Endocytosis of Synaptic Vesicle Membrane at the Frog Neuromuscular Junction

THOMAS M. MILLER and JOHN E. HEUSER
Department of Physiology, University of California School of Medicine, San Francisco, California 94143. Dr. Miller's present address is Department of Anatomy, Harvard Medical School, Boston, Massachusetts 02115; and Dr. Heuser's present address is Department of Physiology and Biophysics, Washington University School of Medicine, St. Louis, Missouri 63110.

ABSTRACT Frog nerve-muscle preparations were quick-frozen at various times after a single electrical stimulus in the presence of 4-aminopyridine (4-AP), after which motor nerve terminals were visualized by freeze-fracture. Previous studies have shown that such stimulation causes prompt discharge of 3,000–6,000 synaptic vesicles from each nerve terminal and, as a result, adds a large amount of synaptic vesicle membrane to its plasmalemma. In the current experiments, we sought to visualize the endocytic retrieval of this vesicle membrane back into the terminal, during the interval between 1 s and 2 min after stimulation. Two distinct types of endocytosis were observed. The first appeared to be rapid and nonselective. Within the first few seconds after stimulation, relatively large vacuoles (~0.1 μm) pinched off from the plasma membrane, both near to and far away from the active zones. Previous thin-section studies have shown that such vacuoles are not coated with clathrin at any stage during their formation. The second endocytic process was slower and appeared to be selective, because it internalized large intramembrane particles. This process was manifest first by the formation of relatively small (~0.05 μm) indentations in the plasma membrane, which occurred everywhere except at the active zones. These indentations first appeared at 1 s, reached a peak abundance of 5.5/μm^2 by 30 s after the stimulus, and disappeared almost completely by 90 s. Previous thin-section studies indicate that these indentations correspond to clathrin-coated pits. Their total abundance is comparable with the number of vesicles that were discharged initially. These endocytic structures could be classified into four intermediate forms, whose relative abundance over time suggests that, at this type of nerve terminal, endocytosis of coated vesicles has the following characteristics: (a) the single endocytotic event is short lived relative to the time scale of two minutes; (*b*) earlier forms last longer than later forms; and (c) a single event spends a smaller portion of its lifetime in the flat configuration soon after the stimulus than it does later on.

The discovery of synaptic vesicles (27, 30, 33, 34, 38) and the evidence for the quantal release of transmitter (5) led to the hypothesis that transmitter release is accomplished by the exocytosis of synaptic vesicles (6). Electron microscopy of chemically-fixed nerve terminals revealed pockets in the presynaptic membranes suggestive of exocytosis (9, 22, 31), but it was not possible to make temporal and quantitative comparisons between these structures and the number of transmitter quanta released.

A quick-freezing machine, based on earlier designs by Van Harreveld (40–42) was developed specifically so that these comparisons could be made (23). Exocytosis was difficult to catch even when the nerve terminals could be frozen within a few milliseconds of nerve stimulation (12), but, by using 4-aminopyridine (4-AP)[1] to augment release, exocytosis was caught (14). Subsequent analysis illustrated that exocytosis of synaptic vesicles occurs next to the active zones[2] and at

[1] *Abbreviations used in this paper:* 4-AP, 4-aminopyridine.

[2] Couteaux (9) defined an active zone as a specialized area of the nerve terminal located directly across from a fold in the postsynaptic membrane and characterized by a density next to the presynaptic membrane and an accumulation of synaptic vesicles.

THE JOURNAL OF CELL BIOLOGY · VOLUME 98 FEBRUARY 1984 685-698

roughly the same time that transmitter is released; in addition, the number of exocytotic figures compares favorably with the number of quanta released (19, 21). Further experiments have demonstrated that once a vesicle begins exocytosis, its membrane completely merges with the plasmalemma (20). Thus, although the interpretation of these results is still controversial (3, 25), quick-freezing experiments provide strong evidence in favor of the vesicle hypothesis.

Secretion by exocytosis ought to result in an increased surface area and fewer vesicles; hence, it was proposed that a compensatory process of membrane retrieval must function to provide for a viable cell economy (28, 29). In fact, vesicle depletion associated with increased surface area has been demonstrated (8, 17, 32, 37), and tracer experiments have shown that there is a stimulation-dependent uptake of extracellular materials into synaptic vesicles (8, 17, 37).

Coated vesicles have been implicated in this retrieval of synaptic vesicle membrane at synapses (10, 17, 43), and are known to be endocytotic in a number of other cells as well (1, 2, 35, 36). However, a controversy has arisen concerning membrane retrieval at the neuromuscular junction; some workers believe that retrieval is accomplished via endocytosis of uncoated vesicles at the active zones (7, 8), while others argue that endocytosis of coated vesicles away from the active zones (and particularly beneath Schwann cell fingers) is the significant process (17). Thus far, no information has been available on the time course of compensatory endocytosis at the neuromuscular junction.

To study membrane recycling at the neuromuscular junction in more detail, we quick-froze synapses at different periods of time after a single stimulus. The transmitter release associated with a single stimulus was augmented ~50-fold with the drug 4-AP (21). Normally, only 100–200 quanta are released from each motor nerve terminal upon stimulation (21, 26), and even the small frogs used in this study possess some 420 μm of active zone on each terminal (21). Hence, only one synaptic vesicle would be expected to undergo exocytosis (or endocytosis) in every 2–4 μm of active zone. Unfortunately, only a small portion of a given endplate can be studied by freeze-fracture, so infrequent endocytotic events cannot be accurately quantitated. To overcome this problem, the exaggerated release in 4-AP can be used to elicit a profound endocytotic response that can be followed with quick-freezing.

Previous papers have given detailed accounts of the structural events taking place at the height of transmitter release (21) and between 3 ms and 1 s after nerve stimulation (20). This paper considers the time interval from 1 s to 2 min. The aim was to confirm or refute previous indications that compensatory endocytosis of synaptic vesicle membrane does occur at the neuromuscular junction, and that it occurs mainly away from the active zones and over an extended time course.

MATERIALS AND METHODS

Cutaneous pectoris muscles were dissected from frogs (*Rana pipiens*) (Hazen Farms, VT) of 1-in body length. The nerve-muscle preparations were refrigerated in a Ringer's solution composed of 116 mM NaCl, 2 mM KCl, 2 mM $CaCl_2$, 0.5 mM NaH_2PO_4, and 5 mM HEPES buffer pH 7.0 (5% glucose added) until 45 min before quick-freezing. At that time they were transferred to room temperature 4-AP Ringer's composed of 116 mM NaCl, 2 mM KCl, 10 mM $CaCl_2$, 1 mM 4-AP, and 5 mM HEPES buffer pH 7.0 (5% glucose added). Each muscle was then quick-frozen against a copper block cooled to 4°K with liquid helium, at a specified time after a single supramaximal stimulus to the nerve. After freeze-fracture, the replica of each muscle was examined with the electron microscope. Every well-frozen endplate was photographed for later analysis. The techniques used for stimulation, quick-freezing, freeze-fracturing, and electron microscopy have been previously described in detail (21).

Quantitative Analysis and Statistical Methods: For quantitative analysis, electron micrographs of neuromuscular junctions were printed at ×90,000. The nerve terminals' surfaces were divided into three areas: (*a*) the active zones and the immediate regions on either side of them in which exocytosis occurs (I; [21]); (*b*) the open areas between active zones (II); and (*c*) the areas beneath Schwann cell fingers that embrace the terminals (III) (Fig. 1). Areas II and III were outlined on each micrograph, and the length of each active zone was marked. The amount of area I was then determined by summing the active zone lengths and multiplying that by their overall width (190 nm). A Lasico rolling desk planimeter and auto-scaler (Los Angeles Scientific Instrument Co., CA) was used to measure areas II and III. To determine the amount of area II, areas II and I were measured in total, and the calculated value of area I for that micrograph was then subtracted from the total.

Endocytotic events were counted and classified as to their appearance and location. Concentrations of endocytotic figures were calculated individually for each area of each endplate studied. This yielded a distribution of concentrations for each area at each time point. Individual concentrations were used as data points for comparison of the different times. The concentrations of endocytotic figures at a given time point were not normally distributed and the distributions of concentrations at different time points did not have comparable variances. For this reason, parameters of the normal distribution such as mean and standard deviation were not appropriate for presenting the data, and it was necessary to use a nonparametric test (Mann-Whitney U test) to test for statistically significant differences (44). The Student's t test was used where specifically indicated.

RESULTS

Table I lists the time points studied and the number of muscles, number of nerve terminals, and total area studied at each time point.

Qualitative Effects of Stimulation

Fig. 1 shows a nerve terminal that was soaked in 4-AP but not stimulated before freezing (all fracture faces presented are P faces). There are no distinguishable morphological differences between a terminal so treated and one that is not exposed to 4-AP. Note the complement of intramembrane particles present on the presynaptic membrane; the active zones are seen as bands lined by double rows of large particles.

Fig. 2 illustrates that when a terminal is stimulated in 4-AP and quick-frozen during the height of transmitter release (~5 ms after the stimulus), both sides of the active zones are lined by pits in the presynaptic membrane, all in area I (cf. Materials and Methods and Fig. 1 for our definition of areas). These structures are presumed to represent synaptic vesicles caught in the act of releasing their transmitter by exocytosis (an interpretation borne out by thin sectioning [14, 21]).

