Graded Activation in Frog Muscle Fibers

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ABSTRACT The membrane potential of frog single muscle fibers in solutions containing tetrodotoxin was controlled with a two-electrode voltage clamp. Local contractions elicited by 100-ms square steps of depolarization were observed microscopically and recorded on cinefilm. The absence of myofibrillar folding with shortening to striation spacings below 1.95 μm served as a criterion for activation of the entire fiber cross section. With depolarizing steps of increasing magnitude, shortening occurred first in the most superficial myofibrils and spread inward to involve axial myofibrils as the depolarization was increased. In contractions in which the entire fiber cross section shortened actively, both the extent of shortening and the velocity of shortening at a given striation spacing could be graded by varying the magnitude of the depolarization step. The results provide evidence that the degree of activation of individual myofibrils can be graded with membrane depolarization.

The force of contraction in voltage-clamped vertebrate muscle fibers is dependent on membrane potential over a range of depolarization of about 20 mV (Heistracher and Hunt, 1969; Bezanilla, Caputo, and Horowicz, 1971). Although the most straightforward explanation for this observation is that the release of activator calcium can be graded with membrane depolarization, an alternative possibility is that activator release at the myofibrillar level is all-or-none, and that progressive depolarization of the surface membrane simply increases the cross-sectional area of the fiber that is activated. Surface membrane depolarization does spread decrementally along the T-system of voltage-clamped muscle fibers (Adrian, Costantin, and Peachey, 1969), but it is not clear whether attenuation along the T-system would be sufficient to account for the observed gradation of contractile response. Similar voltage-dependent variations in the force output of single fibers have been found in potassium-induced contractures (Hodgkin and Horowicz, 1960; Lüttgau, 1963; Frankenhaeuser and Lännergren, 1967). Under these conditions, the radial gradient of depolarization after depolarization of the surface membrane should be abolished as extracellular potassium diffuses

into the T-system. Since this would be a relatively slow process, however, accomodation might occur in the activation mechanism, and less than maximal activation might result. A slow component of the potassium contracture, which presumably arises from diffusion of potassium into the T-system, has been reported (Costantin, 1971 *a*).

In the present experiments, the contraction of voltage-clamped single muscle fibers was examined by high speed cinephotography. The results provide further evidence that activation at the myofibril level can be graded with membrane depolarization.

METHODS

Principle of the Method

When active propagation of depolarization along the T-system is prevented by tetrodotoxin (TTX), the contractile response of a voltage-clamped single muscle fiber can be graded by the magnitude of the applied depolarization (Adrian, Costantin, and Peachey, 1969); a just-threshold depolarization elicits shortening of only a superficial annulus of myofibrils, and an increment of a few millivolts beyond the contractile threshold is required to produce extensive shortening of both superficial and axially located myofibrils. Adrian, Costantin, and Peachey (1969) have pointed out that the shortening of axial myofibrils seen under these conditions need not necessarily represent active shortening but might instead result from lateral mechanical coupling with actively contracting superficial myofibrils. Although the presence of shortening alone is inadequate to distinguish between active and passive myofibrils at striation spacings[1] above rest length (about 2.1 μm), a visual criterion for active shortening of individual myofibrils is available at shorter striation spacings. In a study of single fibers in which passive shortening below rest length was produced by compression of isolated fibers set in gelatin, Brown, Gonzalez-Serratos, and Huxley (1971) have shown that "the sarcomeres shorten by sliding to a length S_{min}" (2.1–1.95 μm) and "further shortening is by bending of the fibrils." In the present experiments, contractions to striation spacings below 1.95 μm were examined, and the *absence* of myofibrillar bending was employed as a criterion for active myofibrillar shortening. In particular, shortening of the entire fiber cross section to striation spacings below 1.95 μm without the appearance of myofibrillar folding was taken to indicate that all the myofibrils in the fiber cross section were to some degree active. It should be emphasized that the results of Brown, Gonzalez-Serratos, and Huxley (1971), obtained with relaxed muscle fibers, indicate only that myofibrils cannot shorten passively below striation spacings of 1.95 μm without folding; their results do not imply that active myofibrils cannot fold or become wavy. In the present experiments, waves were in fact seen in myofibrils which were at least partially active (see Fig. 1).

[1] The term striation spacing will be employed to refer to sarcomere spacing measured along the long axis of the *fiber*, and the term sarcomere length to sarcomere spacing measured along the long axis of the *myofibrils*. In wavy myofibrils, where the axes of the fiber and of the myofibrils are not parallel, these two measurements are different.

Fiber Preparation

Rana temporaria were usually employed in preference to the more readily available *Rana pipiens*. The diameter of the individual fibers in the semitendinosus muscle is rather larger in *R. temporaria* (personal observation), and fibers of 100–120 μm diameter can be readily isolated; fibers larger than 90 μm in diameter are uncommon in *R. pipiens*. *R. temporaria* were obtained from Gerrard & Haig, Ltd., Surrey, England and were kept at room temperature without feeding in a tank with running water.

Single fibers dissected from the semitendinosus muscle were studied by a two-electrode point voltage clamp method (Adrian, Costantin, and Peachey, 1969). After the isolated fiber had been transferred to the experimental chamber, a few twitches were elicited with the fiber at slack length. A section of the fiber was then immobilized by stretching it slightly across two vaseline-coated Lucite pedestals separated by 2–8 mm. The region of the fiber chosen for study was located between these two pedestals and thus was freely suspended in bathing solution. Fibers were bathed in a normal Ringer solution containing 115 mM NaCl, 2.5 mM KCl, 1.8 mM $CaCl_2$, and 1.5 mM sodium phosphate at pH 7.1. TTX (1 μg/ml) was added to prevent action potentials. Experiments were performed at room temperature, usually 21°–23°C; in two experiments (19 Apr F1 and 29 Feb F1 in Table I), the room temperature was 26°–27°C.

Stimulation

The single fiber was impaled by two microelectrodes; one served to record the membrane potential and the second, inserted diametrically opposite the first, served to pass current from an appropriate feedback circuit. Fibers were clamped at a holding potential of −90 mV, and rectangular depolarizing pulses 100 ms in duration were applied every 15 s. In one fiber (4 Feb F2 of Table I), 200-ms pulses were employed. Contractions in the clamped region were observed visually and recorded on cine film. The feedback circuit had an open loop gain of 7000–20,000 and a maximum output of 25–80 V. We are indebted to Dr. W. Almers for suggesting this circuit (Almers, 1971).

