Structural Changes after Transmitter Release at the Frog Neuromuscular Junction

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ABSTRACT The sequence of structural changes that occur during synaptic vesicle exocytosis was studied by quick-freezing muscles at different intervals after stimulating their nerves, in the presence of 4-aminopyridine to increase the number of transmitter quanta released by each stimulus. Vesicle openings began to appear at the active zones of the intramuscular nerves within 3-4 ms after a single stimulus. The concentration of these openings peaked at 5-6 ms, and then declined to zero 50-100 ms later. At the later times, vesicle openings tended to be larger. Left behind at the active zones, after the vesicle openings disappeared, were clusters of large intramembrane particles. The larger particles in these clusters were the same size as intramembrane particles in undischarged vesicles, and were slightly larger than the particles which form the rows delineating active zones. Because previous tracer work had shown that new vesicles do not pinch off from the plasma membrane at these early times, we concluded that the particle clusters originate from membranes of discharged vesicles which collapse into the plasmalemma after exocytosis. The rate of vesicle collapse appeared to be variable because different stages occurred simultaneously at most times after stimulation; this asynchrony was taken to indicate that the collapse of each exocytotic vesicle is slowed by previous nearby collapses. The ultimate fate of synaptic vesicle membrane after collapse appeared to be coalescence with the plasma membrane, as the clusters of particles gradually dispersed into surrounding areas during the first second after a stimulus. The membrane retrieval and recycling that reverse this exocytotic sequence have a slower onset, as has been described in previous reports.

This paper considers the fate of the membranes of synaptic vesicles after they fuse with the surface of nerve terminals at frog neuromuscular junctions. Previous work has shown that vesicle-size pockets, which have been identified as synaptic vesicle openings, appear in the surface membrane of nerve terminals after stimulation (12, 18). Recently, quantitative evaluation of freeze-fracture replicas has illustrated that vesicle openings are just as abundant as the number of quanta released by a single nerve impulse, over a wide range of quantal release (11).

To investigate whether exocytosis by a single synaptic vesicle is the structural basis for the release of a quantum of transmitter, two unusual procedures were combined (11). First, a rapid arrest of structural change was provided by freezing muscles against a copper block cooled with liquid helium. Physical measurements of freezing rates indicated a freezing time of 0.5-2.0 ms with this method (11, 23). Second, quantal release was increased with 4-aminopyridine (4-AP), because during normal release so few vesicle openings occur that quantitative analysis was impractical; each nerve impulse ought normally to discharge only 100-200 vesicles, or 1-2% of the 10,000 or more that line up in the cytoplasm above the active zones (11). At maximal doses, 4-AP increased vesicle openings over 60-fold at 5-6 ms after a stimulus (11, 13). At lower doses, 4-AP's effect was graded, so quantal release could be varied over a wide range; and at every level of release, the number of vesicle openings compared favorably with the number of quanta discharged (11).

In the present experiments, we applied the same techniques to determine the fate of vesicle openings after quantal release. The distinguishing feature of the present approach was that the concentration of 4-AP was kept constant while the interval...
between stimulation and freezing was varied over a wide range, from <3 ms to 1 s. In this way, we could examine both the beginning and the aftermath of vesicle exocytosis, from the early moments of quantal release until the time when vesicle recycling begins, several hundred ms later. The following report presents in detail the freeze-fracture data that were mentioned in previously published reports on the overall process (6, 7, 10).

MATERIALS AND METHODS

Cutaneous pectoris muscles from small, adult Rana pipiens (Hazen Farms, Vermont; formerly called Lake Champlain Frog Farm) were dissected in Ringer's and quick frozen in a freezing press which we have described previously (11). This machine was equipped with a timing circuit so that single shocks in 1 mM 4-AP could be delivered to the nerves of these muscles at different intervals before freezing. The interval between stimulation and contact with the cold copper block that initiated freezing was always monitored to within ±100 μs with the resistance circuit described previously (11).

After freezing, most muscles were taken directly to a Balzers freeze-fracture apparatus (Balzers Corp., Hudson, N. H.) where they were fractured and replicated. Alternatively, they were freeze-substituted by a slow warm-up in 4-5% osmium tetroxide in anhydrous acetone, allowing at least 4-6 h to elapse from the time when the acetone melted (at -95°C) until it reached room temperature. These freeze-substituted muscles were then block stained with 1% uranyl acetate in acetone or ethanol for 2-4 h, and embedded in Araldite for thin sectioning. Techniques for measuring micrographs and performing statistical evaluations were described previously (11) or are elaborated in Results. Ranges are standard deviations unless otherwise stated, and Student's t test was used to test for differences between groups where applicable.

RESULTS

Nature of Vesicle Openings

The basis for believing that the freeze-fracture images that are the subject of attention in this paper were indeed vesicle openings is evident from examination of freeze-substituted nerve terminals like those shown in Figs. 1 and 2. These terminals were frozen 5-6 ms after a stimulus, which was found to be the optimal interval for catching exocytosis (cf. Fig. 10, later). Thin sections of such freeze-substituted nerves displayed large numbers of plasmalemmal pockets, precisely lined up

