The Relationship between Contractile Force and Intracellular [Ca^{2+}] in Intact Rat Cardiac Trabeculae

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ABSTRACT The control of force by [Ca^{2+}] was investigated in rat cardiac trabeculae loaded with fura-2 salt. At sarcomere lengths of 2.1–2.3 μm, the steady state force-$[Ca^{2+}]_i$ relationship during tetanization in the presence of ryanodine was half maximally activated at a $[Ca^{2+}]_i$ of 0.65 ± 0.19 μM with a Hill coefficient of 5.2 ± 1.2 (mean ± SD, $n = 9$), and the maximal stress produced at saturating $[Ca^{2+}]_i$ equalled 121 ± 35 mN/mm^2 ($n = 9$). The dependence of steady state force on $[Ca^{2+}]_i$ was identical in muscles tetanized in the presence of the Ca^{2+}-ATPase inhibitor cyclopiazonic acid (CPA). The force-$[Ca^{2+}]_i$ relationship during the relaxation of twitches in the presence of CPA coincided exactly to that measured at steady state during tetani, suggesting that CPA slows the decay rate of $[Ca^{2+}]_i$ sufficiently to allow the force to come into a steady state with the $[Ca^{2+}]_i$. In contrast, the relationship of force to $[Ca^{2+}]_i$ during the relaxation phase of control twitches was shifted leftward relative to the steady state relationship, establishing that relaxation is limited by the contractile system itself, not by Ca^{2+} removal from the cytosol. Under control conditions the force-$[Ca^{2+}]_i$ relationship, quantified at the time of peak twitch force (i.e., $dF/dt = 0$), coincided fairly well with steady state measurements in some trabeculae (i.e., three of seven). However, the force-$[Ca^{2+}]_i$ relationship at peak force did not correspond to the steady state measurements after the application of 5 mM 2,3-butanedione monoxime (BDM) (to accelerate cross-bridge kinetics) or 100 μM CPA (to slow the relaxation of the $[Ca^{2+}]_i$ transient). Therefore, we conclude that the relationship of force to $[Ca^{2+}]_i$ during physiological twitch contractions cannot be used to predict the steady state relationship.

INTRODUCTION

Accurate determination of the dependence of force on the $[Ca^{2+}]_i$ in cardiac muscle is essential for understanding the final stage of excitation-contraction coupling. The relationship between force and $[Ca^{2+}]_i$ has been studied and characterized in great

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detail in cardiac muscle in which the sarcolemma is hyperpermeabilized to allow direct activation of the myofilament lattice with solutions of known $[Ca^{2+}]$ (Fabiato and Fabiato, 1975; Bers, 1991; Moss, 1992). The steady state force-$[Ca^{2+}]_i$ relationship measured in such "skinned" preparations differed significantly from that recorded in other studies using intact ferret (Yue, Marban, and Wier, 1986; Okazaki, Suda, Hongo, Konishi, and Kurihara, 1990) and human (Gwathmey and Hajjar, 1990) cardiac muscles microinjected with aequorin. Changes in the Ca^{2+} sensitivity and cooperativity of the contractile filaments have recently been confirmed by direct comparison of the same preparations before and after skinning (Gao, Backx, Azan-Backx, and Marban, 1994).

While the steady state force-$[Ca^{2+}]_i$ relationship reveals considerable information on the role of Ca^{2+} in controlling force production, the dependence of force on $[Ca^{2+}]_i$ during a twitch, when the $[Ca^{2+}]_i$ transient is very brief, is further complicated by the dynamics of Ca^{2+} binding to the contractile system and the subsequent delay in force development (see Blinks [1993] for review). Previous results using aequorin in ferret papillary muscles suggested that the $[Ca^{2+}]_i$ returns to diastolic levels by the time of peak force (Yue, 1987). However, $[Ca^{2+}]_i$ transients derived from accurately calibrated aequorin light signals have demonstrated an elevation of the $[Ca^{2+}]_i$ throughout the twitch in both cardiac (Allen and Kurihara, 1980) and skeletal muscle (Ashley, Mulligan, and Lea, 1991) similar to that recorded using fura-2 (Backx and ter Keurs, 1993). The discrepancies observed using aequorin probably reflect the nonlinear dependence of the light output on $[Ca^{2+}]_i$ (Blinks, Wier, Hess, and Prendergast, 1982; Blinks, 1993) and the low finite bioluminescence when $[Ca^{2+}]_i < 180$ nM (Allen and Kurihara, 1982).

In single cells loaded with indo-1, it has been argued that, during the relaxation phase of the twitch, cell shortening comes into steady state with the declining $[Ca^{2+}]_i$ (Spurgeon, duBell, Stern, and Lakatta, 1992). Although cell shortening does not necessarily mirror contractile force, these observations raise the possibility that force and $[Ca^{2+}]_i$ could also be in dynamic equilibrium at some point during the cardiac cycle. Indeed, Blinks (1993) has argued force and $[Ca^{2+}]_i$ recorded at the time of peak force (F) during a twitch, when dF/dt is zero, should correspond closely to that measured in steady state. If this were the case, then quantitative evaluation of the control of force by Ca^{2+} and the modification of this control by physiological and pharmacological interventions could be studied easily without the need for tetanization.

In light of the importance of the control of force by Ca^{2+}, we have investigated the force-$[Ca^{2+}]_i$ relationship in thin rat cardiac trabeculae during twitches and tetani using microinjected fura-2 to measure $[Ca^{2+}]_i$ (Backx and ter Keurs, 1993). In particular, we have addressed the following questions: (*a*) How does the force-$[Ca^{2+}]_i$ relationship measured in rat cardiac trabeculae with fura-2 compare with the results obtained in papillary muscles microinjected with aequorin? (*b*) How does the force-$[Ca^{2+}]_i$ relationship measured during the time course of a twitch relate to the steady state relationship? In achieving these objectives, fura-2 fluorescence signals were calibrated in the intact muscle in order to minimize possible artifacts associated with changes in the fura-2 fluorescence and Ca^{2+} binding properties when fura-2 is in the cytosol (Baylor and Hollingworth, 1988).

A preliminary report of this work has appeared (Backx, Azan-Backx, and Marban, 1993).

MATERIALS AND METHODS

Muscle Preparation, Dissection Procedure, and Solutions

Hearts were excised from 2–6-mo old rats of either sex (LBN-F1 strain, Harlan, Indianapolis, Indiana), after gaseous diethylether anesthesia, by making a midsternal incision and rapidly severing the vascular attachments. The heart was routinely removed in <10 s using this procedure. The aorta of the freshly excised heart was cannulated and the heart was arrested by retrogradely perfusing with a high-K^+ modified Krebs-Henseleit solution (see below). Long, thin trabeculae ($2.22 \pm .75$ mm in length, 194 ± 45 μm in width and 52 ± 12 μm in thickness [mean ± SD, $n = 16$]), were dissected from the right ventricle. The trabeculae were mounted between a force transducer (AE801, AME, Sensonor, Horten, Norway) and a micromanipulator in a perfusion bath ($40 \times 2 \times 4$ mm^3) located on the stage of an inverted microscope (TMD, Nikon, Tokyo, Japan). The trabeculae were stimulated using an electrical pulse stimulator and platinum electrodes running down either side of the bath.

Sarcomere length was measured using laser diffraction by illuminating the muscle with a 5-mW He-Ne diode laser (Edmund Scientific, Barrington, NJ). The diffraction pattern was transmitted through the objective to the front optical port of the microscope and projected onto a video camera using a telephoto lens focused onto the back plane of the objective. The first order diffraction pattern was calibrated using a diffraction grating and was used to set the end-diastolic sarcomere length to 2.1–2.3 μm.