Optics

The voltage-clamped region of the fiber was observed under bright field illumination with green light. The light source was a Xenon 150 watt lamp (Carl Zeiss, Inc., New York), and the intensity of illumination was adjusted with green filters. A Zeiss (Carl Zeiss, Inc., New York) ×40 water immersion objective (numerical aperture = 0.75) was employed, and the condenser was stopped down so that the striation pattern was clearly visible (numerical aperture ~ 0.25). The depth of focus was sufficiently shallow so that the microelectrode passed out of focus with a 10 μm vertical displacement. Fiber thickness was measured as the range of movement of the microscope in focusing from the top to the bottom of the fiber. The plane of focus for observation was set at the midpoint of this range, and the fiber width was measured in this plane. The smaller of these two measurements was taken as the fiber diameter in Table I.

Cine-recordings were made with a Locam 16 mm cine camera (Model no. 51-

0002, Redlake Corp., Santa Clara, Calif.) at 160 frames/s (exposure time, 2 ms). Closure of a relay supplied power to the camera for about 700 ms, and the depolarizing pulse was delivered to the fiber a fixed time after the camera was activated. Each run required about 100 frames of film so that an entire sequence of progressive depolarizations could be recorded on a single reel of film. Film speed was calibrated under conditions similar to those used for the experimental recordings by photographing the oscilloscopic display of both the command depolarization and a pulse synchronous with the camera shutter. In nine sequential determinations, the mean interval between shutter sync pulses during the 100 ms command pulse was 6.2 ms. The interval between sync pulses varied between 5.9 and 6.4 ms during the 100 ms pulse, but the variation between two successive intervals was 0.1 ms or less. In two fibers (4 Feb F2 and 16 Feb F3 of Table I), the film speed was 100 frames/s.

The microscope image was projected on the film by a ×12.5 eyepiece and a 25 mm camera lens focused at infinity. Magnification of the specimen in the film plane was 54 times. Measurements of striation spacing were made on prints with an overall magnification of about 800 times, and calibrations were performed by photographing a grid with 10-μm spacings. There was some "pincushion" distortion of the image; the magnification at the periphery of the frame was about 10% larger than at the center. Most measurements were made over portions of the frame where the variation in magnification was 2–3%; no correction was made for this effect.

Striation spacings were determined by measuring the distance between 10 successive striations and dividing by 10. The accuracy of this measurement was variable and dependent upon the appearance of the striation pattern. Under optimal conditions of sharply defined striations perpendicular to the long axis of the fiber, shortening of 0.01 inches on the photograph, corresponding to a 0.03 μm change in striation spacing, was readily detected; in regions where the striations were angulated or where verniers appeared, the uncertainty of the spacing measurement was perhaps double this value. In some fibers, the distance between two points along one of the longitudinally oriented beaded structures (presumably a chain of mitochondria) which lie between the myofibrils was also measured during contraction; the initial separation of the points (about 30–40 μm) was normalized to the resting striation spacing. The estimate of shortening obtained from these measurements was in good agreement with the directly measured striation spacings (See Figs. 2 and 3). Measurements of the beaded structures were particularly useful at very short striation spacings where the striation pattern itself was difficult to measure accurately.

An intense local contraction is regularly seen at the site of electrode impalement in this two-electrode voltage clamp preparation; Adrian, Costantin, and Peachey (1969) have considered this phenomenon in some detail. In the present study, this local response was ignored, and no measurements of striation spacing were made in this region. The contraction threshold was taken as the depolarization which produced a just visible shortening of superficial myofibrils on the side of the fiber impaled with the voltage-recording electrode.

RESULTS

Depolarizing pulses were appled to the segment of the fiber under microscopic observation, and the pulse amplitude was increased in 2- to 4-mV steps.

At a just-threshold depolarization, the superficial myofibrils shortened only 0.1 μm or less, frequently without any apparent shortening of axial myofibrils. Shortening was rapid and readily visible as a decrease in the striation spacing over 1–2 frames of cine film (6–12 ms), but the slower relaxation phase was difficult to detect. In one fiber, partial relaxation definitely occurred before the end of the 100 ms pulse and in another fiber, the contraction was biphasic. With larger depolarizations, the entire fiber cross section shortened, and the decrease in striation spacing was clearly sustained for the duration of the depolarizing pulse. The extent of shortening also increased with increasing depolarization; shortening to striation spacings of about 2.1 μm was produced with depolarizations of 2–7 mV beyond the contraction threshold. When the extent of shortening was confined to this range, the striation spacing of axial and superficial myofibrils usually differed by less than 0.1 μm, that is, shortening appeared to be more or less uniform through the fiber cross section. With larger depolarizations and more extensive shortening, however, a difference in the pattern of shortening between superficial and axial myofibrils became evident, since the myofibrils in the core of the fiber were thrown into waves. The initial appearance of waves was readily detected in the cine film as a sudden buckling and lateral shift of the longitudinal striation pattern.

Readily visible longitudinal striations arise from longitudinally oriented beaded structures 2–4 μm wide and many sarcomeres in length; the folding of these structures produces the characteristic wavy appearance in cine records (See Fig. 1 B and D). With the resolution achieved in the present experiments, the fine longitudinal striations arising from discrete myofibrils about 1 μm in diameter could not be seen in all portions of the fiber. In regions where these fine striations were visible, their orientation paralleled that of the larger beaded structures.

TABLE I

LOCAL CONTRACTIONS IN UNSTRETCHED FIBERS

Fiber	Diameter	Initial striation spacing	Contraction threshold	Onset of waves	Limiting velocity of shortening	ΔV	ΔV_T
	μm	μm	mV	μm	μm/s per sarcomere	mV	mV
31 May F2*	46	2.28	41.3	1.61–1.58	18.6	6.7	—
31 May F1*	59	2.42	42.2	1.68–1.63	19.8	9.3	—
4 Feb F2*	76	2.24	38.4	1.91–1.86	13.3	14.6	6.6
7 Mar F2	83	2.51	38.4	2.00–1.97	—	>14.8	>6.8
19 Apr F1	83	2.18	32.6	1.65–1.60	22.6	13.6	7.6
21 Apr F1	83	2.22	38.4	1.77–1.75	19.5	9.7	1.7
7 Mar F3	93	2.47	35.4	1.93–1.90	21.2	15.6	7.6
16 Feb F3	100	2.52	34.0	2.06–2.01	—	>10.2	>4.2
23 Feb F2	107	2.46	35.0	2.02–1.97	—	>15.0	>9.0
29 Feb F1	121	2.28	32.8	1.98–1.94	—	>10.0	>4.0
23 Feb F3	130	2.57	34.0	2.14–2.10	—	>19.9	>11.9
3 Mar F3	141	2.34	33.5	1.91–1.88	20.4	9.2	1.2

* Fibers isolated from *Rana pipiens*.

