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Isolation of functional giant smooth muscle cells from an invertebrate: Structural features of relaxed and contracted fibers

(ctenophores/enzymatic dissociation/myofilaments/calcium/mitochondria)

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ABSTRACT **The giant smooth muscle fibers of a ctenophore were isolated by enzymatic digestion. These fibers are multinucleated cells, up to 50 μm in diameter and 2 cm in length. Their ultrastructure and membrane electrical properties are similar to those of *in situ* fibers. Relaxed, coiled (partially contracted), and fully shortened states were distinguished in isolated cells and studied by scanning and transmission electron microscopy. Calcium-containing mitochondrial granules were found in the coiled cells but not in either the relaxed or the fully shortened cells. The relaxed cell is characterized in cross section by the density of myosin filaments (457 ± 15 per μm^2) and the thin-to-thick filament ratio (5.2 ± 0.2). In the coiled cell, the muscle lattice does not expand uniformly, as shown by the variability of myosin spacing, and the thin-to-thick filament ratio decreases. Both clockwise and counterclockwise coiling occur along the same fiber. The implications of these findings with respect to the structure of the contractile apparatus are discussed.**

Giant smooth muscle fibers up to 50 μm in diameter and from 0.5 mm to several cm in length occur in the marine planktonic invertebrate *Beroe ovata*. Their ultrastructural features and basic electrophysiological properties have been described (1, 2). However, the anatomical organization of *Beroe* has hindered our understanding of these muscle cells when studied *in situ*. An unequivocal characterization of the contracted muscle at the ultrastructural level has yet to be done, and the electrophysiological study could not take full advantage of the extreme length of the longitudinal muscles due to complex overlapping of the cells. In addition, the biophysical properties of the intercellular matrix may interfere either by limiting the diffusion of various substances or by restricting the access of electrodes to deeply situated fibers. These latter difficulties were partly overcome by incomplete enzymatic digestion of the mesoglea (1).

A new insight into the properties of smooth muscle has arisen from the use of cells isolated from various vertebrates (3–5). In this report, we describe a simple technique for isolating the giant muscle fibers of *Beroe* in viable form and for processing them for ultrastructural studies. A description of the isolated cell in relaxed, coiled, and fully shortened states is given. The comparison of these states leads us to propose a possible arrangement of the myofilaments in this particular muscle cell.

MATERIAL AND METHODS

Specimens of *Beroe ovata* were collected at the Station Zoologique, Villefranche-sur-Mer, France.

Cell Preparation. The procedure adopted takes advantage of the resealing properties of the muscle fiber of *Beroe* (1). Thin slices of the body wall (2–4 cm long) from any level of the organism were dissected out. The length of the strip was parallel to the longitudinal axis of the animal so as to include intact longitudinal muscle fibers. The tissues were kept for 15 min in cooled artificial sea water (ASW; 500 mM NaCl/10 mM KCl/58 mM $MgCl_2$/10 mM $CaCl_2$/50 mM Tris·HCl, pH 7.8) to allow sectioned muscles to reseal. After a short rinse in Ca^{2+}-free ASW, the tissues were incubated at 30°C for 20–35 min in 0.05% trypsin (type III; Sigma) and 0.1% hyaluronidase (type III; Sigma) in Ca^{2+}-free ASW. When a few free muscle fibers were observed in the medium, digestion was stopped by transferring pieces of tissues to cold Ca^{2+}-free ASW. The pieces were briefly shaken, and released muscle cells were immediately transferred to Ca-containing ASW. From this stage on, siliconized glassware was used. The cells were used during the ensuing 12 hr, during which time they were redistributed in appropriate media, each cell being observed under a dissecting microscope before and after each transfer.

Electron Microscopy. Glutaraldehyde (5%) in cacodylate-buffered ASW (pH 7.8) was added in excess to the incubation medium. After a few minutes, the liquid was pipetted and replaced by fresh fixative. Once fixed, the cells were rather rigid. Thus, they were processed as pieces of tissue (see ref. 2) and were individually embedded. Thin sections from such cells were stained with uranyl acetate and lead citrate. For scanning electron microscopy, osmium tetroxide-treated fibers were dehydrated with acetone and critical point-dried with acetone/carbon dioxide. The dried cells were stuck on holders coated with double-faced adhesive tape, and sputter-coated with gold before observation.

