ASSOCIATION OF CALCIUM WITH MEMBRANES OF SQUID GIANT AXON

Ultrastructure and Microprobe Analysis

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ABSTRACT

Giant axons from the squid, *Loligo pealei*, were fixed in glutaraldehyde and postfixed in osmium tetroxide. Calcium chloride (5 mM/liter) was added to all aqueous solutions used for tissue processing. Electron-opaque deposits were found along the axonal plasma membranes, within mitochondria, and along the basal plasma membranes of Schwann cells. X-ray microprobe analysis (EMMA-4) yielded signals for calcium and phosphorus when deposits were probed, whereas these elements were not detected in the axoplasm.

INTRODUCTION

Many cellular processes appear to be mediated by reversible interactions of ionized calcium with the plasma membrane. We have been interested in using the electron microscope to locate membrane areas with high affinity for calcium. Addition of calcium ions to the solutions used for fixing and processing tissues for the electron microscope causes opaque deposits to be formed along the plasma membranes of a variety of cells (1). In insect intestine, for example, deposits occur on apical membranes (microvilli) and along septate but not along gap junctions (2). Apparently, particular regions of the plasma membrane, or some structure closely associated with the membrane, can sequester enough calcium to make the region adjacent to the membrane opaque to electrons. Although we do not know precisely how the deposits form, we suspect that the presence of calcium ions during processing keeps intracellular binding sites saturated. The deposits were not observed readily in the past because of the widespread use of osmium, which decalcifies tissues unless excess calcium is present in the fixative.

To assess the significance of the deposits adjacent to the membranes, one must know their composition and how they form. The present study was undertaken to utilize the rapidly developing technique of electron-probe X-ray microanalysis (3, 4) to study the composition of the deposits. The analytical electron microscope is a transmission electron microscope fitted with X-ray spectrometers that analyze X rays generated when a micro-area of the specimen is bombarded by the electron beam. Since each element produces X rays of characteristic energy and wavelength, information is obtained about the composition of the specimen. Microprobe analysis is well suited for analyzing bound ions, since conventional fixation and embedding techniques can be used. Our initial attempts were made on intestinal

tissue, the subject of our previous studies. The deposits in intestine proved too small to produce an adequate signal. In the meantime, Hillman and Llinás (personal communication) had observed similar but larger deposits in squid axon, and previously Villegas and Villegas in 1968 (5) had also observed deposits in squid axon. We found that the deposits in squid axon were too small to be analyzed individually (spot size in the microprobe is about 0.2 μm), but groups of several deposits along the membranes and within mitochondria could be probed. Deposits yielded good signals when the spectrometers were set at the Bragg angles for calcium and phosphorus. When the spectrometers were offset slightly from these angles, the signals were much smaller. When the probe was moved to the axoplasm, the count rate also dropped sharply. Energy analyses of the X rays yielded sharp spectral peaks for Ca and P which could be compared with those from a dentine standard.

The results confirm that the deposits contain calcium as well as phosphorus, suggesting that phosphate may be the anion. The results obtained by probing mitochondria, while preliminary, confirm that mitochondria of squid axon, like mitochondria from many other tissues (6, 7), sequester calcium.

A preliminary report of this work has been published (8). Some aspects of this study were independently demonstrated by Hillman and Llinás.[1]

MATERIALS AND METHODS

Electron Microscopy

Axons were taken from living squids (*Loligo pealei*) at Woods Hole. Axons were fixed overnight in *s*-collidine-buffered 2.4% glutaraldehyde containing 5 mM/liter $CaCl_2$ and 25.5% sucrose. Axons were then washed thoroughly in buffered sucrose and postfixed in osmium tetroxide. Calcium chloride (5mM/liter) was also added to the rinse and osmium solutions. In some cases postfixation in osmium was omitted. Glutaraldehyde and collidine buffer were obtained from Polysciences, Inc., Warrington, Pa., because we had found in our previous study (1) that these reagents have very low electrolyte content. Specimens were rapidly dehydrated in ethanol and embedded in Spurr resin. Thin sections cut on a Huxley microtome were mounted on copper grids, stained with uranyl acetate and lead citrate, and examined either with a JEOL EM6B or with a Philips EM 300. Measurements of size and frequency of deposits on membranes were made after calibration with a carbon replica of a diffraction grating, 28,800 lines per inch.

