FREEZE-FRACTURE STUDIES OF FROG NEUROMUSCULAR JUNCTIONS DURING INTENSE RELEASE OF NEUROTRANSMITTER

III. A Morphometric Analysis of the Number and Diameter of Intramembrane Particles

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ABSTRACT

The intramembrane particles on the presynaptic membrane and on the membrane of synaptic vesicles were studied at freeze-fractured neuromuscular junctions of the frog. The particles on the P face of the presynaptic membrane belong to two major classes: small particles with diameters <9 nm and large particles with diameters between 9 and 13 nm. In addition, there were a few extralarge particles with diameters >13 nm. Indirect stimulation of the muscle, or the application of black widow spider venom, decreased the concentration of small particles on the presynaptic membrane but did not change the concentration of large particles.

Three similar classes of particles were found on the P face of the membrane of the synaptic vesicles. The concentrations of large and extralarge particles on the vesicle membrane were comparable to the concentrations of these particles on the presynaptic membrane, whereas the concentration of small particles on the vesicle membrane was less than the concentration of small particles on the presynaptic membrane. These results are compatible with the idea that synaptic vesicles fuse with the presynaptic membrane when quanta of transmitter are released. However, neither the large nor the extralarge particles on the P face of the presynaptic membrane can be used to trace the movement of vesicle membrane that has been incorporated into the axolemma.

Several investigators have studied the effects of stimulation on the distribution of intramembrane particles at the presynaptic membrane of nerve terminals (2, 3, 8, 11, 17, 18). The results of such studies are of interest since they may provide direct morphological evidence that the membrane of synaptic vesicles becomes incorporated into the axolemma when quanta of neurotransmitter are secreted. If the synaptic vesicles contained particles that could be distinguished from those in the axolemma, then these particles might be used to trace the movements of vesicle membrane after it had been incorporated into the axolemma (9, 11).

This report analyzes the changes in the distribution of intramembrane particles in the presynaptic membrane of frog neuromuscular junctions

that had been stimulated indirectly for 20 min at 10/s or had been treated for 1 h with black widow spider venom (BWSV). Both of these treatments cause a profound depletion of synaptic vesicles (4–7, 10, 14, 15). Therefore, if the membrane of the depleted vesicles were incorporated into the membrane of the nerve terminal, then the distribution of intramembrane particles in the axolemma might change, and the extent of the change would depend upon the difference between the distributions of particles in the two membranes in resting preparations.

MATERIALS AND METHODS

Cutaneous pectoris nerve muscle preparations were dissected from frogs, *Rana pipiens*, and mounted in chambers as described previously (14). Some muscles were fixed at rest in Ringer's solution (pH 7.0, composition in mM: Na^+, 116; K^+, 2.1; Ca^{2+}, 1.8; Cl^-, 118; phosphate buffer, 3); some muscles were fixed at rest after soaking for 3 h in a modified Ringer's solution that contained no Ca^{2+}, 4 mM Mg^{2+}, and 1 mM EGTA; some muscles were fixed after 20 min of indirect stimulation at 10/s in Ringer's solution; and some muscles were fixed 1 h after crude BWSV had been applied in a modified Ringer's solution containing 0.5 mM Ca^{2+} and 4 mM Mg^{2+}. The fixative contained 0.5% formaldehyde (freshly prepared from paraformaldehyde) and 1% glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.2. After 1 h of fixation at room temperature, small pieces of muscle containing suspected end-plate regions were cut out and transferred through a graded series of solutions of glycerol in 0.1 M sodium phosphte buffer (pH 7.2) at room temperature. The schedule was: 10 min in 10, 20 min in 20, and 30 min in 30% glycerol. The small pieces of fixed and glycerinated muscle were frozen and then fractured and replicated in a Balzers BAF 301 apparatus (Balzers Corp., Nashua, N. H.), as described previously (2). A quartz crystal thin-film monitor was used to prepare replicas of constant thickness, which were examined in a Philips EM 400 electron microscope. Particles were counted on the P faces of the various membranes.

Many of the replicas of the presynaptic membranes of the nerve terminals came from previous experiments in which cutaneous pectoris muscles were fractured longitudinally so that we could study the distribution of the sites of fusion between synaptic vesicles and the presynaptic membrane (2, 3).

