The Structure of *Mytilus* Smooth Muscle and the Electrical Constants of the Resting Muscle

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ABSTRACT The individual muscle fibers of the anterior byssus retractor muscle (ABRM) of *Mytilus edulis* L. are uninucleate, 1.2–1.8 mm in length, 5 μm in diameter, and organized into bundles 100–200 μm in diameter, surrounded by connective tissue. Some bundles run the length of the whole muscle. Adjacent muscle cell membranes are interconnected by nexuses at frequent intervals. Specialized attachments exist between muscle fibers and connective tissue. Electrical constants of the resting muscle membrane were measured with intracellular recording electrodes and both extracellular and intracellular current-passing electrodes. With an intracellular current-passing electrode, the time constant τ, was 4.3 $\pm$ 1.5 ms. With current delivered via an extracellular electrode τ was 68.3 $\pm$ 15 ms. The space constant, λ, was 1.8 mm $\pm$ 0.4. The membrane input resistance, R_{eff}, ranged from 23 to 51 MΩ. The observations that values of τ depend on the method of passing current, and that the value of λ is large relative to fiber length and diameter are considered evidence that the individual muscle fibers are electrically interconnected within bundles in a three-dimensional network. Estimations are made of the membrane resistance, R_m, to compare the values to fast and slow striated muscle fibers and mammalian smooth muscles. The implications of this study in reinterpreting previous mechanical and electrical studies are discussed.

INTRODUCTION

Previous reports indicated that individual muscle fibers in the anterior byssus retractor muscle (ABRM) of *Mytilus* run the length of the muscle, up to 4 cm (Fletcher, 1937; Twarog, 1954). As studies of the passive electrical properties of *Mytilus* muscle progressed, it appeared that this assumption was incorrect. In this paper evidence is presented that the muscle fibers are short and that they are electrically connected by nexal junctions. In addition, measurements

of the passive electrical properties of the muscle are presented, and possible implications of these measurements are discussed.

METHODS

To determine the length of individual muscle fibers, the muscle was fixed on a frame at approximately 60% of the *in situ* length and then soaked in a macerating solution. Two kinds of macerating solutions were used: In one case, a solution of 10% nitric acid in seawater and, in the other, a saturated solution of boric acid in seawater. Nitric acid maceration was more successful. After 3 days of treatment in the macerating solution, small bundles of muscle were briefly sonicated (20 s) in a Petroff-Hauser bacterial sonicator. The individual cells separated from one another and samples of the resulting suspension were pipetted out and examined microscopically.

Conventional histological preparations were made. The muscle was fixed in Bouin's solution, embedded in paraffin, and stained with hematoxylin and eosin.

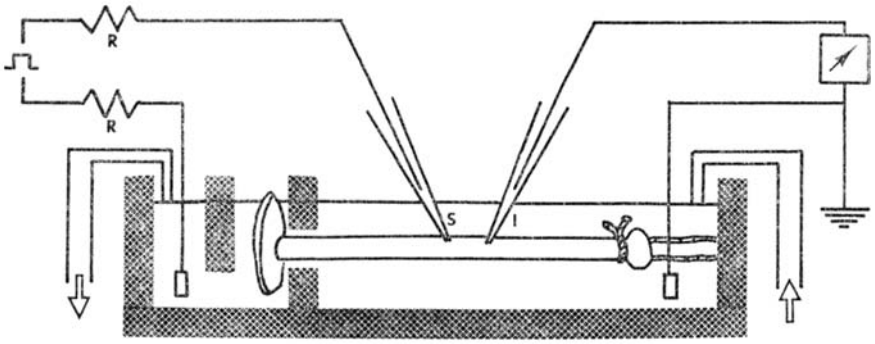

FIGURE 1. Diagram of the chamber for mounting ABRM and passing current via a suction electrode (S); recording via an intracellular electrode (I). Resistors (R) are 20 MΩ. Open arrows indicate flow of ASW.

For electron microscopy, two methods of fixation were used. Some muscles were fixed for 4 h at room temperature in a solution containing 25% sucrose, 0.5% $CaCl_2$, 0.1 M cacodylate buffer at pH 6.5 or pH 7.5, and 3% glutaraldehyde. After rinsing overnight in distilled water, the tissue was postfixed for 4 h in 2% osmium. The preparation was embedded in epon; sections were stained with uranyl acetate and lead citrate, then examined. Other muscles were fixed in 0.6% permanganate in sodium veronal buffer with 3.5 g/100 ml NaCl for ½ h. The tissue was washed in seawater overnight and postfixed in 2% osmium in seawater for 2 h. All specimens were embedded in epon, sectioned, stained, and examined.

Two methods were used for measurements of the passive membrane properties. A modified Araki-Otani bridge (Hidaka, Osa, and Twarog, 1967) permitted passage of current and recording from the same intracellular microelectrode. A second method is diagrammed in Fig. 1. The suction electrode had an orifice of about 100 μm. A portion of a muscle bundle was drawn by suction into the electrode and current was passed from the suction electrode through the muscle bundle. In measuring time constants and space constants, hyperpolarizing current was used because the

voltage current curve is linear with hyperpolarizing currents of the order of 10^{-9} A and because any possibility of exciting the muscle was to be avoided.

