Developmental morphological changes in protrusions in *Artemia* intestinal circular muscles

Masaki Ueno and Takeshi Kimura

Abstract.—This study describes the electron microscopic features of protrusions on the intestinal circular muscle surface of *Artemia* nauplius larvae and adults. The distribution, thickness, and form of the protrusions changed with growth and those of the adults were thicker, forming a bundle of fine protrusions. The budding form of the protrusion from the circular muscle surface was observed, and junctions were detected both at the parts in contact among the protrusions and the connection between the protrusion and the circular muscle cell. These results suggest that the network of protrusions allows communication among circular muscle cells.

Introduction

The alimentary tract of *Artemia* is divided into three morphologically distinct parts: esophagus, midgut, and hindgut. The epithelium of the alimentary tract is separated from the underlying circular muscle layer by a basement membrane. Criel (2002) described the midgut as being mainly surrounded by a layer of circular muscle, whereas the esophagus and hindgut are enclosed by circular and longitudinal muscles, as well as a dilator muscle. Schrehardt (1987) previously reported that midgut innervation exclusively involves its posterior part and is stimulated by multipolar neurons. While chemical synapses occur through vesicles on the side of the nerve, a synapse-like structure has been reported in Crustacea at the neuromuscular junction (Govind & Derosa, 1983; Msghina et al., 1998; Johnstone et al., 2011). Tisdale & Nakajima (1976) reported that synaptic vesicles vary in size. The morphology of synapses in the cardiac ganglion (Mirolli et al., 1987), motor axon terminals (Atwood & Marin, 1983), and stretch receptor organs (Tisdale & Nakajima, 1976) have been described; however, details on the intermuscular synapse connection of the midgut muscle cells remains elusive. Although transmission electron microscopy (TEM) analysis of *Derocheilocaris typica* (Crustacea: Mystacocarida) by Elofsson & Hessler (2010) revealed that the presence of several protrusions on the circular muscle surface, ultrastructural details and the function of the protrusions in *Artemia* are currently unknown. Since these protrusions exist among the circular muscles rather than nerve cells, we hypothesized that these structures function in the transmission of signals among circular muscles. This motivated our fine structure analysis of the developmental change, joining, and attachment of protrusions and circular muscles in *Artemia* by using scanning electron microscopy (SEM) and TEM-based approaches.

Materials and Methods

Animals

Nauplii and adult *Artemia franciscana* specimens were used for this study. Diapause eggs, used as feed for tropical fish, were purchased and allowed to hatch in 2.0% salt solution at 28°C.

Fixation

The nauplii and adult *Artemia* were fixed in a solution containing 0.5% glutaraldehyde and 0.5% paraformaldehyde diluted with 0.1 M phosphate buffer (pH 7.3) for 4 h at room temperature. Thereafter, they were washed with phosphate buffer and postfixed...
in 2% osmium tetroxide for 2 h at room temperature. At the beginning of aldehyde fixation, the samples were irradiated by microwave for 4 min (output, 350W) (MI–77; Azumaya Co., Tokyo, Japan).

**SEM observation**

Samples were dehydrated in a graded ethanol series, followed by isoamylacetate for drying. The samples were dried in a critical–point dryer (CP–5; Topcon Co., Tokyo, Japan), then mounted on an aluminum base using carbon paste. The body wall was dissected using a micro–scalpel to expose the alimentary tract. The mounted samples were coated with a 20 nm thick osmium film using an osmium plasma coater (Neo osmium coater Neoc–ST; MeiwaFosis Co., Ltd. Osaka, Japan). Low–magnification micrographs were obtained using a conventional SEM (DS130S; Topcon Co., Tokyo, Japan) operating at a 10 kV accelerating voltage and the high–resolution SEM (S–5000H; Hitachi, Ltd, Tokyo, Japan) used at a 4 kV accelerating voltage for the high–magnification observations.

**TEM observation**

The dehydrated samples were embedded in epoxy resin and sectioned using an ultramicrotome. The ultra–thin sections were stained with uranyl acetate and lead citrate and observed by TEM (JEM–100SX; JEOL, Ltd, Tokyo, Japan).

**Results**

**Distribution and form of the protrusions of the intestinal circular muscle of nauplius**

The outermost layer of the alimentary tract consists of fine hoop–shaped circular muscles. The interval between each circular muscle is approximately 2 µm (Fig. 1a). The longitudinal muscle and nerve fiber, which are expected to run outside the circular muscles, were not observed in the majority of the midgut. A strong constriction was observed at the lower end of the midgut, and some longitudinal muscles were observed in this region. The circular muscles are arranged in parallel narrow intervals to the intestinal wall. Part of the circular muscle was swollen and was assumed to contain the nuclei of the muscle cells (Fig. 1b). The muscle cells were of constant width and arranged in parallel with no observed intersecting muscles. Several protrusions originating from the surface of the muscle were observed (Fig. 1c). Muscle fibers were approximately 1.5–µm thick and arranged at 2–µm intervals. The thickness of the protrusions ranged from 0.08 to 0.4 µm, and additional protrusions were observed from the lateral side of the circular muscle. The protrusions contained several branches, some of which were at the terminal ends. The thickness of the protrusions varied and both branched and terminal forms were observed (Fig. 1d). A connection among the protrusions and an invasive form to the absorptive–epithelium layer was also detected.

