RETRIEVAL AND RECYCLING OF SYNAPTIC VESICLE MEMBRANE IN PINCHED-OFF NERVE TERMINALS (SYNAPTOSOMES)

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ABSTRACT

The morphological features of pinched-off presynaptic nerve terminals (synaptosomes) from rat brain were examined with electron microscope techniques; in many experiments, an extracellular marker (horseradish peroxidase or colloidal thorium dioxide) was included in the incubation media. When incubated in physiological saline, most terminals appeared approximately spherical, and were filled with small (~400-Å diameter) "synaptic vesicles"; mitochondria were also present in many of the terminals. In a number of instances the region of synaptic contact, with adhering portions of the postsynaptic cell membrane and postsynaptic density, could be readily discerned. ~20–30% of the terminals in our preparations exhibited clear evidence of damage, as indicated by diffuse distribution of extracellular markers in the cytoplasm; the markers appeared to be excluded from the intraterminal vesicles under these circumstances. The markers were excluded from the cytoplasm in ~70–80% of the terminals, which may imply that these terminals have intact plasma membranes. When the terminals were treated with depolarizing agents (veratridine or K-rich media), in the presence of Ca, many new, large (600–900-Å diameter) vesicles and some coated vesicles and new vacuoles appeared. When the media contained an extracellular marker, the newly formed structures frequently were labeled with the marker. If the veratridine-depolarized terminals were subsequently treated with tetrodotoxin (to repolarize the terminals) and allowed to "recover" for 60–90 min, most of the large marker-containing vesicles disappeared, and numerous small (~400-Å diameter) marker-containing vesicles appeared. These observations are consistent with the idea that pinched-off presynaptic terminals contain all of the machinery necessary for vesicular exocytosis and for the retrieval and recycling of synaptic vesicle membrane. The vesicle membrane appears to be retrieved primarily in the form of large diameter vesicles which are subsequently reprocessed to form new "typical" small-diameter synaptic vesicles.

KEY WORDS synaptosomes · presynaptic nerve terminals · synaptic vesicles · membrane retrieval · vesicle recycling · thorium dioxide

The quantal nature of neurotransmitter release, first recognized by del Castillo and Katz (13), has now been repeatedly documented by electrophys-

iological studies in numerous laboratories. Early ultrastructural studies, especially those by De Robertis and Bennett (14, 15), led to the identification of a morphological correlate, the presynaptic terminal vesicles or "synaptic vesicles", which are generally believed to be the storage sites of transmitter "quanta". According to the "Vesicle Hypothesis", each synaptic vesicle contains one multimolecular packet, or quantum, of transmitter; the quantum is released by exocytosis when the vesicle is triggered to fuse with the plasmalemma of the presynaptic terminal. This hypothesis, of course, required morphological verification; a number of electron microscopists (e.g., references 12, 24, 41) have observed images which appear to represent the opening of synaptic vesicles into the synaptic cleft. It soon became clear that this process of fusion of vesicle membrane to plasma membrane could not be a one-way street, and that sooner or later some membrane must be retrieved and returned to the interior of the terminals. One suggestion (e.g., reference 9) was that the vesicles open to the surface only transiently, and then reseal and return to the interior; in studies employing extracellular markers, the markers were found within synaptic vesicles at the frog neuromuscular junction after a period of stimulation and recovery. However, Heuser and Reese (26, 24) have reached a somewhat different conclusion on the basis of studies with extracellular markers and with freeze-fracture techniques: their data indicate that vesicle membrane at the frog neuromuscular junction is initially retrieved from the plasmalemma in the form of coated vesicles and large vacuoles and flattened "cisternae". The membranes from these organelles are then apparently reprocessed, by poorly understood mechanisms, to form small, uncoated "synaptic vesicles" which are able to refill and again release their contents upon stimulation (9, 26, 21).

Most of the evidence for quantal (vesicular) release of transmitter, and for recycling, has been obtained from studies on the peripheral nervous system (e.g., references 9, 26–28, 42, 47). However, a few studies have provided evidence for quantal release (cf. reference 38), vesicle fusion with the plasmalemma (e.g., reference 41), and vesicle membrane retrieval (39, 43, 44) in the vertebrate central nervous system.

The acquisition of detailed information on the intermediate stages of synaptic vesicle membrane retrieval and recycling in the mammalian central nervous system (CNS) would be desirable. Unfortunately, this quest has been hampered by the lack of suitable experimental preparations in which presynaptic terminals are abundant and readily identifiable in the electron microscope; moreover, synaptic activity should, ideally, be under the control of the experimenter, so that an entire population of terminals can be maintained in a synchronous state of activity. These requirements may be met, in large measure, by preparations of pinched-off presynaptic terminals ("synaptosomes") obtained from mammalian brain homogenates and maintained in physiological salt solutions. Studies from many laboratories, including our own, have shown that synaptosomes incubated in physiological salt solutions exhibit a considerable degree of functional integrity (6, 7). Synaptosomes have membrane potentials that respond to depolarizing agents in the same way as the membrane potentials of intact neurons (5). In accordance with the "Calcium Hypothesis" (34), depolarizing agents enhance Ca uptake and trigger Ca-dependent transmitter release by synaptosomes (3). The latter observations prompted us to ask whether or not this transmitter release is vesicular; this provided the initial impetus for the present ultrastructural investigation. Although we have not observed any images of synaptic vesicles opening into the synaptic cleft region, we have been able to demonstrate, with the use of extracellular markers, that "stimulated" synaptosomes do retrieve membrane from the surface in the form of coated vesicles and large vesicles and vacuoles. Our results are, in general, consistent with the retrieval and recycling hypothesis put forth by Heuser and Reese for the frog neuromuscular junction. Moreover, the fact that the majority of terminals in our synaptosome preparations exhibit intermediate stages in the retrieval and recycling process leads us to suggest that most types of terminals at chemical synapses, and not just cholinergic terminals, function in a similar if not identical fashion. Preliminary reports of some of our findings have already been published (17, 18).

MATERIALS AND METHODS

Solutions

The standard physiological saline ("Na + 5 K") used for incubation of synaptosomes contained: 145 mM NaCl, 5 mM KCl, 1.2 mM $CaCl_2$, 1.3 mM $MgCl_2$, 1.2 mM NaH_2PO_4, 10 mM glucose, and 20 mM *N*-2-hydrox-

yethylpiperazine-*N*-2-ethane sulfonic acid (HEPES); the solution was buffered to pH 7.65 at 20°C with Tris base. In some instances a "K-rich" incubation solution was used; this solution usually contained 37 mM KCl and only 113 mM NaCl (see Results), but was in all other respects identical to Na + 5 K. In the "Ca-free" incubation media, $CaCl_2$ was replaced by 1.4 mM ethylene glycol-bis(β-aminoethyl ether)*N*,*N*'-tetraacetic acid (EGTA). Details regarding the addition of veratridine (Aldrich Chemical Co., Inc., Milwaukee, Wis.) and tetrodotoxin (TTX, Calbiochem, La Jolla, Calif.) will be given in Results.

Extracellular Markers

Horseradish peroxidase (HRP, type VI, Sigma Chemical Co., St. Louis, Mo.) was suspended in Na + 5 K (10 mg in 1 ml) and dialyzed against Na + 5 K (150 ml) for 24 h at 4°C. The final HRP concentration in the incubation fluids was 5 mg/ml.

Colloidal thorium dioxide (ThO_2, Thorotrast, in 25% suspension from Fellows Medical Division, Chromalloy Pharmaceuticals, Oak Park, Mich., a gift from Dr. A. Enders and Dr. B. King) was diluted with Na + 5 K to give a final concentration of 3.3% in the incubation solutions.

