Measurement of myofilament calcium sensitivity at physiological temperature in intact cardiac trabeculae

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Varian, Kenneth D., Sripriya Raman, and Paul M. L. Janssen. Measurement of myofilament calcium sensitivity at physiological temperature in intact cardiac trabeculae. Am J Physiol Heart Circ Physiol 290: H2092–H2097, 2006; doi:10.1152/ajpheart.01241.2005.—Cardiac contraction-relaxation coupling is determined by both the free intracellular calcium concentration ([Ca2+]i) and myofilament properties. We set out to develop a technique where we could assess these parameters (twist and steady-state force [Ca2+]i) under near physiological conditions. Bis-fura-2 was iontophoretically introduced into ultrathin rat trabeculae preparations to monitor the [Ca2+]i, and steady-state contractures were achieved by using a modified Krebs-Henseleit solution containing high K+. During K+ contractures, the very slow changes in [Ca2+]i, and force development were in equilibrium and allowed for the construction of a steady-state, force-[Ca2+]i, relationship. Twitch contractions before and after this myofilament calcium sensitivity assessment were unaltered, and this protocol could be repeated several times. For the first time, this novel protocol allows us to measure myofilament calcium sensitivity under physiological temperature. Not only do the data so obtained allow us to assess myofilament calcium sensitivity, the data also will allow us, in the same preparation under nearly identical conditions, to compare the dynamic to the steady-state, force-calcium relationship. To test whether the steady-state relationship between force and calcium in our novel protocol reproduces expected changes, we determined this relationship in the presence of isoproterenol and under acidosis. As expected, β-adrenergic stimulation resulted in an increase of calcium amplitude and twitch force and a desensitization of the myofilaments as indicated by a rightward shift of the obtained steady-state, force-[Ca2+]i relationship. An increase in pH shifted the curve leftward, whereas a decrease in pH resulted in the expected rightward shift.

rat; intracellular calcium concentration; β-adrenergic stimulation

CARDIAC CONTRACTION-RELAXATION coupling is linked to both the rise and fall of the intracellular calcium concentration ([Ca2+]i) and intrinsic properties of the myofilaments (1). Although changes in calcium handling alone or myofilament properties alone can each have effects on the dynamic cardiac twitch, there is bilateral feedback between calcium concentration and myofilament properties. Up until now, these two determinants of contractile function have only been assessed independently or under nonphysiological conditions.

In a dynamic cardiac twitch, the myofilaments and intracellular calcium are virtually never in a steady-state equilibrium (1); the only time during the cardiac cycle at which a pseudo-equilibrium exists is right before stimulation, when active force development is absent and the [Ca2+]i is nearly stable at diastolic values (50–100 nM). After stimulation of a cardiac myocyte, the [Ca2+]i, rapidly rises, whereas force production is much slower. When peak [Ca2+]i is reached, force development is still rising, and usually only at ~50% of peak force development. Well before the time of peak force production, the [Ca2+]i, declines sharply to nearly resting values, whereas during the most part of the relaxation phase, [Ca2+]i, has already reached and remains at resting levels (9). This implies that although the [Ca2+]i, and myofilament response are linked, changes in calcium and force production are decoupled in the time domain. Myofilament calcium sensitivity reflects the contractile response of the myofilaments to a given calcium concentration at steady state. Thus this sensitivity plays a major role in determining the contractile response to the intracellular calcium transient. To elucidate how force production depends on [Ca2+]i, under physiologically relevant conditions, we set out to develop a technique where myofilament calcium sensitivity could be assessed at body temperature in intact cardiac muscle.

Assessment of myofilament calcium sensitivity has almost exclusively been done in skinned (demembranized) fibers where myofilaments are exposed to a series of buffered calcium solutions (3). Our goal is to assess myofilament calcium sensitivity in intact muscle under near physiological conditions; this would have the advantage in which factors that are known to influence myofilament calcium sensitivity, such as pH, temperature, and protein phosphorylation status (3, 11, 18), are close to their in vivo settings. Although myofilament calcium sensitivity has been assessed in intact muscle before, these experiments have thus far been limited to room temperature.

