ULTRASTRUCTURAL AND PHYSIOLOGICAL STUDIES ON THE LONGITUDINAL BODY WALL MUSCLE OF DOLABELLA AURICULARIA

I. Mechanical Response and Ultrastructure

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ABSTRACT

The physiological properties of mechanical response and the ultrastructure in the longitudinal body wall muscle (LBWM) of the opisthobranch mollusc Dolabella auricularia were studied to obtain information about excitation-contraction coupling in somatic smooth muscles responsible for smooth and slow body movement of molluscs. The contracture tension produced by 400 mM K was not affected by Mn ions (5-10 mM) and low pH (up to 4.0), but was reduced by procaine (2 mM). The K-contracture tension was not readily eliminated in a Ca-free solution containing ethylene glycol-bis(β-aminoethyl ether)N,N,N',N'-tetraacetate (EGTA). A large contracture tension was also produced by rapid cooling of the surrounding fluid from 20° to 5°-3°C even when the preparation showed no mechanical response to 400 mM K after prolonged (more than 2 h) soaking in the Ca-free solution. These results indicate that the LBWM fibers contain a large amount of intracellularly stored Ca which can be effectively released by membrane depolarization. The fibers were connected with each other, forming the gap junctions, the desmosomes, and the intermediate junctions. The sarcoplasmic reticulum (SR) consisted of vesicular and tubular elements, and was mostly located near the fiber surface. The plasma membrane showed marked tubular invaginations of 600-800 Å in diameter, with many branches (surface tubules), extending inwards for ~2 µm. These surface tubules were closely apposed to the SR, and the bridgelike structures analogous to those in the triadic junction of vertebrate skeletal muscle were observed in the space between the surface tubules and the SR. It is suggested that the influence of membrane depolarization is transmitted inwards along the surface tubules to cause the release of Ca from the SR.

KEY WORDS molluscan smooth muscle · mechanical response · excitation-contraction coupling · ultrastructure · cell junctions · sarcoplasmic reticulum

It is generally accepted that, in vertebrate fast-striated muscle, the contraction-relaxation cycle is regulated by the release of Ca from, and uptake
by, the sarcoplasmic reticulum (SR) (8). In various kinds of smooth muscles, on the other hand, the relative contribution of the intracellularly released Ca and the inward moving extracellular Ca to the activation of the contractile mechanism is not firmly established, though the intracellular structures such as the SR and the mitochondria are known to accumulate Ca in the presence of ATP (21, 28).

Recent physiological experiments on the anterior byssal retractor muscle (ABRM) of a lamellibranch mollusc, Mytilus edulis, showed that the ABRM contains the intracellularly stored Ca in an amount enough to fully activate the contractile mechanism (24, 25). Further ultrastructural and histochemical studies on the ABRM indicated that the Ca localized at the inner surface of the plasma membrane, the SR and the mitochondria is released into the myoplasm during mechanical activity (1, 2, 22), providing evidence that these intracellular structures are involved in the contraction-relaxation cycle.

While Mytilus ABRM has been studied by many investigators in relation to its extremely prolonged contraction (28), little information is at present available about the intracellular structures and their role in excitation-contraction coupling in other molluscan somatic smooth muscles, which are responsible for slow and smooth body movement.

The present work was undertaken to study the ultrastructure of the longitudinal body wall muscle (LBWM) of an opisthobranch mollusc, Dolabella auricularia, and to relate it to the physiological properties of mechanical activity.

MATERIALS AND METHODS

Physiological Studies

The specimens of D. auricularia (relaxed body length, 16–20 cm) were collected at the Misaki Marine Biological Station, and kept in circulating seawater. The specimen was opened by a longitudinal incision in the dorsal body wall, and the inner surface of the body wall was exposed by removing visceral organs. Then, a strip of the longitudinal body wall muscle (LBWM, 1–3 mm in width, and ~10 mm in length) was dissected from the yellow-colored musculature that runs in the form of flat bands from the anterior to the middle region of the specimen (6, 7).

