

The T-SR Junction in Contracting Single Skeletal Muscle Fibers

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ABSTRACT The junction between the T system and sarcoplasmic reticulum (SR) of frog skeletal muscle was examined in resting and contracting muscles. Pillars, defined as pairs of electron-opaque lines bounding an electron-lucent interior, were seen spanning the gap between T membrane and SR. Feet, defined previously in images of heavily stained preparations, appear with electron-opaque interiors and as such are distinct from the pillars studied here. Amorphous material was often present in the gap between T membrane and SR. Sometimes the amorphous material appeared as a thin line parallel to the membranes; sometimes it seemed loosely organized at the sites where feet have been reported. Resting single fibers contained 39 ± 14.3 (mean \pm SD; $n = 9$ fibers) pillars/ μm^2 of tubule membrane. Single fibers, activated by a potassium-rich solution at 4°C , contained 66 ± 12.9 pillars/ μm^2 ($n = 8$) but fibers contracting in response to 2 mM caffeine contained $33 \pm 8.6/\mu\text{m}^2$ ($n = 5$). Pillar formation occurs when fibers are activated electrically, but not when calcium is released directly from the SR; and so we postulate that pillar formation is a step in excitation-contraction coupling.

The membrane systems of skeletal muscle have distinct functions. The plasma membrane separates the muscle fiber from the extracellular space. The plasma membrane consists of the outer membrane, which conducts the action potential down the length of the fiber, and the transverse tubular system (T system for short), which conducts the action potential radially into the interior of the fiber. The sarcoplasmic reticulum (SR) is an intracellular membrane system, contained entirely within the muscle fiber, consisting of terminal cisternae (TC) and longitudinal SR. The SR stores, releases, and reaccumulates the calcium ion which is the activator of troponin and the chemical controller of contraction. The junction between the T system and the SR (the T-SR junction) links the outer and intracellular membrane systems. It is the only site where the potential change across the outer membranes interacts with the intracellular membrane system, the SR, to control calcium release and thus contraction.

The spread of excitation from T system to SR is probably associated with a structural change at the T-SR junction. But the extent of that structural change and the resolution necessary to observe it are unknown. If the spread of excitation from T system to SR were caused by an increase in the

conductance of a channel linking the systems, the associated structural change would be molecular, indeed atomic, and invisible to present-day microscopy. On the other hand, if the spread of excitation were through the motion of a rigid macromolecule, mechanically coupling a voltage sensor in the T membrane to calcium release from the TC (Schneider and Chandler, 1973; Chandler et al., 1976), the associated structural change might be visible in the electron microscope. If T-SR coupling involved the *formation* of new links from T to TC, instead of a movement of existing links or change in their conductance, the structural change should be visible with present-day microscopy.

The experiments reported here were designed to examine the structure of the T-SR junction in contracting muscle in order to investigate the structural basis of T-SR coupling. Single muscle fibers were activated by prolonged depolarization generated by the sudden application of a solution rich in potassium. The fiber was fixed with glutaraldehyde during the plateau of tension. Pillars, which are seen and defined as parallel electron-opaque lines bounding an electron-lucent interior, frequently spanned the gap between T and TC membranes (Eisenberg and Eisenberg, 1980). Structures similar to pillars have been observed for many years (for example, Birks, 1965; Kelly, 1969; Fawcett and McNutt, 1969; Walker et al., 1971; Spray et al., 1974; Eisenberg et al., 1979 *a* and *b*; Sommer et al., 1980 *a*; Forbes and Sperelakis, 1980); but the images were considered unconvincing until Somlyo (1979) examined resting fibers fixed with glutaraldehyde, then treated with tannic acid.

The convincing images of structures spanning the T-TC gap (Somlyo, 1979; Eisenberg et al., 1979 *a*) show pillars with crisp boundaries and electron-lucent interiors that distinguish them from the "feet" seen as solid structures with electron-opaque interiors in heavily stained preparations (Franzini-Armstrong, 1970). Amorphous fuzzy material as well as pillars are present in the junctions of resting and electrically activated fibers. The preparative procedures used here did not stain the fuzzy material as much as in earlier work and, probably for this reason, we rarely observed feet in resting or electrically activated muscle. Feet were often seen in tannic acid preparations, which in fact contained both pillars and feet, as previously shown by Somlyo (1979; see particularly her Figs. 4 and 6).

Muscle fibers fixed with calcium in all solutions, up through the early stages of dehydration, have electron-opaque precipitates at the T-SR junction (Polittoff et al., 1974), which raises the possibility that pillar formation is a side effect of an increase in sarcoplasmic calcium rather than an integral step in T-SR coupling. Sarcoplasmic calcium concentration can be increased, and contracture produced, by applying caffeine to a muscle fiber. We found that fibers fixed during caffeine contractures contained about the same number of pillars as resting muscle, which suggests that pillar formation is a cause, not a result, of calcium release.

Pillar formation thus seems to be a step in excitation-contraction (EC) coupling, although the functional role, if any, of the formed pillars cannot be determined by the methods used here. Pillars might be the rigid rods postu-

lated to couple charge movement in the T membrane to calcium release from the TC (Schneider and Chandler, 1973; Chandler et al., 1976) or they might be the structures that allow ionic current to flow from T system to TC (Mathias et al., 1980; Mathias et al., 1981).

METHODS

Preparation

A variety of preparations of skeletal muscle from *Xenopus laevis* were used. Whole sartorius muscles and large bundles of fibers from the toe muscle of the fifth digit were used for some studies of structure. All experiments involving contracting muscle were done on dissected single fibers of the toe muscle kept at a temperature of $\sim 4^{\circ}\text{C}$, unless otherwise indicated. Fibers were usually left overnight in the cold after dissection.

Stimulation

The health of fibers was checked by the size of a twitch, then by a contracture produced by rapid application of a potassium-rich solution (100 mM K^+ , 1.8 mM Ca^{++} , 20.15 mM Na^+ , 115 mM methanesulfonate $^-$, 3.6 mM Cl^- , 0.85 mM H_2PO_4^- , 2.15 mM HPO_4^{--}) with normal $[\text{K}][\text{Cl}]$ product (Fig. 1 A). The potassium-rich solution was kept at 4°C to prolong the peak of tension (Caputo, 1972). We could not keep fibers cooler, because a too potent Peltier element produced layers of supercooled, and thus lethal, solution near the bottom of the bath. After a recovery period, fibers were stimulated again and the twitch was checked. Fibers showing visual signs of damage or deviant tension records at any stage of the procedure were rejected.

