Force Velocity Relations of Single Cardiac Muscle Cells

Calcium Dependency

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ABSTRACT Cellular cardiac preparations in which spontaneous activity was suppressed by EGTA buffering were isolated by microdissection. Uniform and reproducible contractions were induced by iontophoretically released calcium ions. No effects of a diffusional barrier to calcium ions between the micropipette and the contractile system were detected since the sensitivity of the mechanical performance for calcium was the same regardless of whether a constant amount of calcium ions was released from a single micropipette or from two micropipettes positioned at different sites along the longitudinal axis of the preparation. Force development, muscle length, and shortening velocity of either isometric or isotonic contractions were measured simultaneously. Initial length, and hence preload of the preparation were established by means of an electronic stop and any additional load was sensed as afterload. Mechanical performance was derived from force velocity relations and from the interrelationship between simultaneously measured force, length, and shortening velocity. From phase plane analysis of shortening velocity vs. instantaneous length during shortening and from load clamp experiments, the interrelationship between force, shortening, and velocity was shown to be independent of time during the major portion of shortening. Moreover, peak force, shortening, and velocity of shortening depended on the amount of calcium ions in the medium at low and high ionic strength.

INTRODUCTION

In chemically skinned cardiac fibers (Winegrad, 1971; Winegrad, 1973; Solaro et al., 1974) and electrically stimulated papillary muscle (Brutsaert et al., 1973; Goethals et al., 1975) tension and maximum unloaded velocity of shortening have been shown to be sensitive to calcium. Yet the exact contribution of calcium in controlling these contractile events is still unclear, as studies on the mechanical behavior of these multicellular preparations are complicated by several factors such as unpredictable structural heterogeneity (Winegrad, 1974) and high compliance, resulting from branching (Muir, 1965), spiral arrangement of the fibers (Rodbard, 1970), damage of tissue adjacent to the clips (Krueger and Pollack, 1975), and shearing through Z-lines and intercalated disks (Abbott and Gordon, 1975). Only recently, less complex single cardiac cells (Fabiato and Fabiato, 1972;

Fabiato and Fabiato, 1975*a, b*; Bloom, 1970; Bloom et al., 1974; Kerrick and Best, 1974) with either nonfunctional or skinned (Fabiato and Fabiato, 1975*a, b*) sarcolemma were obtained, but the mechanical properties of these preparations have been only incompletely characterized. Mechanical analysis of contraction of heart muscle requires consideration of at least four variables: force, length during shortening, velocity of shortening, and time after activation. Yet simultaneous measurement of these variables has not proved technically feasible in isolated cardiac cells.

In the present study the mechanical performance of manually dissected single adult cardiac cells was characterized by the simultaneous measurement of force, length, and velocity of shortening of pre- and afterloaded isotonic and isometric contractions. Uniformity and reproducibility of the contractions was ascertained by activating the preparations with calcium ions released by iontophoresis in the presence of EGTA buffering. The sensitivity to calcium of the mechanical variables was examined by the iontophoretic release of various amounts of calcium ions.

MATERIALS AND METHODS

Solutions and Perfusion Chamber

The following solutions were used (Fabiato and Fabiato, 1972). Solution 1 (mM): NaCl, 132; $MgCl_2 \cdot 6H_2O$, 4; Na_2ATP, 5; glucose, 7; EGTA, 25 × 10^{-3}; imidazol, 18; no calcium was added, pH 7.0. Spontaneous contractions occurred in this solution. Solution 2 (mM): NaCl, 132; $MgCl_2 \cdot 6H_2O$, 4; Na_2ATP, 5; glucose, 7; EGTA, 125 × 10^{-3}; imidazol, 18; pH 7.0. This solution was the same as Solution 1 but EGTA was augmented to abolish spontaneous activity. Experiments were carried out at room temperature. pH was constantly checked by adding phenol red (±5 × 10^{-6} M).

NaCl, $MgCl_2 \cdot 6H_2O$, $CaCl_2 \cdot 2H_2O$, glucose, EGTA, and imidazol were analytical grade reagents obtained from Merck. Na_2ATP was low-calcium ATP obtained from Sigma Chemical Co., St. Louis, Mo. $SrCl_2 \cdot 6H_2O$ was obtained from Riedel-De Haën, A. G. Caffeine was obtained from Merck. When solutions were prepared care was taken to avoid calcium contamination. All solutions were made with double-distilled water. Glassware was specially treated and stored apart from other glassware used in the laboratory. pCa of the solutions was calculated by means of a computer program (Reuben et al., 1971) in which the following apparent stability constants were used (pH 7.0, 22°C): CaEGTA, 4.9 × 10^6 M^{-1} (Schwartzenbach et al., 1957); MgEGTA, 40 M^{-1} (Portzehl et al., 1964); CaATP, 5 × 10^3 M^{-1} (Nanninga, 1961); MgATP, 11.4 × 10^3 M^{-1} (Nanninga, 1961). For an assumed calcium contamination between 5 × 10^{-6} M and 5 × 10^{-5} M, the values were calculated as in Table I.

A depression slide was adapted as an overflow perfusion chamber with infusion (connected to a Harvard infusion withdrawal pump) at the right upper corner and suction by capillary forces at the left lower corner.

Dissection of Preparation

To assure maximum functional integrity manual dissection was preferred over homogenization or enzymatic disruption as previously used (Fabiato and Fabiato, 1972; Fabiato and Fabiato, 1975*a, b*; Bloom, 1970; Bloom et al., 1974; Kerrick and Best, 1974).

Adult rats were decapitated and their hearts were excised quickly and washed in Solution 1. A ventricular strip or a papillary muscle was dissected. For further dissection

the papillary muscle had the advantage of a more or less parallel alignment of its fibers. Dissection was continued in Solution 1 with glass tools. A thin tissue strip was then transferred to the perfusion chamber on the gliding stage of a Reichert-Biovert microscope (×160) for microdissection. This multicellular muscle strip contracted cyclically in Solution 1 with asynchronous contractions spread over the entire strip. In Solution 2, the strip became totally quiescent. The strip was immobilized against the bottom by two glass microtools, made with a de Fonbrune microforge, and mounted on micromanipulators. With a third micromanipulator with microtool, the eventual cellular preparation (Fig. 1) was dissected. Again no cyclical activity was observed in Solution 2, but cyclical contractions reappeared after changing to Solution 1 or to a perfusion medium without EGTA or by adding $CaCl_2$. The time between the change to the new solution and the start of spontaneous activity was dependent on the duration of the preceding perfusion with Solution 2. A few preparations were isolated by homogenization (Fabiato and Fabiato, 1972) (VirTis blender, VirTis Co., Gardiner, N. Y.) instead of manual dissection; as described previously (Fabiato and Fabiato, 1972) these preparations showed cyclical contractions in Solution 1. These preparations were not used for further mechanical analysis. Cells separated by enzymatic disruption (trypsin 0.1%) (Fabiato and Fabiato, 1972) were not suitable for the study of muscle mechanics.