The intracellular microelectrode (30–50 MΩ resistance) was connected to a Bioelectric NF-1 high impedance preamplifier (Bioelectric Instruments Div., General Microwave Corp., Farmingdale, N. Y.) and was moved relative to the suction electrode. The intracellular electrode recorded the membrane potential in muscle cells as current was passed via the suction electrode.

The experiments were performed at 18° or 19°C. Artificial seawater solution flowed through the chamber at a rate of about 1 ml per 2 min and the water level was kept constant by a suction device. The artificial seawater contained Na^+, 428 meq; K^+, 10 meq; Mg^{++}, 40 meq; Ca^{++}, 20 meq; Cl^-, 548 meq; with tris(hydroxymethyl)aminomethane-HCl buffer, 50 mM at pH 7.3.

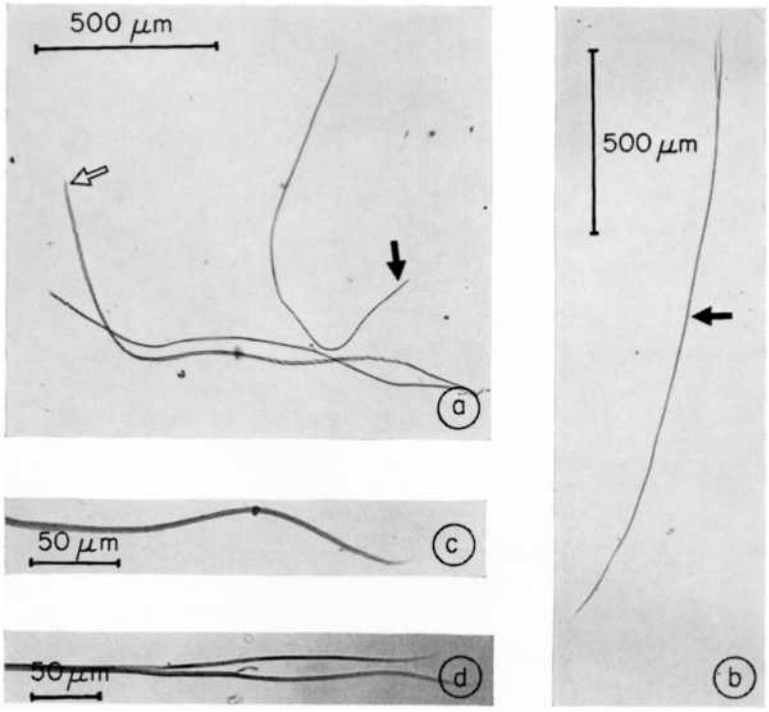

FIGURE 2. Fibers obtained in one preparation. In (*a*) the marker indicates 1 mm; three fibers are seen, one is intact (filled arrow) and two have broken ends (open arrow). In (*b*) one can see a fiber which apparently has a single and a double tapered end and a single central nucleus (arrow). In (*c*) there is a view of the tapered end at high magnification and in (*d*), a higher magnification view of an apparently double tapered fiber. The frequency of this kind of forking is high and the forking occurs only at one end of an otherwise uninterrupted apparently uninucleate fiber. This may well be a characteristic form of these fibers since electron micrographs show branched profiles. Calibration: 500 μm in (*a*) and (*b*); 50 μm in (*c*) and (*d*).

RESULTS

Muscle Fiber Structure

After maceration, fibers which were uninucleate, approximately 5 μm in diameter, and 1.2–1.8 mm in length were isolated. Some fibers appeared broken with frayed blunt ends. Others showed a fine tapered end (Fig. 2). After sonication more than 90% of the cells showed tapered ends and very

