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The Cytoskeletal Architecture of the Presynaptic Terminal and Molecular Structure of Synapsin 1

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Abstract. We have examined the cytoskeletal architecture and its relationship with synaptic vesicles in synapses by quick-freeze deep-etch electron microscopy (QF·DE). The main cytoskeletal elements in the presynaptic terminals (neuromuscular junction, electric organ, and cerebellar cortex) were actin filaments and microtubules. The actin filaments formed a network and frequently were associated closely with the presynaptic plasma membranes and active zones. Short, linking strands ~30 nm long were found between actin and synaptic vesicles, between microtubules and synaptic vesicles. Fine strands (30–60 nm) were also found between synaptic vesicles. Frequently spherical structures existed in the middle of the strands between synaptic vesicles. Another kind of strand (~100 nm long, thinner than the actin filaments) between synaptic vesicles and plasma membranes was also observed. We have examined the molecular structure of synapsin 1 and its relationship with actin filaments, microtubules, and synaptic vesicles in vitro using the low angle rotary shadowing technique and QF·DE. The synapsin 1, ~47 nm long, was composed of a head (~14 nm diam) and a tail (~33 nm long), having a tadpole-like appearance. The high resolution provided by QF·DE revealed that a single synapsin 1 cross-linked actin filaments and linked actin filaments with synaptic vesicles, forming ~30-nm short strands. The head was on the actin and the tail was attached to the synaptic vesicle or actin filament. Microtubules were also cross-linked by a single synapsin 1, which also connected a microtubule to synaptic vesicles, forming ~30 nm strands. The spherical head was on the microtubules and the tail was attached to the synaptic vesicles or to microtubules. Synaptic vesicles incubated with synapsin 1 were linked with each other via fine short fibrils and frequently we identified spherical structures from which two or three fibrils radiated and cross-linked synaptic vesicles.

We have examined the localization of synapsin 1 using ultracryomicrotomy and colloidal gold-immunocytochemistry of anti-synapsin 1 IgG. Synapsin 1 was exclusively localized in the regions occupied by synaptic vesicles. Statistical analyses indicated that synapsin 1 is located mostly at least ~30 nm away from the presynaptic membrane. These data derived via three different approaches suggest that synapsin 1 could be a main element of short linkages between actin filaments and synaptic vesicles, and between microtubules and synaptic vesicles, and between synaptic vesicles in the nerve terminals. The longer strands (~100 nm) associated with presynaptic membrane could consist of other proteins, most probably fodrin, judging from its structure. Because phosphorylation of synapsin 1 by Ca^{++}/calmodulin-dependent kinase detaches synapsin 1 from vesicles it could release synaptic vesicles from actin filaments, microtubules and other synaptic vesicles, and thus increase the mobility of synaptic vesicles to the presynaptic membrane after depolarization dependent influx of Ca^{++} into the presynaptic terminal.

According to recent studies concerning the phenomena involved in transmitter release at nerve terminals, it has been shown that depolarization dependent Ca^{++} influx into the nerve terminal through Ca^{++} channel triggers exocytosis of synaptic vesicles (18). However, the processes between Ca^{++} influx and vesicle fusion to the presynaptic membranes are totally unknown. Similar processes could occur on the occasion of exocytosis induced by Ca^{++} influx into secretory cells.

Recently several proteins associated with synaptic vesicles or localized in the presynaptic terminals such as synapsin 1 and fodrin (calspectin), were identified biochemically or immunocytochemically (6, 7, 11, 23–26, 28, 31, 36, 42–46). Previous biochemical studies indicated that synapsin 1 binds to actin, tubulin, neurofilaments, and synaptic vesicles (1, 2, 12, 24, 33, 36). The binding of synapsin 1 and cytoskeletal elements or membranes was reported to be regulated by phosphorylation of synapsin 1 via Ca^{++} calmodulin-depen-

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dent protein kinase II (1, 33). A recent physiological study has shown that indeed phosphorylation of synapsin 1 could regulate the release of the transmitter (30). In contrast to the biochemical and physiological works about the elements of presynaptic terminals, structural data of the cytoskeleton in the presynaptic terminals, especially concerning the relationship between the cytoskeleton and membranes at a molecular level, is lacking, although some previous studies did indicate the existence of actin filaments and microtubules in the presynaptic terminals (3, 9, 14, 20, 29).

This study was designed to elucidate the cytoskeletal architecture in the presynaptic terminal at the molecular level, with special emphasis on the structure of synapsin 1 and its possible localization in the presynaptic terminal. We will present evidence to suggest that synapsin 1 and fodrin could actually capture synaptic vesicles in the network of the presynaptic cytoskeleton composed of actin filaments and microtubules and thus could play an important role in the process of transmitter release.

Materials and Methods

Quick-Freeze, Deep-Etch Electron Microscopy of Synapses

Rat cerebellum, frog neuromuscular junctions, and the electric organ of electric rays were chosen for this part of our study.

After anesthesia rat cerebella were dissected out and 100–200-μm sections were cut by microslicer or vibratome in Ca^{++}-free rat Ringer's solution (155 mM NaCl, 5 mM KCl, 5 mM Hepes, 10 mM glucose, 0.5 mM NaH_2PO_4, 4 mM EGTA, 6 mM $MgCl_2$ pH 7.4). Some sections were fixed with 2% paraformaldehyde in Ringer's for 2 h, washed with distilled water briefly, and quick frozen. Others were quick frozen immediately. In other cases cerebella were homogenized by a glass homogenizer in Ca^{++}-free Ringer's or in Ca^{++}-free Ringer's solution containing 0.5 mg/ml subfragment 1 of myosin. These were centrifuged at 3,000 rpm for 10 min. By this method large fragments of nerve cells could be obtained. The resulting fragments were quick frozen. Cutaneous pectoris muscles from tiny frogs (*Rana pipiens*) were dissected. After brief saponin treatment as described previously (19, 19a), the muscles were quick frozen. Electric organs of *Narke Japonica* or *Torpedo Californica* were dissected and homogenized in the buffer with or without S1. After centrifugation, the pellets were quick frozen as described previously (17, 19).

Purification of Synapsin 1

Synapsin 1 was prepared from calf brain membrane fractions according to a method described by Okabe and Sobue (33). The final concentration of synapsin 1 was 0.32 mg/ml of 0.1 M KCl, 0.1 mM DTT, 0.1 mM EGTA, 10 μM Tris HCl, pH 7.5.

Measurement of the Phosphate Contents in Synapsin 1

The amount of synapsin 1–bound phosphate were assayed as follows. The synapsin 1 preparations were precipitated and washed repeatedly with cold trichloroacetic acid. The resulting precipitates were extracted with hot trichloroacetic acid to remove noncovalently bound phosphate and nucleic acids (4). After dryness of precipitates at 90°C in the presence of magnesium nitrate, inorganic phosphate in the synapsin 1 preparations was measured by the method of Hess and Derr (13). Phosphorylation of synapsin 1 by Ca^{++}- and calmodulin-dependent protein kinase II and cyclic AMP-dependent protein kinase was carried out under the same conditions as described previously (33).

Production and Purification of Anti-Synapsin 1 Antibody

Antibodies against synapsin 1 were prepared and IgG fractions were obtained by a method described previously (26, 33). The preparation of a synapsin 1 affinity column was performed by the conjugation of the purified protein with cyanogen-activated Sepharose 4B. By this method, 2–3 mg of synapsin 1 was coupled to 1.0 ml of Sepharose gel. The IgG fraction obtained was passed through a synapsin 1–coupled Sepharose 4B affinity column and extensively washed with PBS. The specific antibody against synapsin 1 was eluted with 0.1 M glycine-HCl, pH 2.3. The eluted material was immediately neutralized with 1 M Tris base and then dialyzed against PBS containing 1 mM NaN_3.

Preparation of Synaptic Vesicle Fractions

Synaptic vesicles were prepared from rat brains by sucrose density gradient centrifugation according to a method described by Ueda et al. (44). The final vesicle fractions were centrifuged at 78,000 *g* for 60 min and the resulting pellets were resuspended into small volumes of a similar buffer to that used for suspension of synapsin 1. The protein concentration was 7.1–12.5 mg/ml.

In Vitro Reconstitution Experiments

Tubulin was purified from hog brains by the phosphocellulose column chromatography procedure of Shelanski et al. (37). Actin was prepared from chicken as described previously (38).

Actin plus Synapsin 1

G actin was incubated in K^+ buffer (0.1 M KCl, 2 mM ATP, 2 mM 2 mercaptoethanol, 2 mM Tris HCl, pH 7.5) at 37°C for 15 min. After brief centrifugation at 20,000 *g* for 10 min to remove aggregates, the suspension was mixed with synapsin 1 at a weight ratio of 1:1.3 (actin/synapsin 1). The suspension was incubated at 37°C for 15 min and then centrifuged at 27,000 *g* for 20 min. The resulting pellets were resuspended into a small volume of buffer, dropped on mica flakes, and quick frozen (15, 22).

Synaptic Vesicles plus Synapsin 1

Synaptic vesicles were mixed with synapsin 1 at a weight ratio of 5:3 (synaptic vesicles/synapsin 1) incubated at 37°C for 10 min in 0.1 M KCl, 0.1 mM DTT, 0.1 mM EGTA, 10 μM Tris HCl pH 7.5, and centrifuged at 27,000 *g* for 30 min. Synaptic vesicles without adding synapsin 1 were processed similarly. Pellets were resuspended in a small volume of the same buffer. The suspension was applied to mica flakes and quick frozen.

Microtubule plus Synapsin 1

PC tubulin was incubated in PEM buffer (0.1 M Pipes, 1 mM EGTA, 1 mM $MgCl_2$, pH 6.8) containing 1 mM GTP, 20 μM taxol (Drug Synthesis & Chemistry Branch, Division of Cancer Treatment, National Cancer Institute) at 37°C for 10 min. The synapsin 1 was added to the suspension at a weight ratio of 2:1 (tubulin/synapsin 1). The suspensions were incubated at 37°C for 5 min and centrifuged at 40,000 *g* for 30 min. The resulting pellets were resuspended into a small volume of PEM containing 1 mM GTP and 10 μM taxol. The suspensions were applied to mica flakes, quick frozen, and deep etched.

Actin Plus Synapsin 1 plus Synaptic Vesicles

Actin filaments were prepared as described previously (38). Filaments were mixed with synapsin 1 and synaptic vesicles at a weight ratio of 1:0.3:2 (actin/synapsin 1/synaptic vesicles), incubated in K^+ buffer without ATP at 37°C for 10 min, and centrifuged at 27,000 *g* for 30 min. Pellets were resuspended in a small volume of K buffer without ATP. The suspension was applied to mica flakes and quick frozen.

