High-throughput assessment of calcium sensitivity in skinned cardiac myocytes

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Lim, Chee Chew, Michiel H. B. Helmes, Douglas B. Sawyer, Mohit Jain, and Ronglih Liao. High-throughput assessment of calcium sensitivity in skinned cardiac myocytes. Am J Physiol Heart Circ Physiol 281: H969–H974, 2001.—Isolated permeabilized cardiac myocytes have been used in the study of myofilament calcium sensitivity through measurement of the isometric force-pCa curve. Determining this force-pCa relationship in skinned myocytes is relatively expensive and carries a high degree of variability. We therefore attempted to establish an alternative high-throughput method to measure calcium sensitivity in cardiac myocytes. With the use of commercially available software that allows for precise measurement of sarcomere spacing, we measured sarcomere length changes in unloaded skinned cardiac myocytes over a range of calcium concentrations. With the use of this technique, we were able to accurately detect acute increases or decreases in myofilament calcium sensitivity after exposure to 10 mM caffeine or 5 mM 2,3-butanedione monoxime, respectively. This technique allows for the simple and rapid determination of myofilament calcium sensitivity in cardiac myocytes in a reproducible and inexpensive manner and could be used for high-throughput screening of pharmacological agents and/or transgenic mouse models for changes in myofilament calcium sensitivity.

myofilament; sarcomere length

ISOLATED CARDIAC MYOCYTES have become widely used in the study of cell excitation-contraction coupling, because they allow for precise control of the extracellular environment and preclude any hormonal, structural, or vascular effects associated with multicellular preparations. Excitation-contraction coupling in myocytes is governed by calcium cycling and myofilament calcium sensitivity (2). These two major components are intricately linked, therefore making it difficult to attribute changes in contractile status to either system.

Cardiac “skinned” myocytes have been previously used to separate the contractile status of the myofilament from calcium cycling. In this preparation, isolated cells are exposed to a detergent that permeabilizes the cell membrane, allowing for control of intracellular calcium concentrations via the bathing solution. Cells are attached (loaded) to a force transducer, and calcium sensitivity is ascertained by measuring isometric contraction force over a range of calcium concentrations. Determining this force-pCa relationship in skinned myocytes is relatively cumbersome and time consuming, requiring a high level of expertise and an expensive mechanical setup (3). We therefore attempted to establish an alternative high-throughput methodology to measure calcium sensitivity in cardiac myocytes.

With the use of commercially available software that allows for precise measurement of sarcomere spacing, we measured sarcomere length changes in unloaded skinned cardiac myocytes over a range of calcium concentrations. This technique allows for the rapid determination of myofilament calcium sensitivity in cardiac myocytes in a reproducible and inexpensive manner and could be used for high-throughput screening of pharmacological agents and/or transgenic mouse models for changes in myofilament calcium sensitivity.

METHODS

Myocyte isolation. The present study was performed in accordance with the guidelines of the Animal Care and Use Committee of the Boston University School of Medicine and the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Left ventricular (LV) myocytes were isolated according to a previously described protocol (16). Male Wistar rats weighing 200–250 g were anesthetized (50 mg/kg ip pentobarbital

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sodium) and heparinized (200 IU iv). The hearts were rapidly excised and immersed in an ice-cold modified cardioplegic Krebs-Bulbring (KB) solution containing (in mM) 85 KOH, 30 KCl, 30 KH$_2$PO$_4$, 3 MgSO$_4$, 0.5 EGTA, 10 HEPES, 50 l-glutamic acid, 20 taurine, 10 2,3-butanedione monoxime (BDM), and 10 glucose. The hearts were cannulated via the aorta and perfused in a retrograde fashion at a constant perfusion pressure of 90 cmH$_2$O. The hearts were first perfused for 5 min with nonrecirculating 1.8 mM Ca$^{2+}$ Tyrode solution containing (in mM) 137 NaCl, 5.4 KCl, 1.8 CaCl$_2$, 0.5 MgCl$_2$, 10 HEPES, and 10 glucose; pH 7.4] followed by Ca$^{2+}$-free Tyrode solution containing (in mM) 135 NaCl, 4 KCl, 1 MgCl$_2$, 10 HEPES, 0.33 Na$_2$HPO$_4$, and 10 glucose; pH 7.2] for another 5 min. Hearts were then perfused with a digestion solution containing 0.08% collagenase A (Collagenase A, Boehringer-Mannheim; Indianapolis, MN) and 0.02% protease XIV (Sigma; St. Louis, MO). After the hearts were palpably flaccid, the digestion solution was washed out with Ca$^{2+}$-free Tyrode solution for 30 s. The hearts were then removed from the cannula, and the LV (including septum) was separated, minced, and gently agitated, allowing the myocytes to be dispersed in KB solution.

