Redistribution of Synaptophysin and Synapsin I During α-Latrotoxin-induced Release of Neurotransmitter at the Neuromuscular Junction

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Abstract. The distribution of two synaptic vesicle-specific phosphoproteins, synaptophysin and synapsin I, during intense quantal secretion was studied by applying an immunogold labeling technique to ultrathin frozen sections. In nerve-muscle preparations treated for 1 h with a low dose of α-latrotoxin in the absence of extracellular Ca^{2+} (a condition under which nerve terminals are depleted of both quanta of neurotransmitter and synaptic vesicles), the immunolabeling for both proteins was distributed along the axolemma. These findings indicate that, in the presence of a block of endocytosis, exocytosis leads to the permanent incorporation of the synaptic vesicle membrane into the axolemma and suggest that, under this condition, at least some of the synapsin I molecules remain associated with the vesicle membrane after fusion. When the same dose of α-latrotoxin was applied in the presence of extracellular Ca^{2+}, the immunoreactivity patterns resembled those obtained in resting preparations: immunogold particles were selectively associated with the membrane of synaptic vesicles, whereas the axolemma was virtually unlabeled. Under this condition an active recyling of both quanta of neurotransmitter and vesicles operates. These findings indicate that the retrieval of components of the synaptic vesicle membrane is an efficient process that does not involve extensive intermixing between molecular components of the vesicle and plasma membrane, and show that synaptic vesicles that are rapidly recycling still have the bulk of synapsin I associated with their membrane.

THE most widely accepted hypothesis to explain the quantal nature of transmitter release holds that each quantum (multimolecular packet) is confined within one synaptic vesicle and is released by exocytosis when the vesicle membrane fuses with the axolemma. This fusion process is balanced by a reverse process of membrane removal from the axolemma that tends to conserve the vesicle population in the face of active secretion of neurotransmitter (for reviews see Zimmermann, 1979; Ceccarelli and Hurlbut, 1980*a*; Meldolesi and Ceccarelli, 1988). However, the precise sequence of membrane movements and interactions and the nature of the molecular events occurring during the exo-endocytotic cycle are still poorly understood.

The study of nerve terminal membrane proteins has demonstrated that the synaptic vesicle membrane has a molecular composition different from that of the nerve terminal plasma membrane (for reviews see Reichardt and Kelly, 1983; De Camilli and Jahn, 1990). Several synaptic vesicle-specific proteins have been identified and characterized (Ueda and Greengard, 1977; Matthew et al., 1981; Buckley and Kelly, 1985; Jahn et al., 1985; Wiedenmann and Franke, 1985; Walker et al., 1986; Trimble et al., 1988; Baumert et al., 1989). These proteins can be used as markers to follow the movements of the vesicle membrane during synaptic activity and to identify the mechanisms responsible for these processes.

Recycling of one of these proteins, synaptophysin (a transmembrane glycoprotein of synaptic vesicles; Jahn et al., 1985; Wiedenmann and Franke, 1985), has already been investigated at the light microscope level in frog motor nerve terminals (Valtorta et al., 1988*a*). In that study, when quantal secretion was accompanied by a block of endocytosis, synaptophysin was found to become incorporated into the axolemma. On the contrary, in the presence of active recycling, synaptophysin was found to be confined exclusively inside the nerve terminal (Valtorta et al., 1988*a*). These findings provide support for the vesicle hypothesis of quantal release, and suggest that the retrieval mechanism is selective for synaptic vesicle components. However, a more detailed analysis at the electron microscope level is required in order to gather information about the molecular mechanisms underlying these processes.

Synapsin I, a peripheral membrane phosphoprotein of synaptic vesicles (De Camilli et al., 1983*a*,*b*; Huttner et al.,

1983) has recently been localized in resting frog neuromuscular junctions (Valtorta et al., 1988*b*). Synapsin I is known to interact with various cytoskeletal elements (Baines and Bennett, 1985, 1986; Goldenring et al., 1986; Bähler and Greengard, 1987; Petrucci and Morrow, 1987). Phosphorylation of this protein by Ca^{2+}/calmodulin-dependent protein kinase II regulates its binding to both synaptic vesicles and actin filaments (Schiebler et al., 1986; Bähler and Greengard, 1987; Petrucci and Morrow, 1987). Because of these properties and of the results of direct microinjection experiments (Llinas et al., 1985), synapsin I has been proposed to play a crucial role in the regulation of neurotransmitter release, possibly by controlling the initial steps of exocytosis (i.e., the movement to the fusion sites). High resolution studies of synapsin I distribution within nerve terminals stimulated to release under controlled experimental conditions are therefore expected to provide new information about the process of transmitter release.

In the present work we carried out immunogold labeling for both synaptophysin and synapsin I on ultrathin cryosections of frog neuromuscular junctions obtained from preparations stimulated to massively release neurotransmitter by exposure to α-latrotoxin (α-Ltx)[1] under conditions in which recycling is either maintained in balance with exocytosis or completely blocked.

Materials and Methods

Electrophysiology

Cutaneous pectoris nerve-muscle preparations were dissected out from frogs (*Rana pipiens*) and mounted in lucite chambers containing standard Ringer's solution (in mM: 116 Na^+; 2.1 K^+; 1.8 Ca^{2+}; 117 Cl^-; 2 HPO_4^{2-}; 1 $H_2PO_4^-$; pH 7.0). α-Ltx was purified from black widow spider venom, stored at −70°C, and its activity was tested as previously described (Frontali et al., 1976; Ceccarelli and Hurlbut, 1980*b*). Immediately before use, α-Ltx was diluted to a final concentration of 0.2 μg/ml in either 4 mM Mg^{2+}-standard Ringer's solution or in Ca^{2+}-free (4 mM Mg^{2+}, 1 mM EGTA) Ringer's solution.

All experiments were performed at room temperature. Standard electrophysiological techniques were used for intracellular recording of the miniature end plate potentials from single neuromuscular junctions (Ceccarelli et al., 1973). These records were used to ascertain that the overall patterns of secretion corresponded to those previously described.

Antibodies

Rabbit antisera against frog synaptophysin were generated as previously described (Valtorta et al., 1988*a*). For immunoelectron microscopy, a purified IgG fraction was prepared by precipitation from the serum with 37.5% ammonium sulfate, followed by extensive dialysis against 20 mM Tris-HCl, pH 7.2, and chromatography on a DEAE-Affigel blue column (Bio-Rad Laboratories, Richmond, CA). IgGs were eluted from the column by applying a continuous NaCl gradient (0–100 mM).

