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Rashid Nassar
Duke University and Veterans Administration Medical Centers

Nancy R. Wallace
Duke University and Veterans Administration Medical Centers

Isaiah Taylor
Duke University and Veterans Administration Medical Centers

Joachim R. Sommer
Duke University Medical Center

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THE QUICK-FREEZING OF SINGLE INTACT SKELETAL MUSCLE FIBERS AT KNOWN TIME INTERVALS FOLLOWING ELECTRICAL STIMULATION

Rashid Nassar, Nancy R. Wallace, Isaiah Taylor, and
Joachim R. Sommer*

Departments of Pathology and Physiology, Duke University and
Veterans Administration Medical Centers
Durham, N.C. 27710

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Abstract

Single intact frog skeletal muscle fibers quick-frozen after known time intervals following electrical stimulation are examined electron microscopically in thin sections, after freeze-substitution, in freeze-fracture/etch preparations, and in cryosections prepared for x-ray microprobe analysis. Techniques are described to perform these operations on a single fiber. Notable morphological differences between conventionally fixed and cryopreserved muscle fibers, and between fibers quick-frozen at different post-stimulation intervals are demonstrated.

Key Words: Single skeletal muscle fibers, cryopreservation, cryosections, junctional granules, core cylinder, excitation-contraction coupling, muscle ultrastructure, freeze-substitution, skeletal muscle, junctional sarcoplasmic reticulum, junctional processes, quick-freezing.

***Address for correspondence**
Joachim R. Sommer
Duke University Medical Center
Pathology Department, Box 3548
Durham, NC 27710
Phone No. (919) 286-0411 ext. 6570

Introduction

Recent advances in quick-freezing procedures and technology (5,8,9,10,40) suggest approaches for establishing a direct correlation in a single cell between ultrastructural and microchemical alterations on the one hand, and corresponding physiological events on the other, even as such events proceed at very fast rates, as for example in excitation-contraction-coupling (ECC) in muscle.

Here, we describe our first findings concerning the ultrastructure of single intact frog skeletal muscle fibers that were quick-frozen at various known time intervals after electrical stimulation, and then further processed by freeze-substitution and freeze-fracture/etching. We also give an account of the techniques and instrumentation which we developed for this purpose, including techniques that make it possible to cut cryosections for energy dispersive X-ray microanalysis on the same specimen from which thin sections and freeze-fracture/etch preparations are obtained. In all, our efforts show that it is possible to stop and then correlate certain ultrastructural and functional events at any chosen time within a single muscle twitch.

Materials and Methods

Definitions, Criteria and Dimensions

The anatomical nomenclature we employ has been published (27). The following abbreviations are used: **JSR** = Junctional sarcoplasmic reticulum. **JGs** = Junctional granules. **JPs** = Junctional processes. **T** = Transverse tubules. **JPits** = Junctional pits. **SR** = Sarcoplasmic reticulum. **CC** = Core cylinder. **JM** = Junctional membrane proper; it is apposed to the T, is covered on the outside by JPs and, on the inside of the JSR, by the **coextensive line** = **CL** (41) which is composed of a row of electron densities attached to the inside of the JM and staggered with the position of the JPs on the outside of the JM, in both skeletal (41) and cardiac muscle (12,43). **EPMA** = Electron Probe Microanalysis. **QFFsub** = Quick-freezing with subsequent freeze-substitution, and **QFFe** = quick-freezing with subsequent freeze-etching. **PS** = time interval between the beginning of the electrical stimulus and the impact of the advancing edge of the specimen on the copper block. Cryoartifacts are thought to be absent when the single fibers show none of the features described in detail below (see "Cryoartifacts").

Magnifications of the electron micrographs are approximations derived from measurements taken from 8x10 prints of negatives obtained from a routinely calibrated Zeiss 10A electron microscope.

The Specimen

Single intact skeletal muscle fibers (between 30 and 80 micrometers in diameter) are isolated from frogs (R. temporaria) obtained from Charles Sullivan and Co., Nashville, TN, and kept at 6 °C until used. The frogs are double pithed and the ventral belly of each semitendinosus muscle is removed, with black 7-0 silk attached to each tendon on either end. The muscle bundles are stored in Ringer's solution at least overnight in a refrigerator; such storage seems to facilitate isolation of single fibers the next day. The muscle is observed with dark field illumination under a dissecting microscope. The single fibers are isolated by sharp dissection with 1 - 3 mm steel knives broken from hard surgical "Prep Blades" (Edward Weck & Co. Inc., Research Triangle Park, N.C., 27709; cat.# 450205). The knives are either glued to wooden handles or fastened in the chuck of a mechanical pencil (0.5 Pentel), and are dipped in melted dental wax (to keep them from rusting during storage and to cover all rough edges). During the dissection, the emerging single fibers are repeatedly monitored for viability by electrical stimulation, and the Ringer's solution is frequently changed. A slot is cut into each tendon at the fiber's ends. Although many are used within hours of their isolation, most fibers are stored in the refrigerator for one to five days. For each experiment, a single fiber is transferred under Ringer's solution to the specimen holder, and from there to the "Slammer".

Quick-freezing

The physics and experimental conditions governing the process of quick-freezing for suitable cryopreservation of biological specimens have been discussed in detail in the literature (1,2,3,5,6,15,18, 20,21). For our experiments we have chosen van Harreveld's cold metal block method (39), because it gives better results than other methods on both theoretical and empirical grounds, and also because it affords a unique opportunity to determine the precise time at which a specimen contacts the coolant. In addition, uniform timing and cryopreservation can be obtained in very long specimens, such as skeletal muscle fibers. Many of the techniques we use have been adapted, and then modified, from others described in the literature (3,7,9,19,20,21,39).

The Quick-freezing Device

We are using the "Slammer" (Polaron Instruments Inc., Hatfield, PA.) (Fig. 1), which is a commercial modification of the device described by Heuser et al. (10). For our purposes, the electronics of the "Slammer" were modified (see below). We have retained the mechanical components and the differential thermometer, the latter providing a rather reproducible endpoint, if not absolute temperature, at which to release the specimen for its descent toward the copper block. At that point the last-second twitch-response test (Fig.1) (30) is performed. During the copper block's cool-down time (usually about three minutes), the specimen is shielded against cold He vapors emanating from the copper block by a Styrofoam box (Fig.1) which is quickly withdrawn before dropping the specimen.

The Specimen Holder

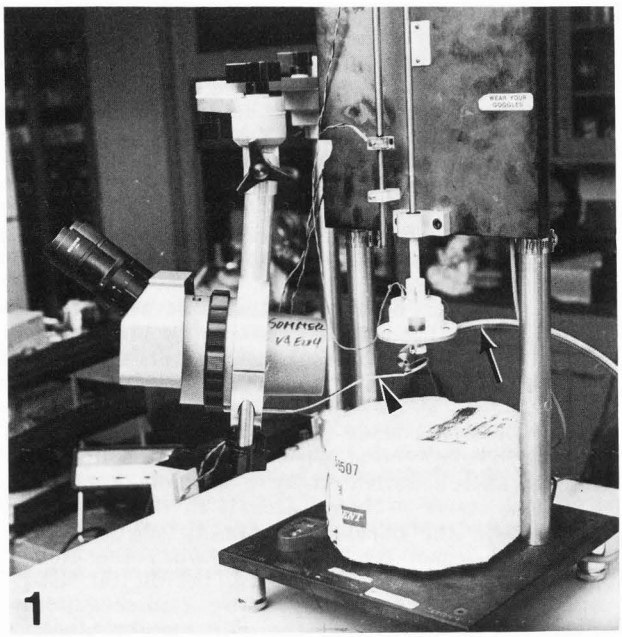
The purpose of the specimen holder (Figs.1-5) is to permit electrical stimulation at known time intervals before quick-freezing, and processing of the quick-frozen single muscle fibers for a) ultrastructural analysis following freeze-substitution, b) freeze-fracture/etching and, c) EPMA following cryosectioning. The specimen holder is the same for each of these operations, all of which can be performed on a single muscle fiber (Fig.4); it consists of a Delrin base (Fig.2) with stimulation and retaining wires, a removable aluminum disc with filter paper and 20% gelatin (Fig. 3), and a spacer ring. After quick-freezing, the stimulation wires are cut, the spacer ring is removed, and the aluminum disc is pried from the Delrin base. For freeze-substitution, the muscle fiber is separated from the aluminum disc by sharp dissection; for freeze-fracture/ etching, the aluminum disc with the frozen muscle fiber is transferred to a holder that fits the Balzers freeze-fracture device, and for cryosections to a holder designed to fit the Reichert cryoultramicrotome (Fig.4)(25,36). Transfers are all performed under liquid nitrogen.

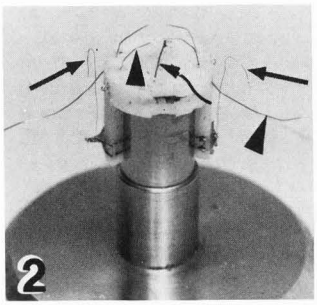
The Delrin base (Figs.2,5) fits the head piece of the "Slammer". The specimen holder itself must be put together with different glues in different combinations depending on the nature of the experiment. As a general rule: cyanoacrylic glues tend to shatter at low temperatures whereas epoxy glues do not.

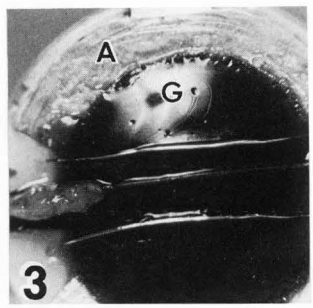
The aluminum disc (Polaron Instruments Inc.) can be clamped on the Delrin base (Figs.2,3) and the specimen holders for the Balzers freeze-etch device and the Reichert cryoultramicrotome. It is also part of the electrical circuit that records the time of impact of the specimen holder on the cold copper block (Fig. 5). The aluminum disc is cut to a dumbbell geometry to keep it from touching the stimulating wires as they emerge from holes in the Delrin base (Fig.3).

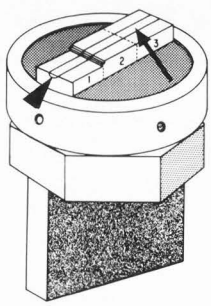
The filter paper (polypropylene filter, Gelman Sciences, Ann Arbor, Mich.), rough side up, is attached with epoxy glue to the top of the aluminum disc. The threads of the fabric from the rough side project into the gelatin and, when frozen, form a very tough combination that virtually eliminates shattering of the muscle fibers later, notably when cryosections or freeze-fractures are prepared. It is even possible to trim a muscle fiber together with the filter paper and frozen gelatin (under liquid N_2), turn it and clamp it directly into the chuck of a Reichert Cryoultramicrotome for transverse sections without having to resort to cryoglues (13).

The gelatin cushions the muscle fiber against being crushed when it slams into the copper block. It also covers all potential rough spots on the specimen holder with a very smooth and highly hydrated surface necessary to keep the fiber alive. A few drops of a warm 20% gelatin solution (Gelatin Powder, J.T. Baker Chemical Co., Phillipsburg, NJ) in distilled water are put on the filter paper on top of the aluminum disc. After setting, the apogee of the gelatin mound with the muscle fiber should be about 1 mm above the level of the spacer ring that will be added later (see below). The gelatin solution also contains 2 to 3 drops of carbon black (Carbon Black Dispersion #8, Faber Castell Corp., Newark, NJ) per 20 ml to provide contrast for better visibility of the single muscle fiber. We have









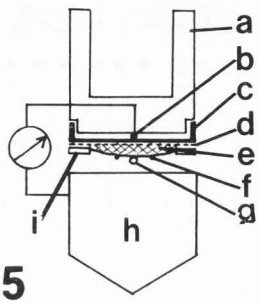


Fig. 1: The "Slammer" with binocular swung in for last-second twitch-response test, fiber optics for illumination (arrow), dental mirror (arrowhead), and Styrofoam shield.

Fig. 2: Delrin base of specimen holder with wires for stimulating (arrowheads), holding (arrows), and timing (curved arrow).

Fig. 3: Specimen holder with single muscle fiber ready to be dropped for quick-freezing. Gelatin: G. Aluminum disc: A. Note meniscus of Ringer's solution around stimulating wires and single fiber.

Fig. 4: Specimen holder fitting the Reichert Cryo-ultramicrotome. The ridge faced from the frozen gelatin contains the single muscle fiber (arrow), parts of which can be processed in three different ways. The fiber portion underneath the last frozen section (arrowhead) is also processed for freeze-substitution.