Microtubules plus Synapsin 1 plus Synaptic Vesicles

Tubulin was incubated at 35°C for 15 min in the presence of 20 μM taxol and 1 mM GTP. Then synapsin 1 and synaptic vesicles were added to the solution at a weight ratio of 2:0.3:2 (tubulin/synapsin 1/synaptic vesicle). After incubation at 30°C for 10 min, the solution was centrifuged at 27,000 *g* for 30 min. The resulting pellets were resuspended in a small volume of PEM containing 1 mM GTP and 10 μM taxol, then dropped on mica, and quick frozen.

Low Angle Rotary Shadowing

The molecular structure of synapsin 1 was examined by the low angle rotary

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shadowing technique developed by Tyler and Branton (41). Purified synapsin I was dissolved in 50% glycerol PEM and sprayed on mica and dried by vacuum evaporation. The samples were rotary shadowed with platinum in a freeze-fracture machine (model 301; Balzers, Furstentum Liechtenstein) at an angle of 6°. The replicas were detached from mica with hydrofluoric acid, washed with distilled water, and collected on Formvar carbon-coated grids.

Immunocytochemistry of Synapsin 1 Using Ultrathin Cryosections

Procedures for immunolabeling of ultrathin cryosections were performed according to Tokuyasu (39) with slight modifications as follows. Adult rats were perfused with 0.1% glutaraldehyde and 2% paraformaldehyde in a 0.1 M phosphate buffer, pH 7.3. The cerebellum was dissected out, cut into small ~8 mm^3 size pieces, and stored overnight in the above fixative. These were washed with 7% sucrose in a 0.1 M phosphate buffer, followed by treatment with 1 mg/ml sodium borohydride in the above buffer containing 7% sucrose. The samples were infiltrated with 2.3 M sucrose in a 0.1 M phosphate buffer and quickly frozen with liquid Freon 22. Ultrathin sections were cut with a cryomicrotome at −100°–−90°C (Ultracut OmU4 equipped with FC-4; Reichert Jung, Vienna, Austria). Gold sections were picked up by a drop of 2.0 M sucrose and 0.75% gelatin in 0.1 M phosphate buffer (pH 7.3) and attached to Formvar-coated grids. The sections were then stained for immunocytochemistry by the following procedure. The sections were washed with 50 mM glycine in PBS (five changes, total of 10 min), blocked with 2% normal goat serum in BSA-PBS, then incubated with 40 times diluted anti-synapsin I antibodies at 37°C for 30 min. After incubation, the sections were washed on 5 drops of 0.2% BSA in TBS pH 8.2 for 5 min each. They were then incubated with a 20-fold dilution of colloidal gold labeled second antibody (goat anti-rabbit IgG:G10; Janssen Life Sciences Products, Beerse, Belgium) for 30 min at 37°C. They were washed with 0.2% BSA-TBS thoroughly. After washing with 0.2% BSA-TBS, pH 8.2, and PBS, sections were fixed with 2% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.3). They were washed with distilled water, stained with uranyl acetate-oxalate (pH 7.0) for 10 min, washed again, and then transferred to the surface of a uranyl acetate-methyl cellulose mixture. After being left to stand for 10 min, sections were embedded in the same mixture without washing. Nonspecific rabbit IgG instead of the first antibody was used as control.

Others

Protein concentration was determined by the method developed by Bradford et al. (5). Western blotting was performed as described by Towbin et al. (40). SDS-PAGE was carried out using 7.5% running gel and 5% stacking gel according to Laemmli (27).

Electron Microscopy and Measurement

The replicas and sections were observed by a JEOL 2000 EX electron microscope at 100 kV. Stereo electron micrographs were taken by tilting the replicas ± 10°. Low angle shadowing replica and ultracryosections were observed without tilting the samples. For measurement the negatives were printed at 200,000× magnification and measured with a magnifying glass. As for the distance of gold particles representing the localization of synapsin I from the presynaptic membrane, the area opposite the postsynaptic membranes was determined. Then a line from a gold particle perpendicular to the presynaptic membrane was drawn and its length was measured.

Results

Quick-Freeze, Deep-Etch View of the Presynaptic Cytoskeleton and its Relation to the Synaptic Membranes

Fig. 1, *A* and *B* demonstrate inside views of presynaptic terminals of the electric organ of electric rays. These samples were homogenized and incubated briefly with the subfragment 1 of myosin then quick frozen after washing. A network of filaments could be observed inside the presynaptic terminal. These filaments were shown to be actin filaments by S1 decoration, revealing ropelike structures after the decoration (16). These micrographs clearly show the existence of a network of randomly running actin filaments in the presynaptic terminal, and that synaptic vesicles are captured in this network.

Fig. 2 *A* highlights the relationships between actin filaments and synaptic vesicles and between synaptic vesicles at high magnification in the neuromuscular junction. This sample was briefly treated with saponin to release soluble proteins. It is clearly shown here that an actin filament runs close to the presynaptic membrane and that short fine strands (~30 nm long) exist between actin filaments and synaptic vesicles. These strands appear to be thicker at the ends where they attach to the actin filaments. Fine short strands link synaptic vesicles with each other as well (Fig. 2 *A*). Frequently spherical structures appeared to connect two or three fine strands associated with synaptic vesicles (Fig. 2 *A*). Basically similar structures were also observed in the presynaptic terminals of mammalian central nervous system (Figs. 2 *B*, 3, and 4 *A*).

Fig. 3, *A–D* show mossy fiber synapses inside the presynaptic terminals of the rat cerebellar cortex. These micrographs display networks of actin filaments (9 nm diam) showing 5-nm banding patterns (16) (Fig. 3, *A–D*). This was confirmed by S1 decoration which clearly exhibited decorated actin filaments associating with the presynaptic membrane (Fig. 3 *E*), and this was sometimes seen to take place at the possible releasing area (active zones) (Fig. 3 *B*). The important findings are that fine short strands (<~30 nm) existed between actin filaments and synaptic vesicles (Fig. 2 *B*) and short, fine fibrils were also found between synaptic vesicles (Fig. 3, *C* and *D* and Fig. 4) and that there was another kind of fine filament, which was thinner than actin filaments but longer (>~100 nm) than the short strands described above, mainly between synaptic vesicles and presynaptic membranes (Fig. 3, *C* and *D*). We found fine short linkages (~30 nm long) between microtubules and synaptic vesicles as well (Fig. 4). Frequently we found spherical structures at the end of the short linkages on the microtubules (Fig. 4).

Single Molecular Structure of Synapsin 1 and In Vitro Reconstitution of the Relationship among Synapsin 1, Actin, Microtubules, and Synaptic Vesicles. Single Molecular Structure of Synapsin 1

Purified synapsin 1 from the calf brain can be identified in Fig. 5 *A*, lane *1*. A single molecule of synapsin 1 revealed by the rotary shadow technique looks like a tadpole composed of a spherical head (~14 nm diam) and tail (~37 nm long) (Fig. 6).

Phosphorylation State of the Purified Synapsin 1

We measured the phosphate contents in synapsin 1 according to the method of Hess and Derr (13). As shown in Table I, the unphosphorylated synapsin 1 preparations used in these experiments contain <0.15 mol of bound phosphate per mole of polypeptide. On the other hand, Ca^{2+}- and calmodulin-dependent protein kinase II and cyclic AMP-dependent protein kinase maximally phosphorylate synapsin 1 at ratios of 3.01 and 0.94 mol of phosphate per mole of protein, respectively. The values of phosphate incorporation by two protein kinases are good agreement with previous observation determined by [^{32}P]γATP as phosphate donor (33). Therefore,

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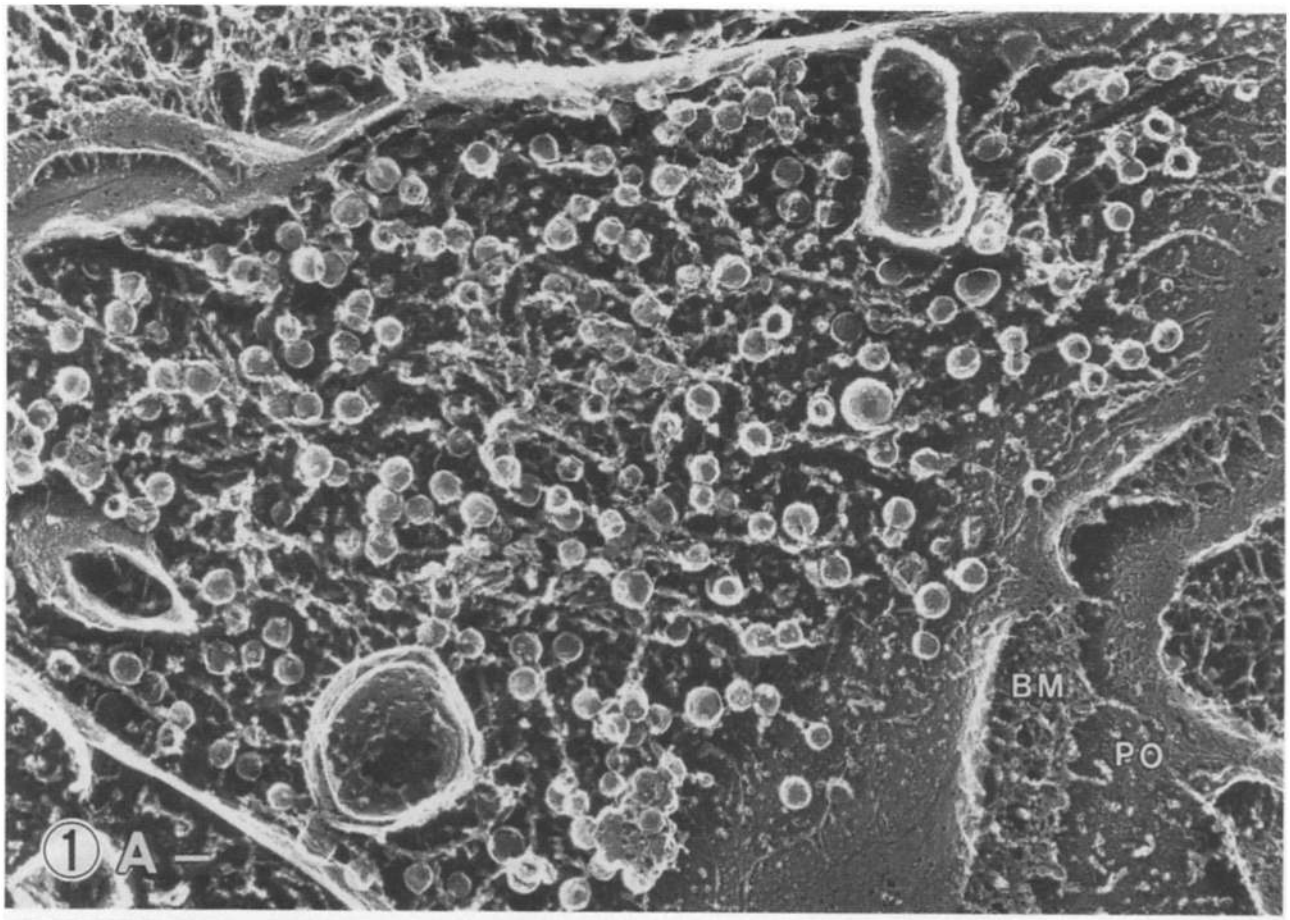

