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

II. Effects of Electrical Stimulation and High Potassium

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ABSTRACT

Frog cutaneous pectoris nerve muscle preparations were studied by the freeze-fracture technique under the following conditions: (a) during repetitive indirect stimulation for 20 min, 10/s; (b) during recovery from this stimulation; and (c) during treatment with 20 mM K^+.

Indirect stimulation causes numerous dimples or protuberances to appear on the presynaptic membrane of the nerve terminal, and most are located near the active zones. Deep infoldings of the axolemma often develop between the active zones. Neither the number nor the distribution of dimples, protuberances, or infoldings changes markedly during the first minute of recovery. The number of dimples, protuberances, and infoldings is greatly reduced after 10 min of recovery. Since endocytosis proceeds vigorously during the recovery periods, we conclude that endocytosis occurs mostly at the active zones, close to the sites of exocytosis.

20 mM K^+ also causes many dimples or protuberances to appear on the axolemma of the nerve terminal but they are distributed almost uniformly along the presynaptic membrane. Experiments with horseradish peroxidase (HRP) show that recycling of synaptic vesicles occurs in 20 mM K^+. This recycling is not accompanied by changes in the number of coated vesicles. Since both exocytosis and endocytosis occur in 20 mM K^+, it is difficult to account for this unique distribution. However, we suggest that K^+ causes dimples or protuberances to appear between the active zones because it activates latent sites of exocytosis specified by small numbers of large intramembrane particles located between active zones. The activation of latent release sites may be related to the complex effects that K^+ has on the quantal release of neurotransmitter.

KEY WORDS membrane fusion · active zones · potassium · endocytosis · vesicle retrieval

Our previous paper showed that black widow spider venom (BWSV) induces the appearance of many dimples (P face) or protuberances (E face)

on the presynaptic membrane of freeze-fractured frog motor nerve terminals (1). The dimples or protuberances, which indicate the sites where synaptic vesicles are connected to the axolemma through a short neck of membrane, were found mainly alongside the double rows of large intramembrane particles that normally line the active zones. Since BWSV causes a profound depletion of synaptic vesicles, we assumed that it inhibited endocytosis and we suggested that the dimples or protuberances seen on terminals treated with BWSV marked the sites where vesicles fused with the axolemma to release transmitter by exocytosis (1).

This paper presents the results of our studies of the distributions of dimples or protuberances seen on frog neuromuscular junction fixed under the following conditions: (a) during the final minute of a 20-min period of repetitive indirect stimulation at 10/s; (b) during recovery from this period of stimulation; and (c) during treatment with 20 mM K^+.

The rate at which membrane is added to the axolemma during prolonged stimulation at 10/s exceeds the rate at which it is removed, and this imbalance leads to a reduction in the number of synaptic vesicles and an increase in the area of the axolemma (2, 4, 9, 17, 18). After 20 min of stimulation, quantal secretion seems to be in a steady state (4) and therefore the rates of addition and removal of membrane may be roughly in balance. When stimulation is stopped, the rate of removal of membrane exceeds the rate of addition, and this imbalance leads to a recovery in the number of synaptic vesicles and a reduction in the area of the axolemma (2, 4, 9, 17, 18). We will show below that both addition and removal of membrane occur in 20 mM K^+. Therefore, if the sites at which membrane is removed were separated spatially from the sites at which membrane is added, then we would expect to obtain different spatial distributions of dimples or protuberances under these different experimental conditions. The greatest differences should exist between the distribution seen on recovering terminals and the distribution seen on terminals treated with BWSV (1).

MATERIALS AND METHODS

Electron Microscopy

The general procedures for freeze-fracture and thin sectioning were carried out as described previously (1) except that in the experiments with 20 mM K^+ this same concentration of K^+ was included in all the fixatives.

In some of the experiments with 20 mM K^+, horseradish peroxidase (HRP), Sigma Type VI (Sigma Chemical Co., St. Louis, Mo.), was added to the bathing medium to determine whether the extracellular tracer was incorporated into the vesicles during the secretion of transmitter. 2.5 ml of Ringer's solution with 1.5–3% HRP was put on a resting muscle in the recording chamber. The solution was removed after 1 h, and 50 λ of 1 M KCl was added to increase the K^+ concentration by 20 mM. The K^+-rich HRP solution was reapplied to the muscle, and the muscle was fixed 1 h later with 2% glutaraldehyde in 0.1 M phosphate buffer, pH 7.2. Controls were treated according to the same schedule except that the bathing solution contained no Ca^{2+} and 4–5 mM Mg^{2+}. After 20-min fixation, the muscles were cut into small pieces containing suspected end-plate regions and put into fresh fixative for a total fixation time of 1.5–2 h. The pieces of muscles were treated according to the procedure of Graham and Karnovsky (8) to reveal the sites of peroxidase activity and then postfixed for 1 h with 2% OsO_4 in 0.1 M phosphate buffer, pH 7.2.

Electrophysiology

End-plate potentials (epp's) or miniature end-plate potentials (mepp's) were recorded intracellularly from each muscle used in this investigation. In most of the experiments with nerve stimulation (10/s), neuromuscular transmission was blocked with d-tubocurarine (3–4 μg/ml) and the curare was washed out after 10 min of stimulation so that both epp's and mepp's could be observed. Stock solutions of curare (200 μg/ml) were made fresh each day and diluted as required. In a few experiments, not used for freeze-fracture, curare was not used and muscle fibers were impaled after ~10 min when the twitch had become very weak.

Statistical Treatment of Data

Histograms were constructed as described in the previous paper (1). We computed from each histogram the cumulative frequency distribution of the number of dimples or protuberances as a function of the distance from the center of the active zone. The statistical significance of the differences between pairs of these frequency distributions was computed from the Kolmogorov-Smirnov two-sample test with the n's equal to the total numbers of dimples or protuberances counted. The Kruskal-Wallis analysis of variance by rank was used to compare individual intervals from various histograms.

