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

I. Effects of Black Widow Spider Venom and Ca^{2+}-Free Solutions on the Structure of the Active Zone

B. CECCARELLI, F. GROHOVAZ, and W. P. HURLBUT, with the technical assistance of N. IEZZI

From the Department of Pharmacology, National Research Council (CNR) Center of Cytopharmacology, University of Milan, 20129 Milano, Italy, and The Rockefeller University, New York 10021

ABSTRACT

Black widow spider venom (BWSV) was applied to frog nerve-muscle preparations bathed in Ca^{2+}-containing, or Ca^{2+}-free, solutions and the neuromuscular junctions were studied by the freeze-fracture technique. When BWSV was applied for short periods (10–15 min) in the presence of Ca^{2+}, numerous dimples (P face) or protuberances (E face) appeared on the presynaptic membrane and ~86% were located immediately adjacent to the double rows of large intramembrane particles that line the active zones. When BWSV was applied for 1 h in the presence of Ca^{2+}, the nerve terminals were depleted of vesicles, few dimples or protuberances were seen, and the active zones were almost completely disorganized. The P face of the presynaptic membrane still contained large intramembrane particles.

When muscles were soaked for 2–3 h in Ca^{2+}-free solutions, the active zones became disorganized, and isolated remnants of the double rows of particles were found scattered over the P face of the presynaptic membrane. When BWSV was applied to these preparations, dimples or protuberances occurred almost exclusively alongside disorganized active zones or alongside dispersed fragments of the active zones.

The loss of synaptic vesicles from terminals treated with BWSV probably occurs because BWSV interferes with the endocytosis of vesicle membrane. Therefore, we assume that the dimples or protuberances seen on these terminals identify the sites of exocytosis, and we conclude that exocytosis can occur mostly in the immediate vicinity of the large intramembrane particles. Extracellular Ca^{2+} seems to be required to maintain the grouping of the large particles into double rows at the active zones, but is not required for these particles to specify the sites of exocytosis.

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Freeze-fracture studies of several synapses and some protozoa have shown that the secretory portions of their plasma membranes contain large intramembrane particles that are often arranged in characteristic patterns (1, 2, 14, 15, 23, 33, 34, 36, 39, 41–43, 47, 49). When secretion is stimulated, the portions of the plasmalemma delineated by the arrays of particles become marked by dimples (P face) or protuberances (E face) which have been shown in several systems to be connections between secretory vesicles and the plasmalemma. Secretion from many cells is dependent upon extracellular Ca^{2+}. Hence, some workers have suggested that the fusions between secretory vesicles and the plasmalemma occur near the large intramembrane particles because these particles are Ca^{2+} ionophores and regulate membrane fusion by controlling the local intracellular concentration of Ca^{2+} (21, 41, 42, 49).

At the frog neuromuscular junction, large intramembrane particles are arranged in transverse double rows at the regions of the presynaptic membrane opposite the troughs of the postjunctional folds (1, 14, 23, 33). These double rows of particles are intramembrane components of the active zones seen in thin sections (3, 11, 12). Several workers have suggested that these zones determine where vesicles fuse with the axolemma to release transmitter by exocytosis. In addition to controlling the fusion of vesicles with the axolemma at the active zones, nerve terminals also retrieve membrane by endocytosis from the axolemma (5–8, 17, 22, 24–26, 29, 38, 44, 46, 48, 50, 51). There is disagreement about whether endocytosis at the frog neuromuscular junction also occurs near the active zones (7, 25) or whether endocytosis occurs mainly in regions of the axolemma well removed from the active zones and requires the participation of clathrin coats (21, 22, 32).

The purpose of the studies described here and in the following paper (4) is: (*a*) to test whether the correlation between the location of the large intramembrane particles and the location of the sites of exocytosis were dependent upon extracellular Ca^{2+}, and (*b*) to determine whether the sites of exocytosis were separated spatially from the sites of endocytosis. Since black widow spider venom (BWSV) can stimulate the release of quanta of acetylcholine in the absence of extracellular Ca^{2+} (28), we examined the first point by studying the distributions of dimples or protuberances obtained when BWSV was applied to frog muscles bathed in solutions that contained Ca^{2+} at concentrations of 0, 0.5, or 1.8 mM. These results are presented in the present paper.

Since BWSV causes the depletion of synaptic vesicles (10, 18, 19), it presumably interferes with endocytosis and we assume that the distribution of fusion sites seen in the presence of BWSV reflects mainly the distribution of the sites of exocytosis. The results of experiments dealing with the separation of the sites of endocytosis and exocytosis are presented in the following paper.

Some of this work has been published in abstract form (9).

MATERIALS AND METHODS

Cutaneous pectoris nerve muscle preparations from frogs, *Rana pipiens*, were used. Pairs of muscles, each with a short stump of nerve, were dissected from a frog and pinned out in separate lucite chambers that contained 2–3 ml of bathing solution at room temperature. The Ringer's solution contained (in mM): Na^+, 116; K^+, 2.1; Ca^{2+}, 1.8; Cl^-, 116; HPO_4^{2-}, 2; $H_2PO_4^-$, 1; pH 7.0. When the ionic composition of the Ringer's solution was modified, the concentration of NaCl was adjusted to maintain the tonicity constant. Ethyleneglycol-bis(β-aminoethyl ether)*N*,*N*,*N'*,*N'*-tetraacetic ecid (EGTA) was dissolved in excess NaOH, titrated with HCl to pH 7.2, and diluted to make a concentrated stock solution isotonic with Ringer's. The appropriate volume of this stock was added to the experimental solutions to give the desired concentrations of EGTA.

Preparation of BWSV

BWSV was prepared fresh each week by homogenizing eight glands from four black widow spiders (*Latrodectus tredecimguttatus*) in 1.0 ml of 120 mM NaCl and was stored at ~3°C. The BWSV was diluted before use by adding 100–200 μl of the homogenate to 5–6 ml of the bathing solution. This dilution corresponds roughly to the dose we have routinely used in the past (50 μl of BWSV added directly to the 2–3 ml of bathing fluid in the recording chamber) and it contains ~7–16 μg of the total glandular protein/ml and ~0.7–1.6 μg of the active neurotoxin/ml (18). The muscle chambers were drained and 2.5–3 ml of the diluted BWSV was added. If done carefully, it was usually possible to drain and refill the chamber without dislodging the recording micropipette. Muscles were fixed at the appropriate times by draining the chambers and adding fixative solutions.

Preparation for Freeze-Fracture

The fixative contained 0.5–0.75% formaldehyde (freshly prepared from paraformaldehyde) and 1% glutaraldehyde in 0.1 M phosphate buffer, pH 7.2. After 1

h of fixation, small pieces of tissue containing suspected end-plate regions were cut from the muscle and transferred through a graded series of solutions of glycerol in 0.1 M phosphate buffer at room temperature. The schedule was: 10 min in 10%, 20 min in 20%, and 30 min in 30% glycerol.