We developed a protocol and technique based on iontophoresis coupled with K+ contractures to construct the steady-state relationship, while still allowing for assessment of the dynamic force-[Ca2+]i relationship measurements at multiple times under different conditions all in the same trabeculæ preparation (to eliminate intermuscle variation). The results show that we can repeatedly assess both the dynamic as well as steady-state, force-[Ca2+]i relationships under physiological load, frequency, and temperature in intact myocardium and that the obtained force-calcium relationships shift in the expected directions under β-adrenergic stimulation, alkalosis, and acidosis.

MATERIALS AND METHODS

Muscle preparation, solutions, and experimental set-up. The investigation conforms to the Guide for the Care and Use of Laboratory Animals, published by the National Institutes of Health (NIH Publi-

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cations No. 85-23, Revised 1996). All protocols were approved by the Institutional Animal Care and Use Committee. LBN-F1 rats (male, 175–200 g) were anesthetized with intraperitoneal injection of pentobarbital sodium. After bilateral thoracotomy and intracardiac heparinization, the hearts were rapidly excised and placed in Krebs-Henseleit (K-H) buffer containing (in mM) 137 NaCl, 5 KCl, 1.2 NaH2PO4, 1.2 MgSO4, 20 NaHCO3, 0.25 CaCl2, and 10 glucose. 2.3-,Butanedione monoxime (BDM, 20 mM) was added to the dissection buffer to prevent cutting injury. Exposure to BDM for a short time has shown to be reversible (8, 14). Hearts were cannulated via the ascending aorta and retrogradely perfused with K-H + BDM buffer in equilibrium with 95% O2-5% CO2, resulting in a constant pH of 7.4. After blood was washed out, the right ventricle was opened and thin, uniform, nonbranched trabeculae were removed, leaving a block of right ventricular free wall tissue on one end and a piece of tricuspid valve on the other (19). The dimensions of the trabeculae (n = 8) were measured with a reticle on the inverted fluorescence microscope (100×) and were 0.17 ± 0.03 mm wide, 0.08 ± 0.01 mm thick, and 2.0 ± 0.2 mm long. The cross-sectional area was calculated by assuming ellipsoid shapes. Muscle dimensions have been shown to be a determinant of contractile performance (due to diffusion distance), and thus only trabeculae with a smallest dimension of <150 μm were selected and included (16).

With the use of a dissection microscope, muscles were mounted between a platinum-iridium, basket-shaped extension of a force transducer (KG7, Scientific Instruments, Heidelberg, Germany), and a hook (valve end) was connected to a micromanipulator. Muscles were superfused with the same buffer (as above) at 37.5°C (with the exception that BDM was omitted) and stimulated at 4 Hz using 3 ms of stimuli of ~150% threshold (typically 3–5 V). Extracellular [Ca2+] ([Ca2+]o) was raised to 1.5 mmol/l, and muscles were allowed to stabilize for at least 30 min before the experimental protocol was initiated. Muscles were stretched to a length where a small increase in length resulted in nearly equal increases in resting tension and active developed tension. This length (optimal length) is slightly below the length where active force development is maximal and was selected to be comparable to the maximally attained length in vivo at the end of diastole (~2.2 μm sarcomere length) (17).

At room temperature, cardiac muscle can be tetanized by rapidly pacing the muscle (at 15 Hz) (15). However, at a physiological temperature, even at pacing rates as fast as 20 Hz, healthy, well-perfused muscles almost fully relax at each beat. We thus resorted to K+ contractures to obtain levels of steady-state force development, which are needed to construct the steady-state, force-[Ca2+] relationship. After normal twitches were recorded at 4 Hz, the superfusion solution was switched to a modified K-H solution containing (in mM) 142 KCl, 0 NaCl, and 3 CaCl2. The rest of the contents were identical to that of the normal K-H buffer. The high K+ solution was applied for 30 s and then washed out.

