Synaptophysin (p38) at the Frog Neuromuscular Junction: Its Incorporation into the Axolemma and Recycling after Intense Quantal Secretion

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Abstract. Recycling of synaptophysin (p38), a synaptic vesicle integral membrane protein, was studied by the use of antisera raised against the protein purified from frog brain. When frog cutaneous pectoris muscles were fixed at rest, a bright, specific immunofluorescent signal was observed in nerve-terminal regions only if their plasma membranes had been previously permeabilized. When muscles were fixed after they had been treated for 1 h with a low dose of α-latrotoxin in Ca^{2+}-free medium, an equally intense fluorescence could be observed without previous permeabilization. Under this condition, α-latrotoxin depletes nerve terminals of their quantal store of acetylcholine and of synaptic vesicles. These results indicate that fusion of synaptic vesicles leads to the exposure of intravesicular antigenic determinants of synaptophysin on the outer surface of the axolemma, and provide direct support for the vesicle hypothesis of neurotransmitter release. After 1 h treatment with the same dose of α-latrotoxin in the presence of 1.8 mM extracellular Ca^{2+}, immunofluorescent images were obtained only after permeabilization with detergents. Under this condition, the vesicle population was maintained by an active process of recycling and more than two times the initial store of quanta were secreted. Thus, despite the active turnover of synaptic vesicles and of quanta of neurotransmitter, no extensive intermixing occurs between components of the vesicle and presynaptic plasma membrane.

THE fusion of the synaptic vesicle membrane with the axolemma leads to neurotransmitter release by exocytosis. This process is followed by an active process of membrane retrieval that tends to maintain the population of synaptic vesicles constant and to prevent an increase in the surface area of the terminal. Two different models have been proposed to account for the functional recovery of synaptic vesicles from the nerve-terminal plasma membrane, mainly on the basis of experimental evidence obtained at the frog neuromuscular junction. The first model postulates a complete collapse of the vesicle membrane into the axolemma, followed by intermixing of molecular components and membrane recovery via coated vesicles (Heuser and Reese, 1973; Miller and Heuser, 1984). Alternatively, the opening of the fused vesicle may be a very transient process, the vesicle membrane being removed directly, without intermixing of membrane components (Ceccarelli et al., 1973; Ceccarelli and Hurlbut, 1980a; Meldolesi and Ceccarelli, 1981; Torri-Tarelli et al., 1987). According to the latter hypothesis, a coated-vesicle mechanism is not required for the retrieval of the synaptic vesicle membrane. Efforts to distinguish between these two possibilities have so far been hampered by the lack of suitable markers for the synaptic vesicle membrane.

Recently synaptophysin, an integral membrane protein of the synaptic vesicle, has been purified from mammalian brain and localized in peripheral and central nerve terminals as well as in neuroendocrine cells (Jahn et al., 1985; Wiedenmann and Franke, 1985; Wiedenmann et al., 1986; Navone et al., 1986). In this report, we describe studies of the recycling of synaptic vesicles at the frog neuromuscular junction using antibodies raised against purified frog brain synaptophysin. Quantal secretion of acetylcholine (ACh)[1] has been stimulated using alpha-latrotoxin (α-LTx), the purified active component of black widow spider venom, which is known to induce a massive release of neurotransmitter independently of extracellular Ca^{2+} (Frontali et al., 1976; Hurlbut and Ceccarelli, 1979; Ceccarelli and Hurlbut, 1980b; Meldolesi et al., 1986). The low doses of α-LTx used in this study stimulate neurotransmitter release in both Ca^{2+}-free and Ca^{2+}-containing media. However, in Ca^{2+}-

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1. *Abbreviations used in this paper:* ACh, acetylcholine; α-LTx, α-latrotoxin; m.e.p.p., miniature endplate potential.

free medium the enhanced quantal secretion is accompanied by a rapid depletion of synaptic vesicles because of a block of endocytosis, whereas in Ca^{2+}-containing medium exocytosis is sustained for longer periods of time by an active process of membrane recycling. α-LTx is therefore a useful tool for studying the redistribution of vesicular antigens during the exo-endocytotic cycle.

Materials and Methods

Materials

Materials were purchased from the sources indicated: 2-in-long frogs (*Rana pipiens*), from Connecticut Valley Biological Supply Co., Southampton, MA; phenylmethylsulfonyl fluoride (PMSF), sperm whale skeletal muscle myoglobin type II, and horseradish peroxidase type VI from Sigma Chemical Co., St. Louis, MO; pepstatin from Chemicon, El Segundo, CA; aprotinin (Trasylol) from Bayer, Leverkusen, FRG; CNBr-activated Sepharose 4B (Pharmacia Fine Chemicals, Uppsala, Sweden); complete Freund's adjuvant from Calbiochem-Behring Corp., La Jolla, CA; nitrocellulose membranes (pore size, 0.22 μm) from Schleicher & Schuell, Keene, NH; ^{125}I-Protein A from New England Nuclear, Boston, MA. Rhodamine-conjugated goat anti-rabbit IgGs, purchased from Cooper Biomedicals, Malvern, PA, were bound onto a Sephadex DE52 column (Pharmacia Fine Chemicals) and eluted with 100 mM NaCl/10 mM sodium phosphate buffer, pH 7.3. All other reagents were of analytical grade. Rat brain synaptophysin and mAbs were prepared as described (Jahn et al., 1985).

Purification of Synaptophysin from Frog Brain

Synaptophysin was purified from frog brain using a modification of the procedure described for the purification of rat synaptophysin (Navone et al., 1986). Briefly, 120 frogs were decapitated, and the brains were removed and homogenized with a glass-Teflon homogenizer in 90 ml of an ice-cold solution containing 0.25 M sucrose, 5 mM Hepes-NaOH, pH 7.4, 1 mM EDTA, 0.1 mM PMSF, 2 μg/ml pepstatin, and 1 U/ml aprotinin. The homogenate was centrifuged at 800 g for 10 min; the supernatant was saved and the pellet was resuspended in 50 ml of the same buffer used for the homogenization and was spun again. The combined supernatants were centrifuged for 60 min at 120,000 g and the resulting pellet was homogenized in 110 ml of sodium PBS, 1% (vol/vol) Triton X-100, 0.1 mM PMSF, 2 μg/ml pepstatin, and 1 U/ml aprotinin. After being stirred on ice for 30 min, the extract was centrifuged for 60 min at 120,000 g. The pellet was resuspended in the extraction medium and the suspension was respun. The combined supernatants were loaded onto a column of activated Sepharose 4B coupled to mAbs (C7.2 and C7.3) against rat brain synaptophysin. The washing and elution steps were performed as described (Navone et al., 1986), to yield the purified frog brain synaptophysin.