A terminal frozen 1 s after a stimulus in 4-AP is shown in

TABLE I

Quantity of Data Considered in this Study

Seconds after stimulation	No.	No. of nerve terminals	Total area studied
			μm^2
0	3	3	65.9
1	2	4	104.4
5	2	6	179.5
10	3	8	280.4
30	2	10	256.4
60	3	12	134.0
90	3	7	162.3
120	4	4	35.4

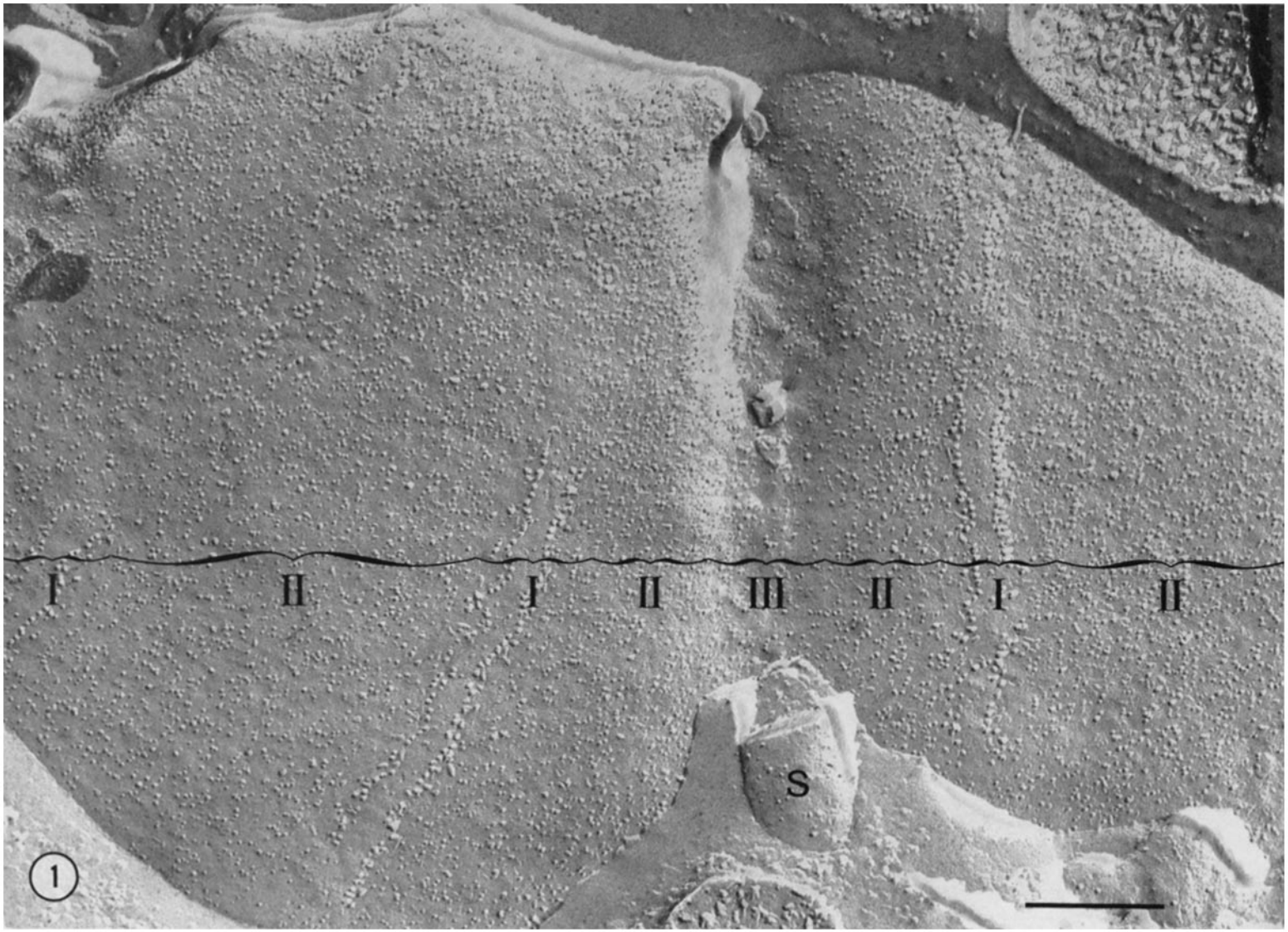


FIGURE 1 A nerve terminal soaked in 4-AP Ringer and quick-frozen without stimulation. The morphology is unchanged from that of a terminal not exposed to 4-AP. For the purposes of this study the nerve terminal was divided into three areas. The active zones, which appear as bands bordered by double rows of large intramembrane particles, and the regions on either side of them where exocytosis occurs compose area *I*. Also visible is an open area between active zones in which typical numbers of intramembrane particles are scattered; this is area *II*. A Schwann cell finger (*S*) has broken away, revealing an example of the part of the nerve terminal surface termed area *III*. Bar, 0.25 μm. × 90,000.

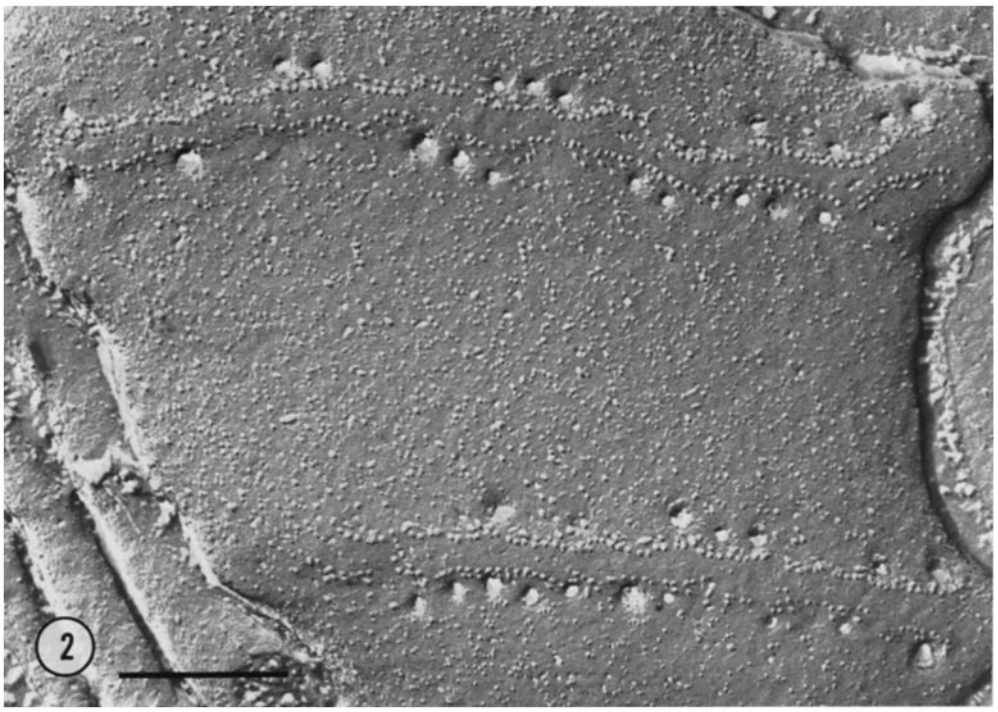


FIGURE 2 A nerve terminal soaked in 4-AP Ringer and stimulated 5 ms before quick-freezing. Deformations in the plasma membrane have appeared on both sides of the two active zones shown here. These presumably represent synaptic vesicles undergoing exocytosis. Bar, 0.25 μm. × 90,000.

Fig. 3. At this time, the structures thought to be exocytotic vesicles at the active zones are no longer visible (cf. reference 20 for a description of their time course of disappearance). Present instead are two new sorts of membrane deformities. First, consider the structures marked by arrows in Fig. 3. These are dents in the presynaptic membrane that are larger than a synaptic vesicle and do not appear to concentrate or exclude intramembrane particles. They are located all along the terminal, both at the active zones (in area I) and away from them (in areas II and III). Correlative thin-section studies (19) have revealed two important characteristics of these structures: (*a*) they take up the extracellular tracer ferritin and are therefore endocytotic; and (*b*) they do not have the fuzzy cytoplasmic coat characteristic of endocytotic structures in many systems. Hence, these structures are here termed "uncoated pits".

A second sort of membrane differentiation present at 1 s after a discharge is enclosed by circles in Fig. 3. These structures are present neither on unstimulated terminals (Fig. 1) nor on acutely stimulated terminals (Fig. 2). They are located only in areas II and III and are not seen at the active zones. They are generally smaller than the aforementioned "uncoated pits", and they usually contain a cluster of large intramembrane particles. It is possible to classify these and related membrane differentiations into four related forms (Fig. 4). Form *1* is a cluster of large intramembrane particles (at least two) that touch one another and invariably include at least one particle that is larger than those that line the active zones. Form *2* is a cluster of large particles in a shallow depression, while form *3* is a cluster of large particles in a deeper depression (the distinction between these two forms being somewhat arbitrary).[3] Form *4* is an ice-filled depression in the presynaptic membrane that is interpreted to be a pit so deep that the group of large particles can no longer be seen.

Correlative thin-section studies of frog neuromuscular junctions have indicated that these structures probably correspond to coated pits of various depths. Treatment of neuromuscular junctions with the presynaptic neurotoxin β-Bungarotoxin results in a maximal concentration of the four forms on freeze-fractured terminals, and, when similarly treated terminals are thin-sectioned, numerous coated pits are seen (19). Also, repeated stimulation of neuromuscular junctions leads to the appearance of these structures in freeze-fracture (13, 14) and to the appearance of coated pits in thin-sections (17). The large intramembrane particles that are seen clustered in the freeze-fracture view of coated pits (Fig. 4) are similar in size and number to the large particles that are found in the membranes of the synaptic vesicles (20). Presumably, they are macromolecular components of the synaptic vesicle membrane that are being recycled along with the membrane itself (18, 20).

When terminals were frozen 5 or 10 s after a stimulus in 4-AP, a moderate concentration of coated pits was found in areas II and III, but none in area I. By 30 s after the stimulus (Fig. 5), a very high concentration of coated pits was found in areas II and III. Again, there was no endocytosis at the active zones, nor were any larger uncoated pits found anywhere on the terminal. By 60 s after the stimulus, the endocytotic response had waned, in that a lesser concentration of coated pits was found; area I was still free of activity. By 90 s after the stimulus, the response appeared to be nearly complete: endocytotic figures were rarely found.

[3] There was some difficulty in identifying the groups of large intramembrane particles before any indentation of the membrane occurred, but we believe they represented part of the same process because all of the particle groups we saw, indented or not, contained the same number of particles. The means ± SD are as follows: the number of particles in form *1* = 5.8 ± 3.5, n = 162; the number of particles in form *2* = 5.6 ± 2.0, n = 60; and the number of particles in form *3* = 5.9 ± 1.7, n = 40. In previous studies, synaptic vesicles have been shown to possess a similar number of particles in their membranes (5.3 ± 0.7, n = 275; reference 20).

