Intracellular Calcium Transient of Working Human Myocardium of Seven Patients Transplanted for Congestive Heart Failure

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Abstract The afterload dependence of the intracellular calcium transient in isolated working human myocardium was analyzed in both donor and recipient hearts of seven patients undergoing transplantation because of dilated cardiomyopathy. The intracellular calcium transient (recorded by the fura 2 ratio method), force development, and muscle shortening were simultaneously recorded in small (0.6x4.0-mm) electrically driven (60 beats per minute) trabeculas contracting at constant preload against varying afterloads. When the fibers contracted under isometric conditions, the intracellular calcium transients of normal and failing myocardium were similar. However, in dilated cardiomyopathy, stepwise afterload reduction and the concomitant increase in shortening amplitudes were associated with extraordinary alterations in the shape of the calcium transients: the amplitude rose, the time to peak was delayed, and at minimal afterloads, a long-lasting plateau was observed, and the diastolic decay was retarded. The calcium-time integral during shortening against passive resting force was 124±5% of the isometric control in normal myocardium and 172±12% in end-stage heart failure (P<.0001). We conclude that adequate interpretation of intracellular calcium transients requires simultaneous recordings of force and shortening. The extraordinary afterload dependence of the calcium transient in end-stage heart failure may be attributed to increased dissociation of calcium from the contractile proteins, a reduced calcium reuptake rate of the sarcoplasmic reticulum, or an increased calcium inflow due to altered permeabilities of the calcium channels during shortening. A potential role of mechanosensitive calcium channels has to be considered. (Circ Res. 1994;74:952-958.)

Key Words • dilated cardiomyopathy • intact myocardium • Ca²⁺ transients • fura 2 • human myocardium

The intracellular calcium transient of normal human myocardium is modulated during the performance of mechanical work.¹,² The ability to perform mechanical work (to shorten against a given afterload) was depressed in skinned myocardial fibers of patients with dilated cardiomyopathy, although the crossbridge cycling rate was normal.³ The intracellular calcium transient of intact working human myocardium of patients with dilated cardiomyopathy had not yet been analyzed. Measurements of calcium transients during the performance of mechanical work have now become possible, since new fluorescent dyes are available.⁴,⁷ For the measurement of intracellular calcium transients, the dual wavelength excitation method ("ratio method") and the fluorescent dye fura 2 were selected to minimize shortening artifacts.¹,⁴,⁶,⁸ Combined measurements of calcium, force, and shortening show for the first time that extraordinary alterations of the intracellular calcium transient occur in dilated cardiomyopathic myocardium during shortening.

Materials and Methods

Muscle Specimens

Left ventricular myocardium excised from the free ventricular wall was obtained from seven recipient hearts of patients undergoing heart transplantation because of dilated cardiomyopathy. None of the patients had a history of drug addiction or alcoholism. In none of these patients was there evidence of acute myocarditis. All patients had been healthy and active individuals without exposure to toxic substances or radiation before transplantation. Preoperative angiographic examination was carried out in all patients and showed no evidence of significant coronary artery stenosis. The waiting period until transplantation ranged between 1 and 14 months. Two patients had intermittent ventricular tachycardia. All patients were evaluated as New York Heart Association class IV when undergoing transplantation.

Left ventricular hypertrophy was present in all explanted hearts. The hearts were enlarged, as detected by chest radiography, with a mean heart-to-thoracic ratio of 0.78. Neither aortic nor mitral valve stenosis was present. The muscle specimens were excised from the fibrillating heart within 6 minutes after interruption of coronary perfusion. Normal human ventricular myocardium was obtained from the right ventricle of each of the donor hearts (n=7). Right ventricular donor heart trabeculas were routinely excised as initial control specimens for right ventricular biopsies after successful transplantation. In three of these patients, additional trabeculas adherent to the free left ventricular wall were also excised.

