The Journal of Neuroscience, June 1989, 9(6): 1937–1942

The Relationship Between the Number of Synaptic Vesicles and the Amount of Transmitter Released

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The relationship between the number of synaptic vesicles and the amount of transmitter released from identified synapses was investigated in the dorsal longitudinal flight muscle (DLM) of the temperature-sensitive endocytosis mutant of *Drosophila melanogaster, shibire*$^{ts\text{-}1}$ (*shi*). In the *shi* fly at 29°C, vesicle recyling is blocked, but transmitter release proceeds normally. Thus, by inducing transmitter release at 29°C, *shi* synapses gradually become depleted of synaptic vesicles. In this way it was possible to regulate the number of vesicles in a synapse. Intracellular recordings were made from individual fibers of the DLM in *shi* flies after various periods at 29°C while stimulating at 0.5 Hz. The amplitude of the evoked excitatory junction potential (ejp), gradually decreased with longer exposure and was brought to various levels. The fiber was then rapidly fixed for electron microscopy. The number of vesicles per synapse was compared with the amplitude of the ejp at the time of fixation. It was observed that the smaller the ejp amplitudes became, the fewer vesicles were in the synapses. Also, as the ejp amplitude decreased, an increased number of synapses contained no vesicles. It is concluded that synaptic vesicles are directly involved in the release process.

The vesicle hypothesis, which proposes that transmitter substance is released into the synaptic cleft by exocytosis of synaptic vesicles upon stimulation, has been a well accepted explanation for transmitter release for 3 decades. Since it was first proposed (Del Castillo and Katz, 1957), innumerable experiments have been performed aimed at demonstrating the predictions set forth by this theory. One popular approach has been to demonstrate a correlation between the number of vesicles in the synapse and the amount of transmitter released. The difficulty with this approach has been in controlling the number of vesicles in the terminal. It has been possible to cause vesicle depletion of the terminal by using treatments that cause massive transmitter release such as excessive stimulation (Heuser and Reese, 1973; Zimmermann and Whittaker, 1974), high K^+ (Gennaro et al., 1978), various venoms [Clark et al., 1972 (BWSV); Chen and Lee, 1970 (β-BTX); Dai and Gomez, 1978 (tityustoxin)], or 4-aminopyridine (Heuser et al., 1979). However, these treatments are quite severe biologically, and in many cases irreversible, so that it could be argued that the loss of transmission is due to a general disruption of the synapse, rather than a result of the vesicle depletion itself. This is suggested by the fact that the time course of transmission failure does not always coincide with the loss of vesicles (Chang et al., 1973). More moderate treatments, e.g., moderate stimulation, have not induced significant changes in vesicle number (Ceccarelli and Hurlbut, 1980; Tremblay et al., 1983). This may be because vesicle recycling immediately replenishes the population (Heuser and Reese, 1973; Hurlbut and Ceccarelli, 1974; Heuser, 1977). Thus, it has not been possible to clearly demonstrate the correlation between vesicle number and transmitter release that is predicted by the vesicle hypothesis.

It is now possible to regulate precisely the number of vesicles in the synapse without using extreme methods of stimulation and without the interference of recycling. This can be done by using the temperature-sensitive endocytosis mutant of *Drosophila, shibire*$^{ts\text{-}1}$ (*shi*). The *shi* mutant carries a single base pair change in the DNA of a gene coding for an as yet unidentified protein involved in the process of endocytosis. The altered protein functions normally at 19°C but becomes nonfunctional at 29°C. As a result, the process of endocytosis is blocked at 29°C at the stage when membrane is retrieved from the plasma membrane. Thus, in the neuron, synaptic vesicle recycling is blocked at the stage when pits are formed on the plasma membrane (Kosaka and Ikeda, 1983). The mutation is specific to endocytosis, so that no other effect, for example, on excitability, nerve impulse conduction, or the transmitter release process itself, is seen (Ikeda et al., 1976). Consequently, the nerve terminal becomes gradually depleted of synaptic vesicles if synaptic activity is induced (Koenig et al., 1983). Without transmitter release, no depletion occurs (Salkoff and Kelly, 1978).

