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Specializations for Excitation-Contraction Coupling in the Podial Retractor Cells of the Starfish *Stylasterias forreri* *

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Summary. Ultrastructural examination of the podium of the asteroid echinoderm *Stylasterias forreri* has revealed that cells of the coelomic epithelium and cells of the retractor muscle should be considered as components of a single epithelium. The podial retractor cells are, therefore, myoepithelial in nature. This report concentrates on those ultrastructural features of the retractor cells that are most likely involved with excitation-contraction coupling. The spatial arrangement of the sarcoplasmic reticulum, the couplings between the sarcoplasmic reticulum and sarcolemma, and an intramembranous specialization of the sarcolemma are documented and discussed. Current concepts regarding the innervation of the retractor cells of the podium and the protractor cells of the ampulla are reviewed, and specific proposals for further investigation of podial innervation are outlined.

Key words: Excitation-contraction coupling – Podium – Retractor cells – Starfish – Ultrastructure

A companion paper has documented that the coelomic lining in the podium of the starfish *Stylasterias forreri* is a bipartite epithelium which encompasses cells of the coelomic epithelium and retractor muscle. The *retractor cells* are highly differentiated, longitudinally oriented cells occurring in deep regions of the coelomic lining (Wood and Cavey 1981). Since retractor cells and the basal processes of *adluminal cells* anchor to a common basal lamina, the coelomic lining of the podium qualifies as a pseudostratified epithelium. Podial retractor cells, formerly judged to be smooth muscle cells, are reclassified as myoepithelial cells.

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The considerable interest in the control of podial contractility owes to the complex motility of these appendages and to the inability to detect neuromuscular junctions between axons and retractor cells (Florey and Cahill 1977). Our observations on the podia of *S. forreri* confirm the relative isolation of the epidermal nerve plexus from the retractor cells of the coelomic lining, and they further indicate that retractor cells may lack conventional communicating (gap) junctions (Wood and Cavey 1981).

In this paper, we describe the ultrastructure of the sarcoplasmic reticulum in the podial retractor cell and its relationship to the sarcolemma. Structural peculiarities of the retractor cells suggest a primitive mechanism for excitation-contraction coupling which obviates the requirement for intercellular communicating junctions. Our morphological findings are discussed from the viewpoint of physiological implications and related to current interpretations of podial innervation.

Portions of this material have appeared in abstract (Cavey and Wood 1979; Wood and Cavey 1980).

Materials and Methods

Specimens. Adult specimens of *Stylasterias forreri* de Loriol, 1887, were obtained by dredging in the San Juan Archipelago, Washington, U.S.A. Animals were maintained in aquaria supplied with running sea water.