RESULTS

Effects of Indirect Electrical Stimulation

When frog neuromuscular junctions are stimulated indirectly at a rate of 10/s, the rate of secretion of quanta of transmitter declines within 10 min to rates of about 100/s and then remains
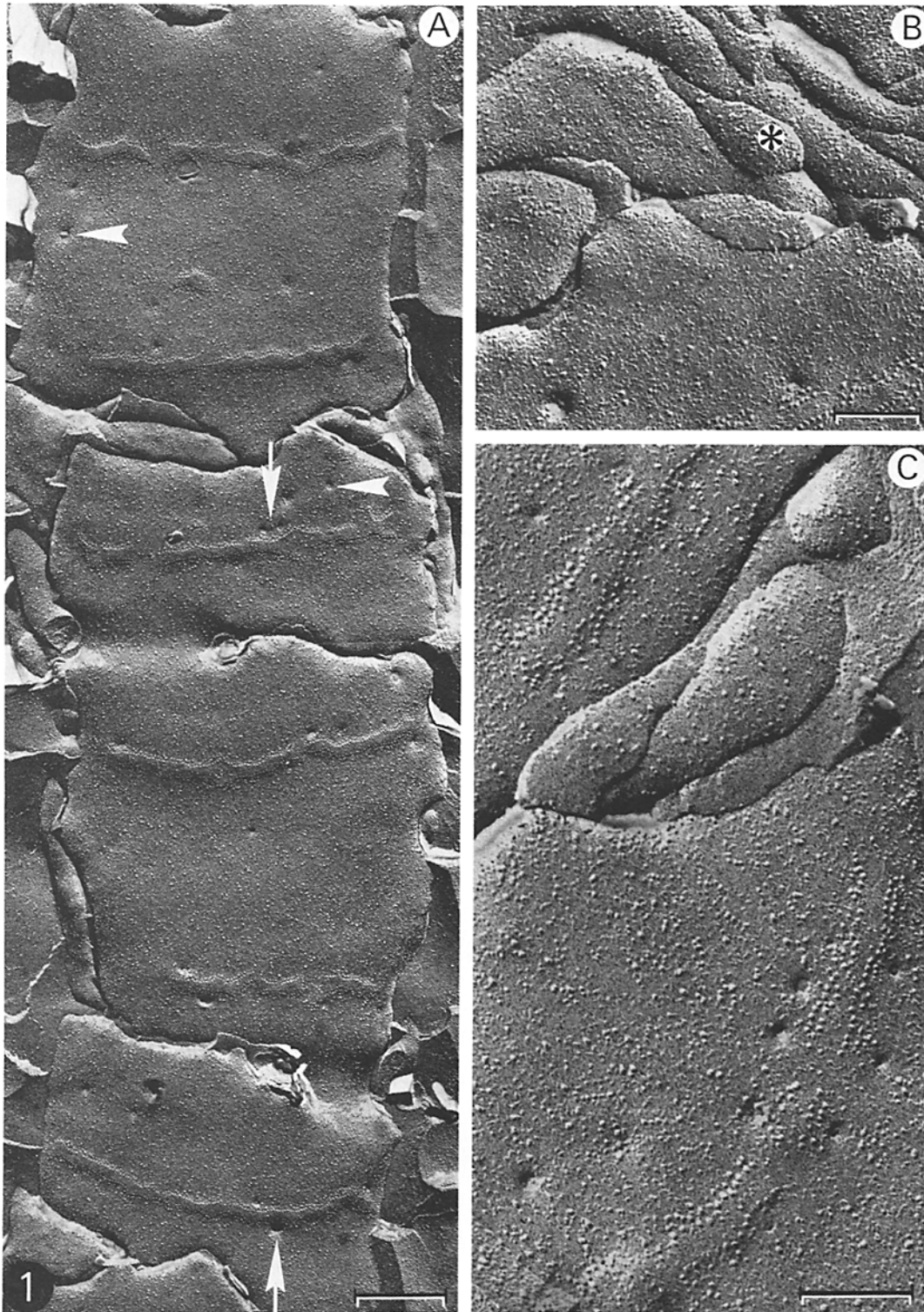
FIGURE 1   Micrographs of replicas of the P faces of freeze-fractured terminals from preparations that had been stimulated at 10/s for 20 min and fixed during the final minute of stimulation. (*A*) Low power micrograph showing several active zones. Numerous dimples are seen, both at the active zone (arrows) and in regions of the axolemma between active zones (arrowheads). (*B* and *C*) Higher magnifications of portions of active zones from different neuromuscular junctions showing elaborate infoldings (*) and several dimples, most of them located alongside the active zones. (*A*) Bar, 0.5 μm. × 29,000. (*B*) Bar, 0.2 μm. × 66,000. (*C*) 0.2 μm. × 86,000.

relatively constant for the next several minutes (4). After 20 min of stimulation, the terminals are partially depleted of synaptic vesicles and deep infoldings have developed in the presynaptic membrane (4, 9). Since the rate of secretion seems to be in a steady state near the end of this period of stimulation, we assume that endocytosis and exocytosis are occurring at approximately equal rates.

Fig. 1 shows P face of three nerve terminals that had been fixed during the final minute of a 20-min period of stimulation at 10/s. Several dimples are seen alongside the active zones, and some are seen in the regions between active zones. The zones are not disorganized, but deep infoldings of the presynaptic membrane occur in the regions between zones.

Fig. 3 shows a histogram of the distribution of dimples or protuberances found on terminals that had been fixed during stimulation. The highest density of the dimples or protuberances was found within 90 nm of the centers of the active zones; few dimples or protuberances were found near the entrances to infoldings or on regions of presynaptic membrane that had been isolated by elaborate infoldings (Fig. 1). A similar distribution of dimples or protuberances has been observed at frog neuromuscular junctions stimulated briefly in fixative (10). The dimples and protuberances found on these stimulated preparations could mark either sites of exocytosis or sites of endocytosis, and we cannot identify any morphological criteria for distinguishing between these two possibilities. The mean diameter of the dimples or protuberances located within 90 nm of the center of the active zones was not significantly different from the mean diameter of dimples or protuberances located at greater distances from the active zones. Similar results were previously reported (10).

Preparations Fixed during Recovery from Indirect Stimulation

After 20 min of stimulation at 10/s in Ringer solution, the mean rate of secretion of quanta of transmitter due to the epp's is about 100/s and the mean rate of secretion due to mepp's is about 20/s (4). These observations were corroborated in the present experiments. When stimulation is stopped, the epp's cease immediately, but the mepp frequencies remain unchanged for several minutes. Therefore, stopping stimulation reduces the rate of exocytosis of quanta of transmitter by

about sixfold on the average. Endocytosis, however, continues and leads within 15-60 min to the retraction of the infoldings and the recovery of the population of synaptic vesicles (4, 9). Most of the recovered vesicles originate from the axolemma (4, 9). Therefore we assume that the distribution of dimples or protuberances seen early in the recovery period reflects mostly the distribution of the sites of endocytosis. This assumption is substantiated by the finding in locust neuromuscular junctions that the poststimulation rate of endocytosis is extremely vigorous and causes within 2 min the complete recovery of the population of synaptic vesicles (18).

Fig. 2 shows the P faces of two terminals that had been stimulated for 20 min at 10/s and then rested for 30 s before being fixed. In general, the terminals resemble those that were fixed during the final minute of stimulation. Numerous dimples are seen, most of them located alongside the active zones, and many infoldings are still present.

