LOCALIZATION OF CALCIUM IN PRESYNAPTIC NERVE TERMINALS

An Ultrastructural and Electron Microprobe Analysis

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ABSTRACT

Ultrastructural techniques and electron probe microanalysis were used to determine whether or not the smooth endoplasmic reticulum (SER) within presynaptic nerve terminals is a Ca-sequestering site. The three-dimensional structure of the SER was determined from serial sections of synaptosomes. The SER consists of flattened cisterns that may branch and are frequently juxtaposed to mitochondria.

To investigate intraterminal Ca sequestration, synaptosomes were treated with saponin to disrupt the plasmalemmal permeability barrier. When these synaptosomes were incubated in solutions containing Ca, ATP, and oxalate, electron-dense Ca oxalate deposits were found in intraterminal mitochondria, SER cisterns, and large vesicular profiles. Saponin-treated synaptosomes that were incubated in the presence of mitochondrial poisons contained electron-dense deposits within SER cisterns and large vesicular profiles, but very rarely in mitochondria. Similar deposits were observed within saponin-treated synaptosomes that were not post-fixed with OsO_4, and within saponin-treated synaptosomes that were prepared for analysis by freeze-substitution. Electron-probe microanalyses of these deposits confirmed the presence of large concentrations of Ca.

When oxalate was omitted from the incubation solutions, no electron-dense deposits were present in saponin-treated synaptosomes. In other control experiments, either the Ca ionophore A23187 or the Ca chelator EGTA was added to the incubation media; electron-dense deposits were very rarely observed within the intraterminal organelles of these saponin-treated synaptosomes. The data indicate that presynaptic nerve terminal SER is indeed a Ca-sequestering organelle.

Neurotransmitter release at chemical synapses appears to be critically dependent upon the level of free intraterminal calcium ($[Ca^{2+}]_i$) (35). The entry of Ca during presynaptic depolarization, and the consequent rise in $[Ca^{2+}]_i$, is the immediate trigger for evoked release (cf. 36, 40). The main sites of

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Ca entry are at or near the "active zones", or regions of synaptic contact (28). Some of the Ca may be immediately extruded after repolarization, but much of the Ca is apparently retained within the terminals for many seconds or minutes (27, 55, 56). This residual Ca must be redistributed and buffered by intraterminal components until it is finally extruded across the plasma membrane.

One aspect of Ca buffering is the binding of Ca to various cytoplasmic proteins and to the surfaces of organelles. A variety of proteins with low affinity for Ca have been isolated from nerve cells (32). Probably more important is the type of high-affinity Ca-binding system characterized by Baker and Schlaepfer (2) in the axoplasm of squid giant axons. Although such Ca-binding ligands may have a high affinity for Ca, they are likely to have a low capacity, with only one or a few Ca-binding sites per molecule; furthermore, the concentrations of the Ca-binding ligands may be limited. Much more efficient buffering can be accomplished by organelles that accumulate Ca.

There is substantial evidence that brain mitochondria are capable of accumulating Ca at the expense of either ATP hydrolysis or the energy derived from electron transport (7, 33). This has led to the suggestion (1) that the mitochondria in nerve terminals play an important part in restoring and maintaining cytoplasmic Ca^{2+} levels.

Several investigators have shown that a nonmitochondrial component of brain preparations can also accumulate Ca at the expense of ATP hydrolysis (29, 53). Recently, Ca sequestration has been studied in preparations of presynaptic nerve terminals (synaptosomes) with plasma membranes disrupted by osmotic lysis or treatment with saponin (7, 8). In these preparations, the nonmitochondrial ATP-dependent Ca^{2+} sequestration system has a higher affinity for Ca^{2+} than does the mitochondrial Ca uptake system; the kinetic properties of the nonmitochondrial Ca transport system are strikingly similar to those of skeletal muscle sarcoplasmic reticulum (8).

A number of investigators have suggested that smooth endoplasmic reticulum (SER) in nerve terminals may sequester Ca^{2+}, possibly at the expense of ATP hydrolysis (7, 17, 34, 43). There is evidence that SER in squid axoplasm (25) and SER in various vertebrate cells (20, 23, 52) can sequester Ca^{2+}. Thus, cytoplasmic Ca buffering may be an important function of SER in many types of cells.

In the present study, we used transmission electron microscopy and electron probe microanalysis to identify the organelles responsible for Ca sequestration in presynaptic nerve terminals. In view of the evidence that $[Ca^{2+}]_i$ may be buffered by the SER, the ultrastructural details of the SER and its relationship to other intraterminal organelles were investigated in both normal, untreated ("intact") and saponin-treated synaptosomes.

MATERIALS AND METHODS

Preparation of Synaptosomes

Presynaptic nerve terminals (synaptosomes) were isolated from Wistar rat brains according to the one-step sucrose gradient method of Hajos (22). The synaptosomes were recovered in a 0.8 M sucrose suspension. The suspension was diluted with 3–4 vol of ice-cold, Ca-free 145 Na + 5 K, over a 20–30-min period, to return the terminals to a more physiological environment. The 145 Na + 5 K solution contained (in millimoles per liter): NaCl, 145; KCl, 5; $MgCl_2$, 1.4; glucose, 10; KH_2PO_4, 2; and HEPES, 20. The solution was adjusted to pH 7.5 with TRIS buffer. After equilibration with the Ca-free 145 Na + 5 K, the synaptosomes were pelleted and then resuspended either in 145 Na + 5 K containing 1.2 mM $CaCl_2$ or in a Ca-free 5 Na + 145 K solution (see below) containing 0.25 mg/ml of saponin. Subsequent handling of the synaptosomes is described below.

Electron Microscopy

NORMAL, UNTREATED SYNAPTOSOMES: Some suspensions of synaptosomes in 145 Na + 5 K + 1.2 mM $CaCl_2$ were incubated for 10 min at 30°C. They were then diluted 1:1 with ice-cold Karnovsky's fixative (26) containing 4% paraformaldehyde, 5% glutaraldehyde and 0.05% $CaCl_2$ in a 0.08 M Na cacodylate buffer adjusted to pH 7.4. The suspensions were fixed for 0.5–2 h, or overnight, at 4°C. The terminals were then pelleted and the pellets were rinsed several times with 0.1 M Na-cacodylate buffer. The pellets were postfixed for 0.5–1 h with 2% OsO_4 in 0.1 M Na cacodylate buffer. The tissue was subsequently dehydrated with an ascending series of alcohols, followed by propylene oxide; it was left overnight in a mixture (1:1, vol/vol) of propylene oxide and Epon-Araldite, and then embedded in Epon-Araldite for 1–2 d at 60°C.

Serial sections, 70-nm thick, were prepared from the embedded tissue. They were poststained with uranyl acetate and lead citrate, and examined in a Siemens 1A electron microscope.

The volume of the SER was obtained by integration, by weight (13, 49), of portions of SER cut from micrographs; the data are expressed as a percentage of the total synaptosomal volume, or a percentage of the total synaptosomal volume minus the mitochondrial volume.

