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Ultrastructural Correlates of Transmitter Release in Presynaptic Areas of Lamprey Reticulospinal Axons[1]

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Abstract

The ultrastructure of presynaptic areas of lamprey reticulospinal axons was studied before, during, and after periods of elevated transmitter release produced either by repetitive action potential activity or depolarization by elevated extracellular potassium. Controls for possible effects of these procedures per se were done by replacing extracellular Ca with Mg to block transmitter release. In some experiments the time course of ultrastructural changes during K depolarization and subsequent recovery were studied by fixing tissue samples at various times.

Transmitter release produced by action potential activity (20/sec for 15 min) in the presence of extracellular Ca significantly and reversibly decreased the number of synaptic vesicles, the area occupied by the vesicles, and the density of synaptic vesicles. An unexpected finding was a reversible decrease in the length of the differentiated membrane during periods of increased transmitter release. Transmitter release significantly and reversibly increased the number of coated vesicles, expanded the presynaptic membrane, and increased the number of pleomorphic vesicles. K depolarization (50 mM K for 15 min) produced identical, reversible effects, except that the expansion of the presynaptic membrane, although significant, was relatively small and there was no change in the number of pleomorphic vesicles. Raising the temperature of the saline from 2°C (K depolarization experiments) or 7°C (action potential experiments) to 20°C did not change the results qualitatively but did produce somewhat larger effects during stimulation and appeared to increase the speed of recovery. Action potential activity or K depolarization in control experiments with the Ca in the saline replaced by Mg had little or no effect on synaptic ultrastructure.

Synaptic vesicles in lamprey reticulospinal axons never contacted the axonal membrane anywhere other than at the differentiated membrane. During periods of elevated transmitter release, although the absolute number of vesicles in contact with the differentiated membrane decreased, the percentage of total vesicles in contact with the differentiated membrane increased dramatically. This suggests that (1) the differentiated membrane is the site of vesicle release and (2) there is an active process of vesicle movement to this membrane.

In the course of this work it was observed that presynaptic areas closer than approximately 2 mm to the site of axonal transection, regardless of the composition of the saline or the experimental conditions, showed ultrastructural changes typical of increased transmitter release. The changes were profound after only several minutes following transection of the axon and were progressively greater for synaptic areas nearer the cut end. Intracellular recording from transected axons confirmed that their cut ends do not seal for at least 24 hr after transection, and, therefore, nearby synapses are depolarized and exposed to the extracellular fluid.

These results from vertebrate central nervous system synapses suggest that during transmitter release synaptic vesicles are drawn to and fuse with the presynaptic membrane, causing it to expand, and are "recycled" in a process requiring minutes, possibly involving coated vesicles and pleomorphic vesicle formation. The differentiated presynaptic membrane (active zone) appears to be the actual site of vesicle fusion, and its overall length is reversibly decreased by release, suggesting a transient depletion of some specialized membrane component.

Synaptic ultrastructural changes correlated with transmitter release have been well characterized at the vertebrate neuromuscular junction (Ceccarelli et al., 1972, 1973; Heuser and Reese, 1973, 1981; Heuser et al., 1974, 1979; Peper et al., 1974) and sympathetic ganglia (Pysh and Wiley, 1974; Dickinson-Nelson and Reese, 1983). Results with thin section and freeze fracture techniques have generally been interpreted to support the vesicle recycling hypothesis originally proposed by Heuser and Reese (1973). In this scheme synaptic vesicles release their contents by exocytosis at the active zone of the presynaptic terminal, add their membrane to the terminal membrane which expands, are recaptured sometime later, probably via the endocytosis of coated vesicles, and finally are re-formed either directly from coated vesicles or from irregular cisternae. Experiments relevant to this hypothesis have been the subject of several reviews both critical (Israel et al., 1979; Tauc, 1982) and supportive (Ceccarelli and Hurlbut, 1980).

Previous work on lamprey giant reticulospinal axons (Wickelgren, 1975; Christensen, 1976; Kershaw and Christensen, 1980) and hatchetfish Mauthner axon synapses (Model et al., 1975) has indicated that vertebrate central nervous system synapses can show ultrastructural changes during transmitter release that are similar to

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those reported at peripheral neuromuscular and ganglionic synapses. The work to be reported here was done to provide detailed quantitative information about the changes in synaptic ultrastructure that occur in lamprey giant reticulospinal axons (Muller and Mauthner axons) during and after periods of elevated transmitter release under a variety of conditions and with controls not present in previous work. In the course of these experiments it was observed that within minutes of spinal transection the synapses near the cut ends of the axons showed profound depletion of synaptic vesicles and other features characteristic of stimulation. Therefore, in the experiments involving increased transmitter release, only synapses more than 5 mm from cut ends were analyzed. The data show that highly significant, reversible changes in synaptic ultrastructure, similar to those observed at the frog neuromuscular junction (Heuser and Reese, 1973), occur during action potential activity or K depolarization of lamprey giant axons. However, the changes in structure produced by action potential activity versus K depolarization, although in many ways similar, were sufficiently different to suggest that the method of inducing transmitter release does affect the nature of the structural changes seen.

Materials and Methods

Adult American brook lamprey (*Lampetra lamottenii*) approximately 15 cm in length were obtained from Michigan and maintained in aerated freshwater aquaria at 4°C until use. For experiments an animal was anesthetized in fresh water containing tricaine methanesulfonate (Finquel, Ayerst; 0.1 gm/liter) and then transferred to a dissecting chamber containing iced lamprey saline of the following composition (in millimolar concentration): NaCl, 108; KCl, 2; $CaCl_2$ (or $MgCl_2$), 10; HEPES, 2; glucose, 4; titrated to pH 7.4. The animal was decapitated and a 7-cm length of spinal cord with its supporting notochord, extending from the last gill opening to the cloaca, was removed. The segments of giant reticulospinal axons (Muller and Mauthner axons) in this preparation will remain in excellent condition with overshooting action potentials in response to electrical stimulation and the ability to release transmitter from their synapses onto spinal motoneurons and interneurons for several days at room temperature and below (Wickelgren, 1977; Rovainen, 1979), but no experiment lasted longer than about 3 hr.

Action potential experiments. For these experiments the spinal cord-notochord preparation was pinned through the notochord (Fig. 1*A*) to Sylgard at the bottom of a Plexiglas dish. The composition of the saline and its temperature, maintained by electrothermal units beneath the dish, were varied depending on the experiment (7 or 20°C, see "Results"). After 15 min a 1.5-cm segment of the spinal cord was removed and fixed (see below) to serve as a prestimulation control. Then platinum wire electrodes were placed at one end of the spinal cord (Fig. 1*A*) and 2-msec bipolar electrical pulses at a frequency of 20 Hz were applied at approximately twice threshold intensity for activation of the giant axons. During stimulation the evoked activity of the spinal cord, the short-latency components of which represent the summed action potential activity of the giant axons, was monitored by a suction electrode on the other end of the cord. Also the activity of single giant axons was sampled by an intracellular micropipette (Fig. 1*C*). After 15 min of stimulation a 1.5-cm section of cord was removed and placed in a separate beaker for a recovery period of 1 hr before fixation. Stimulation was continued for the remaining segment of cord while the saline was drawn off and cold fixative flooded into the chamber to continue transmitter release from the axons until the fixation process ended it. The speed of fixation, as reflected by the time required to abolish action potentials, varied from 1 to 3 min.

K depolarization experiments. In a separate series of experiments transmitter release from giant axons was increased by steady depolarization in a saline containing 50 mM KCl (see Fig. 1*D*) with corresponding reduction in NaCl. For these experiments the spinal cord-notochord preparation was cut into segments slightly longer than 1 cm, one of which was fixed after 15 min in normal saline to serve as a prestimulation control. The other segments were exposed for various lengths of time (30 sec to 30 min) to the K depolarization and then fixed. In order to examine recovery, the remaining segments were removed from the depolarizing solution, immersed in a large volume of normal saline, and fixed at various times during the following 60 min. To control for the possible effects on synaptic structure of elevated K per se, experiments were run in which the Ca was replaced by Mg in the depolarizing solution to eliminate transmitter release (Rovainen, 1967, 1974). Experiments were run either in the cold (2°C) or at room temperature (20°C).

Electron microscopic techniques. Spinal cord-notochord segments were fixed for 2 to 4 hr in a solution of the following composition: 2.5% glutaraldehyde, 2.0% formaldehyde, 0.1 M sodium cacodylate at pH 7.4, 1°C. Then the spinal cord was gently lifted off the notochord, rinsed in 0.1 M sodium cacodylate with 2% sucrose, and postfixed for 1 hr in buffered 1% osmium tetroxide. The tissue was then rinsed in distilled water, stained *en bloc* in 2% aqueous uranyl acetate, dehydrated in graded acetone series, and embedded in Spurr low-viscosity resin (Polysciences, Inc). Pale gold cross-sections of the entire spinal cord were obtained from regions approximately 5 mm away from the cut ends (see "Results") and examined with an electron microscope.

Data collection and analysis. These were carried out in a blind procedure. The tissue samples were randomly assigned by the experimenter to coded vials containing fixative and turned over to another person for processing, thin sectioning, electron microscopy, and scoring of the micrographs. Giant axons are unmistakable in lamprey spinal cord cross-sections because of their large size (40 to 80 μm in diameter, see Fig. 1*B*) and characteristic ultrastructural appearance (Wickelgren, 1977; Rovainen, 1979). Synaptic areas in axons are of the *en passant* type with characteristic pre- and postsynaptic membrane specializations and accumulations of round, clear-centered vesicles presynaptically. The axons in thin sections from each tissue sample were randomly scanned and the first 20 to 25 synaptic areas encountered were photographed, regardless of whether they could be considered "typical." The micrographs were printed at a final magnification of × 47,500 and mixed together randomly before they were scored. This procedure ensured that micrographs from one experimental condition were not scored as a group and further diminished the possibility of unconscious bias. For each synapse a number of the features of presynaptic ultrastructure were quantified (see Fig. 2 for detailed explanation): (*1*) number of synaptic vesicles; (*2*) vesicle area; (*3*) vesicle density; (*4*) number of coated vesicles; (*5*) presynaptic curvature index; (*6*) number of pleomorphic vesicles; (*7*) length of differentiated membrane ("active zone"); and (*8*) whether or not the presynaptic element completely encircled the postsynaptic element. Vesicle counts were done manually with a pen-activated electric counter. Length and area measurements were made using a Hewlett-Packard digitizing pad connected to an H-P 9845 computer. After all of the micrographs had been scored, the code was broken and the average values of all of the synapses in each condition were calculated and statistical analysis was carried out (Student's *t* test, two-tailed).

Results

Effect of action potential activity. The typical appearance of synapses in unstimulated axons or in axons that have recovered from stimulation (Fig. 3, *A* and *C*) is a tight cluster of round, clear-centered vesicles immediately adjacent to a presynaptic membrane region of increased density, the differentiated membrane or "active zone." Although there is variability in the precise geometry of the pre- and postsynaptic membranes, the appearance is generally that of an *en passant* synapse in which the postsynaptic element abuts a relatively straight region of the presynaptic membrane. In such large axons these synaptic regions with their tight clustering of synaptic vesicles are striking and encourage the speculation that some cytoskeletal apparatus must exist to hold the vesicles in place.

