Relation Between Steady-State Force and Intracellular [Ca\(^{2+}\)] in Intact Human Myocardium

Index of Myofibrillar Responsiveness to Ca\(^{2+}\)

Judith K. Gwathmey and Roger J. Hajjar

A novel approach was developed allowing the measurement of steady-state force and intracellular calcium concentration ([Ca\(^{2+}\)]\(_i\)) in tetanized human ventricular trabeculae carnea without pharmacological intervention. We compared and contrasted three methods of assessing calcium sensitivity of the myofilaments: 1) force-[Ca\(^{2+}\)]\(_i\) relations in skinned fiber preparations, 2) peak twitch force-peak [Ca\(^{2+}\)]\(_i\) relations, and 3) steady-state force-[Ca\(^{2+}\)]\(_i\) relations in intact muscles. Steady-state contractile activation was achieved rapidly by tetanizing intact human trabeculae, loaded with aequorin, a Ca\(^{2+}\)-sensitive bioluminescent protein, at a stimulation frequency of 15–20 Hz. Steady-state force and [Ca\(^{2+}\)]\(_i\) were measured during tetani, and the force versus [Ca\(^{2+}\)]\(_i\) relation was obtained by varying the extracellular calcium concentration ([Ca\(^{2+}\)]\(_o\)). Force-[Ca\(^{2+}\)]\(_i\) relations obtained from control and myopathic hearts were fitted to the Hill equation: %Force=\([\text{Ca}^{2+}]_{i}^{nH}/([\text{Ca}^{2+}]_{i}^{nH}+[\text{Ca}^{2+}]_{i}^{h})\), where \(n_H\) is the Hill coefficient, and [Ca\(^{2+}\)]\(_{i}^{h}\) is the [Ca\(^{2+}\)]\(_i\) required for 50% activation. The curves of tetani had Hill coefficients of 5.21±0.20 (n=6) and 5.61±0.60 (n=10) and [Ca\(^{2+}\)]\(_{i}^{h}\) of 0.56±0.05 \(\mu\)M (n=6) and 0.54±0.09 \(\mu\)M (n=10) in control and myopathic muscles, respectively. We also constructed peak force-peak [Ca\(^{2+}\)]\(_i\) relations using isometric twitches from the same muscles. These curves were shifted toward higher [Ca\(^{2+}\)]\(_i\), compared with the steady-state force-[Ca\(^{2+}\)]\(_i\) curve derived from tetani. Ryanodine (1 \(\mu\)M), which increased the time course of the Ca\(^{2+}\) and force transients, shifted the peak force-peak [Ca\(^{2+}\)]\(_i\) relation to the left, without affecting the steady-state force-[Ca\(^{2+}\)]\(_i\) relation. Exposure to 10 mM caffeine shifted the steady-state force-[Ca\(^{2+}\)]\(_i\) relation to the left, whereas exposure to 3 \(\mu\)M isoproterenol shifted this relation to the right. Experiments using skinned fiber preparations were performed in parallel with experiments on intact muscles from the same hearts. The force-pCa (−log[Ca\(^{2+}\)]) relations in saponin-skinned trabeculae from control and myopathic tissue were superimposable. Ryanodine (1 \(\mu\)M) had no effect on the force-pCa relation in skinned fibers. Maximal tension was evoked by the posttetanic twitch, which was larger than the tetanus. This potentiation was abolished in the presence of ryanodine, a sarcoplasmic reticulum inhibitor. We propose that the changes in the steady-state force-[Ca\(^{2+}\)]\(_i\) relations are correlated with alterations in the sensitivity of the myofilaments to Ca\(^{2+}\), whereas changes in the peak force-peak [Ca\(^{2+}\)]\(_i\) relations represent temporal changes in the twitch transient. In both intact aequorin-loaded trabeculae and saponin-skinned fibers, there were no differences in the force-[Ca\(^{2+}\)]\(_i\) relation between control and myopathic hearts, indicating that the Ca\(^{2+}\) sensitivity of the myofilaments is unaltered in the diseased myocardium. This leads us to the conclusion that the steady-state force-[Ca\(^{2+}\)]\(_i\) relation can be used reliably to estimate sensitivity changes at the level of the myofilaments in lieu of the peak force-peak [Ca\(^{2+}\)]\(_i\) relation. (Circulation 1990;82:1266–1278)

The strength of contraction in the heart is related to intracellular calcium concentration ([Ca\(^{2+}\)]\(_i\)). The relation between free calcium in the vicinity of the myofilaments and force development is crucial to understanding the regulation of force by the contractile proteins. The tradi-
tional approach to characterizing this relation has been to measure steady-state [Ca\textsuperscript{2+}]-force relations in “skinned” cardiac muscle preparations in which the sarcolemma has been either mechanically removed,\textsuperscript{1} rendered hyperpermeable,\textsuperscript{2} or chemically disrupted.\textsuperscript{3} [Ca\textsuperscript{2+}] and other constituents thought to affect contraction then can be controlled in the myofibrillar space. By sampling the entire range from resting to maximal force, force-[Ca\textsuperscript{2+}] relations can be obtained. This approach has yielded considerable information about the relation between [Ca\textsuperscript{2+}] and force and about the effects of different intracellular constituents on this relation. This technique also has been used to examine the effects of catecholamines and cyclic nucleotides on the calcium activation of the myofilaments.\textsuperscript{2} One of the most straightforward ways of obtaining evidence about how new inotropic agents alter the myofibrillar response to Ca\textsuperscript{2+} is through the use of these skinned fibers.\textsuperscript{4,2} Because in skinned fiber preparations, the sarcolemma and the sarcoplasmic reticulum are disrupted and other sarcomemmal regulatory proteins are solubilized, it has been difficult to determine whether the findings obtained from skinned preparations can be directly extrapolated to intact myocardium. Another limitation is the fact that the intracellular constituents are simulated in the solutions that activate skinned muscle fibers. These constituents, namely magnesium, MgATP, inorganic phosphate, pH, creatine phosphate, and creatine phosphokinase, have profound effects on the relation between force and [Ca\textsuperscript{2+}].\textsuperscript{6,7} Another major source of error in skinned preparations is that the ion species required for physiological activation of the contractile apparatus are provided from outside the preparation, and complex diffusional problems can create ionic gradients. New inotropic agents, in addition to having a Ca\textsuperscript{2+}-sensitizing action, have various effects on intracellular structures (i.e., inhibition of phosphodiesterase, activation of ionic channels). These effects, like the phosphorylation of troponin I by cyclic AMP-dependent protein kinases, can in turn influence the relation between force and calcium.