Electron Probe X-Ray Microanalysis. After conventional fixation and embedding, dark gold sections were collected on aluminum grids with no supporting film. Staining with heavy metals was omitted. The sections were analyzed on an analytical electron microscope CAMEBAX + TEM, equipped with two wavelength-dispersive spectrometers (vertical and inclined). The vertical spectrometer was used for the Kα line of Ca, and the inclined spectrometer was used for the Mα1 line of Os, each with pentaerythritol crystals. The accelerating voltage was set at 50 kV, and the probe current was at 100 nA.

Electrophysiological Recordings. For intracellular recording and stimulation, double-barrelled microelectrodes were used. These were constructed from paired pieces of borosilicate glass tubing with inner fibers. Both barrels, filled with 3 M KCl, had an initial resistance in the range of 30–50 MΩ. Only microelectrodes with a coupling resistance <200 kΩ were selected for

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Abbreviation: ASW, artificial sea water.

experiments. Ag/AgCl wires were inserted into the barrels and connected, one to a high impedance, capacity-compensated preamplifier and the other to a current source. Injected current was measured across a 2×10^8-Ω resistor in series with the current-injecting barrel of the microelectrode. Signals were amplified with a Tektronix 5A 22N amplifier and displayed on a Tektronix 51302 N storage oscilloscope. The experiments were performed at room temperature (20°C) in ASW.

RESULTS

Viability of Isolated Fibers. Each piece of body wall may yield ≈100 fibers. If allowed to stand in control ASW at 4°C, the fibers remain relaxed, even flaccid, for several hours. From now on, we shall consider relaxed fibers to be those fibers that have the shape of straight, smooth-surfaced cylinders. Their length varied from 0.5 mm up to 2 cm, lengths of 5 mm being rather frequent. The diameters ranged between 20 and 50 μm. These cells were passively bent and twisted by the slightest current, but such profiles differed markedly from those of contracted fibers (Fig. 1).

At room temperature, the isolated fibers might contract either "spontaneously" or under any mechanical stimulus. When it occurred, contraction was long lasting (several hours!), often resulting in a coiled shape, but was reversible. Contraction began by a localized thickened coil and spread from the initiation site at a variable rate. The wave of contraction might stop, so that only a part of the cell was coiled (Fig. 1 *C* and *D*). All of these events were slow enough to be followed under the dissecting microscope and, thus, allowed one to literally fix the cell at the chosen state of contraction.

Coiled cells could undergo further contraction and finally achieved full shortening, at which time they had a thick cylindrical shape with no coils. A fully contracted fiber might be one tenth of its initial length. Full shortening might be reached directly, for example, by exposure of relaxed fibers to 100 mM external K^+, the whole process taking from 1 to 3 sec. The contraction started usually in one region, which thickened markedly (Fig. 1*D*), and seemed to pull on the rest of the cell. In a single freshly isolated sample, fibers could be found in various states of contraction. Most of the fully shortened cells were unable to relax and take up trypan blue, whereas relaxed cells excluded the dye, unless their membrane was obviously damaged. In this manner, it was possible to select fibers that appeared relaxed and that had no irregular swelling or any blebbing of the sarcolemma that might be indicative of damage.

Freshly isolated cells presented no specific ultrastructural alterations that could be attributed to enzymatic dissociation. The cell organelles and the membrane systems were well preserved (Fig. 2), and the general organization of the cells did not differ markedly from that of cells in intact tissue (2).

