Video Article

Assessment of Sarcoplasmic Reticulum Calcium Reserve and Intracellular Diastolic Calcium Removal in Isolated Ventricular Cardiomyocytes

Jing Gao*[1], Xiaolu Shi*[2], Hong He[1], Juhong Zhang[1], Ding Lin[3], Guosheng Fu[1], Dongwu Lai[1]

[1]Department of Cardiology, Sir Run Run Shaw Hospital, Zhejiang University School of Medicine

[2]Experimental Research Center, China Academy of Chinese Medical Sciences

[3]Department of Cardiology, The Third Hospital of Hangzhou City

*These authors contributed equally

Correspondence to: Dongwu Lai at ldw.zju@gmail.com

URL: https://www.jove.com/video/55797
DOI: doi:10.3791/55797

Keywords: Cellular Biology, Issue 127, Sarcoplasmic reticulum, calcium homeostasis, calcium reserve, intracellular calcium removal, SERCA, cardiomyocytes

Date Published: 9/18/2017

Citation: Gao, J., Shi, X., He, H., Zhang, J., Lin, D., Fu, G., Lai, D. Assessment of Sarcoplasmic Reticulum Calcium Reserve and Intracellular Diastolic Calcium Removal in Isolated Ventricular Cardiomyocytes. *J. Vis. Exp.* (127), e55797, doi:10.3791/55797 (2017).

Abstract

Intracellular calcium recycling plays a critical role in regulation of systolic and diastolic function in cardiomyocytes. Cardiac sarcoplasmic reticulum (SR) serves as a Ca^{2+} reservoir for contraction, which reuptakes intracellular Ca^{2+} during relaxation. The SR Ca^{2+} reserve available for beats is determinate for cardiac contractibility, and the removal of intracellular Ca^{2+} is critical for cardiac diastolic function. Under some pathophysiological conditions, such as diabetes and heart failure, impaired calcium clearance and SR Ca^{2+} store in cardiomyocytes may be involved in the progress of cardiac dysfunction. Here, we describe a protocol to evaluate SRCa^{2+} reserve and diastolic Ca^{2+} removal. Briefly, a single cardiomyocyte was enzymatically isolated, and the intracellular Ca^{2+} fluorescence indicated by Fura-2 was recorded by a calcium imaging system. To employ caffeine for inducing total SR Ca^{2+} release, we preset an automatic perfusion switch program by interlinking the stimulation system and the perfusion system. Then, the mono-exponential curve fitting was used for analyzing decay time constants of calcium transients and caffeine-induced calcium pulses. Accordingly, the contribution of the SR Ca^{2+}-ATPase (SERCA) and Na^{+}-Ca^{2+} exchanger (NCX) to diastolic calcium removal was evaluated.

Video Link

The video component of this article can be found at https://www.jove.com/video/55797/

Introduction

Intracellular calcium ($[Ca^{2+}]_i$) recycling plays a critical role in regulation of systolic and diastolic function in cardiomyocytes[1]. As we know, the calcium-induced Ca^{2+} release initiates the excitation-contraction coupling, which translate the electrical signal to contraction. Membrane depolarization activates the sarcolemmal L-type Ca^{2+} channels, which induce Ca^{2+} release from SR into the cytoplasm via ryanodine receptors 2 (RyR2). The transient elevated cytoplasmic Ca^{2+} initiates contraction of myofibrils. During the diastole, cytoplasmic Ca^{2+} is reuptaken into the SR by means of the SR Ca^{2+}-ATPase 2 (SERCA2) and pumped out of the cardiomyocyte via the Na^{+}-Ca^{2+} exchanger (NCX)[2]. This process leads to contraction-relaxation recycling in the cardiomyocyte.

The cardiac SR is an intracellular membrane network that surrounds the contractile machinery. It serves as a Ca^{2+} reservoir for contraction, and it reabsorbs intracellular Ca^{2+} during relaxation. The SR Ca^{2+} reserve available for beats is determinate for cardiac contractility. Meanwhile, the removal of intracellular Ca^{2+} is critical for cardiac diastole. Under some pathophysiological conditions, such as diabetes and heart failure, impaired Ca^{2+} clearance and depressed SR Ca^{2+} store in cardiomyocytes may be involved in the process of cardiac dysfunction[2,3,4].