It has been shown in *shi* flies that when the temperature is raised to 29°C while stimulating at 0.5 Hz, a gradual reduction in the amplitude of the excitatory junction potential (ejp) occurs, until the response is almost completely abolished (Ikeda et al., 1976). At this point, the frequency of spontaneous release has decreased drastically, and vesicle depletion is also observed (Koenig et al., 1983). The loss of vesicles appears to be the result of exocytosis (transmitter release) proceeding normally while endocytosis (recycling) is blocked. Thus, the number of vesicles in the synapse can be controlled by bringing the temperature to 29°C and inducing transmitter release until the desired degree of depletion is reached. The great advantage of this method of depletion over other methods such as excessive stimulation is that the vesicle population is not being continuously replenished

Received July 18, 1988; revised Nov. 8, 1988; accepted Nov. 11, 1988.

We wish to thank Mr. David Gibbel and Ms. Grace Hong for their excellent technical help, and Ms. Sharyn Webb for her secretarial help. This work is supported by USPHS, NIH Grants NS-18856 and NS-18858, and NSF Grant BNS-8415920

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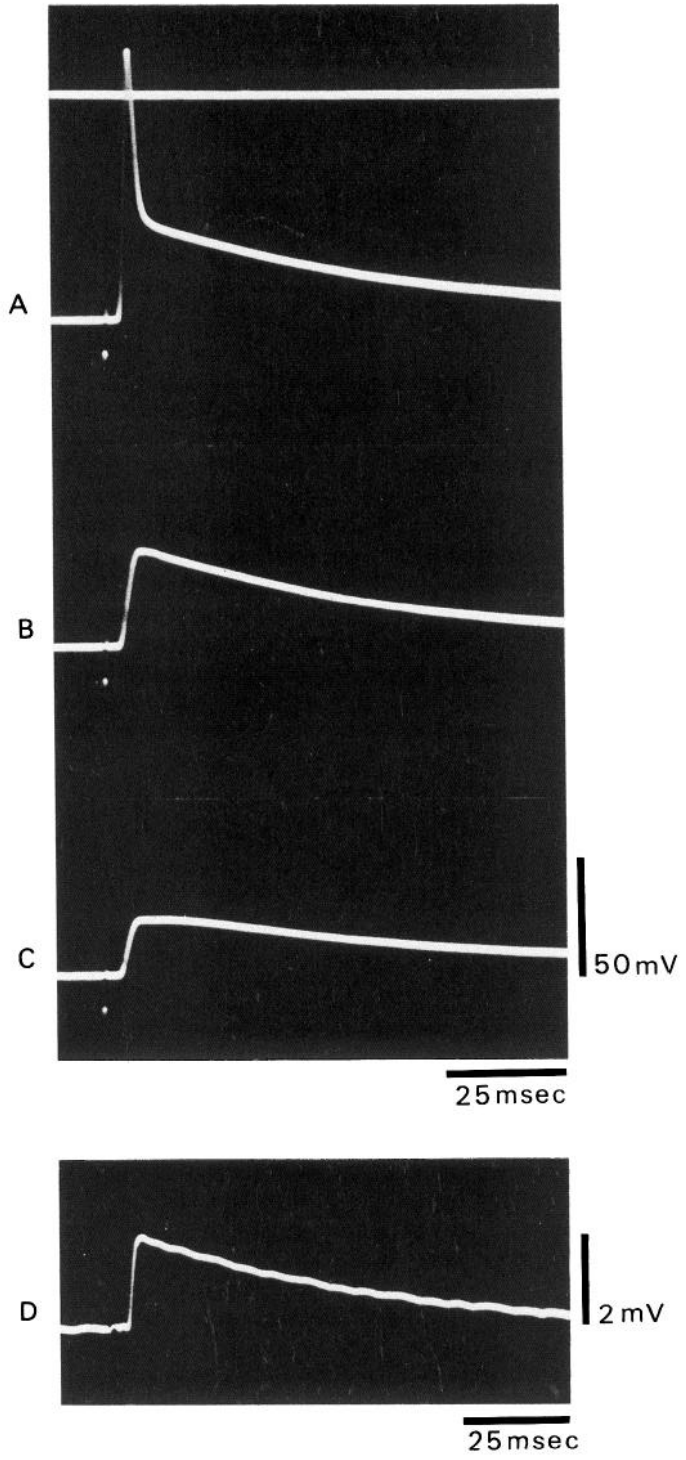