Intracellular calcium measurements. Trabeculae were iontophoretically injected with bis-fura-2 (Texas Fluorescence) as described previously (2, 9). Bis-fura-2 was chosen due to its higher signal to calcium buffering ratio (allowing for a loading of the dye to 5–10 times background without impacting buffering) and its slightly higher Kd (390 nM in vitro) than fura-2, allowing for still accurate diastolic values but a better resolution at higher [Ca2+]. Iontophoretic loading of the dye was performed at room temperature (at body temperature, the rate of dye leak is higher). We loaded the bis-fura-2 until the photomultiplier output at baseline 380 nm excitation was between 6 and 10 (average, 8.7 ± 1.0) times over background. After loading and spreading of the dye was completed at room temperature (1 Hz), the system was returned to 37°C. The muscle was stimulated to contract at 4 Hz while force and fluorescent emission measurements (excitation, 340 and 380 nm) were recorded. The superfusion solution was then switched to the one with 142 mM K+. Excitation wavelength was switched back and forth between 340 and 380 nm so that the 340-to-380 ratio could be taken at given points along the contracture.

After the contracture relaxed and twitches at 4 Hz were resumed, 1 μmol/l isoproterenol was administered to the K-H solution and the protocol was repeated. In a separate set of experiments, the pH values of the K-H and high K+ solutions were set at 7.7 and 7.1 to mimic alkalosis and acidosis, respectively. K+ contractures were performed at pH 7.7, 7.1, and 7.4, and force was plotted against the 340-to-380 ratio of fluorescence. Data were stored on a computer for off-line analysis using custom-written software.

Data analysis and statistics. All force values were normalized to cross-sectional area, and comparisons were done within each muscle to eliminate intermuscle variations. Paired and unpaired t-tests were performed with a two-tailed P value, with a value <0.05 being considered significant.

RESULTS

Defining the relationship between the dynamic cardiac twitch force-[Ca2+]i, relationship and the steady-state force-[Ca2+], required exploring an approach to reversibly “tetanize” cardiac muscle while measuring calcium under physiological conditions. Exposing cardiac muscle to high K+ solutions causes a slow depolarization of myocyte membranes (6) due to the dependence of resting potential on the K+ gradient. This most likely results in the opening of voltage-dependent calcium channels. We found that brief exposure (30 s) to a modified K-H solution with 142 mM K+ elicited a slow-forming contracture in the muscle resembling a twitch but occurring around 200–400 times slower (data not shown). Unlike tetani at room temperature (5, 10), the level of contracture could not be regulated by varying [Ca2+]o. However, the contracture occurred very slowly, and compared with the extreme rapid changes in [Ca2+]i in a twitching muscle, the rise in [Ca2+]i was K+ contractures is >1,000 to 1,200 times slower (data not shown). From these observations we concluded that during the K+ contracture, we may consider the so-obtained values for force and [Ca2+]i, to be in a steady-state equilibrium. Figure 1 shows a representative tracing of such a K+ contracture. The muscle is twitching at 4 Hz, while the solution flowing through the bath is switched to that with high K+. Of note, the twitches
begin to rise in amplitude before stimulation is stopped. This is most likely because the K+ solution contains a higher calcium concentration (3 mmol/l) to reach a near maximal myofilament activation during the contracture. Figure 2, A and B, shows that both twitch amplitudes and 50% relaxation time before and after the contracture were not significantly different, indicating the reversibility and absence of significant rundown. Other contractile and kinetic parameters were likewise unaltered after a K+ contracture. We have repeated this K+ contracture several times in the same muscle, because it is completely reversible. Twitch forces and kinetics return to precontracture values within minutes after the contracture. Currently, the limitation of life span of the muscles was not the contractile and kinetic parameters were likewise unaltered after a K+ contracture. We have repeated this K+ contracture several times in the same muscle, because it is completely reversible. Twitch forces and kinetics return to precontracture values within minutes after the contracture. Currently, the limitation of life span of the muscles was not the contractile

