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Organization of the cortical endoplasmic reticulum in the squid giant axon

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Summary

The organization of the cortical endoplasmic reticulum in the squid giant axon was investigated by rapid freeze and freeze-substitution electron microscopy, thereby eliminating the effects of fixatives on this potentially labile structure. Juvenile squid, which have thinner Schwann sheaths, were used in order to achieve freezing deep enough to include the entire axonal cortex. The smooth endoplasmic reticulum is composed of subaxolemmal and deeper cisternae, tubules, tethers and vesicles. The subaxolemmal cisternae make junctional contacts with the axolemma which are characterized by filamentous-granular bridging structures approximately 3 nm in diameter. The subaxolemmal junctions with the axolemma resemble the coupling junctions between the sarcoplasmic reticulum and the T-tubules in muscle. Reconstruction of short series of sections showed that a number of the elements of the endoplasmic reticulum were continuous but numerous separate vesicles were present as well. The morphology of endoplasmic reticulum as described here suggests that it is a highly dynamic entity as well as a Ca^{2+} sequestering organelle.

Introduction

The cortical endoplasmic reticulum (ER) in the squid giant axon appears to be a Ca^{2+} sequestering organelle and therefore might have a central role in buffering local Ca^{2+} concentrations (Henkart *et al.*, 1978). Since the action potential as well as the transmission of information from the axonal surface into the axoplasm are Ca^{2+} dependent (Bootman & Berridge, 1995), the cortical ER presumably has an important role in these events. However, it remains unclear whether the cortical ER consists of a single branching membrane system or several separate membrane systems. Furthermore, the important question as to whether any of the cortical membrane systems make coupling junctions with the axolemma is still open. The ER of the muscle, the sarcoplasmic reticulum, contacts the transverse (T) invaginations of the sarcolemma at the junctional feet (Block *et al.*, 1988). The possible existence of analogous structures and mechanisms in the neuron is an important question in the understanding of neuronal organization. The present paper presents evidence supporting the existence of junctional feet between the ER and the axolemma in squid giant nerve fibre.

A serial reconstruction of the ER in chemically fixed axons from another mollusc, the octopus, has suggested that peripheral elements of the ER contact the axolemma and are continuous with the tubular network of ER (Ducros, 1974), but no junctional structures were reported. Hodge and Adelman (1980) have observed intricate relationships between the ER and the axolemma in squid giant axon fixed by internal irrigation with the fixative. However, neither these authors nor the studies of Tsukita and colleagues (1986) reported junctions similar to those described in the present report. Ca^{2+} has been localized in dense bodies associated with the axolemma and with fine filamentous elements in squid giant axon (Hillman & Llinás, 1974; Oschman *et al.*, 1974; Henkart *et al.*, 1978; Metuzals *et al.*, 1981). However, the structure of the cortical ER and its relationship to the axolemma are likely to be altered during chemical fixation, for instance by the influx of Ca^{2+} when the fixative arrives at the surface of the axon (Smith & Reese, 1980), so the exact structure of the cortical ER remains in doubt. This conclusion is supported by comparing the results