Viability of isolated muscle fibers also was confirmed by preliminary electrophysiological observations. Most of these experiments were carried out on long fibers, either intact or resealed, which very likely correspond to longitudinal muscle fibers (see ref. 2). The resting potential averaged -55.2 ± 4 mV (SEM; $n = 15$). The membrane potential at rest remained steady for several hours (up to 3 hr and 30 min) and no long-lasting oscillations were observed. Passive changes of the membrane potential were induced by injections of weak depolarizing and hyperpolarizing currents. The voltage responses to depolarizing currents were less than those to equivalent hyperpolarizing currents (Fig. 3). Action potentials could be triggered by depolarizing current. Increasing the membrane potential 15–25 mV above the resting potential triggered an action potential (Fig. 3). The amplitude of the spikes varied between 44

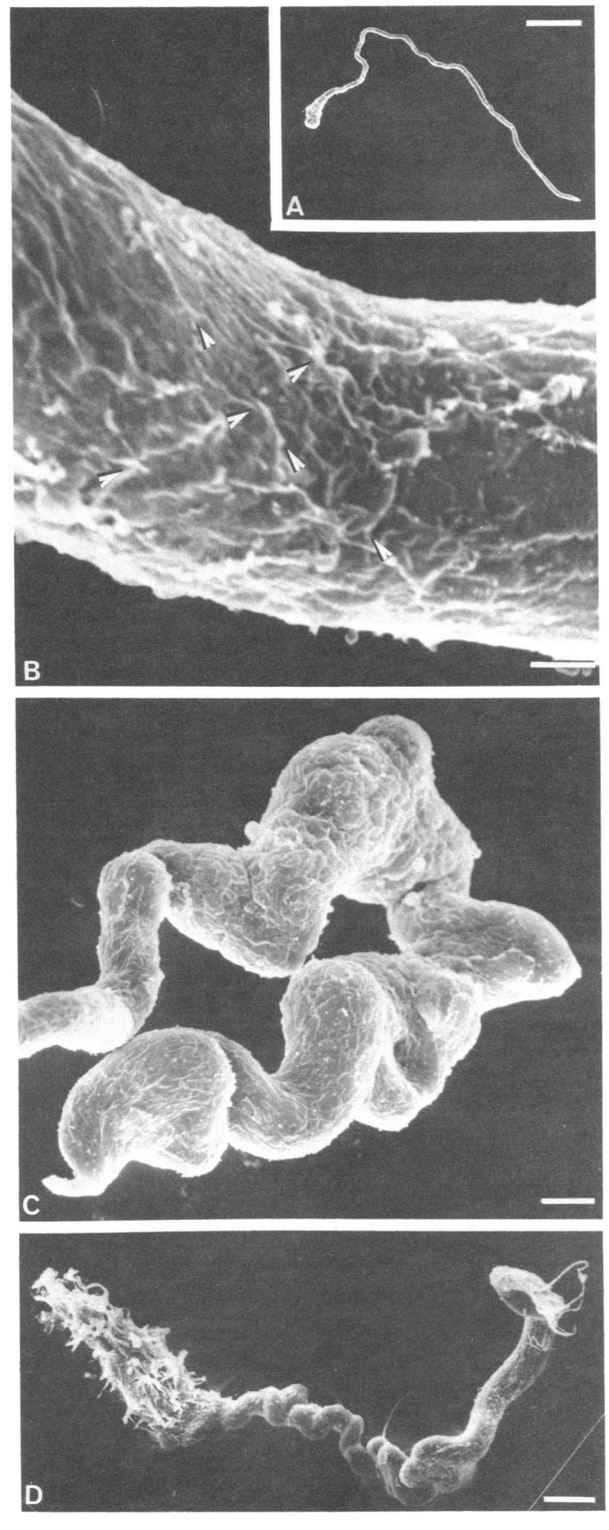

FIG. 1. Scanning electron micrographs of isolated muscle fibers. (*A*) Relaxed cell with an intact tapered extremity and a resealed (enlarged) stump. (Bar = 100 μm.) (*B*) Higher magnification of a relaxed cell. The surface of the cell exhibits ridges ordered in opposite helical arrays (arrowheads). (Bar = 2 μm.) (*C*) Coiled end of a cell undergoing contraction at the time of fixation. The lower part is coiled clockwise; the upper part, counterclockwise. (Bar = 10 μm.) (*D*) The relaxed, coiled, and fully contracted states can be seen simultaneously on this cell. Note the enlarged diameter and ruffled surface of the fully contracted part. (Bar = 20 μm.)