The dissection and perfusion solutions consisted of a modified Krebs-Henseleit solution which included (in millimolar): 120 NaCl, 5 KCl, 1.2 $MgCl_2$, 1.2 NaH_2PO_4, 19 $NaHCO_3$, 1 $CaCl_2$ and 10 glucose. The dissection solution contained an additional 20 mM KCl. All solutions were equilibrated with 95% O_2/5% CO_2 to obtain a pH of 7.4. Ryanodine (Calbiochem Corp., LaJolla, CA) was added as required at a concentration of 1 μM from a 1 mM stock in H_2O, cyclopiazonic acid (CPA) (Sigma Chemical Co., St Louis, MO) was added from a 10-mM stock in dimethylsulfoxide (DMSO) to a final concentration of 100 μM, isoproterenol (Sigma Chemical Co.) was added from a 10-mM stock dissolved in H_2O and 2,3-butanedione monoxime, BDM (Sigma Chemical Co.) was added from powder. All the experiments were performed at room temperature (20–22°C).

The fluorescent properties of fura-2 in vivo were determined as outlined previously (Li, Altschuld, and Stokes, 1987; Backx and ter Keurs, 1993). In brief, preparations were first metabolically inhibited with a solution containing (in millimolar): 120 NaCl, 4 KCl, 0.5 $CaCl_2$, 1.2 $MgCl_2$, 10 HEPES (pH 7.4, NaOH), 4 NaCN and 2 iodoacetate. After rigor development, solutions containing (in millimolar): 120 KCl, 5.8 NaCl, 1.2 $MgCl_2$, 10 HEPES (pH 7.4, KOH), 10 EGTA/CaEGTA, 0.05 ionomycin, 4 NaCN, and 2 iodoacetate were applied for up to 60 min in order to clamp the [Ca^{2+}]$_i$ to fixed levels, as described below.

The skinned fiber solutions contained (in millimolar): 100 KCl, 25 HEPES, 5 MgATP, 15 disodium creatine phosphate (Na_2HCP), 1.2 $MgCl_2$, and either 10 EGTA or 10 CaEGTA. The pH was adjusted with KOH to 7.10. The preparations were skinned by the addition of 1% Triton X-100 to the relaxing solution (see below).

Fluorescence Measurements

Excitation ultra-violet (U-V) light from a 75-W mercury lamp (Oriel Corp, Stratford, CT) was passed through bandpass filters (Omega Optical, Brattleboro, VT) centered at 340, 357, or 380 nm (band width 10 nm) located in a computer-controlled filter wheel. The filtered light was

projected onto the muscle via a 10X objective (10X Fluor, Nikon, Tokyo, Japan) in the inverted microscope using a dichroic mirror (400DPLC, Nikon, Tokyo, Japan). The use of a large field of illumination minimizes Ca^{2+}-independent changes in the fluorescent signal associated with movement of the preparation during a twitch and reduces the amount of excitation light required to measure Ca^{2+}-dependent changes in fluorescence with a high signal-to-noise ratio.

The emitted fluorescent light was collected by the objective and transmitted through a bandpass filter at 510 nm (10 nm band width) to a photomultiplier (R2368, Hamamatsu, Bridgeport, NJ) which was attached to the side camera port of the microscope. The photomultiplier output was filtered at 100 Hz (3 dB), recorded using an A/D data acquisition board (2801A, Data Translation, Marlboro, MA) and stored in the computer for later analysis. The preparations were only illuminated with the excitation light for short periods of time with the use of a filter wheel. Brief illumination of the preparation reduces the effects of photo bleaching which could otherwise interfere with the accurate calibration of the fluorescence signals. With this method, fura-2 fluorescence could be routinely and reliably recorded for periods > 5 h.

Fura-2 Loading Method

Fura-2 was loaded into trabeculae with a micropipette containing 1 mM fura-2 salt by a single impalement. The micropipette holder was back filled with a solution containing 140 mM KCl. The pipette resistance was 150–250 MΩ (<0.2-μm tip diam) when placed in the modified Krebs-Henseleit solution. The use of tip diameters of this size was imperative because larger tips resulted in significant damage to thin trabeculae as evidenced first by local spontaneous activity and later by the development of a contracture in the region of the impalement. After impalement of an unstimulated muscle, the measured membrane potential was between −65 and −95 mV. After achieving a stable impalement, 2–10 nA of hyperpolarizing current was passed for ~15–35 min. During the injection period, the fura-2 diffuses from the impalement site into the adjacent cells via gap junctions, as described previously (Backx and ter Keurs, 1993). After the injection period, the preparations were stimulated at 1 Hz for 0.5–1 h to enhance the rate of spread of fura-2 from the site of injection thereby producing a uniform distribution of the dye within the muscle.

Calibration Procedure for Fura-2

Dual excitation of fura-2 allows the determination of $[Ca^{2+}]_i$ using the ratiometric technique independent of the amount of dye present in the preparation. Because fura-2 slowly leaks out of trabeculae, albeit at a slow rate, the ratio method allows accurate estimates of the $[Ca^{2+}]_i$ to be made throughout the time course of our experiments. In all the preparations studied, the autofluorescence was recorded before loading with fura-2, and again at the end of the experiment by the application of 20–50 μM ionomycin and 0.5 mM Mn^{2+} in a nominally Ca^{2+}-free Krebs-Henseleit solution. The autofluorescence estimates at the beginning and end of the experiment differed by <15% in all the preparations used in this study.

The measured ratio was used to estimate $[Ca^{2+}]_i$ by the equation (Grynkiewicz, Poenie, and Tsien, 1985):

$$[Ca^{2+} = K'_D * (R - R_{min})/(R_{max} - R) \quad (1)$$

where K'_D is the apparent dissociation constant, R is the ratio of the fluorescence at 340 nm excitation to that at 380 nm excitation, R_{min} is R evaluated at zero $[Ca^{2+}]_i$ and R_{max} is the value of R obtained at a saturating $[Ca^{2+}]_i$. The accurate determination of the constants in Eq. 1 is essential to determine $[Ca^{2+}]_i$. Because the optical and binding properties of fura-2 depend on the constituents in the aqueous environment (Baylor and Hollingworth, 1988), we estimated