The LBWM preparation was mounted horizontally in an acrylic chamber (5 ml), which was divided into three compartments by removable partitions with Vaseline-lined slits (25). The central compartment was filled with isotonic sucrose solution, while the two end compartments were filled with the experimental solution and isotonic KCl solution, respectively; the demarcation potential between the KCl-depolarized and the experimental solution segments of the preparation was recorded with a pair of Ag-AgCl electrodes. In the resting preparation, this potential reached a steady level of 20–30 mV within 30–60 min, and the changes in the potential were regarded as a measure of true membrane potential changes (25, 27). The isometric tension developed in the experimental solution segment was recorded with a strain gauge (U-gauge, Shinko Tsushin Co., Zushi-shi, Kanagawa-ken, Japan).

The standard experimental solution (artificial seawater) had the following composition (mM): NaCl, 497; KCl, 10; CaCl2, 20; MgCl2, 50 (pH adjusted to 7.2 by NaHCO3). Natural seawater was also used occasionally with similar results. The preparation was stimulated to contract by the application of high [K]o (25–400 mM, substituted for Na) or acetylcholine (ACh, 10−3–10−4 M), and the resulting mechanical and electrical responses were recorded on an ink writing oscillograph. The solutions in the chamber were exchanged with a water-vacuum suction tube at a rate of 50–100 ml/min. When the factors affecting the mechanical responses were examined, the preparation was previously equilibrated for 5–15 min in solutions providing the same experimental conditions as those of the contracture solutions. The experiments were performed at room temperature (20°–27°C), unless otherwise stated.

Electron Microscopy

The LBWM preparation at its in situ length was fixed with a 6% glutaraldehyde solution containing 2 mM CaCl2 (pH 7.2 by 0.1 M cacodylate buffer), or with a 2.5% glutaraldehyde solution containing 0.6 M sucrose and 2 mM CaCl2 (pH 7.2 by 0.1 M cacodylate buffer). The tissue was then cut into small pieces, postfixed in 2% OsO4 (unbuffered), dehydrated with a graded series of ethanol, and embedded in Epon 812. Ultrathin sections were cut on a Porter-Blum MT-1 ultramicrotome (DuPont Instruments-Sorvall, DuPont Co., Wilmington, Del.) with a glass knife, and double-stained with uranyl acetate and lead acetate.

For freeze fracturing, the small pieces of the preparation fixed with the 2.5% glutaraldehyde solution were rinsed in either artificial seawater or 0.1 M cacodylate buffer (pH 7.2), kept in 20% glycerol for 2 h, and then frozen in liquid nitrogen at −196°C. The frozen specimen was fractured and etched with a Hitachi HFZ-1 freeze-etching device. The etched surface was replicated with platinum and carbon.

Both the ultrathin sections and the freeze-fractured replicas were examined with a Hitachi HU-12AS electron microscope.

RESULTS

Electrical and Mechanical Responses to ACh and High [K]o

Fig. 1 shows the electrical (upper traces) and
the mechanical (lower traces) responses of the LBWM fibers to ACh and high [K]. The fibers showed depolarization and development of contracture tension in response to ACh (10^{-5}-10^{-3} M) (Fig. 1a) and to high [K]o (25-400 mM) (Fig. 1b). Since the length of the fibers is ~200 μm (23) and is much shorter than the distance between the two end compartments (3 mm), the existence of the demarcation potential indicates that, as with many other kinds of smooth muscles, the LBWM fibers are electrically continuous. Both ACh- and K-induced contractures started to relax spontaneously, while the fibers remained depolarized.

Though the contracture tension produced by a supramaximal concentration of ACh (10^{-5}-10^{-3} M) was nearly as large as that produced by a supramaximal concentration of K ions (300-400 mM), the magnitude of ACh-contractures was not stable and tended to decrease with repeated application of ACh. Meanwhile, the magnitude of K-contractures remained almost constant, provided the preparation was kept in the standard solution for 5-10 min before each application of high [K].