Selection of Presynaptic Membranes

All of the presynaptic membranes found on the replicas were photographed in the electron microscope and prints were prepared. For each experimental condition, those micrographs showing high resolution were reprinted at a final magnification of

× 200,000. The particles were measured and counted only on the flat portions of the presynaptic membranes, i.e., the portions where the angle of shadowing was constant. The constancy of the angle of shadowing was judged by the constancy of the ratio of light to dark areas on the large intramembrane particles. Areas of indirectly stimulated terminals that contained either dimples or infoldings were avoided (see Fig. 1 B).

### Counting Particles on the Presynaptic Membrane

The selected portions of the prints were divided into three regions of 150 × 150 nm. The first region was centered over an active zone, the second covered an area 75–225 nm from the center of an active zone, and the third covered an area 225–375 nm from the center of an active zone. The second and third regions were marked off on both sides of the active zones whenever possible. The active zones of frog motor-nerve terminals are 90–100 nm wide (2, 3, 13, 16). Hence the region that was centered on these zones extended ~25–30 nm beyond their edges. This overextension allowed for variations in the width of the zones and ensured that all the particles associated with a zone were counted in those cases where the double rows were not perfectly aligned. The third region extended close to the midpoint between successive active zones: in fact, the average distance between successive zones is ~0.7 μm (16). The chosen regions were examined through a dissecting microscope and the diameters of the particles were measured normal to the direction of shadowing. The diameters were estimated to the nearest 0.1 mm (corresponding to 0.5 nm) with the aid of a reticule placed over the print. Particles with diameters <0.7 mm (3.5 nm) could not be resolved and were not counted.

An hour of soaking in BWSV, or several hours of soaking in a Ca^{2+}-free solution, causes a disorganization of most of the active zones (2). Therefore, in the experiments in which these treatments were used, we counted particles only on those regions of the replicas where remnants of active zones could be clearly identified (Figs. 1 C and 3 A).

Counting Particles on the Membrane of Synaptic Vesicles

Measurements of particle diameters and densities are difficult on synaptic vesicles because the degree of metallization and the angle of shadowing vary even within a single vesicle. To standardize the conditions of shadowing and metallization, we considered only the P faces of vesicles whose membrane had been adequately shadowed by platinum over >50% of the surface. About half of the vesicles with exposed P faces satisfied these requirements. Among these vesicles an average of ~10% of the total exposed surface was poorly metallized so that particles could not be seen even if they had been present. In some instances, the synaptic vesicles lay close to particlelike structures