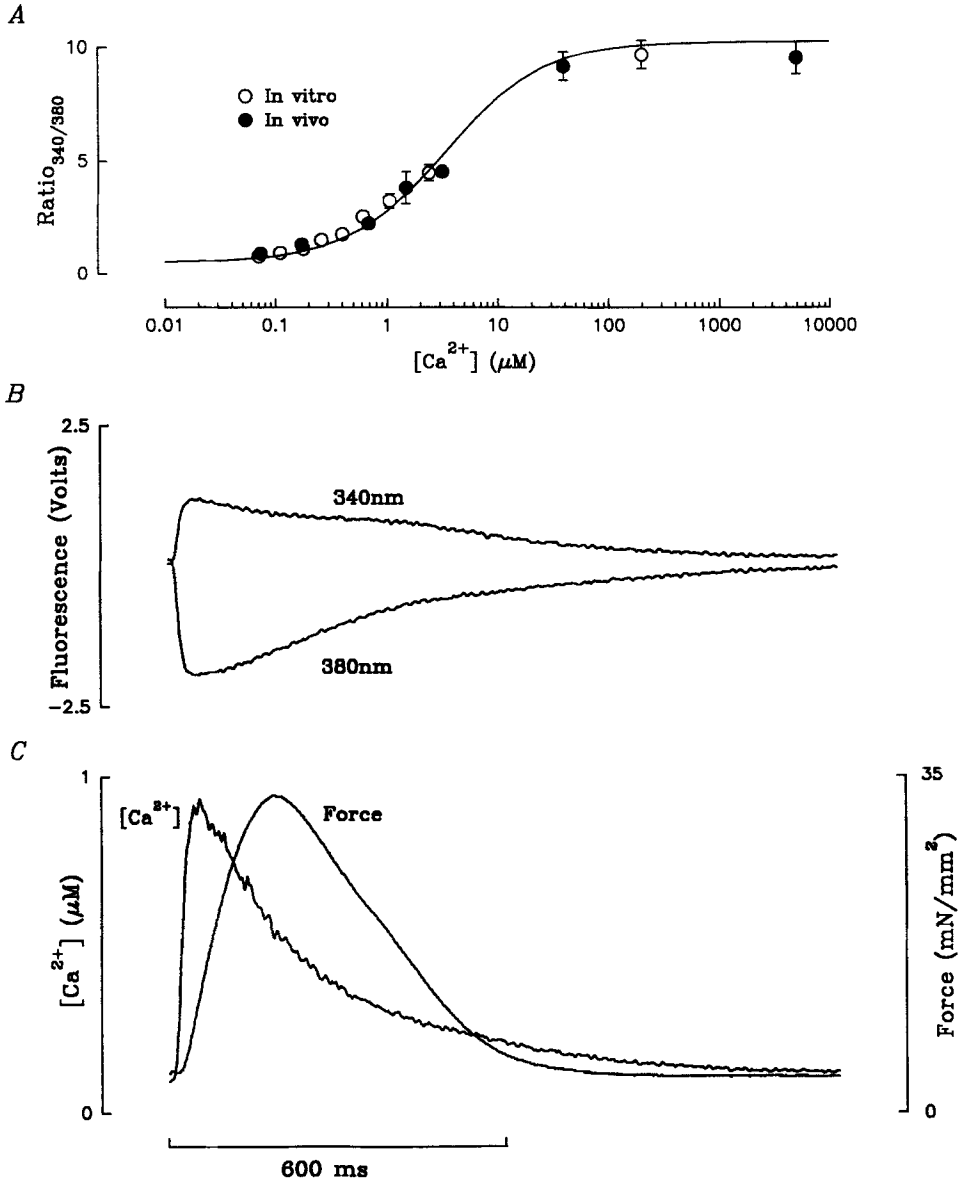

FIGURE 1. In vivo and in vitro properties of fura-2 fluorescence. (*A*) The in vivo dependence of the ratio (340/380) of fluorescence on the applied $[Ca^{2+}]$ in trabeculae metabolically inhibited using 2 mM CN^- and 0.5 mM iodoacetate is represented as filled circles. Open circles show results from calibrating solutions. Details of the method for clamping the intracellular $[Ca^{2+}]$ at known levels is outlined in the text. The results were fit using a least-squares algorithm to the fura-2 binding equation (Eq. 1) assuming a single Ca^{2+}-binding site (*solid line*). The best-fit parameters were: K'_D = 3.3 μM, R_{max} = 10.21 ± 2.33 and R_{min} = 0.47 ± 0.12 (n = 8). Also shown are the fluorescence ratios for varied $[Ca^{2+}]$ in vitro. There was no difference between the in vivo and in vitro dependence of the fluorescence on the $[Ca^{2+}]$. (*B*) After microinjection of fura-2 into a muscle, the fluorescence at 380 nm and 340 nm excitation was about three times that recorded before loading. During a twitch, the 380 and 340 nm fluorescence decrease and increase in a manner expected for the increase in $[Ca^{2+}]_i$. (*C*) The estimated Ca^{2+} transient derived from the fluorescence recordings shown in *B* are shown along with the twitch force. Preparation 271092.

these constants by performing an in vivo calibration in eight trabeculae (Li, Altschuld, and Stokes, 1987). This method involved metabolic inhibition of the muscles followed by the application of a depolarizing solution containing 50–100 μM ionomycin and 10 mM EGTA in order to buffer the $[Ca^{2+}]_i$ to various known levels. The results of such in vivo calibrations are shown in Fig. 1 *A*. The apparent dissociation constant, K'_D, was 3.15 μM while the measured values of R_{max} and R_{min} were 10.2 ± 2.33 and 0.47 ± .12 (n = 8).

Fig. 1 *B* shows typical fluorescence recordings during a twitch at 380 and 340 nm excitation wavelengths after fura-2 injection. In this preparation the fluorescence level at 340 nm excitation increased ~3.5-fold, relative to the autofluorescence, after loading which corresponds to a $[fura\text{-}2]_i$ of ~50–70 μM (Backx and ter Keurs, 1993). The estimated Ca^{2+} transient and the force recorded during this twitch are shown in Fig. 1 *C*. The $[Ca^{2+}]_i$ was estimated using Eq. 1 with the values of K'_D, R_{max}, and R_{min} obtained from our in vivo calibrations described above. For this particular muscle, the signal recorded from the fluorescence was produced by ~1,800 cells (assuming an average cell volume of 5 fl per cell, while the preparation's illuminated dimensions were 40 × 160 × 1,200 μm).

Skinned Muscle Measurements

In some experiments, we measured and compared the maximal force produced by these muscles before and after skinning. Muscles were skinned by superfusing with a "relaxing solution" containing 1% Triton X-100 for 20–30 min (Kentish, 1984). Subsequent to the skinning period, the preparations were maximally activated with skinned fiber solutions containing 10 mM total $[Ca^{2+}]$ and 10 mM EGTA (i.e., free $[Ca^{2+}]$ = 22 μM) for periods lasting 30–120 s (Gao et al., 1994). Between the periods of activation the muscles were maintained in a relaxed state by superfusion with skinned fiber solutions containing 10 mM EGTA without Ca^{2+}.

Steady State Force-$[Ca^{2+}]_i$ Relationships during Tetani Using Ryanodine or CPA

The dependence of force on the $[Ca^{2+}]_i$ of the contractile system in intact trabeculae was examined by stimulating the muscle, in the presence of 1 μM ryanodine or 100 μM CPA, at a rate of 8–10 Hz in order to achieve pseudo steady state levels of activation. Force-$[Ca^{2+}]_i$ relationships obtained in this manner were fit with the Hill equation:

$$F = F_{max}[Ca^{2+}]^N/(K^N_{1/2} + [Ca^{2+}]^N) \quad (2)$$

where F_{max} is the maximal force, $K_{1/2}$ is the dissociation constant and N is the Hill coefficient which is a measure of the amount of cooperativity (Moss, 1992).

Statistics

Pooled data are presented as mean ± SD. Statistical significance was determined using a paired t test (Glantz, 1992). A confidence limit of 95% was used to determine significance.