SAPONIN-TREATED SYNAPTOSOMES: For these studies, synaptosome pellets were resuspended in a Ca-free 5 Na + 145 K solution containing 0.25 mg/ml saponin; these suspensions were preincubated at 0°C for 30 min. The composition of the 5 Na + 145 K solution was (in millimoles per liter): NaCl, 5; KCl, 145; $MgCl_2$, 1.4; KH_2PO_4, 2; and HEPES-TRIS, 20 (pH 7.5). The saponin was employed because it renders cholesterol-rich membranes (e.g., the plasma membrane [10, 30]) permeable to molecules and ions such as ATP and oxalate (16); cholesterol-poor membranes (e.g., those of mitochondria and smooth endoplasmic reticulum [30]) are much less affected (cf. 16). With this disrup-

tion of the plasmalemmal permeability barrier, the intraterminal organelles are bathed directly by the incubation medium.

After the preincubation with saponin, aliquots of the suspensions were added to 5 Na + 145 K solutions (pH 7.5) that also contained 10–20 μM $CaCl_2$, 10–20 mM K oxalate, 2 mM ATP, 5 mM phosphoenol pyruvate, and 5–15 U/ml of pyruvate kinase. The final protein concentration was ~0.4 mg/ml. Saponin-treated synaptosomes were incubated with Ca^{2+}, ATP, and K oxalate to promote Ca^{2+} accumulation in the Ca-sequestering organelles (7). Oxalate enhances Ca^{2+} sequestration, presumably by precipitating Ca within the organelles; the free Ca^{2+} within the organelles is thereby lowered so that more can be accumulated (7).

A variety of experimental conditions were tested. Oxalate was omitted from some incubation media and in some instances the incubation media contained other constituents, such as 10 μM FCCP (carbonylcyanide *p*-trifluoromethoxyphenyl-hydrazone; E. I. DuPont de Nemours, Wilmington, Del.), or a combination of 1.2 mM DNP (2,4-dinitrophenol), 1.2 mM NaN_3 (sodium azide), and 0.7 μg/ml oligomycin. In other experiments, the incubation solutions contained 10 μM A23187 (Eli Lilly, Indianapolis, Ind.), or sufficient EGTA to reduce the free Ca^{2+} levels to $<10^{-8}$ M. Details are given under Results.

The diluted suspensions were incubated for 10 min at 30°C. Incubations were terminated by adding an equal volume of ice-cold, Ca-free Karnovsky's fixative (26) with 10–20 mM K oxalate. Tissue was processed for electron microscopy as described above, with the following exceptions: (*a*) all buffers and fixatives contained 10–20 mM K oxalate, (*b*) some preparations were not postfixed with OsO_4 (see Results), and (*c*) thin sections were not poststained.

Unstained, carbon-coated, 100-nm-thick sections prepared from these tissue samples were subjected to electron probe microanalysis.

Ca Localization by Electron Probe Microanalysis

Elemental analyses were made on a Philips EM400 microscope equipped with a field emission gun operated at 80 kV, and interfaced with a 30-mm^2 Si (Li) energy dispersive detector and model 7000 multichannel analyzer (Kevex Corp., Foster City, Calif.). The electron beam, focused to 50–70 nm Diam, was used for analyses of SER, mitochondria, and cytoplasm in the synaptosomes. Details of the method, its validation, and the sensitivity of the instrumentation have been published elsewhere (46, 47).

Ca was identified by its characteristic K_α peak at 3.69 keV. For comparison of SER, mitochondria, and cytoplasm, all analyses (within a single synaptosome) were made with the same probe parameters (i.e., current, spot diameter, and analysis time). This procedure allows a comparison between two regions analyzed, on the basis of the absolute number of counts in a given peak, which is proportional to the amount of that element in the volume analyzed. Quantitation, such as that used on freeze-dried thin sections (50, 51), and based on the ratio of x-ray characteristic peak counts to the x-ray continuum counts, was not performed because the synaptosomes were embedded in plastic and, in most experiments, were stained with OsO_4.

RESULTS

Morphology of SER

A majority of the synaptosomes incubated in physiological salt solutions were spherical structures and had diameters in the range of 0.5–0.8 μm. These terminals contained a variety of organelles including SER, mitochondria (usually one or two per terminal), numerous small synaptic vesicles, occasional coated vesicles, and microtubules. Fig. 1 shows a set of serial sections of a single nerve terminal in which most of these organelles are observed.

In single planes of section, the SER appeared as membrane-bounded tubular profiles and isolated vesicles. However, serial sections of synaptosomes (such as those in Figs. 1, 3, and 10) showed that the profiles were usually parts of flattened sacs or cisterns, or cross sections of cistern branches. The SER cisterns usually extended through several consecutive serial sections (as in Figs. 1, 3, and 10). They were frequently, although not always (e.g., Fig. 3), situated in juxtaposition to mitochondria, as in Fig. 1. Fig. 2 shows drawings of the SER and the large, horseshoe-shaped mitochondrion reconstructed from the serial sections of the synaptosome in Fig. 1. These drawings depict the close structural relationship between the two organelles.

A noteworthy, but unusual, finding was the beaded appearance of the SER cistern in the first section (*a*) of the synaptosome in Fig. 3. This type of structure could be misinterpreted: in the single thin section, it has the appearance of a string of neurotransmitter-storing synaptic vesicles. A more significant concern is the probability that cross sections of SER branches (cf. Fig. 1) resemble synaptic vesicles. In such cases, which may be quite common, the profiles would not be recognized as branches of SER; this would lead to an underestimate of the distribution and volume of SER. Oblique orientation of the narrow SER cisterns would also result in an underestimate of SER volume in single views of synaptosomes. The volume of the SER in Fig. 1 was 1.8% of the total synaptosome volume and 2.1% of the synaptosome volume minus the mitochondrial volume. Similar volumes were obtained for the SER of the synaptosomes in Fig. 3 (2.0 and 2.7% of the total and mitochondria-free volumes, respectively) and Fig. 10 (1.7 and 2.0%, respectively).

Calcium Localization

PUNCTATE, ELECTRON-DENSE PRECIPITATES: Some tissue was fixed in Ca-free Karnovsky's solution (26) at 4°C, and then left overnight either in fixative or in Na cacodylate buffer at 4°C, and postfixed in OsO_4; this tissue was

