Myofilament properties comprise the rate-limiting step for cardiac relaxation at body temperature in the rat

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Myofilament properties comprise the rate-limiting step for cardiac relaxation at body temperature in the rat. Among the studies aimed at characterizing basic contractile mechanisms, we investigated contractile function and calcium handling in ultrathin rat cardiac trabeculae. Active developed tension was unaltered between 22.5 and 30.0°C (from 89 ± 10 to 86 ± 11 mN/mm²; P = not significant) but steeply declined at 37.5°C (30 ± 5 mN/mm²). Meanwhile, the speed of relaxation (time from peak force to 50% relaxation) declined from 22.5 to 30.0°C (from 360 ± 40 to 157 ± 17 ms) and further declined at 37.5°C to 76 ± 13 ms. Phase-plane analysis of calcium versus force revealed that, with increasing temperature, the relaxation phase is shifted rightward, indicating that the rate-limiting step of relaxation tends to depend more on calcium kinetics as temperature rises. The force-frequency relationship, which was slightly negative at 22.5°C (0.1 vs. 1 Hz: 77 ± 12 vs. 66 ± 7 mN/mm²), became clearly positive at 37.5°C (1 vs. 10 Hz: 30 ± 5 vs. 69 ± 9 mN/mm²). Phase-plane analyses indicated that, with increasing frequency, the relaxation phase is shifted leftward. We conclude that temperature independently affects contraction and relaxation, and cross-bridge cycling kinetics become rate limiting for cardiac relaxation under experimental conditions closest to those in vivo.

METHODS

Muscle preparation and experimental setup. Male LBN-F1 rats weighing 200–250 g (Harlan Sprague Dawley; Indianapolis, IN) were anesthetized by intraperitoneal injection of 1.0 ml/kg pentobarbital sodium (360 mg/ml). After intracardiac heparinization, the hearts were rapidly excised and placed in Krebs-Henseleit buffer containing (in mM) 120 NaCl, 5 KCl, 2 MgSO₄, 1.2 NaH₂PO₄, 20 NaHCO₃, 0.25 Ca²⁺, and 10 glucose (pH 7.4). Additionally, 20 mM 2,3-butanedione monoxime (BDM) were added to the dissection buffer to prevent cutting injury. The effects of BDM after brief exposure have been found to be reversible (18, 25). Hearts were cannulated via the ascending aorta and retrogradely perfused with the same buffer equilibrated with 95% O₂-5% CO₂. Blood was

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thoroughly washed out, and thin, uniform, nonbranched trabeculae from the right ventricle (along the tricuspid valve) were carefully dissected, leaving a block of tissue at one end from the right ventricular free wall and a small part of the valve at the other end to facilitate mounting. The average dimensions of the muscles \((n = 21)\) were measured using a calibration reticle in the ocular of the dissection microscope \((\times 40, \text{ resolution } \sim 10 \mu \text{m})\) and were \(0.170 \pm 0.02 \text{ mm wide, } 0.102 \pm 0.01 \text{ mm thick, and } 2.7 \pm 0.26 \text{ mm long. The cross-sectional area was calculated assuming an ellipsoid shape.} \]

All experimental protocols conformed to institutional guidelines regarding the use and care of animals.

With the use of the dissection microscope, muscles were mounted between a platinum-iridium basket-shaped extension of a force transducer (tissue block end) and a hook (valve end) connected to a micromanipulator. This method has been shown \((4,18,19,23,28)\) to minimize end damage compliance of the muscle and to prevent excessive loss of force throughout the experimental protocols. Muscles were perfused with the same buffer at 22.5°C as above (with the exception that BDM was omitted and stimulated at 0.5 Hz. Extracellular calcium concentration \([\text{Ca}^{2+}]_0\) was raised to 0.50 mM, 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 about equal increases in resting tension and active developed tension. This is a length (optimal length) that is slightly below the length where active force development is maximal and was selected to be comparable to the maximally attained length in vivo \((\sim 2.2 \mu \text{m sarcomere length})\) .