Fig. 3 shows the histogram of the distribution of dimples seen in terminals that had recovered for 0.5-1.0 min. This distribution does not differ significantly from the distribution seen on terminals that were fixed during stimulation ($P >$ 0.15). However, both distributions differed significantly ($P < 0.05$) from that observed on BWSV-treated terminals (1); the differences occurred mainly in the interval nearest the active zones.

After 10 min of recovery, the mepp frequency had fallen to about 10/s at most junctions. The nerve terminals showed few dimples or protuberances, and the number and extent of the infoldings appeared to be greatly reduced (Fig. 2). These observations suggest that most of the vesicle membrane had been recovered from the axolemma by this time.

Effects of Modified Ringer's Solution with 20 mM K^+

High concentrations of K^+ induce a Ca^{2+}-dependent, rapid, asynchronous release of quanta of neurotransmitter (13). When a modified Ringer's solution with 20 mM K^+ was applied to frog neuromuscular junctions, the muscle fibers depolarized to ~40 mV in a few minutes, the mepp amplitude declined by ~50%, and the mepp frequencies increased to 100-300/s after ~5 min (Fig. 4). The membrane potential of the muscle fibers then remained relatively constant over the next hour. The mepp frequencies continued to
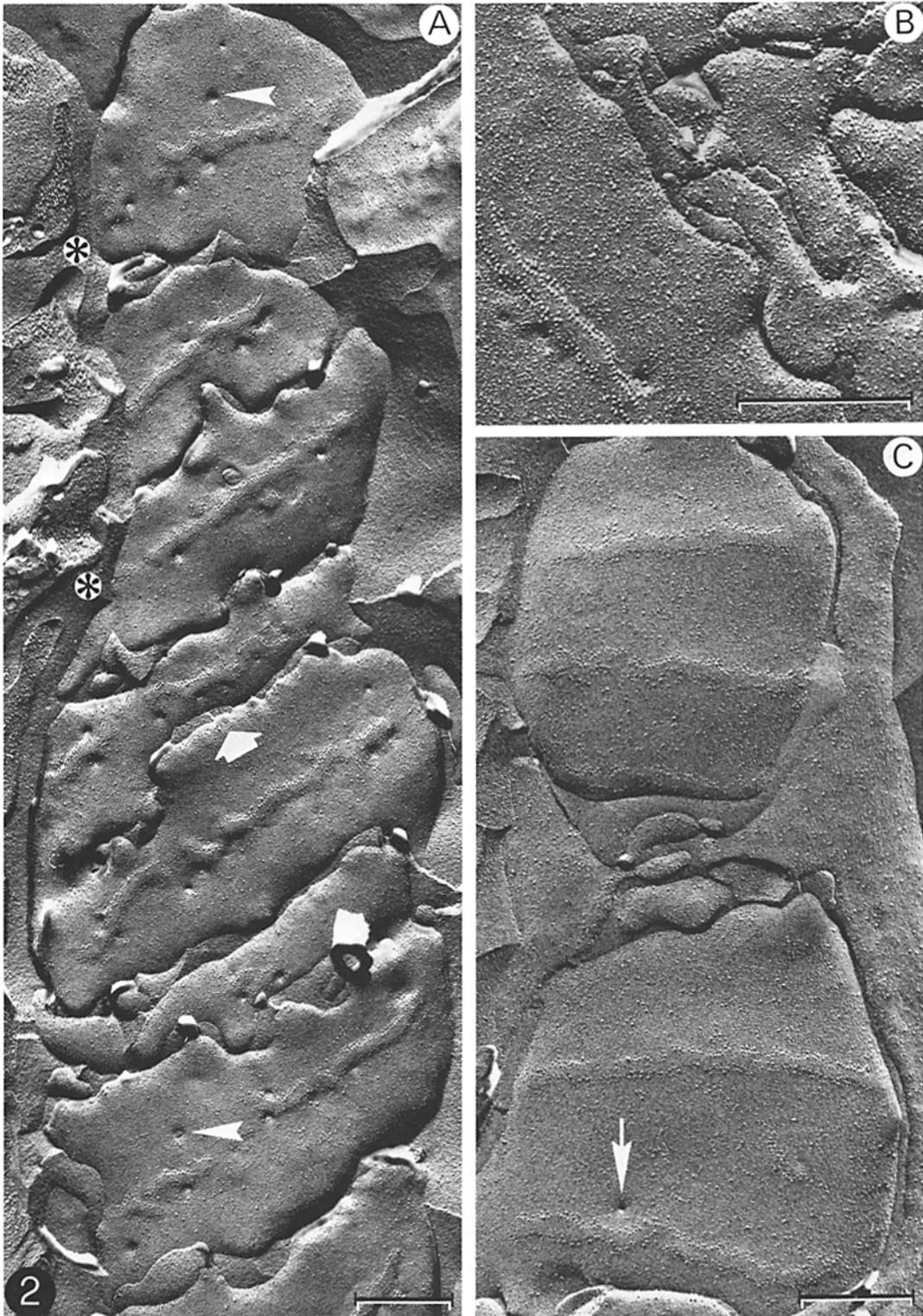
FIGURE 2 Micrographs of replicas of the P faces of freeze-fractured terminals that were fixed during recovery periods after stimulation for 20 min at 10/s. (*A*) Terminal fixed after 30-s recovery. Several active zones are seen with unusually large numbers of dimples located mainly alongside the double rows of particles. A few dimples (arrowheads) are located between active zones. The zones are not disorganized and appear to be aligned with the postjunctional fold (*). Many infoldings are evident (arrow). (*B*) High magnification of a portion of an active zone fixed after 30-s recovery. Some dimples and elaborate infoldings of the axolemma are evident. (*C*) Portion of a neuromuscular junction fixed after 10 min of recovery. One dimple (arrow) is seen on the presynaptic membrane. (*A*) Bar, 0.5 μm. × 30,000. (*B*) 0.5 μm. × 54,000. (*C*) 0.5 μm. × 36,000.
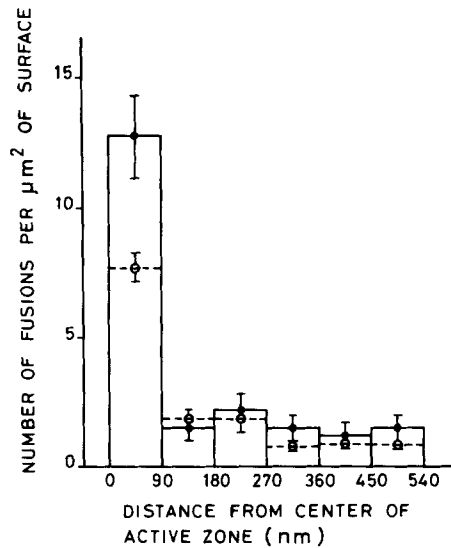
FIGURE 3 Histograms of the distributions of dimples or protuberances found on micrographs of preparations fixed during the final minute of stimulation (20 min, 10/s [○]) or fixed after 0.5- to 1.0-min recovery after this period stimulation (●). In the stimulated preparations, 408 dimples or protuberances were counted on 94 active zones comprising 143 μm^2 of presynaptic membrane on five terminals. Mean (±SEM) density of fusions sites: 2.9 ± 0.28/μm^2. In the recovery preparations, 456 dimples or protuberances were counted on 95 active zones comprising 108 μm^2 of presynaptic membrane on 12 nerve terminals. Mean (±SEM) density of fusion sites: 4.3 ± 0.56/μm^2.

