

Patterns of Sarcomere Activation, Temperature Dependence, and Effect of Ryanodine in Chemically Skinned Cardiac Fibers

A. LUNDBLAD, H. GONZALEZ-SERRATOS, G. INESI,
J. SWANSON, and P. PAOLINI

From the Department of Biology and the San Diego State University Heart Institute, San Diego, California 92182, and the Departments of Biophysics and Biological Chemistry, University of Maryland School of Medicine, Baltimore, Maryland 21201

ABSTRACT Functionally skinned and electrochemically shunted myocytes were prepared by perfusing rat hearts with collagenase in order to obtain a technically improved measurement of sarcomere dynamics and to evaluate the role of sarcoplasmic reticulum in situ with respect to contractile activation. In the presence of micromolar calcium, the myocytes exhibited phasic and propagated contraction waves beginning at one end and proceeding along the myocyte. Beating rates, the propagation velocity of the activation wave, and single sarcomere shortening and relaxation velocities were obtained by manual or automated analysis of 16-mm film recorded at 170 frames/s from a camera attached to a microscope that was equipped with a temperature-controlled stage. In parallel experiments, calcium accumulation by the sarcoplasmic reticulum of the myocytes in situ was measured by direct isotopic tracer methods. The frequency ($10\text{--}38\text{ min}^{-1}$) of spontaneous contractions, the velocity ($1.9\text{--}7.4\ \mu\text{m}\cdot\text{s}^{-1}$) of sarcomere shortening, and the velocity ($1.7\text{--}6.8\ \mu\text{m}\cdot\text{s}^{-1}$) of sarcomere relaxation displayed identical temperature dependences ($Q_{10} = 2.2$), which are similar to that of the calcium pump of sarcoplasmic reticulum and are consistent with a rate limit imposed by enzyme-catalyzed mechanisms on all these parameters. On the other hand, the velocity ($77\text{--}159\ \mu\text{m}\cdot\text{s}^{-1}$) of sequential sarcomere activation displayed a lower temperature dependence ($Q_{10} = 1.5$), which is consistent with a diffusion-limited and self-propagating release of calcium from one sarcomere to the other. The phasic contractile activity of the dissociated myocytes was inhibited by $10^{-8}\text{--}10^{-6}\text{ M}$ ryanodine (and not by myolemmal calcium blockers) under conditions in which calcium accumulation by sarcoplasmic reticulum in situ was demonstrated to proceed optimally. The effect of ryanodine is attributed to an interaction of this drug with sarcotubular structures, producing inhibition of calcium release from the sarcoplasmic retic-

Address reprint requests to Dr. P. Paolini, Dept. of Biology, San Diego State University, San Diego, CA 92182.

ulum. The consequent lack of sarcomere activation underlines the role of sarcoplasmic reticulum uptake and release in the phasic contractile activation of the electrochemically shunted myocytes.

INTRODUCTION

Skinned cardiac fibers have been used extensively for the study of excitation-contraction coupling in cardiac muscle (see Best, 1983, and Fabiato, 1983, for recent reviews). In addition to mechanical skinning, functional skinning (i.e., increased permeability) can be obtained by incubation in low-Ca²⁺ media (Winegrad, 1971). We have previously described (Fry et al., 1979; Dani et al., 1979) structural and functional features of chemically skinned fibers obtained by enzymatic dissociation of adult cardiac tissue. Even though the myolemmal membrane is electrochemically shunted, these fibers undergo spontaneous contractile cycles with a pattern dependent upon a calcium ion concentration in the micromolar range. A useful characterization of this phenomenon can be obtained by recording the dynamics of sarcomere shortening. Our earlier efforts (Rieser et al., 1979) using videomicrography were limited by poor time resolution of sarcomere motion. Better temporal resolution can be obtained by high-speed cinemicrophotography. High-speed film recording yields images of low contrast and insufficiently sharp sarcomere boundaries to allow absolutely objective manual measurements. We therefore decided to combine cine and video methods for the benefits afforded by each technique, in order to obtain high-resolution patterns of single sarcomere shortening and relaxation, as well as patterns of sequential activation of neighboring sarcomeres. Furthermore, we obtained direct measurements of calcium transport by the sarcoplasmic reticulum (SR) of the myocytes in situ, in order to study the role of the SR in the contractile activation of electrochemically shunted cardiac fibers. Information on the mechanism of contractile activation in these fibers was then derived from experiments on the temperature dependence of contractile and transport parameters, as well as on their sensitivity to appropriate pharmacological tools.

MATERIALS AND METHODS

Cardiac Myocyte Preparation

Hearts from adult rats (Long-Evans strain) were used for the preparation of dissociated cardiac myocytes. The animals were given light ether anesthesia, the heart was exposed, and heparin was injected directly into the left ventricle. The heart was then excised and mounted by its aortic stump to a Langendorff apparatus and subjected to retrograde perfusion with a series of solutions saturated with 95% O₂-5% CO₂ at 37°C in the following sequence: (a) perfusion for 10 min with 150 ml of medium A, which contained 118 mM NaCl, 4.7 mM KCl, 12 mM NaHCO₃, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄, 1.3 mM CaCl₂, 50 mM 2-(*N*-morpholino)propane-sulfonic acid (MOPS), pH 7, and 5.6 mM glucose; (b) perfusion for 10 min with 150 ml of medium A without the CaCl₂ (medium B); (c) perfusion for 30 min with 50 ml (recirculated) of medium B to which 30 mg of collagenase (type IV, 165 μU/mg, Sigma Chemical Co., St. Louis, MO) was added; (d) perfusion for 10 min with 100 ml of ice-cold medium B containing 0.1 mM EGTA. After these perfusions, the ventricles were cut away from the heart and transferred to a petri

dish containing 5 ml of medium B to which 0.3 mM EGTA, 50 mg bovine albumin (A-4378, Sigma Chemical Co.), 500 μg creatine phosphokinase, 2 mM creatine phosphate, and 1–2 mM ATP were added. The ventricles were cut open and the dissociated myocytes were obtained by gently scraping the tissue with a dull rod. Tissue debris was removed by filtration through multilayer gauze, and the cell suspension was stored on ice. In most cases, 10 mM malate, glutamate, and pyruvate were added, and the suspension was kept under oxygen while stored on ice.

