Positive force- and \([\text{Ca}^{2+}]\)-frequency relationships in rat ventricular trabeculae at physiological frequencies

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Layland, Joanne, and Jonathan C. Kentish. Positive force- and \([\text{Ca}^{2+}]\)-frequency relationships in rat ventricular trabeculae at physiological frequencies. Am. J. Physiol. 276 (Heart Circ. Physiol. 45): H9–H18, 1999.—The isometric force-frequency relationship of isolated rat ventricular trabeculae (diameter <250 µm) was examined at 24, 30, and 37°C at stimulation frequencies (0.1–12 Hz) encompassing the physiological range. Some muscles were microinjected with fura PE3 to monitor the diastolic and systolic intracellular concentration of \([\text{Ca}^{2+}]\) ([Ca\text{\textsuperscript{i}}]). At a near-physiological external \([\text{Ca}^{2+}]\) concentration ([Ca\text{\textsuperscript{o}}]) of 1 mM, a positive force-frequency relationship was demonstrated at all temperatures. The force-frequency relationship became negative at high frequencies (e.g., >6 Hz at 30°C) at 1 mM [Ca\text{\textsuperscript{o}}], or at low frequencies at 8 mM [Ca\text{\textsuperscript{o}}]. The twitch and \([\text{Ca}^{2+}]\) transient became shorter as stimulation frequency increased; these changes were related to changes in systolic, rather than diastolic, [Ca\text{\textsuperscript{i}}], and were not blocked by inhibitors of \([\text{Ca}^{2+}]\)/calmodulin-dependent protein kinase II. The positive force-frequency relationship of rat trabeculae was caused by a frequency-dependent loading of the sarcoplasmic reticulum (SR) with \([\text{Ca}^{2+}]\). We suggest that at high frequencies, or under conditions of \([\text{Ca}^{2+}]\) overload, this loading saturates. Processes that tend to decrease SR \([\text{Ca}^{2+}]\) release will then predominate, resulting in a negative force-frequency relationship.

fura PE3; calcium uptake by sarcoplasmic reticulum; calcium/calmodulin-dependent protein kinase

IN ISOLATED MYOCARDIAL preparations from most mammalian species an increase in the frequency of stimulation produces an increase of steady-state twitch force. This positive force-frequency relationship is generally attributed to a net uptake of \([\text{Ca}^{2+}]\) into cells as the frequency of stimulation is increased (26). The \([\text{Ca}^{2+}]\) loading is caused not only by the greater number of \([\text{Ca}^{2+}]\) currents (\(I_{\text{Ca}}\)) per unit time (from the increased frequency of action potentials) but also to the greater number of \([\text{Na}^{+}]\) currents (\(I_{\text{Na}}\)) per unit time, which raises the intracellular \([\text{Na}^{+}]\) activity and thereby promotes \([\text{Ca}^{2+}]\) entry and decreases \([\text{Ca}^{2+}]\) efflux via \([\text{Na}^{+}]\)/\([\text{Ca}^{2+}]\) exchange (26, 35). The enhanced \([\text{Ca}^{2+}]\) loading of the cell results in a greater uptake of \([\text{Ca}^{2+}]\) into, and \([\text{Ca}^{2+}]\) release from, the sarcoplasmic reticulum (SR).

Rat cardiac muscle is generally considered to be unusual among mammalian preparations because it usually exhibits a negative force-frequency relationship, i.e., steady-state isometric force is reduced as stimulation frequency is increased (4, 8, 20). This would seemingly contradict to its physiological requirement, because resting heart rates of rats are within the range of 5–7 Hz at 37°C (40) and the frequency at which cardiac muscle produces maximum force would be expected to fall within the physiological range of heart rates (24). However, there have been a few reports of positive force-frequency relationships in rat cardiac muscle (7, 15, 17, 33). Furthermore, a negative force-frequency relationship in rat cardiac preparations can be reduced, or even converted to a positive staircase, by reducing the external \([\text{Ca}^{2+}]\) concentration ([Ca\text{\textsuperscript{o}}]) (8, 14) or by inhibiting normal SR function with ryanodine (38). Indeed, the nature of the rat cardiac muscle force-frequency relationship appears to be extremely sensitive to the experimental conditions, including the adequacy of metabolic support (19, 33), the [Ca\text{\textsuperscript{o}}] (14, 29), and the frequency range examined (7).

Many studies with rat myocardium were conducted at stimulation frequencies, temperatures, and [Ca\text{\textsuperscript{o}}] that bear little resemblance to the physiological condition. Therefore, in the present study we aimed to reexamine the isometric force-frequency relationship in thin rat ventricular trabeculae using a range of frequencies (0.1–12 Hz) encompassing the physiological range, a range of temperatures (24, 30, and 37°C), and a [Ca\text{\textsuperscript{o}}] of 1 mM, which is close to the physiological [Ca\text{\textsuperscript{o}}] of 1.3 mM in rat plasma (11, 14). In addition, the concentration of Ca\text{\textsuperscript{i}} in the cytosol ([Ca\text{\textsuperscript{i}}]) was monitored simultaneously with isometric force, using the Ca\text{\textsuperscript{2+}}-sensitive fluorescent indicator fura PE3 (K\textsuperscript{+} salt), to determine to what extent the rate-dependent changes in twitch magnitude and time course were caused by changes in the Ca\text{\textsuperscript{i}} transient. In a few experiments we also examined the force- and [Ca\text{\textsuperscript{2+}}]-frequency relationships at elevated [Ca\text{\textsuperscript{2+}}] (8 mM). The SR Ca\text{\textsuperscript{2+}} load at different frequencies of stimulation was also assessed. A preliminary account of this work has been presented in abstract form (25).