Another approach to characterize the relation between force and calcium is using preparations of living cardiac muscle in which Ca\textsuperscript{2+} transients are detected with the Ca\textsuperscript{2+}-sensitive bioluminescent protein aequorin.\textsuperscript{5,6,9} Investigators historically have related peak force development to peak intracellular Ca\textsuperscript{2+} reached during a twitch. By varying extracellular Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]\textsubscript{o}), they have been able to obtain various amplitudes of twitch force and peak [Ca\textsuperscript{2+}], enabling them to construct peak [Ca\textsuperscript{2+}] vs. peak force relations. As in skinned fibers, this technique was used to gain information about the sensitivity of the myofilaments to Ca\textsuperscript{2+} in intact cells. It has been used in the investigation of the effects of various inotropic and pharmacological interventions on myofilament responsiveness to Ca\textsuperscript{2+}.\textsuperscript{9} However, the use of this technique is fraught with problems. The relation between the peak [Ca\textsuperscript{2+}] and associated peak force is derived from measurements that are transient and not in a steady state, making this relation hard to interpret. Also, the peak [Ca\textsuperscript{2+}] signal is temporally different from the peak force signal, severely limiting the validity of relating these two measurements. Finally, during a twitch, sharp spatial gradients in [Ca\textsuperscript{2+}] occur, overestimating the true [Ca\textsuperscript{2+}].\textsuperscript{10}

It has become clear that it was not suitable to derive information about the sensitivity of the myofilaments to Ca\textsuperscript{2+} using peak [Ca\textsuperscript{2+}] vs. peak force relations.\textsuperscript{11,12} It was therefore important to develop a technique that would allow us to investigate the myofilament responsiveness to Ca\textsuperscript{2+} in intact muscle. Yue et al\textsuperscript{11} and Marban et al\textsuperscript{13} described a new experimental approach that enabled them to measure maximal Ca\textsuperscript{2+}-activated force by tetanizing ryanodine-treated intact hearts and to thereby deduce myofilament Ca\textsuperscript{2+} sensitivity. Our own experience with human myocardium had revealed that human muscles at 30°C could be tetanized, producing steady-state force without pharmacological intervention. Using aequorin-loaded human muscles, we were able to measure simultaneously steady-state force and steady-state [Ca\textsuperscript{2+}]. By varying [Ca\textsuperscript{2+}]\textsubscript{o}, we obtained different levels of steady-state force and steady-state [Ca\textsuperscript{2+}], allowing us to relate these two measurements. The result was a steady-state force-[Ca\textsuperscript{2+}] relation in intact human myocardium, yielding direct information about events at the level of the myofilaments. To validate this technique, we compared it with skinned fiber preparations from the same human hearts. We specifically showed that information obtained about the sensitivity of the myofilaments to Ca\textsuperscript{2+} from skinned fibers also can be obtained by this new technique in intact muscle preparations. We verified that known effects of isoproterenol and caffeine on the sensitivity of the myofilaments to Ca\textsuperscript{2+} were present in the intact muscle preparations. We examined the effect of a new inotropic agent, DPI 201-106, in intact preparations and convincingly demonstrated that previous results about the activity of this drug on the myofilaments in skinned fiber preparations\textsuperscript{14} was obtained in the intact preparations. We also showed that using peak force versus peak [Ca\textsuperscript{2+}] to derive information about events at the level of the myofilaments was not valid. Finally, we found some interesting results about posttetanic twitches in the human trabeculae.

Methods

**Muscle Preparation**

Informed consent was obtained from all transplant recipients and from the families of all prospective heart donors. Hearts not suitable for transplantation were obtained from organ donor patients and used as controls (n=6). Muscles were obtained from patients undergoing transplantation due to end-stage heart failure (n=10). Six of the 10 from the end-stage heart failure group were from patients with idiopathic dilated cardiomyopathy, and four were from patients with ischemic cardio-
myopathy. Experimental protocols have been reviewed and approved by the Institutional Review Board. Trabeculae carneae were removed from the right ventricle of these 16 human hearts. For control and myopathic muscles, mean fiber diameters were \(0.63 \pm 0.27\) and \(0.73 \pm 0.22\) mm, and mean lengths were \(5.83 \pm 1.67\) and \(7.92 \pm 1.15\) mm, respectively. Calcium concentration response curves have revealed no significant differences in responsiveness to calcium between myopathic groups; for this reason, we did not differentiate between the dilated cardiomyopathy and ischemic myopathy groups.

At the time of excision, the hearts were placed in an oxygenated physiological salt solution at room temperature (see composition below). The hearts were rapidly transported to the laboratory where suitable muscles were dissected free. The time from excision to being placed in the physiological salt solution was immediate, and transport to the laboratory was within 15–45 minutes. The hearts then were placed into a superfusate solution with the following composition (mM): \(\text{NaCl} 120, \text{KCl} 5.9, \text{NaHCO}_3 25, \text{NaH}_2\text{PO}_4 1.2, \text{MgCl}_2 1.2, \text{CaCl}_2 2.5, \text{and dextrose} 11.5\). The solution was bubbled with 95\% \(O_2\) and 5\% \(CO_2\) at 30\°C to a pH of 7.4. The base of each muscle was attached to a muscle holder while the other end was tied to a force transducer/ergometer (Cambridge Technology Inc., Watertown, Mass.) and allowed to equilibrate at \(L_0\) (a length at which maximal force developed) for 1 hour at a frequency of 0.33 Hz. The muscles then were stimulated with a square wave pulse 5 msec in duration at threshold voltage.

**Measurements of Intracellular Calcium Concentration**

Aequorin was introduced intracellularly by a chemical-loading technique described previously. The light emitted by the aequorin was detected with a photomultiplier tube (9635QA, Thorn EMI Inc., Fairfield, N.J.) attached to a collecting apparatus similar to that described by Blinks et al.