![Figures 1 and 2](https://jcb.rupress.org/article-pdf/565/1/1/5390565/565.pdf)
FIGURES 3-8 Freeze-fracture views of single active zones selected to show activity typical of various times after a single stimulus in 4-AP: Fig. 3, 3.8 ms; Fig. 4, 3.7 ms; Fig. 5, 5.2 ms; Fig. 6, 20 ms; Fig. 7, 50 ms; Fig. 8, 250 ms. Vesicle openings first appear at ~4 ms (Fig. 4), reach peak concentration at 5–6 ms (Fig. 5), and mostly disappear again by 50 ms (Fig. 7). Arrow in Fig. 5 indicates rim of membrane leaflet which shows that this vesicle opening has been cross-fractured. Vesicle openings leave behind clusters of large particles (arrow, Fig. 7) which scatter by 250 ms (arrows, Fig. 8). X 120,000.
next to presynaptic dense bars (not shown), which is exactly where openings are found in freeze-fracture replicas (11, and Figs. 4-6). Because many of these pockets were the same size and curvature as synaptic vesicles, we concluded that they were synaptic vesicles caught in the act of exocytosis. Shallow pockets were also present, consistent with the idea that the vesicle membrane progressively flattens into the surface.

These two sorts of plasmalemmal pockets were also seen in freeze-fracture replicas. Some were smooth membrane fractures through relatively wide-open plasmalemmal pockets, while others were narrower and more complex (Figs. 4-6). The apex of this second type of deformation was etchable; that is, 30 s of sublimation or "etching" at -100°C before replication excavated its center, as if the fracture had crossed a narrow water-filled opening in the plasma membrane. The rim visible on the walls of many of these excavations was interpreted to be the point where the fracture plane had left the plasmalemma (Fig. 5). Occasionally, views were also obtained of the outer plasmalemmal leaflet of nerve terminals which showed these two types of plasmalemmal pockets even more clearly (Fig. 9). There was little doubt that these were membrane-bound pockets in the plasmalemma; most likely, they were exocytotic synaptic vesicles.
Close scrutiny of nerves that were freeze-substituted after stimulation convinced us that some of the omega profiles seen in thin section had mouths as narrow as the narrowest openings seen in freeze-fracture (compare Fig. 2 with Fig. 4). The narrowness of their mouths dictated that their membrane joined the plasma membrane at a relatively acute angle. This was the configuration required by our interpretation of the freeze-fracture images; namely, that the etchable openings were places where the fracture jumped from the interior of the plasma membrane across the orifice of a vesicle opening. This consistency—plus the similarity in the size, position, and time of appearance of the plasmalemmal openings seen in freeze-fracture to the omega-shaped profiles seen in thin sections—led us to conclude that we were witnessing the same structures with the two techniques, and that they were exocytotic synaptic vesicles.

The difficulties involved in obtaining enough thin sections of freeze-substituted nerve terminals discouraged us from using this approach to reconstruct the sequence of events leading up to and following synaptic vesicle exocytosis. The freeze-fracture technique produced expansive views of the presynaptic plasmalemma and therefore provided much more useful data.

**Origin and Fate of Vesicle Openings**

When nerve terminals were examined at different intervals after a single stimulus in 1 mM 4-AP, the most obvious progression was an increase in the number of vesicle openings beginning at 3 ms, increasing up to 6 ms, and then decreasing more slowly (Figs. 3-8). To measure this increase, active zones

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1 In our previous report on vesicle exocytosis, we said that vesicle openings were not visible in nerves that were stimulated at 3 ms or less before freezing, yet we provided a physiological record that showed that transmitter release in 4-AP started at ~2.5 ms. We argued that this did not represent a fundamental discrepancy between the anatomical and physiological data, because the samples prepared for anatomy had been treated differently. They were mounted in the freezing machine, and they cooled a bit in the moments just before transmitter release, which slowed them down. However, some critics have focused.
FIGURES 14 and 15  Single active zones from two different nerves quick-frozen 50 ms after stimulation, selected to show that contrary to the average results in Fig. 11, vesicle openings can differ in diameter at the same times after freezing, as a consequence of other variables discussed in the text. x 120,000.

were photographed at magnifications of 15,000–30,000. Of these, 331 from 13 muscles were selected for measurement on the basis of good quality of freezing and replication. The length of each active zone was measured and the number of vesicle openings along it counted, yielding a value for the number of vesicle openings per micrometer for each individual active zone considering both sides of the ridge formed in the plasmalemma at the active zone (Fig. 10). These measurements documented our visual impression that the number of vesicle openings rose to a peak at 5–6 ms after a stimulus, and then declined progressively, practically to disappear by 100 ms.

Another impression from micrographs such as Figs. 3–8 was that the vesicle openings enlarged as time passed, although a wide range of sizes occurred at every time. To substantiate this impression, measurements were made of the diameters of vesicle openings from the same groups of nerve terminals, stimulated from 3 to 250 ms before freezing. The results did show a tendency for vesicle openings to increase in size as time passed (Fig. 11). Examples of the wide variations in size, out of which this overall tendency emerged, are given in Figs. 12–15.

A reasonable interpretation of this rise and fall in the number of vesicle openings, and their tendency to increase in size with time, was that each vesicle opening begins as a small pore in the surface membrane and enlarges until the synaptic vesicle membrane is entirely collapsed into the plasmalemma. If this were so, then the progressive increase in the number of vesicle openings at early moments would represent a transient accumulation of these structures, and the fall in number would occur when the rate of appearance of new openings dropped below the rate of vesicle collapse; that is, when openings began to disappear faster than they appeared.