rise over the next 10–15 min (7), achieved maximum rates of 200–400/s after 15–20 min, and then gradually declined so that after 1 h they reached levels of 50–100/s. At the end of the hour in 20 mM K^+, many junctions seemed to exhibit very small amplitude mepp's (Fig. 4). In some junctions, this could be confirmed by polarizing the muscle fibers to membrane potentials of 80–100 mV. The peak frequencies recorded in 20 mM K^+ were generally less than the peak frequencies recorded in BWSV. However, secretion persisted for at least 1 h in 20 mM K^+, and the total number of mepp's recorded in these two situations were similar (5–7 × 10^5 mepp's).

Figs. 5 and 6 show the P and E faces of prejunctional membrane from preparations that had been fixed 15 min after the application of the modified Ringer's with 20 mM K^+. Large numbers of dimples or protuberances are seen, and they are located all over the prejunctional membrane rather than mainly near the active zones. When 20 mM K^+ was applied in Ca^{2+}-free solu-

tions with 4 mM Mg^{2+}, the mepp frequencies did not increase and very few dimples or protuberances were found.

Fig. 7 shows a histogram of the distribution of dimples or protuberances found on terminals that had been treated for 15 min with 20 mM K^+. The distribution is almost uniform and is significantly different ($P < 0.01$) from the distributions obtained with BWSV, stimulation, or stimulation followed by recovery.

When preparations were fixed after soaking for 1 h in 20 mM K^+, the nerve terminals still showed many dimples or protuberances located all over the presynaptic membrane (Figs. 8 and 9). Tortuous infoldings often had developed in the axolemma at this time (Fig. 9). The active zones were not disorganized, but where extensive infoldings of the axolemma had developed, the zones were frequently displaced from their usual locations opposite the troughs of the postjunctional folds (Fig. 9). All of these results obtained with freeze-
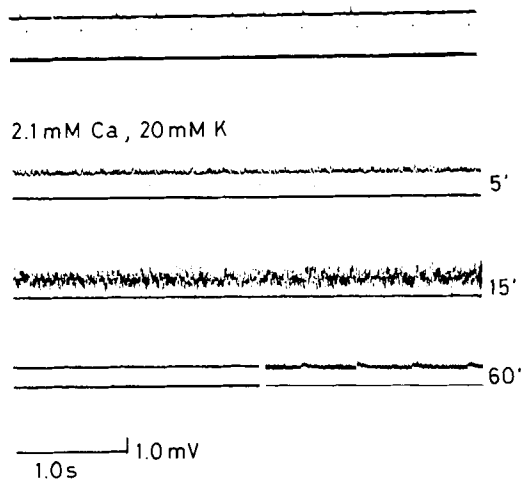

FIGURE 4 Effect of modified Ringer's solution with 20 mM K^+ on mepp frequency. For each pair of records, the upper trace is a high gain AC-coupled record of mepp's and the lower trace is a low gain DC-coupled record. The separation of the traces is a measure of the resting potential of the muscle fiber. The voltage calibration applies to the upper traces. The top record shows mepp's recorded in Ringer's immediately before adding the 20 mM K^+, and the other records were taken from the same junction 5, 15, and 60 min after applying the solution. The right-hand portion of the lowermost record was obtained immediately after the left, and the gain was increased fivefold. Note what appears to be the peaks of many small mepp's. The four bumps in the trace were caused by the time calibration signal.
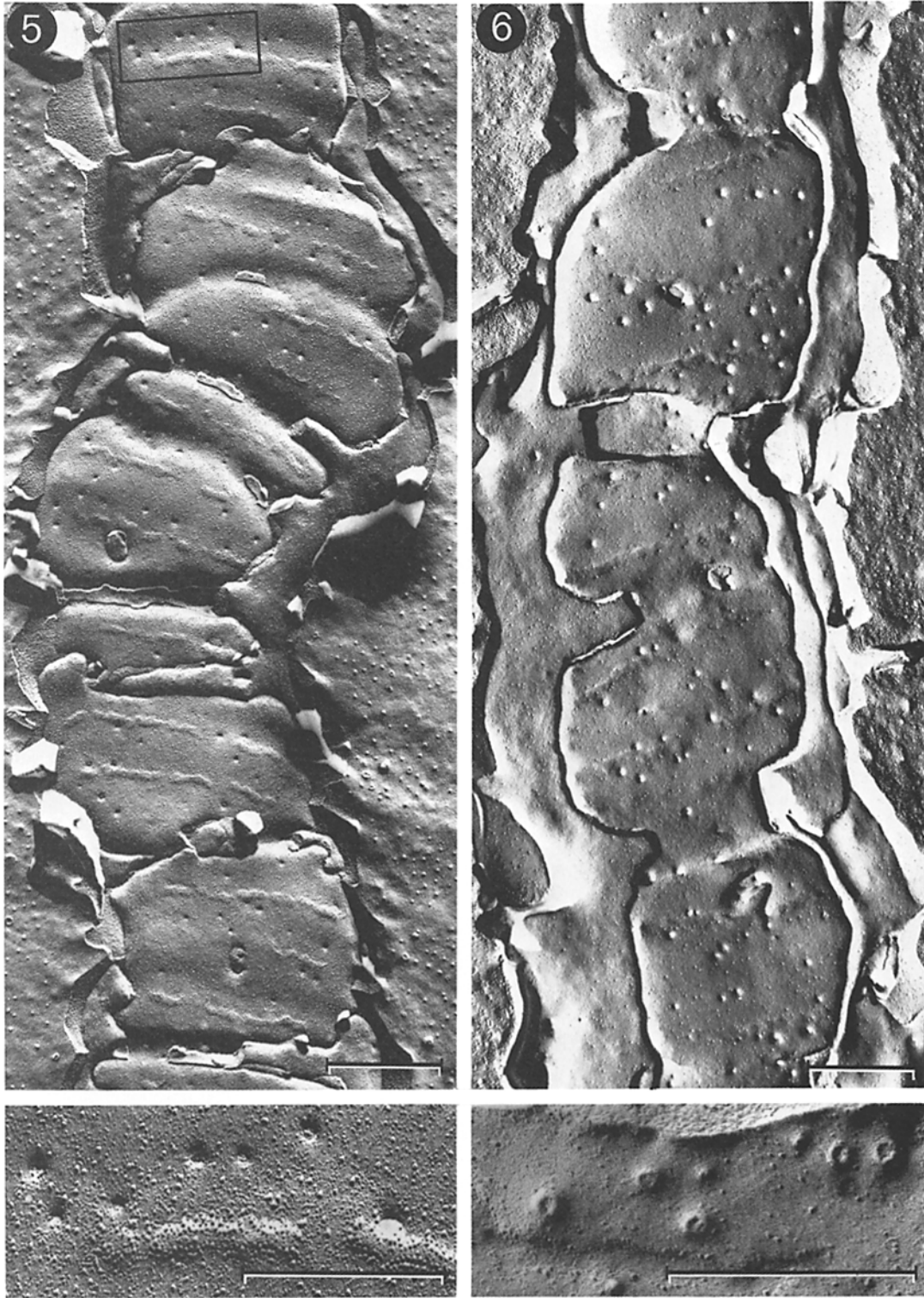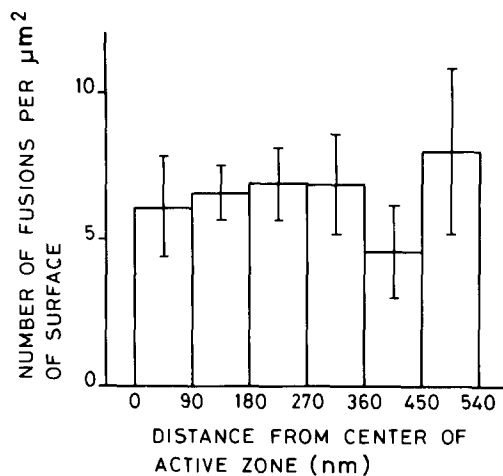
FIGURE 7 Histogram of the distribution of dimples or protuberances found on nerve terminals soaked for 15 min in 20 mM K^+. 505 fusions were counted on 111 active zones comprising 93 μm^2 of presynaptic membrane of 12 terminals. Mean ($\pm$SEM) density of fusion sites: 6.5 $\pm$ 1.1/μm^2.