FIGURE 1 Micrographs of replicas of the P faces of three different nerve terminals from control (A), indirectly stimulated (B), and BWSV-treated (C) cutaneous pectoris muscles. The three squares (150 × 150 nm) on each of the micrographs indicate the three regions of the presynaptic membrane on which the intramembrane particles were measured and counted. The left square was always centered over an active zone. The disorganization of the active zone caused by BWSV is evident in C. Arrow indicates the site of fusion between the membrane of a vesicle and the axolemma. Bar, 0.1 μm. × 200,000.
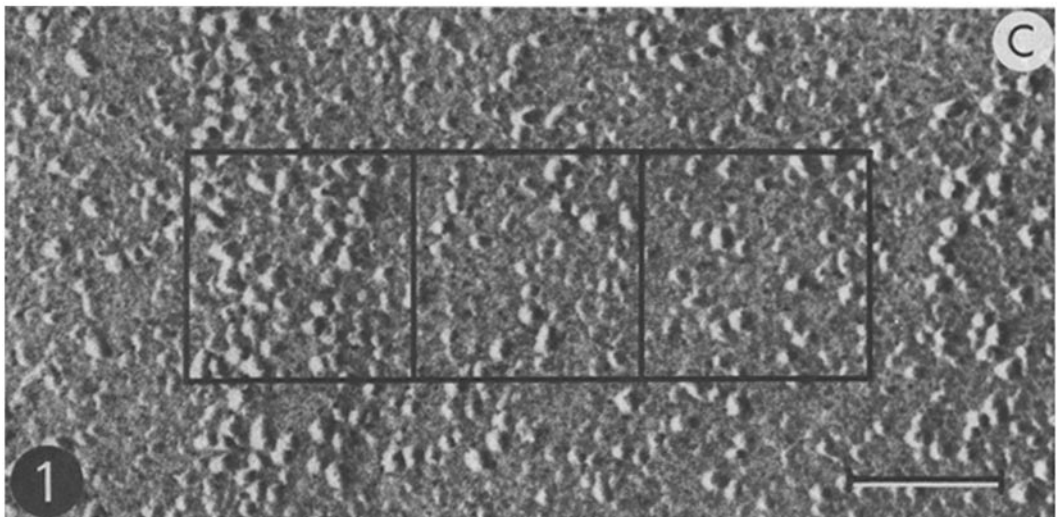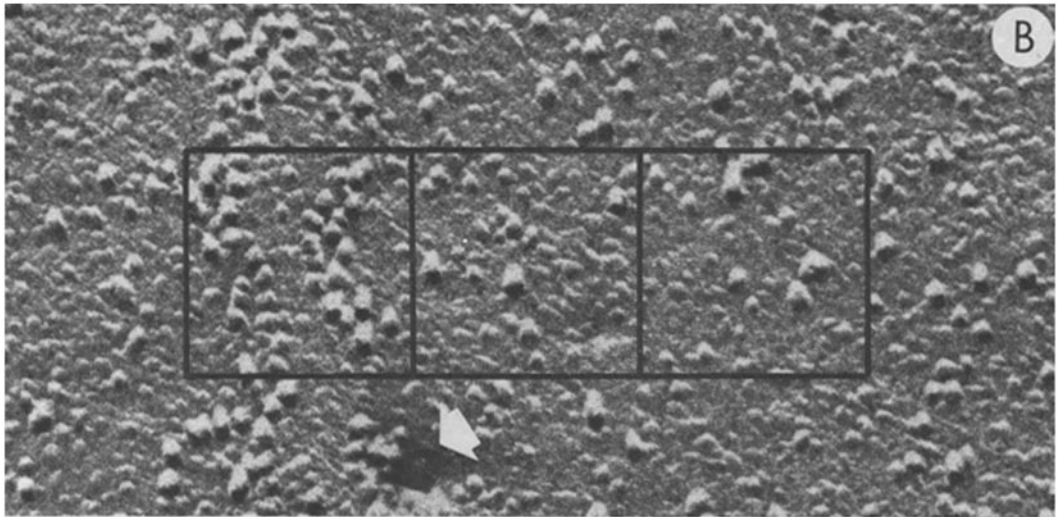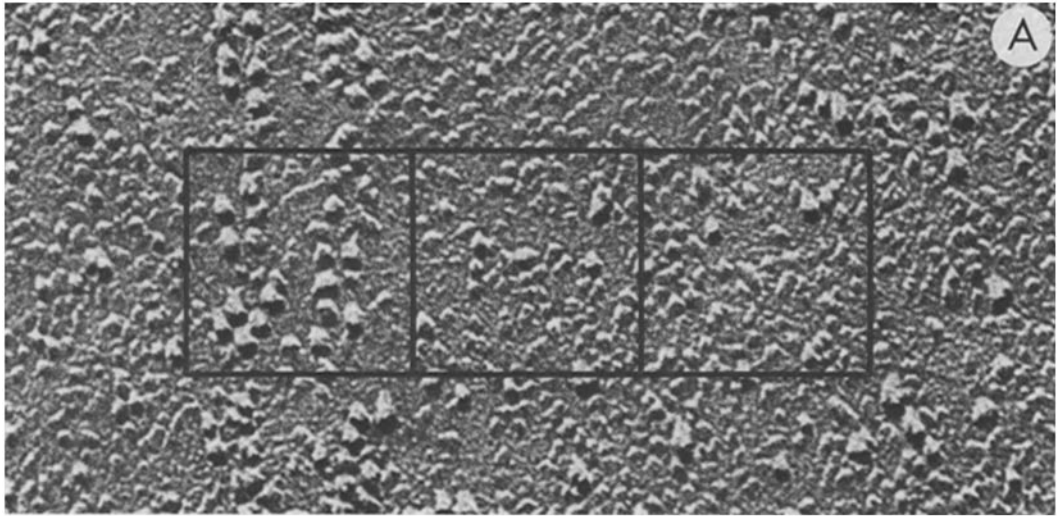
within the axoplasm. To avoid including these axoplasmic structures in our particle counts, we considered only particles that lay entirely within the rim of the craters left by vesicles that the fracture scooped from the axoplasm.