Figure 1. *A*, Example of a typical full-sized action potential in a DLM fiber of a *shi* fly at 19°C. This is also typical of a wild-type response at both 19°C and 29°C. *B*, Example of a 40 mV ejp in a DLM fiber of a *shi* fly at 29°C (≈3 min stimulation). The ejp amplitude is now below threshold for the electrogenic response. *C*, Example of a 20 mV ejp in a DLM fiber of a *shi* fly at 29°C (≈6 min stimulation). *D*, Example of a 2 mV ejp in a DLM fiber of a *shi* fly at 29°C (≈10 min stimulation).

during the experiment. In this way, the number of vesicles/synapse is correlated with the amount of transmitter released (as expressed by the amplitude of the ejp).

Materials and Methods

The experimental animals were 4-d-old adult mutant, *shibire*$^{ts-1}$, and wild-type (Oregon) *Drosophila melanogaster*. The dorsal longitudinal flight muscle (DLM), which contains 6 singly innervated, identifiable fibers, was used for this study. The neuromuscular junctions of the most dorsally located fiber, muscle fiber no. 6, were used. This fiber receives thousands of *en passant*-type synapses from a single excitatory motor neuron, which sends its axon through the posterior dorsal mesothoracic nerve (PDMN) (Ikeda et al., 1980; Ikeda and Koenig, 1988).

The fly was mounted in Tackiwax over an opening in a plastic tube so that its underside could remain exposed to the air in the tube while the fly was covered with saline (128 mM NaCl; 4.7 mM KCl; 1.8 mM $CaCl_2$; buffered to pH 7.4 with 5 mM Tris-aminomethane HCl). The lateral surfaces of the DLM and the PDMN were exposed by dissection. The PDMN was cut approximately 20 μm from the thoracic ganglion and sucked into a glass capillary electrode filled with saline for stimulation. The temperature was quickly raised to 29°C by replacing the 19°C saline with 29°C saline, and then maintained at 29°C using a Peltier heating device, and was monitored by a thermistor placed in the bath. The nerve was then stimulated with a 0.1 msec square pulse at a rate of 0.5 Hz, which caused the ejp to gradually diminish in amplitude. The nerve was thus stimulated until the desired ejp amplitude was reached. The ejp's were recorded via an intracellular electrode inserted into the muscle fiber at its lateral surface, and observed on an oscilloscope. The length constant of the muscle (3 mm) far exceeds the length of the entire muscle (350 μm), so that the ejp represents the summation of all the synaptic inputs to the fiber. When the desired ejp amplitude was reached, the saline in which the fly was immersed was rapidly replaced with fixative (2% glutaraldehyde–2% paraformaldehyde). The fixative was poured directly onto the exposed muscle. The muscle fiber was monitored intracellularly as the fixative was applied to determine if further transmitter release occurred as a result of the application. If the nerve was not cut, a great deal of activity caused by the excitation of the CNS was observed as a result of fixative application. However, with the nerve cut, no activity was observed when the fixative was applied. Data were taken only from flies that showed at least −90 mV resting potentials both at the beginning and the end of the experiment. After fixation for 30 min in the aldehyde mixture, the fixative was replaced with 4% glutaraldehyde for 2 hr. The fly was then postfixed in 2% OsO_4 in 0.1 M cacodylate buffer (pH 7.4), block-stained in 1% aqueous uranyl acetate, dehydrated in alcohol, and embedded in Epon 812. Thin sections of the same muscle fiber that had been monitored intracellularly were observed on a Philips 301 electron microscope and photographed. The number of vesicles/synapse were counted from the EM prints. Only those synapses with a single, clearly definable presynaptic dense body were counted.

Results

The ejp of the DLM fiber was brought to a particular amplitude by stimulating the PDMN at 29°C until the desired amplitude was achieved. The amplitudes chosen for this experiment were 40 mV (≈3 min stimulation), 20 mV (≈6 min stimulation), and 2 mV (≈10 min stimulation). A complete reduction of the ejp was not attempted because the length of heat exposure necessary to induce this state greatly stresses the fly, which in turn might influence the experimental results. Thus, the exposure to 29°C was kept to 10 min or less, from which it is known that the fly can fully recover. The full-sized action potential in a DLM fiber is shown in Figure 1*A*. It is composed of an ejp of approximately 60 mV on which is superimposed an electrogenic response that brings the full action potential to about 110 mV over the initial resting level of −95 mV (Ikeda, 1980). When the PDMN is stimulated in a *shi* fly at 29°C, the ejp of a DLM fiber gradually diminishes until it is below the critical firing level for the electrogenic response, which unmasks the ejp itself. The ejp's of 40, 20, and 2 mV are shown in Figure 1, *B*, *C*, and *D*, respectively.