Fig. 5: Specimen holder with wiring for electrical stimulation and registering impact on copper block. a) Delrin base, b) timing wire, c) aluminum disc, d) filter paper, e) gelatin, f) stimulation wires, g) muscle fiber, h) copper block, i) spacer ring.

abandoned agarose as a hydrated fiber cushion (31) because it shatters prohibitively when frozen.

The stimulating wires are Teflon-coated Pt wires 0.005" in diameter (Medwire Corp., Mt. Vernon, NY) (Figs.2,3,5). The insulation is removed where the wires are coextensive with the muscle fiber. The wires are threaded through two very small opposing holes in the top part of the Delrin base (Figs.2,3) and pulled just taut over the gelatin, but without cutting into it. They are held in their final position by sharply bending them against the outside of the Delrin base. For reasons of precise timing, the stimulating wires lie a fraction of a millimeter lower than the advancing edge of the muscle fiber on the gelatin mound. One end of each stimulating wire is connected to an appropriate electrical terminal when the specimen head is finally put on the "Slammer".

The spacer ring (Polaron Instruments Inc.; actually, two stuck together) (Fig. 5) is glued with a cyanoacrylate glue (e.g., Crazyglue, Superglue) onto the specimen holder last, with a viable single muscle fiber already in place. Care must be taken lest the glue spread over the wet muscle fiber and destroy it. The spacer ring prevents the muscle fiber from being crushed on impact with the cold copper block, and provides enough height for the specimen to clear the screw cap that holds the aluminum disc in the Balzers device and the cryoultramicrotome.

Attaching the Single Fiber to Specimen Holder

Each isolated single skeletal muscle fiber is carefully floated in a dish with Ringer's solution onto the gelatin mound of the specimen holder. Together, they are lifted from the dish and placed under a dissecting microscope. Thin Pt wires (0.003"diameter, uncoated Pt wire, Medwire Corp., Mt. Vernon, NY), permanently attached to the Delrin base (Fig.2) are threaded through slots in the tendons of the muscle fiber. The wires are bent over to retain the muscle fiber on the holder in a just taut position (Fig.3). For technical reasons, no attempts were made to determine the precise sarcomere length. After the single fiber is tested for viability, the spacer ring is glued on, and the copper block is cooled down. The head piece holding the specimen holder with the specimen is then put on the carriage of the "Slammer", upside down, and the electrical connections are made (Fig.1). At this point, the muscle fiber is covered by a large hanging drop of Ringer's solution which is frequently replenished.

Testing Fiber Viability

With a hand-held probe, the viability of the single intact skeletal muscle fibers is repeatedly

tested during the isolation procedure and, later, at every transfer step, by observing that a vigorous all-or-none twitch occurs in response to an electrical stimulus applied at any point along the entire length of the muscle fiber. Once the specimen has been mounted on the holder, field stimulation is accomplished through the Pt wires running parallel to the fiber, and the threshold voltage is found. A last-second twitch-response test is performed within 1 to 5 seconds of freezing when the copper block has just reached operating temperature (30): a dental mirror attached to the front of a binocular dissecting microscope head is swung beneath the muscle fiber (Fig.1) covered by a large hanging drop of Ringer's solution. Under direct observation, the Ringer's solution is removed with a Pasteur pipette so that only a minute amount remains: not enough to prohibitively retard the freezing rate, but sufficient to sustain an effective electric field for stimulation. Ideally, a barely visible meniscus remains around the single fiber and the stimulating wires (Fig.3). The fiber is then given a final test by stimulating it at 1.5x threshold voltage. If a vigorous twitch is observed, the binocular is instantly swung out of the way and the specimen dropped on the cold copper block. We have made no attempt to design instrumentation for recording the twitch just prior to impact because of the unsuitable geometry of the "Slammer" and the magnitude of its mechanical noise as compared with the force developed by a single skeletal muscle fiber.

Electrical Stimulation

The muscle fiber is stimulated with a Grass Instruments Model S48 Stimulator through an isolating transformer, using two platinum wires (see above) placed 2 mm parallel on either side of the muscle fiber. The stimulator is externally triggered by the control box (see below). Single twitches are elicited by electrical impulses of 0.5 ms duration at 1.5 x threshold potential. For tetanus, the stimulator delivers 50 Hz pulses at 1.5 x threshold potential for one second, enough to cover the programmed stimulus-to-freezing interval set at 0.9 sec.

Control box. The specially-built control box is used to relate the release of the specimen carriage of the "Slammer" to the time of application of the electrical stimulus to the muscle fiber, allowing a wide range of stimulation-to-freezing intervals to be studied. It also provides a means of measuring those intervals accurately.

The stimulus-to-freezing interval is measured using a digital timer (Heath Co. Model SM-4100). The digital timer is started by the pulse from the control box that also stimulates the fiber, and is stopped by the pulse generated by a comparator circuit upon impact of the specimen on the copper block. The sensitivity of the circuit is adjusted such that the current passing on closing the circuit is less than 10 μA. Any variation in the operating time of the release solenoid and in the time taken for the specimen carrier to drop (time between release and impact) will be included in the measurement given by the digital timer: thus, whereas with a constant dial setting the stimulus-to-freezing interval may vary slightly between successive experiments, in each experiment the actual stimulus-to-impact time is recorded. The only significant error remaining is the possible time difference caused by fortuitous elevations on the gelatin mound that might make contact with the copper block and, thus, stop the digital timer before the muscle fiber touches the block. This possibility is minimized by proper positioning of the fiber before quick-freezing. The control box itself consists of circuits using type 555 timers. There are two basic modes: in mode I (long times between stimulation and freezing) pushing the START button begins the timing of a "preset interval" before the release solenoid is activated. The setting of the stimulus-to-freezing interval dial determines the time of application of the stimulus during the "preset interval". This mode allows the stimulus-to-freezing interval chosen to be longer than the time between release of the specimen carriage and impact with the copper block.

For stimulus-to-freezing intervals shorter than the dropping time of the specimen carriage, the control box is switched to the medium or short interval range, Mode II. In this mode the START button releases the specimen carriage. A Hall effect digital switch activated by a magnet fixed to the carriage starts a timing circuit in the control box as soon as the carriage begins to move. After an interval, preset by the timing dial, the fiber is stimulated and, simultaneously, the digital timer is started. The timer is stopped by a pulse generated when the specimen hits the cold copper block. In mode II, the operation of the device excludes any variable delays caused by the operation of the release solenoid but would still include variation in the time taken for the carriage to drop (e.g., due to variation in frictional force). This variation, as in mode I, will affect the reproducibility obtainable with any given setting of the timing dial but, again, since the stimulus-to-impact interval is measured independently, does not affect the accuracy of the interval measured by the digital timer.

Processing After Quick-freezing

Following quick-freezing per se, the specimen is transferred to a container of liquid nitrogen. Under a dissecting microscope, the spacer ring is pried off and the stimulating wires are cut and pulled off from the ice. Then, the aluminum disc carrying the frozen muscle fiber is transferred to a weighted plastic scintillation vial with multiple perforations for storage in liquid nitrogen.

For definitive processing, the aluminum disc is first moved under liquid nitrogen to the specimen holder designed to fit the Reichert cryoultramicrotome (Fig.4) (36). The holder is put into the chuck of the microtome at -135°C, and a ridge containing the single muscle fiber in its center is faced from the frozen gelatin with a glass knife. The ridge is divided roughly into three parts (Fig.4). One way of proceeding is to cut cryosections from one third of the fiber, then carefully remove the adjacent third for freeze-substitution (see below), returning the last third remaining attached to the aluminum disc to storage in liquid nitrogen for freeze-fracture/etching at a later time. In other cases the entire length of the quick-frozen muscle fiber is processed in one way only. There are many options, due to the relatively great length of well-frozen fiber obtainable (up to 8 mm).

Freeze-substitution. For freeze-substitution (31) the frozen muscle fibers are removed from the aluminum disc under liquid nitrogen by sharp dissection with a minute steel knife, and then transferred into a scintillation vial containing about 1 ml of a frozen solution of 1% osmium tetroxide in

acetone beneath a layer of liquid nitrogen. The specimens are placed in a -80°C freezer for at least 24 hours but no longer than 3 days. Thereafter, the acetone/OsO_4 solution containing one single fiber is poured into a small petri dish inside a fume hood at room temperature. Under a dissecting microscope the tissue is transferred as quickly as possible to an aqueous solution of 2% osmium tetroxide in 0.1 M cacodylate buffer at 0 - 6 °C, and post-fixed for 1 1/2 hours, followed by staining in block with 4% aqueous uranyl acetate, dehydration in ascending ethanol concentrations, rinsing in propylene oxide, and embedding in Epon 812. The muscle fiber is cut into fragments which are oriented such that sections cover the muscle fiber's entire thickness from the advancing to the trailing edge (See later, e.g. fig.15). Sections are cut with diamond knives on a Reichert OMU III ultramicrotome and stained on the grid with lead citrate. The fiber portion remaining beneath the last cryosection is also processed for freeze-substitution followed by thin sectioning.

In a few cases, the muscle fibers were transferred from a mixture of acetone and 1% osmium tetroxide at -80°C to acetone at 0 to 4°C for a few minutes and then embedded in Epon 812 directly from the acetone.

Freeze-fracture/etching. The aluminum disc with the single muscle fiber, or portions thereof (see Fig.4), is transferred under liquid nitrogen onto a Balzers fourfold specimen table which is then placed in the Balzers BAF 301 freeze-etch device. The freeze-fracture plane must be within the uppermost 5 micrometers of the muscle fiber. All freeze-fracturing is performed at -100°C (as measured by a thermocouple on top of a specimen holder), including etching for three minutes followed by Pt/C replication. Pt is evaporated with an electron gun. The thickness of the film is controlled by a quartz monitor. In order to minimize thermal exposure prior to actual Pt evaporation, the specimen is covered by the cold knife while the gun is heating. The carbon is evaporated by resistive heating of carbon rods (Balzers Graphit-Electroden). When muscle fibers are warmed up after quick-freezing and Pt/C replication, actomyosin interaction results in a contracture that destroys the replicas. This is avoided by transferring the still frozen fiber quickly to a bath of partly frozen ethanol (acetone is not effective) which is then allowed to warm to room temperature in about 15 minutes. During that time, the muscle fiber can be peeled off from the gelatin on the aluminum disc. After an additional 15 minutes in ethanol at room temperature, which stiffens the fiber, the replica is covered by collodion and processed as described earlier (28). In most specimens, the entire replica over the tissue is preserved this way. Since the fiber is rarely oriented exactly parallel to the fracturing knife, the fracture plane displays a gradient of depths within the fiber from which areas fractured more superficially can be chosen for analysis.

Cryosectioning is condicio sine qua non for EPMA. The sections are cut from either end of the ridge faced from the gelatin (Fig.4) at -100°C, and as thin as possible (about 200 nm). They are picked up by pressing them between the carbon film of a 150 or 200 mesh grid and the glass knife, or between two cold grids. The sections are numbered as they come off the knife, and immediately put into a small container made of solid copper which rests in the well of the Reichert cryoultramicrotome and is kept at about -180°C. After approximately ten numbered sections have been cut, the copper container with its lid is transferred to a large copper container (6 kg) cooled to, and held at, liquid nitrogen temperature. This container, its lid kept open by a wooden applicator stick, is transferred to a Denton Vacuum Evaporator with a vacuum of at least 10^{-3} mbar. The cryosections in the container are freeze-dried until the copper container has warmed up to room temperature. The vacuum is broken and the sections, with or without an additional carbon coat,are very quickly put into a vacuum for storage. From there, individual sections are transferred to, and then viewed and photographed with either a JEOL 1200 EX or a Zeiss 10A electron microscope. The images from the cryosections are then compared with adjacent thin sections from freeze-substituted fiber segments. EPMA on these same preparations will be the subject of future communications.