These clues that vesicle openings enlarged as time passed mandated a closer look at small vesicle openings at early times, on this time discrepancy, and have argued that we were not witnessing exocytosis, but some sort of endocytosis that occurs in the moments after transmitter release. This encouraged us to examine more nerves stimulated –3 ms before freezing in the present study. We found that, contrary to our earlier conclusion, these often did turn out to show the first few examples of what we believe were exocytotic synaptic vesicles. The analysis of these nerves is incorporated in Fig. 10, and a nerve showing a number of vesicle openings just 3.7 ms after stimulation is shown in Fig. 4. These data, compared with the temporal dissection of the whole process presented in this report, and combined with our previous pulse-tracer studies which showed no sign of endocytosis until hundreds of milliseconds after transmitter release (7), will settle this controversy, we hope.
to see how vesicle opening begins. At 3–5 ms, when the smallest class of vesicle openings was most prevalent, none were narrower than 20 nm in diameter, and none fractured or etched in an unusual way which might have indicated that they were intermediates in exocytosis (Fig. 4). There were no signs of any of the prodromata that have been described in other secretory FIGURE 16 Cross fracture through a field of synaptic vesicles inside a nerve terminal, which exposes their convex external leaflets or their concave protoplasmic leaflets. The concave protoplasmic leaflets display one to three unusually large intramembrane particles (arrows); in addition, smaller particles occur on both fracture faces. × 250,000.

FIGURE 17 Active zone from a nerve stimulated 20 ms before freezing, shown at the same high magnification as Fig. 16. The particles that have appeared beside the active zones after exocytosis (arrows) are distinctly larger than those that make up the double rows at the edges of the active zone itself, but are similar to those in the synaptic vesicle membranes of Fig. 16. × 250,000.
cells, such as particle clearings or shallow depressions around diaphragms in the plasma membrane (16, 17, 19). Apparently, the time resolution of the freezing method used here was not good enough to capture whatever event led up to the 20-nm openings. All we could see was the progressive enlargement of the openings after that time.

**Fate of Collapsed Vesicles**

Our earlier work on chemically fixed nerve terminals illustrated that, during nerve stimulation, the plasma membrane becomes progressively "splattered" with large intramembrane particles like those that form rows at the active zones (7, 9). We noted that this change was not caused by disturbance of the active zone particles, but was actually caused by a net addition of new particles to the plasma membrane. We noted also that synaptic vesicle membranes possess similar large particles (7, 10); so we proposed that vesicles contribute their large particles to the plasma membrane when they collapse into it.

In replicas of quick-frozen nerve terminals it has become possible to substantiate our previous observations, thanks to the fortunate circumstance that the particles added during stimulation and the particles in the membranes of synaptic vesicles have turned out to be slightly larger than those that form the rows beside active zones. Figs. 16 and 17 show ourclearest views of these three types of particles. Fig. 16 shows how large some of the particles are on the inner (concave) leaflet of synaptic vesicle membranes. Fig. 17 shows that similar unusually large particles can be discerned beside the rows of particles at the active zone, and that they are even larger than most of the particles in these rows.

Measuring the diameters of the platinum heads on the intramembrane particles at these three locations confirmed this visual impression. The large particles in synaptic vesicle membranes and the largest ones scattered randomly in the plasma membrane had a mean diameter 10-15% larger than those that formed the rows at the edges of the active zones (Table I). Furthermore, measuring the diameters of all intramembrane particles around 10 short segments of active zone (chosen from nerves whose replication was optimal) illustrated that the presynaptic plasmalemma contained a subpopulation of particles that the eye could pick out as distinctly larger (Fig. 18).

To gather further evidence that such large particles were added to the plasma membrane by collapse of synaptic vesicles, we sought to correlate the number of particles added with the number of vesicles discharged. This required determining, first, how concentrated these particles were in synaptic vesicle membranes, relative to the plasma membrane at the active zone. For this measurement, we were limited to areal views of the curved surfaces of fractured synaptic vesicles. The area of one of the cups of vesicle membrane left after fracturing is $2\pi R^2 - 2\pi R \sqrt{R^2-r^2}$, where $r$ is the radius of the cup and $R$ is the true radius of the vesicle. Because we could not measure $R$ directly for every vesicle, we determined an average $R$ for the 60 largest and most deeply concave cups in a field of fractured vesicles, assuming that these were vesicles fractured near their meridian and thus most representative of mean vesicle size. These vesicles were $41.5 \pm 5$ nm in diameter. Using half this value for $R$, we could determine the areas of large numbers of fractured vesicles (if $r > R$, which was rare, the area of the cup was taken as $2\pi R^2$). The results indicated that large particles in synaptic vesicle membrane were 5-20 times more concentrated than in the plasma membrane adjacent to resting active zones, where they ranged from 22 to 30/µm² (Table II). Thus, collapse of synaptic vesicles at an active zone ought to result in a local increase in the concentration of large particles.

Indeed, active zones examined at intervals of 4-50 ms after a single stimulus did possess numerous clusters of large particles in their vicinity. As expected from the counts of particles in intact synaptic vesicles, these clusters typically contained a total of four to seven particles, with two to four large ones (Figs. 7, 13, 17, and 20), though the assignment of exact borders to a particle cluster was somewhat arbitrary.