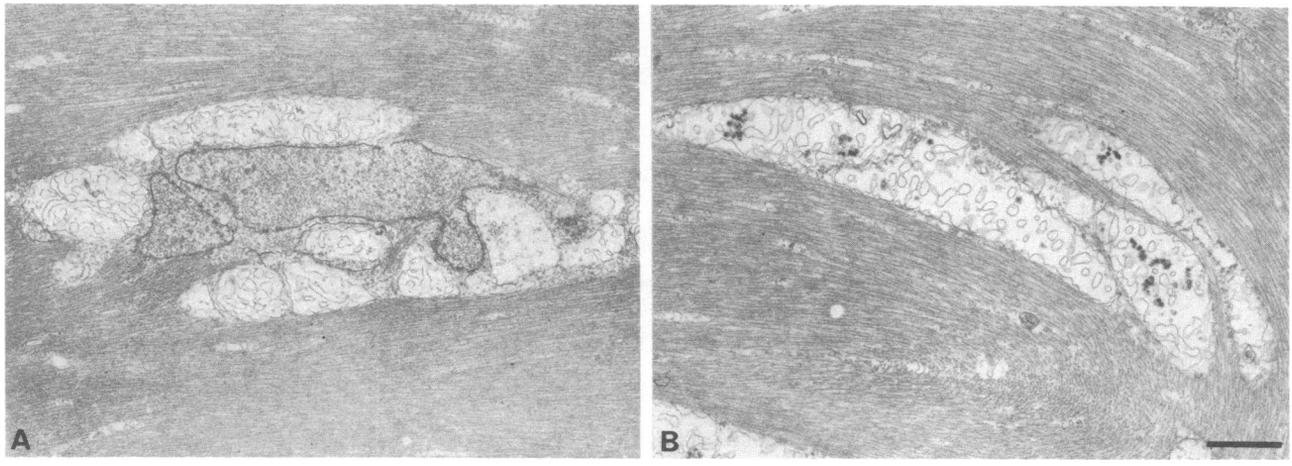

FIG. 2. Transmission electron micrographs of a relaxed (*A*) and a contracted (*B*) cell. The ultrastructures are well preserved. The myofilaments have an overall longitudinal orientation in *A* and are coiled in *B*. The mitochondrial matrix of the contracted cell contains larger and more numerous electron-opaque granules. (Bar = 1 μm.)

and 98 mV. The mean amplitude of overshoot was 23.0 mV ± 3 (SEM; n = 13) versus 11.2 mV for *in situ* fibers (1).

The contractile activity of the isolated muscle fiber during intracellular current injection was observed through the dissecting microscope. Action potentials could be associated with twitch, characterized by a fast contracting phase immediately followed by relaxation. Action potentials also were associated with different patterns of long-lasting events. First, a local contraction might originate from the stimulation site and propagate along the fiber or, second, a local, sustained, nonpropagated contraction might be observed at any point of the fiber. In the latter case, when the fiber was repetitively stimulated (1–2 Hz), the fiber coiled up and then underwent full shortening. Fully shortened fibers could produce action potentials in the absence of perceptible contractile activities. Contractions induced by intracellular repetitive current injections might be followed by a long-lasting relaxation, which began at any time after the end of stimulation. Third, a single action potential could be accompanied by a slow (2–4 sec), continuous shortening of the fiber originating from the stimulation site. No modifications of the membrane resting potential were observed during this contraction.