Myocyte skimming procedures. Myocytes were permeabilized according to the method described by Granzier and Irving (10). Briefly, myocytes were washed four times with KB solution containing 10 mM BDM and 0.5 mM phenylmethylsulfonyl fluoride (PMSF) to remove trace contaminants of calcium or proteases. Myocytes were then Skinning by suspension in relaxation solution (containing (in mM) 10 EGTA, 5.9 MgAc, 5.9 Na$_2$ATP, 10 creatine phosphate, 40 imidazole, 70 potassium propionate, 5 NaN$_3$, and 1 dithiothreitol and 50 U/ml creatine phosphokinase, 0.5 PMSF, and 0.004 leupeptin; pH 7.0) with 1% Triton (Sigma). Cells were kept on ice throughout the skinning procedure. After 50 min, the cells were washed twice with relaxing solution to wash out Triton.

Experimental setup. The cell perfusion setup was adapted from the method described by Spitzer et al. (22a), and a schematic of the system is shown in Fig. 1 (6). A single myocyte was gravity perfused using a three-barrel pipette (Fig. 1A) with a fast-step solution switcher (SF-77B, Warner Instruments; Hamden, CT). Each of the barrels was made of square glass tubing, 800-μm wide. The pipette and stepper motor were attached to a micromanipulator to allow precise positioning of the pipette opening over the myocyte. The three-barrel pipette was attached to a stepper motor (Fig. 1B) that could quickly switch the adjacent barrel over the myocyte (in 20 ms), effectively switching the solution bathing the myocyte. An eight-channel valve-controlled gravity perfusion system (Cell MicroControls; Virginia Beach, VA) was connected via manifolds (Fig. 1C) to the fast-step solution switcher. This allowed for quick switching of the myocyte to the adjacent barrel while changing the solution in the previous barrel using the valve-controlled system. The flow through the barrels was held constant at ~250 μl/min by adjusting the height of the manifold system. This flow was high enough to prevent perfusion cross-contamination from adjacent barrels, thus allowing for exclusive perfusion of a myocyte by a single barrel. The combination of the three-barrel pipette and the manifold system made it possible to cycle through nine different pCa solutions in several minutes. The pCa solutions were prepared according to the Fabiato computer program (7).

In conjunction with the fast-step solution switching system, the microscope’s coverslip was replaced with a three-barrel custom-made coverslip (Fig. 1C). The coverslip (Piranha-SC, Scientific Instruments Pittsburgh, PA) was attached with Dow Corning vacuum grease to the bottom of a custom-designed chamber made out of polycarbonate (Fig. 1D). The chamber had one slanted side to let in the three-barrel pipette at a 45° angle. The adjacent side had an opening to a reservoir to aspirate excess solution. This geometry allowed for the placement of a second glass coverslip on top of the chamber that covered the tip of the pipette and the myocyte under study but left an opening at the surface to allow entrance and movement of the pipette. Surface tension made the solution in the chamber adhere to the top coverslip, eliminating the meniscus and assuring even illumination. This arrangement had the additional advantage of preventing excess aspiration; the level in the aspiration reservoir would drop before the solution detached from the top coverslip. The chamber was mounted on an inverted microscope (Nikon Diaphot epifluorescence microscope), and cells were visualized using a Nikon ×40 (numerical aperture 1.3) oil-immersion fluorescence objective.

Sarcomere length measurement. Cells were imaged using a variable frame rate (60–240 Hz) charge-coupled device camera, and the images were digitized and displayed on the computer screen at a sampling speed of 240 Hz. Frame grabber, acquisition, and analysis software for sarcomere length measurements were obtained from IonOptix (Milton, MA). The algorithm for measuring sarcomere spacing was adapted from Gannier et al. (9). First, the user aligns the cell image horizontally, after which a user-defined rectangular region of interest (ROI) within the myocyte is selected. Because of the horizontal alignment of the cell, the line optical density of each video line in the ROI shows the sinusoidal dark-light pattern of the alternating I and A bands of the sarcomeres of the myocyte. The program then performs a fast Fourier transform for each video line in the ROI, and the area under the resulting power spectra are then summed to get an average power spectrum for that ROI. Undesirable high- and low-frequency components are filtered out by user-defined upper and lower boundaries for sarcomere length. The maximum value of the remainder of the power spectrum is found, and, using the two neighboring values, the peak of the power spectrum is found by curve fitting these points with a second-order polynomial. This peak represents the sarcomere spacing and is converted from pixel frequency to sarcomere length. The real-time analysis gives the user instantaneous feedback of the signal quality, thus allowing the ROI to be adjusted accordingly. A ROI that encompassed 50–60% of the total cell surface area typically gave the sharpest power.
spectrum peak and hence the most accurate measurement of sarcomere length. This method of calculating the average sarcomere length allowed us to resolve changes in sarcomere spacing as small as 5 nm.