X-Ray Microprobe Analysis

Specimens for microprobe study were fixed and embedded as described above. Thick sections (blue or green interference colors, ca. 1,900–2,800 Å thick) (9) were placed on nickel grids, and a layer of carbon was evaporated upon them. To minimize fourth-order copper interference with the phosphorus K_α line, a titanium specimen holder was used. Specimens were examined without staining in the EMMA-4 microprobe analyzer. Column voltage was set at 40 kV. A dentine standard was used to calibrate the spectrometers for calcium and phosphorus. One spectrometer was set for the calcium K_α radiation, using a lithium fluoride diffracting crystal. The other spectrometer was set for the phosphorus K_α radiation, using a penta-erythritol (PET) crystal. The regions of interest were probed for intervals of 20, 40, or 100 s. Background counts were determined by probing the same areas with the spectrometers offset by 0.080 inch. In some instances a length of membrane was probed with an astigmatic elliptical spot. After microprobe analysis the areas probed were photographed with the EM 300 to provide a record of the precise location of the probe spot, which could be recognized because of the beam damage done to the specimen.

The results of probing different areas were compared by computing the values for R, given by:

$$R = \frac{P - b}{W - W_b}$$

where P is the peak count; b is the count obtained from the spectrometer offset by 0.080 inch; W is the "white" count obtained from a third, nondispersive detector receiving radiation directly from the specimen; and W_b is the white count obtained by probing a specimen-free region of the grid. The latter value permits correction for extraneous X rays such as those produced by backscattered electrons hitting parts of the column. The value of R is a relative measure of elemental concentration within an entire analyzed microvolume. These volumes typically were larger than individual deposits and did not consist solely of deposits.

Separate analyses were done with a Kevex energy dispersive X-ray spectrometer with a Si-(Li) detector attached to the EMMA-4. Specimens were prepared as described above except that osmium treatment was omitted. This technique facilitated spectrum scans to determine if other elements were present in

[1] Hillman, D. E., and R. Llinás. 1974. Calcium-containing electron-dense structures in the axons of the squid giant synapse. *J. Cell Biol.* 61:146.