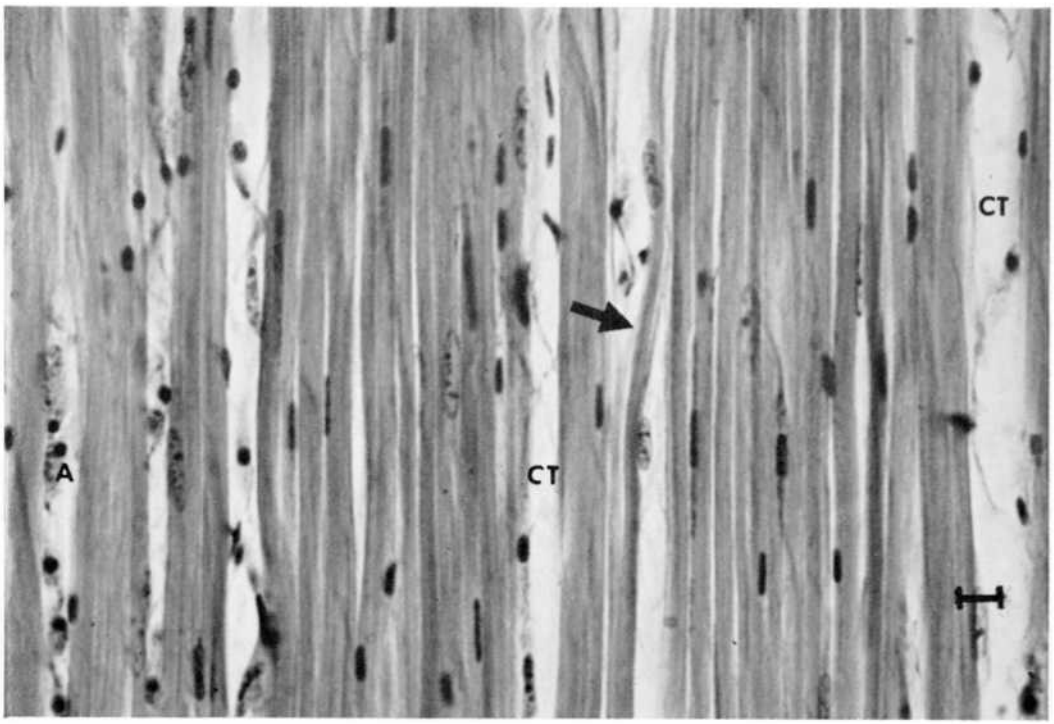

FIGURE 3. A longitudinal section of the muscle (hematoxylin and eosin) showing clustering of muscle fibers in bundles. Arrow indicates fiber strand crossing from one bundle to the next. Calibration 10 μm. A, amoebocyte (blood cell); CT, connective tissue.

few were broken. When macerated muscle was teased with needles instead of separated by sonication, it was very difficult to separate individual fibers. In fact, long thin bundles were obtained in which the individual fibers could not be distinguished. Further attempts to reduce the bundle resulted in tearing the fibers. This is, no doubt, the reason why previous work based on teasing with needles suggested that the fibers run from end to end.

Fig. 3, a hematoxylin-eosin stained section, displays the cylindrical shape of the individual fibers; the arrow indicates a point at which the tapered ends

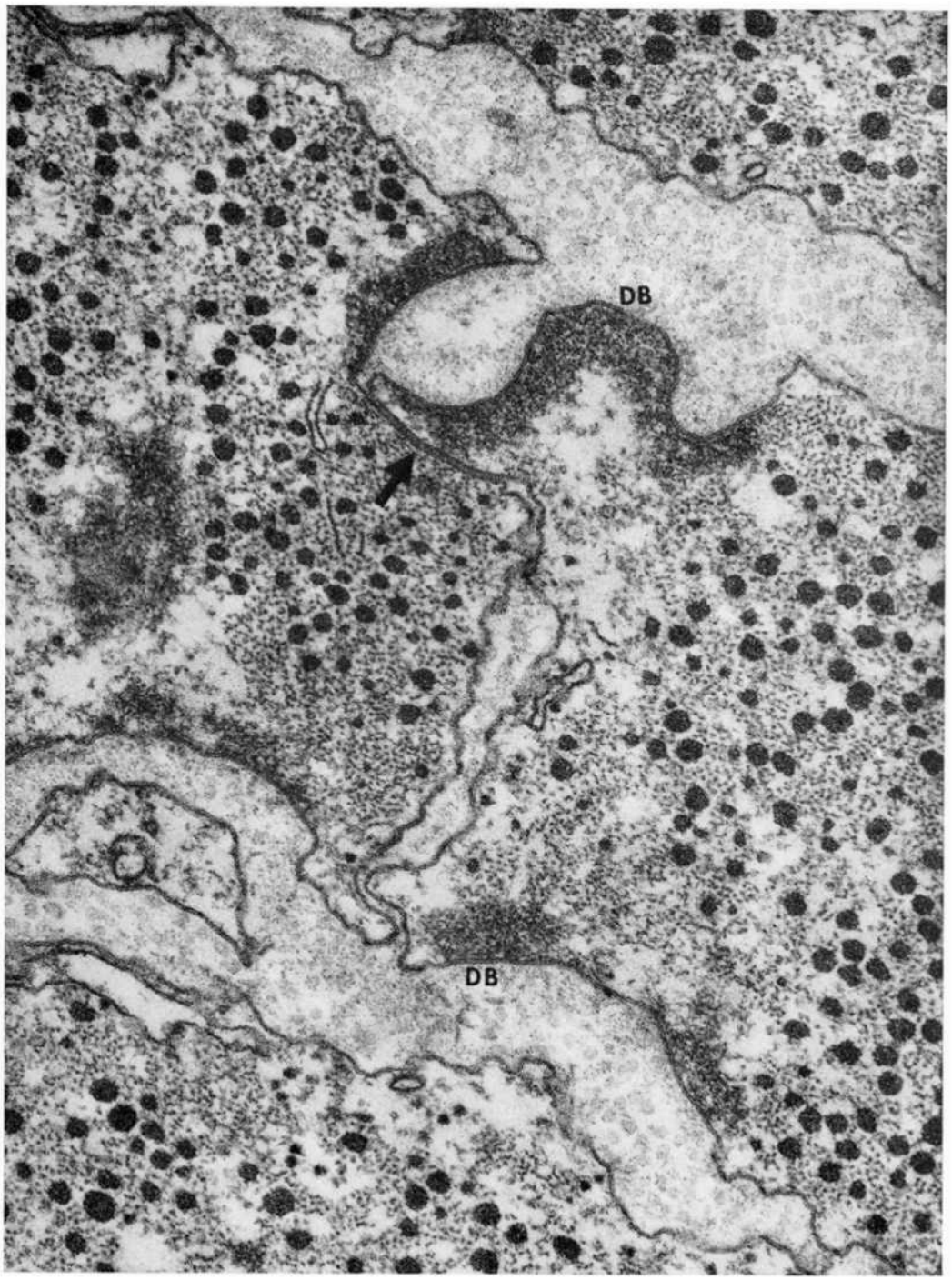

FIGURE 4. Cross-section of glutaraldehyde-fixed relaxed muscle showing the form of contact between muscle cells. A typical nexus occurs at the arrow. Regions of increased membrane density, marked DB, join fibers and connective tissue. × 70,000.

of two fibers appear to overlap. In this figure there are areas in which connective tissue is relatively more abundant between the fibers. The muscle is organized into bundles, some of which run the length of the muscle and which range from 100 to about 500 μm in diameter; each bundle is invested by connective tissue. It is easy to separate these small bundles without damaging the fibers within (Twarog, 1967).