Preparations of Synaptosomes

Crude mitochondrial fractions ("P_2" of Gray and Whittaker [20]) were prepared from homogenates of rat cerebral cortex by a slight modification (4) of the original Gray and Whittaker procedure. The P_2 fraction was resuspended in 0.32 M sucrose and subjected to further fractionation on a one-step discontinuous sucrose gradient (22) to obtain nerve-terminal-enriched preparations ("synaptosomes"). The synaptosomes were recovered in the 0.8 M sucrose fraction. To return the synaptosomes to a more physiological, normotonic environment, small volumes of ice-cold Ca-free Na + 5 K were added to the sucrose suspensions over a 20–30-min period, to give a final volume ~3–5 times the initial sucrose volume. These "equilibrated synaptosome suspensions" were centrifuged at 9,000 *g* (max) for 5 min at 3°C. The supernatant solutions were discarded and the the synaptosome pellets were resuspended in Na + 5 K (or, in some cases, Ca-free Na + 5 K) and incubated for 10–12 min at 30°C, to bring the terminals to a physiological steady state; the protein concentrations in the suspensions were in the range of 1–5 mg/ml, as measured by nephelometry. After the initial incubation, solutions containing extracellular markers and/or drugs were added and the suspensions were incubated for an additional period; further details will be given in Results.

Procedures for Electron Microscopy

Upon completion of the incubation, the synaptosome suspensions were diluted with 1 vol of ice-cold Na + 5 K and two vol of ice-cold Karnovsky's cacodylate-buffered fixative (31), and then stored overnight at 2°–3°C. The suspensions were then centrifuged at 14,000 *g* (max) for 6 min at 3°C; the supernatant solutions were discarded and the pellets were washed in three changes of 0.1 M Na cacodylate buffer (pH 7.0) over a 30-min period.

When HRP was employed as the extracellular marker, the pellets were washed with two more changes of the cacodylate buffer, and then reacted for 1–2 h with a freshly prepared solution of 0.05% diaminobenzidine in 0.1 M Na cacodylate buffer with 0.01% hydrogen peroxide (19). The diaminobenzidine solution was then removed and the pellets were washed with three changes of 0.1 M Na cacodylate buffer.

All the buffer-washed pellets were fixed and stained in potassium ferrocyanide-reduced OsO_4 (32) for 1 h, followed by 2 h in either 2 or 4% OsO_4. The stained pellets were dehydrated in increasing concentrations of ethanol, and embedded in Araldite. Thin sections were stained for 20 min with uranyl acetate (saturated solution in 50% ethanol) and for 15 s with lead citrate (45), and were examined in either a Philips 300 or a Siemens Elmiskop 1A electron microscope.

Statistical Analysis

Quantitative information about the size of vesicles and the frequency of labeled vesicles was obtained in a number of experiments. For these analyses, grid squares were chosen at random, and synaptosomes were photographed at both low and high magnifications. Only profiles of terminals with cross-sectional diameters ≥0.4 μm were included in the statistical analyses. The mean diameters of vesicles were calculated from two perpendicular measurements. Agranular vesicular structures with diameters in excess of 900 Å were arbitrarily termed "vacuoles," as opposed to "vesicles." Vesicle counts were made on sections of approximately equal thickness, to avoid biasing the results. Standard χ^2 and Student's *t* test analyses were performed on some of the data, as indicated in Results, Table I footnotes, and the figure legends.

RESULTS

Morphology of Normal Synaptosomes

When suspensions of synaptosomes are incubated in normal physiological saline, and then fixed and prepared for electron microscopy, a majority of the terminals have the appearance of small spheres full of smaller spheres, the synaptic vesicles (Figs. 1 and 2). In addition to the small ("synaptic") vesicles, the terminals often contain mitochondria (Fig. 1) and, on occasion, large vesicles (600–900) Å in diameter; see Fig. 1) and vacuoles (agranular vesicles >900 Å in diameter; Figs. 1 and 2). Jones (29), too, has commented on the presence of vacuoles in the cytoplasm of

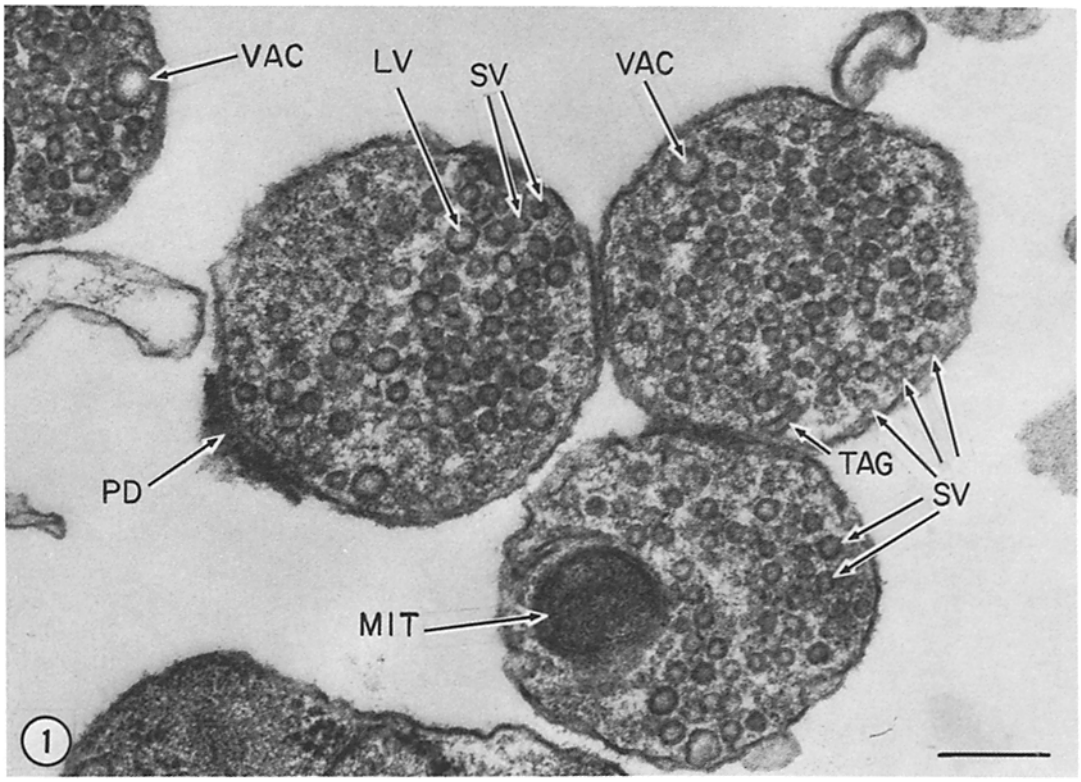


FIGURE 1 Electron micrograph of "normal" synaptosomes obtained from a one-step sucrose gradient (reference 22; and see Materials and Methods), and incubated in Na + 5 K for 10 min at 30°C. The terminals are filled with small synaptic vesicles (*SV*), ~400 Å in diameter, although occasional large vesicles (*LV*) or vacuoles (*VAC*) are observed. A tubule of agranular membrane (*TAG*), perhaps representing a cross section through smooth endoplasmic reticulum, is occasionally seen. Mitochondria (*MIT*) are frequently seen within the terminals. Synaptic contact regions, when present in the plane of section, can be detected by the adherence of portions of the postsynaptic cell plasmalemma and postsynaptic density (*PD*). The spherical shape of the terminals seen in this and many of the succeeding figures was by far the most frequently observed shape. Bar, 0.2 μm.

some synaptosomes; because such vacuoles are not often present in directly fixed specimens of brain tissue, it seems possible that these vacuoles may be artifacts which are formed during the homogenization and fractionation procedures. High-power electron micrographs (e.g., see Fig. 2) generally reveal a poorly defined meshwork of filamentous material in the cytoplasm (29). Elements which have the appearance of tubules of agranular membrane, presumed to be fragments of smooth endoplasmic reticulum, are occasionally observed (Fig. 1). In general, the morphological features of these pinched-off terminals appear comparable to those of intact central (40) and peripheral (2) presynaptic endings.