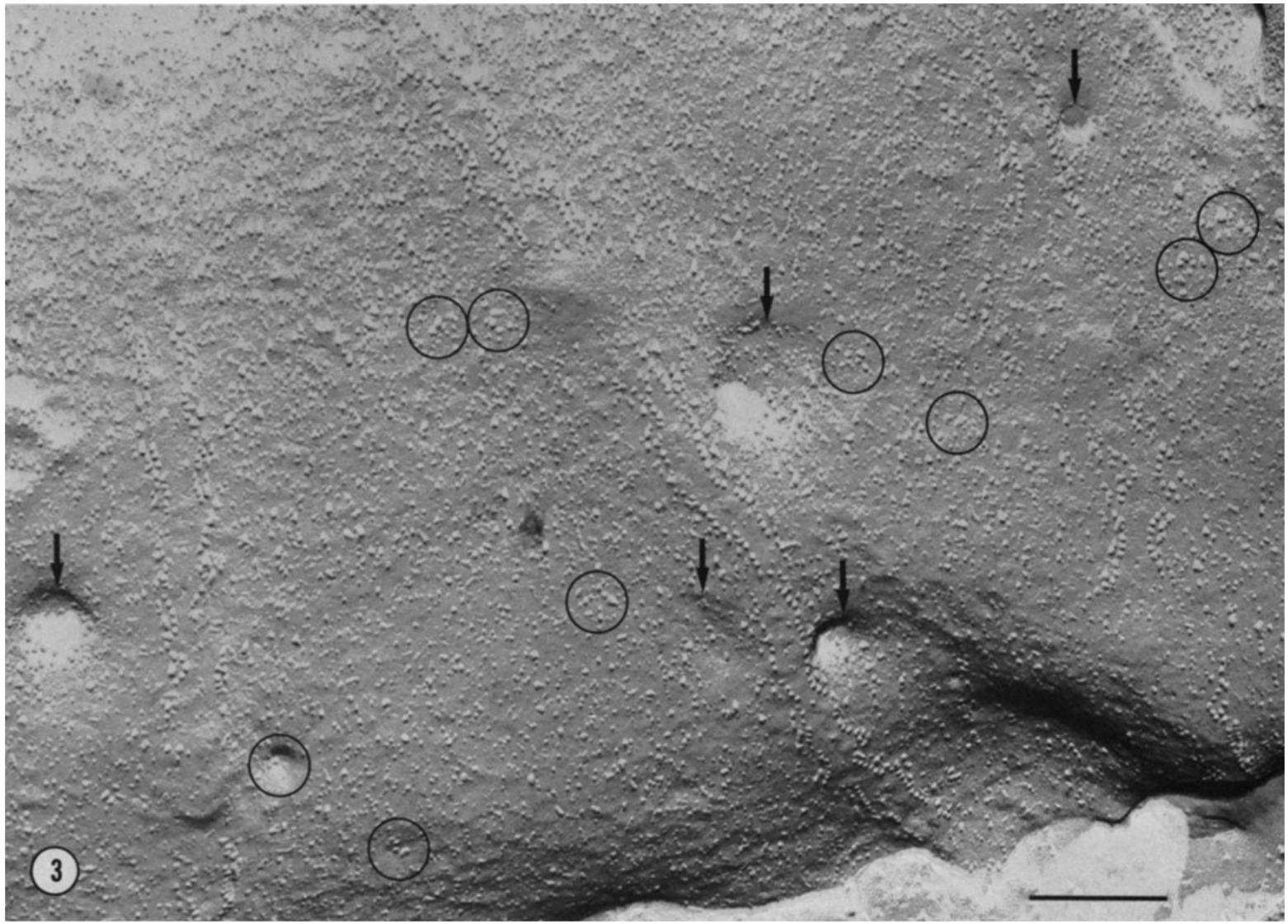


FIGURE 3 A nerve terminal quick-frozen 1 s after a stimulus in 4-AP. The structures pointed out by the arrows are presumed to be "uncoated pits" and the structures enclosed by circles "coated pits", both involved in endocytosis. Note that exocytosis of synaptic vesicles is absent by this time. Area *I* can be identified by the rows of intramembrane particles lining the active zones (see Fig. 1); there is no area *III* on this micrograph. Bar, 0.25 μm. × 90,000.

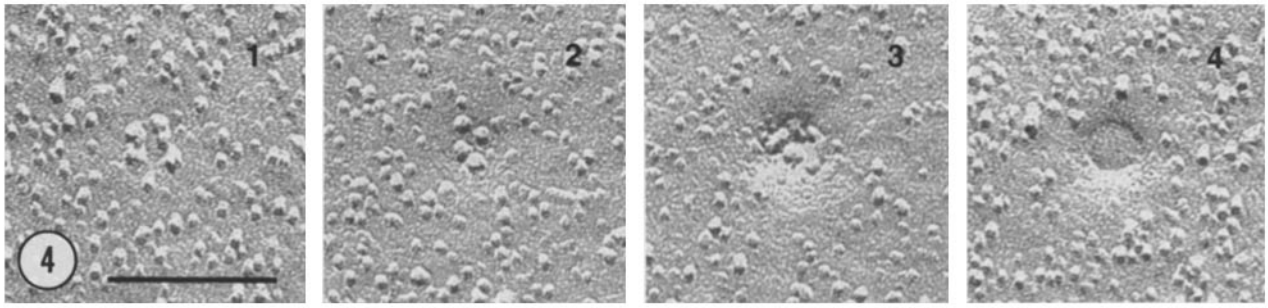


FIGURE 4 Four examples of the plasmalemmal dimples seen on nerve terminals after a burst of transmitter discharge. These structures are thought to be the freeze-fracture views of various stages in coated vesicle formation. They are arranged, from left to right, in what is believed to be the correct temporal sequence, and are referred to in the text as forms one through four. Bar, 0.125 μm. × 180,000.

Quantitative Effects of Stimulation

A quantitative analysis of the data was carried out as described in Materials and Methods. The overall abundance of endocytosis and its time course is summarized in Fig. 6 (a plot of endocytotic figures per square micrometer vs. time after a single stimulus in 4-AP). This plot presents the median values at each time point and includes data from all of the nerve terminals that we analyzed. It illustrates that a statistically significant increase in endocytotic figures is already present by 1 s ($p < 0.005$, t-test). It also displays an apparent drop in their abundance between 1 and 5 s ($p < 0.2$), no change from 5 to 10 s ($p > 0.2$), and then a dramatic increase in their abundance at 30 s ($p < 0.005$) when a peak of 5.5 endocytotic figures per square micrometer is reached. This is followed by a significant decline between 30 and 60 s ($p < 0.001$) and a further drop at 90 s ($p < 0.001$), followed by no further change at 120 s ($p > 0.2$).

Figs. 7, 8, and 9 show these data for areas I, II, and III, respectively, again as median values. It is clear from Fig. 7 that the active zones are not the predominant sites of endocytosis. In fact, there is no activity found in area I at all, except at 1 s when uncoated pits are present. In contrast, both areas II and III show a pattern of activity very different than that seen at the active zones, but are themselves very similar except for the absolute magnitude of their activity. As Figs. 8 and 9 illustrate, both these areas show a prompt onset of endocytosis within the first second after stimulation, no change between 1 and 5 s or between 5 and 10 s, and then a further rise to a peak of endocytosis at 30 s after stimulation. This activity then declines back to baseline over the next minute. The complete sets of data for areas I, II, and III, not just the median values, are shown in Figs. 10, 11, and 12, respectively. In these figures each bar represents the concentration of endocytotic figures on a single nerve terminal. There are several advantages to looking at the data in this way. It quickly displays the range of the data as well as the variance and relative variances from time point to time point. The shapes of the distributions are also evident, and it should be noted that, regardless of whether the extreme values are included or excluded, the trends are very clear cut.

We have already mentioned that coated pits could be classified into four forms according to their appearance in freeze-fracture (Fig. 4). Although it is not possible to watch the growth and development of a single coated pit, it is not unreasonable to assume that these four forms are sequential intermediates in the process. (Certainly, since the process is endocytosis, the deeper forms ought to come later.) To gather evidence for this assumption, and to learn more about the mechanism of coated vesicle formation, we compared the relative concentrations of these various forms over time. Fig. 13 shows the results of this analysis for area II. The points plotted are medians but the shapes of the curves were the same regardless of whether we plotted medians, means, or the total numbers of endocytotic figures divided by the total area studied at each time point. Note in particular that forms *2*, *3*, and *4* follow very similar time courses, with a gradual rise to 10 s and a fall from 30 s on. (There may be a peak between 10 and 30 s that was missed here.) These matching time-courses for the later forms suggest that a single endocytotic event is short-lived relative to the overall time scale of 2 min. If, instead, a single event were long-lived relative to 2 min, we would have expected to see form *2* peaking first, then form *3*, and then form *4*.

A further trend that emerges from the data in Fig. 13 is that at the time of peak abundance of endocytosis (30 s) there is definitely more form *1* than *2* ($p < 0.001$), slightly more *2* than *3* (though this difference was not statistically significant, $p > 0.2$), and clearly more *3* than *4* ($p < 0.05$). Assuming that the four forms are indeed sequential, these data would suggest that each succeeding form is briefer than its predecessors, i.e., that the individual endocytotic event begins slowly and then proceeds more rapidly as the invagination gets deeper.

As pointed out previously (Fig. 7), the only activity in area I occurs at 1 s, at which time three forms have median values greater than zero: uncoated pits ($0.49/\mu m^2$), form *1* ($0.12/\mu m^2$), and form *4* ($1.31/\mu m^2$). Form *1* may be discharged synaptic vesicles that have not yet dispersed. Most particle groups disperse by 225 ms after the stimulus (Fig. 21, reference 20); those seen here could be some groups that dispersed more slowly than normal, or they could be the remnants of the last exocytotic events to occur. The form in highest concentration is form *4*. These we believe to be a late stage in the abscission of uncoated pits (Fig. 3, upper right hand arrow), because they are present when there is no form *2* or form *3*.[4] Hence there does not appear to be any evidence for coated vesicle formation immediately at the active zones.

DISCUSSION

Time Course of Endocytosis

The endocytotic response to the addition of a large amount

[4] Note that at later times (Fig. 13), when no uncoated pits are present, form *4* is always associated with higher concentrations of forms *2* and *3*. Similar reasoning leads us to believe that the small peak of form *4* at 1 s in area II (Fig. 13) also represents uncoated pits.