The area of exposed vesicle membrane is difficult to measure exactly. One estimate (probably an overestimate) is obtained if each crater is assumed to be a hemisphere with a diameter equal to the measured diameter. Another estimate (probably an underestimate) is obtained if each vesicle is assumed to be a sphere with a diameter equal to the diameter of the largest vesicle crater found in any of the nerve terminals. Any particular crater then is assumed to be a portion of a similar sphere and its surface area is computed from the formula: Area $= (\pi D^2/2)[1 - \sqrt{1 - (d/D)^2}]$, where $d =$ diameter of a particular crater, and D $=$ diameter of the largest crater.

These two estimates of the total area of the exposed membrane of the synaptic vesicles were reduced by 10% to account for the portions of the craters that were not adequately metallized, and the values so obtained were used to draw the histogram in Fig. 4 B.

The Kruskal-Wallis analysis of variance by ranks was used to determine the statistical significance of the changes in the densities of intramembrane particles.

RESULTS

Presynaptic Membrane

Fig. 1 shows representative micrographs of replicas of the P faces of terminals from (A) a resting preparation in Ringer's, (B) a preparation stimulated indirectly for 20 min at 10/s in Ringer's and fixed without rest, and (C) a preparation treated for 1 h with BWSV in modified Ringer's solution with 0.5 mM Ca^{2+} and 4 mM Mg^{2+}. The three regions selected for the analysis of the particles are indicated on each micrograph. BWSV ultimately causes a profound disorganization of the active zone, and well-delineated double rows of large intramembrane particles often cannot be found. However, large particles are often found concentrated in patches at regions of the terminals aligned opposite the postjunctional folds in the muscle surface (2). These regions were assumed to be remnants of former active zones, and the first square was centered on these regions when the particle counts were performed.

Fig. 2 shows histograms of the diameters of the intramembrane particles in the three regions of the nerve terminals in the three experimental conditions. The distributions of particle diameters are skewed to the right in all three regions of the nerve terminals. In the regions of the axolemma containing the active zone, two distinct classes of particles seem to be present. One class is composed of particles with diameters that range from 4 to 8 nm and whose mean diameter is ~6 nm. The second class is composed of particles with diameters >9
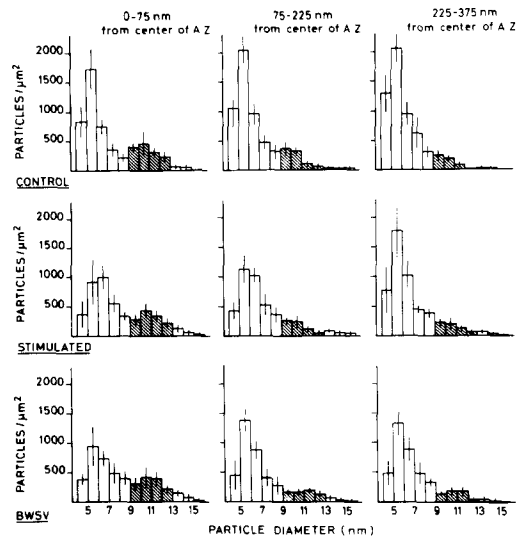

FIGURE 2 Histograms of the distributions of the diameters of the intramembrane particles on the three regions of the presynaptic membrane (P faces) of control (top), indirectly stimulated (middle), and BWSV-treated (bottom) nerve terminals. The crosshatched areas of the histograms indicate the range of the diameters of the particles that are aligned in double rows at the active zones (A Z). For any one histogram the number of particles counted ranged from 645 to 1,264, the area of membrane examined ranged from 0.16 to 0.26 μm^2, and the number of active zones examined ranged from 6 to 12. Total presynaptic membrane: Control, 0.6 μm^2, four muscles; Stimulated, 0.77 μm^2, four muscles; BWSV, 0.47 μm^2, three muscles.

nm. Most of the particles in this second class are the particles aligned in double rows along the edges of the active zones (2, 3, 12, 13, 16). The diameters of these particles range from 9 to 13 nm, and the average is ~11 nm (3). This class of particle is indicated by the crosshatched areas on the histograms. In addition, there are a few particles with diameters >13 nm. These three classes of particles will be referred to as small, large, and extralarge, respectively.

Small particles are distributed uniformly over the presynaptic membrane of control nerve terminals at an average density of 4,538 ± 151 particles/μm^2. Large particles are also found in all regions of the presynaptic membrane. Their average density in the region centered on the active zone is 1,413 ± 302 particles/μm^2; their average density over the other two regions is 684 ± 222 particles/μm^2. The density is higher at the active

zone mainly because many of the particles are packed into double rows in these regions.

