CALCIUM-CONTAINING ELECTRON-DENSE
STRUCTURES IN THE
AXONS OF THE SQUID GIANT SYNAPSE

D. E. HILLMAN and R. LLINÁS

From the Division of Neurobiology, Department of Physiology and Biophysics,
University of Iowa, Iowa City, Iowa 52242

ABSTRACT

Following the Oschman and Wall technique, electron-dense structures (EDS) were found on unstained, unosmicated membranes of squid giant synapse axons. These densities contain high concentrations of calcium and phosphorus as identified by energy dispersive X-ray analysis. Based on the signal strength, the quantity is significantly greater than that of other regions of the membrane or tissue spaces. The calcium EDS occur as plaques or globules along the axonic membrane, and small globules are found between sheath cell processes. EDS also occur at the synaptic site. These densities were correlated with the opacity change seen in giant axons. It is proposed that these structures represent sites where the calcium-binding protein found by other investigators has become nearly saturated with calcium.

INTRODUCTION

Calcium ions are essential for the activation of the depolarization secretion coupling system including chemically mediated synaptic transmission (1–9) as well as for the regulation of membrane permeability (10) and muscle contraction (11). Although morphological studies have failed to demonstrate the presence of calcium binding sites, the axoplasm of the squid giant axon is known to contain 0.4–0.5 mM calcium (12), of which only 0.0003 mM is in the free form (13). The major question of where this inorganic substance is bound has an important bearing on the site of action of calcium during synaptic transmission. Thus, while in the past some findings indicated that a rapid influx of calcium which elevates the intracellular calcium concentration, $[Ca^{++}]_i$, is necessary for synaptic transmission (14, 15), others favored the hypothesis that calcium could act through a special receptor in the external surface of the presynaptic membrane without actually reaching the axoplasm. The latter hypothesis was based on the fact that synaptic transmission could not be triggered by an intracellular calcium injection into the presynaptic terminal (16). The possibility that such injection was ineffective due to a rapid intracellular binding of calcium was to be expected from the findings of Alema et al. (17). They demonstrated by immunological means that a calcium-binding protein is present in large quantities in squid axoplasm. In fact, Hodgkin and Keynes (18) and Baker and Crawford (19) have shown that calcium has a very small mobility in agreement with the presence of a strong calcium-sequestering system in the axoplasm. More recently, however, new results indicate that intracellular calcium injection apparently does trigger the release of synaptic transmitter (20). This is in keeping with the first hypothesis (14, 15) and establishes a

transient variation of $[Ca^{++}]_i$ as an important event in the depolarization-secretion coupling mechanism.

The possibility of localizing calcium by ultrastructural means has recently been suggested. The method of Oschman and Wall (21) is believed to localize calcium of either endogenous or exogenous origin; however, the identification of calcium in these sites has not, so far, been directly demonstrated. The present study was undertaken in order to determine whether the Oschman-Wall method could be utilized in the squid giant synapse and, if so, whether the resulting densities are indeed calcium rich.

MATERIALS AND METHODS

Stellate ganglia from 32 squid (*Loligo pealii*) were prepared for electron microscopy. The ganglia were dissected from the decapitated mantle in a flowing sea water bath, and then fixed by rapid immersion in cold (5°C) fixative. Of this material, 20 pairs of ganglia were immersed in 0.08 M collidine, 2.4% glutaraldehyde, and 1.2% sucrose, and then either postfixed in osmium tetroxide or embedded directly in Spurr (22) or DER Epon. Four pairs were immersed directly in osmium tetroxide and then dehydrated for embedding. Alternatively, a series of ganglia was fixed by whole animal perfusion through the vascular system. The perfusion solution consisted of 5.75% glutaraldehyde in 0.39 M cacodylate buffer at pH 7.2–7.3. The fixatives in both cases contained either 0.015 or 0.005 M calcium chloride.

In a separate experimental series, four ganglia were immersed in sea water with 50 mM calcium for periods of 1–2 h until the giant axons became cloudy. Such cloudiness was apparent only when a certain amount of stretch was applied to the preparation during dissection. These preparations were fixed by immersion in the collidine-buffered glutaraldehyde solution. Another four ganglia were fixed after a microelectrode injection of 0.5 M calcium into the center of the pre- and postsynaptic fibers.

Sections of the tissue were mounted on single-hole grids with a Formvar support membrane; some were stained with uranyl acetate and lead citrate. Unstained thick sections up to 0.5 μm were used for energy dispersive X-ray analysis for inorganic elements, and thin sections were viewed in an Hitachi HS-7 transmission electron microscope.

The analytical system consisted of a JEM 100B transmission microscope with a scanning attachment and solid state Ortec or EDAX energy dispersive spectrometer. The scanning attachment (JEM-ASID) allowed viewing of the image and localization of the beam for excitation of a spot about 200 Å in diameter.

