Release and Recycling of the Readily Releasable Vesicle Population in a Synapse Possessing No Reserve Population

J. H. Koenig and Kazuo Ikeda
Division of Neuroscience, Beckman Research Institute of the City of Hope, Duarte, California
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Koenig JH, Ikeda K. Release and recycling of the readily releasable vesicle population in a synapse possessing no reserve population. J Neurophysiol 97: 4048–4057, 2007. First published March 28, 2007; doi:10.1152/jn.01258.2006. We previously demonstrated that the tergotrochanchal muscle (TTM) of Drosophila is innervated by unique synapses that possess a small readily releasable/recycling vesicle population (active zone population), but not the larger reserve vesicle population observed at other neuromuscular junctions in this animal. Using light and electron microscopic techniques and intracellular recording from the G1 muscle fiber of the TTM, the release and recycling characteristics of the readily releasable/recycling population were observed without any possible contribution from a reserve population. Our results indicate 1) the total number of vesicles in synapses presynaptic to the G1 fiber correlates with the total number of quanta that can be released onto this fiber; 2) the number of quanta released by a single action potential onto the G1 fiber is about one half of quanta that can be released onto this fiber; 3) the number of quanta correlated well with the number of vesicles present in the terminals as determined by EM. It was found that the number of quanta correlated well with the number of vesicles present in the terminals as determined by EM. It was found that the number of quanta correlated well with the number of vesicles present in the terminals as determined by EM. It was found that the number of quanta correlated well with the number of vesicles present in the terminals. Also, an electrophysiological estimate of the number of quanta released by a single action potential onto fiber G1 (i.e., the docked/primed population) was compared with the number of docked vesicles at the time of the stimulus as determined by EM. It was found that the number of quanta correlated well with the number of vesicles present in the terminals. Also, an electrophysiological estimate of the number of quanta released by a single action potential onto fiber G1 (i.e., the docked/primed population) was compared with the number of docked vesicles at the time of the stimulus as determined by EM. It was found that the number of quanta correlated well with the number of vesicles present in the terminals. Also, an electrophysiological estimate of the number of quanta released by a single action potential onto fiber G1 (i.e., the docked/primed population) was compared with the number of docked vesicles at the time of the stimulus as determined by EM. This was also done in a condition of partial depletion using the temperature-sensitive Drosophila mutant, shibire (shi), which is normal at 19°C, but in which synaptic vesicle recycling is blocked at 29°C, to investigate recycling of the active zone vesicle population. By comparing the difference in the number of quanta released by a single action potential onto fiber G1 (i.e., the docked/primed population) was compared with the number of docked vesicles at the time of the stimulus as determined by EM. It was found that the number of quanta correlated well with the number of vesicles present in the terminals.

Recently we discovered that the synapses onto the tergotrochaanal muscle (TTM) are very unusual in that they possess the active zone population, but not the nonactive zone population (Koenig and Ikeda 2005). For purposes of this paper, the population that is immediately releasable when an action potential invades the terminal (presumably docked and primed) will be referred to as the readily releasable pool (RRP) and the rest of the active zone population (docked but not primed and/or tethered to the dense body) will be referred to as the recycling population. The TTM synapses allow a unique opportunity to study the release and recycling characteristics of the active zone population without the complication of a contribution by the reserve population. Herein, we use the temperature-sensitive Drosophila mutant, shibire (shi), which is normal at 19°C, but in which synaptic vesicle recycling is blocked at 29°C, to investigate recycling of the active zone vesicle population. By comparing the difference in the number of quanta released by a single action potential onto fiber G1 (i.e., the docked/primed population) was compared with the number of docked vesicles at the time of the stimulus as determined by EM. It was found that the number of quanta correlated well with the number of vesicles present in the terminals.

Because of the small size of this muscle it was possible, by ultrathin serial sectioning for electron microscopy (EM), to make an accurate estimate of the total number of active zones and the total number of vesicles in these active zones for a single TTM fiber, the G1 fiber. With this information, an estimate of the number of vesicles recycled per active zone could be made. An estimate of the total number of quanta released under conditions of blocked recycling was then made by intracellular recording from this fiber and this was compared with the estimate of the total number of vesicles present in the terminals as determined by EM. It was found that the number of quanta correlated well with the number of vesicles present in the terminals. Also, an electrophysiological estimate of the number of quanta released by a single action potential onto fiber G1 (i.e., the docked/primed population) was compared with the number of docked vesicles at the time of the stimulus as determined by EM. This was also done in a condition of partial depletion using the shi mutant.

Finally, spontaneous release of miniature excitatory junction potentials (MEJPs) from the active zone population onto the G1 fiber was characterized and an unusual relationship between high-frequency evoked release and spontaneous release was observed.

INTRODUCTION

We previously showed that two vesicle populations that are replenished by two distinct recycling pathways exist in Drosophila synapses (Koenig and Ikeda 1996). The small active zone population is recycled very quickly (after complete fusion with the plasma membrane) by invagination and direct pinch-off from the presynaptic membrane. It is composed of vesicles either docked at the presynaptic membrane under the dense body or closely (within 150 nm) tethered to the dense body at the active zone. The much larger nonactive zone population is recycled more slowly from presynaptic membrane located intermediates and this population is dispersed throughout the terminal cytoplasm. We also showed that in the dorsal longitudinal muscle (DLM) the reserve population can be experimentally eliminated and the active zone vesicle population alone can maintain normal evoked release rates (Koenig and Ikeda 1999). These two vesicle populations appear to be equivalent to what was referred to as the readily releasable/recycling and reserve vesicle pools reported in the synapses of various other organisms (see review by Rizzoli and Betz 2005).