Properties of K-Induced Contractures

The contracture tension in response to 400 mM K was not affected in the presence of Mn ions (5-10 mM), which are believed to block Ca influx in various kinds of excitable membrane (12) (Fig. 2a), while it was reduced by 50-70% in the presence of procaine (2 mM), which is known to inhibit Ca release from the SR in vertebrate skeletal muscle (10, 32) (Fig. 2b). Meanwhile, the submaximal contracture tension produced by 25-150 mM K was sometimes decreased to a certain extent in the presence of Mn ions (23). The K-contracture tension started to decrease after the removal of [Ca], (Fig. 2c), but a noticeable tension (5-10% of the maximum value) could still be produced by 400 mM K even after a prolonged soaking (up to 2 h) with a Ca-free solution containing ethylene glycol-bis(β-aminoethyl ether)N,N′,N′′,N′′-tetraacetate (EGTA) (2 mM). The height of K-contractures was not affected by high pH of the external medium (up to 10.0 by adding NaOH) or by low pH (up to 4.0 by adding HCl) which is known to inhibit Ca influx in vertebrate smooth and cardiac muscles (13, 23). Meanwhile, K-induced depolarizations were not affected by the above factors.

These results may be taken to indicate that the LBWM fibers can be fully activated by a supramaximal concentration of K ions even when the influx of extracellular Ca is inhibited by Mn ions or low pH, suggesting that they contain a large amount of intracellularly stored Ca ions available for the activation of the contractile mechanism. The inhibitory effect of procaine on the K-contracture tension and the persistence of K-contractures in the Ca-free solution may also be consistent with this view, since procaine is expected to reduce the Ca release from the intracellular structures (10, 32) and the internal Ca may not be readily removed in Ca-free medium.

Contractures Induced by Rapid Cooling

As shown in Fig. 3, the LBWM fibers exhibited depolarization and tension development when the
temperature of the standard solution was rapidly lowered from 20°C to 5°C within 5-10 s. The contracture tension induced by rapid cooling was as large as the maximum K-contracture tension, and also started to relax spontaneously at low temperatures, while the membrane remained depolarized (Fig. 3a). The contracture tension by rapid cooling could also be produced in the preparation which had almost relaxed in 400 mM K (Fig. 3b), or in the preparation in which K-contracture tension had been eliminated after a prolonged soaking in a Ca-free solution containing EGTA (2-3 mM) (Fig. 3c). These results indicate that the primary cause of the mechanical response is the rapid cooling of the external medium but not the accompanying depolarization; the depolarization by cooling might be explained in terms of the inhibition of electrogenic Na-pump. As with K-induced contractures, the tension development by rapid cooling was reduced by ~60% in the presence of procaine (2 mM), but was not affected by Mn ions (5-10 mM).

In vertebrate skeletal muscle, the subthreshold concentrations of caffeine cause marked contractures by rapid cooling (15, 19). This effect of rapid cooling has been accounted for as a result of the reduction in the capacity of Ca-binding and Ca-pump by the SR, whereas the release of Ca from the SR by caffeine (32) is less affected. The mechanical response to rapid cooling in the LBWM fibers may also be explained as being a result of the reduced Ca binding capacity and Ca uptake in some intracellular structures, since these phenomena are expected to cause an increase in the myoplasmic Ca concentration. It may be that the intracellular structures in the LBWM are more sensitive to temperature than those in vertebrate skeletal muscle, so that a large amount of Ca can be released to fully activate the contractile mechanism by simply cooling the LBWM without any additional agent facilitating the Ca release. The inhibitory effect of procaine on the mechanical response to rapid cooling is also consistent with the view that procaine reduces the Ca release from the intracellular structures.