In an in vitro calibration curve was used to convert normalized light signals obtained from the preparations into calcium concentrations as shown by the following equation:

\[
\frac{L}{L_{\text{max}}} = \left[\frac{1 + K_R[Ca^{2+}]}{1 + K_{TR}[Ca^{2+}]}\right]^3
\]

where \(L\) is the luminescence signal, \(L_{\text{max}}\) is the maximal light emitted after exposing the muscle preparation to a solution containing 2\% Triton X-100 and a saturating \([Ca^{2+}]_o\), and \(K_R\) and \(K_{TR}\) are constants. The conditions of the in vitro medium were 150 mM KCl, 2 mM MgCl\(_2\), and 5 mM 1,4-piperazine-diethanesulfonic acid (PIPES), pH 7.1, at 30\°C. The rate constant for aequorin consumption measured in vitro in 10 mM \([Ca^{2+}]_o\), 150 mM KCl, 2 mM MgCl\(_2\), pH 7.1, at 30\°C, was 1.98 sec\(^{-1}\). This rate constant was taken into account when measuring \(L_{\text{max}}\) and converting light signals to fractional luminescence. [\(Ca^{2+}\)]\text{\textit{e}} obtained during isometric contractions provide upper-limit estimates of the spatial-averaged concentrations.

We also verified that the addition of micromolar concentrations of ryanodine did not affect the in vitro calibration of the aequorin signal.

**Tetanization of Muscles**

Muscle tetani were induced by stimulating intact muscles at 15–20 Hz for 4–6 seconds using stimulus pulses 50 msec in duration. Steady-state measurements were made during the first 4–6 seconds of the tetan. Tetani were reproducible when they were separated by an interval of at least 3 minutes during which muscles were stimulated at 0.33 Hz. Tetani were elicited at varying \([Ca^{2+}]_o\), from 0.5 to 16 mM in the presence or absence of several agents as described below. Phosphate was removed from the bathing medium during these determinations to avoid calcium precipitation.

**Skinning Procedure**

Suitable trabeculae (diameter,<250 \(\mu\)m) obtained from the same human hearts were chemically skinned by exposure to a solution containing saponin, 250 \(\mu\)g/ml; \(\text{Na}_2\text{ATP}, 5 \text{mM}; \text{MgCl}_2, 7 \text{mM}; \text{EGTA}, 5 \text{mM}; \text{KCl}, 60 \text{mM}; \text{imidazole}, 60 \text{mM}; \text{creatine phosphate}, 12 \text{mM}; \text{creatine phosphokinase}, 15 \text{units/ml}; \text{pH} 7.1 \text{ at 20°C}. This high concentration of saponin was chosen as it has been reported that it destroys the integrity of both the sarcolemma and sarcoplasmic reticulum.

The total salt concentrations necessary for obtaining the desired pCa, pMg, pMgATP, and pH at a constant ionic strength were calculated using the program described by Fabiato and Fabiato. The absolute stability constants used for calculating the compositions of the solutions were as reported by Fabiato. The solutions were prepared at a temperature of 20\°C, with a pMg of 2.5, a pMgATP of 2.5, an EGTA concentration of 10 mM, an ionic strength of 0.16 M, and a pH of 7.1 adjusted using 30 mM \(N\)-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES). The solutions also contained 12 mM creatine phosphate and 15 units/ml creatine phosphokinase. The skinned muscles initially were subjected to a relaxation-activation cycle using the method of Moisescu and Thieleczek. The relaxation solution had a pCa greater than 8.0, and EGTA was replaced with HDTA (hexamethylenediamine \(N,N,N',N'\)-tetraacetic acid). The activation solution had a pCa of 4.0. During the relaxation cycle, the muscle length was adjusted to a length at which an increase in resting tension first was observed as described by Maughan et al.

**Force-[\(Ca^{2+}\)] Analysis**

The force versus \([Ca^{2+}]\) curves were fit to a modified Hill equation:
F = \frac{F_{\text{max}}}{\left[\text{Ca}^{2+}\right]_{50\%} + \left[\text{Ca}^{2+}\right]_{100\%}}

where F is developed force, F_{\text{max}} is the maximal force developed, n_h is the Hill coefficient, and \left[\text{Ca}^{2+}\right]_{50\%} is the \left[\text{Ca}^{2+}\right] required for 50\% activation.

Chemicals
Ryanodine was kindly donated by Merck Sharp & Dohme, West Point, Pa. DPI 201-106 (4-[3-(4-diphenylmethyl-1-piperazinyl)-2-hydroxypropoxy]-1H-indole-2-carbonitrile) was generously supplied by Sandoz Pharmaceuticals Corp., East Hanover, N.J. All other chemicals were purchased from Sigma Chemical Co., St. Louis. The aequorin used in these experiments was purchased from the laboratory of Dr. J.R. Blinks in Rochester, Minn.

Statistical Analysis
Results are presented as mean±SE. Statistical significance was determined by the Student's t test or one-way analysis of variance (ANOVA). The level of statistical significance was set at a probability of 0.05. In skinned fiber preparations, there were no significant differences between control or muscle from end-stage heart failure patients with either etiology (i.e., ischemic or idiopathic cardiomyopathy). We therefore pooled the data for diseased myocardium. When comparing the Hill parameters of the force-[Ca^{2+}] relations, differences between means of the half-maximally activating [Ca^{2+}] were tested for significance using one-way ANOVA.

Results
Steady Levels of Intracellular Calcium Concentration and Force
Trabeculae carneae from both control and myopathic human hearts were tetanized at 30°C without chemical interventions, producing steady-state levels of force and [Ca^{2+}]. Both force and aequorin luminescence signal decreased upon exposure of muscle preparation to solution of 2% Triton X-100 and saturating [Ca^{2+}] (see "Methods").
nescence reached a plateau level. At a given [Ca\textsuperscript{2+}]\textsubscript{o}, the tetani were superimposable when separated by periods of more than 3 minutes, during which the muscles were stimulated at 0.33 Hz. The level of force and light signals during the plateau phase of the tetanus were determined from a single tetanus.