Caffeine Contractures

Caffeine contractures were induced at a temperature of 4°C by a solution of normal Ringer to which 2 mM caffeine had been added (Fig. 1 B). This dose was chosen to ensure full activation at this temperature, following preliminary experiments at other concentrations (see Sakai et al., 1970; Yu et al., 1979). The fibers analyzed were fixed in the glutaraldehyde solution designed for resting fibers (described later) without addition of caffeine to the fixative. In preliminary experiments, a few fibers had been fixed in cold fixative containing 2 mM caffeine. The structure of these fibers was grossly disordered and so fixative-containing caffeine was not used in the experiments analyzed here.

Other Procedures

Some bundles of fibers were placed in 1% tannic acid (code A-310; 79226; lot 764674; Fisher Scientific, Fair Lawn, N. J.) after glutaraldehyde fixation (method D of Somlyo, 1979). Other resting bundles were fixed and processed as previously described, but 5 mM CaCl_2 was added to each processing solution, through the early steps of dehydration (Oschman and Wall, 1972; Politoff et al., 1974).

Fixation

Resting fibers were fixed sometime after relaxation from a twitch with a solution of 5% (wt/vol) glutaraldehyde in a buffer (pH 7.2) of 100 mM Na^+ cacodylate $^-$ and 1.8 mM CaCl_2 (Eisenberg and Gilai, 1979). All glutaraldehyde was "Ultrapure TEM Grade" from Tousimis Research Co., Rockville, Md. "Ultrapure" glutaraldehyde was

used to decrease the amount of electron-dense particulate matter on the sections. Fibers electrically activated by a potassium-rich solution were fixed during the plateau of tension (Fig. 1 A) with a solution of 5% glutaraldehyde, 100 mM K^+ cacodylate $^-$, 15 mM Na^+ methanesulfonate $^-$, 1.8 mM $CaCl_2$, 0.85 mM Na^+ $H_2PO_4^-$, 2.15 Na^+ HPO_4^{2-} . After overnight fixation, the single fibers were removed from the bath and washed in a buffer (pH 7.2) of 100 mM Na^+ cacodylate $^-$, 10% sucrose, and 1.8 mM $CaCl_2$. Fibers were cut into segments and processed as previously described (Eisenberg and Gilai, 1979). Preservation of filaments and membranes was qualitatively similar in resting and activated fibers. Some variability in the volume of the longitudinal SR is always found in micrographs. Indeed, Sommer et al. (1980 *b* and references cited therein) have suggested that changes in SR volume have a specific functional role. The range of volume appeared similar in resting and activated fibers.

Sectioning and Photographing of Sections

Sections were cut parallel to the longitudinal axis of the fiber with an LKB-Huxley microtome (LKB Instruments, Inc., Rockville, Md.) using a diamond knife. Most of

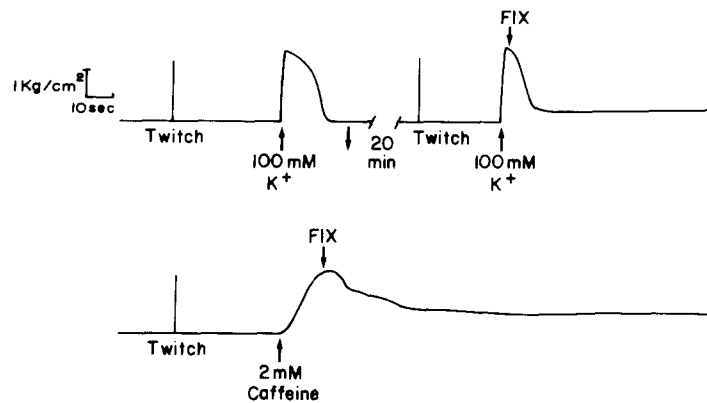


FIGURE 1. Upper trace: the tension record of a typical experiment fixing a single muscle fiber during a potassium contracture. Lower trace: the tension record of a typical experiment fixing a single muscle fiber during a caffeine contracture.

the sections examined in the electron microscope were very thin and gray in color when floating on water; some blocks were difficult to cut and yielded somewhat thicker silver sections. Sections were stained with Reynolds' (1963) lead citrate for 2 min and 1% aqueous uranyl acetate for 10 min at room temperature. Sections were photographed either with a Philips 300 (Philips Electronic Instruments, Inc., Mahwah, N. J.) or a JEOL 100CX (JEOL USA, Electron Optics Division, Peabody, Mass.) electron microscope at an accelerating voltage of 60 or 80 kV with an objective aperture of 20 μm . Accurate and frequent calibration was made using the same waffle calibration grid (Polysciences, Inc., Warrington, Pa.) in both electron microscopes. The nominal scope magnification was 20,00 \times and about three times photographic enlargement was used.

Areas to be photographed were chosen by a systematic rule, designed to produce a random sample of the structure of the muscle fiber. Nine sites were defined within each square opening of the copper grid used to support the section in the electron beam, namely the four corners, the four regions halfway along each side of the grid

square, and finally the point in the center of the square grid opening. Each of these sites was photographed, if occupied by muscle, progressing from grid square to grid square sequentially (without omission), until 15 micrographs were taken from a single section. Since the location of the section on the grid is thought to be random (i.e., uniformly distributed), micrographs taken this way select different locations in an unbiased and statistically reproducible manner. An additional 15 photographs were taken, in a similar manner, from a second section cut from the same block placed on another grid. The second section was not contiguous to the first; it was usually $\sim 1 \mu\text{m}$ further through the fiber.

Precautions to randomize the structures photographed are essential because subjective selection of T-SR junctions may well introduce bias: selected junctions are likely to emphasize the regular structure, stored in the mind's eye of the investigator, at the expense of the irregular structure actually present in the muscle.

The thickness of three typical sections was measured by embedding the grid supporting the section within a new block of Epon. A glass knife was used to cut the re-embedded grid and section at right angles to the original plane of section. The original section was located by scanning between the profiles of the copper grid. The sections measured were 50, 70, and 90 nm thick.