**Determination of contraction and relaxation.** To assess the effect of muscle length on relaxation time, the following protocol was performed. First, slack length (length of muscle without any preload) and optimal length were determined. The difference was divided into nine equal steps, and the muscle was stretched sequentially, with each step increasing the length of the muscle until the baseline length was achieved. Parameters were recorded when the muscle had stabilized at each length. To determine the individual and synergistic effects of calcium and muscle length on relaxation time, this protocol was performed during exposure to 0.5 and 1.0 mM Ca\(^{2+}\) at 22.5°C. Additionally, this length-step experiment was repeated at 37.5°C \((1.0 \text{ and } 2.0 \text{ mM Ca}^{2+}; \text{ stimulation frequency, } 4 \text{ Hz})\).

We assessed the effects of increasing stimulation frequencies at 22.5 and 37.5°C. In the first set of experiments at 22.5°C, muscles were stimulated at 0.1, 0.25, 0.5, and 1 Hz. These low stimulation frequencies were necessary because, at low temperatures, high frequencies lead to fused contractions because both force generation and calcium removal systems are significantly slowed. At 37.5°C, muscles were stimulated between 0.5 and 10 Hz. At each frequency, forces were allowed to reach steady state before data were recorded. To determine the effects of increasing or decreasing the temperature from 22.5 to 37.5°C, force was recorded while the temperature was slowly increased at 2.5°C intervals. The changes in temperature were slow enough to be considered steady state \((<0.5°C/\text{min})\). To ensure the latter, this experiment was repeated in the opposite direction (from 37.5 to 22.5°C) with no hysteresis. Contractile behavior of the preparations at 37.5°C was stable; under identical conditions \((1.0 \text{ mM Ca}^{2+}, 4 \text{ Hz})\), in three different protocols \((>30 \text{ min apart})\) throughout the experiment, the developed force \(F_{\text{dev}}\) amounted to 54.7 ± 7.1, 56.2 ± 9.4, and 55.0 ± 7.6 mN/mm², respectively. However, a small injury would lead to an immediate decay of \(F_{\text{dev}}\) and development of aftercontractions, whereas these small injuries would not immediately lead to such strong decay in behavior at room temperature. Thus, at body temperature, assessment of contractility is potentially more dependent on preparation quality.

**Measurement of \([\text{Ca}^{2+}]_i\), in right ventricular trabeculae.** In a subset of experiments, the \([\text{Ca}^{2+}]_i\) was measured as previously described \((3,4,10,17)\). Briefly, after the muscle had stabilized \((at 1.0 \text{ mM Ca}^{2+}, 22.5°C, \text{ and } 0.5 \text{ Hz})\) for 30 min, the \([\text{Ca}^{2+}]_i\) was reduced to 0.25 mM and stimulation was stopped. Background fluorescence at 340-, 358-, and 380-nm excitation was collected at both 22.5 and 37.5°C. Fura 2 pentapotassium salt \((\text{Molecular Probes; Eugene, OR})\) was microinjected iontophoretically into one cell and allowed to spread throughout the muscle via the gap junctions. After fura 2 loading, stimulation was resumed, and \([\text{Ca}^{2+}]_i\) was determined by alternating illumination at 340 and 380 nm. We used a heat-exchange system to achieve rapid \(<2 \text{ s}\) switching to 37.5 from 22.5°C. Limiting exposure to high temperatures to short intervals \((4–6 \text{ min})\) enabled us to measure \([\text{Ca}^{2+}]_i\), at body temperature by virtually eliminating the loss of dye, which has thus far undermined the assessment of calibrated \([\text{Ca}^{2+}]_i\) transients at 37.5°C. Additionally, this length-step experiment was repeated in the opposite direction (from 37.5 to 22.5°C) with no hysteresis. Thus, at 37.5°C, \([\text{Ca}^{2+}]_i\) was measured throughout the protocol to monitor the amount of dye in the muscle. Calcium transients were calculated using in situ calibration parameters as previously obtained \((4,10)\). It has been shown that the dissociation constant \(K_d\) for Ca\(^{2+}\) binding to fura 2 displays very little temperature dependence \((12,29)\). We were not able to record steady-state tetani at body temperature and were therefore restricted to use the \(K_d\) value previously obtained in the present experimental setup \((3,10)\) (also see DISCUSSION). In these experiments, we measured force and calcium transients over a broad range \((0.5–10 \text{ Hz})\) of stimulation frequencies at 37.5°C. Fluorescence was collected at 510 nm by a photomultiplier tube \((R1527\text{ Hamamatsu})\). The output of the photomultiplier was filtered at 300 Hz, collected by an analog-digital converter, and stored in a computer for off-line analysis. \([\text{Ca}^{2+}]_i\) was calculated as described previously \((3,4,10,17)\).