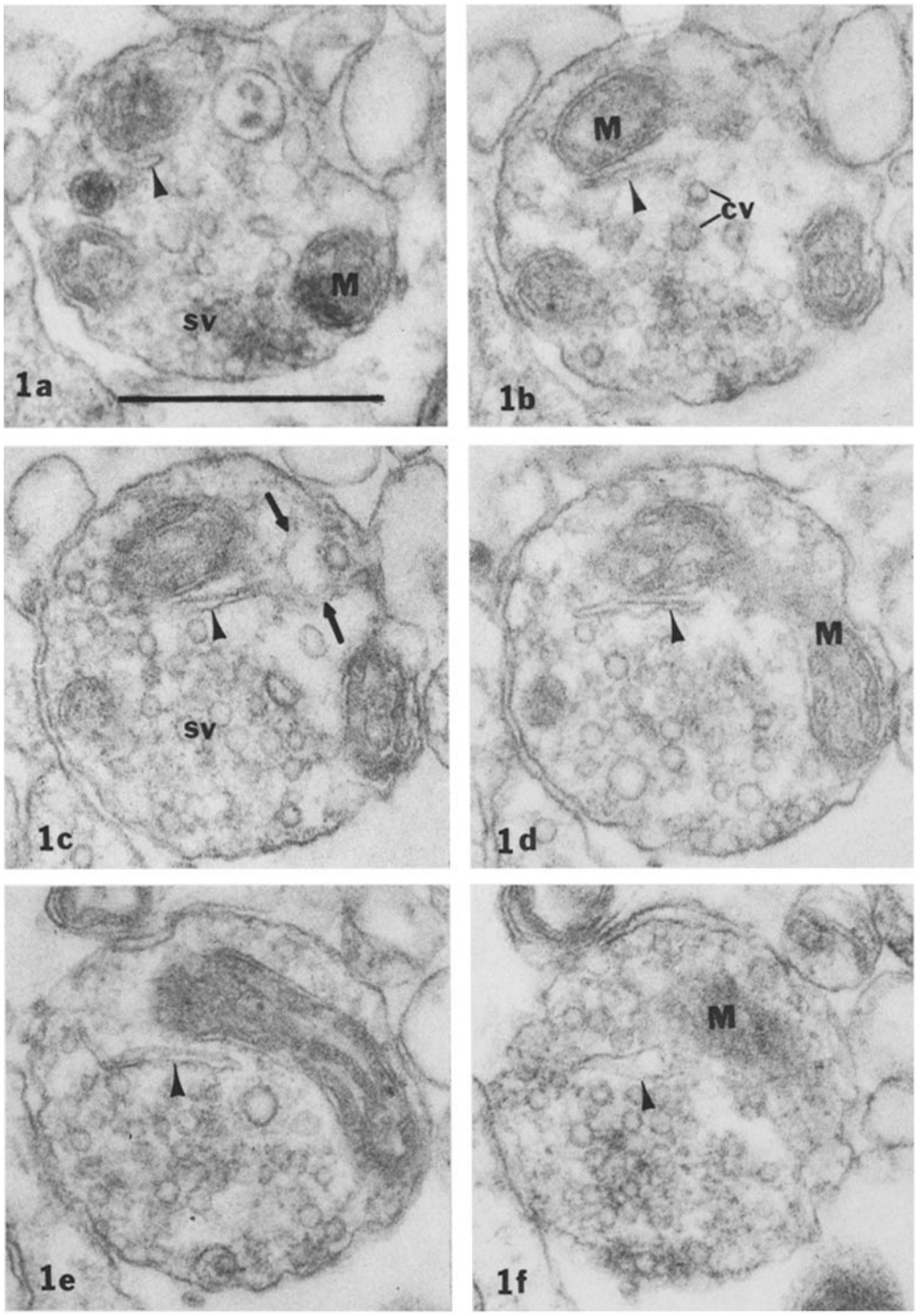


FIGURE 1 (*a–f*) Consecutive serial thin sections (70-nm thick) of an untreated synaptosome containing an SER cistern (arrowheads) in close proximity to a mitochondrion (*M*), synaptic vesicles (*sv*), and coated vesicles (*cv*). In section 1 *c*, the SER cistern has two branches that extend to the plasmalemma (arrows). Stained with uranyl acetate and lead citrate. Bar, 0.5 μm on all micrographs. × 77,000.

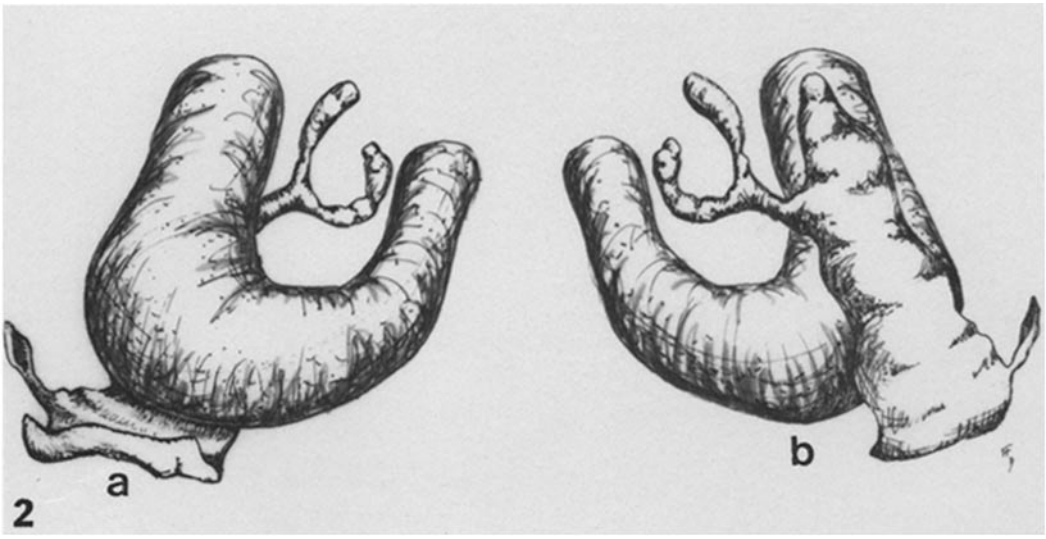


FIGURE 2 Drawings of a three-dimensional reconstruction of the SER and mitochondrion of the synaptosome in Fig. 1. (*a*) The sections were "stacked" so that the mitochondrial arms and SER profiles of serial section 1 *a* are in the background, whereas the curve of the horseshoe-shaped mitochondrion and the SER profile of section 1 *f* are depicted in the foreground. (*b*) The mitochondria and SER were rotated 180° around an axis parallel to the plane of the paper. Thus, the SER cistern, lodged against one of the mitochondrial arms, can be visualized in its entirety.

frequently found to contain small, punctate, electron-dense precipitates similar to those described by Gray and Paula-Barbosa (21). As illustrated in Fig. 4, these precipitates appeared to line the inner surfaces of the SER membranes; they were also associated with some synaptic vesicle membranes, extrasynaptosomal vesicular membranes, and contaminating myelin. The precipitates were observed in normal, untreated synaptosomes as well as in saponin-treated synaptosomes, whether or not Ca and oxalate were included in the incubation media.

Structures containing the small punctate precipitates were probed for elemental content. Fig. 5 *a* shows a representative electron probe spectrum obtained from an SER cistern lined with precipitates (as in Fig. 4). The spectrum contains a prominent osmium peak and a small, but significant, peak at 3.69 keV, the K_α line characteristic for Ca. The osmium peak is considerably larger than that in the spectrum obtained from the adjacent cytoplasm (Fig. 5 *b*), and there is no detectable Ca peak in the latter spectrum.