RESULTS

Cooperativity and Ca^{2+} Sensitivity in Intact Trabeculae

Fig. 2 *A* shows typical force and $[Ca^{2+}]_i$ records during ryanodine tetani in a trabecula at a sarcomere length of 2.25 μm for four different $[Ca^{2+}]_o$. The corresponding steady state force-$[Ca^{2+}]_i$ relationship for the same muscle is plotted in Fig. 2 *B* where the force and $[Ca^{2+}]_i$ were measured 3 s after the start of the tetanus. Note that the force saturates at intracellular Ca^{2+} concentrations above 0.8 μM, which is strikingly

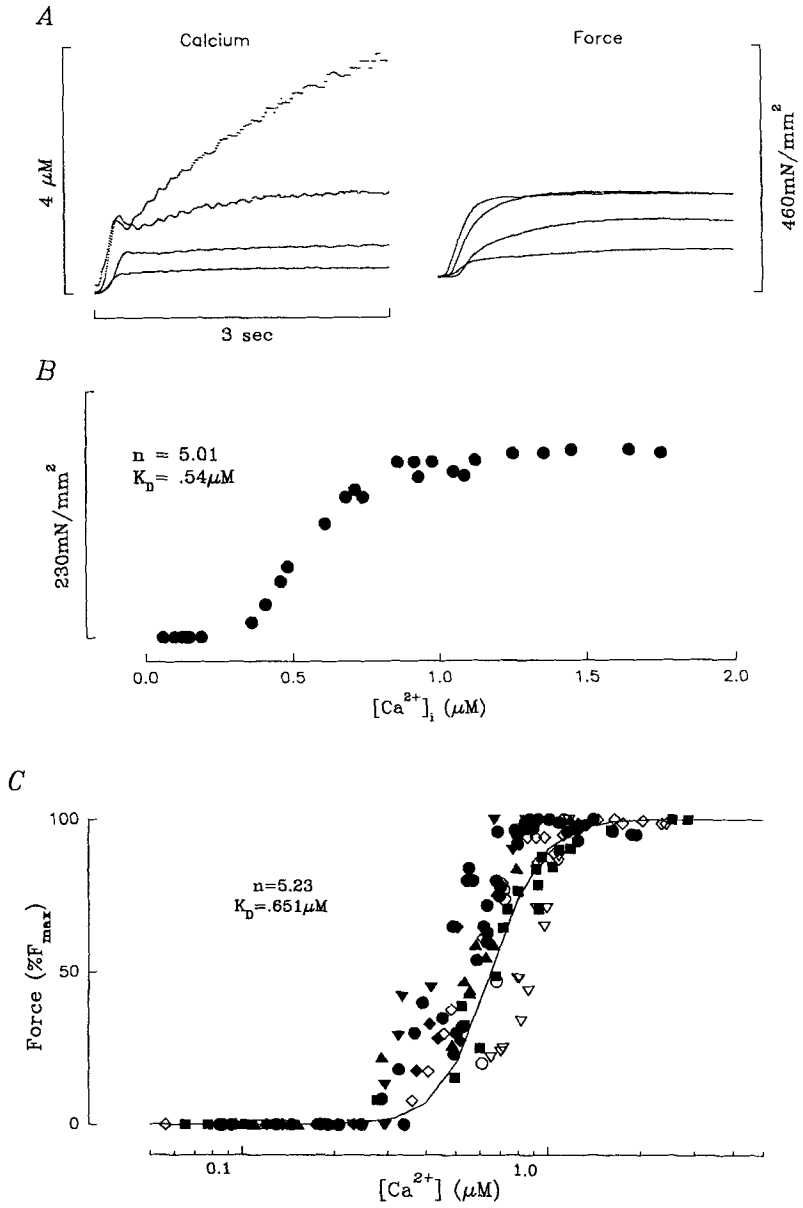

FIGURE 2. Calcium and force recorded during tetanization in the presence of ryanodine. (*A*) Typical $[Ca^{2+}]_i$ (*left graph*) and force (*right graph*) records during ryanodine tetani at four different $[Ca^{2+}]_o$. Increasing the $[Ca^{2+}]_o$ resulted in concurrent increases in the tetanic level of $[Ca^{2+}]$ and force. (*B*) Plot of $[Ca^{2+}]_i$ and force measured 2 s after the beginning of the tetanus. The parameters for the Hill fit for this muscle (Eq. 2) were: $K_{1/2}$ = 0.54 μM and N = 5.01. Preparation 030792. (*C*) The dependence of force on $[Ca^{2+}]_i$ during ryanodine tetani for nine preparations. The results for each preparation were fit individually to Eq. 2. The average values for the parameters were: $K_{1/2}$ = 0.65 ± 0.15 μM and N = 5.2 ± 1.1 (n = 9). The average maximal stress for these nine preparations was 121 ± 35 mN/mm². Preparations: 050692, 290692, 030792, 070792, 200792, 041192, 031192, 181192, 210292.

similar to the behavior observed previously (Yue et al., 1986; Okazaki et al., 1990; Gwathmey and Hajjar, 1990) in papillary muscles using aequorin to measure the $[Ca^{2+}]_i$.

Pooled results for the steady state force-$[Ca^{2+}]_i$ relationship obtained from nine preparations are shown in Fig. 2 *C*. Separately fitting the observed force-$[Ca^{2+}]_i$ results to the Hill equation (Eq. 2) yielded a $K_{1/2}$ of 0.65 ± 0.19 and a Hill coefficient (N) of 5.23 ± 1.18 (n = 9). These mean values for $K_{1/2}$ and N in our rat trabeculae using fura-2 correspond closely to previous results obtained in intact muscle using aequorin but differ markedly from most of the results observed in skinned cardiac muscle (see Bers, 1991, for references). The maximal stress developed for the preparations shown in Fig. 2 *C* was 121 ± 35 mN/mm^2 (n = 9) at saturating $[Ca^{2+}]_i$.

Maximal Ca^{2+}-activated Force before and after Skinning

In spite of observing clear saturation of the developed force as the $[Ca^{2+}]_i$ was raised sufficiently, we examined a number of preparations before and after chemical skinning to verify that maximal force had indeed been achieved (Godt and Maughan, 1981). Fig. 3 shows typical force tracings recorded in an intact tetanized trabecula (*A*) and in the same skinned muscle at maximally activating levels of Ca^{2+} (*B*). As has been observed previously, saturating forces recorded during tetanizations at high levels of activation with ryanodine tetani were not different from those recorded after skinning (Gao et al., 1994; Kentish, ter Keurs, Ricciardi, Bucx, and Noble, 1985). The average maximal stress in the intact muscle of 113 ± 33 mN/mm^2 was statistically identical to that in the same muscles after skinning (112 ± 33 mN/mm^2, n = 5). The maximal stress levels are similar to those observed previously in skinned rat cardiac trabeculae (Kentish et al., 1985; Gao et al., 1994).

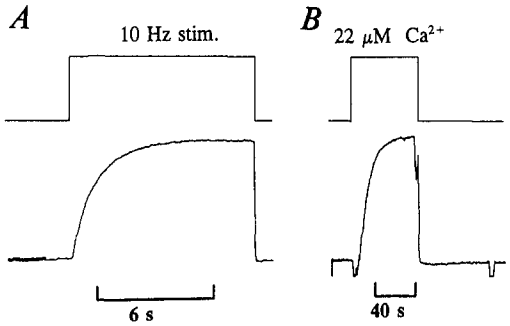

FIGURE 3. Comparison of the maximal force generated in a trabecula before (*A*) and after (*B*) skinning. (*A*) Typical force records at maximal activation in the intact trabecula superfused with 10 mM external $[Ca^{2+}]$ during tetanization in the presence of 1 μM ryanodine. This same preparation was skinned and maximally activated (*B*). The maximal force was not different between the intact and skinned trabeculae (~120 mN/mm^2). Preparation 031193.