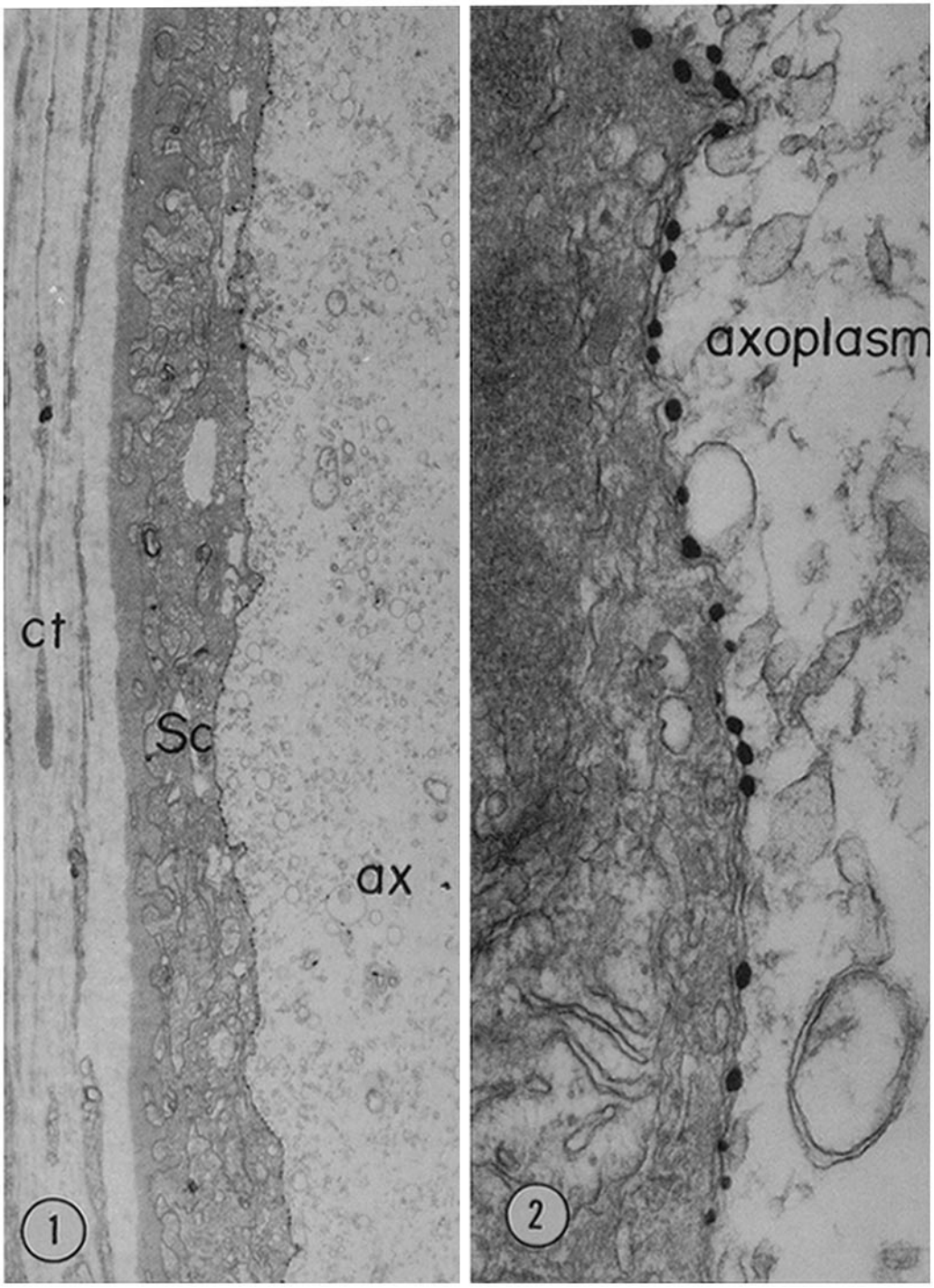

FIGURE 1 Survey view of axon (*ax*), Schwann cell sheath (*Sc*), and connective tissue (*ct*). Deposits occur at interface between axoplasm and Schwann cell. × 9,000.

FIGURE 2 Detail of calcium deposits along axolemma. Schwann cell cytoplasm shown at left. Glutaraldehyde, osmium, stained section. Average size of deposits is about 0.05 μm. × 61,000.

the deposits, and provided data on the relative amounts of Ca and P.

For quantitative estimation of Ca/P ratios, background-corrected integral counts were obtained from quantum-energy bands around the calcium and phosphorus spectral peaks.

RESULTS

Electron Microscopy

When calcium ions are present in the solutions used for processing squid axons for electron microscopy, electron-opaque deposits occur along the interface between axoplasm and Schwann cell sheath. Figs. 1 and 2 illustrate these deposits at low and high magnifications, respectively. The deposits appear to have a globular shape and are most frequently associated with the axonal membrane rather than with the nearby Schwann cell membrane. Some deposits appear to be located within the narrow interspace between axonal and Schwann cell membranes, but this could be due to curvature of the membrane within the thickness of the section.

An unstained specimen that was fixed only in glutaraldehyde is illustrated in Fig. 3. Since no heavy metals were added to this specimen during preparation, the deposits must be due to calcium, the only cation present, along with tissue components with intrinsic electron-scattering power.