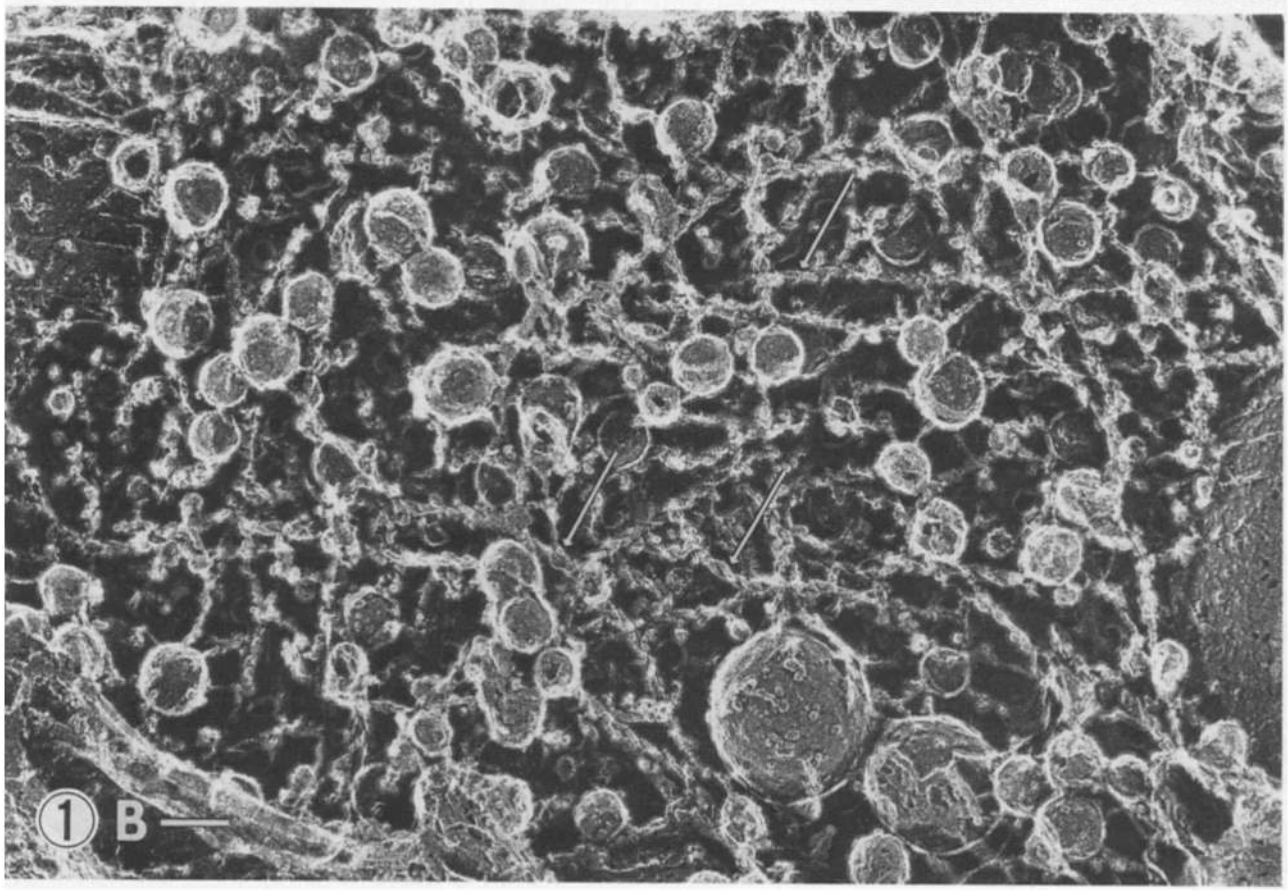

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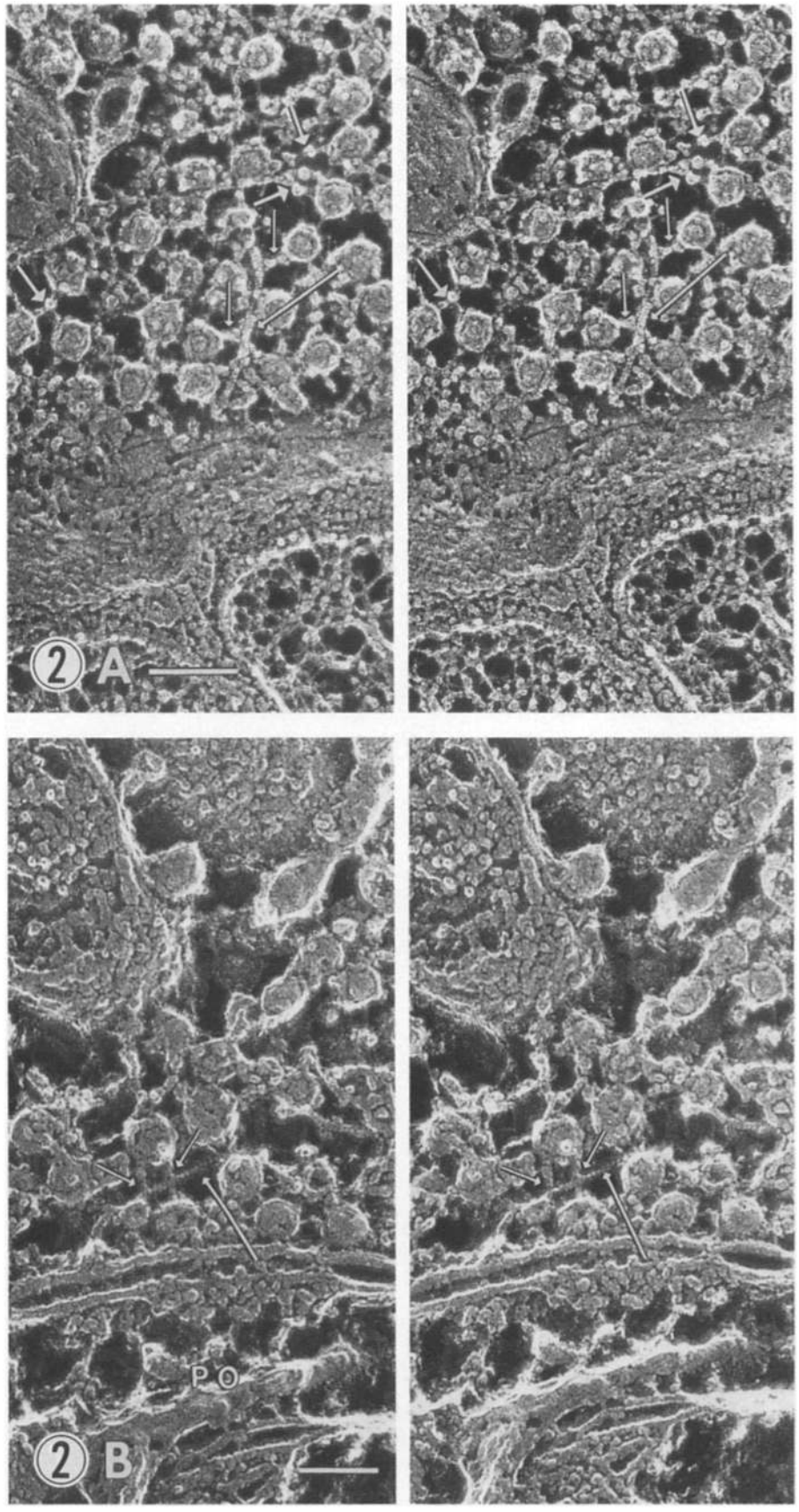

Figure 2. (*A*) Inside the presynaptic terminal of frog neuromuscular junction. These stereo micrographs show clearly that there are short strands (20–30 nm) (*short arrows*) between actin filament (*long arrow*) and synaptic vesicles. Short linking strands (30–60 nm) are found between synaptic vesicles as well (*thick arrows*). Frequently globular structures are observed in the middle of linking strands between synaptic vesicles (*thick arrows*). (*B*) Inside a mossy fiber terminal of a rat cerebellar cortex. An actin filament (*long arrow*) runs close to the presynaptic membrane (active zone). Short strands (*short arrows*) link the synaptic vesicle to the actin filament. *PO*, postsynaptic part. Structures comparable to the postsynaptic density observed by ultrathin section method is present opposite the presynaptic membrane. Bar, 0.1 μm.

Figure 1. (*A*) Quick-frozen, deep-etched *Torpedo* electric organ treated with S1. The presynaptic terminal is filled with synaptic vesicles and actin filaments. *BM*, Basement membrane; *PO*, postsynaptic membrane. (*B*) A higher magnification view of the inside of the presynaptic terminal of *Torpedo* electric organ. Actin filaments decorated with S1 (*arrows*) exist as a network in the presynaptic terminal. Bar, 0.1 μm.

