SYNAPTIC VESICLE DEPLETION AND RECOVERY IN CAT SYMPATHETIC GANGLIA ELECTRICALLY STIMULATED IN VIVO

Evidence for Transmitter Secretion by Exocytosis

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ABSTRACT

This study examined the ultrastructure of presynaptic terminals after short periods of vigorous acetylcholine (ACh) secretion in the cat superior cervical ganglion in vivo. Experimental trunks of cats anesthetized with chloralose-urethane were stimulated supramaximally for periods of 15–30 min and at several frequencies including the upper physiological range (5–10 Hz). Stimulated and contralateral control ganglia from each animal were fixed by intra-arterial aldehyde perfusion, processed simultaneously, and compared by electron microscopy. Stimulation produced an absolute decrease in the number of synaptic vesicles, an enlargement of axonal surface membrane, and distinct alterations in the shape of presynaptic terminals. Virtually complete recovery occurred within 1 h after stimulation at 10 Hz for 30 min. These results support the hypothesis that ACh release at mammalian axodendritic synapses occurs by exocytosis of synaptic vesicles resulting in the incorporation of vesicle membrane into the presynaptic membrane and that synaptic vesicles subsequently are reformed from plasma membrane.

INTRODUCTION

Recently, we have observed striking alterations in the ultrastructure of presynaptic terminals in the cat superior cervical ganglion after prolonged high frequency electrical stimulation of the cervical sympathetic trunk in vivo. Stimulation produced a reduction in synaptic vesicles, an enlargement of surface membrane, and a distinct alteration in the shape of nerve terminals (22). Such morphological changes may be interpreted to result from the persistent secretion of transmitter by a mechanism of exocytosis of synaptic vesicles, in which synaptic vesicle membrane is incorporated into the presynaptic membrane during stimulation and subsequently recovered from it during rest. The purpose of the present study was to determine (*a*) whether such morphological alterations of presynaptic terminals occur under more physiological conditions of stimulation and (*b*) whether they are reversible. This study establishes that synaptic vesicle depletion and axonal surface enlargement occur in the cat superior cervical ganglion during more physiological stimulation and slowly reverse during a subsequent rest period. Preliminary reports in abstract form of some of these findings have appeared elsewhere (23, 24).

MATERIALS AND METHODS

22 adult mongrel cats of both sexes weighing 1.6–3.5 kg were used in these experiments. They were anes-

thetized by an intraperitoneal injection of a mixture of chloralose (40 mg/kg) and urethane (400 mg/kg). Both cervical sympathetic trunks were surgically exposed low in the neck. One side served as a sham-operated control while the other trunk was placed on a platinum bipolar-stimulating electrode and packed in petroleum jelly. In this study, the sympathetic trunks were not sectioned so as not to interfere with recovery. The right sympathetic trunk was stimulated in 12 experiments and the left in another 10 experiments.

Stimulation, several times supramaximal, consisted of a continuous train of rectangular pulses each 1 ms in duration and 20 V in amplitude (0.7–3.2 mA). Periodically the electrode was advanced toward the ganglion to insure continually effective stimulation. Stimulus current was monitored continuously and to assess the effectiveness of stimulation, nictitating membrane contractions were recorded using force-displacement transducers connected to a Grass polygraph (Grass Instrument Co., Quincy, Mass.). The various combinations of stimulus frequencies and duration, and the timing of fixation, are detailed with the Results. These included stimulation at 10 Hz which lies within the physiological range (13).