The regions of synaptic contact are seen in a number of the terminals (Figs. 1 and 2). Portions of the postsynaptic cell membrane and postsynaptic density usually remain attached to the presynaptic terminal.

Not all of the terminals in our preparations are spherical. Another typical but less common shape is illustrated in Fig. 12. These terminals have irregular outlines and are characterized by having their synaptic contact regions located on deep invaginations. Presumably, these terminals synapsed onto dendritic spines in the brain, and retained their original shapes (at least near the synaptic contact region) through all the homogenization, centrifugation, and incubation steps. In some instances (Fig. 7, and perhaps Fig. 5), a fingerlike protrusion from the postsynaptic neuron appears to indent the presynaptic terminal; thus, in cross section, a spherical or cup-shaped portion

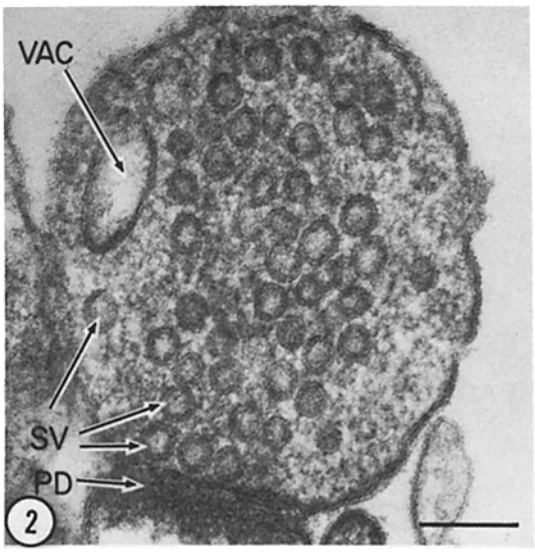


FIGURE 2 A terminal from the same preparation as those of Fig. 1, shown at higher magnification. The synaptic contact region with postsynaptic density (*PD*) is clearly seen. This terminal contains numerous synaptic vesicles (*SV*) as well as a large vacuole (*VAC*). Bar, 0.1 μm.

of the postsynaptic cell may lie within the presynaptic terminal.

In all of our earlier morphological (17) and physiological studies (3–6), synaptosomes were separated on the two-step sucrose gradient described by Gray and Whittaker (20). More recently, we have employed the much simpler and faster one-step gradient procedure outlined by Hajos (22). We have been unable to detect any significant differences in the physiological properties or morphological features (compare Figs. 1 and 2 with Fig. 1 in reference 17) of terminals prepared by these methods. The more convenient Hajos method has therefore been used for all of the studies described below.

Permeability of the Terminals to Extracellular Markers

An important aspect of our experiments is the integrity of the nerve terminal surface membrane. On the one hand, some terminals may, in cross section, appear to have damaged or incomplete surface membranes. Many of these terminals may, nevertheless, be impervious to extracellular markers such as HRP, ferritin, or ThO_2 (see below); the membrane defect might then represent a sectioning (e.g., too thick) or viewing (e.g., bad angle) artifact. On the other hand, some terminals may appear (in cross section) to have intact surface membranes, and yet have cytoplasm diffusely labeled with extracellular marker (e.g., see the right-hand terminal in Fig. 3*a*). The explanation for the latter observation is that a small surface defect, out of the plane of section, may be sufficient to allow marker to gain access to all of the cytoplasm (see Fig. 3*b*). This indicates that it may be misleading to conclude that a terminal is intact simply because it has an uninterrupted surface membrane in a single cross-sectional profile (cf. reference 30), unless an extracellular marker is used to test for surface defects. Of course, some surface defects may be too small to permit passage of markers the size of HRP (~50-Å diameter [10]) or ThO_2 (~25 Å diameter [46]); thus, marker-free cytoplasm is not necessarily synonymous with an intact terminal. Nevertheless, with the foregoing considerations in mind, it seems reasonable to use the markers to obtain an estimate of the fraction of resealed terminals with intact surface membranes in our preparations. In three experiments employing ThO_2 as a marker, of 294 synaptosomes examined (diameter $\geq$ 0.4 μm in cross section), ~22% (range = 15–26%) had the marker distributed throughout the cytoplasm (as in the right-hand terminal in Fig. 3*a* and *b*). When HRP was used as a marker, 32% of 86 terminals had diffusely labeled cytoplasm.

An interesting finding with regard to the ThO_2 particles is that, although the individual particles are only ~25 Å in diameter (46), they often appear in aggregates ~100 Å in diameter (2, 46). We routinely observed ThO_2 aggregates of about this size or larger attached to the plasmalemma, free in the extracellular space, or in retrieved vesicles and vacuoles (Fig. 3 *et seq.*). In damaged terminals, however, the size of many of the ThO_2 particles dispersed in the cytoplasm was <100 Å (Fig. 3). The explanation for this difference in the distribution of large and small ThO_2 aggregates is not clear.

The Effects of "Depolarization" on Marker Uptake

As noted in the Introduction, depolarizing agents (e.g., veratridine or K-rich media) enhance Ca uptake and promote Ca-dependent transmitter release by synaptosomes. The morphological changes induced by these agents were examined in a number of experiments; in most of the experiments, extracellular markers were included