**Data analysis and statistics.** In all the experiments performed, the parameters of \(F_{\text{dev}}\) and diastolic force \(F_{\text{dia}}\) were determined and normalized to the cross-sectional area of the muscle. Additionally, as a model-independent parameter of force decay kinetics, time from peak force to 50% relaxation \((\text{RT}_{50})\) was determined. Parameters were calculated off-line and also on-line to facilitate immediate judgement of prepa-

**RESULTS**

**Effect of temperature on contraction and relaxation.** We observed a large acceleration of relaxation \((\text{RT}_{50}, to 55.8 \pm 4.1\%; n = 10, P < 0.01; \text{ Fig. 1, A and B})\) when temperature was increased from 22.5 to 30.0°C. However, over this range of temperatures, \(F_{\text{dev}}\) did not significantly change \((to 97.4 \pm 8.2\%\). This is in close agreement with a previous study \((8)\) on rat trabeculae.
in which temperature between 20 and 30°C had little effect on F_dev. However, when the temperature was further increased to 37.5°C, F_dev sharply decreased to 35.3 ± 5.6%, whereas the duration of relaxation continued to decrease to 22.6 ± 2.7%. Thus comparing 22.5 with 37.5°C, F_dev fell about threefold, whereas relaxation was accelerated by a factor of 4–5. When this temperature protocol was reversed, i.e., lowering the temperature from 37.5 to 22.5°C, similar results were obtained (data not shown). These results indicate that temperature exerts distinctive and different effects on F_dev and on duration of the twitch, with a highly nonlinear relationship between these two parameters.

To further probe the mechanism of the temperature dependence of twitch contractions, we measured cytosolic calcium transients in a subset of preparations. Similar to the data obtained by Layland and Kentish (23), who showed that the amplitude of the fluorescence ratio slightly decreases from ~2.4 at 24°C to 2.0 at 30°C (1 Hz, 1 mM [Ca²⁺]), we also observed a slight decrease in the fluorescence ratio amplitude from ~2.6 at 22.5°C to ~2.4 at 30°C (very similar conditions, n = 4, noncalibrated ratio not shown; for a calibrated example, see Fig. 1C). In addition, when we further increased temperature to the physiological range, this ratio dropped even further, and much more steeply, to only ~1.3 (at 37.5°C). Thus the temperature dependence of calcium handling is most prominent between 30 and 37.5°C, with little change between 22.5 and 30°C.

With the use of the K_d for fura 2 obtained at room temperature, we converted the measured fluorescent ratios to free cytosolic calcium transients. As can be clearly seen in Fig. 1C, the amplitude and kinetics of the calcium transients parallel changes in F_dev and duration of the twitch. With increasing temperature, the amplitude of the transient was unaltered between 22.5 and 30.0°C (1.41 ± 0.21 vs. 1.33 ± 0.18 μM). However, it declined to 0.73 ± 0.29 μM at 37.5°C. Similar to F_dev, timing of the calcium transient (50% decay time) was affected by temperature over the entire range (110 ± 14 to 73 ± 12 to 60 ± 8 ms). However,
the changes in calcium transient duration are less pronounced than the observed changes in timing of the twitch.