**Experimental protocol.** Skinned cells were placed on a coverslip affixed to the myocyte chamber and superfused with relaxing solution (9.0 pCa). After 5 min of stabilization time, the relaxing solution was switched to a new pCa solution, and sarcomere length was measured when the steady state was achieved. The myocyte was subsequently reperfused with relaxing solution until slack sarcomere length was achieved. This process was repeated for various pCa solutions.

**Statistical analysis.** Data are means ± SE. Group comparisons were made using ANOVA followed by a Bonferroni-Dunn post hoc test. \( P < 0.05 \) was considered statistically significant.

**RESULTS**

**Myocyte quality and sarcomere length.** High-quality myocytes were found to be critical for reproducible results, and great care was taken during the isolation procedure and subsequent skinning of the cells to avoid protein degradation. This preparation led to a high percentage (>90\% after skinning) of rod-shaped cells with a clear striation pattern and a consistent sarcomere length of >1.9 \( \mu \)m. The average resting sarcomere length of 84 cells from 5 hearts was 1.95 ± 0.01 \( \mu \)m. Cells with resting lengths below 1.85 \( \mu \)m were excluded from the study. Figure 2A shows a representative bright-field image of a myocyte exposed to 9.0, 5.7, and 5.1 pCa solutions. The myocyte striation pattern remained clearly visible at all calcium levels, including 5.1 pCa, allowing for accurate detection of sarcomere length changes at each pCa solution. Figure 2B shows a representative tracing of sarcomere length in a cardiomyocyte exposed to increasing pCa solutions. Sarcomere shortening could be detected at a pCa of 7.0, and, with increasing pCa, sarcomere length progressively decreased. In preliminary experiments, we simultaneously measured sarcomere shortening and cell shortening (by video edge detection) and observed that with increasing calcium concentration, parallel relative shortening was observed in sarcomere and cell length, suggesting that changes in sarcomere length with calcium accurately represent changes in cell shortening.

**Sarcomere length-pCa curve.** Figure 3 demonstrates the relationship of sarcomere length changes versus pCa. The data show remarkable reproducibility among 38 cells from 3 animals. With increasing calcium concentration, the sarcomere gradually shortened, and at 5.1 pCa, sarcomere length decreased to 1.41 ± 0.03 \( \mu \)m. At calcium concentrations below 5.1 pCa, however, the unloaded myocytes never reached steady state, and sarcomere length continued to shorten until the cell reached hypercontracture.

To assess the sensitivity of this technique to detect acute changes in myofilament calcium sensitivity, we determined the sarcomere length-pCa relationship after exposure to 10 mM caffeine, a calcium sensitizer (13, 25), or 5 mM BDM, which inhibits cross-bridge cycling (11, 13) (Fig. 4). Caffeine caused a leftward shift of the sarcomere length-pCa curve, resulting in greater sarcomere shortening relative to control cells from 6.5 to 5.25 pCa. At a pCa below 5.25, cells exposed to caffeine would irreversibly hypercontract. In contrast, exposure of myocytes to BDM resulted in a rightward shift of the sarcomere length-pCa relationship, with less sarcomere shortening at a given pCa value from 6.0 to 5.1 pCa.

**DISCUSSION**

In this paper, we described a technique employing a rapid solution switcher and sarcomere length detection...
system to assess myofilament calcium sensitivity in skinned cardiac myocytes. We determined changes in sarcomere length on exposure to increasing calcium concentrations. With the use of this technique, we were able to accurately detect acute changes, both an increase with caffeine and a decrease with BDM, in myofilament calcium sensitivity (11, 13, 20, 25). BDM has been used at high concentrations (30 mM) to completely block cross-bridge formation and hence inhibit force generation (20). In our experiment, a low BDM concentration (5 mM) resulted in a significant reduction in sarcomere shortening, similar to previously reported decreases in maximal force generation in skinned cardiac muscle treated with 5 mM BDM (13, 20).

After exposure to calcium, unloaded skinned myocyte underwent shortening and rapidly reached a new steady-state sarcomere length, where sarcomere shortening was opposed by an equal but opposite restoring force (12, 17, 24). At pCa values below 5.4, we were able to resolve a clear striation pattern of the myocyte (see Fig. 2A) and were able to record sarcomere lengths well below the thick filament length of 1.6 μm, as has similarly been reported by Roos and Brady (22). It is unclear why I bands were resolvable at sarcomere lengths at which the Z disk would be expected to collide against the thick filaments, although several potential mechanisms exist. Because of decreased molecular packing at the ends of thick filaments, individual myosin projections are thought to be free at each tapered end of the thick filament (18). Thus the presence of I bands at sarcomere lengths <1.6 μm may represent A band shortening due to a lack of myosin heads at the region of the Z disks as a result of “folding” or “bending” back of the free myosin projections onto the thick filament stalk (14). Another potential mechanism for A band shortening includes cross-bridge disarray at the thick filament ends, possibly due to terminal cross-bridges falling out of register (6, 19).