These results are summarized in Table I. The contraction threshold is given as the magnitude of the depolarizing pulse (from a holding potential of −90 mV) which was sufficient to just activate the superficial myofibrils. The striation spacing at which waves were first seen is given in the column headed "Onset of waves" in Table I, together with the striation spacing in the cine frame immediately preceding the appearance of waves. Usually, waves were first seen at axial striation spacings of 1.9 μm or greater, but in a few fibers, shortening progressed well beyond 1.9 μm before bending of the axial myofibrils was seen. When the magnitude of the depolarizing pulse was further increased, waves appeared at shorter striation spacings. In the fiber shown in Fig. 1, for example, waves were first apparent at a striation spacing of 1.88 μm. When the magnitude of the depolarizing pulse was increased by 3 mV, the axial myofibrils shortened without waves to 1.85 μm (Fig. 1A); waves were clearly present at a striation spacing of 1.77 μm (Fig. 1B). With a 3 mV larger depolarizing pulse, no waves developed at a striation spacing of 1.73 μm (Fig. 1 C); definite waves were present at 1.58 μm (Fig. 1 D).

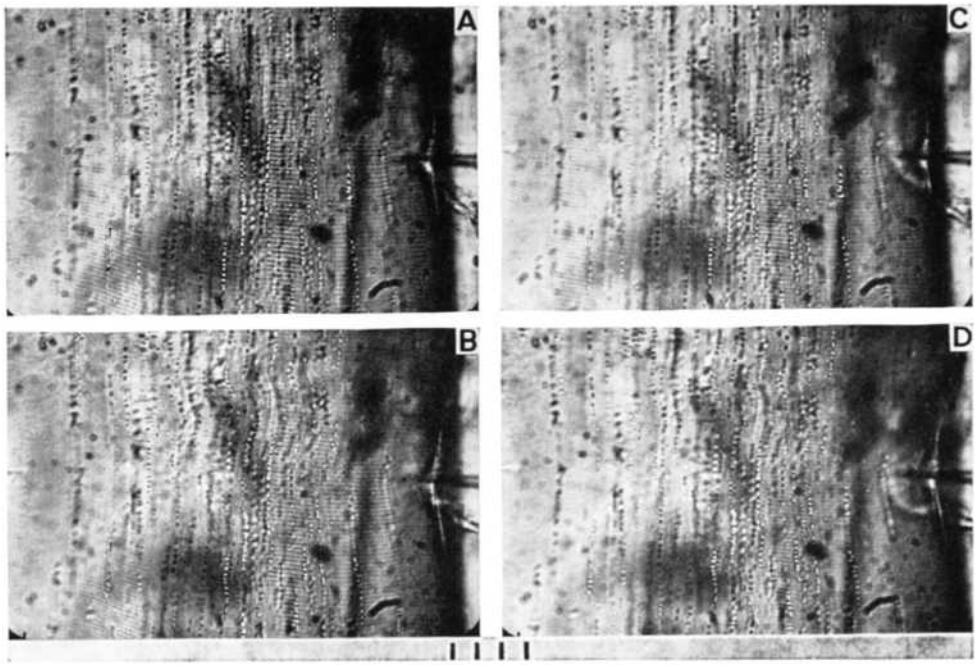

FIGURE 1. Selected cineframes from two successive contractions in fiber no. 3 Mar F3. In panels (A) and (B), contraction was elicited by a 100 ms depolarizing step of 40 mV, and in panels (C) and (D), by a step of 43 mV. Holding potential −90 mV. The voltage-recording electrode can be seen on the right in each panel; the tip of the current-passing electrode is just visible on the left. Striation spacing in axial myofibrils: 1.85 μm in (A), 1.77 μm in (B), 1.73 μm in (C), and 1.58 μm in (D). Note the appearance of longitudinal waves in the core of the fiber in panels (B) and (D). Grid spacing 10 μm.

Gradation of Shortening Velocity

When the amplitude of the depolarizing pulse was increased, the fiber not only shortened further before the appearance of wavy myofibrils in the core but it also shortened more rapidly. The result is illustrated in Fig. 2. Shortening of axial myofibrils without waves has been plotted as a solid line; further shortening with folding of axial myofibrils is shown as an interrupted line. In each of the contractions plotted in Fig. 2, the solid curve of shortening without waves continues to striation spacings below 1.95 μm, i.e., each curve describes

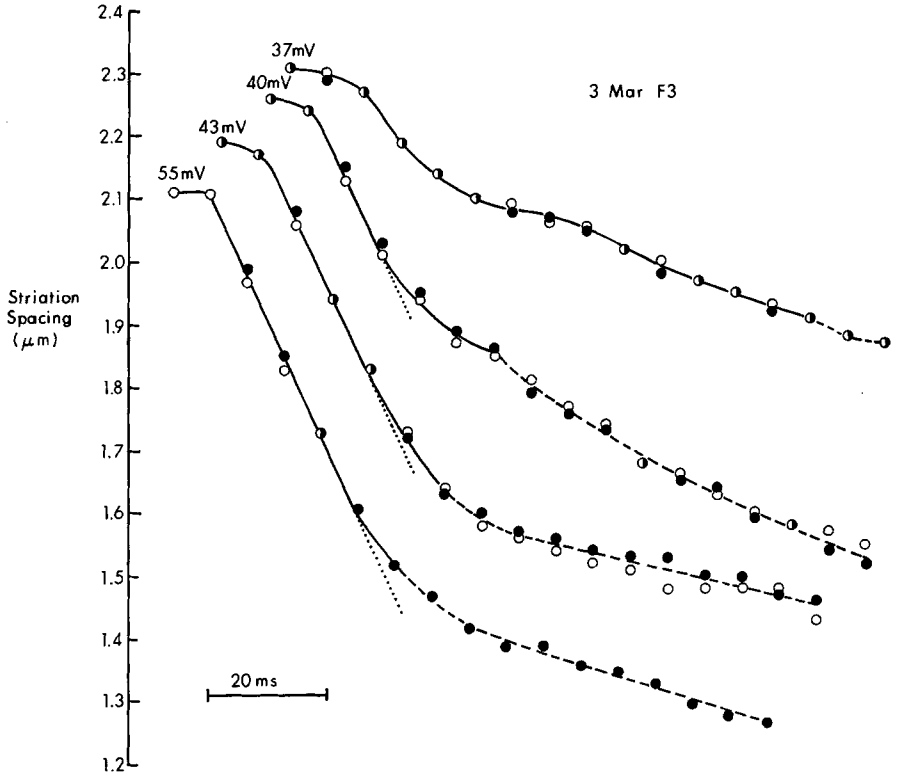

FIGURE 2. Time-course of shortening with increasing depolarization. The magnitude of the 100 ms depolarizing step is shown above each curve. The open circles represent the striation spacing of axial myofibrils measured in successive frames during each depolarization, beginning with the frame immediately before the onset of shortening. Each successive shortening curve has been displaced to the left for clarity. The filled circles are estimates of axial striation spacing obtained from measurements of extramyofibrillar structures (see Methods). Smooth curves have been fitted to the data points by eye. Solid curves indicate shortening without waves; interrupted curves indicate shortening accompanied by folding of axial myofibrils. The dotted line extending from the linear segment of the shortening curves has a slope of 20.4 μm/s per sarcomere.