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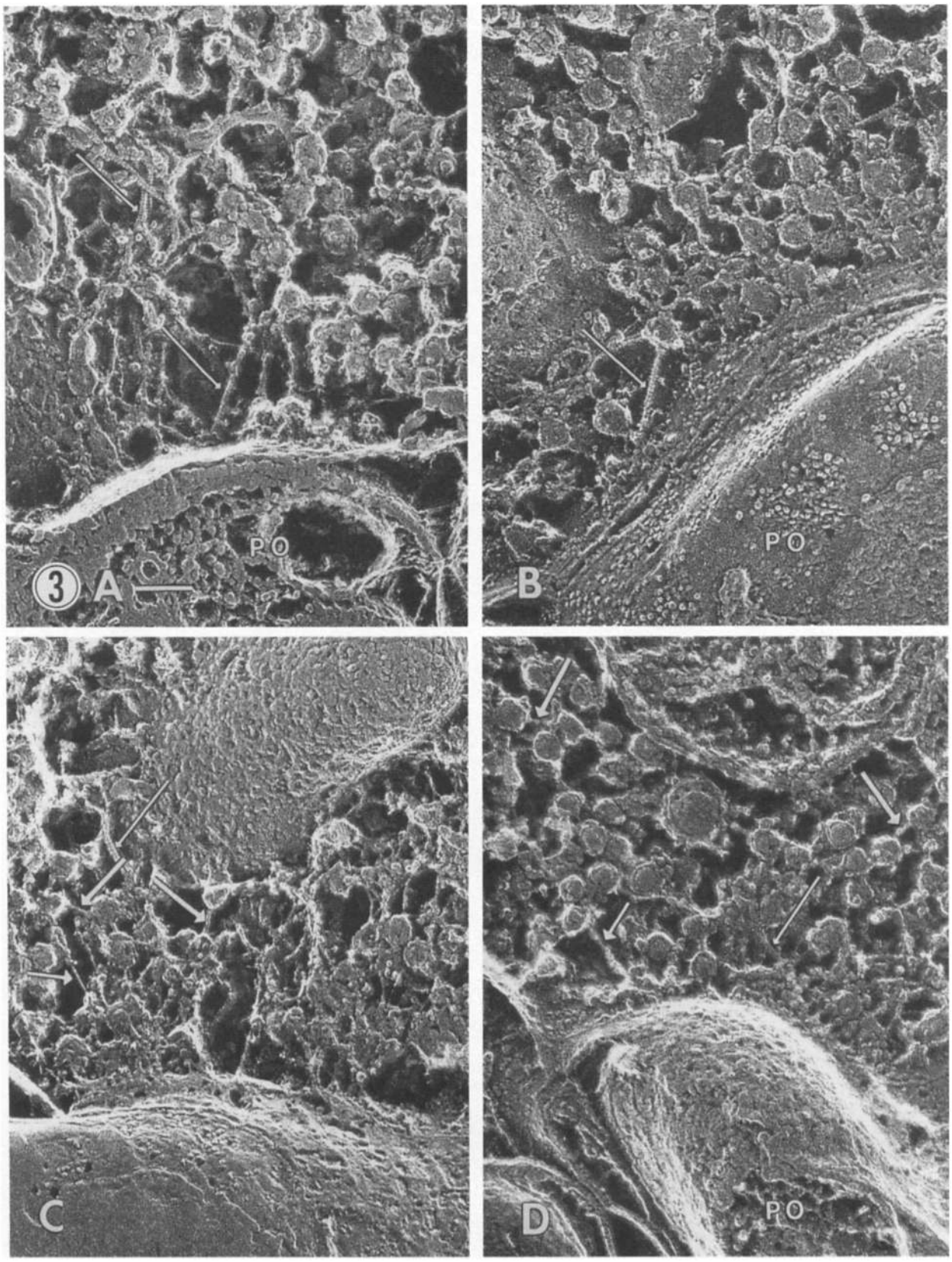

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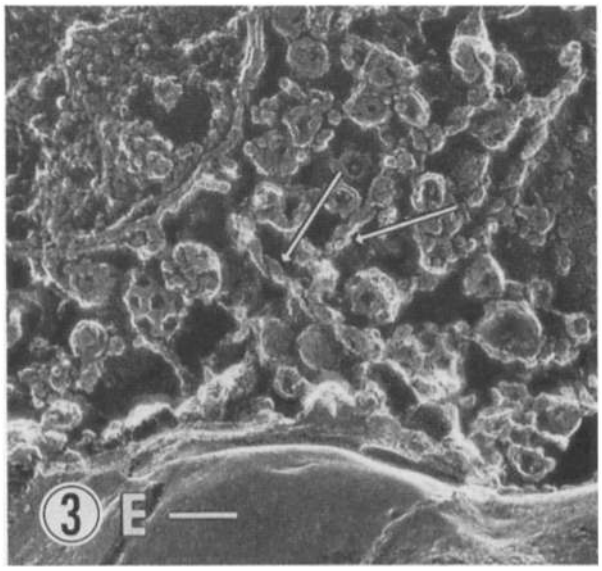

Figure 3. (*A*, *B*, *C*, and *D*). Presynaptic terminals of rat cerebellar mossy fibers. There are actin filaments (*long arrows*) showing 5-nm banding patterns in the presynaptic terminals. Actin filaments sometimes run very close to the presynaptic membranes. Long strands (>~100 nm) (*short arrows*) thinner than actin filaments link synaptic vesicles with the presynaptic membrane. Synaptic vesicles are linked with each other via fine short fibrils (*thick long arrows*) *PO*, postsynaptic part. (*E*) A presynaptic terminal of a rat cerebellar mossy fiber. This sample is treated with S1. Decorated actin filaments (*arrows*) run very close to the presynaptic membrane. Bar, 0.1 μm.

synapsin 1 used in these experiments was considered to be less significantly phosphorylated preparation.

Actin plus Synapsin 1

After mixing actin with synapsin 1, actin filaments formed bundles. The suspension of actin filaments was centrifuged and pellets were resuspended into a small volume of buffer solution (Fig. 5 *A*, lanes *2–4*). The resuspended solution was dropped on mica flakes and quick frozen and deep etched. This way we could observe the actin and synapsin 1 complex in suspension.

Fig. 7, *A* and *B* show actin filament bundles formed after adding synapsin 1 to actin filaments. Numerous fine short bridges (20–30 nm long) were seen to cross-link actin filaments. Frequently we observed a spherical head on an actin filament and only a tail on the adjacent actin filament (Fig. 7, *A* and *B*), indicating that a single synapsin 1 can cross-link actin filaments.

Synaptic Vesicles plus Synapsin 1

Synaptic vesicles were purified from rat brains by density gradient centrifugation according to Ueda et al. (44). After incubation in buffer solutions with or without synapsin 1, synaptic vesicles were centrifuged. The resulting supernatants and pellets were examined by SDS-PAGE. As shown in Fig. 5 *B* pellets of synapsin 1 plus synaptic vesicles contain a large amount of synapsin 1 compared with the pellets of synaptic vesicles only (Fig. 5 *B*). Therefore, synapsin 1 specifically bound to synaptic vesicles. The pellets were resuspended into a small volume of the buffer, dropped on the mica flakes, and quick frozen deep etched. This way we could observe the synaptic vesicles and synapsin 1 complex in suspension. The synaptic vesicles incubated with synapsin 1 were much more frequently linked with each other via fine short strands than synaptic vesicles incubated without synapsin 1 (Fig. 8). The length of strands varied from 30–60 nm. Frequently we recognized spherical head-like structures from which two or three fine strands radiated and cross-linked synaptic vesicles (Fig. 8). We assume that heads of synapsin 1 can associate with each other to link adjacent synaptic vesicles.

Actin plus Synapsin 1 plus Synaptic Vesicles

The suspension of actin and synapsin 1 and synaptic vesicle was centrifuged and the pellet was quick frozen, deep etched after resuspending into a small volume of the buffer (Fig. 5 *C*, lane *3*). 20–30-nm short strands were observed between synaptic vesicles and actin filaments (Fig. 7 *C*). Often a spherical head was found on the actin filament and a tail attached to synaptic vesicles (Fig. 7 *C*). Thus, a single synapsin 1 is able to link an actin filament to a synaptic vesicle. In this case the head always seems to be on the actin and the tail is attached to the synaptic vesicle.

Microtubules plus Synapsin 1

PC tubulin from hog brains was combined with synapsin 1 in the presence of taxol and 1 mM GTP. The suspension was incubated at 37°C for 10 min and centrifuged. The resulting pellets were quick frozen, deep etched after resuspending into a small volume of the buffer (Fig. 5 *A*, lane *6*). Microtubules were mainly arranged in a parallel fashion. Between adjacent microtubules numerous fine short strands (20–30 nm long) were found (Fig. 9, *B* and *C*). Because a spherical head was often localized on one microtubule while on the adjacent microtubule a tail was found, a single synapsin molecule seemed to cross-link adjacent microtubules with each other (Fig. 9, *B* and *C*).

Interestingly, when adjacent microtubules were further apart we found globular masses from which fine strands radiated and cross-linked microtubules. From this we assume that synapsin 1 molecules can connect with each other at their heads.

Microtubule plus Synapsin 1 plus Synaptic Vesicles

We purified synaptic vesicles from rat brains and mixed synaptic vesicles and synapsin 1 with microtubules polymerized by taxol from PC tubulin. The aliquots were centrifuged and the pellets were resuspended with a small amount of buffer solution and quick frozen, deep etched (Fig. 5 *C*, lane *4*). Numerous synaptic vesicles were attached to microtubules and short fine strands (10–30 nm long) linked synaptic vesicles with microtubules (Figs. 9 *A* and 10). In this case spherical heads located on the microtubules and tails attached to synaptic vesicles (Fig. 10).

Thus a single synapsin 1 molecule linked a microtubule with a synaptic vesicle. The tail ends can be considered to be the binding sites of synapsin 1 to the synaptic vesicles.