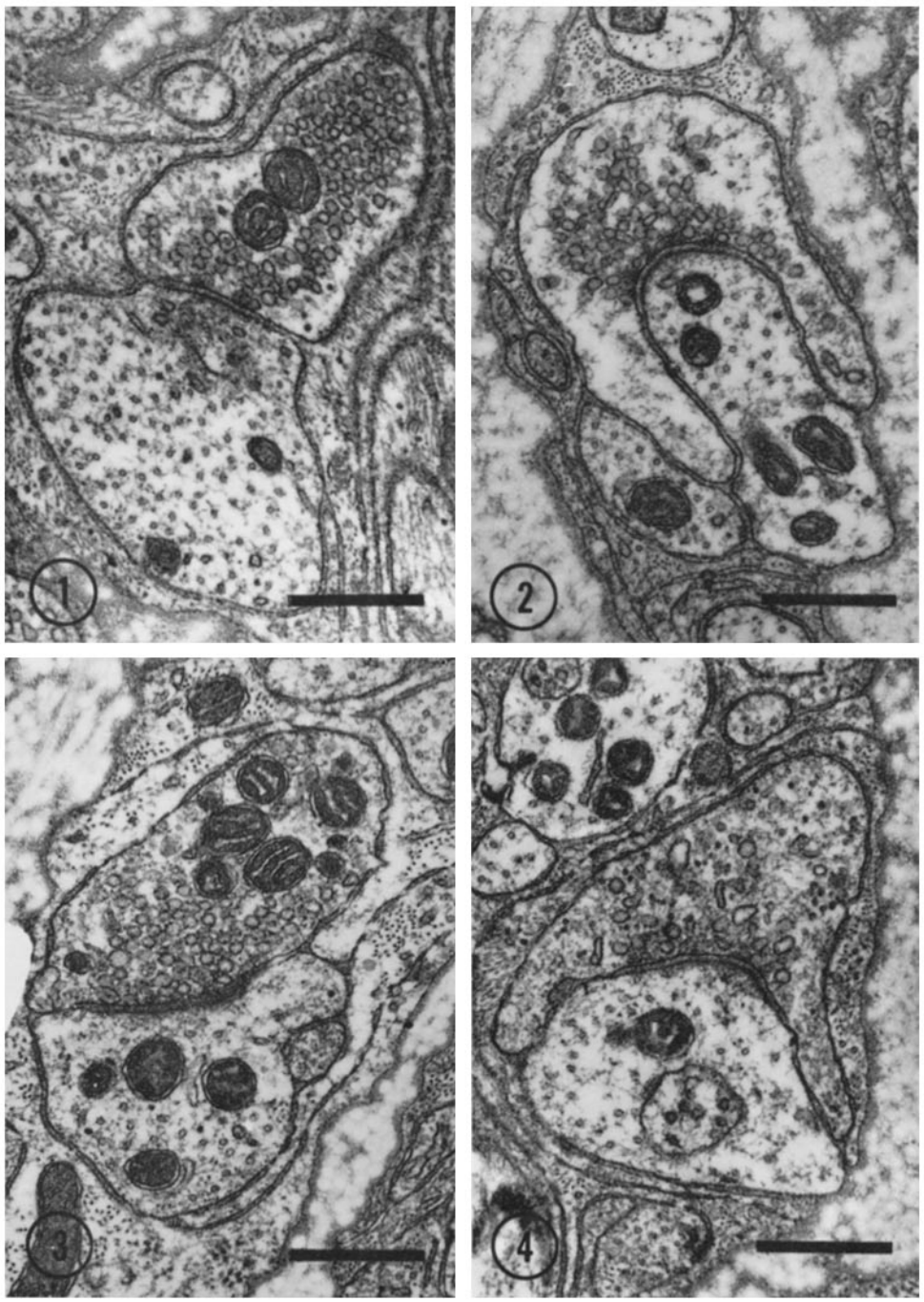
Primary fixation was accomplished by arterial perfusion of a fixative consisting of 4% formaldehyde (freshly prepared from paraformaldehyde) and 2.5% glutaraldehyde in a phosphate buffer (17) at pH 7.35 and at room temperature. Approximately 30 s before the termination of each experiment, a polyethylene catheter for perfusion of fluids was positioned in the root of the ascending aorta while the animal was artificially ventilated. After a 10-s saline wash, the fixative was perfused at a pressure of 140 cm H_2O for 10 min and experimental and control ganglia from each experiment were removed and processed simultaneously. After storage overnight in fixative at 4°C, the ganglia were cut into 1-mm transverse slices, washed in cold 0.1 M phosphate buffer for 1 h, postfixed in 1% OsO_4 in Veronal-acetate buffer for 1 h, dehydrated in ascending concentrations of ethanol, and embedded in Araldite 502. Thick sections (0.75 μm) stained with methylene blue were examined by light microscopy to check tissue preservation and large, thin silver sections cut in the transverse plane through the middle of the ganglion were used for electron microscopy.