Morphometry

It was important to develop criteria that would objectively select the T-SR junctions to be examined for pillars, rejecting sections cut at such an angle that pillars would certainly be obscured by tilted membranes or overhanging material in the thickness of the section. The criteria, which had to be easily applicable to the more than 10,000 junctions examined, were (a) that the T membrane appear with a crisp boundary, with visible signs of its bilayer substructure, and (b) that the gap between T and TC membrane be clearly visible.

Boundary lengths, B_T , were measured by the method of Buffon (1777) (see analysis in Weibel, 1979 and 1980; Solomon, 1978) using the formula

$$\frac{B_T}{A_f} = M \frac{\pi}{2} \frac{I_T}{L_f} \quad (1)$$

I_T is the number of intersections that a test line makes with the boundary of the T membrane. L_f is the length of the test line on the test grid placed over the muscle fiber. M is the magnification factor at which the photograph of the muscle was taken. A_f is the fiber area in which intersections are counted.

Since the T membrane is partially oriented, a semicircular test grid (Merz, 1967) was used to remove orientation effects by averaging over the various possible orientations (Fig. 2). The test grid consists of continuous connected semicircles, with diameters $\hat{d} \approx 1 \text{ cm}$. The angle between the tangent to the semicircles and the myosin filaments of the fiber is distributed uniformly. Measurements made with this grid are interpreted with the formulae

$$L_f = \frac{\pi}{2} d P_f \quad A_f = P_f d^2 \quad (2)$$

where P_f is the number of semicircles that fall over a muscle fiber and $d = \hat{d} \div M \approx 200 \text{ nm}$ is the diameter of the semicircles.

The surface area, S_T , of the T system is approximately given by

$$S_T \approx t d I_T \quad (3)$$

where t is the section thickness and the boundary of T membrane is assumed to be

perpendicular to the section thickness. The formula is approximate because the sections are not perfectly perpendicular and the section thickness is not measured for every section.

Estimates were made of the amount of obliquely oriented T membrane, the membrane excluded from our analysis of pillar density. Intersections between the test lines and oblique T membrane (called I_{T0}) were counted to estimate the boundary length of oblique membrane (cf. Eq. 1). The ratio $I_{T0}/(I_{T0} + I_T)$ is used as an estimate of the amount of membrane rejected in our analysis of pillar density, although the surface area of oblique membrane cannot be accurately calculated from Eq. 3.

Counting of Pillars

Each T-SR junction was examined in a photographic print with a hand magnifier ($7\times$) giving a final magnification of $400,000\times$. Perpendicular junctions (selected by the criteria just described), were colored yellow and the number of pillars was counted. A pillar was defined as a structure with two dark (electron-opaque) parallel sides surrounding a pale (electron-lucent) interior. A pillar was counted only if it crossed the entire gap between T and TC membranes. Single filaments (i.e., single dark lines) were sometimes seen crossing the gap; they were not counted. Half pillars, projecting from the TC membrane but not spanning the gap, were often seen but were not counted. The images of half pillars seen were sufficiently distinct to distinguish between fully formed pillars and half pillars, but they were not always sufficiently distinct to distinguish among half pillars, fuzzy material, and bulges of the TC membrane.

Micrographs were identified only by negative number and not by experimental procedure, so subjective bias on the part of the observer could be reduced. Some of the contracting fibers had short sarcomeres, however, and were thus recognizable. The number of pillars in each micrograph was counted twice on widely separated (~ 9 mo) occasions without the observer being aware of the previous count (see Discussion). Tables report the mean value of the two counts.

RESULTS

Resting Fibers

A variety of distinct and amorphous structures were seen at the active and resting T-SR junction (Figs. 3 and 4). The T membrane itself was smooth, but the TC membrane had a more complex structure. Long pillars, crossing the T-SR gap, and knobs, apparently short pillars that did not cross the gap, were found at some of the sites where "feet" have been reported (Franzini-Armstrong, 1970). We remind the reader that pillars (by definition) appear with an electron-lucent interior, whereas feet are apparently solid structures

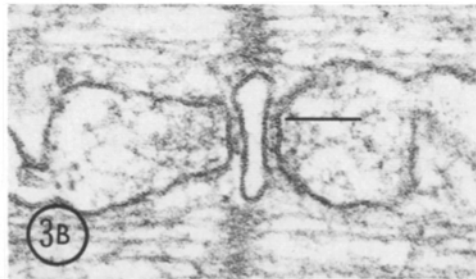
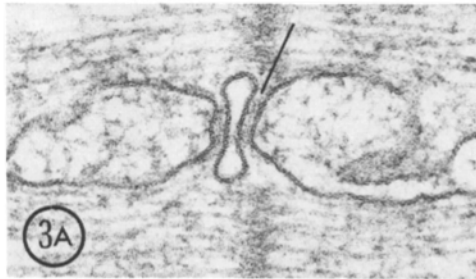
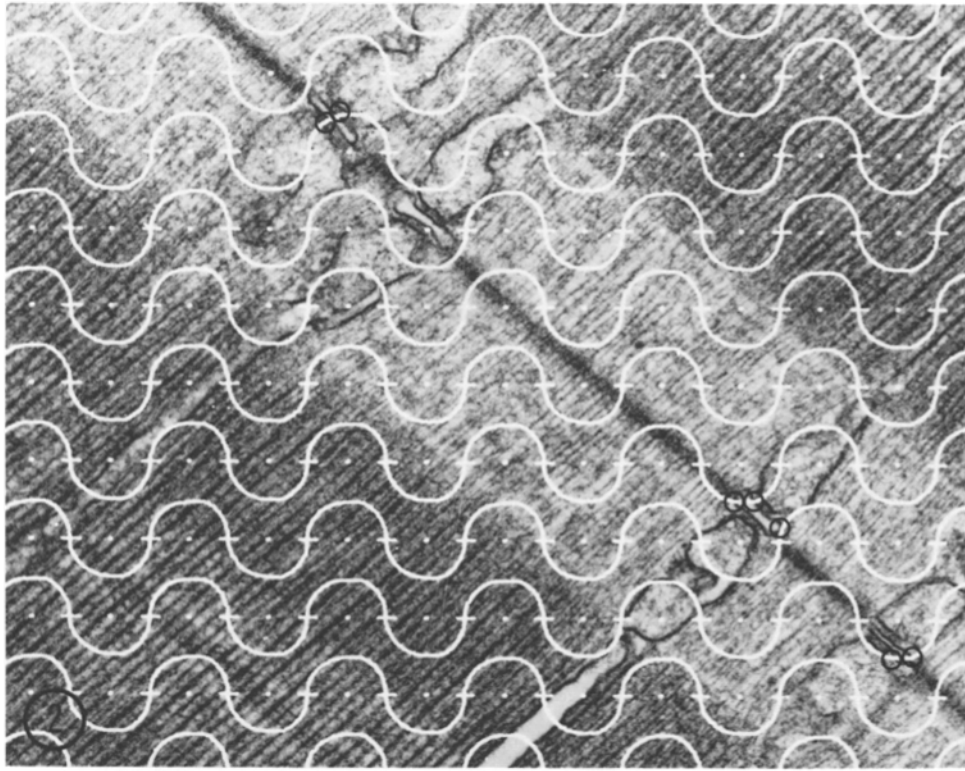
FIGURE 2. The semicircular grid used to estimate morphometric parameters is shown overlying a longitudinal section of resting muscle. Circles mark the intersections I_T of the grid with the boundary of T tubules. $\times 50,000$.