1. *Abbreviation used in this paper*: α-Ltx, α-latrotoxin.

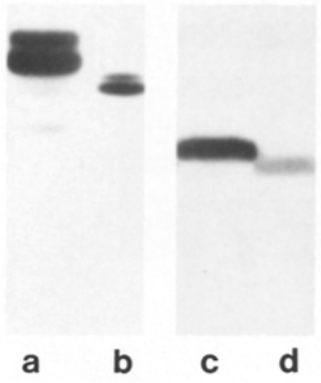


Figure 1. Autoradiograph of immunoblots showing the specificity of the antisera against synaptophysin and synapsin I. The protein samples were separated on SDS–10% polyacrylamide gels and transferred to nitrocellulose membranes. The blots were labeled with anti–bovine synapsin I (lanes *a* and *b*) or anti–frog synaptophysin (lanes *c* and *d*) antisera, followed by ^{125}I-protein A. (*a* and *c*) Frog brain homogenate, 10 μg; (*b*) rat brain homogenate, 2 μg; (*d*) rat brain homogenate, 10 μg.

Antisera against synapsin I were generated by cross-linking purified bovine synapsin I with 0.1% glutaraldehyde. The cross-linked protein was dialyzed against sodium PBS, emulsified in complete Freund's adjuvant, and injected intradermally at multiple sites into New Zealand female rabbits. A booster injection (containing one third of the original amount of protein) was administered 3 wk after the initial injection. Antibodies directed against synapsin I were purified by affinity chromatography on a bovine synapsin I–Sepharose CL4B column and eluted with 0.2 M glycine-HCl, pH 2.2. The eluted fractions were immediately brought to pH 8.0 by adding 1 M Tris.

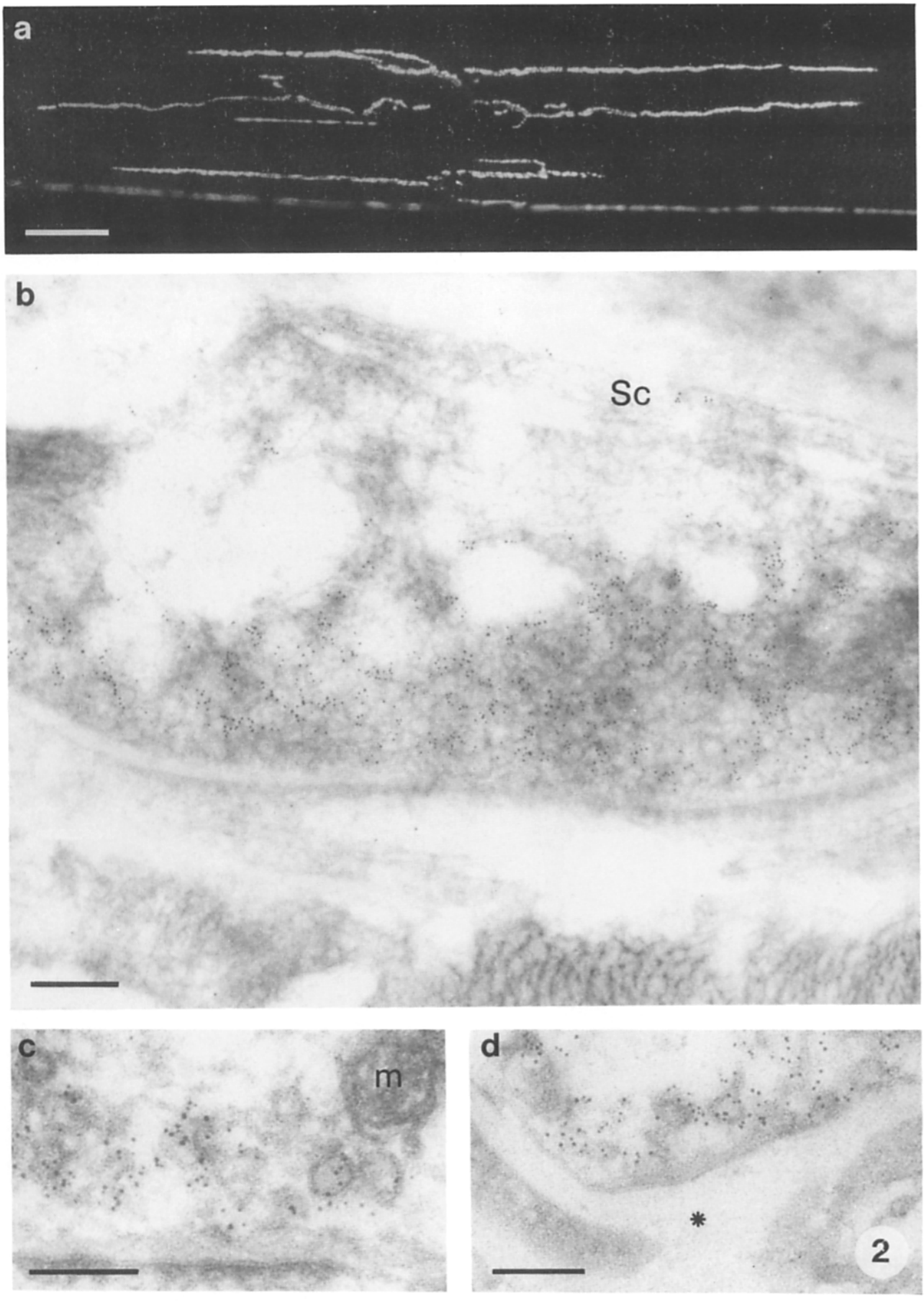
Immunofluorescence

Cutaneous pectoris nerve-muscle preparations were fixed for 1 h at room temperature with a mixture of 0.2% glutaraldehyde/2% formaldehyde (freshly prepared from paraformaldehyde) in 0.1 M sodium phosphate buffer, pH 7.3. After fixation the muscles were washed with sodium phosphate buffer, pH 7.3, and the muscle fibers were then dissociated under a dissection microscope. Single fibers were transferred to test tubes and processed for rhodamine indirect immunofluorescence as previously described (Valtorta et al., 1988*b*). Before incubation for 3 h with either rabbit antisynaptophysin, antisynapsin I or preimmune serum (1:10–1:40), the fibers were incubated for 10 min on ice in 1% $NaBH_4$ and then washed for 10 min with 0.1 M sodium phosphate buffer, pH 7.3. The single muscle fibers were subsequently mounted in 95% glycerol/10 mM sodium phosphate buffer, pH 7.3, and examined in a photomicroscope (model III; Carl Zeiss, Inc., Thornwood, NY) equipped with epifluorescence optics.