Generation of Antisera

Purified frog brain synaptophysin ($\sim$3 mg) was boiled for 2 min in stop-solution (3% sodium dodecyl sulfate [SDS], 5% [vol/vol] β-mercaptoethanol, 1 mM EDTA, 8% [wt/vol] sucrose, 62 mM Tris-HCl [pH 6.7]) and loaded on an SDS-10% polyacrylamide preparative gel. After electrophoresis, the unfixed gel was soaked for $\sim$10 min in 4 M sodium acetate (Higgins and Dahmus, 1979). The visualized protein band was cut out and the excised gel piece was homogenized, emulsified in complete Freund's adjuvant, and injected intradermally at multiple sites into New Zealand female rabbits. After the initial injection, three booster injections (containing one third of the original amount of protein) were given at weekly intervals. The animals were bled 1 wk after the administration of the final booster.

Preparation and Pronase Digestion of a Crude Synaptic Vesicle Fraction from Frog Brain

A fraction enriched in frog brain synaptic vesicles was prepared by a modification of a new procedure developed for the purification of synaptic vesicles from rat brain (Hell, Maycox, Stadler, and Jahn, manuscript in preparation). 10 frog brains (total wet weight: 0.5 g) were quickly frozen in liquid nitrogen and crushed into powder using a cold steel mortar. All the following steps were carried out at 10°C. The powder was quickly resus-

pended in 10 ml of 0.32 M sucrose, 1 mM EGTA, 1 μg/ml pepstatin, 0.2 mM PMSF, and 10 mM Tris-Cl (pH 7.4 at 4°C) and homogenized with 10 strokes (1,000 rpm) in a tight-fitting glass-Teflon homogenizer. The homogenate was centrifuged at 47,000 g_{max} for 10 min. The resulting supernatant was layered over a 5-ml sucrose cushion (0.6 M sucrose, 1 mM EGTA, 1 μg/ml pepstatin A, 0.2 mM PMSF, and 10 mM Tris-Cl, pH 7.4) and centrifuged for 2 h at 260,000 g_{max} in a rotor (Ti 60; Beckman Instruments, Fullerton, CA). The resulting pellet was resuspended in a small volume of 150 mM NaCl, 5 mM Hepes-NaOH, pH 7.4, and subjected to pronase digestion. Digestion of the vesicles by pronase was performed for different periods of time (up to 60 min) as described (Jahn et al., 1985), using 1 mg/ml vesicle protein and 0.1 mg/ml pronase. At different time points, aliquots were removed and either solubilized in stop solution, boiled for 2 min and used for electrophoresis and immunoblotting, or negatively stained with 1% uranyl acetate and observed in the electron microscope.

Electrophoresis and Immunoblotting

Protein samples, solubilized in stop-solution, were boiled for 2 min and separated on SDS-10% polyacrylamide gels using the discontinuous system described by Laemmli (Laemmli, 1970). After electrophoresis, the gels were either stained with Coomassie Brilliant Blue or transferred to nitrocellulose membrane sheets (0.22 μm pore size) as described (Towbin et al., 1979). The protein blots were processed for synaptophysin immunoreactivity either by an immunoperoxidase procedure (Jahn et al., 1985) or by using ^{125}I-labeled protein A. In the latter case, immunolabeling was performed as follows: (a) incubation for 1 h in PBS, 5% (wt/vol) nonfat dry milk, 0.1% Triton X-100; (b) incubation for 2 h in the same solution containing anti-synaptophysin antiserum (1:500-1:3,000); (c) washing with five changes of PBS, 0.1% Triton X-100 over a total period of 30 min; (d) incubation for 30 min in PBS, 5% nonfat dry milk, 0.1% Triton-X-100; (e) incubation for 45 min in the same solution containing ^{125}I-protein A (200 cpm/μl); and (f) extensive washing with several changes of PBS, 0.1% Triton-X-100 (3 h to overnight).

Electrophysiology

Cutaneous pectoris nerve-muscle preparations were dissected from frogs, mounted in Lucite chambers, and bathed in standard Ringer's solution containing: Na^{+}, 116; K^{+}, 2.1; Ca^{2+}, 1.8; Cl^{-}, 117; HPO$_4^{2-}$, 2; H$_2$PO$_4^{-}$, 1 mM; pH 7.0. When the ionic composition of the bathing solution was to be changed, the concentration of NaCl was modified to maintain the tonicity constant. α-LTx (a gift from Dr. J. Meldolesi, University of Milan, Milan, Italy) was stored at -70°C and its activity was tested as previously described (Ceccarelli and Hurlbut, 1980b). Immediately before use, α-LTx was diluted to a final concentration of 0.2-0.4 μg/ml in either 4 mM Mg^{2+}-standard Ringer's solution or Ca^{2+}-free (4 mM Mg^{2+} and 1 mM EGTA) Ringer's solution; the recording chamber was drained and refilled with 2.5 ml of this solution. Paired muscles from individual frogs were used in all experiments in which the effects of Ca^{2+}-free and Ca^{2+}-containing solutions were compared. All experiments were performed at room temperature. Endplate regions were impaled with glass microelectrodes filled with 3 M KCl (resistance 10-30 mΩ) and membrane potential was recorded through a conventional high-impedance preamplifier. The amplified signal was saved on tape both as a high gain AC record (band width 0.3-1,250 Hz) and as a low gain DC record (band width 1,250 Hz), using a tape recorder (Racal 4DS; Rank Precision Ltd., London, UK) for subsequent acquisition and analysis by a computer (Micro-PDP 11/73; Digital Equipment Corp., Maynard, MA).

Fluctuation Analysis

Intense asynchronous quantal secretion of ACh generates, at postjunctional sites, a random signal that can be studied by fluctuation analysis (Segal et al., 1985). This analysis allows the measurement of the rate of quantal secretion (and therefore of exocytosis) when its value is high, so that single events cannot be distinguished individually. The stochastic procedure used is insensitive to changes in membrane potential that may occur during prolonged experiments and yields reliable estimates of the time course, amplitude, and rate of occurrence of miniature endplate potentials (m.e.p.p.s) even in the presence of nonlinear summation, nonhomogeneous amplitudes, and rapid variations of m.e.p.p. rate and amplitude (Fesce et al., 1986). We summarize here only the main features of the procedure. The entire time course of the m.e.p.p.s is accurately described by the product of an amplitude factor (h) times a dimensionless waveform factor, $w(t) = e^{-t/\Theta_1}$

$- e^{-t/\Theta_2}$. The two time constants, Θ_1 and Θ_2, were deduced from the power spectra of the voltage signal recorded at the endplate, computed at intervals of a few minutes. m.e.p.p. rate and the amplitude factor "h" were measured from the variance and skew of endplate noise (Segal et al., 1985), computed on successive 10-s records and corrected for nonlinear summation of m.e.p.p.s. The following equations were used:

$$\text{m.e.p.p. rate} = VAR^3/SKEW^2 \times I_3^2/I_2^3 \qquad (1)$$

$$h = SKEW/VAR \times I_2/I_3 \qquad (2)$$

where I_2 and I_3 represent the integrals of the square and the cube of m.e.p.p. waveform, respectively. A high-pass resistance capacitance filter with a 2-ms time constant was used to eliminate contributions of nonstationarity to the variance (VAR) and skew (SKEW) prominent especially in the bursting pattern of secretion induced by α-LTx in the presence of Ca^{2+} (Fesce et al., 1986).