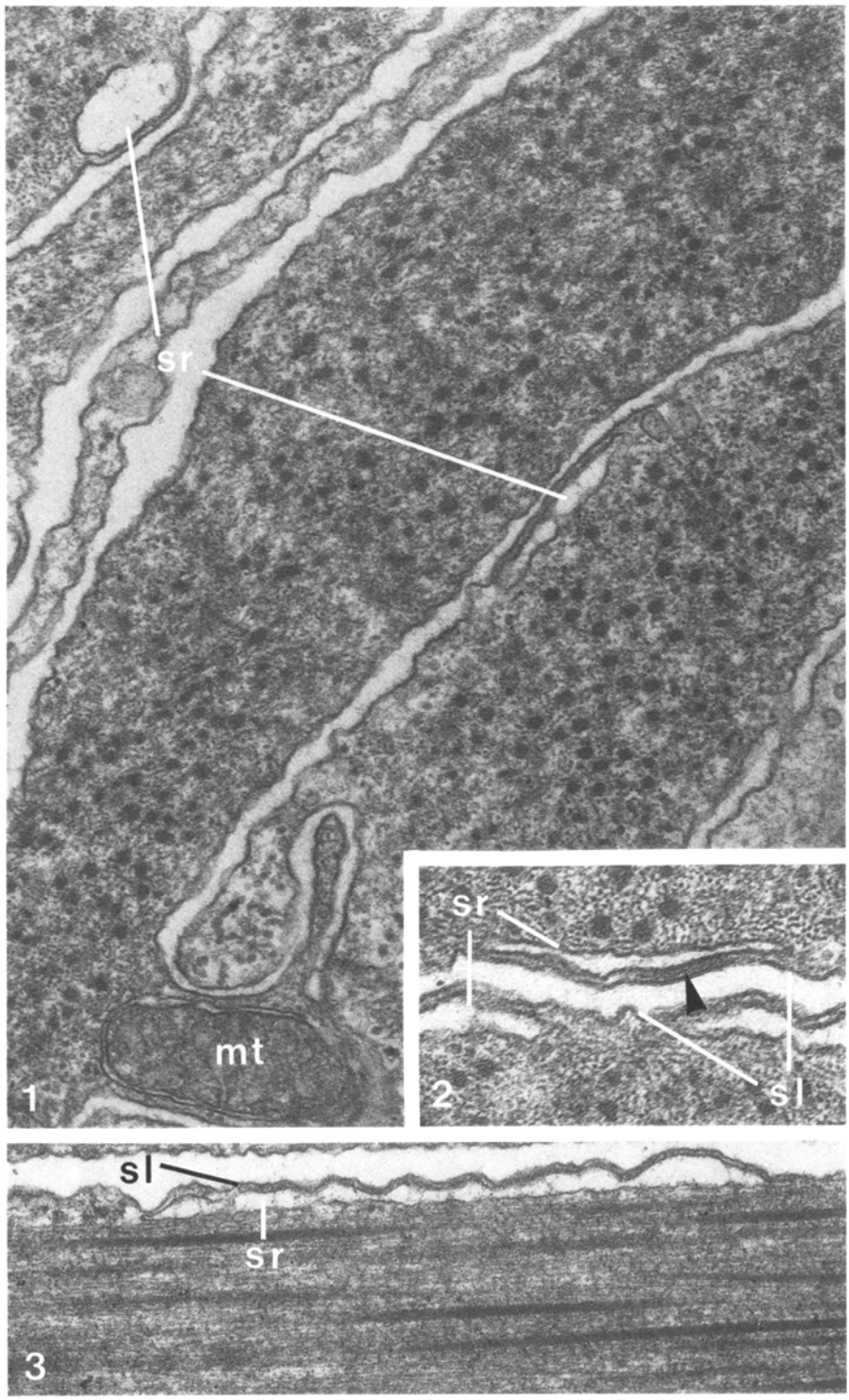
Protracted podia were immobilized by ligature, severed, and placed in phosphate-buffered glutaraldehyde (Cloney and Florey 1968). After 3–5 min of exposure to the fixative, the podial shafts (stems) were cut into short cylindrical segments and removed to fresh fixative. Podial segments for microtomy remained in the aldehyde-containing fixative for 1–2 h. Segments for freeze-fracture replication remained in the fixative for 30 min.

Sectioned Tissues. Aldehyde-fixed podial segments were secondarily treated with bicarbonate-buffered osmium tetroxide for 45–60 min (Wood and Luft 1965). The segments were then dehydrated and embedded in Epon (Luft 1961). Light microscopic sections (1 μm in thickness) were used for orientational purposes. These sections were cut with glass knives on a Sorvall MT-2B ultramicrotome and stained with azure II-methylene blue (Richardson et al. 1960). Electron microscopic sections (40–70 nm in thickness) of suitably oriented cells were cut with diamond knives, collected on naked or carboncoated grids, and stained with uranyl acetate and lead citrate (Reynolds 1963).