Electron micrographs were obtained in two ways. In some experiments, survey electron micrographs (× 4,000) were taken at the corners of grid holes to sample systematically all tissue of single sections from individual blocks. In all experiments, electron micrographs taken at × 20,000 and enlarged photographically to × 71,000 were obtained of *all* axodendritic synapses encountered in a single section from each block studied. Random electron micrographs at this higher magnification, selected by lottery, were used for all morphometric analyses. Profiles of presynaptic terminals were identified on the basis of the presence of characteristic synaptic densities and synaptic vesicles. Because transverse sections were used exclusively, almost all nerve terminals measured in our electron micrographs were cut transverse or slightly oblique to the dendrite as judged by dendritic microtubular shape. A few presynaptic profiles cut longitudinal to the dendrite were not measured. Electron micrographs were unlabeled and the experimental condition for any given synapse was not identified until all measurements were completed. All agranular vesicles ranging in size from 300 to 650 Å in outside diameter (mean OD = 500 Å) were counted. Electron micrographs were occasionally subjected to repeat vesicle counts to check reproducibility. Circumferences of axonal profiles were estimated with a map measure (Keuffel & Esser Co.) and areas of profiles were measured using a planimeter. Profile circumference and area were studied as two-dimensional analogues of surface area and volume, respectively.

RESULTS

In nonstimulated superior cervical ganglia, axodendritic synapses had a typical ultrastructure as seen in Figs. 1 and 3. They possessed synaptic densities and presynaptic terminals containing synaptic vesicles. Vesicles were primarily of the small agranular spheroidal variety, ranging in diameter from 300 to 650 Å. Dense-cored vesicles, 700–1,000 Å in diameter comprised a smaller fraction of the vesicle population. Profiles of presynaptic terminals in transverse section generally were oval in shape with a regular contour and ranged in diameter from 0.5 to about 2.5 μm or rarely larger. Presynaptic terminals varied in vesicle content. Some endings were virtually completely filled with densely packed or overlapping vesicles while other endings were only partially filled with loosely packed vesicles. Such variability seemed greater generally among different animals than within individual ganglia. The present observations of presynaptic terminals in unstimulated ganglia agree closely with those derived from electron microscope examination of serial sections of normal superior cervical ganglia of the cat (9).

In stimulated superior cervical ganglia, under all conditions examined, two striking alterations in axodendritic synapses were evident in electron micrographs. Many presynaptic terminals showed both a change in shape and a reduction in vesicle numbers (Figs. 2 and 4). Such altered nerve terminals, when viewed in sections cut transverse to



FIGURES 1 and 3 Axodendritic synapses from two different control ganglia. Axon terminals were generally bulbous shaped and variably packed with spheroidal, clear-cored synaptic vesicles.

FIGURES 2 and 4 Examples of altered axodendritic synapses in ganglia stimulated at 10 Hz for 30 min from the same two cats as in Figs. 1 and 3. Stimulation produced the following prominent changes in some presynaptic terminals: an absolute reduction in number of vesicles; an increase in surface membrane; and an enlargement of the zone of axodendritic apposition. Figs. 1 and 2 from cat no. 15; Figs. 3 and 4 from cat no. 16; Bars = 0.5 μm. × 37,500.

the postsynaptic dendrite, appeared irregular and often crescent shaped. The surface membrane of the normally bulbous presynaptic terminal appeared to expand along a plane between the dendrite and the overlying Schwann cell, to form an extended crescent-shaped contact with the dendrite (Figs. 2 and 4). These ultrastructural alterations, some quite marked in degree, occurred at 60–80% of all axodendritic synapses in stimulated ganglia, whereas crescent-shaped axonal profiles, most only slightly altered, occurred at only 4–6% of all axodendritic synapses in unstimulated control ganglia.

In stimulated nerve endings that showed prominent alterations in contour there were clearly reduced numbers of synaptic vesicles (Figs. 2 and 4). Because the normal interanimal variability in vesicle content was substantial, it was not always possible to arrive at a subjective judgment of the effect of stimulation on vesicle numbers from examination of electron micrographs of only stimulated ganglia. However, comparison of electron micrographs of stimulated ganglia with those of contralateral controls revealed that stimulation increased the proportion of endings containing fewer vesicles. Stimulation also produced synaptic vesicles which were often pleomorphic or flattened in shape. Comparisons, in addition, revealed that a subpopulation of endings (20–40%) was apparently unaltered in vesicle content by stimulation.

No alterations in mitochondria were seen after 10 Hz stimulation. After 20 and 40 Hz stimulation many nerve terminals contained swollen mitochondria, some appearing two to three times larger with a relatively less dense matrix.

The above alterations in nerve terminal shape and vesicle numbers were most prominent in ganglia stimulated at 40 Hz for 30 min and 20 Hz for 15 min and least prominent in ganglia stimulated at 5 Hz for 15 min.