Immunocytochemistry

For frog nerve-muscle preparations, after intracellular recording of m.e.p.p.s, the muscles were fixed for 1 h in the recording chamber. In preliminary experiments, when antibodies were applied to resting preparations fixed with 4% formaldehyde, 0.1 M sodium phosphate buffer, pH 7.4, occasionally patches of fluorescence could be observed in nerve-terminal branches even without previous permeabilization. We attributed this observation to partial permeabilization of the axolemma induced by incomplete membrane fixation and/or by the hypertonicity of the fixative solution. We thus tested different fixative solutions and found that the mixture of 0.25% glutaraldehyde with 1% formaldehyde in 0.1 M sodium phosphate buffer, pH 7.4, represented a good compromise between preserving membrane integrity and retaining adequate immunoreactivity. After fixation, the muscles were washed with 0.1 M sodium phosphate buffer (pH 7.4) and the muscle fibers were then dissociated under a dissecting microscope as previously described (Valtorta et al., 1984). Single fibers were transferred to test tubes and processed for synaptophysin immunoreactivity through the following steps (at room temperature, unless otherwise indicated): (a) washing for 20 min in 0.1 M glycine-NaOH, pH 7.4; (b) washing for 10 min in 0.1 M sodium phosphate buffer, pH 7.4; (c) incubation for 3 h with rabbit anti–synaptophysin antiserum or preimmune serum diluted (1:40–1:100) with 0.5 M NaCl, 17% normal goat serum, and 20 mM sodium phosphate buffer, pH 7.4; (d) several changes of 0.5 M NaCl, 20 mM sodium phosphate buffer, pH 7.4; (e) 1 h incubation with rhodamine-conjugated goat anti–rabbit IgGs diluted 1:20 with the same solution used for the first antiserum supplemented with 0.1% Triton X-100; and (f) overnight washing at 4°C with 0.5 M NaCl, 20 mM sodium phosphate buffer (pH 7.4), 0.1% Triton X-100. In the detergent-treated samples, 0.1% Triton X-100 was included in the solutions during the incubation with the primary antiserum and during all the washing steps. In some experiments, after the final wash, ACh receptors were labeled by incubating the muscle fibers either for 3 h at room temperature or overnight at 4°C with fluorescein-conjugated α-bungarotoxin (diluted 1:1,000 with the same buffer used for the dilution of the antibodies), followed by washing with several changes of 0.5 M NaCl, 20 mM sodium phosphate buffer (pH 7.4) and 0.1% Triton X-100.

For frozen sections from rat brain, $\sim$10-μm-thick frozen sections from different regions of rat brain were prepared and immunostained as previously described (De Camilli et al., 1983).

Electron Microscopy

Muscles to be processed for electron microscopy were fixed in the recording chamber for 1 h at 4°C with 2% OsO_4 in 0.1 M sodium phosphate buffer, pH 7.4. Small specimens of tissue containing endplate regions were dissected out, dehydrated, and flat embedded in Epon 812 according to standard procedures (Ceccarelli et al., 1973). Silver-gray sections were cut on a Reichert-Jung Ultracut microtome, double-stained with uranyl acetate and lead citrate, and examined in either a H-600 or a H-7000 Hitachi electron microscope. In some experiments, 1.6% (wt/vol) horseradish peroxidase (or 1.6% horseradish peroxidase, 0.5% sperm whale myoglobin) was added $\sim$30 min before the addition of α-LTx. This preincubation did not affect m.e.p.p. frequency. After 1 h in α-LTx and extracellular tracers, these muscles were fixed for 1 h with 1% glutaraldehyde, 0.25% formaldehyde (freshly prepared from paraformaldehyde) in 0.1 M sodium phosphate buffer, pH 7.4. Small pieces of muscles were treated to reveal peroxidase activity and further processed as previously described (Graham and Karnowsky, 1966; Ceccarelli and Hurlbut, 1980b).

Protein Determinations

Protein was determined by the method of Bradford (1976), using the protein assay reagent (Bio-Rad Laboratories, Richmond, CA) and IgGs as standards.

Results

Antisera to Frog Synaptophysin

Preliminary experiments performed with both monoclonal and serum antibodies generated against rat synaptophysin demonstrated the presence in the frog of a protein cross-reacting with antibodies to mammalian synaptophysin (Jahn et al., 1986). However, the degree of cross-reactivity was low, and attempts to use these antibodies for the immunocytochemical localization of synaptophysin in the frog were unsuccessful. We therefore decided to purify synaptophysin from frog brain and to use it to raise antibodies. We followed a modification of the procedure described by Navone et al. (1986) to purify synaptophysin from rat brain; for the affinity-column step, we took advantage of the cross-reactivity towards the frog of the mAb against the rat protein. By this procedure, we obtained a material that appeared to consist of a single polypeptide when analyzed by Coomassie Brilliant Blue staining of an SDS-10% polyacrylamide gel (Fig. 1). This polypeptide was specifically recognized on blots by antibodies against rat brain synaptophysin (data not shown). The antisera raised against the purified frog protein specifically recognized synaptophysin on immunoblots of proteins from frog brain homogenates (Fig. 2). Frog brain synaptophysin appears to have a slightly higher molecular weight than the mammalian homologue and like the mammalian protein (Wiedenmann et al., 1985), it can also exist in a dimeric form (Fig. 2, lane b). The ratio between the dimeric and the monomeric form was dependent on the amount of reducing agent present in the buffer used for the solubilization of the sample. As can be seen in Fig. 2, the affinity of the antisera for the antigen was much higher in frog than in rat. Nevertheless, bright nerve-terminal staining could be seen when antisera against frog synaptophysin were
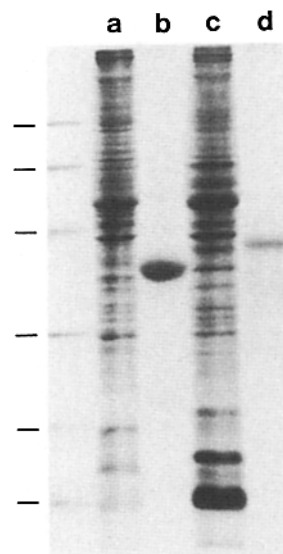

Figure 1. Purification of synaptophysin from frog brain. Lane *a*, rat forebrain homogenate (50 μg); lane *b*, purified rat brain synaptophysin (2 μg); lane *c*, frog brain homogenate (50 μg); lane *d*, frog brain synaptophysin (2 μg). Molecular weight standards: 94,000; 68,000; 45,000; 36,000; 29,000; and 24,000. The samples were separated on an SDS-10% polyacrylamide gel and stained with Coomassie Brilliant Blue.
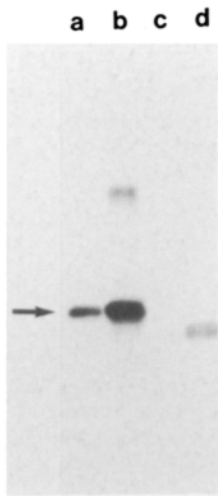
a b c d