**Effects of Varying Extracellular Calcium Concentration on Steady-State Activation**

Increasing [Ca\textsuperscript{2+}]\textsubscript{o} from 1 to 16 mM increased the amplitude of the twitch and tetanus forces. Figure 1 illustrates the effect of increasing [Ca\textsuperscript{2+}]\textsubscript{o} on force and [Ca\textsuperscript{2+}], in a preparation from a myopathic heart. As [Ca\textsuperscript{2+}]\textsubscript{o} was increased, a progressive increase occurred in the magnitude of the steady-state levels of [Ca\textsuperscript{2+}], along with the steady-state force levels, which increased from 1 to 8 mM [Ca\textsuperscript{2+}], but reached a plateau at 10 mM [Ca\textsuperscript{2+}], in both control and myopathic muscles. Developed force with tetani and peak twitch force were plotted as a function of [Ca\textsuperscript{2+}]\textsubscript{o}. Pooled results from experiments in control and myopathic muscles are presented in Figure 2. The tetanic force at 16 mM [Ca\textsuperscript{2+}], was used to normalize all other force levels in the control and myopathic experiments. At 16 mM [Ca\textsuperscript{2+}], the normalized peak twitch force in the control muscle (63±3%) was significantly different (p<0.001) from the normalized peak twitch force of the myopathic muscle (83±4%). Gwathmey et al\textsuperscript{16} previously have reported that intracellular calcium transients are prolonged in myopathic human myocardium and have two components. The associated isometric contractions also are prolonged. The left panel of Figure 3 illustrates the peak force–peak [Ca\textsuperscript{2+}] relations in a control and a myopathic muscle. The force-[Ca\textsuperscript{2+}] curve of the myopathic heart is shifted to the left from the force-[Ca\textsuperscript{2+}] curve of the control myocardium. Similar results were obtained when peak force–peak [Ca\textsuperscript{2+}] relations were compared in the six control muscles and the 10 myopathic ones. These data reflect the longer [Ca\textsuperscript{2+}] transient in myopathic muscles.

The right panel of Figure 3 shows a plot of developed steady-state force as the percent of maximal force at [Ca\textsuperscript{2+}],=16 mM versus steady-state [Ca\textsuperscript{2+}],. The points were fitted to a modified Hill equation. During tetani, both the force of contraction and [Ca\textsuperscript{2+}], reached a plateau, allowing us to plot steady-state force versus [Ca\textsuperscript{2+}], relations. Because there was no significant difference among the muscles from end-stage heart failure patients, data in the myopathic group were pooled. The force-[Ca\textsuperscript{2+}] curves for control and myopathic muscles essentially were superimposable (Figure 3, right panel) with no significant differences in Hill parameters (p>0.2, ANOVA).
**Intracellular Calcium Levels**

The mean [Ca\(^{2+}\)] was measured during tetani at various levels of [Ca\(^{2+}\)]. The data obtained from control and myopathic muscles first were pooled. Separate linear fits then were made for the control and myopathic values (Figure 4). The slope of the fitted lines and the intercepts were significantly different (p<0.01) between the control and myopathic groups.

We also measured the resting [Ca\(^{2+}\)], at a [Ca\(^{2+}\)] of 2.5 mM. In the control muscles, resting [Ca\(^{2+}\)], was 0.225±0.052 \(\mu\)M (n=6), whereas in the myopathic muscles, resting [Ca\(^{2+}\)], was significantly higher (p<0.01) at 0.361±0.068 \(\mu\)M (n=10).

**Comparisons of Relations Between Force and Intracellular Calcium Concentration Obtained From Tetani and From Twitches**

To investigate whether the presence of ryanodine (as used by Yue et al\(^{11}\)) altered the tetanic force-calcium relation, we examined steady-state force-[Ca\(^{2+}\)], relations in the presence and absence of ryanodine. We found that 1 \(\mu\)M ryanodine had no effect on the force-pCa curve or on the maximal force generated in skinned fibers, suggesting that ryanodine has no effect on actin-myosin interactions.\(^{23}\) Another question was whether the relation between peak force and peak [Ca\(^{2+}\)] derived from isometric contractions predicted the relation between force and [Ca\(^{2+}\)] obtained from tetani. In Figure 5, the curves for tetani in the presence and absence of ryanodine are compared with the curves for isometric contractions in the presence and absence of ryanodine. As with skinned fiber preparations, the tetani curves in the absence and in the presence of ryanodine are superimposable. However, with isometric contractions in intact muscle, the force-pCa curve shifted to the left in the presence of ryanodine. Time to peak force and peak [Ca\(^{2+}\)] were 206±45 and 40±13 msec before exposure to ryanodine and 516±65 and 411±39 msec after exposure to ryanodine in control muscles (n=5) (p<0.01 comparing pre- and postryanodine). These results indicate that although twitch force may be affected by changes in myofilament sensitivity, force-[Ca\(^{2+}\)], relations derived from isometric contractions are not always predictive of changes occurring at the level of the myofilaments. However, these data are important in predicting differences in the time course of [Ca\(^{2+}\)] signals.

**Effects of Caffeine and Isoproterenol on the Force-[Ca\(^{2+}\)], Relation**

Experiments on skinned muscle preparations of mammalian myocardium have shown that caffeine in the concentration range of 1–10 mM increases the sensitivity of myofilaments to Ca\(^{2+}\), whereas isoproterenol decreases the sensitivity of myofilaments to Ca\(^{2+}\).\(^{2}\) Figure 6 illustrates the effect of 10 mM caffeine on the force-[Ca\(^{2+}\)], relation in a tetanized myopathic human muscle. A clear shift to the left is observed in the presence of caffeine, indicating an increase in the sensitivity of the myofilaments to Ca\(^{2+}\), consistent with findings in skinned fiber experiments. Isoproterenol shifts the force-[Ca\(^{2+}\)], relation to the right (Figure 6B), a finding again consistent with results in skinned fiber experiments. Similar effects of caffeine and isoproterenol were observed on the steady-state force-[Ca\(^{2+}\)], relation in two other intact preparations. These results clearly show that steady-state force-[Ca\(^{2+}\)], relations derived from tet-
FIGURE 6. Panel A: Effect of 10 mM caffeine on steady-state force-[Ca\(^{2+}\)]\(_i\) relation in myopathic muscle. Panel B: Effect of 3 \(\mu\)M isoproterenol on steady-state force-[Ca\(^{2+}\)]\(_i\) relation.