The most obvious changes produced by 15 min of action potential activity were a reduction in the number of synaptic vesicles with a nearly corresponding reduction in the area they occupy in the axon, plus the appearance of coated vesicles, the latter seen either attached to the presynaptic membrane or free in the cytoplasm (see Fig. 3*B*, Fig. 4, *A* and *B*, and Table I). Typically an area of presynaptic cytoplasm in the vicinity of the differentiated membrane in a stimulated synapse was devoid of neurofilaments, which are common throughout the axonal cytoplasm, and contained a fuzzy substance (see Figs. 3*B* and 6*B*). We assume this area is the space occupied by the cluster of synaptic vesicles before their release; thus, the fuzzy substance could represent some aspect of the cytoskeletal structure that normally holds the synaptic vesicles in such focused arrays.

Another change in the presynaptic morphology of stimulated synapses was an apparent outpouching of the presynaptic membrane, which was quantified as the "presynaptic curvature index" (see Fig. 2). This change in the usual flattened *en passant* relationship of the pre- and postsynaptic elements was particularly obvious in those synapses in which the postsynaptic element was engulfed

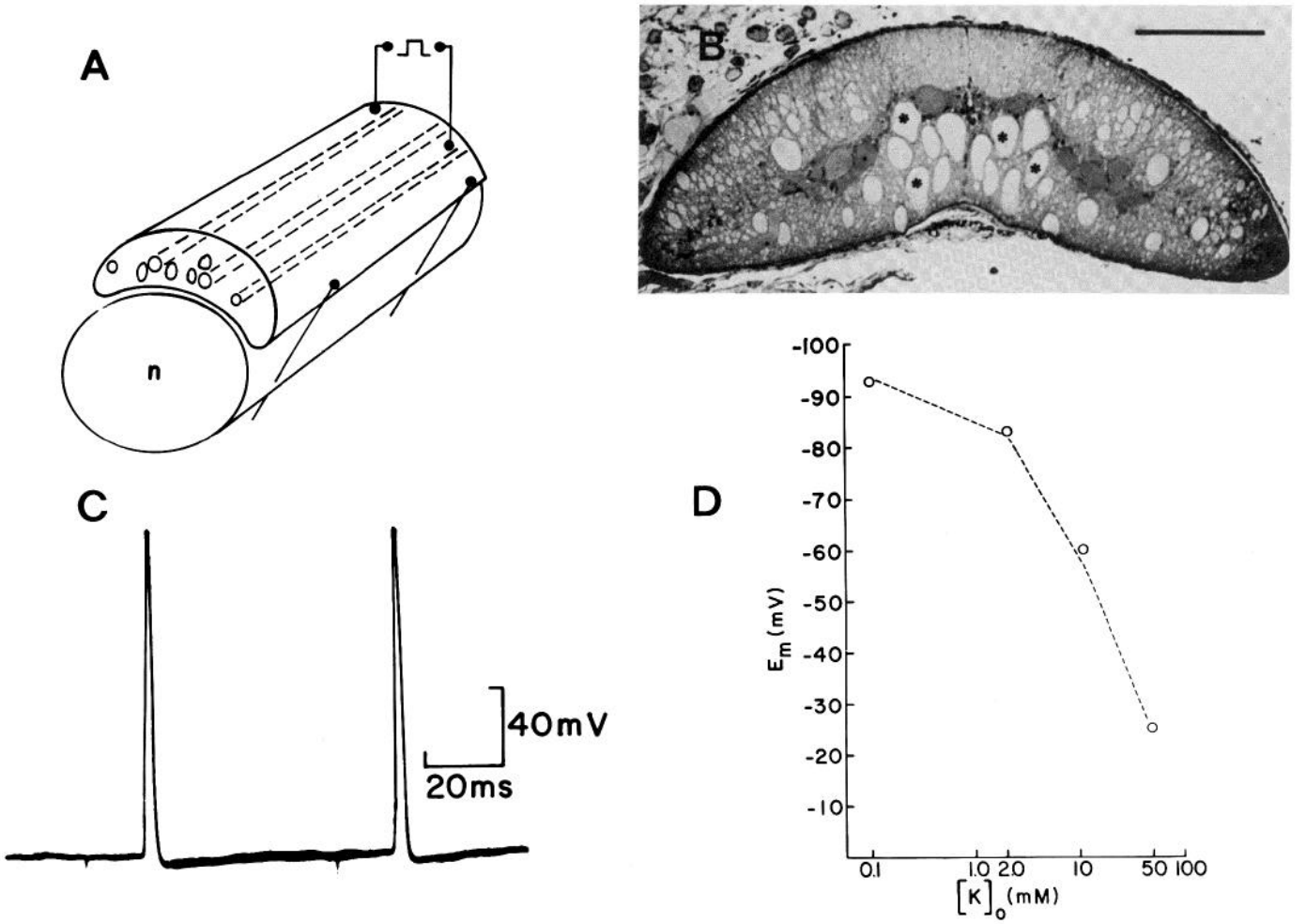

Figure 1. Giant reticulospinal axon morphology and physiological response to the two different methods of stimulation employed. *A*, Schematic drawing of the lamprey spinal cord-notochord segment. Positions of giant axons are indicated by *circles* at the *left end* of the spinal cord; *dashed lines* represent their longitudinal unbranched nature (only three were drawn in for simplicity). For direct electrical stimulation, the preparation was pinned as illustrated through the underlying notochord (*n*) to Sylgard and was stimulated with platinum wires as indicated. *B*, Light micrograph of a 1-μm-thick transverse section of lamprey spinal cord after routine fixation. Two of the nine giant axons on each side of the midline are marked by *asterisks*. Toluidine blue stain. *Bar* = 200 μm. *C*, Typical action potentials recorded by an intracellular microelectrode in a giant axon during repetitive electrical stimulation. *D*, Effect of extracellular K concentration on giant axon membrane potential. Giant axons depolarized to about −25 mV within seconds after washing in saline containing 50 mM K (K depolarization saline).

by the presynaptic axon (see Fig. 10). These so-called "surround synapses" were never seen in control or recovered synapses but were relatively frequent after 15 min of action potential activity (see Table I).

Action potential activity also produced an increase in the number of presynaptic "pleomorphic vesicles." This was a heterogeneous classification of single membrane-bound structures that varied from round vesicles too large to be synaptic vesicles to highly irregular structures similar to the "cisternae" reported by Heuser and Reese (1973), who suggested that they may represent one stage in the recycling of synaptic vesicle membrane. The relevance of these structures to vesicle recycling in our experiments is unclear since, although they were much increased by action potential activity (Table I), their numbers, as described below, were unchanged by release produced by K depolarization (see Table III).

A somewhat surprising finding was that, despite the apparent expansion of the presynaptic membrane during transmitter release, the length of the presynaptic differentiated membrane (active zone) was reversibly decreased (Table I). If, as seems likely, this region of increased membrane density is the site where vesicle release occurs, then its transient shrinkage during release suggests that its structure is not static and that perhaps the recycling of vesicle membrane is paralleled by a process that removes and then replaces the presynaptic membrane components to which the vesicles attach during exocytosis.

To be certain that the changes in synaptic structure described above were a result of increased transmitter release and not a consequence of action potential activity per se, several experiments identical to those above were performed but with the Ca in the saline replaced by 10 mM Mg, which blocks evoked transmitter release from these axons (Rovainen 1967, 1974). The results of these control experiments are shown in Figure 4 and Table II. Under these conditions action potential activity had little or no effect on synaptic ultrastructure. The small changes that did occur appeared to reflect a progressive effect of exposure to the high Mg saline rather than any reversible effect of action potential activity. Thus, we conclude that the reversible changes in synaptic ultrastructure produced by action potential activity in Ca-containing saline were a result of increased transmitter release.

Effect of K depolarization. Transmitter release can also be increased by the steady depolarization of nerve terminals in a saline with elevated extracellular K concentration (Liley, 1956; Pfenninger and Rovainen, 1974). When the K concentration of the saline bathing a segment of lamprey spinal cord was raised from 2 mM to 50 mM,

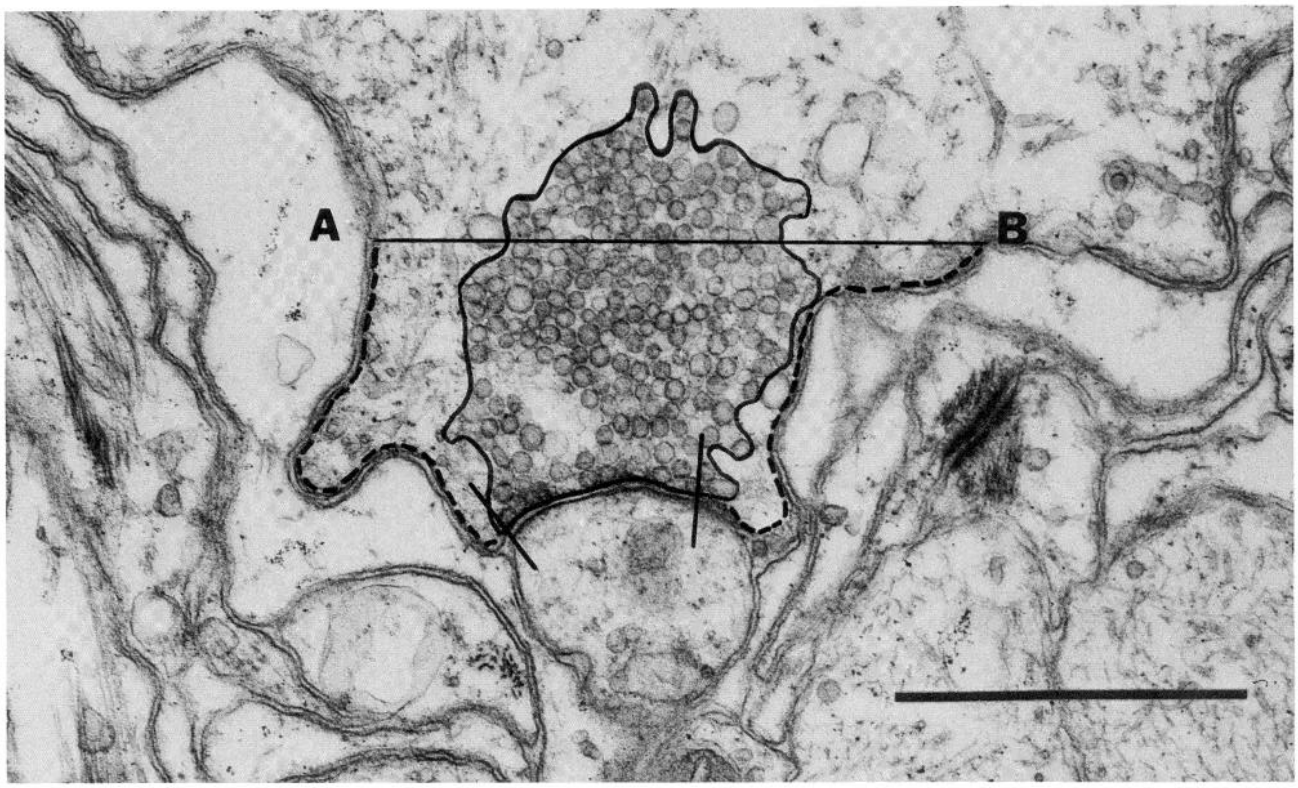

Figure 2. Parameters measured in scoring micrographs. The *outline* which encircles synaptic vesicles delimits the area occupied by the synaptic vesicles clustered at the synapse (*vesicle area*). All synaptic vesicles within the cluster were counted at each synapse (*number of synaptic vesicles*). Dividing the number of synaptic vesicles at each synapse by the vesicle area at each synapse yields the *vesicle density* for that synapse. The *differentiated membrane* (the region with increased electron density sometimes referred to as the active zone) at this synapse is located between the *two short line segments*. Two points (*A* and *B*) were always marked 1.5 μm from each end of the differentiated membrane to define the stretch of axolemma associated with this synapse (*dashed line* from *A* to *B*). The *presynaptic curvature index* (a measure of the presynaptic membrane expansion) was calculated as follows. The difference between the *straight line* distance from *A* to *B* and the path along the axolemma from *A* and *B* (*dashed line*) was divided by the sum of these two distances (Kershaw and Christensen, 1980). This equation yielded numbers that varied from 0 (for a completely straight presynaptic membrane) to 1 (where the presynaptic membrane completely surrounded the postsynaptic process). Other parameters scored were *number of coated vesicles* (either attached to the presynaptic membrane as "coated pits" or free in the cytoplasm), *number of pleomorphic vesicles* (any single membrane-bound structure in the vicinity of the presynaptic area that was either too large or irregularly shaped to be classified as a synaptic vesicle; this classification included structures termed "cisternae" by Heuser and Reese, 1973 (see Fig. 3)), and *number of surround synapses* where the presynaptic element completely surrounded the postsynaptic element (see Fig. 10). *Bar* = 1 μm.