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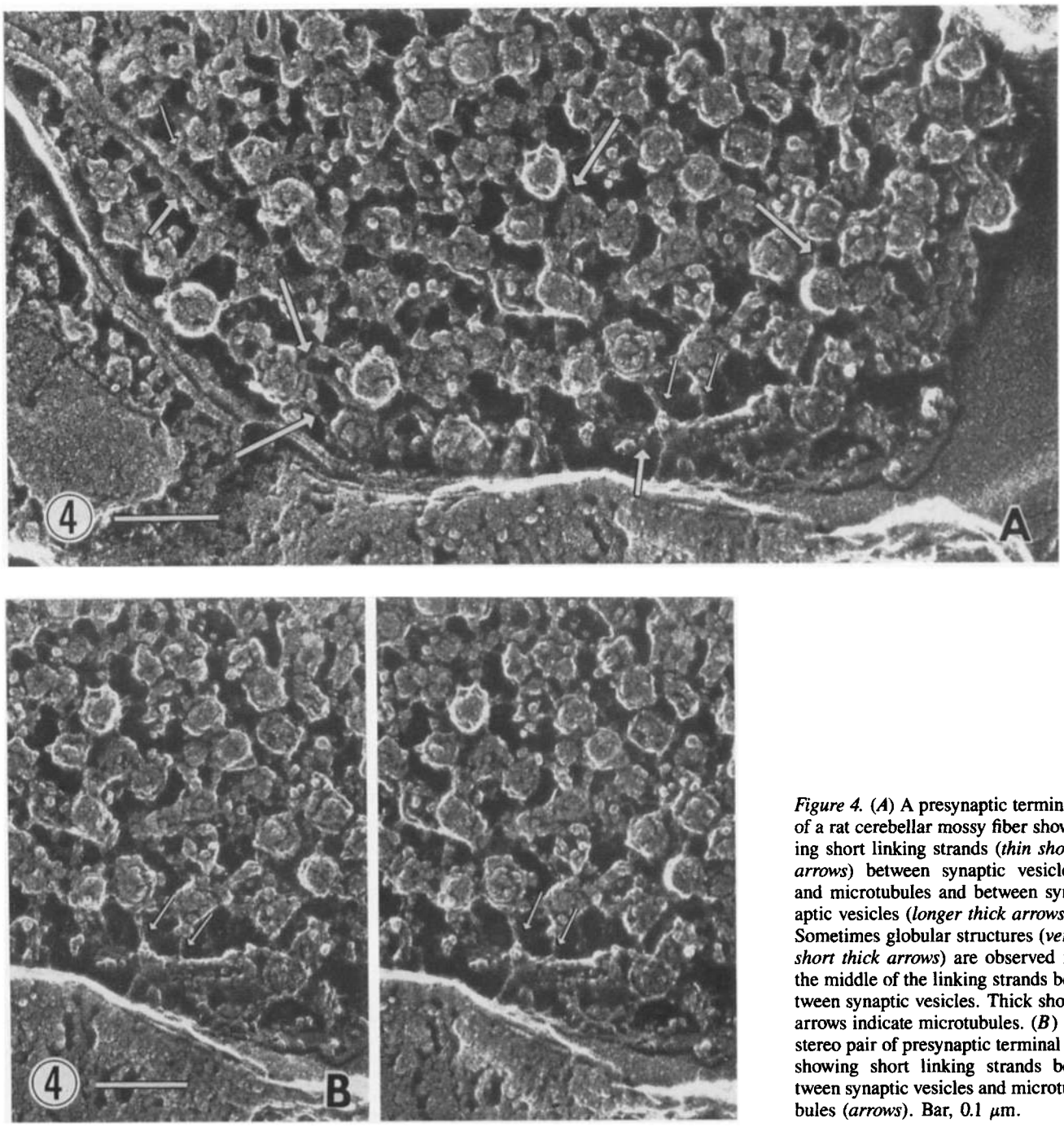

Figure 4. (*A*) A presynaptic terminal of a rat cerebellar mossy fiber showing short linking strands (*thin short arrows*) between synaptic vesicles and microtubules and between synaptic vesicles (*longer thick arrows*). Sometimes globular structures (*very short thick arrows*) are observed in the middle of the linking strands between synaptic vesicles. Thick short arrows indicate microtubules. (*B*) A stereo pair of presynaptic terminal *A* showing short linking strands between synaptic vesicles and microtubules (*arrows*). Bar, 0.1 μm.

Immunocytochemical Localization of Synapsin 1 Revealed by Anti-Synapsin 1 and Gold Label Second Antibody Decoration of Ultrathin Cryosections

The localization of synapsin 1 has been previously studied via two major approaches. De Camelli et al. (6) examined immunofluorescence localization of synapsin 1 using affinity-purified antibody on the cryostate sections which were washed with a solution containing Triton X-100. They also very impressively illustrated the association of synapsin 1 with synaptic vesicles by incubating osmotically ruptured synaptosomes with antisynapsin 1 and ferritin-labeled second antibody (7). In contrast, Goldenring et al. (12) have reported that synapsin 1 immunoreactivity is specifically associated with the neuronal cytoskeleton as well as synaptic vesicles. In their study Goldenring and co-workers (12) used vibratome sections of fixed rat brains and they omitted Triton X-100 from the reaction medium. They concluded that synapsin 1 could attach to microtubules and neurofilaments in the neuropile and that this group of synapsin 1 could be released if the reaction solution contained Triton X-100.

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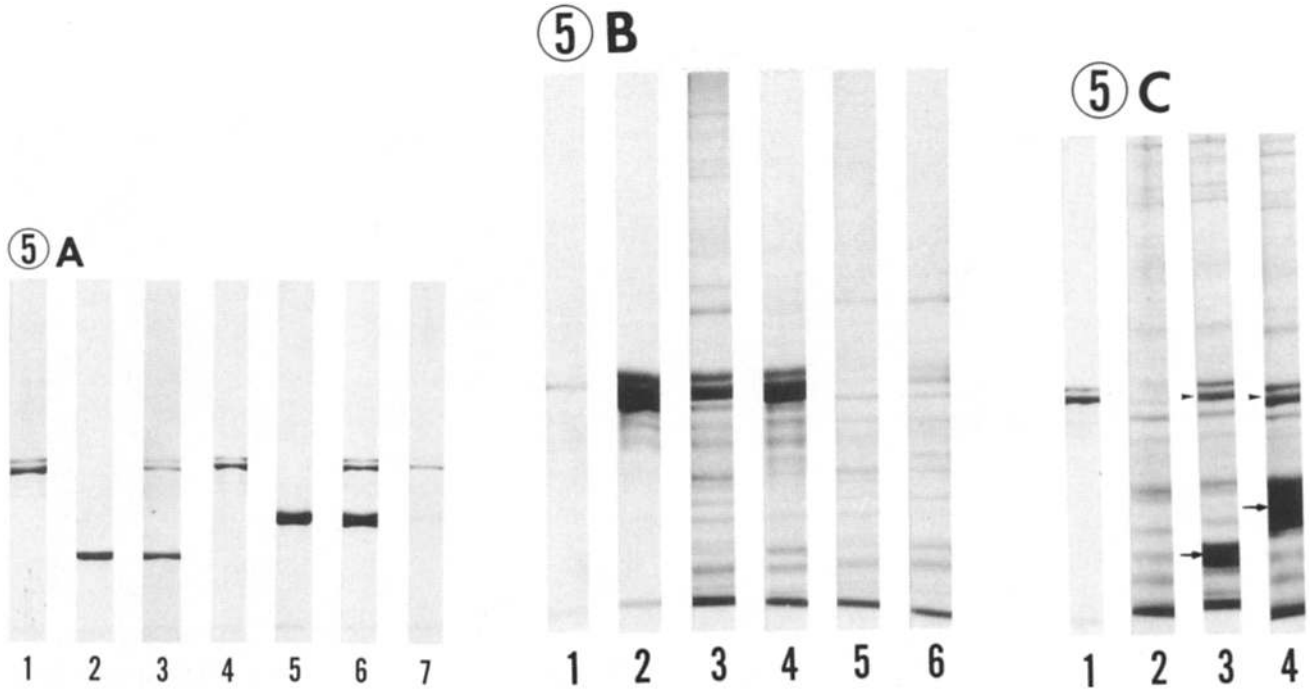

Figure 5. (*A*) SDS-PAGE of binding experiments of synapsin 1 and actin or microtubules. Lane *1*, purified synapsin 1 composed of 1a and 1b; lane *2*, actin; lane *3*, pellet of actin plus synapsin 1; lane *4*, supernatant of actin plus synapsin 1; lane *5*, tubulin; lane *6*, pellet of microtubules plus synapsin 1; lane *7*, supernatant of microtubules plus synapsin 1. Synapsin 1 bound to actin and microtubules. (*B*) SDS-PAGE of binding experiments of synapsin 1 and synaptic vesicles. Lane *1*, pellet of synapsin 1 only; lane *2*, supernatant of synapsin 1 only; lane *3*, pellet of synaptic vesicles plus synapsin 1; lane *4*, supernatant of synaptic vesicles plus synapsin 1; lane *5*, pellet of synaptic vesicles only; lane *6*, supernatant of synaptic vesicles only. Synapsin 1 bound to synaptic vesicles. (*C*) SDS-PAGE of binding experiments of synapsin 1 and synaptic vesicles and actin or microtubules. Lane *1*, purified synapsin 1, lane *2*, synaptic vesicles; lane *3*, pellet of synapsin 1 plus synaptic vesicles plus actin (*arrow*, actin; *arrowhead*, synapsin 1); lane *4*, pellet of synapsin 1 plus synaptic vesicles plus microtubules (*arrow*, tubulin; *arrowhead*, synapsin 1). Synaptic vesicles bound to actin and microtubules.

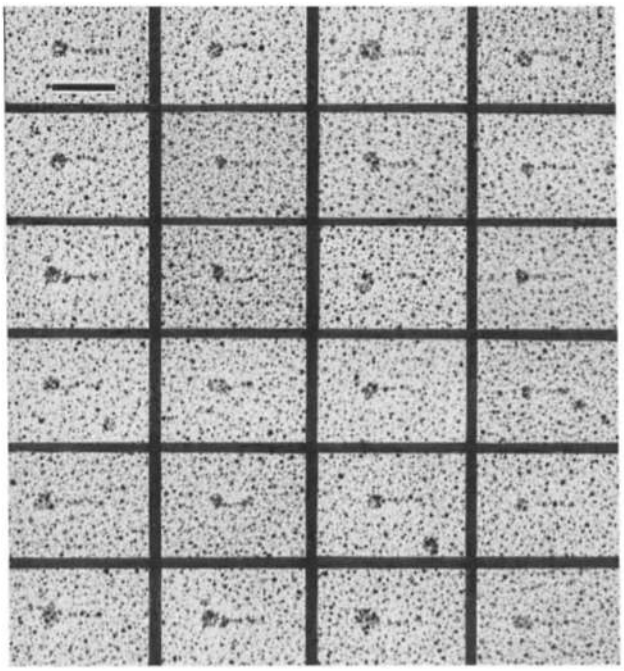

Figure 6. A gallery of the structure of synapsin 1 molecules revealed by the low angle rotary shadowing technique. Bar, 50 nm.

We attempted to analyze the localization of synapsin 1 using different approaches which could reflect natural localization of synapsin 1. For this purpose we used ultrathin cryosections for immunostaining. With these sections we thought we could omit Triton X-100 in the reaction medium because the penetration of antibodies would be much better than in previous studies. In addition, by this method the membrane is not necessarily permeabilized, meaning that the natural relationship between the presynaptic membrane and localization of synapsin 1 could be studied.