Fig. 1. Transverse section of retractor cells in the coelomic lining of the podium. The contractile apparatus of thick and thin myofilaments occupies the medullary sarcoplasm along the main cellular axis. Cisternae of the sarcoplasmic reticulum (*sr*), mitochondria (*mt*), and occasional glycogen granules lie peripheral to the contractile apparatus. × 60,600

Fig. 2. Transverse section through the cortices of apposed retractor cells. Agranular cisternae of sarcoplasmic reticulum (*sr*) closely approximate the sarcolemmata (*sl*). Note the parallel configuration of cisternal and cellular membranes in the upper cell and the occurrence of an amorphous plaque (*arrowhead*) in the intervening sarcoplasmic gap. × 80,800

Fig. 3. Longitudinal section of the cortical and subcortical regions of a retractor cell. A cisterna of sarcoplasmic reticulum (*sr*) appears between the sarcolemma (*sl*) and the thick and thin myofilaments of the contractile apparatus. Observe the conformation of sarcolemmal inflections and cisternal contours. × 44,400


sr
mt
1
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sl
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Replicated Tissues. Aldehyde-fixed podial segments were rinsed with 0.05 M Millonig's phosphate buffer (pH 7.4) for 30 min and refrigerated in fresh buffer until needed. Prior to freeze-fracture replication, the podial segments were sliced into small sectors. Over a period of 1–2 h, the sectors of the podial wall were cryoprotected by transfers through 10%, 20%, and 30% solutions of glycerol in 0.05 M sodium cacodylate buffer (pH 7.4). Glycerinated sectors were oriented on gold-alloy disks and plunged into liquid Freon 22 held near its freezing point with liquid nitrogen. Frozen tissues were fractured and replicated in a Balzers BAF 301 freeze-etch plant. On retrieval, the replicas were cleaned with absolute methanol, commercial bleach, and 50% sulfuric acid. The replicas were then rinsed in distilled water and transferred to parlodion-coated grids (Wood and Kuda 1980).

Micrography. Sections and replicas were photographed with JEOL JEM-100S and JEM-100C transmission electron microscopes operated at 60 or 80 kV. The microscopes were calibrated with a replica of a grating of 21,600 lines/cm.

Observations

The contractile apparatus of a retractor cell from the coelomic lining of the starfish *Stylasterias forreri* is a longitudinal array of unregistered thick and thin myofilaments (Wood and Cavey 1981). The myofilaments occupy the medullary sarcoplasm along the main cellular axis, and they frequently occur in the longitudinal keels and axial branches of a cell. In the keels and branches, the contractile apparatus may consist of either thick and thin myofilaments or thin myofilaments only. Thin myofilaments of the retractor cell associate with the subsarcolemmal densities of symmetric, asymmetric, and anchoring intermediate junctions.

Along the main axis of the retractor cell, the sarcoplasmic reticulum (SR) is a network of flattened, agranular cisternae lying peripheral to the contractile apparatus (Figs. 1, 3). Cisternae of SR make simultaneous contacts with the sarcolemma and the contractile apparatus. Mitochondria and glycogen granules are interspersed with the cisternae of SR. There is a tendency for the mitochondria to orient parallel to the myofilaments of the contractile apparatus.

Cisternal profiles are generally more expansive in longitudinal sections (Fig. 3) than in transverse sections (Fig. 1). It is logical to conclude, therefore, that the sarcoplasmic reticulum is a plexiform system of cisternae and that the predominant orientation of cisternae is longitudinal. Cisternal configuration and orientation are dramatically confirmed in freeze-fracture replicas of the retractor cells (Fig. 4). Although the plane of fracture cleaves the sarcolemma, the irregular contours of the subjacent cisternae are readily visible. The accuracy of this interpretation is confirmed by careful analysis of thin sections (Figs. 1, 3). In sectioned cells, the cisternae of SR seem to induce inflections of the overlying sarcolemma. Sarcolemmal inflections would account for the profiles observed in the freeze-fracture replicas. In replicas where the plane of fracture deviates from the sarcolemma and crosses the sarcoplasm (Fig. 5), the sarcolemmal inflections can be directly correlated with the presence of subsarcolemmal cisternae of SR.