RESULTS

General Observations

Specimens of squid ganglia prepared by either perfusion or immersion in glutaraldehyde dis-
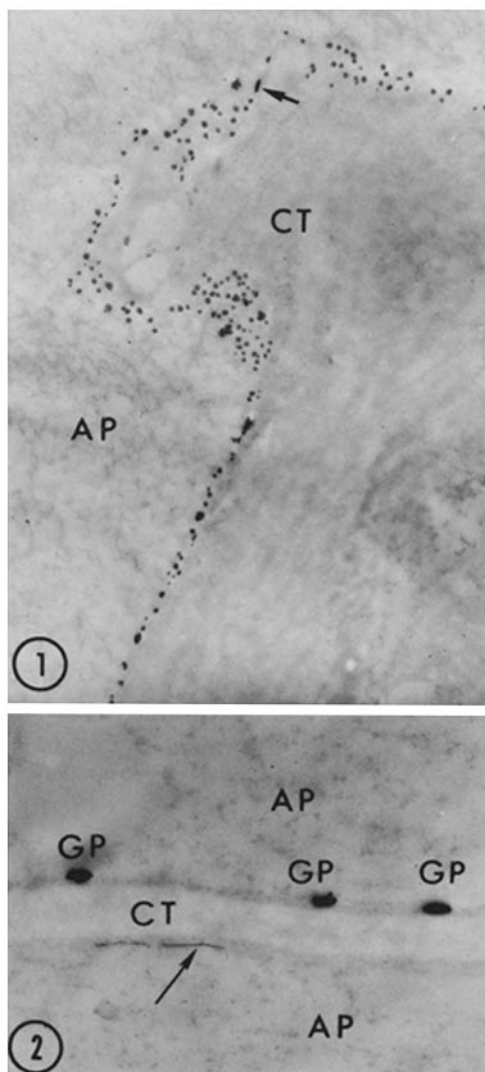

FIGURE 1 Electron micrograph of squid giant axon showing dense globules and plaques (arrow) which line the internal surface of the axonic membrane. Axoplasm, AP. Connective tissue, CT. Collidine-glutaraldehyde fixation, nonosmicated and unstained. × 6,000.

FIGURE 2. Electron micrograph of two adjacent axons. The upper giant axon has globular plaques (large arrow) while the lower smaller axon has a long thin plaque (small arrow). Connective tissue, CT. Collidine-glutaraldehyde fixation, nonosmicated, and unstained. × 10,000.

played electron-dense structures (EDS) in the pre- and postsynaptic giant axons. Postfixation in osmium tetroxide did not alter these deposits; however, when the preparations were pre-fixed in osmium tetroxide, densities were never found. In unstained nonosmicated material, the EDS clearly delineated the margin of the axons whereas the axonic membranes and sheath cells were faintly visible due to the lack of electron-opaque material (Figs. 1 and 2). In stained preparations, the EDS could be visualized on the membranes of the giant axon and on the membranes of the surrounding sheath cells.

The EDS vary in shape from rounded globules to flattened plaques (Fig. 2). Profiles of globules were as small as 10 nm and ranged up to 120 nm in diameter. The plaque profiles range up to 1 μm in length. The ultrastructural image suggested that the plaques were composed of smaller subunits which appeared to protrude into the axoplasmic space (Figs. 3, 4, and 6). The size of the globular EDS, though variable, was in the range of the plaque subunits. In most axonic profiles the densities occurred on the intracellular side of the membrane and were thus separated from the sheath cell by intercellular space and axonal membranes (Figs. 3 and 4). Occasionally very thin, dense structures could be seen, as illustrated in Fig. 2 (arrow). Others had an overall light appearance (Fig. 5). Globular densities were dispersed between the plaques or occurred separately.

Identification of Inorganic Constituents by Energy Dispersive Spectrographic Analysis

The comparison of X-ray spectrometer signals from various areas of the tissue within a given sec-

tion demonstrated that the EDS were significantly higher in calcium than any other region. The energy dispersive X-ray spectrograph in Fig. 8 a was obtained from the electron-dense plaque (a) illustrated in the scanning transmission micrograph in Fig. 7, and shows a distinct emission at the 3.7 keV energy level (Ca) which clearly indicates calcium. The relative difference in the amount of calcium between such EDS and the rest of the tissue is indicated by the small signal seen in trace b taken from the membrane site (b) which is free of densities. A small signal was obtained in all other areas but never reached the comparative magnitude seen in the dense plaque structure. Phosphorus was also found in the EDS.

Density Numbers and Distribution

Axonic profiles displayed a variable number of EDS; they could be totally lacking in some preparations, while in others they were distributed at close intervals (Fig. 1) throughout the length of the fibers. This variability was noted between specimens and also within individual axons. Within axonal cross-section, plaques were seen to occur on one side only or in patches, while the other side could be totally void of obvious dense structures. Likewise, longitudinal sections of both the pre- and post-axons of the giant synapse demonstrated some variability along their course. In those axons where EDS were observed, the small globular EDS were most commonly found in the second-order presynaptic axons, whereas the large dense plaques were more often associated with the third-order giant axon. Densities were also seen on the neuronal bodies and axons of other than giant fibers.