Interfiber Junctions

Fig. 4 shows a typical junction as it appears after glutaraldehyde-osmium fixation. Two ajdacent muscle cells are seen in Fig. 4. Thick and thin filaments within the muscle cells are apparent; the muscle membranes are distinct and the extracellular space is filled with collagen except where two fibers abut. At the junction between two fibers the extracellular space appears nearly occluded. Fig. 6 *b* shows the unit membranes of both fibers and the very close approximation of the membranes.

The results of permanganate fixation are shown in Fig. 5 and Fig. 6 *a*. Fig. 5 shows the invagination of a fiber into an adjacent fiber. Also, simple abutment of cells is a common feature of intercellular contacts in this muscle. Fig. 6 *a* shows at higher magnification a region of close approximation of two muscle fibers where apparent fusion of the outer leaflets of the membrane is evident. These are nexal junctions as defined by Dewey and Barr (1962).

Fiber–Connective Tissue Junctions

Except for nexuses, no other muscle to muscle junction was observed. However an entity exists along the muscle membrane which may relate to mechanical connection between muscle fiber and connective tissue fibers. Fig. 4 and Fig. 7 illustrate such regions. At these regions, thin filaments insert into an area of increased density at the fiber membrane. In addition, there appears to be a thickening of the basement membrane at this point and connective tissue fibers seem to be embedded in this material. When cells are poorly fixed as in Fig. 7 the sarcolemma detaches from the surrounding connective tissue components except at such junctions. Apparently these sites act as regions of mechanical adherance of muscle cell to connective tissue.

Passive Electrical Properties

Fig. 8 shows recordings made from the muscle when currents of different intensities were applied. In Fig. 8 *a*, current was passed through the suction electrode and in Fig. 8 *b*, through the intracellular electrode, using the bridge circuit. The resting membrane potential was near 65 mV in most cases. The time constant of the muscle was measured and proved to be much shorter when current was passed via an intracellular electrode than when the current was applied to muscle bundles via the suction electrode.