the time-course of a contraction in which all of the myofibrils in the fiber cross section are to some degree active. With depolarizing pulses of 37 and 40 mV, the velocity of active shortening clearly fell as the striation spacing decreased. When the magnitude of depolarization was increased, the fiber segment under observation shortened more rapidly over the same range of striation spacings. Since all the myofibrils in the fiber cross section were shortening actively even with the smaller depolarizing pulses, the increase in shortening velocity must have been due either to an increase in the intrinsic force of contraction of individual myofibrils or to a decreased load on the actively contracting segment. The external load, the stiffness of the portions of the fiber in series with the segment under observation, presumably increased with larger depolarizations; with the activation of longer lengths of fiber, the inactive segments of fiber between the impalement site and the pedestals on which the fiber rested would have been stretched further and would have exerted greater passive force. Thus if the intrinsic force of contraction in the fiber segment under observation were constant, the speed of shortening should have decreased with larger depolarizations; the observed increase in speed, therefore, can be taken as evidence for an increase in the contractile force of individual myofibrils.

Although it seems likely that the external load on the actively contracting segment increases with increasing depolarization, it should be noted that the interpretation of a graded velocity of shortening as evidence for graded myofibrillar activation need not rest on this assumption. Even if one postulates that some decrease in resistance to shortening occurs with increasing depolarization, the decrease which would be required to account for the observed variation in shortening speed is unrealistically large. In Fig. 2, for example, the velocity at a striation spacing of 1.95 μm increased 5.8-fold above that produced by a 37 mV depolarization; such a change in velocity in a fully activated fiber would require a change in load of 0.5 P_o or more (Hill, 1938). If the length constant of the fiber in Fig. 2 is assumed to be 2 mm, the length of fiber depolarized beyond the contraction threshold by the 37 mV depolarizing pulse is only about 350 μm. Since the length of fiber between the two pedestals was always 2 mm or more, the passive segments of fiber were at least 5 times longer than the actively contracting segment. Thus shortening of the active segment to 1.95 μm stretched the passive regions of the fiber by less than 0.1 μm to striation spacings of less than 2.5 μm. The static load exerted by inactive sarcomeres at this striation spacing is at least an order of magnitude less than 0.5 P_o (Rapoport, 1972), and any velocity-dependent load should presumably be greater in the more rapid contractions.

Reversibility of Extreme Shortening

Since experiments were routinely performed by applying progressively increasing depolarizing pulses, one possible explanation for the increase in ex-

tent and speed of active shortening with increasing depolarization is that each contraction produced some irreversible or slowly reversible decrease in the internal resistance to shortening so that subsequent contractions proceeded under a lighter internal load. To examine this possibility, two series of depolarizing steps were applied to the same fiber; in both series, the extent and speed of shortening without folding of axial myofibrils was graded over a range of depolarizing pulses. One experiment of this type is illustrated in Fig. 3; similar results were obtained in two other fibers. The order in which the

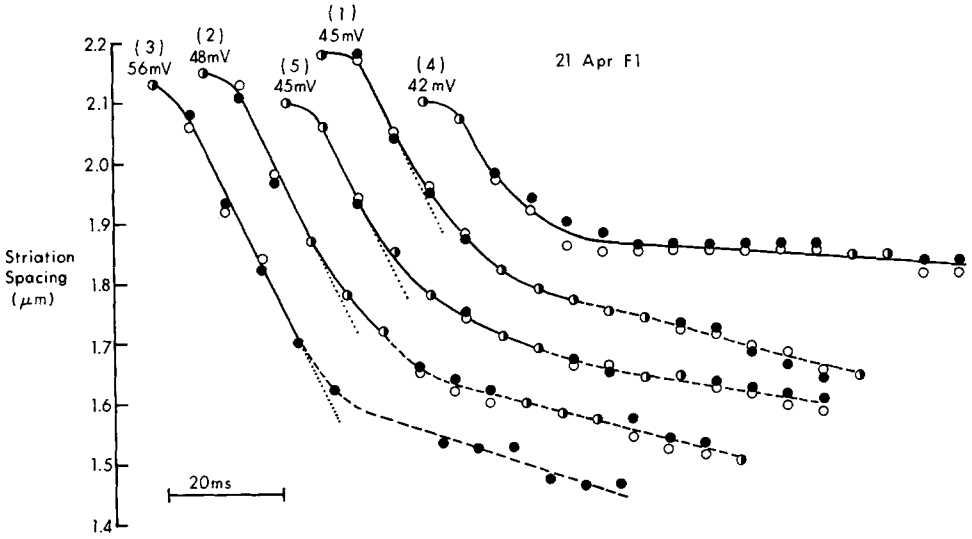

FIGURE 3. Reversibility of graded shortening. Symbols as in Fig. 2. Shortening curves are illustrated for two series of depolarizing steps. The order in which the contractions were elicited is indicated by the number in parentheses above each curve. Both the extent of shortening and the velocity of shortening below 1.85 μm are less in curves 1 and 5 than in curve 3 which was obtained with a larger depolarizing step. The dotted line extending from the linear segment of the shortening curves has a slope of 19.5 μm/s per sarcomere.

records were obtained is indicated on each curve. The largest depolarizing pulse in the first series (Pulse no. 3, 56 mV) produced shortening without waves to 1.7 μm and subsequent shortening with folded axial myofibrils to less than 1.5 μm, while the smaller depolarizations (Pulses no. 4 and no. 5) applied subsequently produced slower and less extreme shortening. Some irreversible effect of extreme shortening does appear to be present in that waves first appeared in this fiber at a striation spacing of 1.75 μm (Pulse no. 1) while in the second series, waves first appeared at 1.67 μm (Pulse no. 5).