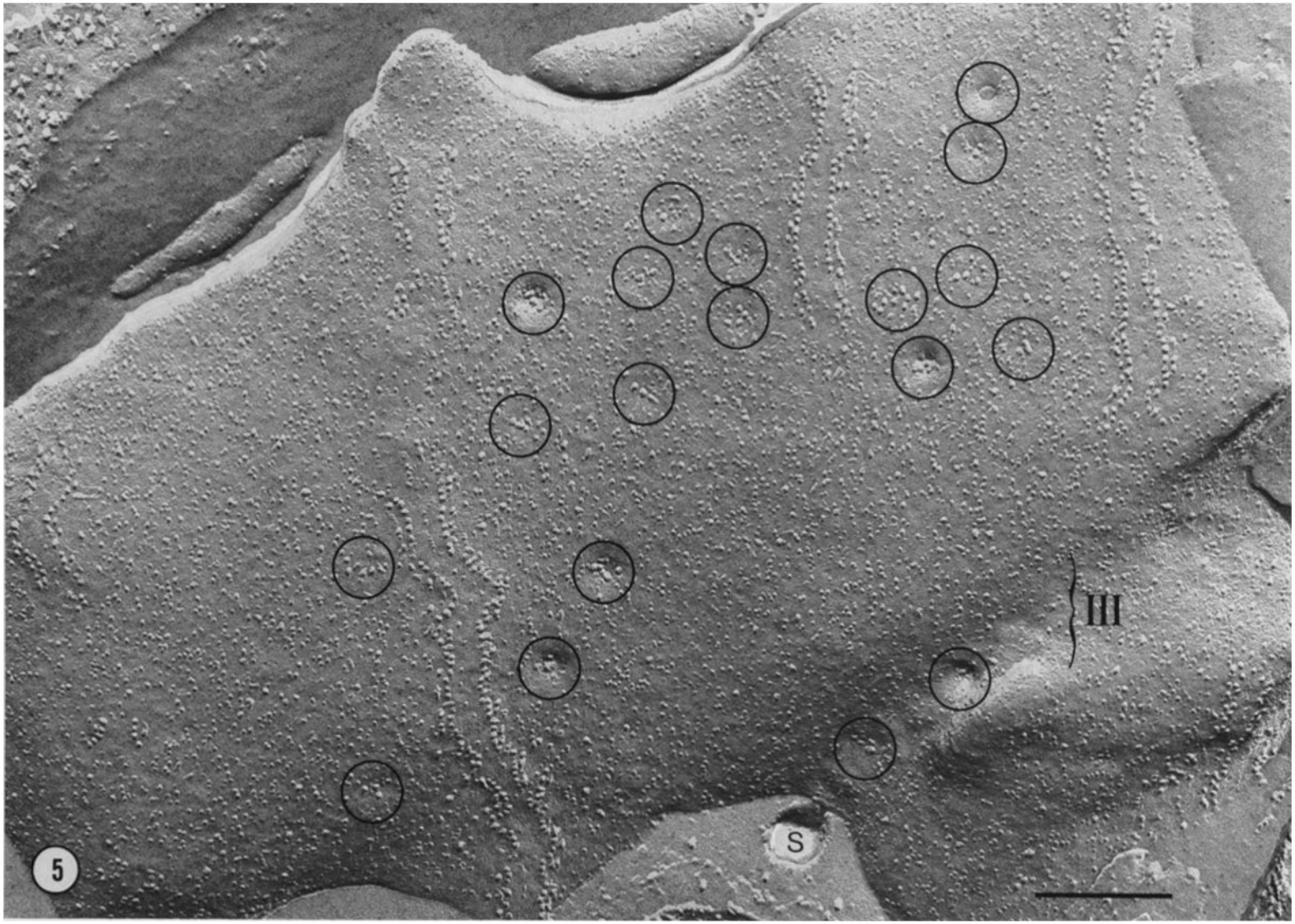


FIGURE 5 A nerve terminal quick-frozen 30 s after a stimulus in 4-AP. The active zones are clear of activity and now a large number of membrane dimples thought to be nascent coated pits are seen. The active zones can be identified as in Fig. 1. There is some area *III* visible here; it is wider at the right and narrows to the point where a cross-fractured Schwann cell finger (*S*) is visible. Bar, 0.25 μm. × 90,000.

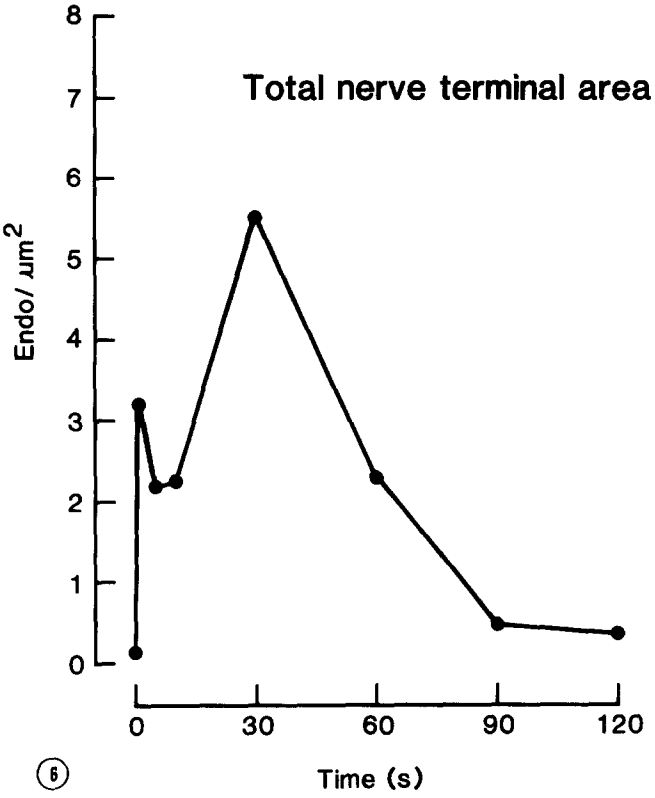


FIGURE 6 A plot of endocytotic figures per square micrometer, vs. time (in seconds) after a single stimulus in 4-AP, considering the nerve terminal as a whole. Points are median values. Details of this endocytotic response are discussed in the text.

of synaptic vesicle membrane to the surface of the motor nerve terminal was found to follow an extended time-course. Even though synaptic vesicles join the axolemma and discharge their transmitter within ~8 ms under the conditions we studied (21), the retrieval of their membrane went on for more than a minute. Considering the entire presynaptic surface as a whole (Fig. 6), it is clear that endocytosis starts up within the first second after transmitter discharge. An apparent decline after ~5 s was due to: (*a*) the completion of an early round of formation of large "uncoated" membrane compartments, and (*b*) a decrease in the number of particle groups (form *1*). A peak abundance occured at ~30 s after discharge. From that time on, endocytosis declined but it did not appear to be complete until another minute had passed. Thus, under the experimental conditions of unusually abrupt and massive transmitter discharge, endocytosis lags behind exocytosis and is very prolonged.

Location of Endocytosis

The location of endocytosis is interesting in terms of the organization of membrane recycling machinery in the nerve terminal, and has been an area of some controversy (7, 8, 17). Resolution of the disagreements may be provided by our demonstration in the present report that two different sorts of endocytosis occur, one mediated by uncoated pits and the other mediated by coated pits. Uncoated pits were found both at the active zones and away from them; hence, Ceccarelli and co-workers (7, 8) are partially correct in implicating the active zones as one potential site of membrane retrieval. On the other hand, Heuser and Reese (17) argue that the region beneath Schwann cell fingers is the most common site of membrane retrieval. This requires the postulate that the sites of endocytosis can be separated from those for exocytosis. The present observations indicate that this conclusion is also partially correct, in that the process that appears to be the major pathway of endocytosis (the formation of coated pits and vesicles) is not found at the active zones but is found both on the open area of the terminal and under Schwann fingers. Moreover, this sort of endocytosis does occur in greatest abundance beneath Schwann cell fingers, as can be seen by comparing Fig. 8 with Fig. 9.

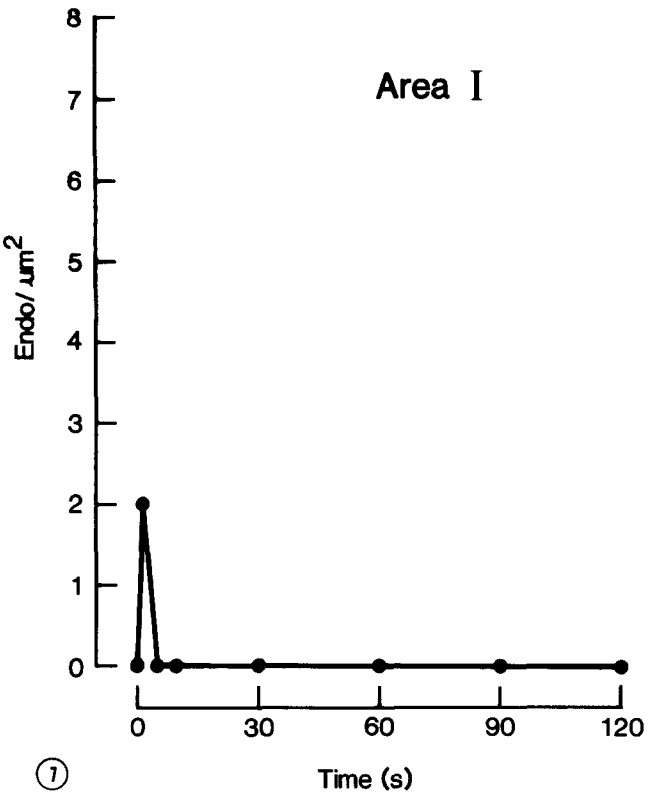
However, in the present experiments we analyzed only that part of the nerve terminal that directly faces the postsynaptic element. In this region, the amount of area covered by Schwann cells (area III) is relatively small compared with the general open area (area II), so the total number of coated vesicles formed in area II (which is equal to concentration times area) is in fact larger than the number formed under the Schwann fingers. This might at first seem to argue against the importance of the Schwann-nerve interface, but it should be remembered that more than two-thirds of the circumference of the nerve is normally enveloped by Schwann cells (4) and even a higher proportion is enveloped during prolonged activity (17, 32). The earlier thin-sectioning work (17) would argue that coated vesicles can form anywhere within these enveloped domains, but due to the characteristics of freeze-fracture we never saw these domains in the present study.