Cells prepared in this manner were typically 100–150 μm long and 15–30 μm wide (Fig. 1). The myocytes remained in a relaxed state while maintained at a $[\text{Ca}^{2+}]$ below 10^{-7} M. Cell populations within a suspension droplet appeared quite homogeneous, with a viability (i.e., the ability to develop phasic contractile behavior) of $\geq 90\%$. When exposed

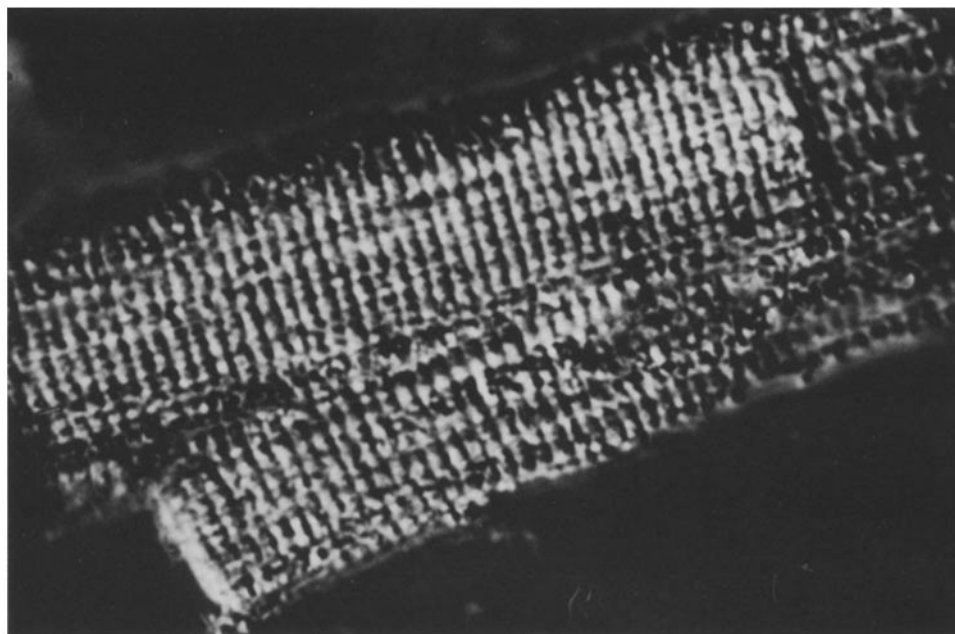


FIGURE 1. Photomicrograph of rat cardiac myocytes; differential interference contrast optics, 63 \times objective, $\times 1,000$. The single myocytes occasionally cluster in groups of two or three, which can be easily dispersed by gentle mechanical perturbations.

to $[\text{Ca}^{2+}]$ in the 10^{-6} M range, the cells underwent phasic contractions with a wavelike spread of activation beginning at one end of the cell and proceeding along the full cell length.

Total Soluble Calcium

Total calcium in the cell suspensions was determined by titrating excess EGTA with Ca^{2+} , using murexide as a metallochromic indicator. The absorption changes undergone by murexide were monitored by double-wavelength spectrophotometry, as previously described by Dani et al. (1979). The $[\text{Ca}^{2+}]$ in reaction mixtures was calculated using a computer program that took into account the Ca^{2+} binding constants of EGTA and ATP, as well as the pH and Mg^{2+} and K^+ concentrations (Fabiato and Fabiato, 1979; D. G.

Allen et al., 1977). It should be pointed out that a MOPS buffer was used for pH control, since this buffer is largely unaffected by temperature. Furthermore, the pH was checked at all experimental temperatures.

Cinemicrophotography

Cells were observed under a 40× bright-field objective (Carl Zeiss, New York), on a microscope (E. Leitz, Inc., Rockleigh, NJ) equipped with a high-speed (0–500 frames/s) 16-mm film camera. In order to observe the striations clearly, the substage condenser iris diaphragm was closed so as to decrease the illuminating cone given a numerical aperture (NA) of 0.26–0.24 (Gonzalez-Serratos, 1971). Although this reduces the amount of light that is effectively passing through the objective, it is possible to see the muscle fiber with ordinary light. If the substage iris diaphragm is more open, instead of having a narrow pencil of light composed of parallel rays, one has a wider pencil with rays coming from different directions. Thus, when the rays reach the muscle fiber, they are diffracted in several directions and it is not possible to obtain an interference of the light, which makes the fiber visible (Shillaker, 1963). With an NA of 0.24–0.26, the depth of focus is 11–13 μm , which is in the myocyte thickness range. If there were small changes in the diameter of the cell caused by shortening, the relative appearance of dark and clear bands of the cell would not be affected, since the whole cell remains attached to the bottom of the experimental chamber and within the depth of focus. Care was taken to set the focus of the microscope midway between the top and bottom of the myocyte.

Droplets of calcium-activated cell suspension were placed in a simple chamber fabricated from cut rectangles of 100- μm -thick cover glass mounted on a glass slide. The microscope stage was equipped with a Peltier-effect thermoelectric module capable of rapidly changing and then maintaining a stable stage temperature over a range of 0–40°C. Once a cell in a suspension droplet was selected for examination, its contractile behavior was recorded on film for 2–10-s periods at a rate of 170 frames/s. A light flash recorded on the film edge at 10-ms intervals permitted calibration of the film speed.

In initial experiments, cinemicrographic data were analyzed using two different techniques of measurement, one a manual analysis and the other an automated computer analysis. In the manual analysis, sequential frames were projected from a vertically mounted film-strip projector onto an opaque white screen on a table. Micrometer calipers were used to estimate the spacing of five selected contiguous sarcomeres. This method was limited by the ability of the observer to consistently identify the location of each “fuzzy” striation, which was taken to be the center of the I-band.

The automated method of analysis involved subjecting the film frames to computer digitization, image enhancement (to improve striation contrast), and determination of striation position by the use of a program that determined the weighted center of I-bands in the cell region being analyzed. In this procedure, the image from a stop-frame 16-mm-film projector was focused onto the ground-glass rear screen of a uniplexer field-flattening lens. A vidicon TV camera (model 602, Dage-MTI, Cincinnati, OH) was focused on this image, which measured $\sim 5 \times 7.5$ cm. The projector, lens assembly, and video camera were aligned on a 4-ft optical bench (Oriel Optical Co., Stamford, CT). Film frames could be advanced manually in sequence and captured by the image-analysis system described below. The improvement obtained by digital image-processing in the determination of individual sarcomere length in cardiac myocytes has been well documented by Roos and Brady (1982).

Image-Analysis Instrumentation

The electronic apparatus used in these studies consisted of a model 83/20 computer (Dual Systems, Berkeley, CA), with an S-100 card cage, an 8-MHz MC68000 CPU, 512 kilobytes

of high-speed RAM, four serial I/O ports, a clock, a floppy disk controller and driver, and a 21-megabyte Winchester disk, with a CRT terminal and printer. Resident software consisted of the UNIX operating system, utility packages (editors, debuggers, etc.), and compilers for the "C," FORTRAN 77, and 68000 assembly languages. Analysis software was written in the "C" language.