Electron Microscopy Immunocytochemistry

Cutaneous pectoris nerve-muscle preparations were fixed for 1 h at room temperature with a mixture of 0.2% glutaraldehyde/2% formaldehyde (freshly prepared from paraformaldehyde) in 0.1 M sodium phosphate buffer, pH 7.3. Small tissue blocks were dissected out from regions of the muscles with a high chance to contain end plates, washed for 1 h in 0.1 M phosphate buffer, pH 7.3, rinsed in PBS (2.6 mM KCl; 136 mM NaCl; 10 mM sodium phosphate buffer), pH 7.3, infiltrated in 2.3 M sucrose and frozen in Freon 22 cooled with liquid nitrogen. Ultrathin frozen sections were prepared with an ultracut microtome equipped with an FC4 attachment (Reichert Jung, Austria), collected onto Formvar-coated nickel grids and sequentially floated over drops of 2% gelatin/PBS for 15 min followed by 1%

Figure 2. (*a*) Fluorescence micrograph showing the distribution of synaptophysin immunoreactivity at a resting neuromuscular junction on a single fiber teased from frog cutaneous pectoris muscle. Note the regions of intense fluorescence which are arranged in the branching pattern characteristic of frog motor nerve endings. Little fluorescence is associated with the muscle fiber or with the myelinated axon. (*b–d*) Electron micrographs of ultrathin frozen sections of three resting neuromuscular junctions immunolabeled for synaptophysin by a colloidal gold technique. Gold particles are concentrated almost exclusively inside the nerve terminal. The muscle fiber, Schwann cell (*Sc*), synaptic cleft (*), and postjunctional folds are virtually devoid of labeling. In the portions of terminals in *c* and *d* the selective association of the immunoreactivity with the membrane of synaptic vesicles can be seen: gold particles decorate synaptic vesicles, whereas mitochondria (*m*) and the presynaptic membrane are free of immunolabeling. Bars: (*a*) 40 μm; (*b*) 0.2 μm; (*c* and *d*) 0.1 μm.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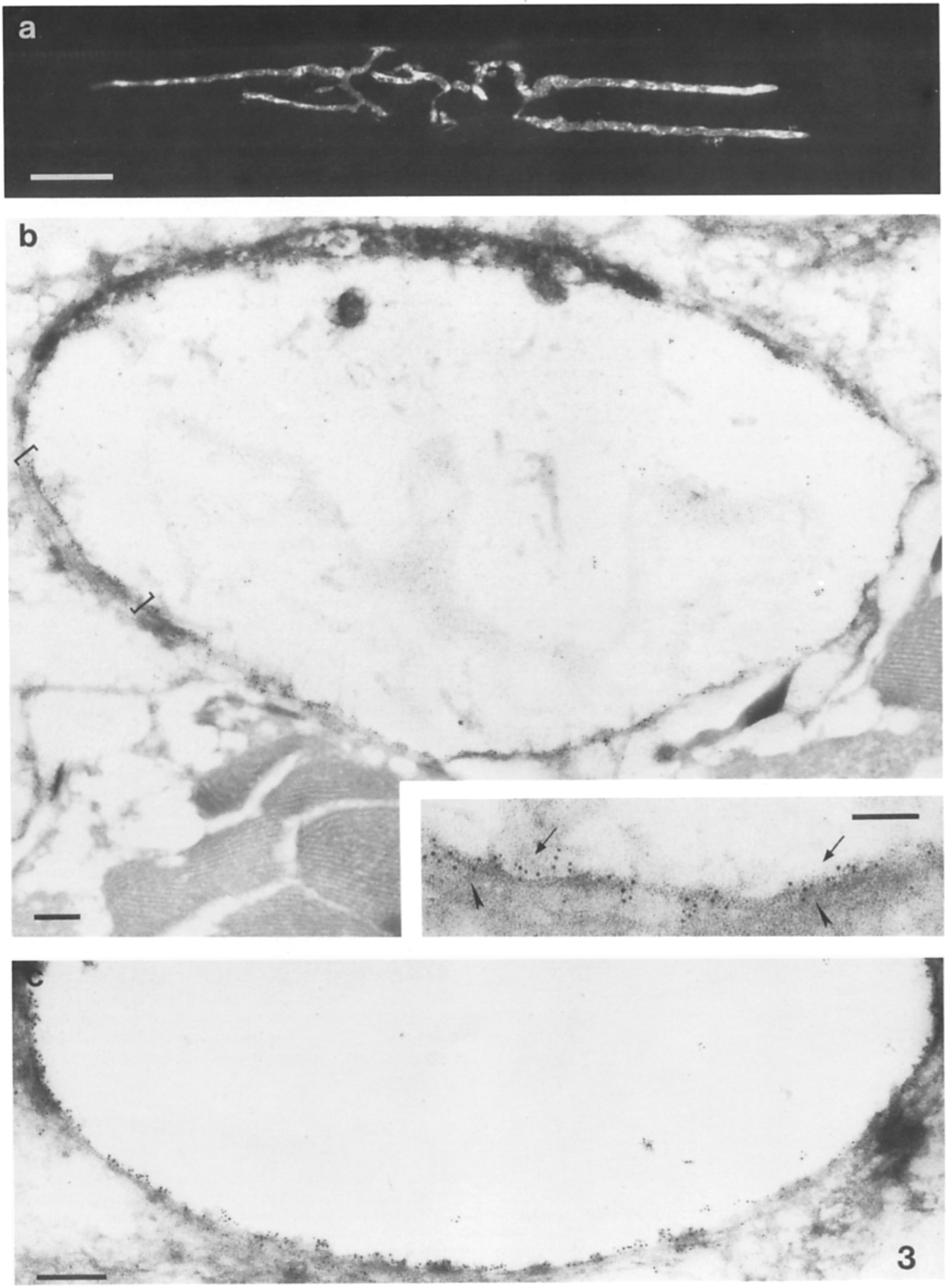
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chicken egg albumin/0.1 M glycine/PBS for 20 min. The sections were then immunolabeled for 1 h at 37°C with either affinity-purified antisynapsin I antibodies (final concentration, 35 μg/ml) or IgGs prepared from either antisynaptophysin or preimmune sera (final concentration, 20 μg/ml). IgGs were diluted in 1% chicken egg albumin/0.1 M glycine/PBS. After washing in 1% chicken egg albumin/0.1 M glycine/PBS for 30 min at room temperature, the sections were exposed for 45 min at 37°C to goat anti-rabbit IgGs conjugated with 4-nm colloidal gold particles diluted in the same solution used for the dilution of the primary antibodies. After washing in PBS for 30 min, the grids were floated on a 2% solution of glutaraldehyde in PBS and then rinsed in PBS. Contrasting and plastic (LR White resin) embedding of the labeled grids were performed according to the procedure described by Keller et al. (1984). Finally, the sections were double stained with uranyl acetate and lead citrate and observed in an electron microscope (H-7000; Hitachi Ltd., Tokyo).