The pieces of fixed, glycerinated tissue were trimmed to fit raised specimen holders (No. 12003, Balzers S. p. A. Milan, Italy) and one to three pieces of the thin muscle were stacked on a holder. The specimens were frozen in liquid Freon 22, stored in liquid nitrogen, and fractured in a Balzers BA 360 M or BAF 301 apparatus. The tissues were fractured at −100°C in the 360 M apparatus and at −110°C in the F301 and replicated using electron beam guns. The platinum-carbon replicas were cleaned in sodium hypochlorite (concentrated laundry bleach) for 2 h, taken up on uncoated grids, and examined in Philips EM200, EM300 or EM400 electron microscopes.

In describing our micrographs of the replicas, we have used the current nomenclature, i.e. the protoplasmic fracture face is called the P face (formerly the A face) and the extracellular fracture face is called the E face (formerly the B face). The micrographs have been oriented with the source of platinum shadowing below.

Fixation for Thin Sectioning

Some of the preparations treated with venom were fixed with aldehydes according to the procedure used for freeze-fracture. Others were fixed for 1 h with cold 2% OsO_4 in 0.1 M phosphate buffer, pH 7.2. Small pieces of the fixed muscles containing suspected end-plate regions were cut out for embedding (25).

Preparation for Thin Sectioning

The small pieces of fixed muscle were dehydrated and flat-embedded in Epon 812. The blocks were oriented and trimmed under a light microscope so that longitudinal or transverse sections could be cut with a diamond knife (6, 8, 18). Sections were double-stained with uranyl acetate and lead citrate.

Construction of Histogram of the Distribution of Dimples or Protuberances around Active Zones

Prints (× 45,000) of the P or E faces of BWSV-treated terminals were prepared and covered by 4 × 4 mm mesh grid. On each print, we counted the number of dimples or protuberances occurring in successive 4-mm (90-nm) intervals from the centers of the active zones. The total area of exposed axolemma in each interval was also determined. The counting was carried out on each side of each active zone and extended to the point midway between successive zones, or to a point 540 nm from the center of a zone. The counts and areas of symmetrically located intervals were combined. Thus, the histogram shows single-sided distributions (assumed to be symmetrical for a large population of active zones) extending from the center of a zone to a point halfway to the next active zone.

To compute the standard errors of the histogram, we combined the data for any one terminal to give an average for that terminal. We then combined these average results for individual terminals, each terminal being given equal weight, to compute a grand mean and its standard error. The results are plotted in the histogram (Fig. 6).

Dimples or protuberances were defined as smoothly outlined, approximately circular deformations of the presynaptic membrane with diameters ranging from 6 to 60 nm (23).

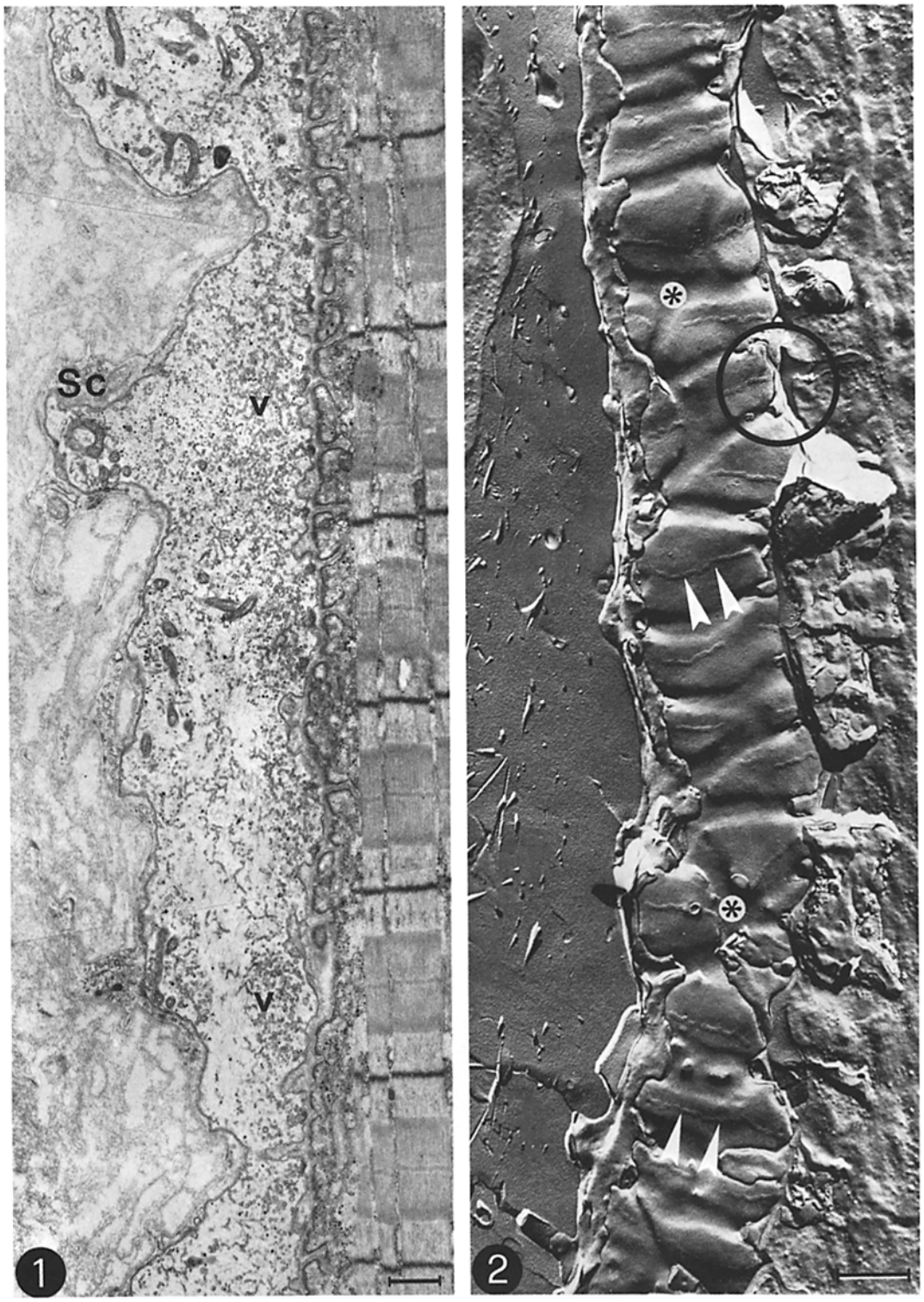
Electrophysiology

Intracellular records of end-plate potentials (epp's) and miniature end-plate potentials (mepp's) were made by standard electrophysiological techniques (6, 8). Usually, electrical activity was monitored in at least one fiber of each muscle used, but on some occasions, when both muscles from the same frog were treated identically, electrical activity was monitored in only one chamber of a pair. In some experiments we recorded from a single end plate; in others we briefly sampled the activity of many different end plates.