One persistent effect of large contractions was a decrease in the resting striation spacing, as can be seen in Figs. 2 and 3. The effect was small, how-

ever; relaxation usually progressed to striation spacings greater than 2 μm, and waves disappeared. Relaxation to striation spacings below 2 μm and persistently wavy axial myofibrils were only seen if a large contracture developed at the electrode impalement site; the experiment was terminated in these cases.

Limiting Velocity of Shortening

With very large depolarizations, large longitudinal movements of the fiber occurred, and the microelectrode impalements could no longer be maintained. Presumably, as the length of fiber activated was increased, the stretching of unequal lengths of relaxed fiber between the active region and the two pedestals on which the fiber rested resulted in different stiffnesses of the two relaxed segments. Since no attempt was made to choose a site for electrode impalement equidistant between the two pedestals, observations could be continued only as long as the external load on the active segment was small enough so that little longitudinal movement occurred during contraction. Nevertheless in a number of fibers it was possible to produce a series of increasingly vigorous contractions in the segment under observation. In these fibers, the speed of shortening appeared to approach a limiting value as the depolarization was increased. In Fig. 2, for example, the maximum measured velocity of shortening over a range of depolarizations from 40 to 55 mV was about 20 μm/s per sarcomere. With larger depolarizations, the shortening speed was more or less constant down to striation spacings below 1.75 μm. It should be noted that at these rapid shortening speeds, the striation spacing decreased more than 0.1 μm per frame of cinefilm; under these conditions, some variation in shortening velocity, perhaps as large as 25%, would not have been detected due to the uncertainty in the measurement of striation spacings. For those fibers in which active shortening of the entire fiber cross section could be followed to striation spacings below 1.75 μm, the maximum observed velocity of shortening has been recorded in Table I.

Three fibers from *R. pipiens* (See Table I) were also examined; the results were similar to those already described, that is, in contractions where the entire fiber cross section was active, both the extent and the velocity of shortening were graded with progressive depolarizations.

Distribution of Waves in Axial Myofibrils

In most of the fibers examined, folding of axial myofibrils first appeared, not in the region immediately between the two electrodes, but about 20–40 μm in either direction along the fiber axis; with further shortening, all the axial myofibrils became wavy, but the amplitude of the waves was variable and somewhat larger at some distance from the electrodes. In the fiber of Fig. 1, for example, the most prominent waves are present in the upper portion of panels B and D. A more striking illustration of this effect can be seen in Fig. 4. Waves

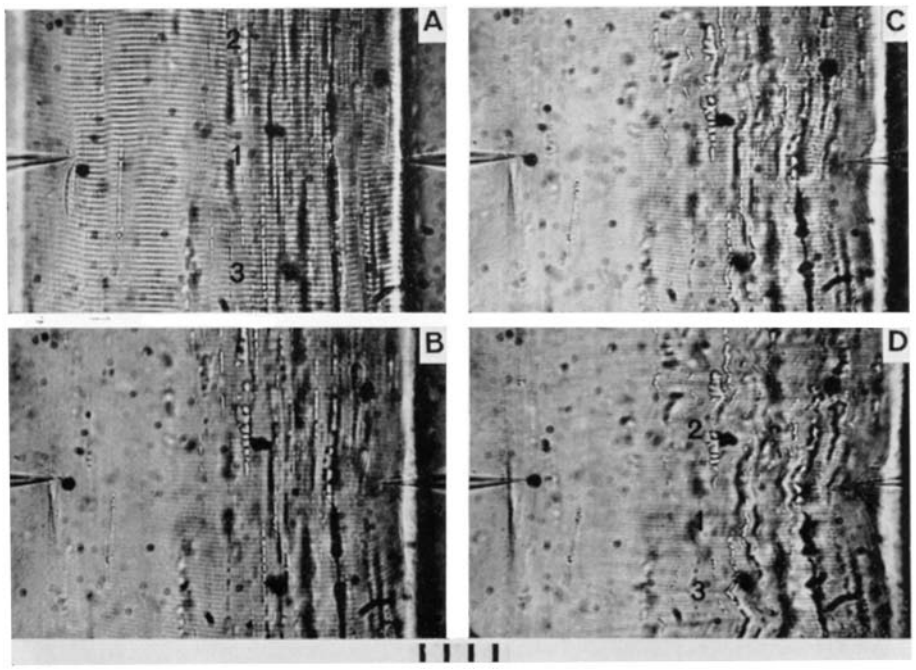

FIGURE 4. Selected cineframes from a contraction in fiber no. 7 Mar F3 in response to a 100 ms depolarizing step of 36 mV. (A), relaxed fiber voltage-clamped at a holding potential of −90 mV. Voltage-recording electrode on right; current-passing electrode on left. (B), cineframe immediately preceding the appearance of waves in axial myofibrils. (C), folding of axial myofibrils is evident in regions above and below the site of microelectrode impalement, but the axial myofibrils between the two electrodes appear straight. (D), with further shortening, axial myofibrils along the entire length of the contracting segment under observation have been thrown into waves. The numbers along the fiber axis in (A) and (D) correspond to the same regions of the fiber in the two frames; a slight shift of the contracting segment toward the bottom of the cineframe is evident during shortening. The time-course of shortening of the axial myofibrils in the numbered regions is shown in Fig. 5, and the frames selected for Fig. 4 are indicated by arrows on the shortening curve. Grid spacing 10 μm.

first appear in the axial myofibrils slightly removed from the impalement site (regions 2 and 3) while the axial myofibrils between the electrodes (region 1) are still straight; with further shortening, all of the axial myofibrils become wavy, but the waves in regions 2 and 3 are larger in amplitude than those in region 1. This difference in the pattern of waves persisted even with very large depolarizations, so that the effect probably is not due to a difference in activation of the axial myofibrils along the length of fiber under observation. One possible explanation for this observation is that the axial myofibrils between the two electrodes shorten with the formation of waves perpendicular to the plane of focus so that the waves are not visible in this region. This is not the

case, however; the delayed appearance of waves in the region between the two electrodes is associated with a temporary cessation of shortening in this region. This result is illustrated in Fig. 5 in which the striation spacing in the numbered regions of Fig. 4 are plotted against time. Shortening is quite uniform along the length of the fiber until a striation spacing of about 1.9 μm is reached. At this point the myofibrils in regions 2 and 3 fold with further shortening; shortening in region 1 lags behind momentarily. About 20 ms later, as