Quantity of Endocytosis

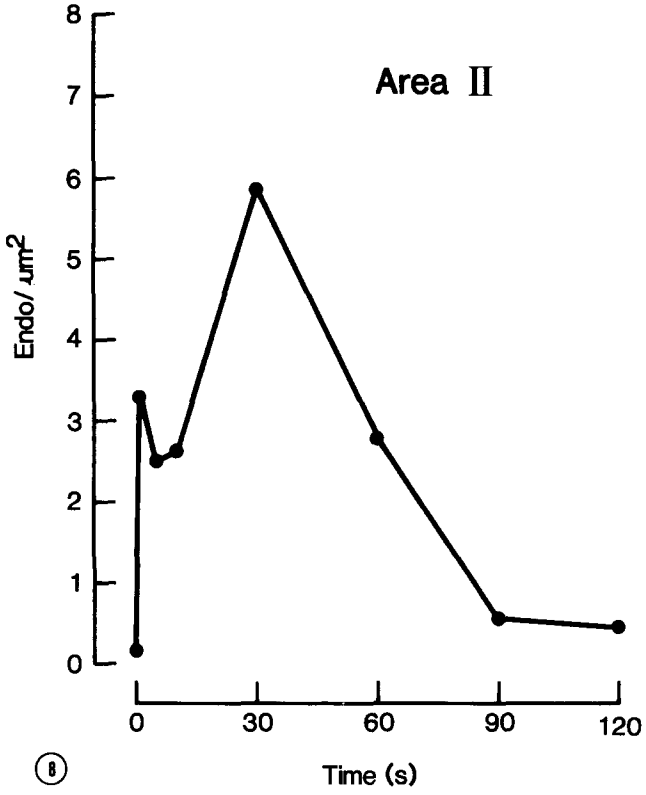
Previous use of quick-freezing to catch exocytosis has led to the estimate that, in 1 mM 4-AP, ~5,500 synaptic vesicles join the nerve terminal surface in response to a single stimulus. Here, we would like to determine whether an equivalent amount of membrane is retrieved after such a discharge. However, it is not possible to make accurate estimates of the amount of membrane retrieved. Because uncoated pits vary greatly in size and shape, and because they were observed at only one early time point, it is difficult to estimate their average surface area or how many occur in total. The coated pits apparently occur in an asynchronous wave, so use of the peak concentration of coated pits as an estimate of the total number of events tends to overlook the very earliest and very latest events. In thin-sections it is seen that coated pits form all over the nerve terminal surface (17), but in the present study we have obtained only fractures of the nerve terminal surface facing the muscle, which also complicates an estimate of the total number of coated pits. Thin-section views of uncoated pits indicate that they do not tend to form on the side of the nerve terminal away from the muscle (19).

With such potentially large errors it would be misleading to suggest that we can make any precise comparisons of the quantity of membrane in the uncoated pits, the coated pits, or the synaptic vesicles that discharged. However, gross comparisons give an indication that the amount of membrane retrieved from the nerve terminal surface was enough to compensate for the redundancy in the membrane resulting from exocytosis.

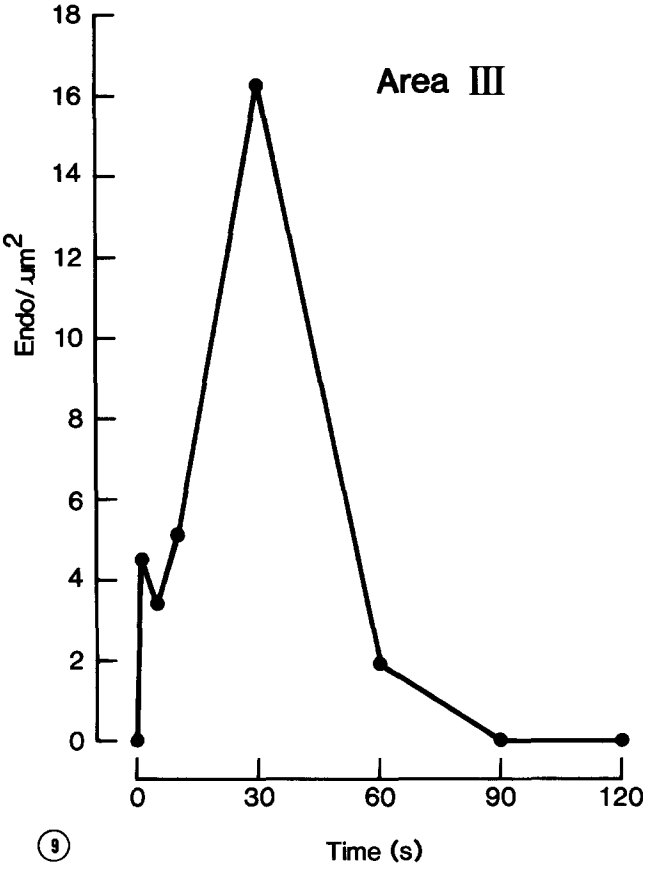
If we assume the nerve terminal to be a cylinder with a diameter (d) of 1.2 μm (17, 39), and a typical length (l) of 300 μm (21), the surface area (πdl) is ~1,100 μm^2, approximately one-half of which faces the muscle. It is our impression from thin-sections that a typical vacuole resulting from an



7



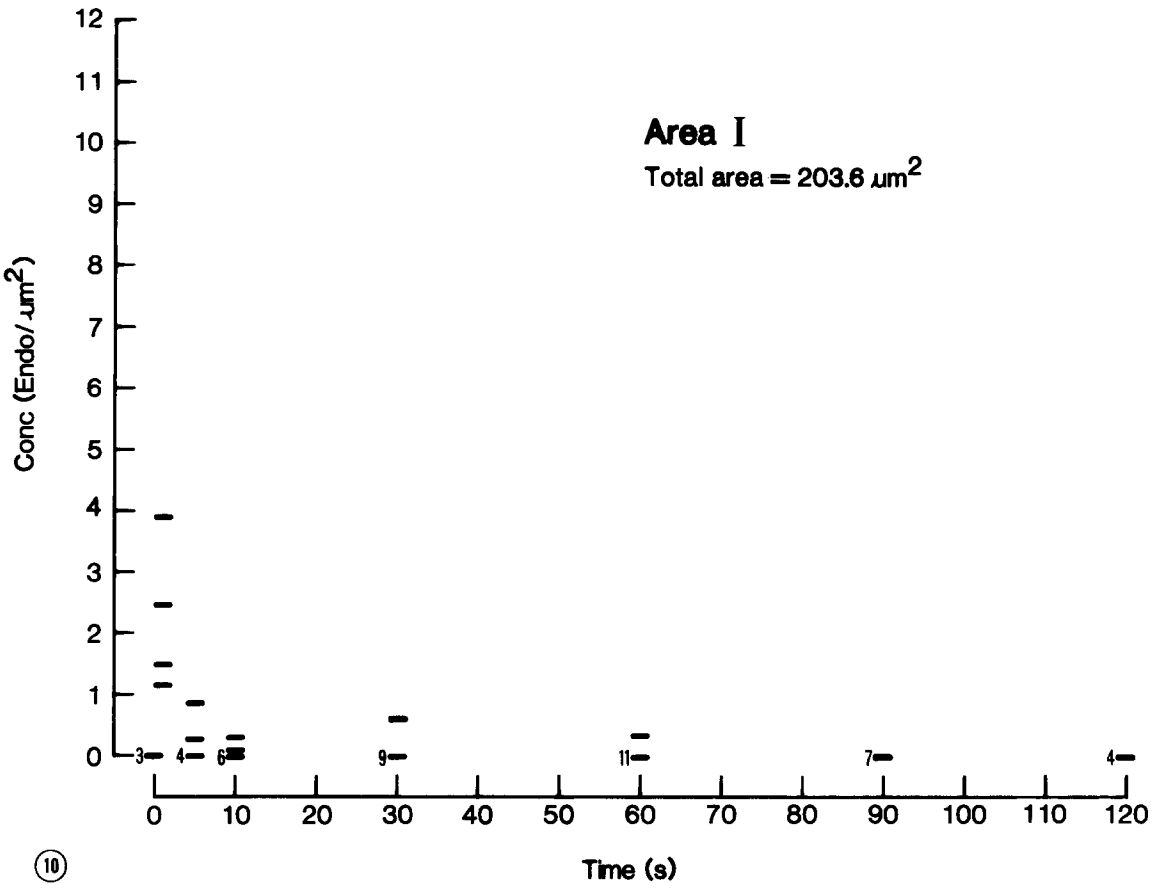
8



9

FIGURES 7–9 Median values of the endocytotic responses for areas *I*, *II*, and *III*, respectively. Fig. 7 illustrates that in area *I* there is a transient but significant rise in activity at 1 s ($p < 0.05$, t-test) followed by a prompt and permanent decline by 5 s (significant at $p < 0.005$, t-test). Fig. 8 illustrates that in area *II* there is a rise at 1 s ($p < 0.001$, t-test), no change at 5 s ($p > 0.2$) or 10 s ($p > 0.2$), a further rise at 30 s ($p < 0.02$), a drop at 60 s ($p < 0.001$) and 90 s ($p < 0.001$), and no change from then until 120 s ($p > 0.2$). Fig. 9 illustrates that in area *III* the same temporal pattern occurs, but the peak concentration is much greater (note the change in ordinate). There is a rise at 1 s ($p < 0.02$, t-test), no change at 5 s ($p > 0.2$) or 10 s ($p > 0.2$), an increase at 30 s ($p < 0.005$), a decrease at 60 s ($p < 0.001$) and 90 s ($p < 0.05$), and then no further change at 120 s ($p > 0.2$).

uncoated pit, when spherical, has a diameter roughly double that of a synaptic vesicle, and, therefore, a surface area (πd^2) four times that of a synaptic vesicle. There are ~80 μm^2 of area I (active zone) per nerve terminal (21) and a peak concentration of 1.8 uncoated pits per square micrometer (the sum of the concentrations of uncoated pits and form *4* at 1 s in area I). This means that ~150 uncoated pits, which are equivalent in membrane area to ~600 synaptic vesicles, form



FIGURES 10–12 All of the data for areas *I*, *II*, and *III*, respectively. Each horizontal bar represents the concentration of endocytotic figures on a single nerve terminal. Looking at the data in this way gives an immediate sense of its range, its variances, and relative variances from time point to time point, as well as the shapes of the distributions of data. The small numbers represent the number of observations in the zero category.