TABLE I

EFFECTS OF ASSUMED CALCIUM CONTAMINATIONS OF SOLUTIONS

Solutions	Assumed calcium contamination	Free EGTA	pCa	pMg
	(M)	(M)		
1	5×10^{-6}	2.01×10^{-5}	7.33	3.58
	10^{-5}	1.57×10^{-5}	6.92	3.58
	5×10^{-5}	1.32×10^{-6}	5.44	3.58
2	5×10^{-6}	1.19×10^{-4}	8.07	3.58
	10^{-5}	1.14×10^{-4}	7.75	3.58
	5×10^{-5}	7.5×10^{-5}	6.86	3.58

Table II shows the basic characteristics of the 20 most suitable out of a total of 124 preparations in which satisfactory results were obtained in this study. Total force development (f_0) at l_{max} amounted to 530 ± 50 µg (MSE). This force is similar to the value of 570 ± 40 µg reported by Fabiato and Fabiato (1975a). Since the thickness of our preparations was not measured, these force values were not normalized for cross-sectional area. Peak velocity of shortening (0.35 ± 0.03 muscle lengths/s at l_{max} preload) was lower in these single cells at room temperature than a corresponding measurement (2.90 ± 0.25 muscle lengths/s) in rat papillary muscle at higher temperatures (Henderson et al., 1969).

Length Transducer (Fig. 2)

Length was measured by a television (tv) tracking technique. This technique was modified after a similar television tracking system previously described (Intaglietta and Tompkins, 1972). The image of the preparation was recorded by a tv camera through a beam-splitting device incorporated in the microscope. The video signals were processed in a control unit and visualized on a tv monitor. The control unit generated an X coordinate on the tv screen by means of a timing circuit (one-shot multivibrators) triggered by the line-sync pulses. The Y coordinate was generated in the same way, but triggered by the frame-sync pulses. These coordinates controlled a reference point which was visualized as a small black rectangle. The coordinates were manually set to focus the tracking on the

most favorable portion of the picture, thereby increasing the signal-to-noise ratio and ensuring a stable tracking which was a prerequisite for a reliable force measurement. When the scanning spot reached the reference point, a trigger pulse was generated to set a flip-flop. Reset action occurred when the video signal exceeded a preset level, e.g. white
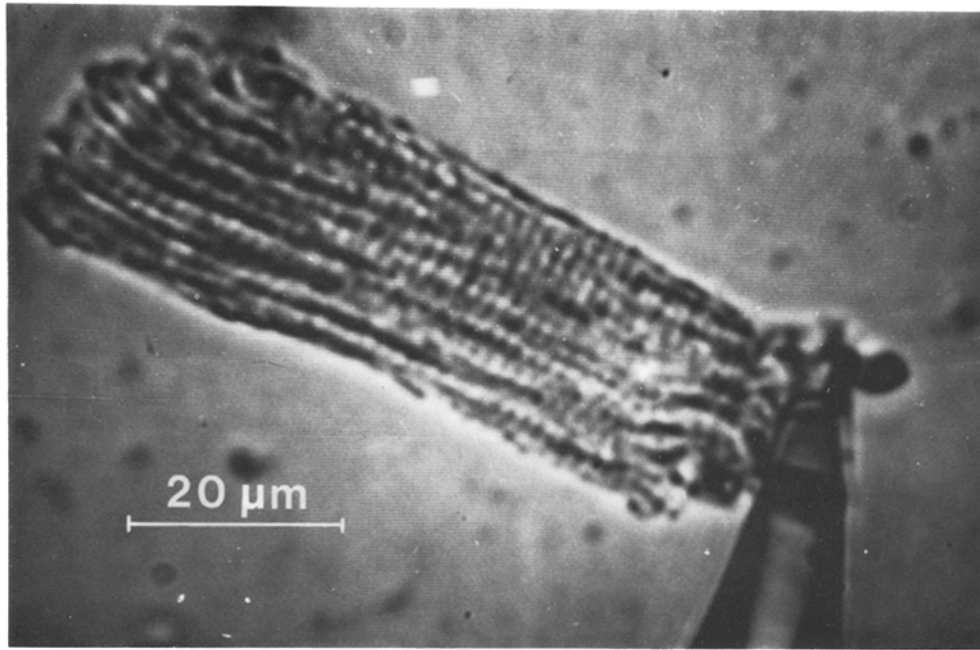

FIGURE 1.   Photographic illustration of a representative, freshly dissected preparation obtained by microdissection under microscope.

TABLE II

BASIC CHARACTERISTICS OF THE 20 MOST SUITABLE PREPARATIONS USED FOR THIS STUDY

	Length	Width	Peak velocity of shortening of entire preparation		Total force (f_0)	Ratio of preload to f_0	Time to peak shortening	Time to peak velocity	Time to peak force
	μm	μm	$\mu m/s$	lengths/s	μg		ms	ms	ms
Mean ± SE	80±4	16±1	26±2	0.35±0.03	530±50	0.14±0.01	3,800±350	670±50	3,400±600
n	20	20	20	20	10	10	17	15	7

All contractions were recorded with a preload at l_{max}, i.e. the length at which the preparation developed maximal force. The ratio of the preload at l_{max} to f_0 is comparable to known values in more complex cardiac preparations in literature, in both cat (Brutsaert et al., 1973; Brutsaert and Claes, 1974) and rat (Henderson et al., 1969). The dimensions of the preparations along with this ratio were used as criteria for selection of the preparations. n indicates the number of preparations.

to black transition at the adhering site of the mobile microtool to the preparation. The output pulse from the flip-flop was converted into a voltage proportional to the pulse length and was memorized in a sample-and-hold circuit. The time between set and reset (e.g., measured length) was depicted on the screen as a black (or white) line connecting reference point and site of measurement, thus providing a visual control of the tracking quality and the area of measurement. Length was sampled at each frame in the course of