Figure 3A shows the unfiltered output of the photomultiplier during a K+ contracture, with excitation filter being alternated between 340 and 380 nm roughly every half second. As expected, calcium enters the sarcoplasm, slowly indicated by the slow decrease in fluorescence emission at 380 nm excitation and an increase at 340 nm. In Fig. 3B, raw recordings of force and analyzed calcium fluorescence ratios are depicted. It can be seen that calcium and force rise simultaneously. When ratios were plotted against force up to the peak of the K+ contracture, a sigmoidal curve is observed, typical of a myofilament calcium sensitivity curve. An example of such a curve is shown in Fig. 4. In pilot experiments, with the use of the indicator BCECF, we observed no changes in intracellular pH until peak contracture was reached. We did not observe open-loop behavior similar to the dynamic calcium transient versus force relationship. Instead, either there was no hysteresis at all (downstroke fell right on top of upstroke) or there was a very small amount of open-loop behavior but in the opposite direction as the twitch (calcium still high but force falling). The latter behavior coincided with a drop in intracellular pH. In addition, when K+ contractures were applied for a short time (15 s), the up and down strokes of both force and calcium were identical, underlining the fact that during K+ contractures, force and calcium are in equilibrium.

With the ability to repeat the protocol and compare the steady-state force [Ca2+]i, plots to the twitch force [Ca2+]i, we used a set of preparations to show that the observed force-calcium curve truly represents myofilament responsiveness. We used the β-adrenergic agonist isoproterenol to evoke an expected change in myofilament sensitivity [reduction in sensitivity due to PKA mediated phosphorylation of troponin I (TnI)]. Figure 5 shows the simultaneous measurement of [Ca2+]i (as 340-to-380 ratio) and force during a cardiac twitch with and with out a maximal isoproterenol (1 μM) response. Figure 6A shows the resulting phase-plane plot when [Ca2+]i is plotted against force in a single cardiac trabeculae with and without isoproterenol. We averaged the contractures-derived myofilament calcium sensitivity curves (n = 8 muscles) to show the reproducible rightward shift in the curve upon iso-

Fig. 2. Twitch amplitudes (A) and time from peak tension to 50% relaxation (RT50) (B) before and after K+ contracture are not significantly different. Retention of muscle of its twitch characteristics suggests that K+ contractures are fully reversible.

Fig. 3. A: recording of intracellular calcium concentration during K+ contracture, depicted by photomultiplier output during K+ contracture. Excitation wavelength is switched every half second between 340 and 380 nm. Vertical lines represent time taken to switch filter. The 340-to-380 (340/380) ratio taken at each filter switch was used as an indication of calcium concentrations increasing slowly throughout contracture. B: simultaneous measurement of K+ contracture force with 340/380 ratios taken throughout contracture. Solid line represents force, whereas dotted line represents fluorescence 340/380 ratio. It can be seen that calcium and force rise and fall at nearly the same rate. Entire contracture lasts ~30 s and can be shortened or prolonged.
proterenol administration (Fig. 6B). The average 340-to-380 ratio at 50% of maximal developed force was 0.65 and shifted to a higher calcium value of ratio 0.85 upon administration of isoproterenol. When comparing the two steady-state curves, we observed a clear rightward shift upon administration of isoproterenol, probably resulting from myofilament protein phosphorylation, such as TnI (11).

Assessment of dynamic and steady-state calcium relationships now allows us to better analyze the phase-plane loops of force versus calcium. We can now relate the active twitch curves to the steady-state values and determine, for instance, at what isochrone the dynamic force-calcium value equals that curves to the steady-state values and determine, for instance, at what isochrone the dynamic force-calcium value equals that obtained in the steady-state relationship. We termed this point the contraction-relaxation coupling point (CRCP; in ms) from initiation of stimulation. Before reaching this point, the [Ca$^{2+}$], exceeds that predicted by steady-state behavior, whereas after the opposite holds: force is higher than predicted from the steady-state relationship. As expected, the CRCP after isoproterenol administration shifted to lower values, from 43 ± 2 to 29 ± 2 ms (n = 8 muscles, P < 0.0001).