Counts of these particle clusters were made at 97 active zones from five muscles stimulated at five different intervals before freezing. These were the five best-replicated examples of the 13 nerves previously used for measuring these sequential changes in the diameters of vesicle openings. Only one muscle was selected from each time point, to insure that conditions in the different nerve terminals at that time would be as homogeneous as possible. To improve observation of patches of large particles, terminals were rephotographed at a magnification of

![FIGURE 18 Frequency histogram of the diameters of all particles found around 10 stimulated active zones (frozen 20-50 ms after stimulation). The second peak in this distribution suggests that there is a distinct class of large particles present around the active zone after stimulation. Indeed, when impartial observers were asked to circle all particles that looked unusually large, and we determined the mean diameter of the ones they circled, it turned out to correspond closely to the second peak in this overall distribution (arrow at right). (The arrow on the left indicates the mean size of all particles in the vicinity of active zones.)](https://jcb.rupress.org/doi/abs/10.1083/jcb.88.5.572)

### Table I

| Large-Particle Diameters | Synaptic vesicle | Plasma membrane | Inner row of active zone |
|--------------------------|------------------|------------------|--------------------------|
| Mean, nm                 | 12.2             | 12.9*            | 11.0†                    |
| SD                       | 1.1              | 1.5              | 1.2                      |
| n                        | 15               | 34               | 69                       |

* Not significantly different from particles in synaptic vesicle membrane.
† Significantly smaller than particles in synaptic vesicle membrane ($P < 0.01$).

### Table II

| Particles in Synaptic Vesicle Membrane (Protoplasmic Leaflet) | Particles counted | Particles/µm² (m ± SD) | Particles/vesicle (m ± SD) |
|--------------------------------------------------------------|-------------------|------------------------|---------------------------|
| Micrographs | Large particles | 8 | 300 | 400 ± 48 | 2.8 ± 0.3 |
| Small particles | 8 | 274 | 395 ± 68 | 2.5 ± 0.4 |

* Assuming a diameter of 42 nm (see text)
 southeastern United States. The study of birdsong has been a favorite topic for many years, with researchers exploring the intricate details of courtship and territorial behaviors. The songs themselves are incredibly diverse, ranging from simple chirps to complex multi-trill motifs. Understanding the evolution and function of these songs is crucial for grasping the larger picture of avian communication and behavior. The data collected from these experiments can contribute significantly to our understanding of bird song evolution and the importance of these sounds in the animal kingdom.
maximum of 60 outer-row particles per micrometer of active zone, as was described above. Nevertheless, we reconsidered this alternative later and looked for evidence that outer-row particles were derived from the rows at active zones—because the peak in large forms occurs after the peak in vesicle openings; and the peak number of individual particles occurs in turn, after the peak number of particle clusters. This fits with the sequence of exocytosis proposed in the text.

**Table III**

Increases in Large Particle Concentration (above the Control Level of 22-30 Particles/μm²)

| Zones (cf. Fig. 22) | 5 ms | 250 ms | 1,000 ms |
|---------------------|------|--------|----------|
| 20-80 nm            | 95   | 4.1    | 20       |
| 50-160 nm           | 15   | 0.9    | 24       |
| 161-240 nm          | 3    | 0.2    | 44       |
| 241-320 nm          | 4    | 0.2    | 21       |
| Total collapsed vesicles | 5.4 | 7.4    | 6.1      |
| Vesicles still open | 7.8  | 0.1    | 0.0      |

* Concentration of large particles per μm² of active zone, plotted in Fig. 22.
‡ Calculated number of vesicles (per running μm of active zone) that would have had to collapse to provide the observed number of large particles, assuming 2.8 large particles per synaptic vesicle (from Table II), and taking into account that each zone is <1 μm² in area.

surrounding plasma membrane by diffusion.

We initially rejected the alternative explanation—that these particles were derived from the rows at active zones—because we could detect a small difference in the size of these two types, as was described above. Nevertheless, we reconsidered this alternative later and looked for evidence that outer-row particles might have been depleted by stimulation in 4-AP. The closest spaced particles in the active zone rows averaged 17.1 ± 0.5 nm center-to-center, so there were a maximum of 116 inner-row particles per μm of active zone (considering both sides of a ridge). Because there were approximately half as many particles in the outer rows as in the inner, there was a maximum of 60 outer-row particles per micrometer of active zone. After a single stimulus in 4-AP, we found increases in large particles of 10-45 per μm of active zone, depending on the interval between stimulation and freezing, the largest being 45 particles per μm of active zone at 50 ms (Fig. 21). If these particles had been drawn from the adjacent outer row, then its original complement of 60 particles should have been diminished by more than half. We looked for such a decrement by comparing the number of outer-row particles to the number of inner-row particles. We chose this measurement to get around the problem of gross discontinuities in the active zones, which occur often. Our control mean was 53 outer-row particles per 100 inner-row particles, measured at 20 active zones from terminals given a single stimulus without 4-AP (which produced little enough change from the resting condition to be considered a control). Experimental values were: 42 at 5 ms, 48.5 at 50 ms, and 54 at 250 ms after a single shock in 4-AP. That is, we were unable to demonstrate any decrease in the outer-row particles which might account for the increase in particles around the active zones that occurs after stimulation. This was an additional reason to reject the possibility that the active zone particles were a major source of the added intramembrane particles.

Although this coming and going of large particles was hard to see against the rich background of smaller particles in the plasma membrane, it seemed to represent a substantial addition of vesicle membrane to the plasmalemma. That is, when we translated the total number of large particles we saw into an equivalent number of vesicles, assuming an average of 2.8 particles per vesicle, it emerged that a large proportion of the discharged synaptic vesicles must have left their particles behind in the plasma membrane (Table III). Even by 5 ms there were already enough large particle clusters present to amount to almost as many vesicles as were caught in the open configuration. More importantly, by 250 ms, when vesicle openings were almost gone, there were enough large particles left to account for more than half of all the vesicles that had discharged. (We presume that, by that time, the rest of the particles must have moved out of the zones we measured; their fate is considered in the Discussion.)