Table I. Phosphate Contents in Synapsin I

Samples	Phosphate contents (mole of phosphate/mole of synapsin I)
Unphosphorylated synapsin I	0.15 ± 0.02
Phosphorylated synapsin I (Ca^{2+}- and calmodulin-dependent protein kinase II)	3.01 ± 0.13
Phosphorylated synapsin I (cAMP-dependent protein kinase)	0.94 ± 0.08

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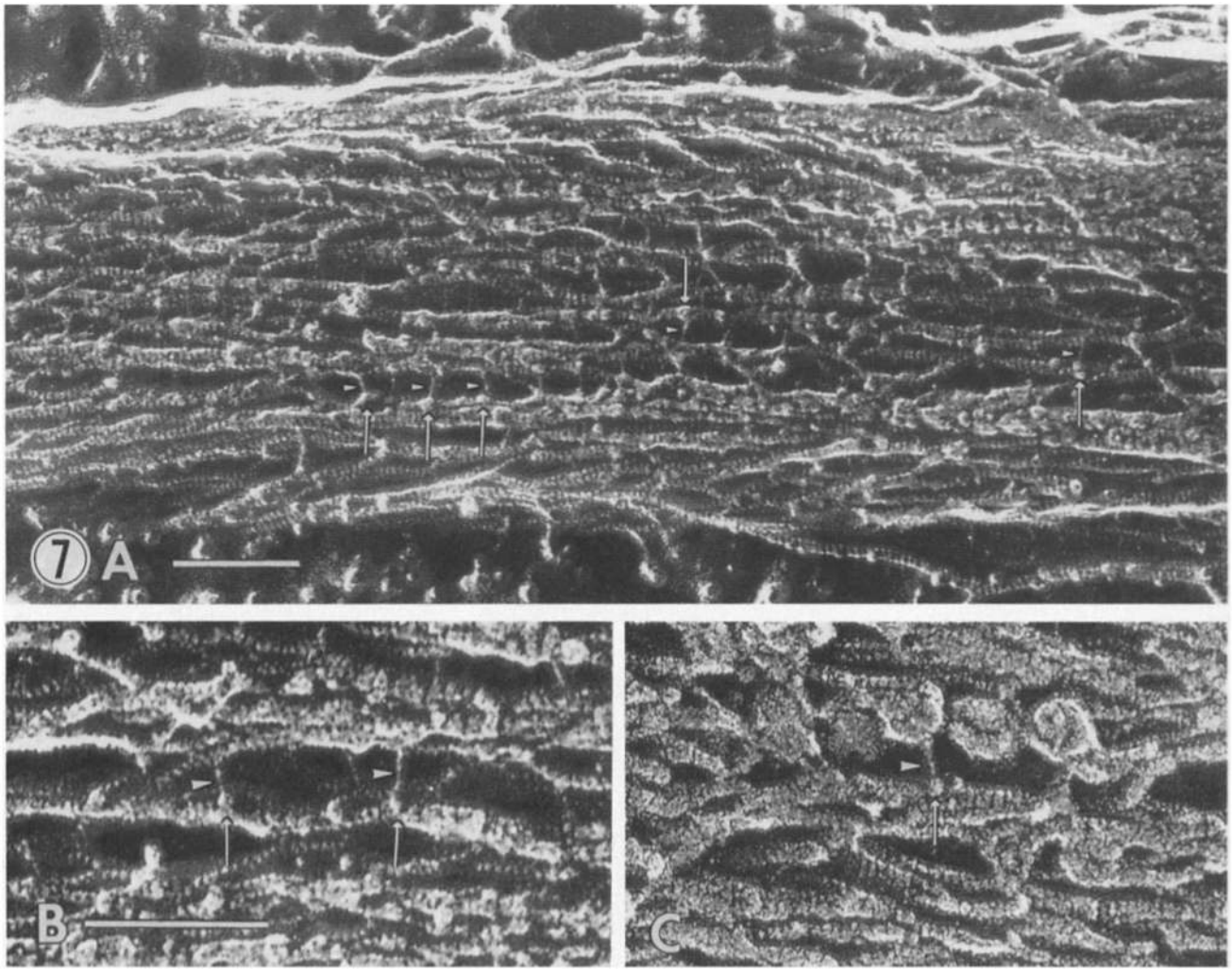

Figure 7. (*A*) Actin bundles formed by synapsin 1. Numerous fine short strands (20–30 nm long) link actin filaments. Frequently heads (*arrows*) and tails (*arrowheads*) are recognizable. (*B*) A high magnification view of actin filaments linked by synapsin 1. Spherical heads (*arrows*) attach to an actin filament and tails (*arrowheads*) attach to the adjacent actin filament. (*C*) Actin bundle incubated with synaptic vesicle and synapsin 1. A synaptic vesicle is linked with an actin filament via a fine strand. Spherical head (*arrow*) and tail (*arrowhead*) are recognizable. Bar, 0.1 μm.

Rat cerebellar cortices fixed with 2% paraformaldehyde and 0.1% glutaraldehyde were used as materials and affinity-purified rabbit anti-synapsin 1 as the first antibody are seen in Fig. 11. Most of the label was exclusively localized in the area where synaptic vesicles exist (Fig. 12). However, postsynaptic densities and dendrites containing microtubules were not significantly labeled (Fig. 12). In addition only a few gold particles existed very close to the presynaptic membranes as shown in a histogram of the distance between presynaptic membranes and gold particles (Fig. 13). Most of the labels were found at least 30 nm from the plasma membrane (Fig. 13). In the controls only a few gold particles were found on the sections.

Discussion

Primary Structure of Amino Acid Sequence Vs. Molecular Structure of Synapsin 1

Our low angle rotary shadowing study revealed that synapsin

Figure 8. (*A* and *B*) Synaptic vesicles incubated with synapsin 1. Synaptic vesicles are very frequently linked with each other via fine short strands. Sometimes spherical head-like structures (*short thick arrows*) are found to connect two or three short strands which bind to synaptic vesicles. Bar, 0.1 μm.

Figure 9. (*A*) Microtubule bundles incubated with synaptic vesicles and synapsin 1. Microtubules (*thick arrows*) form bundles. Synaptic vesicles (*thin arrows*) are specifically attached to microtubules. (*B* and *C*) Microtubules bundled by synapsin 1. Microtubules are cross linked by fine short strands. Frequently spherical heads (*arrows*) and tails (*arrowheads*) are recognized. Bar, 0.1 μm.

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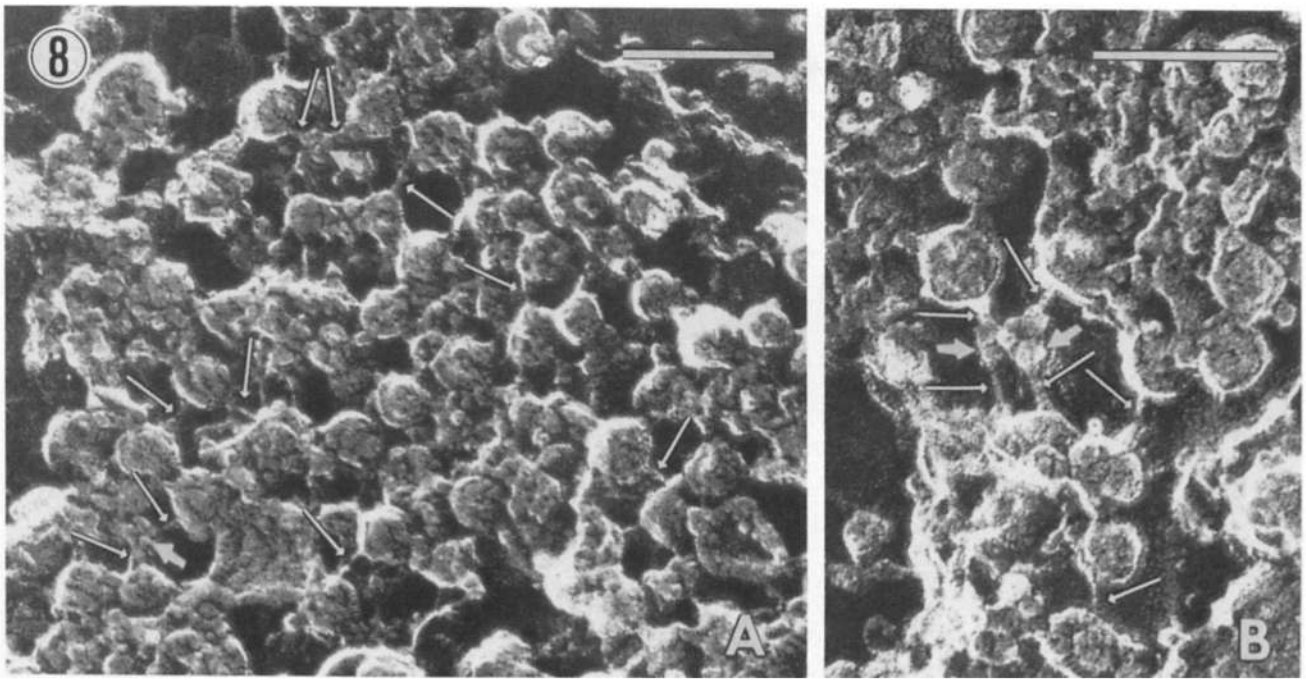