For measuring SR Ca^{2+} release and diastolic Ca^{2+} removal in cardiomyocytes, there are two widely used approaches: the integrity of the NCX current based on patch-clamp[5,6], and the caffeine-induced Ca^{2+} pulse based on Ca^{2+} fluorescence imaging[7,8,9]. The former approach depends on the fact that the Ca^{2+} released from the SR is largely pumped out of the cell by NCX. However, this approach is limited by its requirement of advanced equipment and skillful operation. In the present study, we describe a convenient approach to assess SR Ca^{2+} reserve and Ca^{2+} removal in myocytes by measuring a caffeine-induced Ca^{2+} pulse based on a Ca^{2+} fluorescence imaging system. Briefly, intracellular Ca^{2+} fluorescence is indicated by Fura-2. By interlinking the stimulation system and perfusion system, we present a program for switching the perfusion and pacing system automatically. 10 mM caffeine was employed to rapidly induce total Ca^{2+} release in the SR. The exponential decay time constants (Tau) of calcium transients and caffeine-induced calcium pulses were obtained from mono-exponential curve fitting, which reflect the contribution of SERCA and NCX to diastolic Ca^{2+} removal accordingly.

Protocol

All animal experiments were performed in accordance with protocols approved by the Institutional Animal Care and Use Committee at Experimental Research Center, China Academy of Chinese Medical Sciences and Zhejiang University.

1. Solution Preparation

1. Prepare all solutions as described in **Table 1**.

2. Isolation of Left Ventricular (LV) Cardiomyocytes

NOTE: LV cardiomyocytes are isolated enzymatically using a Langendroff perfusion system as previously described[7,8,9,10,11].

1. Set up the Langendroff perfusion system. Fill the perfusion system with Normal Tyrode (NT) solution, set the temperature at 36.5 °C, and eliminate any air bubbles in the tube.
2. Weigh and anesthetize a Sprague-Dawley rat by intraperitoneal injection (ip) of chloral hydrate (400 mg/kg). Confirm the anesthetic status by evaluating tail or toe pinch response after 5 min.
3. Open the abdominal cavity under the xiphoid process with a surgical scissor, lift the xiphoid process, and open the chest with the scissor. Remove the pericardium, slightly lift the heart with curved forceps, identify the aortic arch, and cut off the heart by scissors from the root of the aorta.
4. Transfer the heart to a 60-mm dish and wash it with NT solution. Hold the aorta with two micro-dissecting forceps, and mount it onto the Langendorff perfusion cannula, and then firmly ligate the aorta onto the cannula using surgical sutures.
   NOTE: A skilled operator can finish this process within 15 s.
5. Perfuse the heart with 30 mL NT solution to recover circulation of the coronary arteries. Then, switch the perfusate to 30 mL Ca^{2+}-free Tyrode solution (10 mM Taurine, 1 mg/mL BSA) to stop the heartbeat. Next, switch the perfusate to Collagenase A isolation solution (0.6 mg/mL) for enzyme digestion.
   NOTE: Use Collagenase A for experiments with diabetic rats, or Collagenase II for experiments without diabetic rats.
6. After digestion for 20-25 min, quickly change the perfusion solution to the Ca^{2+}-free Tyrode solution to stop further digestion. Then, hold the heart with forceps, cut it off from the cannula, and place the heart in a 35-mm dish containing KB solution (see **Table 1**).
7. Dissect the LV wall with scissors and forceps. Cut off the atrium, right ventricle and atrioventricular junction area. The remaining tissue is the LV; transfer it into a new 35-mm dish with KB solution. Mince the LV tissue into small pieces.
8. Gently pipette the pieces with a filtered plastic dropper, and re-suspend in 10 mL KB solution.
9. Filter the cells with 150 μm aperture stainless steel filter, and transfer to a 15 mL centrifuge tube. Centrifuge at 150 x g for 30 s and discard the supernatant. Re-suspend the myocytes in 10 mL KB solution, free settle for 6 min, discard the supernatant, and re-suspend the pellet in 10 mL KB solution.
   NOTE: All steps were performed at 36 °C in solutions gassed with 100% O_2.