The image-processing hardware installed in this computer consisted of an eight-bit real-time frame-grabber (a 256 gray level flash digitizer), a 256-kilobyte video buffer (for storage of one 512×512 pixel image), a 7-MHz microprocessor, a 32-kilobyte ROM chip set containing numerous callable routines, a RAM color lookup table, and RGB output circuitry, which drove an external high-resolution, 19-in. RGB color monitor. The ROM firmware contained numerous "primitives" to control various graphics and image-manipulation functions. Calls to such firmware routines can be made from user programs in "C."

Computer Software for Analysis of Cell Images

The image-processing algorithm operates within an "analysis window," a region of the digitized image chosen by the experimenter according to the orientation of the cell being examined (Fig. 2A). This window is a parallelogram with long sides parallel to the cell axis, and short sides parallel to the striations. The computer establishes the position of each I-band within the window by superimposing a straight line over the band; this line is parallel to the short sides of the window. The placement of these lines by the computer agrees very well with human judgments based on visual estimation of the I-band (light) position.

As the first step in the processing of each frame, a previously stored defocused image was subtracted pixel by pixel from the digitized image of the frame (Castleman, 1979). A fixed constant was added to the resulting difference for each pixel to produce positive values for all pixels. The same constant was used for all pixels of all frames within a contraction record. This procedure corrected effectively for nonuniform illumination over the window area, so that absolute pixel intensities were relatively consistent from striation to striation.

The next step was a linear contrast enhancement using a procedure similar to that described by Gonzalez and Wintz (1977), where the gray level intensity of each pixel is replaced by a different value. The result of this process is that the initial image, where only a fraction of the possible range of gray levels is populated, is transformed into an image in which the set of replacement pixel values spans the entire range of gray levels (Fig. 2B).

Next, pixel intensities within the window were projected onto a long side of the analysis window, which was parallel to the cell axis, to produce a one-dimensional array of numbers. Local maxima in this array were then detected; maxima below a selected threshold were not sensed. The median spacings between maxima were calculated, local maxima spaced closer than a given fraction of this median value were compared, and the smaller of the paired values was discarded.

The analysis window was next subdivided into regions about retained maxima. The boundaries of these regions were positioned on either side of the retained maxima at a distance that was a fixed fraction of the median separation of the retained maxima. No neighboring regions were observed to overlap. Centroids of the projection values within each region about a maximum were calculated, and a line passing through this centroid and parallel to the short side of the analysis window was evaluated. These lines could be displayed either within the cleared window (Fig. 2C) or superimposed upon the original image to permit observation of the goodness of fit to the original I-bands (Fig. 2D). Line

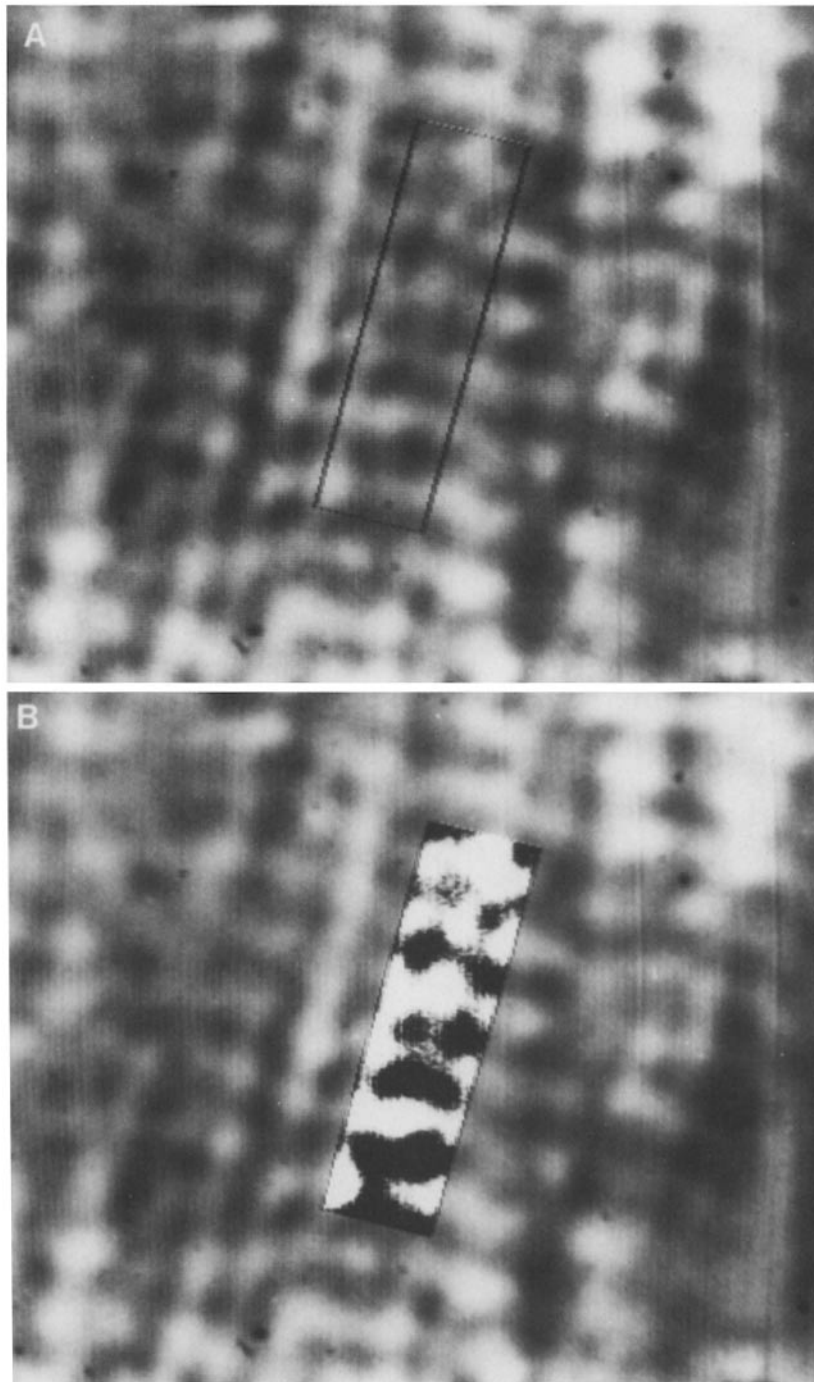
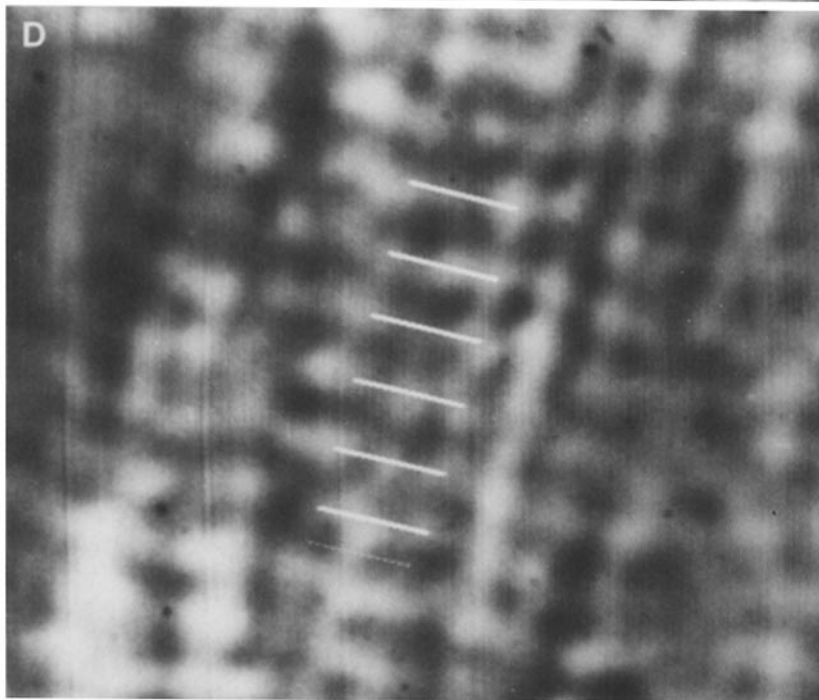
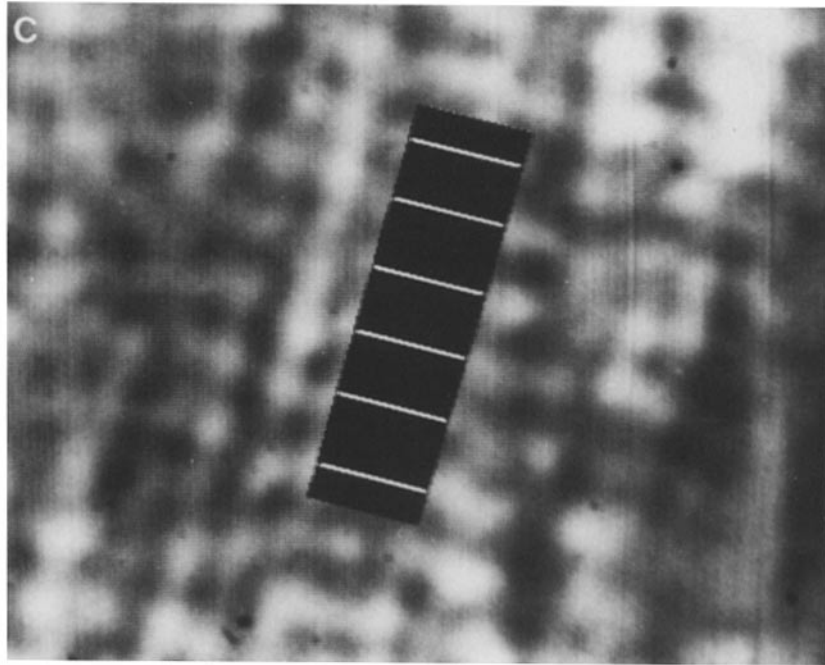