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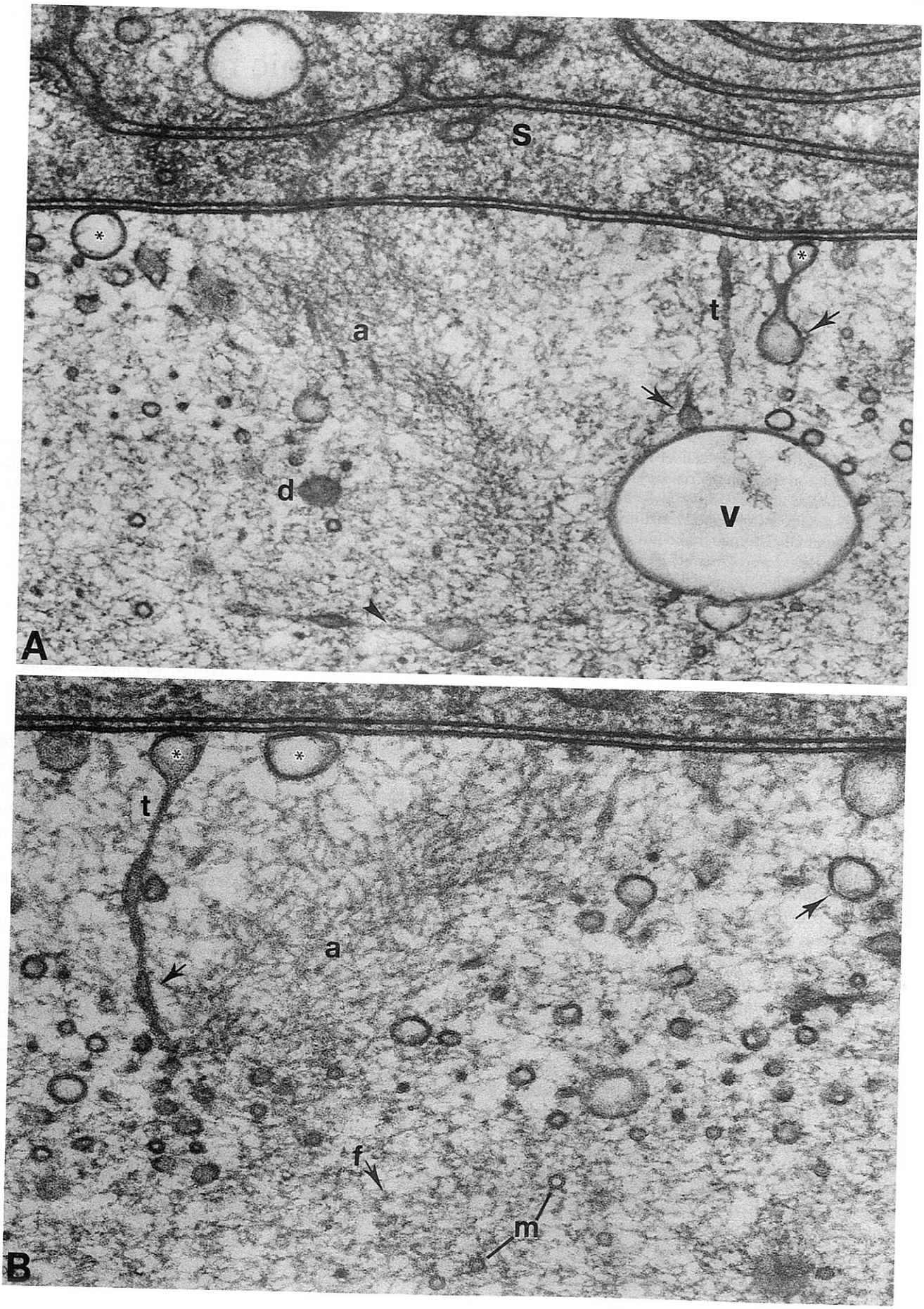
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presented in the following with the results obtained using chemical fixation.

The effects of chemical fixatives on labile or Ca^{2+} sensitive structures can be avoided if tissues are prepared for thin section electron microscopy by rapid freezing and freeze substitution, physically arresting them on a millisecond time scale (Heuser *et al.*, 1979). However, it is not possible to achieve sufficiently rapid freezing in fully adult squid because the Schwann cell layer surrounding the giant axon is relatively thick. Fortunately, the Schwann sheath is much thinner in juvenile squid permitting more rapid freezing of the axon. The present approach provided the first views of the internal membranes near the axolemma in freeze-substituted axoplasm. It was necessary systematically to classify these membranes, requiring a large number of micrographs. The relationships between the different membrane classes could then be reconstructed from a relatively small number of short series of sections. Thus, this study identifies and characterizes a system of cortical ER forming junctions with the axolemma, that is likely to be a major element in coupling the action potential to events inside the axon.

Materials and methods

Giant axons and associated smaller axons and blood vessels were removed from the mantle of juvenile specimens of the squid, *Loligo pealei* (Marine Biological Laboratory, Woods Hole, MA) in running sea water. Axons were pinned out so that the outer layers of sheath around them could be meticulously removed along approximately 1 cm of the axon. Any axons showing evidence of damage (white spots) were discarded. Even with the smaller axons, 200–300 μm in diameter rather than 400–600 μm, a very thorough final 15 min dissection was needed to bring the axolemma close enough to the surface of the tissue to achieve useful freezing of the axon. The cleaned segment of dissected axon was then draped over a piece of gelatin on an aluminum disk that attached to the head of the freezing apparatus, and immediately slam-frozen against a copper block cooled with liquid helium (Heuser *et al.*, 1979). The time elapsed between transection of the end of the cleaned fibre and the slam-freezing was approximately 15 s. Specimens were transferred under liquid nitrogen to the surface of 5 ml of a frozen solution of 4% osmium tetroxide in reagent grade acetone in a plastic 20 ml scintillation vial, and warmed up to room temperature over 16 hours on a gently rocking table. The freeze-substituted specimens were then washed in acetone, and stained in acetone saturated with uranyl acetate for 2–6 h at room temperature. Specimens were embedded in Araldite, sectioned transversely at approximately 70 nm and photographed in a Philips 400 electron microscope. Over 4000 micrographs were made singly, or in short series of 5–8 sections, selecting areas where displacements by ice crystals did not impinge on the structures being examined. The illustrations shown here are from diverse areas of one axon in which the freezing was particularly favourable. From short series of micrographs the axolemma, ER, vesicles, tethers and vacuoles were traced on a transparent paper placed on the micrograph. For reconstruction the drawings were superimposed and a composite drawing (Fig. 5) was made from consecutive traces.

