, which represents the number of signals the FFT has calculated. One sharp peak means a clean sarcomere recording. **(B)** Simultaneous recordings of sarcomere length and the Fura -2 ratio in response to field stimulation (2Hz). **(C)** Phase-plane plot of the Fura 2 ratio *vs.* sarcomere length of the same LV myocyte (note that both the actual length/Fura 2 ratio and delta changes of these parameters are analyzed). EC_{50} (Fura 2 ratio at 50% relaxation, circle indicated by arrow) is the qualitative comparison of myofilament Ca^{2+} sensitivity between the groups. Please click here to view a larger version of this figure.

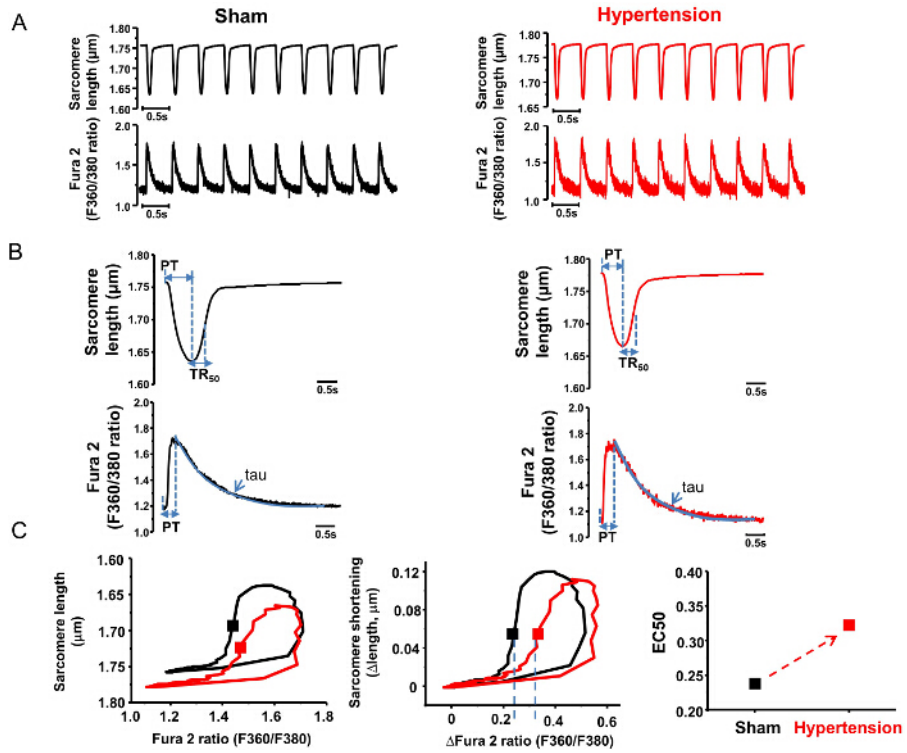

Figure 3. Representative results of the analysis of LV myocyte contraction in sham and hypertensive rats. (A) Raw traces of sarcomere shortening and Fura - 2 ratio measurement in LV myocytes from sham and hypertensive rats. **(B)** Average traces of sarcomere length and Fura - 2 signals. Parameters analyzed in the averaged traces are shown in Table 1. **(C)** Phase-plane plots of the Fura - 2 ratio *vs.* sarcomere length (both the actual length and Fura - 2 ratio and delta changes of these parameters) in the two groups. The trajectory loop is shifted to the right and EC_{50} tends to be higher in hypertension, suggesting myofilament Ca^{2+} desensitization. PT, time to peak (sec); Tau, time constant of Ca^{2+} transient decay (sec) (obtained by fitting the decline phase of the Fura - 2 ratio with an exponential function).TR_{50}: time to 50% relaxation (sec). $\int(t) = \sum_{i=1}^{n} A_i\, e^{-t/\tau_i} + C$ Please click here to view a larger version of this figure.

	Parameters	Sham	Hypertension
Intracellular Ca^{2+}	Diastolic Ca^{2+}	1.189	1.124
	Systolic Ca^{2+}	1.71	1.691
	Amplitude (Δ ratio)	0.521	0.567
	Time to peak (PT, s)	0.021	0.031
	Tau (s)	0.079	0.076
Sarcomere	Diastolic	1.758	1.78
	sarcomere length (μm)		
	Sarcomere	0.122	0.115
	Shortening (Δ length,mm)		
	Time to peak (PT, s)	0.064	0.055
	Time to 50%	0.032	0.03
	Relaxation (TR_{50},s)		
EC50	$[Ca^{2+}]_i$ (Fura-2 ratio) for 50% sarcomere relengthening	0.2382	0.3224

Table 1. Analysis of the Fura - 2 ratio (intracellular Ca^{2+}) and sarcomere length measurements.

Discussion

Here, we describe the protocols to assess changes of myofilament Ca^{2+} sensitivity in single isolated cardiac myocyte and emphasize the importance of measuring this parameter alongside electrophysiological properties, intracellular Ca^{2+} transients, and myofilament dynamics. This is because the recordings of one or two of the parameters may not explicate the mechanisms underlying cardiac contraction and relaxation.

Unlike conventional methods that measure myocyte contraction and the intracellular Ca^{2+} profile individually[1], the present method examines both parameters simultaneously in the same cardiac myocytes.