Donor hearts were transported in a 4°C cold HTK-cardioplegic solution composed of (mmol/L) NaCl 15.0, KCl 9.0, MgCl₂·6H₂O 4.0, potassium hydrogen-2-ketoglutarate 1.0, histidine·HCl·H₂O 180.0, tryptophan 2.0, mannitol 30.0, and CaCl₂ 0.015 (osmolarity, 310 mOsm/L). In none of the donor hearts did the period of cold ischemia before excision of muscle specimen exceed 3 hours. After sampling of the muscle fibers, both donor and recipient heart muscle specimens were stored in a 4°C Krebs-Henseleit solution composed of (mmol/L) NaCl 119.0, NaHCO₃ 25.0, KCl 4.6, KH₂PO₄ 1.2, MgSO₄ 1.2, CaCl₂ 1.3, and glucose 11.0, which contained 30 mmol/L 2,3-butanedione monoxime (BDM). Small trabeculas (diameter, <0.6 mm; length, =4 mm) were selected and carefully prepared so that they could be directly used for

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experiments, thus minimizing any cutting injury to the tissue. The study was reviewed and approved by the Committee of Medical Ethics in Human Research of the University of Heidelberg.

**Mechanical Measurements**

An experimental design was used that was similar to that in a previous study. After transfer from the 4°C Krebs-Henseleit–BDM solution, the fibers were incubated for 30 minutes in the same solution without BDM. The muscles were then placed under a constant level of passive force (1.5 mN) by adjusting the preload (set by holding the muscle at a constant length). After the onset of relaxation, the muscle was restretched at constant velocity to its initial length. Resting force was 1.5 mN. ML indicates muscle length; MD, muscle diameter. The preparation consisted of normal human right ventricular myocardium.

**Calcium Measurements**

After the initial control measurements were performed, muscle strips with adequate mechanical performance were incubated in darkness in an oxygenated Krebs-Henseleit solution containing 5 μmol/L fura 2-AM for 6 hours at 26°C. The nontoxic detergent Cremophor EL (0.5%) was added to the incubation solution to increase the solubility of fura 2-AM. After the fura 2 loading, the muscle fiber was rinsed with normal oxygenated Krebs-Henseleit solution for 15 minutes at 37°C and mounted between the vibrator and force transducer at 1.5-mN passive resting force. Then, electrical stimulation was carried out until a steady-state force output was obtained. Fibers were included in experiments when isometric force amplitude after fura 2 loading was at least 90% of the initial control before fura 2 loading.

The excitation light was obtained from a xenon lamp. A rotating filter wheel (rotation frequency, 125 Hz) allowed alternating excitation at wavelengths of either 340 or 380 nm. The emitted fluorescence signal resulting from the excitation with one of these wavelengths was recorded at 510 nm and sorted in the respective channels of a photomultiplier. After background fluorescence subtraction (fluorescence measured before fura 2 loading under isometric conditions at optimal length), the ratio of both fura 2 fluorescence signals, which is proportional to the intracellular calcium concentration, was continuously recorded, as previously described. Those minimal alterations of background fluorescence occurring during muscle shortening were not subtracted.

For the determination of maximum fluorescence at maximum intracellular calcium concentration, myocardial muscle strips were tetanized at the end of the experiment by electrical stimulation (100 Hz; duration, 5 milliseconds; amplitude, 10% above threshold; mode, square wave) in the presence of a Krebs-Henseleit solution containing 3 mmol/L CaCl2. Under these conditions, the value of the amplitude of the steady-state tetanic force exceeded by about four times the maximum isometric twitch contraction. Minimal free calcium concentrations were measured after adding 3% Triton X-100, a substance used to lyse membranes, as, for example, during skimming procedures. After 30 minutes, the Triton X-100–containing solution was washed out and substituted by a Krebs-Henseleit solution without calcium and containing 50 μmol/L fura 2 and 5 mmol/L EGTA. According to Grynkiewicz et al., the intracellular calcium concentration ([Ca2+]i) can be calculated on the basis of the following equation:

\[ [Ca^{2+}] = \frac{R - R_{\text{min}}}{R_{\text{max}} - R_{\text{min}}} \cdot K_d \cdot s \]

where \( R \) is the measured ratio of fluorescence occurring during steady-state tetanic force, \( R_{\text{min}} \) is the minimal intracellular free calcium concentration, \( R_{\text{max}} \) is R at maximal intracellular free calcium concentration, \( K_d \) is the dissociation constant of the fura 2–calcium complex, and s is the ratio of fluorescence calcium-free solution to fluorescence in calcium-containing solution. According to Grynkiewicz et al., \( K_d \) was assumed to be 224 nmol/L for measurements at 37°C. An “in vivo calibration” required for direct measurement of \( R_{\text{min}} \) and \( R_{\text{max}} \) could not be carried out, because the intracellular calcium transient of human myocardial tissue could not be “clamped” at minimal and maximal levels under physiological conditions.