RESULTS

Control Neuromuscular Junctions

Fig. 1 is a micrograph of a longitudinal thin section of a portion of a neuromuscular junction from a control preparation, and Fig. 2 is a micrograph of a replica of the P face of a portion of a prejunctional membrane from a different control preparation. The primary features of the P face of the prejunctional membrane are the regularly spaced 90-nm wide ridges that usually run perpendicular to the longitudinal axis of the nerve terminal and are bordered on each side by a double row of large intramembrane particles (Figs. 11 and 12). These ridges and their bordering particles represent intramembrane components of the active zones, structures first described in thin sections as discrete portions of the axolemma lying opposite the troughs of the postjunctional folds and characterized by the presence of a feltwork of fibrillar material associated with the inner aspect of the presynaptic membrane (3, 11, 12). Figs. 1 and 2 reveal the close correspondence between the ridges seen in freeze-fracture and the active zones seen in thin sections. In agreement with the results of other workers, we rarely found dimples or protuberances in our resting control preparations (23).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Effect of BWSV Applied in Solutions with Ca^{2+}

Our first experiments were done with solutions that contained 0.5–0.7 mM Ca^{2+} and 4 mM Mg^{2+} because most of our previous work with BWSV had been done with these solutions. When BWSV (100 μl/6 ml) was applied to the muscles, the mepp frequencies recorded from the surface fibers began to increase after 1–3 min, reached maximum rates within 6 min, and then began to subside (Fig. 3). When preparations were fixed 3–8 min after BWSV had been applied, few of the nerve terminals showed large numbers of dimples or protuberances; when muscles were fixed 8–15 min after BWSV had been applied, a high proportion of the terminals showed large numbers of dimples or protuberances. The lag between the time at which the mepp frequencies reached peak levels in the surface fibers and the time at which dimples or protuberances were found to be abundant in freeze-fractured terminals is probably the time required for BWSV to penetrate into the interior of the muscle where most of the fractured terminals lay. Dimples or protuberances were not observed at every active zone. On a single nerve terminal some of the zones could show numerous dimples while others showed none. These observations of highly localized action of BWSV correlate well with the electrophysiological observations of Del Castillo and Pumplin (13) who showed that brown widow spider venom often caused bursts of mepp's that originated from discrete portions of the nerve terminals.

Fig. 4 is a replica of the P face of a prejunctional membrane from a preparation that had been fixed 14 min after BWSV had been applied. Numerous dimples have appeared immediately adjacent to the two double rows of particles that line the ridges, but the junction appears normal in all other respects. In all of the replicas we examined from preparations fixed under these conditions, the dimples occurred almost exclusively on the borders of the ridges near the double rows of particles; few were found in the areas of membrane between the ridges (Fig. 6). The portions of the P face between the ridges appeared undistorted, as in the controls, and showed no evidence of infolding or lifting.

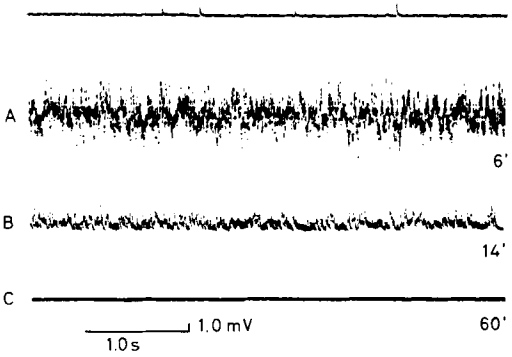
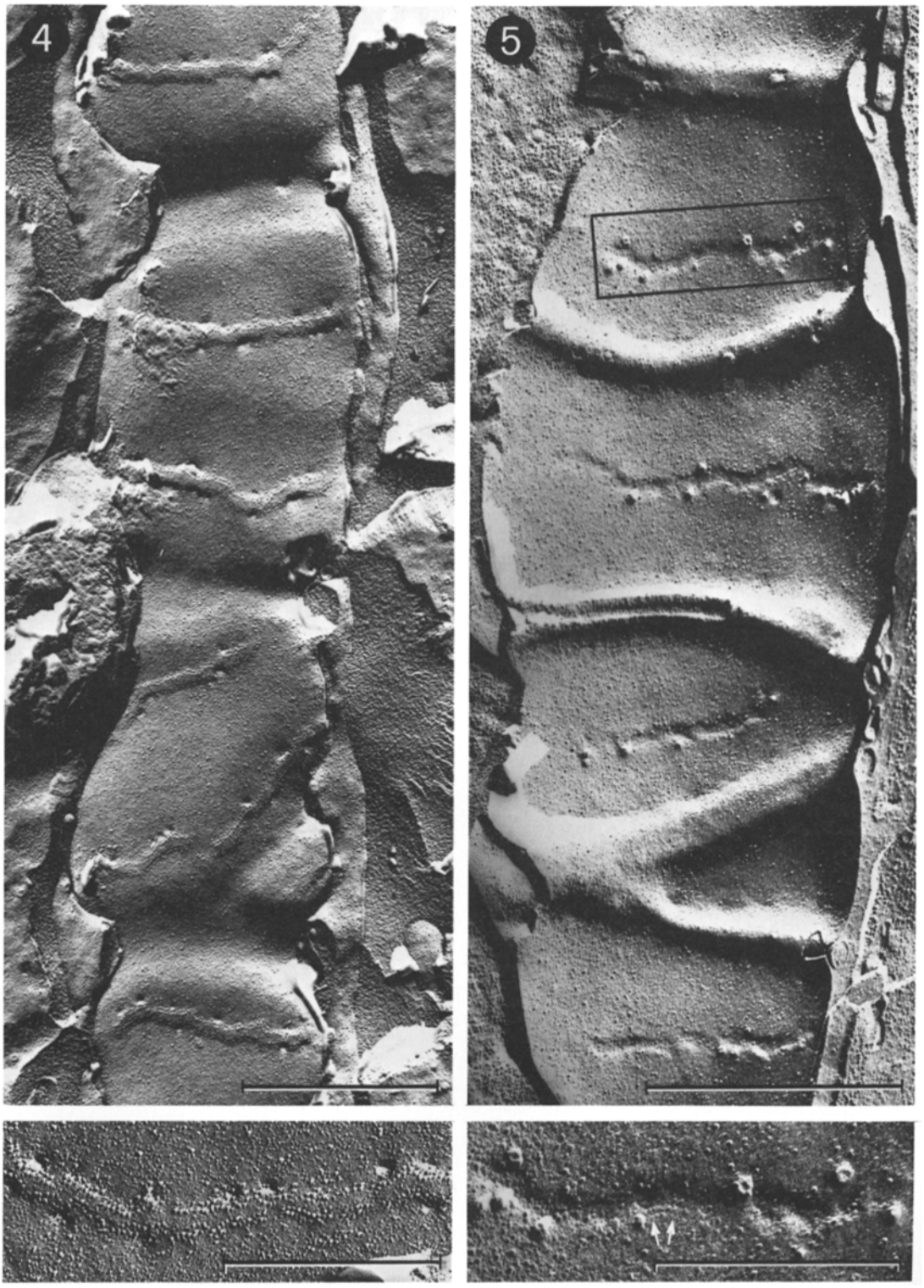


FIGURE 3 Mepp's recorded before (top row), and 6 (*A*), 14 (*B*), or 60 (*C*) min after the application of BWSV (100 μl/5 ml) in modified Ringer's with 0.5 mM Ca^{2+} and 4.0 mM Mg^{2+}. The figure illustrates the time-course of BWSV-induced release of quanta of neurotransmitter. Each record was made from a different preparation, and the membrane potentials of the four muscle fibers ranged from 75 to 95 mV.