Figure 2. Autoradiograph of an immunoblot showing the specificity of the antiserum against frog synaptophysin. The protein samples were separated on an SDS-10% polyacrylamide gel and transferred to a nitrocellulose membrane. The blot was labeled with anti-frog synaptophysin antiserum (dilution 1:3,000), followed by ^{125}I-Protein A. (lane *a*) Frog brain homogenate, 10 µg; (lane *b*) purified frog brain synaptophysin, 0.5 µg; (lane *c*) rat forebrain total homogenate, 10 µg; and (lane *d*) purified rat brain synaptophysin, 1 µg. *Arrow,* the single band of immunoreactivity corresponding to the synaptophysin band in the frog brain total homogenate. The upper band in lane *b* corresponds to the dimeric form of synaptophysin.

main. A fragment generated by pronase digestion of intact synaptic vesicles was indeed recognized by the antiserum against frog synaptophysin (Fig. 4). These findings are in agreement with previous findings obtained in rat brain synaptic vesicles, in which a major 27-kD proteolytic fragment of synaptophysin is found (Jahn et al., 1985).

Immunolocalization of Synaptophysin in Resting Terminals

Frog neuromuscular preparations fixed at rest were exposed to anti-frog synaptophysin antisera in the presence of detergent and subsequently exposed to a rhodaminated secondary antibody. The distribution of immunofluorescence for synaptophysin (Fig. 5 *b*) was similar to the distribution of ACh receptors as revealed by fluoresceinated α-bungarotoxin (Fig. 5 *a*), indicating that synaptophysin was highly concentrated only in the nerve-terminal region. Indeed, the branching patterns revealed by the two stainings were virtually superimposable. The individual terminal branches revealed by antisynaptophysin immunostaining often exhibited a segmented pattern of immunofluorescence. This pattern might be due to a higher concentration of synaptic vesicles in the region of the nerve terminals between adjacent Schwann cell invaginations (Valtorta et al., 1988). When preparations were similarly exposed to antisynaptophysin antisera in the absence of detergent (so that intraterminal components were not accessible to antibodies), no specific immunofluorescence was detected (Fig. 5 *d*) and neuromuscular junction regions
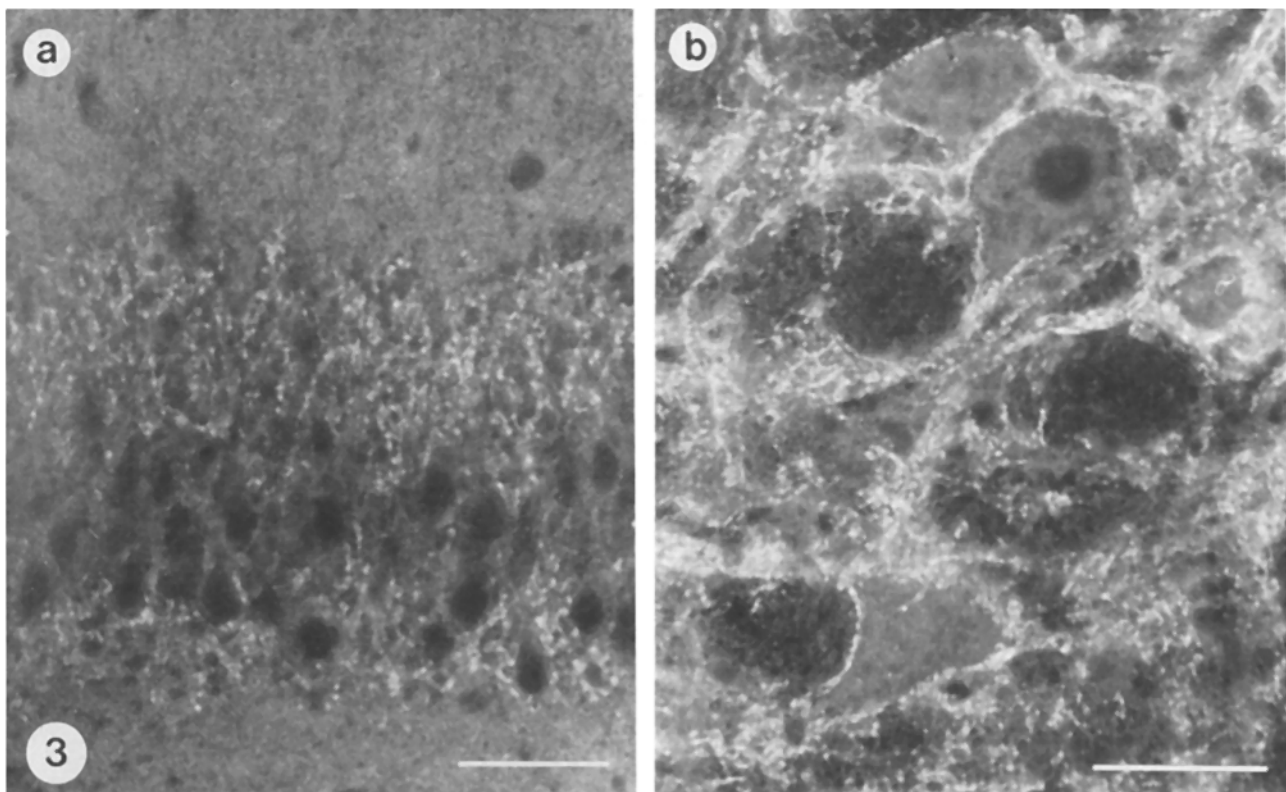
used in immunofluorescence experiments on frozen sections of rat brain (Fig. 3).