FIGURES 3–5 Electron-dense plaques are related to the plasma membrane with an irregular surface toward the axoplasm (AP). Note intercellular space between EDS and sheath cell (SC). Collidine-glutaraldehyde fixation, postosmicated, and stained. × 50,000.

FIGURE 6 Electron micrograph showing usual plaque distribution along a giant axon. AP, axoplasm; SC, sheath cell; CT connective tissue; BM, basement membrane. Collidine-glutaraldehyde fixation, postosmicated, and stained. × 20,000.

FIGURES 7 and 8 Transmission electron micrograph from an 0.2 μm thick section with corresponding records for energy levels 1.8–3.8 keV from an EDAX energy dispersive X-ray analyzer. Record 8 a from the plaque in Fig. 7 shows a distinct signal at energy level 3.7 keV, which is the calcium peak (Ca). In record 8 b from an area of the membrane free of densities (Fig. 7 b), only a small indication of calcium is seen in the respective traces at the calcium Kα energy level. Phosphorus (Kα 2.0 keV) is also found in high concentrations at these densities. AP, axoplasm; P, phosphorus; Cl, chloride. Collidine-glutaraldehyde fixation, nonosmicated, and unstained. × 8,000.
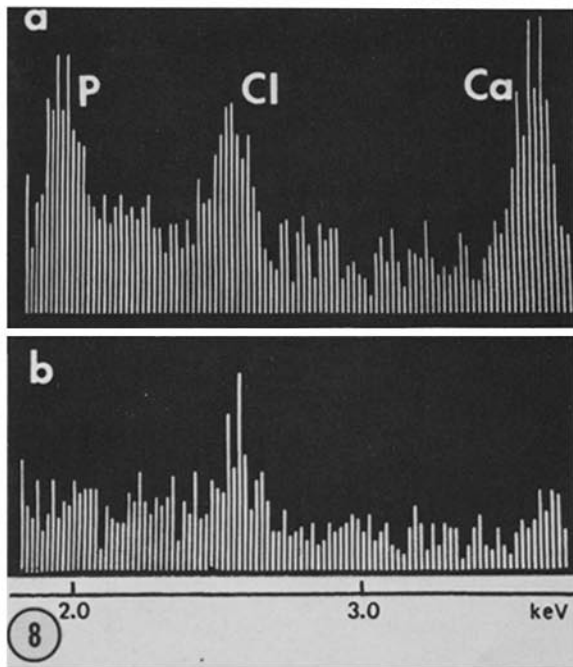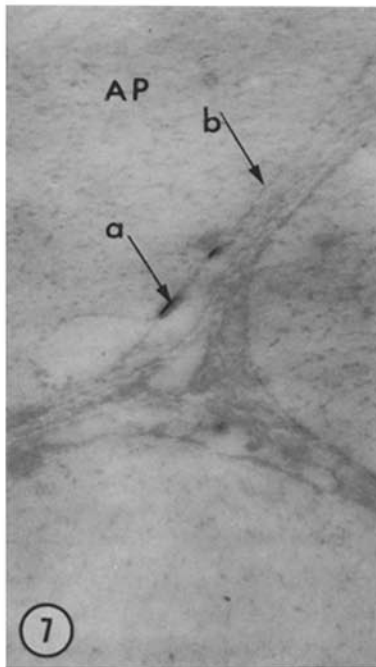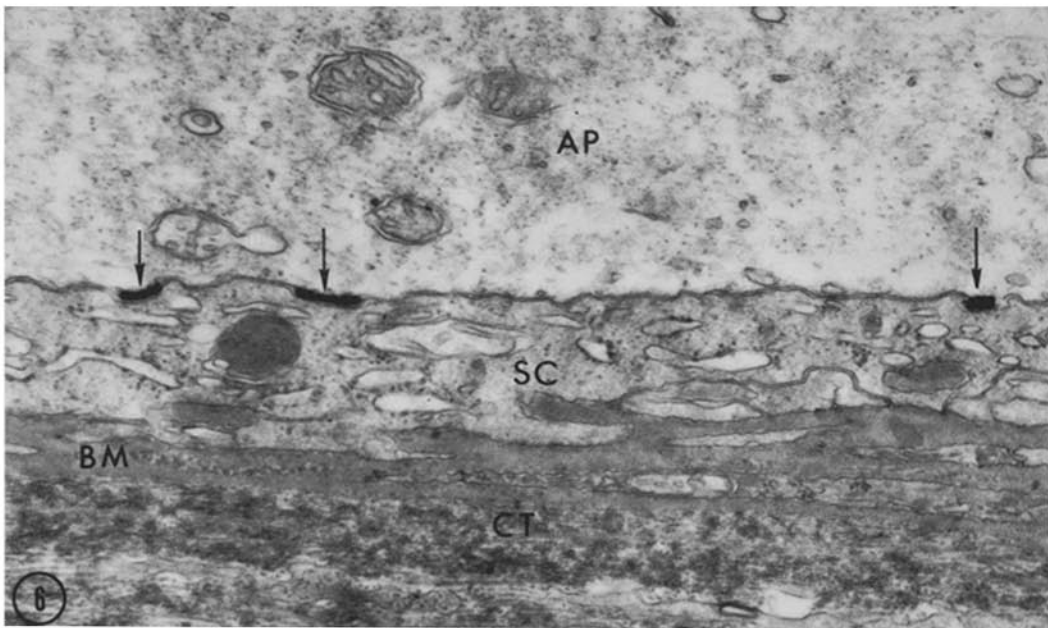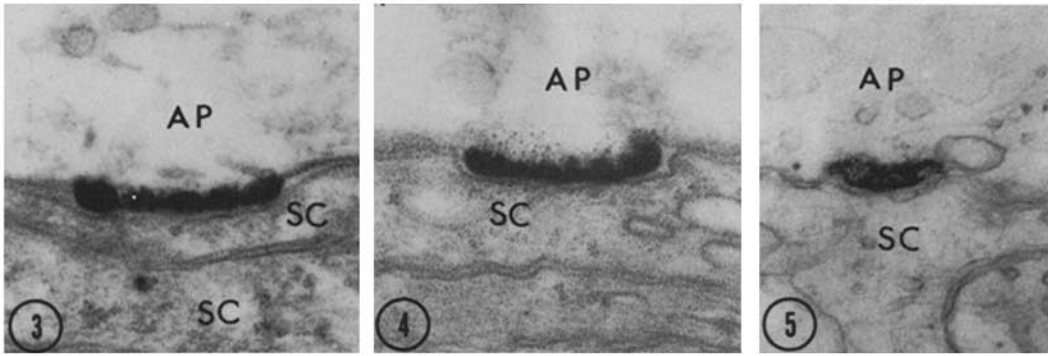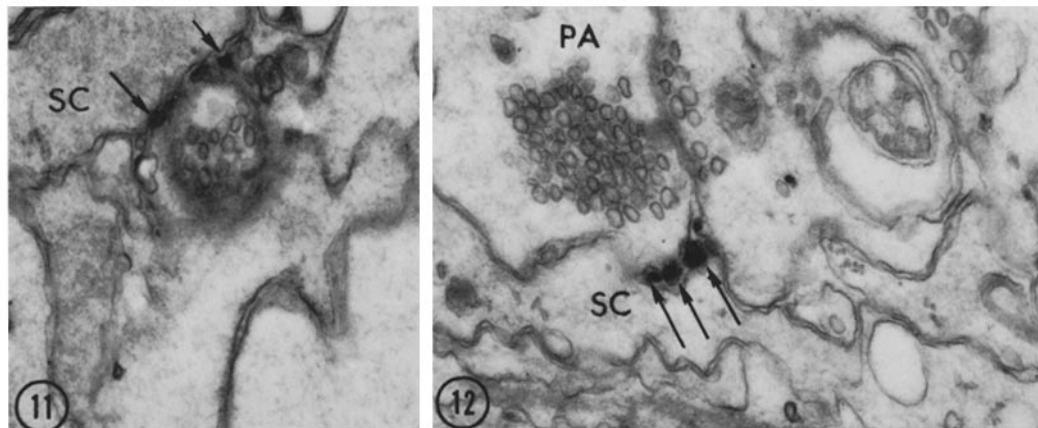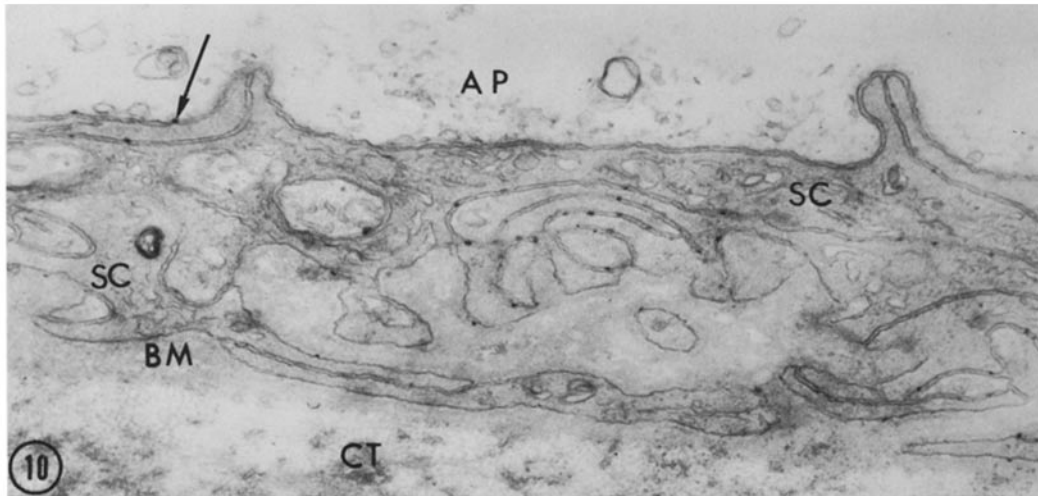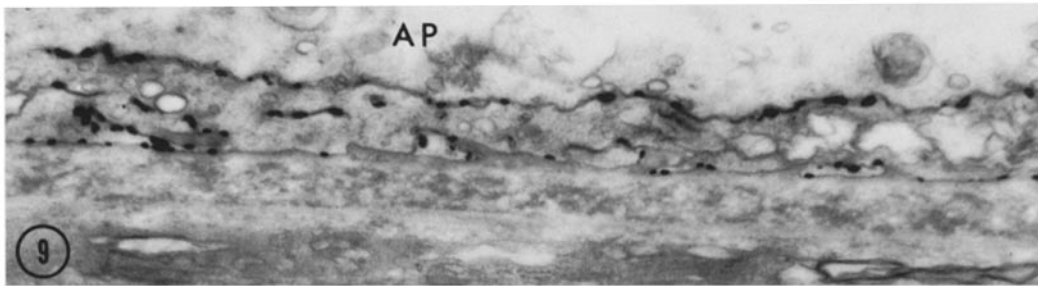
FIGURE 9 Prominent EDS on giant axon plasma membrane and on sheath cell process (*SC*). *AP*, Axoplasm. Thick section with collidine-glutaraldehyde fixation and postosmication, but unstained. × 10,000.