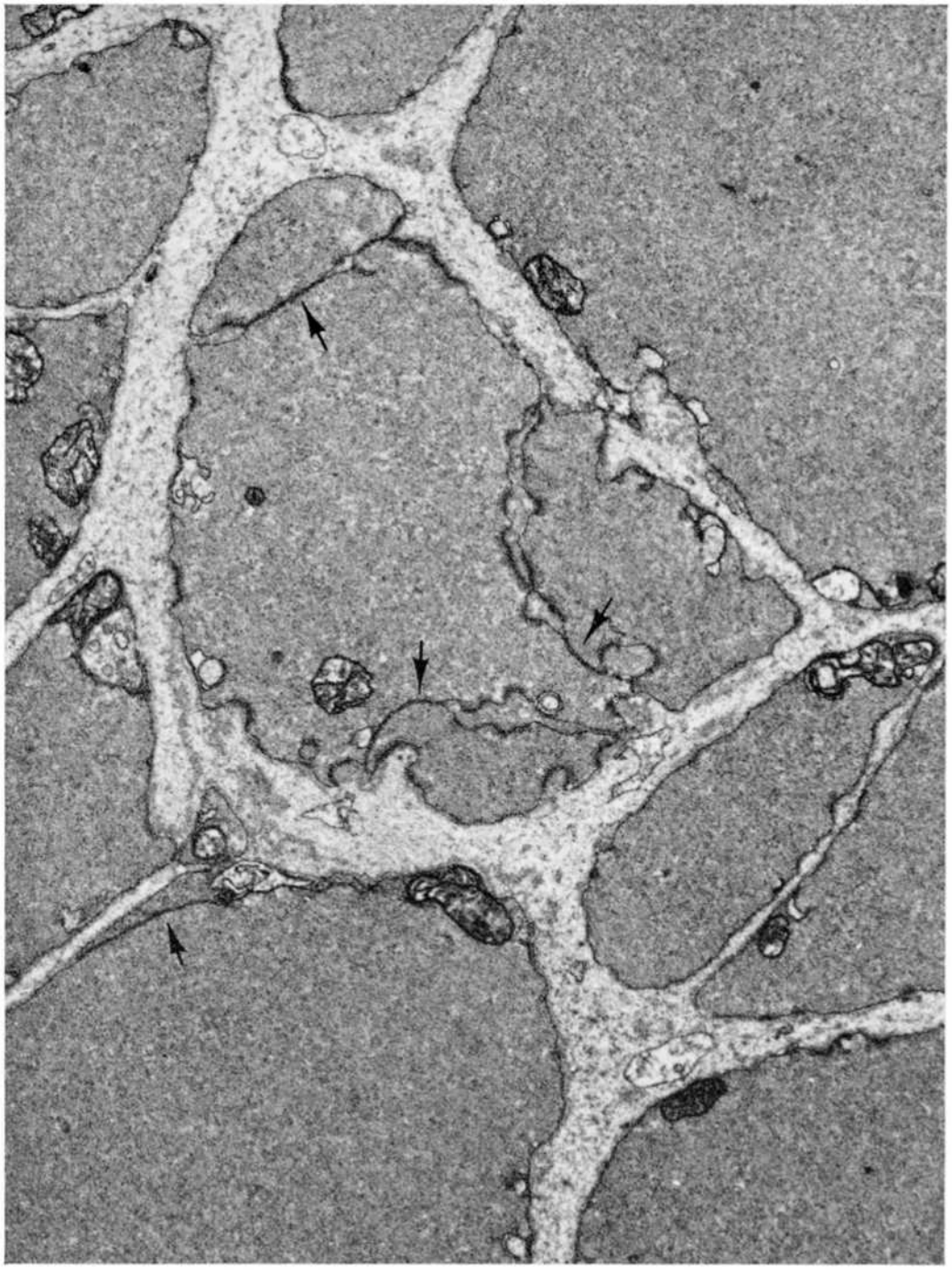

FIGURE 5. Cross-section of permanganate-fixed relaxed muscle. Arrows indicate nexal junctions. × 22,000.

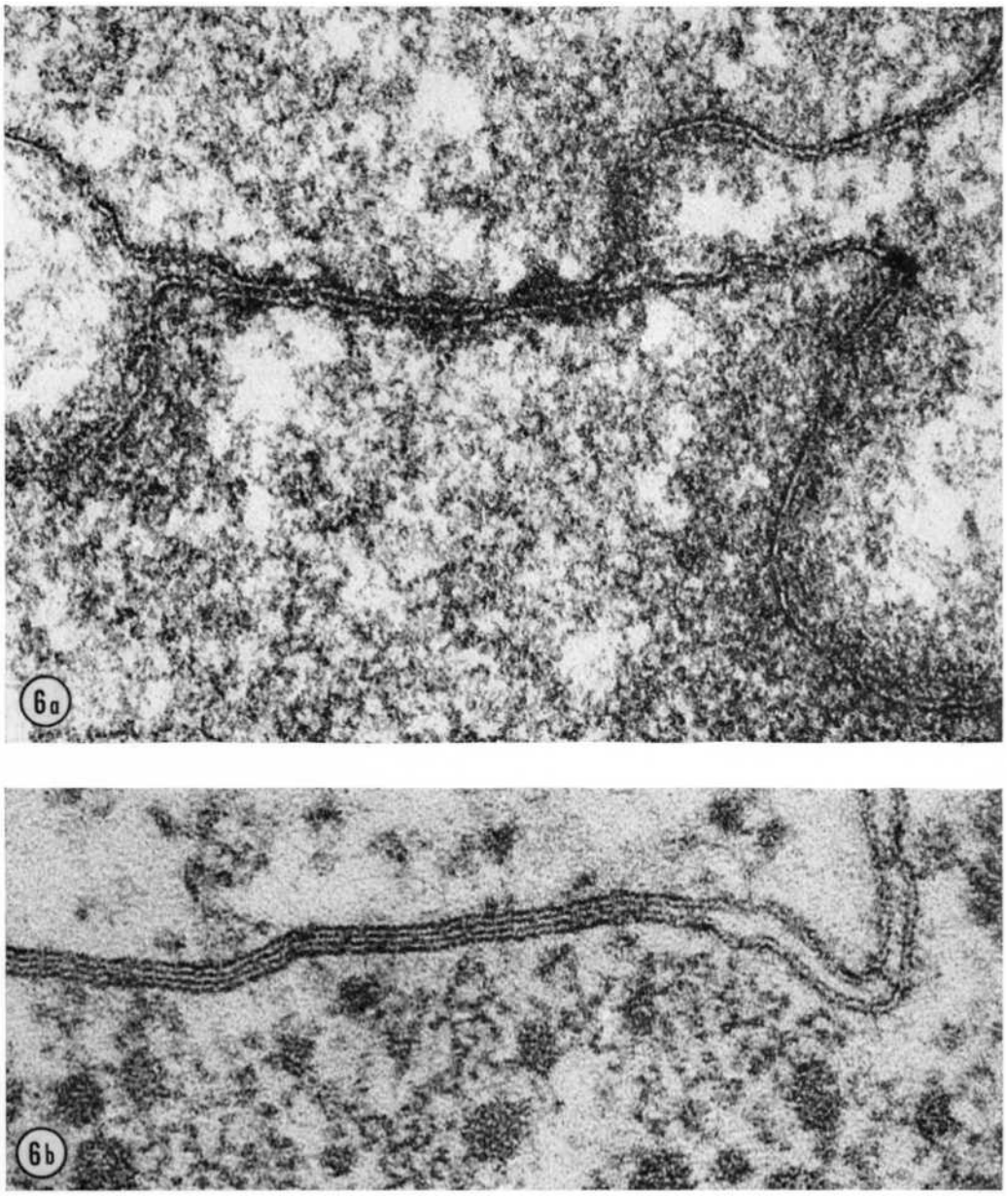

FIGURE 6. (*a*) Permanganate-fixed muscle, illustrating nexus in higher magnification. Thickness of nexus is ~ 150 Å. (*b*) Glutaraldehyde-fixed muscle, illustrating nexus in higher magnification. While a narrow intercellular gap appears to be present, the total thickness of the nexuses (~ 150 Å) is less than the thickness of two opposed membranes. The thickness of the sarcolemma is ~ 75 Å. × 270,000.