Although it was described that the ratio method neither depends on the indicator concentration nor on alterations of cell thickness as, for example, during shortening, movement artifacts were also experimentally excluded: stretches and releases with an amplitude of 20% of muscle length (ML) were carried out at resting force while the calcium transient was recorded. The calcium transient always remained unaffected under these conditions. Furthermore, an additional direct estimation of the potential presence of mechanical artifacts was carried out during the experimental performance of afterloaded contractions: the fibers were initially allowed to shorten against a given load. Immediately after the onset of diastole, the fibers were rapidly mechanically restretched at a constant velocity to their initial length while the calcium transient was continuously recorded. The shape of the calcium
transient was not affected by this kind of standardized mode of length alteration.

The calcium transient always remained stable for periods exceeding 60 minutes. For any given afterload, the force, shortening, and calcium transients obtained during a 1-minute sampling period were digitized, averaged, and printed (Figs 2 through 4). The calcium transient remained constant throughout this collecting period. The calcium-time integral was measured to compare the exposition time of intracellular structures with calcium under isotonic and isometric conditions. For that reason, the calcium transient obtained under isometric measurement conditions of any individual fiber was normalized as 100%. For any given afterload, the calcium-time integral was measured and expressed as percentage of the control. This allowed comparison of different preparations.

Statistical Analysis

Data are expressed as mean±SD. To evaluate statistical significance between the various groups, one-way ANOVA followed by Student's t test was carried out. For on-line data analysis, special software was used (Guth Scientific Instruments). A value of P<0.01 was considered to indicate a statistically significant difference.

Results

Mechanical Measurements

In normal human right ventricular myocardium, force development was 20 to 26 mN/mm² (mean, 22.29±2.63 mN/mm²; n=7), and maximal shortening amplitude was 18% to 25% ML (mean, 22.86±2.54% ML; n=7). Force development of normal left ventricular myocardium of three donor hearts ranged between 17 and 29 mN/mm² (mean, 23.67±6.11 mN/mm²; n=3). In these muscle samples, the shortening amplitude was 19% to 24% ML (mean, 22.33±2.89% ML; n=3). Whereas the reduction of isometric force failed to be statistically significant in patients with dilated cardiomyopathy (range, 8 to 25 mN/mm²; mean, 15.57±6.55 mN/mm²; n=7), the shortening amplitude was significantly reduced as compared with normal left and right ventricular muscle specimens (P<0.001) (Table).

Calcium Transients

Calcium transients of myocardium from donor and recipient hearts were similar under isometric conditions (Figs 2 through 4). Under the given assumptions (see "Materials and Methods" and "Limitations"), diastolic calcium was estimated to be 178±68 nmol/L (n=7) in normal right ventricular myocardium, 195±80 nmol/L (n=3) in normal left ventricular myocardium, and 234±75 nmol/L (n=7) in dilated cardiomyopathic myocardium. Systolic calcium transients were calculated under the given assumptions to be 1650±190 nmol/L (n=7) in normal right ventricular myocardium, 1435±230 nmol/L (n=3) in normal left ventricular myocardium, and 1562±172 nmol/L in dilated cardiomyopathic myocardium. Under isometric conditions, the mean values for diastolic and systolic calcium were similar in all groups.

To analyze the modulation of the calcium transient during shortening, the amplitude of the systolic calcium transient was set to be 100% to allow direct comparison between different muscle preparations. Any alteration of the calcium transient occurring during shortening was analyzed in relation to the respective isometric control.

In both recipient and donor heart myocardium, the intracellular calcium transient was altered during shortening. Only slight alterations of the intracellular calcium transient were observed in normal human myocardium (Fig 2). The calcium-time integral during shortening against passive resting force averaged 124±5% of the isometric control value.