Comparison of Ryanodine and Cyclopiazonic Acid Effects

The ability to tetanize cardiac muscle after the application of 1 μM ryanodine is a consequence of the alteration of the functional properties of the SR Ca^{2+} release channel which results in a marked prolongation of the rate of decay of $[Ca^{2+}]_i$ and thus force (Rousseau, Smith, Henderson, and Meissner, 1987). Fig. 4 shows an

example of the profound alterations in the kinetics of the twitch force and $[Ca^{2+}]_i$ transient induced by ryanodine. In the control twitch shown in Fig. 4 *A*, the $[Ca^{2+}]_o$ was 0.5 mM while in the presence of ryanodine (Fig. 4 *B*), the $[Ca^{2+}]_o$ was elevated to 4 mM. After the application of ryanodine, the peak of the twitch force and the $[Ca^{2+}]_i$ transient are very similar in spite of the fact that the external $[Ca^{2+}]$ had been increased eightfold in the presence of ryanodine. On the other hand, the relaxation phase of the $[Ca^{2+}]_i$ transient with ryanodine is markedly biphasic when compared to control. The changes are particularly prominent given the difference in time scales between Figs. 4, *A* and *B*. The early portions of the relaxation period for the control and ryanodine twitches are very similar, while the late phase of relaxation is distinctly slowed in ryanodine. This biphasic behavior of the $[Ca^{2+}]_i$ transient is expected from the known mechanism of action of ryanodine. That is, ryanodine reduces the average unitary conductance and increases the open probability of the sarcoplasmic reticulum (SR) Ca^{2+}-release channel while not affecting the SR Ca^{2+}-ATPase. Presumably, Ca^{2+}

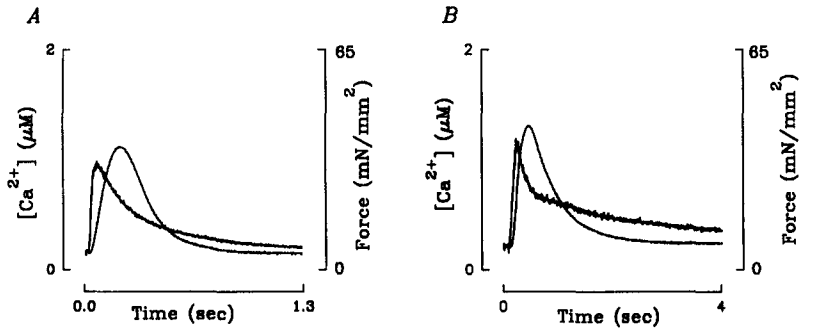

FIGURE 4. Twitch force and $[Ca^{2+}]_i$ transients measured before and after the addition of 1 μM ryanodine. (*A*) Twitch force and $[Ca^{2+}]_i$ transient in control at an external $[Ca^{2+}]$ of 0.5 mM. (*B*) Records from the same trabecula after the application of 1 μM ryanodine. Note the different time scales. Preparation 290692.

taken up by the SR during early relaxation leaks through the SR Ca^{2+} release channels thereby slowing the rate of relaxation of the $[Ca^{2+}]_i$ transient.

With continued uptake of the Ca^{2+} by the SR during ryanodine tetani, significant $[Ca^{2+}]_i$ gradients might occur within each sarcomere which would impair the measurement of the true steady state force-$[Ca^{2+}]_i$ relationship (Wier and Yue, 1985; Blinks, 1993). In light of the potential complications associated with the use of ryanodine, we reevaluated the force-$[Ca^{2+}]_i$ relationships in our rat trabeculae using cyclopiazonic acid (CPA). CPA, like thapsigargin, is a potent inhibitor of the Ca^{2+}-ATPase and therefore blocks Ca^{2+} uptake by the SR; this uptake is expected to contribute significantly to the $[Ca^{2+}]_i$ gradients within the cell (Blinks, 1993). Like ryanodine, the application of 100 μM CPA results in a marked slowing of the rate of relaxation of the twitch force and allows tetanization of cardiac muscle. Fig. 5 shows typical $[Ca^{2+}]_i$ and force recordings (*A*) in response to 8 Hz stimulation in the presence of 1.0 mM $[Ca^{2+}]_o$, a concentration sufficient activate this trabecula to ~25% of maximal force. The force-$[Ca^{2+}]_i$ relationships from five preparations

tetanized with CPA are shown in Fig. 5 *B*. Fits of the force-$[Ca^{2+}]_i$ relationships yielded values for $K_{1/2}$ and N of 0.64 ± 0.11 μM and 4.59 ± 1.26 (n = 6), statistically identical to estimates obtained in the presence of ryanodine. Generally, fused tetani were more easily achieved in the presence of CPA compared to ryanodine and the external $[Ca^{2+}]$ required to obtain the maximal force (F_{max}) was reduced to 6–8 mM versus 15–20 mM with ryanodine. In addition, the effect of CPA on the twitch kinetics

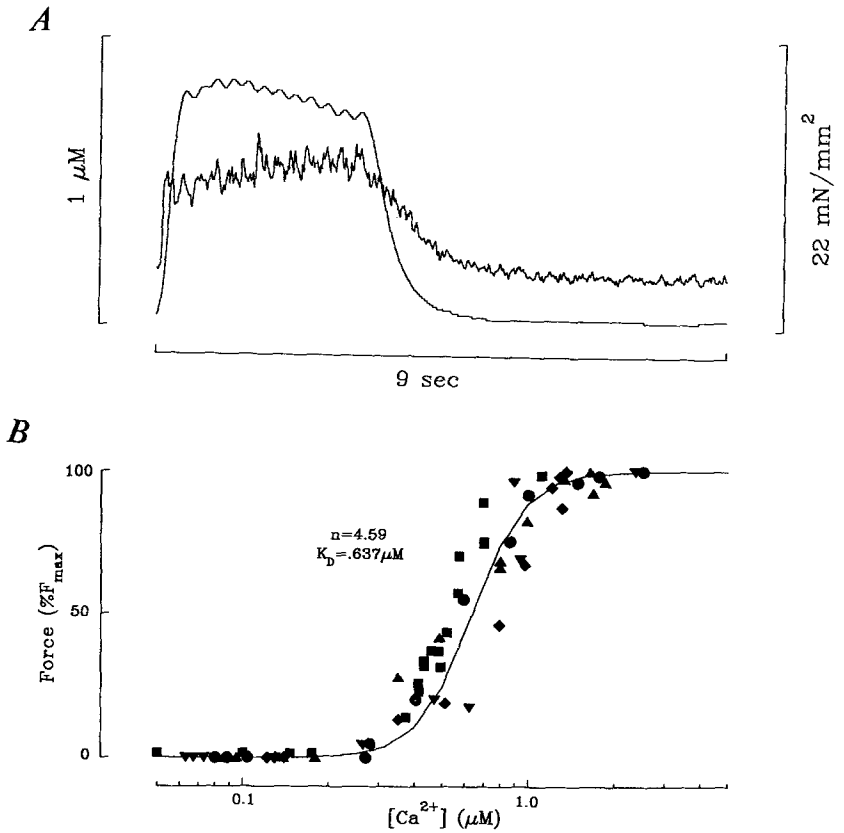

FIGURE 5. The dependence of force on $[Ca^{2+}]_i$ during CPA tetani. (*A*) $[Ca^{2+}]_i$ and force records in response to 8 Hz stimulation in the presence of 100 μM CPA at an external $[Ca^{2+}]$ of 8.5 mM. (*B*) Pooled steady state force-$[Ca^{2+}]_i$ relationships from five muscles exposed to CPA. As in Fig. 4, force and $[Ca^{2+}]_i$ were recorded 2 s after the onset of tetanic stimulation. Preparations: 250193, 230193, 210193, 091092, 230293.

and the ability to generate tetani requires ~10 min and is reversible, versus 30–60 min with ryanodine which is irreversible. It is reassuring that the force-$[Ca^{2+}]_i$ relationship in intact heart muscle is nearly identical whether measured using fura-2 in the presence of ryanodine or CPA in rat myocardium, or measured using aequorin in ferret and human heart muscle.