For our imunofluorescence experiments (see below), it was important to use an antiserum recognizing intravesicular epitopes, which are expected to become exposed to the extracellular space upon fusion of the vesicle membrane with the axolemma. If this were the case, (an) immunoreactive fragment(s) of synaptophysin should be found in synaptic vesicles subjected to proteolytic digestion of the cytoplasmic do-

Figure 3. Light micrographs showing the distribution of immunofluorescence for synaptophysin in two regions of rat brain. Frozen sections (~10 µm thick) were stained by immunofluorescence using antiserum against frog brain synaptophysin (1:100) and rhodaminated goat anti-rabbit IgGs (1:50). (*a*) CA3–CA4 region of hippocampus; (*b*) deep mesencephalic nucleus. Immunoreactivity is specifically localized to regions containing nerve terminals. Bars, 50 µm.
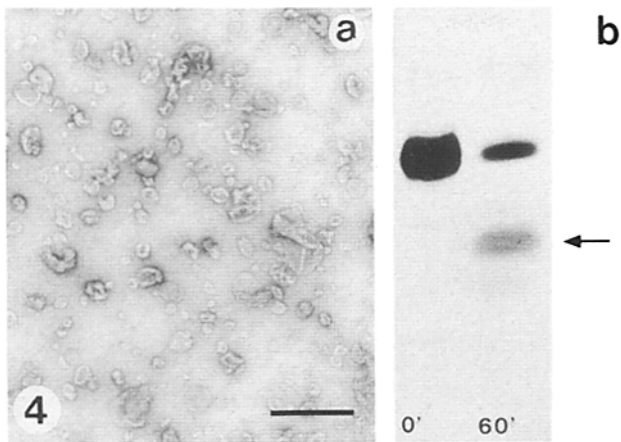
Figure 4. (a) Electron micrograph of a negatively stained crude synaptic vesicle fraction purified from frog brain and subjected to pronase treatment for 60 min. (b) Identification of an intravesicular fragment recognized by antifrog synaptophysin antiserum. A crude synaptic vesicle fraction purified from frog brain was subjected to pronase digestion. The digestion was either immediately stopped (left lane) or continued for 60 min (right lane). Proteins were separated by SDS-PAGE, transferred to a nitrocellulose membrane, incubated with antiserum against frog synaptophysin, and the immunoreactive bands were visualized by immunoperoxidase. The antiserum recognized both the intact protein and a fragment generated by proteolytic digestion (arrow). Bar, 0.5 μm.

could be identified only by fluoresceinated α-bungarotoxin (Fig. 5 c). These findings are consistent with the notion that synaptophysin is an integral membrane protein of synaptic vesicles, which in resting preparations is expected to be confined within the nerve-terminal axoplasm and not to be exposed to the extracellular space.

Effects of α-LTx in Ca²⁺-free and Ca²⁺-containing Medium

To study the redistribution of synaptophysin at neuromuscular junctions upon exocytosis we have stimulated secretion with α-LTx. Low doses of toxin exert different effects on the exo-endocytotic process when applied in the absence rather than in the presence of extracellular calcium. In the absence of extracellular calcium (4 mM Mg^{2+}), 0.2 μg/ml α-LTx induced an enormous but transient discharge of quanta of ACh accompanied by complete depletion of synaptic vesicles that was achieved within 1 h, and by enlargement of the transverse dimension of nerve-terminal branches (Fig. 6 a). A total of 830,000 ± 120,000 quanta were secreted under this condition, as measured by fluctuation analysis applied to intracellular endplate recordings (Fig. 7 a). This figure is in agreement with previous estimates of the initial store of quanta and vesicles present at rest in frog motor nerve terminals (Ceccarelli and Hurlbut, 1975; Haimann et al., 1985; Fesce et al., 1986; Ceccarelli et al., 1988). Experiments with the extracellular tracers horseradish peroxidase and myoglobin showed that, under these conditions, no extracellular tracer reaction products could be detected inside the nerve terminal, indicating that the plasma membrane was not grossly permeabilized (Fig. 6 b). These findings confirm previous findings obtained with crude homogenates of black widow spider venom (Ceccarelli and Hurlbut, 1980b). When

the same concentration of toxin was applied in the presence of 1.8 mM Ca^{2+} and 4 mM Mg^{2+}, the quantal secretion rate remained high and constant for at least 1 h, 1.8 million quanta being secreted in that time (Fig. 7 b), and neither marked depletion nor swelling of the nerve terminals was observed (Fig. 6 c). In this condition, most of the synaptic vesicles were labeled with extracellular tracer reaction product (Fig. 6, d and e), indicating that the vesicle population was maintained by active membrane recycling.

Immunolocalization of Synaptophysin in α-LTx-treated Terminals

The distribution of synaptophysin in nerve terminals treated for 1 h with 0.2 μg/ml α-LTx in Ca^{2+}-free solution is shown in Fig. 8. In contrast to the situation observed in control preparations (Fig. 5), bright fluorescence staining of nerve terminals was present without previous permeabilization of the plasma membrane with detergent. A marked increase in the apparent transverse dimension of the nerve terminal was always observed. The average transverse dimensions (micrometers ± SD) were 2.4 ± 0.6 for the control preparations and 4.9 ± 1.6 for the preparations treated with α-LTx in Ca^{2+}-free medium. These figures represent an overestimate of the true diameters, as they include the fluorescence halo (Valtorta et al., 1984) and are reported only to provide an estimate of the degree of swelling that occurred in the α-LTx-treated preparations.

When nerve-muscle preparations were treated with the same dose of α-LTx in the presence of extracellular calcium, the fluorescent images observed were indistinguishable from those observed under resting conditions (Fig. 9). Omission of detergent from the solution used for dilution of the anti-synaptophysin antisera resulted in the lack of specific staining of nerve terminals, which could be identified by double-labeling with fluoresceinated α-bungarotoxin. No increase in the apparent transverse dimension was observed in the presence of calcium (Fig. 9).

Discussion

Morphometric analysis of changes in ultrastructure induced by prolonged stimulation and the use of extracellular tracers to follow the recovery of synaptic vesicle membrane from the axolemma have provided evidence for the existence of the exo-endocytotic cycle (Heuser and Reese, 1973; Ceccarelli et al., 1972; Ceccarelli et al., 1973; Ceccarelli and Hurlbut, 1980a). However, these approaches cannot be used to study the selectivity of endocytosis of vesicle membrane relative to components of the plasma membrane. We have now attempted to study vesicle membrane movements using specific antibodies against a synaptic vesicle membrane protein, synaptophysin, purified from frog brain.

mAbs to rat synaptophysin were used to affinity purify a related protein from frog brain. In SDS–polyacrylamide gels, this protein had a higher apparent molecular weight than the mammalian homologue. The slight difference in molecular weight might reflect a more general phenomenon, since in the frog another synaptic vesicle protein, synapsin I, also appears to have a higher molecular weight (Valtorta et al., 1988). In agreement with what has been observed in mammals (Wiedenmann and Franke, 1985; Navone et al., 1986), in the frog synaptophysin appears to consist of a