These results, together with the properties of K-contractures, indicate the presence of intracellularly stored Ca in the LBWM fibers in an amount enough to fully activate the contractile mechanism.

**Muscle Fiber Dimensions and Interfiber Relationships**

The LBWM fibers consisted of uninucleate unstriated muscle fibers of 10-20 μm diameter and ~200 μm long with tapered ends (Figs. 4 and 6), and contained thick (diameter, ~350 Å) and thin (diameter, ~70 Å) myofilaments. Mitochondria were located mostly at the center of the fiber. The fibers ran along the long axis of the LBWM, and came into close contact with each other at their tapered ends (Fig. 4). At these interfiber junctions, an interfiber space (400-500 Å) was occupied by a homogeneous and amorphous material of moderate density, and associated with more electron-opaque myoplasmic components along the plasma membrane (Fig. 5). Such a type of interfiber junction has been classified as a kind of the intermediate junction (fascia adherens) (9).

In addition to the above end-to-end junctions, the LBWM fibers came also into contact with...
each other at four different kinds of interfiber junction, while at the nonjunctional regions the fibers were normally separated by a space of >0.1 \( \mu m \). The first type was the gap junction (or the nexus) where the plasma membranes of the adjacent fibers showed a fused appearance (Fig. 6). Closer examination of the gap junction revealed that it formed a seven-layer membrane system
Figure 6 Low-magnification micrograph of the LBWM in transverse section. Arrows indicate the interfiber junctional regions characteristic of the LBWM. The gap junction is seen in the area enclosed in the rectangle. Bar, 1 μm. × 14,900.

Figure 7 High-magnification view around the area of gap junction shown in Fig. 6. A seven-layered appearance of the gap junction is clearly seen. The plasma membranes at the junctional region are ~80 Å thick, being closely apposed with a gap space of ~30 Å. Thus, the overall thickness of the gap junction is ~190 Å. Bar, 0.1 μm. × 213,000.
with an overall thickness of ~190 Å as a result of close apposition of the adjacent plasma membranes (Fig. 7). The plasma membrane consisted of the inner and outer dense layers ~25 and 20 Å thick, respectively, and the intermediate light layer ~30 Å thick. The gap space between the apposed plasma membranes was 30–40 Å. The presence of the gap junctions was also shown by the observation of freeze-fractured replicas (Figs. 8 and 9). The aggregates of intermembrane particles characteristic of the gap junction (14) were variable both in shape and in size (diameter, 0.05–1 µm). The diameter of the gap junctional particles was 60–80 Å.

The second type of interfiber junction seemed to correspond with the desmosome (16). This type of interfiber junction had an interfiber space of 400–500 Å filled with a homogeneous and amorphous material of moderate density, and electron-opaque, amorphous and fibrillar material was present at areas subjacent to the apposed plasma membranes (Fig. 10).

The third type of interfiber junction was composed of adjacent plasma membranes closely apposed over a considerable distance (up to 3 µm), with an interfiber space of 60–120 Å (Fig. 11). The interfiber space was also occupied by a homogeneous and amorphous material of moderate density. These features indicate that this type of junction may be regarded as a typical intermediate junction, except for the absence of the densification of the myoplasm at the junctional area.

The fourth type of interfiber junction was characteristic of the LBWM, and was most frequently observed in transverse sections (Fig. 6). At this type of junction, the adjacent plasma membranes were apposed with an interfiber space of 400–800 Å filled with a material of moderate density (Fig. 12). The apposed plasma membranes were markedly wavy in both transverse (Fig. 12) and the longitudinal (Fig. 13) sections, and sometimes the interfiber space was as small as 100 Å (Fig. 12).

These structural observations indicate that there are several types of interfiber junction between the LBWM fibers. Since these junctions are believed to provide not only the mechanical connection but also the electrical continuity between the smooth muscle fibers, these results may serve as the structural basis for the electrical continuity between the fibers within the LBWM.