Results

Despite the removal of the connective tissue sheath with associated blood vessels and small axons from long stretches of the giant fibres, there was no apparent damage to the giant axons and their investing Schwann cells. The covering of the giant axons was reduced in such stretches to a thin layer of interdigitating Schwann cells and the basement membrane. There was no extensive vacuolation of Schwann cell cytoplasm and all the membrane systems in such cells appeared smooth and continuous (Fig. 1A). These stretches showed little or no evidence of ice crystals at the magnification needed to study the distribution of ER and the cytoskeleton elements in the cortical axoplasm.

The squid giant axon is differentiated into a peripheral dense and ordered cortical zone and a central, less dense core endoplasm (Chambers, 1947; Chambers & Kao, 1952; Baker *et al.*, 1962; Metuzals & Izzard, 1969; Metuzals & Tasaki, 1978). The cortical zone was also called ectoplasm (Metuzals & Tasaki, 1978) or the subaxolemmal zone (Hodge & Adelman, 1980; Tsukita *et al.*, 1986). The cortical axoplasm, recognized by its content of organelles and distinct cytoskeletal elements, extends approximately 4–5 μm into the axoplasm. The cortical axoplasm is easily differentiated from the flattened Schwann cell processes enveloping the axon. A variety of cisternae, vesicles, membrane tubules and vacuoles, as well as distinct cytoskeletal elements, are evident in the cortical axoplasm. The junctions between the subaxolemmal cisternae of the ER and the axolemma, the pleomorphic organization of the ER and the architecture of the cytoskeleton are described in more detail below.

Subaxolemmal cisternae and their axolemmal junctions

Figure 1A and B illustrates the architecture of the cortical zone of the squid giant axon and the narrow

Fig. 1. (A, B) Architecture of the cortical zone of the squid giant axon surrounded by Schwann cell cytoplasm (S), showing subaxolemmal cisternae (asterisks), deeper cisternae (arrows), thin tubules or tethers (t), large vacuole (V), strands of actin (a), neurofilamentous network (f), microtubules (m), round dense profiles (d), tether arising from a spindle-shaped cisterna (arrowhead). A × 85 000; B × 140 000.

layer of the Schwann cells typically apposing the axon. The axolemma is separated from the Schwann cell by the extracellular space. Various components of the smooth endoplasmic reticulum (ER) can be identified: subaxolemmal cisternae (asterisks), deeper cisternae (arrows), membrane tubules, thin tubules or tethers (t) and vesicles. Figure 1B, on the left, shows an example of the continuity of the subaxolemmal cisterna with underlying tubule and vesicles to form a sinuous structure. The undulating and varicose architecture of this structure suggests that it is undergoing continuous dynamic shape changes. Other cisternae of varying size and shape are distributed throughout the whole cortical axoplasm. They have different densities, which might depend on their content, functional state, or the plane of the section.

The subaxolemmal cisternae make junctional contacts with the axolemma. Due to the thickness of the section and the low resolution of the micrograph, these junctions appear in Fig. 1A and B as dense, amorphous material. At better resolution and higher magnification (Fig. 2A–F), it is evident that these contacts are junctions, as they are crossed by fine filamentous-granular structures approximately 3 nm in diameter. These structures are oriented perpendicular to the plane of the membrane except at the periphery of the functional assembly where they are typically oriented obliquely (Fig. 2D). These junctional filaments are either evenly distributed or aggregated in small groups. The junctional structures are also clearly seen in a grazing section showing a surface view of the cisterna in Fig. 2A and F. No coated