FIGURE 3. Images of the T-SR junction in resting fibers, without pillars. In A, note the central plate indicated by a line. In B, note the half pillar protruding from the TC, also indicated by a line. $\times 100,000$.

FIGURE 4. A and B: images of the T-SR junction in potassium-activated fibers, with pillars marked by arrows. $\times 100,000$.

with an electron-opaque interior. We sometimes found pillars surrounded by amorphous material, but that was not always the case.

Some junctions contained amorphous material that, if heavily stained,



would probably resemble feet. In other junctions the amorphous material was organized into a very thin (5–10 nm thick) plate, presumably oriented almost exactly parallel to the long face of the T tubule and perpendicular to the section. The plate can be compared to the central dense lamina seen in desmosome junctions (Fawcett, 1966, p. 369).

On occasion, *single* electron-dense lines were seen extending all the way from T membrane to TC. Although the single lines resembled each of the parallel dark lines that form a pillar, the correct interpretation of the single line image is uncertain.

Table I shows that resting muscle contains $\sim 2.7 \pm 1.0$ (mean \pm SD; $n = 9$ fibers) pillars per μm of tubule perimeter (i.e., the boundary length B_T). If the section thickness is 70 nm, the corresponding density of pillars N_p/S_T is $\sim 39/$

TABLE I
PILLARS IN RESTING AND K^+ -ACTIVATED FIBERS

B_T	Resting N_p	N_p/B_T	B_T	K^+ -activated N_p	N_p/B_T
Perimeter of T membrane	Number of pillars	Pillars per length	Perimeter of T membrane	Number of pillars	Pillars per length
μm		μm^{-1}	μm		μm^{-1}
49.6	122	2.5	42.2	221	5.2
40.8	79	1.9	54.5	269	4.9
65.9	120	1.8	58.5	223	3.8
58.7	142	2.4	33.0	151	4.6
40.5	197	4.9	40.9	231	5.6
47.4	142	3.0	44.4	206	4.6
43.1	144	3.3	36.2	191	5.3
47.4	117	2.5	42.4	118	2.8
45.0	87	1.9	—	—	—
	Mean	2.7		Mean	4.6
	SD	1.0		SD	0.9

Values are the mean of two determinations from the same micrographs.

The number of pillars per unit area of T membrane is $(N_p/B_T)/t$, where t is the section thickness, ~ 70 nm.

μm^2 of T membrane, where N_p is the number of pillars found on an area S_T of tubule membrane.

The sarcomere length was measured in 20 sarcomeres from each fiber. The mean sarcomere length was $2.3 \pm 0.16 \mu\text{m}$, $n = 9$ fibers. The nonuniformity of sarcomere length within one fiber can be estimated from the ratio of the standard deviation of the 20 measurements to the mean sarcomere length of that fiber. The mean of this index of nonuniformity was 2.6% in the nine fibers.

Resting Muscle, Observed with Other Methods

Small bundles of resting fibers were fixed and treated with tannic acid using method D of Somlyo (1979). The results were strikingly variable from bundle

to bundle and from fiber to fiber within a bundle. Many areas were overstained and few structural details could then be seen, at least at the T-SR junction. In some areas, the lumen of the T system and basement membrane of the sarcolemma were stained black.

Densely stained images, resembling those reported by Somlyo (1979), were observed in still other areas (Fig. 5). Pillars were apparent at many T-SR junctions in the densely stained regions; most junctions in such areas also contained feet. Despite the general impression of dense staining, pillars could often be found free of amorphous material.

Because pillars and feet were only visible in regions with dense staining, estimates of the frequency of the structures will be biased if the frequency is correlated with staining density. The estimates nonetheless have some interest. In one bundle we found 81.3 pillars and 197 feet/ μm^2 of T membrane in the densely staining regions.

Our raw estimate of the density of feet, made from longitudinal sections, is lower than the more reliable figure of 790/ μm^2 of Franzini-Armstrong (1975; Table 3) determined from longitudinal and transverse sections and freeze fracture replicas of frog muscle. The effects of section thickness are substantial when structures as small and frequent as feet are counted in longitudinal sections. An image, apparently of just one foot, is often the composite image of two or even three feet stacked on top of each other within the thickness of the section. We expect that the actual number of feet is between two and three times the number we counted, giving a figure of 400–600/ μm^2 , close to the figure of Franzini-Armstrong.

Fibers fixed with calcium present in all solutions, through the early stages of dehydration, contained dense spots, presumably calcium precipitates, within the T-SR junction (Fig. 6 A and B). No sign of periodicity was apparent in the dense spots. A few dense spots appeared on the membrane of nonjunctional SR, and the thick and thin filaments were peppered with a light nonperiodic precipitate.

Fibers Contracting in Potassium-rich Solutions

The mean sarcomere length of contracting fibers was $2.2 \pm 0.25 \mu\text{m}$, $n = 8$ fibers. The index of nonuniformity for these fibers is 8.3%, which suggests that the sarcomere length of these contracting fibers is three times less uniform than the sarcomere length of resting fibers. Fibers contracting in potassium-rich solutions contained small regions of asymmetrical sarcomeres (Fig. 7) in which the thick filaments appear to have been pulled preferentially towards one Z line (see Fig. 4 of Page and Huxley, 1963). The associated movements of the filament lattice and muscle fiber might produce stepwise shortening, creep in tension, or other exotic mechanical records, not representing the properties of a single sarcomere of controlled length or tension.