However, in any left ventricular myocardium from patients with dilated cardiomyopathy, we found a characteristic modulation of the intracellular calcium transient during shortening. There was a pronounced increase of the amplitude, a delay of the time to peak, a long-lasting plateau at minimal afterloads, and a retardation of the diastolic decay (Fig 3). A stepwise alteration of the afterload always induced respective alterations of the calcium transient in dilated cardiomyopathic myocardium. Thus, the shape of the intracellular calcium transient could be determined by the selection of the afterload. The calcium-time integral during shortening against passive resting force averaged 172±12% of the isometric control value. In normal myocardium, the calcium peak always preceded maximum force development (isometric conditions) or maximum shortening amplitude (isometric conditions). In contrast, in dilated cardiomyopathy, however, we found a stepwise retardation of the peak value for the calcium transients with decreasing afterloads. During shortening against minimal loads, peak values for shortening amplitudes and for intracellular calcium transients were sometimes simultaneously observed (Fig 4). Similarly, as in the normal reference preparations, the calcium-time integral (area below the calcium-time curve) during the control isometric twitch contraction was normalized as 100% in any preparation. The calcium-time integral measured at different afterloads and expressed as percentage of the isometric control is presented as a function of the afterload in Fig 5, illustrating the differences between normal (right ventricular trabeculae of donor hearts) and failing myocardium (left ventricular trabeculae of recipient hearts).
Calcium % Control

**FIG 2.** Digitized tracings of force, muscle length (ML), and intracellular calcium transient of normal left ventricular myocardium. 1 and 2 represent isometric control contractions at the beginning and the end of the experiment. 3 shows an isotonic contraction against passive resting force. Note that the intracellular calcium transient is only slightly modulated by shortening. ML was 4.0 mm; muscle diameter, 0.5 mm.

Note that in none of the preparations were alterations of the diameter of the muscle, such as occurs during length alterations, associated with changes in the calcium transients: passive rapid alteration of the muscle length, such as occurs during restretching of the muscle fibers after the onset of relaxation (Fig 4), did not alter the shape of the calcium transient.

**Discussion**

The extraordinary afterload dependence of the intracellular calcium transient in dilated cardiomyopathy is a new finding. Most measurements of intracellular calcium transients have been carried out either under isometric measurement conditions or in isolated myocytes. However, in none of these preparations did the muscle perform mechanical work. In myocytes, the additional problems of a nondirected contraction without defined preload and a heterogeneity of calcium transients exist. At a given preload, a slight afterload dependence of the intracellular calcium transient has recently been described in isolated healthy working human myocardium. Rapid reduction of the muscle length during an isometric twitch ("quick release") was associated with alterations of the calcium transient in normal rat cardiac trabeculae. The present analysis confirms these findings. It was suggested that in normal myocardium this process may reflect a dissociation of calcium from the contractile proteins during shortening.

It is an interesting finding in the present analysis that differences of the calcium transient between normal and recipient hearts with dilated cardiomyopathy were minimal or even absent under isometric conditions. This indicates that the performance of mechanical work was crucial for inducing a pathological regulation of the intracellular calcium homeostasis in dilated cardiomyopathy.

The time course of the fura 2 light transient can be influenced by the kinetics of calcium entry into the sarcoplasm, the characteristics of calcium diffusion and binding to myofilaments, the dissociation rate from the myofilaments, and the rate of calcium uptake into the sarcoplasmic reticulum. Additional potential methodological factors are discussed in "Limitations." The intracellular calcium homeostasis depends on the total amount of released calcium, on intracellular calcium buffers exchanging calcium, and on the kinetics of calcium removal from the cytosol.

An abnormal calcium release of the sarcoplasmic reticulum was described in dilated cardiomyopathy. Limas et al found that calcium uptake was reduced in heart failure. However, other authors have observed an unchanged calcium uptake rate. Phosphorylation of phospholamban significantly increases the rate at which the sarcoplasmic reticulum can resequester calcium, thereby enhancing relaxation. Thus, a reduced phosphorylation of phospholamban could decrease the rate at which calcium is resequestered by the sarcoplasmic reticulum, hence accounting for the prolonged calcium...
transients and delayed relaxation. However, detailed studies in normal and failing human left ventricles of the function of sarcoplasmic reticulum proteins involved in the modulation of the calcium uptake did not reveal alterations related to the calcium-transporting ATPase, phospholamban, or cGMP-inhibited cAMP phosphodiesterase activity. Recordings of human calcium channels were similar, regardless of the etiology of cardiac dysfunction, and resembled normal sheep and canine recordings. Thus, Holmberg and Williams suggested that other factors may contribute to abnormalities of the calcium homeostasis in failing myocardium.