Fig. 5 shows the E face of a terminal that had been fixed 12 min after BWSV had been applied. In this case, the prejunctional membrane contains numerous small protuberances that occur immediately adjacent to 90-nm wide furrows, the structures that are complementary to the ridges on the P face. Occasionally, the furrows were seen to be bordered by short parallel rows of small particles,

FIGURE 1 Low power micrograph of a longitudinal thin section of a control neuromuscular junction that had been soaked in Ringer's solution for 1 h. The terminal is covered by a thin projection of a Schwann cell (*Sc*) and contains mitochondria, neurofilaments, elements of smooth endoplasmic reticulum, glycogen particles, and numerous synaptic vesicles (*v*). Groups of vesicles are clustered around the active zones which are recognized as local thickenings of the axolemma at regions opposite the troughs of the postjunctional folds. Bar, 1 μm. × 9,500.

FIGURE 2 Low power micrograph of a replica of the P face of a control nerve terminal. The figure shows the regularly spaced ridges (arrowheads), ~90 nm wide, that run perpendicular to the longitudinal axis of the terminals and are located in exact register with the troughs of the postsynaptic folds (circle). The broad depressions (asterisks) between the ridges are impressions left by fingers of the Schwann cell that embrace the terminal at these regions and were broken away during fracture. It is nlear that the location of the ridges on the P face corresponds to the location of the active zones in thin section. Bar, 1 μm. × 12,000.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and the protuberances were seen to contain central craters (*inset*, Fig. 5). The prejunctional membrane between the furrows was undisturbed and showed no protuberances or infoldings.

Similar results were obtained when BWSV was applied in normal Ringer's solution (1.8 mM Ca^{2+}). When preparations were fixed after 12–15 min, many dimples (or protuberances) were seen adjacent to the ridges (or furrows) on the P (or E) face of the prejunctional membrane. The areas of membrane between the ridges (or furrows) showed neither infoldings, dimples (or protuberances), nor other gross alterations of structure.

Fig. 6 shows a histogram of the distribution of all the dimples or protuberances that we found on the junctions that had been treated with BWSV in the presence of Ca^{2+}. About 86% of the dimples or protuberances occurred within 90 nm of the centers of the active zones, i.e., immediately adjacent to the double rows of large intramembrane particles. The average number of dimples or protuberances found beside both edges of an active zone was 3.6/μm. Although our figure for the average number of dimples or protuberances per micron of active zone is somewhat less than the figure reported by Pumplin and Reese for brown widow spider venom (37), our results are in general agreement with theirs.

Our results show that large numbers of dimples or protuberances are found near the active zones when the rate of secretion of quanta of transmitter is high, and this correlation between the occurrence of dimples or protuberances and the release of quanta of transmitter indicates that the two phenomena are causally related. To test further the correlation between the occurrence of dimples or protuberances and the release of quanta of transmitter, we examined terminals that had been treated with venom for 1 h and fixed after the mepp frequencies had fallen to low levels.

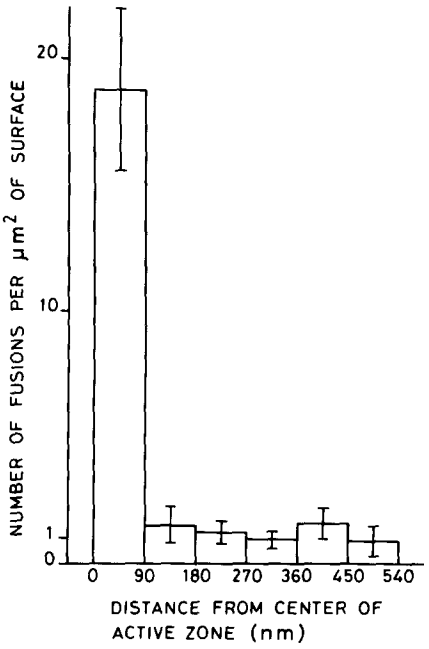


FIGURE 6 Histogram of the location of dimples or protuberances seen on replicas of terminals treated with BWSV for 12–15 min in solutions with Ca^{2+}. The histogram extends in one direction from the center of an active zone to a point halfway to the next active zone. A total of 80 active zones comprising ~64 μm^2 of presynaptic membrane and containing ~400 dimples or protuberances were analyzed.

Fig. 7 shows a thin section of a terminal from a preparation that had been treated with BWSV (200 μl/6 ml) for 1 h. The terminal is completely depleted of vesicles and appears to be swollen, in agreement with previous observations (10, 18, 19). Figs. 8–10 and 13 show the P and E faces of terminals that had been treated with BWSV for 1 h. The surfaces appear undisturbed and show no dimples or protuberances, as one would expect of

FIGURE 4 P face of a neuromuscular junction from a preparation that had been fixed 15 min after the application of BWSV. The membrane of the nerve terminal is marked by a large number of dimples that occur almost exclusively alongside the active zones, but the junction appears normal in all other respects. *Inset*: High magnification of the P face of an active zone from another preparation treated with BWSV. Note the close association between the dimples and the double rows of particles that line the edges of the active zone. Bar, 1 μm. × 32,000. *Inset*: bar, 0.5 μm. × 70,000.

FIGURE 5 E face of a neuromuscular junction that had been treated for 12 min with BWSV. The surface of the nerve terminal is marked by a large number of protuberances located almost exclusively at the edges of furrows which are structures complementary to the ridges on the P face. The junction appears normal in all other respects. *Inset*: High magnification of the portion of the junction outlined by the rectangle. Some of the protuberances contain well-defined craters. Short, parallel rows of small particles appear at the arrows. Bar, 1 μm. × 42,000. *Inset*: bar, 0.5 μm. × 79,000.