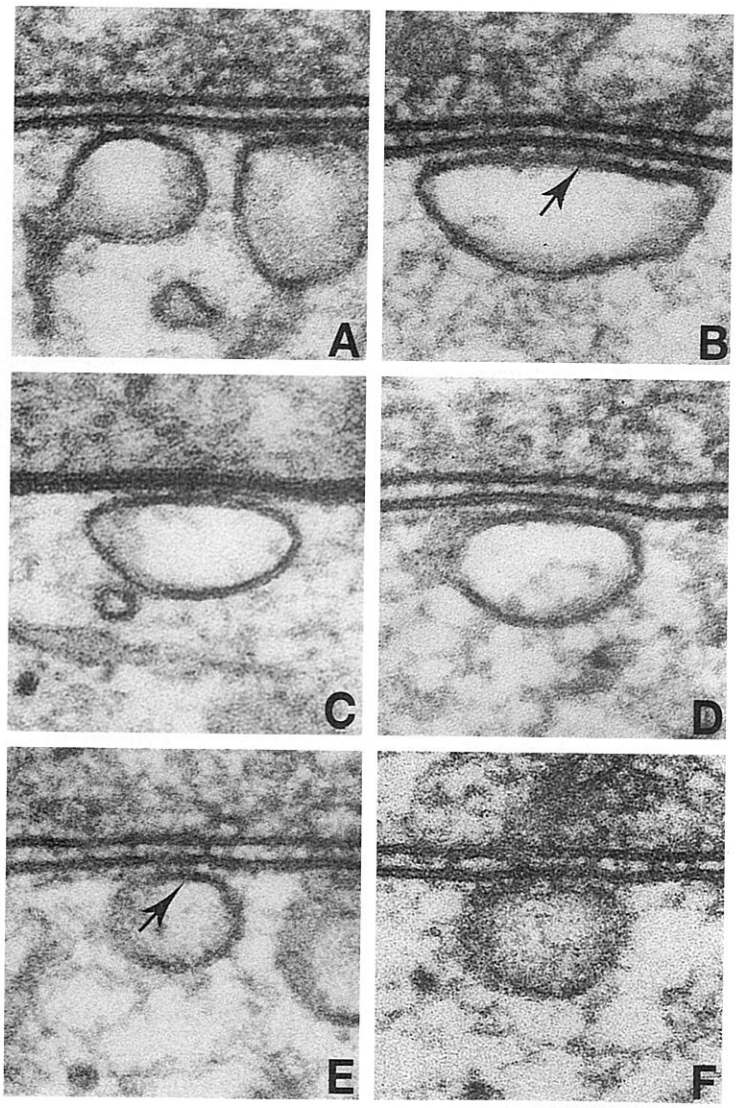


Fig. 2. (A–F) High-resolution micrographs of filamentous-granular junctions bridging the space between the subaxolemmal cisternae and the axolemma. These junctional structures, approximately 3 nm wide, appear individually (arrow, B) or in groups (arrow, E). A, B, E × 190 000; C, D, F × 170 000.

pits of the type associated with clathrin have been observed.

Tubular–vesicular endoplasmic reticulum

Tubular connections between subaxolemmal cisternae and intracortical compartments of the ER are frequent. Figure 3A–D shows examples of vertically oriented connections while Fig. 3H–K shows horizontal interconnections. Among the components of the same system the oblique connections have either a right or left-handed twist (Fig. 3D–G) or extend parallel to the plane of the axolemma (Fig. 3H–K). Tubules of ER oriented parallel to the surface of the axoplasm may be found anywhere from close to the axolemma down to the boundary between the cortex and the endoplasm. They often form T or Y-shaped junctions with other ER tubules (Fig. 3C–G).

Vesicles may be arranged in rows which appear to be continuations of ER tubules, suggesting an interconversion between the vesicular and tubular types of organization. In other instances, the continuation of a single tubule appears as a dense filament 20 nm or less in diameter (Figs 2B and 4F). These tubules seem to correspond to the tethers that are formed when ER membranes are stretched beyond a certain limit (Dabora & Sheetz, 1988). Tethers arise from a length of spindle-shaped ER (arrowhead, Figs 1A and 4F).

Large vacuoles

Large vacuoles (0.3–0.5 μm in diameter) are irregularly distributed in the axonal cortex. Some regions of the cortex lack vacuoles while others show densely packed rows of vacuoles (Fig. 4). The vacuoles have little content but typically have ER cisternae contacting their surfaces at junctions characterized by the same type of bridging filaments as those associated with the subaxolemmal cisternae (Fig. 4B–D). Lamellar membrane profiles (Fig. 4E) may represent a collapsed form of the large vacuoles. Like the large vacuoles they occasionally have ER contacts on their surface. Vertical membrane tubules may end as cisternae in the boundary zone between cortical ER and deeper axoplasm (asterisk, Fig. 4F).

Cytoskeleton

The cytoskeleton is a prominent element of the cortical axoplasm. Three components – the neurofilamentous network, the actin strands, and the microtubules – can be identified. The actin strands were particularly evident as 8 nm wide filaments interwoven in strands which are twisted into bundles (a, Fig. 1). Microtubules appear mostly in cross-section, approximately 20 nm in diameter (m, Fig. 1B). The subunit structure in the wall of the microtubules can often be used as a marker to distinguish them from the tubular profiles of the ER. The neurofilamentous network (f, Fig. 1B) appears as a dense matrix consisting of neurofilaments, mostly in cross-section, and their sidearms. A more detailed report on the three dimensional organization of the cytoskeleton will be presented elsewhere.