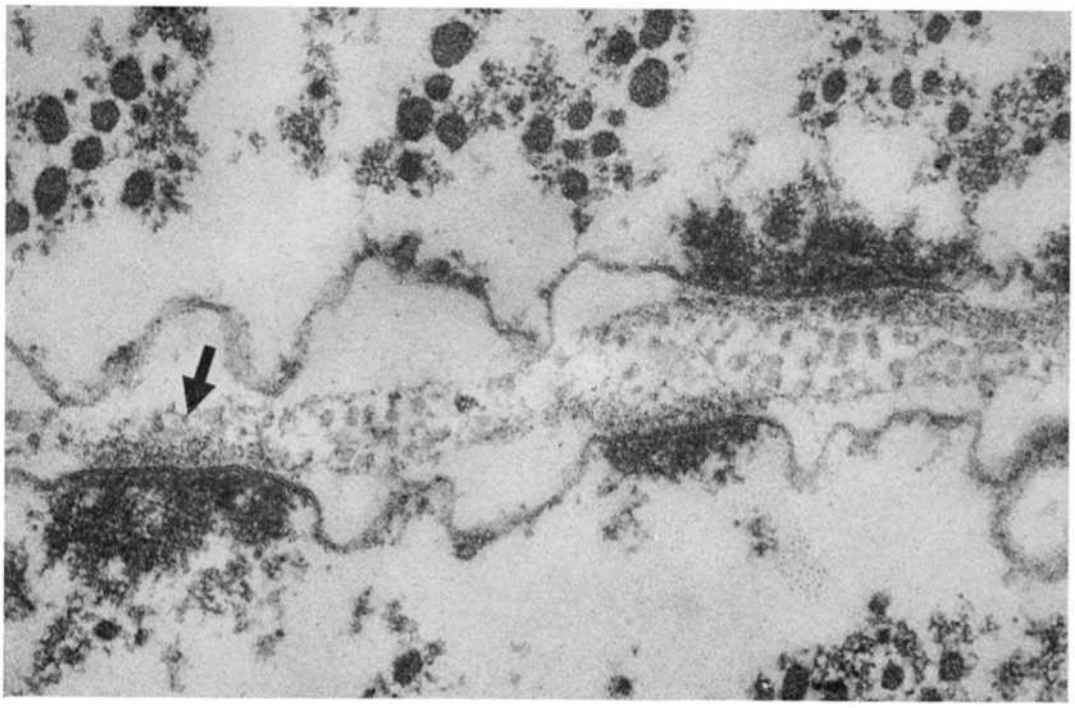

FIGURE 7. Poorly fixed muscle, illustrating separation of sarcolemma from connective tissue except at regions of thin filament attachment at the sarcolemma. Note the increased thickness of the external lamina of the membrane at these regions (arrow). × 28,000.

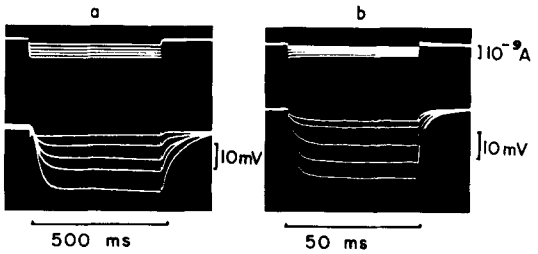

FIGURE 8. Responses to anodal current pulses of increasing intensity, recorded with an intracellular microelectrode, calibration 10 mV: (*a*) Current applied via suction electrode; current calibration 7×10^{-7} A; time 500 ms. (*b*) Current applied via intracellular microelectrode (bridge method); current calibration 10^{-9} A; time 50 ms.

Table I shows values of the time constant τ in a number of independent experiments on muscle cells in different preparations. The time constant was determined as the time required to reach 84% of the final electrotonic potential. The column τ_1 shows time constants obtained by passing current via an intracellular electrode; these averaged 4.3 ms with a standard devia-

tion of ± 1.2 ms. τ_2 was obtained when current was applied via a suction electrode. The mean was 68.3 ms with a standard deviation of ± 15 ms.

Fig. 9 shows potentials recorded intracellularly at the indicated distances from the orifice of a current-passing suction electrode. The graph shows the

TABLE I

VALUES OF TIME CONSTANT IN A NUMBER OF INDEPENDENT EXPERIMENTS ON MUSCLE CELLS IN DIFFERENT PREPARATIONS

τ_1		τ_2	
Obtained by intracellular stimulation (bridge method)		Obtained by extracellular stimulation (suction electrode)	
ms		*ms*	
5.5		60	
4.9		71	
5.8	Mean = 4.3 ms	54	Mean = 68.3 ms
3.9		68	
4.3	SD = ±1.2	93	SD = ±15.3
3.3		91	
2.5	SE = ±0.5	53	SE = ±4.8
		61	
		57	
		75	

τ, Time in milliseconds to reach 84%.