Soaking in Ca^{2+}-free solutions does not change the particle densities in those portions of the terminals where active zones could still be clearly recognized (Fig. 3). In those cases, the average density of small particles was 4,132 ± 852 particles/μm^2; the density of large particles at the remnants of active zones was 1,298 ± 221 particles/μm^2, and the average density of large particles in the two regions between active zones was 587 ± 219 particles/μm^2.

Extralarge particles are relatively sparse and never form a definite peak on the histograms. On
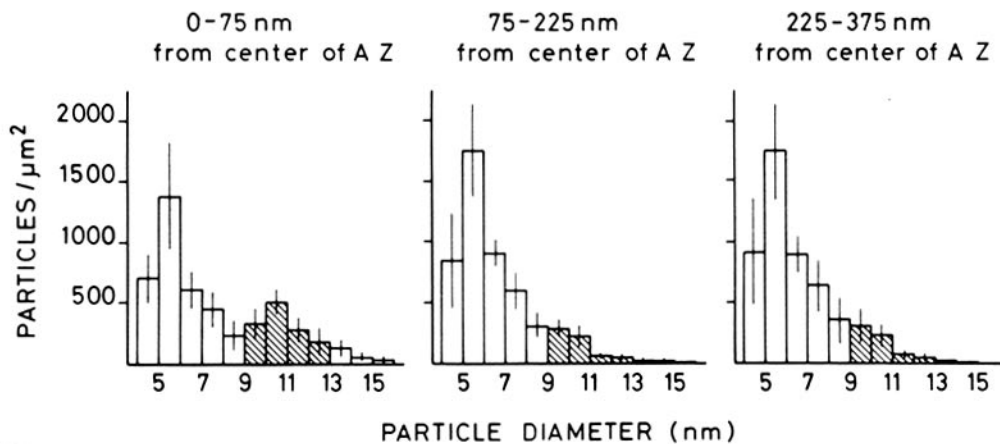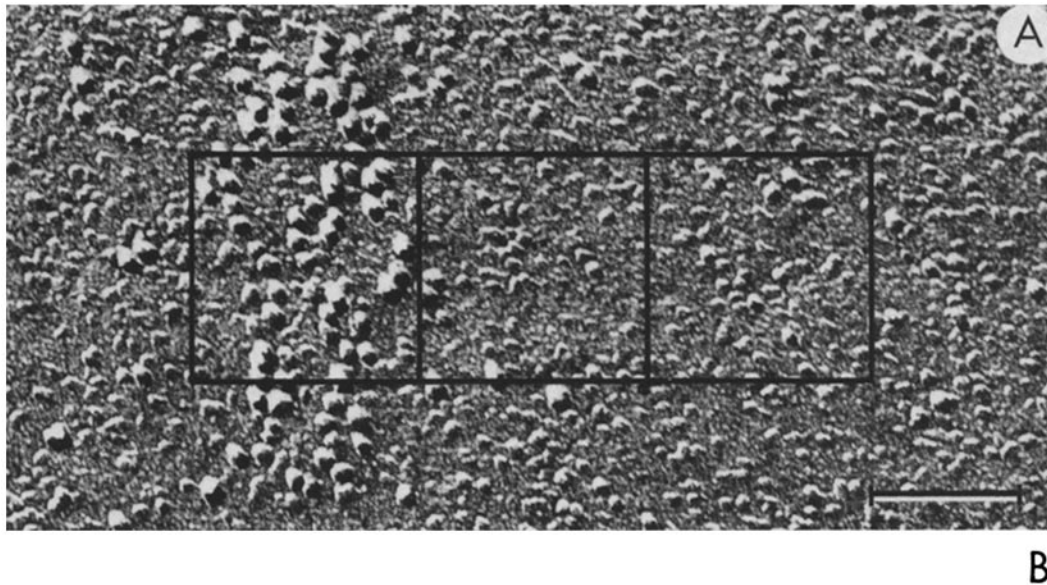
FIGURE 3   (A) Micrograph of a replica of the P face of a nerve terminal from a muscle that had been bathed for 3 h in a Ca^{2+}-free Ringer's solution with 4 mM Mg^{2+} and 1 mM EGTA. A clear remnant of a disorganized active zone lies within the first square. Bar, 0.1 μm. × 200,000. (B) Histogram of the distribution of the diameters of the particles in each of the three designated areas of the presynaptic membrane. We counted 1,700 particles on the areas of presynaptic membrane containing remnants of five active zones from three muscles. Total presynaptic area analyzed: 0.34 μm^2.

preparations soaked in Ringer's or in Ca^{2+}-free Ringer's, the densities of these particles ranged from 100 to 200 particles/μm^2 at the active zone and from 7 to 20 particles/μm^2 in the region most distant from the active zone.