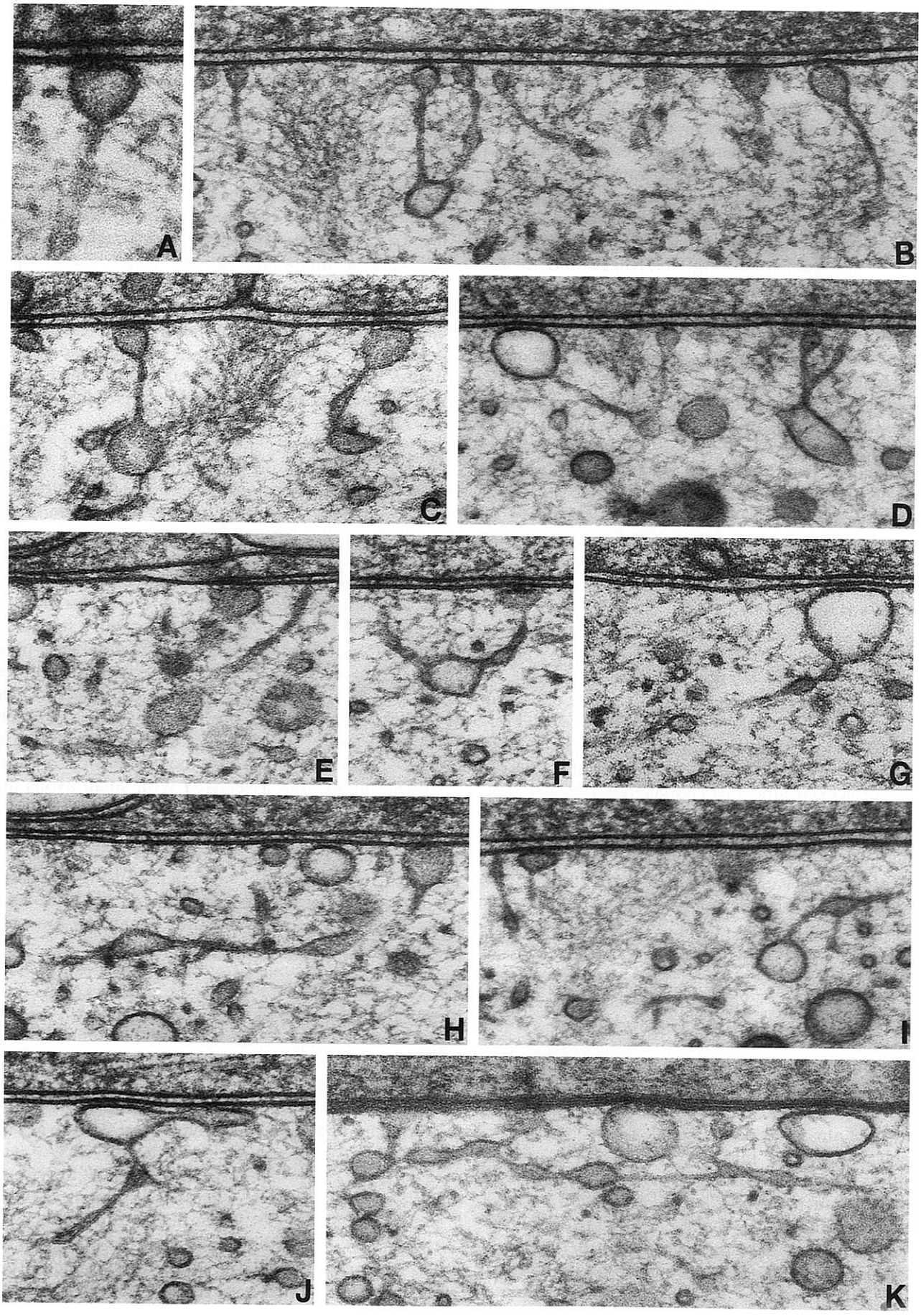
Serial reconstruction of the ER

The serial reconstructions of cortical structures revealed interconnections between the different types of membranous components of the cortex (Fig. 5). One of the sections used for this reconstruction is shown in Fig. 1. The three-dimensional relationships of the structures demonstrated in Fig. 1 are evident upon comparison of Fig. 1 with Fig. 5. This reconstruction shows a partly continuous, pleomorphic network of ER next to independent cisternae and vesicles. The vesicles are ordered linearly at the right side of the figure. The large vacuole is separate from the ER, and may be derived from the axolemma (see Discussion).

Discussion

This investigation depended on the ability of freezing methods to preserve structures as close to the living state as possible because the ER membranes in the axon, as elsewhere, appear to be highly labile (Dabora & Sheetz, 1988; Lee & Chen, 1988). For rapid freezing to be effective in avoiding damage from ice crystals, the tissue to be examined must be within a few μm of a natural or undamaged surface. The number of preparations of axons which meet this criterion is limited, and this is a particularly difficult condition to meet with myelinated axons (Schnapp & Reese, 1982). The giant axon of the juvenile squid was well suited for this purpose because the loose connective tissue sheath around and associated with small axons and blood vessels could be removed. The difficulty of achieving satisfactory freezing with adult squid is attributable to its much thicker sheath. High pressure freezing (Galway *et al.*, 1995) is a promising alternative approach, but existing equipment is far from being able to accommodate structures the size of the squid giant axon.

It has been assumed from many studies that isolation of the giant axon has negligible effect on its excitability (Chang, 1986). Nevertheless, it is still possible that the cleaning of the axon by dissecting the surrounding blood vessels, connective tissue and small axons could affect the organization of the ER. Cutting of small side branches during cleaning of the fibre is unavoidable, though these may reseal (Moore & Cole, 1954) if left long enough. Thus, the presence of large vacuoles in the cortex of the axon must be interpreted with caution. Indeed, extensive vacuolation of the axoplasm of the giant nerve fibre can be induced by transection of the fibre in sea water containing Ca^{2+} (Fishman *et al.*, 1990). The giant vacuoles, perhaps in response to local damage, tended to occur in groups,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leaving other areas of the axon without vacuoles. Furthermore, deep penetration of the axolemma into the axoplasm occurs where the Schwann cells have been removed (Metuzals *et al.*, 1981). In any instance, the relationships of the ER membranes to the vacuoles closely resemble the relationship of ER membranes to the axolemma, suggesting that the vacuoles are ultimately formed from the axolemma.