Figure 5. Fluorescence micrographs of resting neuromuscular junctions on single muscle fibers teased apart from frog cutaneous pectoris muscle. The fixed preparation shown in *a* and *b* was treated with 0.1% Triton X-100 and double stained with (*a*) fluoresceinated α-bungarotoxin and with (*b*) antisynaptophysin antiserum followed by rhodamine-conjugated goat anti–rabbit IgGs. Similar patterns of nerve terminal
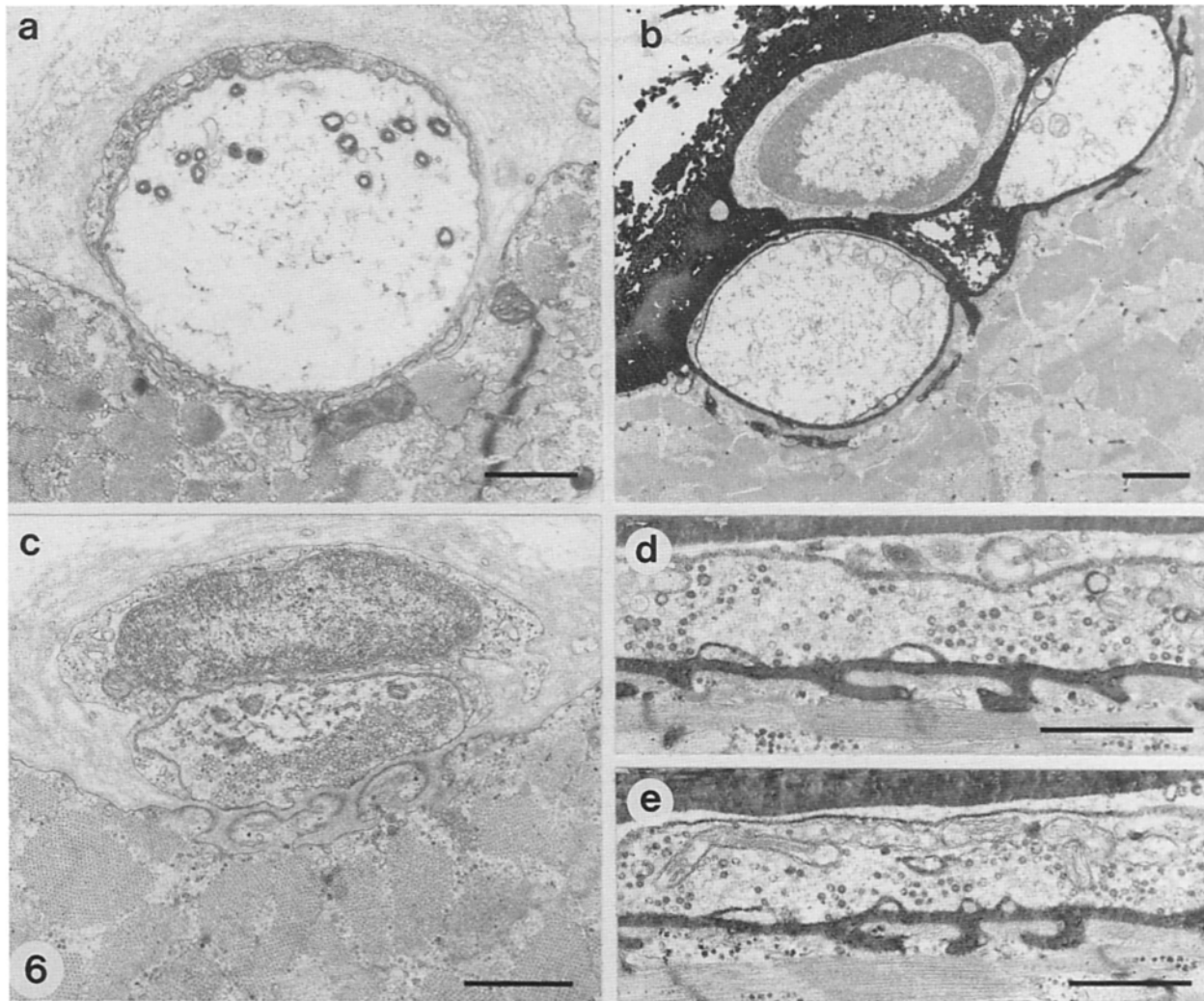
Figure 6. Effects of α-LTx on the ultrastructure of neuromuscular junctions. (*a* and *b*) Electron micrographs of cross-sectioned terminals treated for 1 h with 0.2 μg/ml α-LTx in Ca^{2+}-free solution. The terminal branches shown in *b* come from an experiment in which 1.6% horseradish peroxidase and 0.5% myoglobin were present. (*c*) Electron micrograph from a cross-sectioned terminal treated for 1 h with the same concentration of α-LTx in Ca^{2+}-containing Ringer's solution. (*d* and *e*) Longitudinal sections of terminals treated as in *c* in the presence of 1.6% horseradish peroxidase. Notice the normal appearance of the terminal in *c* and the depletion of synaptic vesicles and the swelling of the terminal in *a* and *b*. In *d* and *e*, most of the synaptic vesicles are loaded with horseradish peroxidase reaction product. Bars, 1 μm.

disulfide-linked homooligomer. When tested by immuno-blotting, the antisera raised against the purified frog protein specifically recognized synaptophysin from both frog and rat. When tested by immunofluorescence on frozen sections from rat brain, these antisera reproduced the fluorescence pattern previously described using antisera raised against rat synaptophysin (Wiedenmann and Franke, 1985; Navone et al., 1986). In immunofluorescence experiments with frog nerve-muscle preparations, the entire nerve-terminal arbori-zation was uniformly decorated by these antisera. Im-munolabeling was highly specific for nerve terminals and no fluorescence was found associated with other portions of

muscle fibers, with myelinated axons, or with unmyelinated preterminal axons. The lack of immunostaining observed when the antisera were applied before membrane permea-bilization indicates that synaptophysin was confined within the axoplasm. Together, these findings are those expected for immunostaining with antibodies to a synaptic vesicle antigen.

Antibodies specific to secretory granule membrane com-ponents have been previously used in other systems for the study of membrane recycling. Evidence for a selectivity of the retrieval process has been provided in chromaffin cells, a widely used model for the study of exocytosis, by the use

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branching are revealed by the postsynaptic (*a*) and presynaptic (*b*) markers. The nerve terminal shown in *c* and *d* is from a preparation double stained as in *a* and *b* (*c*, α-bungarotoxin; *d*, synaptophysin), except that no detergent was used. In this condition, synaptophysin labeling is undetectable and the nerve terminal region can be identified only by the distribution of α-bungarotoxin labeling. Bar, 50 μm.
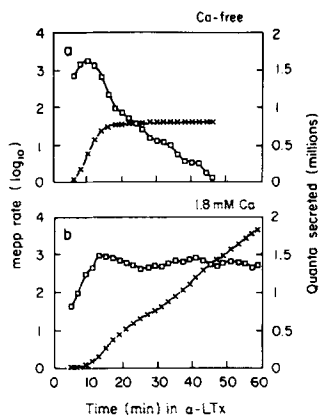
Figure 7. Time course of m.e.p.p. rate (□) and cumulative quantal secretion (×) computed from fluctuation analysis of endplate recordings during exposure to 0.2 μg/ml α-LTx in either Ca^{2+}-free or Ca^{2+}-containing Ringer's solution. Each point is the average of the corresponding values from three different experiments. m.e.p.p. rate (s^{-1}) is given on a logarithmic scale.

properties of the exo-endocytotic cycle in that system, in which the half-life of the fused granules has been estimated to be of the order of minutes (von Grafenstein et al., 1986). At the frog neuromuscular junction, it appears that the half-life for the fused vesicle is <1 s (Ceccarelli et al., 1979; Ceccarelli and Hurlbut, 1980a). Thus, when high rates of secretion are maintained by a balance between exocytosis and endocytosis, a selective retrieval process would prevent the incorporation of relevant amounts of vesicle membrane into the axolemma. A vesicle antigen would be detectable in the axolemma only when the rate of exocytosis far exceeded the rate of endocytosis. Hence, we studied the distribution of synaptophysin under two extreme conditions: (*a*) after intense quantal secretion in the presence of a block of endocytosis; and (*b*) during intense quantal secretion with active recycling of synaptic vesicles.