FIGURE 10 Very small globules on sheath cell membranes which appear to be both in and between opposed sheath cells (*SC*). A few (arrow) are seen on the axoplasmic membrane of the giant fiber. *AP*, axoplasm; *BM*, basement membrane; *CT*, connective tissue. Cacodylate-glutaraldehyde perfusion fixation. × 20,000.

FIGURES 11 and 12 Dense globules adjacent to synapses. The calcium binding structures are between sheath cell processes (*SC*) and the presynaptic axon (*PA*), as well as on the membrane within the presynaptic axons. Collidine-glutaraldehyde fixation, postosmicated, and stained. × 30,000.

Sheath Cell-Related EDS

EDS associated with sheath cells had a small globular shape and a diameter of 10–80 nm (Figs. 9 and 10). They were particularly present in the cytoplasm of the sheath cell processes surrounding the axons. Others were extracellular particles which seemed to be attached to the outer surface of the sheath cell processes (Fig. 10). Some of these latter EDS were located between the axon and the sheath cell.

Densities at the Giant Synapse

EDS were commonly found in both pre- and postsynaptic axons of the giant synapse. Presynaptically, plaques and globules were seen along the membrane at the vicinity of the synaptic junctions.[1] The EDS were often seen in the membrane infoldings, which resemble the cisternae described by Heuser and Reese (23), as well as surrounding the synaptic attachment itself and on the presynaptic membrane in the vicinity of synaptic vesicles (Figs. 11 and 12). However, no EDS were observed at the pre- or postsynaptic subsynaptic thickening.

Opaque Axonal Reaction

Normally, giant axons have a glossy transparent appearance when viewed under indirect light. After standing in a sea water bath for 1–2 h, the axons take on a white opaque appearance if the membrane has been over-stretched during dissection. Usually this opacity appears as an evenly distributed, fine-grained material. With time, or manipulation of the axon, this pattern tends to change to a conglomerate of distinctly particulate appearance.

The ultrastructural changes corresponding to the early opacity were characterized by a distinct increase of EDS on the axonal membrane. Later, as the axonal opacity changed to the particulate pattern, or after intracellular calcium injection, the preparations had distinct EDS in the axoplasm. Some were scattered globules or aggregates of globules (Fig. 13), while others were located in

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[1] The synaptic junctions were recognized by: the direct apposition of the small branches of the postsynaptic protrusions onto the presynaptic axons, the presence of synaptic membrane densities, and the presence of synaptic vesicles.

vacuolar structures (Fig. 15). Mitochondria contained these densities in the form of small globules (Fig. 14) as well as larger masses (Fig. 15, arrow).

In the calcium-injected specimens, globular EDS, generated by the injected solutions, lined cisterns in the axoplasm (Fig. 16). At the presynaptic zone, vesicles contained dense structures in the form of an eccentrically located dot (Fig. 17, arrows). These vesicles were of both the coated and noncoated variety, though most were of the former type.

DISCUSSION

The Oschman and Wall (21) observation that glutaraldehyde fixatives are capable of demonstrating inorganic material believed to represent calcium deposits offers new possibilities for the structural localization of inorganic elements. The densities were first described by these authors in relation to intestinal cells of the cockroach. Similar EDS are now demonstrated in squid giant axons, and can be shown to contain high calcium as well as phosphorus concentrations; however, EDS have not been described in tissues such as in the barnacle muscle (24) which contain large quantities of calcium. Nevertheless, the delicate granules described by Oschman and Wall (21) could easily be undetected if their appearance was similar to that of the fine particles found in the cockroach intestinal muscle or those seen on sheath cell membranes of the squid giant axons, which are faintly visible.