METHODS

Muscle preparation. The experimental procedure and apparatus were similar in most respects to those described in detail previously (23). The strain of rats used was either Wistar (male, ~250 g) or LB/Mr1, i.e., first progeny of a cross between female Lewis and male Brown Norway rats (either sex, ~250 g). Rats were stunned and then killed by cervical dislocation (Schedule 1 procedure in accordance with United Kingdom Home Office regulations). The hearts were removed and rinsed free of blood with modified Krebs-Henseleit solution, which contained (in mM) 93 NaCl, 20 NaHCO\textsubscript{3}, 1 Na\textsubscript{2}HPO\textsubscript{4}, 1 MgSO\textsubscript{4}, 5 KCl, 1 CaCl\textsubscript{2}, 10 glucose, and 20 Na-acetate with 5 U/l insulin, bubbled with 95% O\textsubscript{2}-5% CO\textsubscript{2}, pH 7.4. Strongly beating, unbranched trabeculae (diameter 80–250 µm) were identified in the right ventricle. The Krebs-Henseleit solution was then exchanged for Krebs to which 25 mM 2,3-butanedione monoxime (BDM) had been added, to minimize irreversible muscle damage during dissection. After 30 min in this solution a trabecula was carefully
dissected out, with a piece of valve at one end and a small chunk of ventricular wall at the other. After a further 15- to 30-min period in BDM-Krebs, the preparation was transferred to a horizontal flow-through chamber on the stage of a Nikon Diaphot inverted microscope. The wall end of the muscle was secured in a wire loop attached to an isometric force transducer (SensoNor, Horten, Norway), and the valve end was impaled on a hook attached to a micromanipulator. Initially, the muscle was bathed in normal Krebs solution (1 mM Ca\(^{2+}\)) at 24°C. The temperature was later raised to 30 or 37°C if required. The muscle was stimulated via a platinum field electrodes connected to a Digitimer D4030 and DS2 isolated stimulator. Force and fluorescence signals (see Measurement of [Ca\(^{2+}\)]) using fura PE3) were recorded on a four-channel chart recorder and, via a 12-bit analog-digital board (Digidata 1200, Axon Instruments), on a PC running pCLAMP software (Axon). The preparation was stimulated at 0.33 Hz for >1 h to allow equilibration, during which time muscle length was gradually increased to 95% of the optimum length for active force generation. Passive force was then ~10% of active force. Muscle length and width were measured, and the cross-sectional area was estimated assuming that the preparation was cylindrical.

A video camera attached to the microscope allowed us to see the muscle (illuminated with red light of wavelength >700 nm) on a video monitor. In this way, any spontaneous oscillations of the preparation resulting from damage during the isolation procedure could be observed clearly. The experimental protocol was not commenced until such oscillations had disappeared and force had stabilized.

All chemicals were analytic grade and were obtained from BDH (Poole, UK) or Sigma (Poole, UK), except for KN-62 and KN-95, which were obtained from Calbiochem-Novabiochem (Beeston, UK).

Measurement of [Ca\(^{2+}\)], using fura PE3. Because higher temperatures greatly accelerate the loss of fluorescent Ca\(^{2+}\) indicators such as fura 2 from the cell (39), we chose to monitor [Ca\(^{2+}\)] at 30°C using fura PE3 (K+ salt, Texas Fluorescence). Fura PE3 has fluorescence and Ca\(^{2+}\)-binding properties similar to those of fura 2 and can be loaded into the cells in the same way but, as a zwitterion, it resists compartmentalization and leakage from the cell (41). Even so, we found that at 37°C fura PE3 was lost too rapidly from the cells for the extended experiments of the present study. Therefore, [Ca\(^{2+}\)] could only be measured at 24 or 30°C.

To measure fura PE3 fluorescence, light from a xenon arc lamp was passed through 340- and 380-nm bandpass filters in a six-filter rotating wheel system (Cairn Research, Faversham, UK), directed onto a 400-nm dichroic mirror, and then focused on the muscle via a ×10 Nikon CF-Fluor objective. Emitted light was collected via the objective, passed through a 510-nm emission filter (bandwidth 50 nm), and measured by a photomultiplier. A shutter in the excitation light path was kept closed when fluorescence measurements were not being made, to minimize photobleaching. The autofluorescences of the preparation at 340 and 380 nm were first recorded during steady-state contractions at 0.33 Hz. Preparations were then loaded with fura PE3 by iontophoresis into several cells, as described previously for iontophoresis of fura 2 (1, 23). In summary, a cell in the muscle was impaled with a microelectrode containing 2 mM fura PE3 dissolved in H2O (and back-filled with 140 mM KCl), and a negative current (4–8 nA) was injected for 10–20 min. Iontophoresis was conducted at two to four different locations to achieve a fluorescence level that was three to four times the autofluorescence level.

Iontophoresis was followed by a 45- to 60-min period of equilibration with the trabecula superfused in normal Krebs solution at 30°C and stimulated at 0.33 Hz. This allowed fura PE3 to spread from cell to cell via the gap junctions. Because of the larger molecular weight of fura PE3 (mol wt 1,054), spreading of the dye was not as complete as with fura 2 (mol wt 832). This was assessed by measuring fura PE3 fluorescence while scanning a small window along the muscle. However, because the present study uses a ratiometric method to estimate [Ca\(^{2+}\)], inhomogeneities in the amount of dye present in different regions of the muscle are unlikely to significantly alter the results obtained. To check this, in preliminary studies we examined the Ca\(^{2+}\) transients in different regions of the injected muscle after the equilibration period and found no appreciable difference between regions.