one line time. The number of samples corresponded to 50 frames/s. In order to improve the frequency response, especially for proper operation of the force sensing system, the time base frequency of both tv camera and monitor was increased to 100 frames/s. Although line frequency was unchanged, quality of the picture was acceptable. The signal from the time-to-voltage converter was filtered by a second-order Butterworth filter (cut-off frequency 5 c/s) to make smooth stepwise transitions in the length signal.
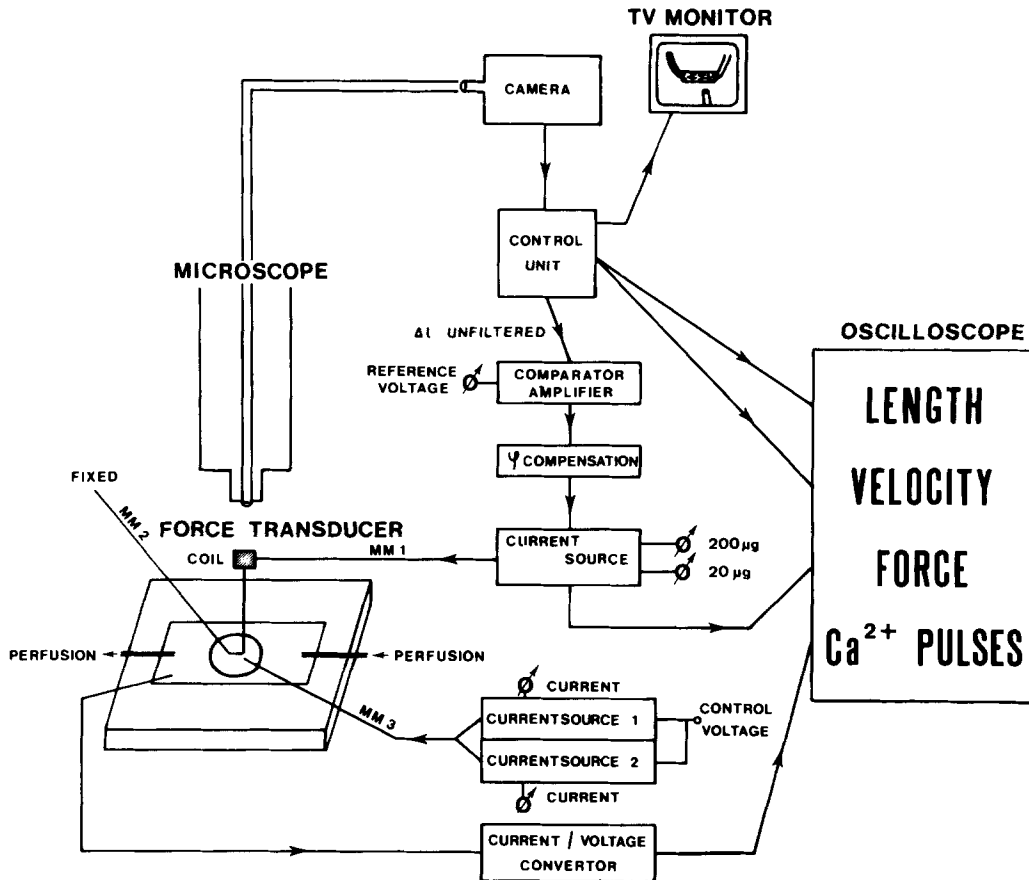

FIGURE 2. Diagrammatic illustration of the experimental set-up (see text). *MM*, micromanipulator. Iontophoretic micropipettes (one or two) were mounted on *MM*3.

Velocity of Shortening and of Lengthening

The nonfiltered length signal was composed of discrete steps. Every 10 ms, voltage changed abruptly and remained on the established level in the sample-and-hold circuit. Velocity was evaluated by subtracting the preceding voltage level from the next. The former level was memorized by a second sample-and-hold circuit of which the acquisition time occurred a few milliseconds before the next length sampling. The two voltage levels were thus memorized, and the velocity could be computed as

$$\frac{dl}{dt} = \lim_{\Delta t \to 0} \frac{\Delta l}{\Delta t} \simeq \frac{l_2 - l_1}{\Delta t},$$

with l_2 next length value, l_1 preceding value, t small increment of time (10 ms). The velocity signal was amplified and scaled. Filtering was the same as for the length signal in order to avoid distortion of the phase-plane analysis of velocity vs. length relationship and to make smooth stepwise transitions. An output of 100 mV corresponded to a velocity of 25 μm/s.

Force Generation

A movable microtool was fixed on a tiny lever with a droplet of paraffin. This lever was an extended pointer of a miniature panel meter, Eagle SB 11 (Eagle-Picher Industries, Inc., Joplin, Mo.) (sensitivity = 100 μA; internal resistance = 600 Ω) with center zero, mounted on a micromanipulator. This electromagnetic force generator had sapphire bearings with negligible friction as measured at the tip of the lever. The small change in force which occurred as a function of displacement was due to two spiral springs which conducted the electric current to and from the coil and which held the lever in midposition. The compliance of these springs was only 4 μm/μg. A current source controlled the current through the coil and hence the load imposed on the preparation. The current was calibrated and set by decade switches in steps of 20 μg and 200 μg up to a total of 1,980 μg. Load clamp experiments were performed by switching the current to a second current level controlled by a second set of decade switches.

Force Measurement

Simultaneous measurement of force was achieved by feedback control of the current through the coil of the force generator. This feedback force-sensing system consisted of the length transducer, a unidirectional comparator and amplifier, a phase compensation circuit, the current source, and the force generator (Fig. 3). The unfiltered length signal was compared to a preset reference level. As long as this signal was higher than the preset level, no feedback action occurred, and the preparation carried the full load set by the decade switches. As the preparation lengthened the signal level decreased. When it became equal to the preset level, current through the coil was decreased by the feedback action, which tended to keep the microtool at the length established by the reference level. This voltage level functioned as an "electronic stop," visualized on the tv screen as a small white line, and acted in an analogous way as a mechanical stop in classical muscle lever systems. By changing the reference voltage, the stop could be "moved." By adjusting the stop, resting length and hence the preload of the preparation were established; any additional load represented afterload. Load carried by the preparation was measured by monitoring the voltage applied to the current source driving the coil. To avoid oscillation in a feedback system, loop gain must be smaller or equal to unity at the frequency where the phase lag becomes 180°. The phase compensation extended this critical frequency and ensured the stability of the feedback loop. Gain and frequency roll-off of the loop were adjusted to obtain good static and dynamic behavior. The open loop gain determined the static compliance of the lever held against the stop. Ideally, no displacement should occur when a load is imposed on the lever which is pushed against the stop. Due to the finite open loop gain, there was a small displacement proportional to the imposed load. The resulting static compliance amounted to 6 μm/mg. The roll-off point influenced the dynamic compliance, i.e. a displacement proportional to the rate of force development by the muscle preparation because the loop gain declines at higher frequencies. At optimal phase compensation, a displacement of 12 μm was obtained when a rate of force change of 1 mg/s was imposed, resulting in a small but transient (5 μm for 440 μg/s in most isometric contractions) length change at the onset of force development in the isometric contractions when the rate of force development was greatest (see also Fig. 4B, contraction d). Further extension of the phase shift correction was not possible

without the risk of oscillations, because the phase lag became excessive at still higher frequencies due to the vidicon lag (smearing of the picture) and the sampling system.