Subaxolemmal junctions

In muscle the T-tubules make close contacts with the sarcoplasmic reticulum (SR). Evenly dispersed components, called feet, span the junctional gap (Franzini-Armstrong, 1970; Henkart *et al.*, 1976). Considerable advances in the understanding of the functional coupling at T tubule–SR junctions have recently come from the identification of the molecular components of this junction.

Two proteins, which are likely to play a major role in the process of signal transduction have been isolated from SR and T-tubule membranes, and one of these, the ryanodine receptor, is associated with the junctional feet (Franzini-Armstrong, 1970). The hydropyridine receptor may be the molecule which initiates transduction of the voltage change across the T-tubule membrane to trigger release of Ca^{2+} from the SR (Block *et al.*, 1988). Ryanodine receptor-like immunoreactivity occurs in neurons of the CNS of fish (Zupanc *et al.*, 1992), and these neuronal ryanodine-like receptors appear to function as Ca^{2+} channels which may gate the release of Ca^{2+} from intracellular pools (Bootman & Berridge, 1995).

The junctions bridging the gap between the axolemma and the subaxolemmal cisternae in the cortex of the giant axon resemble the bridging filaments in muscle that are thought to be sites of ryanodine receptors, and the topological relationships are the same as in some muscles with surface junctions (Henkart *et al.*, 1976). Profiles of smooth ER are also in close contact with the axolemma in gastropod nerves (Schlote & Hanneforth, 1963), where the axolemma with the Schwann cell forms deep tubular infoldings into the axon, which in turn are contacted by cisternae of ER, just as T-tubules make contact with SR. Similar infoldings are also seen in serial reconstructions of vertebrate nerves (Reiter, 1966) where complicated cavities interweave with the axoplasm either diffusely or in labyrinthine formations. Thus, it is likely that the cisternal junctions in the squid giant axon are sites where action potentials are coupled to internal axoplasmic events by Ca^{2+} release from subaxolemmal ER.

Dynamic tubulo-vesicular ER

The ER near the axolemma forms a three-dimensional network of membrane-bound interconnected cisternae, tubules and tethers. The serial reconstructions also show that there is a population of vesicles that is not directly connected to the ER. While some of these might be Golgi-derived vesicles travelling to the axolemma, or endocytic vesicles leaving it, the tendency of some vesicles to occur in lines as well as their associations with the ends of tethers, suggests that they are components of the ER that are only intermittently incorporated into the ER network. In fact, the numbers of vesicles and ER cisternae typically are inversely related in other mollusc axons (Ducros, 1974).

Cytoskeleton

The application of freeze substitution to the giant axon has shown that there is a continuous cytoskeletal matrix in the cortical axoplasm consisting of neurofilaments, their side arms, and microtubules with their associated proteins (Alberts *et al.*, 1994). Strands of actin filaments continuously joined by single actin filaments along their side, giving them a Christmas tree profile, are embedded in this matrix and curve obliquely towards the axolemma. Actin and tubulin domains are known components of the axolemmal cytoskeleton (Metuzals & Tasaki, 1978; Kobayashi *et al.*, 1986; Tsukita *et al.*, 1986). The present findings suggest that the list of domains should be extended to include the domain defined by clusters of ER cisternae making junctions with the axolemma. The surrounding cytoskeletal elements contact the ER as well as the axolemma where they might modulate sodium (Undrovinas *et al.*, 1995) and potassium (Wang *et al.*, 1994) channels (see also Luna & Hitt, 1992). Furukawa and colleagues (1996) have demonstrated functional linkage of the K^+ channels to the assembly and disassembly states of the actin cytoskeletal network, which provides a mechanism for regulating this channel. We note here that the cytoskeletal organization in the cortex also offers an oriented system of actin strands that could be involved in displacements of the ER in response to local changes in Ca^{2+}. Oval protuberances of approximately the same size as the subaxolemmal cisternae have been observed by scanning electron microscopy of the surface of desheathed giant axons (Metuzals *et al.*, 1981, Fig. 5). The surface of these axons reveals interwoven ridges on which the protuberances are located. The axes of the protuberances and the ridges deviate by 10° from the long axis of the axon.

Fig. 3. (A–K) Tubular connections between subaxolemmal cisternae and intracortical components of the ER. A × 190 000; B–K × 110 000.