When the desired ejp amplitude was achieved, the muscle fiber was rapidly fixed for electron microscopy. A typical ex-

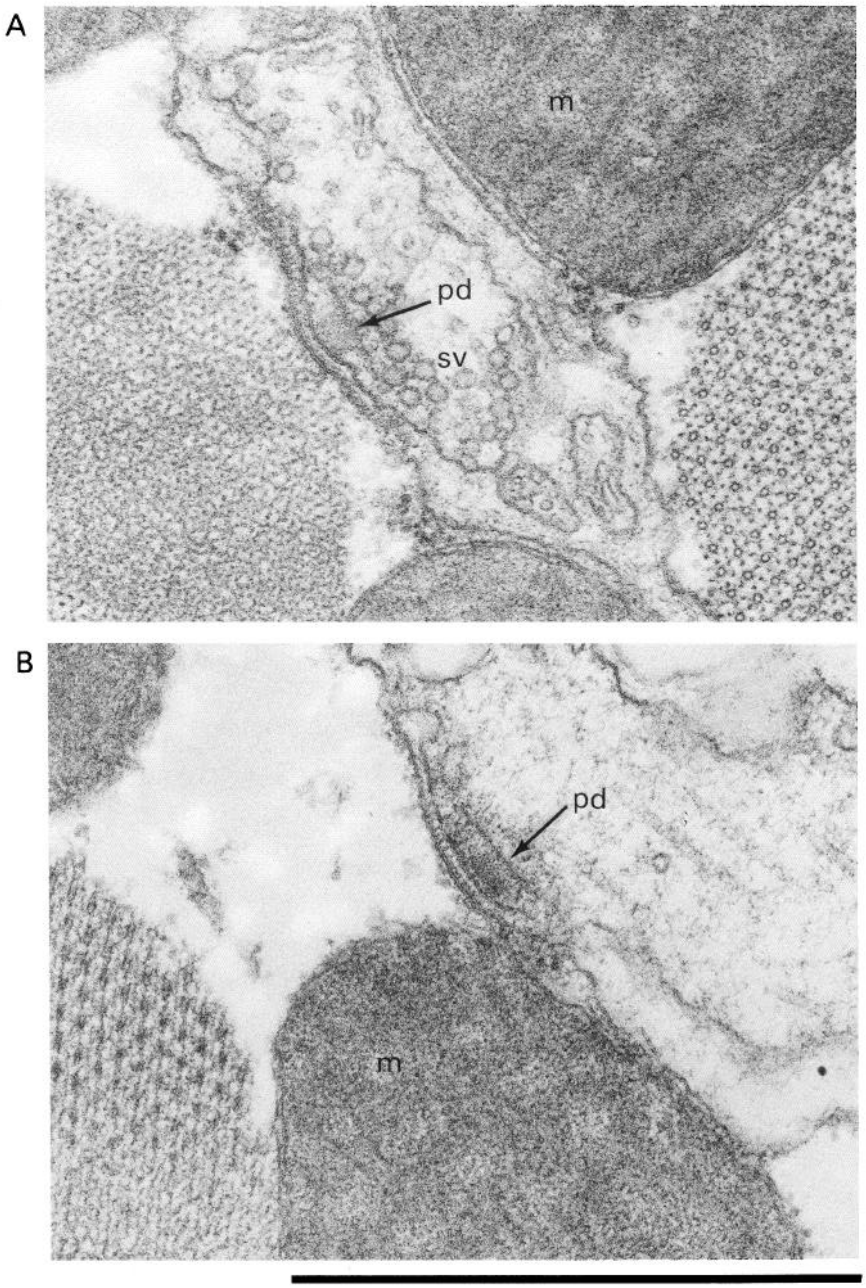

Figure 2. *A*, Example of a typical DLM synapse from a *shi* fly at 19°C. This is also typical of a wild-type synapse at 19°C. *B*, Example of a typical DLM synapse from a *shi* fly after 10 min exposure to 29°C while stimulating the PDMN at 0.5 Hz. *sv*, synaptic vesicles; *pd*, presynaptic dense body; *m*, mitochondrion. Scale bar, 1 μm.