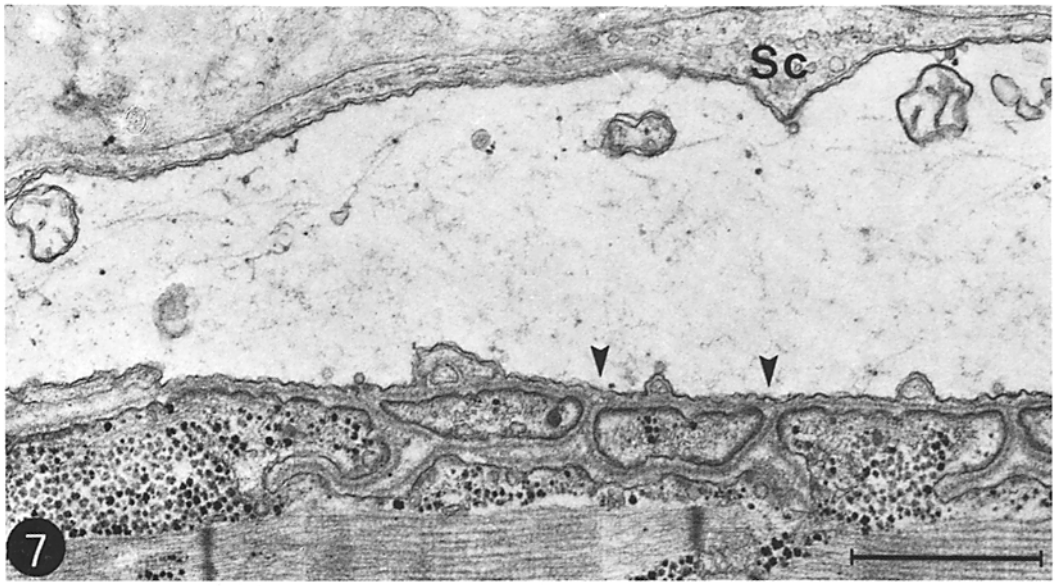


FIGURE 7 Thin section of a neuromuscular junction from a preparation treated for 1 h with BWSV. The terminal appears to be swollen, devoid of synaptic vesicles, and a few neurofilaments and mitochondria are present. Note that the feltwork of fibrillar material that normally covers the inner aspect of the presynaptic membrane at the active zones is largely absent (arrowheads). The muscle fiber and Schwann cell process (*Sc*) appear normal. Bar, 1 μm. × 26,000.

FIGURE 8 P face of neuromuscular junction from a preparation treated for 1 h with BWSV. The active zones are highly disorganized, but traces of one zone remain (arrowheads) still roughly aligned with a trough of a postjunctional fold (asterisk). No dimples are emident on the presynaptic membrane. This micrograph also shows a cross-fracture of the axoplasm (*A*) which is devoid of synaptic vesicles. A compressed portion of a Schwann cell (*Sc*) can also be seen. Bar, 1 μm. × 43,000.

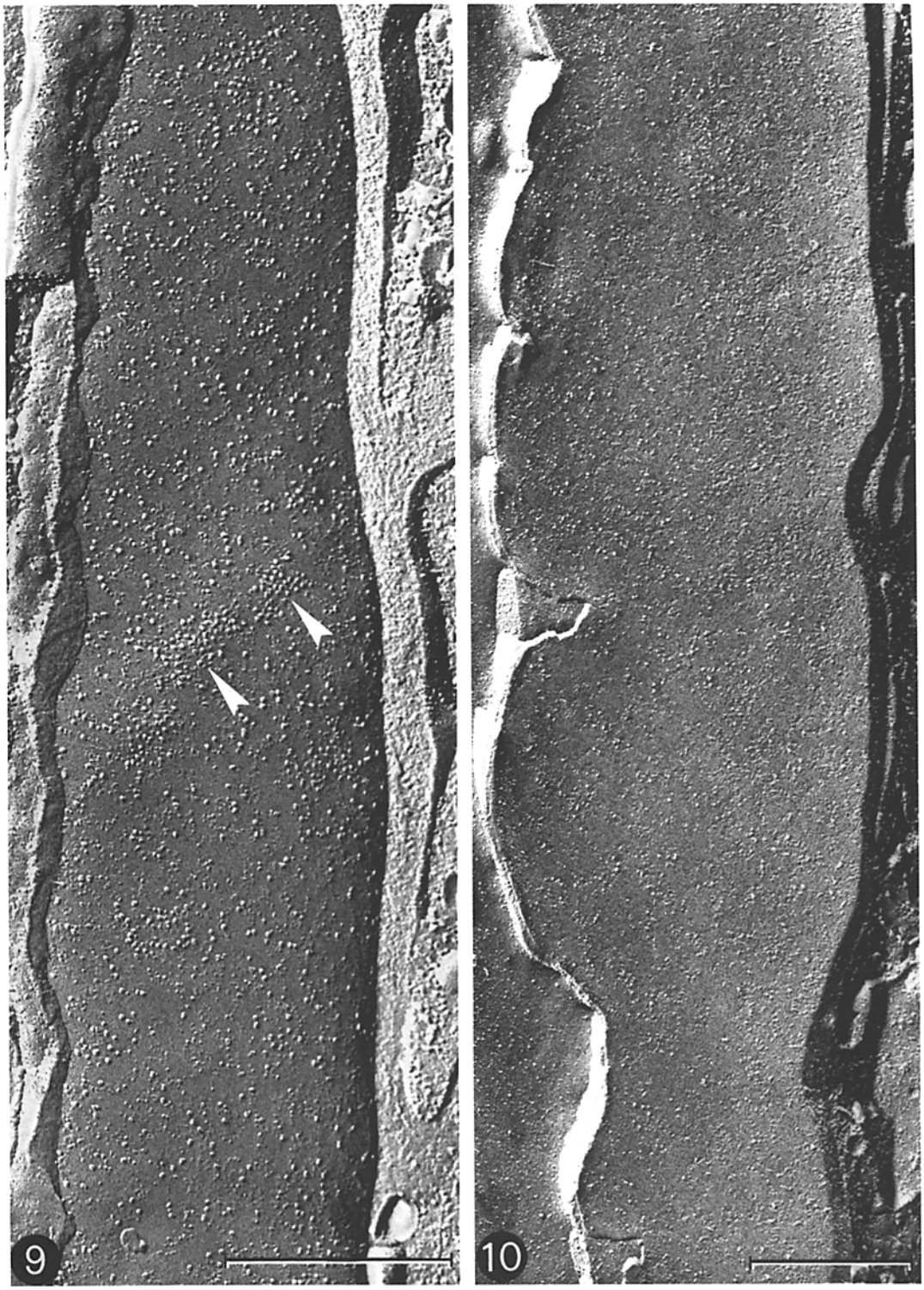
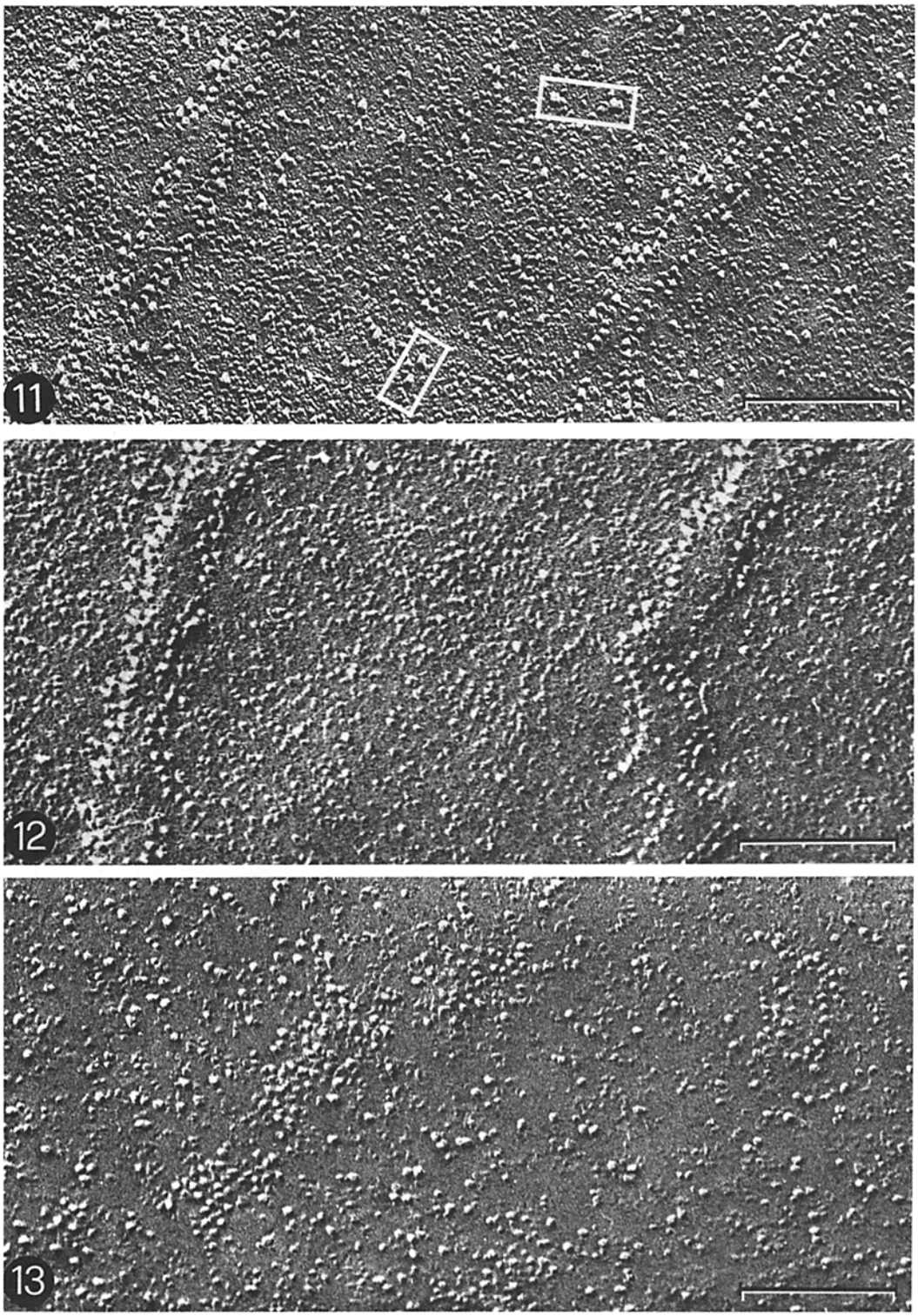