Since the morphological localization of EDS is variable, it seems apparent that the structural phenomena demonstrated here are the result of particular changes in a set of structural entities present in the general area where the EDS were observed. Indications that such changes may be related to alteration in the regional calcium concentration are suggested by the structural variability or complete lack of EDS in response to changes in $[Ca^{++}]_o$. In addition, it has been shown that giant axons take on a cloudy appearance when they are injured and that such a phenomenon does not occur in the absence of calcium (18, 25–27). The most important point here, however, is the correct assessment of the calcium-binding entities.

EDS may be considered to be formed as an inorganic precipitate of Ca^{++} with phosphorus; however, the structure and localization of the EDS suggest their relation to an organic substrate.
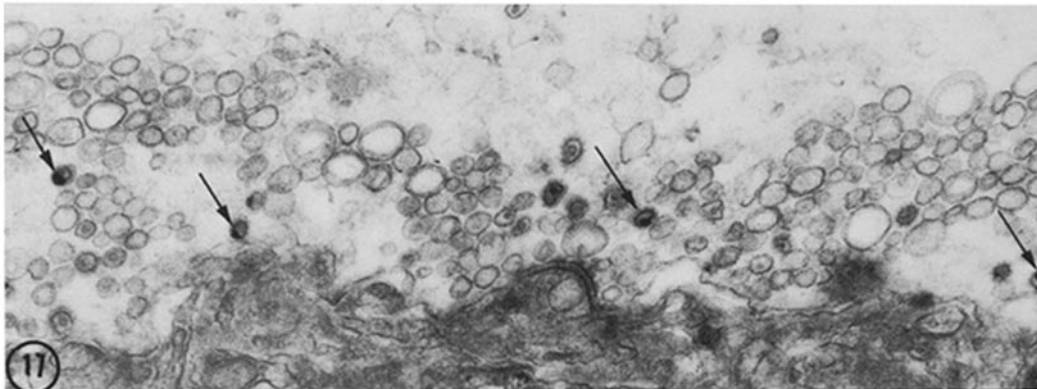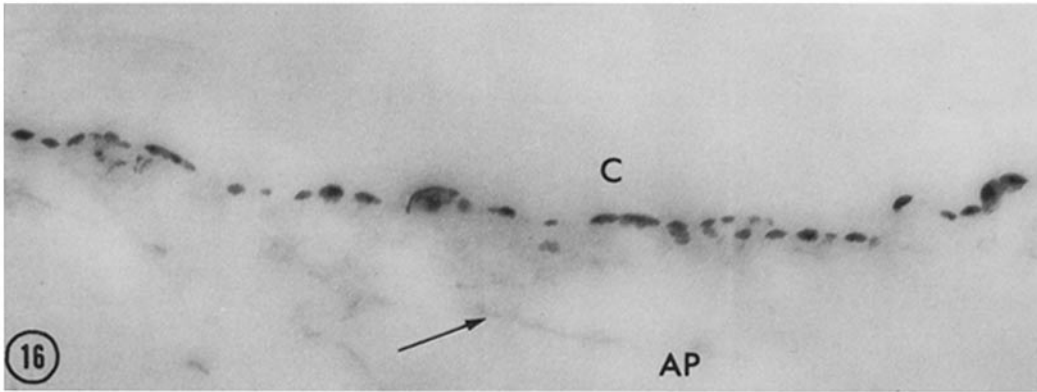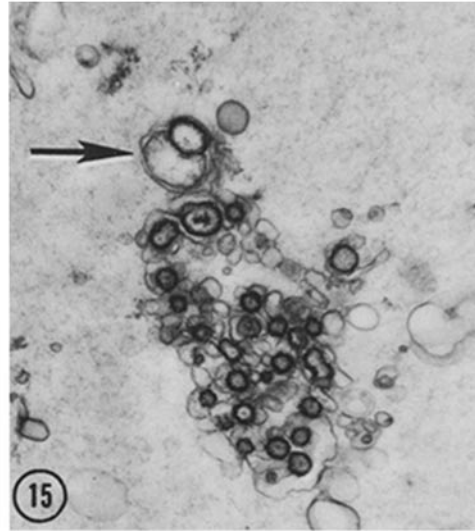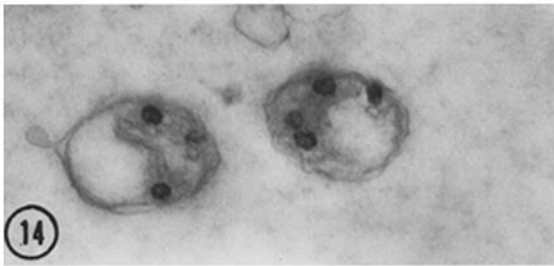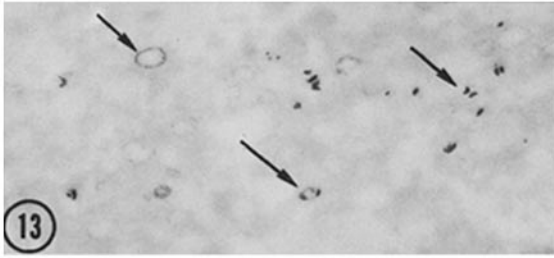
Although the calcium-binding protein described by Alema et al. (17) cannot be definitely linked to the EDS, it seems probable that the two may be closely related, given the extra-organelle location of the EDS. Assuming that the EDS are sites of calcium-binding protein which are marked by accumulations of calcium, numerous possibilities arise as to the nature of the EDS and their variability. For example, the changes could be due to shifts of the protein which aggregate into plaques or large globular accumulations in the presence of calcium. On the other hand, if the protein were to be located in the periphery of the axoplasm or on the inner surface of the axonic membrane itself, the variability could be due to the actual amounts of calcium bound within a particular structure. This may represent the normal fluctuation of calcium stores or, more likely, fixation changes in the axon which could correspond to the opacity change described by Hodgkin and Keynes (18), and would suggest EDS as being due to a rapid entry of calcium into the giant fiber. However, EDS are also found in the interior of the axoplasmic mass in a nonmembranous substrate after prolonged immersion in a high calcium medium and particularly after membrane injury. Since a similar situation occurs after intracellular calcium injection, it must be concluded that the calcium-binding protein is not necessarily associated with the axonal membrane in an exclusive manner. This also suggests that the location of EDS in the periphery of the axoplasm may simply indicate that during fixation, calcium penetrates through the membrane and thus is bound at the periphery of the axoplasmic mass.