the membrane potential of the giant reticulospinal axon decreased from approximately −80 mV to −25 mV within 5 to 10 sec (see Fig. 1). When the K concentration was subsequently returned to 2 mM, the axons repolarized within 1 min. Segments of lamprey spinal cord were fixed at various times before, during and after steady depolarization in a saline with 50 mM KCl. The ultrastructural effects of K depolarization were, with two exceptions, very similar to those produced by action potential activity. The results of a single experiment, showing synaptic vesicle depletion and coated vesicle accumulation during 15 min of K depolarization and subsequent recovery following a return to normal saline, are presented in Figure 5. The decline in number of synaptic vesicles and the increase in number of coated vesicles are evident as early as 1 min after exposure to the 50 mM K saline and are greater at the end of the 15-min stimulation period. The number of synaptic vesicles is still depressed after 5 min in the recovery saline but appears to have recovered after 30 min. The increase in the number of coated vesicles appears maximal after 5 min in the 50 mM K saline (the 5- and 15-min values are not significantly different) and has returned to almost control levels after 5 min in recovery saline. Although the detailed time course of changes in synaptic vesicles and coated vesicles during and after K depolarization varied somewhat from experiment to experiment, it was generally true that at room temperature (20°C) the number of coated vesicles returned to nearly control levels during recovery sooner than did the number of synaptic vesicles.

Table III summarizes the combined data for all of the ultrastructural variables scored for all of the K depolarization experiments. For simplicity only the control, 15-min K depolarization and 60-min recovery data are presented. During the K depolarization period there were highly significant decreases in the number of synaptic vesicles, the area occupied by the synaptic vesicles, the density of synaptic vesicles, and the length of the differentiated membrane. Also there was a highly significant increase in the number of coated vesicles during K depolarization. These effects are similar to those reported for increased transmitter release produced by action potential activity.

Two measures of synaptic ultrastructure, however, were not altered by K depolarization in the same manner as by action potential activity: (*1*) the number of irregular or "pleomorphic" vesicles, which in our classification includes both large, round vesicles and irregular "cisternae," was not changed by K depolarization, and (*2*) the presynaptic curvature index was only slightly increased by K depolarization over control levels (see Table III). We were concerned by the low level of statistical significance ($p < 0.05$) of this increase from 0.17 (control) to 0.20 (K depolarization), especially because there was no significant recovery during the 60-min rest period. However, when we added the data from another 82 control synapses and another 39 K-depolarized synapses (these data were not included in Table III because they were done for another purpose that did not require a recovery period; see Table V), the presynaptic

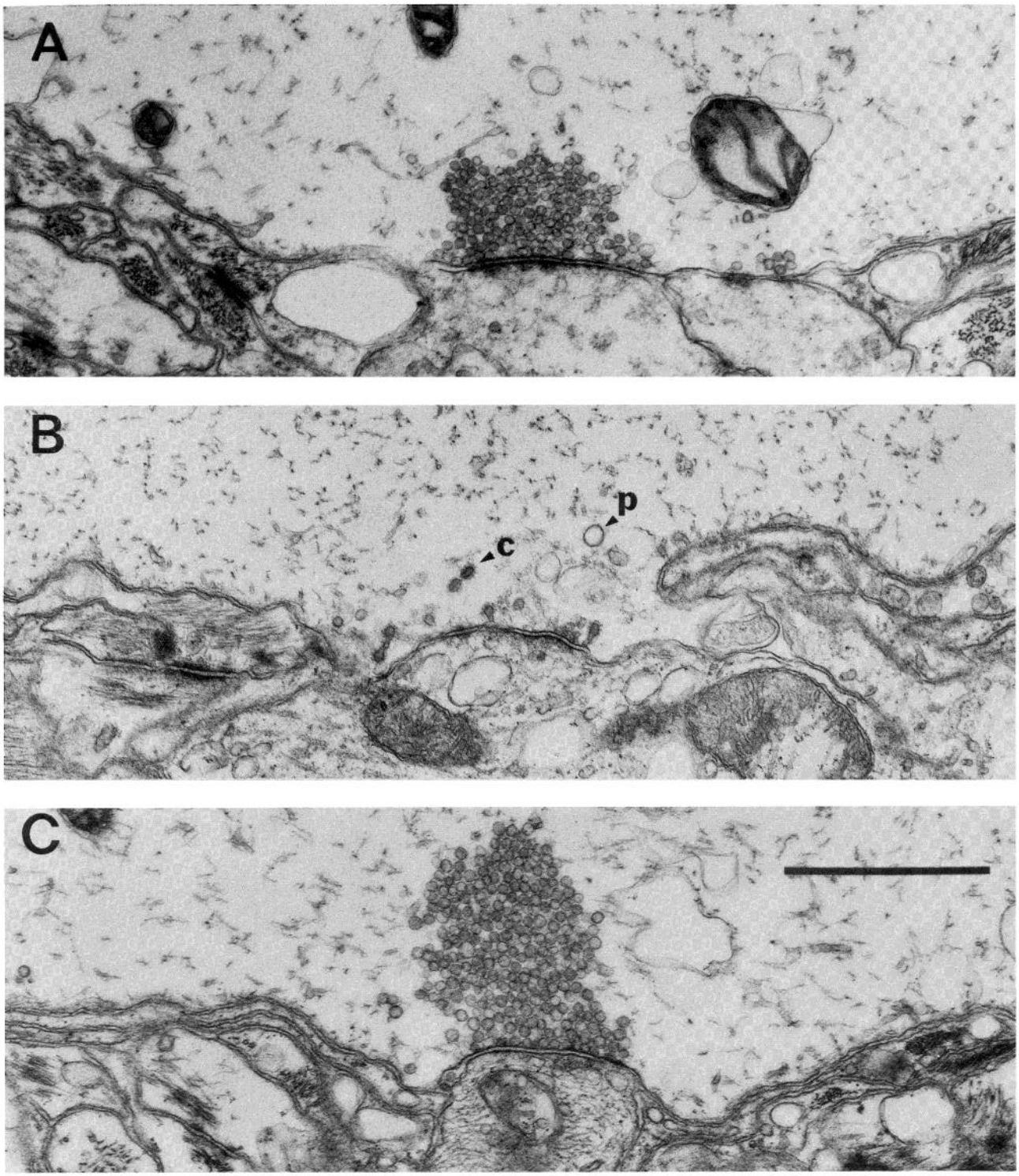

Figure 3. Reversible effects of action potential activity on presynaptic ultrastructure of giant axons. *A*, Control synapse fixed after 15 min in saline containing 10 mM Ca (Ca saline). Note the large, high density cluster of synaptic vesicles at the presynaptic membrane and the lack of coated vesicles. *B*, Synapse fixed after 15 min of 20 Hz electrical stimulation of the spinal cord. Note the depletion of synaptic vesicles, the presence of coated vesicles (*c*) and pleomorphic vesicles (*p*), and the more complex geometry of the presynaptic membrane. *C*, Synapse fixed 60 min after cessation of stimulation. Note similarities to control synapse. *Bar* = 1 μm.

curvature index for the control, K-depolarized, and recovery periods were 0.18 ± 0.01, 0.22 ± 0.01, and 0.19 ± 0.01, respectively (mean ± SEM). The increase during the K depolarization was now significantly different from both the control and recovery values ($p < 0.01$). From this we conclude that there is a very small but significant expansion of the presynaptic membrane during transmitter release induced by K depolarization. A further indication that transmitter release induced by elevated K does not produce the same change in presynaptic membrane morphology as does action potential activity was the complete absence of "surround synapses" in K-depolarized tissue (Table III).

We have no explanation for the absence of a change in pleo-

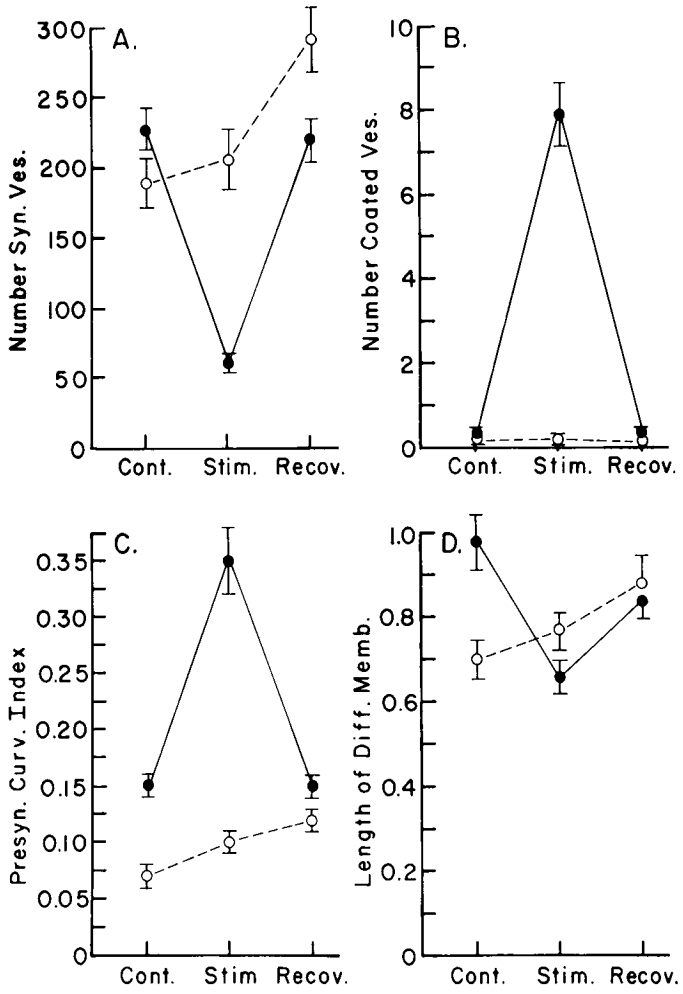

Figure 4. Effects of repetitive action potential activity depend on extracellular Ca. *Solid circles* represent results obtained using saline containing 10 mM Ca (Ca saline). *Open circles* represent results obtained using saline containing 10 mM Mg (Mg saline). The temperature was 20°C. *Cont.*, control spinal cord segment fixed after 15 min in saline; *Stim.*, different segment from same spinal cord; *Recov.*, segment fixed 60 min after cessation of stimulation. *A*, The number of synaptic vesicles per synapse decreased after 15 min stimulation in Ca saline then recovered to the control level after 60 min of rest. In contrast, stimulation in Mg saline did not deplete the number of synaptic vesicles, which increased slightly with time. *B*, The number of coated vesicles increased during stimulation in Ca saline and fell to control levels after a 60-min rest period. Stimulation in Mg saline had no effect on the number of coated vesicles. *C*, Presynaptic curvature index increased during stimulation in Ca saline and returned to the control level after 60 min of rest. In Mg saline the presynaptic curvature index showed a small continuous increase over time. *D*, The length of differentiated membrane (active zone) at the synapse decreased during stimulation in Ca saline and then partially recovered during a 60-min rest period. In Mg saline the length of differentiated membrane showed a small monotonic increase. Data points are mean ± SEM. For complete results of these experiments see Tables I and II.

morphic vesicles in the K depolarization experiments or for the very small expansion in the presynaptic membrane. Even in those single experiments in which K depolarization produced as much vesicle depletion as in any action potential experiment, there was no change in the number of pleomorphic vesicles and the increase in presynaptic curvature index was very small (and frequently not statistically significant due to the lower number of synapses in the samples).