Morphometry

Random cryosections of nerve endings were photographed at a primary magnification of 20,000–30,000 and then printed at a fourfold enlargement. Gold particles associated with synaptic vesicles, the axolemma, and the postsynaptic membrane were counted and the corresponding membrane profiles were measured with a quantitative digital image analyzer (Model MOP 1; Carl Zeiss, Inc.). The data presented represent ratios of total numbers of gold particles to total membrane length. The total lengths (in μm) of membrane profiles measured were (*a*) for synaptic vesicles, 40.65 (resting) and 28.7 (α-Ltx $+Ca^{2+}$) in preparations immunostained for synaptophysin; 40.72 (resting) and 30.12 (α-Ltx $+Ca^{2+}$) in preparations immunostained for synapsin I; (*b*) for axolemma, 18.14 (resting), 26.16 (α-Ltx $+Ca^{2+}$), and 35.6 (α-Ltx no Ca^{2+}) in preparations immunostained for synaptophysin; 12.23 (resting), 22.26 (α-Ltx $+Ca^{2+}$), and 47.9 (α-Ltx no Ca^{2+}) in preparations immunostained for synapsin I; and (*c*) for postsynaptic membrane, 15.12 (resting), 18.53 (α-Ltx $+Ca^{2+}$), and 22.3 (α-Ltx no Ca^{2+}) in preparations immunostained for synaptophysin; 12.96 (resting), 14.95 (α-Ltx $+Ca^{2+}$), and 14.58 (α-Ltx no Ca^{2+}) in preparations immunostained for synapsin I.

Other Procedures

SDS-PAGE and immunoblotting were performed as described (Valtorta et al., 1988*a*). Protein was determined by the method of Bradford (1976), using the protein assay reagent (Bio-Rad Laboratories) and IgGs as standards.

Results

The antisera against synapsin I and synaptophysin were characterized by immunoblotting. When tested against frog and rat brain homogenates, the antiserum against frog synaptophysin recognized a single band of immunoreactivity, whereas in the case of the antisynapsin I antiserum a doublet, corresponding to the two isoforms of the protein, was observed (Fig. 1). In agreement with previous data (Valtorta et al., 1988*a*,*b*), both frog synaptophysin and frog synapsin I appeared to have a higher apparent molecular weight than their mammalian homologues (compare lanes *a* and *c* with lanes *b* and *d* in Fig. 1).

Distribution of Synaptophysin in Resting Motor Nerve Terminals

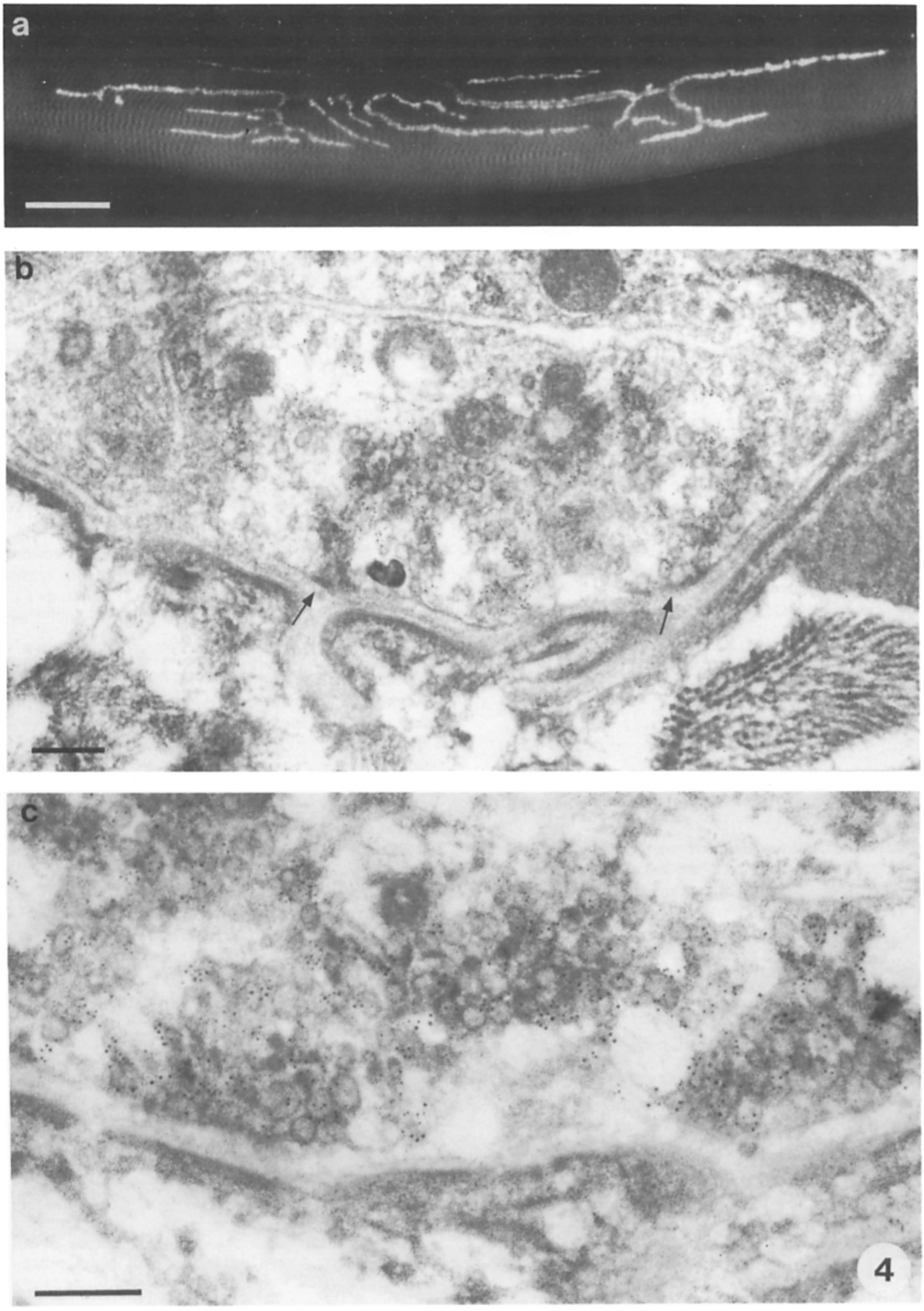
Fig. 2 shows the distribution of synaptophysin in resting nerve–muscle preparations. At the light microscope level, a bright specific signal was observed along the entire nerve-ending arborization (Fig. 2 *a*). The distribution of synaptophysin was determined at the electron microscope level by colloidal gold immunolabeling of ultrathin cryosections. As shown in Fig. 2, *b–d*, synaptophysin was selectively localized in the nerve terminal compartment; no labeling of muscle fibers, postjunctional infoldings, synaptic clefts, or Schwann cells was observed. Within the nerve terminal, synaptophysin immunoreactivity was associated with the synaptic vesicle membrane, whereas mitochondria, large vesicular structures, and the axolemma were virtually free of immunogold particles (see Fig. 2, *c* and *d*).