FIGURE 9 P face of a nerve terminal from a different preparation treated for 1 h with BWSV. The active zone which would be expected to appear just above the scale-marker cannot be recognized. Particles with diameters in the range of those that normally line the active zone are scattered over the presynaptic membrane. In some places the particles occur in clusters or patches (arrowheads) that may be remnants of previous active zones. Bar, 0.5 μm. × 71,000.

FIGURE 10 E face of a nerve terminal from a preparation treated for 1 h with BWSV. The furrows of the active zones and the elevations caused by the Schwann cell fingers are absent. No protuberances are seen on the presynaptic membrane. Bar, 0.5 μm. × 50,000.



FIGURES 11–13 High magnifications of the P faces of two control (Figs. 11 and 12) and one BWSV-treated (Fig. 13) terminal to illustrate the number, size, and distribution of large membrane particles seen in these preparations. Note that many large particles occur between the active zones in the two control preparations (rectangles). Fig. 13 is a portion of the terminal shown in Fig. 9. Bars, 0.2 μm. × 120,000.

terminals that had been depleted of vesicles (Fig. 8). Most of these terminals were also completely devoid of ridges, furrows, or other structures characteristic of the active zone. Some, however, showed groups of large intramembrane particles that appeared to be residues of the active zones still aligned opposite the troughs of the postjunctional folds (Fig. 8). On a few replicas, rare dimples were observed. The fingers of Schwann cell cytoplasm that interdigitate between the nerve terminal and the muscle fiber frequently seemed to be compressed into thin layers (Fig. 8). This may have been a consequence of the swelling of the terminal that occurs in preparations treated with BWSV. Thin sections (Fig. 7) also showed that prolonged exposure to BWSV altered the structure of the active zone so that the characteristic feltwork on the inner aspect of the presynaptic membrane was no longer evident. The loss of this feltwork may be related to the dispersion of the double rows of the large intramembrane particles.

Effects of Venom Applied in Ca^{2+}-Free Solutions

When muscles were soaked for 2–3 h in Ca^{2+}-free solutions with 1 mM EGTA and 4–5 mM Mg^{2+}, several profound changes in the structure of the active zones were observed, some of which are illustrated in Fig. 14. In some junctions the double rows of particles became interrupted or disoriented but retained the general outlines of an active zone; in others, the rows seemed to form divergent columns leading away from the active zones. In the most extreme cases, all association between the particles and the ridges seemed to be lost, and small clusters of particles, arranged as remnants of double rows, were scattered about the prejunctional membrane. Some signs of disorganization were seen at nearly every terminal, but within a given terminal the degree of disorganization was different at different active zones, and a few zones appeared normal.

The effects of the Ca^{2+}-free solution on the organization of the active zones were not readily reversible. Essentially the same states of disorganization were seen in terminals that had been soaked for 1 h in Ringer's solution after they had been previously soaked for 3 h in the Ca^{2+}-free solution. The disorganization of the active zones did not prevent neuromuscular transmission since the amplitude of the compound action potential of the muscle in response to stimulation of the nerve was almost fully restored during 15 min of washing in Ringer's.