Analyzed muscle parallel findings in skinned fiber experiments and that modulation at the level of the myofilaments can be predicted by changes in the steady-state force-[Ca\(^{2+}\)]\(_i\) relation.

**Effect of the Inotropic Agent DPI 201-106 on the Force-[Ca\(^{2+}\)]\(_i\) Relation**

A potential use of the steady-state force-[Ca\(^{2+}\)]\(_i\) relation lies in its ability to predict whether proposed inotropic agents affect the sensitivity of myofilaments to Ca\(^{2+}\). Using skinned fiber preparations, we previously reported that the inotropic agent DPI 201-106, a cardioactive agent with calcium-sensitizing properties, differentially increases the sensitivity of myofilaments to Ca\(^{2+}\) in myopathic but not control human myocardium.\(^{14}\) Figure 7 shows force-[Ca\(^{2+}\)]\(_i\) relations obtained from tetani in myopathic and control human muscles in the presence of 1 \(\mu\)M DPI. In control muscle, DPI does not affect the force-[Ca\(^{2+}\)]\(_i\) relation, whereas in myopathic muscle, DPI shifts the force-[Ca\(^{2+}\)]\(_i\) relation to the left, indicating an increase in the sensitivity of myofilaments to Ca\(^{2+}\). Similar differential effects of 1 \(\mu\)M DPI on the steady-state force-[Ca\(^{2+}\)]\(_i\) relations were observed between control and myopathic muscles for two other preparations. These data are in accordance with our previous findings in skinned fiber preparations where a small leftward shift (0.20 pCa units) was detected in the myopathic muscles.\(^{14}\)

**Comparisons of Relations Between Force and Calcium Concentration Obtained From Tetani and From Skinned Fiber Preparations**

An essential question is whether the relation between force and [Ca\(^{2+}\)]\(_i\), obtained from tetani is a measure of the sensitivity of the myofilaments to Ca\(^{2+}\). To address this question, we used saponin-skinned fiber preparations to construct force-[Ca\(^{2+}\)]\(_i\) relations in control and myopathic muscles. Figure 8 illustrates force-pCa relations from a control versus a myopathic muscle. The force-pCa curves were superimposable, consistent with the steady-state force-[Ca\(^{2+}\)]\(_i\) relations obtained from tetani. Because muscles of different hearts were used, we compared the Hill parameters for the Hill parameters for skinned fiber preparations and intact muscle tetani in both control and myopathic groups. As shown in Table 1, in skinned fiber preparations, force-pCa relations were shifted toward higher [Ca\(^{2+}\)] and had a lower Hill coefficient as compared with force-[Ca\(^{2+}\)]\(_i\) relations obtained from tetani. Table 1 also demonstrates that [Ca\(^{2+}\)] at 95% maximal force, which is a more reliable parameter of the force-[Ca\(^{2+}\)]\(_i\) relation as shown by Yue et al.,\(^{11}\) also is larger in skinned fiber as compared with
posttetanic twitch amplitude was similar or less than the amplitude of the steady-state [Ca^{2+}] signal. Table 2 represents the pre- and posttetanic time to peak tension and time to peak light in control and myopathic muscles. It is evident that the posttetanic [Ca^{2+}] transient and accompanying twitch are significantly briefer than pretetanic [Ca^{2+}], transient and twitch.

The upper panel of Figure 10 compares the decay in posttetanic twitch force at two extracellular Ca^{2+} concentrations in five control and eight myopathic hearts. The beat-dependent decay was slower at higher [Ca^{2+}]_o and in myopathic muscles.

To investigate the potential role of the sarcoplasmic reticulum in posttetanic potentiation, we studied the effects of ryanodine. The addition of ryanodine resulted in posttetanic depression (Figure 10, lower panel). There was a beat-dependent increase in posttetanic twitch amplitude in the presence of ryanodine as opposed to a beat-dependent decay in the absence of ryanodine. The relation between peak force and peak [Ca^{2+}], for the posttetanic twitch was shifted to the right compared with the pretetanic twitch, as illustrated for a control muscle in Figure 11. This shift is consistent with the large increase in [Ca^{2+}], associated with the first potentiated beat.

Some myopathic muscles with excessively prolonged force and calcium transients did not exhibit posttetanic potentiation. In these muscles, the sarcoplasmic reticulum was impaired; therefore, the behavior was similar to ryanodine-treated muscles.

**Discussion**

**Tetanization of Human Myocardium**

It generally has been held that mammalian myocardium cannot be tetanized because of the temporal overlap between the cardiac action potential and the associated contraction. However, in ventricular muscles from certain mammalian species, for example, the shrew and the rat, where the action potential is short, it is possible to induce tetanus. In this case, voltage-dependent calcium channels can be activated and deactivated fast enough to allow the repetitive stimulation to gradually increase intracellular Ca^{2+}.

Interventions such as the addition of ryanodine, which impairs the sarcoplasmic reticulum, have been used previously to tetanize mammalian ventricular muscle. In the presence of an impaired or poorly developed sarcoplasmic reticulum, the time course of the twitch becomes very long. Subsequent stimulations introduce more Ca^{2+}, consequently increasing the myofibrillar Ca^{2+} interaction, which results in summation and tetanus.

We propose that tetani can occur if calcium handling by the sarcoplasmic reticulum is slow or impaired and/or the refractory period of action potential is short enough to produce an almost continuous entry of Ca^{2+}. The present experiments show that human cardiac muscle, despite having relatively prolonged action potentials, can be tetanized in the absence of pharmacological agents. In

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**Figure 8.** Force vs. [Ca^{2+}] relation in saponin-skinned human trabeculae from control (n=6) and myopathic (n=10) muscles. Curves were fitted to Hill equation. [Ca^{2+}] required for 50% activation, Hill coefficient, and maximal force developed were 1.56±0.22 μM, 2.05±0.33, and 0.30±0.09 g/mm² in control muscles, and 1.44±0.16 μM, 2.30±0.23, and 0.34±0.08 g/mm² in myopathic muscles, respectively.