The major change in the distributions of particles produced by indirect stimulation was a reduction in the density of small particles. This reduction was significant ($P < 0.001$) only in the intermediate region of the terminal (75–225 nm from the centers of the active zones), and in this region the average reduction was 28%. The density of the large particles was not changed by indirect stimulation at any of the regions of the presynaptic membrane.

The major change observed on BWSV-treated terminals also was a reduction in the density of small particles. The reduction was significant ($P < 0.01$) at all distances from the active zone, and the average reduction was ~30% (from 28 to 32%). Significant changes in the densities of large particles did not occur.

The number of extralarge particles was not great enough to allow us to draw conclusions about changes in density resulting from indirect stimulation or from the application of BWSV.

Synaptic Vesicles

Fig. 4A shows a micrograph of a replica of a cross-fractured nerve terminal. Small numbers of particles are found on the P faces of the membranes of synaptic vesicles. The diameters of the particles on the vesicle membrane fall in the same range as the diameters of the particles on the presynaptic membrane (Figs. 2 and 3 B). The density of the large particles on the membrane of the synaptic vesicles ranged from 311 to 507 particles/μm^2, depending upon which estimate of the total surface area was used. These densities are comparable to the density of the large particles on the regions of the axolemma >75 nm away from the centers of the active zones. The density of small particles on the membrane of the vesicles ranged from 727 to 1,188 particles/μm^2. These densities are <27% of the density of small particles on the presynaptic membrane. The density of the extralarge particles on the membrane of the synaptic vesicles ranged from 71 to 117 particles/μm^2. These values are comparable to the densities of extralarge particles on the axolemma at the active zones.

The number of particles on the P face of the

membrane of individual vesicles ranged from 0 to 10, and the average number was 3.4. About two-thirds of these particles were small and about a third were large or extralarge (Fig. 4B). On the average, a single synaptic vesicle contained seven intramembrane particles, and two or three of these particles had diameters in the same range as those of particles that line the active zones.

DISCUSSION

The most significant feature of our results is the failure to find changes in the distribution of large intramembrane particles on the presynaptic membrane of frog neuromuscular junctions after exhaustive indirect stimulation in Ringer's solution or after 1 h of treatment with BWSV in a modified Ringer's solution containing 0.5 mM Ca^{2+} and 4 mM Mg^{2+}. Thus, contrary to suggestions made by others (8, 9, 11), we conclude that these large particles cannot be used as tracers for vesicle membrane that has become incorporated into the presynaptic membrane. We also find that the membranes of the synpatic vesicles contain large intramembrane particles that are similar in diameter and in numerical density to the large particles found on the regions of the presynaptic membrane between the active zones. Thus, fusion of the vesicles with the presynaptic membrane should not lead to changes in particle density, unless there is selective retrieval of portions of the vesicle membrane.

Venzin et al. (18) reported that the density of large intramembrane particles on nerve endings from unanesthetized rats was greater then the density on endings from anesthetized rats. This suggests that the particle density increases as a result of stimulation of these synapses. The increase in density that they observed was small, however (208 vs. 137 paticles/μm^2), and because the added particles were similar in size to those normally present in the active zone, they could not be used as tracers for vesicle membrane incorporated into the presynaptic membrane.