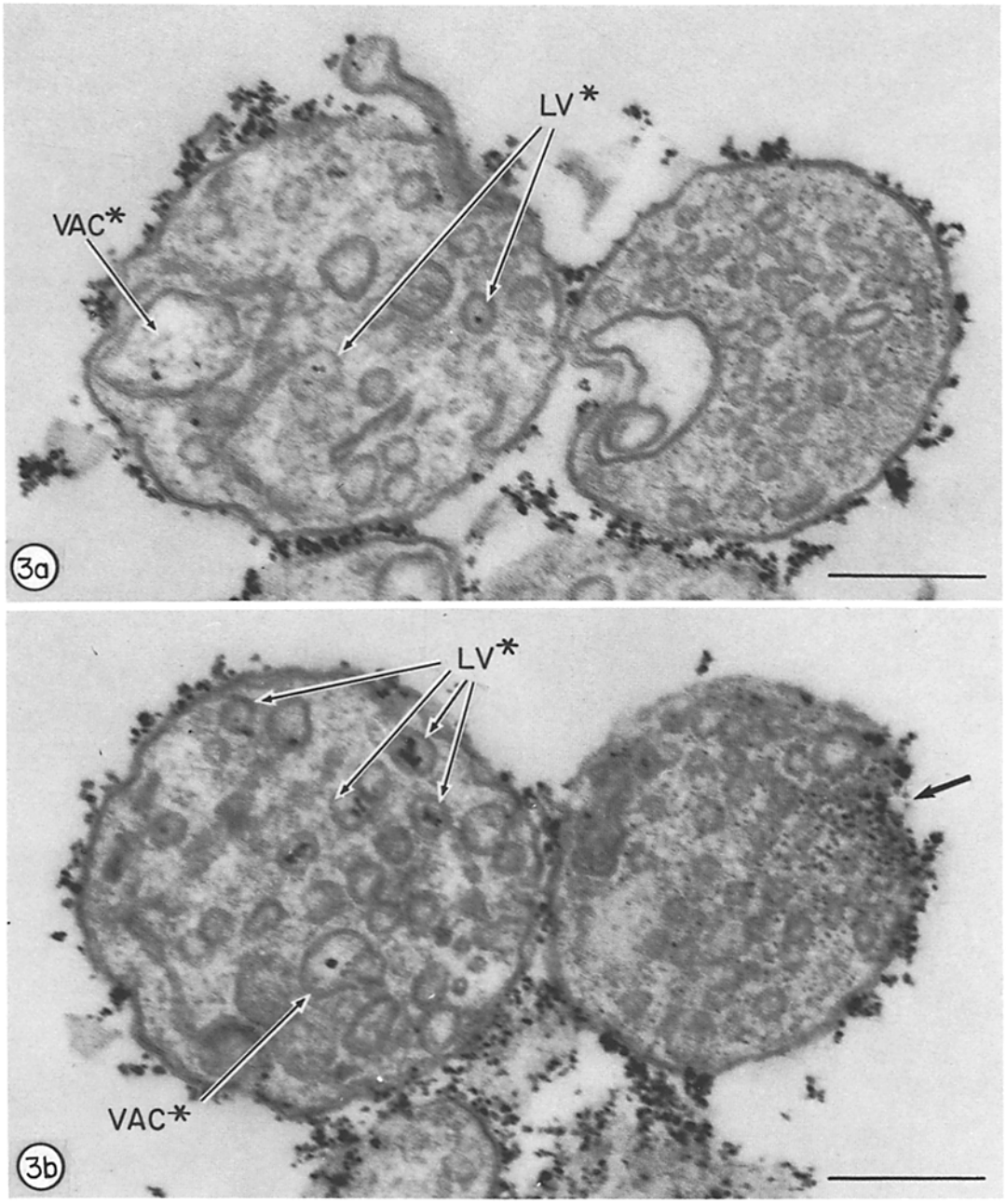


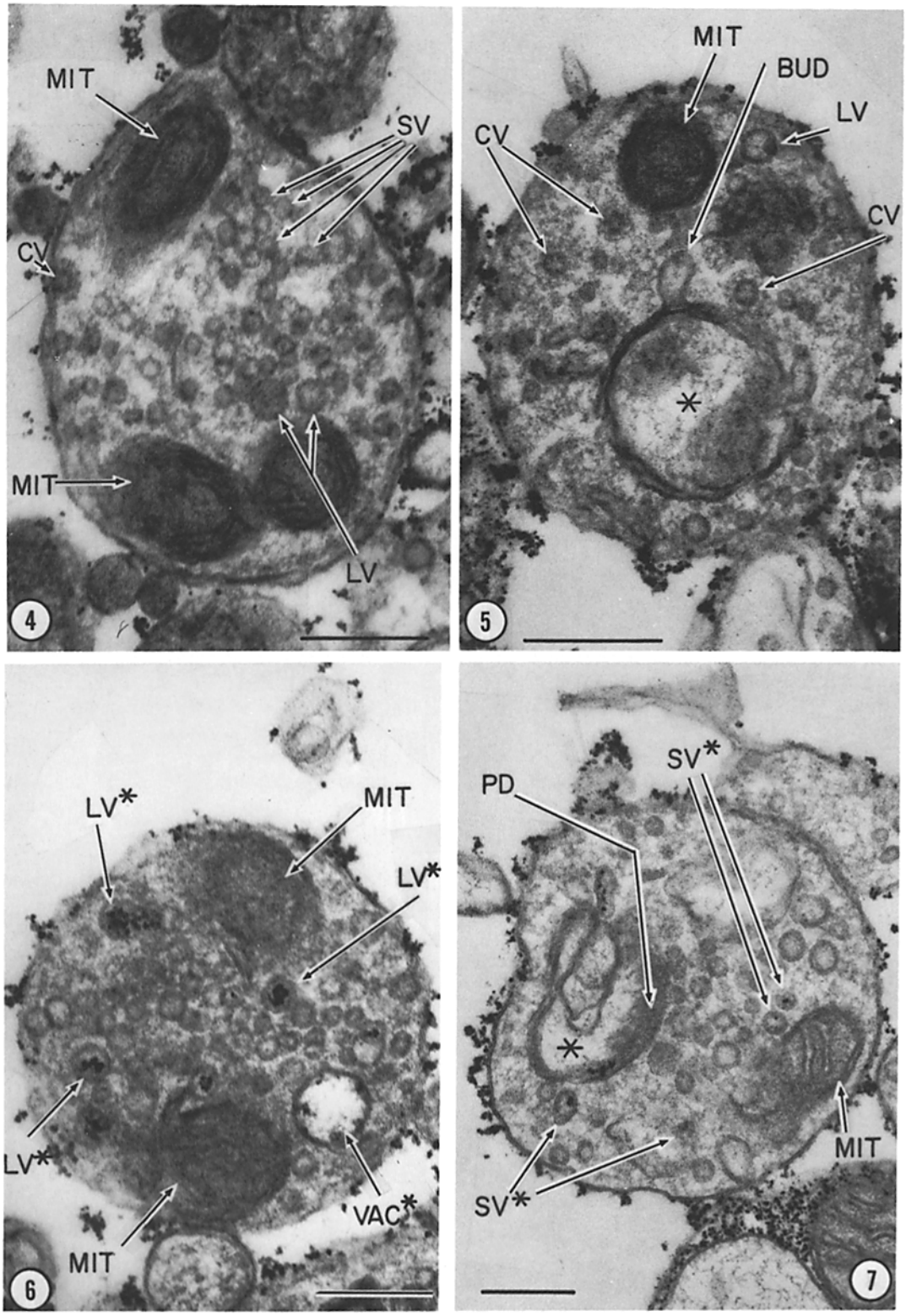
FIGURE 3 Comparison of "intact" and damaged synaptosomes. These terminals were incubated in solutions containing 3.3% ThO_2 (the 100-Å-diameter, electron-opaque particles in the micrographs), and were "stimulated" for 2 min with 75 μM veratridine. Note the large vesicle (*LV**) and vacuoles (*VAC**) in the left-hand terminal; as indicated by the asterisks, many of these structures contain ThO_2. In the left-hand terminal, the ThO_2 is confined to the intraterminal organelles, whereas in the right-hand terminal the ThO_2 is distributed diffusely in the cytoplasm and even appears to be excluded from the vesicles. This is clear evidence that the right-hand terminal is damaged. Fig. 3*a* and *b* are near-serial sections; the unlabeled arrow in Fig. 3*b* points to the defect in the plasmalemma of the right-hand terminal where the ThO_2 appears to be entering. Bars, 0.2 μm.

in the incubation media to detect damaged terminals and to determine whether or not any organelles were formed by endocytosis. Colloidal ThO_2 was the marker used in all of the experiments to be described in detail below, because it is readily visualized in the electron microscope without further chemical reaction; however, in several other experiments, comparable results were obtained with either HRP (17) or ferritin. In a few instances, the terminals were examined after a prolonged exposure (10–30 min) to a depolarizing agent (see Fig. 2 in reference 17). However, a somewhat different protocol was generally employed for the present studies on the sequence of events associated with membrane retrieval and recycling. Synaptosomes were suspended in Na + 5 K and incubated for 10–12 min at 30°C, to allow the terminals to approach a physiological steady state; the suspensions were then diluted with additional Na + 5 K containing one of the extracellular markers. A small volume of Na + 5 K containing veratridine was then added (giving a final veratridine concentration of 75 μM) to depolarize the synaptosomes (5). 1 min later, a small aliquot of a concentrated TTX solution was added (final TTX concentration, 200 nM) to block the Na channels opened by veratridine (cf. reference 5) so that the synaptosomes could repolarize. In some "control" samples, the TTX was added before the veratridine to prevent depolarization; alternatively, veratridine and TTX were both omitted from the incubation fluids in some cases. In other instances, Ca was omitted from all the solutions, and 1.4 mM EGTA was added to block Ca uptake and Ca-dependent transmitter release (3). In all experiments, incubation at 30°C was continued for up to 60–90 min, and samples from the suspensions were fixed (see Materials and Methods) at various times during the incubation to assess the progression of morphological changes.