Iontophoretic Current Source

As in homogenized preparations (Fabiato and Fabiato, 1972), manually dissected cells could not be paced electrically by field stimulation, and contractions occurred equally well despite equimolar substitution of Na^+ by K^+ in the perfusion solution. In the present study synchronous, uniform, and reproducible mechanical activation of the preparation
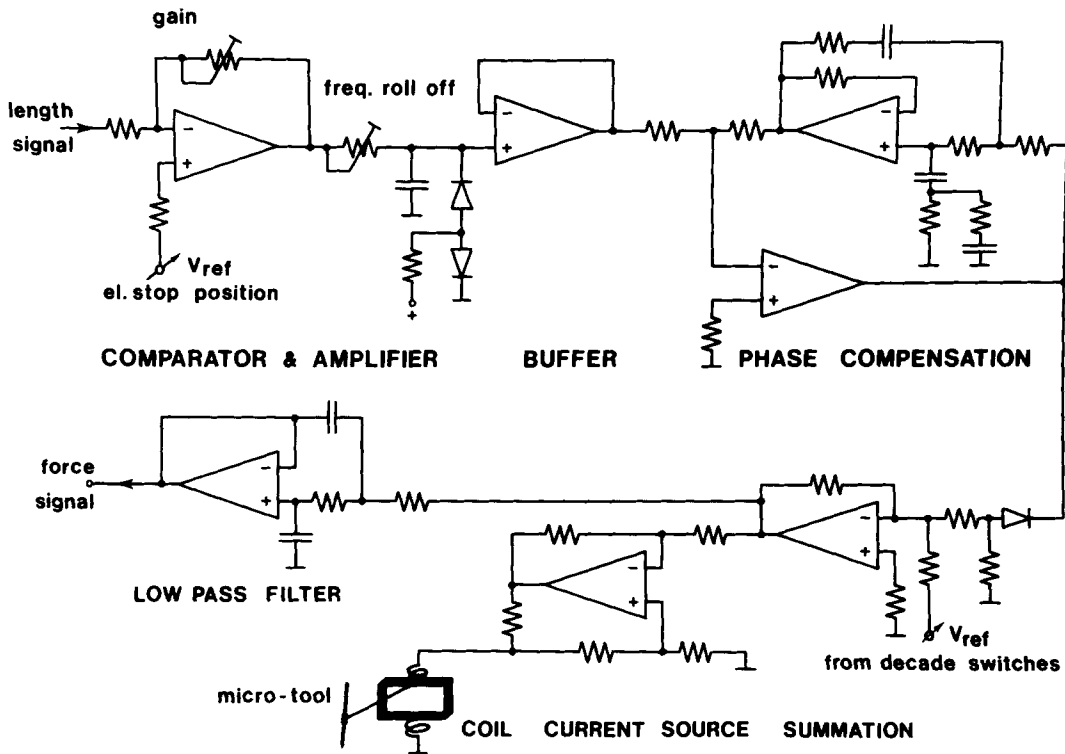

FIGURE 3. Feedback force sensing system.

was obtained by means of calcium pulses. These pulses were applied to the preparation by iontophoresis (Krnjevic et al., 1963) from micropipettes filled with calcium chloride (0.1 M). The micropipettes were pulled with a vertical microelectrode puller (David Kopf Instruments, Tujunga, Calif.) and filled by boiling at low pressure. One or two micropipettes were mounted on a micromanipulator and connected to the current source by a silver wire. Iontophoretic ejection of calcium ions from these micropipettes was controlled by small electric pulses. The current must be stable in the course of an experiment despite large differences in the electric resistance of the micropipettes containing the calcium ions. Accordingly, the output impedance of the current source must be high, i.e. preferably 100 times larger than the resistance of the micropipettes, and the source must have a high voltage compliance in order to overcome the large voltage drops across the micropipettes. A modified Howland current source, which combined operational amplifier performance with high voltage and ultra low leakage transistors, showed an output impedance in excess of 10 GΩ and a voltage compliance of 130 V. The current could be

set with a 10-turn potentiometer up to 1 μA. The source was switched on and off by a control pulse from a stimulator (Grass S4, Grass Instrument Co., Quincy, Mass.). The current through the micropipette could be monitored with an FET operational amplifier connected as a current-to-voltage converter, scaled to deliver 1V/μA; the input of this circuit was connected to a silver reference electrode mounted in the perfusion chamber.

In most experiments calcium pulses of different amplitudes but of a constant duration of 500 ms were used. The amount of released calcium ions was expressed as the product of measured current (nA) i.e. the amplitude of the pulse, and the duration of the pulse (500 ms).

RESULTS

Iontophoretically Triggered Contractions

As spontaneous cyclical contractions observed in Solution 1 were not reproducible for a given loading and contractile state, these contractions were not suitable for further mechanical analysis. Reproducible contractions were elicited by iontophoretic release of calcium ions under continuous perfusion with Solution 2 (Fig. 4 A). Each calcium pulse was immediately followed by a single contraction. Upstroke and amplitude were uniform and reproducible for a given amount of calcium released (nA × s). No spontaneous activity was seen between these triggered contractions. Contraction a was induced by a calcium pulse with a duration of 500 ms and an amplitude sufficiently high to induce a maximal response of the preparation. With a diminished calcium pulse, developed isometric force in contraction b was correspondingly lower. Contraction c was again elicited by a maximal calcium pulse and the same tension as in contraction a was resumed. No differences of the contractile response were seen when the time interval between the pulses was altered. Also, after long resting periods no differences were observed between the first triggered contraction and subsequent beats triggered by the same amount of calcium. These findings indicated an absence of frequency potentiation, postextrasystolic potentiation, and post-rest potentiation. When the frequency of the calcium pulses was augmented beyond the point where the preparations could fully relax, summation and eventually a contracture-like response ensued (contraction d). The maximum amplitude of this response did not significantly exceed peak isometric force in twitch contractions triggered by a single maximal calcium pulse.

Force Velocity Relation

Twitch contractions with different loadings are illustrated in Fig. 4 B. Contraction a was an isotonic contraction shortening with preload only. Contractions b and c shortened with increasing afterloads, the preload being maintained at a constant value throughout the experiment. Contraction d was an isometric contraction. Peak velocity of shortening decreased with increasing afterloads describing a classical force velocity relation (Fig. 4 C). Similar force velocity curves were obtained in 10 preparations. In contrast to observations in more complex cardiac muscle preparations (Parmley et al., 1969), adjustment of the load to a new value always resulted in an immediate, appropriate, and reproducible response of the first subsequent triggered contraction without any transitory contractile performance due to some long term memory for the loading condi-