Phase-plane analysis of calcium versus force reveals that, with increasing temperature, the relaxation phase moves to the right, indicating a movement toward the steady-state force-calcium relationship, displayed by the dashed line representing pooled data previously obtained in the same setup by tetanizing the intact muscle (Fig. 1D). This suggests that, at 37.5°C, cross-bridge kinetics may no longer be the rate-limiting step in cardiac relaxation at this rate of stimulation (0.5 Hz), as they are at room temperature (3, 16). This experiment was repeated in three additional trabeculae, rendering nearly identical results (data not shown).

Effect of length on contraction and relaxation. To study cardiac relaxation under a variety of loading conditions, we modulated the length of the preparation. Stretching the muscle from slack length to near-optimal length (covering the entire range of sarcomere length achieved in the in vivo beating heart) resulted in an increase in contractile force and an increase in the duration of the twitch (Fig. 2, B and D) at both 22.5 and 37.5°C, as is evident from the twitch shape (Fig. 2, A and D). Interestingly, at 22.5°C, an increase in calcium concentration from 0.5 to 1.0 mM seemed to flatten this relationship. At 37.5°C, an increase in calcium concentration from 1.0 to 2.0 mM resulted in a slight parallel upward shift of this relationship (data not shown). It has previously been shown that calcium transients do not prolong in response to muscle stretch (2). Thus, under any load that exceeds slack length, the rate-limiting step of the relaxation phase resides in the myofilaments.

Effects of stimulation frequency on contraction and relaxation. At 22.5°C, an increase in stimulation frequency from 0.1 to 1 Hz led to a small decrement of F_{dev}, from 77.5 ± 12.0 to 65.6 ± 7.1 mN/mm² (n = 9, typical example shown in Fig. 3A), resulting in a so-called negative force-frequency relationship. Over this range of frequencies, twitch timing parameters accelerated (Fig. 3B), leading to a positive correlation between F_{dev} and duration of relaxation, similar to the behavior during length-dependent activation. In sharp contrast, at 37.5°C, an increase of the stimulation frequency from 0.5 to 10 Hz resulted in a clear increase in F_{dev} (from 30.4 ± 4.6 to 68.7 ± 8.9 mN/mm², n = 9; Fig. 3D), whereas relaxation became progressively faster over the same range of frequencies.
Thus, in the physiologically relevant range for the rat (5–9 Hz; 300–350 beats/min at rest to 550 beats/min under stress conditions, e.g., full β-adrenergic stimulation), the force-frequency relationship is positive.

As shown in Fig. 3, \( F_{\text{dev}} \) appeared to reach an “optimal” frequency in many preparations. It is conceivable that, at these high frequencies (but achievable in the rat in vivo), diffusion of nutrients and oxygen on one hand, and removal of metabolites like phosphate on the other hand, can become limiting for muscle function. Hence, we plotted the relationship between thickness of the preparation and the frequency at which \( F_{\text{dev}} \) was maximal (Fig. 4). Thickness and optimal frequency were related such that preparations that exceeded 100 μm in thickness tended to display an optimal frequency below the maximal in vivo achievable frequency of the rat (8–10 Hz). This would indicate that generation of contractile force at high frequencies may be hampered at diffusion distances exceeding ~50–75 μm. For this reason, the analysis of the force-frequency data was restricted to muscles ≤100 μm in thickness (n = 10).

For technical reasons, calcium transients from iontophoretically loaded muscle have not yet been assessed at body temperature: at high temperature, the rapid loss of dye prevents extended measurements (23). We developed a protocol/system whereby calcium transients can now be recorded at physiological temperature.

Fig. 3. A: at 22.5°C, increasing stimulation frequencies lead to decreased \( F_{\text{dev}} \) and abbreviated twitch timing. Average data for \( F_{\text{dev}} \) and \( RT_{50} \) at 22.5°C (B; n = 11) and at 37.5°C (D, n = 9) are shown. C: at 37.5°C, \( F_{\text{dev}} \) increased, whereas twitch duration decreased with increasing stimulation frequency.