**Table 1.** Comparisons of Hill Parameters Between Skinned Muscle Preparations and Tetanizations in Control and Myopathic Muscles

<table>
<thead>
<tr>
<th>Preparation and group</th>
<th>[Ca^{2+}]_{50%} (μM)</th>
<th>[Ca^{2+}]_{95%} (μM)</th>
<th>n_h</th>
<th>F_{max} (g/mm²)</th>
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<tr>
<td>Intact</td>
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<tr>
<td>Control</td>
<td>0.56±0.05</td>
<td>0.97±0.05</td>
<td>5.21±0.20</td>
<td>1.23±0.49</td>
</tr>
<tr>
<td>Myopathic</td>
<td>0.54±0.09</td>
<td>0.98±0.03</td>
<td>5.61±0.60</td>
<td>1.84±0.74</td>
</tr>
<tr>
<td>Skinned</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>1.56±0.22</td>
<td>6.56±0.93</td>
<td>2.05±0.33</td>
<td>0.30±0.09</td>
</tr>
<tr>
<td>Myopathic</td>
<td>1.44±0.16</td>
<td>5.88±0.67</td>
<td>2.30±0.23</td>
<td>0.34±0.08</td>
</tr>
</tbody>
</table>

Values are mean±SE.

[Ca^{2+}]_{50%}, calcium concentration required for 50% activation; [Ca^{2+}]_{95%}, calcium concentration required for 95% activation; n_h, Hill coefficient; F_{max}, maximal force developed.
human myocardium, the sarcoplasmic reticulum is relatively slower compared with other mammalian cardiac tissue and is impaired in muscles from diseased hearts. Delayed inactivation of calcium channels also would result in a continuous entry of Ca$^{2+}$, producing tetanization.

Differences in Myofilament Sensitivity to Calcium as Determined by Tetanized Intact Muscle Versus Skinned Fiber Preparations

Comparison of the Hill parameters for force-[Ca$^{2+}$], relations from intact and skinned preparations showed that there are differences in F$_{max}$ [Ca$^{2+}$]$_{50\%}$, [Ca$^{2+}$]$_{95\%}$, and n$_o$. It is important to note that the Hill coefficient and [Ca$^{2+}$]$_{50\%}$ obtained from the Hill equation are less reliable than the [Ca$^{2+}$]$_{95\%}$ because of the few data points in the critical steep portion of the force-[Ca$^{2+}$] curve. At 95% Ca$^{2+}$ activation, it is likely that all the cells in our multicellular preparations are close to maximal activation, unlike at 50% activation, where different cells can have different levels of activation averaging 50%. As shown in Table 1, we found that maximal Ca$^{2+}$-activated force is higher in intact tetanized muscles than in skinned fiber preparations. Because skinning with saponin causes the fibers to swell, as shown in histological stains, it can be argued that the skinning procedure increases the intrafilament spacing and therefore decreases the developed force. Recently, Matsubara et al. have shown that there is an optimal intrafilament spacing at which skinned fibers develop maximum force that correlates well with the intrafilament spacing in the intact tissue.

Another difference between skinned fiber preparations and intact muscle is the calcium concentration required for 95% activation. We found that the steady-state force-[Ca$^{2+}$], relation from tetanized intact muscles approaches maximal force by 1 μM [Ca$^{2+}$], consistent with the results of Yue et al. However, skinned muscle preparations from the same human hearts exhibited a lower calcium sensitivity. The discrepancy between [Ca$^{2+}$]$_{95\%}$ in intact versus skinned preparations from the same hearts may be due to depletion of membrane bound phospholipids, peptides, amino acids, and soluble cofactors that determine the sensitivity of the myofilaments to calcium. We also should note that our skinned fiber experiments were performed at 20°C, whereas the intact preparations were tetanized at 30°C. Recently, Harrison and Bers showed that cooling led to a shift in the force-pCa curve toward higher [Ca$^{2+}$], but the shift of [Ca$^{2+}$]$_{95\%}$ from 29°C to 22°C was only 0.15 pCa units, which cannot solely explain the discrepancy in [Ca$^{2+}$]$_{95\%}$ between skinned fibers and intact muscles.

![Record of potentiated contraction evoked after tetanus in control human muscle at 2.5 mM [Ca$^{2+}$].](http://circ.ahajournals.org/)

**FIGURE 9.** Record of potentiated contraction evoked after tetanus in control human muscle at 2.5 mM [Ca$^{2+}$]. L/L$\text{max}$, fractional luminescence (see ‘Methods’).

**TABLE 2.** Time Course of Light and Tension Transients for Pretetanic and Posttetanic Twitches From Control and Myopathic Muscles

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of preparations</th>
<th>Pretetanic</th>
<th>Posttetanic</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>TPT (msec)</td>
<td>TPL (msec)</td>
</tr>
<tr>
<td>Control</td>
<td>4</td>
<td>301±55</td>
<td>27±6</td>
</tr>
<tr>
<td>Myopathic</td>
<td>5</td>
<td>497±68</td>
<td>51±12</td>
</tr>
</tbody>
</table>

Values are mean±SE. Muscles superfused at [Ca$^{2+}$]$_o$ of 2.5 mM. TPT, time to peak tension; TPL, time to peak light.