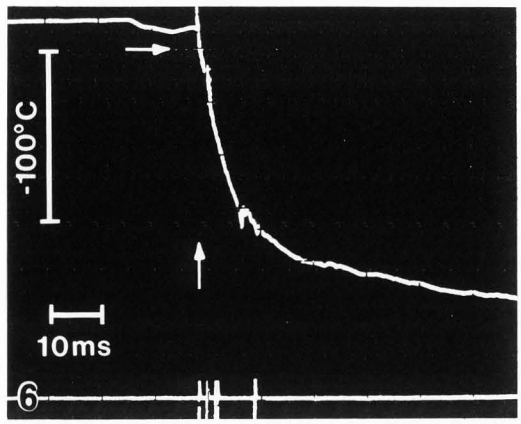


Fig. 6: Freezing curve (time constant: 6.8 ms) from thermocouple descending and impacting (vertical arrow) on cold copper block. Horizontal arrow = 0°C. There is some bouncing of the specimen holder after the initial impact (see lower trace). Precooling is negligible.

Temperature Measurements

For the general operation the empirical readings of the differential thermometer are used: the specimen is dropped when the thermometer is close to (but not at) the NULL position (about 10 - 30K). The wetting by liquid He of the polished copper surface must be averted.

Measurements were performed to determine both actual temperatures and rates of precooling and freezing. Temperature changes are measured in a moving thermocouple along its descent up to and including its impact on the polished cold copper block (Fig.6). Copper-constantan (25 µm"Ø) thermocouples (Omega Engineering, Inc., Stamford,CT) connected to a Tektronix 5115 Storage oscilloscope with a Dual Differential Amplifier are placed, just like the single muscle fibers, on the gelatin mound of a specimen holder attached to the specimen head of the "Slammer". When the appropriate operating temperature of the copper block is reached, the specimen carriage is dropped. The voltage output of the thermocouple is recorded photographically from the oscilloscope, which also displays the impact of the thermocouple on the copper block (Fig.6). It

should be emphasized that it is not possible to implant a thermocouple into a living muscle fiber.

Water-loss Measurements

There is danger of loss of water from, and thus, increase of solute concentration in, the film of Ringer's solution covering the muscle fiber prior to quick-freezing. In order to gauge the magnitude of this possible concentration increase, we measured with a microbalance (Cahn 27 Electrobalance) the time course of water loss from a specimen holder. A droplet of Ringer's solution is put on the gelatin mound of an inverted specimen holder, equilibrated with the gelatin matrix, and then the excess is withdrawn with a Pasteur pipette. The water evaporation, expressed in loss of weight, is recorded every 5 seconds for three minutes. After measuring the residual amount of water, the resulting percentage increase in solute concentration per second is calculated.

Conventional Fixation and Processing

For purposes of comparison, single intact skeletal muscle fibers, and minute bundles thereof, were processed conventionally also. They were put into 3 % glutaraldehyde in 0.1 M cacodylate buffer for three to five hours at room temperature, and then washed in 0.1 M cacodylate buffer or distilled water at 0 to 6 °C overnight, followed the next day by dehydration in ascending concentrations of ethanol and embedding in Epon 812 or Spurr's resin. Sections were cut on a Reichert OM III ultramicrotome and viewed with a Zeiss Electron microscope EM 10A at 80 kV.

Results

General

Our results are based on the quick-freezing of almost 500 muscle preparations of which about 350 were single fibers. Of these about 250 were suitable for evaluation. Both technical mishaps that precluded accurate timing and poor cryopreservation accounted for experimental failures. The most likely causes for poor cryopreservation were: a) indeterminate and unavoidable variations in the size of the water jacket remaining around the muscle fibers at the time of freezing, b) possible variations in the actual temperature of the copper block, c) variations in the precise placement of the fibers on top of the gelatin mound and, d) frosting of the copper block. Small amplitude bouncing of the spring-loaded specimen holder on impact with the copper block, which was observed on almost every oscillographic record during the temperature measurements (see below), had no notable effect on cryopreservation (Fig.6). Cryopreservation turned out to be excellent even in some cases in which the specimen head had come loose from its magnetic attachment in a bounce following the first impact.

Temperature measurements show that precooling was not a major problem and that freezing rates were sufficiently high to account for the superior cryopreservation that we obtained. Figure 6 shows a temperature record obtained from a 25 μm Copper-Constantan thermocouple. It shows that the thermocouple temperature remained above freezing until the moment of impact with the copper block: after impact, the thermocouple voltage dropped with a time constant of 6.8 ms (initial cooling rate 39,000 °C/s), the average cooling rate from room temperature to -100 °C being about 17,000 °C/s.

Water-loss measurements indicate the average loss to be 5 micrograms/sec. For the critical holding time (up to 5 sec) just before quick-freezing, this corresponds to less than a 0.5% increase in solute concentration, given a residual water jacket around the fiber proper of 1 mg. Most actual measurements were well above 1 mg, with a mean of 5.3 and a range of 0.8 to 10.7 mg. These data agree with the observation that in most of our muscle preparations, transverse tubules, which act as osmometers, were not dilated.

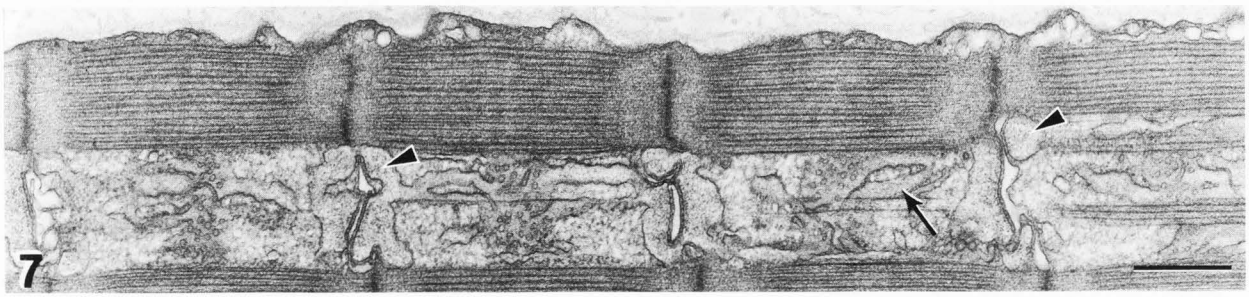
Ultrastructure

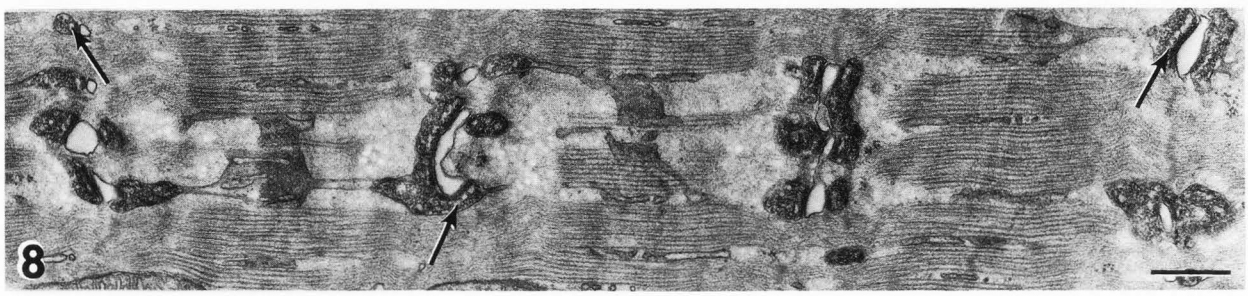
Thin sections after glutaraldehyde fixation showed the usual ultrastructure of frog skeletal muscle fibers (Figs.7, 9,13) (17,27). The granular material inside the JSR and free SR had a random distribution. It was not as electron-dense as that in quick-frozen muscle fibers and, occasionally, revealed cribriform substructures (11,14). CCs (22, 29), first described in frog seletal muscle fibers treated with ruthenium red and other cationic substances (23,27,42), were ill defined. The JPs were prominent and, under favorable conditions, showed their typical substructures.

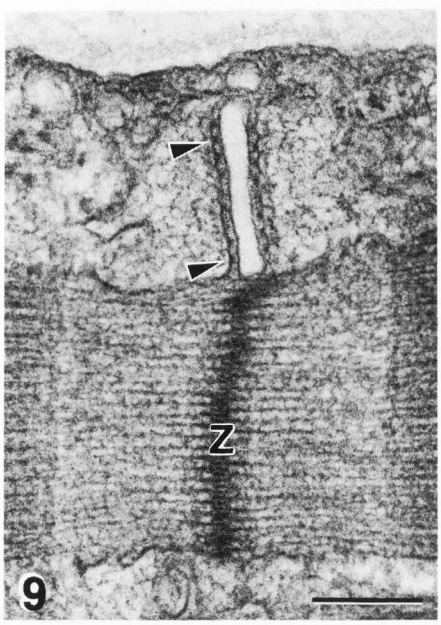
Quick-freeze preparations. Freeze-substitution followed by thin sectioning yielded electron microscopic images within the upper 5 micrometers of the fiber almost indistinguishable from those obtained after conventional fixation of muscle fibers (Figs.7-14). The contrast of the stained cell components was always uniform throughout the thickness of the fibers. Unit membranes of different organelles were well resolved in regions without cryoartifacts.

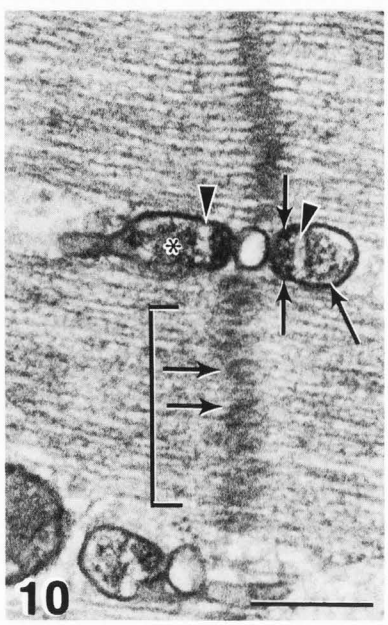
Cryoartifacts appeared in a gradient of increasing severity starting at the advancing surface of the cell, which contacts the copper block (Figs.10,12,15-18). This gradient was helpful in identifying the nature of the observed structural alterations. Cryoartifacts manifested themselves usually by the disruption of the orderly geometry of the cell constituents, notably the contractile material, and conisted of clefts of increasing size, usually oriented along the longitudinal cell axis (Figs.12, 15). Myofilaments and the granular material in the SR tended to clump together. Even in regions of the cell that were markedly distorted by cryoartifacts, the CCs (see below) and the surrounding JGs remained visible. The JSR appeared hydropic. The SR and mitochondrial membranes became angular. In the nuclei, the nucleoplasm became foamy in appearance.

Minimal cryoartifacts were very difficult to define. The most subtle morphological changes that we could detect consisted of alterations in the architecture of the Z line (Figs.10,16,19) (21). However, we were able to accept this criterion as valid only in the face of its total absence in regions in which perfect cryopreservation could in fact be demonstrated (Figs.10,12,16,18). Next in sensitivity, another change occurred in the alignment of the myosin filaments, especially at the A/I junction (Figs.12, 15,17, 29). We judged, as one must, the quality of cryopreservation electron microscopically at high power as can be appreciated in Figs. 17 and 18. Although the low power picture of fig.17 would