3. Calcium Reintroduction

1. After settling for 20 min in KB solution, discard the supernatant, and re-suspend the myocytes with calcium reintroduction solution A (0.3 mM Ca^{2+}, 4.5 mL Ca^{2+}-free Tyrode solution, 1.5 mL NT solution) for 10 min.
2. Repeat the above procedure with calcium reintroduction solution B (0.3 mM Ca^{2+}, 3 mL Ca^{2+}-free Tyrode solution, 3 mL NT solution).
3. Repeat the above procedure with NT solution (1.2 mM Ca^{2+}) to purify the available myocytes. Store the isolated LV myocytes in this solution and study them within 4-6 h.

4. Set Up of Perfusion System

1. Connect the inflow tube with the NT solution for chamber perfusion (**Figure 1A**).
2. For needle perfusion of the target myocyte, connect the multi-barrel manifold tip (*e.g.*, perfusion pencil, referred to as pencil henceforth), fixed in the micromanipulator, to the valve controlled perfusion system. Add NT solution and 10 mM caffeine solution to each column of the pencil (**Figure 1A**).
3. Evacuate any air bubbles in the tubes to avoid air blockage.
4. Count the drip number from the micron tip of the pencil for 10 s, and manually adjust the flow regulator to keep the flow speed at an approximate velocity of 3 drip/10 s.

5. Measurements of Intracellular Ca^{2+} Transient and Sarcomere Shortening [7,8,9]

1. Pipette 10 μL cell suspension on the slide, count cell numbers by a cell counter under the microscope, and calculate the density. Dilute myocytes to an approximate concentration of 50,000 cell/mL.
2. Add the Fura-2 acetoxymethyl (AM) stock (a calcium sensitive dye) into a 1 mL suspension of myocytes to bring the final concentration to 2 μM. Keep in the dark for 20 min at room temperature.
3. Centrifuge at 150 g for 30 s, and re-suspend the cardiomyocytes with NT solution 2 times.
4. Turn off the light and keep the cells in the dark. Place the myocytes in the perfusion chamber for 15 min. Then start the chamber perfusion (1.5 mL/min) with NT solution. Pace the myocytes with 1 Hz field stimulation using a stimulator (wave duration 4.0 ms, pulse amplitude 8.0 V) for 5 min.

5. Select a myocyte with good shape (rod shape, sharp edge, and clear cross striations) and stable stimulated twitch (no spontaneous contraction) under the 10x microscopic objective lens. Next, change the microscopic magnification to 40x, and rotate the CCD camera orientation to keep the myocyte in a horizontal position.
6. Frame the single myocyte by adjusting the cell framing adapter. Ensure that the background is clear.
7. Expose the myocytes to the light emitted by a Xenon lamp, with 340 or 380 nm wavelength excitation filters, and image the myocytes through a 40x objective. Detect fluorescence signal at 510 nm. In the meantime, note the sarcomere length changes of the myocytes and record using the soft-edge module simultaneously.
8. Record fluorescence by a dual-excitation fluorescence photomultiplier system. Run the recording program for the calcium imaging system, click "File/new File" to create a new recording file, and click the "start" button to synchronously record fluorescence and sarcomere length.