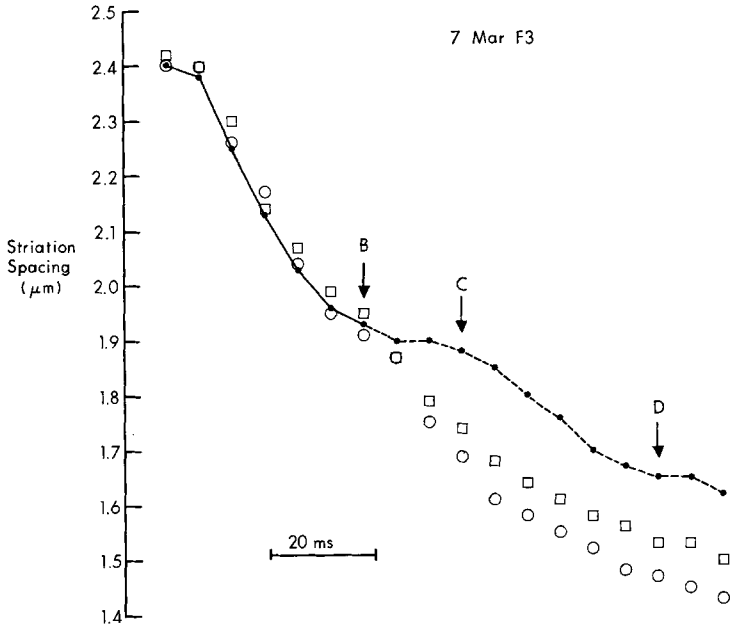

FIGURE 5. Time-course of shortening in the contraction illustrated in Fig. 4. The axial striation spacing was measured in successive frames during contraction in the three numbered regions in Fig. 4 A. The striation spacing in region 1 is plotted as points connected by straight line segments; the spacing in region 2 is plotted as open squares and in region 3 as open circles. The cineframes corresponding to panels (B), (C), and (D) of Fig. 4 are indicated by arrows. In this contraction, folding of axial myofibrils was first noted in regions 2 and 3 in the cineframe immediately after arrow B.

the axial myofibrils in region 1 develop obvious waves, the striation spacing again begins to decrease rapidly.

The microelectrodes provide some resistance to lateral expansion of the fiber since deep indentations develop at the impalement sites during vigorous contraction and lateral expansion of the fiber, and it seems plausible that the microelectrodes, or perhaps the intense local contraction at the impalement sites (see Adrian, Costantin, and Peachey, 1969), could act as a mechanical constraint to folding of the adjacent axial myofibrils. One difficulty with this explanation is that, because the myofilament lattice volume is normally con-

stant, at least above resting length, (Elliott, Lowy, and Millman, 1967), lateral expansion of the fiber also accompanies shortening with straight myofibrils. Thus one might expect some interference with shortening even before waves appear. As Fig. 5 illustrates, this was not found; little variation in striation spacing was present along the core of the fiber until waves appeared. It may be that shortening by myofibril folding entails somewhat greater lateral expansion of the fiber than shortening by myofilament sliding, but no evidence is available on this point.

Fibers at Long Initial Striation Spacings

In another series of experiments, fibers were stretched about 40% beyond slack length before microelectrode impalement. As was the case in less stretched fibers, a just-threshold depolarization produced only slight shortening of superficial myofibrils, and a few millivolts larger depolarizing pulse was required for shortening of the entire fiber cross section. Folding of axial myofibrils with shortening below 2 μm was much less pronounced in these stretched fibers; in two instances, shortening of axial myofibrils below 1.5 μm was obtained without the appearance of waves (See Table II). Since the external load was somewhat greater in these stretched fibers, the shortening produced by the superficial myofibrils with only slightly suprathreshold depolarizations may not have been sufficient to drive the axial myofibrils into coarse longitudinal waves, while larger depolarizations resulted in active shortening of the entire fiber cross section.

Gordon, Huxley, and Julian (1966) found that the velocity of shortening in lightly loaded intact fibers decreased slightly at striation spacings below 1.9 μm. Similar results were obtained in the present experiments. With sufficiently large depolarizing pulses, the fiber shortened from its initial stretched length to striation spacings of about 2.1 μm at an apparently constant speed. With further shortening, longitudinal movement of the actively contracting

TABLE II

LOCAL CONTRACTIONS IN STRETCHED FIBERS

Fiber	Diameter	Initial striation spacing	Contraction threshold	Onset of waves	Limiting velocity of shortening		ΔV	ΔV_T
					Above 2.1 μm	Below 2.1 μm		
	μm	μm	mV	μm	$\mu m/s$ *per sarcomere*		mV	mV
21 Apr F2	71	2.86	40.3	1.88–1.82	18.9	—	>13.2	>5.2
26 Apr F1	77	2.83	39.4	None*	18.4	14.5	10.6	2.6
26 Apr F2	77	2.74	38.8	1.40–1.37	24.9	18.6	8.3	0.3
28 Apr F2	93	3.02	40.3	1.88–1.80	19.1	—	—	—

* Maximum shortening of axial myofibrils to 1.43 μm.

fiber segment sometimes obscured the striation pattern. In two fibers, however, it was possible to follow the time-course of shortening down to striation spacings below 1.75 μm. The limiting velocity over the range of striation spacings from 2.1 to 1.75 μm was about 25% less than at the longer striation spacings. These results are summarized in Table II.

DISCUSSION

The present experiments indicate that at least two distinct processes contribute to graded local contractions in voltage-clamped muscle fibers with progressively larger depolarizations. The first is the recruitment of more myofibrils within the depth of the fiber and the second is the gradation of activation of individual myofibrils over a range of membrane potential. It should be emphasized that these two processes can be distinguished only under very specific conditions. With a barely suprathreshold depolarization, the contraction threshold is exceeded only in the most superficial T-tubules due to attenuation of the applied depolarization along the T-system, and only a superficial annulus of myofibrils shortens (See also Adrian, Costantin, and Peachey, 1969). With larger depolarizations, shortening of the entire fiber cross section is seen, and with sufficiently large depolarizations, all the myofibrils in the fiber cross section shorten without folding to striation spacings below 1.95 μm. Under these conditions, all the myofibrils must be activated, since passive shortening to these striation spacings occurs only by folding of the myofibrils (Brown, Gonzalez-Serratos, and Huxley, 1970, 1971). Thus the change in the pattern of contraction from shortening of only superficial myofibrils with no movement of axial myofibrils to active shortening of the entire fiber cross section reflects the recruitment of myofibrils in the core of the fiber as the surface depolarization is increased.