Strict localization of the sarcoplasmic reticulum in the cellular cortex enhances an intimate relationship between the cisternae and the sarcolemma. A narrow zone of sarcoplasm, usually devoid of ultrastructural detail, separates the cisternal membranes from the inner surface of the sarcolemma (Fig. 2). Occasionally, the apposed membranes are rigidly parallel, producing a uniform gap which measures

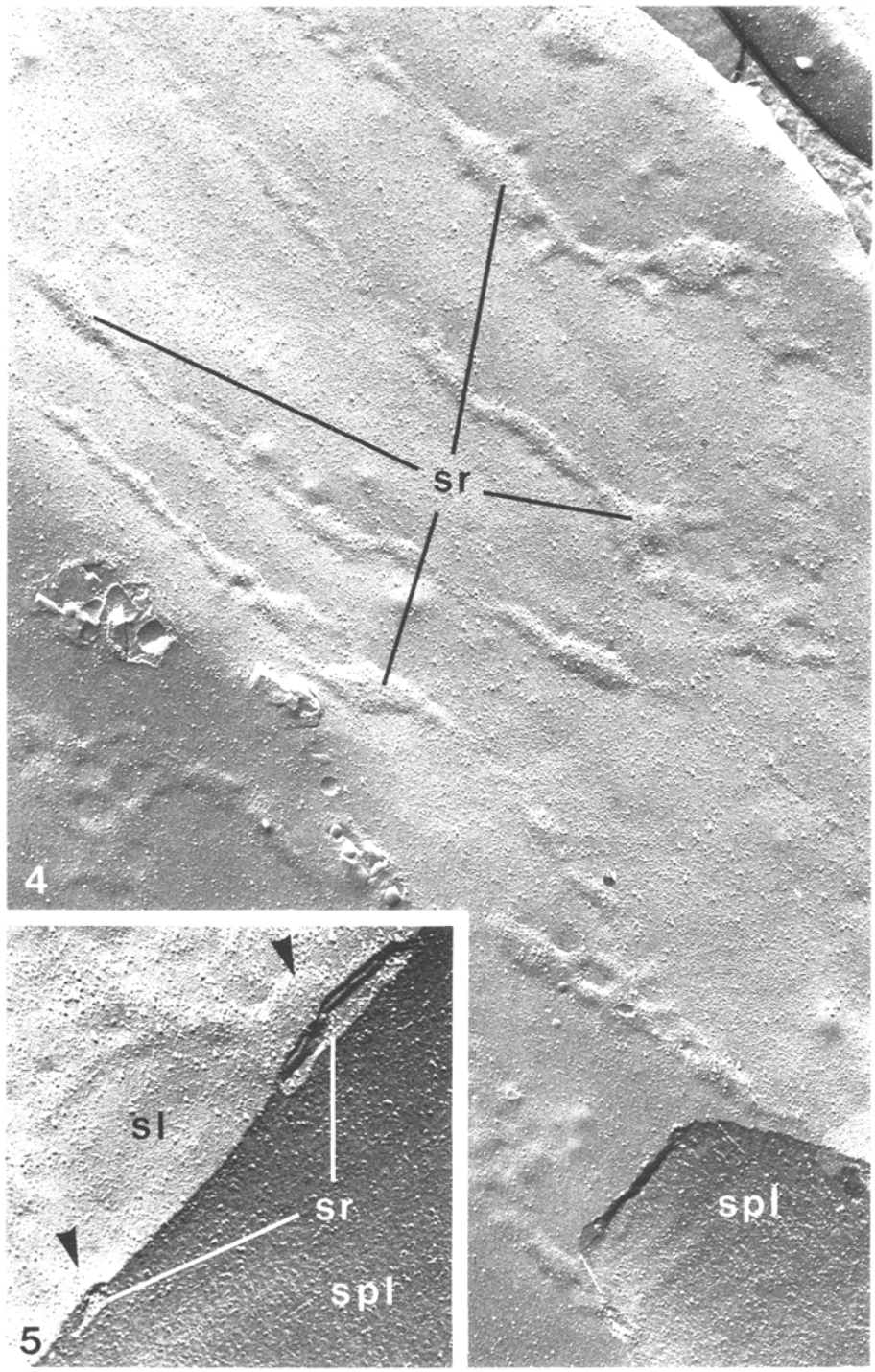

Fig. 4. Freeze-fracture replica of a retractor cell revealing the sarcolemmal P face. Cisternae of the sarcoplasmic reticulum (*sr*) which underlie the sarcolemma are obvious. The plane of fracture enters the sarcoplasm (*spl*) in the lower right corner of the micrograph. × 39,000