Structure of the Relaxed Fiber. When living relaxed fibers were observed under Nomarski optics, numerous axial nuclei and mitochondria could be seen. The cytoplasm of the cells contained fine longitudinal or slightly coiled fibrils. From our ultrastructural observations, a relaxed cell was characterized by its axial symmetry: the oval, euchromatic nuclei were aligned along the cell axis together with closely packed mitochondria. This axial core was surrounded by the thick sheath of obliquely oriented myofilaments (Fig. 2*A*). The sarcoplasmic tubules ran parallel to the myofilaments and appeared unchanged by the dissociation. Microtubules were regularly interspersed among the myofilaments, particularly in the outer part of cross sections of fibers that had been incubated in Ca-free ASW prior to fixation. The periphery of a given muscle cell might present alternatively either a smooth outline with occasional finger-shaped myofilament-containing evaginations or an intricate infolding that created an alveolated subsarcolemmal space. Under the scanning electron microscopy, the outer surface of relaxed cells was slightly ruffled: it presented ridges that had an overall helicoidal pattern (Fig. 1).

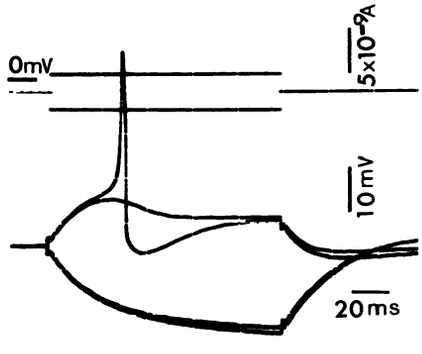

FIG. 3. Effects of depolarizing and hyperpolarizing current pulses (upper trace) on the membrane activity of an isolated muscle fiber (lower trace).

In all of the sections examined, two types of myofilaments were found. The thick, myosin-like filaments had a minimum diameter of 16.11 nm ± 2.12 (SD). Their density was 457 ± 15 (SD) filaments per μm^2 of organelle-free contractile cytoplasm in cross section (from a sample of six cells, with two sections from each cell). The thin, actin-like filaments had a diameter of 6.3 ± 0.6 nm. In the same sections as above, the ratio of thin-to-thick filaments varied from 4.65 to 5.47, with a mean value of 5.22 ± 0.21 (SD) when counted on the basis of 200 myosin filaments per section.

Special attention was paid to mitochondria, as we know that they are able to accumulate Ca^{2+} which is present outside the cell (6). Mitochondria had the same electron-lucent matrix as those in the *in situ* cells, and they contained well-ordered tubular cristae. Despite the use of a Ca^{2+}-containing fixative, mitochondria of isolated cells contained only a few small intramitochondrial granules.

Structure of the Coiled Fibers. The coiled shape of freely contracting cells was the most striking feature of these isolated muscle cells. This coiling was not observed *in situ*, in the mesoglea of the intact living animal. Both clockwise and counterclockwise coilings were usually observed along a given fiber (Fig. 1*C*). Longitudinal and transverse sections of contracted fibers gave the impression that the contractile subunits were coiled like the threads of a cable. On longitudinal sections, the myofilaments, the dense contorted nuclei, the strands of mitochondria, and the sarcoplasmic tubules followed an overall zigzag pattern. The ultrastructure of the periphery of the coiled cells was rather similar to that of relaxed fibers. However, in some cases, the sarcolemmal evaginations might develop into a system of intricate folds. Microtubules were particularly abun-