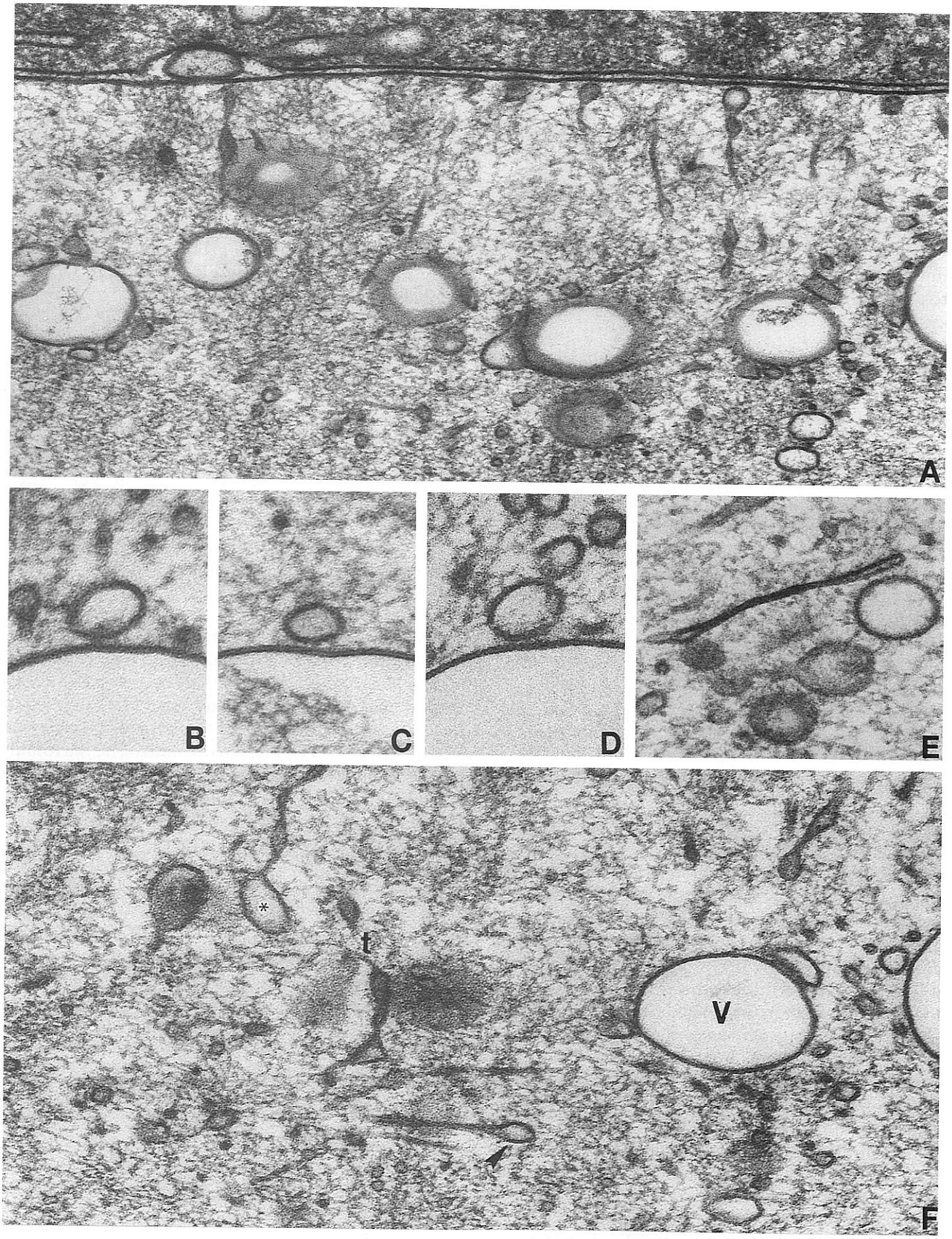

Fig. 4. (A–F) (A) a row of seven, closely spaced large vacuoles at the inner limit of the cortical zone of tubular and vesicular ER. (B–D) note the presence of cisternae making junctional contacts with the surface of the vacuoles. (E) lamellar membrane profiles. (F) twisted membrane tubule ends as a cisterna (asterisk), and two cisternae contact the surface of a large vacuole (V). A tether arises from a length of spindle-shaped ER (arrowhead). A × 70 000; B–D × 190 000; E × 140 000; F × 100 000.