Fig. 5. Freeze-fracture replica of a retractor cell illustrating the sarcolemmal P face and underlying sarcoplasm (*spl*). Cisternae of sarcoplasmic reticulum (*sr*) are situated in the cortical sarcoplasm immediately beneath the sarcolemma (*sl*). Sarcolemmal inflections (*arrowheads*) are induced by the subsarcolemmal cisternae of sarcoplasmic reticulum. × 45,400

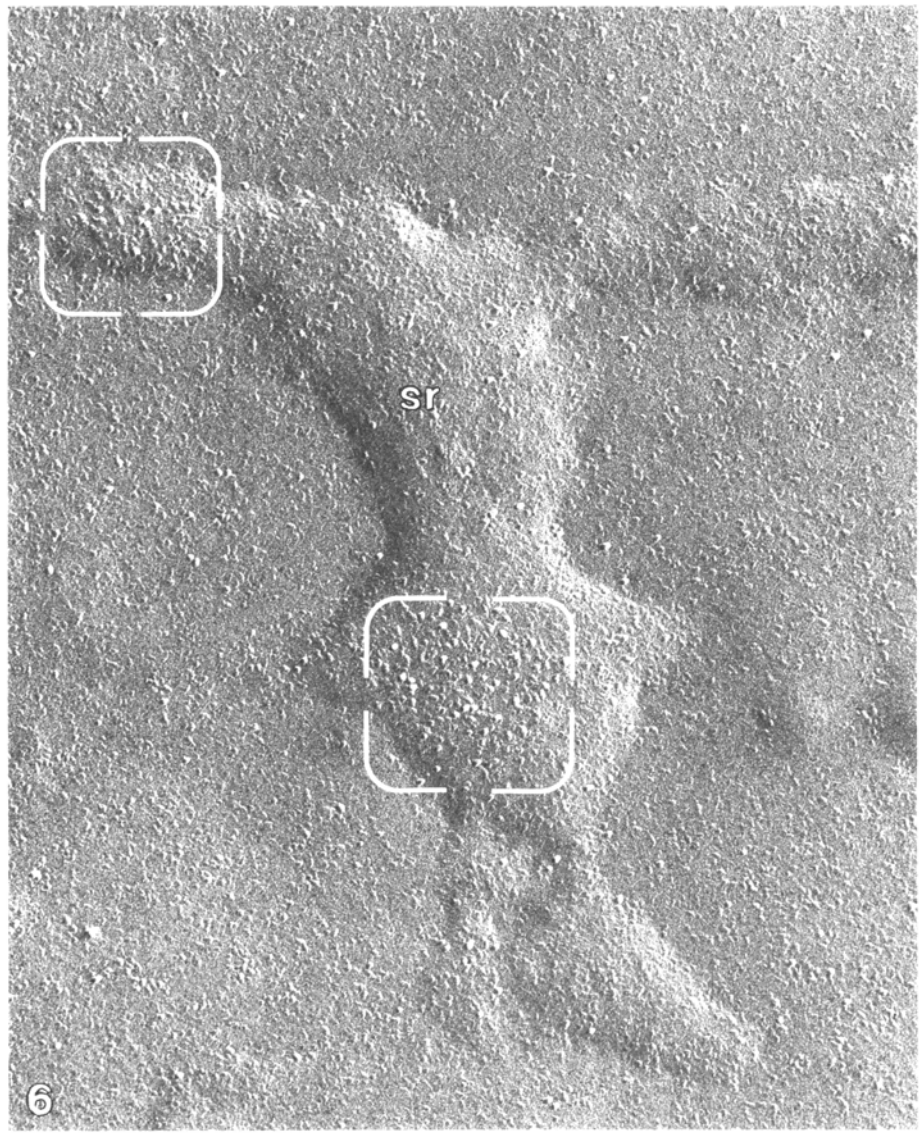

Fig. 6. Freeze-fracture replica of a retractor cell showing intramembranous particles on the sarcolemmal P face. Note the clusters of particles (*circumscriptions*) and their preferential positions over cisternae of the sarcoplasmic reticulum (*sr*). Preferential disposition of the clustered particles is also evident in Fig. 4. × 74,000