EARLY STAGES OF MEMBRANE RETRIEVAL AND RECYCLING: Some of the most dramatic morphological changes are observed within the first 30–120 s after a 60-s depolarization. One notable finding is that depolarization, in the presence of Ca, leads to a reduction in the number of synaptic vesicles per unit area in the terminals. This effect may be visualized by comparing the terminals in Figs. 3–7 (during or shortly after "stimulation") with those in Figs. 1 and 2 (normal controls) and in Figs. 10–13 (terminals permitted to "recover" for 15–90 min after a brief "stimulation"). The change in synaptic vesicle density, after "stimulation", was quantitated in two experiments. In one experiment, synaptosomes incubated in Na + 5 K for 10 min were found to have 189 ± 19 synaptic vesicles/μm^2 of cytoplasm (mean ± SEM; n = 16 terminals examined); by comparison, synaptosomes exposed to 37 mM K medium (see Materials and Methods) for 10 min contained only 77 ± 11 synaptic vesicles/μm^2 of cytoplasm (n = 11 terminals). In another experiment, synaptosomes were depolarized with veratridine for 1 min, and then repolarized with TTX for 1 min; when Ca was omitted (to prevent transmitter release), the terminals were found to have 215 ± 14 vesicles/μm^2 (n = 9), whereas those incubated with Ca-containing solution had only 104 ± 11 vesicles/μm^2 (n = 22). The Student's t test indicates that the decrease in synaptic vesicle density, under conditions in which transmitter release may be expected, is highly significant ($P < 0.001$) in both experiments. These results are consistent with data from the frog neuromuscular junction, where tetanic stimulation causes depletion of synaptic vesicles (9, 26). On the other hand, Jones and Bradford (30) have reported that electrical stimulation of synaptosome suspensions does not alter the number of vesicles per terminal; unfortunately, there is no evidence that the terminals were actually depolarized, and that vesicle recycling was effected in these experiments.

Coincident with the decrease in synaptic vesicle density, during "depolarization" and the early period of recovery, we noted an increase in numbers of coated vesicles and of large vesicles (>500 - Å diameter) within the terminals. When the synaptosomes were incubated with extracellular markers, these markers frequently appeared within the large vesicles and, less often, coated vesicles (see Fig. 2*b* in reference 17). Typical examples of these morphological features are illustrated in Figs. 3–7.

Some early stages in the membrane retrieval process are shown at high magnification in Fig. 8; these micrographs illustrate the formation of a coated vesicle (Fig. 8*a*) and large labeled vesicles (Fig. 8*b–d*). An interesting feature is that these retrieval events do not appear to take place in the specialized regions of synaptic contact, where synaptic vesicles are believed to fuse with the surface membrane (11, 23, 24). The data are consistent with the idea that membrane retrieval occurs elsewhere on the surface of the presynaptic termi-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nal, and does not involve the transient opening of a vesicle to the extracellular environment followed by closure of the hole and immediate retrieval of the vesicle.

Table I provides some quantitative information about the increase in the numbers of labeled vesicles. Note the particularly large increment (three- to fourfold) in the number of terminals containing two or more labeled vesicles early on after stimulation. The electron micrographs (Figs. 3–7, and 8*c* and *d*) clearly show that many of the vesicles in these stimulated terminals, especially the marker-labeled vesicles, are much larger than the average vesicles observed in resting terminals (Figs. 1 and 2); in fact, at this early stage (1 min) of recovery, few of the labeled vesicles are 300–500 Å in diameter (the size of the "normal" vesicles in resting terminals). This striking difference in vesicle size is illustrated graphically in Fig. 9. Marker was also taken up by some vesicles with diameters in excess of 900 Å (see Figs. 3 and 6). These structures were arbitrarily termed "vacuoles", however (see Materials and Methods). Consequently, the distribution of labeled vesicles, after 1 min of recovery, may actually be skewed farther to the right (i.e., toward larger diameters) than is apparent in Fig. 9. These findings on the uptake of extracellular marker are consistent with the aforementioned vesicle depletion data: taken together, the observations suggest that the depolarization causes numerous small vesicles to fuse with the surface membrane, and that subsequently the vesicle membrane is retrieved in the form of (a few) large vesicles and vacuoles. Our observations on marker uptake may be contrasted with those of Zimmerman and Denston (48) who found that, when *Torpedo* electric organ was stimulated for a prolonged period, extracellular marker (dextran) appeared mainly in a new, smaller than normal, synaptic vesicle population. However, Zimmerman and Denston stimulated their preparations for about 5 h (at a frequency of 0.1 s^{-1}) and did not attempt to study the early stages of vesicle membrane retrieval.

The veratridine-treated terminals were "repolarized" with TTX before fixation in most of the experiments illustrated here; the retrieval of membrane did not require repolarization, however. For example, as shown in Fig. 3, extracellular-marker-labeled vesicles and vacuoles could be formed during the two min of exposure to veratridine alone. As noted in our preliminary report (17), numerous marker-labeled vacuoles and vesicles (including coated vesicles; see Fig. 2 in reference 17) were observed in terminals which were maintained in veratridine-containing or K-rich media for as long as 30 min. Moreover, the morphological changes did not depend upon the depolarizing agent used: both K and veratridine induced the formation of large vesicles and coated vesicles of (cf. Fig. 8b and c, and reference 17).

A central feature of the "Calcium Hypothesis" (33, 34) is that the depolarization-induced increase in Ca conductance causes Ca to move into the terminals, down its electrochemical gradient. Consequently, the intracellular ionized Ca^{2+} concentration increases, and this triggers fusion of vesicles with the plasmalemma and exocytosis of transmitter. These effects should all be abolished in Ca-free media, as we have, in fact, observed in synaptosomes (3; and see vesicle depletion data, above). Furthermore, if the fusion of vesicle mem-

FIGURE 4 Synaptosome "stimulated" with 75 μM veratridine for 30 s in the presence of ThO_2. The majority of the vesicles are small (~400-Å diameter; *SV*), although some large vesicles (*LV*) are seen. A coated vesicle (*CV*) appears to be budding off from the plasmalemma. *MIT* = mitochondria. No ThO_2-labeled structures are seen in this terminal. Bar, 0.2 μm.

FIGURES 5 and 6 Synaptosomes "stimulated" with veratridine for 1 min, and then "repolarized" with TTX for 1 min, in the presence of ThO_2. A number or large vesicles (*LV*), some of them containing ThO_2 (*LV**), and coated vesicles (*CV*) are seen. The terminal in Fig. 5 contains a large membrane-lined structure (*); as noted in the text, this may be the cross section of a fingerlike projection of the postsynaptic cell which forms an invagination in the presynaptic terminal. A vesicle is seen fusing with, or budding off (*BUD*) from, this structure. A ThO_2-containing vacuole (*VAC**) is seen in Fig. 6. *MIT* = mitochondria. Bars, 0.2 μm.