Measurements of the size and frequency of deposits along the axolemma are given in Table I. Although the data are based on only three axons, enough deposits have been measured to give reliable values of the size and spacing of the deposits. The values for the distance between deposits are for comparative purposes only, as they have not been corrected for the thickness of the sections, which is about 3–4 times the average diameter of the deposits.

Deposits are sometimes observed on the basal membranes of Schwann cells (Fig. 4), but they are generally smaller (0.01–0.028 μm) than those along the axonal membranes. Deposits were also observed within mitochondria, and, occasionally, free within the cytoplasm (inset, Fig. 4). Similar deposits within mitochondria are amply illustrated in the literature (reviews 6, 7), and are thus not described in detail here.

Microprobe Analysis

The results obtained with the analytical microscope are shown in Table II and summarized in

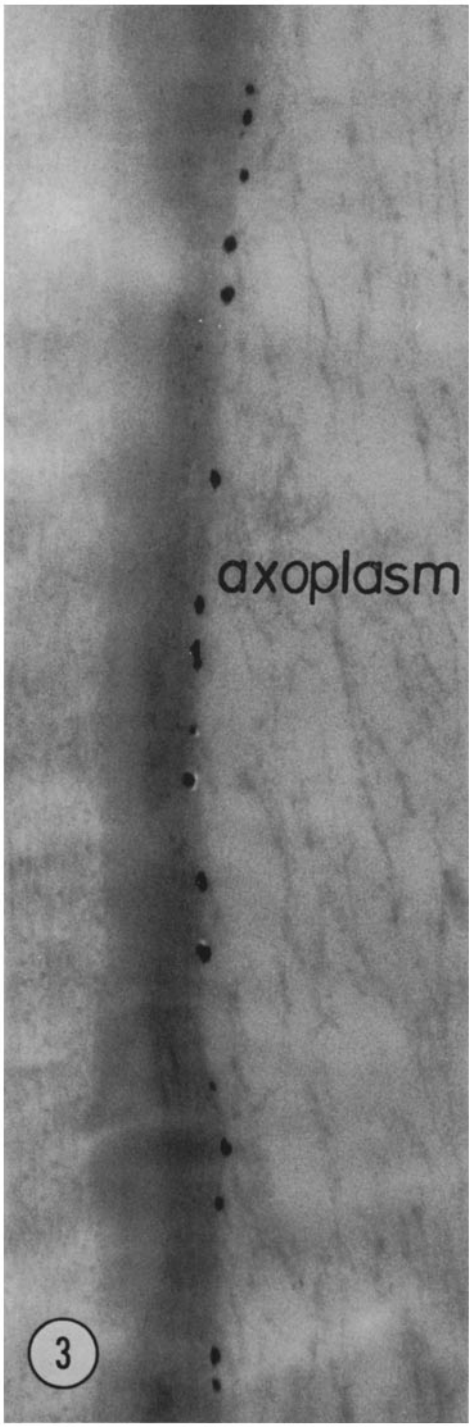

FIGURE 3 Fixation in glutaraldehyde only. Unstained section. Deposits are of similar size and shape to those in osmium-treated specimens. × 33,000.

TABLE I

Measurements of Deposit Size and Frequency

Axon	Fixation	Length of membrane examined	Number of deposits	Average diameter	Range in diameter	Average frequency*	Range of separation*
		μm		μm	μm		μm
1	Glutaraldehyde, osmium	72	181	0.053	0.03–0.08	2.5/μm	0.02–1.43
2	Glutaraldehyde, osmium	323	2,932	0.031‡	0.014–0.128	9/μm	0.02–1.16
3	Glutaraldehyde	52	147	0.045	0.021–0.081	2.8/μm	0.05–1.43

* Not corrected for section thickness.

‡ Based on 138 deposits.