fracture were corroborated by results obtained from thin sections.

Fig. 10 shows electron micrographs of neuromuscular junctions that had been soaked for 1 h in modified Ringer's solution with 20 mM K^+. These nerve terminals still contained many synaptic vesicles although a partial depletion may have occurred. The mitochondria within the terminal were swollen, and many extensive infoldings of the axolemma had developed along the presynaptic membrane. In addition, the local thickenings of presynaptic membrane and clusters of synaptic vesicles that identify active zones were often found displaced from their usual positions opposite the troughs of the postjunctional folds (Fig. 10A, B, and D). As was the case with freeze-fractured terminals, displaced active zones were usually

found near regions of the axolemma with extensive infoldings.

The persistence of secretion, the continuing occurrence of dimples or protuberances, and the retention of numerous synaptic vesicles throughout the hour of soaking in 20 mM K^+ all suggest that a recycling of synaptic vesicles had occurred. To obtain direct evidence that membrane was retrieved from the axolemma, we performed some experiments with HRP.

Figs. 12–14 show terminals that had been soaked for 1 h in a modified Ringer's solution that contained 20 mM K^+ and HRP. Several of the vesicles in these terminals contain HRP reaction product, indicating that these vesicles had been open to the extracellular space. Control terminals that had been soaked for 1 h in a modified Ringer's solution with 20 mM K^+, 0 mM Ca^{2+}, and 4 mM Mg^{2+} contained only a few HRP-labeled vesicles and their mitochondria were not swollen (Fig. 11). These observations fully corroborate our suggestion that membrane is retrieved from the axolemma when terminals are soaked in solutions with 20 mM K^+. This retrieval of membrane is not accompanied by an obvious increase in the numbers of coated vesicles. Coated vesicles were found in thin sections of K^+-treated terminals (Figs. 10 and 13), but their numbers were no greater than in controls (2–4).

Intramembrane Particles

The mean diameter of the large intramembrane particles that line the edges of the active zones is 112 $\pm$ 12 Å (n = 200). The regions of the presynaptic membrane between active zones in resting preparations contain many intramembrane particles with diameters in this range (see Figs. 11 and 12 in reference 1). Because these particles are numerous and distributed randomly over the areas

FIGURE 5 Low power micrograph of a replica of the P face of a freeze-fractured nerve terminal that had been soaked for 15 min in 20 mM K^+. Several active zones are seen; many dimples are present and seem to be uniformly distributed over the presynaptic membrane. *Inset:* high magnification of the region of the terminal outlined by the rectangle. Several dimples are located more than 90 nm from the center of the active zone. Bar, 1 μm. × 18,000. *Inset:* bar, 0.5 μm. × 63,000.

FIGURE 6 Low power micrograph of a replica of the E face of a freeze-fractured terminal that had been soaked for 15 min in 20 mM K^+. Numerous protuberances are seen and they appear to be uniformly distributed along the axolemma. *Inset:* high magnification of an active zone from a different neuromuscular junction. Many protuberances containing small craters are seen, and most are located more than 90 nm from the center of the furrow. Bar, 1 μm. × 17,000. *Inset:* bar, 0.5 μm. × 78,000.
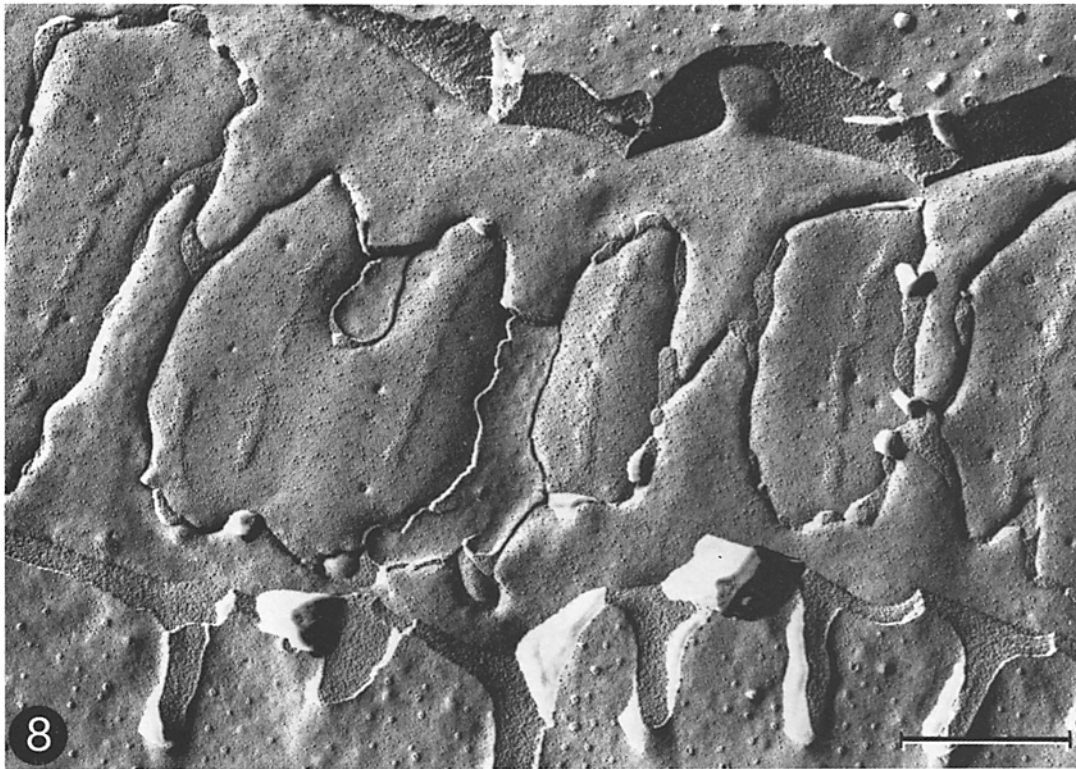
FIGURE 8 Low power micrograph of a replica of the P face of a freeze-fractured terminal that had been soaked for 1 h in 20 mM K⁺. The active zones seem not disorganized; many dimples can be seen and they appear to be distributed uniformly over the presynaptic membrane. Bar, 1 μm. × 23,000.