8.5 nm in width. In these associations, an amorphous plaque, situated equidistant from the apposed membranes, occupies the sarcoplasmic gap. Similar plaques typify the dyadic peripheral couplings of myoepithelial and muscle cells in many other invertebrates. In the retractor cell of the starfish podium, we assume that the plaque also indicates the site of a functional contact between the sarcoplasmic reticulum and sarcolemma.

Neuromuscular junctions were never observed in or near the coelomic lining of the podial shaft (stem) in any of our preparations. The closest axons to the coelomic lining belong to the nerve plexus of the epidermis, but these axons are separated from the retractor cells by a formidable layer of collagenous connective tissue (Wood and Cavey 1981). We have never observed axons to breach the basal lamina of the epidermis and enter the connective tissue. Since our study was confined to the podial shaft, we obviously cannot rule out the possibility of direct innervation of retractor cells at other levels.

Freeze-fracture replicas of retractor cells were inspected for intramembranous specializations which might reflect the positions of intermediate junctions, but none were found (Wood and Cavey 1981). Similarly, a search of extensive areas of replicated sarcolemma failed to reveal aggregations of intramembranous particles indicative of conventional communicating (gap) junctions. Clusters of intramembranous particles were observed, however, on the protoplasmic (P) face of the sarcolemma (Fig. 6). These clusters generally localized over distended cisternae of sarcoplasmic reticulum, and we postulate that they might relate to sarcolemmal regions coupled to the SR. Based on distribution and frequency, the clustered particles correlate well with the putative cortical couplings already described (Fig. 2). Our search for intramembranous specializations of the sarcoplasmic reticulum, which might reflect cisternal regions coupled to the sarcolemma, has thus far been unsuccessful.

Discussion

The sarcoplasmic reticulum (SR) of the podial retractor cell of *Stylasterias forreri* is modestly developed and highly localized. The plexiform network of cisternae, sandwiched between the sarcolemma and contractile apparatus, is structurally quite simple. Restriction of cisternae to the margin of the retractor cell facilitates intimacy of the SR and cellular membrane. Despite the closeness of all cisternae of SR to the sarcolemma, only certain cisternal regions appear to participate in the formation of couplings. In the podial retractor cell, the putative cortical coupling can be morphologically defined by three parameters: 1) the parallel configuration of the apposed membranes; 2) the uniform sarcoplasmic gap between the apposed membranes; and 3) the amorphous plaque situated equidistant from the apposed membranes within the sarcoplasmic gap. The organization of the sarcoplasmic reticulum in the retractor cell of *S. forreri* seems more elaborate than that described in the podial retractor cells and the ampullar protractor cells of other asteroids (Bargmann and Behrens 1963; Dolder 1972).

In our study of the coelomic lining in the podium of *S. forreri,* freeze-fracture replication has provided three important advances toward better appreciation of the mechanisms of excitation-contraction coupling in this tissue. First, it permitted better visualization of the distribution of SR in the retractor cell than was possible with sections and showed a more extensive cisternal system than was predicted from sections alone. Second, it was instrumental in our conclusion that all cells of the coelomic lining lack conventional communicating (gap) junctions (Wood and Cavey 1981). And third, it led to the discovery of clustered particles within the sarcolemma of the retractor cell. We speculate that these particles may relate to the

sarcolemmal events in excitation-contraction coupling, as they are restricted to segments of the membrane which overlie the sarcoplasmic reticulum. Since clusters of intramembranous particles tend to localize over sites of cisternal confluency, it is our impression that sarcolemmal excitation may modulate contraction by topical effects on the sarcoplasmic reticulum.