6. Assessment of SR Ca^{2+} Reserve and Diastolic Ca^{2+} Removal Function [7,8,9]

1. Interlink the "Aux Out" port in the stimulator with the "Analog In" port on the valve control system (*e.g.*, valve commander, see **Table of Materials**) by a BNC cable (to synchronize the TTL signal).
2. Preset a program for switching the perfusion valves automatically as previously described[8,9]; Detailed steps for operation are listed as following.
    1. Preset the parameters in the valve control software as per **Table 2**, and click the "Download" button to download the sequence loaded in the program. Click the "Trigger" button to enable the trigger function.
    NOTE: The valve control system can execute the sequence after receiving TTL signal.
    2. Preset the stimulator to sequence mode, and set the parameters as per **Table 3**.
3. Set the stimulator at "S1 step" to pace the LV myocytes at 1 Hz. Start the needle perfusion at the speed of 1.5 mL/min with NT solution.
NOTE: Because the speed of the needle stream is faster than chamber perfusion, the light refraction of the needle stream is different from the light refraction of chamber perfusion. The difference of light refraction indicates the range of effective needle perfusion, which surrounds the target myocyte.
4. Select a target myocyte under the low power microscopic view (in the sequence of downstream to upstream), and make sure it can be reached by the micro tip of the pencil. Change the microscope magnification to 400x. Rotate the CCD camera orientation to keep the myocyte in the horizontal position. Adjust the rectangular aperture under the cell framing adapter to a suitable window that fills with the myocytes. Ensure that there is minimal background; do not allow other myocytes or dead cell debris in this window.
5. Adjust the position of the pencil fixed on the micromanipulator, and carefully place the micro tip at the distance of the radius of vision field to the target myocyte under 400x microscope magnification.
6. Adjust the needle stream range to mostly cover the target myocyte and make sure that the myocyte cannot be swept away by the needle flow.
7. After 60 s basic pacing, roll the stimulator cursor to the "D2 step".
NOTE: The rest of protocol will be executed automatically by the stimulator and valve control system. Based on the above setting, the protocol could be executed automatically as in **Figure 2A**, 1 Hz basic pacing with NT solution for 60 s. Then pause pacing and delay for 2 s, rapidly switch to 10 mM caffeine perfusion for 15 s (to functionally release Ca^{2+} storage in the SR), and then switch back to NT solution.
8. At the end of the recording, detect the background fluorescence for individual myocytes. Click the "pause" button to pause the file recording, move the microscopic view to a nearby blank area. Click the "record" button to resume recording for seconds, and record numerical 340 and 380 nm intensity values for background correction.
9. Open the "Operation/Constant" dialog box and fill the values into the "background" form, respectively; the software can correct Fura 2 ratio values by subtracting the background.

7. Data Analysis [9]

1. For measurements of calcium transients and sarcomere shortening at the basic pacing stage, average the twitch pulses, and then analyze dynamic parameters, such as calcium transients, the time constant of calcium transient decay (Tau-1 Hz), using the software automatically.
NOTE: If the software does not fit the decay segment well, export the decline trace for manual mono-exponential curve fitting.
2. For caffeine-induced calcium pulses, select only the pulse with a steep wave for analysis of calcium removal function. Exclude cells with signal disturbance, abnormal pulse, or those that flowed away midway.
3. Measure the amplitude of the caffeine-induced calcium pulse (Ca-caffeine, an index of $SRCa^{2+}$ reserve).
4. Obtain the decay time constant of caffeine-induced calcium pulse (Tau-caffeine) by mono-exponential curve fitting (10 s duration) manually from software (**Figure 2C**).

Representative Results

Here, we illustrate streptozotocin (STZ)-induced diabetic rats (DM) and age-matched Sprague-Dawley (SD) rats for example. 8-week-old male SD rats (200 ± 20 g) received a single intraperitoneal injection of STZ (70 mg/kg, ip) for the DM group or citrate buffer for the control group. One week after STZ administration, rats with blood glucose > 16.7 mmol/L were considered diabetic. After 8 weeks, the LV myocytes were enzymatically isolated. After calcium reintroduction, there is about 70 - 80% of myocytes that remain in the survival state. Only myocytes with a rod-shape, clear striations, and stable contractions were selected for recording (**Figure 1B**). As shown in **Figure 1A**, we set up the chamber perfusion and needle perfusion for myocytes. Perfusion valves and pacing system were automatically switched by a preset program as described in **Figure 2A**. The intracellular calcium fluorescence was recorded by a calcium imaging system. Values were reported as Mean ± SEM.

Compared with the SD group, the DM group showed significant lower amplitude of the caffeine-induced calcium pulse (Ca-caffeine) (0.332 ± 0.008 vs. 0.276 ± 0.008, t-test: $p < 0.05$) and a lower fractional calcium release SR (Ca-1 Hz/Ca-caffeine) (78.5 ± 1.5% vs. 72.1 ± 1.0%, t-test: $p < 0.05$) (**Figure 2B**). The Tau-1 Hz and Tau-caffeine were obtained from mono-exponential curve fitting; the DM group showed remarkable longer decay time constants of caffeine-induced calcium pulse (Tau-caffeine) (1.822 ± 0.07 ms vs. 2.896 ± 0.088 ms, t-test, $p < 0.05$), and a higher level in Tau-1 Hz/Tau-caffeine ratio (0.076 ± 0.003 vs. 0.086 ± 0.003, t-test, $p < 0.05$) than the SD group (**Figure 2D**). These results indicated the depressed SR Ca^{2+} reserve and impaired Ca^{2+} clearance in diabetic cardiomyocytes.