FIGURE 9 Low power micrograph of a replica of the E face of a terminal that had been soaked for 1 h in 20 mM K⁺. Many protuberances are seen, and they seem to be uniformly distributed over the presynaptic membrane. Several infoldings are present, and some of the furrows (arrow) have been displaced from their usual positions opposite the troughs of the postjunctional folds (*). Bar, 1 μm. × 20,000.

of presynaptic surface between active zones, some are almost always found near dimples or protuberances that occur in these regions. This is illustrated in Fig. 15 which is a composite of micrographs at high magnification of dimples found at points distant from the active zones in stimulated preparations.

Several authors have reported that the number of large intramembrane particles in the presynaptic membrane increases as a result of stimulation (16, 20). This is an important point and requires a complete morphometric analysis of the number and diameter of the particles measured on replicas of controlled thickness. It will be the subject of a future report.

DISCUSSION

The fusion of the membrane of a synaptic vesicle with the axolemma to release a quantum of transmitter by exocytosis, and the subsequent removal of membrane from the axolemma mark the beginning and end, respectively, of a continuum of states that occur while the vesicle membrane is incorporated into the axolemma. If this overall process is fast compared to the speed of fixation, then terminals fixed during intense secretion will display vesicles caught in all possible states. If no clear morphological differences exist among the various states, then it is impossible to determine from micrographs where membrane is added to the axolemma and where it is removed. However, a distinction between the sites of addition (exocytosis of quanta of transmitter) and the sites of removal (endocytosis) could be made, in principle, if conditions were found under which one of these processes occurred while the other was suppressed.

This paper, and the previous one (1), describe an attempt to achieve such conditions. BWSV is assumed to create a condition in which the rate of addition of membrane is fast while the rate of removal is slow. Recovery from prolonged repetitive indirect stimulation is assumed to create a condition under which the rate of removal of membrane is fast while the rate of addition is slow. In each of these experimental conditions, the dimples or protuberances occurred mainly at the active zones. In each of these conditions, also, both membrane addition and membrane removal undoubtedly occurred concurrently; however, the balance between the two opposing processes should have been very different in the various experimental conditions. Since the distribution of dimples or protuberances was independent of which process predominated, we conclude that both processes occur primarily at the active zones and that vesicle membrane need not migrate from the active zone to be removed from the axolemma.

Membrane accumulates in the axolemma during prolonged intense stimulation and gives rise to infoldings between the active zones. The accumulation of membrane between zones under these conditions presumably occurred because membrane was added at the active zones faster than it could be removed. However, the accumulation of membrane between the active zones seems not to be a prerequisite for removal since dimples and protuberances occurred mainly at the active zones even in terminals with extensive infoldings.

The correlation between the locations of the dimples and protuberances and the locations of the active zones is broken when transmitter release is stimulated by 20 mM K^+. The uniform distribution of dimples or protuberances seen under this condition has not been seen on resting control terminals, terminals stimulated either briefly (10) or for prolonged periods, terminals recovering from stimulation, or terminals soaked in Ca^{2+}-free solutions containing 20 mM K^+. This unique distribution seems to be due to a real effect of high concentrations of K^+ on the location of the sites of exocytosis, the sites of endocytosis, or both. Since both exocytosis and endocytosis occur in solutions with 20 mM K^+, we cannot be sure which process accounts for most of the dimples or protuberances seen between the active zones.