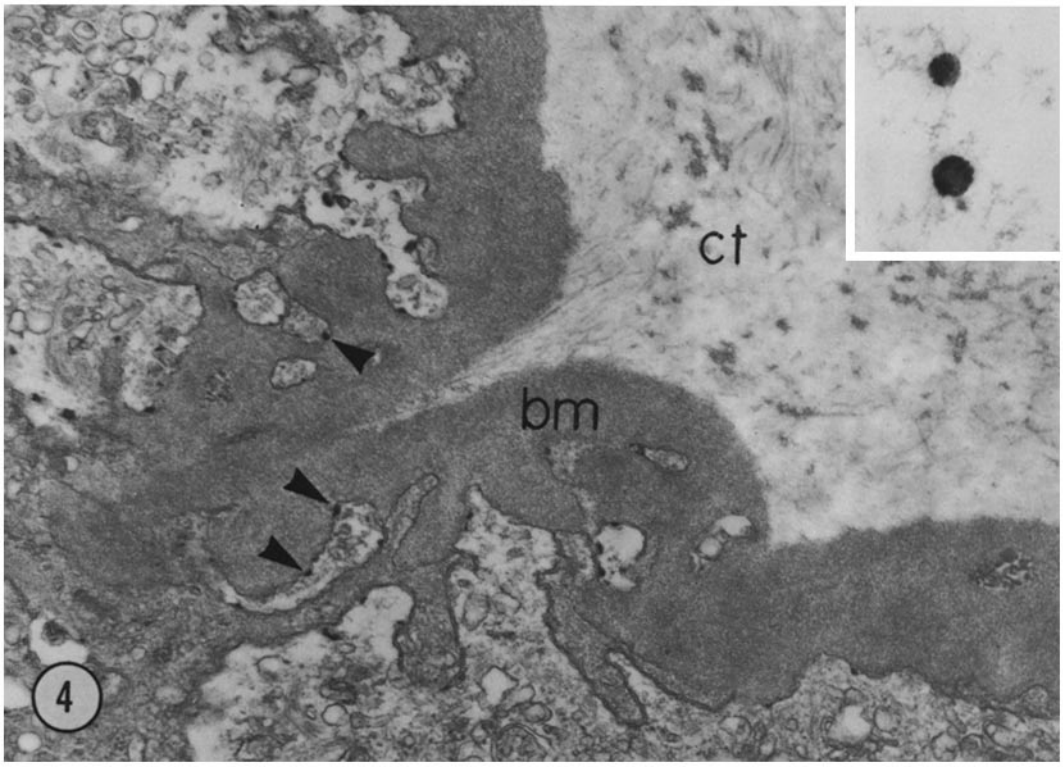

FIGURE 4 Schwann cell basal surface, basement membrane (*bm*), and connective tissue (*ct*). Deposits are present on basal plasma membrane (arrows). These are much smaller than those on axonal plasma membrane. × 26,000. *Inset.* Deposits found within the axoplasm. Probes of these deposits also gave strong signals for Ca and P. × 50,000.

Table III. Probes of the membrane consistently gave strong signals for calcium and for phosphorus, whereas the axoplasm did not. Signals varied in intensity because of differences in the number of deposits and the plane of section through the membrane. One area with a large accumulation of deposits gave very strong signals (spot 11). Probes of the Schwann cells are more difficult to interpret, as the spot size was not small enough to avoid some overlap with the Schwann cell plasma membranes, which also have small deposits. The Schwann cells appear to interdigitate extensively, and we were unable, with the unstained sections used in the microprobe, to distinguish the cytoplasm of the Schwann cells from their thick basement membranes, as these areas were of similar

TABLE II

Microprobe Data

Spot	Location	Calcium	Phosphorus
		R × 10^{-5}	
1	Membrane	28	26
2	"	31	6
3	"	23	19
4	"	86	20
5	"	67	43
6	"	44	32
7	"	39	18
8	"	75	66
9	"	76	24
10	"	80	77
11	"	293	161
12	Axoplasm	3	3
13	"	3	5
14	"	10	—
15	Schwann cell	22	27
16	"	17	24
17	"	29	17
18	"	23	16
19	"	6	—
20	"	75	64
21	"	85	45
22	Mitochondria	20	6
23	"	6	2
24	"	189	52
25	"	351	93
26	Granule	186	77

TABLE III

Averages of Microprobe Analyses

Location	Calcium	Phosphorus
	R × 10^{-5}	
Membrane	76	45
Axoplasm	5	2
Schwann cell	37	27
Mitochondria	141	38

density. When a Schwann cell which appeared to have a large area free of deposits was selected, the probe yielded a signal that was very low in both Ca and P radiation (spot 19).