FIGURE 2. (A) Region in rat cardiac myocyte. 16-mm film was frame-digitized and stored in a video buffer (480×512 pixel \times eight-bit resolution), with an analysis window drawn. (B) Image within window subjected to contrast enhancement ("stretch") and subtraction of background. (C) Window contents replaced by straight



lines fitted through the center of the mass of the projection of a binary image along the window edge. (D) Lines representing sensed striation patterns superimposed upon the original halftone image.

positions were recorded and the spacing between each of these sensed "striations" was calculated.

Each frame in a given contraction was digitized and processed in sequence using the same analysis window. The positions of the lines representing the I-bands were recorded automatically on the hard disk for each successive frame. The calibrated distances between adjacent pairs of lines plotted vs. time yielded the computer-evaluated sarcomere shortening curves.

This procedure for detecting striations was independent of I-band striation shape and thus more successful than edge-detection algorithms, which we used initially in these studies. One problem occasionally limited the effectiveness of this procedure: if, during the contraction, striations became obviously tilted with respect to the short edges of the window, the projection maxima were broader and less distinct, making the striations more difficult to detect. It should also be pointed out that under unfavorable conditions, i.e., when the resolution was of the same order as the sarcomere period, there may have been phase shifts between the apparent striations and the true ones (because of some overlap in images of successive sarcomeres), which may not have been apparent but could have biased the estimation of single sarcomere length. In our experiments, however, the resolution estimated from $R = 0.61\lambda/\text{NA}$ for a λ of $0.55\ \mu\text{m}$ (green-yellow) would be $1.29\ \mu\text{m}$ (for 0.26 NA), which is below the length of a thick filament and the observed sarcomere length.

Computer Data Reduction

Striation spacing data from each analyzed film frame could be transferred to a VAX11/750 minicomputer (Digital Equipment Corp., Maynard, MA) for further processing. Reduced data generated by the computer consisted of graphs of striation position vs. time, or plots of individual sarcomere length vs. time. These curves were produced by a program that tracked the initial striation position from frame to frame by a nearest-neighbor analysis. A further analysis of these shortening curves gave values for the sarcomere initial shortening kinetics, the percent contraction, the relaxation kinetics, and activation wave propagation velocities. Sarcomere records were calibrated from analyzing pixel spacing in images of ocular micrometer grating lines spaced $10\ \mu\text{m}$ apart and recorded on the original film.

Calcium Uptake by Isolated Myocytes

Calcium transport by the myocytes as a function of temperature was measured by following the uptake of $[^{45}\text{Ca}]\text{CaCl}_2$ from the suspension medium. The cells were suspended in the same medium described above, with the following additions: 10 mM pyruvate, 10 mM glutamate, 10 mM malate, and 5 mM oxalate. Sufficient cold calcium was added first to induce optimum contractile activity. The suspension was kept on ice and under a stream of 95% O_2 –5% CO_2 . Aliquots of the suspension were incubated in a water bath at various temperatures; at appropriate intervals, samples were collected and filtered by suction on HA 0.45- μm Millipore filters. The samples were then washed with ice-cold medium B and dissolved in 5 ml of scintillation liquid, and the radioactive calcium associated with the cells on the filter was measured with a scintillation spectrometer.

RESULTS

Observation of large fields by light microscopy revealed a large number of single, dissociated myocytes undergoing phasic contractions when suspended in media

containing $1 \mu\text{M Ca}^{2+}$. This concentration of Ca^{2+} in the medium was insufficient to activate the myofibrils directly, when calcium uptake or release by the SR was prevented with ionophores or ryanodine, respectively (see below). Although the beating rates are not identical for all myocytes under the same conditions, the average values collected from numerous myocytes showed a clear temperature dependence (Table I), with a Q_{10} of 2.2 within the temperature range $10\text{--}20^\circ\text{C}$. When the temperature was increased to 35°C , the rate of contraction changed to a pattern of very high frequency and incomplete relaxation, apparently because of a qualitative change in the mechanism controlling the occurrence of contractions. It is noteworthy that Fabiato (1985*b*) also observed a large effect of temperature on the beating frequency of skinned cardiac Purkinje fibers within this higher temperature range. All our subsequent observations were carried out within the lower temperature range ($6\text{--}23^\circ\text{C}$).