During shortening, dissociation of calcium from the contractile apparatus may occur if there is a concomitant decrease of calcium sensitivity at lower muscle length as suggested by Backx and ter Keurs and Allen and Kentish. It may be hypothesized that the length dependence of calcium binding to troponin is altered in dilated cardiomyopathy, resulting in an increased amount of calcium released from the contractile apparatus during shortening. However, for two reasons it seems unlikely that such a mechanism alone can explain the altered calcium transient during shortening observed in dilated cardiomyopathy: (1) The shortening amplitudes in dilated cardiomyopathy are much smaller than those in normal myocardium (Table). (2) In skinned myocardial muscle fibers, there was no evidence for an altered length dependence of the calcium sensitivity of the contractile proteins in diseased human myocardium including dilated cardiomyopathy.

The modulation of calcium metabolism by mechanical influences had been described in smooth muscle and myocardial tissue. In addition to other mechanisms, the presence of mechanosensitive calcium channels in the heart may help to explain the dependence of intracellular calcium transients on mechanical performance. Increased permeability of the sarcoplasmic reticulum membrane during shortening or impeded calcium reuptake due to mechanically induced alterations of membrane permeability may contribute to cytosolic calcium overload during shortening. The hypothesized mechanosensitive calcium channels could at least explain the finding that reduced shortening amplitudes are related to a disturbed calcium reuptake rate. Gamble et al described that the loading capacity of the sarcoplasmic reticulum in rat ventricular muscle depends on muscle length. These authors concluded in a detailed study that diastolic muscle length differentially influences sarcoplasmic reticulum calcium storage and release processes. In support of this hypothesis, Allen and Kurihara have shown that when superimposed, the rise of the calcium transient was unchanged at both long or short muscle lengths, whereas the time course of the fall in the calcium transient was abbreviated at longer muscle lengths. In chick embryo cardiac myocytes, stretch-sensitive channels for potassium, calcium, and magnesium had been described. However, further analysis is required to analyze the relation between mechanical influences and intracellular calcium transients in human myocardium in more detail.

The present results confirm findings of other groups that observed with aequorin unchanged diastolic and systolic calcium levels in control and dilated cardiomyopathic myocardium. The observations of Beuckel-
mann et al. may also support our data. These authors showed that there was a long-lasting systolic-diastolic calcium plateau of the intracellular calcium transient (lasting more than 900 milliseconds) in isolated myocytes of patients with dilated cardiomyopathy. The fact that in these preparations shortening occurs against minimal load may play a crucial role. Their recordings of the calcium transient may be interpreted as an extreme example of the length dependence of the intracellular calcium transient. According to Backx and ter Keurs, the duration of the diastolic calcium transient may be also partly due to the calcium off-rate from the dye and the intracellular fura 2 concentration. The different absolute values of the calcium transient in the study of Backx and ter Keurs and the present study may be at least partly due to the different types of preparations. Methodological factors including potential damage to the preparation during the isolation procedure of myocytes may also be considered. Force responses at 1-Hz stimulation frequency were not different in control and myopathic tissue in the present study, confirming the results of Gwathmey et al.

**Limitations**

The interpretation of the present results requires careful consideration of several potential limitations: (1) For the present experiments, a stimulation frequency of 60 beats per minute was used. At this frequency, the effects of the inverted force-frequency relation in dilated cardiomyopathy on intracellular calcium transients and isometric force may not be detectable. Although the present results and conclusions are valid at the given experimental conditions, the effects of higher stimulation frequencies on the modulation of the intracellular calcium transients remain to be analyzed. (2) Sarcomere length was not measured in the present study. Therefore, an isometric twitch contraction was defined as contraction at constant muscle length. Modulation of the calcium transient by internal sarcomere shortening during isometric twitches has to be considered. However, internal sarcomere shortening potentially associated with greater displacement of calcium from troponin C occurs in normal and failing myocardium. If it was more pronounced in failing myocardium, the isometric calcium transient should differ from the normal control value. However, no differences were present under isometric conditions between control and myopathic myocardium. Thus, the effects of internal sarcomere length alterations occurring at constant muscle length are assumed to be minimal compared with those changes of the calcium transient observed during afterloaded contractions. (3) Sarcomere length at passive resting force may be different in normal and diseased myocardium. If so, the reduced shortening amplitude could be the result of different points on the length-force relation of both preparations. However, experiments were carried out at optimal length in all types of preparations. Furthermore, it is known from skinned fibers of patients with dilated cardiomyopathy that force development and crossbridge cycling rate are the same as in control preparations at optimal length. The present data confirm this observation in intact tissue. The decrease of shortening capability was also observed in skinned muscle fibers from patients with dilated cardiomyopathy and was assumed to be due to an increased internal resistance against shortening.