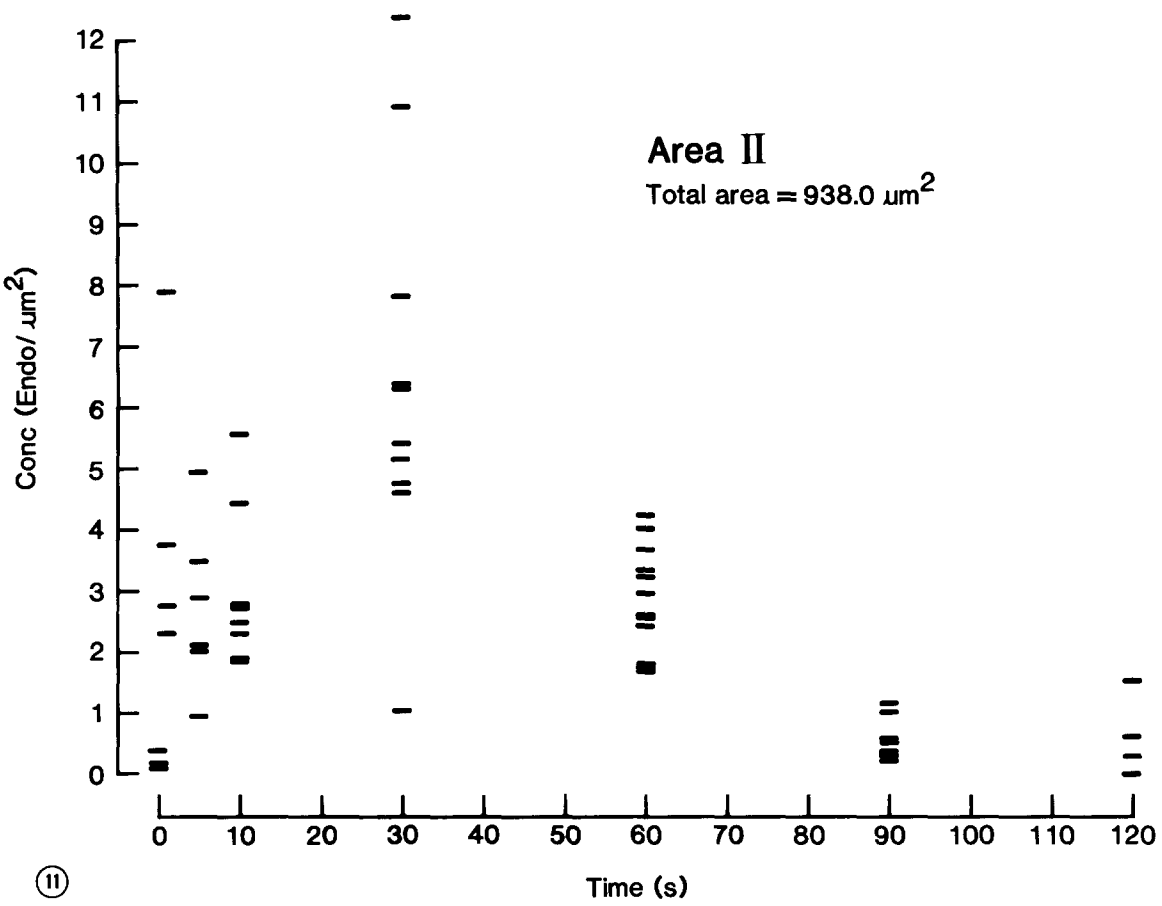
at the active zones. That leaves ~470 μm^2 of nerve terminal surface facing the muscle with ~0.8 uncoated pits forming per square micrometer (the sum of the concentrations of uncoated pits and form *4* at 1 s in area II, which is nearly identical to that in area III), which means that a total of ~375 uncoated pits form away from the active zones, or the equivalent of 1,500 synaptic vesicles.

Synaptic vesicles and coated vesicles are of about the same size, implying equal amounts of membrane. A simple way to estimate the number of coated pits is to use the peak median concentration of endocytosis for the whole terminal, which is ~5.5 endocytotic figures per square micrometer of surface, and assume that most events are represented as an early or late form (there are no uncoated pits at 30 s to cloud the issue further). When this concentration is multiplied by the calculated surface area of the terminal, an estimate of ~6,000 is obtained for the number of coated pits formed. It appears from these very rough estimates that an amount of membrane equivalent to ~8,100 synaptic vesicles was retrieved from the nerve terminal surface by endocytosis in the 2 min after a single stimulus in 4-AP. This value compares favorably with earlier estimates (21) of the number of synaptic vesicles released (which was 5,500) or the number of quanta released (which varied between 3,000 and 6,000). Hence, it argues that the endocytosis we observed was sufficiently abundant to be truly compensatory.

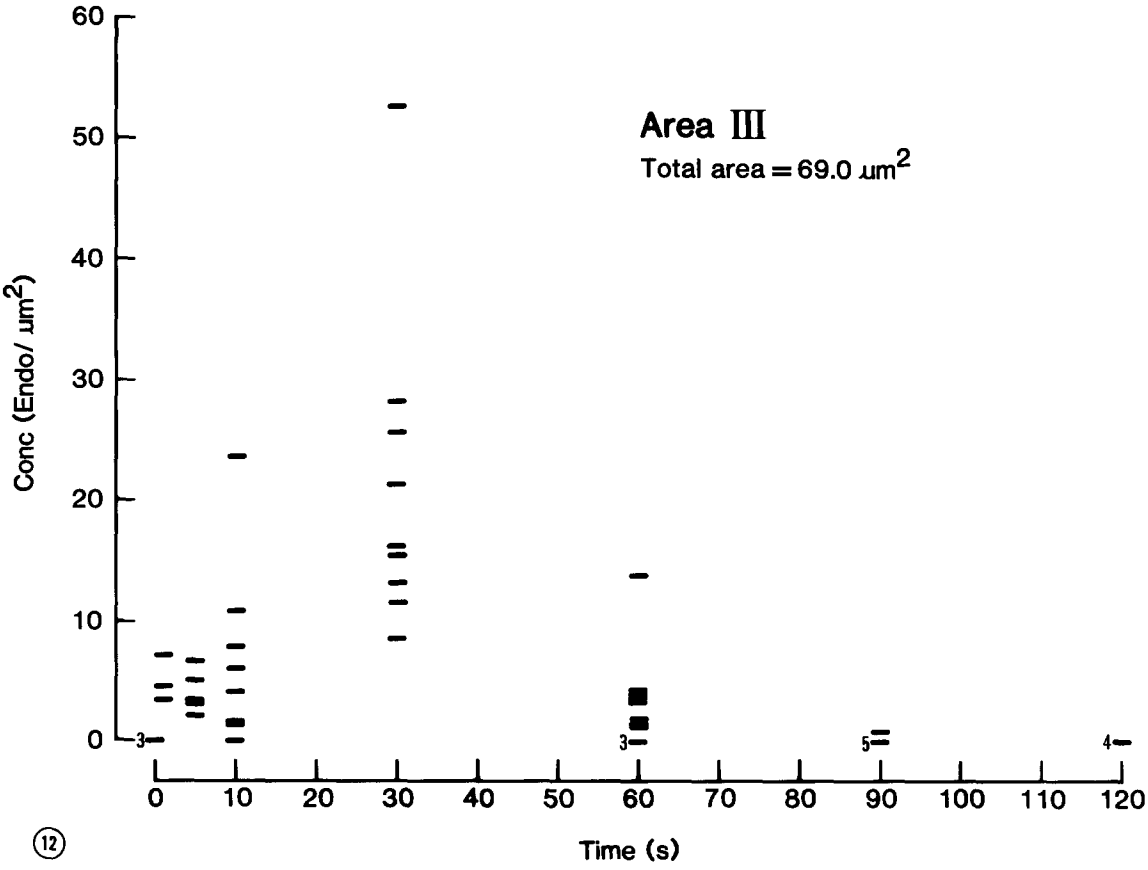
Forms of Endocytosis

Two distinct types of endocytosis were observed. The first was mediated by uncoated pits. This process appeared to be nonselective because when the membrane of uncoated pits was fractured there was no indication that intramembrane particles were concentrated or excluded (Fig. 3). The second type of endocytosis was mediated by coated pits, which do seem to be selective because large intramembrane particles like those found in synaptic vesicles were concentrated into such pits. These pits can be further subclassified into four forms (Fig. 4), and the relative abundance of these forms over time allows certain predictions to be made about the time-course of formation of each individual coated pit.

The distribution of these forms versus time in area II, which is the data-rich area, is plotted in Fig. 13. Form *1* (particle groups) rises quickly between 0 and 1 s, then drops off transiently between 1 and 10 s ($p < 0.01$), then rises to a peak at 30 s and finally gradually disappears. We cannot fully explain the early transient rise in this form, but two possibilities come to mind. On the one hand, a large fraction of the particle groups present at 1 s might simply be remnants of synaptic vesicles that had been added to the surface and had not yet dispersed. If this were true, then we would have the first evidence for a delayed, ectopic discharge of synaptic vesicles after 4-AP, which we actually expected in our earlier studies of exocytosis in the presence of this drug, due to the


Area II
Total area = 938.0 μm²
Conc (Endo/ μm²)
Time (s)
0
1
2
3
4
5
6
7
8
9
10
11
12
0
10
20
30
40
50
60
70
80
90
100
110
120

(11)


Area III
Total area = 69.0 μm²
Conc (Endo/ μm²)
Time (s)
0
10
20
30
40
50
60
3
3
5
4
0
10
20
30
40
50
60
70
80
90
100
110
120

(12)