Morphometric Analysis

In order to specify more precisely the extent and frequency of the synaptic alterations described above, certain measurements were performed on ganglia stimulated at 10 Hz for 30 min. To assess the degree of increase in axonal surface membrane, the circumferences of presynaptic profiles were measured in stimulated and control ganglia. This parameter is dependent on the size and shape of axonal terminals; endings which are large or irregular in shape will have a large profile circumference. Data from experiments stimulated at 10 Hz for 30 min are illustrated in Fig. 5. In the control ganglia (Fig. 5 A) the range of normal values for presynaptic profile circumference is due to the normal range of sizes of axonal endings, since larger endings have greater profile circum-

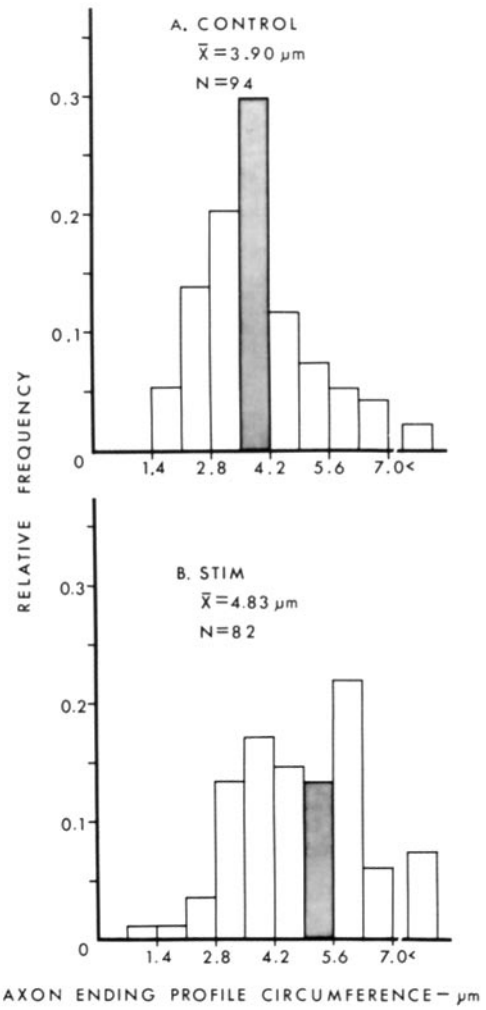


FIGURE 5 Relative frequency histograms of measurements of presynaptic profile *circumferences*, pooled from three experiments. Fig. 5 A, control ganglia; Fig. 5 B, contralateral ganglia stimulated at 10 Hz for 30 min. Histogram (B) shows that stimulation produced greater numbers of axonal profiles with larger circumferences. The far right column represents all axonal profiles with circumferences larger than 7 μm. Shading indicates location of median values, $\bar{X}$ the means, and N the number of presynaptic profiles measured for each condition.

TABLE I

Axon Ending Profile Circumferences (Microns)—Individual Ganglion Means

Condition	Exp. no.	Stimulated	Control	
40 Hz × 30 min	1	5.11 (22)	3.91 (10)	1.21
	2	5.23 (15)	3.90 (13)	1.33
	3	5.27 (17)	3.69 (24)	1.57
		$\overline{X}$ = 5.20	3.83	1.37
20 Hz × 15 min	10	5.56 (12)	4.18 (16)	1.38
	11	4.68 (16)	3.66 (18)	1.02
	12	5.44 (15)	3.74 (15)	1.72
		$\overline{X}$ = 5.23	3.86	1.37
10 Hz × 30 min	15	4.87 (26)	3.46 (35)	1.41
	16	4.84 (31)	3.97 (36)	0.87
	17	4.75 (24)	4.27 (23)	0.48
		$\overline{X}$ = 4.82	3.90	0.92

Each entry represents the mean of all presynaptic profiles measured from a particular ganglion. The numbers in parentheses indicate the number of presynaptic profiles used to obtain each mean. The last column (far right) shows the differences between each pair of ganglia from the same animal (stimulated vs. control). The mean difference for the nine experiments stimulated without recovery is significantly different from zero ($P < 0.02$, $t = 3.213$, 8 df).