From a physiological point of view, the densities may probably represent sites of calcium binding which, after having sequestered calcium, move towards the membrane in order to have the cation actively extruded via a calcium pump (28). Calcium-binding protein could also transfer this ion to mitochondria or smooth endoplasmic reticulum. Such a protein in the axon could bind calcium at the membrane surface to serve as a calcium-sequestering system for the calcium influx-associated action potentials such as that shown with aequorin by Baker et al. (29) and in the presynaptic axon in the squid stellate ganglion during synaptic transmission (15, 30).

Given that small EDS have been found in synaptic vesicles in this and other synapses (23, 31, 32), it may be possible that such EDS also represent calcium-binding sites. This supposition is strengthened by the fact that vesicular dark spots are found if high calcium (0.02 M) is added to the aldehyde fixative, but are not present if the calcium is replaced by magnesium (0.02 M) (Heuser, personal communication). Whether this synaptic vesicular site plays an important role in the release of transmitter by the transient intracellular change in calcium concentration (15, 20, 30) is yet to be determined.

FIGURE 13 Squid axoplasm which was fixed after the development of the opacity in the axon. Dense membranes of vacuoles were commonly noted. Free globular densities were irregularly distributed in the axoplasm. Collidine-glutaraldehyde fixation, nonosmicated, and unstained material. × 8,000.

FIGURE 14 Mitochondrial EDS of the globular type after injection of 0.5 M calcium chloride. Collidine-glutaraldehyde fixation with postosmication. × 25,000.

FIGURE 15 Vacuolar EDS in axoplasm after the opacity change and aggregation of the white substance. Mitochondrion (arrow) has vacuolar density. Collidine-glutaraldehyde fixation, osmicated, and stained. × 17,000.

FIGURE 16 Cisterns (C) in axoplasm (AP) lined with EDS after injection of 0.5 M calcium chloride. Arrow indicates neurofilaments. Collidine-glutaraldehyde fixation, nonosmicated, and unstained. × 37,000.

FIGURE 17 After calcium injection, the synaptic complex contained a moderate number of vesicles which had dark, eccentrically located dots (arrows). Collidine-glutaraldehyde fixation, osmicated, and stained. × 30,000.

REFERENCES

1. DOUGLAS, W. W., and R. P. RUBIN. 1961. The role of calcium in the secretory response of the adrenal medulla to acetylcholine. *J. Physiol. (Lond.).* 159:40.

2. DOUGLAS, W. W., and R. P. RUBIN. 1963. The mechanism of catecholamine release from the adrenal medulla and the role of calcium in stimulus-secretion coupling. *J. Physiol. (Lond.).* 167:288.

3. HUKOVIC, S., and E. MUSCHOLL. 1962. Die Noradrenalin-Abgabe aus dem isolierten Kaninchenherzen bie sympathischer Nervenreizung und ihre pharmakologische Beeinflussung. *Naunyn-Schmiedeberg's Arch. Exp. Pathol. Pharmakol.* 244: 81.

4. BOULLIN, D. J. 1967. The action of extracellular cations on the release of the sympathetic transmitter from peripheral nerves. *J. Physiol. (Lond.).* 189:85.

5. KIRPEKAR, S. M., and Y. MISU. 1967. Release of noradrenaline by splenic nerve stimulation and its dependence on calcium. *J. Physiol. (Lond.).* 188:219.