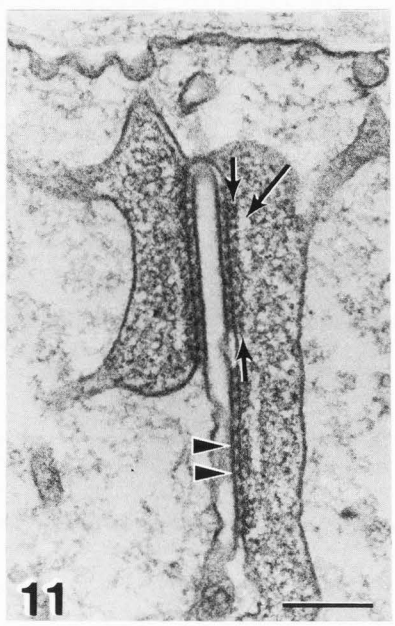


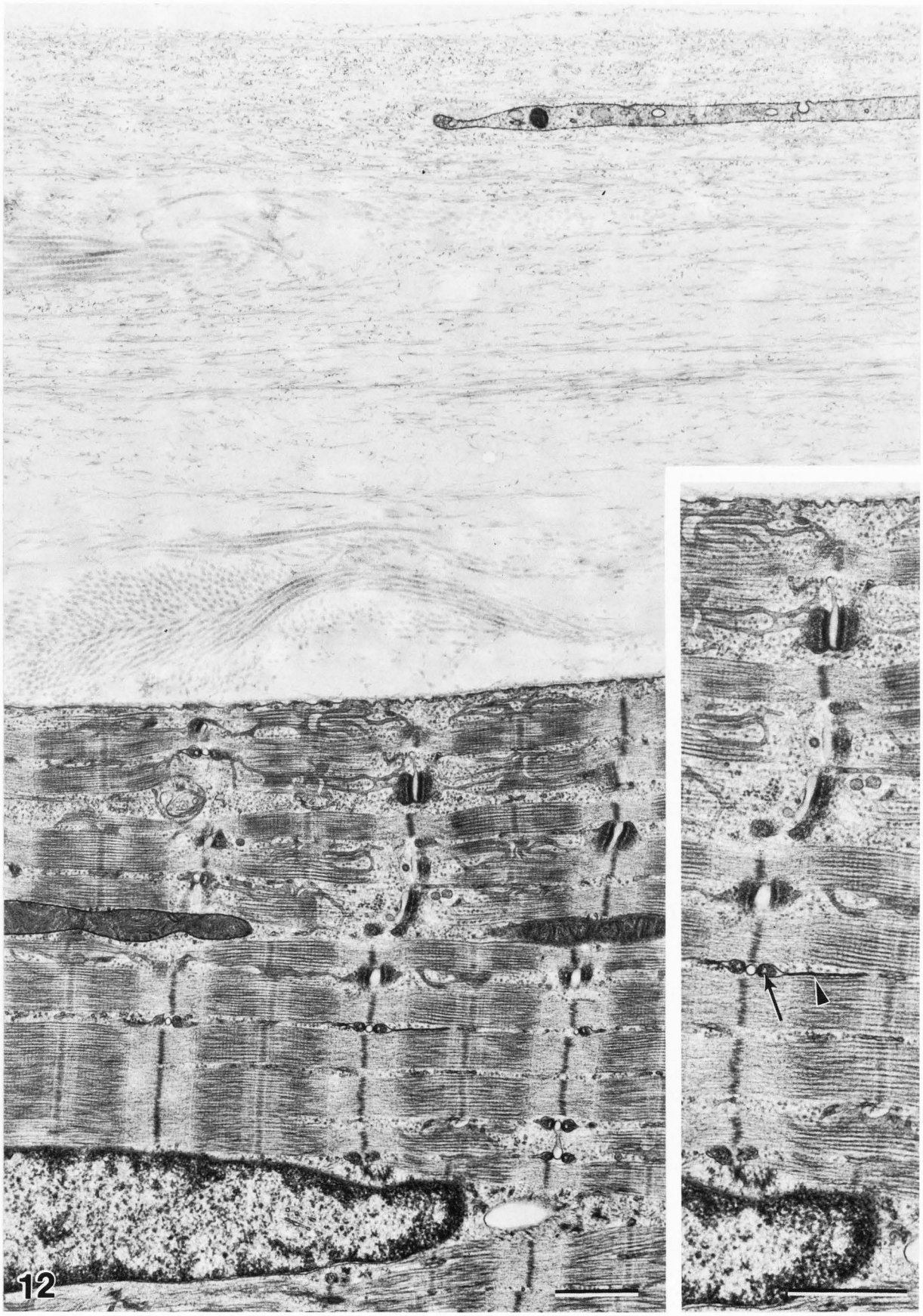
Fig. 7: Conventional glutaraldehyde fixation. Both the junctional (arrowheads) and the free SR (arrow) have a fluffy, granular content of low electron-density. Bar: 0.5µm.

Fig. 8: Quick-frozen freeze-substituted (**QFFsub**) unstimulated muscle fiber with very electron-dense granular contents and prominent septate CCs (arrows). Bar:0.5µm.

Fig. 9: Conventional glutaraldehyde fixation showing JSR with JPs (arrowheads), as well as Z-line architecture (Z); cf. Fig.10. Bar: 0.2 µm.

Fig. 10: QFFsub.10.5ms post-stimulation **(PS)**. CC (arrowheads) connecting with peripheral halo (slanting arrow), JGs (asterisk), CL (between opposing arrows), and earliest cryodamage in Z line (arrows in bracket). Bar: 0.2 µm.

Fig. 11: QFFsub. 0.47 ms PS. CC (arrow) in JSR and JPs (arrowheads). Inside the JSR, the JPs that define the JM proper of the JSR, are matched by the CL, a staggered row of similar densities (between opposing arrows). Bar: 0.2µm.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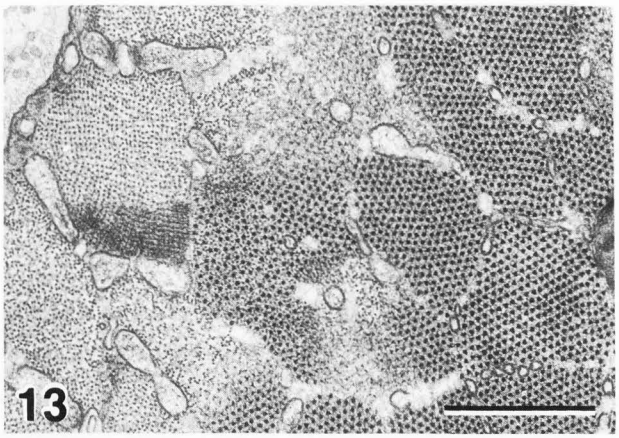


Fig. 12: QFFsub. 15.5 ms PS. Excellent cryopreservation over unusual distance (from top of picture to about the row of mitochondria, see inset). The CCs in the JSR are very prominent. Bar: 1 μm. Inset: Higher magnification to show CCs (arrow), gradient of Z line alterations, and occasional SR collapse (arrowhead), which also occurs in the total absence of cryoartifacts (not show). Bar: 1 μm.

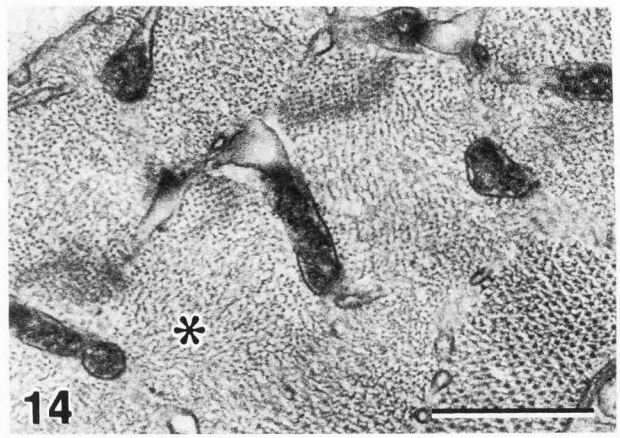


Fig. 13: Conventional glutaraldehyde fixation. Note loose granular material in SR. Cf. Fig.14. Bar: 0.5 μm.

Fig. 14: QFFsub. Unstimulated. Very dense granular material in the SR. Note typical Z line architecture except for some distortions (asterisk) perhaps due to fiber impact on copper block. Bar: 0.5 μm.

suggest superb cryopreservation throughout the thickness of the fiber, the preservation, actually, leaves much to be desired when viewed with higher power (Fig.18). Most of our preparations had a zone of from 1 to 5 and, rarely, up to 10 micrometers (Fig.12) without Z line or myosin filament alterations.

In some instances, there were focal, sharply circumscribed accumulations of rather large cryoartifacts at the impact surface of the fiber with excellent cryopreservation underneath and next to them. Other fibers had such accumulations deeper inside the cell. In neither instance did we find the cause for it.

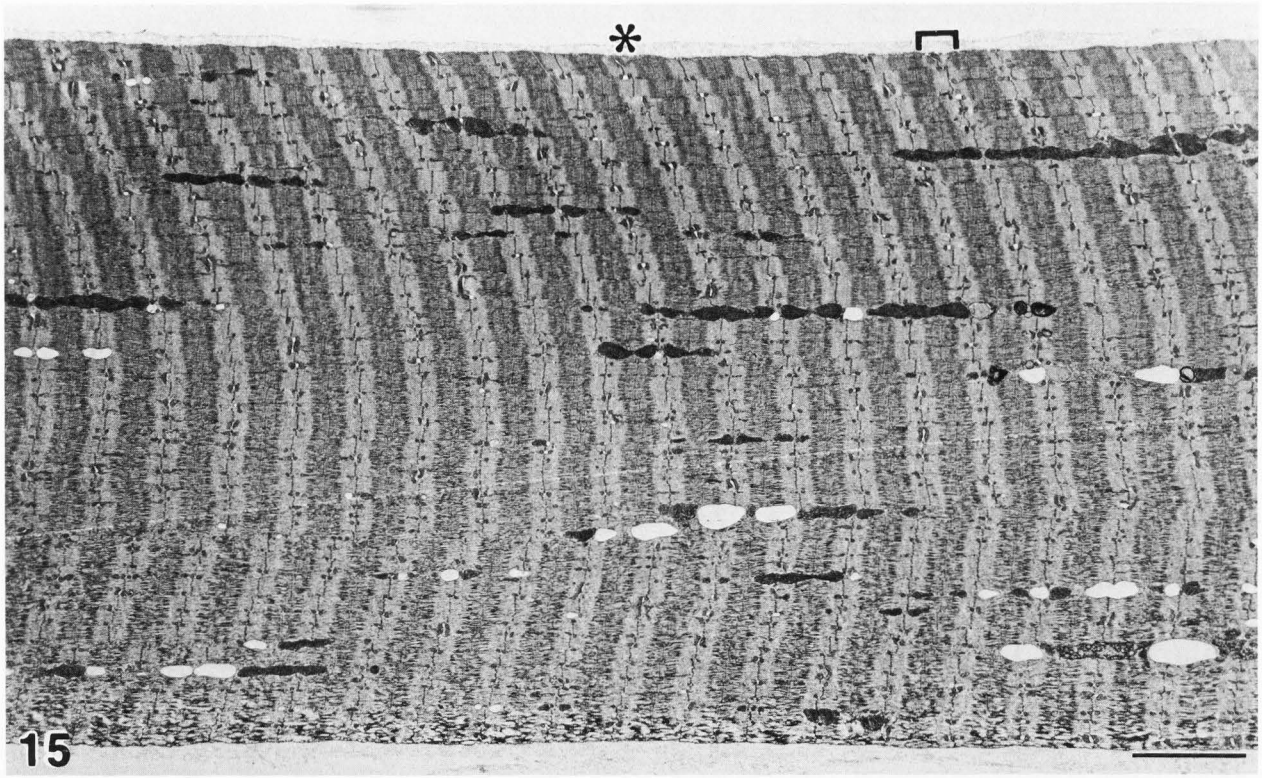
The sarcoplasmic reticulum (SR) was essentially similar to the corresponding images from conventionally fixed muscle fibers (Figs.7-11). As with conventional fixation, both JSR and free SR contained large amounts of electron-dense granular material(Figs.7-16). In the JSR of quick-frozen fibers, however, this material (the JGs; ref.27) was arranged in a characteristic pattern: it often formed a rosette (Figs.19-22) with an electron-lucent halo just underneath the SR membrane, and an electron-lucent region in the shape of an eccentric, round or flattened cylinder (CC,Figs.8,10,12,19,21,24) (29) in the core of the JSR near the junctional membrane (JM) proper, i.e. where the JSR abuts the transverse tubule (Figs.10,11,24). CCs were an invariant feature of the JSR in the absence of any evidence of cryoartifacts either in the JSR or in adjacent structures. At its ends, the CC appeared continuous with the peripheral halo (Figs.10,21,24) surrounding the electron-dense granular material in the JSR (29). In cross sections, the CCs displayed either a circular (Figs.19,21) or a flared (Figs.10,21,24) profile. In longitudinal sections the CCs were segmented (Figs. 8,16,24). Overall, there was considerable variation in the geometric expression of the CCs. The perimeter of the CCs inside the JSR corresponded, roughly, to the location of the JPits on the E faces of the JSR as seen after freeze-fracture (cf.Figs.23,25,26, 31) (29). The JPs (Figs.11,19,20) could not be distinguished from those observed after glutaraldehyde fixation (Fig.9). They were often conical in shape, and occasionally touched the cytoplasmic surface of the plasma membrane, which sometimes showed dimples in register with the JPs. Several of the JPs were found to have electron-lucent cores (17).

Collapse of the SR (34) was found focally and randomly distributed in intact unstimulated fibers (Fig.12) as well as after various time intervals following stimulation, including tetanus, i.e. it was unrelated to the time course of ECC (24). In intact fibers such foci were very few in number and very small in size; in damaged fibers they were large and common.