The Cairn spectrophotometer control box subtracted the autofluorescences at 340 and 380 nm (recorded at 0.33 Hz before iontophoresis) from the appropriate fluorescence signals and calculated the 340-to-380 fluorescence ratio, which is a measure of [Ca\(^{2+}\)]. Results are given in terms of 340/380 ratio rather than [Ca\(^{2+}\)], because 1) there are difficulties with the calibration of fura fluorescence in intact cells, and 2) we were interested chiefly in the relative changes in [Ca\(^{2+}\)], rather than in its absolute measurement.

A potential problem was that the Cairn system subtracted a constant autofluorescence from the fluorescence signals, yet muscle autofluorescence, which is largely caused by cellular NAD(P)H, might be expected to vary with frequency. In pilot experiments (at 30°C) we found that the autofluorescence of noninjected muscles decreased progressively as the stimulation frequency was increased over the range 0.33–4 Hz but showed little further change above 4 Hz. At 4 Hz the autofluorescence signals at 340 and 380 nm were 82.0 ± 2.1 and 92.8 ± 1.5%, respectively, of those recorded at 0.33 Hz (means ± SE, n = 4 muscles). We calculate that the inability to subtract the true autofluorescence from the total fluorescence signals would produce an error in the peak fluorescence ratio at 4 Hz of <5%, with changes in autofluorescence having smaller effects at lower frequencies. These small changes would not affect the results qualitatively.

Steady-state force-frequency and [Ca\(^{2+}\)]-frequency relationships. From the equilibration frequency of 0.33 Hz, stimulation frequency was first reduced to 0.1 Hz for 3 min. Steady-state force and Ca\(^{2+}\) transients at 0.1 Hz (average of 10 contractions) were then recorded using pCLAMP. Stimulation frequency was then returned to the control of 0.33 Hz, and the average steady-state force and Ca\(^{2+}\) transients at 0.33 Hz were recorded after 3 min. This protocol was repeated for successively increasing test frequencies (0.1–3 Hz at 24°C, 0.1–8 Hz at 30°C, and 0.1–12 Hz at 37°C). The highest frequency investigated was dependent on the bath temperature and was limited by the appearance of incomplete relaxation. The protocol was then repeated with decreasing stimulation frequency. For the corresponding frequencies on the ascending and descending frequency protocols, force or [Ca\(^{2+}\)] records were averaged.

Assessment of SR Ca\(^{2+}\) load. In some muscles at 24°C, the SR Ca\(^{2+}\) load after different frequencies of stimulation was assessed by rapid application of caffeine (40 mM) dissolved in Krebs solution. Electrical stimulation was stopped, and the caffeine solution was applied directly to the muscle bath within 3 s. This elicited a caffeine contracture. The amplitude of the fura PE3 fluorescence ratio in response to caffeine application was used as an index of SR Ca\(^{2+}\) content. Preliminary experiments confirmed a previous report (15) that
varying the time of caffeine application after the last stimulus had little effect on the size of the caffeine-induced rise of [Ca\(^{2+}\)].

Changes in force and [Ca\(^{2+}\)], immediately after a frequency change. The pCLAMP software was set up to automatically cycle between 1- and 4-Hz stimulation (3 min at each rate) and to record 1) steady-state force and Ca\(^{2+}\) transients (average of 10 contractions) at 1 and 4 Hz and 2) force and Ca\(^{2+}\) transients recorded for individual contractions during the first 30 beats after the increase or decrease in frequency (transitional period). These experiments were performed at 30°C only. The two frequencies, 1 and 4 Hz, were chosen for examination because the changes in force and Ca\(^{2+}\) transient between these frequencies were very distinct. For each trabecula, the frequency change protocol was repeated 10 times and the corresponding recorded files were averaged using Clampfit software (Axon). Averaging considerably improved the signal-to-noise ratio of the Ca\(^{2+}\) transients, allowing individual beats of the transitional period to be analyzed more accurately.

Limitations in the software meant that the delay in changing between frequencies was more than the required delay (i.e., 0.8 s when changing from 1 to 4 Hz and 1.6 s when changing from 4 to 1 Hz). This delay was entirely reproducible, and it was considered more useful to use this constant delay and ignore the first beat after the change than to introduce a variable delay by manual operation.

Data analysis. Results were analyzed off-line using Clampfit software. For steady-state and transitional period data, we measured diastolic force (force immediately preceding a stimulus) and active force (i.e., peak force minus diastolic force), together with the half-width (time from 50% of activation to 50% relaxation of active force) from the twitch records for each frequency. Ca\(^{2+}\) transients (340-to-380-nm ratio) were fitted with double exponential functions (exponential rise and exponential decay), and the fits were used as guides to allow a more accurate measurement of the peak systolic ratio, the diastolic ratio (the ratio immediately preceding the stimulus), the amplitude (peak systolic ratio minus diastolic ratio), and the half-width of the Ca\(^{2+}\) transient (time from 50% of peak Ca\(^{2+}\) on the rising phase to 50% on the declining phase).

**RESULTS**

Effects of frequency on steady-state force and [Ca\(^{2+}\)]. Twitch records from a typical muscle at 30°C (Fig. 1A) show that steady-state contractions exhibited an increase in active force with increasing frequency in the range 0.33–6 Hz and then declined at 8 Hz. The increase in frequency also reduced the time to peak force and the overall duration of the twitch. Diastolic force was relatively constant up to 6 Hz but increased at higher frequencies because of incomplete relaxation of the twitch. Figure 1B illustrates the averaged steady-state relationships between active force and frequency recorded at 24, 30, and 37°C. To facilitate comparison between preparations of different dimensions, force is expressed per unit of cross-sectional area, i.e., as stress (mN·mm\(^{-2}\)). At 1 mM [Ca\(^{2+}\)], rat ventricular trabeculae always demonstrated a positive force-frequency relationship over most of the frequency range studied. Increasing the temperature from 24 to 30 and 37°C produced a decrease in the absolute value of maximum force achieved and shifted the force-frequency relationship to a higher frequency range (Fig. 1B). At 24°C a positive force-frequency relationship was observed in the range 0.1–3 Hz, but incomplete relaxation prevented the study of higher frequencies. At 30°C peak force was achieved at 6 Hz and was 3.8 times higher than the force at 0.33 Hz. At 37°C, peak force was achieved at 10 Hz and was 5.2 times higher than the force at 0.33 Hz.