tions of the preceding beats. Since force velocity analysis of twitch contractions is limited by the continuously changing length and time (Brutsaert, 1974), the study of contractile performance was further extended to an analysis of length and time. In Fig. 5 two afterloaded contractions were superimposed. For both contractions, force, velocity of shortening, and length were recorded as a
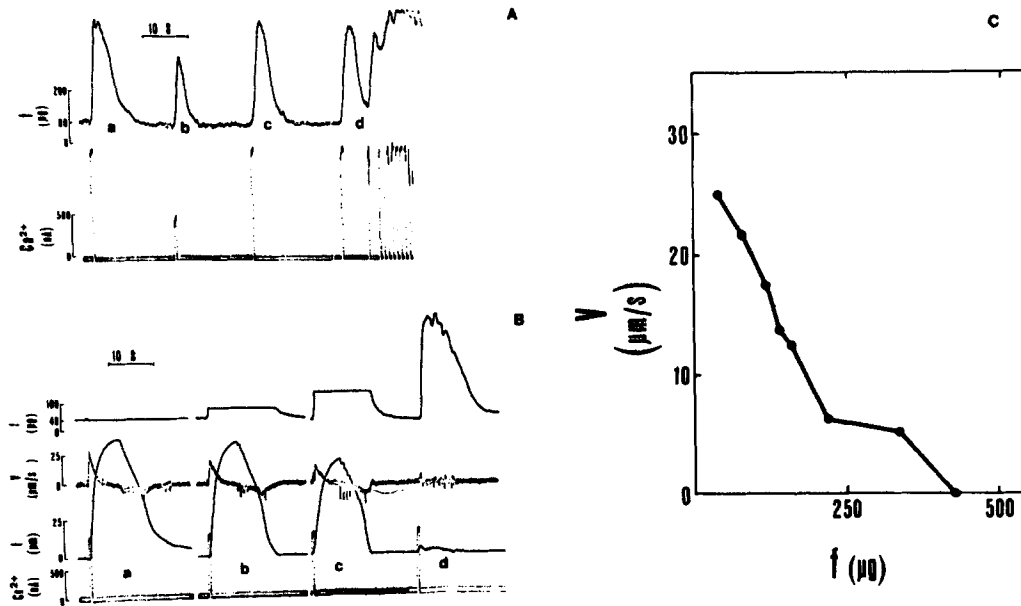

FIGURE 4.   A, Iontophoretically triggered isometric contractions. *Lower*, Calcium pulses of 500-ms duration (Ca^{2+}); *upper*, isometric force (f). Initial muscle length was set by the preload at l_{max}; isometric conditions were obtained by increasing the afterload. *Contr.* (a), isometric twitch at a maximal calcium pulse; *contr.* (b), instantaneous and proportional response to a smaller calcium pulse; *contr.* (c), isometric twitch when maximal calcium pulse was resumed; *contr.* (d), summation and contracture-like response when frequency of calcium pulses was increased. B, Triggered isotonic contractions at increasing afterloads. *Lower*, Calcium pulses (Ca^{2+}); *middle*, shortening (l) and shortening velocity (v); *upper*, force (f). *Contr.(a)*, isotonic contraction at l_{max} preload; *contr.* (b) and (c), isotonic contractions with increasing afterloads; *contr.* (d), isometric contraction. C, Peak velocity of shortening (v) plotted as a function of load (f). The ratio of the preload to f_0 at l_{max} preload amounted to only 9% (see also Table II).

function of time and simultaneously as phase plane analysis of the velocity of shortening as a function of instantaneous length during shortening. Initial length of the preparation was readjusted during the interval between the two contractions by displacing the electronic stop, thus changing the initial length by altering the preload:afterload ratio while maintaining total load constant. Both phase plane velocity length tracings coincided except for the initial velocity rise. In contrast, shortening tracings as a function of time were distinctly dissociated. For the same total load at a given length (e.g., at arrow) during shortening, velocity of shortening of both contractions was identical despite a difference in

time of 125 ms. This time independency of the interrelationship between force, length, and velocity during the major portion of the shortening phase was also shown in experiments in which load alterations, i.e. load clamps, were imposed on the shortening muscle (Fig. 6). After the clamp to the higher load, the phase plane velocity length trajectory of the load-clamped contraction coincided with the phase plane of the control contraction carrying that same load from the onset of shortening, and this despite a distinct dissociation of the shortening
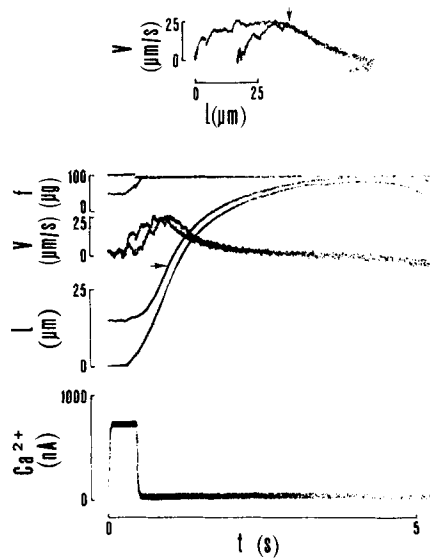

Figure 5. Fast sweep time tracings of force (f), velocity of shortening (v), shortening (l), and calcium pulse of two superimposed isotonic contractions, shortening with the same total load from two different initial lengths. In the contraction starting from the longer length, total load equalled the preload at l_{max}. In the second contraction for the same total load, preload to afterload was appropriately altered by adjusting the electronic stop (see force tracing). The simultaneously recorded phase plane velocity length tracings are illustrated above. The phase of relaxation is not shown. The arrow indicates an arbitrarily chosen length illustrating the time independency of the interrelationship between force, velocity, and length (see text).

time tracings of both contractions. Similar load clamps performed at various instants during shortening showed this time-independent force-velocity-length relationship to pertain throughout a major portion of the shortening phase.

Effect of Calcium

Increasing amounts of calcium ions ($nA \times s$) released by single calcium pulses enhanced peak force of isometric twitch contractions, and peak shortening and velocity of shortening of isotonic preloaded twitch contractions at l_{max} (Fig. 7). Force, shortening, and shortening velocity reached a maximum which could not be exceeded by further augmenting the amplitude of the single calcium pulses. This value was taken as 100%. The values obtained by imposing a high-fre-

quency train of maximal calcium pulses were similar. This latter value for force is indicated by the open circle symbol in Fig. 7.

Force velocity relations were obtained at three different activation levels, i.e. 50%, 75%, and 100% of the curve relating force to increasing amounts of released calcium ions (Fig. 8). Each point on the force velocity curves represented an average velocity of three contractions. The curves shifted to the right
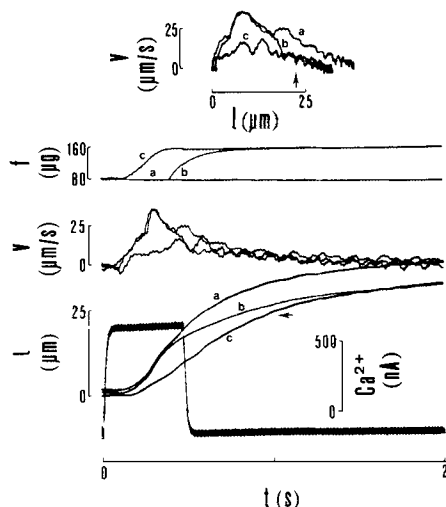