As previously noted (Rieser et al., 1979), the wave of contractile activation always begins at one end of the myocytes, where the intercalated disk and gap junctions are located. It should be pointed out that some of the gap junctions

TABLE I
Temperature Dependence of Rat Cardiac Myocyte Sarcomere Kinetics and Propagation Velocity

Tempera- ture	Beat rate	Initial sar- comere length	Percent shortening	Velocity of shortening	Velocity of relaxation	Contract duration	Propaga- tion veloc- ity
$^\circ\text{C}$	<i>beats/min</i>	μm		$\mu\text{m/s}$	$\mu\text{m/s}$	<i>ms</i>	$\mu\text{m/s}$
6.0	10 ± 2.8	1.84 ± 0.12	26 ± 4	1.86 ± 0.6	1.7 ± 0.1	563 ± 15	77 ± 15
10.0	14 ± 0.6	1.91 ± 0.01	29 ± 1	3.40 ± 0.49	2.6 ± 0.3	475 ± 21	94 ± 6
16.5	21 ± 3.2	1.90 ± 0.18	25 ± 4	4.90 ± 0.71	4.4 ± 0.4	242 ± 25	110 ± 20
23.0	38 ± 2.1	1.91 ± 0.05	22 ± 4	7.40 ± 0.25	6.8 ± 1.4	134 ± 13	158 ± 24
Q_{10}	2.2			2.2	2.2	2.4	1.5

Beating rates were derived from the observation of 30–50 cells per temperature point. Measurements of sarcomere dynamics were carried out on five to eight cells per temperature point, with sarcomeres chosen within the central portion of the myocytes and the same myocyte used at different temperatures.

follow one of two complementary myocytes upon dissociation and some follow the other (while the membrane discontinuities caused by gap junction removal reseal) and provide prominent permeability pathways across the myolemma (Fry et al., 1979). Microscopic studies of the diffusion of fluorescent dyes of various sizes (Leong, J., and G. Inesi, unpublished observations) show that the myocytes allow the entry of approximately round molecules up to 500 mol wt. This size is consistent with the limit imposed by gap junction channels. It is of interest that a constant origin of activation waves from the intercalated disk was also observed by Fabiato (1985*d*) in chemically skinned and “calcium-tolerant” cardiac myocytes.

More detailed information was obtained through our analysis of sarcomere dynamics. The results reported here are all derived from unedited direct measurements of single sarcomere motion observed under the microscope.

Fig. 3 illustrates representative cycles of single sarcomere shortening and

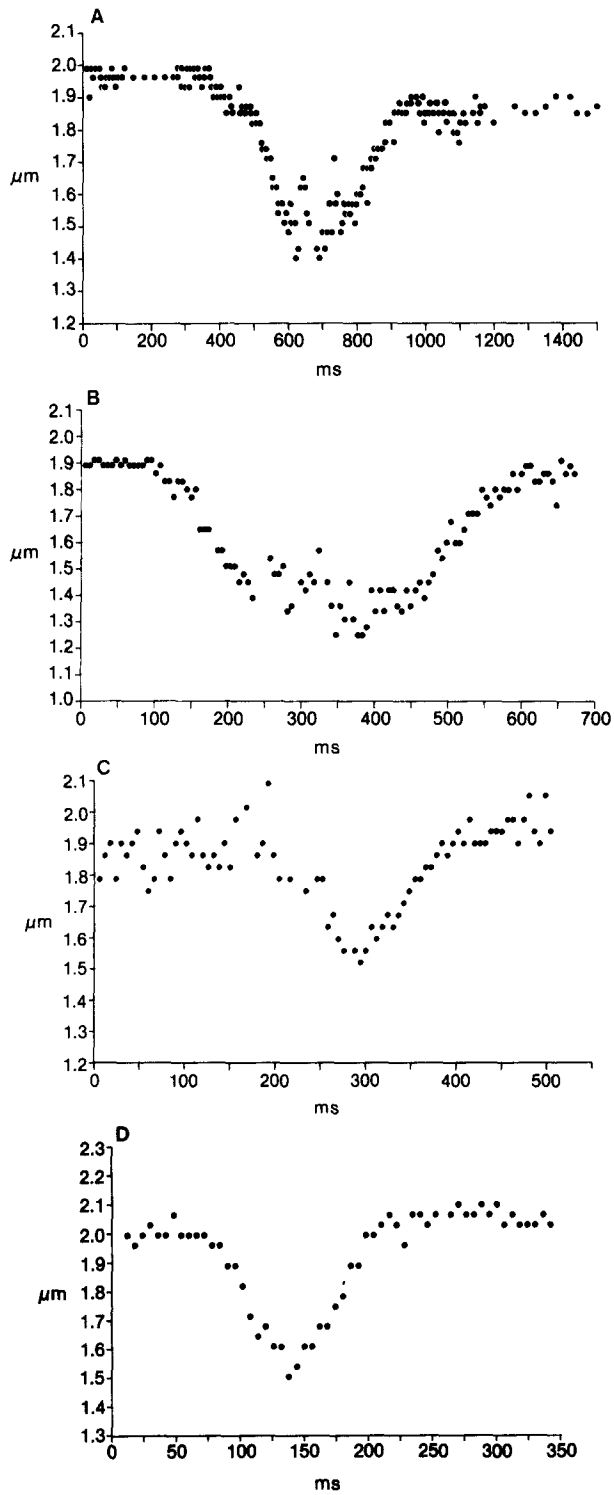


FIGURE 3. Representative sarcomere shortening curves at 6 (A), 10 (B), 16.5 (C), and 23°C (D).

relaxation at various temperatures. Since the shortening phase of a single sarcomere activation cycle presents a prominent linear portion, the shortening velocities were obtained by linear regression analysis and were found to vary from 1.86 to 7.40 $\mu\text{m/s}$, within the temperature range between 6 and 23°C (Table I). We also derived relaxation velocities (1.7–6.8 $\mu\text{m/s}$) by linear regression analysis, since we did not identify significant segments requiring exponential fitting. With respect to the pattern of relaxation, it should be noted that the single myocytes used in our experiments were free of surrounding connective tissue, and were observed without restraint at either end; therefore, the origin of linear forces determining sarcomere elongation was likely to be internal (Gonzalez-Serratos, 1971), and quite different from that in whole fibers. It is of interest that identical temperature dependences ($Q_{10} = 2.2$) were observed for the velocity of shortening and the velocity of relaxation, as well as for the beating rates (Table I). The duration of the shortening and relaxation cycle of each sarcomere varied between 563 and 134 ms within the temperature range 6–23°C, manifesting a temperature dependence very similar to that of the shortening and relaxation velocities (Table I).