Tissue that was fixed with aldehydes for 0.5–2 h, immediately rinsed in Na cacodylate buffer, and postfixed with OsO_4 contained only a very few punctate deposits. When postfixation with OsO_4 was omitted, the aldehyde-fixed tissue did not contain these punctate precipitates. Unfixed tissue that was frozen in liquid Freon-22, and then freeze-substituted with an osmium-acetone solution at −80°C (19, 54), also did not contain punctate precipitates. These observations suggest that the punctate precipitates may be an artifact, possibly caused by the combination of treatment with aldehydes and postfixation with OsO_4 (cf. 21).

CA OXALATE DEPOSITS: Saponin-treated synaptosomes that were incubated in the presence of Ca, ATP, and oxalate contained large electron-dense deposits that were readily distinguishable from the punctate precipitates and were presumed to be Ca oxalate. The majority of the large deposits were located in the mitochondria (Fig. 6). Many SER cisterns (Fig. 7) and vesicular profiles of various sizes (Fig. 8), perhaps cross sections of SER cisterns or branches (see above), also contained deposits. On rare occasions, deposits appeared to be free within the cytoplasm. In one typical experiment, of 100 intraterminal electron-dense deposits counted in single thin sections, 71 deposits were localized in the mitochondria, 27 deposits were localized in SER cisterns or large vesicular profiles, and 2 deposits did not appear to overlie any visible organelle.

A number of the large, electron-dense deposits were analyzed for elemental content, and the presence of Ca was confirmed. The electron probe spectra obtained from the dense deposits within the two mitochondrial profiles of Fig. 6 *a* both have large Ca peaks (Fig. 6 *b* and *c*). There was no detectable Ca in the cytoplasm (region labeled *cyt* in Fig. 6 *a*) between the mitochondria (see Fig. 6 *d*). All of the spectra have significant osmium (1.9–2.0 keV) and chlorine (2.62 keV) peaks because the tissue was postfixed with OsO_4 and the embedding medium contained chlorine. Large Ca peaks are also seen in the spectra (Figs. 7 *b* and 8 *b*, respectively) obtained from the deposits illustrated in Figs. 7 *a* and 8 *a*. On the other hand, no Ca peaks are observed in the spectra from nearby cytoplasm or from a mitochondrion without a dense deposit (Fig. 7 *c*). Spectra obtained from regions adjacent to electron-dense deposits within mitochondria also did not have Ca peaks. Ca that was not precipitated (i.e., by oxalate) within synaptosomes during the incubation was probably extracted during the fixation and dehydration procedures. This would explain why Ca was not detected in the regions of Ca-sequestering organelles without deposits.

The mitochondria in the synaptosome of Fig. 9 contain two large holes (indicated by arrowheads); these holes may be the sites occupied by electron-dense deposits that were lost during preparation of the sections (25). Electron probe analyses frequently indicated the presence of Ca at the edges of holes, such as those shown in Fig. 9.

Fig. 10 shows a set of serial sections of a syn-

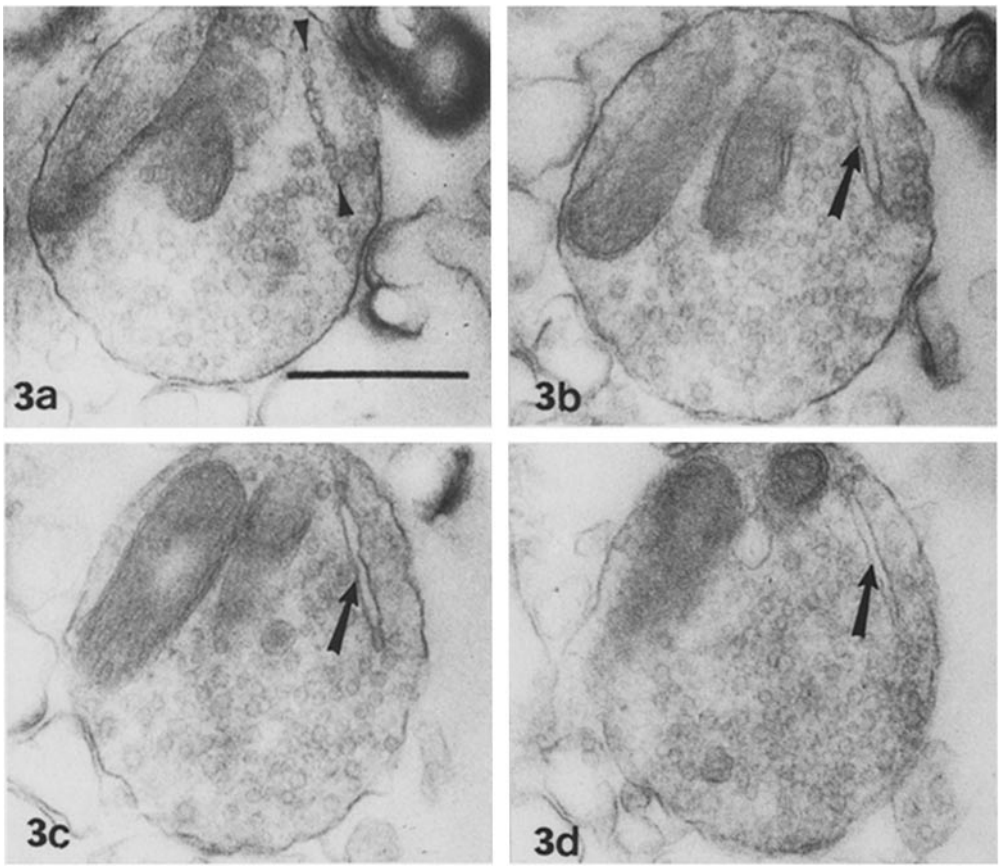
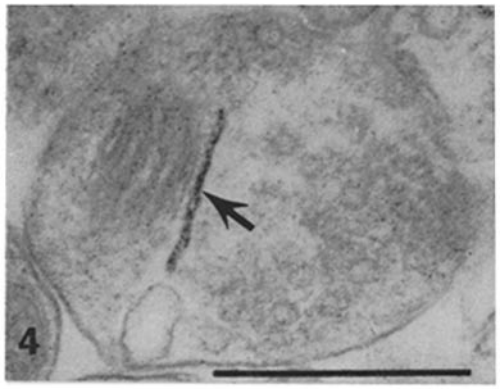


FIGURE 3 Serial thin sections (70-nm thick) of a saponin-treated synaptosome containing an SER cistern. (*a*) The cistern appears beaded and may be mistaken for synaptic vesicles (between arrowheads). (*b–d*). However, the consecutive serial sections illustrate that these "synaptic vesicles" make up one end of the SER cistern (arrows) that is not entirely in the plane of section. (*a–d*). Unstained. × 47,800.



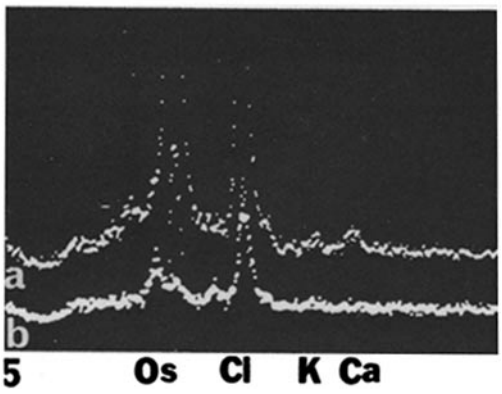


FIGURE 4 Small punctate precipitates line the SER cistern (arrow) and some synaptic vesicle membranes of a saponin-treated synaptosome. Unstained, 70-nm-thick section. Tissue was fixed with aldehydes and postfixed with OsO_4. × 70,200.