$p<0.01$ compared with pretetanic values.
Our results also show that the steady-state force versus \([\text{Ca}^{2+}]\) relation is steeper in intact myocardium than in skinned fiber preparations, with a correspondingly larger Hill coefficient (5.3±0.2 versus 2.3±0.2). It is important to note that the Hill coefficient does not correlate to the \(\text{Ca}^{2+}\) binding sites, and a Hill coefficient that is greater than the number of calcium binding sites (believed to be three) is not necessarily unexpected. Kentish et al.\(^{32}\) have obtained Hill coefficients as large as 5.5 in mammalian cardiac muscle when sarcomere length is fixed. The Hill coefficients we obtained in our skinned preparations are, however, similar to those previously reported.\(^{3,14,19}\) Force–[\(\text{Ca}^{2+}\)] relations do not allow direct conclusions about affinity and cooperativity of calcium binding to troponin C. A high Hill coefficient does not necessarily indicate long-range cooperativity among a large number of calcium binding sites located along the thin filaments but probably results from altered isometric cross-bridge turnover kinetics.\(^{33–35}\)

**Differences Between Steady-State Force–[\(\text{Ca}^{2+}\)] and Peak Force–Peak [\(\text{Ca}^{2+}\)] Relations**

Many investigators using aequorin\(^{8,9,36,37}\) have tried to make inferences about the force–[\(\text{Ca}^{2+}\)] relations in intact heart muscle using peak force and peak calcium measurements obtained during isometric twitches. As our results illustrate, peak force–[\(\text{Ca}^{2+}\)] relations during twitches cannot be used to examine the sensitivity of myofilaments to \(\text{Ca}^{2+}\). Interventions that increase the time course of the [\(\text{Ca}^{2+}\)] transient and twitch contraction shift the peak force–peak [\(\text{Ca}^{2+}\)] relation to the left (Figure 3), whereas interventions that decrease the time course of twitch contraction and the accompanying [\(\text{Ca}^{2+}\)] transient shift the peak force versus peak [\(\text{Ca}^{2+}\)] relation to the right. The slower the change in the time course of the twitch contraction and [\(\text{Ca}^{2+}\)], transient, the more

**Figure 10.** Upper panel: Effect of low and high \([\text{Ca}^{2+}]_o\) on normalized beat-dependent decay of posttetanic potentiated contraction. Baseline frequency of stimulation was 0.33 Hz in control (n=5) and myopathic (n=8) muscles. \(F_1\) developed force at a certain beat; \(F_0\), first beat after tetanus; \(F_0\), pretetanic twitch force. Lower panel: Effect of 1 \(\mu\text{M}\) ryanodine on posttetanic contractions at 2.5 mM \([\text{Ca}^{2+}]_o\).

**Figure 11.** Relation between peak force and peak [\(\text{Ca}^{2+}\)], obtained from twitches before and just after tetani in control muscle. Also plotted, the steady levels of force and [\(\text{Ca}^{2+}\)], derived from tetani. Maximal force developed was 1.23 g/mm\(^2\). Similar results were obtained in four other control muscles.
closely the relation between peak force and peak calcium approaches the steady-state force-[Ca\textsuperscript{2+}] relation. During the plateau phase of a tetanus, [Ca\textsuperscript{2+}], is spatially uniform, as demonstrated by two models of [Ca\textsuperscript{2+}], dynamics in muscle fibers.\textsuperscript{38,39} Ryanodine does not affect the force-[Ca\textsuperscript{2+}] relation derived from either tetani or skinned preparations. However, ryanodine, which significantly prolongs the time course of the twitch and the [Ca\textsuperscript{2+}], transient, shifts the peak force-peak [Ca\textsuperscript{2+}] relation to the left. These data suggest that peak force-peak [Ca\textsuperscript{2+}], relations obtained from twitches may be misleading when used to arrive at conclusions about events occurring at the level of the myofilaments.

Blinks and Endo\textsuperscript{3} extensively explored mechanisms that can alter the time course and amplitude of the aequorin signal. They specifically looked at the roles of sarcoplasmic reticulum and troponin C in regulating [Ca\textsuperscript{2+}]. They came to the conclusion that there was no satisfactory way of distinguishing between contributions of time course changes in the [Ca\textsuperscript{2+}], transient and changes in responsiveness of myofibrils to Ca\textsuperscript{2+} by examining the [Ca\textsuperscript{2+}], transient. Because the [Ca\textsuperscript{2+}], transient peaks before the development of tension, there are severe limitations to interpreting plots of peak tension versus peak light as quantitative indexes of myofibrillar responsiveness to Ca\textsuperscript{2+}.

As illustrated in Figure 2, peak tetanus force was similar for control and myopathic tissue. However, there was an upward shift in the peak twitch force for myopathic muscles as compared with control muscles. This probably is due to a slower time course of the myopathic calcium transient and twitch. Furthermore, it has been demonstrated that there is enhanced transsarcolemmal calcium flux and impaired sarcoplasmic reticulum function in myopathic human myocardium, resulting in a second component of the calcium transient.\textsuperscript{16} The presence of this slower second component in addition to an elevated resting [Ca\textsuperscript{2+}], would allow for increased force activation.

It is important to note that the aforementioned explanations do not account for temporal dissociation between [Ca\textsuperscript{2+}], transients and force signals. An important example of this phenomenon is the effect of muscle length on the [Ca\textsuperscript{2+}], and force transients.\textsuperscript{40} In this case, plotting peak force versus peak [Ca\textsuperscript{2+}] would lead to a leftward shift at the longer length, where the time course of the twitch is longer. Another example of this phenomenon is the differential effect of hypoxia and reoxygenation on [Ca\textsuperscript{2+}], and isometric force.\textsuperscript{41} Plotting force versus [Ca\textsuperscript{2+}], would result in a shift to the left during reoxygenation (with the longer contraction) as compared with the curve for hypoxia (with the briefer contraction). It would seem that the time course of the twitch governs the relative changes of the peak force-peak [Ca\textsuperscript{2+}], relation as noted earlier.\textsuperscript{11,12}