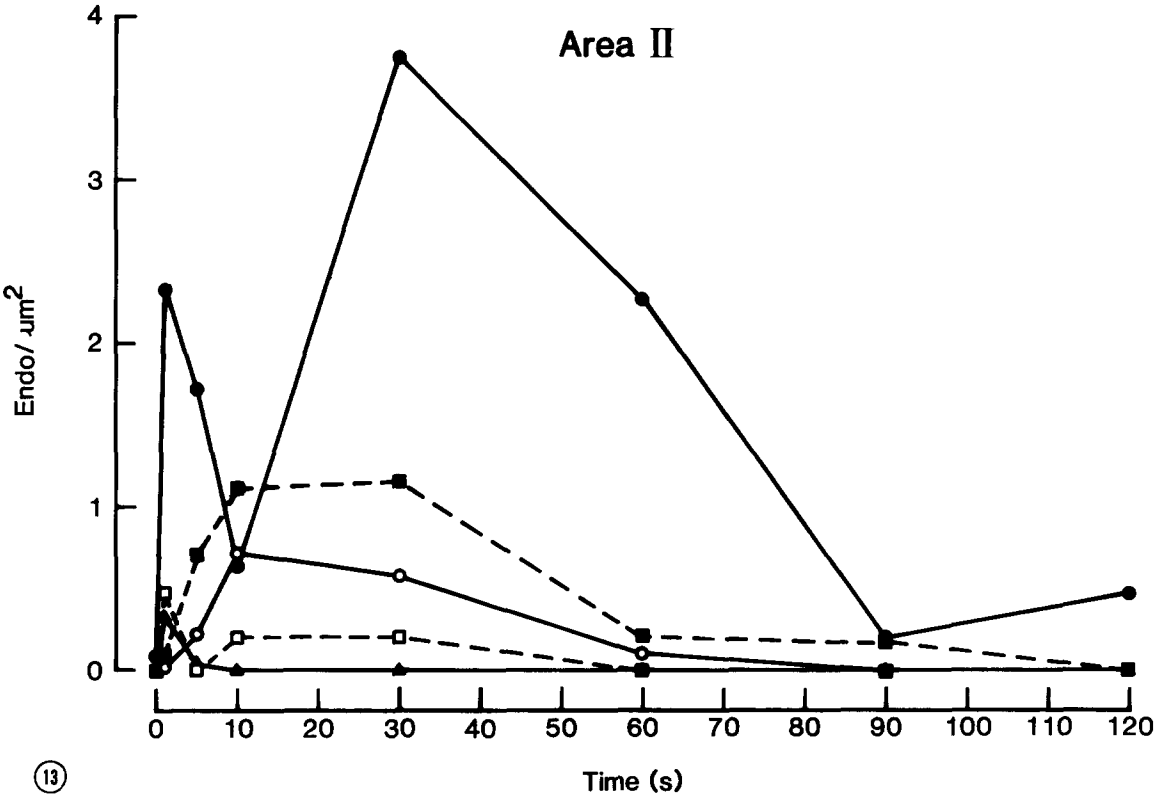


FIGURE 13 A plot of the abundance of each stage in endocytosis vs. time, as observed in the very active area *II*. Important points are discussed in the text. ▲, uncoated pits; ●, form 1; ■, form 2; O, form 3; □, form 4.

massive influx of calcium that it permits, but which we never saw. The current observations might suggest that we did not look for such ectopic exocytosis late enough.

On the other hand, the rise and fall of form *1* between 0 and 10 s might represent a first wave of endocytosis via coated pits. This could be true if there were a certain amount of coat material present at the time of the stimulus that was immediately available for action, and that by 1 s had already concentrated some of the particles from synaptic vesicles into groups. In this case, the transient drop in particle groups would be due to their rapid uptake into the nerve terminal. The subsequent rise in this form might then indicate a second wave, mediated by recruitment of additional coat material from a cytoplasmic pool, as well as reuse of the coat material involved in the first wave of coated vesicle formation.

Further information about the duration of a single event came from the time-courses of forms *2*, *3*, and *4*. These later forms all parallel one another in their abundance over time, so it would appear that a single event must be short-lived relative to the total 2 min observed. Were this not so, form *2* should have peaked first, then form *3*, then form *4*.

The relative abundance of the forms is also important to note. Assuming that the forms are sequential intermediates in a single process, and further assuming that quick-freezing stops the process very rapidly at whatever form it is in (and that the formation of each coated pit is independent of other events), then the higher the concentration of a form, the longer it must last. In fact, at the peak of the endocytotic response, the relative abundance of the different forms is $1 > 2 = 3 > 4$; hence, each form may last longer than the form that follows it. This implies that a coated pit begins to form slowly but then accelerates as it invaginates more deeply. This suggests a picture of the event as follows. The coat lies flat on the cytoplasmic surface of the axolemma and may take part in the gathering of specific vesicle components. Stress is then applied by the coat to create a membrane invagination. (How stress might be applied has been discussed in reference 16.) The actual invagination starts slowly because interfacial tension forces must be overcome, but, once the coat has initiated membrane curvature, it can act more efficiently and can conclude the event rapidly, thus making later forms relatively transient. A further point of interest in Fig. 13 is that the proportion of form *1* relative to the later forms becomes progressively greater as time elapses (compare the 30 and 60 s data), which indicates that early forms last a progressively longer time relative to the later forms. In other words, each coated pit would appear to spend a greater portion of its lifetime in the flat configuration once the bulk of membrane retrieval has already occurred, as might be expected if it had a harder time overcoming interfacial tension forces.

On the basis of the data presented here and in earlier publications, we have proposed a model of membrane recycling in motor nerve terminals, which is shown in Fig. 14. It suggests that, upon stimulation, synaptic vesicle membrane is added to the nerve terminal surface, thereby changing its plasmalemma in two ways: first, by expanding its surface area and second, by adding relatively more lipid to its cytoplasmic leaflet than to its external leaflet. Both of these effects would tend to make the membrane buckle back into the terminal. Uncoated pits and vesicles are thought to be a simple consequence of this buckling inward[5], and would be expected to

[5] The tendency for membrane to buckle back into the terminal would also affect the coated pits. We believe that this explains the shorter duration of form *1* relative to the later forms soon after the stimulus, as compared with later on (see above).

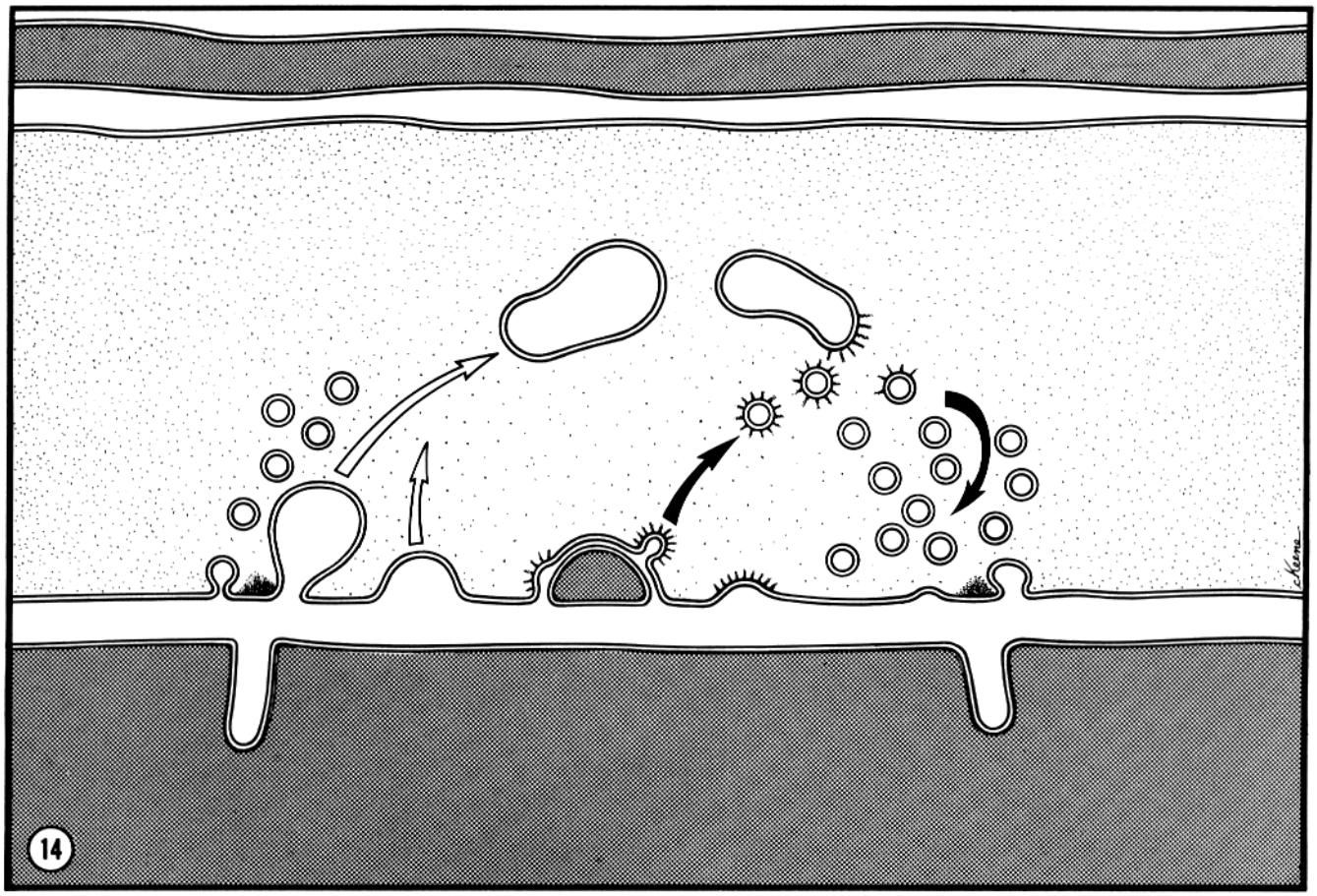


FIGURE 14 This diagram summarizes our interpretation of the membrane dynamics at the frog neuromuscular junction. Synaptic vesicles undergo exocytosis at the active zones, which results in plasmalemmal redundancies. Some of this excess membrane reenters the nerve terminal via the abscission of uncoated pits at the active zones or away from them. These structures are nonselective in that they do not concentrate or exclude intramembrane particles; they are formed in highest concentration at the active zones, and in approximately equal concentrations on the open area of the terminal and beneath Schwann cell fingers. Nearly all of the uncoated pits form during the first few seconds after exocytosis has occurred; they seem to be a simple consequence of having extra membrane on the nerve terminal surface. Other redundant membrane is retrieved over the next 90 s via coated pits. These structures are selective in that they do concentrate intramembrane particles. They are not found at the active zones; they are found on the open area of the terminal, and at an even higher concentration beneath Schwann cell fingers. The coated pits are also affected by the redundancy of the nerve terminal membrane in that they tend to spend proportionally more time beginning the membrane indentation after a great deal of endocytosis has occurred, as compared with soon after exocytosis. The path of the coated pits is shown by solid arrows because our observations taken together seem to indicate that they are physiologically more significant. The uncoated pits may not occur at all during the normal functioning of the synapse. Membrane compartments are not drawn to scale. Although the two endocytotic processes have been separated here for purposes of illustration, as far as we know, active zones are equivalent (21). The long-term relationships between the vacuoles formed by uncoated pits, the coated vesicles formed by coated pits, and other membrane compartments in the nerve terminal have been discussed previously (14, 15, 24).

internalize random bits of membrane nonselectively. In the present paper, we have shown that such structures form very rapidly, mostly within the first few seconds after stimulation, and often form right at the active zones where synaptic vesicles normally discharge. In fact, we have the impression that some of them may grow from synaptic vesicles that fail to collapse after exocytosis and, instead, swell. Formation of uncoated pits may be a very abnormal manifestation of the unusually massive amounts of vesicle membrane added to the nerve terminal surface in 4-AP. Although we have no evidence that this process ever occurs in vivo, it may explain the presence of "cisternae" after an unphysiological train of stimuli (17).