It is worth stressing that this increase in large particles after stimulation was not a peculiarity of 4-AP. We observed a gradient, identical to the one seen 250 ms after a single shock in 4-AP, when a rapid tetanus of 20 stimuli was delivered in 200 ms to muscles that were bathed simply in normal Ringer's (data not shown).

**Factors Affecting Rate of Collapse of Vesicle Openings**

Having recognized these sequelae of exocytosis, we found it possible to look for factors that altered the time-course of
vesicle collapse. In a previous study, we proposed that vesicles collapse more slowly under conditions of intense discharge. This was because we found that early forms were relatively abundant in higher concentrations of 4-AP, when greater numbers of vesicles were discharged (11). The data on which this conclusion was based were reevaluated in the present study, to determine whether the proportion of later forms (i.e., clusters of particles) also varied as expected. These data consisted of 440 active zones from 19 muscles frozen 5–6 ms after a single stimulus, in which the level of discharge had been varied by stimulating in different concentrations of 4-AP. The new results, plotted in Fig. 23, illustrate that the proportion of late forms decreased markedly as the overall level of activity (i.e., total number of vesicle openings) increased. This substantiated the idea that the rate of collapse into late forms is slowed as the concentration of vesicle openings increases, which implies that the collapse of one exocytotic vesicle slows the collapse of ones around it.

To investigate whether this inhibitory effect is caused simply by bulk expansion of the plasma membrane resulting from collapse of discharged vesicles, we proceeded to increase the surface-to-volume ratio of the nerve by another means, in order to see whether this change would also slow vesicle collapse. That means was to shrink the nerve with hypertonic Ringer’s before stimulation. (Of course, we recognized that a relative membrane excess produced in this manner might not have an effect equivalent to membrane added by vesicle exocytosis, as vesicle membrane would be much more asymmetric because of its extreme curvature, and changes in membrane asymmetry could also affect the tendency of vesicles to collapse.) For these experiments, muscles were exposed for 15 min to Ringer’s made hypertonic by adding 50 mM sucrose and then were given the usual single stimulus in 4-AP.

The only problem with soaking nerve-muscle preparations in hypertonic sucrose was that it causes a dramatic increase in spontaneous release of quanta (14), and we found that this treatment itself resulted in the appearance of nearly one vesicle opening per micrometer of active zone, even without stimulation in 4-AP. Furthermore, the hypertonic treatment decreased the abundance of vesicle openings after stimulation in 4-AP. Less than three vesicles per micrometer of active zone were caught at 5 ms after one shock, instead of the usual 10–12 (Figs. 10 and 21). Nevertheless, these vesicle openings seemed to last much longer than normal; 100 ms later, an average of two openings could still be found at the active zones of the hypertonic muscles, whereas 11 out of 12 would have disappeared by that time, if the experiment had been done in normal Ringer’s without sucrose (Fig. 24).

Interestingly, the persistent vesicle openings found in hypertonically treated muscles were usually small, of a size which we previously identified as the earliest stages (Figs. 11, 24, and 25). Moreover, the number of vesicle openings present in hypertonic saline, even in the absence of stimulation, was about one per active zone or ~500 per whole nerve-muscle junction. This concentration of vesicle openings, at a time when the rate of spontaneous quantal discharge was ~100/s, would imply that each vesicle opening was lasting ~5 s during the hypertonic treatment. Such profound slowing of the collapse of synaptic vesicles, we presume, was caused by the increased surface-to-volume ratio of the nerve terminal. It suggests that the retardation in the rate of collapse that develops during the massive release of vesicles in 4-AP may also result from a transient increase in the surface-to-volume ratio of the terminal (cf. Figs. 13 and 15).

Shrinkage in hypertonic Ringer’s appeared to affect vesicle collapse equally over the whole terminal. In contrast, stimulation in 4-AP appeared to result in local variations in the stages of vesicle collapse along individual nerves, which suggested that local factors might also be important (Fig. 26). We docu-

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**Figure 23** Percent of activity seen as late forms (left) at four different levels of activity resulting from stimulation in different concentrations of 4-AP. The absolute numbers of late forms per micrometer of active zone (right) are shown for each activity level. Activity refers to sum of the vesicle openings and late forms per micrometer of active zone.

**Figure 24** Diameters of vesicle openings at 5 and 100 ms after stimulation in Ringer’s made hypertonic with sucrose, compared to the diameters of vesicle openings 100 ms after stimulation in normal Ringer’s (dotted line). The solid lines for hypertonic Ringer’s represent the increase above the background number of vesicle openings induced by the hypertonic Ringer’s in the absence of stimulation. At 100 ms after stimulation in hypertonic Ringer’s, more vesicle openings remained than normally, but fewer of these were large ones (cf. dotted line). (The peak number of vesicle openings that would be seen 5 ms after stimulation in normal Ringer’s would be off scale if this graph had the same coordinates as Fig. 11.) Based on 30 active zones from 12 muscles.