The contractile material was very well resolved, including the cross bridges and their periodicities, even in fairly thick sections that were not especially designed to study cross bridges in detail (Figs. 27,28). Although we made an effort to position the fibers straight and just taut, in many cases they emerged curved after the impact on the copper block (Fig.12). Whereas such curvature did lead to loss of resolution, preliminary optical diffraction studies hold great promise for the future elucidation of changes in cross bridge geometry at known time points during a single twitch (cf.Figs. 12,27,28) (cf.32, 38).

The Z line geometry depended on the plane of sectioning but was, in aggregate views, indistinguishable from its appearance in conventionally fixed material (e.g., Figs. 9, 10, 12, 14, 27, 28, 29). The significance of slight distortions in the structure of the Z lines has been mentioned above.

Other organelles did not show morphological differences from those in conventionally prepared muscle fibers. The clumping of the nuclear heterochromatin and the lamellar whorls of bits of membranes that are often seen in glutaraldehyde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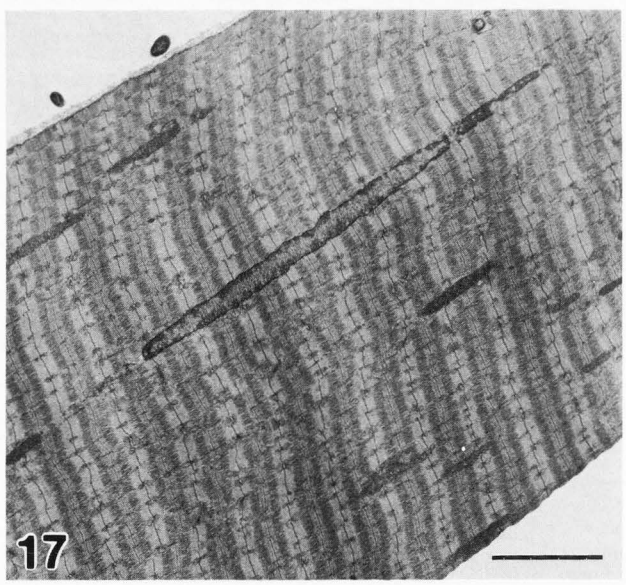
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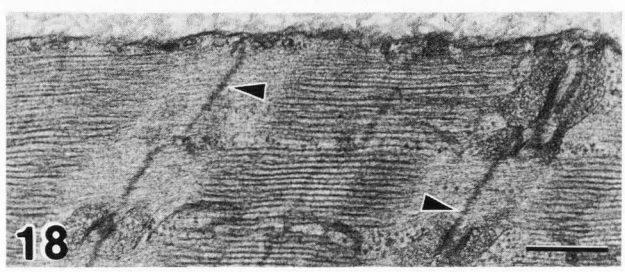
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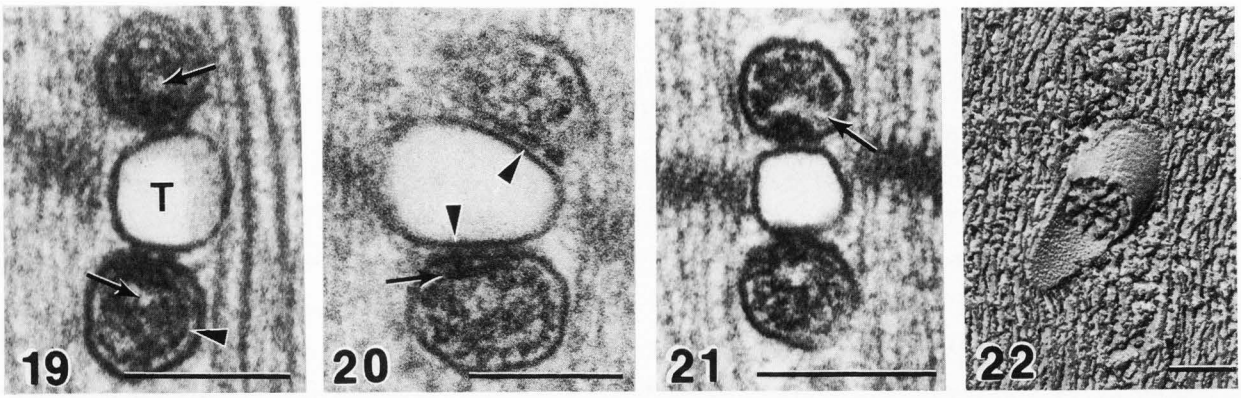


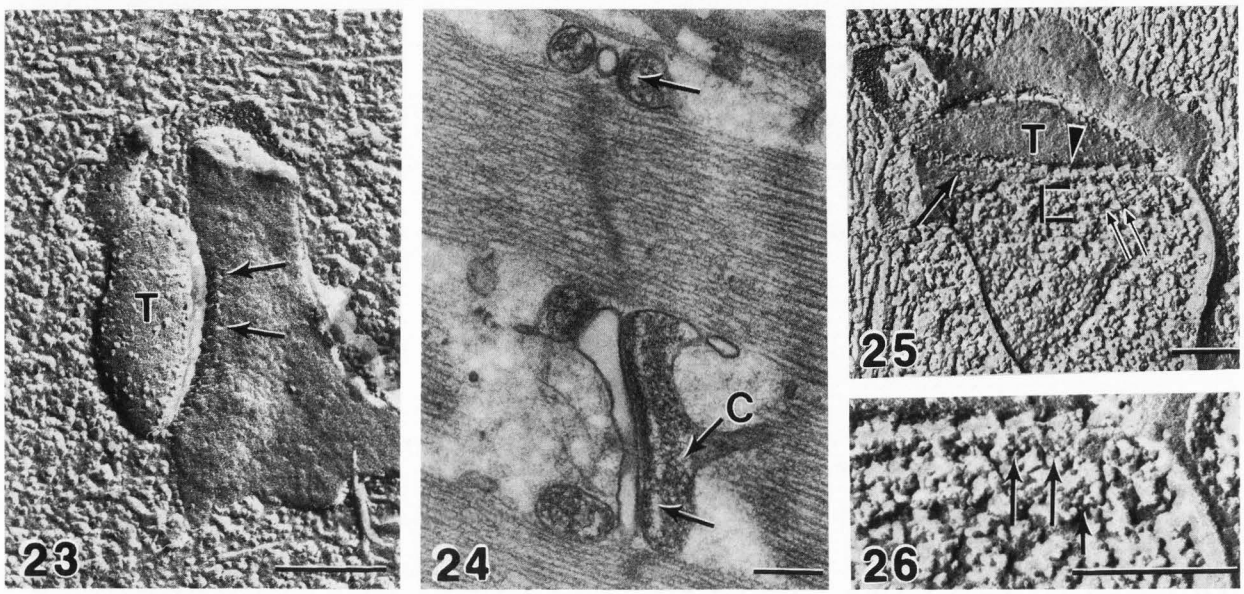


Fig. 15: QFFsub. 3.7 ms PS. Increasing cryoartifacts starting at the advancing surface of the fiber (asterisk). Only the upper two micrometers are free of any cryoartifacts (cf. Fig. 16). Bracket: see Fig. 16. Bar: 5 μm

Fig. 16: Higher power of region marked in Fig. 15. Note earliest cryoartifacts (arrows). Bar: 0.5 μm.

Fig. 17: QFFsub. 11.7 ms PS. "Excellent" (cf. Fig. 18) cryopreservation in low power from the advancing (bacteria) to the far surface. Bar: 5 μm.

Fig. 18: High power of advancing surface in Fig. 17 showing early cryoartifacts (arrowheads). Bar: 0.5 μm.

Fig. 19: QFFsub. Unstimulated. A triad with JGs delineating rosettes, CCs (arrows) and a peripheral halo (arrowhead). Transverse tubule: T. Bar: 0.2 μm.

Fig. 20: QFFsub. 10ms PS. Triad. Prominent rosette formation of JGs. JPs: arrowheads. CL: arrow. Bar: 0.2 μm.

Fig. 21: QFFsub. Unstimulated. Note CCs and rosettes of JGs. One CC flares into the peripheral halo (arrow). Bar: 0.2 μm.

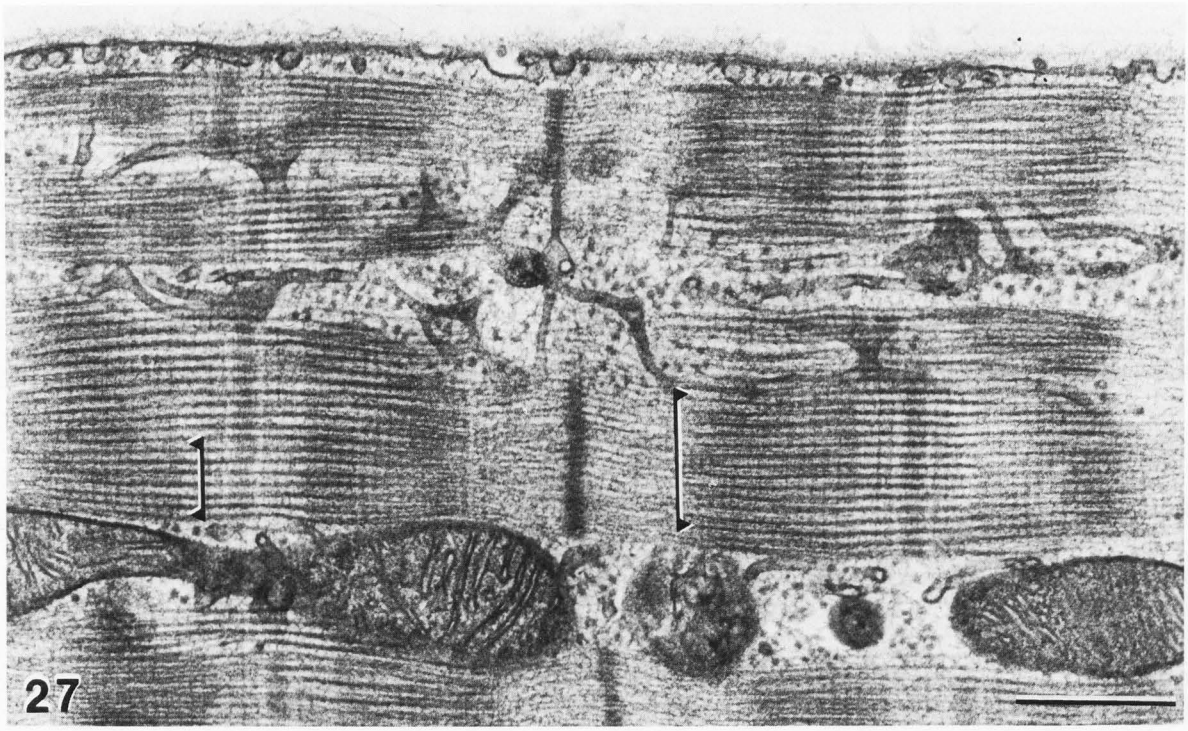
Fig. 22: QFFe. 0.5ms PS. Note rosette formation. Bar: 0.2 μm.

Fig. 23: QFF-fracture. Unstimulated. Note JPits (arrows). Transverse tubule: T. Bar: 0.2 μm.

Fig. 24: QFFsub. 1.5 ms PS. CC: arrows. Note that the topography of the CC roughly matches that of the JPits (cf. Fig. 23). The JGs show a cribriform pattern (c-with arrow). Bar: 0.2μm.

Fig. 25: QFFe. 0.6 ms PS. Triad with transverse tubule (T), JSR and its JM (arrow) with periodic connections (arrowhead) to T. The CL has a periodic architecture (bracket) with connections (thin arrows) to luminal face of the JM proper that are staggered with the JPs on the outside (cf.Fig.26). The JM also shows a peculiar square array of pits, particles and ridges just above and to the right of the thin arrows (cf.Fig.26). Bar: 0.2 μm.