In the coelomic lining of the podium, it is possible that excitation-contraction coupling is accomplished in the absence of communicating junctions. This could mean that retractor cells are individually innervated, are excited by diffusable neurotransmitters, or are stimulated to contract by non-neural agents. An understanding of cellular innervation is central to analysis of excitation-contraction coupling within the coelomic lining, but the paucity of information on echinoderm neuroanatomy and neurophysiology is a serious impediment toward realization of this goal (Pentreath and Cobb 1972). To illustrate some of the analytical perplexities, a brief historical review on the echinoderm podium and its ampulla is in order. The ampulla and the lining of the water vascular canal are synergetic components which control protraction and retraction of the podium. Contraction of the ampullar protractor cells forces coelomic fluid into the water vascular canal of the podium, resulting in hydraulic extension of the appendage. Contraction of the podial retractor cells returns fluid to the ampulla and concurrently shortens the appendage.

An elaborate histological study by Smith (1950) was an early effort to study innervation of the podial and ampullar linings. After vital staining with methylene blue, he observed broad "ribbon axons" emanating from a branch of the radial nerve and infiltrating among the retractor cells of the podium and the protractor cells of the ampulla. Based on these light microscopic findings, it seemed that contractile cells of the podium and ampulla were richly innervated.

Early ultrastructural studies on the podial lining (Dolder 1972; Florey and Cahill 1977; Kawaguti 1964) and the ampullar lining (Bargmann and Behrens 1963; Cobb 1967; Kawaguti 1965) failed to find neuromuscular junctions or, for that matter, any evidence of "ribbon axons" among the retractor and protractor cells. The concept of "ribbon axons", formulated on the basis of vital staining (Smith 1950), had to be reassessed, since the identification of axons with methylene blue was obviously unreliable.

With the failure to corroborate the existence of "ribbon axons", the search began anew for mechanisms of podial and ampullar innervation. Cobb (1967), by sampling tissues from a variety of locations in and near the ampulla, satisfactorily explained the innervation of the protractor cells. He determined that axons do not project to the protractor cells of the ampulla but, instead, the protractor cells send processes to the nerve supply which is situated near the base of the podium. The extensions of the protractor cells mingle with the axons but do not participate in neuromuscular junctions with them. The absence of distinctive neuromuscular junctions resembles autonomic innervation of vertebrate smooth muscle (Pentreath and Cobb 1972).

It is tempting to analogize the protractor cells of the ampulla with the Purkinje fibers of the vertebrate heart. For both organs, innervation appears to involve specialized contractile cells. In the heart, however, the Purkinje fibers are thought to depolarize ordinary myocardial cells through communicating junctions. In-

tercellular spread of excitation in the myocardium can also be attributed to communicating junctions. In the echinoderm ampulla, it is not known whether each protractor cell sends a process to the podial base to receive innervation, but this possibility seems unlikely. A mechanism is needed, therefore, to explain how innervated cells elicit the contractions of their neighbors. Secondary excitation of the protractor cells must be accomplished without the participation of typical communicating junctions since these junctions are apparently lacking in the ampullar lining (Bargmann and Behrens 1963).

A unified proposal covering innervation of the retractor cells in the coelomic lining of the podium is not available. As mentioned by others and confirmed by us for *S. forreri*, there are no neuromuscular junctions or axons within the coelomic lining. The nearest axons occur in the proximal and distal nerve rings of the podium (Coleman 1969a, 1969b; Engster and Brown 1972; Nichols 1959a, 1959b, 1961) and in the epidermal nerve plexus (Cobb and Raymond 1979; Florey and Cahill 1977; Kawaguti 1964; Weber and Grosmann 1977).

From an ultrastructural examination of the echinoid podium, Kawaguti (1964) hypothesized that excitation of the retractor cells could be a non-neural phenomenon. He inferred that adluminal cells of the coelomic lining might fulfill the motor functions normally assigned to axons. Justification for such a proposal rests largely on the absence of axons among the retractor cells, the isolation of the coelomic lining from the epidermal nerve plexus, and the close contacts between retractor cells and the basal processes of adluminal cells. Although adluminal cells might serve as sensory receptors (Nørrevang and Wingstrand 1970), it is difficult to perceive how they could control the intricate movements of the podium or how they alone could coordinate the activities of the podium and ampulla.