Figure 1C illustrates superimposed Ca\(^{2+}\) transients recorded from a typical muscle at 30°C during steady-
state contractions at different frequencies. As frequency was increased to 6 Hz, the transient increased in magnitude and the time to peak [Ca\textsuperscript{2+}]\textsubscript{i} was shortened. Diastolic [Ca\textsuperscript{2+}]\textsubscript{i} also increased. Averaged steady-state data for the peak systolic, diastolic, and amplitude (peak systolic ratio – diastolic ratio) values of the fluorescence ratio at 30°C are given in Fig. 1D. As with the force-frequency relationship at 30°C (Fig. 1B), peak systolic [Ca\textsuperscript{2+}]\textsubscript{i} was greatest at 6 Hz, where it was 3.1 times that measured at 0.33 Hz. Diastolic [Ca\textsuperscript{2+}]\textsubscript{i} increased gradually up to 4 Hz, beyond which the increase became more dramatic. At 6 Hz, the diastolic ratio was 1.8 times greater than at 0.33 Hz.

Because many other studies have shown a negative force-frequency relationship in rat cardiac muscles, we tried to find conditions that produced a negative force-frequency relationship in our experiments. One such condition was elevated [Ca\textsuperscript{2+}]\textsubscript{o}. In four muscles at 24°C, the positive force-frequency relationship (0.33–2 Hz) at 1 mM [Ca\textsuperscript{2+}]\textsubscript{o} (Fig. 2A) was converted to a negative force-frequency relationship by increasing [Ca\textsuperscript{2+}]\textsubscript{o} to 8 mM (Fig. 2B). At 8 mM [Ca\textsuperscript{2+}]\textsubscript{o}, these muscles demonstrated spontaneous oscillations indicative of Ca\textsuperscript{2+} overload. At 1 mM [Ca\textsuperscript{2+}]\textsubscript{o}, peak systolic ratio increased with increasing stimulation frequency, in accordance with the positive force-frequency effect (Fig. 2C). However, despite the negative force-frequency effect at 8 mM [Ca\textsuperscript{2+}]\textsubscript{o}, peak systolic ratio showed little change with increasing frequency (Fig. 2D). Nevertheless, at 8 mM [Ca\textsuperscript{2+}]\textsubscript{o}, the amplitude of the Ca\textsuperscript{2+} transient decreased at the higher stimulation frequencies because the diastolic ratio dramatically increased (Fig. 2D).

The positive force-frequency relationship at 1 mM bathing [Ca\textsuperscript{2+}] can be attributed to an increase in SR Ca\textsuperscript{2+} load with increasing stimulation frequency, because the release of Ca\textsuperscript{2+} produced by rapid caffeine application increased as the preceding stimulation frequency was increased (Fig. 2E). In contrast, at 8 mM [Ca\textsuperscript{2+}]\textsubscript{o} the SR load was relatively high even at the lowest stimulation frequencies (compare Fig. 2, E and F) and showed little increase as stimulation frequency was increased. Note that in our experiments the caffeine-induced increase of [Ca\textsuperscript{2+}] was always smaller than the Ca\textsuperscript{2+} transients produced by electrical stimulation. This probably reflects the difficulty in achieving a rapid and uniform application of caffeine in a multicellular preparation (37).

Force and [Ca\textsuperscript{2+}] after frequency changes. We also measured the changes in active force, Ca\textsuperscript{2+} transient amplitude, and diastolic [Ca\textsuperscript{2+}] during the first 30 beats...
after an increase (from 1 to 4 Hz, Fig. 3A) or decrease (from 4 to 1 Hz, Fig. 3B) in stimulation frequency at 30°C. Increasing the frequency produced an initial small drop in active stress, followed by a gradual increase over 8 s to achieve the higher steady-state stress at 4 Hz (Fig. 3A, top). The amplitude of the Ca^{2+} transient (Fig. 3A, middle) showed a monophasic increase, reaching double its initial value over this period. In contrast, there was an immediate dramatic increase in diastolic [Ca^{2+}], followed by complex secondary changes; these consisted of a small decrease and then an increase in diastolic [Ca^{2+}], during the first 8 s, then a gradual decline to a steady-state diastolic [Ca^{2+}] at 4 Hz that was ~25% higher than that at 1 Hz (Fig. 3A, bottom). In general, reducing the stimulation frequency from 4 to 1 Hz produced the opposite changes: an initial increase in stress followed by a progressive decline (Fig. 3B, top) and a gradual decrease in the amplitude of the Ca^{2+} transient (Fig. 3B, middle). However, diastolic [Ca^{2+}] showed a sudden fall, followed by a steady level for ~30 s and then a secondary increase over the course of 3 min to achieve its new lower steady-state level at 1 Hz (Fig. 3B, bottom). In addition, the gradual reductions in force and amplitude of the Ca^{2+} transient after the decrease in frequency to 1 Hz were about four times slower than the corresponding increases when frequency was raised to 4 Hz; thus the time course of the changes of force and Ca^{2+} transient amplitude were dependent on the twitch number after the frequency change (Fig. 3) rather than on time itself.