The T-SR junctions of fibers contracting in potassium-rich solution were qualitatively similar to the junctions of resting fibers, although pillars spanning the T-TC gap were apparently more common in contracting fibers. The distribution of pillars within a fiber seemed reasonably uniform: although the

pillars were frequently found in patches, the patches were apparently distributed uniformly throughout the core and periphery of the fiber.

Morphometric analysis confirmed the qualitative impression that contracting fibers contain more pillars than resting fibers. The number of pillars in

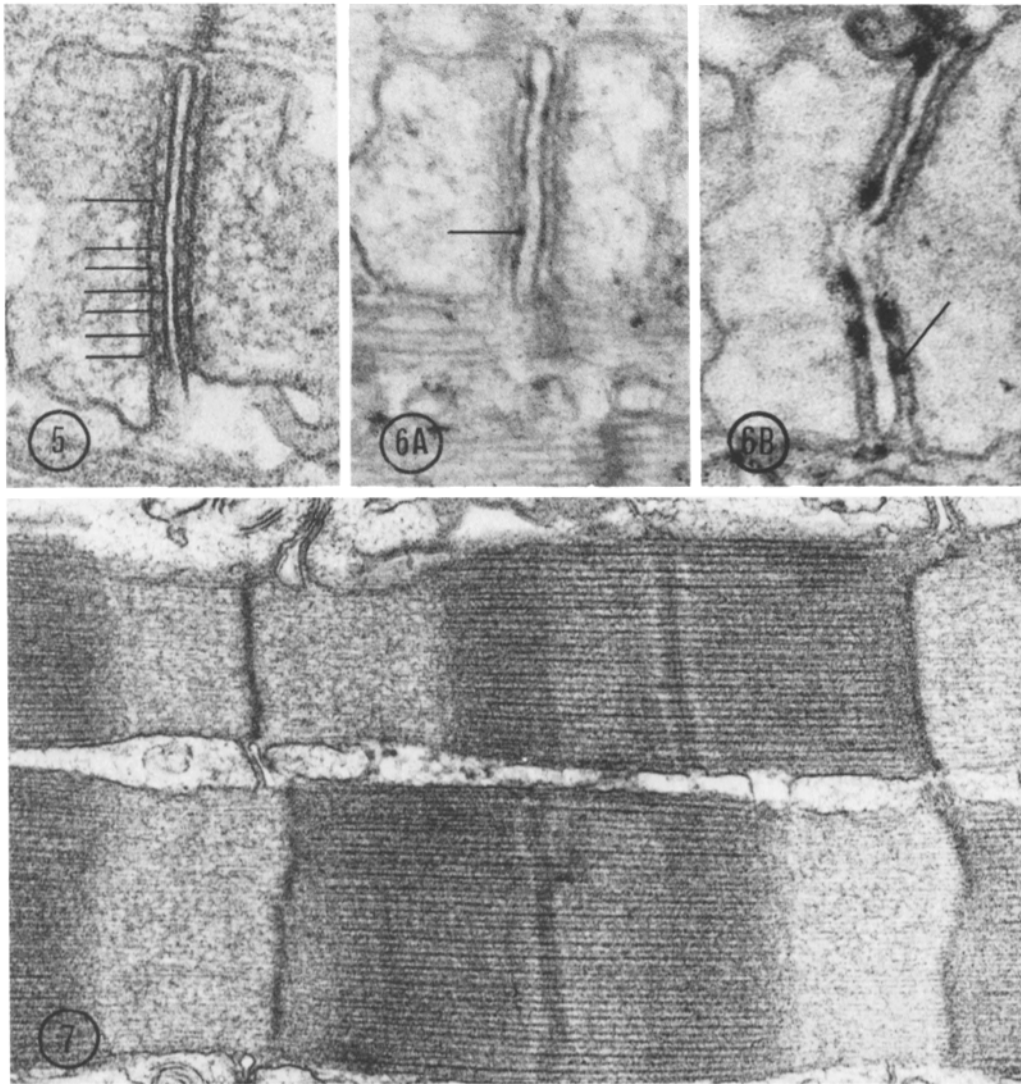


FIGURE 5. An image of a densely stained region of tannic acid treated preparations. A periodic row of pillars is marked by lines. $\times 100,000$.

FIGURE 6. Images of T-SR junctions in resting fibers, prepared for electron microscopy with calcium in all solutions through the early stages of fixation. A shows precipitate at the T membrane. B shows precipitate filling the T-TC gap. $\times 100,000$.

FIGURE 7. Sarcomere nonuniformity in potassium-activated fibers. $\times 40,000$.

muscles contracting in potassium-rich solution increased to 4.6 per μm of tubule perimeter, $\sim 66/\mu\text{m}^2$ of T membrane if the section were 70 nm thick (Table I). A glance at the histogram (Fig. 8) shows that the pillar density seen in contracting muscle differs significantly from that in resting muscle. Because the variances of the two populations are almost equal, Student's two-tailed *t*