ferences. In stimulated ganglia, measurements revealed increased numbers of presynaptic profiles with larger circumferences (Fig. 5 B); some were two- to three-fold larger suggesting a considerable growth of the surface membrane of some axonal terminals. However, normal variability and the 20–40% of profiles unaltered by stimulation made it difficult to judge the statistical significance of the changes seen in Fig. 5. Therefore, additional circumference measurements were made on nerve endings from other stimulation conditions. These data are shown in Table I. It can be seen that stimulation under various conditions produced a statistically significant increase in the mean profile circumference of axon endings ($P < 0.02$, $t = 3.213$, 8 df) indicating that stimulation increased the surface membrane of axon terminals in cat superior cervical ganglia. The mean increase in profile circumference of axon endings was 0.92 μm for ganglia stimulated at 10 Hz for 30 min. Stimulation also appeared to produce an increase within some presynaptic terminals of nonvesicular membranous profiles consisting of vacuoles (0.1–0.2 μm in diameter) and cisternae (0.2–0.4 μm long) resembling smooth endoplasmic reticulum. The linear extent of such nonvesicular membrane in presynaptic terminals was measured with a map measure in three ganglia stimulated at 10 Hz for 30 min and in their contralateral control ganglia. The pooled results showed that stimulation produced a mean linear increase in nonvesicular membrane of 0.31 μm (range 0.25–0.43 μm) per presynaptic profile based upon a comparison of means for each ganglia. Combining the mean increases in circumference of axonal profiles (0.92 μm) and nonvesicular membrane (0.31 μm) showed that stimulation at 10 Hz for 30 min produced a total mean increase in axonal membrane of 1.23 μm per presynaptic profile.

To assess the decrease in synaptic vesicles, the number of vesicles in each presynaptic profile was divided by the profile area to obtain the synaptic vesicle packing density (no./μm²). Fig. 6, the pooled results of measurements from three experiments, shows that stimulation produced a marked decrease in the mean synaptic vesicle packing density of presynaptic terminals. In stimulated ganglia, 80% of axonal terminals were included in the three lowest categories whereas only 22% of the control endings appeared in these same categories (Fig. 6). A two-way analysis of variance revealed a highly significant effect of stimulation ($F = 20.1$ $1/168$ df, $P < 0.01$). It is possible that the packing of synaptic vesicles in presynaptic terminals might be reduced by swelling of axon endings resulting from stimulation. Enlarged

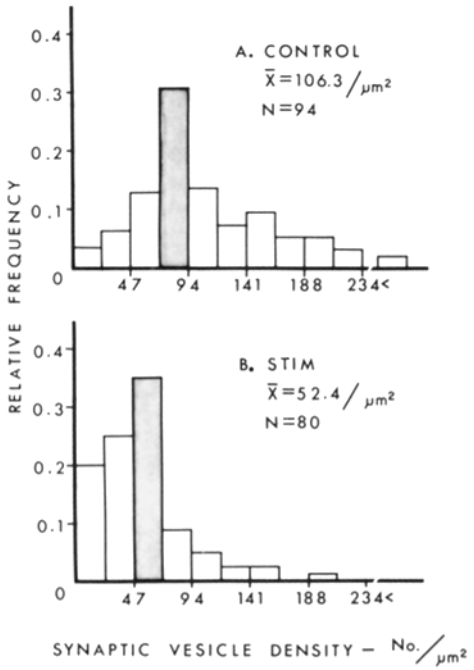


FIGURE 6 Relative frequency histograms of pooled measurements of presynaptic profiles from same experiments as in Fig. 5 plotted according to *vesicle packing density*. Histogram (B) shows increased numbers of presynaptic profiles with lower synaptic vesicle packing resulting from stimulation at 10 Hz for a period of 30 min. The far right column in control histogram (A) represents all endings having packing densities greater than 234 vesicles/μm^2. Symbols same as in Fig. 5.

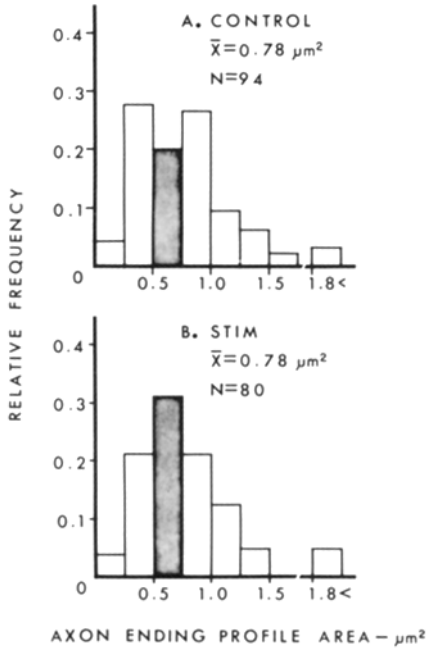


FIGURE 7 Relative frequency histograms of pooled measurements of presynaptic profile *areas* from same experiments as Figs. 5 and 6 (stimulation at 10 Hz for 30 min), showing no effect of stimulation on profile areas. The far right column represents all profiles with areas greater than 1.8 μm^2. Symbols same as in Fig. 5.