FIGURE 5 Electron probe spectra obtained from an SER cistern (*a*) and from adjacent cytoplasm (*b*). The cistern contained punctate precipitates (similar to those in Fig. 4). The spectrum from the SER cistern has a large osmium peak, which overlaps with a P peak, and a much smaller but significant Ca peak. There is no significant Ca present in the cytoplasm (*x*-axis = keV; *y*-axis = number of counts in all electron probe spectra).

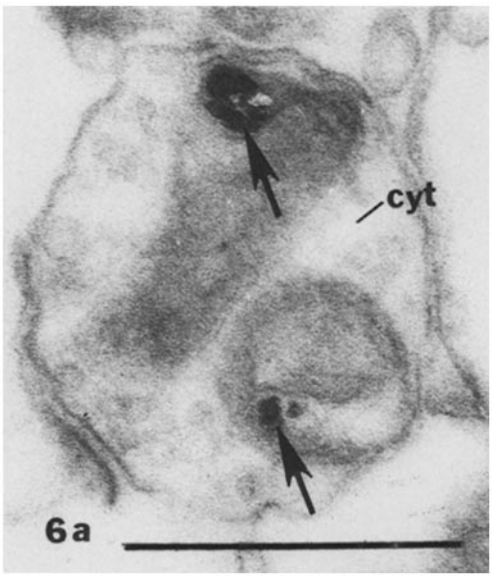
aptosome that has an electron-dense deposit located in a SER cistern. Typically (as in this figure), an electron-dense deposit is observed in only one plane of section of the cistern. A possible explanation for this is that the electron-dense deposits form around nidi and are not distributed diffusely within the SER and mitochondria.

The appearance and electron diffraction pattern of the dense Ca deposits were typical of Ca oxalate (3). The deposits lost mass as the electron beam was focused on them; this "Swiss cheese" effect of the electron beam on Ca oxalate crystals has been described by Carsten and Reedy (12). The Ca was not associated with phosphorus or sulfur (Figs. 8*b*, 11, and 12*b*) or with any other inorganic anion of atomic number >11. Although large errors are introduced as a result of the overlap of the P-K and Os-M x-ray lines, the osmium peaks were stripped from each spectrum and in no instance were the Ca deposits associated with phosphorus. This was confirmed in analyses of deposits in unosmicated tissues (Fig. 11). Moreover, electron-dense deposits were not present in synaptosomes incubated in the absence of oxalate. These considerations provide strong evidence that the large electron-dense deposits are composed of Ca oxalate.

Unlike the punctate precipitates described previously, the Ca oxalate deposits were observed in unosmicated, aldehyde-fixed tissue and in synaptosomes that were freeze-substituted in an osmium-acetone solution. In both preparations the Ca oxalate deposits were observed in the mitochondria and other regions of the synaptosomes. The precise subcellular localization of nonmitochondrial deposits could not be determined because the tissue prepared by these methods exhibited very little contrast in the electron beam. Electron probe analyses of these deposits provided spectra (see dotted spectrum in Fig. 11) similar to those spectra obtained from the electron-dense deposits in aldehyde-fixed, osmicated synaptosomes (e.g., Figs. 6*b* and 7*b*). These data indicate that the formation of the Ca oxalate deposits is not an artifact of the fixation methods.

EFFECTS OF VARIOUS INCUBATION MEDIA ON THE LOCALIZATION OF CA DEPOSITS:

As already noted, synaptosomes incubated in the presence of Ca, ATP, and oxalate contain electron-dense Ca deposits in mitochondria and elements of SER. When the mitochondrial uncoupler FCCP or a combination of three mitochondrial poisons (DNP, NaN_3, and oligomycin) was added to the incubation media, the electron-dense deposits were found primarily in SER cisterns and vesicular profiles; very rarely were they seen in mitochondria when the inhibitors were present. In one representative experiment, we examined single thin sections to determine the effects of mitochondrial poisons on the frequency of electron-dense deposits. We observed deposits in 68 of 500 (13.6%) intraterminal mitochondria of Ca-loaded synaptosomes, whereas, in the presence of poisons, only 12 of 1500 (0.8%) intraterminal mitochondria



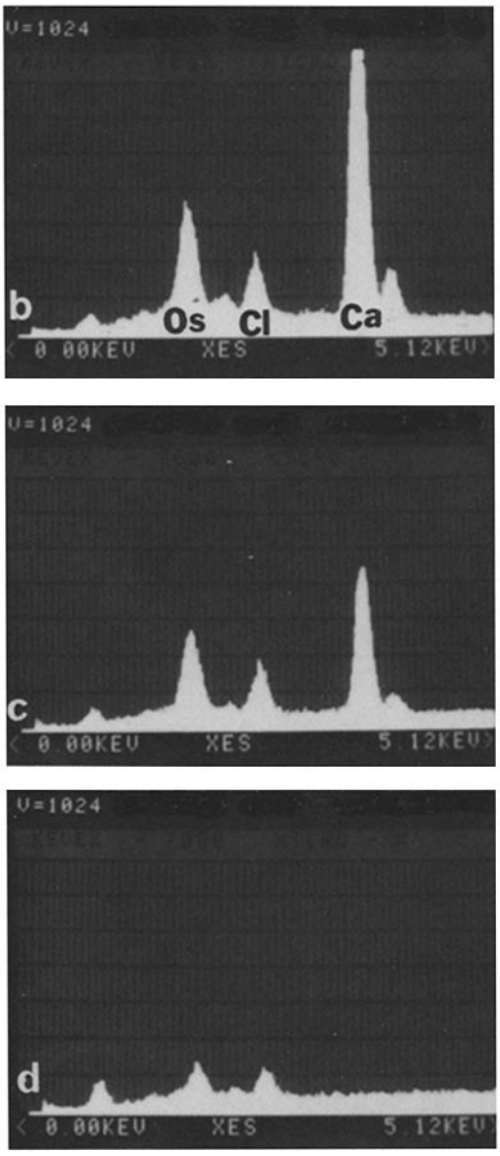
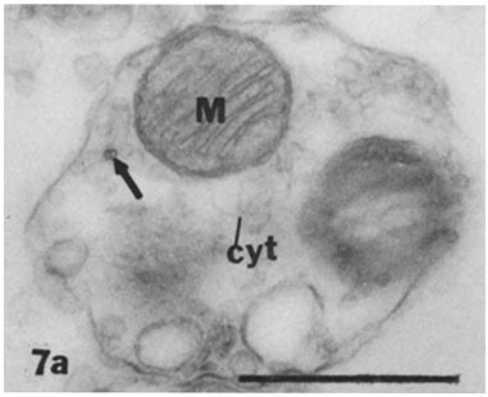
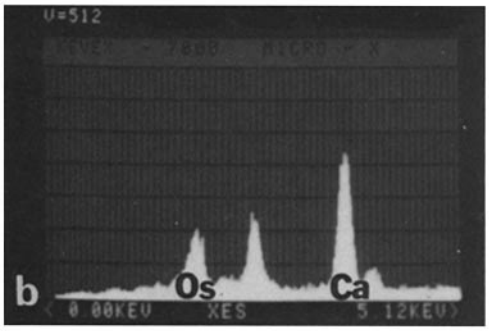