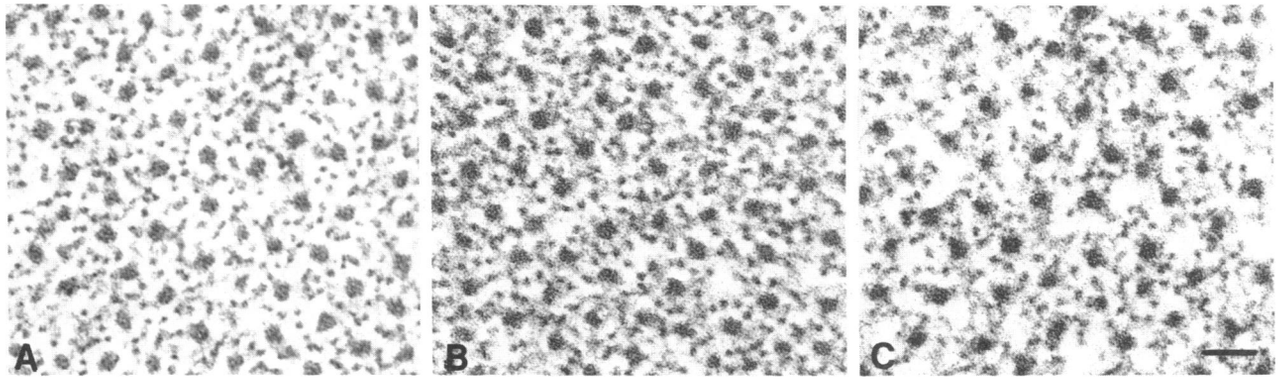

FIG. 4. Transmission electron micrographs of transverse sections of relaxed (*A*) and coiled (*B*, *C*) fibers. In the field *A*, myofilaments are regularly distributed in a nearly hexagonal pattern. The lattice of myofilaments is condensed in *B* and expanded in *C*. (Bar = 50 nm.)

dant at the periphery of contracted cells. Under the scanning electron microscope, the outer surface was ruffled along helicoidal ridges. Thick and thin filaments were always present in cross sections of myofilament bundles. Their minimum diameters were not significantly different from those of the relaxed muscles. The density of myosin-like filaments was extremely variable from one cell to another and within a given cell. In our sample (six cells, two fields from each cell), this density varied from 350 to 660 filaments per μm^2. In a coiled cell, there were areas where the lattice expanded (as in the contracting sarcomere of striated muscle) adjacent to regions where this lattice was comparable to, or even denser than, that of relaxed fibers. In the same sample the ratio of thin-to-thick filaments varied from 4.12 to 4.88, with a mean value of 4.88 ± 0.29 (SD). This value was rather constant and significantly different from the value found in relaxed fibers ($t < 2$ for $\alpha = 0.05$); it was calculated from areas containing 200 myosin filaments. Within each area the ratio was variable: it might be <4 in a region showing an expanded myosin lattice as in Fig. 4*C*.

The mitochondria of coiled cells presented a clear matrix with few dilated cristae. They usually contained electron-dense granules that were larger and always more numerous than in the relaxed cells. X-ray microanalysis of these granules indicated that they contained important amounts of Ca^{2+} (Fig. 5). Their electron density was essentially due to that high Ca^{2+} content because the Os signal over the granules was not different from that over adjacent myofilaments. Although a quantitative correlation would be difficult to establish, the size, opacity, and number of the mitochondrial granules under the probe seemed to be proportional to the Ca^{2+} signal. This is also true for the relaxed fibers, the mitochondria of which, therefore, contain much less Ca^{2+}.

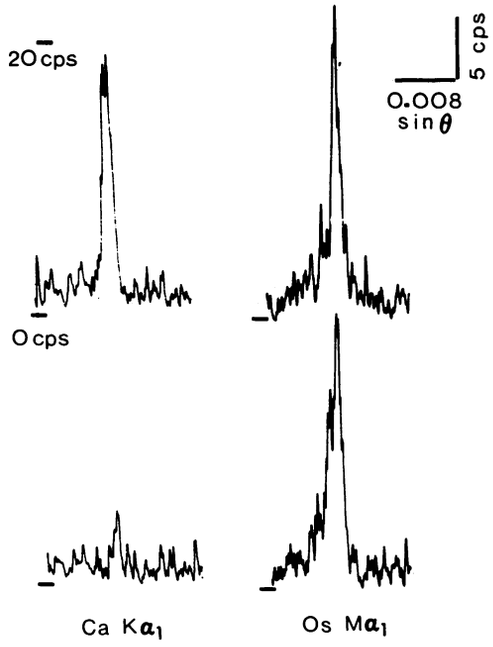

FIG. 5. X-ray wavelength spectra showing the Ca and Os signals from intramitochondrial dense granules (upper traces) and an area of myofilaments in the same coiled cell (lower traces).