The gradation of activation at the level of an individual myofibril can be demonstrated by examining contractions in which all myofibrils in the fiber cross section are known to be activated. At striation spacings below 1.95 μm where the absence of myofibrillar folding can be employed as a visual criterion for active shortening of the entire fiber cross section, the speed of shortening was found to increase with an increase in the magnitude of the applied depolarization (See Figs. 2 and 3); thus the intrinsic force of contraction of individual myofibrils increased with increasing depolarization.

LIMITING VELOCITY OF SHORTENING The velocity of shortening in the semitendinosus muscle of *R. temporaria* or *R. esculenta* at 24°C is given by Buchthal and Kaiser (1951 p. 145) as 9.1 muscle lengths/s at a load of 0.05 P_o, that is, about 18–21 μm/s per sarcomere. In the present experiments, the maximum observed velocity of shortening averaged 20.3 μm/s per sarcomere in the four fibers examined at striation spacings above 2.1 μm (see Table II). Passive tension in these stretched fibers was probably about 4–5% of P_o

(Rapoport, 1972 and personal communication) so that the behavior of the short segment of fiber in the present study appeared quite comparable to the behavior of intact muscle. The maximum observed velocity over striation spacings of 2.1–1.75 μm averaged 18.7 μm/s per sarcomere; where measurements over both long and short striation spacings were made in the same fiber, the velocity was about 25% less at short striation spacings (see Table II). This relatively small decrease in velocity indicates that the change in the internal resistance to active shortening over the range of striation spacings from 2.9 to 1.75 μm is quite small. Similar results were obtained by Gordon, Huxley, and Julian (1966) in lightly loaded intact single fibers; the velocity of shortening at 1.7 μm was about 80–90% of the velocity above 2 μm.

FOLDING OF MYOFIBRILS AT SHORT STRIATION SPACINGS Taylor and Rüdel (1970) reported that previously active myofibrils in the core of a fiber can become wavy with extreme shortening; in their experiments with externally stimulated intact fibers, waves appeared at striation spacings of about 1.6 μm, which roughly corresponds to the length of the thick filaments. Thus it seemed to them that the axial myofibrils buckled when they encountered the resistance produced by the thick filaments hitting the Z lines. Relatively low concentrations of caffeine prevented the appearance of these waves and significantly increased the ability of the fibers to produce force at short lengths (Rüdel and Taylor, 1971). Accordingly, Rüdel and Taylor concluded that the folding of initially active myofibrils was due to inactivation induced by shortening.

In the present experiments where the possibility of active propagation within the T-system was eliminated by TTX and where the magnitude of the applied depolarization could be more finely graded than in the Taylor and Rüdel study, folding of previously active axial myofibrils was sometimes seen at spacings well above 1.6 μm (see Fig. 1). With larger depolarizations, waves appeared at progressively shorter striation spacings. In two fibers (31 May F2 and 31 May F1 in Table I), waves first appeared at striation spacings of 1.58–1.63 μm, and larger depolarizations produced shortening of axial myofibrils to below 1.20 μm without folding. Taylor and Rüdel (1970) suggested that the inactivation of the axial myofibrils occured because of failure of electrical transmission along the T-system. If this explanation is applicable to the present results, then the spread of depolarization along the T-system must be progressively impaired over a range of striation spacings.

It is possible, however, to account for the appearance of waves in these TTX-treated fibers at striation spacings above 1.6 μm without assuming that inactivation of axial myofibrils has occured. Since the applied depolarizations spread decrementally along the T-system, the degree of activation was presumably greater in superficial than in deep myofibrils over the range of depolarizing pulses where only partial activation was produced, e.g. the 37 and

40 mV depolarizations of Fig. 2. If the internal resistance to shortening increases progressively below striation spacings of 1.95 μm, shortening without waves of axial myofibrils would cease at a striation spacing at which the developed force was just equal to this internal load; further shortening of the more vigorously contracting superficial myofibrils would then result in folding of the axial myofibrils.

Quantification of the Graded Contractile Response

Two distinct stages of contraction of the voltage-clamped fiber can be recognized in the present experiments: (1) a just-threshold contraction of superficial myofibrils, and (2) active contraction of the entire fiber cross section to below 1.75 μm at a more or less constant speed of shortening; the response of the fiber in Fig. 2 to a 43 mV depolarization is an example of a stage 2 contractile response. The increment in depolarization above threshold which was required to elicit a stage 2 response has been recorded as ΔV in Table I. In some experiments, fiber movement or voltage clamp failure limited the magnitude of the applied depolarization, and in these cases, ΔV has been recorded as greater than the largest increment in depolarization achieved in the experiment. Increments in depolarization beyond ΔV produced no further increase in shortening velocity, and the extent of shortening of axial myofibrils without waves was only slightly increased (Compare the response to a 43 and a 55 mV depolarization in Fig. 2). Since distortion of the thick filaments probably occurs with shortening below 1.65 μm (Gordon, Huxley, and Julian, 1966), it seems possible that the slight increase in extent of contraction seen with very large depolarizations was the result, not of a further increase in myofibrillar activation, but rather of some irreversible change in the internal resistance to shortening with repetitive contractions to very short striation spacings. If this is the case, then the stage 2 response can be taken to represent nearly maximal activation of the entire fiber cross section. The observation that, in a stage 2 contraction, the velocity of shortening, in the presence of a finite internal load, was close to the maximum velocity expected for a frog twitch fiber offers some support to this suggestion.

If the entire fiber cross section is maximally activated in a stage 2 response, then the magnitude of ΔV should reflect gradation of the contractile response by both graded myofibrillar activation and graded spread of depolarization. If the contribution of graded myofibrillar activation to ΔV could be estimated independently, it should be possible to calculate the voltage gradient along the T-system. Since the voltage gradient along the T-system should contribute little to ΔV in the smallest fibers studied, one possible estimate of the range of depolarization over which myofibrillar activation is graded is the value of ΔV in small fibers. From Table I, ΔV in the two smallest fibers is about 8.0 mV; if this value is taken as the range of depolarization over which myofibrillar acti-

vation is graded in all the fibers studied, then the voltage gradient along the T-system (ΔV_T) in the larger fibers is simply ΔV-8. The values of ΔV_T obtained in this manner are shown in the last column of Table I. If this analysis were valid, ΔV_T should increase progressively with increasing fiber diameter; this is clearly not the case. Most strikingly, one of the smallest values of ΔV_T was obtained in fiber no. 3 Mar F3, the largest fiber examined. In fiber no. 23 Feb F3, on the other hand, folding of the axial myofibrils appeared below striation spacings of 1.93 μm even with a depolarization 19.9 mV beyond the surface threshold.