(H503RELAXATION OF CARDIAC TRABECULAE AT BODY TEMPERATURE)

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Fig. 4. With increasing thickness, the optimal frequency of contraction is reduced in rat cardiac trabeculae. As a result of this phenomenon, only muscles with a diameter of <100 μm were included in the final analysis of the data regarding force-frequency. *Optimal frequency of that preparation may be actually higher than 10 Hz; the protocol was routinely only measured from 0.5 to 10 Hz, although in some pilot experiments, increasing the stimulation to even higher frequencies showed that the optimal frequency can be as high as 15 Hz.

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perature. By rapidly (2–3 s) switching from 22.5 to 37.5°C, waiting for steady state (30–60 s), measuring a force-frequency protocol (0.5–10 Hz), and returning (again in a few seconds) to 22.5°C, loss of dye (assessed by fluorescence at 358-nm excitation) was limited to only 2–5% (n = 4). Figure 5 shows the results so obtained; with increasing frequency, the amplitude of the calcium transient increases in parallel with force of contraction (Fig. 5A). Meanwhile, the calcium transient decays more rapidly. Compared with the changes in twitch duration, the calcium transient acceleration is proportionally larger.

Phase-plane analysis of calcium versus force (Fig. 5B) reveals this more clearly and shows that, when frequency increases, the amount of force produced during the relaxation phase increases for a given calcium level, indicating enhanced responsiveness of the myofilaments. Thus, with increasing frequency, it appears that the relaxation plane moves away from the steady-state force-calcium relationship (dashed line), indicating that the myofilaments have become rate limiting for relaxation. An enhanced responsiveness of the myofilaments can also be seen in the activation phase; when frequency increases, the slope of this phase increases, indicating that this enhancement of myofilament responsiveness persists throughout the entire twitch.

DISCUSSION

Under conditions of physiological temperature, frequency, and load, the rate-limiting step of cardiac relaxation resides in the myofilaments. Although calcium handling is clearly involved in the initiation of relaxation, in isometric contractions, the slowest, rate-limiting step in the cascade of events facilitating force decay is cross-bridge detachment. In addition, we have shown that the relationships among temperature, force of contraction, and speed of relaxation are complex and that extrapolations and interpretation of data obtained at lower temperatures, or at lower frequencies, may be undermined by these complex relationships.

**Temperature dependence of force development and twitch duration.** Temperature has distinctly different effects on force development and on the duration of the twitch. These effects are likely due to a different temperature dependence of myofilament properties on one hand and calcium handling on the other. It appears that the dynamic contractile responses measured in this study (steep decrease in force over the same temperature range) cannot simply be explained by changes in steady-state myofilament calcium sensitivity. Although steady-state cardiac myofilament calcium sensitivity is only modestly temperature dependent (14), this observation of increased calcium sensitivity (0.14 pCa units from 22 to 36°C) with increasing temperature was directionally opposite to what we observed in intact twitching muscles in this study. In skinned cardiac rabbit fibers, myofilament sensitivity increased from 22 to 29°C, whereas maximal force development slightly (~10%) increased; no further change in myofilament calcium sensitivity was observed from 29 to 36°C, whereas maximal force development went up by ~10% (13). However, at room temperature, and in the absence of sarcomere length control, force-calcium relations in intact muscle have been shown to be steeper and more sensitive to calcium compared with those in skinned myocardium (9, 10). This difference may persist at body temperature and may possibly contribute to the difference between the dynamic and the steady-state force-calcium relation. Clearly, our data underline the fact that the relaxation phase does not simply follow steady-state myofilament force-calcium relationship at room temperature. This finding is in close agreement with previous analyses of phase-plane plots of calcium versus force, in which the relaxation phase of force-bearing preparations at subphysiological temperature does not follow the steady-state force-calcium relationship (11, 16). We have now shown that, at physiological temperature, the relaxation phase may be governed by the speed of decline of the calcium transient as its rate-limiting step at low stimulation frequencies; however, such frequencies are so low that
they are not observed in vivo. Moreover, at temperatures below 37.5°C, and at physiologically relevant temperatures at 37.5°C, the relaxation phase appears to be governed by rate-limiting myofilament properties.