Redistribution of Synaptophysin after Depletion

Fig. 3 shows the distribution of synaptophysin in nerve terminals stimulated for 1 h with 0.2 μg/ml α-Ltx in Ca^{2+}-free solution. Under these conditions, α-Ltx induced very high initial rates of quantal secretion. After a short period of time (less than 20 min) the miniature end plate potential frequency declined to very low levels because of a concomitant block of endocytosis (not shown; see also Ceccarelli and Hurlbut, 1980*b*; Valtorta et al., 1988*a*). In agreement with previous results (Valtorta et al., 1988*a*), after such treatment synaptophysin immunofluorescence revealed nerve terminals that showed a marked increase in their transverse dimension (compare Fig. 3 *a* with Fig. 2 *a*).

An ultrathin cryosection of a typical nerve–muscle preparation treated with α-Ltx in Ca^{2+}-free medium and labeled for synaptophysin by immunogold is shown in Fig. 3 *b*. The terminal appears swollen and devoid of synaptic vesicles. The gold particles are selectively associated with the axolemma which appears uniformly decorated along its entire cross-sectional profile. The specificity of plasma membrane labeling was confirmed by the morphometric analysis carried out on axolemma and postsynaptic membrane profiles. The average density of gold particles per micrometer on the two membranes was 35.28 and 0.58, respectively. This result demonstrates that, under these experimental conditions, the synaptic vesicle membrane becomes permanently incorporated into the axolemma and that it or its components can diffuse away from the presynaptic regions. The observation of high magnification images revealed that the immunolabeling was preferentially, but not exclusively, associated with the inner surface of the axolemma, with about 10% of the gold particles being associated with the extracellular side (Fig. 3, *b* and *c*). Since the inner surface of the axolemma

Figure 3. Fluorescence micrograph showing the distribution of synaptophysin immunoreactivity at a frog neuromuscular junction treated for 1 h with 0.2 μg/ml α-Ltx in Ca^{2+}-free (4 mM Mg^{2+}, 1 mM EGTA) solution. Note the marked increase in the transverse dimension of the nerve terminal branches. (*b* and *c*) Electron micrographs of ultrathin frozen sections of two neuromuscular junctions from preparations treated with 0.2 μg/ml α-Ltx in Ca^{2+}-free medium. The terminals appear swollen with the axoplasm totally devoid of synaptic vesicles. Immunogold particles selectively decorate the axolemma along its full extension. The inset shows a high magnification of the portion of synaptic membrane outlined in *b*. Note that the immunolabeling is associated with both the intracytoplasmic (*arrows*) and the external (*arrowheads*) side of axolemma. Bars: (*a*) 40 μm; (*b* and *c*) 0.2 μm; (*inset*) 0.1 μm.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corresponds to the cytoplasmic side of the fused synaptic vesicle membrane, and the outer surface of the axolemma to the intravesicular side, this finding corroborates previous results indicating that the frog antisynaptophysin antisera recognize both extra and intravesicular antigenic determinants (Valtorta et al., 1988*a*).

Distribution of Synaptophysin during Intense Recycling

Treatment of nerve-muscle preparations with 0.2 μg/ml α-Ltx in the presence of extracellular Ca^{2+} induced a rapid increase in the frequency of miniature end plate potentials, which remained high and constant for more than 1 h (not shown). This was shown to result from active recycling of both synaptic vesicles and quanta (Ceccarelli and Hurlbut, 1980*b*; Valtorta et al., 1988*a*). Under these conditions, the pattern of synaptophysin immunoreactivity was very similar to that observed in resting preparations (compare Fig. 4 with Fig. 2). The terminals were not swollen, and by electron microscopy they were shown to maintain their normal complement of synaptic vesicles. Immunogold particles were associated with the membrane of synaptic vesicles, whereas other structures were not labeled (compare Fig. 4, *b* and *c*, with Fig. 2 *b*). In particular, no significant labeling of the axolemma was found even in the presynaptic regions where active zones are located (see arrows in Fig. 4 *b*), indicating that no extensive intermixing of molecular components occurred between the vesicle and the axolemma membrane. The morphometric analysis confirmed these observations. Synaptic vesicles showed a specific labeling of 47.18 and 45.78 particles/μm in resting and α-Ltx–treated preparations, respectively, whereas the axolemma showed 0.99 and 0.95 particles/μm in the same two conditions. Background labeling can account for the axolemma decoration, since a similar density of gold particles was found lining the postsynaptic membrane (1.12 and 0.97 particles/μm in resting and α-Ltx–treated preparations, respectively).