On the other hand, coated vesicles are thought to be a more selective form of membrane retrieval in nerves (17, 18) as they are in other cells (11). The data in the present paper support this notion by showing that the freeze-fracture appearance of the membrane they incorporate is different than the bulk of the plasma membrane but very similar to the membrane of synaptic vesicles (20). Furthermore, the present data show that this process can be distinguished from the more random, bulk uptake of membrane described above by its time-course and distribution on the nerve terminal surface. Specifically, coated vesicle formation has been found to occur in areas where synaptic vesicle exocytosis normally does not occur, and has been found to proceed relatively slowly, as might be expected from its leisurely rate in other cell systems (11). These observations, as well as the overall abundance of coated pits, support earlier claims that coated vesicle formation is normally the major pathway for retrieval of synaptic vesicle membrane after exocytosis.

We would like to thank Sylvia Colard-Keene for drawing Fig. 14.

This work was supported by grants from the Muscular Dystrophy Association of America.

Received for publication 16 November 1982, and in revised form 11 October 1983.

REFERENCES

1. Anderson, R. G. W., J. L. Goldstein, and M. S. Brown. 1976. Localization of low density lipoprotein receptors on plasma membrane of normal human fibroblasts and their absence in cells from a familial hypercholesterolemia homozygote. *Proc. Natl. Acad. Sci. USA.* 73:2434–2438.
2. Anderson, R. G. W., E. Vasila, R. J. Mello, M. S. Brown, and J. L. Goldstein. 1978. Immunocytochemical visualization of coated pits and vesicles in human fibroblasts: relation to low density lipoprotein receptor distribution. *Cell.* 15:919–933.
3. Birks, R. I. 1974. The relationship of transmitter release and storage to fine structure in a sympathetic ganglion. *J. Neurocytol.* 3:133–160.
4. Birks, R., H. E. Huxley, and B. Katz. 1960. The fine structure of the neuromuscular junction of the frog. *J. Physiol.* 150:134–144.
5. del Castillo, J., and B. Katz. 1954. Quantal components of the end-plate potential. *J. Physiol.* 124:560–573.
6. del Castillo, J., and B. Katz. 1955. Local activity at a depolarized nerve-muscle junction. *J. Physiol.* 128:396–411.
7. Ceccarelli, B., F. Grohovaz, and W. P. Hurlbut. 1979. Freeze-fracture studies of frog neuromuscular junction during intense release of neurotransmitter. II. Effects of electrical stimulation and high potassium. *J. Cell Biol.* 81:178–192.
8. 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.
9. Couteaux, R., and M. Pecot-Dechavassine. 1970. Vesicules synaptiques et poches au niveau des "zones actives" de la jonction neuromusculaire. *Comptes Rendus Hébdomadaires des Séances de l'Academie des Sciences, Series D.* 271:2346–2349.
10. Douglas, W. W., J. Nagasawa, and R. Schulz. 1971. Electron microscopic studies on the mechanism of secretion of posterior pituitary hormones and significance of microvesicles ("synaptic vesicles"): evidence of secretion by exocytosis and formation of microvesicles as a by-product of this process. *Mem. Soc. Endocrinol.* 19:353–383.
11. Goldstein, J. L., R. G. W. Anderson, and M. S. Brown. 1979. Coated pits, coated vesicles, and receptor-mediated endocytosis. *Nature (Lond.).* 279:679–685.
12. Heuser, J. E. 1976. Ultrafast freezing to capture the structural changes that underlie neuromuscular transmission. *In* Pathogenesis of Human Muscular Dystrophies. Excerpta Medica International Congress Series No. 404. L. P. Rowland, editor. Excerpta Medica, Amsterdam. 61–72.
13. Heuser, J. E. 1976. Morphology of synaptic vesicle discharge and reformation at the frog neuromuscular junction. *In* Motor Innervation of Muscle. S. Thesleff, editor. Academic Press, Inc., London. 51–115.
14. Heuser, J. 1977. Synaptic vesicle exocytosis revealed in quick-frozen frog neuromuscular junctions treated with 4-aminopyridine and given a single electrical shock. *In* Society for Neuroscience Symposia. W. M. Cowan, and J. A. Ferrendelli, editors. Society for Neuroscience, Bethesda, Maryland. 2:215–239.
15. Heuser, J. E. 1978. Synaptic vesicle exocytosis and recycling during transmitter discharge from the neuromuscular junction. *In* Transport of Macromolecules in Cellular Systems. S. C. Silverstein, editor. Dahlem Konferenzen, Berlin. 445–464.
16. Heuser, J. E. 1980. Three-dimensional visualization of coated vesicle formation in fibroblasts. *J. Cell Biol.* 84:560–583.
17. 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.
18. Heuser, J. E., and T. S. Reese. 1975. Redistribution of intramembranous particles from synaptic vesicles: direct evidence for vesicle recycling. *Anat. Rec.* 181:374.
19. Heuser, J. E., and T. S. Reese. 1979. Synaptic vesicle exocytosis captured by quick-freezing. *In* Fourth Intensive Study Program in the Neurosciences. F. O. Schmitt, editor. M.I.T. Press, Cambridge. 573–600.
20. Heuser, J. E., and T. S. Reese. 1980. Structural changes following transmitter release at the frog neuromuscular junction. *J. Cell Biol.* 88:564–580.
21. 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.
22. Heuser, J. E., T. S. Reese, and D. M. D. Landis. 1974. Functional changes in frog neuromuscular junctions studied with freeze-fracture. *J. Neurocytol.* 3:109–131.
23. Heuser, J. E., T. S. Reese, and D. M. D. Landis. 1976. Preservation of synaptic structure by rapid freezing. *Cold Spring Harbor Symp. Quant. Biol.* 40:17–24.
24. Llinas, R. R., and J. E. Heuser. 1977. Depolarization-release coupling systems in neurons. *Neurosci. Res. Program Bull.* 15:557–687.
25. Marchbanks, R. M. 1978. The vesicular hypothesis questioned. *Trends Neurosci.* 1:83–84.
26. Martin, A. R. 1955. A further study of the statistical composition of the end-plate potential. *J. Physiol.* 130:114–122.
27. Palade, G. E. 1954. Electron microscope observations of interneuronal and neuromuscular synapses. *Anat. Rec.* 118:335*a*. (Abstr.)
28. Palade, G. E. 1956. The endoplasmic reticulum. *J. Biophys. Biochem. Cytol.* 2:85–98. (Suppl.)
29. Palade, G. E. 1959. Functional changes in the structure of cell components. *In* Subcellular Particles. T. Hayashi, editor. Ronald Press, New York. 64–80.
30. Palay, S. L. 1954. Electron microscope study of the cytoplasm of neurons. *Anat. Rec.* 118:336*a*. (Abstr.)
31. Pfenninger, K., K. Akert, H. Moor, and C. Sandri. 1972. The fine structure of freeze-fractured presynaptic membranes. *J. Neurocytol.* 1:129–149.
32. Pysh, J., and R. G. Wiley. 1974. Synaptic vesicle depletion and recovery in cat symapthetic ganglia electrically stimulated in vivo. Evidence for transmitter release by exocytosis. *J. Cell Biol.* 60:365–374.
33. de Robertis, E. D. P., and H. S. Bennett. 1954. Submicroscopic vesicular component in the synapse. *Fed. Proc.* 13:35*a*. (Abstr.)
34. Robertson, J. D. 1956. The ultrastructure of a reptilian myoneural junction. *J. Biophys. Biochem. Cytol.* 2:381–394.
35. Rosenbluth, J., and S. L. Wissig. 1964. The distribution of exogenous ferritin in toad spinal ganglia and the mechanism of its uptake by neurons. *J. Cell Biol.* 23:307–325.
36. Roth, T. F., and K. R. Porter. 1964. Yolk protein uptake in the oocyte of the mosquito *Aedes aegypti* L. *J. Cell Biol.* 20:313–332.
37. Schaeffer, S. F., and E. Raviola. 1978. Membrane recycling in the cone cell endings of the turtle retina. *J. Cell Biol.* 79:802–825.
38. Sjostrand, F. S. 1953. The ultrastructure of the retinal rod synapses of the guinea pig eye. *J. Appl. Phys.* 24:1422*a*. (Abstr.)
39. Steinbach, J. H., and C. F. Stevens. 1976. Neuromuscular transmission. *In* Frog Neurobiology. R. Llinas and W. Prect, editors. Springer-Verlag, New York. 33–92.
40. Van Harreveld, A., and J. Crowell. 1964. Electron microscopy after rapid freezing on a metal surface and substitition fixation. *Anat. Rec.* 149:381–385.
41. Van Harreveld, A., J. Crowell, and S. K. Malhotra. 1965. A study of extracellular space in central nervous tissue by freeze-substitution. *J. Cell Biol.* 25:117–137.
42. Van Harreveld, A., J. Trubatch, and J. Steiner. 1974. Rapid freezing and electron microscopy for the arrest of physiological processes. *J. Microsc. (Oxf.).* 100:189–198.
43. Westrum, L. E. 1965. On the origin of synaptic vesicles in cerebral cortex. *J. Physiol.* 179:4P–6P.
44. Zar, J. H. 1974. Biostatistical Analysis. Prentice-Hall, Englewood Cliffs, New Jersey.