Unfortunately many factors may contribute to the variability of ΔV, and it is not possible to evaluate their relative importance from these experiments. Thus the range of membrane potential over which myofibrillar activation is graded may vary from fiber to fiber. Furthermore the internal resistance to shortening below 2.1 μm is clearly different in different fibers (Brown, Gonzalez-Serratos, and Huxley, 1971) and the development of a stage 2 response may require different degrees of activation in different fibers, rather than essentially complete activation in all fibers as was assumed in the above analysis. Still another possibility which must be considered is that the cable properties of the T-system are affected by extreme shortening (Taylor and Rüdel, 1970). If changes in the geometry of the T-system with shortening below slack length were to result in significant decreases in the radial conductance of the tubules, the electrical properties of the T-system calculated under conditions of extreme shortening might have no physiological significance. The values of ΔV and ΔV_T were apparently independent of initial striation spacing (compare Tables I and II), so that extensive shortening above slack length did not appear to markedly affect the spread of depolarization along the T-system.

RELATION TO PREVIOUS STUDIES Adrian, Costantin, and Peachey (1969) have studied the radial spread of contraction in fibers stretched to striation spacings beyond 3 μm, and have employed simultaneous shortening of superficial and axial myofibrils as a criterion for activation of the entire fiber cross section. In their experiments, the first detectable shortening of axial myofibrils was seen with depolarizations 1–2 mV smaller than were required for vigorous shortening, and they attributed this to passive shortening of axial myofibrils secondary to vigorous contraction of superficial myofibrils. The finding of graded myofibrillar activation in the present study raises the possibility that this initial slight shortening may have represented a just-threshold activation of axial myofibrils, so that their estimate of the voltage gradient along the T-system may have been 1–2 mV too large. If this smaller gradient is accepted as correct, the tubular length constant for a 100 μm fiber, as calculated in their experiments, would be about 90 μm instead of 60 μm.

Graded Myofibrillar Activation and the Calcium Release Mechanism

There is much evidence to indicate that normal activation of the frog twitch muscle fiber results from an increase in the myofibrillar calcium concentration of about 0.1 mM (Ebashi and Endo, 1968). The absence of a calcium influx of this magnitude from the extracellular medium has led naturally to the assumption that the source of activator calcium is an intracellular calcium store in the sarcoplasmic reticulum (SR), and that calcium release results from an increase in the calcium permeability of the SR following *T*-tubule depolarization.

Two somewhat different mechanisms have been proposed to explain this increase in calcium permeability (see Costantin, 1971 *b*). One is that the calcium permeability of the SR membrane is voltage-dependent, and that current flow across the SR-*T* junction depolarizes the SR to some voltage threshold at which a regenerative calcium current develops. The second mechanism involves a chemical trigger of the permeability change. A calcium-induced release of calcium from the SR has been reported in skinned muscle fibers (Endo, Tanaka, and Ogawa, 1970; Ford and Podolsky, 1970), and it has been proposed that a small amount of calcium in the myofibrillar space can trigger SR calcium release. Both of these mechanisms predict that, after the development of a threshold calcium flux across the SR membrane, an all-or-none increase in calcium permeability should occur. Although graded increases in calcium permeability and thus the graded activation seen in the present experiments could be explained by assuming that calcium release was limited by a time-dependent inactivation process, the available evidence indicates that the time-course of inactivation is much too slow for significant inactivation to occur during the 100 ms depolarizing pulses employed in the present study. Hodgkin and Horowicz (1960) have reported that, with sustained depolarizations produced by high K, the duration of contraction, and presumably of calcium release, is at least 1–2 s with depolarizations to about 0 mV and 5 s or more with smaller depolarizations.

Attenuation of the regenerative response with distance could also serve to prevent an all-or-none increase in SR calcium permeability throughout an entire muscle fiber. Ford and Podolsky (1972) have attributed the graded force spikes seen in skinned fibres in response to added calcium to a failure of propagation into the core of the fiber of a regenerative calcium release from the superficial myofibrils. Similarly, one might account for the demonstration by Huxley and Taylor (1958) that activation can be confined to a single half-sarcomere, if calcium release were assumed to decrease along the longitudinal elements of the SR and thus fail to excite the adjacent lateral sac. In the present experiments, however, the entire fiber cross section over a length of the fiber

many sarcomeres long has been shown to be capable of graded activation in response to progressive surface and presumably *T*-tubule depolarization; failure of propagation of a regenerative response within a single sarcomere is unlikely under these conditions. Thus, the present results indicate that the release of calcium in amounts sufficient to activate the contractile mechanism need not trigger an all-or-none increase in SR calcium permeability. Instead, in the intact fiber, the amount of calcium released during the activation process appears to be regulated by the transmembrane potential across the *T*-tubules.

One possible explanation for the presence of a calcium-induced calcium release in the skinned fiber and the absence of a similar phenomenon in intact muscle is that the structure upon which calcium acts is not readily accessible to myofibrillar calcium in the intact fiber, but that, under appropriate conditions in the skinned fiber, the structure becomes exposed to the myofibrillar space. For example, if *T*-tubule depolarization caused the release of calcium from the myofibrillar surface of the tubule and if this calcium acted upon a calcium-sensitive receptor within the SR-*T* junction to cause calcium release from the lateral sacs of the SR, then disruption of the SR-*T* junction, by making the calcium-receptor accessible to the myofibrillar space, would permit myofibrillar calcium to trigger calcium release. It seems quite possible that in intact and in freshly skinned fibers, a portion of the junctional gap is not accessible to the myofibrillar space (Franzini-Armstrong, 1971); in such fibers the application of calcium to the myofibrillar space does not result in a propagated contraction (Niedergerke, 1955; Podolsky and Costantin, 1964). With immersion of a skinned fiber in relaxing solution, however, a calcium-induced calcium release becomes evident, and electron microscopy of such fibers reveals that in many triads the *T*-tubules and the lateral sacs of the SR have become separated (personal communication by M. Endo and by S. Hatchett and R. J. Podolsky).

We are grateful to Dr. E. Corpus for excellent technical assistance. In addition, L. L. Costantin wishes to acknowledge the invaluable assistance of Dr. K. A. Forde in the initial stages of this study. This investigation was supported by U. S. Public Health Service Research Grants No. 5R01-AM 12071 (National Institute of Arthritis and Metabolic Diseases) and No. 7 R01-NS10327 (National Institute of Neurological Diseases and Stroke).

Received for publication 3 October 1972.

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