Fig. 4 illustrates a computer printout of relative sarcomere positions within the user-defined window followed during a single phasic contraction of one myocyte. The wave of contraction involves the activation of a single sarcomere at a time. A contracting sarcomere pulls on the adjoining sarcomere ahead of it, but only one sarcomere at a time achieves maximum shortening. Previous studies of dissociated myocytes by direct video recording (without preliminary fast film recording) did not detect any variation in the sarcomere activation pattern from one end of a myocyte to the other (Rieser et al., 1979). In our present study, we were able to demonstrate a slight and progressive reduction in the velocities of sarcomere shortening and relaxation as the activation wave proceeded along the length of the myocyte, undergoing a maximal reduction of 50% by the time that the activation wave reached the far end of the myocyte. Consequently, the velocity of the self-propagating wave was also correspondingly reduced as it proceeded along the cell. A similar slowing of the propagation wave was also noted by Fabiato (1985*d*) in dissociated, "calcium-tolerant" cardiac myocytes. In our preparation, the propagation velocity was 150–175 $\mu\text{m/s}$ at the center of the cell at 23°C. The temperature dependence of the propagation velocity, measured between 6 and 23°C, was quite low (Table I), with a Q_{10} of 1.5 between 10 and 20°C.

It should be pointed out that in calcium-tolerant myocytes isolated from Wistar rats (Kort et al., 1985), a similar range of propagation velocities, but a somewhat higher Q_{10} (2.1), was found between 25 and 29°C. However, Kort et al. found, as we did, that the propagation velocity increased with temperature to a lesser extent than did wave frequency.

Table II presents measurements of initial and final values for the velocity of propagation of the activation wave. A mean reduction in velocity of 39% (SD = 16%) was observed over the range 6.0–23.5°C. Such a reduction was apparently independent of the temperature within the 6–23°C range. An additional set of measurements on three cells (11 contractions) studied at a constant temperature (19°C) yielded a mean decrease of 36% in propagation velocity (SD = 5%).

It is possible that the reduction of sarcomere shortening and relaxation velocities that was observed as the activation wave proceeded through the myocyte was related to a slight impairment of the metabolic adequacy of the isolated cells observed under the microscope, within a single cycle. The metabolic balance was then restored during the relaxation phase, so that a new shortening cycle could occur with the original characteristics. Fibers maintained under optimal oxygenation until immediately before microscopic observation consistently displayed faster propagation velocities.

A useful feature of our preparation is the yield of single and chemically skinned myocytes in quantities sufficiently large to be used for measurements of calcium uptake by radioactive tracer and filtration methods (Fig. 5). It was demonstrated by electron probe microanalysis that the energy-dependent calcium uptake observed in the presence of micromolar Ca^{2+} specifically produced calcium loading of SR cisternal spaces (Chiesi et al., 1981). An example of the temperature dependence exhibited by this phenomenon is shown in Fig. 5. Consistent with

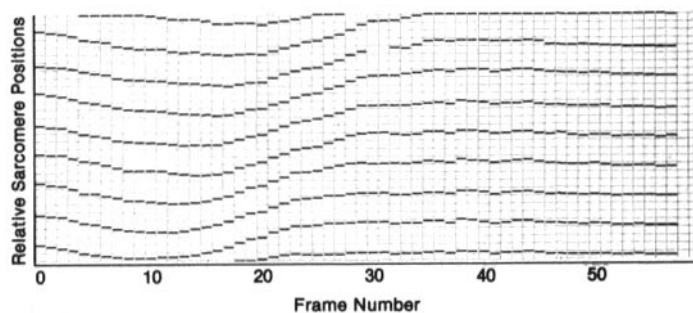


FIGURE 4. Striation map of contraction in rat cardiac myocyte at 23°C; each frame = ~6 ms; output from electrostatic printer/plotter.

previous work on isolated SR vesicles (Inesi et al., 1973), we found that the temperature dependence of calcium transport into the SR of dissociated myocytes in situ decreased as the temperature was raised from 5 to 30°C, with a Q_{10} that varied between 2 and 3.

Taking advantage of the possibility of evaluating both the contractile activation of the myocytes and calcium accumulation by the SR in situ, we tested the effect of ryanodine on these two parameters. Ryanodine is known as an agent that interferes with contractile activation to produce permanent relaxation in cardiac muscle (Bianchi, 1983; Frank and Sleator, 1975a; Grossman and Furchgott, 1964; Hadju and Leonard, 1961; Hillyard and Procita, 1959; Nayler, 1963; Nayler et al., 1970; Penefsky and Kahn, 1970; Sleator et al., 1964; Lakatta et al., 1985). This effect has been attributed to inhibition of calcium release from the SR (Jones et al., 1979; Sutko and Kenyon, 1983; Sutko et al., 1979, 1985; Sutko and Willerson, 1980; Fabiato, 1985a; Marban and Wier, 1985), based on experimentation with muscle preparations and isolated SR vesicles. On the other hand, other mechanisms have also been proposed (Frank and Sleator, 1975b;

Nayler et al., 1970; Hilgemann et al., 1983; Hunter et al., 1983), such as interference with the calcium pump of the SR by either uncoupling or passive leak.

It is shown in Fig. 6A that ryanodine, within a concentration range between 10^{-8} and 10^{-6} M, inhibited the spontaneous contractile activation of the dissociated myocytes. The data in Fig. 6 were collected after 5 min incubation of the myocytes with ryanodine in the presence of Ca^{2+} , at which time the effect of ryanodine had reached the appropriate maximum for any of the concentrations

TABLE II
*Decline in Propagation Velocity as the Activation
Wave Spreads from One End of the Cell to the Other*

(A) 11 contractions at varied temperatures				
Temperature	Initial velocity	Terminal velocity	Percent change	
$^{\circ}\text{C}$	$\mu\text{m/s}$	$\mu\text{m/s}$		
6.0	95	55	42	
	140	80	43	
17.5	170	135	21	
21.0	218	107	53	
22.4	230	130	43	
	240	155	35	
	170	95	44	
23.0	220	80	64	
	200	100	50	
23.5	160	120	25	
	170	160	6	
Mean decline:		39% ($n = 11$)		
(B) 11 contractions at the same temperature (three different cells)				
Temperature	Initial velocity	Terminal velocity	Percent change	Number
$^{\circ}\text{C}$	$\mu\text{m/s}$	$\mu\text{m/s}$		
19.0	145	85	41	4
	160	107	33	4
	114	76	33	3
Mean decline:		36% ($n = 11$)		

used. We noted that the inhibition was produced more quickly by the higher ryanodine concentrations (within the 10^{-8} – 10^{-6} M range).