Four mitochondria were analyzed, and two of them gave very strong calcium signals, although phosphorus signals were proportionately lower compared to membrane associated deposits and Schwann cell probes. The mitochondria that provided the strong signals (spots 24 and 25) contained large dense deposits.

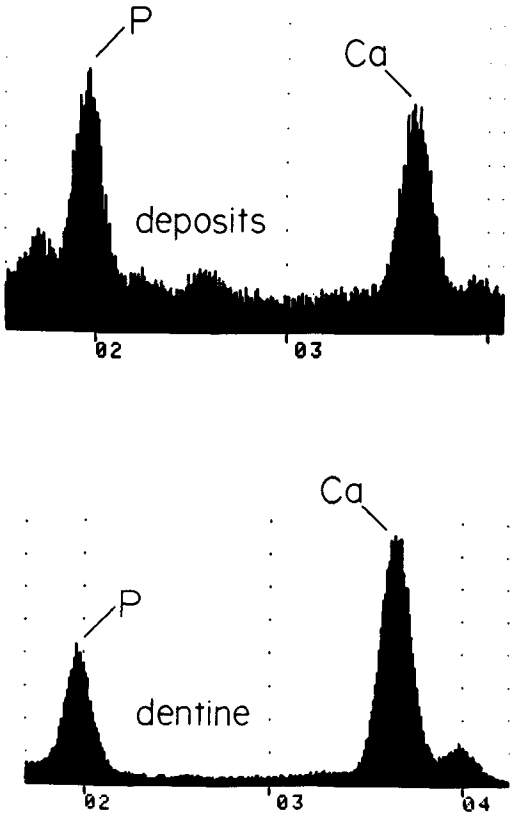

FIGURE 5 X-ray energy spectra from deposits associated with membrane compared with dentine (apatite) standard. Axon fixed in glutaraldehyde-calcium, with no heavy metals (Os, Pb, U) added, as in Fig. 3. Ca/P atomic ratio for deposits is about 7:10, based on average of eight separate background-corrected counts obtained from narrow energy bands around the Ca and P spectral peaks. Spectra obtained with energy dispersive X-ray analyzer during 40 s counting time.

We also found occasional small granules unassociated with either mitochondria or membranes (see inset, Fig. 4). These cytoplasmic granules also gave strong signals for Ca and P (spot 26).

Energy dispersive X-ray analysis confirmed the presence of Ca and P in the deposits and permitted graphic representation of the signals (Fig. 5) relative to those from a dentine standard. The deposits appear to have lower Ca/P than dentine. The Ca/P signal ratio for dentine was about 3:1, corresponding to an atomic ratio of 5:3. The signal ratio for the deposits was about 5:4, corresponding to an atomic ratio of 7:10. The spectra did not reveal other X-ray lines except for those from Cl (probably from the embedding resin) and Si

(probably from the detector), and those expected from the specimen rod and grids.

DISCUSSION

Composition of the Deposits

It is worthwhile to review previous evidence indicating that the deposits adjacent to the membranes contain calcium atoms. We draw upon studies of other tissues, but it must be kept in mind that the properties of the deposits may vary from one tissue to another.

(*a*) Electron-opaque granules are found along apical and lateral membranes of various intestinal epithelia when calcium is present in the fixative, but the deposits are difficult to find or are absent when calcium-free solutions are used (1, 2).

(*b*) Deposits are removed when the tissue is treated with EDTA (1).

(*c*) When autoradiography is used to follow intestinal uptake of orally administered ^{45}Ca in rats, the autoradiographic grains as well as the electron-opaque deposits are found mainly in the microvilli and mitochondria (10).