Maximal Twitch Tension Evoked by Posttetanic Potentiation

At a [Ca\textsuperscript{2+}] of less than 8 mM, the first posttetanic twitch was larger than the force developed during tetanus. As shown in Figure 9, the posttetanic potentiation was associated with an increase in the [Ca\textsuperscript{2+}], transient, which was larger than either the pretetanic or tetanic [Ca\textsuperscript{2+}], level. Increasing [Ca\textsuperscript{2+}], caused an increase in posttetanic potentiation and an increase in the amplitude of the [Ca\textsuperscript{2+}], transient. At higher [Ca\textsuperscript{2+}], (>8 mM), the posttetanic twitch was similar or smaller in amplitude than the developed tetanus. This is important to note because the tetanus force at a [Ca\textsuperscript{2+}], of 16 mM was used as the maximal Ca\textsuperscript{2+}-activated force. These results suggest that posttetanic potentiated twitches at low [Ca\textsuperscript{2+}],, which are larger than the steady-state levels of force developed by tetanized muscles, more accurately represent the maximal force that intact cardiac muscle can generate at a certain extracellular calcium concentration. It can be argued that the rapid stimulation necessary to produce tetani allows a large buildup of cytoplasmic [Ca\textsuperscript{2+}], which then equilibrates with internal calcium stores (i.e., calcium stored in the sarcoplasmic reticulum and/or mitochondria). In the presence of ryanodine (which interferes with calcium handling by the sarcoplasmic reticulum), the posttetanic twitch was not potentiated and actually was smaller than the pretetanic twitch, similar to the findings of Yue et al.\textsuperscript{11} This suggests that the sarcoplasmic reticulum provides the calcium that is released during posttetanization.

Although the posttetanic twitch largely was potentiated as compared with the pretetanic twitch and the tetanus, the relation between peak force and peak [Ca\textsuperscript{2+}], for the posttetanic twitch was shifted to the right as compared with either the pretetanic twitch or the tetanus. The shift to the right is caused by the decrease in twitch and [Ca\textsuperscript{2+}], transient duration. This would suggest that during posttetanic potentiation, there is not enough time for intracellular Ca\textsuperscript{2+} to reach equilibrium because of a large efflux of calcium from the sarcoplasmic reticulum, even though the large increase in [Ca\textsuperscript{2+}], level produces a larger force. The reduced time course of the signals can be explained by postulating an accumulation of metabolic products during tetanus. The balance between energy production and energy use could be disturbed during tetanus as cytoplasmic [Ca\textsuperscript{2+}] rises, but Kusuzuka et al\textsuperscript{42} showed metabolites were not significantly affected by tetani. Another explanation could be that during the repeated stimuli, a large flux of Ca\textsuperscript{2+} enters the cell through the sarcolemma, activating the myofilaments and loading the sarcoplasmic reticulum. After the tetanus, the sarcoplasmic reticulum accumulates the Ca\textsuperscript{2+}, activating the myofilaments in addition to the transsarcolemmal Ca\textsuperscript{2+}, which would lead to a larger and faster release of the stored Ca\textsuperscript{2+} during the posttetanic twitch.\textsuperscript{43}

These results would suggest that at specific small [Ca\textsuperscript{2+}], it is possible to evoke a maximal twitch that is larger than the steady-state level of the tetanus. This requires a large buildup of [Ca\textsuperscript{2+}], inside internal stores, such as the sarcoplasmic reticulum, and a subsequent release of this calcium. The steady-state
force-[Ca\(^{2+}\)] relation therefore probably represents a more stable Ca\(^{2+}\) activation curve.

The Steady-State Force-[Ca\(^{2+}\)] Relation: Index of Myofilibril Response to Calcium

We have described a strategy for producing steady activation in human myocardium and deriving steady-state force-[Ca\(^{2+}\)] relations. To validate this new approach, we verified the effects of agents with known pharmacological action at the level of the myofilaments on the steady-state force-[Ca\(^{2+}\)] relation. We have shown that agents known to alter the sensitivity of myofilaments to Ca\(^{2+}\) in skinned cardiac fibers affect the steady-state force-[Ca\(^{2+}\)] relation in tetanized human cardiac muscle in the same way. Caffeine, which increases the sensitivity of myofilaments to Ca\(^{2+}\) in skinned fiber preparations, shifts the steady-state force-[Ca\(^{2+}\)] relation to the left, whereas isoproterenol, which decreases the sensitivity of myofilaments to Ca\(^{2+}\) in skinned fiber preparations, shifts the steady-state force-[Ca\(^{2+}\)] relation to the right. Recently, inotropic agents such as sulmazole, DPI 201-106, and MCI 1544 have been reported to act, at least partially, by increasing the sensitivity of myofilaments to Ca\(^{2+}\). We further tested the validity of the steady-state force-[Ca\(^{2+}\)] relation as a measure of myofilament Ca\(^{2+}\) sensitivity by examining the effects of the inotropic agent DPI 201-106. We have demonstrated previously that DPI differentially increases the sensitivity of the myofilaments to calcium in skinned myopathic human muscle when compared with control human myocardium.

Figure 7, the steady-state force-[Ca\(^{2+}\)] relation reveals an increase in the sensitivity of myofilaments to Ca\(^{2+}\) in intact myopathic human muscle but not in human control muscle. These data indicate that steady-state force-[Ca\(^{2+}\)] relations can potentially detect differential responses to pharmacological agents between control and myopathic tissues of various etiologies. This approach also revealed that there were no differences in sensitivity of the myofilaments to Ca\(^{2+}\) in maximal Ca\(^{2+}\)-activated force between control and myopathic hearts. This suggests that the dysfunction underlying diseased myocardium does not reside at the level of the myofilaments.

Even though [Ca\(^{2+}\)], is calculated using a highly nonlinear relation between the aequorin signal and [Ca\(^{2+}\)], the steady-state force-[Ca\(^{2+}\)] relation has the advantage of being able to detect changes in myofilament Ca\(^{2+}\) sensitivity in intact muscle, as opposed to the skinned fiber preparation, in which the sarcolemma and the sarcoplasmic reticulum are destroyed. In conclusion, we have for the first time unambiguously shown that the use of muscle tetanization to construct a steady-state force-[Ca\(^{2+}\)] relation in aequorin-loaded cardiac muscle provides a valid alternative to the use of skinned muscle preparations for examining the responsiveness of the contractile proteins to Ca\(^{2+}\). Furthermore, use of aequorin-loaded intact muscle preparations offers the advantage of allowing simultaneous examination of calcium handling by the sarcoplasmic reticulum and other intracellular organelles during experimental interventions.

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KEY WORDS: tetanization • force-[Ca²⁺] relation • skinned fibers • human myocardium • aequorin
Relation between steady-state force and intracellular [Ca2+] in intact human myocardium. Index of myofibrillar responsiveness to Ca2+.

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