presynaptic profiles were not observed by visual inspection, nevertheless, in order to detect possible smaller degrees of swelling of axon terminals resulting from stimulation, the areas of presynaptic profiles in control and stimulated ganglia were measured and plotted in frequency distribution histograms as illustrated in Fig. 7. It can be seen that no change in the mean area of presynaptic profiles or in the pattern of the population distributions resulted from stimulation indicating that stimulation did not produce swelling of axon terminals. It is also possible that a reduction in the number of vesicles in profiles of stimulated presynaptic terminals might result from a redistribution of vesicles away from the points of synaptic densities producing an increase in vesicle packing in profiles of axon endings without synaptic densities. Such profiles were searched for and examined in our electron micrographs. Simple visual examination sugested that stimulation produced a slight decrease in vesicle packing in profiles of axonal terminals without synaptic densities, nevertheless, to make a better assessment, counts of vesicles and measurements of axonal profile areas were made of terminal profiles without synaptic densities. These data indicated that stimulation produced a slight but statistically insignificant decrease in synaptic vesicle packing and no change in the size of these parts of the nerve terminals. Therefore, we conclude from these morphological observations that repetitive electrical stimulation resulted in an absolute decrease in the number of synaptic vesicles in presynaptic terminals of the cat superior cervical ganglia.

Calculations were performed on the data from ganglia stimulated at 10 Hz for 30 min to compare the decrease in vesicle membrane to the increase in surface and nonvesicular membrane. First, the theoretical number of synaptic vesicles needed to produce the observed increase in axonal membrane was calculated. We observed a mean linear increase in axonal surface and nonvesicular

membrane of 1.23 μm per profile of axon terminal. In a section 700 Å thick, this indicates the production of 8,610,000 (Å)^2 of membrane per section of axon terminal. The membrane area of a synaptic vesicle was estimated to be 541,000 (Å)^2 using the formula for the surface of a sphere, SA = πd^2 (vesicle diameter was taken as the average of the mean inner and outer diameters or 415 Å). Thus, the theoretical number of synaptic vesicles needed to produce the observed increase in axonal membrane was found to be 16 vesicles per section of axon terminal. Next, the actual number of vesicles lost per section of axon terminal was calculated from the observed data. Observed vesicle numbers are an overestimation of the actual number of vesicles in sections because of the inclusion in the tissue sections of profiles of vesicles whose centers are outside, but not more than one radius away from, the plane of section (16). Therefore, we multiplied the observed means of vesicle numbers per axon profile for control and stimulated ganglia by a correction factor $\frac{T}{T + 2R}$ where T is the section thickness (700 Å) and $2R$ is the mean vesicle diameter (500 Å). The actual number of vesicles that disappeared during stimulation was found to average 24 vesicles per section of axon terminal. Thus, we conclude that the loss of vesicle membrane after stimulation is at least sufficient to equal the increase in axonal surface and nonvesicular membrane. While our analysis suggests that somewhat more vesicular membrane is lost than recovered in other membrane, variability in the data, uncertainty over whether vesicle membrane surface area would be conserved during fusion, and lack of knowledge of movement of surface membrane out of the plane of sections studied render this calculation an approximation only and a more detailed study will be required to resolve this point.

Recovery

In order to evaluate the reversibility of the above ultrastructural alterations, ganglia were stimulated and allowed to rest for various periods before fixation. After 40 Hz stimulation for 30 min, a substantial but incomplete return toward normal morphology occurred after a rest period of 30 min. After a rest period of 90 min, most synapses appeared normal while a small number were still somewhat altered.

After 10 Hz stimulation for 30 min and a rest period of 60 min, virtually all nerve endings appeared normal (Figs. 8 and 9) although vesicles sometimes appeared flattened or pleomorphic. Morphometric data supported these visual observations indicating that almost all presynaptic terminals after rest were indistinguishable from unstimulated controls in vesicle content, or profile circumference.