At the neuromuscular junction, α-LTx is known to induce an increase of several orders of magnitude in the rate of occurrence of m.e.p.p.s that ultimately leads to a complete depletion of quanta of ACh and of synaptic vesicles. It has been shown that high doses of crude black widow spider venom homogenate cause depletion independently of the
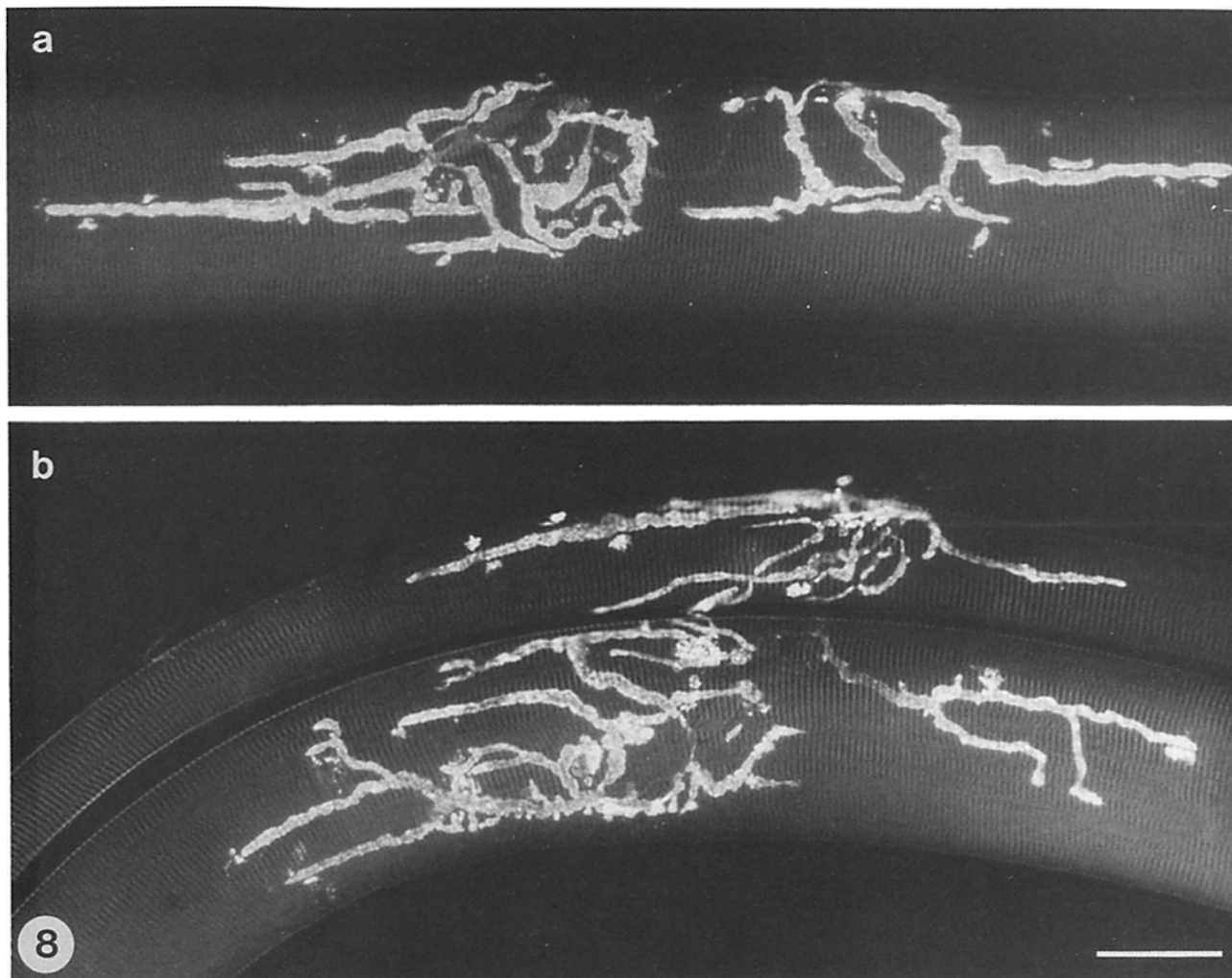
of antisera against glycoprotein III and dopamine-β-hydroxylase (Patzak et al., 1984; Patzak and Winkler, 1986). In that system, patches of immunoreactivity for vesicle antigens could be detected on the cell surface for up to 45 min after the end of stimulation. Several factors can account for this relatively slow recovery process; among them are the kinetic

Figure 8. Two examples of fluorescence micrographs showing synaptophysin immunoreactivity in neuromuscular junctions treated for 1 h with 0.2 μg/ml α-LTx in Ca^{2+}-free Ringer's solution. No detergent was used. In this condition, no permeabilization was necessary to reveal synaptophysin immunoreactivity and the nerve terminal branches show a marked increase in their transverse dimension. Bar, 50 μm.