Redistribution of Synapsin I during Synaptic Activity

The fate of synapsin I upon synaptic vesicle exocytosis was investigated under the same conditions of stimulation used for the study of synaptophysin. Synapsin I is an extrinsic protein of the synaptic vesicle membrane which, under in vitro conditions, can be dissociated from the vesicle membrane (Huttner et al., 1983; Schiebler et al., 1986). We therefore investigated its localization at both the light and the electron microscope level in order to establish whether or not it remains associated with the vesicle membrane during the secretory activity induced by α-Ltx.

The patterns of immunofluorescence obtained with antisynapsin I antibodies were very similar to those observed with antisynaptophysin antibodies in resting preparations, as well as in nerve terminals treated with α-Ltx in both the presence and the absence of Ca^{2+} (compare Fig. 5 with Figs. 2–4).

Labeling patterns similar to those produced by antisynaptophysin antibodies were also observed at the electron microscope level by immunogold labeling of ultrathin cryosections. In terminals treated for 1 h with α-Ltx in Ca^{2+}-free medium, gold particles were found in association with the whole perimeter of the axolemma, a situation similar to that described in the case of synaptophysin immunogold staining (30.66 particles/μm against a background labeling of the postsynaptic membrane of 1.23 particles/μm). However, under these experimental conditions, synapsin I immunoreactivity seemed to be less uniformly distributed than synaptophysin immunoreactivity. Gold particles were found to line the axolemma either continuously, or in small clusters spaced by unlabeled areas of axolemma (see arrows and arrowheads in Fig. 6 *b*, respectively). In nerve–muscle preparations fixed after treatment with α-Ltx in the presence of Ca^{2+}, as well as in resting terminals, gold particles specifically decorated synaptic vesicles (Fig. 6, *a* and *c*). In both conditions, the density of labeling for synapsin I associated with synaptic vesicles was similar (34.33 particles/μm in resting preparations and 35.59 particles/μm in α-Ltx–treated ones). The nerve terminal plasma membrane showed 1.06 and 1.03 particles/μm, and the postsynaptic membrane showed 0.69 and 0.8 particles/μm in resting and α-Ltx–treated preparations, respectively.

Discussion

α-Ltx, the purified active component of black widow spider venom, is an experimental tool that has been extensively used to stimulate neurotransmitter release from synapses and neurosecretory cells (Hurlbut and Ceccarelli, 1979; Meldolesi et al., 1986). At the frog neuromuscular junction low doses of α-Ltx are known to exert different effects on the exo-endocytotic process depending on the composition of the extracellular medium. When the toxin is applied in Ca^{2+}-containing solutions it induces high rates of quantal secretion (~1,000 quanta/min) which are maintained for hours; neither marked depletion of synaptic vesicles nor swelling of the nerve terminals is observed. This occurs because, under this condition, quantal secretion is coupled with active recycling of vesicles and quanta. On the other hand, when α-Ltx is used in Ca^{2+}-free solutions, it induces a massive (~2,000 quanta/min) but transient secretion concomitant with a block of endocytosis, leading to depletion of both quanta and synaptic vesicles. After this treatment nerve terminals appear

Figure 4. (*a*) Fluorescence micrograph showing the distribution of synaptophysin immunoreactivity at a single neuromuscular junction treated with 0.2 μg/ml α-Ltx in Ca^{2+}-containing solution. The immunofluorescence pattern is similar to that observed in resting nerve terminals (see Fig. 2 *a*). (*b* and *c*) Electron micrographs of ultracryosections of two neuromuscular junctions from preparations fixed after a 1-h exposure to 0.2 μg/ml α-Ltx in Ca^{2+}-containing medium and immunogold stained for synaptophysin. The axoplasm still contains a full complement of synaptic vesicles and the terminal is normal in diameter. The distribution of immunolabeling appears similar to that shown in resting preparations: gold particles are present only in regions where vesicles are concentrated. Arrows indicate active zones. Mitochondria and the axolemma are virtually free of gold particles. In the portion of terminal in *c* the lack of labeling of the presynaptic membrane is evident. Bars: (*a*) 40 μm; (*b* and *c*) 0.2 μm.