We assumed that the effect of K depolarization on synaptic ultrastructure was a result of a depolarization-induced influx of Ca, which increased transmitter release. But to control for the possible effects of the membrane depolarization itself or, for example, a change in intracellular Cl produced by an increase in extracellular K, we did a K depolarization experiment with Mg substituted for Ca. As with the action potential experiments in Mg saline, this treatment did not produce the ultrastructural changes produced by K depolarization in Ca-containing saline (see Table IV), and, therefore, we conclude that the ultrastructural changes produced by K depolarization in Ca-containing saline are the result of an increase in transmitter release.

Active association of synaptic vesicles with differentiated membrane. It is generally assumed that the presynaptic differentiated membrane represents the site of vesicle exocytosis. This idea seems reasonable for lamprey giant axons, since in thin sections synaptic vesicles seldom, if ever, contact the presynaptic membrane anywhere other than in this region of increased electron density. It has been suggested that fixation during increased transmitter release should show selective depletion of vesicles near this region of membrane (Kershaw and Christensen, 1980). However, this is true only if the process that moves vesicles from the interior of the axon to the presumed release sites in the differentiated membrane (vesicle mobilization) is relatively slow compared to the rate of vesicle exocytosis and if, during fixation, vesicle exocytosis and mobilization are arrested simultaneously.

In the experiments on the effects of action potential activity and K depolarization described above we were struck by the apparent tendency for a disproportionate number of the remaining vesicles in stimulated axons to be clustered near or touching the differentiated membrane (see Fig. 6). To quantify this impression we counted in several experiments the number of synaptic vesicles which appeared to physically contact the differentiated membrane, expressed this number as a percentage of the total vesicle population, and normalized it to a unit length of differentiated membrane. The resulting number can be viewed as a measure of the tendency of synaptic vesicles to associate with the differentiated membrane. In the experiment for which data are plotted in Figure 7, segments of spinal cord were fixed before stimulation, after 15 min of action potential activity, and after 60 min of recovery. Electrical stimulation of the axons was continued while fixative was washed into the chamber in order to continue transmitter release for as long as possible during the 1 to 3 min required for the fixative to eliminate action potentials in all axons. Nevertheless, the percentage of vesicles in contact with a unit length of differentiated membrane was actually much greater during the stimulation period than during the control or recovery periods, suggesting that as vesicles are released at the differentiated membrane, cytoplasmic vesicles are moved relatively quickly to replace them. We considered that we may have been prevented from seeing selective depletion of vesicles attached to the differentiated membrane because action potential activity and/or transmitter release may have been arrested by the fixative before vesicle mobilization was affected, a possibility made more likely by the absence of Ca in the fixative solution. Also, we considered that selective depletion of vesicles near the differentiated membrane might be observed in synapses that were fixed at the beginning of a period of increased transmitter release when the rate of transmitter release was presumably higher. Therefore, we fixed segments of previously unstimulated (control) lamprey spinal cord in normal fixative, in fixative + 10 mM Ca, and in fixative + 10 mM Ca and 50 mM K and compared the appearance of the synaptic areas (Table V). Since the fixative requires a minimum of 1 min to halt regenerative electrical activity in the axons but an increase in K concentration depolarizes the axons within seconds, we thought that the third fixative, which provides the opportunity for at least a minute of increased transmitter release before fixation takes place, might reveal selective vesicle depletion near the differentiated membrane. However, there were no differences among the results with the different fixatives (see Table V). Furthermore, when we fixed tissue that had been depolarized by 50 mM K for 15 min in Ca saline, the same significant increase in the percentage of synaptic vesicles in contact per unit length of differentiated membrane was observed as

TABLE I

Reversible changes in synaptic ultrastructure in lamprey giant axons prouced by action potential activity in Ca saline

Values in this and subsequent tables are mean ± SEM. All values at the end of 15 min of stimulation (20 action potentials/sec) are significantly different (t test, two tailed) from both control and 60-min recovery values at $p < 0.001$, except stimulation versus recovery for length of differentiated membrane, where $p < 0.01$. Data are from three experiments, two at 20°C and one at 7°C. Parameters are explained under "Materials and Methods" (see Fig. 2).

Experimental Condition	Total No. of Synapses	No. of Synaptic Vesicles	Vesicular Area (μm^2)	Vesicles/μm^2	No. of Coated Vesicles	Presynaptic Curvature Index	Total No. of Surround Synapses	No. of Pleomorphic Vesicles	Length of Differentiated Membrane (μm)
Control	64	229 ± 16	0.64 ± 0.04	357 ± 3	0.3 ± 0.1	0.15 ± 0.01	0	3.9 ± 0.3	0.98 ± 0.07
Stimulation	71	62 ± 7	0.23 ± 0.03	278 ± 6	7.9 ± 0.8	0.35 ± 0.03	14	14.6 ± 1.6	0.66 ± 0.04
Recovery	64	221 ± 17	0.67 ± 0.06	331 ± 4	0.4 ± 0.1	0.15 ± 0.01	0	4.6 ± 0.5	0.84 ± 0.04

TABLE II

Lack of effect on synaptic ultrastructure in lamprey giant axons of action potential activity in Mg saline

Stimulation (20 action potentials/sec for 15 min) did not produce significant changes in any of the parameters except for small changes in vesicles per square micrometer and presynaptic curvature index ($p < 0.05$). However, prolonged exposure to the 0 mM Ca, 10 mM Mg saline appeared to increase the number of synaptic vesicles, vesicle area, and presynaptic curvature index, since the parameters were significantly greater at the end of the recovery period than in controls ($p < 0.001$). Also, the length of differentiated membrane was slightly increased over control in the recovery period ($p < 0.05$). Data are from two experiments, one at 7°C and one at 20°C.

Experimental Condition	Total No. of Synapses	No. of Synaptic Vesicles	Vesicular Area (μm^2)	Vesicles/ μm^2	No. of Coated Vesicles	Presynaptic Curvature Index	Total No. of Surround Synapses	No. of Pleomorphic Vesicles	Length of Differentiated Membrane (μm)
Control	42	190 ± 18	0.52 ± 0.05	366 ± 6	0.1 ± 0.1	0.07 ± 0.01	0	3.3 ± 0.8	0.70 ± 0.05
Stimulation	45	207 ± 22	0.59 ± 0.06	344 ± 7	0.2 ± 0.1	0.10 ± 0.01	0	2.6 ± 0.4	0.77 ± 0.05
Recovery	43	292 ± 24	0.83 ± 0.07	361 ± 7	0.1 ± 0.1	0.12 ± 0.01	0	2.7 ± 0.3	0.88 ± 0.07

in the action potential experiment, and, once again, it made no difference whether the fixative contained Ca and K or not (Table V). Note that the absolute number of synaptic vesicles contacting the differentiated membrane was reduced in the K-depolarized axons compared to the controls, but the reduction was much less than that of the vesicle population as a whole. This, combined with the decrease in the length of the differentiated membrane which is characteristic of stimulated synapses, resulted in the much increased percentage of vesicles contacting the differentiated membrane per unit length. Therefore, these data are consistent with the idea that synaptic vesicles are released at the differentiated membrane and replaced there by an efficient process of mobilization from the cytoplasmic pool of vesicles.

Temperature effects on stimulation-induced changes in synaptic ultrastructure. A previous report on the hatchetfish giant synapse (Model et al., 1975) noted a qualitative change in the appearance of stimulated synapses at room temperature versus 12 to 14°C. We failed to observe the striking qualitative differences seen in the hatchetfish synapses but did find quantitative differences that are illustrated for two variables in Figure 8. The data in this figure are part of the data in Tables I and III where data from experiments in the warm and cold were combined (see table legends). At the warmer temperature (20°C), both action potential activity and K depolarization caused greater vesicle depletion and a greater increase in the number of coated vesicles, both attached to the membrane and free in the cytoplasm, than at the lower temperature, which was 7°C for the action potential experiment and 2°C for the K depolarization experiments. (The reason for the 7°C temperature in the action potential experiment is that it was not possible to maintain action potential activity in giant axons at 20/sec for 15 min at a lower temperature.) Despite the fact that the stimulation-induced changes were greater at 20°C, reversal of these changes at 60 min in the recovery period was complete, whereas the lesser changes at the lower temperatures were not completely reversed at 60 min; thus, at the low temperatures vesicle depletion and recovery from depletion were both diminished.

Since Model et al. (1975) reported that, in the cold, the coated vesicles they observed were nearly always attached to membrane as coated pits, we wondered whether in our experiments the failure of the number of synaptic vesicles to recover completely by 60 min in the cold might be attributed to a failure of coated pits to pinch off into coated vesicles, inhibiting this presumed step in the recycling process. However, when we divided our data on number of coated vesicles in the K depolarization experiments into open vesicles still attached to the axolemma and vesicles free in the cytoplasm for the experiments at 20°C versus those at 2°C, we found no differences in the percentage in each category. In 58 K-depolarized synapses at 2°C, 59% of the coated vesicles were attached to the axolemma, and in 38 synapses at 20°C, 61% of the coated vesicles were attached to the axolemma. Similar analysis was not done for the action potential experiments since there were relatively few coated vesicles seen in the single experiment at 7°C. However, we did find a difference between K depolarization and action potential stimulation in the percentage of coated vesicles seen as coated pits. Although approximately 60% of coated vesicles were attached to the axolemma in the combined K depolarization experiments (and, therefore, 40% of the coated vesicles were free in the cytoplasm), these numbers were almost exactly reversed in the combined action potential experiments in which 41% of the coated profiles were attached and 59% were free in the cytoplasm (differences significant at $p < 0.001$). We have no explanation for this alteration in the distribution of coated pits and vesicles between stimulation of release by action potentials and that by K depolarization, but it represents a third difference in the ultrastructural effects of these two methods of increasing release, along with the differences in pleomorphic vesicle formation and expansion in the presynaptic membrane.