Figure 4 plots the averaged data for diastolic ratio against peak systolic ratio at each frequency. For steady-state contractions up to 4 Hz, the peak systolic ratio was linearly related to the diastolic ratio, but

Fig. 3. Changes in active stress (top), Ca^{2+} transient amplitude (peak ratio/diastolic ratio, middle), and diastolic ratio (bottom) caused by change in frequency from 1 to 4 Hz (A) and from 4 to 1 Hz (B) at 30°C. The x-axes illustrate twitch number and time after frequency change. ●, Data for twitches 2–30 after frequency change; ○, initial and final steady-state twitches at 1 or 4 Hz. First twitch after frequency change is not shown because first interstimulus interval after change was longer than desired interval. Symbols represent means ± SE (n = 5 muscles).

Fig. 4. Relationship between peak systolic and diastolic fluorescence ratios at different frequencies during steady-state conditions at 30°C. Symbols represent means ± SE (n = 7 muscles). Numbers next to symbols indicate stimulation frequency (Hz).
above 6 Hz the systolic ratio saturated whereas the diastolic ratio continued to increase.

Time course of force and [Ca\(^{2+}\)] transient. An increase in stimulation frequency was accompanied by a marked reduction in the time to peak force and relaxation time at all temperatures studied (see, e.g., Fig. 1A). A reduction in the duration of the Ca\(^{2+}\) transient was also observed (examined at 30°C only; Fig. 1C). Figure 5A illustrates this frequency-dependent decrease in twitch and Ca\(^{2+}\) transient duration for steady-state conditions at 30°C, using half-width as an index of twitch and Ca\(^{2+}\) transient duration. The shortening of the Ca\(^{2+}\) transient was most dramatic between 0.33 and 3 Hz, with the half-width at 0.33 Hz being reduced by 50% at a stimulation frequency of ~1.5 Hz. The initial phase of the decline of the Ca\(^{2+}\) transients at each frequency appeared to follow a common curve (see Fig. 1C), suggesting that, over this period at least, the rate of decline may be dependent only on [Ca\(^{2+}\)] (see DISCUSSION). The reduction in twitch half-width was less dramatic than for the Ca\(^{2+}\) transient. During the transitional periods half-width decreased gradually when stimulation frequency was increased (from 1 to 4 Hz, Fig. 5B) and increased gradually when stimulation frequency was reduced (from 4 to 1 Hz, Fig. 5C). The time course of these changes in half-width were similar to the time course of the changes in the amplitude of the Ca\(^{2+}\) transient (Fig. 3) but were different from the rapid changes in diastolic Ca\(^{2+}\) (Fig. 3). In addition, there was an inverse relationship between half-width and peak systolic ratio, which was similar for both increases and decreases of frequency (Fig. 5D); there was, however, no correlation between half-width and diastolic ratio under these conditions (data not shown).

Inhibition of Ca\(^{2+}\)-calmodulin-dependent protein kinase. To investigate whether phosphorylation of phospholamban by Ca\(^{2+}\)/calmodulin-dependent protein kinase (CaMKII) was involved in the frequency-dependent shortening of the twitch and the Ca\(^{2+}\) transient, in separate experiments we used two selective inhibitors of CaMKII, KN-62 and KN-93. These drugs were dissolved in DMSO (final concn 0.1% in Krebs), which by itself had no significant effect on either the force-frequency or [Ca\(^{2+}\)]-frequency relationships (n = 5 muscles, data not shown). Addition of 10 μM KN-62 (n = 3) or KN-93 (n = 2) in 0.1% DMSO decreased both force (by ~60%) and the Ca\(^{2+}\) transient (by ~25%) at each stimulation frequency studied (0.33–8 Hz), although the positive force-frequency and Ca\(^{2+}\)-frequency relationships were preserved (data not shown). A similar decrease in the amplitude of the Ca\(^{2+}\) transient with CaMKII inhibitors (KN-62 and KN-93) was observed by Bassani et al. (3) and Li et al. (28). Bassani et al. (3) attributed this to a decrease in the amplitude of the I\(_{Ca}\) with KN-62. It may be that the 25% decrease in the Ca\(^{2+}\) transient is sufficient to explain the 60% decrease in force because of the steepness of the force-Ca\(^{2+}\) relationship of the myofibrils (see, e.g., Ref. 23). Despite these effects, inhibition of CaMKII in the present study appeared to have little effect on the frequency-dependent shortening of the twitch or the Ca\(^{2+}\) transient. Normalization of the Ca\(^{2+}\) transients, recorded for a typical muscle at each frequency in the presence of KN-93 (Fig. 6A), clearly demonstrates that the frequency-dependent shortening of the Ca\(^{2+}\) transient remains in the presence of CaMKII inhibitors. Figure 6B shows that the decrease in the half-width of the Ca\(^{2+}\) transient with increasing stimulation fre-
abnormal trabeculae. This is the first study to use thin transients were not reported. Thin trabeculae (250-µm diameter) are advantageous because they reduce the diffusional limitations associated with large multicellular preparations but are more robust than single cells and unlike single cells require no enzymatic isolation procedure, which may alter their properties. We used a [Ca2⁺], of 1 mM (~0.8 mM ionized Ca2⁺) that was lower than in previous studies [e.g., 2.7 mM [Ca2⁺], (20). 2.5 mM [Ca2⁺], (19, 40)]. The physiological concentration of free, ionized Ca2⁺ in rat plasma is ~1.3 mM (11, 14), which corresponds to a total Ca2⁺ concentration in Krebs of 1.5 mM (11). In additional experiments (data not shown) we confirmed that the positive force-frequency relationship of rat trabeculae observed at 1 mM total Ca2⁺ was preserved at a true physiological [Ca2⁺] (1.5 mM total Ca2⁺).

Under our experimental conditions, at 1 mM [Ca2⁺], rat trabeculae demonstrated distinct positive force-frequency relationships at all temperatures (24, 30, and 37°C) and over most of the frequency range investigated, including the physiological range. Indeed, at 37°C the force increased with frequency from 0.1 to 10 Hz (Fig. 1B). Increasing the temperature shifted the frequency for maximum force to a higher frequency but produced a decrease in the maximum force attained. These effects may be explained by an acceleration of Ca2⁺ reuptake by the SR and Ca2⁺ extrusion via Na⁺/Ca2⁺ exchange with increasing temperature (12).