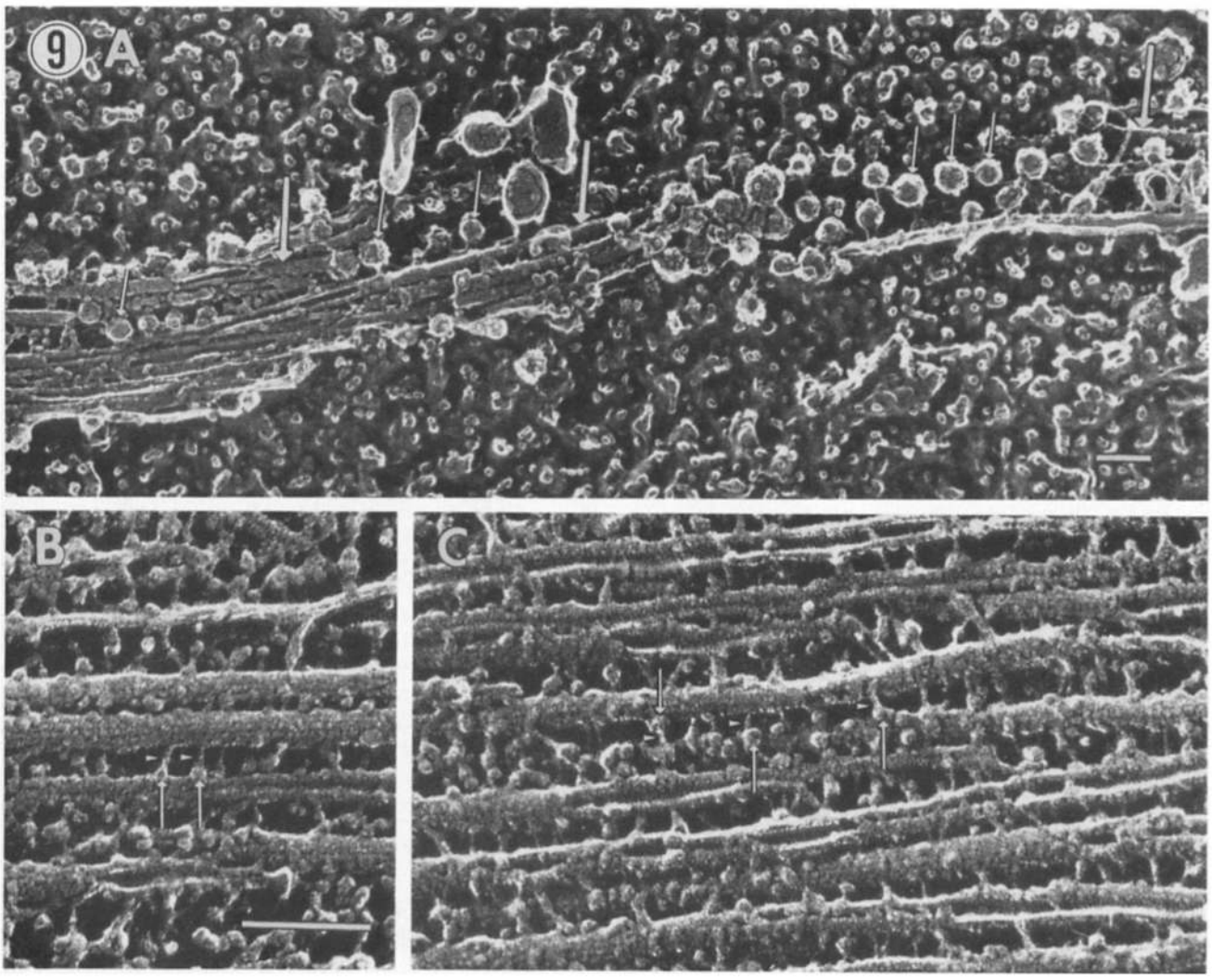

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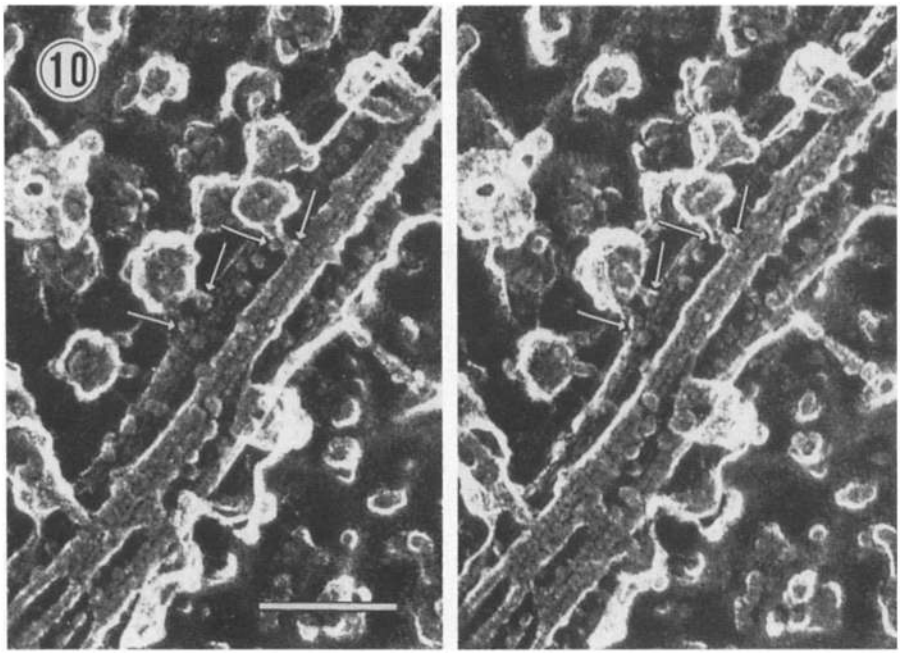

Figure 10. A stereo pair of microtubule bundle incubated with synaptic vesicles and synapsin 1. Synaptic vesicles are linked with microtubules via fine short strands. Spherical heads (*arrows*) attached to microtubules and tails attached to synaptic vesicles. Bar, 0.1 μm.

1 is composed of a head ~14 nm diam and a tail ~33 nm long. Our in vitro reconstruction studies indicated that a single synapsin 1 molecule cross-links adjacent actin filaments as well as an actin filament to a synaptic vesicle. In the latter case the spherical head was on the actin filament. A single synapsin 1 molecule also cross-links microtubules and a microtubule to a synaptic vesicle. In this case the spherical head was on the microtubule. These observations suggest that both ends of synapsin 1 possess binding sites to actin filaments and microtubules and that the binding site of synapsin 1 to a synaptic vesicle is localized at the tail domain. Our in vitro study indicated also that synaptic vesicles were cross-linked with each other via synapsin 1. Because we frequently found that spherical head-like structures associated with each other and tails attached to synaptic vesicles, we assume that multiple synapsin 1 molecules could link synaptic vesicles with each other.

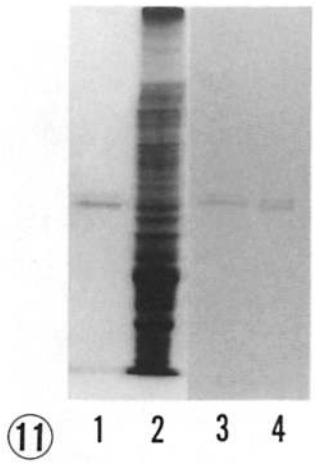

Figure 11. Antigenic specificity of affinity-purified anti-synapsin 1 polyclonal antibody. Purified synapsin 1 (lanes *1* and *3*) and rat brain crude extracts (lanes *2* and *4*) were subjected to SDS-PAGE. The antibody showed specific staining against synapsin 1. Lanes *1* and *2* were gels stained with Coomassie Brilliant Blue R250; lanes *3* and *4* were nitrocellulose replicas stained with anti-synapsin 1 antibody.

Previous biochemical studies have suggested that synapsin 1 is composed of a globular collagenase-resistant domain and a highly elongated collagenase-sensitive domain (42, 43). The primary structure of the amino acid sequence of synapsin 1 based on a cDNA study indicates that the protein is composed of 691 amino acids (32). The collagenase resistant fragment has been defined as the amino-terminal 439 amino acids of the molecule which contain sites for the cAMP dependent protein kinase phosphorylation (23, 43). On the other hand collagenase-sensitive carboxy-terminal 252 amino acids of the molecule contain sites for Ca^{++}/calmodulin-dependent protein kinase phosphorylation (23).

Based on our morphological data, the ~14-nm spherical head domain and ~33-nm tail domain should correspond to the collagenase-resistant amino-terminal 439 amino acids and collagenase sensitive carboxy-terminal 252 amino acids, respectively. The length of the tail domain, if it is completely composed of only the alpha helix, would be ~37.8 nm (252 amino acids × 0.15 nm spacing/amino acid residue in alpha helix). If all the amino acid residues are extended as in the beta sheet, the length would be 88.2 nm (252 amino acids × 0.53 nm/amino acid residue in beta sheets). Our observed value of 33 nm long for the tail suggests the possibility of some packing by the secondary structure. Petrucci and Morrow (35) recently reported that two complementary peptide fragments of synapsin generated by 2-nitro-5-thiocyanobenzoic cleavage and which map to opposite ends of the molecule participate in the actin-bundling process, either by binding directly to actin or by binding to other synapsin 1 molecules. Although previous studies (1, 35) did not differentiate between the presence of two actin-binding sites on a single synapsin 1 vs. one actin-binding site and one self-association site, the present study shows that a single synapsin 1 has binding sites to the actin at both its ends, namely the head and tail, and could cross-link actin filaments. Head and tail links were seen to be true for the microtubules as well. Previous biochemical studies indicated that synapsin 1

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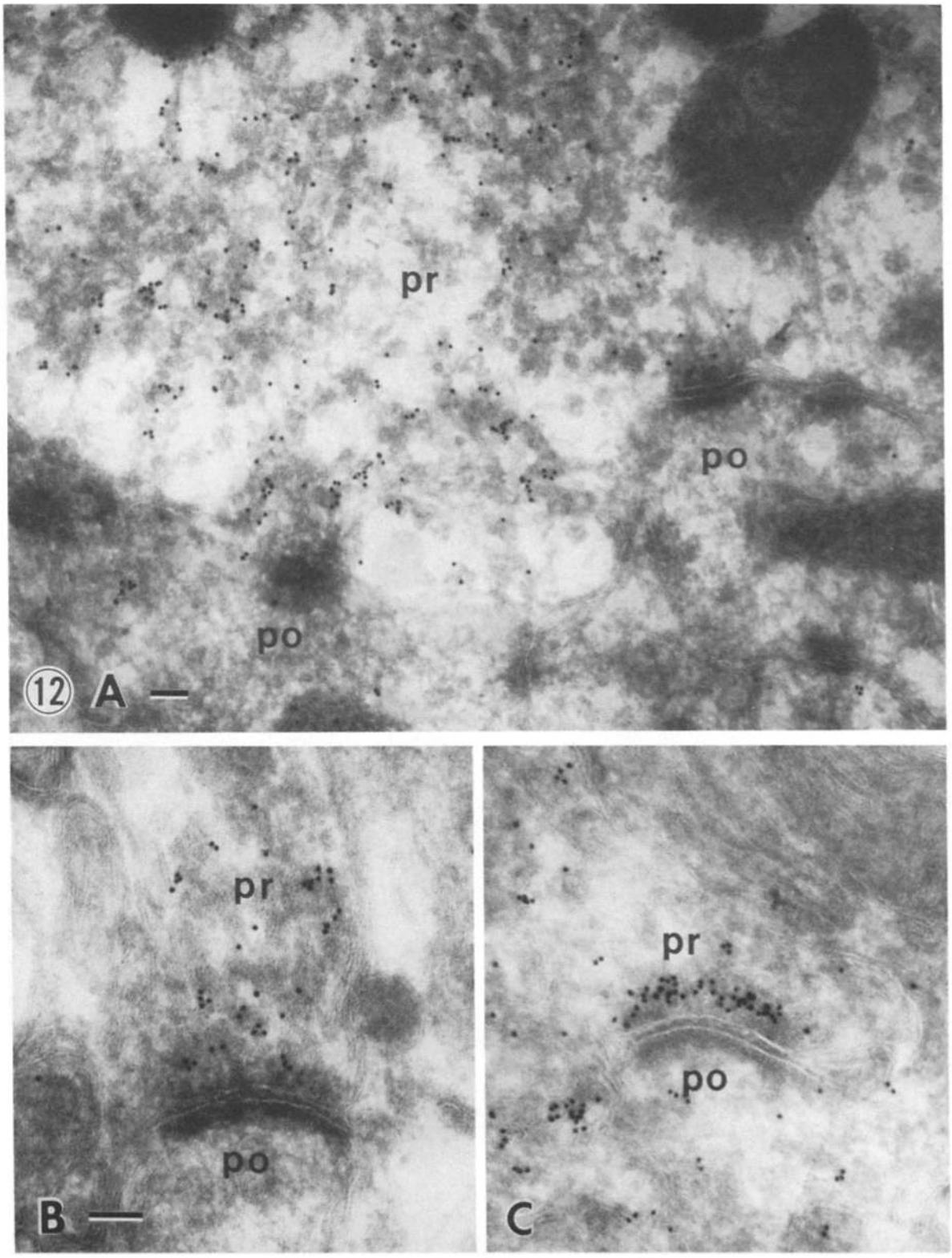