The maximal shortening response, however, was difficult to assess in these myocytes. High calcium concentrations were often damaging to the preparation, as previously reported (15). Moreover, at a pCa below 5.1, prolonged contraction lead to irreversible shortening and hypercontraction. For calcium concentrations that did not exceed 5.5 pCa, however, the preparation was remarkably stable, allowing for 20–30 contractions at pCa values between 6 and 5.5 over a time span of an hour without visible deterioration of the preparation or decline in contractility. An inability to accurately assess maximum shortening response precluded normalization of the data and Hill plot analysis. The high reproducibility of the pCa-sarcomere shortening relation, and similar initial sarcomere lengths, however, allowed for direct comparison of the absolute values of sarcomere shortening between cells and within a cell between treatments.

One of the determinants of myofilament calcium sensitivity is sarcomere length. It has been shown that at shorter sarcomere lengths calcium binding of tropo-nin C is reduced (1, 8). To avoid the confounding effects of sarcomere length on calcium sensitivity, each myocyte was perfused with relaxing solution to ensure that cells contracted from a comparable sarcomere length, i.e., resting sarcomere length (as seen in Fig. 2B). Myocytes with inherently greater calcium sensitivity shortened to a smaller sarcomere length and will therefore exhibit reduced length-dependent calcium sensitivity. Although this may blunt the leftward shift of the sarcomere length-pCa curve in cells with increased myofilament calcium sensitivity, we were still able to detect changes in the sarcomere length-pCa curve with caffeine treatment (Fig. 3).

Experiments measuring calcium sensitivity in skinned preparations of muscle strips or isolated myocytes have traditionally used mechanical setups in which the muscle specimen or cell is attached to a

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Fig. 3. Plot of sarcomere length versus pCa in control skinned myocytes (n = 38). Each symbol represents one individual myocyte.

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Fig. 4. Plot of sarcomere length versus pCa in control skinned myocytes (circles; n = 38), myocytes exposed to 10 mM caffeine (triangles; n = 26), and myocytes exposed to 5 mM 2,3-butanedione monoxime (BDM; diamonds; n = 16). Each point is the mean ± SE of the number of cells. *P < 0.05, caffeine versus control; †P < 0.05, BDM versus control.
motor and a force transducer to generate force-pCa curves. This approach has led to invaluable contributions to our understanding of muscle function. The experimental characterization of the basic contractile element in multicellular preparations is inherently problematic, however, due to the viscoelastic nature and nonuniformity of sarcomere shortening of these preparations and variable diffusion distances (3). To circumvent many of the confounding factors associated with multicellular preparations, methods using loaded isolated skinned cardiac myocytes to measure isometric contraction have been employed. Measurement of force-pCa curves in isolated myocytes requires expensive mechanical equipment and has relatively large variations between preparations. This is in part due to problems with transducer attachment procedures that require strong enough attachment to endure cell contraction without influencing cell function (3). Limited sampling size combined with large variation reduces the sensitivity of this approach to detect differences between experimental populations (3).

The variation in sarcomere shortening was much smaller using our myocyte preparation than has previously been reported for isometric force in skinned cardiac myocyte preparations (21, 23). Variation was reduced mainly by selecting a relatively large ROI in the cell from which the sarcomere length was calculated. The ROI in an average cell encompassed 50–60% of the total surface. As a result, the sarcomere shortening on calcium exposure was found to be extremely consistent and reproducible within a population of myocytes or between animals.

Cell shortening-pCa curves have previously been derived in skinned cardiac myocytes, utilizing video edge detection to monitor changes in cell length at different calcium concentrations (21). We have observed that sarcomere and cell shortening were comparable when measured simultaneously in a cell; however, assessment of cell shortening can be inconsistent relative to sarcomere shortening due to nonlinearities in cell movement and geometry (5). In addition, assessment of sarcomere shortening may be preferable to video edge detection in instances where cells loose a clear edge, as in long-term adult cardiac myocyte culture (4).

Our main objective was to establish a high-throughput method to measure calcium sensitivity in cardiac myocytes. Assessment of the sarcomere length-pCa relationship, described in this paper, is low in cost, time of development, and maintenance and can be readily adapted by many laboratories. This high-throughput highly sensitive methodology provides a relatively easy alternative to screen pharmacological agents or transgenic animals for alterations in calcium sensitivity.

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REFERENCES