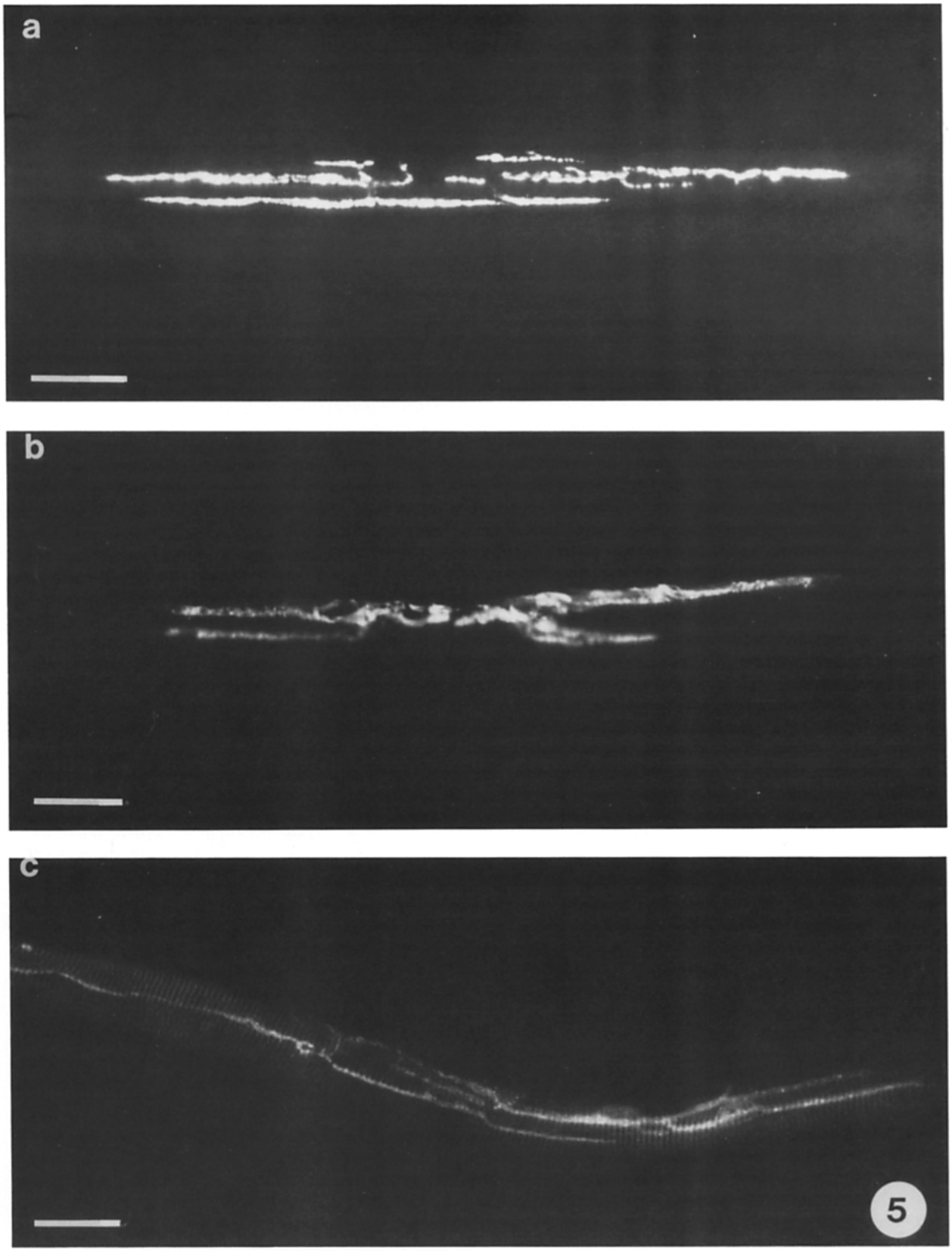

Figure 5. Gallery of fluorescence micrographs showing the immunoreactivity for synapsin I at neuromuscular junctions on single muscle fibers teased apart from frog cutaneous pectoris muscle preparations fixed respectively (*a*) at rest; (*b*) after a 1-h treatment with 0.2 μg/ml α-Ltx in Ca^{2+}-free solution; (*c*) after a 1-h treatment with 0.2 μg/ml α-Ltx in Ca^{2+}-containing solution. Note that in each of the three experimental conditions the immunostaining for synapsin I is very similar to that observed using antisynaptophysin antibodies (compare with Figs. 2 *a*, 3 *a*, and 4 *a*). Bars, 40 μm.

virtually empty and exhibit an increase in their transverse dimension (Ceccarelli and Hurlbut, 1980*b*; Valtorta et al., 1988*a*). These two experimental paradigms, when coupled to high-resolution immunocytochemistry, appear particularly appropriate to extend at the molecular level the study of the different steps of the exo-endocytotic pathway.

Previous studies had already dealt with this topic, but the results obtained had been limited by the low resolution of the techniques employed (indirect immunofluorescence: von Wedel et al., 1981; Valtorta et al., 1988*a*; immunoperoxidase: Robitaille and Tremblay, 1987).

In the present work, when we analyzed by immunogold labeling the distribution of synaptophysin and synapsin I in nerve terminals treated with α-Ltx in the presence of extracellular Ca^{2+}, the patterns of immunoreactivity for both proteins resembled those observed in resting preparations: immunogold particles specifically decorated synaptic vesicles and the axolemma was virtually free of labeling. The failure to detect significant immunogold labeling for synaptophysin, an integral vesicle membrane protein, in association with the axolemma of terminals fixed during active secretion indicates that the membrane retrieval mechanism is very fast and selective for vesicle components, so that no extensive intermixing of the vesicle and plasma membrane occurs (see also Valtorta et al., 1988*a* and references therein). Evidence for the selectivity of the retrieval process had already been provided by Patzak et al. (1984) and Patzak and Winkler (1986) in another secretory system, the chromaffin cell. However, in that system the exo-endocytotic process has a much slower time course (von Grafenstein et al., 1986) and antigens of the chromaffin granule membrane were detectable in the plasmalemma of stimulated cells.

Extensive data suggest that the peripheral membrane protein, synapsin I, plays an important regulatory role in the exocytotic process. Because of its ability to interact in vitro with actin filaments, synapsin I has been proposed to mediate the interaction of synaptic vesicles with the actin-based cytoskeletal matrix of the nerve terminal (Bähler and Greengard, 1987; Petrucci and Morrow, 1987; Valtorta, F., M. Bähler, and P. Greengard, manuscript submitted for publication). On the other hand, phosphorylation of synapsin I by Ca^{2+}/calmodulin-dependent protein kinase II decreases both its affinity for the vesicle surface and its actin-bundling activity. Therefore, any treatment that produces a rise in free cytosolic Ca^{2+} in nerve terminals (as is the case with most treatments inducing neurotransmitter release) is expected to favor the dissociation of synapsin I from synaptic vesicles and/or cytoskeletal elements. This could increase the number of vesicles available for exocytosis.

The association of synapsin I immunoreactivity with synaptic vesicles of terminals intensely stimulated in the presence of extracellular Ca^{2+}, indicates that vesicles which are rapidly recycling still have synapsin I associated with their membrane. This suggests that, if indeed synapsin I dissociates from the vesicle surface before exocytosis as a result of Ca^{2+}-mediated phosphorylation, it rapidly reassociates with the vesicle after its retrieval.

The main result obtained in the terminals exposed to α-Ltx in Ca^{2+}-free medium is the demonstration of the translocation of the two proteins synaptophysin and synapsin I from the vesicle compartment to the axolemma. This finding corroborates previous results indicating that, when endocytosis is blocked, exocytosis leads to the permanent incorporation of the synaptic vesicle membrane into the axolemma, causing an increase in the nerve terminal surface area (Ceccarelli and Hurlbut, 1980*b*; Haimann et al., 1985; Valtorta et al., 1988*a*).

Two aspects of this process revealed by our present study appear to be of considerable interest. First, the distribution of both synaptophysin and synapsin I in the axolemma was not limited to the region facing the synaptic cleft, where exocytosis has been shown to occur (for review see Ceccarelli et al., 1988; Rash et al., 1988), but was spread over the entire nerve terminal plasma membrane. These results demonstrate that when recycling is blocked, the vesicle membrane diffuses in the plane of the plasma membrane and possibly intermixes with the components specific to the axolemma. The lack of intermixing observed in Ca^{2+}-containing media could be due to the very transient nature of the fusion between synaptic vesicles and the plasmalemma, which might involve interactions between the synaptic vesicle membrane and cytoskeletal structures. Stimulation conditions that lead to the permanent incorporation of the synaptic vesicle membrane might disturb these interactions, thereby destabilizing the fused membrane. The difference in the distribution of the staining along the axolemma observed in Ca^{2+}-free media for the two proteins could be due to the persistence of some interaction between synapsin I and actin after fusion. This interaction could keep the synapsin I molecules organized in clusters.