In our previous paper, we showed that highly organized active zones were not required for the exocytosis of quanta of transmitter and that BWSV could induce exocytosis at isolated remnants of double rows of large particles (1). The minimum number of large particles required to specify a site for exocytosis at neuromuscular junctions is not known, but under some conditions one may be sufficient. The regions of the presynaptic membrane between the active zones are normally studded with large, isolated particles similar in size to those at the active zones. Some of these particles may represent intramembrane components of isolated sites for the quantal release of transmitter that are less effective than several particles collected into small groups. It is therefore possible that such isolated particles are
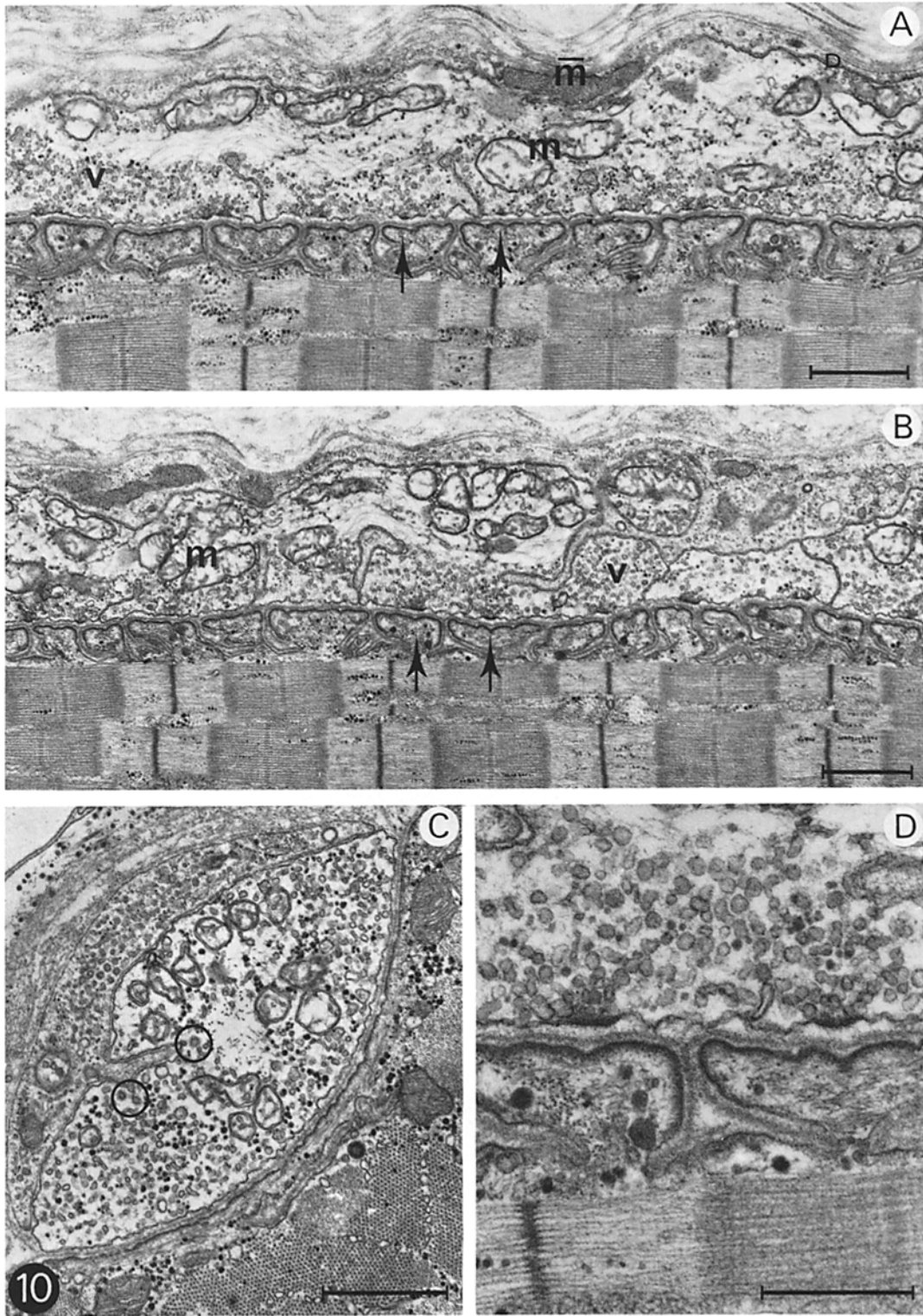
not activated by the brief depolarizations produced by action potentials in the nerve terminal but are activated by high concentrations of K^+ or by prolonged depolarization of the presynaptic membrane. K^+ has complex and still poorly understood effects on release of quanta of transmitter (6, 7, 19). Furthermore, the release of transmitter induced by K^+ is less strongly dependent on extracellular Ca^{2+} than is the release induced by indirect electrical stimulation (5). It would be intriguing if some of these effects of K^+ on quantal release of transmitter were due to the activation of latent release sites located between the active zones.

We did not observe obvious increases in the numbers of coated vesicles in our K^+-stimulated preparations as compared with resting controls. The failure to detect increases in the number of coats seems not to be due to the inadequency of our fixation and staining procedures because clear coats were seen surrounding some vesicles in thin sections of resting (3, 4), K^+-stimulated or indirectly stimulated terminals (4). However, if coats were associated with vesicles for only a small fraction of the time required for endocytosis, then one would not expect them to become very numerous even if endocytosis was accelerated.

APPENDIX

Elementary Model for the Accumulation of Synaptic Vesicles in the Axolemma

If the addition of synaptic vesicles to the axolemma were an irreversible process, then during secretion at a constant rate, R, the number of vesicles, n, accumulated in the axolemma would increase linearly with time, t, so that $n = R \times t$.

If vesicles are also removed from the axo-lemma, and if τ is the average time that elapses between the moment a vesicle is added and the moment it is removed, then the number of vesicles accumulated will approach asymptotically a steady state value given by: $n = R \times \tau$. The approach to the steady state would be 95% complete at times equal to 3 τ. This model assumes that τ is independent of R and n.

Table I presents for various values of τ the number of vesicles that would have accumulated in the steady state if the rate of secretion of quanta were 1,000/s. This is roughly the maximum rate of secretion achieved with BWSV (14). The number of vesicles accumulated per active zone was computed by assuming a terminal contains 500 active zones (15).

The figure of 1 ms corresponds to the average synaptic delay at frog neuromuscular junctions at 20°C (12) and represents the upper limit of the time required for the exocytosis of transmitter. The figure of 100 s corresponds to a recent estimate of the fixation time (11). Neuromuscular junctions can be stimulated for several hours at a rate of 2/s without suffering an obvious depletion of synaptic vesicles or increase in the area of the axolemma (2–4). This indicates that whatever membrane is added to the axolemma during the exocytosis of transmitter is largely removed during the interval between successive stimuli, and it suggests that τ is <1 s in moderately stimulated terminals. If τ were 1 s, and were independent of the rate of stimulation, then stimulating at 10/s, which raises the rate of secretion to initial values of about 2,000 quanta/s (4), would cause 2,000 vesicles to accumulate in the axolemma in the steady state. The approach to this steady state would be 95% complete after 3 s. The predicted accumulation is only ~1% of the total number of