A stimulation-like effect of axonal transection on the ultrastructure of nearby synapses. In the course of this work we observed that synaptic areas of giant axons near sites of spinal transection appeared stimulated (depletion of synaptic vesicles, increase in coated vesicles, increase in presynaptic membrane curvature, etc.), regardless of the experimental conditions or composition of the saline. In preliminary experiments such synapses contributed considerable variability to the data, since we were unaware of this effect and sampled synapses without regard to their proximity to the cut ends of the axons, which we assumed sealed over rapidly after spinal transection. However, measurements of their resting potentials es-

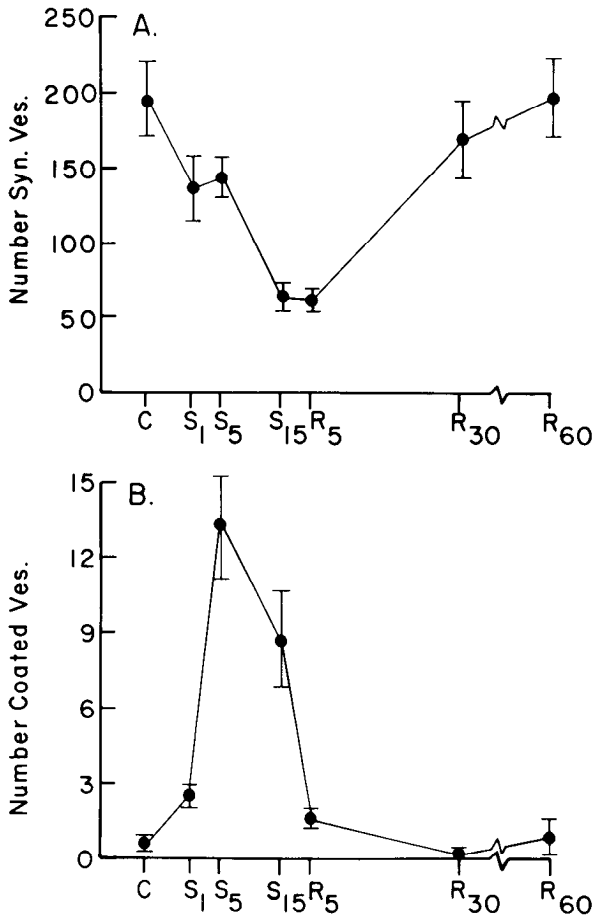

Figure 5. Time course of the changes in number of synaptic vesicles and coated vesicles produced by K depolarization of Muller axons in Ca-containing saline and their subsequent recovery. The temperature was 20°C. *C*, control (2 mM K); *S*, 50 mM K depolarization for the number of minutes indicated by the subscripts. *R*, recovery in 2 mM K for the times indicated (minutes). *A*, Progressive depletion of synaptic vesicles during 15 min in 50 mM K saline. Complete return to the control number of synaptic vesicles occurred by 60 min in recovery saline. *B*, Increase in number of coated vesicles during K depolarization reversed on return to normal low K saline. The recovery of coated vesicles to the control level normally occurred more quickly than for synaptic vesicles. Each *data point* represents the average (±SEM) of at least 20 synapses.

tablished that giant axons do not seal for at least 24 hr after transection (Clark and Wickelgren, 1982). Figure 9 shows the exponential decay of a giant axon resting potential as its cut end is approached during multiple intracellular penetrations 2 hr after transection. The space constants of giant axons are approximately 1 mm and, thus, the stable resting potential of an axon was not reached until the micropipette was inserted several millimeters from the cut end. The stimulated appearance of giant axon synapses appeared to parallel this recovery of resting potential, since the changes were most obvious within 1 mm of the cut end and were absent at a distance of 5 mm or more. Figure 10 shows a "surround synapse" approximately 0.25 mm from the cut end of an unstimulated giant axon that was placed in fixative 1 min after transection in Ca saline. Note the small number of synaptic vesicles and the large number of coated vesicles. Also note the relatively normal appearance of the axoplasm. Near cut ends there frequently were large numbers of vacuoles and, in places, separation of the axoplasm from the plasma membrane, but these gross changes in axonal structure were not necessary for the synapses in a region to show a stimulated appearance.

The progressive changes in synaptic areas of giant axons that occurred as tissue was sampled at increasing distance from the cut end were quantified for one experiment as shown in Figure 11 and Table VI. In this experiment a segment of lamprey spinal cord was transected and bathed without stimulation in Mg saline for 15 min before fixation. The changes in the numbers of synaptic vesicles and coated vesicles for synapses at 0.25, 0.5, 1, and 5 mm from the cut end are graphed in Figure 11, and the results for all of the variables are presented in Table VI. With the exception of the length of differentiated membrane, where there was an unexpected decrease at 5 mm, all of the ultrastructural variables scored showed progressive changes from "very stimulated" to "unstimulated" appearance as the distance from the cut end increased. Particularly striking were the large numbers of surround synapses near the cut ends. In this regard these axonal regions were more similar to axons that had been stimulated by action potential activity in Ca saline than to stimulation by K depolarization in Ca saline, where the expansion of the presynaptic membrane was minimal and surround synapses were absent.

Not only did synapses near cut ends appear stimulated, they also were much less frequent, as indicated by the approximately 4-fold increase in the number of sections needed to locate 20 synapses. Thus, it is apparent that synaptic areas of giant axons near cut ends begin to "disappear" within minutes of transection, leaving regions of closely opposed pre- and postsynaptic membrane that appear to have been synapses but now lack both differentiated membranes and synaptic vesicles. Occasionally a few coated vesicles are seen in these areas, lending credence to the idea that they may have once been synaptic areas. Since the disappearance of synaptic areas at a given distance from the cut end appears to increase with time after transection, we suggest that there is a temporal sequence of action on synaptic areas in which first they are intensely stimulated until all synaptic vesicles are depleted and then lose their differentiated membranes.

We do not know what factors contribute to the stimulation and disappearance of synaptic areas near the cut ends of giant axons. However, the fact that synapses near cut ends are affected in both

TABLE III

Reversible changes in synaptic ultrastructure in lamprey giant axons produced by K depolarization in Ca saline

The values of the following parameters at the end of the 15-min K depolarization period are significantly different from both control and recovery (60 min) values at the following levels: number of synaptic vesicles, $p < 0.001$; vesicles per square micrometer, $p < 0.001$; number of coated vesicles, $p < 0.001$; and length of differentiated membrane, $p < 0.01$. Also, recovery values for vesicles per square micrometer and number of coated vesicles are significantly different from control values ($p < 0.001$), indicating incomplete recovery. The only other significant difference is a slight rise over control in the presynaptic curvature index ($p < 0.05$) after K depolarization that did not reverse during the recovery period. Data are from eight experiments, five at 2°C and three at 20°C.

Experimental Condition	Total No. of Synapses	No. of Synaptic Vesicles	Vesicular Area (μm^2)	Vesicles/ μm^2	No. of Coated Vesicles	Presynaptic Curvature Index	Total No. of Surround Synapses	No. of Pleomorphic Vesicles	Length of Differentiated Membrane (μm)
Control	160	213 ± 9	0.60 ± 0.03	354 ± 3	0.4 ± 0.1	0.17 ± 0.01	0	4.2 ± 0.3	0.73 ± 0.02
K depolarization	154	129 ± 12	0.51 ± 0.06	284 ± 6	7.5 ± 0.6	0.20 ± 0.01	0	3.5 ± 0.3	0.63 ± 0.02
Recovery	156	191 ± 12	0.58 ± 0.04	330 ± 4	1.8 ± 0.3	0.19 ± 0.01	0	4.4 ± 0.4	0.75 ± 0.03

TABLE IV

Lack of effect on synaptic ultrastructure in lamprey giant axons of K depolarization in Mg saline

There were no significant differences among any of the values for the parameters except for a slight increase in presynaptic curvature index in the recovery period over control ($p < 0.05$) and a slight decrease in the number of pleomorphic vesicles after K depolarization that did reverse during recovery ($p < 0.05$). Data are from one experiment at 2°C.

Experimental Condition	Total No. of Synapses	No. of Synaptic Vesicles	Vesicular Area (μm^2)	Vesicles/ μm^2	No. of Coated Vesicles	Presynaptic Curvature Index	Total No. of Surround Synapses	No. of Pleomorphic Vesicles	Length of Differentiated Membrane (μm)
Control	21	152 ± 14	0.49 ± 0.05	317 ± 9	0.1 ± 0.1	0.05 ± 0.01	0	5.3 ± 0.9	0.91 ± 0.08
K depolarization	22	158 ± 25	0.48 ± 0.07	318 ± 8	0.1 ± 0.1	0.07 ± 0.01	0	2.7 ± 0.5	0.76 ± 0.08
Recovery	20	205 ± 34	0.64 ± 0.10	321 ± 8	0.6 ± 0.3	0.10 ± 0.02	0	5.1 ± 0.9	0.83 ± 0.11

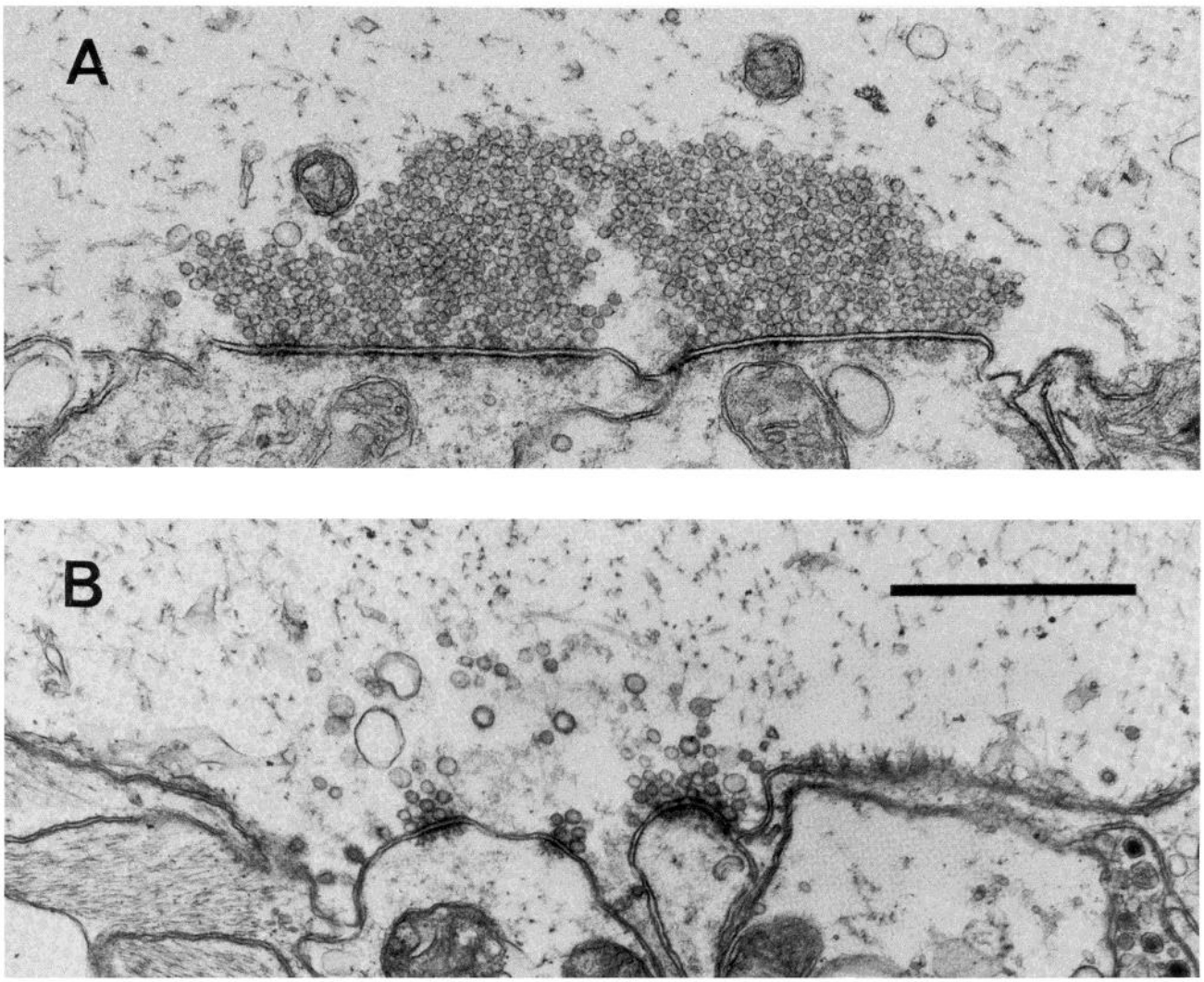

Figure 6. Association of synaptic vesicles with differentiated membrane (active zone). *A*, Control synapse. Two clusters of synaptic vesicles are focused on two segments of differentiated membrane. Such vesicles are essentially absent from the interior of giant axons (diameter = 80 μm). *B*, After 15 min of 20 Hz action potential activity, synaptic vesicles are still clustered near the differentiated membrane. Also note the typical lack of neurofilaments and the presence of a fuzzy substance in the area which was presumably occupied by a cluster of synaptic vesicles before stimulation of the axon. *Bar* = 1 μm.