(4) The diameters of muscle fibers used in these experiments were only half the size of the diameters used by Gwathmey and Morgan, who reported mean diameters of 1.0 to 1.2 mm. Because resting force remained constant throughout the experiments, there was no evidence of the formation of rigor bridges due to limited oxygen supply. Because the experimental conditions were standardized and the same for normal and diseased myocardium, it seems unlikely that the effects were due to an increased proportion of damaged cells in dilated cardiomyopathic myocardium. (5) The fura 2–loading procedure seems to be the most critical point of the experiments. Effects of pathologically altered myocardial function due to an action of high intracellular concentrations of fura 2 acting as a calcium buffer were minimized by including only fibers in the experiments when the isotropic twitch amplitude and the shortening amplitude decreased not more than 10% from the control value before fura 2 loading. During the measurement period, force and shortening amplitudes were always constant. (6) Fura 2 is susceptible to photobleaching. Although Grienkiewicz et al. argued that the ratio method is immune to bleaching artifacts, Becker and Fay showed that at intense light a considerable amount of photobleaching may occur. In the present study, strategies to minimize bleaching included minimal exposure time to light and storage and fura 2 loading out of light. Exposure to fluorescent light never exceeded 30 minutes in any experiment. The intracellular fura 2 concentration is not known. However, both the amplitude and kinetics of the diastolic force and calcium decay are modulated by the intracellular fura 2 concentration. High intracellular fura 2 concentrations may well contribute to a retarded relaxation rate, because the fura 2 that is bound to calcium will act as a source of calcium during relaxation.

(7) Because no in vivo calibration could be carried out, a considerable error regarding the estimation of absolute values for the intracellular calcium concentrations may be present. Steady-state background fluorescence at optimal length before fura 2 loading was subtracted. However, the value for background fluorescence may not be exactly the same when the muscle is slightly “twisted,” as when it is remounted between force transducer and vibrator after the loading period. Furthermore, because those slight changes of background fluorescence occurring during shortening were not subtracted, this may also complicate calculation of absolute values for intracellular calcium concentrations. However, the calcium transient was unaffected during the period of rapid restretching of the muscle. This may indicate that length-dependent alterations of background fluorescence did not seem to interfere severely with the measurements. Accumulation of fura 2 in other compartments besides the cytosol, including mitochondria and sarcoplasmic reticulum, may occur. However, because the ratio method was applied, this effect should basically not disturb the measurements. It is known that the dynamic range of the indicator fura 2 may be reduced inside cells compared with that of thin solutions of fura 2 observed in the microscope. Therefore, we acknowledge the possibility that the absolute values calculated in the present study may have to be revised when the effects of fura 2 within an intact intracellular myocardial environment are completely known. However, it was not the aim of the present study to make quantitative comparisons of intracellular calcium transients in normal and diseased myocardium. Calculations of the intracellular calcium tran-
sients were carried out to ensure that the recorded calcium concentrations were within a range that could be detected by fura-2. Because both donor and recipient heart fibers were obtained from the same heart transplantation and analyzed the same day under the same experimental conditions, it is reasonable to assume that despite the discussed limitations, similar calcium transients in normal and recipient hearts under isometric conditions indicate a real similarity of calcium transients in normal and failing myocardium under isometric conditions.

However, although the determination of absolute values of intracellular calcium transients may remain a matter of controversial discussion related to the problems of fura-2 in vivo calibration,4,7,8,10 the two new main findings of the present study remain unaffected by these considerations: (1) There is an afterload-dependent modulation of the intracellular calcium transient during muscle shortening. (2) Modulation of the calcium transient during shortening is characteristically altered in end-stage dilated cardiomyopathy. On the basis of the present data, we conclude that adequate interpretation and comparison of intracellular calcium transients in human myocardium require simultaneous recordings of force, shortening, and calcium transients at a defined stimulation frequency, preload, and afterload.

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