FIGURE 6.   Load clamp experiment: fast sweep time tracings of force (f), velocity of shortening (v), shortening (l), and calcium pulse (Ca^{2+}) (lower) and phase plane velocity length relations (upper) of three superimposed isotonic contractions; phase of relaxation is not shown; contractions (a) and (c) were control contractions at l_{max}; contraction (a) shortened with the preload only (80 μg); contraction (c) shortened with an additional afterload of 80 μg; in contraction (b), after an initial shortening with preload only for about 400 ms, the load was augmented to a total load of 160 μg. In order to avoid detachment of the force microtool from the preparation by a too abrupt load clamp, the transition from the lower to the higher load was imposed smoothly. The arrow, pointing to an arbitrarily chosen length during contractions (b) and (c) where their respective load had become the same, indicated that at the same length and for this same total load, velocity of shortening was the same on the phase plane velocity length tracing, despite a distinct difference in time of 180 ms on the shortening time tracings. All three contractions were triggered with supramaximal calcium pulses. Dimensions of the preparation were 16 $\times$ 66 μm.

with increasing calcium. When the curves were plotted in a normalized way as a function of the relation of the actual force (f) in the afterloaded contractions to total peak isometric force (f_0), a distinct divergence of the curves towards lower loads was observed.

On augmenting the concentration of NaCl from 132 to 200 mM in Solution 2, thus increasing the ionic strength of the medium by about 40%, calcium sensitivity of force, shortening, and velocity of shortening was markedly depressed (Fig. 7) as was evident from the diminished slope of the three curves and from the depressed maximal force obtained with a high-frequency train of calcium pulses.

Effect of Double vs. Single Calcium Pulses and the Morphology of the Calcium Pulses

Iontophoretically released calcium ions reached the preparation after a short time lag depending on the position of the micropipettes. The micropipettes were
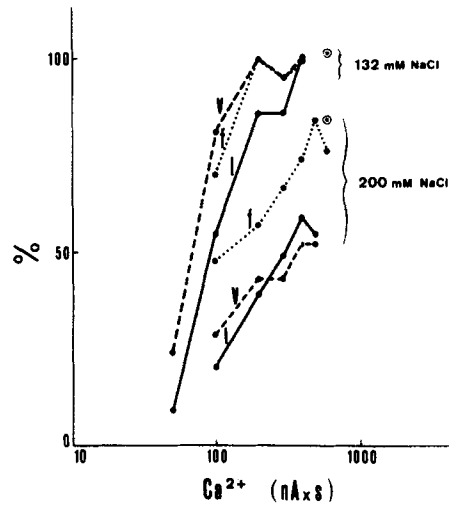

FIGURE 7. Force (f), shortening (l), and velocity (v) expressed as percentage of the maximum attained value were plotted as a function of the released calcium ions (Ca^{2+}). The upper curves were from contractions in Solution 2; in the lower curves ionic strength was increased by adding 68 mM NaCl. The symbol ⊙ indicates the force value obtained with a high-frequency train of calcium pulses in both conditions.
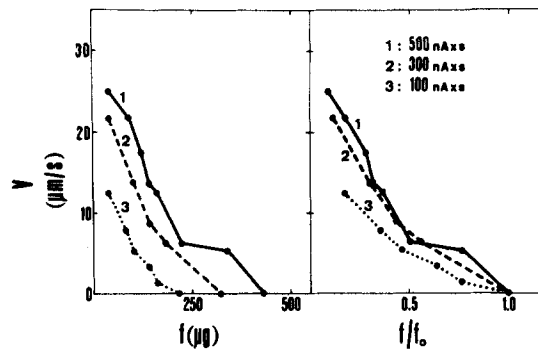

FIGURE 8. Force peak velocity relations at three different degrees of activation, i.e., at 50% (curve 3), 75% (curve 2), and 100% (curve 1) of the force development obtained with maximal calcium pulses (500 nA × s). Force was expressed both in absolute values (left) and after normalization for f/f_0.

always placed perpendicularly to the preparation to facilitate diffusion of the calcium ions equally well to both ends of the preparation. In order to examine the contribution of diffusion, double calcium pulses were applied from two micropipettes positioned at different sites along the preparation (Fig. 9). The

same amount of calcium ions was released either through micropipette 1 or 2 or through both. No significant differences were seen among the effects of these three types of calcium pulses on force, rate of rise of force development, shortening, and velocity of shortening.
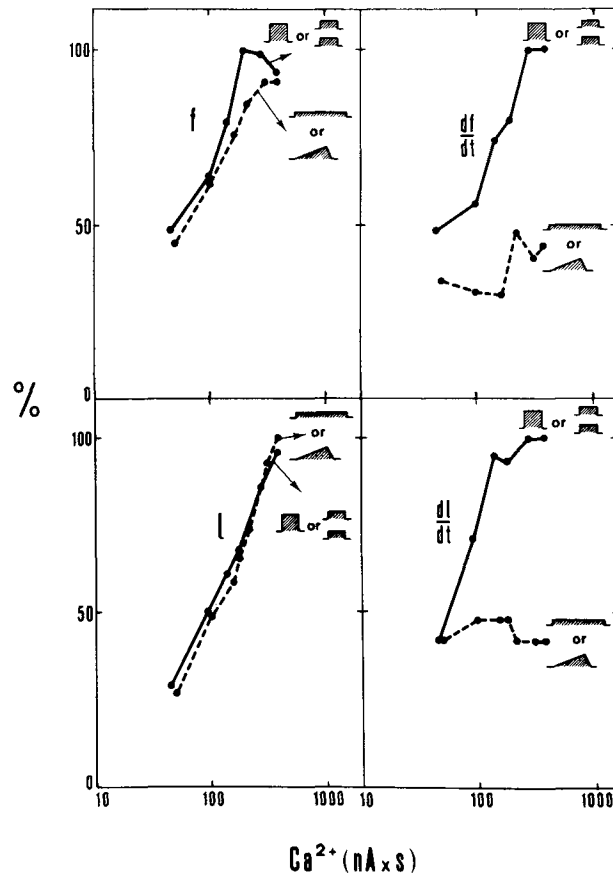

$$Ca^{2+}(nA \times s)$$

FIGURE 9. Calcium dependency of peak force (f)and peak rate of rise of force development (df/dt) of isometric twitch contractions, and of peak shortening (l) and peak velocity of shortening (dl/dt) of isotonic preloaded contractions at l_{max}. Control curves (●———●) were obtained by either single (🔲) or double (🔲🔲) calcium pulses for the same amount of calcium ions (nA × s). No significant differences were observed between the effects on the mechanical responses induced by both modes of calcium release. The dotted curves (●----●) were obtained by releasing given amounts of calcium ions (nA × s) either by slowly rising pulses (_◿◺_) or by long-lasting pulses of low amplitude (▱▱▱).

In addition, when contractions initiated either by double instead of single pulses or by single pulses of constant duration but increasing amplitude were analyzed at high sweep speed of the oscilloscope, variations of less than 50 ms were seen in the delay between the beginning of the calcium pulse and the onset of the contraction.