Ca saline and Mg saline would appear to rule out the possibility that the changes are the direct or indirect result of the membrane depolarization, since a similar magnitude of depolarization produced by 50 mM K in Mg saline had no effect on synaptic structure in axonal regions away from the cut ends. Rather, it appears that the changes seen are somehow the result of direct exposure of the inside of the axon to the bathing saline.

Discussion

Action potential activity and depolarization by elevated extracellular K were used to induce changes in the structure of synaptic areas of giant reticulospinal axons (Muller and Mauthner axons) of lamprey. The changes produced were shown to be reversible and to depend on the presence of extracellular Ca and, thus, appear to be the result of increased transmitter release. Action potential activity in Ca saline decreased the number of synaptic vesicles and the length of the presynaptic differentiated membrane or active zone. There was a concomitant increase in the curvature of the presynaptic membrane, in the number of coated vesicles, and in the number of pleomorphic vesicles, which includes structures others have termed cisternae (Heuser and Reese, 1973). These changes in the synaptic ultrastructure of lamprey giant axons during action potential activity,

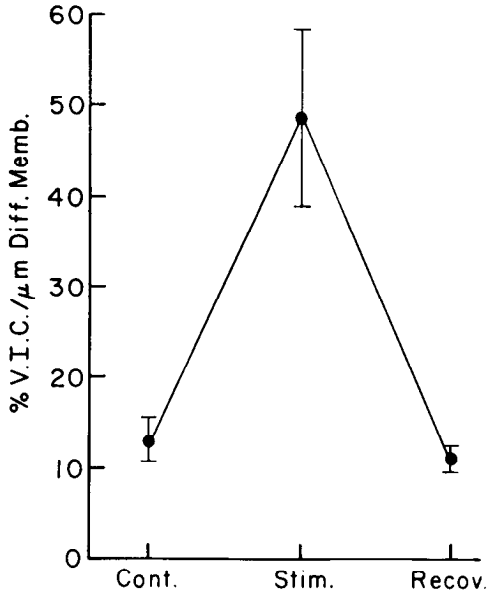

Figure 7. Reversible increase in the percentage of synaptic vesicles in contact with the differentiated membrane (*%V.I.C./μm Diff. Memb.*) during repetitive action potential activity in Ca-containing saline. *Cont.*, tissue fixed after 15 min in saline; *Stim.* tissue fixed after 15 min of 20 Hz electrical stimulation (with stimulation continued during fixation); *Recov.*, tissue fixed 60 min after cessation of stimulation. Each *data point* is the mean (±SEM) of 20 synapses.

some of which were also reported previously by Kershaw and Christensen (1980), are virtually identical to those reported at the frog neuromuscular junction by Heuser and Reese (1973). They interpreted their results to suggest that synaptic vesicles are released via exocytosis, adding their membrane to that of the presynaptic terminal and causing it to expand. They further suggested that synaptic vesicles are recaptured via endocytosis of coated vesicles, which shed their coats and coalesce into cisternae, which, in turn, bud off new synaptic vesicles. Our results show that similar changes in synaptic structure during and after action potential-evoked transmitter release occur in vertebrate CNS but do not, of course, establish the validity of this specific vesicle recycling hypothesis.

Although in many ways K depolarization and action potential activity produced similar effects, there were two striking differences. K depolarization produced only a very small, although statistically significant, expansion of the presynaptic membrane, and there was no change in the number of pleomorphic vesicles. The small quantitative effect of K depolarization on presynaptic expansion was also reflected in the complete absence of "surround synapses," which we assume to reflect extreme instances of expansion where the postsynaptic process is engulfed by the presynaptic element. But if expansion of the presynaptic membrane is simply the physical result of addition of vesicle membrane to it and since K depolarization in many cases produced reductions in synaptic vesicle number comparable to that in action potential experiments, why was there such a difference in the apparent expansion of the presynaptic membrane with the two methods of increasing transmitter release? We speculate that the difference may lie in the physical ability of the giant axon membrane to show a local expansion. In this regard, it is apparent that the plasma membrane of many cells, including nerve cells, is lined by fodrin, a protein that is similar or identical to spectrin, the cytoskeletal protein that binds actin and is responsible for the shape of erythrocytes (Levine and Willard, 1981). Fodrin binds both calmodulin and actin (Davies and Klee, 1981; Carlin et al., 1983), and there is evidence that the interaction of fodrin with actin is Ca sensitive (Sobue et al., 1983), which suggests that the deformability of the membrane may be a function of the intracellular Ca concentration (see Lazarides and Nelson, 1982). Therefore, the relatively small expansion of the presynaptic membrane during transmitter release induced by K depolarization may be a result of a different level of internal Ca produced by this treatment compared to action potential activity.

The absence of an increase in pleomorphic vesicles following release by K depolarization is also puzzling. If these heterogeneous structures represent a stage of vesicle recycling intermediate between coated vesicles and synaptic vesicles, they should have been increased by K depolarization as well as by action potentials, since recovery of the ultrastructural effects of both kinds of stimulation was complete by 60 min at 20°C and appeared to occur at comparable rates. Thus, we have no explanation for the failure of K depolarization to increase the number of pleomorphic vesicles, although the same processes that inhibited expansion of the presynaptic membrane could be involved. Alternatively, it may be that K depolarization does induce the formation of pleomorphic vesicles but at sites several micrometers further from the synapse than during repetitive action potential activity and these vesicles would have been overlooked. This problem reflects the difficulty in deciding where an *en passant* synapse ends.

One unexpected change in synaptic ultrastructure that occurred during stimulation in our experiments was the decrease in the length of the differentiated membrane (active zone) during periods of increased transmitter release. Kershaw and Christensen (1980) observed a similar result in synaptic areas in a particular Muller axon, I_2 (although they did not see the effect in another group of unidenti-

TABLE V

Active association of synaptic vesicles with the differentiated membrane

There are no significant differences among the control parameters in the different fixatives. Also, there are no significant differences between the K depolarization parameters for the two types of fixative. K depolarization (15 min), regardless of fixative composition, produced a large reduction in total number of synaptic vesicles ($p < 0.001$), a lesser reduction in those vesicles in contact with the differentiated membrane ($p < 0.001$), and a decrease in the length of the differentiated membrane ($p < 0.05$). When the synaptic vesicles in contact with the differentiated membrane were expressed as a percentage of the total vesicle population and normalized to the length of differentiated membrane (column 7), a large increase occurred after K-depolarization in both normal fixative ($p < 0.001$) and fixative containing 10 mM Ca + 50 mM K ($p < 0.02$), indicating a movement of vesicles to the differentiated membrane during transmitter release.

Experimental Condition	Fixative	Total No. of Synapses	No. of Synaptic Vesicles	No. of Vesicles in Contact with Differential Membrane	Length of Differential Membrane (μm)	Percentage of Vesicles in Contact/ μm of Differential Membrane
Control	Normal	20	277 ± 46	7.6 ± 0.8	0.94 ± 0.11	5.6 ± 1.0
Control	+10 mM Ca	22	293 ± 48	8.7 ± 0.9	0.92 ± 0.07	5.8 ± 1.1
Control	+10 mM Ca, 50 mM K	20	256 ± 42	8.2 ± 0.8	0.92 ± 0.08	5.4 ± 1.0
K depolarization	Normal	19	61 ± 14	4.1 ± 0.8	0.73 ± 0.05	14.3 ± 2.0
K depolarization	+10 mM Ca, 50 mM K	20	64 ± 12	3.4 ± 0.1	0.73 ± 0.07	11.6 ± 2.2

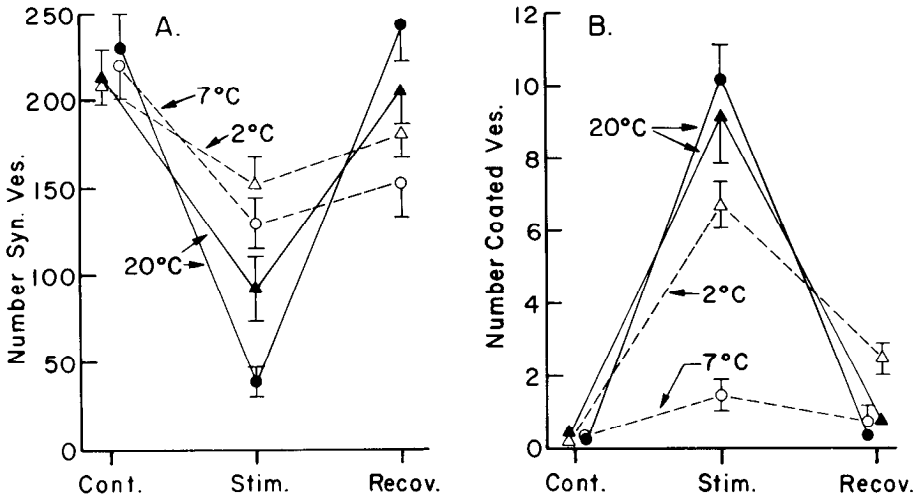

Figure 8. Temperature dependence of changes in number of synaptic vesicles (*A*) and number of coated vesicles (*B*) during control, stimulation, and recovery periods for action potential stimulation (*circles*) and K depolarization (*triangles*). *Open symbols* represent data obtained at 2°C (K depolarization experiments) and 7°C (action potential experiments). *Solid symbols* represent data obtained at 20°C for both K depolarization and action potential experiments. There was no effect of temperature on control values. There was less depletion of synaptic vesicles in the cold for both action potential stimulation ($p < 0.001$) and K depolarization ($p < 0.05$) and less increase in coated vesicles in the cold for both action potential stimulation ($p < 0.001$) and K depolarization ($p < 0.05$). Return to control values during the 60-min recovery appeared to be more complete in the warm than in the cold following both action potential stimulation ($p < 0.05$ for synaptic vesicles) and K depolarization ($p < 0.01$ for coated vesicles), although the difference did not reach statistical significance for coated vesicles in action potential experiments and synaptic vesicles in K depolarization experiments. Each *data point* represents mean ± SEM of at least 20 synapses.