Temperature has a profound effect on calcium handling between 30 and 37.5°C but not between 22.5 and 30°C. At room temperature, myofilament properties have been shown to be the rate-limiting step in the relaxation of cardiac muscle (for a review, see Ref. 16). However, until now, no data were available at physiological temperature in combination with physiological frequency. Our data indicate that, as body temperature is approached, the “extra force,” i.e., the force in excess of that predicted by the steady-state force-calcium relationship, diminishes. Thus cardiac calcium handling becomes more important in the regulation of cardiac relaxation (at low stimulation frequencies) as temperature increases. It is well known that both myofilament properties and calcium handling are processes that contribute to cardiac relaxation. The fact that these processes have different temperature dependencies complicates extrapolation of data obtained at one temperature to the other. Especially at a temperature in between room and body temperature (≈30°C), the difference between force of contraction and duration of the twitch is most pronounced (no change in \( F_{\text{dev}} \), whereas duration of relaxation is more than halved). Although this temperature (30°C) is closer to body temperature than room temperature, the observed discordance between contraction and duration of relaxation may be particularly nonphysiological.

Effect of temperature on the Frank-Starling mechanism. The Frank-Starling mechanism dictates that force development increases when sarcomeres are stretched within the physiological range. Twitch \( F_{\text{dev}} \) has been shown to be a major determinant of the duration of relaxation in cardiac trabeculae where the sarcomere length was kept constant by means of laser diffraction-based feedback at room temperature (18). In the absence of tight sarcomere length control, the relationship between force of contraction and duration of relaxation also exists at room temperature under different calcium concentrations (this study). At room temperature, the prolongation of relaxation as a result of increasing sarcomere length has been postulated to reside in the myocardium itself as an intrinsic property of the myofilaments (1). These mechanisms include force-dependent enhancement of Ca\(^{2+}\) binding to troponin C (7, 15) and cooperation among cross-bridges to keep tropomyosin in its cross-bridge attachment permissive state (18). Although the current study cannot distinguish between these mechanisms, it does underline that a near-linear relationship between length-induced alterations in force and duration of relaxation exists. Thus the Frank-Starling mechanism increases contraction and duration of relaxation independent of temperature. Given the fact that calcium transients do not prolong or may even slightly shorten when a muscle is stretched (2), in combination with the large increase in both force and duration of relaxation, the rate-limiting step of relaxation at different lengths resides in the myofilaments.

Effect of temperature on the force-frequency relationship. Under physiological conditions, the force-frequency relationship in rat myocardium is positive. Although reports (5, 6, 24) have previously reported this relationship to be negative, biphasic, or flat, the conditions under which these were measured differed from the physiological situation either by a subphysiological frequency range, subphysiological temperature, or calcium concentrations exceeding those in vivo. This study confirms the findings of Layland and Kentish (23); the force-frequency relationship in rat is positive if the experimental conditions chosen are close to physiological.

A longer diffusion distance for oxygen and nutrients leads to incomplete energy supply to the core of the preparation and/or accumulation of metabolites such as inorganic phosphate (21), resulting in loss of contractile force. An upper limit for dimensions of a preparation of −0.2 mm was reported by Schouten and ter Keurs (27) for room temperature. We now add the recognition that the dimensions of the experimental preparations are more strict under physiological temperature and already seem to come into play at a thickness of −100 μm. Thus unambiguous results can only be obtained with ultrathin preparations when studied under physiological conditions.

Also, we have now, for the first time, assessed the intracellular calcium transients associated with the increased force generation at higher frequencies and at body temperature. These data allow for the central observation of our work; myofilament properties contain the rate-limiting step in the cardiac relaxation process. The increase in the speed of relaxation at increasing frequencies is caused by a frequency-induced acceleration of the calcium transient. We were not able to evoke steady tetani at 37°C even in presence of ryanodine and thapsigargin when stimulated at 20 Hz. Although the exact quantification of the results may be hampered by the absence of steady-state data at 37°C, conclusions can be drawn regardless of where this steady-state relationship resides. Phase-plane analysis clearly shows a leftward shift of the twitch relaxation phase, indicating that myofilament properties play an increasingly important role in the duration of the contraction as frequency increases toward the physiological heart rate. This observation is reminiscent of the behavior of mouse myocardium at room temperature (11), albeit over a much lower frequency range in the mouse study. Thus it appears that force-frequency behavior is determined by both calcium handling and myofilament properties and not solely by enhanced SR function.