FIGURE 6 The electron-dense deposits (arrows) in mitochondrial profiles and the adjacent cytoplasm (*cyt*) in a saponin-treated synaptosome incubated with Ca, ATP, and oxalate were analyzed by electron probe. (*a*) Unstained, 100-nm-thick section. × 84,250. (*b*, *c*) Spectra from both mitochondrial electron-dense deposits illustrated in (*a*) have large Ca peaks. (*d*) The spectrum from the region of the cytoplasm labeled in (*a*) has no Ca peak.

contained deposits. The mitochondrial poisons did not affect the frequency of Ca deposits in the SER profiles. In one experiment, of 300 SER cisterns or large vesicular profiles counted for each preparation, 6.3% contained deposits in the unpoisoned preparation as compared to 8.3% in the poisoned preparation. As stated previously (see Fig. 10), electron-dense deposits are not distributed uniformly within mitochondria or SER profiles and are not observed in every plane of section of these organelles. Therefore, it seems certain that these percentages markedly underestimate the total number of organelles that contain deposits. Examples of the deposits in vesicular profiles and cisterns of poisoned terminals are shown in Figs. 12*a* and 13*a*, respectively. Elemental analyses of these deposits confirmed the presence of Ca, as illustrated by the spectra in Figs. 12*b* and 13*b*.

When saponin-treated synaptosomes were incubated with the Ca chelator EGTA, or the Ca ionophore A23187, electron-dense deposits were very rarely seen in any intraterminal organelle (~1% of the terminals contained an electron-dense deposit).





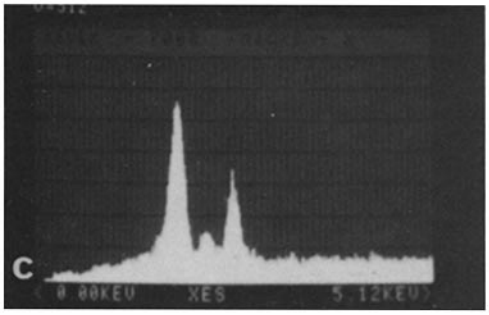
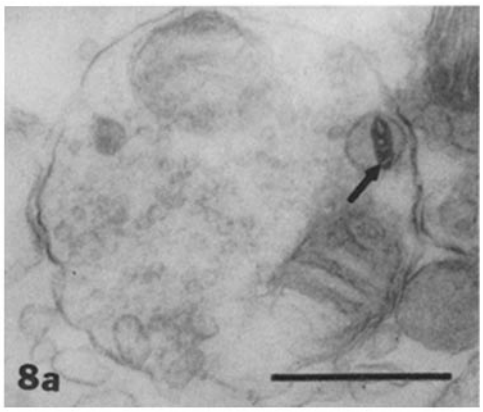
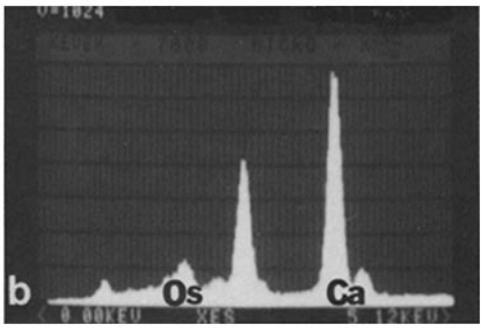


FIGURE 7 (*a*) Electron-dense deposit (arrow) in SER cistern of a saponin-treated synaptosome incubated in presence of Ca, ATP, and oxalate. *M*, mitochondrion; *cyt*, cytoplasm. Unstained, 100-nm-thick section. × 49,150. (*b*) Electron probe spectrum obtained from analysis of the deposit illustrated in (*a*) has a large Ca peak, whereas the spectrum (*c*) obtained from the mitochondrion has no significant Ca peak.





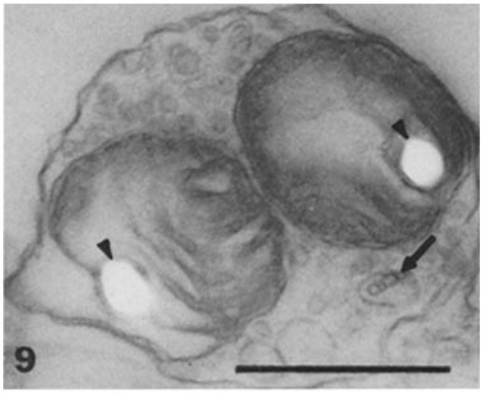


FIGURE 8 (*a*) An electron-dense deposit (arrow) in a vesicular profile within a saponin-treated synaptosome incubated in Ca, ATP, and oxalate. Unstained, 100-nm-thick section. × 49,150. (*b*) Analysis of the electron-dense deposit in the vesicular profile confirms the presence of Ca.

FIGURE 9 Electron-dense deposit (arrow) in a vesicular profile within a saponin-treated synaptosome incubated in the presence of Ca, ATP, and oxalate. Two large holes (arrowheads) in mitochondrial profiles may represent loss of Ca oxalate deposits. Unstained, 70-nm-thick section. × 56,150.

The rationale for these studies is that they parallel biochemical observations on the ATP-dependent sequestration of Ca by an intraterminal nonmitochondrial organelle; this sequestration is insensitive to mitochondrial poisons but inhibited by A23187 or a large excess of EGTA (7, 8).