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FIGURE 10   Micrographs of thin sections of neuromuscular junctions that had been soaked for 1 h in 20 mM K^+. (*A* and *B*) Low power micrographs of longitudinal sections. Many synaptic vesicles (*v*) are still present although a partial depletion may have occurred. Mitochondria (*m*) of the terminal appear to be swollen, whereas mitochondria in the thin Schwann cell process (*ṁ*) are normal. Numerous elaborate infoldings are evident, and in many places local thickenings of the presynaptic membrane, with clusters of synaptic vesicles, are located between postjunctional folds (arrows). Presumably, these structures represent displaced active zones. (*C*) Cross section of a neuromuscular junction. The terminal contains swollen mitochondria, synaptic vesicles, glycogen, elements of smooth endoplasmic reticulum, and a few coated vesicles (circles). Note the unusually large number of coated vesicles in the Schwann cell process. Mitochondria in the muscle fiber appear normal. (*D*) High magnification of a portion of Fig. 10*B* showing the details of two displaced active zones. Many of the synaptic vesicles have a polymorphic shape. (*A*) Bar, 1 μm. × 16,000. (*B*) 1 μm. × 14,500. (*C*) Bar, 1 μm. × 19,500. (*D*) Bar, 0.5 μm. × 48,000.
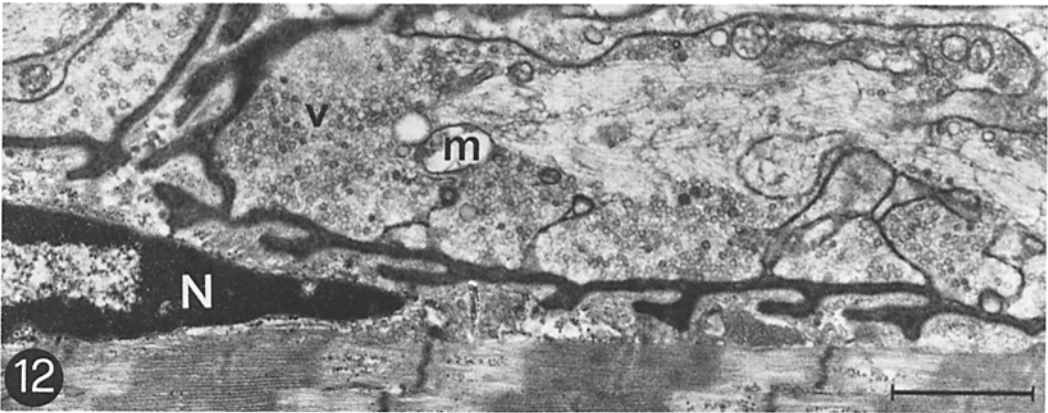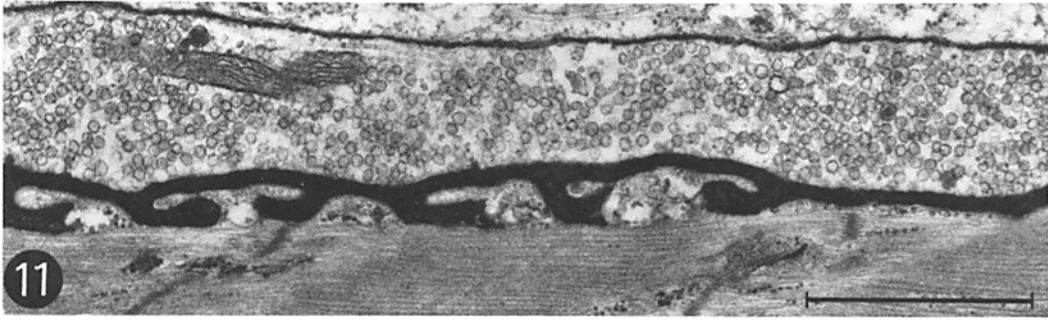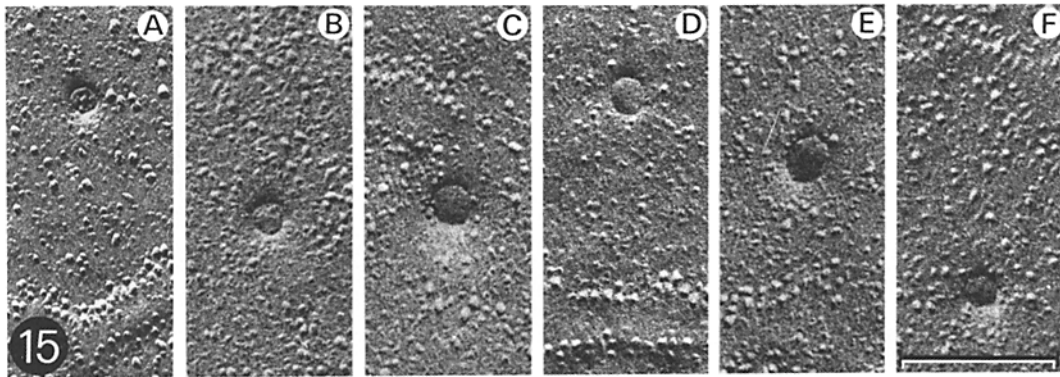
FIGURE 15   Composite of high magnification micrographs of replicas of P faces of several different nerve terminals showing dimples removed from active zones. (A) 15 min; (B) 1 h in 20 mM K$^+$; (C and D) electrical stimulation without recovery; (E and F) electrical stimulation followed by 0.5- to 1-min recovery. Large intramembrane particles similar in size to those at the active zones are scattered over the presynaptic membrane. A few individual large particles occur near almost every dimple; in Fig. 15 C, the large particles may be a tail extending from an active zone. Bar, 0.2 μm. $\times$ 105,000.

vesicles in a frog neuromuscular junction (2, 4) and would not be detected in thin sections. The fact that depletion is observed after more prolonged stimulation at 10/s implies that τ may increase as a result of intense activity.

We found four to five dimples or protuberances per active zone on terminals that had been stimulated in various ways. The rates of secretion under the various conditions of stimulation ranged from 100 to 1,000/s and the calculated values of τ range from 2 to 25 s. These large values of τ may not be real, but may occur because the fixative interferes with the removal of membrane before it blocks secretion (10). Therefore micrographs of chemi-

TABLE I

	No. of vesicles accumulated (n)	
τ	Entire junction	Active zone
s		
10^{-3}	1	0.002
1	10^3	2
100	10^5	200

cally fixed preparations probably do not represent instantaneous views of the states of the presynaptic membrane, but they show an integral of the secretory events that occurred during the time required for complete fixation.

FIGURE 11   Electron micrograph of a longitudinal thin section of a terminal soaked for 1 h in Ca^{2+}-free solution with 4 mM Mg^{2+} and HRP and then soaked for an additional hour in the same solution plus 20 mM K$^+$. The terminal appears normal and the synaptic cleft is heavily stained, but no vesicles contain reaction product and no infoldings are present. Bar, 1 μm. $\times$ 31,000.

FIGURE 12   Low power electron micrograph of a longitudinal thin section of a terminal bathed for 1 h in Ringer's solution with HRP and then soaked for an additional hour in the same solution plus 20 mM K$^+$. The mitochondria (m) of the terminal appear to be swollen, numerous elaborate infoldings are evident, and many synaptic vesicles contain reaction product (v). N, nucleus of muscle fiber. Bar, 1 μm. $\times$ 20,000.

FIGURE 13   Electron micrograph of a thin transverse section of a terminal treated as described in Fig. 12. The tortuous outline of the terminal may be due to the deep infoldings of the axolemma that develop in 20 mM K$^+$. The terminal contains many synaptic vesicles loaded with reaction product and also contains a few coated vesicles, two of them without reaction product (circle). Bar, 1 μm. $\times$ 39,000.

FIGURE 14   Electron micrograph of a thin transverse section of another terminal treated as described in Fig. 12. This terminal with smooth outlines contains many synaptic vesicles with HRP reaction product. Bar, 1 μm. $\times$ 31,000.

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