(*d*) Microincineration of sections of rat intestine leaves the granules intact, indicating high mineral content (10).

(*e*) Histochemical studies employing potassium pyroantimonate show opaque deposits along the plasma membranes of a variety of tissues (e.g., 11–14). Calcium pyroantimonate is relatively insoluble (11, 15) and, in one study (12) microprobe analysis revealed that the antimonate deposits contain large amounts of calcium.

The microprobe data reported here confirm that calcium is present in the deposits found in squid axon. In addition, significant phosphorus signals were obtained. Use of nickel grids and titanium specimen holder enabled us to rule out fourth-order copper interference as the source of the signals obtained when the spectrometer was set for the phosphorus K_α line. It is not yet clear, however, how much of the phosphorus signal comes from structures adjacent to the deposits (e.g., membrane phospholipids and Schwann cell cytoplasm). A useful control would be to probe deposit-free areas of the membrane to see if a phosphorus signal could be obtained. Since areas without deposits occurred where the cells and membranes appeared damaged, it seemed that these regions would not provide valid controls. We suspect that much of the phospholipid is extracted during dehydration, particularly after fixation in glutaraldehyde alone (16, 17). However, the deposits may contain some organic matter such as membrane phospholipid or phosphoprotein.

One can estimate the number of calcium atoms in each granule. The average diameter of the deposits is 0.053 μm (axon 1, Table I), corresponding to about 5×10^{-17} g of calcium (on the basis of calcium phosphate) or about 800,000 calcium atoms. Since the deposits probably contain some organic matter (based on the decrease in size upon microincineration) (10), the estimate is a high one.

The deposits in mitochondria had higher Ca/P ratios than those on the membranes. These results, which are considered preliminary, could indicate that some of the mitochondrial calcium is combined with groups other than phosphate.

Calcium Fluxes During the Action Potential

Calcium entry during the action potential is closely associated with the Na^+ and K^+ conductance increases that, respectively, depolarize and repolarize the membrane (18, 19). However, the precise role of calcium in propagation of the action potential is not understood.

Hodgkin and Keynes (20) studied the effects of electrical stimulation on the inward calcium flux. They stimulated squid axons in ^{45}Ca sea water and then extruded the axoplasm and measured its isotope concentration. They estimated that during each action potential calcium enters in the amount of 36 $Ca^{++}/\mu m^2$, which is roughly $\frac{1}{700}$ of the net Na^+ entry. However, our findings indicate that inwardly diffusing calcium ions could be sequestered at the plasma membrane. After fixation the concentration of calcium contributed by the deposits is about 0.1 mM/kg wet weight of axoplasm.[2] The concentration of ionized calcium is

[2] For example, an axon 5.1 cm long and 0.465 mm in diameter has a wet weight of 6.6 mg and contains about 6.0 mg of axoplasm. This axon has a surface area of 74.6 mm^2 and has 6.25×10^6 deposits per mm^2 if one assumes 2.5 deposits per μm (see Table I). Average diameter of deposit is 0.053 μm, corresponding to 5×10^{-17} g Ca per deposit (on basis of calcium phosphate). With these values one obtains the figure 2.33×10^{-8} g Ca per 74.6 mm^2 axon surface or per 6.0 mg axoplasm; this corresponds to 0.096 mM Ca/kg wet weight of axoplasm (Prusch, R. D., B. J. Wall, and J. L. Oschman. Calcium ions associated with the plasma membrane in squid axon. Biol. Bull. [Woods Hole]. **145**:451. [Abstr.]).

probably much lower (about 0.003 mM/kg wet weight in *Loligo forbesi*) (18). If the deposits provide an accurate indication of the binding capacity of the membrane, the membrane may aid in buffering the intracellular calcium concentration. If this is correct, tracer fluxes may underestimate the movement of calcium across the membrane since labeled calcium may become adsorbed on the inner surface of the plasma membrane, where it would be unable to contribute to the radioactivity of the extruded axoplasm. The significance of tracer measurements is thus open to doubt until we know how the deposits form.