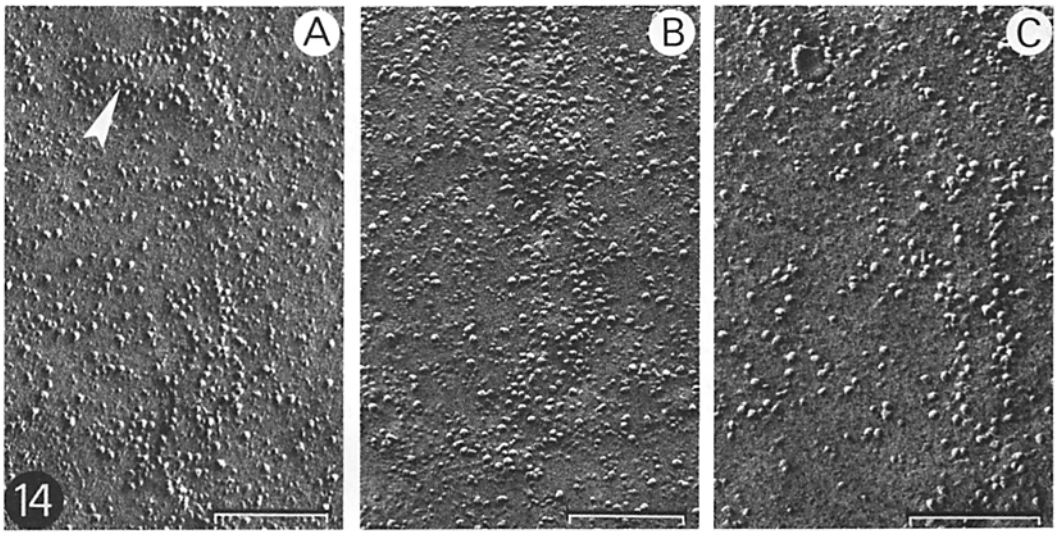


FIGURE 14 High magnifications of disorganized active zones from three preparations that had been soaked for 2 h in Ca^{2+}-free solution. In Fig. 14*A*, the diffuse outlines of an active zone can be recognized in the lower portion of the figure. The zone is interrupted in the middle, and then there appears a short segment whose axis of symmetry has been rotated ~90° (arrowhead). Fig. 14*B* shows an active zone whose particles seem to have randomly dispersed into the adjacent regions of the presynaptic membrane. Fig. 14*C* shows an active zone whose bordering particles have been reduced from double to single rows. (*A*) Bar, 0.2 μm. × 79,000. (*B*) Bar, 0.2 μm. × 83,000. (*C*) Bar, 0.2 μm. × 90,000.

When BWSV was applied to preparations that had been treated for 2 h in Ca^{2+}-free solution, the quantal secretion of transmitter still occurred and dimples or protuberances still appeared on the presynaptic membrane (Fig. 15). Many dimples occurred near double rows of particles that were still loosely associated into recognizable active zones, but equal numbers occurred near short segments of the double rows of particles that were isolated from other recognizable elements of the active zones. We counted 179 dimples on 62 μm^2 of presynaptic membrane from preparations fixed 10–15 min after BWSV had been applied. The average number of dimples per unit area, about 3/μm^2, was half that obtained when BWSV was applied in the presence of Ca^{2+}. This difference may not be significant. About 40% of the dimples were located alongside fairly well-defined active zones (Fig. 15*D* and *E*), ~46% were scattered about the presynaptic surface but located within 45 nm of arrays of particles that clearly were displaced remnants of active zones (Fig. 15*A*, *B*, and *C*), and ~14% were either not associated with the particles or were located where the association was ambiguous. This correlation between the locations of the dimples and the locations of arrays of large intramembrane particles detached from the active zones strongly suggests that it is the particles themselves, or some structure tightly linked to them, that determine the sites of exocytosis. The arrangement of the particles into the highly organized patterns characteristic of the active zones is not necessary for them to specify the sites of exocytosis. Since the dimples occur near the particles even in Ca^{2+}-free solution, it appears that an influx of extracellular Ca^{2+} also is not required for the particles to specify the sites of exocytosis.

Another characteristic feature of nerve terminals treated with BWSV after prolonged soaking in Ca^{2+}-free solutions was the appearance of numerous particle-free bulges on the P face of the presynaptic membrane (Fig. 15). These bulges were never seen when BWSV was applied in the presence of Ca^{2+}, and they were never seen when Ca^{2+}-free solution without BWSV was applied. The bulges usually occurred interspersed among groups of dimples near the remnants of the active zones. Their diameters were ~100 nm. It is difficult to be sure of the significance of these bulges, but they may be caused by vesicles in the underlying axoplasm pressing against the protoplasmic face of the axolemma.

Particle-free regions in the plasmalemma of secretory cells have been observed before (27, 30, 35). In these cases, the particle-free domains seem to represent prefusion states of association between vesicles and the plasmalemma, and fusion of the two membranes usually occurs within the particle-free zones. This seems not to be the case at the neuromuscular junction, however, since dimples were always observed near the edges of, and never within, the particle-free bulges (Fig. 15). Since the release of neurotransmitter provoked by BWSV in Ca^{2+}-free solutions is dependent on extracellular Mg^{2+} (footnote 1 and reference 31), the bulges that we see under these conditions may be related to the bulges seen by Heuser (21) in frog muscles soaked at 4°C in isotonic solutions of $MgCl_2$.

DISCUSSION

Our results clearly demonstrate that when the rate of secretion of quanta of transmitter is increased by BWSV, then the presynaptic membrane of the nerve terminal becomes marked with a large number of dimples or protuberances. Since the appearance of dimples or protuberances is generally correlated with the secretion of quanta of transmitter, we assume, as have several previous workers (1, 14, 15, 21, 23, 34, 37, 47, 49), that the dimples or protuberances are sites where the membrane of the synaptic vesicles is continuous with the presynaptic membrane through a short neck. The results confirm early EM studies of the effect of BWSV (10) and give additional support to the idea that quantal secretion occurs by the discharge of transmitter from vesicles that have fused with the axolemma.

Vigorously secreting neuromuscular junctions retrieve membrane by endocytosis from the axolemma, and this membrane may be used to reform functional synaptic vesicles (5–8, 21, 22, 50, 51). Freeze-fracture images of dimples or protuberances on the axolemma do not reveal whether membrane is being added or removed at these sites. However, since BWSV causes the depletion of synaptic vesicles, we assume that it interferes with the removal of membrane from the axolemma and that the dimples or protuberances seen in the presence of BWSV mark primarily the sites

[1] Misler, S., and W. P. Hurlbut, Action of black widow spider venom on quantized release of acetylcholine at the frog neuromuscular junction: Dependence upon external Mg^{2+}. Manuscript submitted for publication.