A number of methods are generally used to assess the myofilament Ca^{2+} sensitivity of muscles or myocytes[15,16,17], *e.g.,* examination of the interactions between exogenous Ca^{2+} and sarcomere/cell length/tension in skinned myocytes (treated with detergents such as saponin or β-esin to permeabilize the membrane). Lengths are measured with photo-diode and laser beam diffraction techniques, and tension with force transducers or carbon fibers). These techniques are widely used in muscle studies because they enable quantitative assessments of myofilament Ca^{2+} sensitivity. However, endogenous ion channel activities in the plasma membrane and intracellular Ca^{2+} handling, those are required to initiate the mechanics of myofilament, are neglected in these techniques. In addition, the muscle strips or myocytes under study are pre-stretched to a certain diastolic length, and under these conditions, the initial sarcomere length can differ from the actual diastolic length of the myocytes (especially in disease conditions and with interventions). This highlights the advantage of the current measurement where cellular organelles remain relatively unperturbed, thereby, enabling the comprehensive evaluation of myocyte contractile function.

Understandably, quality of the myocytes (Ca^{2+}-tolerating) and the optimal loading of Fura - 2AM are two key elements for successful assessment of myofilament Ca^{2+} sensitivity. Accordingly, several modifications are applied to the protocol in order to obtain viable rod-shaped myocytes (60-80% of total cells, as described previously[18,19,20]). First, a low dose of Ca^{2+} is added to the solutions during the digestion processes (*e.g.,* the Ca^{2+} concentration is 50 µM in the collagenase solutions and 200 µM in the storage solution). Second, during the digestion process, BSA is added to the collagenase solutions to enhance membrane stability. Third, most of the solutions are oxygenated during isolation, which enhances the percentage of functional myocytes and the duration of the experiments (6-8 hr). Fourth, isolated myocytes are kept in storage solution containing 200 µM Ca^{2+}.

To obtain optimal loading with Fura 2AM, two different Ca^{2+} concentrations (250 and 500 µM) are used and poloxamer 407 (2 µl, 20% prepared in dimethyl sulfoxide) is added to enhance the permeability of Fura-2AM through the membrane and its stability in myocytes. It should be noted that all Ca^{2+} indicator dyes are Ca^{2+} buffers[21]. Therefore, researchers should avoid using a high concentration of Fura-2 AM or a longer incubation to avoid excessive buffering and reduced myocyte contractility. It is recommended that myocyte contraction without Fura - 2 loading is routinely checked, which is an essential step to estimate the quality of myocyte and the loading status. Variability in loading is inevitable. Therefore, it is important to check the variability of myocyte contraction, specifically, whether the number of contracting myocytes in the chamber is similar before and after loading. Analysis of hyper- or hypo-contracting myocytes should be avoided because they do not represent the average status of myocytes and may cause biased results and evaluations.

It should be noted that the measured changes in the Fura 2 ratio are reflections of the intracellular Ca^{2+} level, rather than the actual chemical concentration of Ca^{2+}. Therefore, the method described here evaluates qualitative changes of myofilament Ca^{2+} sensitivity, rather than the actual sensitivity of myofilaments to Ca^{2+}. Calibration of Fura - 2 signal to the intracellular Ca^{2+} concentration is the solution to acquire reliable Ca^{2+} concentrations in individual myocytes. The calibration procedure requires loading myocytes with various concentrations of Ca^{2+}-conjugated Fura - 2 AM (Ca^{2+} concentration ranging from 0 to 39 µM), and the obtained Fura - 2 ratios are calculated with the following formula: $[Ca^{2+}]_i$ = Kd * (R - Rmin) / (Rmax - R) * F380max / F380min[21]. It is possible to derive the pooled average values of Ca^{2+} concentrations through such a calibration procedure. However, because the loading of Ca^{2+} indicators is variable among myocytes and calibration is not performed in an individual cell, Ca^{2+} concentrations may not be acquired accurately. Furthermore, if F360/F380 is measured rather than F340/F380 (as in the present study), the Fura 2 ratio is less accurate (because F360 is the isobestic wavelength of Fura-2 fluorescence[22]). Nevertheless, it is still a valid method to assess qualitative changes in myofilament Ca^{2+} sensitivity in physiological experiments, especially in diseased human hearts, where myofilaments can be concomitantly altered after changes in the intracellular environment. It is recommended that this method is combined with alternative methods (as described previously in the Discussion section) to precisely analyze the true sensitivity of myofilaments to Ca^{2+}.

The other limitation of the method in the study of myocyte contraction is the unloaded condition. It may underestimate or overestimate the changes in myofilament Ca^{2+} sensitivity. Therefore, to accurately evaluate myofilament Ca^{2+} sensitivity, analysis should be performed with alternative measurements for both qualitative and quantitative assessment.

The technique is applicable for assessing myocardial function in healthy and diseased hearts, including human cardiac samples, where the cellular redox environment and post-transcriptional modifications are changed, resulting in concomitant alterations in myofilament functions. In particular, ion channels, intracellular ion homeostasis and regulatory proteins in myofilaments are interactive; therefore, all these components function in concert to determine heart performance *in vivo* (*e.g.*, as measured in the echocardiography).

In conclusion, we describe a method to evaluate the changes of myofilament Ca^{2+} sensitivity and emphasize the importance of analyzing this parameter in conjunction with cardiac electrophysiology, intracellular Ca^{2+} handling, and myocyte contraction to obtain a full profile of myocyte function. Myofilament Ca^{2+} sensitivity should be measured routinely in mechanistic studies using diseased heart models, where changes in these parameters as well as various intracellular signaling pathways are interrelated.

Disclosures

The authors declare that they have no competing financial interests.

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