The distribution of particle diameters we observed on nerve endings at frog neuromuscular junctions is similar to the distribution observed on nerve endings in the spinal cord of rats (18). Both types of ending contain two major classes of intramembrane particles; small particles with a mean diameter of ~6 nm and large particles with a mean diameter of ~11 nm. The large particles occur all over the presynaptic membrane but are more
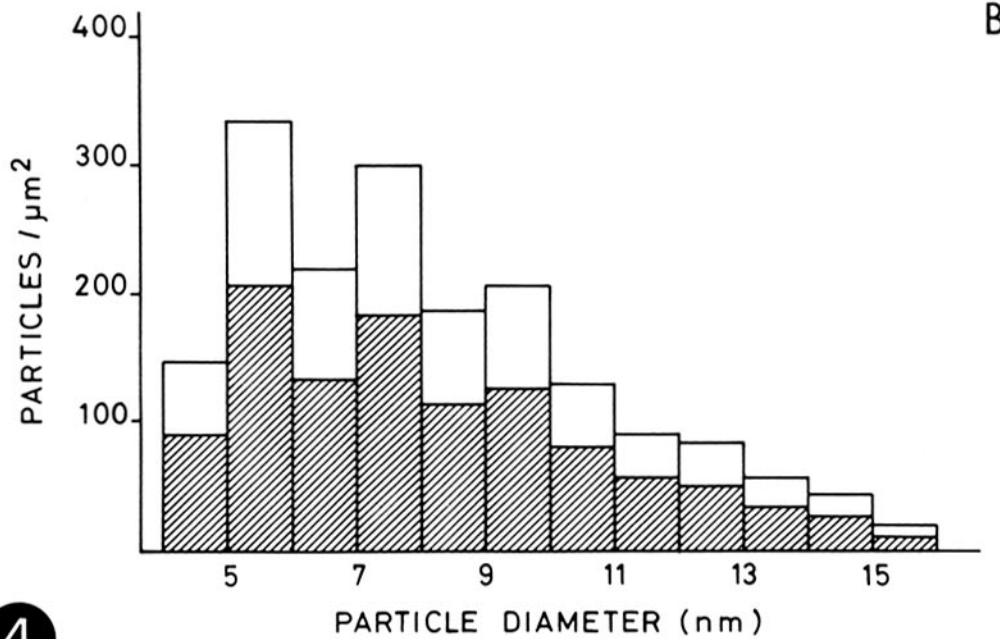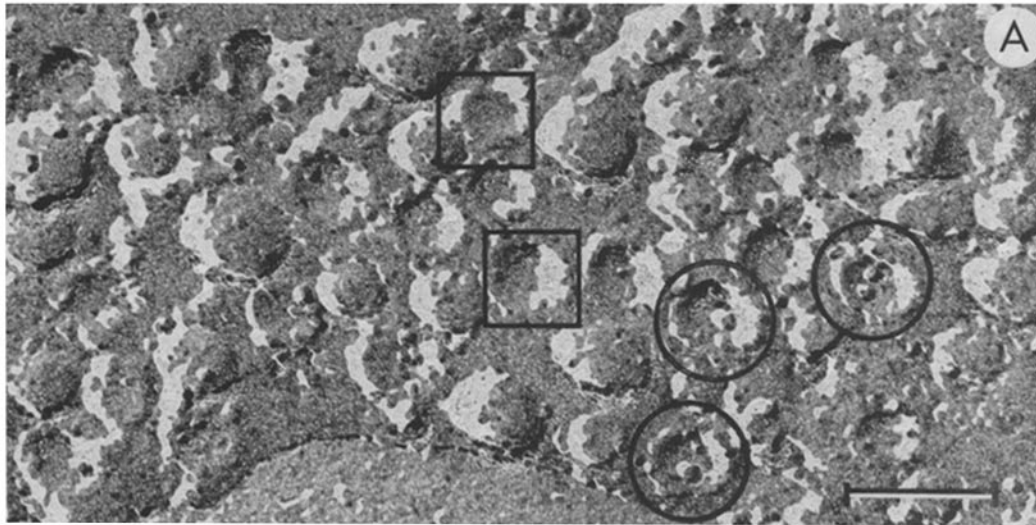
FIGURE 4   (A) Micrograph of a replica of a cross-fractured nerve terminal from a cutaneous pectoris muscle at rest. Small numbers of large intramembrane particles can be seen on the P faces of the membranes of some of the synaptic vesicles (circles); other vesicles show only small particles, and a few vesicles show no particles (squares). Bar, 0.1 μm. × 200,000. (B) Histogram of the distribution of the diameters of the intramembrane particles on the P face of the membrane of synaptic vesicles. The highest set of bars indicates the densities obtained when the smaller of two estimates of the total exposed area of vesicle membrane was used; the crosshatched bars indicate the densities obtained when the larger of the two estimates was used (see Materials and Methods). We counted 434 particles in 122 vesicles from 17 terminals. The average diameter of the synaptic vesicles was ~45 nm; the diameter of the largest vesicle was 80 nm.

highly concentrated at the active zone, whereas the small particles are spread uniformly over the presynaptic membranes. The absolute values of the particle densities were 4–5 × higher on the frog nerve endings than on the rat nerve endings (small particles, 4,538 vs. $1,177/\mu m^2$; large particles in regions away from the active zones, 684 vs. $137/\mu m^2$), but because the two kinds of terminals are from different species of animals and different regions of the nervous system, there is no reason to expect the densities to be similar.