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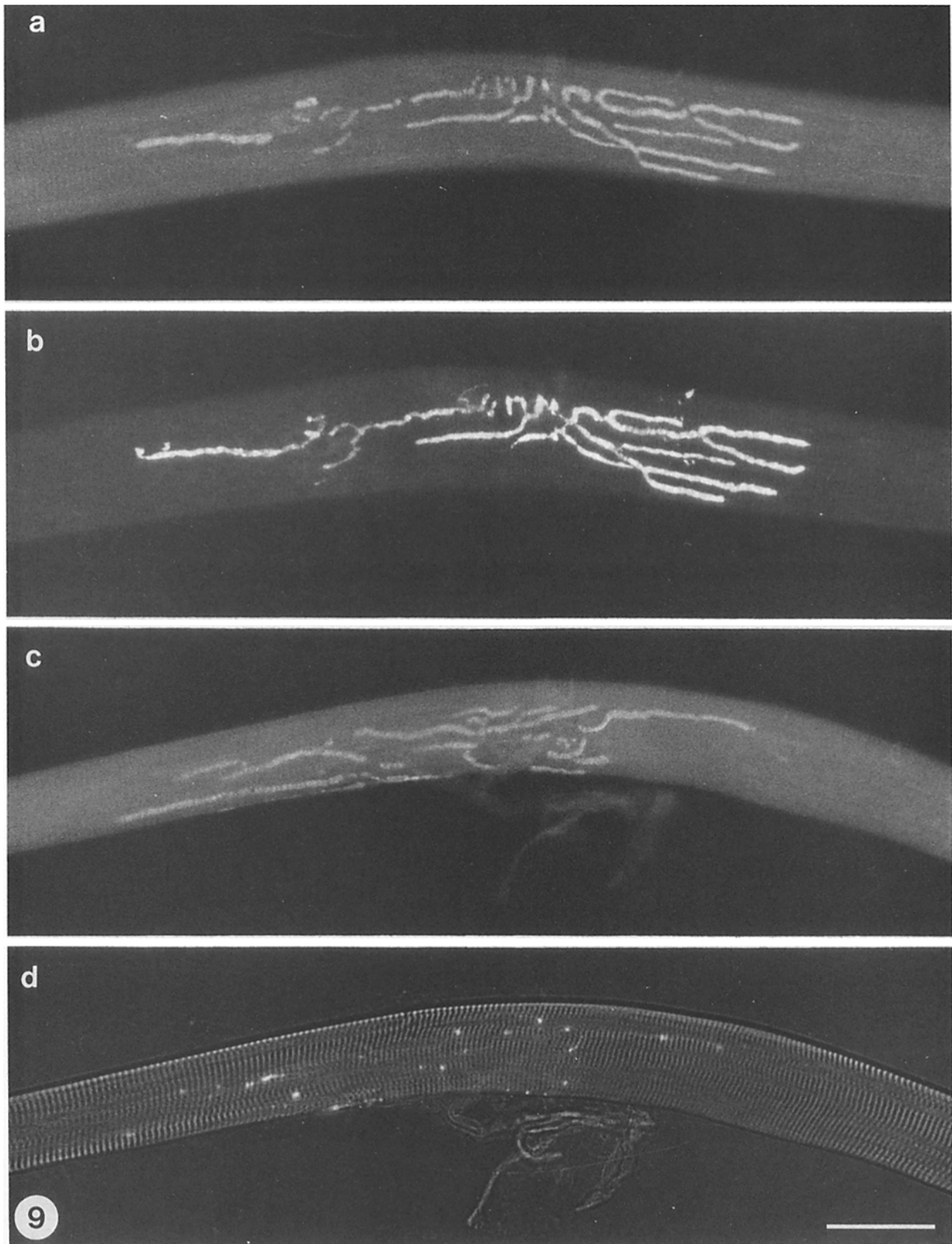*Figure 9.* Fluorescence micrographs of neuromuscular junctions exposed for 1 h to 0.2 μg/ml α-LTx in Ca^{2+}-containing Ringer's solution and double stained as in Fig. 5. (*a* and *b*) Neuromuscular junction double stained (*a*, α-bungarotoxin; *b*, synaptophysin) after permeabilization with 0.1% Triton X-100. No obvious difference can be seen between the synaptophysin immunofluorescence pattern of this nerve terminal and that of a nerve terminal fixed at rest (Fig. 5). (*c* and *d*) Neuromuscular junction not treated with detergent. Note that synaptophysin immunoreactivity (*d*) is not revealed without permeabilization, whereas α-bungarotoxin labeling (*c*) shows the normal appearance of the endplate region. Bar, 50 μm.

presence of extracellular calcium; however, when low doses are used, a complete depletion can be obtained only in the absence of calcium (Ceccarelli and Hurlbut, 1980b).

In these experiments, low doses of purified α-LTx, when applied in Ca²⁺-free solution, induced very high initial rates of secretion that were maintained for only a few minutes, subsiding to very low levels within 20 min. In these preparations, nerve terminals were swollen and depleted of synaptic vesicles; measurements by fluctuation analysis revealed that the total number of quanta secreted roughly corresponded to previous estimates of the quantal store (Haimann et al., 1985; Fesce et al., 1986) and to the estimated average number of synaptic vesicles present in resting frog neuromuscular junctions (Segal et al., 1985; Haimann et al., 1985; Ceccarelli et al., 1988). These results indicate that recycling of synaptic vesicles and continuous secretion of quanta of ACh were blocked and suggest that the synaptic vesicle membranes were incorporated into the axolemma. When antisera to frog synaptophysin were used to stain these preparations, fluorescent nerve terminal branching was revealed even without previous permeabilization with detergent. This finding is in agreement with previous findings by von Wedel et al. (1981), who showed that a polyspecific antiserum to elasmobranch synaptic vesicles bound to exhaustively stimulated intact frog nerve terminals. It is very unlikely that our finding is due to penetration of antibodies through an axolemma damaged by exposure to α-LTx in Ca²⁺-free medium. In fact, α-LTx is known to exert its effects by interacting with a high affinity receptor localized in the axolemma (Meldolesi, 1982; Valtorta et al., 1984) and it is devoid of proteolytic and phospholipolytic activity (Frontali et al., 1976). Furthermore, after 1 h of exposure to α-LTx in Ca²⁺-free medium, the muscle fiber retains its normal resting potential indicating that the toxin does not produce unspecific permeabilization of membranes. Patch-clamp experiments have shown that α-LTx causes the opening of small cation channels with a conductance of 15 pS (Wanke et al., 1986) corresponding to a calculated pore radius <0.5 nm (Hille, 1984). The present experiments show that in α-LTx-treated preparations, the axolemma is not permeable to macromolecules such as horseradish peroxidase and myoglobin (M_r 40,000 and 17,000, respectively). It is therefore very unlikely that larger molecules like antibodies could penetrate into these terminals.

These results provide strong evidence that synaptophysin is incorporated into the axolemma of the swollen terminal and that, as a result of vesicle fusion, some intravesicular antigenic determinants become exposed to the extracellular space. Previous findings have shown that the most antigenic portions of mammalian synaptophysin are exposed on the outer (cytoplasmic) surface of synaptic vesicles (Jahn et al., 1985). However, based on the amino acid sequences predicted from rat and human cDNA and genomic clones, synaptophysin appears to have two intravesicular domains (Suedhof et al., 1987; Buckley et al., 1987; Leube et al., 1987). The results reported here show that the anti–frog synaptophysin antisera recognize intravesicular epitopes.

The same concentration of α-LTx, when applied in the presence of extracellular calcium, induced high rates of secretion that were maintained throughout the entire hour of exposure to the toxin. After this period, a total of nearly two million quanta had been secreted. This figure represents more than two times the initial quantal store. These terminals, which were still secreting at a high steady rate, retained their complement of synaptic vesicles and no swelling of the terminal was evident. When horseradish peroxidase was present in the medium, many of the vesicles appeared to be loaded with the tracer. Thus, this multiple quantal turnover was maintained by an active recycling process. Under these conditions, no synaptophysin immunostaining could be detected unless plasma membranes had been previously permeabilized with detergent. In permeabilized preparations, the pattern of fluorescence was similar to that observed in preparations fixed at rest. These results indicate that, in spite of active membrane recycling, the bulk of synaptophysin was still confined within the axoplasm, suggesting that membrane retrieval was selective for the vesicle components, without detectable intermixing between the two membranes.

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