FIGURE 7 Synaptosome "stimulated" with veratridine for 1 min, and then "recovered" with TTX for 2 min, in the presence of ThO_2. Note the mitochondrion (*MIT*) and the ThO_2-labeled vesicles (*SV**). A cup-shaped projection (*) from the postsynaptic neuron, with postsynaptic density (*PD*) still attached, appears to have invaginated the presynaptic terminal (see text). Bar, 0.2 μm.

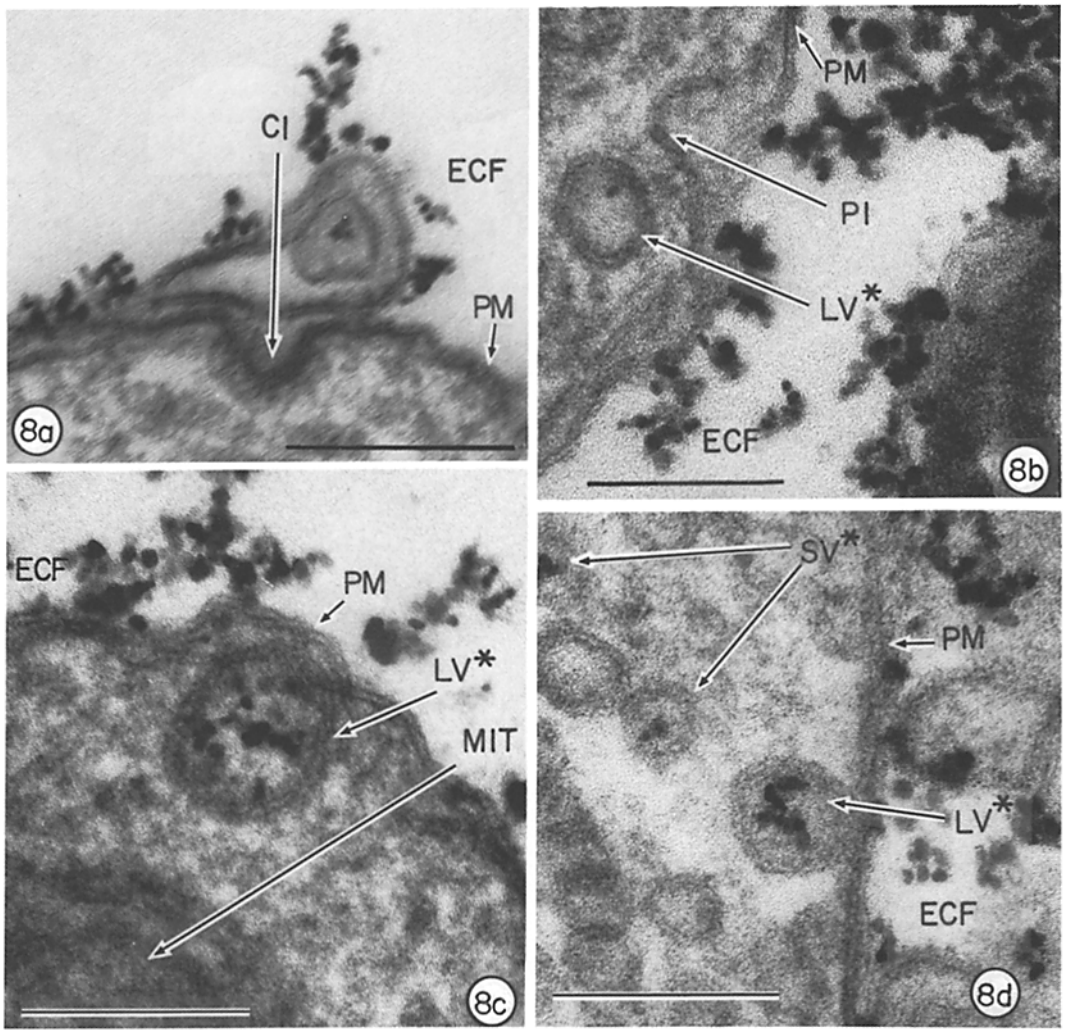


FIGURE 8 Electron micrographs illustrating early stages in the vesicle membrane recycling process. The preparations were all incubated with ThO_2. (*a*) Synaptosome "stimulated" with veratridine for 2 min and "recovered" with TTX for 30 s. A coated indentation (*CI*) is seen invaginating the plasmalemma. (*b* and *c*) Synaptosomes "stimulated" with K-rich media (37 mM Na in Na + 5 K replaced by 37 mM K) for 1 min. Fig. 8*b* shows a deep invagination of the plasmalemma (*PI*) which may represent an early step in the formation of a vesicle or vacuole. The terminals in both Fig. 8*b* and *c* have large ThO_2-containing vesicles (*LV**); in Fig. 8*c*, the large vesicle appears to have just budded off from the plasmalemma, with which it is still in contact. (*d*) Synaptosome "stimulated" with veratridine for 1 min, and "recovered" with TTX for 1 min. This synaptosome has several ThO_2-containing vesicles (*SV**); one large, labeled vesicle (*LV**) is still in contact with the plasmalemma. *ECF* = extracellular fluid. *PM* = plasmalemma. All bars, 0.1 μm.

brane and plasma membrane is blocked in Ca-free media, membrane retrieval should be reduced, as well. The data in Table I bear out this prediction: in the (virtual) absence of extracellular Ca, exposure to veratridine does not increase the percentage of terminals containing labeled vesicles, as compared to controls.

LATE STAGES OF MEMBRANE RECYCLING: If the veratridine-treated terminals are permitted to recover for 15 min or longer, additional changes in morphology are observed. The most obvious new feature is the appearance of numerous small (300–500-Å diameter) marker-containing vesicles. A few of these small labeled

TABLE I

Statistics on ThO_2-Labeling of Synaptic Vesicles

Incubation Conditions*	Number of synaptosomes counted‡	Percentage of synaptosome profiles with: ≥1 ThO_2-labeled vesicles	≥2 ThO_2-labeled vesicles	≥8 ThO_2-labeled vesicles	Experiment§
Control:					
TTX + veratridine, 1 min	132	19	5	0	A, B
TTX + veratridine, 60 min	86	17	6	0	B
Na + 5 K, 2 min	202	24	7	NC‖	C, D
Ca-free veratridine, 1 min	54	16	6	NC‖	A
Ca-free veratridine, 5 min	38	11	5	NC‖	A
"Stimulated" (with Ca):					
Veratridine, 1 min: TTX, 1 min	133	36¶	19¶	2	A, B
Veratridine, 1 min; TTX, 15 min	77	52¶	34¶	8¶**	A, B
Veratridine, 1 min; TTX, 60 min	141	62¶	47¶	20¶‡‡	B

* Incubated at 30°C for the time indicated (see Materials and Methods).

‡ Counts were made on profiles of terminals with diameters ≥0.4 μm in cross section (see Materials and Methods). "Damaged" terminals (see text) were not included in the tabulated data.

§ Each letter indicates a different synaptosome preparation.

‖ NC = not counted.

¶ Significantly greater than respective "control" values by χ^2 analysis; $P < 0.001$.

** Significantly greater than value after 1 min of recovery; $P < 0.001$.

‡‡ Significantly greater than value after 15 min of recovery; $P < 0.001$.

vesicles are seen within the first 15 min of recovery (Figs. 9 and 10), and by 60 min many of the terminals contain large numbers of these vesicles (Figs. 9, 11, and 12); terminals observed after 90 min of recovery (Fig. 13) are not significantly different from those "recovered" for 60 min. Most of these terminals (Figs. 11–13) are, in fact, indistinguishable from normal controls (Figs. 1 and 2) except for the presence of marker within many of the vesicles.