Structure of the Fully Shortened Fibers. On random longitudinal and cross sections, the filaments appeared in considerable disarray. However, a zigzag pattern with a short period was recognizable in axial longitudinal sections. Thus, it was impossible to observe myofilaments in cross section on a scale large enough to allow an accurate count, but the two types of myofilaments were clearly recognizable. The nuclei were denser than in less contracted states and were always axially located. The mitochondria were swollen, and their matrix granules were less electron-dense than were those of the coiled fibers. The periphery of the cells appeared highly folded. Under the scanning electron microscope, the outer surface of the cell was often arranged in deep, thin folds or covered by finger-like processes (Fig. 1*D*).

DISCUSSION

Based upon these results, we believe that the enzymatically isolated muscle cells of *Beroe* are viable cells, as (*i*) their membrane appears to be intact and functions normally, (*ii*) it is possible to induce their contraction by electrical stimulation or excess K_0^+, and (*iii*) the cell ultrastructures appear unaltered.

The isolation of functional giant smooth muscle fibers will permit fuller use of this preparation. With the method described here, it is possible to obtain numerous fibers (intact cells or resealed fragments) and distribute them in homogeneous aliquots for any experimental purpose (biochemical, pharmacological, cytochemical studies) while controlling and processing them individually (if necessary) under the dissecting microscope.

The present study shows that *Beroe* muscle cells contract in different patterns and describes the ultrastructural correlates of relaxed, coiled, and fully shortened single cells. Similar features have been described for *in situ Beroe* muscle cells (2), but in that situation the state of contraction could not be assessed. In the latter study, it was proposed that the actin-like filaments attach directly upon the cell membrane and that the "attachment sites could be located along several longitudinal strands." Dense bodies were not visualized after the conventional fixation and staining procedures used, but the actin filaments would

have to be extremely long if there were no cytoplasmic attachment sites. It is possible that such attachment sites are no more conspicuous than those that must exist on the plasma membrane. It is certain, however, that the filaments are oblique to the axis of the relaxed, cylindric-shaped cell and that they display a definite zigzag pattern in sections of contracted, coiled cells. Such a zigzag pattern typically occurs in contracted helical striated muscles (7). The attachment of actin filaments on the sarcolemma could be along helices as hypothesized for other smooth muscles (4, 5). Furthermore, the ripples observed on the muscle surface in scanning electron microscopy, together with the fact that successive lengths of the same fiber coil both clockwise and counterclockwise, suggest the existence of two, or two sets of, opposite helices, along which the actin filaments would insert.

The distribution of thick and thin filaments in the coiled (contracted) state indicates that the coiling is induced by a sliding of the filaments. The variations of the thin-to-thick filament ratios and of myosin density between the cylindric (relaxed) and coiled (contracted) states are again reminiscent of the figures known for helical striated muscles (7, 8) and indicate that staggering and shearing of myosin filaments may occur during contraction. However, there are instances in which *Beroe* muscle fibers maintain a cylindric profile while contracting either locally or in totality. In such cases, it can be assumed that all the contractile units are simultaneously activated. Thus, the uneven distribution of myosin filaments (i.e., densities inferior, equal, or superior to that of the relaxed muscle) in adjacent small areas of a cross-sectioned coiled fiber could indicate that the coiled cell is a cell in which a series of helically arranged contractile units has been activated.

The formation of Ca^{2+}-containing granules in the mitochondrial matrix of coiled cells was most probably consecutive to a large increase in intracellular free Ca^{2+}, possibly induced by cell damage (see ref. 9). Then the fully shortened cells might be expected to display still more mitochondrial Ca^{2+} accumulations; however, this is not the case. Considering our tissue processing for conventional electron microscopy, it is not possible to tell whether the mitochondrial Ca^{2+} was mobilized during full contraction or if it was in a less stable form and, therefore, lost during fixation or embedding, or both. Even if the mitochondria play the role of a Ca^{2+} source for contraction in these barely physiological conditions, there are now indications that sarcoplasmic reticulum would, contrary to previous speculations (1), be involved in the contraction/relaxation cycle of this giant muscle fiber (10).

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