Caveolae, Surface Tubules, and SR

Bottle-shaped caveolae (600–800 Å in diameter) were distributed along the fiber surface (Figs. 6, 10, 12, 14, and 15). The lumens of the caveolae communicated with the extracellular space and were filled with a material of moderate density. The caveolar ostium was ~200–300 Å in diameter. In addition to the caveolae, an extensive system of plasma membrane invaginations was found within the LBWM fibers. When the fibers were sectioned with slight inclination to their long axis, marked tubular invaginations of the plasma membrane (600–800 Å in diameter) were found to extend inwards for a distance of ~2 µm from the fiber surface (Fig. 14). These tubular invaginations will be called the surface tubules in this paper. The surface tubules had many branches (Fig. 15), and were also filled with a material of moderate density. At the area of the intermediate junction, the surface tubules appeared to form an elaborate network (Fig. 16).

The SR consisted of tubular and vesicular elements of variable size and shape, and was frequently observed near the fiber surface (Figs. 14 and 15), though the SR was also seen at the center of the fiber. The SR was frequently observed to be closely apposed to the plasma membrane, or to the membrane of the surface tubules. The gap between the membrane of the SR and that of the surface tubules was ~140 Å, and electron-opaque bridgelike structures analogous to those in the triadic junction of vertebrate skeletal muscle (30, 31) could be observed.

Neuromuscular Junction

The motor nerve terminals were closely apposed to the muscle fibers with a gap of ~200 Å, and contained mitochondria and two kinds of synaptic vesicles; small vesicles of 300–500 Å diameter and large vesicles of ~1,000 Å diameter with an electron-opaque core (Fig. 17). As with vertebrate smooth muscle (3), the postjunctional membrane of the LBWM fibers did not show the specializations found in vertebrate skeletal muscle, such as the formation of junctional folds and the thickening of the postjunctional membrane.

DISCUSSION

Although molluscan muscles have been studied from time to time by many investigators, few physiological and ultrastructural studies have hitherto been made on the somatic muscles involved in the locomotion or body movement of the gastropods. The present work has revealed some characteristic features in the LBWM of D. aur-
FIGURE 8  Freeze-fractured replica of a nearly circular aggregate of gap junctional particles. Bar, 0.1 μm. × 94,000.

FIGURE 9  Freeze-fractured replica of aggregates of gap junctional particles with irregular shape. Bar, 0.1 μm. × 97,700.

FIGURE 10  Transverse section showing a desmosome (arrow). The interfiber space is occupied by a homogeneous material of moderate density. Dense, amorphous and fibrillar material is observed in the subjacent myoplasm. Bar, 0.1 μm. × 79,400.
FIGURE 11 Electron micrograph showing an intermediate junction with the interfiber space of ~100 Å. The apposed plasma membranes run parallel to one another over a considerable distance. Bar, 0.5 μm. × 45,000.

FIGURE 12 Transverse section showing an interfiber junction characteristic of the LBWM with the interfiber space of 400–800 Å. Note that the plasma membranes are remarkably wavy along the junctional region. The interfiber space is filled with an amorphous material of moderate density. Many caveolae are found at this junctional region. The interfiber space is as small as 100 Å where indicated by arrows. Bar, 0.5 μm. × 41,000.

FIGURE 13 Longitudinal section showing the same type of the interfiber junction as shown in Fig. 12. The interfiber space is 400–800 Å. Bar, 0.5 μm. × 37,000.
FIGURE 14 Electron micrograph of oblique section showing the caveolae (C), the surface tubules (ST) and the vesicular elements of the SR (SR). The SR is closely apposed to the surface tubules formed by tubular invaginations of the plasma membrane. The lumen of caveolae and surface tubules is filled with material of moderate density, and communicates with the extracellular space. The bridgelike structures (arrows) in the gap between the surface tubule and the SR. Bar, 1 μm. × 35,000.