ample of a DLM synapse from a *shi* fly at 19°C (full-sized ejp plus electrogenic response) is shown in Figure 2*A*. At 19°C, many synaptic vesicles are usually seen near the presynaptic dense body. The synapses of wild-type flies are indistinguishable from *shi* synapses at 19°C. A synapse of a *shi* fly after 10 min exposure to 29°C while stimulating at 0.5 Hz (2 mV ejp) is shown in Figure 2*B*. Under these conditions, *shi* synapses are usually completely depleted of synaptic vesicles, as seen here. On the other hand, the synapses of wild-type flies under these conditions do not show any noticeable change in synaptic vesicle number from those at 19°C.

A typical wild-type distribution at 19°C of vesicles/synapse for many synapses in a single fiber in which the nerve was cut is shown in Figure 3*A*. As can be seen, the number of vesicles per synapse varied from 0 to about 30. It should be emphasized that the number of vesicles/synapse in this study represents the number of vesicles in a particular plane of sectioning relative to the presynaptic dense body, i.e., serial sectioning through each synapse was not done. Thus, the distribution in Figure 3*A* means that one can expect from 0–30 vesicles in any particular plane of sectioning through a dense body. Since one active zone covers about 3 thin sections, the actual number of vesicles/synapse will be much higher than the figures in these distributions. What these distributions actually describe is the probability of how many vesicles any single plane of sectioning might contain. The variability in the number of vesicles found at different synapses must therefore depend in part on the particular plane of sectioning. A second factor affecting this variability would be an intrinsic variability in the number of vesicles at different synapses. The limited amount of serial sectioning done

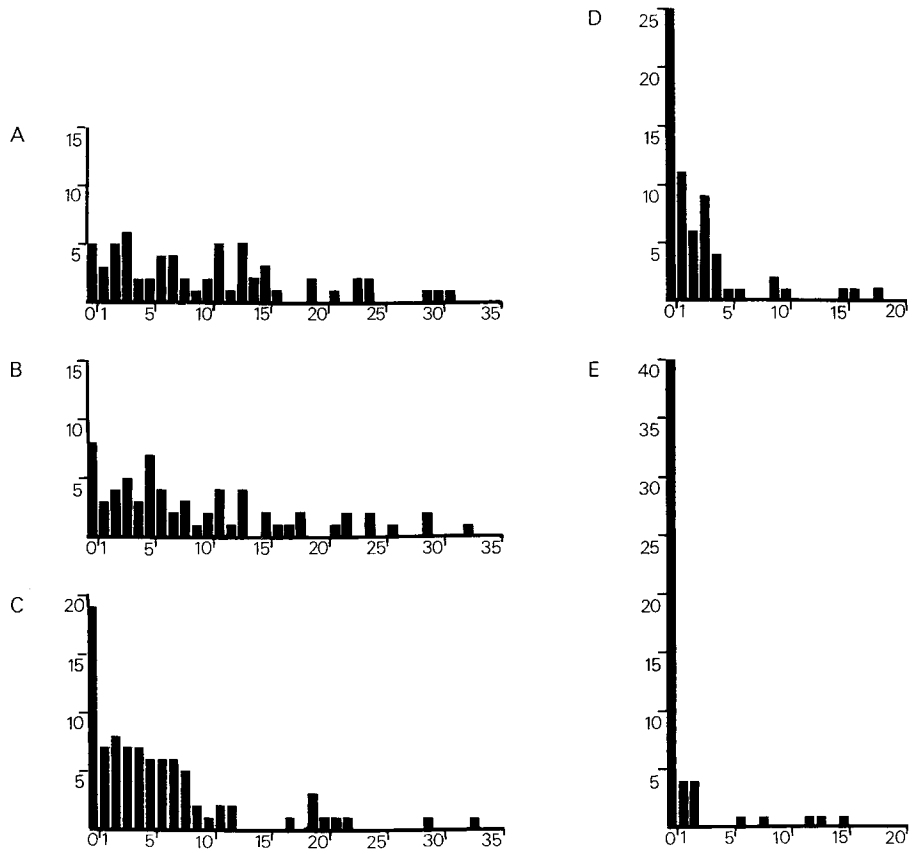

Figure 3. Distributions of vesicles/active site/plane of sectioning for many active sites innervating the same DLM fiber. *A*, Wild-type at 19°C with full-sized ejp in innervated fiber at the time of fixation; *B*, *shi* at 19°C with full-sized ejp; *C*, *shi* at 29°C with an ejp of 40 mV; *D*, *shi* at 29°C with an ejp of 20 mV; *E*, *shi* at 29°C with an ejp of 2 mV. *Ordinate*, number of synapses; *abscissa*, synapses containing x number of vesicles.