Fig. 26: Higher power of array of pits, particles and ridges (arrows) above and to the right of thin arrows in Fig.25. The array appears to be part of the luminal portion of the JM, immediately adjacent to the JPs and subjacent to the JPits. Bar: 0.2 μm.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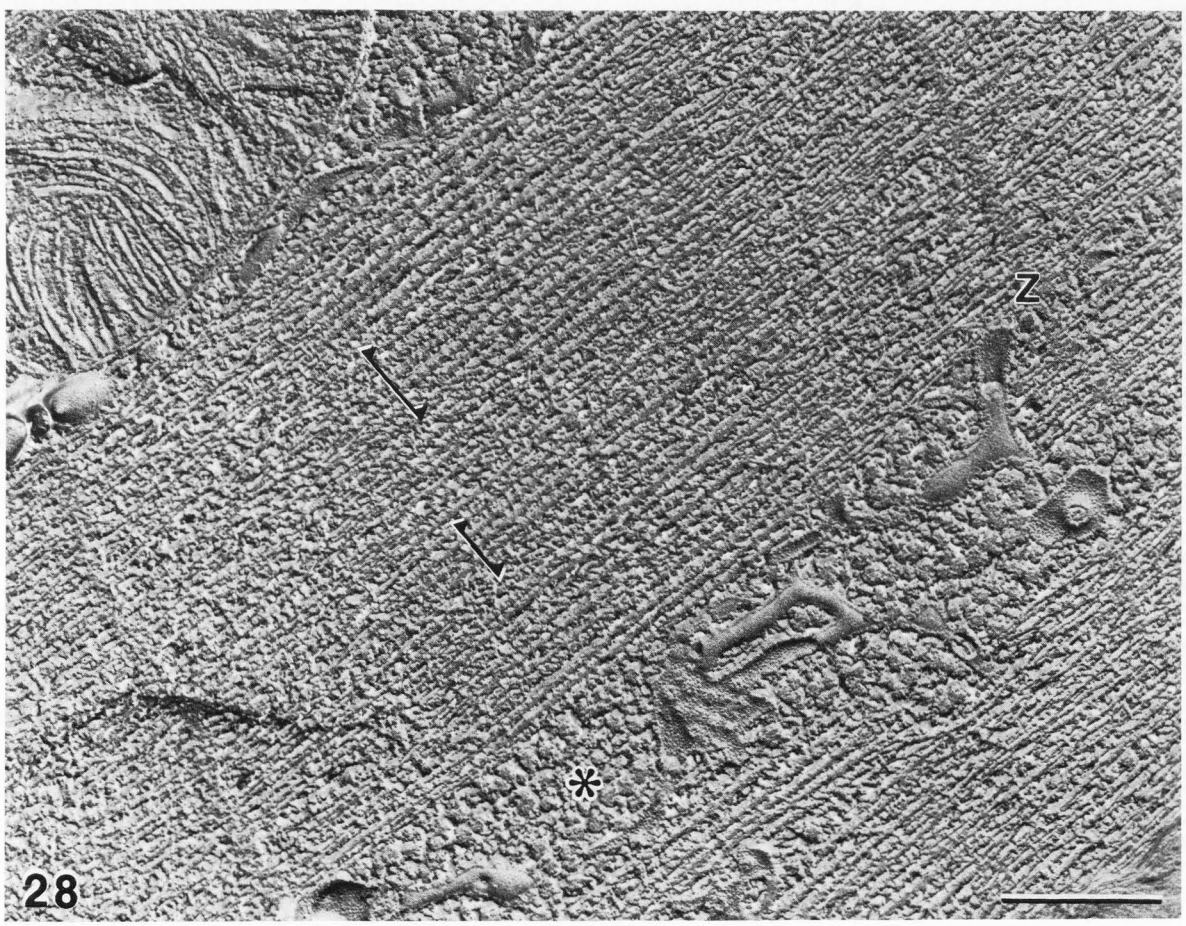
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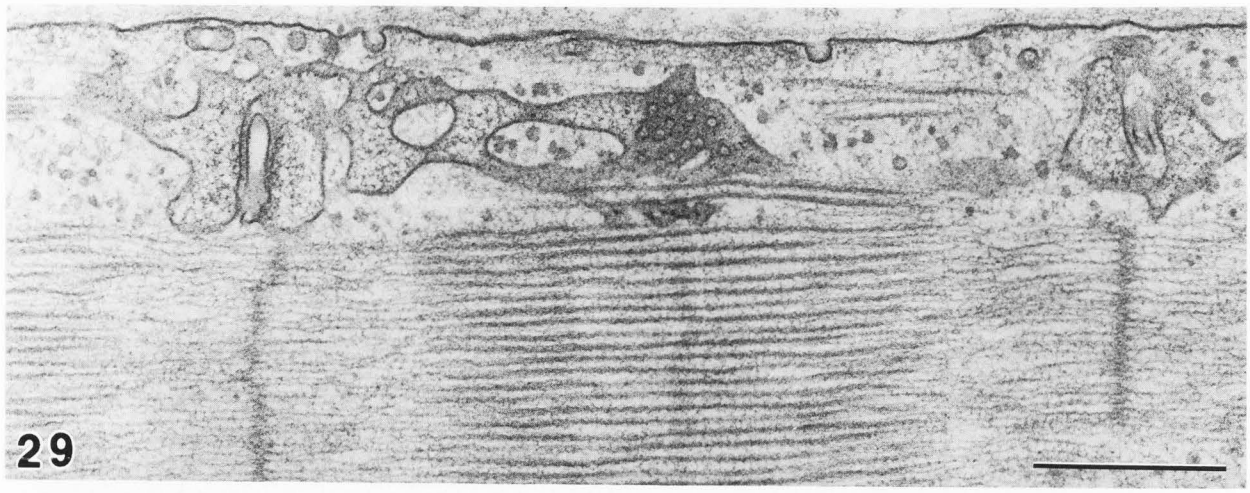

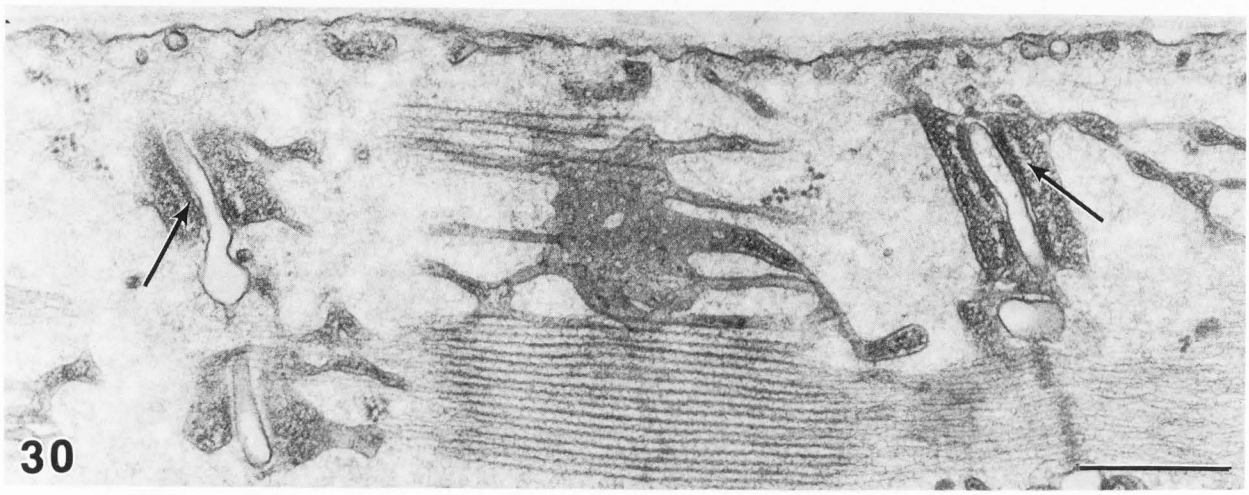

Fig. 27: High power of a region of Fig. 12. Brackets show myofilaments with cross bridges in this routine section. Bar: 0.5 μm.

Fig. 28: QFFe. 0.5 ms PS. Note myosin filaments showing lateral extensions suggesting cross bridges (brackets). Z line = Z. Glycogen = asterisk. Bar:0.5μm.

Fig. 29: QFFsub. Tetanus. Note low contrast, fluffy granular material in JSR and free SR. CCs and CL hardly visible. Note the detailed rendition of actin and myosin filaments. Bar: 0.5μm.

Fig. 30: QFFsub. 6 ms PS. Note highly electron-dense material in JSR and free SR. CC and CL prominent at arrows. Bar: 0.5 μm.

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fixed tissue were absent in quick-frozen freeze-substituted muscle fibers.

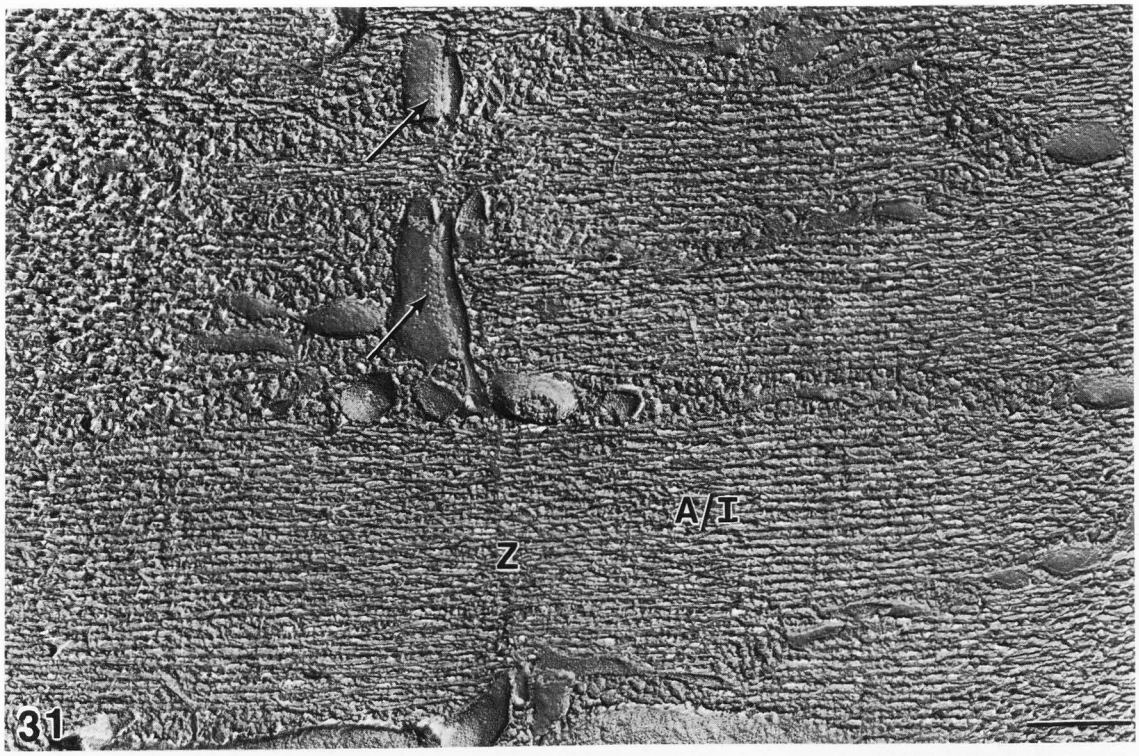
Myo-neural junctions were well preserved in several preparations. The junctional gap was uniform in width, which did not differ from that observed after conventional fixation. There were not enough samples to study differences in the geometry of the myo-neural junction following different time intervals after stimulation.

Freeze-fracture preparations after quick-freezing, with some notable exceptions, were essentially indistinguishable from those performed after slow freezing in the presence of cryoprotectants. We have not as yet obtained fractures that provided face views of the JPs as shown, for example, in isolated triads, especially after rotary shadowing (4).

In the sarcoplasmic reticulum, in both the tubular and cisternal forms (35), the intramembranous particle distribution in both JSR and free SR matched that found in conventional preparations: the P faces were densely studded with particles and the E faces were smooth. The JPits on the JSR were not seen very often but were prominent when present, especially in tetanus.

The contractile material was well delineated and the pattern of the various bands could be distinguished clearly. Cross bridges were suggested by some sculptured extensions of the myosin filaments.