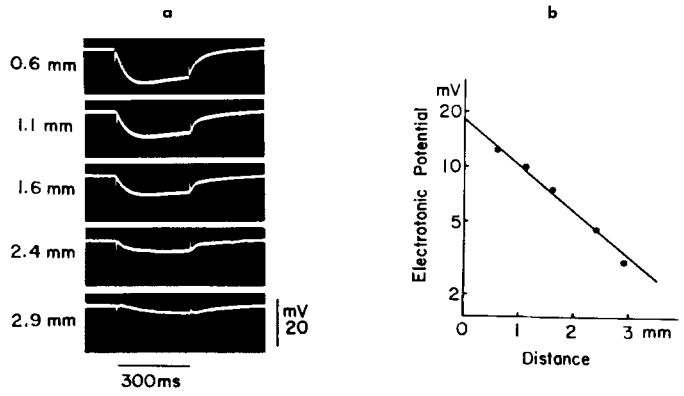

FIGURE 9. Determination of space constant. (*a*) Intracellular recording of electrotonic potential at increasing distances from suction electrode, calibrations 20 mV; 300 ms. (*b*) Log plot of voltage against distance.

membrane potential on a log scale plotted against distance of the intracellular electrode from the orifice of the suction electrode. The potential has fallen to $1/e$ of its initial value at a distance of approximately 2.7 mm.

Table II summarizes data on the space constant from six different preparations. The mean space constant is 1.8 mm, the standard deviation is ± 0.4 mm. When the recording electrode is moved away from the current-passing

electrode in a lateral rather than in a longitudinal direction, the potential recorded falls to zero within 0.1 to 0.3 mm. In an immediately adjacent, optically identifiable bundle, no effect of passing current is detectable although the intracellular recording electrode may be within 0.1 mm of the orifice of the current-passing electrode. In contrast, the voltage is quite high within 2 mm of the orifice in a longitudinal direction. This indicates that one

TABLE II

SUMMARY OF DATA ON SPACE CONSTANT FROM SIX DIFFERENT PREPARATIONS

Space constant λ	
mm	
2.0	Mean = 1.8 mm
1.6	SD = ±0.4
1.7	SE = ±0.2
2.5	
1.2	
1.6	

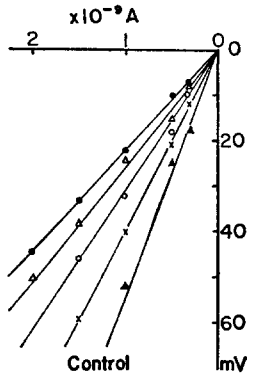

FIGURE 10. Determination of R_{eff}. Hyperpolarizing current applied via intracellular bridge electrode. Voltage of electrotonic response in five cells plotted against applied current.

is not recording current leakage by this method since, at very close lateral distances, no voltage change is recorded. More important, it indicates that only fibers within a muscle bundle are in electrical communication with one another.

An intracellular current-passing electrode was used with intracellular recording via a bridge. Fig. 10 shows a series of current-voltage curves. The current used was all in the hyperpolarizing direction and it was of the order of 10^{-9} A. It can be seen that the current-voltage curve is linear and that the

membrane input resistance is between 20 and 50 MΩ. Individual measurements in one typical muscle were 23, 30, 32, 34, 51, and 56 MΩ.

DISCUSSION

The individual muscle fibers of the *Mytilus* byssus retractor have been assumed by previous authors to be very long and in fact, to run from the origin of the muscle at the byssus to its insertion on the shell, 4 cm in an adult *Mytilus* (Fletcher, 1937; Twarog, 1954). This has proved inaccurate; the individual uninucleate fibers of the muscle are no more than 2 mm long and the average fiber is approximately 1.5 mm. The finding that these fibers are shorter than the total length of the muscle has serious implications for the interpretation of past studies of the mechanical and electrical properties of the muscle.

Mechanical Properties of ABRM

Our electron microscopic studies have shown that there are no mechanical connections between muscle fibers, but rather that the muscle fibers form attachments with connective tissue fibers. This invalidates the assumption in many studies of the mechanical properties of this muscle that the muscle fibers are uninterrupted. Rather, this muscle is a mechanically complex system where fibers are connected to one another via connective tissue components. Especially in studies of the properties of glycerinated muscles, the extra series resistance, the connective tissue, must be considered in interpreting data which previously were considered to come from two mechanically independent systems within long uninterrupted fibers (see Rüegg, 1971).

Electrical Properties of ABRM

The assumption that the fibers ran from end to end of the muscle and were electrically continuous from one end to the other was supported by the observation that demarkation potentials could be measured (Twarog, 1954), and that one could use sucrose gap techniques (Rüegg, Straub, and Twarog, 1963). Although the fibers are not long and the original rationale is in error, the fibers within bundles are in fact electrically continuous. Space constants of 1–2.5 mm were determined. Thus a voltage displacement in one fiber can be detected in a fiber two or more cell lengths distant.