It is of great interest that under identical experimental conditions, calcium accumulation by the SR of the myocytes in situ was not inhibited at all by ryanodine (Fig. 6B). We also confirmed with isolated SR vesicles (not shown) that calcium accumulation was not inhibited by ryanodine. Therefore, our experiments show unambiguously that contractile activation was inhibited by ryanodine in the same preparation and under the same conditions in which calcium accumulation by the SR was not. Since our electrochemically shunted

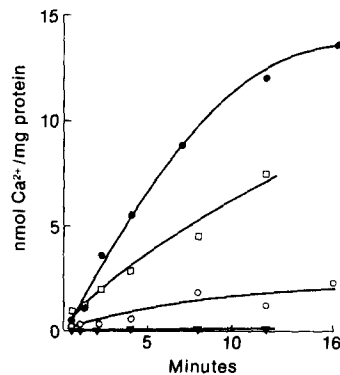


FIGURE 5. Calcium uptake vs. time (min) by isolated cardiac myocytes in suspension at 10 (○), 20 (□), and 30°C (●), expressed in nanomoles calcium ion per milligram of protein; ▼, data obtained in the presence of ionophore A23187 (10 μ M). The experimental conditions are as described in Materials and Methods.

myocytes did not sustain significant electrical activity of the myolemma (Rieser et al., 1979) and since ryanodine does not appear to inhibit the direct activation of myofibrils by calcium (Fabiato, 1985a), it is clear that ryanodine's interference with contractile activation was produced by inhibition of calcium release from the SR.

In parallel experiments, we did not detect any effect of myolemmal calcium channel blockers either on phasic contractile activity or on calcium accumulation by the SR in the dissociated myocytes. The ability to prevent phasic contractile activity either by inhibition of calcium release with ryanodine (Fig. 6) or by

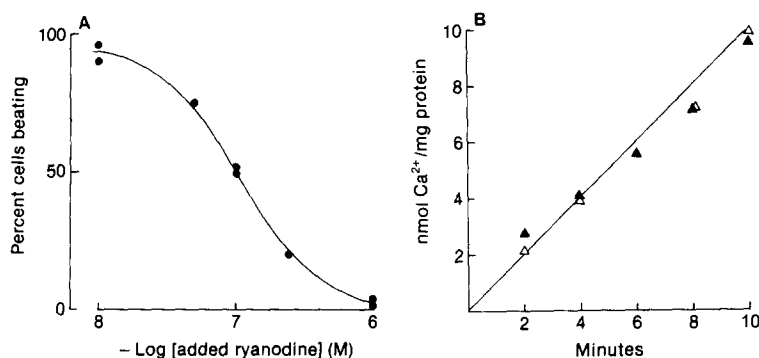


FIGURE 6. Effect of ryanodine on spontaneous beating (A) and SR calcium uptake (B) in dissociated cardiac myocytes. In both cases, the reaction medium contained 4 mg cell protein/ml, 118 mM NaCl, 4.7 mM KCl, 12 mM NaHCO₃, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄, 50 mM MOPS, 5.6 mM glucose, 0.3 mM EGTA, 10 mg/ml bovine albumin, 100 μ g/ml creatine phosphokinase, 2 mM creatine phosphate, 5 mM oxalate, 2 mM ATP·Mg, 10 mM pyruvate, 10 mM maleate, and 10 mM glutamate. The pH was adjusted to 7.0. The reaction was started by the addition of CaCl₂ to yield 0.7 μ M free Ca²⁺. In this experiment, ryanodine was added 2 min before CaCl₂.

inhibition of accumulation uptake by the SR (Dani et al., 1979) underscores the central role of the SR in the mechanism of electrochemically shunted phasic contractile activation of the myocytes, in agreement with the temperature dependence studies described above.

DISCUSSION

The usefulness of light microscopy in quantifying muscle cell contractile dynamics has been limited by factors such as poor time resolution, the blurring of images during cell motion, the time-consuming task of manually analyzing photomicrographs, and the resultant uncertainty in measurements based on small sample sizes. The recent availability of video and computer hardware for capturing and analyzing pictorial information now offers a means to overcome most of these limitations (Walter and Berns, 1981). By using a combination of optical, video, and computer hardware, image-processors can be used to enhance and exaggerate fine differences in the optical density of the original images, so that otherwise visually undetectable features can be observed (R. Allen and Allen, 1983; Inoue, 1981). Computer analysis of muscle cell motion eliminates much of the observer bias that is associated with the subjective placement of cursors to measure sarcomere lengths from video data (e.g., DeClerck et al., 1977).

We have previously reported preliminary attempts on computerized image analysis of isolated contracting rabbit cardiac myocytes (Rieser et al., 1979). Other investigators have extracted a limited amount of pictorial information from videomicrographic (direct CCTV camera or videotaped) records of contracting cells. Examples include the work of Pollack and Krueger (1976), who monitored average sarcomere lengths in cardiac fiber bundles using specialized video circuitry, and that of Tarr et al. (1979), who measured atrial cell sarcomere lengths by stop-frame videotape-recorder playback to a television monitor equipped with positionable cursors and digital coordinate readout. DeClerck et al. (1981) have also described a technique to monitor a selected spot on an isolated cardiac cell using a television "tracking" circuit.

A shortcoming of videomicrographic methods is that there is insufficient time resolution of sarcomere motion to permit individual sarcomeres to be traced from frame to frame (30 frames/s for 480-line vertical resolution, or 60 fields/s for 240-line resolution). Cinemicrophotography allows temporal resolution to be increased sufficiently to allow sarcomere tracking. In this case, however, low contrast and a lack of image sharpness resulted in less than objective manual measurements. The combination of cine and video methods, together with the computer-assisted contrast enhancement and image analysis of the digitized data output, was used for the first time in the experiments reported here. This method resulted in a significant improvement in the temporal resolution and in the objectivity of the final measurements.

The 6-ms time resolution obtained in our experiments was well suited to the measurement of single sarcomere shortening as well as the wave of sequential sarcomere activation through the cardiac myocytes at temperatures between 6 and 23°C. It is of interest that the pattern of sequential sarcomere activation (Fig. 4) and the profiles of single sarcomere shortening and relaxation obtained

with this method were smooth functions (Fig. 3). It is possible that our time resolution and/or the particular mechanism of skinned myocyte activation prevented us from observing the stepwise shortening phenomenon demonstrated by Pollack et al. (1977) using different muscle fiber preparations, in which better than 1-ms resolution was achieved (Delay et al., 1981).