Freeze-etching after quick-freezing produced replicas that revealed considerably more detail than those obtained from conventional cryoprotected freeze-fracture or unetched quick-freeze preparations (Figs.28,31,32).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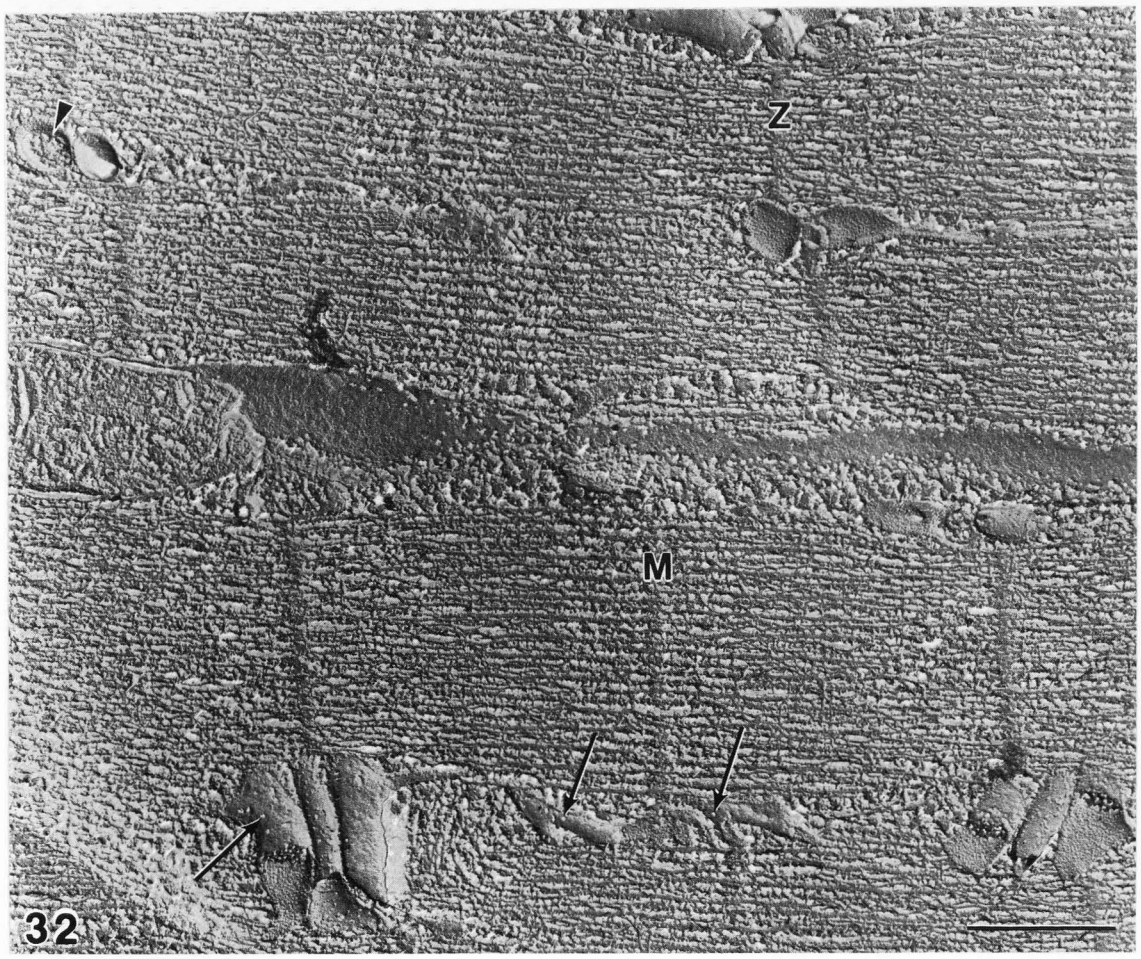
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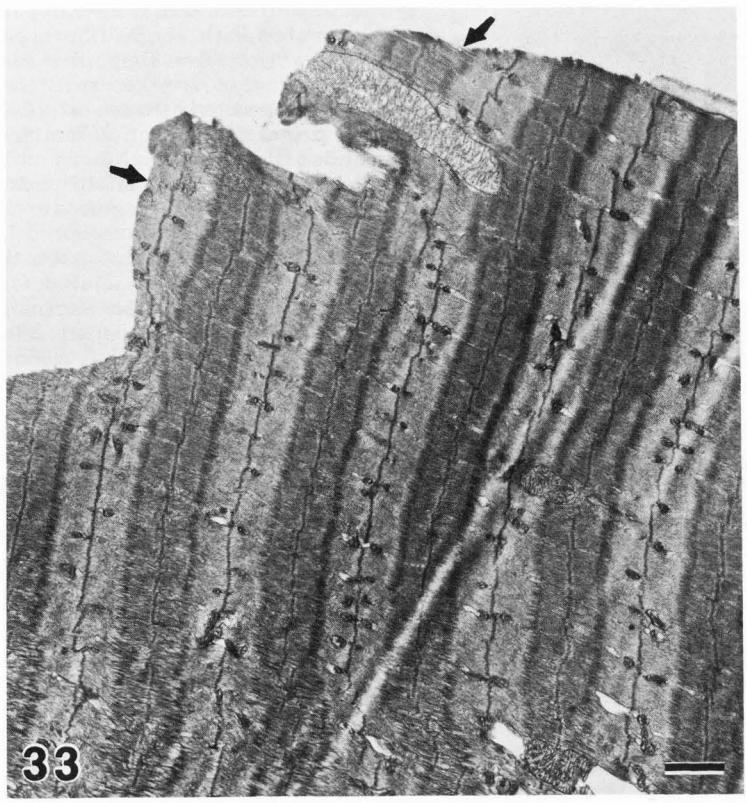

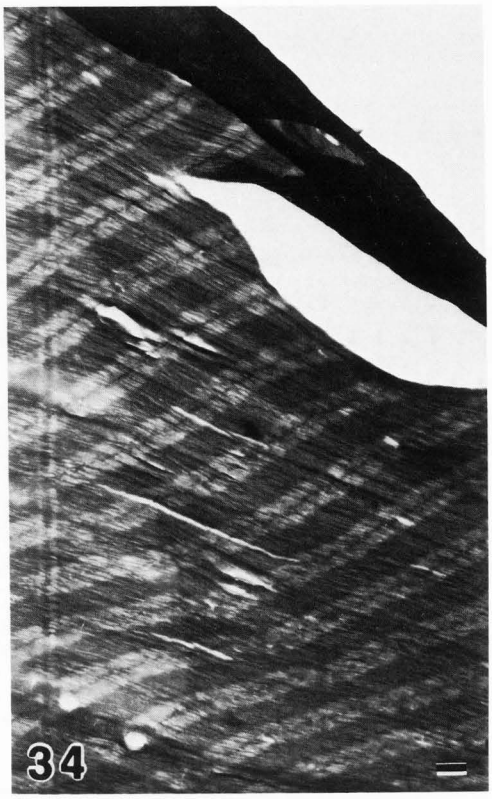

Fig. 31: Quick-frozen freeze-etched single fiber (**QFFe**). Tetanus. Note staggered row of JPits (arrows) and moderate cryoartifacts at Z lines (Z) and A/I junctions. Bar: 0.5 μm.

Fig. 32: QFFe. Unstimulated. Superb cryopreservation, including the Z lines, A/I junctions and actomyosin with the cross bridges. A few deep pits in the JSR and free SR (arrows) and a rosette in the JSR (arrowhead). M lines: M. Bar: 0.5 μm.

Fig. 33: QFFsub. Unstimulated. Junction from which frozen sections (Fig.34) were cut (arrows). Bar: 1 μm.

Fig. 34: One (the fourth) out of nine consecutive cryosections from the same fiber. Bar: 1 μm.

In the sarcoplasmic reticulum, the E faces showed a few randomly distributed deep pits of approximately 13 nm diameter, especially over the JSR. Significantly, a few pits were also seen on the E faces of the free SR (Fig.32). JPits in their usual geometry and location were seen infrequently but more often than in freeze-fracture (i.e., unetched) preparations (Fig.31).

In one case (Figs.25,26), interesting substructures in a triad were exposed by freeze-etching (unidirectional shadow) following quick-freezing. In addition to sculptured images corresponding to the coextensive line with its extensions toward the luminal side of the JM (12, 41,43), there was a faint square lattice close to the JPs, consisting of particles, ridges and pits. The lattice was part of the luminal portion (i.e. part of an E face) of the JSR envelope and was apparently confined to the JM proper.

The contractile material, in short stretches, showed striking three-dimensional representations of the geometry of actin and myosin filaments, including the cross bridges (Figs.28,32). In some areas, surface features suggesting a helical architecture were observed. Elsewhere, the interfilamentous space contained structures reminiscent of chevrons that emerged more clearly after rotary shadowing (not shown). Often there was excellent replication of the Z line architecture. A, I and H bands as well as M lines could be identified (Figs.28,31,32). In contrast to conventional freeze-etch preparations, after quick-freezing followed by etching, intracellular glycogen was very prominent (Figs. 28,31,32). Minimal cryoartifacts were difficult to evaluate in freeze-fracture preparations, although the Z line changes seen in freeze-substituted material could also be identified in freeze-etch preparations (Fig.31).

Stimulated vs. Unstimulated Fibers.

There were a few striking differences between stimulated and non-stimulated muscle fibers after freeze-substitution and freeze-etching, respectively.

1. Freeze-substituted muscle fibers frozen during tetanus displayed much less electron-density in their JGs than was observed in fibers that received a single electrical stimulus or in unstimulated fibers (cf.Figs.29,30) (24). This faint, foamy material was very prominent also in the free SR of tetanized fibers. The CCs were difficult to make out, perhaps because of the reduced contrast overall (24). A similar consistent pattern was not discernible when comparing unstimulated fibers with others frozen after different time intervals following a single stimulus. Although under the

latter conditions there were some notable differences in the electron-density of the SR contents from one experiment to the next, the absence of X-ray microprobe data and rigorous control of section thickness and staining procedures makes any interpretation, however tantalizing, inappropriate at this time.

2. Freeze-etching following quick-freezing after stimulation revealed the E faces of the JSR and free SR to have a few prominent pits of about 13 nm diameter. Such pits are only rarely seen on the JSR, and never on the free SR, in either unetched or conventional, cryo-protected (e.g. glycerol) preparations. The pits seem to be increased in muscle fibers frozen during the first ms, especially in one case in which the SR was studded with pits of about 13 nm in diameter (33). There were differences in the number of pits in the JSR and free SR within different regions of one fiber and from one equivalent fiber to the next. The P faces were occupied by a dense population of particles, but devoid of large, or aggregates of small complementary particles that might match the E face pits mentioned above.

Cryosectioning

Cryosectioning was achieved adjacent to freeze-substituted portions of the muscle fibers (Figs.33,34). The relative position of the cryosections with respect to the advancing edge of the fiber was gauged by numbering the sections consecutively and, in some instances, by the fortuitous, if unsanitary, display of contaminating bacteria marking the surface of both thin sections and the adjacent cryosections (not shown). The high-resolution freeze-substituted thin sections provided a highly sensitive criterion for gauging the state of cryopreservation in adjacent cryosections that by their nature permit only low resolution.

Discussion

Our experiments have shown that isolated single intact skeletal muscle fibers can be quick-frozen with excellent cryopreservation at specific time intervals following electrical stimulation. Segments of each quick-frozen single muscle fiber can be investigated by 1) high resolution electron microscopy following freeze-substitution, 2) freeze-fracture/etching and, 3) EPMA after cryo-sectioning. Quick-freezing with freeze-fracture/etching and cryosectioning is the preparative technique of choice for maintaining the morphology of a living cell closest to its in vivo state for electron microscopic viewing.

Single intact skeletal muscle fibers were used because they can be identified positively and tested individually for viability seconds before freezing and, therefore, give assurance that the timing of the impact of the fiber on the cold copper block and all subsequent structural and microchemical examinations apply to one particular, single, live fiber.

The superior cryopreservation was accomplished by using an optimal quick-freezing method and was unrelated to the size of the specimen. Given, that only a few micrometers of amorphous ice are formed below the surface, the diameter of a single skeletal muscle fiber is effectively infinite by comparison. The specimen holder was made of Delrin, both for electrical insulation and thermal isolation of the very small mass of the specimen from a potentially large heat sink. The latter may be an important consideration especially when liquid coolants are employed. Earlier we had experimented with single fibers and obtained good cryopreservation when they were suspended on wooden applicator sticks and then manually thrust into "supercooled" Freon 22 (34). However, when in preparation for electrical stimulation, we suspended single muscle fibers on Pt wires instead, cryopreservation was invariably poor. We concluded that this was probably due to the disproportionately large heat sink (represented by the Pt wires) in direct thermal contact with the small mass of muscle fiber, and to the related fact that the Pt wires, and not the muscle fiber, actually made the first contact with the liquid coolant. This experience led to the successful use of wooden instead of copper specimen holders in a recent study (16). Ideally, the specimen holder should be very small in mass and of low heat conductivity, whereas the coolant must have opposite properties. Very high cooling rates resulting in excellent cryopreservation can only be reached when a specimen is placed such that its region of interest (to the exclusion of any other structure, e.g., stimulating wires, gelatin mound, etc.) is first to make contact with a coolant of both large heat capacity and high thermal conductivity. A liquid He-cooled copper block serves that purpose best in addition to allowing precise timing of the impact of the muscle fiber after stimulation. By comparison, these requirements are difficult to meet with a liquid coolant because the geometry of its meniscus at first contact with the specimen is a) indeterminate and b) prohibitive of a heat conductivity comparable to that of a large cold copper block at the instant of contact.