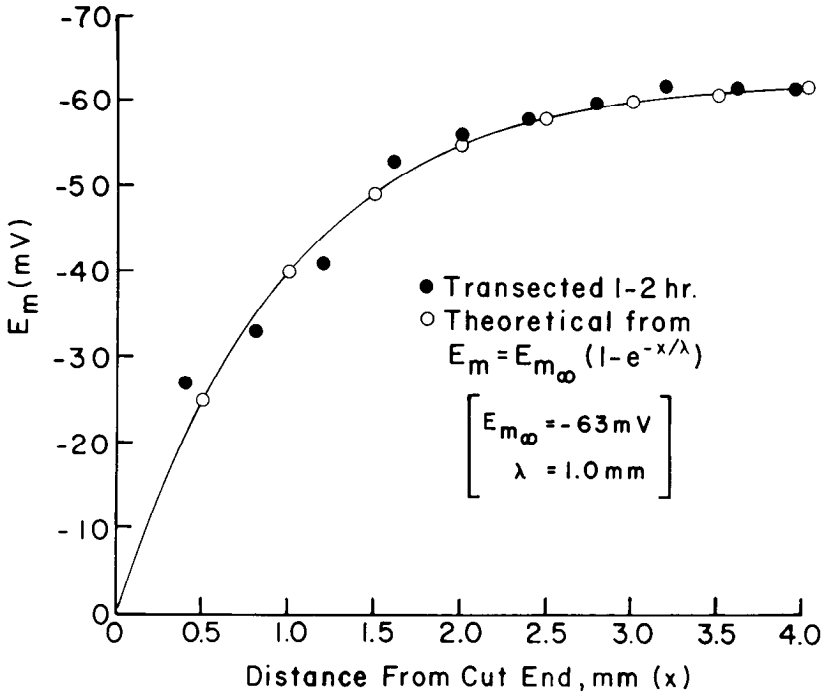

Figure 9. Graph of membrane potential at various distances from the cut end of the transected giant axon. The cut ends of giant axons did not appear to seal as the resting potential increased exponentially with distance from the cut end ($E_m = E_{m\infty}(1 - e^{-x/\lambda})$, where E_m = resting potential at distance x from the cut end, λ = space constant (1 mm), and $E_{m\infty}$ is resting potential at distances greater than 5 mm from the cut end (−63mV)).

fied giant axons), but they considered it a possible artifact of the increased likelihood of tangential sectioning of the more curved presynaptic areas of stimulated synapses. Whether this reasoning is valid or not, it cannot explain our results, since there was only a very small increase in presynaptic curvature during stimulation in the K depolarization experiments, and yet there was a highly significant decrease in the length of the differentiated membrane in these experiments as well as in the action potential experiments in which the increase in curvature of the presynaptic membrane was far greater. The reason for the shrinkage of the differentiated membrane during transmitter release is unclear, but it recovers during rest as do all of the other stimulation-induced changes and, thus, may represent some aspect of the cellular dynamics of the release process. One possibility is that vesicle recognition sites in the differentiated membrane remain attached to synaptic vesicle membrane after exocytosis, are carried into surrounding areas along with the synaptic vesicle membrane, and are eventually recycled back to the active zone. Alternatively, the calcium influx during transmitter release could activate a protease that partially degrades the proteins responsible for the darkened appearance of the differentiated membrane, a process similar to the destruction of Z-bands in skeletal muscle during Ca influx through the acetylcholine channel (Leonard and Salpeter, 1979).

Working on hatchetfish giant synapses, Model et al. (1975) reported that synapses stimulated at cooler temperatures (12 to 14°C) showed many infoldings of the presynaptic membrane and numerous coated vesicles attached to the membrane, whereas at room temperature these were absent and instead there were numerous cisternae within the nerve terminal. It was suggested that the recapture of synaptic vesicle membrane via coated vesicle endocytosis is an energy-requiring step that is blocked at low temperature, leaving the presynaptic membrane in an expanded state from the addition of synaptic vesicle membrane and unable to move it via coated vesicle endocytosis to the next compartment (cisternae) in the

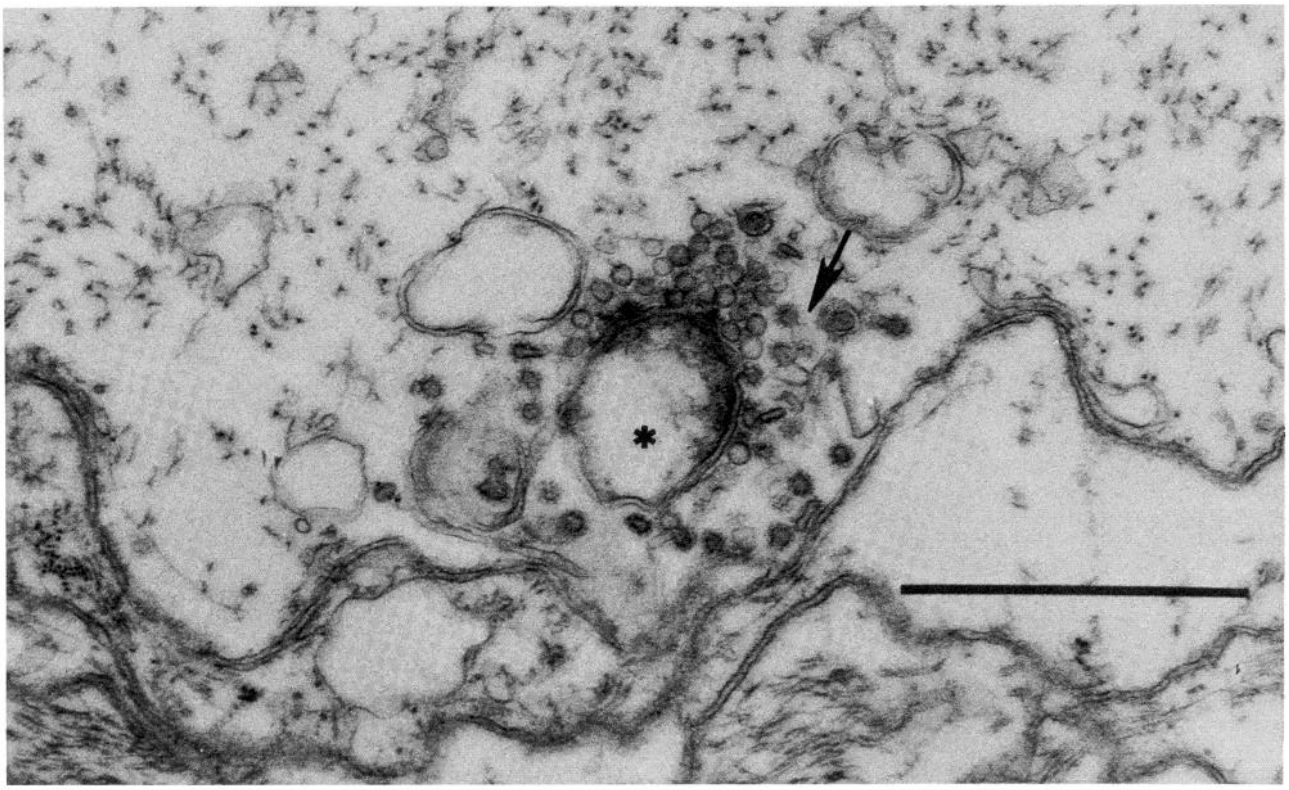

Figure 10. "Surround synapse" 0.25 mm from the cut end of a giant axon in Ca saline. Tissue was fixed 1 min after spinal cord transection. The postsynaptic element (*) is entirely surrounded by the presynaptic giant axon. Such synapses were also found after repetitive action potential activity in Ca-containing saline but were never seen following K depolarization (see Tables I and III). An *arrow* indicates a fusion of several coated vesicles, a common structure in stimulated synapses in both action potential and K depolarization experiments.

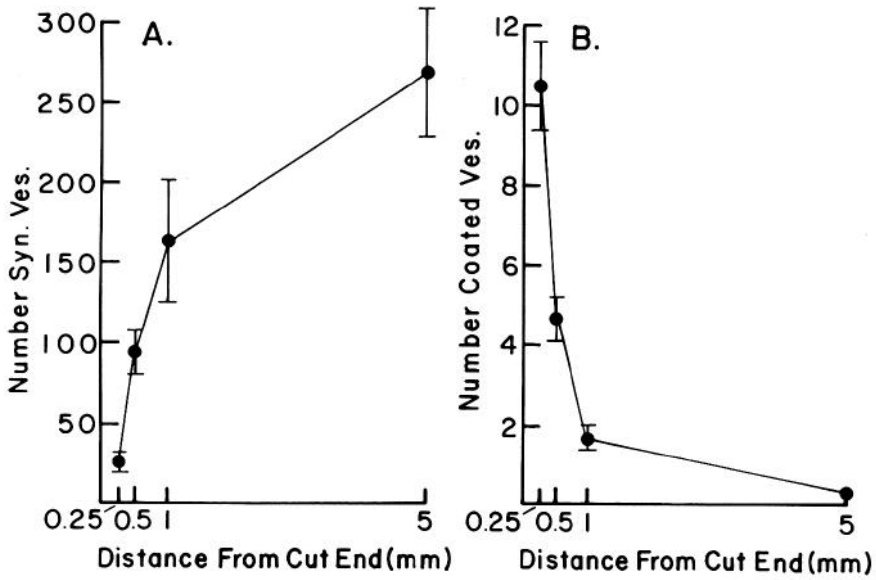

Figure 11. Changes in the number of synaptic vesicles and the number of coated vesicles in synapses located at 0.25, 0.5, 1.0, and 5.0 mm from transected ends of giant axons. *A*, Number of synaptic vesicles per synapse increased with distance away from the cut end. *B*, Number of coated vesicles decreased with distance away from the cut end. To avoid these effects, which occurred in both Ca saline and Mg saline, the electron microscope experiments reported throughout this study were based on sections taken at least 5 mm away from the cut end, except for the experiment of Table VI.

recycling process. We did not observe such qualitative differences at warm and cold temperatures in lamprey giant axons, although the amount of synaptic vesicle depletion and increase in coated vesicles was somewhat less in the cold as was the speed of recovery after stimulation. Coated vesicle endocytosis was certainly not blocked even at 2°C, since the percentages of coated vesicles attached to the membrane and free in the cytoplasm were the same as at 20°C. Perhaps an explanation for this difference in the results for the hatchetfish and lamprey is that the lampreys we used are cold-water fish and are adapted for life at water temperatures approaching freezing, whereas hatchetfish are tropical.

A previous report (Kershaw and Christensen, 1980) suggested that during action potential stimulation there was selective depletion of synaptic vesicles near the differentiated membrane. Although we also found a decline in the absolute number of synaptic vesicles near the differentiated membrane during either action potential activity or K depolarization, quantitative analysis showed that the percentage of vesicles associated with this presumed site of vesicle release was much increased. This result is consistent with several previous reports showing a greater proportion of the total vesicle population located near the synaptic cleft during stimulation (Csillik and Bense, 1971; Korneliussen, 1972; Perri et al., 1972; Model et

TABLE VI

Effect of distance from cut end of axon on synaptic ultrastructure

Data are from a spinal cord transected and soaked for 15 min in Mg-saline at 20°C before fixation. Synapses near (0.25 mm) the cut end show ultrastructural characteristics similar to those of stimulated synapses. Synapses at increasing distances from the cut end appear progressively less stimulated until at 5 mm the synapses have the appearance of unstimulated synapses.

Distance from Cut End (mm)	Total No. of Synapses	No. of Synaptic Vesicles	Vesicular Area (μm^2)	Vesicles/ μm^2	No. of Coated Vesicles	Presynaptic Curvature Index	Total No. of Surround Synapses	No. of Pleomorphic Vesicles	Length of Differentiated Membrane (μm)
0.25	20	26 ± 7	0.10 ± 0.03	279 ± 17	10.5 ± 1.2	0.64 ± 0.08	9	8.4 ± 1.3	0.79 ± 0.08
0.5	21	94 ± 14	0.39 ± 0.06	251 ± 9	4.7 ± 0.6	0.41 ± 0.05	1	6.1 ± 1.3	1.06 ± 0.13
1.0	20	164 ± 39	0.56 ± 0.16	319 ± 11	1.7 ± 0.4	0.22 ± 0.03	0	5.4 ± 0.8	1.15 ± 0.15
5.0	22	270 ± 41	0.71 ± 0.11	385 ± 11	0.3 ± 0.0	0.14 ± 0.02	0	3.1 ± 0.7	0.84 ± 0.09

al., 1975). Working on frog sympathetic ganglia, Dickinson-Nelson and Reese (1983) did find a selective depletion of vesicles near the active zone. They felt that previous failure to see selective depletion along active zones was probably due to the fact that fixative was applied after, rather than during, stimulation. However, our results were the same even when we fixed during action potential stimulation or added 50 mM K and 10 mM Ca to the fixative following K depolarization. Our findings of significant depletion of synaptic vesicles during increased release plus a tendency for the remaining vesicles to be attached to the differentiated membrane suggest that synaptic vesicles are released at the differentiated membrane and are replaced by cytoplasmic vesicles that are actively drawn to this membrane (vesicle mobilization). Whether selective depletion at the active zone is seen at a particular synapse may then depend on the efficiency of the vesicle replacement mechanism versus the rate of transmitter release.