Similar to ryanodine twitches, Fig. 6 shows a marked slowing of the kinetics of twitch force and the $[Ca^{2+}]_i$ transient by CPA (compare *A* with *B*). The external $[Ca^{2+}]$ was 0.5 mM for the control twitch (*A*) and 2.0 mM for the CPA twitch (*B*). Comparison of Figs. 6 *B* and 4 *B* clearly reveals that the biphasic character of the relaxation of the force and the $[Ca^{2+}]_i$ transient seen in the presence of ryanodine (Fig. 4 *B*) was less pronounced in the presence of CPA (Fig. 6 *B*) where the relaxation is more monoexponential.

Force-$[Ca^{2+}]_i$ Relationship during Twitch Contractions

Fig. 6 *C* shows phase plots of the force-$[Ca^{2+}]_i$ relationship during three typical twitches at varied external $[Ca^{2+}]$ in the presence of 100 μM CPA, superimposed upon the steady state relationship measured in the same muscle. During the

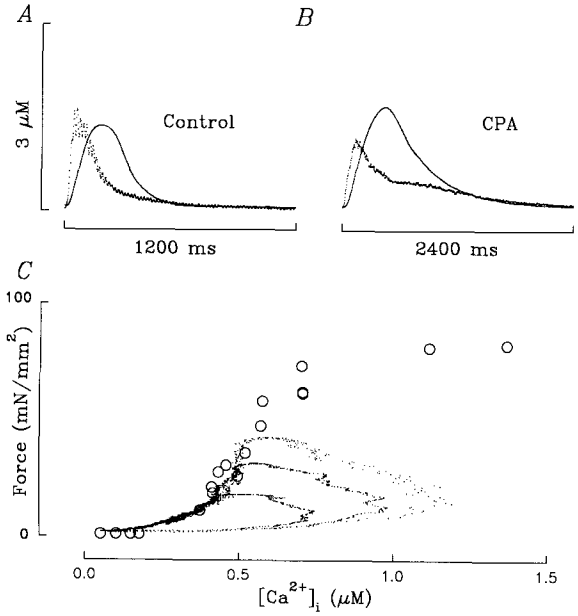

FIGURE 6. The effects of CPA on twitch force and $[Ca^{2+}]_i$ transients. (*A*) Force and $[Ca^{2+}]_i$ transient in control at an external $[Ca^{2+}]$ of 1 mM and (*B*) after the application of 100 μM CPA with an external $[Ca^{2+}]$ of 4 mM. Note the different time scales. (*C*) Phase plots for twitches at three different external $[Ca^{2+}]$. Open circles indicate steady state measurements during CPA tetani in the same preparation. Preparation 210193.

relaxation period there is total overlap of the force-$[Ca^{2+}]_i$ despite twitches with marked differences in the level of activation. Similar results were obtained in three other preparations. Thus, regardless of activation level, the force-$[Ca^{2+}]_i$ relationship is unique during most of the relaxation period. Even more striking is the additional observation that this unique relationship coincides identically with the steady state relationship measured during CPA tetani (*open circles*). The application and utility of this method for examining shifts in the sensitivity of the steady state force-$[Ca^{2+}]_i$ relationship after the application of isoproterenol was recently demonstrated (Dobrunz, Backx, and Yue, 1993).

Previously, many attempts have been made to relate the steady state force-$[Ca^{2+}]_i$ relationship to the twitch force in response to a $[Ca^{2+}]_i$ transient (Yue et al., 1986; Yue, 1987; Blinks, 1993). Until now, no straightforward interpretation has been

proposed due largely to limited information on the kinetics of Ca^{2+} binding to troponin and the dynamic response of the contractile system to rapid changes in $[Ca^{2+}]_i$. This lack of understanding of the dynamics of force generation in response to changing $[Ca^{2+}]_i$ is not surprising given the complex cooperative nature of Ca^{2+} binding and force generation (Hill, 1985). In fact, a satisfactory theory which incorporates the known complexities of the contractile apparatus is lacking even in the steady state (but see Hill, 1985). To understand more fully the dynamic relationship of force to $[Ca^{2+}]_i$, it would be of interest to determine whether the force and $[Ca^{2+}]_i$ during a twitch coincide at any time to those measured during tetani. To address this, Fig. 7 *A* shows a typical force-$[Ca^{2+}]_i$ relationship (*closed circles*) measured in a preparation tetanized in the presence of ryanodine, as well as the relationship between peak force and peak $[Ca^{2+}]_i$ (*open circles*) for twitches in the same prepara-

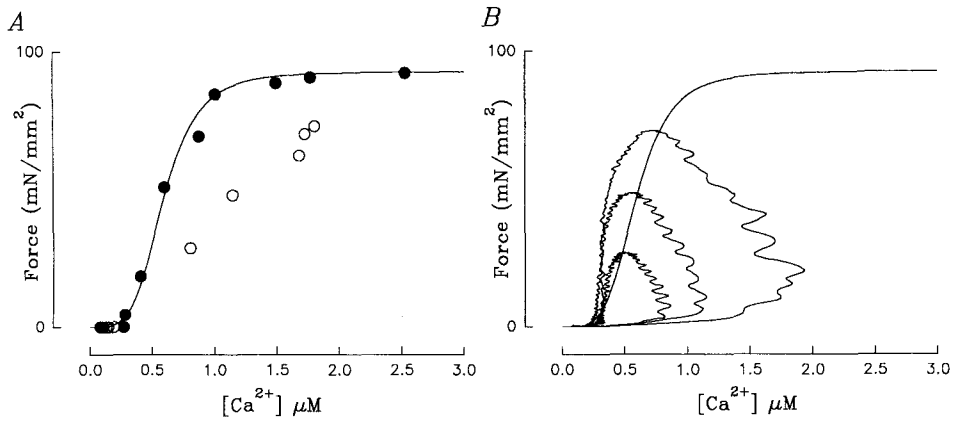

FIGURE 7. Comparison of the force-$[Ca^{2+}]$ relationship during tetani to twitches in control. (*A*) The force-$[Ca^{2+}]_i$ relationship (*closed circles*) measured during ryanodine tetani along with the best fit curve to Eq. 2. Also shown in this panel is the relationship between the peak force and the peak $[Ca^{2+}]_i$ transient (*open circles*), which lies far to the right of the steady state results. Different peak force and peak $[Ca^{2+}]_i$ values were achieved by varying external $[Ca^{2+}]$. (*B*) Phase plots during twitches at three different levels of activation superimposed upon the best fit to the steady state data from *A*. Preparation 210292.

tion in the absence of drug. As expected from previous results in ferret heart muscle (Yue et al., 1986), the peak force vs peak $[Ca^{2+}]_i$ relationship is shifted far to the right of the steady state values. This lack of correspondence is not surprising because $[Ca^{2+}]_i$ and force are changing rapidly during the twitch and therefore the peak force vs peak $[Ca^{2+}]_i$ relationship is unlikely to bear any simple correspondence to the steady state relationship.

Nevertheless, it might be possible that at certain times during the twitch the instantaneous relation of $[Ca^{2+}]_i$ to force coincides with that recorded during tetani. Fig. 7 *B* demonstrates using phase plots that, unlike the situation with CPA (or ryanodine, results not shown), there is little correspondence between the force-$[Ca^{2+}]_i$ relationship in the control twitches and that measured during tetani. Indeed, Fig. 7 *B*

shows that the force-$[Ca^{2+}]_i$ relationship during the relaxation phase of the twitch is shifted leftward relative to the steady state relationship which confirms that relaxation in cardiac muscle is not limited by the decay rate of the $[Ca^{2+}]_i$ transient. Thus, force relaxation lags behind the relaxation of the $[Ca^{2+}]_i$ transient, consistent with the notion that mechanical relaxation is limited by the contractile filaments themselves.