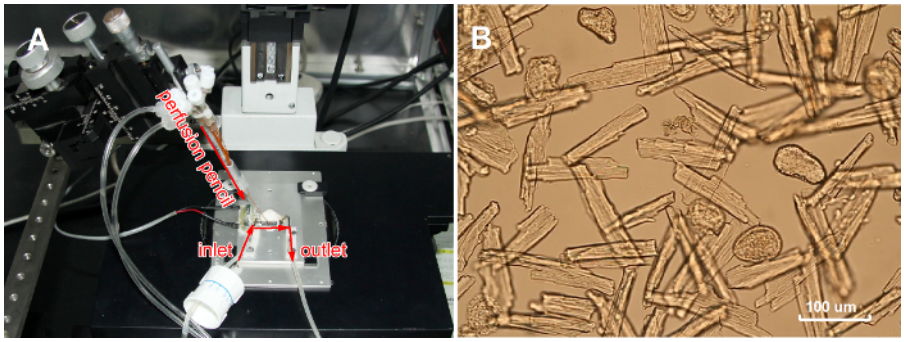

Figure 1. Illustration of perfusion system and isolated LV myocytes. (**A**) Illustration of the chamber perfusion and pencil perfusion. Arrows indicate the flow direction of the NT solution in the perfusion chamber. The pencil provides perfusion surrounding the target myocyte with NT or caffeine solution. The micron tip can be manipulated freely above the chamber. (**B**) Microscopic view of isolated LV myocytes with a rod-shape and clear cross striations. Please click here to view a larger version of this figure.

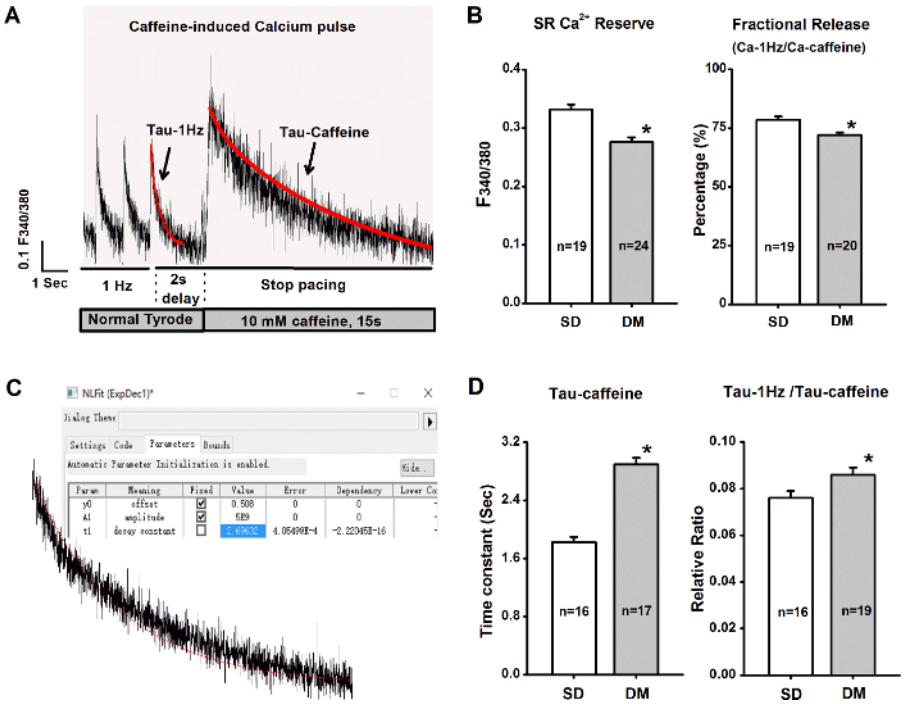

Figure 2. Assessment of SR calcium reserve and calcium removal function. (**A**) Illustration of the Protocol for the switching perfusion valves and pacing system automatically. (**B**) Statistic values of SR Ca^{2+} reserve and SR fractional Ca^{2+} release for 1 Hz stimulated twitches in the DM and SD groups. The DM group showed significant decreased SR Ca^{2+} reserve and SR fractional Ca^{2+} release than the SD group. (**C**) Software panel showing a manually mono-exponential curve fitting for the decay time constant of caffeine-induced calcium pulse (Tau-caffeine). (**D**) Bar graphs comparing the Tau-caffeine and Tau-1 Hz/Tau-caffeine between the DM and SD groups. The DM group showed significant increased values of Tau-caffeine and Tau-1 Hz/Tau-caffeine than the SD group. Value = Mean ± SEM, t-test were performed, *$p < 0.05$ compared with the SD group. Please click here to view a larger version of this figure.