Our initial observations reported here clearly demonstrate that conventional fixatives,glutaraldehyde in particular, preserve the in vivo morphology of skeletal muscle fibers very well as judged by comparing freeze-fracture and freeze-etch images obtained after quick-freezing and after chemical fixation.

Nevertheless, some striking morphological differences were noted between chemical fixation and quick-freezing followed by freeze-substitution. In most quick-frozen freeze-substituted muscle fibers the JGs in both the JSR and free SR were very electron-dense, unveiling consistent substructures in the JSR, such as the "CCs", the "peripheral halos", and the "rosettes". Unless contrasted with certain cationic substances (23,27,34,42), in conventional, chemically fixed muscle cells, the JGs appeared more homogeneous and were much less intensely stained. The CCs, which are not seen in conventionally fixed skeletal muscle preparations, can now be recognized as an invariant anatomical feature of normal frog skeletal muscle JSR. Although one might disregard their earlier descriptions because of the presence in these experiments of certain exotic cations (23,27,42) or cryoartifacts (21), our studies confirm the main geometric features of the CCs in the absence of both, even as they had been established already in the absence of freezing (23,27,42). The varying prominence of the CCs may reflect 'frozen' manifestations of a cation-loaded versus a cation-depleted state of the JGs which might be obscured by conventional chemical fixation. Quick-freezing followed by freeze-substitution, however, and quite apart

from the possible fate of diffusible elements under those conditions, might preserve time-dependent, cation-induced structural rearrangements that could enhance the electron-density of the JGs. It is interesting to note that whereas the rosettes seen in thin sections following freeze-substitution were imaged with considerable fidelity in freeze-fracture/etch preparations, the CCs were not. CCs were seen at most post-stimulation intervals so far studied.

Some morphological differences were found also in cells quick-frozen at different post-stimulation intervals. For example, muscle cells quick-frozen in tetanus were commonly distinguishable from unstimulated cells or cells that had been quick-frozen at various time intervals following electrical stimulation: in tetanus, the JGs were significantly less electron dense than at most other post-stimulation intervals so far tested, and the SR often seemed somewhat distended. One might expect to see similar images immediately following a single stimulus and before calcium returns to the SR. But, although among our muscle preparations following a single stimulus there were some in which the JGs looked similar to those seen in tetanus, many more experiments at different stimulus-to-freezing intervals, controlled by EPMA, are needed to strike a persuasive correlation between electron-density of the JGs and the time at which calcium is released from, or returned to, them following an electrical stimulus.

Our quick-freeze preparations after various post-stimulation intervals, including tetanus, have confirmed the presence of JGs in many regions of the free SR (29) as is found in conventionally fixed striated muscle cells (23,26,27, 34). Evidence exists that the JGs in the JSR represent calsequestrin. But since, electron microscopically, there is nothing to distinguish the JGs in the JSR from those in the free SR, including the cytochemical demonstration that in both locations they carry a prominent negative charge (23,34), the JGs in the free SR may, in fact, be calsequestrin as well. Evidence presented earlier (26,34,35) suggests the possibility that the JGs in the free SR are actually representative of a substantive material, e.g., a protein, which can move freely, if randomly, within the lumens of the SR. In the case of calsequestrin this would be an interesting property in view of its presumed binding (reversible?) to the SR wall. As in the case of SR collapse (24), our present methodology will, eventually, permit the correlation of the spatial distribution of JGs in the free SR with the time course of ECC.

Collapse of small SR segments was occasionally found, randomly it seemed, in unstimulated fibers and after various post-stimulation intervals, including tetanus. It was unrelated to the time course of ECC and, perhaps, is due to transitory, local osmotic gradients. The attending fusion of the opposing inner lamellae of the SR envelope's bilayer into one, remains a fascinating, unexplained phenomenon reflecting some unknown in vivo property of SR membranes in general; it is not confined to the intermediate cisterna.

In a few experiments we have processed the freeze-substituted quick-frozen muscle fibers from cold acetone/ osmium tetroxide directly to Epon embedding. Without exception, the resolution of all components of subcellular structures, especially their membranes at high power, was not as good as with our routine procedure. The reason for the excellent rendition of structural detail which we obtained in all of our preparations is, perhaps, the fact that after freeze-substitution the tissue is effectively rehydrated in OsO_4 in aqueous cacodylate buffer at about 0-10 ^{o}C. Dehydrated proteins are quite denatured. Rehydration may restore their secondary and higher order geometries and, in addition, uncover hydrophilic groups to which osmium tends to bind, resulting in added contrast. The imperceptible transitions in the continuous gradient of increasing sizes of cryoartifacts from the advancing to the trailing edges of the single muscle fibers mitigate against the possibility that rehydration affects the loci of ice crystals in a secondary way resulting in their occurrence being masked.

The abundance of pits which we found in a few freeze-etch preparations on the E faces of the SR in single fibers quick-frozen within the first ms following stimulation (36) defies explanation at the moment. Many more fibers will have to be studied before a final judgment can be made, and before artifacts can be ruled out.

The propagation of excitation along the transverse tubules has an important influence on the time course of physiological events taking place between electrical stimulation and freezing. The comparison of frozen sections with the adjacent freeze-substituted portion of the muscle fiber will provide a reasonable estimate of the distance of each processed portion of the fiber from its surface; it will be very close given the spatial limits for excellent cryopreservation (1-5 micrometers). Such an estimate of distance will be facilitated even more by transverse frozen sections which are possible without the use of cryoglues (see above). We have abandoned using cryoglue (heptane) since we suspected that even at -100 ^{o}C they seemed to alter the intramembranous particle distribution (unpublished observations). The impact of geometry on the time resolution of structural events taking place over distance can be dealt with by changing the mode of stimulation, e.g., point vs. field stimulation, and then following the spread of excitation at different points along the entire length of the fiber's surface. The impact on time resolution of shortening the duration of the electrical stimulus from 0.5 ms, which was used in all present experiments, to 0.1 ms and below needs to be explored.

The quality of cryopreservation, and precision with which freezing following stimulation can be timed, offers an opportunity to study acto-myosin interactions during a single muscle twitch (Figs.12,27-30) (32). One of the difficulties that will have to be overcome is the fiber distortions occurring at impact with the copper block, as they diminish the clarity of signals recorded by optical diffraction. This problem may be amenable to appropriate computer assisted image processing.

The present report has described the feasibility of a number of techniques and procedures for studying the morphology of single intact frog skeletal muscle fibers at known time intervals after electrical stimulation. The proper use of these techniques will most likely lead to a description of additional anatomical features, including cross bridge geometry, that may appear at specific times during ECC. Most importantly, with these techniques we

shall be able to explore the time course of microchemical events during ECC.

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We appreciate the permission to reproduce figures 23 and 33, modified from figures 2 of ref. 29 and 1 of ref. 37, respectively.

The results of these investigations have been reported, in part, at the following occasions: 22nd and 23rd Ann. Meeting of the ASCB, 1982 and 1983, respectively; 41st, 42nd and 43rd Annual Meeting of the EMSA, 1983, 1984, and 1985, respectively; FASEB Meeting, 1985; 13th European Conference on Muscle and Motility, 1984; Biophysical Society Annual Meeting, 1986; FASEB 1986.

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Discussion with Reviewers

G.M. Roomans: It would be very interesting to see whether the elemental composition of the single isolated fibre after storage would be the same as that of the fibre in situ. Have you done any preliminary experiments on this point or are you planning to do such measurements?
Authors: No such experiments have been done but are planned as part of attempts at delineating all possible background noise.

G.M. Roomans: The criterium you use to judge the quality of the freezing is very stringent, more stringent than the commonly used criterium of 'no visible ice crystal damage'. You may be perfectly right in adopting this more stringent criterium.

However, this makes the figures you give for the well frozen part of your material difficult to compare with those given by others using the criterium of 'no visible ice crystal damage'. Could you either using the criterium of 'no visible ice crystal damage' compare your data with those reported by others, or do the same thing using your own criterium?
Authors: This is the crux of the matter, clearly. At the moment, "Quality" of freezing can only be surmised 1. by determining the geometry of the physical state of ice and extrapolating from it the distortions it **might** create in tissue with known dimensions and properties and, 2. by the absence of any distortions in tissue after freezing. In either case, a standard for comparison is needed. The only one available to us is the structure of muscle after chemical fixation in the hydrated state as compared with, say, x-ray diffraction data of living muscle. Such comparisons, including recent optical diffraction data on freeze-substituted muscle following timed quick-freezing, show good agreement. Freeze-fracture replicas made below 140 °C could serve as additional standards for directly studying the structural effects of quick-freezing, allowing for problems of resolution especially in the absence of etching. Given these constraints, our criteria are no different in nature from those used by others, except that we exclude even very delicate structural alterations. Moreover, we can gauge the quality of cryosections by comparing them with adjacent thin sections from freeze-substituted muscle fibers. The Z line alterations that we use as a criterion have already been pointed out by Somlyo et al. We have contributed the new evidence that there is a state after optimal quick-freezing (as opposed to conventional fixation) in which Z line alterations, too, are absent and that, therefore, such alterations do represent artifacts secondary to preparatory procedures in which quick-freezing is a part. An obverse situation is obtained in the case of the so-called "core cylinders". These, too, have been pointed out before in quick-frozen preparations by Somlyo et al. However, we now show that they exist in muscle also in the absence of any evidence of artifactual distortions. This agrees with our earlier observation that "core cylinders" occur in chemically fixed skeletal muscle as well, even if only in the presence of certain cations. It is within that context that the "core cylinders" can be viewed with greater confidence as normal structures of muscle. Without this independent evidence in conventionally fixed, non-frozen muscle, "core cylinders" might be assumed to represent yet another, even more sensitive level (than the Z line damage) of response to the freezing process, per se, displayed by this particular portion of the JSR. All of which suggests that more rigorous, universal criteria to define cryoartifacts must still be developed, especially since the degree to which tissue itself may have a cryoprotectant effect is still poorly understood. Such knowledge is central to all arguments concerning the physical state of water at certain freezing rates and temperatures in, and its effect on, tissue. As of now in each case judgments are only useful within the framework of stated definitions, criteria and dimensions.

H.K. Hagler: How much degradation of ultrastructure is to be expected from the formation of crystalline ice in the specimens as they warm during the freeze substitution process?
Authors: We don't know (see above).

J. Tormey: Does PS refer to the time after the end of a single pulse? Were all pulses 0.5 ms in duration?
Authors: The impulse that starts stimulation of the fiber also starts the timer. All impulses were of 0.5 ms duration.

J. Tormey: In light of the experience of others, it is curious that you obtain good structural preservation in freeze-substituted material only when you osmicate in aqueous solution. What are your images like when you avoided aqueous osmication? Is it possible that the aqueous fixation was necessary to reverse and thereby mask freezing artifacts?
Authors: The absence or presence of cryoartifacts was not influenced by the treatment following freeze-substitution. Actually, the imperceptible transitions in the gradient of cryoartifacts from the advancing to the trailing edge of the muscle fiber would suggest that the treatment of the fiber after quick-freezing preserves existing states, rather than reverses cryoartifacts in one region of the fiber differently from that in another. Our comments concerning the quality of structural preservation following post-fixation in aqueous osmium tetroxide after freeze-substitution apply to the quality and consistency in the rendition of ultrastructural detail at high magnifications (such as unit membranes, cross bridges, filamentous cell components). Our experience, albeit limited, with the other method of processing did not compare favourably. Nevertheless, we do submit that rehydration may reverse artifacts caused by dehydration, although we do not know whether, or to what extent, old artifacts may be retained or new ones introduced this way; it might be recalled that many isolated acetone-dried proteins regain their original structural configuration, as well as their function, upon rehydration.