To further validate this novel assessment of myofilament calcium sensitivity, we demonstrated that it is sensitive to other forms of physiological modifiers of myofilament calcium sensitivity as well. We raised the pH from 7.4 to 7.7 and observed a leftward shift of the steady-state, force-calcium relationship as expected. The 340-to-380 ratio at 50% of maximal developed force decreased by an average of 0.08 ± 0.01 units (n = 3 muscles). When this experiment was repeated in the opposite direction (pH 7.7 to 7.4), identical results were obtained, and when pH was lowered to 7.1 (from 7.4), an expected rightward shift of ~0.12 ratio units was observed.

**DISCUSSION**

Accurate measurements of contraction and relaxation parameters of cardiac muscle are essential as we dive further into the molecular mechanisms regulating excitation-contraction coupling and contractility in health and disease. We cannot fully understand the functional implications of any changes in molecular components of cardiac muscle unless the potentially changed physiological parameters can be measured under conditions that resemble as close as possible the in vivo situation. We set out to develop a technique that would allow simultaneous measurement of the force-[Ca$^{2+}$] relationship (both dynamic and static) in intact muscle under physiological conditions, to allow us to elucidate the cardiac contraction-relaxation coupling process under rates and conditions as they occur in vivo. We show for the first time that a protocol that couples the assessment of [Ca$^{2+}$], and loaded contractions in both the dynamic and static state will enable us to study calcium handling and the myofilament response under physiologically relevant conditions.

Myofilament calcium sensitivity has traditionally been assessed in so-called skinned fibers where membranes are dissolved away and myofilaments are exposed to EGTA-buffered calcium solutions (3). Skinned preparations have been very useful in defining calcium sensitivity changes with respect to mutations at the myofilament level, but they can be neither unambiguously compared with dynamic twitch contractions nor traditionally done at physiological temperature due to technical limitations. In addition, cytosolic factors and protein phosphorylations (and other posttranslational modifications, such as methylations, etc.) that occur physiologically and that can potentially be contributors to the sensitivity of myofilaments to calcium are generally not controlled for in skinned fiber preparations (4). Although assessment of myofilament calcium sensitivity in intact muscle has been performed in the past, it has been limited to experiments at or below room temperature and in the presence of compounds that prevent sarcoplasmic reticulum (SR) cycling (CPA, thapsigargin, etc.) (4, 15). Saw-tooth contraction patterns (i.e., nonsmooth tetani) are, however, still somewhat present when we tried to employ the tetanizing/SR poison method at body temperature, likely due to the refractory period that does not allow a true smooth tetanus to develop even when stimulated at +30 Hz. Because such experiments with an incapacitated SR induce contractures that are not reversible or cannot be repeated multiple times,
they do not lend themselves to assessment of calcium sensitivity, such that data obtained can easily be extrapolated to in vivo conditions. In addition, both calcium-handling rates and myofilament crossbridge kinetics do not change similarly at various different temperatures (18), limiting the extrapolation of room temperature data to the in vivo situation (9, 12).

To validate whether the obtained force-calcium relationship reflects myofilament calcium sensitivity, we used several interventions that would potentially impact on the position of this relationship. It is known that myofilament sensitivity is sensitive to pH (3, 13). Although this data have almost exclusively been generated at room temperature conditions, it is well known that also at body temperature, alkalosis and acidosis only minorly affect the calcium transient but drastically impact on force development; this observation is in line with a pH-dependent myofilament calcium sensitivity. Indeed, when we lowered the pH of the perfusate, not only did twitch force drop as expected, but the obtained steady-state, force-calcium relation also shifted rightward, indicating a loss of calcium sensitivity. Perfusing with a higher pH resulted in the expected opposite, an increase in twitch force, with a leftward shift of the force-calcium relationship, indicating an increase in calcium sensitivity. Another well-known modulation of the position of the force-calcium relation is phosphorylation of TnI, which induces a loss in myofilament calcium sensitivity due to TnI phosphorylation (11). We found a clear and consistent rightward shift in the position of the steady-state, force-calcium relationship upon addition of a maximal dose of isoproteranol to the perfusate. These experiments showed that interventions that are expected to impact on myofilament calcium sensitivity showed our obtained force-calcium relation in the expected direction with roughly the expected magnitude.