Solutions	Contents (in mM)
Normal Tyrode (NT) solution	135 NaCl, 5.4 KCl, 1.2 $MgCl_2$, 10 Glucose, 10 HEPES, 1.2 Na_2HPO_4, 1.2 $CaCl_2$, adjust pH with NaOH to 7.35
Ca^{2+} free Tyrode solution	135 NaCl, 5.4 KCl, 1.2 $MgCl_2$, 10 Glucose, 10 HEPES, 1.2 Na_2HPO_4, 10 taurine, adjust pH with NaOH to 7.35
Collagenase isolation solution	Collagenase A or Collagenase II + Ca^{2+} free Tyrode solution
KB solution	120 KOH, 120 L-Glutamic, 5 MgCl2, 10 HEPEs, 1 EGTA, 10 Glucose, 20 Taurine, adjust pH to 7.35 with KOH
Caffeine perfusion solution	10 Caffeine in NT solution

Table 1. Solutions for LV myocytes isolation and cellular perfusion.

Valve	Time (Second)
2	15
1	60

Table 2. Preset of parameters in the valve control system (valve commander).

Panel display	comment
4.0 ms duration	electrical stimulation field time
8.0 V	electrical voltage
S1 1.0 HZ 999s	find the target myocyte at pacing status
D2 001s	select this step to pause pacing and delay 1s after basal twitch recording
D3 015s *	"*" indicate that the stimulator can output a 5V TTL signal
D4 060s	finish caffeine perfusion and the myocyte recover to normal state
END	rollback to S1 step

Table 3. Preset of parameters in the stimulator.

Discussion

Calcium flux released from the SR is the major Ca^{2+} source for systole in the heart. To some extent, the amplitude of SR Ca^{2+} content and the fractional Ca^{2+} released from the SR reflect the SR Ca^{2+} reserve available for cardiac contraction. On the other hand, the Ca^{2+} reserve of SR depends on the ability of SR Ca^{2+} reuptake, Ca^{2+} leak of SR, and their balance across the SR during diastole[12,13,14]. In our experiment, rapid application of 10 mM caffeine is aimed to induce total Ca^{2+} release and prevent Ca^{2+} reuptake in the SR by opening the RyR channel. Thus, the amplitude of the caffeine-induced Ca^{2+} pulse could be used as an index of SR Ca^{2+} reserve. In addition, with basic pacing in 1 Hz and 3 Hz, we could further investigate the frequency-dependence of SR load and its underlying mechanism[6].

Removal of Ca^{2+} in the cytoplasm is critical for cardiac diastolic function. As shown in **Figure 2A**, at the stage of 1 Hz field stimulation, the decline of calcium transients was attributed to SERCA in the SR, NCX in the cytomembrane, and slow transport systems (mitochondrial Ca^{2+} uniporter and sarcolemmal Ca^{2+}-ATPase). As the contribution of slow mechanisms is negligible, the decay time constant of Ca^{2+} transient (Tau-1 Hz) reflects the combined activity of SERCA and NCX[7,8,9]. At the stage of caffeine perfusion, SERCA failed to build the SR calcium reserve. Thus, the decline of caffeine-induced calcium pulse (Tau-caffeine) was mainly attributed to NCX. Correspondingly, the NCX function is negative as relevant to Tau-caffeine. The NCX contribution to diastolic Ca^{2+} removal is positive as relevant to the ratio of Tau-1 Hz/Tau-caffeine. The SERCA contribution is positive as relevant to the difference between Tau-caffeine and Tau-1 Hz[7,8,9].

To complete the procedure successfully (**Figure 2**), there are some technical key points that should be noted. Firstly, establishing an automated system is critical to switching the pacing and perfusion system precisely. The preset program could control the delay time accurately and apply the caffeine perfusion rapidly. Secondly, during the process, the myocytes are at the risk of being washed away by the perfusion solution. For keeping the myocytes stably adhered to the chamber dish, the cells should be placed in the dish for more than 15 minutes before recording.