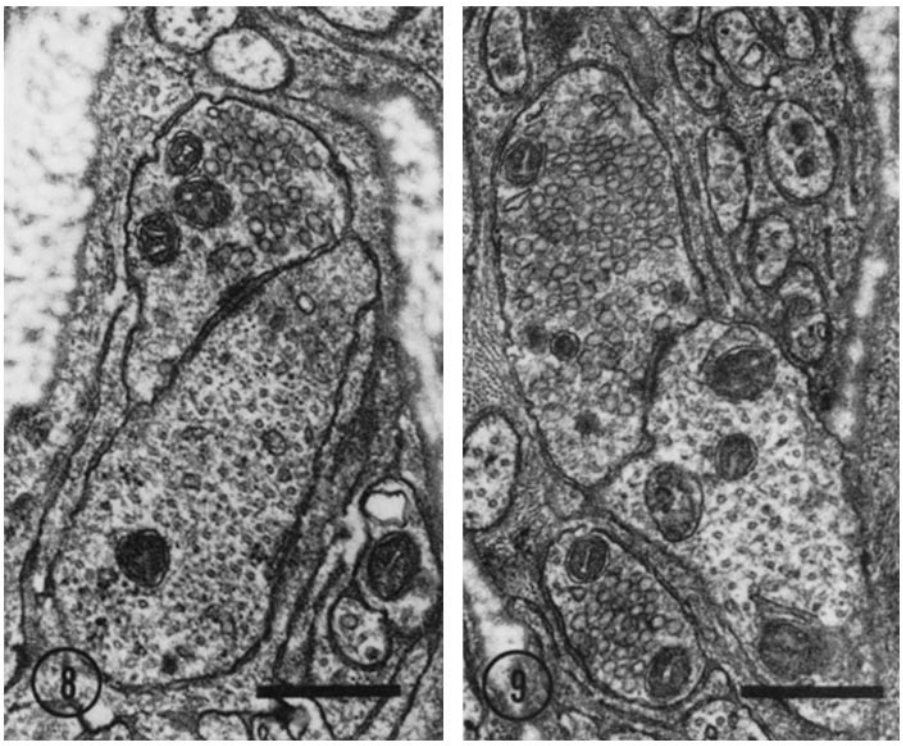
A summary of some of our findings is illustrated in Fig. 10. Electrical stimulation of cat superior cervical ganglia produced an absolute reduction in the number of synaptic vesicles and a concomitant enlargement of surface membrane of presynaptic terminals. After a sufficient period of recovery, vesicle content and surface membrane of axonal terminals returned to normal.

DISCUSSION

These experiments confirm that presynaptic terminals in the cat superior cervical ganglion become depleted of synaptic vesicles and distorted by surface enlargements during in vivo nerve stimulation. Furthermore, these experiments demonstrate that such morphological changes occur even during relatively mild stimulation approaching the normal physiological range, and under these circumstances the morphological changes are completely reversible.

The concident decrease in synaptic vesicles and increase in nerve terminal plasma membrane amounts to a net shift of visible membrane from vesicles to the surface. If nerve stimulation produces transmitter release by evoking exocytosis of synaptic vesicles, the present results would suggest that during stimulation of this synapse the rate of vesicle fusion with the plasma membrane exceeds the rate of vesicle recovery from the surface, while during rest vesicle recovery from the surface continues until normal morphology is restored.

Recently numerous laboratories have reported a reduction of vesicles in various tissues under a variety of experimental conditions which elevate acetylcholine (ACh) release. Vesicle depletion in cholinergic nerve terminals have been reported in: (*a*) in mammalian sympathetic ganglia after electrical stimulation, alone (3, 10, 21–24), in the presence of HC-3 (20), or in the absence of choline (19); (*b*) at mammalian neuromuscular junctions after electrical stimulation, alone (18) or in the presence of HC-3 (16), or after chemical stimulation with K^+ (15); (*c*) at amphibian neuromuscular junctions, after chemical stimulation by lanthanum (11) or black widow spider venom (7), or



FIGURES 8 and 9 Axon terminals from two different ganglia allowed to recover for 1 h after stimulation at 10 Hz for 30 min. Almost all presynaptic terminals returned to normal morphology and were indistinguishable from unstimulated controls in vesicle content, profile circumference, and profile shape. Fig. 8 from cat no. 22; Fig. 9 from cat no. 24. Bars = 0.5 μm. × 37,500.

after electrical stimulation, for short periods at moderate frequencies (6, 12) or for long periods at low frequencies (5); and (*d*) at crustacean neuromuscular junctions after electrical stimulation in the presence of a metabolic inhibitor (2).