Figure 12. Ultrathin cryosections stained with anti-synapsin 1 antibody and colloidal gold-labeled second antibody. (*A*) Mossy fiber synapses. (*B* and *C*) Granule cell axon-Purkinje cell dendrite synapses. *pr*, presynaptic terminals; *po*, postsynaptic parts. Gold particles are specifically observed in the presynaptic terminals and associated with synaptic vesicles. Most gold particles are localized a short distance away from the presynaptic membrane. Bar, 0.1 μm.

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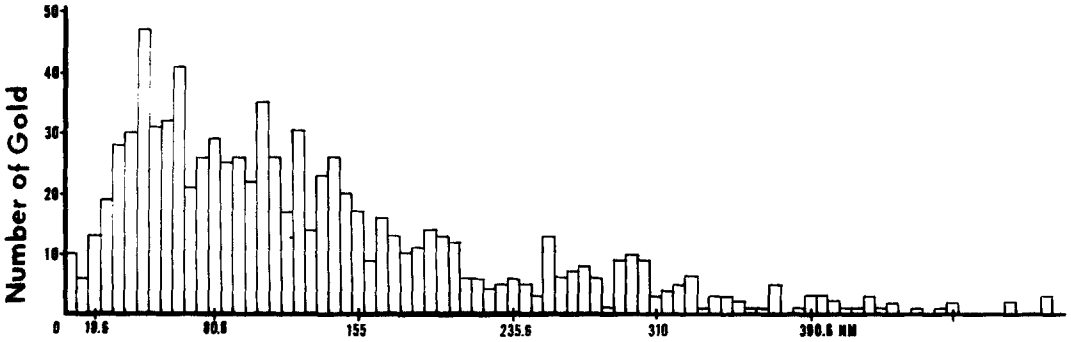

Figure 13. A histogram of the relationship of the distance between the presynaptic membranes and gold particles. Most of the gold particles exist ~30 nm away from the presynaptic membrane.

bundles the microtubules (2). However, it was not clear whether synapsin 1 contains two tubulin-binding sites on a single molecule or one tubulin-binding site and one self-association site. The present study indicated that a single synapsin molecule can cross-link microtubules, meaning that a molecule has binding sites to tubulin at both ends.

From our structural data in vitro synaptic vesicles were cross-linked with both microtubules and actin filaments, and the head domain localized on the cytoskeletons and the tail domain attached to the synaptic vesicles. These findings also indicate that a single synapsin molecule links synaptic vesicle to actin filaments or microtubules. These structural data suggest then that the binding site for the synaptic vesicle is located at the tail domain (COOH terminus) of synapsin 1. This assumption was further supported by the observation that synapsin 1 molecules frequently could associate with each other at their spherical head regions from which tails radiate to link synaptic vesicles. These results coincide well with previous biochemical studies suggesting the binding site for the synaptic vesicle to be at the collagenase-sensitive domain (35). Previous studies have shown that sites for Ca^{++}/calmodulin-dependent kinase phosphorylation are contained in the carboxyl-terminal domain and phosphorylation of synapsin 1 by this enzyme releases synapsin 1 from the vesicles (35). In our reconstruction studies synapsin 1 formed 10–30-nm-short stands between cytoskeletons or between cytoskeletons and synaptic vesicles. We measured the phosphorylation state of the synapsin 1 used in this study and found that synapsin 1 was not significantly phosphorylated and could be phosphorylated by Ca^{++}/calmodulin-dependent kinase and cAMP-dependent protein kinase. This suggests that phosphorylation of synapsin 1 by Ca^{++}/calmodulin-dependent kinase could detach the tail domain of synapsin 1 from synaptic vesicles but that synapsin 1 could remain attached to the actin or microtubules.

Possible Relationship between In Vitro Morphology of Synapsin 1 and In Vivo Cytoskeletal Structure in the Presynaptic Terminals

We found in the presynaptic terminals that synaptic vesicles are linked via 10–30-nm-thin fine strands with actin filaments and microtubules. Frequently the ends of the strands attaching to the actin or microtubules were thicker or appeared to be globular. We observed also that synaptic vesicles are cross-linked via short strands. In these cases frequently we identified spherical structures from which two or three fine strands radiated to link synaptic vesicles. Our immunocytochemistry confirmed that synapsin 1 localized exclusively at the region occupied by synaptic vesicles. These fine short strands existing between synaptic vesicles and actin or microtubules and between synaptic vesicles look very much like synapsin 1 reconstituted in vitro. From these in vivo and in vitro data we conclude that synapsin 1 could be a component of short cross-bridges between synaptic vesicles and actin filaments, between synaptic vesicles and microtubules, and between synaptic vesicles.

However, there are possibilities that other molecules such as calpactin (8) also cross-link synaptic vesicles or that kinesin may to some extent participate in the interaction between microtubules and synaptic vesicles (45). Llinas et al. (30) has injected dephosphosynapsin 1 into the squid giant synapse and found a decrease in the amplitude and rate of rise of the postsynaptic potential generated in response to the presynaptic depolarizing step. Injection of Ca^{++}/calmodulin-dependent kinase increased the rate of rise and amplitude and decreased the latency of the postsynaptic potential. Thus they proposed that calcium entry into the nerve terminal activates Ca^{++}/calmodulin-dependent protein kinase 11, which phosphorylates synapsin 1 and dissociates it from the vesicles and thereby removing a constraint from the release process.

The hypothesis of Llinas et al. coincides well with our structural data (30). There are networks of actin filaments in the presynaptic terminal although in a fracture plane actin filaments do not look very numerous because of their random orientation. Synaptic vesicles are captured in a network of actin filaments and microtubules. They are linked with each other as well. These links could be released by phosphorylation of synapsin 1 induced by Ca^{++} influx which activates Ca^{++}/calmodulin-dependent kinase. Because the transmitter release occurs in millisecond duration, it may not be the case that synaptic vesicles released from synapsin 1 are immediately discharged. It is likely that the released vesicles are free to move and work as reservoirs for the next discharge of the transmitter. In this point the immunocytochemical data indicating that only a few gold particles localize very near the presynaptic membrane is interesting. It is very probable that synaptic vesicles localizing very close to the presynaptic membrane are devoid of synapsin 1.

We found longer strands (>~100 nm) which are thinner

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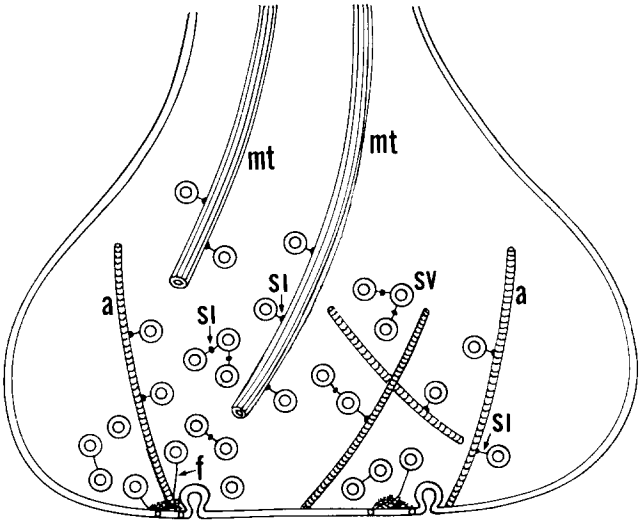

Figure 14. A scheme of the inside of presynaptic terminals based on the present study. Actin filaments (*a*) and microtubules (*mt*) are main cytoskeletal elements in the presynaptic terminal. Short fine strands (probably synapsin 1 [*SI*]) link synaptic vesicles (*SV*) with actin and microtubules. Synaptic vesicles are also cross-linked via fine short strands (probably synapsin 1). There are longer strands (*f*) (>~100 nm) linking synaptic vesicles with the presynaptic membrane. Fodrin could be a component of these longer strands. Ca^{++} influx through the presynaptic Ca^{++} channel activates Ca^{++}/calmodulin-dependent kinase which phosphorylates synapsin 1. Phosphorylated synapsin 1 detaches from synaptic vesicles, which are released from actin, microtubules, and other synaptic vesicles. The synaptic vesicles are free to move to the presynaptic membrane, and thus these vesicles are supplied as reservoirs for the next discharge.

than actin filaments and are associating with the presynaptic membrane. Previous studies have reported the existence of fodrin (or calspectin) inside the axonal membrane (28, 31, 46). Fodrin is a fibrous protein (heterodimer ~120 nm long) localized mainly in the cellular cortex (10, 21). Therefore, from the structural and immunocytochemical data it is reasonable to assume that these thin strands are at least partially composed of fodrin. Recently it has been suggested that fodrin is also involved in the process of exocytosis (34). The cytoskeletal structure based on the present study is drawn as a scheme in Fig. 14. Further structural studies combining a modification process and microinjection studies will be necessary to clarify the mechanism of transmitter release and the possible role of cytoskeletons in this process.

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