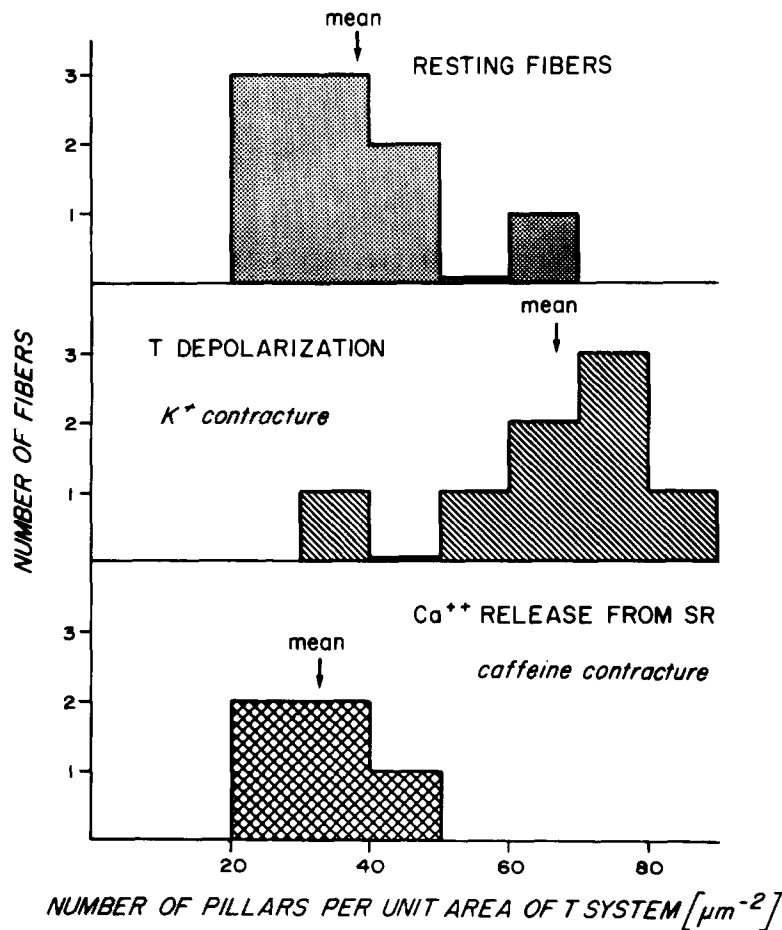


FIGURE 8. Histograms of pillar density (number per unit area of T membrane) in fibers fixed at rest, during a potassium contracture, and during a caffeine contracture.

test can be used to confirm the significance of the difference of the means at a confidence level of 99.9%.

Caffeine Contractures

Preliminary experiments were done with a range of caffeine concentrations from 1-5 mM. At the lower concentrations, the tension recorded was less than

that expected for a fiber of that cross-section and we assumed the entire cross-section of the fiber had not been activated at that concentration and temperature. When 4 or 5 mM caffeine was used, the contraction was visibly nonuniform along the fiber, resulting in supercontracted regions in series with overstretched regions. Occasionally, the fiber was broken in two. The 2-mM concentration used here produced maximal tension without supercontraction at these temperatures in five out of eight experiments. The other three experiments were rejected either because the tension recorded was too low or because sarcomere lengths $<1.6 \mu\text{m}$ were observed in fixed material with the light microscope. The five acceptable experiments were sampled stereologically as described in Methods, exactly as were the resting fibers and potassium-activated fibers.

The fibers contracting in caffeine (Fig. 9 A) had sarcomere length $2.1 \pm 0.4 \mu\text{m}$, $n = 5$ fibers. The index of nonuniformity was 8.7%, which suggests that fibers contracting in caffeine had about the same amount of nonuniformity as fibers contracting in potassium. These fibers showed little modification of structure at the T-SR junction (Fig. 10 B and C), although the gap between TC membrane and T membrane was larger than normal in some junctions (cf. Yoshioka et al., 1981).

The density of pillars in fibers contracting in caffeine was close to the density in resting muscle (Table II and Fig. 8).

DISCUSSION

Analysis of Errors

Our greatest concern was that subjective bias might contaminate our results and so procedures were designed, as described in Methods, to reduce such errors. Some measure of the amount of bias is given by the reproducibility of counts from the same sets of micrographs, counts being taken on two different occasions, ~ 9 mo apart, with the observer unaware of the previous result. The ratio of first estimate of the number of pillars to the second was 1.07 ± 0.27 (mean \pm SD, $n = 9$ fibers) for resting fibers and 0.99 ± 0.18 ($n = 8$) for fibers contracting in a potassium-rich solution. We were surprised by the reproducibility of the results.

The length of T membrane B_T can be measured with as little random error as desired by using large sample sizes and appropriate test systems. Hilliard (1976) estimates the standard deviation of the estimate of B_T as $\sqrt{2}/I_T$ if B_T is measured from discrete profiles of low density sampled so the individual counts I_T are uncorrelated with the measuring grid. The factor of two takes into account the correlation of the entry and exit of each line of the measuring grid through a closed convex profile. Fig. 3 shows that our micrographs and measuring grid meet these conditions. The relative standard deviation for our estimate of B_T is $\sim 7\%$ for each fiber. The error for the ensemble of fibers is, of course, much less. The total error in our estimate of the number of pillars per unit perimeter of T membrane (N_p/B_T) is then given by the root mean square of the relative errors in N_p and B_T . For each fiber, the error is $\sim 15\%$. These