Assessment of both the dynamic and the static force-calcium relationship now allows us to correlate their relationships. The dynamic force-calcium relationship is often analyzed by using phase-plane loops, where calcium and force of a twitch are plotted against one another (20). With the use of these loops, surface areas, specific phases, or measures of “openness” of the loops have been used to gain insight of calcium and myofilament dynamics. The addition of the steady-state, force-calcium relation that is assessed under identical conditions of pH, temperature, frequency, etc., as the dynamic relation, will now allow us to analyze this dynamic behavior in relation to its steady-state behavior. Notably, we were able to define at which point in time during the cardiac cycle the force production switches from below steady-state-predicted levels to above that level. This point, termed the CRCP in a cardiac beat, depends on both myofilament properties and calcium handling. Thus any shift in either parameter will theoretically shift the CRCP. This may make the CRCP potentially useful to verify that changes in the myofilament or calcium-handling level have physiological consequences in the time domain of calcium-dependent activation. In addition, one could measure force-calcium areas, as has been done previously, but now relate them not to peak calcium or peak force but to what part of the area of the curve lies below the steady-state level and to what part above. When both the biochemical and biophysical regulation of contraction-relaxation coupling become better understood in the future, specific processes can potentially be analyzed by using the combined dynamic and steady-state, force-calcium relations.

Limitations of the study. Leakage of the calcium indicator bis-fura-2 and fura-2 is faster at body temperature than at room temperature. We observed a leak rate of roughly 15% per 10 min. This limits the time in which calcium signals can be reliably obtained with minimal effects due to the loss of dye to approximately 20–30 min. This rather short time span currently prevents a full in situ calibration (2), and, as a result, we currently are limited to display the calcium signals as fluorescence ratios. However, depending on the intensity and duration of the various protocols (K⁺ contracture and high frequencies) to accelerate the loss of dye, we envision that a calibration is possible in the near future. In addition, both the dynamic and steady-state relationships, as well as the comparison of two data sets obtained in the same preparation, would be equally affected by a calibration, and thus the relative position of these relationships, including the CRCP, time-to-peak calcium, etc., is not affected by the lack of calibration. Another current limitation is the lack of sarcomere length control. Sarcomere length inhomogeneity can never be avoided altogether and may impact upon not only force development but calcium transients. Previously, it has been shown that clamping sarcomere length at room temperature slightly affects the amplitude of the calcium transient (7), but not the kinetics, and significantly impacts upon both the amplitude and kinetics of twitch force development. In the current study, it was not feasible to control...
for sarcomere length, and thus we used a muscle length (where peak twitch force is optimal) that represents a sarcomere length comparable to end-diastolic values (~2.2 to 2.3 μm). At this sarcomere (muscle) length, the passive forces in the preparation minimize the amount of central segment sarcomere shortening compared with shorter length. Moreover, the inhomogeneity will occur both during the twitch and the K⁺ contractions, possibly limiting this potentially confounding factor, albeit we cannot altogether exclude kinetic differences to cause small variations in the actual central segment shortening magnitude to impact the data.

In conclusion, we have shown for the first time the relationship between force and calcium in intact cardiac trabeculae at body temperature under both dynamic and static conditions. With the use of this protocol, repetitive assessments of myofilament calcium sensitivity can be performed under conditions that very closely mimic those observed in vivo.

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