With few exceptions (22–24) little data has been reported regarding the fate of vesicles during transmitter release at a mammalian *synapse*, although, the correlation of a reduction in the number of synaptic vesicles and an enlargement of the axonal plasma membrane has been noted in the stimulated amphibian neuromuscular junction (5–7, 11, 12). The present findings suggest that the mechanism of ACh release at synapses of mammalian sympathetic ganglia occurs by exocytosis involving the fusion and the incorporation of synaptic vesicle membrane into the presynaptic plasma membrane. Morphological evidence suggests that a similar exocytotic mechanism of secretion occurs at the neuromuscular junction (5–7, 11, 12, 14), in the neurohypophysis (9), in glands (1), and in other tissues (25). Thus, exocytosis with incorporation of vesicle or secretory granule membrane into plasma membrane may be a common mechanism of secretion in a variety of tissues including the cholinergic neuron. Differ-

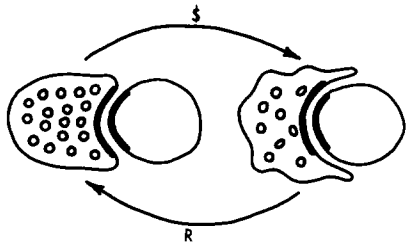


FIGURE 10 Schematic diagram summarizing the effect of electrical stimulation on the ultrastructure of axon terminals in cat superior cervical ganglion. Stimulation at frequencies of 5–40 Hz for 15–60 min produced an absolute decrease in the number of synaptic vesicles, an increase in axon terminal surface membrane area, and a larger zone of axodendritic apposition. Quiescent recovery resulted in reversal of these changes within 60 min after stimulation at 10 Hz for 30 min.

ences between the cholinergic neuron and other secretory cells may reside in the specific mechanism and kinetics of reutilization of vesicular membrane (1, 6, 8, 12).

Our interpretation of the present data predicts that there will be a depletion of synaptic vesicles and an increase in nerve ending plasma membrane whenever the fusion rate (ACh release rate) exceeds the maximum vesicle membrane reutilization rate. This might occur intermittently in the intact animal since maximal reflex sympathetic activation can reach 10–15 Hz (13). However, the degree of vesicle depletion should be determined not only by the difference between the rates of vesicle fusion and vesicle reformation but also by the duration of the stimulation period. This prediction is supported by the present observations that the depletion of vesicles is more pronounced with stimulation at 40 Hz for 30 min than at 5 Hz for 15 min. Under normal conditions of slower, more intermittent activity, 1–2 Hz (13), the reutilization rate of membrane may be adequate to maintain the normal complement of synaptic vesicles in preganglionic nerve terminals. Clearly, the vesicle fusion rate and the reutilization rate may depend upon species and tissue variables and upon the particular choice of experimental conditions.

It is not clear from the present observations whether or not extensive mixing occurs between vesicle and plasma membrane constituents. It would seem possible that vesicle membrane could maintain its apparently distinctive composition during the brief time it is normally fused with the plasma membrane. At least, the reported differences in vesicle and plasma membrane lipid compositions (26) do not rule out the fusion hypothesis without further understanding of the processes of fusion and reutilization and the migration of lipid constituents within intact biological membranes. The present results suggest that vesicles, after exocytosis, do not remain as omega figures attached to the surface membrane but completely merge flat with the plasma membrane and must be reformed completely. These results shed no light on the possible mechanisms of vesicle reformation: whether by a direct reverse at the same point on the surface membrane of all of the membrane movements of exocytosis (5), or by attachment elsewhere to a special cytoplasmic apparatus for vesicle reformation from the surface (12). Furthermore, the present results primarily relate to the issue of the turnover of vesicle membrane during periods of synaptic activity and may have little bearing on the issues of the initial synthesis and final degradation of vesicle membrane.

Birks and MacIntosh (4) have shown that the rate of ACh release from perfused cat superior cervical ganglion stimulated at 20 Hz falls off during the first 5 min of stimulation from an initial high rate to a lower sustained plateau level (steady state) which is relatively constant for up to 60 min of continued stimulation, although the total ACh content of the ganglia remained constant. This suggests that transmitter release is limited not by the total amount of ACh in the terminal but by the rate at which it is made available in some particular form for release. If transmitter is released exclusively by exocytosis of synaptic vesicles, then a decline in their numbers could reduce the rate at which ACh is made available for release. The present results seem entirely consistent with such a scheme.

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