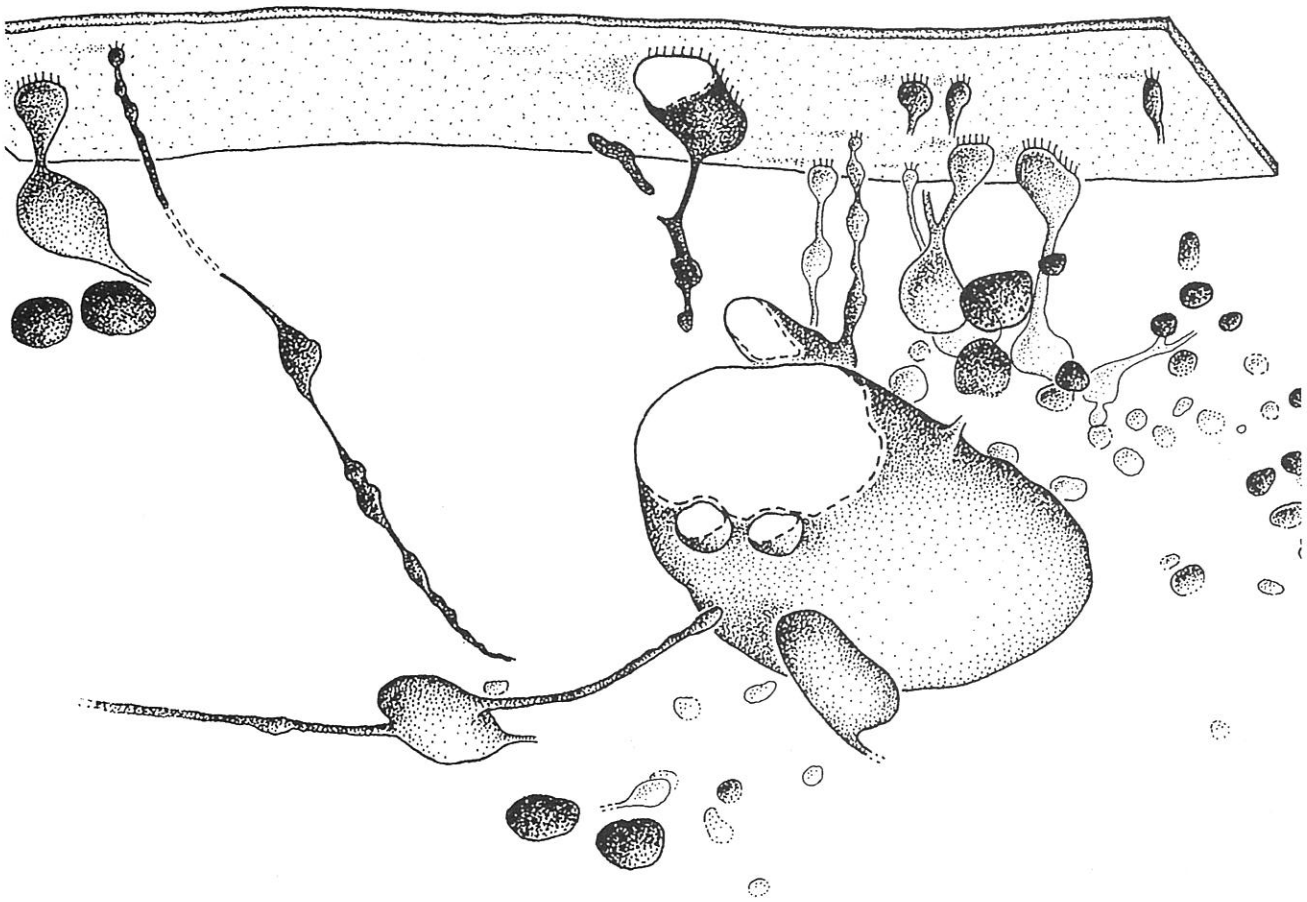

Fig. 5. Three-dimensional reconstruction of the smooth ER in the axonal cortex adjoining the axolemma, made from five serial sections. Many vesicular and tubular elements clustered near the axolemma are interconnected as shown in Fig. 3, forming a system of ER membranes below the axolemma. There are, however, vesicular elements (to the right) that are not interconnected at the instant in time depicted here. A long curved tether has been reconstructed, but a spiral of actin filaments between it and the rest of the ER (see Fig. 1) has not been included. The orientation of the drawing of the reconstruction corresponds to that of the electron micrographs and the conventional shadow orientation of the drawing is upside-down. ×85 000.

The curved structure of the tethers indicates that torquing forces affect the architecture of the ER. The helical organization of the main component of the axoplasm of the squid giant axon – the neurofilamentous network – could be the organizational basis of these forces (Metuzals, 1969; Metuzals & Izzard, 1969; Gilbert, 1972). The curved actin filament bundles might also represent cross-sections of helically organized bundles. Thus, the organization of the ER in the cortex of the giant axon may depend on interactions with a helically organized cytoskeleton as well as the associations of the sub-axolemmal cisternae with the axolemma at the ER-axolemmal junctions. The sinuous form of the tubules constituting the ER and their associations with the neurofilaments have also been observed in olfactory nerve and in squid giant axon (Burton & Laveri, 1985; Metuzals *et al.*, 1995).

We can conclude by speculating that in the course of its function in regulating Ca^{2+} distribution (Henkart *et al.*, 1978; Burton & Laveri, 1985; Bootman & Berridge, 1995; Llinás *et al.*, 1995; Silver, 1996), the organization of the cortical ER is very dynamic, depending on assembly and disassembly of its components. Activity in the form of trains of action potentials might be expected to influence its organization and state of disassembly, either directly or by the secondary effects of Ca^{2+} entry and release on the local cytoskeleton.

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