From a functional standpoint, a most important question is related to the mechanism of spontaneous activation in the functionally skinned myocytes. It was previously demonstrated by electron probe analysis that the myoplasmic composition of these myocytes is nearly identical to that of the medium (Chiesi et al., 1981), and therefore the myolemmal membrane is electrochemically shunted. Furthermore, contractile activation occurs equally well in media containing Na^+ or K^+ , and in the presence of tetrodotoxin (Dani et al., 1979). For these reasons, the dissociated myocytes are a useful system to study the behavior of the SR in situ, with respect to calcium loading, calcium release, and contractile activation, independent of myolemmal excitation. In fact, it was shown with radioactive tracer (Chiesi et al., 1981) and electron probe analysis that the SR accumulates calcium under conditions permitting phasic contractile activity, and if SR loading is prevented, contractile activation does not occur.

Fabiato (1983) has shown by rapid medium substitutions in mechanically skinned fibers that the addition of micromolar Ca^{2+} is a triggering stimulus for Ca^{2+} release from the SR. Fabiato (1985*c, d*) has also indicated that the Ca^{2+} release induced by a rapid increase of Ca^{2+} near the outer surface of optimally loaded SR is a distinct phenomenon with respect to other types of release that may occur spontaneously from overloaded SR. In our preparation, a role of Ca^{2+} on the outer surface of the SR is suggested by the self-propagating pattern of the activation wave, which indicates that the calcium released at the level of any sarcomere is diffusing to the next sarcomere to produce more release (Ford and Podolsky, 1970). In the light of our present observations, a distinction among the rate-limiting steps in the mechanisms of calcium diffusion, calcium release, and calcium uptake can be attempted on the basis of their temperature dependence.

We found, first, that the extent of sarcomere shortening was independent of temperature, which indicates that (in the unrestrained myocytes) maximal sarcomere shortening was always obtained in our conditions, even though the shortening velocity varied with the temperature. On the other hand, it is of interest that beating rates, the velocity of sarcomere shortening, and the velocity of sarcomere relaxation displayed identical temperature dependences (Table I), which were also similar to that of the SR calcium pump (Fig. 5). This behavior would be expected if the opening of release channels and the extent of calcium release were dependent on the SR calcium load and, therefore, on the velocity of the calcium pump. It must be stressed, however, that the rate-limiting steps for sarcomere shortening may be related to cross-bridge kinetics, which could coincidentally display a temperature dependence similar to that of the calcium pump. On the other hand, it is probable that calcium removal upon closure of the release channels in these myocytes is dependent upon the velocity of the calcium pump. At any rate, it is clear that the beating rates and velocities of

sarcomere shortening and relaxation displayed a temperature dependence consistent with enzyme-catalyzed mechanisms. The very low temperature dependence ($Q_{10} = 1.5$) of the propagation velocity of sequential sarcomere activation underscores the importance of diffusion in the propagation of the release phenomenon from one sarcomere to the next. This is consistent with a role of the released calcium in raising the Ca^{2+} concentration near the cytoplasmic SR surface in contiguous sarcomeres. It is possible that the diffusing calcium is simply permissive of greater uptake by the SR in a neighboring sarcomere, so as to reach a "releasing load." On the other hand, the rate of calcium uptake ($Q_{10} = 2-3$) is clearly not rate-limiting in the process of self-propagation, as would be expected (especially in the low temperature range) if calcium uptake were involved in self-propagation. Therefore, it is apparent that the diffusing Ca^{2+} produces perturbations on the outer surface of the SR that are involved in triggering the release of the calcium load. Our observations are consistent with the idea (Fabiato, 1985c, d) that calcium release requires a high Ca^{2+} concentration in the cisternal lumen, as well as a low Ca^{2+} concentration near the cytoplasmic surface of the SR. They also suggest that the SR release sites are localized to one region of the sarcomere and are separated from the release sites of neighboring sarcomeres by regions of inexcitable SR. In fact, the presence of inexcitable SR regions between release sites would enhance the rate-limiting role of diffusion. It is noteworthy in this regard that selective fractionation of SR vesicles (isolated from skeletal muscle) has shown that Ca^{2+} release channels are present in junctional but not in longitudinal SR (Meissner, 1984).

The diffusion of calcium through the myoplasm (Kushmerick and Podolsky, 1969) and its role in triggering further calcium release and contractile activation are subject to multiple interactions. The complexity of these interactions is brought to light by appropriate model analysis (Stern et al., 1984). It should be pointed out that if the SR release sites were homogeneously distributed, the variation of propagation velocity as the square root of the calcium release rate might give rise to a fairly low Q_{10} , even if release rather than diffusion were rate-limiting. This, however, is very unlikely, since the experimental evidence indicates that junctional, and not longitudinal, SR has release channels (Meissner, 1984).

With regard to the inhibition of phasic contractile activity by ryanodine, it is noteworthy that in our experiments this effect was obtained with drug concentrations within the range (10^{-8} – 10^{-6} M) effective on whole cardiac muscle and did not require prolonged incubation of the myocytes with ryanodine. This is in contrast to experiments on ryanodine inhibition of contractile activation in skinned cardiac fibers (Fabiato, 1985a) and, to an even greater extent, to ryanodine binding to microsomal preparations (Pessah, 1985), in which a requirement for relatively high ryanodine concentrations and/or prolonged preincubation in the presence of Ca^{2+} was found. In a parallel set of experiments (not shown), we found that ryanodine (up to 10^{-4} M) was not effective in inhibiting passive Ca^{2+} efflux through "heavy" (Meissner, 1984) SR vesicles possessing the Ca^{2+} release channel. It is possible that, as suggested by Fabiato (1985a), the ryanodine receptor is perturbed by the skinning procedure and, to a greater

extent, by muscle homogenization and differential centrifugation. The nature of the ryanodine effect and the sensitivity of the ryanodine receptor to structural perturbations indicate that the receptor is a complex structure located at the sarcotubular junction, which is in control of the calcium release channel of the SR. In our preparation of myocytes dissociated by the collagenase method, the ryanodine receptor appears to be preserved in its native conformation.

Previous studies (Dani et al., 1979), which showed that the phasic contractile activity of the dissociated myocytes is prevented by ionophore-induced inhibition of calcium accumulation by the SR, indicated a prominent role of the SR in the mechanism of activation. It was not actually demonstrated, however, that calcium release from SR is also required, since the activation of myofibrils could be induced by calcium diffusion from the medium, and then relaxation could be produced phasically by SR calcium uptake. Our finding that ryanodine inhibits contractile activation, but does not interfere with calcium uptake by SR, demonstrates clearly that a repetitive sequence of both uptake and release is required for phasic contractile activation of the dissociated myocytes. The role of calcium diffusion is then related to the self-propagating pattern of contractile activation from one sarcomere to the other. This is consistent with the interpretation given above for the temperature dependence of the various parameters of contractile activation.

Note added in proof: We have just become aware that a primary effect of ryanodine on sarcotubular structures was suggested by Zia Penefsky (1974. *Pflügers Archiv European Journal of Physiology*. 347:173–198) on the basis of comparative observations on developing hearts of different species.

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