Source of the Deposits

In view of the uncertainty about the significance of the deposits and the way they form, it seems worthwhile to mention some of the possibilities.

(*a*) As calcium enters the cell during fixation, it forms an insoluble complex with some diffusible intracellular anion (e.g., phosphate, carbonate, citrate, lactate, oxalate, protein, ATP, or ADP).

(*b*) The fixative exposes negatively charged sites not normally available for calcium binding. For example, it is known that glutaraldehyde reacts mainly with basic residues, rendering proteins more acidic (21, 22). It is also possible that fixation could change protein conformation, exposing charged sites.

(*c*) Deposits may be caused by "tri-complex flocculation" of calcium, osmium, and phospholipid (23). This seems unlikely because the deposits form and are of similar size and shape when tissues are fixed only in glutaraldehyde, and osmium is not present to complex with calcium and lipid.

(*d*) Calcium ions are associated with the membrane surface as part of the diffuse double-layer or Stern layer, but when the tissue is transferred to a nonaqueous environment during dehydration, the membrane components rearrange to form the deposits. This might be expected because glutaraldehyde fixation alone should leave the membrane lipids in a more labile state (16). For example, retention of phosphatidyl ethanolamine in rat liver is 99% after glutaraldehyde-osmium fixation but only 53% after glutaraldehyde alone (17). However, there does not seem to be a difference in the size or shape of the deposits when axons are fixed only in glutaraldehyde (cf. Figs. 2 and 3).

(*e*) Ionized calcium is normally low in the axoplasm due to the relatively low permeability of the axonal plasma membrane to calcium and due to the action of a calcium extrusion pump (reviewed by Baker, reference 18). Glutaraldehyde causes an increase in membrane permeability (e.g. 24), allowing calcium to enter. Sites along the membrane (possibly acidic phospholipids or phosphoproteins) that are normally sparsely occupied by calcium take up the calcium as it enters and form a relatively insoluble complex. In support of this hypothesis is the recent finding of Alemà et al. (25) of a calcium binding protein (CBP) in squid axon. It is possible that the technique we have used actually localizes CBP or precipitates it at the cell surface. The deposits observed in rat intestinal brush border (1, 2) correspond well with the localization by immunofluorescence of CBP in the same tissue (26). The finding of high phosphorus signals from the deposits would be consistent with a P-rich protein. Although the phosphorus content of squid axon CBP has not been determined, a CBP isolated from pig brain is a phosphoprotein, and it is likely that the phosphate groups are responsible for its calcium-binding properties (27).

(*f*) The deposits observed with glutaraldehyde-calcium fixation are similar to those obtained after incubation for adenosine triphosphatase (ATPase) localization (28). It has occurred to us that glutaraldehyde-calcium fixation may localize sites of ATPase activity by using endogenous ATP as the substrate and calcium from the fixative as the trapping agent to precipitate inorganic phosphate as it is released. Squid axons contain about 1 mM ATP per kg of axoplasm (20), a concentration comparable to that used in histochemical reactions for ATPase.

CONCLUSIONS

This study confirms previous evidence that the dense deposits that form adjacent to the membranes during fixation in the presence of calcium ions are indeed made up of calcium atoms. Identification of significant concentrations of phosphorus in the deposits suggests that the anion may be phosphate. Further study will be needed to determine if the deposits are related to increases in density of axonal membranes following electrical stimulation observed by Peracchia and Robertson (29) and to determine how the deposits form. The deposits we observe are strikingly similar to the reaction product obtained with ATPase localization (Sabatini et al., reference 28). However, more information is needed before we can determine if

the deposits localize a differentiated portion of the membrane such as an ion carrier or channel, or if they form by precipitation of calcium with a diffusible intracellular anion. Finally, it is important to consider the relative significance of mitochondria and plasma membranes in buffering intracellular calcium concentration.

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