When, however, for a given amount of calcium ions the morphology of the pulses was altered by using slowly rising pulses or long pulses of extremely low amplitude, no differences were observed with respect to eventual peak force and peak shortening (Fig. 9). In contrast, in accordance to the delayed release of a given amount of calcium ions from the micropipettes, velocity of shortening and the rate of rise of force development had apparently and artifactually become less sensitive to alterations in the amount of released calcium.

Effects of Caffeine

Although the sarcolemma of the present preparations was not functional, an intact sarcoplasmic reticulum could still constitute a physiological diffusion barrier for the calcium ions along their diffusion pathway from the iontophoretic pipettes to the contractile sites. Blocking the uptake of the diffusing calcium ions by a remaining intact sarcoplasmic reticulum by adding caffeine to the solution would be expected to potentiate mechanical responses for any amount of released calcium ions, thus shifting to the left the curves relating this mechanical response to the amount of calcium ions. Yet, adding caffeine (5 or 10 mM) to 16 preparations always resulted in a symmetrical shift to the right. Although the maximum mechanical response to maximum calcium pulses was initially unchanged, a progressive depression of the preparation at any given amount of calcium ions was observed after long incubation in caffeine.

Since addition of caffeine can directly induce mechanical activation of skeletal (Weber, 1968; Weber and Herz, 1968; Endo et al., 1970) and cardiac (Fabiato and Fabiato, 1975a) muscle by releasing up to about 40% of the calcium stored in the sarcoplasmic reticulum, additional experiments with caffeine were carried out in this study to explore to what extent the sarcoplasmic reticulum had been loaded with calcium in the various solutions. If release of such stored calcium could be induced by the calcium pulses, it would act as an amplifier of these pulses. However, adding caffeine (5, 10, or even 25 mM) to Solution 2 did never activate the preparation. In contrast, after transition from Solution 2 to Solution 1 or to a solution without EGTA, adding caffeine resulted in an instantaneous mechanical activation. These responses became progressively more marked when caffeine was added later in time. These results would indicate that in Solution 2 the sarcoplasmic reticulum had been depleted of the calcium that can normally be released by adding caffeine, and that reloading with calcium would recover on transition to Solution 1 or to a solution without EGTA. The slow recovery of spontaneous activity in these latter solutions, which (see also Materials and Methods) depended on the time of perfusion with Solution 2, would also be in line with these observations. Moreover, when the preparations were perfused with Solution 2 with high EGTA (7 mM) in order more fully to deplete the sarcoplasmic reticulum of calcium and were then perfused with Solution 2, again no differences were observed between subsequent contractions activated by iontophoresis.

Strontium vs. Calcium Pulses

When one of the two iontophoretic micropipettes was filled with $SrCl_2$ (0.1 M) instead of $CaCl_2$ and equal amounts of current were imposed alternatively

through the two micropipettes, thus releasing equal amounts of ions, isotonic and isometric contractions induced by strontium pulses coincided with superimposed contractions obtained by calcium pulses (Fig. 10). The same results were obtained by pulsing one-half of a constant amount of ions through a calcium micropipette and the other half through the strontium micropipette.

DISCUSSION

Mechanical Performance of Cellular Preparations

Force development, length during shortening, and velocity of shortening were simultaneously measured in isotonic and isometric twitch contractions of single
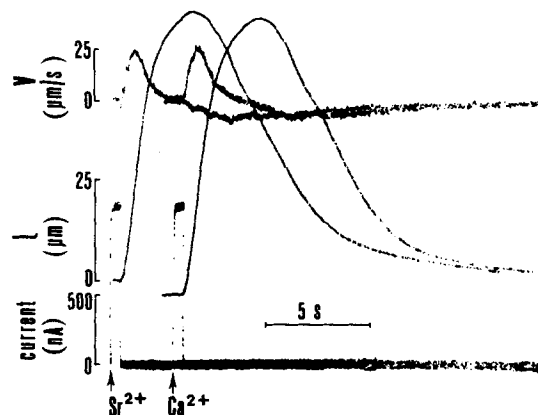

FIGURE 10.   Comparison between strontium- and calcium-triggered isotonic contractions. No differences were seen between onset of shortening, peak shortening, peak velocity of shortening, and the time course of both twitches. Preload was at l_{max}. The amplitude of both calcium and strontium pulses was sufficiently high to induce maximal responses of the preparation.

manually dissected mammalian cardiac cells. Twitch contractions were elicited by iontophoretically released calcium ions. The negligible difference between peak force obtained in isometric twitch contractions when single maximal calcium pulses were applied and peak force of contracture-like responses induced by increasing the frequency of the calcium pulses implied that peak isometric twitch force triggered by single maximal pulses was already at near full activation.

The interrelationship between force, velocity, length, and time was similar to this interrelationship in more complex heart muscle preparations (Brutsaert, 1974). Continuously changing length during shortening did not affect the force peak velocity relation in the present single cells, as peak velocity values occurred at nearly the same length during shortening regardless of the afterload. Although peak velocity at increasing afterloads occurred at progressively later times after the calcium pulse, and hence after the onset of activation, load clamp experiments and phase plane analysis of velocity length relations demonstrated that consideration of time at the instant where peak velocity was measured was less relevant. Over a major portion of the shortening phase, velocity of shorten-

ing at a given total load i.e. pre- and afterload, was determined by the instantaneous length during shortening, regardless of the time at which this length was attained and of the initial length from where the shortening started. Hence, the basic contractile properties of these single cardiac cells were comparable to the mechanical properties of more complex cardiac preparations (Brutsaert et al., 1971; Brutsaert, 1974).

Influence of Calcium Ions

Force, extent of shortening, and peak velocity of shortening varied with the amount of calcium ions released in the medium. The marked divergence towards lower loads of force velocity curves in which peak velocity of shortening was plotted as a function of normalized force, or f/f_0, for various amounts of released calcium ions, suggested a direct effect of calcium on maximum unloaded velocity of shortening (V_{max}). Although the presence of an unrecognized internal load (Wise et al., 1971; Julian, 1971) could not entirely be excluded despite this divergence, a surprisingly high internal load of about $0.3 f/f_0$ ought to be assumed in order to decrease extrapolated V_{max} obtained with maximal calcium pulses (curve 1 of Fig. 7) to a velocity value on the same curve, corresponding to the extrapolated V_{max} obtained with lower pulses, i.e. pulses which induced isometric twitch contractions developing only 50% of the force obtained with maximal pulses (curve 3 of Fig. 7).