Second, our results with neuromuscular junctions stimulated with α-Ltx in Ca^{2+}-free medium demonstrate that synaptic vesicles that have permanently fused with the plasmalemma still retain synapsin I associated with their membrane. However, it should be pointed out that the mechanism by which α-Ltx induces neurotransmitter release in Ca^{2+}-free medium is probably different from that involved in the release triggered by action potentials and depolarizing agents. Meldolesi et al. (1984) demonstrated that the application of α-Ltx under this condition does not result in either an increase of cytosolic Ca^{2+} or the phosphorylation of synapsin I. It is therefore possible that the synapsin I–mediated step in the modulation of transmitter release is bypassed by the action of the toxin and that the lack of phosphorylation accounts for the failure of synapsin I to dissociate from the vesicles.

In conclusion, our results appear consistent with the vesicle hypothesis of neurotransmitter release and add further experimental evidence in support of the suggestion that, under appropriate conditions, the mean residence time of the synaptic vesicle membrane on the surface of the nerve terminal is very short (Ceccarelli et al., 1980*a*; Valtorta et al., 1988*a*). The significance of the persistent association of synapsin I with the permanently fused vesicle membranes remains to be fully understood. The results obtained with α-Ltx in Ca^{2+}-free medium suggest that the dissociation of synapsin I is not a prerequisite in order for the vesicles to fuse. However, the validity of this observation in the case of secretion induced by stimulatory agents other than α-Ltx remains to be proved.

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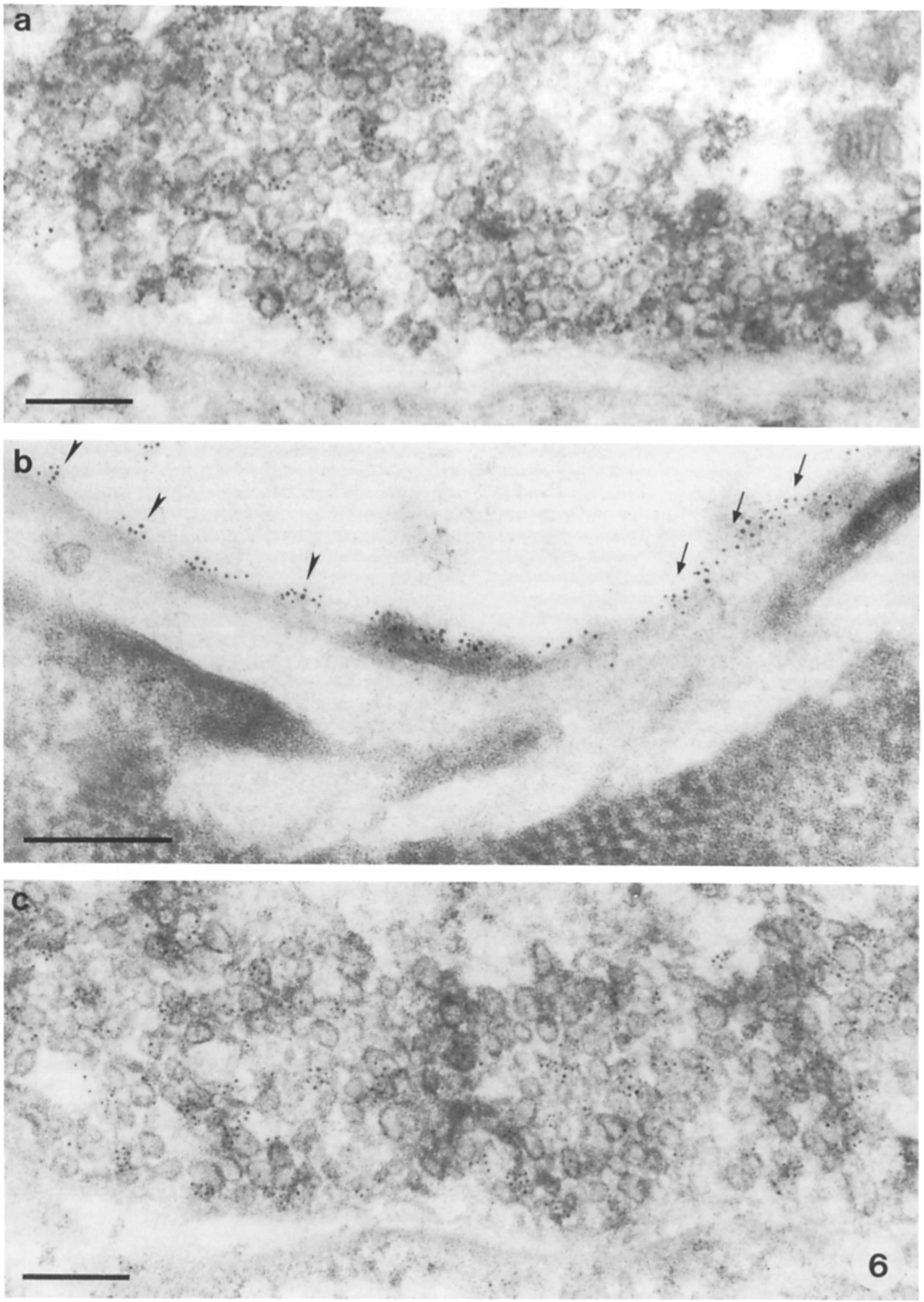
a
b
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Figure 6. Electron micrographs of ultrathin frozen sections of neuromuscular junctions immunolabeled for synapsin I by a colloidal gold technique. (*a*) Portion of nerve terminal from a resting preparation; (*b*) preparation fixed after a 1-h treatment with 0.2 μg/ml α-Ltx in Ca^{2+}-free solution; (*c*) preparation fixed after a 1-h treatment with the same dose of α-Ltx in Ca^{2+}-containing solution. Note the similarity of the distribution of synapsin I immunoreactivity in *a* and *c*; the gold particles are present in regions of the axoplasm where synaptic vesicles are concentrated. In *b*, a portion of presynaptic membrane of a swollen and depleted terminal decorated with gold particles can be seen. The arrows and arrowheads in *b* indicate portions of the axolemma decorated by gold particles continuously and in clusters, respectively. Bars, 0.2 μm.