Frequency-dependent changes in [Ca2⁺]. Like twitch force, the amplitude of the Ca2⁺ transient (Fig. 1D) increased as frequency was raised above 0.1 Hz, and both force and [Ca2⁺] were maximal at 6 Hz at 30°C. Furthermore, after a frequency change, active force and the amplitude of the Ca2⁺ transient changed gradually with similar time courses (Fig. 3). It is therefore likely that the effects of frequency on the magnitude of twitch force can be explained entirely by the effects on the magnitude of the Ca2⁺ transient. The relative changes in force (Fig. 1B) were somewhat greater than for the Ca2⁺ transient (Fig. 1D). This may be because small changes in [Ca2⁺] can produce large changes in the activation of the myofibrils because of the steepness of the force-[Ca2⁺] relationship of cardiac myofibrils (see, e.g., Ref. 23). The positive force-frequency and [Ca2⁺]-frequency relationships observed here for rat ventricular trabeculae are similar to those in most other mammalian species, and there is no reason why they should not be explained in the same way, by increased loading of the cell and of the SR with Ca2⁺ when the frequency of action potentials is increased (see below). Our caffeine release experiments at 24°C confirmed the rise in SR Ca2⁺ loading, at least up to 2 Hz.

Diastolic [Ca2⁺], was also influenced by frequency. Note that "diastolic"[Ca2⁺] was the [Ca2⁺] at the end of the interstimulus interval; this was not a true resting [Ca2⁺], because even at lower frequencies the [Ca2⁺] was still decreasing at this point (Fig. 1C). Increasing the stimulation frequency produced an immediate increase in the diastolic fluorescence ratio (Fig. 3A), presumably because Ca2⁺ influx exceeded the amount of Ca2⁺ that could be removed by SR uptake or by

DISCUSSION

Positive force-frequency relationship in rat ventricular trabeculae. This is the first study to use thin ventricular trabeculae from rats to investigate frequency-dependent changes in force and [Ca2⁺] over an extensive frequency range that includes physiological frequencies (resting heart rate of the rat is 5–7 Hz; Ref. 40). Physiologically relevant force-frequency relationships for the rat were previously investigated using either large multicellular preparations [papillary muscles (20), whole hearts (19), ventricular strips (40)] or single cells (7). However, the accompanying Ca2⁺ transients were not reported. Thin trabeculae (<250-µm
extrusion mechanisms during the reduced interstimulus interval. The resulting increase in \( [\text{Ca}^{2+}]_o \) after a frequency increase could promote a \( \text{Ca}^{2+} \) efflux via \( \text{Na}^+/	ext{Ca}^{2+} \) exchange (although this would be limited by the concomitant increase in \( [\text{Na}^+] \)), a reduction in the amplitude of \( I_{\text{Ca}} \) (9, 36), or an increase in the rate of SR \( \text{Ca}^{2+} \) uptake, the interaction of which may explain the complex secondary changes in diastolic \( \text{Ca}^{2+} \) observed in the transitional periods (Fig. 3A). Changes in the diastolic ratio observed immediately after a reduction in stimulation frequency (Fig. 3B) may be explained by these mechanisms operating in reverse.

We found a linear relationship between peak systolic \( [\text{Ca}^{2+}]_i \) and diastolic \( [\text{Ca}^{2+}]_i \), under steady-state conditions for the frequency range 0.1–4 Hz (Fig. 4). Frampton et al. (17) reported a similar relationship for rat ventricular myocytes at frequencies up to 2 Hz, but here we show that it is true for higher frequencies. The linearity could be because the increase in diastolic \( [\text{Ca}^{2+}]_i \), with increasing stimulation frequency would tend to stimulate the SR \( \text{Ca}^{2+}-\text{ATPase} \) system and promote SR \( \text{Ca}^{2+} \) loading (17). On the other hand, because the diastolic \( [\text{Ca}^{2+}]_i \), corresponded to the tail of the preceding \( \text{Ca}^{2+} \) transient, it could be argued that the greater diastolic \( [\text{Ca}^{2+}]_i \) was the result (rather than the cause) of the enhanced SR \( \text{Ca}^{2+} \) release, coupled with the shorter interstimulus period. Probably both factors contribute to the observed relationship. The peak systolic \( [\text{Ca}^{2+}]_i \) saturated at the highest frequencies (=6 Hz, Fig. 4), because the amplitude of the \( \text{Ca}^{2+} \) transient decreased even though diastolic \( [\text{Ca}^{2+}]_i \) continued to rise (Fig. 1D). Reasons for the reduction in \( \text{Ca}^{2+} \) transient amplitude, resulting presumably from diminished SR \( \text{Ca}^{2+} \) release, are considered in Frequency-dependent decline of \( \text{Ca}^{2+} \) transient.

Possible mechanisms for positive and negative force-frequency relationships of rat cardiac muscle. In our experiments, rat ventricular trabeculae generally demonstrated positive force-frequency behavior at 1 mM \( [\text{Ca}^{2+}]_o \) (Figs. 1B and 2A). However, this could be made negative by raising \( [\text{Ca}^{2+}]_o \) to 8 mM (Fig. 2B). Furthermore, some preparations showed a negative force-frequency relationship at 1 mM \( [\text{Ca}^{2+}]_o \), in the period immediately after mounting in the setup, when spontaneous oscillations were apparent (results not shown), although positive force-frequency behavior was restored after equilibration, when spontaneous oscillations had died away.