The evidence for nexal junctions is strong. After glutaraldehyde fixation close approximations between adjacent muscle cells were seen. At the region of the close approximations the connective tissue fibers were excluded and extracellular space appeared partially occluded. After permanganate fixation it could be seen that the outer leaflets of membranes appeared fused in the regions where the cells touched. Overall dimensions of the nexus in either case had similar dimensions, about 150 Å, including both cytoplasmic laminae of the fused membranes. Such junctions between the adjacent

muscle fibers have been called nexuses (Dewey and Barr 1962, 1964) and are implicated as sites of electrical connectivity between adjacent cells (Barr, Dewey, and Berger, 1965; Barr, Berger, and Dewey, 1968).

Certain electrical constants were measured: membrane input resistance (R_{eff}), time constant (τ), and space constant (λ). τ was different depending on whether current was applied to the muscle through intracellular electrodes using an Araki-Otani bridge (2–6 ms) or through an external suction electrode (50–75 ms). A similar difference in time constant depending upon the method of passing current has been observed in mammalian smooth muscle (Kuriyama and Tomita, 1965; Tomita, 1966). This difference has been explained by Tomita as a function of the electrical interconnection of individual muscle cells in a three-dimensional network. When current is applied to a single cell in the network, the spread of current is quite different from when it is applied via a suction electrode to a group of 20–50 parallel cells in a bundle. When current (of the order of 10^{-9} A) is applied intracellularly to one single muscle fiber, it spreads in three dimensions to adjacent electrically connected muscle cells and thus hyperpolarizes only a small area of membrane. When current is applied through the suction electrode it is essentially equal and simultaneous in all of the muscle cells which lie across the orifice of the electrode and spreads longitudinally from these cells to many cells which lie beyond the suction electrode, thus hyperpolarizing a larger area of membrane.

Measurements of space constant show that potentials can still be recorded at distances which are greater than the length of any one muscle cell, supporting the conclusion that there is electrical connection between the cells. Measurements of the membrane input resistance extended and confirmed previously reported data of Hidaka, Osa, and Twarog (1967).

It seemed useful, in order to make comparisons with other muscles, to calculate R_m, even roughly. Using available data on the size and shape of individual muscle fibers (a diameter ranging from 4 to 8 μm, a fiber approximately cylindrical along most of its length and about 1–2 mm in total length), the R_m calculated from the limited cable equation: $R_m = 2\pi rl \cdot R_{eff}$, ranges from 3700 $\Omega \cdot cm^2$ to 7400 $\Omega \cdot cm^2$. However, since the individual cells are electrically interconnected, R_m has also been calculated using an equation derived from the cable hypothesis: $R_m = (2\ R_i\lambda^2)/a$, where a is the fiber radius, R_m the membrane resistance in ohms·square centimeter and R_i the resistivity of the cell interior in ohms·centimeter. In the calculations in Table III the fiber radius is taken as 3 μm and R_i is assumed to be 60 $\Omega \cdot cm$. This R_i is the value given by Katz (1966) for the internal resistance of a crab nerve fiber. The true R_i in *Mytilus* is probably larger than the 60 $\Omega \cdot cm$ due to the junctional resistance between cells, which makes the calculations underestimate R_m. The space constant, λ, taken at the ends of the ranges measured, is 1 and 2.5 mm.

R_m is thus calculated to lie between 4700 and 29,000 $\Omega \cdot cm^2$. These values

represent reasonable minimal estimates of the true membrane resistance of this muscle which may be comparable to amphibian fast striated muscle fibers where R_m is 4000 $\Omega \cdot cm^2$ or to amphibian slow fibers where R_m has been estimated as 20,000–50,000 $\Omega \cdot cm^2$ (Adrian and Peachey, 1965). These ranges of R_m are similar to the ranges calculated for mammalian smooth muscles (Tomita, 1970).

TABLE III

CALCULATION OF R_m

$$\lambda = \sqrt{\frac{a}{2}\frac{R_m}{R_i}}$$

$$R_m = \frac{2 R_i}{a} \cdot \lambda^2$$

$a = 3 \times 10^{-4}$ cm $R_i = 60\ \Omega \cdot$cm. $\lambda = 0.1$ cm	$R_m \doteqdot 4700\ \Omega \cdot cm^2$
$a = 3 \times 10^{-4}$ cm $R_i = 60\ \Omega \cdot$cm $\lambda = 0.25$ cm	$R_m \doteqdot 29{,}000\ \Omega \cdot cm^2$

R_i possibly larger due to junctional resistance.

TABLE IV

CALCULATION OF C_m

$\tau_m = 50$ ms $R_m = 5000\ \Omega \cdot cm^2$	$C_m = 10\ \mu F/cm^2$
$\tau_m = 50$ ms $R_m = 30000\ \Omega \cdot cm^2$	$C_m = 1.7\ \mu F/cm^2$

τ is probably $\leq$ 50 ms if x is not at point of current application.

Table IV shows calculations of C_m. The time constant τ_m taken from measurements with the suction electrode, probably overestimates the actual value since the intracellular electrode measures the voltage change at a finite distance from the point of current application. R_m was taken from the previous approximations as 5,000 and 30,000 $\Omega \cdot cm^2$, and the calculations of membrane capacitance thus range from 10 $\mu F/cm^2$ to 1.7 $\mu F/cm^2$. If τ_m is overestimated, the true values would actually be smaller than this. Large values of membrane capacitance would not be anticipated in this muscle since it displays none of the infoldings of membrane that are seen in amphibian or crustacean striated muscle, and in fact, there are fewer membrane invaginations than have been noted in many mammalian smooth muscles.

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