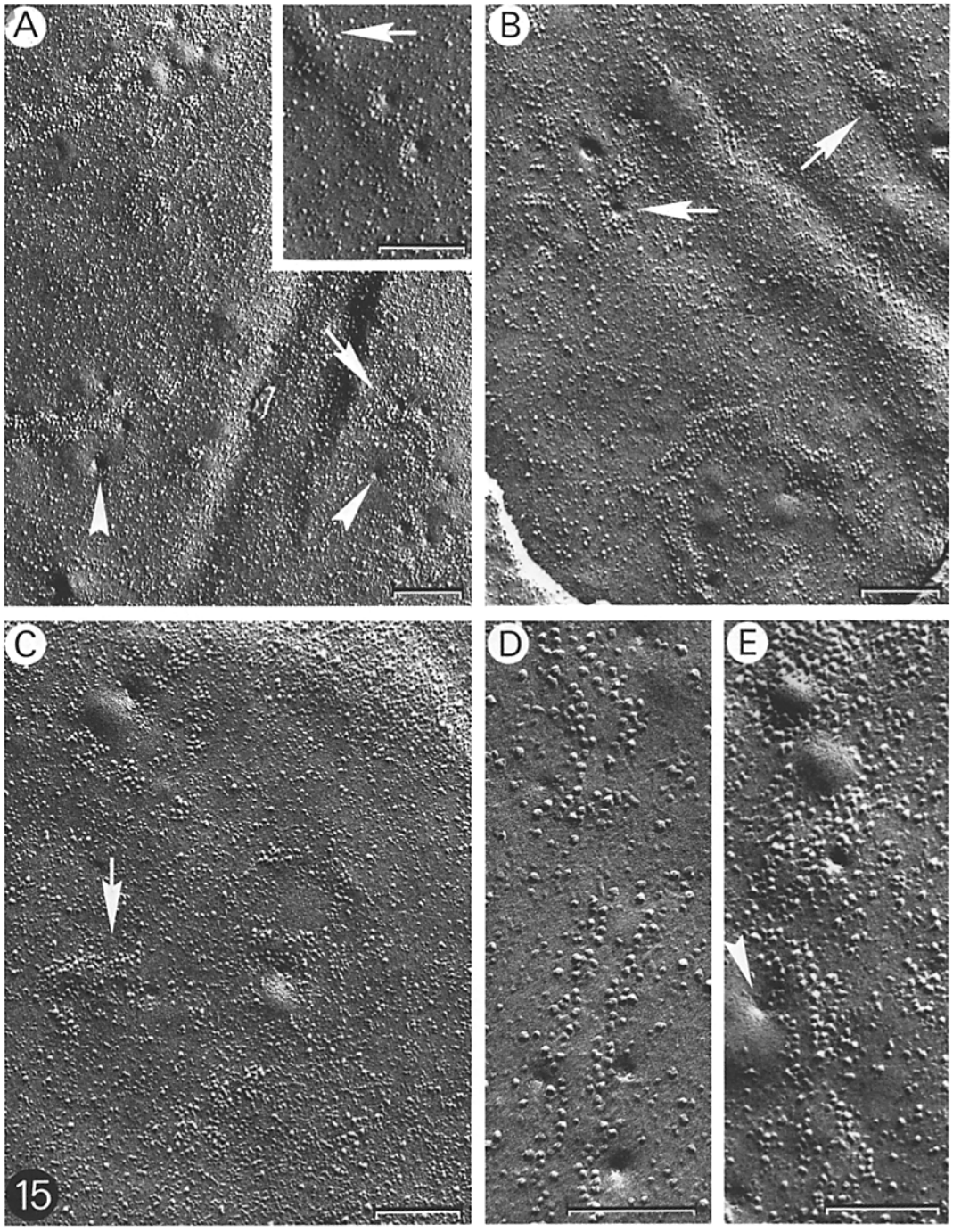


FIGURE 15 P faces of terminals from different preparations bathed for 2 h in Ca^{2+}-free solution and then treated for 15 min with BWSV. (*A–C*) Micrographs showing active zones in various stages of disorganization. Note that, with a few apparent exceptions (arrowheads), all the dimples occur closely associated with clusters of large intramembrane particles that in most cases occur as remnants of the double rows (arrows). Numerous particle-free bulges occur near the clusters of large intramembrane particles. No dimples are seen in the particle-free domains. *Inset*: High magnification of dimples surrounded by rosette-like arrays of large particles that seem to be derived from a disorganized active zone (arrow). (*D*) High magnification view of a partially disorganized active zone. Dimples appear near the large intramembrane particles. (*E*) High magnification view of a highly disorganized active zone. Three particle-free bulges occur along the zone, and one has a dimple at its edge near an array of large particles (arrowhead). (*A*) Bar, 0.2 μm. × 53,000. *Inset*: bar, 0.2 μm. × 68,000. (*B*) Bar, 0.2 μm. × 61,000. (*C*) Bar, 0.2 μm. × 66,000. (*D*) Bar, 0.2 μm. × 100,000. (*E*) Bar, 0.2 μm. × 88,000.

of membrane addition, or exocytosis. These sites seem to be strictly associated with the large intramembrane particles of the active zones, independent of whether these particles are arranged in double rows at the active zones or whether the association of the particles with the zones has been disrupted by soaking in Ca^{2+}-free solutions. It also appears that an influx of Ca^{2+} is not required for these particles to specify a site of exocytosis.

Pumplin and Reese (37) have studied the effects of brown widow spider venom applied to frog neuromuscular junctions bathed in standard, or Ca^{2+}-free, Ringer's. Their findings were similar to ours in that: (*a*) BWSV-induced dimples occurred almost exclusively at the active zones in solutions with Ca^{2+}, (*b*) the total number of dimples/μm^2 was reduced by ~50% in Ca^{2+}-free solutions, and (*c*) in Ca^{2+}-free solutions about half of the dimples occurred in regions of the axolemma between the active zones. They pointed out that if these ectopic dimples marked sites of exocytosis, then their occurrence implied that exocytosis could occur at regions of the axolemma devoid of any of the special structures associated with the active zone. They suggested that a general rise in the level of intracellular Ca^{2+} might be the cause of such ectopic exocytosis.

However, we find that soaking junctions in Ca^{2+}-free solutions leads to the disorganization and dispersal of the active zones so that clusters of large intramembrane particles migrate into regions of the axolemma between the troughs of the postjunctional folds. BWSV induces fusion in these regions, but the fusions occur primarily in the immediate vicinity of the isolated remnants of the double rows. Hence, we suggest that the large intramembrane particles are able to specify the sites of exocytosis even in Ca^{2+}-free solutions. These particles may represent only the intramembrane components of more elaborate structures which completely span the presynaptic membrane and act as complex receptors, or attachment sites, for vesicles, and thereby determine the sites of exocytosis. Extracellular Ca^{2+} may be required to establish or maintain lateral connections between these structures and other intramembrane or extramembrane components of the active zones. The packing of these particles into dense, highly ordered arrays at the active zones may improve the safety factor for neuromuscular transmission by aligning the sites of transmitter release opposite the sites of transmitter action. However, the packing is not required for exocytosis, at least for the exocytosis induced by BWSV.

Although BWSV causes extensive changes in the morphology of nerve terminals, it does not seem to be a lytic or destructive agent. It does not cause obvious changes in the morphology of the muscle fibers or the Schwann cells, and the release of quanta of transmitter that it provokes occurs principally at the active zones which appear to be the normal sites of transmitter release. The loss of the active zones that accompanies prolonged exposure to BWSV seems not to be due to enzymatic destruction of the zones since the remainder of the presynaptic membrane appears to be undamaged. Most of the effects of BWSV on the neuromuscular junction may occur as consequences of relatively specific actions directed at the active zones. It is not clear what these actions may be. One possibility is that BWSV acts as an ionophore to permit cations to enter the axolemma and trigger transmitter release (16, 20, 31, 37, 45, and footnote 1). Another possibility is that BWSV causes a redistribution of membrane components or interacts through the membrane with actin filaments in the underlying cytoplasm (20, 40). A third possibility is that BWSV interacts with the membrane at points where the large intramembrane particles are located and perhaps provokes transmitter release by bypassing the Ca^{2+}-dependent step that controls normal secretion.

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