Many previous experiments have demonstrated a negative force-frequency relationship in rat cardiac muscle (see, e.g., Refs. 4, 8, and 20), and at least four types of explanation have been postulated. 1) An inadequate \( \text{O}_2 \) supply to the central fibers of isolated rat cardiac muscles could mask a true positive force-frequency relationship (24, 33). Although this could well contribute to the negative force-frequency relationship in larger preparations, it is unlikely to be applicable in our experiments, in which all muscles were of sufficiently small diameter (<250 µm) to overcome diffusional limitations. Furthermore, it cannot explain why negative force-frequency behavior has also been observed in isolated rat ventricular myocytes (15, 17). 2) There could be a frequency-dependent decrease in the SR \( \text{Ca}^{2+} \) load of rat ventricular preparations, attributable either to net \( \text{Ca}^{2+} \) efflux via \( \text{Na}^+/	ext{Ca}^{2+} \) exchange during the \( \text{Ca}^{2+} \) transient (35) or to the spontaneous release of \( \text{Ca}^{2+} \) from the SR resulting from \( \text{Ca}^{2+} \) overload, which becomes more likely at higher frequencies (8, 10, 17). However, the negative force-frequency behavior at 8 mM \( [\text{Ca}^{2+}]_o \), was accompanied by no change in SR \( \text{Ca}^{2+} \) load (Fig. 2F), and similar results were reported by Frampton et al. (15) using cardiac myocytes. 3) The negative force-frequency behavior could be caused by a reduction in the fraction of SR \( \text{Ca}^{2+} \) released at higher frequencies (see, e.g., Refs. 5 and 31). The SR \( \text{Ca}^{2+} \)-release channel inactivates or adapts after \( \text{Ca}^{2+} \) release (13, 18), and a reduced fractional release would result from the reduced time between beats for recovery from the adapted state (22). Fractional release would also be depressed by a reduced \( I_{\text{Ca}} \) trigger for SR \( \text{Ca}^{2+} \) release, because of incomplete recovery of \( I_{\text{Ca}} \) from inactivation (see, e.g., Ref. 36). Our results support a reduced fractional release because the negative force-frequency relationship observed at 8 mM \( [\text{Ca}^{2+}]_o \), was associated with a decrease in the \( \text{Ca}^{2+} \) transient amplitude but no change in the \( \text{Ca}^{2+} \) load of the SR (Fig. 2, B, D, and F). 4) Finally, it has been suggested (15) that the acceleration of the \( \text{Ca}^{2+} \) transient at higher frequencies (as shown in Figs. 1C and 5A) may contribute to the negative force-frequency response, because it reduces the time available for \( \text{Ca}^{2+} \) to activate the contractile machinery.

Whether rat cardiac muscle demonstrates a positive or negative force-frequency relationship may be dependent on the complex interaction of numerous frequency-dependent processes. With regard to the SR, these can be grouped into those that tend to decrease SR \( \text{Ca}^{2+} \) release and those that tend to increase SR \( \text{Ca}^{2+} \) release. In our experiments a major factor tending to decrease SR release was the reduction in fractional release (mechanism 3 above). The major factor tending to increase SR \( \text{Ca}^{2+} \) release is the increased loading of the cell with \( \text{Ca}^{2+} \); raising the stimulation frequency increases \( \text{Ca}^{2+} \) entry (via \( I_{\text{Ca}} \)) per unit time and increases \( \text{Na}^+ \) entry (via \( I_{\text{Na}} \)), thereby raising cytosolic \( [\text{Na}^+] \) and promoting \( \text{Ca}^{2+} \) influx and decreasing \( \text{Ca}^{2+} \) efflux, via \( \text{Na}^+/	ext{Ca}^{2+} \) exchange. The increased \( [\text{Ca}^{2+}]_i \) will promote SR \( \text{Ca}^{2+} \) loading, so that more \( \text{Ca}^{2+} \) is available for release. An additional factor is that the rise in the intra-SR \( \text{Ca}^{2+} \) load leads to a greater fraction of this sequestered \( \text{Ca}^{2+} \) being released (2, 31). The results of the present study suggest that the net outcome of these positive and negative frequency-dependent processes may depend on the filling status of the SR at the initial frequency, i.e., whether the SR has the capacity to increase its \( \text{Ca}^{2+} \) load in response to an increase in frequency. In our experiments, at low frequencies (0.1–1 Hz) and near-physiological \( [\text{Ca}^{2+}]_o \) (1 mM) the SR \( \text{Ca}^{2+} \) load is far from saturated, so that there can be a large stimulation of SR \( \text{Ca}^{2+} \) loading as frequency is increased (Fig. 2E). This could more than compensate for the fall in fractional SR \( \text{Ca}^{2+} \) release as frequency is
increased (mechanism 3), so that the amount of Ca\textsuperscript{2+} released from the SR will increase (Fig. 2C) and twitch force will be potentiated (Fig. 2A). As frequency is increased further, however, the capacity of the SR to increase its Ca\textsuperscript{2+} load will be progressively reduced (see below) and the reduced fractional release will then tend to dominate, resulting in a leveling off, and then a fall, in net Ca\textsuperscript{2+} release. This type of mechanism could therefore explain both the positive and negative components of the force-frequency relationship observed at 1 mM [Ca\textsuperscript{2+}]\textsubscript{i} (Fig. 1B). At 8 mM [Ca\textsuperscript{2+}]\textsubscript{i}, the SR Ca\textsuperscript{2+} loading may approach the maximal value at comparatively low frequencies (Fig. 2F). As a result, SR Ca\textsuperscript{2+} release, and hence force, are high even at low frequencies (Fig. 2, A, C, and F). Because the SR cannot further load with Ca\textsuperscript{2+} (Fig. 2F), the dominant influence as frequency is increased is now the reduction in fractional SR Ca\textsuperscript{2+} release. The net effect is therefore that SR Ca\textsuperscript{2+} release decreases with increasing frequency and force is reduced. It is possible that the negative force-frequency relationship seen in other studies is caused by the presence of a high SR Ca\textsuperscript{2+} load even at the lower frequencies used in those studies. The reason for this difference is unknown, but it could be caused by a higher resting [Na\textsuperscript{+}] in the other studies.