Our results on the distribution of large particles on the membrane of synaptic vesicles is also in general agreement with the results of others (1, 8, 9). The general finding is that the membranes of the vesicles contain a small number of particles with diameters in the same range as those of particles found on the presynaptic membrane. Because the diameters of the vesicles are about 400 Å, the average density of the particles is of the order of several hundred per square micrometer.

However, our results disagree with those of Pumplin and Reese (17) and Heuser and Reese (11). These workers also analyzed the distribution of particles on nerve terminals in frog cutaneous pectoris and sartorius muscles. They reported that stimulation of these nerve terminals increased the density of large particles four- to fivefold, and suggested that these particles could be used as tracers of vesicle membrane incorporated into the axolemma (9, 11). The major difference between their results and ours is the density of large particles that were counted on resting preparations. Pumplin and Reese (17) counted particles "as large as, or larger than, those at the inner row at the ridges" of the active zone. These particles should be equivalent to our large and extralarge particles. They reported that on resting terminals, the density of these particles at regions away from the active zone was $43/\mu m^2$. They also reported that on stimulated terminals from botulinum toxin–treated frogs, these particles were present at a density of $43/\mu m^2$. These densities are <10% of the densities we counted on our resting control terminals. Pumplin and Reese (17) did not show a high-power micrograph of a resting control preparation, and on the micrograph of the stimulated, botulinum toxin–treated preparation we can count several hundred large particles per square micrometer. We cannot account for the great difference between our average results and theirs. We think it is unlikely that our fixative caused the difference because we can also count several hundred large particles per square micrometer in the published

micrographs of rapidly frozen terminals (12).

Indirect electrical stimulation in Ringer's, or treatment with BWSV in modified Ringer's with 0.5 mM Ca^{2+} and 4 mM Mg^{2+}, leads to a 20% reduction in the density of small particles on the presynaptic membrane. The change observed on the venom-treated terminals was not a result of the slightly modified ionic environment because the density of small particles was not changed by soaking terminals in Ca^{2+}-free solutions with 4 mM Mg^{2+} and 1 mM EGTA. These results are in agreement with those of Venzin et al. (18), who reported that the density of small particles was ~30% less on terminals from the spinal cords of unanesthetized rats than on terminals from the spinal cords of anesthetized rats.

The density of small particles on the membrane of synaptic vesicles is only ~30% of the density of small particles on the presynaptic membrane. This result, together with the finding that the density of small particles on the presynaptic membrane is reduced in stimulated preparations, is consistent with the idea that vesicle membrane becomes incorporated into the axolemma when quanta of transmitter are secreted. However, it is possible that the true density of small particles on the vesicle membrane has been underestimated because the sharp curvature of the P face of vesicles scooped from the axoplasm prevents uniform shadowing. Hence, another possible interpretation of these results is that the change in density of small particles is unrelated to the fusion of vesicles with the presynaptic membrane, but is caused by the active displacement or alteration of the molecular components of the presynaptic membrane during synaptic stimulation. However, we feel that the results with BWSV, in which vesicles are almost totally gone from the axoplasm and are presumably incorporated into the axolemma, support the interpretation that the density of small particles is less on the vesicle membrane than on the presynaptic membrane.

In conclusion, our results support the idea that the membrane of synaptic vesicles is incorporated into the axolemma during stimulation, thereby reducing the concentration of small particles in the presynaptic membrane. The density of large particles in the presynaptic membrane does not change, however, and no new class of particles appears that can be used to trace the movements of vesicle membrane added into the axolemma.

of N. Iezzi for helping with the experiments.

This work was supported by grants from the Muscular Dystrophy Association of America (B. Ceccarelli) and by National Science Foundation grant BNS 7715808 T (W. P. Hurlbut). F. Grohovaz is a recipient of a fellowship from Fidia Research Laboratories, Padua, Italy.

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