Further, setting up a stable needle perfusion system for application of caffeine is another critical step. There are three key points for this step: (1) Rapid channel switch and smooth needle perfusion, (2) Well-controlled perfusion flow velocity, and (3) Proper distance between the needle tip and target myocyte. Inappropriate setting of flow velocity, tip distance, or interference in the solution channel switch may result in failure of acquiring an acceptable caffeine-induced calcium pulse. For caffeine-induced calcium pulses, only the pulse with a steep wave crest could be selected for analysis of calcium removal function.

The alternative method based on the patch-clamp system can provide relatively accurate data. However, some factors, such as inward-rectifying K^+ current, may influence the accuracy. In addition, a higher requirement of advanced equipment, skilled technician, and time consumption may also limit the application of patch-clamp. To some extent, our protocol has the limitation that it could not provide direct values of SR load or

activity of SERCA and NCX. The parameters (*e.g.*, Ca-caffeine, Tau-caffeine) only reflect changing SR content and contribution to diastolic Ca^{2+} removal indirectly. However, this protocol has the advantage of convenience, less limitation of technique and equipment.

Disclosures

The authors have nothing to disclose.

Acknowledgements

This work was supported by grants from the National Natural Science Foundation of China (No. 81100159, Dongwu Lai; 81401147, Juhong Zhang), the Medical and Health Science Program of Zhejiang Province (No. 201646246, Dongwu Lai), and the Health Science and Technology Plan of Hangzhou City (No. 2013A28, Ding Lin).

References

1. Capel, R. A., Terrar, D. A. The importance of Ca (2+)-dependent mechanisms for the initiation of the heartbeat. *Front Physiol.* **6** 80 (2015).
2. Bers, D. M. Calcium cycling and signaling in cardiac myocytes. *Annu Rev Physiol.* **70** 23-49 (2008).
3. Zhao, S. M., Wang, Y. L., Guo, C. Y., Chen, J. L., Wu, Y. Q. Progressive decay of Ca^{2+} homeostasis in the development of diabetic cardiomyopathy. *Cardiovasc Diabetol.* **13** 75 (2014).
4. Pereira, L., *et al.* Calcium signaling in diabetic cardiomyocytes. *Cell calcium.* **56** (5) 372-380 (2014).
5. Coppini, R., *et al.* Late sodium current inhibition reverses electromechanical dysfunction in human hypertrophic cardiomyopathy. *Circulation.* **127** (5) 575-584 (2013).
6. Ferrantini, C., *et al.* R4496C RyR2 mutation impairs atrial and ventricular contractility. *J Gen Physiol.* **147** (1) 39-52 (2016).
7. Li, L., Chu, G., Kranias, E. G., Bers, D. M. Cardiac myocyte calcium transport in phospholamban knockout mouse relaxation and endogenous CaMKII effects. *Am J Physiol.* **274** H1335-1347, (1998).
8. Cheng, J., *et al.* CaMKII inhibition in heart failure, beneficial, harmful, or both. *Am J Physiol Heart Circ Physiol.* **302** H1454-1465 (2012).
9. Lai, D., *et al.* The Rho kinase inhibitor, fasudil, ameliorates diabetes-induced cardiac dysfunction by improving calcium clearance and actin remodeling. *J Mol Med (Berl).* **95** (2) 155-165. (2017).
10. Lai, D., *et al.* Stretch Current-Induced Abnormal Impulses in CaMKIIδ Knockout Mouse Ventricular Myocytes. *J Cardiovasc Electrophysiol.* **24** (4), 457-63 (2013).
11. Xu, L., *et al.* Alterations of L-type calcium current and cardiac function in CaMKII{delta} knockout mice. *Circ Res.* **107** (3) 398-407. (2010).
12. Roussel, J., *et al.* Palmitoyl-carnitine increases RyR2 oxidation and sarcoplasmic reticulum Ca^{2+} leak in cardiomyocytes: Role of adenine nucleotide translocase. *Biochim Biophys Acta.* **1852** (5), 749-758 (2015).
13. Yaras, N., et al. Effects of diabetes on ryanodine receptor Ca^{2+} release channel (RyR2) and Ca^{2+} homeostasis in rat heart. *Diabetes.* **54** (11) 3082-30888. (2005).
14. Hu, Y., *et al.* Adenovirus-Mediated Overexpression of O-GlcNAcase Improves Contractile Function in the Diabetic Heart. *Circ Res.* **96** (9) 1006-1013 (2005).