The declined slope of the curves relating force, velocity, and shortening to the amount of calcium ions released in a solution with a higher ionic strength indicated a diminished sensitivity for calcium. Calcium sensitivity was not abolished over this range of ionic strengths which clearly abolished calcium sensitivity in skeletal muscle (Thames et al., 1974). This relative independency of calcium sensitivity of changes in ionic strength over this range, would suggest that in contrast to skeletal muscle, V_{max} of cardiac muscle can still be influenced by calcium. Referring to current cross-bridge theories of muscle contraction (Huxley, 1974; Moss et al., 1976) this would mean that mechanical behavior of cardiac muscle would not merely be determined by a simple on and off switch of interactions between actin and myosin filaments, but that the number of force generating sites as well as the kinetic properties of these cross-bridges would be influenced by calcium and by alterations of ionic strength. Obviously, the differences of the response of calcium sensitivity to alterations in ionic strength between cardiac and skeletal muscle could also result from differences in the overall range of ionic strengths that could affect calcium sensitivity. The present results would indicate that a complete inhibition of calcium sensitivity of cardiac muscle would require much larger increases in ionic strength. Moreover, while considering these results, species differences between rat and other mammalian species (Henderson et al., 1969) should be kept in mind.

Iontophoretic Activation

When a constant amount of calcium ions is iontophoretically released by a rectangular pulse from the micropipette, resulting in the ejection of a single bolus, uniform activation of the preparation might be complicated by (a) physi-

cal diffusion of the calcium ions from the micropipette through the preparation, including remnants of the sarcolemma, (b) dilution of the released calcium bolus by the overflowing perfusion and competitive binding by the EGTA in the solution, and (c) competitive binding by a functioning sarcoplasmic reticulum. First, assuming a diffusion constant (D) for calcium through muscle of the order of 10^{-5} cm^2/s (Orentlicher et al., 1974; Kushmerick and Podolsky, 1969; Mulieri, 1972) it would take about 600 ms for the calcium ions to reach both ends if released from a micropipette positioned in the middle of the long axis of a preparation with a length of approximately 80 μm and a width of 15 μm. This time, which is short compared to the time to peak shortening of 3,800 ms (Table II), would roughly account for the latent period between the beginning of the iontophoretic pulses and the onset of the contraction. Only minor differences in the duration of the latent period were observed when single square wave calcium pulses of different amplitude were applied. A major limitation by diffusion could also be excluded since the mechanical response to a given amount of calcium ions could be superimposed independently of whether this amount was ejected by double or single pulses from two micropipettes at different sites along the longitudinal axis of the preparation. The presence of remnants of the sarcolemma, which could either act as a physical hindrance to diffusion or actually bind some of the calcium ions, could not readily be excluded in these mechanically nonskinned preparations. However, the insensitivity to substitution of external sodium by potassium, the insensitivity to electrical stimulation, the presence of EGTA, and absence of any added calcium, along with the inability of the preparation to function without externally added ATP, a much larger molecule than calcium and one which normally does not readily diffuse through cell membranes, would indicate that the sarcolemma was nonfunctional and did not constitute an important diffusion barrier. The experiments with double pulses again excluded a major contribution of such a remaining physical barrier. Second, dilution of the diffusing iontophoretically released calcium ions by the overflowing perfusion solution could theoretically also explain graded activation as a function of various amounts of released calcium ions, the diluting effect being more pronounced for the smaller amounts. Dilution was negligible, however, since contractions obtained by a given amount of released calcium ions were identical, in both delay of onset and rate of rise, when the perfusion was temporarily stopped. An increase of EGTA from 125 to 500 μM resulted in a symmetrical shift to the right of the relation between the mechanical response and the amount of iontophoretically released calcium ions. Such a shift with an unchanged maximal response and an unchanged slope, i.e. an unchanged sensitivity for calcium, would imply that competitive binding of the iontophoretically released calcium ions by EGTA did not influence the overall interpretation of the results. Third, although the sarcoplasmic reticulum could act as a physiological diffusion barrier for calcium ions along their diffusion pathway from the pipettes to the contractile sites, the shift of the curves relating the mechanical response to the amount of released calcium ions in caffeine indicated a depression of the mechanical response and not a potentiation, as would be expected from blocking the sarcoplasmic reticulum by caffeine. On the other hand, it is

still unclear whether activation of the actin and myosin interactions is a direct effect of the calcium ions or is mediated indirectly through regenerative release of calcium from the sarcotubular system (Endo et al., 1970; Ford and Podolsky, 1970; Fabiato and Fabiato, 1975 a). It has also been shown that the threshold for such regenerative calcium release is dependent on the threshold amount of calcium loaded into the sarcoplasmic reticulum (Endo et al., 1970; Ford and Podolsky, 1970; Fabiato and Fabiato, 1975 a). This mechanism could be an alternative explanation for the shift to the right of the calcium response curve after caffeine, as more iontophoretic released calcium would be required to initiate a maximal calcium-induced calcium release. However, from recent experiments with lanthanum (Langer, 1976), it would seem that in cardiac muscle a calcium-induced calcium release is probably not a physiological mechanism. The sarcotubular system would then serve primarily in sequestration and little, if at all, in the release of calcium. Moreover, if the preparations used in the present study were immersed in a solution containing extremely high EGTA (7 mM) without added calcium, resulting in a more thorough depletion of the sarcotubular system, and then perfused with Solution 2, the responses of the iontophoretic calcium pulses were neither graded in time nor dependent on the interval between the pulses. The similarity of these results, after thorough depletion of the sarcotubular system, with the data obtained in Solution 2 with the moderately elevated EGTA (125 μM), would exclude any significant control by the sarcotubular system resulting from subsequent reloading of this system with calcium. These results would suggest that in both conditions the iontophoretic released calcium ions might have activated the contractile proteins directly. The absence of frequency potentiation, postextrasystolic potentiation, and post-rest potentiation could also be in line with this view.

The similarity between the mechanical responses obtained with calcium and strontium pulses in the present preparations was in contrast to the known delayed mechanical performance when strontium is substituted for calcium in intact cardiac muscle (Nayler, 1965; Brutsaert and Claes, 1974). Since the delayed response to strontium has been ascribed mainly to differences of the sarcoplasmic reticulum in handling this ion (Ebashi and Endo, 1968; Edwards et al., 1966), the absence of delayed strontium-induced contractions could again suggest that the sarcoplasmic reticulum in the present preparations was probably bypassed both by calcium and strontium pulses. Still, the similarity of the responses to the calcium and strontium pulses might have been a coincidental result of the different sensitivities of the actomyosin system for calcium and strontium and the difference in affinity between sarcoplasmic reticulum, EGTA, and ATP for calcium and strontium (Ebashi and Endo, 1968; Edwards et al., 1966). Experiments are planned further to investigate this matter.

An important observation in this study was the independency of time of the interrelationship between force, velocity of shortening, and length during shortening in isotonic contractions. This time independency was first described in intact cat papillary muscle and ascribed to complex interactions of multiple activating and deactivating factors (Brutsaert et al., 1971; Brutsaert, 1974). The possible existence of such a time-independent interrelationship between force,

velocity, and length at the level of the interactions between actin and myosin could never be totally excluded. This mechanism should be reconsidered in view of the possibility in the present experiments of a direct activation of the contractile sites with a negligible contribution of the sarcoplasmic reticulum.

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