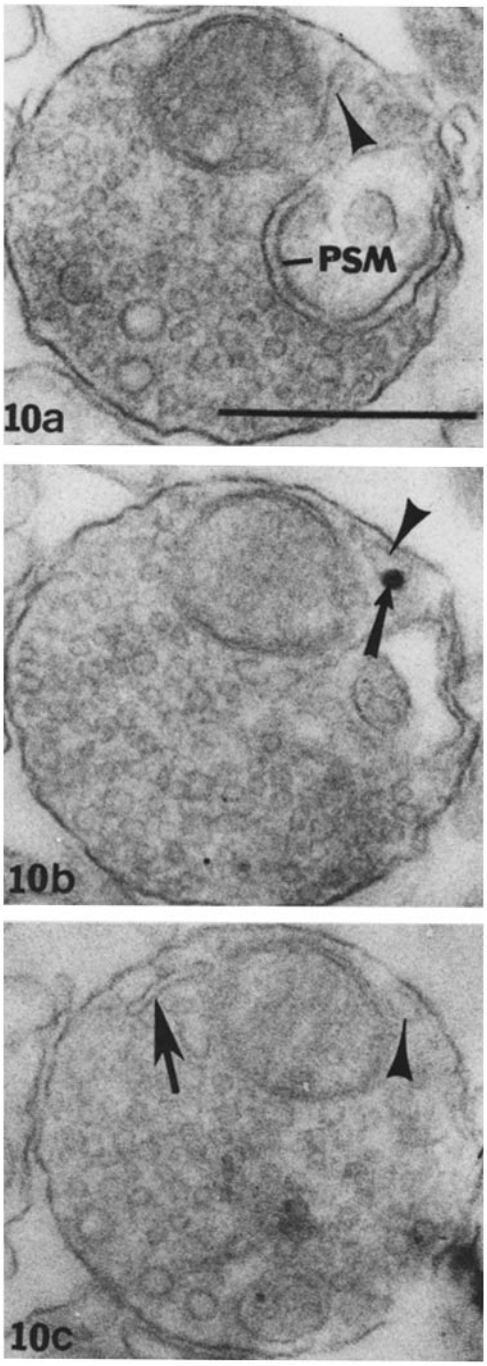


FIGURE 10 (*a–c*) Serial thin sections (70-nm thick) of a saponin-treated synaptosome with an electron-dense deposit (small arrow) located in an SER cistern (arrowheads) in section *b*. The electron-dense deposit is present in only one plane of section. Another SER profile (larger arrow) is present in section *c*. Unstained. × 68,500. (*a*, *b*) The postsynaptic membrane (*PSM*) of a spinous contact is still attached to the synaptosome.

DISCUSSION

Recent biochemical studies have suggested that $[Ca^{2+}]$ in the cytoplasm of nerve terminals is regulated in part by Ca transport across the plasma membrane (cf. 5, 6), and in part by intracellular buffering mechanisms (cf. 7, 8). The intracellular buffering mechanisms may play an especially important role in the short-term buffering of the Ca that enters during a period of neuronal activity. At nerve terminals, the Ca that is retained (and buffered) is probably involved in synaptic modulation: e.g., facilitation and posttetanic potentiation (27, 55, 56).

In the present study we investigated the ultrastructural localization of Ca sequestration sites within presynaptic nerve terminals. Attention was focused on the intraterminal SER to determine whether or not this organelle is, as previously postulated (7), a nonmitochondrial membrane-bounded structure that can sequester Ca (with high affinity) at the expense of ATP hydrolysis (7, 8).

Morphology of SER

Serial thin sections of synaptosomes helped to determine the distribution of the SER and to distinguish elements of SER from other intraterminal organelles. We found that a large fraction of the terminals contained SER characterized by extensive, flattened sacs or cisterns often with branches. The volumes of the SER were ~2% of the synaptosome volume; however, these values may have been underestimated. To correct for membranes not visualized because of their tangential orientation with respect to the incident beam, Loud (37) has suggested that a correction factor of 50% be added to stereologically determined endoplasmic reticulum volumes. A similar correction factor also may be necessary for calculations of synaptosome SER volumes. Comparable fractional volumes (~2%) have been calculated for the sarcoplasmic reticulum (SR) found in some vascular smooth muscles and taenia coli (13, 49) where the Ca released by a single action potential is sufficient for a twitch response. Other similarities between the SER and SR are discussed below.

We observed that the SER was frequently juxtaposed to mitochondria. It is interesting that other investigators have also noted close associations

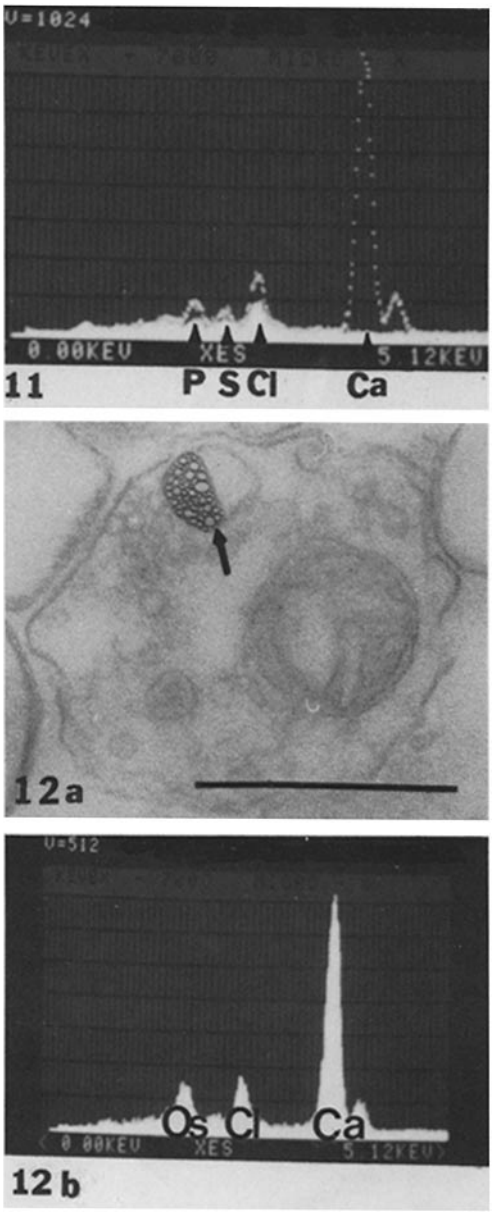


FIGURE 11 A comparison of spectra obtained from an electron-dense deposit (dotted spectrum) and from the adjacent cytoplasm (solid spectrum) of a saponin-treated synaptosome incubated in Ca, ATP, and oxalate. The synaptosome was not postfixed with osmium. A large Ca signal was obtained from the deposit but not from the cytoplasm.

FIGURE 12 (*a*) A large electron-dense deposit (arrow) is seen within a vesicular profile in a saponin-treated synaptosome incubated in presence of Ca, ATP, oxalate, and mitochondrial poisons (DNP, oligomycin, and NaN_3). Unstained, 100-nm-thick section. × 70,200. (*b*) Spectrum obtained from the vesicular electron-dense deposit indicates that Ca is present.

between these organelles in other neuronal preparations (4, 14, 34); this morphological arrangement may have significant physiological implications, as discussed below.

Ca Localization Studies

PUNCTATE, ELECTRON-DENSE PRECIPITATES: Aldehyde-fixed, osmicated tissue contained small, punctate precipitates lining SER membranes. Elemental analyses revealed substantial amounts of Os and some Ca in these precipitates. Krames and Page (31) observed that there is a large uptake of Ca and Os, probably localized to the plasma membrane, in tissue fixed with OsO_4 in the presence of millimolar concentrations of Ca. This finding, coupled with the fact that we did not observe punctate precipitates when the tissue was not postfixed with Os, suggests that the punctate precipitates are artifacts of the fixation process. In addition, various incubation conditions (such as the presence of mitochondrial inhibitors or A23187) did not alter the location of the punctate precipitates; the implication is that these precipitates are not related to Ca sequestration. This finding is of critical importance when considering data obtained from Ca localization studies in which Os is routinely used (as in the "osmium-pyroantimonate" technique [48]); appropriate controls and direct analysis of the deposits are essential for correct interpretation of the presence of Ca.