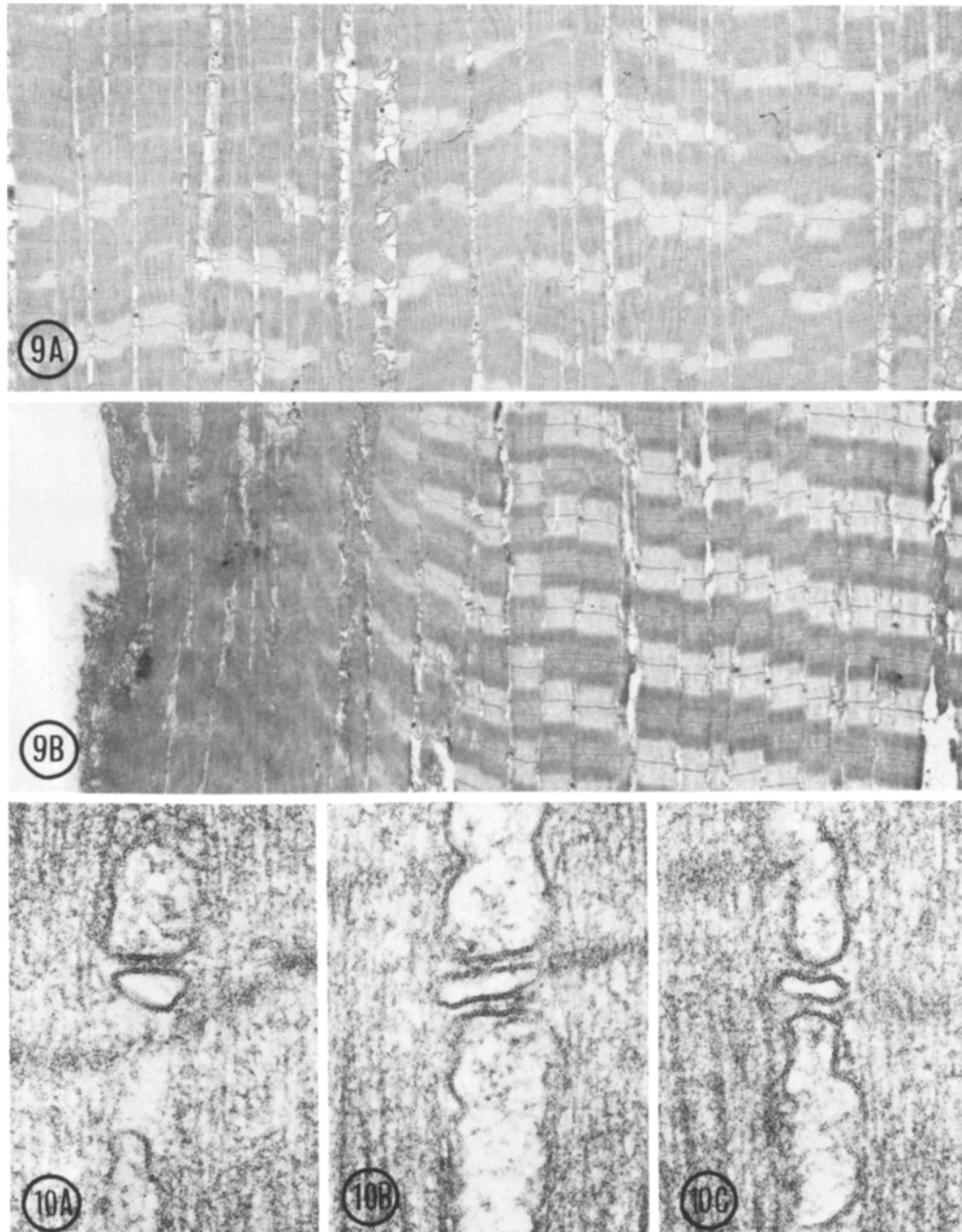


FIGURE 9. A: patterns of irregular sarcomeres in the center of fibers fixed during a caffeine contracture. $\times 5,000$. B: patterns of irregular sarcomeres at the edge of fibers fixed during a caffeine contracture. $\times 5,000$.

FIGURE 10. Images of the T-SR junction in fibers during caffeine contracture. $\times 100,000$.

errors would produce a fraction of the variance shown in the histogram of our results (Fig. 8), the rest of the variance presumably reflecting biological variation in the density of pillars.

Our results are potentially subject to another systematic error because the thickness of the sections was much larger than the size of a pillar or the spacing between potential locations of pillars. If sections were transverse to the longitudinal axis of the T tubule, the section thickness would not affect the comparison of results from resting and stimulated muscle fibers, although it might effect the absolute value of our measurements. On the other hand, if the sections were tilted, pillars might be obscured by overhanging membrane, or associated material. About one-third of the T membrane was rejected as oblique, using the criteria described in Methods, with roughly the same amount rejected in resting and contracting muscles (35% rejected in resting, 43% in potassium contractures, and 38% in caffeine contractures).

TABLE II
PILLARS DURING CAFFEINE CONTRACTURES

B_T	N_p	N_p/B_T
μm		μm^{-1}
36.8	97	2.6
45.0	65	1.5
48.3	138	2.9
34.2	90	2.6
35.7	74	2.1
	Mean	2.3
	SD	0.6

Symbols and notes are the same as in Table I.

The thickness of the section can also lead to underestimates of the number of pillars, if pillars were frequently superimposed in the thickness of the section. In that case, two or three pillars would be counted as only one and the error would itself depend on the frequency of pillars: the more common the pillars, the more the observed count would underestimate the actual pillar frequency. We find, however, that the incidence of pillar images is very low; the incidence of pairs of pillars is presumably much lower, because the probability of occurrence of pairs of pillars can be estimated by the square of the probability of occurrence of individual pillars if the presence of one connected pillar is statistically independent of the presence of another. In that case, only a negligible number of pillars would have been hidden from our view.

A few attempts were made to tilt sections in the electron microscope to look for pairs of stacked pillars and to increase the number of acceptable T-SR junctions. These attempts were abandoned as unproductive. Each time a junction was examined, the tilt stage had to be carefully realigned with respect to the T-TC gap. Because most sections of T-SR junctions do not contain pillars, and those that do can rarely be identified on the fluorescent screen of

the electron microscope, tilting could not be applied to a significant number of junctions containing pillars.

Finally, we are left with the familiar irreducible error of structural measurements: we can report only what we observe after fixation and subsequent processing. The image seen in the electron microscope can properly be used only to form a working hypothesis concerning the actual structure, and possible function, of the tissue. That hypothesis receives support if it correlates well with observations made by other techniques subject to other artifacts, if it aids the design of other experiments, and if it allows insight into how the tissue might function.

Our working hypothesis is that pillar formation accompanies T-SR coupling. Control experiments, suggested by several colleagues, lend support to this hypothesis. The finding that muscle in caffeine contracture has the same number of pillars as *resting* muscle argues that the increased number of pillars observed in electrically activated muscle is neither an artifact nor a byproduct of contraction. Corollaries of contraction such as longitudinal shortening, radial swelling, tension generation, and increased sarcoplasmic calcium concentration are presumably similar in potassium and caffeine contractures, but the number of pillars differs.

The interpretation of the caffeine experiments requires the assumption that the region of the fiber sampled had many of the characteristics of normally contracting muscle, for example, elevated sarcoplasmic calcium concentration. It is difficult to test this assumption directly, but there is reason to expect reasonably uniform activation of cross bridges in our caffeine contractures. (a) Tension records from caffeine contractures indicated that most of the cross-section of the fiber was active at the time fixative was applied. (b) By the time fixative was applied to the fiber (~10 s after application of caffeine), the drug would have reached all the SR of the fiber and presumably released calcium reasonably uniformly from all the SR. (c) Even if the release of calcium from the SR were nonuniform, the time necessary for calcium to diffuse from myofibril to myofibril is $\ll 10$ s. For these reasons, it is difficult to believe that calcium concentration, and therefore activation of cross bridges, was nonuniform at the time fixative was applied.