**Figure 25** Comparison of small vesicle openings (~30 nm in diameter) at different intervals after a single shock in normal or hypertonic Ringer’s. The disappearance of the small vesicle openings between 5 and 100 ms is more complete in normal Ringer’s than in hypertonic Ringer’s. Based on the same data as Fig. 24.
FIGURE 26  Tracings from a series of 43 contiguous active zones on a nerve stimulated 6 ms before freezing. The bars represent the double particle rows, dots represent vesicle openings, and circles represent late forms of vesicle collapse. Late forms predominate in the fifth through ninth active zones from the top, significantly departing from an expected random distribution of stages. Statistical analyses of such series are presented in Results. Spacings between active zones have been compressed.

mented the existence of these local variations by performing statistical tests on the spatial distribution of early and late forms of vesicle collapse along individual nerve terminals. The 15 terminals tested, which had series of 14 to 57 active zones each, were the same as used previously (11). The test was whether the variation that was present, when considered active zone by active zone, was the same as would be predicted by a trinomial distribution generated from the overall activity in that terminal. Specifically, each active zone was considered to be a series of sites, one vesicle wide, each of which could be: empty (i.e., devoid of an opening), occupied by an opening, or occupied by a late form of vesicle collapse. The numbers of sites in each of these states for the whole nerve segment were used to generate a trinomial distribution, which predicted the expected variation from active zone to active zone that would be expected if the three possible states at each site were randomly distributed along the nerve.

In 5 of the 15 lengths of individual nerve terminals that we examined, the actual variation in numbers of late forms from active zone to active zone was significantly greater than expected at the $P < 0.01$ level; in fact, 8 of the 15 varied significantly at the $P < 0.05$ level. This variation could not be explained simply by variations in the total output along individual terminals, because in 13 out of 15 there was no more variability than would be expected from a random process, when both early and later forms were considered to be a single state in a binomial distribution. Thus, only the rate or timing of vesicle collapse appeared to be distributed in a nonrandom manner from active zone to active zone.

Subjectively, this result looked as though it was caused by local variations in the rate of vesicle collapse that affected several active zones in a row along any individual terminal. Photomontages of individual terminals contained some regions with distinct clumps of late forms, next to regions that displayed a prevalence of earlier forms of vesicle opening (Fig. 26). However, this was only a subjective impression, and the eye is notorious for selecting patterns out of random distributions. So we attempted to confirm this visual impression by applying run tests that were designed to show whether active zones in a series had a tendency to be at the same stage of vesicle collapse as their neighbors. Each active zone was scored as to whether it displayed above or below the median number of late forms for its particular nerve; repeated deviations in the same direction constituted a run. All 15 nerves were tested for runs, and the results of each test (expressed as the deviation from the expected number of runs) were compared to the deviations that would have been expected from random sampling. Overall, the results on the 15 terminals were biased toward clumping; that is, runs tended to be longer and more infrequent than expected from a random distribution. Specifically, 74% of a normal distribution fitted to the results from the 15 terminals was biased toward clumping. In only 2 of the 15 terminals could this have been caused by nonrandom variation in the overall output (early and late forms pooled). In the other terminals, regions that were rich in late forms seemed to have fewer early forms, and vice-versa, which is why the summed frequency of both types remained random. These regional variations indicated that there were some regional influences on the rate of vesicle collapse. What these influences might be was not apparent from the morphological data; but if some regions of a nerve terminal began to secrete later than others, the vesicles in these late-comers might be selectively inhibited by the membrane previously added to adjacent active zones.
DISCUSSION

By freezing muscles at different intervals after nerve stimulation, we followed the morphological changes associated with neuronal secretion on a millisecond scale from a time near their beginning, a few milliseconds after the nerve stimulus, until their sequelae disappear hundreds of milliseconds later. The relatively abrupt onset of secretion in response to nerve stimulation has permitted the recognition of a series of stages in this process.

The earliest visible changes were minute pores in the plasma membrane that lined up in rows at active zones 3-4 ms after each nerve impulse. Freeze substitution and thin sectioning of nerve terminals frozen at this stage demonstrated that the pores were complete openings into underlying synaptic vesicles, that is, points where the vesicle membrane had become continuous with the plasma membrane. By 5-6 ms after stimulation the pores were more abundant and larger. Thereafter, in the next 20-100 ms, they decreased in abundance and left, in their aftermath, larger, shallow depressions in the plasma membrane marked by clusters of large intramembrane particles, similar in size to those found in intact synaptic vesicles. By 250 ms, these particle clusters were no longer readily discernible, and over the next second, individual large particles appeared to spread progressively away from the active zones.

This postulated sequence of changes is illustrated in Fig. 27. The events leading to coalescence of the two membranes and pore formation are evidently too fleeting to capture by current freezing methods. The initial pore is small, but it widens almost immediately and in a fraction of a second the vesicle opens completely and collapses flat into the plasma membrane. This shape change can apparently be delayed by shrinking the nerve or by expanding its plasma membrane with added exocytotic vesicles. But normally, vesicle collapse proceeds faster than the rate of mixing in the two membranes and, as a result, flat patches enriched in the particulate components of the vesicle membrane remain behind at the same spots where exocytotic openings had been. These particle clusters persist for several tens of milliseconds but gradually disperse as the particles spread away from the active zone. Such mixing of vesicle and plasma membranes appears to be complete by 1 s, when the first sign of membrane retrieval by coated vesicles is found (7, 10).