Although we have now shown the rate-limiting step of cardiac relaxation to reside in the myofilaments under near physiological conditions, it has long been known that stimuli that accelerate calcium decline can also accelerate relaxation. Indeed, faster intracellular calcium decline would reduce the time in which a cross-bridge can be activated. Hypothetically, a cross-
bridge can be activated as soon as the threshold calcium concentration is reached. Additional cross-bridges can be recruited until the calcium level in the cell falls below the threshold. Decreasing the time that intracellular calcium levels are above threshold level can therefore decrease the time in of contraction. Thus, although the rate-limiting step does reside in the myofilaments according to our data, shortening of the activation pulse (i.e., shortening of the intracellular calcium transient) can also contribute to shortening of contraction.

Methodological considerations and limitations. Although isolated myocytes are routinely employed to characterize cardiac function and have contributed important insights to the study of cardiac function, in this study we chose isolated trabeculae to investigate the relaxation of cardiac muscle, for various reasons. The most important reason is that trabecule enable the study of loaded contractions. This is extremely important, because in unloaded contractions, cross-bridges cycle much faster and will therefore follow the decay in calcium during relaxation promptly. These loading conditions are strongly affected by sarcomere length, which ranges in the vivo beating heart from 1.95 to 2.2 μm (26), and can be achieved in trabeculae but not in isolated cells. The end-diastolic resting length of an isolated myocyte is 1.85–1.95 μm and, upon stimulation, shortens to 1.6–1.7 μm further from the physiological range. In addition, trabeculae allow for the assessment of a larger frequency range, have considerable longevity without deterioration of function, and allow iontophoretic loading of fluorescent indicators. Although most of these myocyte limitations can also be overcome using the aequorin-injected intact heart preparation (22), the isolated trabecula potentially allows for a more tight control of experimental conditions, and the method of iontophoretic fura 2 loading is not hampered by subcellular compartmentalization of the indicator.

Although we have now shown the feasibility of assessing cytosolic calcium transients from iontophoretically loaded cardiac trabeculae at physiological temperature, the short period over which these can be collected unfortunately precludes an in vivo calibration, which typically takes several hours. Thus because it is impossible to obtain an in situ $K_0$, we currently have to rely on the previously assessed $K_0$ for fura-$Ca^{2+}$ obtained at room temperature to calculate free calcium from the fluorescence ratio at 340 to 380 nm. It has been reported that the $K_0$ of fura 2 displays little temperature dependence and may shift as little as 0.04 μM (12, 29). However, even if such a small shift in the $K_0$ with temperature were to occur, this would not affect the central conclusions. A shift in $K_0$ would alter the exact calcium concentration calculated from the ratio, but the central conclusions (shift in the relaxation phase of the phase-plane plots at the same temperature at different frequencies/length) will not be directionally affected. Although comparison between temperatures (as in Fig. 1) may be hampered by a shift in $K_0$ of the indicator, the extremely large shift in the relaxation plane (roughly estimated as 0.4–0.45 pCa units) by far exceeds a possible shift in $K_0$ of the indicator (0.04 pCa units). This implies that a qualitative shift (i.e., a leftward shift of the relaxation plane) is unequivocally present.

Sarcomere length was not controlled by feedback iterations in this study. However, increased central segment shortening has been shown to slightly affect the amplitude but not the kinetics of the cytosolic calcium transient (17). Thus, although internal shortening may have induced small changes in the observed calcium transient amplitudes, our central conclusions regarding kinetics (and therefore rate-limiting steps) are not affected by the absence of sarcomere length control.

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