Table 1. Average number of vesicles/active site for various ejp amplitudes

Experimental condition	Number of active sites analyzed	Average number of vesicles/active site (SD)
A. Wild-type (19°C)	64	10.2 (±8.9)
ejp = full	48	11.2 (±9.3)
(29°C)	32	9.9 (±8.3)
	61	10.2 (±8.6)
	51	10.6 (±9.0)
B. *Shibire* (19°C)	64	9.0 (±8.3)
ejp = full	52	10.3 (±8.7)
C. *Shibire* (29°C)	54	5.2 (±7.1)
ejp = 40 mV	90	6.6 (±7.6)
	73	6.1 (±7.2)
D. *Shibire* (29°C)	63	2.4 (±3.8)
ejp = 20 mV	38	3.1 (±2.9)
E. *Shibire* (29°C)	53	1.2 (±3.3)
ejp = 2 mV	61	0.9 (±2.9)

Average number of vesicles/active site/plane of sectioning for many active sites innervating the same DLM fiber. (A) Wild-type at 19°C (2 flies) and 29°C (3 flies) with a full-sized ejp in the innervated muscle fiber at the time of fixation; (B) *shi* at 19°C with a full-sized ejp (2 flies); (C) *shi* at 29°C with an ejp of 40 mV (3 flies); (D) *shi* at 29°C with an ejp of 20 mV (2 flies); (E) *shi* at 29°C with an ejp of 2 mV (2 flies). Note gradual decrease in the number of vesicles/active site as the ejp amplitude is decreased. The larger SDs seen in *shi* at high temperatures are derived from the higher number of completely depleted synapses under these conditions. In general, the SDs are large because they reflect the wide distributions (see Fig. 3) at any given condition, but do not reflect a large variability in the distributions themselves at any given condition. Thus, the similarity of the SDs at any particular condition indicates that the distributions are similar for any given condition.

suggested that there could be a large difference from one synapse to the next. The relative contributions of these 2 factors cannot be distinguished in this study.

The distributions of vesicles/synapse for many synapses were quite similar among muscle fibers from different flies, with most synapses containing between 0 and 30 vesicles at their particular plane of sectioning. The means of these distributions were also quite similar, as shown in Table 1A. Thus, these distributions represent an accurate description of relative vesicle population per synapse, even though the absolute number of vesicles per synapse is not shown.

Muscle fibers of *shi* flies at 19°C showed the same type of distributions of vesicles/synapse as seen for wild-type fibers (Fig. 3*B*, Table 1B). In *shi* muscle fibers that had been stimulated at 29°C until their ejp amplitudes were diminished to 40 mV, a shift in the vesicles/synapse distribution was seen: More synapses had no vesicles or a small number of vesicles, while fewer had a higher number of vesicles. A typical example is shown in Figure 3*C*. The mean number of vesicles/synapse for these fibers is shown in Table 1C. For muscle fibers with ejp amplitudes of 20 mV, a greater shift toward lower numbers of vesicles/synapse was observed (Fig. 3*D*, Table 1D). In muscle fibers with ejp's reduced to 2 mV, the shift toward fewer vesicles/synapse was even more extreme: Most synapses showed no vesicles, while synapses with higher numbers of vesicles were rare (Fig. 3*E*, Table 1E).

Discussion

The relationship between the number of vesicles at a synapse and the amplitude of the postsynaptic response has been de-

scribed by quantal theory with the equation $m = pn$, where m is the mean quantal content (which determines average ejp amplitude), p is the probability of release, and n is the number of quanta, if the physiological correlate of n is considered to be the number of vesicles at the synapse (del Castillo and Katz, 1957). This equation predicts that as the number of vesicles decreases, the average ejp amplitude will decrease, assuming p remains the same. As outlined in our introductory remarks, this relationship has been difficult to observe in the past because of the ongoing process of vesicle replenishment, i.e., vesicle recycling. Using the *shi* mutant, however, it is possible to gradually deplete the synapse of vesicles with moderate stimulation while blocking the vesicle replenishment mechanism. It was thus possible to regulate the number of vesicles in the DLM synapses and correlate it with the amplitude of the ejp.