The data in Fig. 9 and Table I summarize the progression of changes in vesicle size and labeling during the process of synaptic vesicle membrane retrieval and recycling. Initially, after a period of depolarization, there is a relative depletion of small synaptic vesicles (see above), and a number of (new) large-diameter (600–900 Å) vesicles are formed (Figs. 3–8). In the experiments with extracellular markers, the markers are preferentially taken up by the large vesicles (Figs. 3, 6, and 8*c* and *d*); this occurs, presumably, because the large vesicles are formed by endocytosis (Fig. 8). At this early stage of recycling, very few small vesicles (<600-Å diameter) contain marker (Fig. 9), despite the large increase in the percentage of terminals with marker-filled vesicles (Table I); moreover, only a small fraction of the terminals contain as many as eight labeled vesicles in a cross section (Table I). The implication is that most small vesicles are not formed by endocytosis. However, within about 15 min, a new population of small marker-containing vesicles appears (Figs. 9 and 10); this is coincident with a marked rise in the percentage of terminals containing many (e.g., 8 or more; Table I) labeled vesicles. These data are consistent with the hypothesis that the new vesicles (containing only a few colloidal ThO_2 particles; Figs. 10–13) are somehow formed by subdivision of the large vesicles (which are often filled with numerous large ThO_2 particles; Figs. 3*b*, 5, and 8*c*). The recycling process appears to be completed within about 60 min (Figs. 9, 11, and 12). Unfortunately, we have thus far been unable to determine precisely how the small vesicles are formed from the large ones.

DISCUSSION

The Morphological and Functional Integrity of Synaptosomes

During the past few years considerable interest has been generated in the use of synaptosome preparations for studies of presynaptic function; the present study is an obvious example. It is

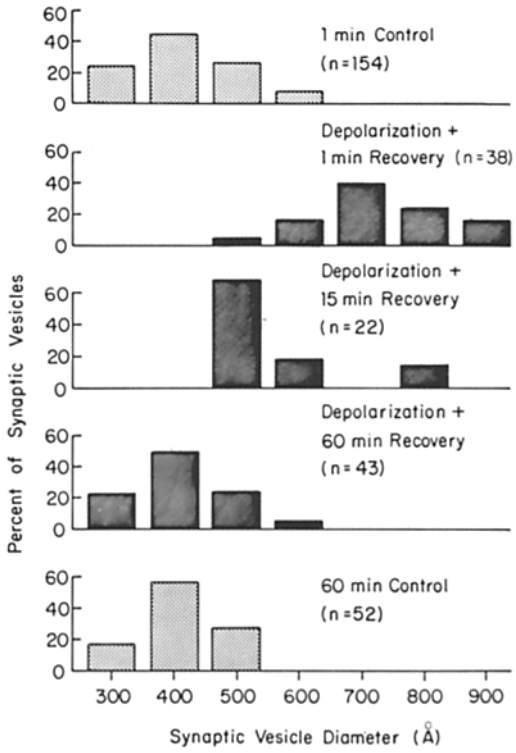


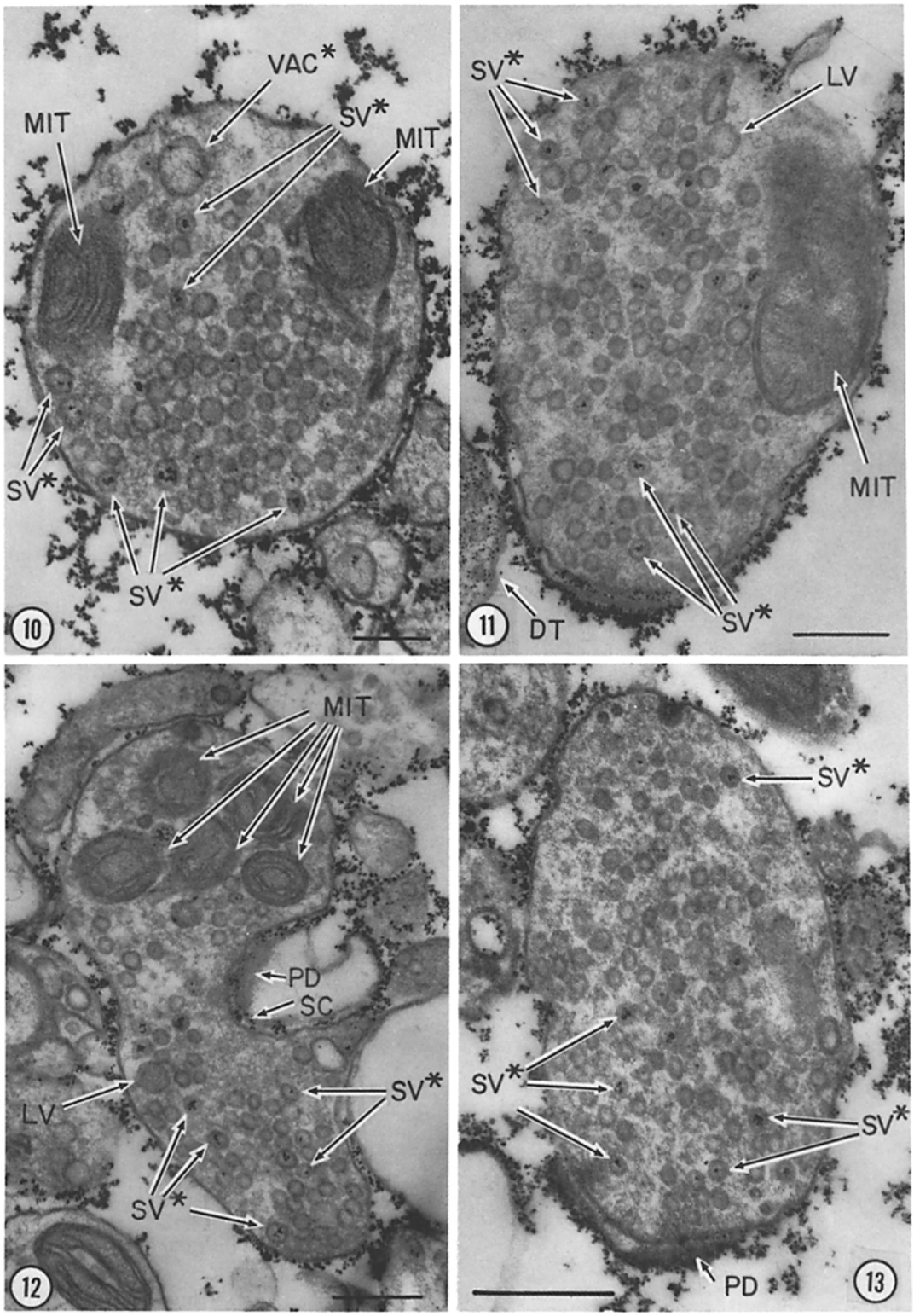
FIGURE 9 Histogram showing the relative frequency of occurrence of vesicles of various sizes in resting terminals, and in terminals "stimulated" with veratridine and "repolarized" with TTX. The stippled bars indicate the diameters of (unlabeled) synaptic vesicles in synaptosomes incubated in Na + 5 K at 30°C for 1 min or 60 min, as indicated. The black bars indicate the diameters of ThO_2-containing vesicles in synaptosomes depolarized with veratridine for 1 min and "recovered" with TTX for 1, 15, or 60 min, as indicated. The control samples were also incubated with ThO_2, but few synaptic vesicles in these terminals contained ThO_2, even after 60 min of incubation (see Table I). The vesicle sizes have been rounded off to the nearest 100 Å for convenience. χ^2 analysis shows that the distribution of ThO_2-labeled vesicle sizes after 1 min of recovery is significantly different from the distribution observed after 60 min or recovery ($P < 0.001$). The distribution of labeled vesicle sizes after 15 min of recovery is significantly different from both the 1-min and 60-min distributions ($P < 0.01$ in both cases). On the other hand, the distribution of labeled vesicle sizes after 60 min of recovery is not significantly different from the (unlabeled) vesicle size distribution in the 1-min or 60-min controls ($P > 0.5$). All of the data are from a single experiment (i.e., from one synaptosome preparation).