A previous study of lamprey giant axon synapses reported a specific association between synaptic vesicles and microtubules in regions of the axon adjoining presynaptic areas (Smith et al., 1970). It was suggested that synaptic vesicles may be supplied to presynaptic regions via transport on microtubules. In more than a thousand synapses examined in our material with fixation procedures similar to those of Smith et al. (1970), we have seen only a few instances of synaptic vesicles associated with microtubules. Thus, the association of synaptic vesicles with microtubules, although striking in the report of Smith et al. (1970), must be considered a rare event, which probably has little role in the local recycling of vesicles. An attractive idea is that microtubules might be involved in the initial transport of vesicles from the cell body to newly forming synaptic areas and perhaps in long-term turnover of vesicles at established synapses.

During periods of elevated transmitter release, the large cluster of synaptic vesicles adjacent to the differentiated membrane is replaced by an area of roughly equal size that is devoid of neurofilaments and contains a fuzzy filamentous material. Lasek and Hoffman (1976) have proposed that the usual absence of neurofilaments in synaptic boutons may be due to the activation of a Ca-activated protease during Ca influx into the presynaptic terminal. Indeed, Roots (1983) has shown that inhibition of Ca-activated protease with leupeptin can induce the appearance of neurofilaments in boutons of the goldfish optic tectum. The *en passant* synapses in lamprey giant axons are surrounded by axoplasm containing neurofilaments, but it is difficult to tell whether there are any neurofilaments in the cluster of synaptic vesicles. Therefore, the absence of neurofilaments after stimulation could be due either to their absence before stimulation or to degradation during stimulation. Less is known about the fuzzy substance which appears to define the area vacated by synaptic vesicles in stimulated synapses. This material appears to be present between vesicles in unstimulated synapses, and one attractive possibility is that it represents a cytoskeletal apparatus for focusing synaptic vesicles onto the active zone.

The stimulated appearance and the decrease in the number of synapses near the cut ends of lamprey giant axons was a striking observation incidental to results of these experiments. We have not specifically investigated this phenomenon other than to demonstrate (*1*) that it gradually declines at increasing distance from the cut end (0 to 5 mm) with the largest changes occurring in the nearest 1 mm, (*2*) that it occurs in Mg saline and, thus, cannot be simply a result of depolarization since K depolarization does not produce any effect in Mg saline, and (*3*) that it begins within 1 min of the axonal transection. Thus, it would appear that the mechanical effect of transection itself or exposure of the inside of the axon to the bathing saline rapidly initiates a process of stimulation and elimination of synaptic areas within several millimeters of the cut end. Following spinal transection in intact lamprey, the giant axons die back several millimeters, and within a week many of them form large end bulbs from which axonal sprouting occurs (Wood and Cohen 1981; Clark and Wickelgren, 1982). What we have observed in the first minute following axotomy is apparently the beginning of this process, and this preparation could be useful in examining the factors influencing post-traumatic axonal degeneration, membrane sealing, and regrowth.

The stimulated appearance of synapses near axonal cut ends may explain one discrepancy between the results of Kershaw and Christensen (1980) and ours. They reported that approximately 15% of unstimulated synapses were of the surround type, in which the postsynaptic element is surrounded by the giant axon presynaptic process. We have never seen a surround synapse in unstimulated axons except near cut ends, where they are very common. Thus, it may be that Kershaw and Christensen (1980) included some synapses from regions near cut ends in their control data. Despite the increase in variability that this would entail, they reported significant decreases in vesicle number and an expansion of the presynaptic membrane during action potential activity, results which our work confirms.

In summary, the results of this paper have provided detailed, quantitative evidence that ultrastructural changes similar to those at vertebrate neuromuscular junctions (Ceccarelli et al., 1972, 1973; Heuser and Reese, 1973, 1981; Peper et al., 1974; Heuser et al., 1974, 1979) and ganglionic synapses (Pysh and Wiley, 1974; Dickinson-Nelson and Reese, 1983) also occur following action potential-evoked release at vertebrate CNS synapses. The changes induced by K depolarization were similar except for much smaller expansion of the presynaptic membrane and no increase in pleomorphic vesicles during release. We have no evidence regarding the reason(s) for these discrepancies. A structural change induced by stimulation that has not been reported in other preparations is the decrease in the length of the active zone, a finding which suggests this structure is a dynamic participant in the release process.

References

Carlin, R. K., D. C. Bartlet, and P. Siekevitz (1983) Identification of fodrin as a major calmodulin-binding protein in postsynaptic density preparations. J. Cell Biol. *96:* 443–448.

Ceccarelli, B., and W. P. Hurlbut (1980) Vesicle hypothesis of the release of quanta of acetylcholine. Physiol. Rev. *60:* 396–441.

Ceccarelli, B., W. P. Hurlbut, and A. Mauro (1972) Depletion of vesicles from frog neuromuscular junctions by prolonged tetanic stimulation. J. Cell Biol. *54:* 30–38.

Ceccarelli, B., W. P. Hurlbut, and A. Mauro (1973) Turnover of transmitter and synaptic vesicles at the frog neuromuscular junction. J. Cell Biol. *57:* 499–524.

Christensen, B. N. (1976) Morphological correlates of synaptic transmission in lamprey spinal cord. J. Neurophysiol. *39:* 197–212.

Clark, R. D., and W. O. Wickelgren (1982) Effects of spinal transection on brain cell bodies and proximal and distal spinal axonal segments of the lamprey. Soc. Neurosci. Abstr. *8:* 914.

Csillik, B., and B. Bense (1971) Function dependent alterations in the distribution of synaptic vesicles. Acta. Biol. Acad. Sci. Hung. *22:* 131–139.

Davies, P. J. A., and C. B. Klee (1981) Calmodulin binding proteins: A high molecular weight calmodulin binding protein from whole brain. Biochem. Int. *3:* 202–212.

Dickinson-Nelson, A., and T. S. Reese (1983) Structural changes during transmitter release at synapses in the frog sympathetic ganglion. J. Neurosci. *3:* 42–52.

Heuser, J. E., and T. S. Reese (1973) Evidence for recycling of synaptic vesicle membrane during transmitter release at the frog neuromuscular junction. J. Cell Biol. *57:* 315–344.

Heuser, J. E., and T. S. Reese (1981) Structural changes following transmitter release at the frog neuromuscular junction. J. Cell Biol. *88:* 564–580.

Heuser, J. E., T. S. Reese, and D. M. P. Landis (1974) Functional changes in frog neuromuscular junctions studied with freeze-fracture. J. Neurocytol. *3:* 109–131.

Heuser, J. E., T. S. Reese, M. J. Dennis, Y. Jan, L. Jan, and L. Evans (1979) Synaptic vesicle exocytosis captured by quick freezing and correlated with quantal transmitter release. J. Cell Biol. *81:* 275–300.

Israel, M., Y. Dunant, and R. Manaranche (1979) The present status of the vesicular hypothesis. Prog. Neurobiol. *13:* 237–275.

Kershaw, P., and B. N. Christensen (1980) A quantitative analysis of ultrastructural changes induced by electrical stimulation of identified spinal cord axons in the larval lamprey. J. Neurocytol. *9:* 119–138.

Kornelliussen, H. (1972) Ultrastructure of normal and stimulated motor endplates. Z. Zellforsch. *130:* 28–57.

Lasek, R. J., and P. N. Hoffman (1976) The neuronal cytoskeleton, axonal transport and axonal growth. In *Cell Motility*, R. Goldman, T. Pollard, and J. Rosenbaum, eds., pp. 1021–1049, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Lazarides, E., and W. J. Nelson (1982) Expression of spectrin in nonerythroid cells. Cell *31:* 505–508.

Leonard, J. P., and M. M. Salpeter (1979) Agonist-induced myopathy at the neuromuscular junction is mediated by calcium. J. Cell Biol. *82:* 811–819.

Levine, J., and M. Willard (1981) Fodrin: Axonally transported polypeptides associated with the internal periphery of many cell types. J. Cell Biol. *90:* 631–643.

Liley, A. W. (1956) The effects of presynaptic polarization on the spontaneous activity at the mammalian neuromuscular junction. J. Physiol. (Lond.) *134:* 427–443.

Model, P. G., S. M. Highstein, and M. V. L. Bennett (1975) Depletion of vesicles and fatigue of transmission at a vertebrate control synapse. Brain Res. *98:* 209–228.

Peper, K., F. Dryer, C. Sandri, K. Akert, and H. Moor (1974) Structure and ultrastructure of the frog motor endplate: A freeze etching study. Cell Tissue Res. *149:* 437–455.

Perri, V., O. Sacchi, E. Raviola, and G. Raviola (1972) Evaluation of the number and distribution of synaptic vesicles at cholinergic nerve endings after sustained stimulation. Brain Res. *39:* 526–529.

Pfenninger, K. H., and C. M. Rovainen (1974) Stimulation- and calcium-dependence of vesicle attachment sites in the presynaptic membrane: A freeze-cleave study on the lamprey spinal cord. Brain Res. *72:* 1–23.

Pysh, J. J., and R. G. Wiley (1974) Synaptic vesicle depletion and recovery in cat sympathetic ganglia electrically stimulated *in vivo*. J. Cell Biol. *60:* 365–374.

Roots, B. I. (1983) Neurofilament accumulation induced in synapses by leupeptin. Science *221:* 971–972.

Rovainen, C. M. (1967) Physiological and anatomical studies on large neurons of central nervous system of the sea lamprey (*Petromyzon marinus*). II. Dorsal cells and giant interneurons. J. Neurophysiol. *30:* 1024–1042.

Rovainen, C. M. (1974) Synaptic interactions of reticulospinal neurons and nerve cells in the spinal cord of the sea lamprey. J. Comp. Neurol. *154:* 207–223.

Rovainen, C. M. (1979) Neurobiology of lampreys. Physiol. Rev. *59:* 1007–1077.

Smith, D. S., V. Jarlfors, and R. Beranek (1970) The organization of synaptic axoplasm in the lamprey (*Petromyzon marinus*) central nervous system. J. Cell Biol. *46:* 199–219.

Sobue, K., K. Kanda, J. Adachi, and S. Kakiuchi (1983) Calmodulin-binding proteins that interact with actin filaments in a Ca^{2+}-dependent flip-flop manner: Survey in brain and secretory tissues. Proc. Natl. Acad. Sci. U. S. A. *80:* 6868–6871.

Tauc, L. (1982) Nonvesicular release of neurotransmitter. Physiol. Rev. *62:* 857–893.

Wickelgren, W. O. (1975) Structural presynaptic changes following repetitive action potential activity in giant reticulospinal axons of lamprey. Soc. Neurosci. Abstr. *1:* 663.

Wickelgren, W. O. (1977) Physiological and anatomical characteristics of reticulospinal neurons in lamprey. J. Physiol. (Lond.) *270:* 89–114.

Wood, M. W., and M. J. Cohen (1981) Synaptic regeneration and glial reactions in the transected spinal cord of the lamprey. J. Neurocytol. *10:* 57–79.