**Distribution and form of the protrusion in adult intestinal circular muscles**

The hoop–shaped circular muscle covered the outer surface of the intestine with no longitudinal muscles or nerve fibers observed (Fig. 1e). Muscular cells were arranged in parallel and no intersection of the structures was observed. The length of the intervals of muscle fiber varied slightly with certain fibers showing extremely narrow intervals. The muscle was approximately 3–µm thick and arranged at 2 to 5–µm intervals. Figure 1f shows the varying intervals of four circular muscles, with that of the lower two muscles being markedly narrower. The number of protrusions decreased significantly and the thicknesses varied extensively. The intersecting image and the ended form were not observed in the protrusion. The thickness of both the muscle and the protrusion increased in comparison to that observed in the nauplii. TEM analysis of the thick protrusion revealed the formation of a bundle of protrusions (Figs. 2a and 2b). Afterward, the protrusions clustered and thin connective tissue was observed to envelop the cluster outside the circular muscle. Because these were embedded in the basal lamina of the absorptive epithelium, they appeared as thick protrusions during SEM observation. A protrusion bundle was found...
Fig. 1. Scanning electron micrographs. a: Alimentary tract in a nauplius. The ventral body wall is removed. Hindgut is depicted on the right. b: Midgut in a nauplius. Arrows; swollen parts that might contain the nuclei of the muscle cells. c: Two circular muscles in a nauplius. Several protrusions originate from the side of each muscle. d: A protrusion in a nauplius. Arrowhead; a connection among the protrusions. e: Adult midgut, showing covered hoop–like circular muscles outside the midgut. Arrowhead; blood cell. f: Adult circular muscle. Arrows; protrusions.
protrusions.

**Budding, joining, and adhesion of a protrusion**

The structure of the budding part of
the muscle surface was revealed by TEM analysis (Fig. 2c). Growth of the protrusion occurred in an upward direction. No distinct, fine structure was detected around the budding structure, as well as within the developing protrusion. While structures such as vesicles and myofibrils were not observed, characteristic structures were observed in the area where the protrusion adhered to the neighbor muscle cell (Fig. 2d). Electron–dense cell membrane was observed in both the muscle cell and the protrusion. The gap measured approximately 0.02 µm. In the middle of the gap, a bead–like structure with high electron density was observed. Similar structures were observed at the junction between protrusions (Fig. 2e).

Discussion

Potential role of the protrusion in the adhesion of circular muscles to the absorptive–epithelial layer

In nauplius larvae, several images show the termini of the protrusion embedded within the basilar membrane of the absorptive–epithelium (Fig. 2f). The protrusions physically combine a circular muscle and the epithelium and shrinkage or relaxation of the circular muscle may facilitate the absorption of nutrients from the intestinal epithelium. Despite the presence of a small vesicle on the termini, no connection between the termini and epithelial cell was observed. Thus, it is impossible for the signals emitted by the circular muscles to be transmitted to the epithelial cell layer.

Potential role of the protrusions in securing an intervening space between the adjoining circular muscles

The circular muscles in the larva were thin and separated by narrow intervals. The protrusion network may prevent adjoining circular muscles from overlapping. This structure may also prevent the dissociation of the epithelium from the circular muscle and facilitate the efficient movement of the circular muscle to the epithelium. The circular muscle thickened in the adult, thereby increasing its contractive power. The protrusion eventually merges with another similar structure, thickens, and integrates into the sub–epithelial connective tissue. It is possible that the structure contributes to signal transmission in circular muscles and reinforces the adhesion of the epithelial layers and the circular muscles.

Potential role for the protrusions in signal transmission in circular muscles

No direct contacts among the adjoining circular muscles were detected. Several minute protrusions (nauplius larvae) or small bundles of protrusions (adult) were observed at the junctions of the muscles. The structures resembled those of neuromuscular junctions (Govind & Derosa, 1983), similar to the morphology of synaptic vesicles, sub–synaptic membrane, and synaptic cleft. The presence of synaptic vesicle–like structures at the junction of the protrusion and the circular muscle or the junction between the protrusions suggests that an exchange of information occurs in these sites. Figure 3 shows a schematic of the two junctions described above: protrusion–protrusion junction (A) and protrusion–muscle junction (B). The structure may be involved in signal exchange between the two adjoining circular muscles; however, the protrusion occurred directly from a muscle cell rather than a nerve cell.
While direct evidence remains to be determined, these observations suggest the participation of the structure in the transmission time-course.

Literature Cited

Addresses: (MU, TK)School of Allied Health Sciences, Department of Basic Medical Science, Kitasato University, 1–15–1 Minami–Ku Kitasato, Sagamihara, Kanagawa 252–0373, Japan.

E-mails: (MU) matt@ahs.kitasato-u.ac.jp; (TK) kimurata@ahs.kitasato-u.ac.jp

Received: 5 November 2013.
Accepted: 17 September 2014.