therefore helpful to have some measure of the quality of these preparations, in terms of their morphological and functional integrity. The use of extracellular markers is important in two ways: the markers may help to show which terminals are structurally damaged (i.e., with ruptured or incompletely resealed surface membranes) and, as demonstrated in Results, they can be used to provide morphological information about functional activity in intact terminals. We have estimated (see Results) that ~20–30% of the terminals in our preparations may be damaged—as indicated by the presence of markers in the cytoplasm. These values are quite close to values obtained from some of our biochemical studies: the data in Fig. 1 of reference 36, and in similar unpublished experiments, imply that extracellular Ca and ATP (which are, of course, much smaller than HRP and colloidal ThO_2) have direct access to the interior of ~15–25% of terminals bathed in normotonic media. In view of the rather harsh treatment to which these terminals are subjected during preparation, a 75%, or so, survival seems remarkably good.

It may be even more important to estimate the fraction of terminals that is functional. The data in Table I may be useful for this purpose: they indicate that ~50% of the nondamaged terminals show evidence of membrane retrieval and recycling. Thus, ~40% of all the terminals in our synaptosome preparations may be functionally intact. Of course, if some terminals do not normally exhibit membrane retrieval and recycling, this 40% value may be an underestimate of the percentage of functionally active terminals. Despite this uncertainty, our data do provide new and useful information about membrane recycling in the mammalian central nervous system.

Membrane Retrieval and Recycling in the Mammalian CNS

In the present study we have attempted to determine whether or not the isolated terminals can release transmitter by exocytosis, and whether or not they can retrieve and recycle vesicle membrane. Although the latter question has been answered in the affirmative, the evidence for exocytosis is only indirect and circumstantial, since no direct communications between small (300–500-Å diameter) vesicles and extracellular space were seen in the synaptic region (the "active zone" of Couteaux, [11]). However, during the first 1–2 min after "stimulation", the number of small vesicles per unit synaptosome area decreased significantly (see Figs. 3–7 and related text), as compared with controls (Figs. 1 and 2



FIGURES 10–13 Synaptosomes "depolarized" with veratridine for 1 min and "recovered" with TTX for 15 min (Fig. 10), 60 min (Figs. 11 and 12), or 90 min (Fig. 13). The terminals were all incubated with ThO_2. *SV** = some of the synaptic vesicles labeled with ThO_2; *LV* = large vesicle; *VAS** = a ThO_2-containing vacuole; *MIT* = mitochondria; *DT* = edge of a damaged terminal; *PD* = postsynaptic density. Note that the ThO_2 has penetrated into the synaptic clefts (*SC*) in the terminals in Figs. 11–13. The shape of the terminal in Fig. 12 suggests that, in vivo, it synapsed onto a dendritic spine. Bars, 0.2 μm.

and related text). No significant reduction in the number of small synaptic vesicles was observed when the terminals were depolarized in Ca-free media; this is consistent with the evidence that Ca is required for vesicular (quantal) transmitter release (e.g., references 33, 35). Only under conditions in which transmitter release is expected (viz. depolarization in the presence of Ca) was there clear evidence of membrane retrieval (Table I and Figs. 3–13). In view of the documentation available from studies on the peripheral nervous system (9, 26), it seems reasonable to conclude that the reduction in the density of small vesicles, which we noted immediately after stimulation, was a consequence of the fusion of vesicles with the plasmalemma.

The observations on the incorporation of extracellular markers are much more direct and compelling. They show that the isolated terminals possess all the machinery necessary for the retrieval and recycling processes. The main unanswered questions concern the mechanisms that underlie these processes. The fact that the small-vesicle population is reduced immediately after stimulation, and that relatively few small (300–500-Å diameter) marker-containing vesicles are observed at this time (Figs. 3–7 and 9), implies that the retrieval process is not a simple reversal of events after transient fusion between the vesicles and the plasmalemma. Figs. 11–13 show that ThO_2 can enter the cleft between the pre- and postsynaptic membranes at the "active zone", so that we might have expected to see rapid labeling of small vesicles if this type of retrieval did occur. In contrast, our data do show (Figs. 3–9) that membrane retrieval occurs in the form of large vesicles (including coated vesicles) and vacuoles. The membranes that form these structures appear to be pinched off from the plasmalemma in regions other than the "active zone". Unfortunately, our studies do not answer the question of how the membranes from these structures are recycled into new small synaptic vesicles; perhaps a detailed study of the morphological changes occurring in the period between 15 and 60 min of recovery, after a brief stimulation, would be helpful in this regard.

The aforementioned observations illustrate the functional competence of the pinched-off terminals; however, it is important to emphasize the fact that these considerations do not eliminate the possibility that, in intact neurons, new synaptic vesicles may also be derived from other sources (e.g., reference 16).

As noted in Results, the marker-labeled structures that are seen in rat brain synaptosomes shortly after a period of stimulation are very similar to those observed at the frog neuromuscular junction (23, 26). A major quantitative difference, however, is that coated vesicles were perhaps not so prevalent in our terminals (Figs. 3–7) as in the neuromuscular junction (possibly because we did not use en bloc staining, which may help to emphasize the coats [25]). However, Bunge (8) has observed that the most prevalent route of membrane retrieval in the growth cones of cultured neurites is via uncoated vesicles; taken in conjunction with our findings, this may indicate that uncoated vesicles play a more important role in membrane retrieval processes than has previously been suspected.

Large-diameter (600–900 Å) vesicles, which were particularly common in our preparations during the early stages of retrieval (Figs. 3–9), were not commented upon by Heuser and Reese (26). Inasmuch as the mean quantal content (i.e., the number of vesicles released by a single nerve impulse) at central synapses is very small (on the order of two–three; cf. reference 38) as compared with the mean quantal content at frog neuromuscular junctions (cf. reference 33), perhaps the large vesicles we observed represent the normal mode of membrane retrieval at central synapses. (Note that the surface area of these large vesicles is approximately the same as that of four 300–500-Å-diameter vesicles, because surface area increases as the square of the diameter).

It is well known that synaptosomes prepared from rat cerebral cortex are very heterogeneous, in terms of neurotransmitter type; transmitter uptake studies suggest that some terminals of each type (cholinergic, noradrenergic, GABAnergic, dopaminergic, etc.) are functional (1, 37). As we have noted, ~50% (Table I) of the structurally intact terminals show evidence of membrane retrieval and recycling after "stimulation"; this finding raises the possibility that most, if not all, of the transmitter-specific types of terminals release transmitter and recycle (vesicle) membranes by similar if not identical mechanisms.

It should be apparent that the observations described in this report do not represent the limits imposed by the use of synaptosome preparations. On the contrary, these studies encourage the view

that a judicious combination of morphological and biochemical methods may eventually give us critical new insight into some as-yet unanswered questions concerning presynaptic function.

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