ELECTRON-DENSE CA OXALATE DEPOSITS: The Ca oxalate deposits, on the other hand, were not artifacts of the chemical preparation; they were observed within tissue that was aldehyde-fixed and OsO_4-postfixed, and were also present when either aldehyde or OsO_4 was omitted. Conditions that did alter the location of these deposits were those that were expected to affect the intracellular distribution of Ca (i.e., incubations in the presence of mitochondrial poisons, EGTA, or A23187). We conclude that these deposits are present at sites of Ca sequestration. Our experimental results, confirmed by electron probe analysis, have established that the mitochondria and cisterns and vesicular profiles of SER within presynaptic nerve terminals sequester Ca in the presence of ATP.

Other investigators have suggested that coated vesicles and neurotransmitter-containing synaptic

vesicles sequester Ca (9, 42), perhaps by an ATP-dependent mechanism (9). We have not observed any electron-dense deposits in either coated vesicles or synaptic vesicles within saponin-treated synaptosomes incubated under conditions promoting Ca sequestration (i.e., in the presence of Ca, ATP, and oxalate). However, in our preparations, saponin treatment would have rendered organelles bounded by cholesterol-rich membranes "leaky" to ions (16) and therefore incapable of sequestering Ca. Because plasmalemma (30), synaptic vesicle membrane (11), and, presumably, coated vesicle membrane (assuming that it is formed by endocytosis of the plasmalemma) have a high cholesterol content, the question of whether or not these organelles do sequester Ca remains unresolved.

Various-sized vesicular profiles that did not resemble coated vesicles or neurotransmitter-containing synaptic vesicles did contain Ca deposits. These profiles were insensitive to saponin, and therefore were probably not derived from the plasmalemma, coated vesicles, or synaptic vesicles. Instead, serial sections have provided evidence that at least some of these vesicular profiles are cross sections of SER cisterns or branches. Henkart et al. (25) have shown that elements of SER become swollen during Ca sequestration. Indeed, if portions of the flattened SER cisterns (cf. Fig. 1) had become swollen during Ca oxalate loading, they may have appeared as large vesicular profiles (cf. Fig. 12*a*) in single thin sections.

Physiological Significance of Ca Buffering

The observations described above demonstrate that at least two intraterminal organelles are capable of sequestering Ca: mitochondria and SER. The mitochondria can accumulate large amounts of Ca, but the mitochondrial transport system exhibits a low affinity for Ca and slow initial rate of uptake (cf. 7, 8). In contrast, the SER ("nonmitochondrial") storage sites have a smaller capacity but higher affinity for Ca (cf. 7, 8). Available evidence (7, 8, 45) indicates that these organelles may be capable of buffering $[Ca^{2+}]_i$ to submicromolar levels. These considerations provide a basis for speculation about the roles of SER and mitochondria in buffering the increased cytoplasmic Ca levels associated with neuronal activity.

One possible consequence of the aforementioned dissimilarities in the Ca transport properties of the two organelles is that the SER may be the initial and, hence, more important Ca-buffering site, especially when the Ca loads are small. A potentially significant finding is the close proximity of SER to mitochondria; the SER may act as a "screen" for the mitochondria, and may damp the fluctuations in $[Ca^{2+}]_i$ before they reach the mitochondria. In the event that the $[Ca^{2+}]_i$ is increased in the vicinity of these organelles, the SER would rapidly sequester Ca, thereby lowering the $[Ca^{2+}]_i$. This would allow the mitochondria to use most of the energy derived from electron transport to phosphorylate ADP, rather than to sequester Ca; thus, the mitochondria might be protected from Ca overload.

The kinetic properties of Ca transport by neuronal SER closely resemble those of skeletal muscle SR (8). In addition, it is possible that the SER is involved in "depolarization-secretion" coupling

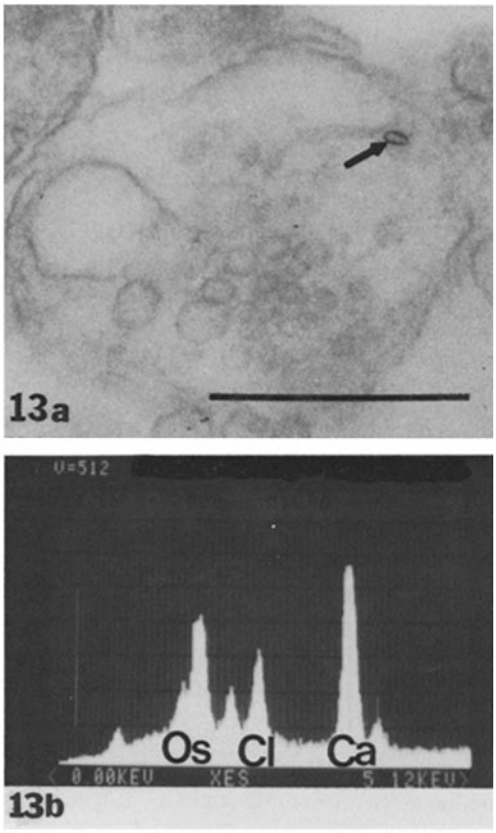


FIGURE 13 (*a*) Electron-dense deposit (arrow) in an SER cistern within a saponin-treated synaptosome incubated in presence of Ca, ATP, oxalate, and FCCP. Unstained, 100-nm-thick section. × 70,200. (*b*) Spectrum obtained from the cisternal electron-dense deposit has a substantial Ca peak.

(36), with a role analogous to that of the SR in excitation-contraction coupling. The intraterminal SER may couple depolarization of the presynaptic membrane to various intracellular activities by releasing sequestered Ca; recent evidence suggests that Ca can be released from presynaptic intracellular stores after depolarization of motor nerve terminals (18). A similar role has also been proposed for specialized regions of neuronal endoplasmic reticulum, the subsurface cisternae, which form "junctions" with the plasmalemma (24).

Ca is the common mediator for many neuronal functions including neurotransmission (cf. 35), regulation of K permeability (15, 39), microtubule assembly (44), and axonal transport (41). The Ca that enters the terminals as a result of depolarization causes an immediate increase in the $[Ca^{2+}]_i$ at the active zones and somehow triggers neurotransmitter release (36). All of the Ca that enters the terminals is probably not instantaneously buffered or extruded; rather, some of it diffuses away from the active zones and is temporarily sequestered by various cytoplasmic components, particularly the SER.

It seems clear that the distribution of Ca^{2+} is not always constant throughout the terminal. Spatial and temporal variations in the $[Ca^{2+}]_i$ may be crucial to the diverse neuronal activities and must be precisely controlled in response to appropriate stimuli. Of considerable importance is the relative role that SER and mitochondria play in regulating $[Ca^{2+}]_i$. As mentioned earlier, the kinetic properties of Ca transport in these organelles are dissimilar (8). Differences in the storage capacity for Ca and rate of Ca uptake would create an environment in which the $[Ca^{2+}]_i$ at specific sites would be time dependent and would differ from that at other intraterminal loci. Thus, by participating in the rapid regulation of the $[Ca^{2+}]_i$, the SER could help to modulate many Ca-dependent neuronal functions. Moreover, differences in the behavior of individual neurons may, in part, be attributable to variations in the size and location of the Ca-buffering organelles in the respective neurons.

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