It is not necessary to review here all the observations that led up to our scheme of synaptic vesicle membrane recycling, but it is important to stress that we have never found any reason to suspect that the critical step of membrane retrieval after exocytosis ever operates as a simple reverse of the exocytotic sequence shown in Fig. 27. Indeed, in the present study, the relative abundance of different stages of vesicle collapse in nerve terminals frozen at different times after one shock is a further indication that the sequence does not reverse, because narrow openings appear before wider ones and collapsed vesicles appear after the wider openings. These observations fit with our earlier study of the uptake of horseradish peroxidase during stimulation, which showed that the first organelles that pinch off from the plasma membrane after stimulation are coated vesicles (8).

Even more direct evidence against the possibility that the sequence seen in Fig. 27 runs in reverse order to reform vesicles has come from observations of ferritin uptake in the first few moments after one shock in 4-AP. In terminals quick-frozen and freeze-substituted 1 s after stimulation in a medium containing ferritin, no ferritin-laden vesicles could be found at the active zones, though they would be expected if vesicles pinched off from the plasma membrane as fast as the openings disappeared. Instead, vesicles were conspicuously absent from the vicinity of the active zones, because of the loss of those that had collapsed (7, 10).

Such local loss of synaptic vesicles in nerves frozen 1 s after a single massive discharge is reminiscent of the overall loss of vesicles seen in terminals chemically fixed after a prolonged period of stimulation (8). In both instances, the loss in vesicles appears to be balanced by membrane addition to the plasma membrane. This was previously recognized as a 20% expansion in the surface area of the plasma membrane of thin-sectioned terminals (8), and as an overall increase in the abundance of large intramembrane particles in the plasma membrane of freeze-fractured terminals (7, 9). Now that quick-freezing can be done instead of chemical fixation, the merging of vesicles with the plasma membrane implied by the previous observations can be seen directly. This method is fast enough to catch clusters of intramembrane particles beside the active zones, just at the time when exocytotic vesicle openings are disappearing. These clusters are sufficiently abundant to suggest that they are the natural outcome of most vesicle discharges; in other words, that most vesicles collapse after exocytosis instead of pinching off from the presynaptic membrane again.

This fate for synaptic vesicles has been a part of our scheme of vesicle recycling at the frog neuromuscular junction since its conception (8). Other investigators have witnessed some of the same structural changes at frog neuromuscular junctions or at other synapses but have reached different conclusions. They have contended that endocytosis occurs at active zones and does not look different than exocytosis running in reverse. They have interpreted their thin-section images as showing direct horseradish peroxidase uptake into synaptic vesicles at the active zone (3); and they have published freeze-fracture images of nerves fixed after a long tetanus (when we imagine that exocytosis would have stopped and endocytosis would have been abundant) which show a considerable number of vesicle openings right at the active zones (1). However, we question whether proper physiological conditions are maintained during chemical fixation and wonder whether these investigators were witnessing a new round of exocytosis induced by their fixative, rather than endocytosis. We prefer to put weight on quick-freezing results, which do not support the idea that exocytosis can run in reverse at the active zone. Instead, these results indicate that reformation of vesicles after discharge is a more complicated process, involving formation of coated vesicles and cisternae, which starts up long after the 100-ms interval during which synaptic openings can be found at the active zone (7, 8, 10).

We should stress that the strongest piece of evidence presented in this paper for the idea that vesicle exocytosis does not run in reverse is the sequence of intramembrane particle changes that occur right next to the active zones after transmitter discharge. However, the chemical identity of the particles involved in these changes remains unknown, and their origin from collapsing synaptic vesicles can only be inferred. Other investigators have seen changes in presynaptic membrane particles during stimulation but have interpreted them differently, concluding that the active zone particles themselves could be displaced during transmitter release (24). They have regarded these displacements as analogous to the intramembrane particle changes seen originally at sites of exocytosis in
protozoa (22).

We do not think this is so, for the following reason. The active zone particles of the frog neuromuscular junction would be particularly suitable for visualizing such a displacement, because they are normally aligned into such orderly double rows. We have documented the development of this order (5), and other investigators have shown that extreme disorder can develop in nerves soaked in calcium-free solutions (2); but we have been unable to recognize any rapid changes in particle alignment before, during, or after exocytosis. Moreover, not only are these active zone particles stationary, but the particles that appear immediately beside the active zones after stimulation are slightly but distinctly larger than those at the active zones. The extreme degree of active zone disorder that has been seen after severe treatments such as black widow spider venom (2) is presumably a result of gross damage, not a physiological process occurring during normal neurotransmission. Ordinarily, the active zone particles remain unperturbed by transmitter release, and are part of a structurally stable complex (21). In fact, recent deep-etching results have illustrated that the active zone particles occur exactly where the presynaptic membrane is attached by delicate threads to the basal lamina in the synaptic cleft (Hirokawa and Heuser, manuscript in preparation). Because of the stability of the active zone particles, the particle changes seen after one shock in 4-AP can be recognized as a separate phenomenon, involving a net addition of particle-rich membrane, evidently derived from synaptic vesicles.

Moreover, because the particles added to the presynaptic membrane could be distinguished by their size from other plasmalemmal particles, their spread away from the active zone could be measured. From these data, presented in Table III, we could calculate a diffusion constant $D$, assuming that the particles move by free diffusion. This constant worked out to be $2 \times 10^{-10}$ cm$^2$/s, which is similar to the diffusion constants that have been determined for other membrane proteins (20, 26).