6. KIRPEKAR, S. M., and A. R. WAKADE. 1968. Release of noradrenaline from the cat spleen by potassium. *J. Physiol. (Lond.).* 94:595.

7. HARVEY, A. M., and F. C. MACINTOSH. 1940. Calcium and synaptic transmission in a sympathetic ganglion. *J. Physiol. (Lond.).* 97:408.

8. KATZ, B., and R. MILEDI. 1965. The effect of calcium on acetylcholine release from motor nerve terminals. *Proc. R. Soc. Lond. Ser. B Biol. Sci.* 161:496.

9. KATZ, B., and R. MILEDI. 1967. Ionic requirements of synaptic transmitter release. *Nature* 215:651.

10. TASAKI, I., A. WATANABE, and L. LERMAN. 1967. Role of divalent cations in excitation of squid axons. *Am. J. Physiol.* 213:1465.

11. EBASHI, S., M. ENDO, and I. OHTSUKI. 1969. Control of muscle contraction. *Q. Rev. Biophys.* 2: 351.

12. KEYNES, R. D., and P. R. LEWIS. 1956. The intracellular calcium contents of some invertebrate nerves. *J. Physiol. (Lond.).* 134:399.

13. BAKER P. F. 1972. Transport and metabolism of calcium ions in nerve. *Prog. Biophys Mol. Biol.* 24:177.

14. KATZ, B., and R. MILEDI. 1969. Tetrodotoxin-resistant electric activity in presynaptic terminals. *J. Physiol. (Lond.).* 203:459.

15. LLINÁS, R., J. BLINKS, and C. NICHOLSON. 1972. Calcium transient in presynaptic terminal of squid giant synapse: Detection with aequorin. *Science (Wash. D.C.).* 176:1127.

16. MILEDI, R., and C. SLATER. 1966. The action of calcium on neuronal synapses in the squid. *J. Physiol. (Lond.).* 184:473.

17. ALEMA, S., P. CALISSANO, G. RUSCA, and A. GIUDITTA. 1973. Identification of a calcium-binding, brain specific protein in the axoplasm of squid giant axons. *J. Neurochem.* 20: 681.

18. HODGKIN, A. L., and R. D. KEYNES. 1957. Movements of labelled calcium in squid giant axons. *J. Physiol. (Lond.).* 138:253.

19. BAKER, P. F., and A. C. CRAWFORD. 1972. Mobility and transport of magnesium in squid giant axons. *J. Physiol. (Lond.).* 227:855.

20. MILEDI, R. 1973. Transmitter release induced by injection of calcium ions into nerve terminals. *Proc. R. Soc. Lond. Ser. B Biol. Sci.* 183:421.

21. OSCHMAN, J., and B. WALL. 1972. Calcium binding to intestinal membranes. *J. Cell Biol.* 55: 58.

22. SPURR, A. R. 1969. A low-viscosity epoxy resin embedding medium for electron microscopy. *J. Ultrastruct. Res.* 26:31.

23. HEUSER, J. E., and T. S. REESE. 1973. Evidence for recycling of synaptic vesicle membrane during transmitter release at the frog neuromuscular junction. *J. Cell Biol.* 57:315.

24. HOYLE, G., P. MCNEILL, and A. SELVERSTON. 1973. Ultrastructure of barnacle giant muscle fibers. *J. Cell Biol.* 56:74.

25. CHAMBERS, R., and C. KAO. 1952. The effect of electrolytes on the physical state of the nerve axon of the squid and of *Stentor*, a protozoan. *Exp. Cell Res.* 3:564.

26. HODGKIN, A. L., and B. KATZ. 1949. The effect of calcium on the axoplasm of giant nerve fibres. *J. Exp. Biol.* 26:292.

27. HODGKIN, A. L., and R. D. KEYNES. 1956. Experiments on the injection of substances into squid giant axons by means of a microsyringe. *J. Physiol. (Lond.).* 131:592.

28. BLAUSTEIN, M. P., and A. L. HODGKIN. 1969. The effects of cyanide on the efflux of calcium from squid axons. *J. Physiol. (Lond.).* 200:497.

29. BAKER, P. F., A. L. HODGKIN, and E. B. RIDGWAY. 1971. Depolarization and calcium entry in squid giant axons. *J. Physiol. (Lond.).* 218:709.

30. LLINÁS, R., C. NICHOLSON, and J. R. BLINKS.

1973. Aequorin detection of calcium concentration changes in the presynaptic fiber of the squid stellate ganglion during synaptic transmission. *J. Gen. Physiol.* **61:**259.

31. BOHAN, T. P., A. F. BOYNE, P. S. GUTH, Y. NARAYANAN, and T. H. WILLIAMS. 1973.

Electron-dense particle in cholinergic synaptic vesicles. *Nature (Lond.).* **244:**32.

32. POLITOFF, A. L., S. ROSE, and G. B. PAPPAS. 1973. Calcium binding sites in synaptic vesicles of the frog neuromuscular junction. *Biol. Bull. (Woods Hole).* **145:**449.