The data show a parallelism between a reduction in the number of vesicles/synapse and reduction in ejp amplitude, as would be predicted by quantal theory and the vesicle hypothesis. Thus, as transmitter is released by moderately stimulating (0.5 Hz) the nerve, the number of vesicles in the synapses decreases. This is accompanied by a decrease in the amplitude of the ejp. As the number of vesicles decreases further, the ejp amplitude also decreases further. These results suggest that the vesicles are being gradually used up as transmitter is being released and that as their number decreases, the quantal content of the response is decreased.

It is interesting to note that at 29°C in *shi* when the ejp amplitude has been reduced to 2 mV, the number of vesicles, or the number of active zones having vesicles associated with them, far exceeds the number of quanta actually released by an impulse at this time. According to the vesicle hypothesis, a 2 mV ejp should be made up of just a few quanta, the amplitude of one mejp being about 0.5 mV in the muscle fiber (Koenig et al., 1983). Thus, if one vesicle equals one quantum, only a few vesicles (or active zones) contribute to the 2 mV ejp, even though about 25% of the synapses observed contained at least one vesicle and often more. Considering that adjacent sections have a certain probability of containing vesicles, and considering the fact that there are thousands of synapses on this fiber, this suggests that hundreds of synapses must contain vesicles at the time when the ejp is only 2 mV in amplitude. This indicates that the probability of release for these many remaining vesicles must be very low.

One explanation for this low probability is that most of these remaining vesicles, although morphologically indistinguishable from other vesicles, represent a subpopulation of vesicles that are nonreleasable and serve primarily a storage function. Some evidence for such an idea has been shown in the *Torpedo* electric organ, where 2 populations of vesicles were isolated, one with a much higher turnover rate (Suszkiw et al., 1978).

Another explanation is that all the vesicles normally have this low probability of release. This would be possible in the muscle since so many active sites exist. Since this muscle is isopotential, if each active site released even a single quantum upon stimulation, an ejp made up of thousands of quanta should occur, many more than would be necessary to bring the fiber to firing threshold. Thus, it may be that for any given stimulus, only a small percentage of the many existing active sites responds. The mechanism responsible for this low probability of release is not known. However, one possibility could be that the electrotonic spread of the nerve impulse might invade only some of the fine axonal branches on which the synapses are located each time it fires. If the quantal content were related to the number of synapses releasing a single quanta, then an increase in the number of completely depleted synapses should cause a decrease in quantal content. This correlation is also seen in these data.

In recent years, evidence has been presented to suggest that release of transmitter may be nonvesicular (for review, see Tauc, 1982). If cytoplasmic transmitter were being released through channels in the presynaptic membrane, then the function of the vesicles becomes obscure. It has been suggested that vesicles might serve as transmitter storage depots, releasing their stores into the cytoplasm when cytoplasmic concentrations become low or that they are not directly involved in transmitter release, but rather function in some related capacity, such as sequesterers of Ca^{2+} (Israel et al., 1979; Tauc, 1982).

Our data, however, suggest that synaptic vesicles are intimately involved with transmitter release. Thus, with very moderate transmitter release (stimulating at 0.5 Hz for 3 min) while blocking recycling, a reduction in the ejp is seen, accompanied by a reduction in the number of vesicles. This indicates that vesicles are being used immediately during transmitter release, rather than coming into play only when cytoplasmic stores become depleted by excessive stimulation. Thus, their function would not seem to be that of storers of transmitter, but rather immediate suppliers of transmitter. The possibility that vesicles might have an indirect role in transmitter release is again unlikely in light of these results. Thus, it is observed that simply by blocking the production of vesicles (recycling), and then reducing their number by exocytosis, transmitter release gradually diminishes. Obviously, whatever their function, the vesicles are necessary for the release process itself. Since transmitter substance has been shown to be associated with vesicles, it still seems the most likely possibility that vesicles contain transmitter and release it upon stimulation.

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