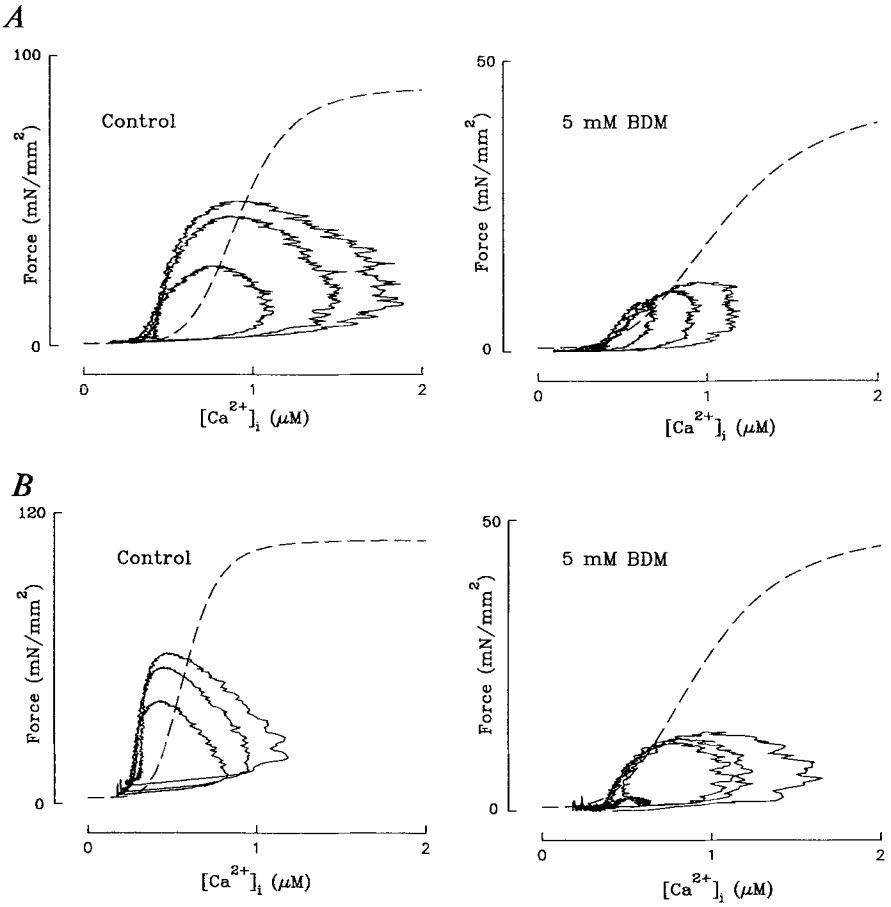

FIGURE 8. The effect of BDM on the force-$[Ca^{2+}]_i$ relationship during twitches compared to steady state. (*A*) Phase plots of the force-$[Ca^{2+}]_i$ relationship recorded during the time course of a twitch at different $[Ca^{2+}]_o$ (*solid lines*) and the estimated steady state curves recorded using ryanodine tetani (*broken line*) in control (*left*) and with 5 mM BDM (*right*). (*B*) Results of the same experimental protocol as in *A* but in a different trabecula. Preparations 211093 and 230293.

Fig. 7 *B* further demonstrates that, during the rising phase of $[Ca^{2+}]_i$, force again lags behind the increases in $[Ca^{2+}]_i$. These results are consistent with various cross-bridge models of heart having kinetic responses which are slower than $[Ca^{2+}]_i$ changes during a twitch. In contrast, the relaxation of cell shortening in isolated myocytes is limited by the decline of the $[Ca^{2+}]_i$ transient (Spurgeon et al., 1992) which highlights

that cell shortening and force generation are not equivalent measures of contractile function.

In a recent comprehensive review article, Blinks (1993) suggested that the relationship between force and $[Ca^{2+}]_i$ at the time of peak force might correspond closely to the steady state relationship. Fig. 7 *B* lends some support to this expectation. In this trabecula, the force-$[Ca^{2+}]_i$ relationship during a twitch, measured at the peak of force (i.e., when the time derivative of the force is zero), coincides well with the relationship estimated from tetani. However, the degree of such correspondence varies markedly among preparations (see Fig. 8).

To examine more fully the potential utility of analyzing the force-$[Ca^{2+}]_i$ relationship at the time of peak force (i.e., the Blinks hypothesis), we examined the effects of the application of 5 mM BDM; this agent significantly inhibits myosin ATPase at this concentration (Herrmann, Wray, Travers, and Barman, 1992; Higuchi and Takemori, 1989) and markedly reduces twitch duration by accelerating cross-bridge cycling without affecting the kinetics of the $[Ca^{2+}]_i$ transient (Backx, Gao, Azan-Backx, and Marban, 1994). Fig. 8 shows force-$[Ca^{2+}]_i$ phase plots in two trabeculae before (*left*) and after (*right*) the application of 5 mM BDM. In the absence of BDM for the trabecula in Fig. 8 *A*, there is reasonable agreement between the force-$[Ca^{2+}]_i$ relationship at the time of peak force and that recorded at steady state, while the agreement is poor for the same trabecula in Fig. 8 *B*. This figure highlights the general finding that the degree of correspondence varied widely among preparations, being approximately correct only in three of seven trabeculae; such variability undermines the general validity of the Blinks hypothesis. Even in those preparations in which the agreement is good under control conditions, the Blinks hypothesis breaks down in the presence of BDM (Fig. 8). In fact, with BDM the relaxation regions of the phase plots coincide rather closely with the steady state relationship, reminiscent of CPA-modified twitches. This relative shift in the relaxation portion of the phase plots towards the steady state relationship is expected for agents which accelerate cross-bridge cycling without affecting the kinetics of the $[Ca^{2+}]_i$ transient.

DISCUSSION

Agreement of Force-$[Ca^{2+}]$ Relationship Obtained Using Aequorin or Fura-2

The force-$[Ca^{2+}]$ relationship we measured using fura-2 in rat cardiac trabeculae is strikingly similar to that recorded previously in ferret papillary muscle (Yue et al., 1986; Okazaki et al., 1990) using aequorin as the Ca^{2+} indicator under conditions that are otherwise almost identical. The mean values for $K_{1/2}$ and the Hill coefficient were 0.65 μM and 5.2 in rat trabeculae using fura-2 while they were 0.50 and 6.08 for ferret muscle using aequorin (Yue et al., 1986). This close agreement is gratifying given that the experimental methodologies have complementary limitations (Blinks et al., 1982; Cobbold and Rink, 1987). Aequorin is difficult to calibrate and tends to overestimate the $[Ca^{2+}]$ under conditions where significant Ca^{2+} gradients exist because of the nonlinear dependence of the luminescence on the $[Ca^{2+}]$ (Blinks et al., 1982). Furthermore, aequorin cannot accurately measure the $[Ca^{2+}]$ below ~0.2 μM corresponding to diastolic levels of Ca^{2+}, but is useful for concentrations of Ca^{2+} up to 100 μM (Allen and Kurihara, 1982). On the other hand, fura-2 binds Ca^{2+} with

one-to-one stoichiometry which minimizes distortions associated with large Ca^{2+} gradients. Due to its high binding affinity for Ca^{2+}, fura-2 is a very reliable Ca^{2+} indicator at low [Ca^{2+}] but is less accurate when the [Ca^{2+}] is $> \sim 3$ μM (Grynkiewicz et al., 1985). The previous results using aequorin were accomplished in relatively thick papillary muscles with the aequorin injected into ~100 surface cells. Potential problems of metabolite buildup (i.e., elevations in phosphate and protons) in the central core of these large papillary muscles due to substantial diffusion distances (Schouten and ter Keurs, 1986), which would not be serious at the surface cells, could further distort the force-[Ca^{2+}] relationship in these muscles. The very small size of our trabeculae, coupled with the fact that the fura-2 is loaded uniformly throughout the muscle, would be expected to minimize problems associated with nonuniformity.