FIGURE 15 Electron micrograph showing the branches of surface tubules and the bridgelike structures (arrows) in the gap between the surface tubules and the SR. Bar, 0.5 μm. × 49,400.
cularia with respect to both physiological and ultrastructural aspects.

The applicability of the sucrose-gap method in recording the electrical response of the LBWM fibers to ACh, high [K], and rapid cooling (Figs. 1 and 3) indicates that the fibers within the LBWM are electrically continuous, as is the case in many other types of smooth muscle. In accordance with this, ultrastructural observations have demonstrated that the LBWM fibers come into close contact with each other, forming several different kinds of interfiber junctions: the inter-
mediate junction at the fiber ends (Fig. 5), the gap junction (Figs. 6–9), the desmosome (Fig. 10), and two other types of junction that may also be regarded as two different kinds of intermediate junction (Figs. 11–13). Though the gap junction is generally believed to be the structural correlate to the electrical coupling between various types of smooth muscle fibers, serving as a low resistance pathway for current flow (5, 11), the possibility that the other types of interfiber junction also contribute to the electrical coupling between the LBWM fibers may not be excluded.

The results of the physiological experiments (Figs. 2 and 3) have shown that the LBWM fibers contain a large amount of intracellularly stored Ca, which can be released by a sufficient membrane depolarization or by rapid cooling, so that the contractile mechanism can still be fully activated by 400 mM K when the Ca influx is inhibited with Mn ions or low pH, or by rapid cooling even when the depolarization-Ca release coupling is severed by a prolonged soaking in Ca-free medium. In contrast with the LBWM, the K-contraction tension in Mytilus ABRM is markedly reduced by Mn ions or low pH, though the ABRM fibers are also shown to contain the intracellularly stored Ca in an amount enough to fully activate the contractile mechanism (25, 28). Furthermore, K-contractions of the ABRM disappeared within 10 min after the removal of [Ca], while K-contractions of the LBWM could be produced for as long as 2 h in Ca-free solution containing EGTA. The above comparison may be taken to indicate that membrane depolarization induced by high [K]o may effectively cause the release of stored Ca in the LBWM but not in the ABRM, and that the depolarization-Ca release coupling in the LBWM may be relatively insensitive to the removal of [Ca].

The LBWM fibers were found to contain abundant SR located mostly near the fiber surface and the surface tubules formed by the extensive invagination of the plasma membrane (9, 10). An analogous but less extensive plasma membrane invagination is observed in the radula protractor muscle of Busycyon canaliculatum (20) and also in the smooth muscle covering the visceral ganglion of Aplysia californica (18). The close apposition of the SR to the surface tubules, together with the presence of bridgelike structures analogous to those in the triadic junction of vertebrate skeletal muscle, strongly suggests that the surface tubules serve as a structure by which the membrane depolarization is transmitted inwards to cause the release of Ca from the SR. In this connection, the surface tubules characteristic of the LBWM may be regarded as a primitive type of transverse tubule in vertebrate skeletal muscle (17). The bridgelike structures have also been observed between the SR and the plasma membrane in Mytilus ABRM (1) and in vertebrate smooth muscles (4). In the LBWM, the presence of the bridgelike structures between the SR and the plasma membrane was not clear. According to Devine, Somlyo and Somlyo (4), there is a correlation between the relative volume of the SR and the degree of persistence of mechanical response in Ca-free solution in various vertebrate smooth muscles.

The main findings of the present study concerning excitation-contraction coupling in the LBWM may be that a sufficient membrane depolarization by high [K]o effectively causes the release of intracellularly stored Ca to fully activate the contractile mechanism under the conditions of reduced Ca influx, and that the LBWM fibers contain abundant SR and extensive surface tubules in close apposition to the SR. The actual localization of Ca in the SR and the other intracellular structures within the LBWM fibers, and the translocation of Ca during mechanical activity will be described in the following paper (26).

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