The sequence of changes proposed in Fig. 27 rests ultimately on the temporal data of Fig. 11. Unfortunately, these data were probably badly smeared, because all exocytotic events do not start at exactly the same moment. At least, that is the most straightforward conclusion from the fact that the end plate potential in 4-AP continues to rise for 2-3 ms after it first begins, then drags on for many more milliseconds (11). This would imply that new vesicle openings are continuously being added in the midst of others that have been open for some time. Such a lack of synchronicity would blur the curves in Fig. 11, and make it difficult to learn from them how long each stage lasted. Particularly for the shortest-lived stages, it would become difficult to know whether their numbers were determined by their rate of formation or their rate of disappearance, so their exact lifetime could be determined.

Such considerations are also critical when attempting to interpret the narrow openings seen 100 ms after a single shock in hypertonic Ringer's. The interpretation we prefer is that these were openings that had appeared almost 100 ms earlier, and that hypertonicity had slowed their collapse. But this interpretation assumes that secretion does not continue for 100 ms after a stimulus in hypertonic Ringer's. In fact, electrophysiological recordings of quantal release have shown no sign
of such delayed release (13). Still, it is conceivable that synaptic vesicles open and close many times after each nerve impulse but discharge a quantum of transmitter only the first time. If this were so, then the continuous one-way scheme in Fig. 27 would have to be modified to allow rapid oscillation back and forth between open and closed vesicles with only occasional progression through stages c-f.

The most frequent argument against our assumption that stimulation in 4-AP should reveal the natural sequence of transmitter discharge, and that Fig. 27 is that sequence, has been that 4-AP may alter the behavior of the nerve membrane directly. This argument has been fueled by our earlier report that synaptic vesicle openings are very rare in terminals stimulated without 4-AP. Such terminals release 100 times fewer quanta than terminals in 4-AP but nevertheless should show a discernible number of vesicle openings. We finally explained this apparent discrepancy to ourselves when we found that an appropriate number of particle clusters appeared at the active zones of nerves stimulated without 4-AP, even though vesicle openings did not appear. This led us to conclude that synaptic vesicles collapse much faster after stimulation in normal Ringer's than they do after stimulation in 4-AP. From this conclusion came the idea that each vesicle opening must slow down the collapse of subsequent openings, perhaps by the slight expansion or asymmetry it produces in the plasma membrane. The experiments using hypertonic Ringer's which resulted from this idea give it further support. Nevertheless, we cannot dismiss definitively the alternate possibility that vesicle openings always resolve rapidly, and that their apparent persistence in 4-AP is caused by a prolongation of the interval during which they begin to open. If this were so, then the total number of vesicle openings in 4-AP would have been much higher than we observed at any one time, and would approach the time-integral of all the openings we saw. Hence, there would be far too many vesicle openings to correspond with the number of quanta released. This alternative interpretation would also require that the number of vesicles which open in hypertonic Ringer's be much greater than the number of quanta that are discharged, but it could accommodate the idea that one quantum results from the simultaneous discharge of several vesicles (15, 25).

Clearly, it is important to determine the exact time-course of quantal discharge in 4-AP under the conditions used in the present morphological study. Only if discharge is sufficiently brief or "pulsatile" can the morphological changes reported here be regarded as a clear temporal sequence revealing the unidirectional nature of vesicle opening. Also, it is clearly essential to improve the method of freezing, so that it can catch the brief vesicle openings in normal Ringer's. Quicker freezing might also catch any transient events that precede vesicle opening. While no such transient states have been seen so far in quick-frozen terminals, vesicles have been seen to form broad and intimate contacts with the plasma membrane in chemically fixed terminals and in other secretory cells. Cytoplasm looks excluded from these contacts in thin sections, and intramembrane particles look excluded in freeze-fracture replicas. These contacts are thought by many observers to be a preliminary step in exocytosis (17, 19) but may also be simply an artifact of chemical fixation (4, 6). We simply do not see them in terminals quick-frozen after one shock. This may mean that vesicle and plasma membranes normally fuse and permute before their point of contact has had time to grow to discernible proportions, except when exocytosis is halted by chemical fixatives. Nevertheless, this would not rule out the possibility that some sort of intimate membrane contact precedes exocytosis. Rather, the results from quick-freezing only indicate that such contact occurs over a limited domain: a tiny spot rather than a broad disk. Exactly what sort of contact might occur at these spots may be learned only when we have faster methods of freezing, better physiological control, and perhaps more favorable preparations.

Though the data reported here stop at 1 s after stimulation, we should mention briefly what occurs in subsequent seconds, which we have reported in previous publications (7, 10). By 1 s, large particles are totally dispersed from the vicinity of the active zone. In subsequent seconds, these large particles reaggregate, over what appear to be coated pits, and then become internalized in coated vesicles. Also operative is a second form of membrane retrieval, which consists of a large vesicle that pinch off from the plasma membrane in regions near active zones, in the first few milliseconds after stimulation. This second form of endocytosis is quite different from coated vesicle formation, in that (a) it does not display a clathrin coat on the cytoplasmic surface of the membrane, (b) it retrieves membrane which looks like a random bite of the plasma membrane, according to its complement of intramembrane particles, and (c) it involves much larger pieces of surface membrane in each event. The vacuoles formed by this route appear to be comparable to the cisternae that we described in our first study of vesicle recycling (8); thus it was probably incorrect to say, in that study, that cisternae form indirectly, by coalescence of coated vesicles. (It remains to be seen whether this second form of rapid, "indiscriminant" membrane retrieval into cisternae occurs normally, or only during the abnormally high rates of secretion produced experimentally.) In any case, we anticipate that the accurate timing afforded by the quick-freezing method will be particularly useful for sorting out the sequence of membrane movements in these later stages of synaptic vesicle recycling.

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