In spite of the agreement of our results using fura-2 in rat trabeculae with those obtained with aequorin in ferret and human papillary muscles, the measured relationship between force and [Ca^{2+}] could be fundamentally inaccurate due to significant inhomogeneities in the [Ca^{2+}] throughout the sarcomere (Wier and Yue, 1986). Indeed, tetani generated in the presence of ryanodine might be expected to have large [Ca^{2+}] gradients because the Ca^{2+}-ATP-ase of the SR is still fully functional (Blinks, 1993). However, the force-[Ca^{2+}] relationship measured in the presence of CPA was identical to that recorded with ryanodine. Since the inhibition of the Ca^{2+} pump by CPA, which is expected to minimize the Ca^{2+} gradients within the sarcomere, did not measurably alter the force-[Ca^{2+}] relationship, it appears that such gradients of $[Ca^{2+}]_i$ do not impact significantly on the force-[Ca^{2+}] relationship.

In many respects the use of CPA to enable tetanization is preferable to the use of ryanodine. The onset of action of 100 μM CPA is rapid and the external [Ca^{2+}] required to achieve a given level of force is much lower than is required with ryanodine (cf. compare Fig. 4 *B* with Fig. 6 *B*). Again, this probably arises from continued activity of the SR Ca^{2+}-ATPase in the presence of ryanodine; the Ca^{2+} pump is still actively sequestering much of the incoming Ca^{2+}. An additional bonus of using CPA is the precise correspondence of the force-$[Ca^{2+}]_i$ relation during most of the relaxation phase of the twitch with the force-$[Ca^{2+}]_i$ relationship recorded during tetani, thereby allowing rapid determination of changes in the responsiveness of the contractile system during pharmacological interventions.

Comparison of Intact to Skinned Force-[Ca^{2+}] Relationships

The values for $K_{1/2}$ and the Hill coefficient obtained in this study and others using intact cardiac muscle differ significantly from the bulk of skinned fiber results. Generally, $K_{1/2}$ is reported to be ~2–10-fold larger and the Hill coefficient is ~2–3 times smaller in skinned muscle compared to our intact results (Gao et al., 1994). These differences could result from the loss of soluble cellular constituents after skinning, which alters force-generating properties of the muscle (Moss, 1992; Bers, 1991; Miller, Lamont, and O'Dowd, 1993; Harrison et al., 1988) and/or associated changes in myofilament lattice spacing (Godt and Maughan, 1981). In addition, many of the previous studies in skinned muscle used free [Mg^{2+}] > 1.5 mM while the concentration in intact cells appears to be closer to 0.5–0.8 mM (Fabiato and Fabiato, 1975; Moss, 1992; Blinks, 1993; Gao et al., 1994). In spite of shifts in $K_{1/2}$ and the Hill coefficient, maximal force was unaffected by skinning (Fig. 3), verifying that we

had indeed achieved maximal force during tetanization of our intact trabeculae at high $[Ca^{2+}]_o$.

During twitches, the peak $[Ca^{2+}]_i$ can reach 2 μM or greater, even though the peak force generally achieves levels which are far $<70\%$ of F_{max}. These observations support the assertion that the steady state force-$[Ca^{2+}]$ relationship in intact muscle is indeed left shifted relative to that in the skinned fiber. Certainly, the peak force versus peak $[Ca^{2+}]_i$ relationship is right shifted when compared to the steady state force-$[Ca^{2+}]_i$ relationship (Yue et al., 1986; Fig. 7) but would actually be left shifted relative to most published force-$[Ca^{2+}]$ curves recorded in skinned fibers. Surprisingly, in the absence of CPA or ryanodine, the relationship of force to the $[Ca^{2+}]_i$ during the relaxation of a twitch (in five out of six muscles) is actually shifted to the left of the relationship recorded during tetani (e.g., Fig. 7 *B*); this is only possible if the time course of relaxation is limited by the off rate of Ca^{2+} from troponin or cross-bridge detachment rather than the rate of Ca^{2+} removal from the cytosol. Therefore, our results establish that relaxation of force in cardiac muscle is controlled by the contractile proteins themselves unlike cell relengthening rates in isolated myocytes which are limited by the rate of relaxation of the $[Ca^{2+}]_i$ transient (Spurgeon et al., 1992). This conclusion is consistent with the observation that rapid reductions in force associated with quick releases in muscle length during the relaxation phase of the twitch result in a rise in the $[Ca^{2+}]_i$. Cross-bridge attachment appears to control the affinity of Ca^{2+} binding to troponin primarily by modifying the off rate for Ca^{2+} unbinding from troponin (Hoffman and Fuchs, 1987; Guth and Potter, 1987), possibly as discussed mechanistically by Hill and co-workers (Hill, Eisenberg, and Greene, 1980; Hill, 1985).

Relationship of Force to $[Ca^{2+}]_i$ during Twitches Compared to Steady State

Previously, it has been argued that the force required to maintain isolated myocytes below slack length during the entire relaxation phase of the twitch, where a small internal load must exist, corresponds to the steady state relationship with $[Ca^{2+}]_i$ (Spurgeon et al., 1992). Although these authors did not report the cell shortening vs $[Ca^{2+}]$ relationship during tetani, they did find an appropriate rightward shift after the addition of isoproterenol (Spurgeon et al., 1992). However, during cell shortening and relengthening, the sarcomere length is, by definition, changing significantly throughout the twitch. In contrast, the force-$[Ca^{2+}]_i$ relationship during the relaxation phase of the twitch at a fixed sarcomere length in trabeculae does not generally coincide with the steady state force-$[Ca^{2+}]_i$ relationship recorded during ryanodine tetani (Figs. 7 *B*, 8, *A* and *B*).

While the steady state force-$[Ca^{2+}]_i$ relationship at the time of peak force did correspond somewhat with that recorded in steady state, precise quantitative agreement varied between preparations. Nevertheless, the correlation was abolished after the application of 5 mM BDM (Fig. 8) as well as CPA (Fig. 6 *C*). These agents did enhance the agreement between force-$[Ca^{2+}]_i$ relationships at steady state and during the relaxation phase of twitch contractions. This suggests that in control the correlation, when observed, between the force-$[Ca^{2+}]_i$ relationship measured in steady state and in twitches at peak force was rather fortuitous. In other words, dynamic steady state between Ca^{2+} and force does not exist during twitches under

control conditions as hypothesized by Blinks (1993). No accurate quantitative analysis is possible with this method.

Force-$[Ca^{2+}]_i$ relationships recorded during the rising or relaxation phases of twitches do not correspond to the steady state relationship recorded in the same muscle. The basis for this lack of correspondence can be understood by recognizing that the kinetics of force development are slow compared to the $[Ca^{2+}]_i$ transient (Backx et al., 1994). As a result, the force-$[Ca^{2+}]_i$ relationships during the rising and relaxation phases of force are shifted rightward and leftward, respectively, compared to the steady state. Therefore, as expected, slowing the rate of relaxation of the $[Ca^{2+}]_i$ transient using CPA results in precise correspondence between the steady state force-$[Ca^{2+}]_i$ relationship and that measured during the relaxation phase. Similarly, accelerating cross-bridge kinetics using BDM also improves the correlation between steady state and relaxation phase force-$[Ca^{2+}]_i$ relationships. Poor correspondence during the rising phase of the force is expected for all the conditions which we studied because $[Ca^{2+}]_i$ is changing too rapidly during this period for a steady state to develop.

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