The question then remains: why are contracting fibers nonuniform in sarcomere length? We interpret this nonuniformity as an example of the well-known tendency of fibers, held only at their tendons, to shorten inhomogeneously during prolonged contractures (Hill, 1953; Gordon et al., 1966; Julian et al., 1978). This inhomogeneity is not thought to reflect inhomogeneity of activation. Rather, it is considered an unavoidable instability of a stretched system of sliding filaments that results from the tendency of some sarcomeres stretched onto the descending limb of their length tension curve to shorten at the expense of others. The nonuniformity interferes with our ability to observe T-TC junctions because it disorders them with respect to our plane of section. We do not believe this type of nonuniformity is likely to bias our results, however, because we see no reason to believe that T-TC junctions that are disordered with respect to our plane of section (and therefore are not included

in our sample) have different numbers of pillars from those that are oriented with respect to our plane of section.

Consider also the observations of tissue treated with tannic acid. Although tannic acid is thought to be a strong crosslinker (Simonescu and Simonescu, 1976 *a* and *b*), the number of pillars counted in tannic acid preparations is close to that found in potassium-activated fibers, which suggests that the same population of structures is being studied in both cases.

Speculations Concerning Excitation-Contraction Coupling

Our results are consistent either with the electrical model of T-SR coupling (Mathias et al., 1980 and 1981) or with a minor modification of the remote control model (Chandler et al., 1976). In the former case, the number of pillars spanning the T-TC gap is taken as proportional to the conductance linking the lumen of tubule with the lumen of the TC. In the latter case, one must

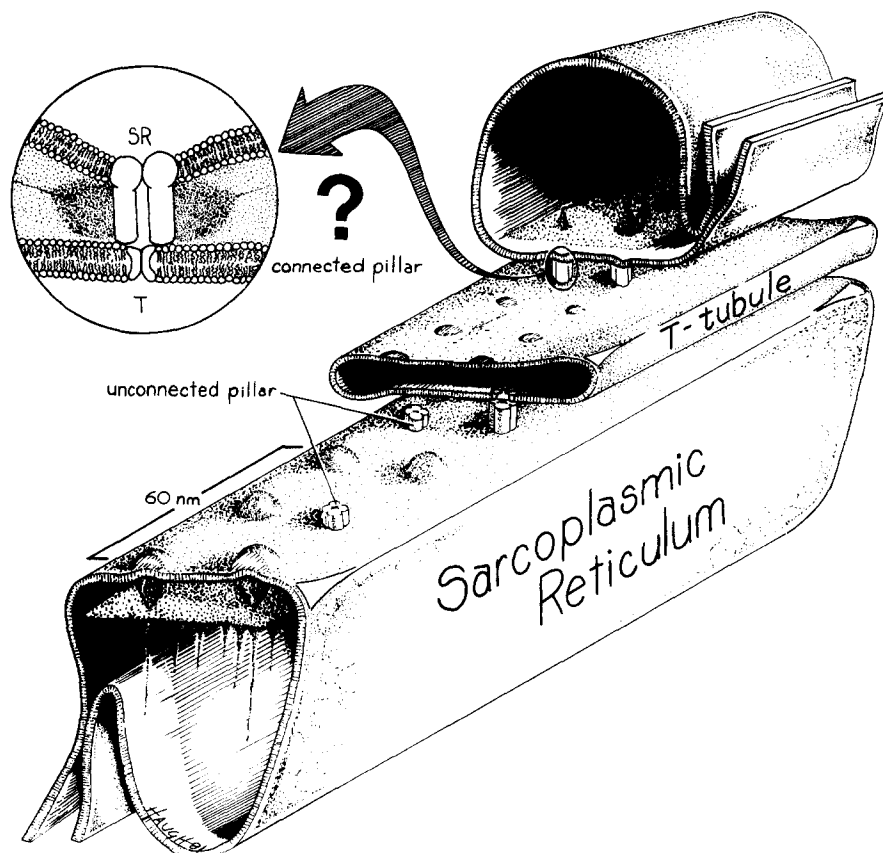


FIGURE 11. A fanciful melding of morphological data (our own and that of others, principally Franzini-Armstrong [1980]; Kelly and Kuda [1979]; and Somlyo [1979]) with the electrical model of T-SR coupling (Mathias et al., 1980).

suppose that pillars (i.e., rigid rods) are assembled upon depolarization. Then, nonlinear charge movement represents the displacement current associated both with assembly of the pillars and with the action of the pillars on the calcium release sites of the TC. This interpretation produces some quantitative difficulties. Schneider and Chandler (1973) and Chandler et al. (1976) estimate that there are 500–600 charged groups per μm^2 of T membrane, using electrical measurements and theoretical considerations, whereas we find only some 40 pillars per μm^2 . But the discrepancy can be easily removed, depending as it does on a specific molecular model of charge movement that might not describe well the substantial conformational changes, binding and unbinding of ions, and internal charge movements involved in pillar formation.

In Fig. 11 we blend the morphological results of Franzini-Armstrong (1980), Somlyo (1979), Kelly and Kuda (1979), and ourselves with the electrical model of T-SR coupling of Mathias et al. (1980), freely drawing on our molecular and artistic imagination to show one way in which pillar formation might produce T-SR coupling. Here pillars are interpreted as *modified connexons* (described in gap junctions by Makowski et al., 1977) that allow ions to flow down their central channel from T to TC lumen. In this interpretation, the pillars must be viewed as highly modified connexons: both their dimensions and their conductance (<0.01 pS) differ substantially from the properties of connexons of gap junctions. Feet are indicated in Fig. 11 by hemispheres of indistinct stippling because of our ignorance of their structure and role. The fine structure of the pillar shown in the inset is particularly fanciful; it is certainly beyond the practical resolution of the electron microscope.

We are indebted to our technical associates for their contributions to this work. The dissections and tension recording were performed by Mr. R. McCarthy. Miss R. Knopf and Mrs. P. Larramendi prepared the tissue for electron microscopy. Cheryl Haugh drew the figure. We thank J. Rae for generously providing us with the semicircular grid and Drs. R. Levis, R. Mathias, and J. Rae for many helpful discussions and comments on the manuscript. The work was supported by grants from the National Institutes of Health (HL-20230), the Muscular Dystrophy Association, and the American Heart Association.

Received for publication 25 May 1981 and in revised form 23 September 1981.

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