Coleman (1969b) proposed that axons from the epidermal plexus and neurosecretory cells were present in the connective tissue layer of the podium, but subsequent studies have not confirmed this view. Profiles in the connective tissue deemed to be axons or neurosecretory cells are probably the processess of migratory coelomocytes (Florey and Cahill 1977). *Granulocytes* in the coelomic lining of *S. forreri* closely resemble the coelomocytes of the podial connective tissue (Wood and Cavey 1981). While the nature of the granulocytes is uncertain, it is clearly evident that these cells do not have the morphology that is appropriate to either neurons or neurosecretory cells. From all available studies on echinoids and asteroids, it is now generally accepted that the connective tissue in the podial shaft (stem) is aneural.

Florey and his associates have closely scrutinized the echinoid podium both ultrastructurally (Florey and Cahill 1977) and pharmacologically (Florey et al. 1975). They have looked to the epidermal nerve plexus for clues concerning the innervation of retractor cells in the coelomic lining. Since the epidermal nerve plexus contains a high concentration of acetylcholine and the retractor cells are cholinoreceptive, it is proposed that retractor cells are subject to indirect excitation. According to this view, axons of the nerve plexus terminate on the basal lamina of the epidermis. Upon release from these nerve terminals, the neurotransmitter diffuses across the connective tissue layer of the podium and acts upon the membranes of the retractor cells in the coelomic lining. During diffusion, the neurotransmitter may also plasticize the connective tissue to lower its resistance to

deformation. Plasticization is a facet of the proposal that digresses from the classical view of the podial connective tissue as a passive elastic counterbody to retraction (Kawaguti 1964).

Indirect excitation of the podial retractor cells, as outlined by Florey and Cahill (1977), involves the epidermal nerve plexus which is a part of the ectoneural division of the echinoderm nervous system. The ectoneural division is chiefly sensory (Pentreath and Cobb 1972; Smith 1965), so the proposal of indirect excitation of the retractor cells is contrary to existing doctrine. Another reservation about the proposal of indirect excitation is purely subjective: it is intuitively difficult to reconcile direct innervation of the ampullar protractor cells (Cobb 1967) with indirect innervation of the podial retractor cells. Since the actions of the ampulla and podium are synergetic and the responses of the latter are more intricate than those of the former, it may be too soon to dismiss the possibility of direct innervation of the retractor cells.

The search for axons that might directly innervate the retractor cells would logically begin at the base of the podium where branches of the radial nerve have ready access to the coelomic lining. The podial base is the site where protractor cells of the ampulla receive innervation (Cobb 1967) and the level where retractor cells of the podium appear the most cholinoreceptive (Babskaya 1977). A mechanism for direct innervation of the retractor cells would be attractive for several reasons: 1) it would normalize the patterns of podial and ampullar innervation; 2) it would approximate the centers controlling the synergetic responses of the podium and ampulla; and 3) it would localize the centers of podial and ampullar innervation to the vicinity of the hyponeural (motor) division of the nervous system. Control of podial motility by directly innervated protractor and retractor cells does not discount the possibility of plasticization of the connective tissue by axons of the epidermal plexus (Florey and Cahill 1977). Plasticization could be an intriguing functional corollary regardless of the pattern of cellular innervation.

For an indirect or direct pattern of innervation, there must be an arrangement between retractor cells to mediate the spread of excitation. Electrical spread of excitation by conventional communicating junctions cannot be invoked for either the protractor cells of the ampulla (Bargmann and Behrens 1963) or the retractor cells of the podium (Wood and Cavey 1981). Mechanical spread of cellular excitation, originally proposed by Bargmann and Behrens (1963), seems a viable alternative for further consideration. Cellular contractions could physically deform the membranes of adjoining cells and trigger sarcolemmal depolarizations. Sarcolemmal depolarization by physical deformation occurs in some vertebrate smooth muscle cells, and it is already known that certain echinoderm muscles are extremely sensitive to stretch (Prosser et al. 1965). The prolific intermediate junctions between protractor and retractor cells are obvious candidates to mediate the intercellular spread of excitation.

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