From our experiments it is not clear what limits the capacity of the SR to load up with Ca\textsuperscript{2+} at higher frequencies. One possibility is that the SR Ca\textsuperscript{2+} load reaches the thermodynamic equilibrium between intras-R [Ca\textsuperscript{2+}] and cytosolic [Ca\textsuperscript{2+}] that is determined by the free energy of ATP hydrolysis (34). However, we observed that the SR Ca\textsuperscript{2+} content at 8 mM [Ca\textsuperscript{2+}]\textsubscript{i}, remained relatively constant with increasing frequency (Fig. 2F) despite an increase in diastolic [Ca\textsuperscript{2+}] (Fig. 2D). This suggests that a thermodynamic equilibrium is not achieved even at lower frequencies (<2 Hz). An alternative explanation is that net SR Ca\textsuperscript{2+} uptake may become limited by the reduced time available for uptake, because at 6 and 8 Hz the next Ca\textsuperscript{2+} transient occurs well before the decline of the previous Ca\textsuperscript{2+} transient has been completed (Fig. 1C).

Frequency-dependent decline of Ca\textsuperscript{2+} transient. It was shown previously, for frequencies below the physiological range, that the rates of decline of force and of the Ca\textsuperscript{2+} transient during the twitch increase with increasing stimulation frequency (see, e.g., Refs. 15, 21, and 32). Here we show that this is true at higher frequencies encompassing the physiological range for the rat (Fig. 5). The shortening of the Ca\textsuperscript{2+} transient at higher frequencies probably accounts for the shortening of the twitch, because both changes occur with similar time courses after a change of frequency (Fig. 5). This abbreviation of the twitch is likely to be very important in vivo, because it would allow time for adequate diastolic filling of the heart at higher frequencies.

The mechanism of the shortening of the Ca\textsuperscript{2+} transient remains unclear. Some of the shortening was caused by the decrease in the time to peak [Ca\textsuperscript{2+}], which may reflect an enhanced Ca\textsuperscript{2+} sensitivity of the SR Ca\textsuperscript{2+} release mechanism when the SR load is high (30). Most of the shortening was, however, caused by a faster decline of the Ca\textsuperscript{2+} transient, which in the rat myocardium is dominated by the SR Ca\textsuperscript{2+}-ATPase activity. One possibility is that the frequency-dependent increase in time-averaged [Ca\textsuperscript{2+}], may lead to activation of CaMKII, which would enhance the rate of SR Ca\textsuperscript{2+} uptake by phosphorylation of phospholamban (3, 32). The relevance of this mechanism is controversial, however, because inhibition of CaMKII has been found to prevent this phenomenon in some studies (see, e.g., Refs. 3 and 28) but not others (see, e.g., Ref. 16 and Fig. 6 in the present study). Furthermore, there is evidence that the frequency-dependent shortening of the Ca\textsuperscript{2+} transient does not involve the phosphorylation of phospholamban (21) and, indeed, persists in cardiac myocytes isolated from phospholamban-knockout mice (27).

Another explanation for the shortening of the Ca\textsuperscript{2+} transient, proposed by Bers and Berlin (6), is that it does not depend on changes in properties of the SR Ca\textsuperscript{2+} pump but merely reflects the characteristics of the stimulation of the pump by Ca\textsuperscript{2+}, i.e., the greater [Ca\textsuperscript{2+}] during the twitch stimulates SR Ca\textsuperscript{2+} uptake in a nonlinear (Michaelis-Menten) fashion. Several observations in the present study support this explanation.
1) The half-width of the Ca\textsuperscript{2+} transient was inversely related to peak systolic [Ca\textsuperscript{2+}] during the transitional periods (Fig. 5D).
2) The fall of half-width reached a minimum at ~6 Hz (Fig. 5A), at which systolic [Ca\textsuperscript{2+}], but not diastolic [Ca\textsuperscript{2+}], reached a maximum (Fig. 1D).
3) The initial declination phase of the Ca\textsuperscript{2+} transients at the different frequencies appeared to follow a common pathway (Fig. 1C), as would be predicted if SR Ca\textsuperscript{2+} uptake depended only on the instantaneous [Ca\textsuperscript{2+}]. However, the relevance of the Bers and Berlin hypothesis is questionable because acceleration of Ca\textsuperscript{2+} decline with increasing frequency persists when there is no increase, or even a decrease, in peak systolic [Ca\textsuperscript{2+}] (see, e.g., Ref. 15 and present results with 8 mM Ca\textsuperscript{2+} (data not shown)). It is clear that further experiments are required to elucidate the mechanisms involved in the rate-dependent acceleration of Ca\textsuperscript{2+} decline.

In conclusion, rat ventricular trabeculae demonstrated a distinct positive force-frequency relationship for a wide range of frequencies encompassing the physiological range. Assessment of the Ca\textsuperscript{2+} transient with fura PE 3 showed that the positive force-frequency relationship could be attributed to a frequency-dependent increase in the Ca\textsuperscript{2+} transient because of greater loading of the SR with Ca\textsuperscript{2+}, as described for other species. The physiological significance of these results are apparent, i.e., the greatest forces are produced at physiological frequencies, at which relaxation between beats is aided by the frequency-dependent decrease in twitch duration. The mechanisms behind the frequency-dependent shortening of the time course of the twitch and Ca\textsuperscript{2+} transient remain to be elucidated.

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