Address for reprint requests and other correspondence: J. H. Koenig, Division of Neuroscience, Beckman Research Institute of the City of Hope, 1450 E. Duarte Rd., Duarte, CA 91010 (E-mail: jkoenig@coh.org).

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METHODS

Four-day-old adult female Drosophila melanogaster of the wild-type strain, Oregon-R, and the single gene, recessive, temperature-sensitive mutant shibire110 (shi) were used for these experiments. The shi gene encodes the protein dynamin, a GTPase involved in the pinch-off process of endocytotic pits from the plasma membrane (for review, see De Camilli et al. 1995). In shi synapses, the mutant dynamin functions normally at 19°C, but becomes dysfunctional at >27°C, thereby blocking endocytosis of synaptic vesicles (Kosaka and Ikeda 1983). Thus at 29°C, vesicle depletion occurs as exocytosis proceeds whereas recycling is blocked. When the temperature is returned to 19°C, recycling proceeds and the vesicle population(s) is restored (Koenig et al. 1989). In some experiments, gynandromorphs (mosaics) possessing heads and abdomens composed of wild-type tissue and thoraces composed of shi tissue were used. To produce the gynandromorphs, the unstable ring X chromosome Inversion (1) wC was used (Lindsley and Grell 1968). For details on this technique, see Koenig and Ikeda (1983).

For details of the light microscopic techniques, see Ikeda et al. (1980). Briefly, the fly was immobilized on its side in Tackiwax, so that respiration could occur from the underside, and was then immersed in saline consisting of (in mM) 128 NaCl, 4.7 KCl, 1.8 CaCl2, and 5 Tris aminomethane HCl (pH 7.4). The right wing and lateral cuticle overlying the TTM were dissected away and the saline was replaced with aqueous Bouin’s solution for fixation. The preparation was then processed for silver impregnation.

For details of the electron microscopic techniques, see Kosaka and Ikeda (1983). Briefly, the fly was dissected as above to expose the TTM fibers. The TTM was fixed by instantly replacing the saline with 2% paraformaldehyde, 2% gluteraldehyde in 0.1 M phosphate buffer, pH 7.4 for 30 min, followed by 4% gluteraldehyde in 0.1 M phosphate buffer, pH 7.4, for 2 h. The fly was postfixed in 2% OsO4 in 0.1 M cacodylate buffer (pH 7.4), block-stained in aqueous uranyl acetate, dehydrated in alcohol, and embedded in Spurr’s medium. Thin sections were stained with 2% uranyl acetate in 70% ethanol and Millonig’s lead hydroxide solution and were observed with a Philips CM-10 microscope and photographed. For details on the intracellular recording techniques from TTM fibers, see Koenig and Ikeda (2005). Briefly, the fly was immobilized in Tackiwax and a glass micropipette (recording electrode) filled with 0.5% Niagira Sky Blue dye in distilled water (for visibility) was inserted into one of the TTM muscle fibers, the G1, where it attaches to the tergum. The ground electrode, a thin silver wire, was inserted very shallowly through the membrane between the thorax and the scutellum. The stimulating electrode was inserted into the neck of the fly. The muscle is composed of 23 muscle fibers arranged in a monolayer cylinder, as shown in the cross section of the TTM in Fig. 2A. The four small fibers located at the anterior median corner of the muscle, designated herein as F1–F4, are distinct from the rest of the fibers of the TTM and will be dealt with separately in a future publication. As can be seen in Fig. 2A, these four fibers are innervated by two fine axons (small arrows), each axon innervating the two fibers it runs between. Here we deal exclusively with the rest of the fibers, which are singly innervated by a giant motor axon (large arrow in Fig. 2A) that provides the input for the jump response. These fibers are designated G1–G19, starting with fiber G1 (outlined in Fig. 2A), located at the anterior, lateral corner of the muscle, next to fiber F4. For the following experiments, the TTM fiber G1 was used exclusively because of its easily determined location. However, it should be stated that the G1 fiber is representative of the other 18 muscle fibers, all of which possess almost exactly the same innervation pattern by the giant motor axon.

Number of vesicles

The number of vesicles in the active zones presynaptic to fiber G1 was determined by obtaining the average number of branches innervating this particular fiber, the average number of active zones on each branch, and the average number of vesicles per active zone. The innervation pattern onto the TTM was particularly amenable to these kinds of estimates because of the exceptional regularity in spacing between branches and between active zones on each branch.

The giant motor axon enters the muscle at a level of about 215 μm dorsal to the trochanter insertion and travels to the center of the cylinder created by the muscle fibers. There it branches into two main trunks, running dorsally and ventrally in the center of the cylinder of fibers, extending almost to the dorsal and ventral extremities of the muscle. From these main trunks, which retain their large diameter, come many fine branches (about 1 μm in diameter) at various levels of the entire muscle length. These branches run perpendicular to the main trunk and between two of the fibers. Each branch innervates both of the fibers it runs between by means of en passant synapses on both sides. An example of one of these branches
running between G1 and G2 is shown in Fig. 2B. These fine branches were observed to come off of the main trunk and run between G1 and G2 at intervals of about 72 / 1006 μm (eight flies). This spacing was very regular, as can be seen in Fig. 1 (arrows). The average length of the G1 muscle fiber, measured from the tergum to the mesothoracic leg insertion, was determined to be 860 / 1006 ± 24 / 1006 μm (eight flies), indicating that the average number of these fine branches onto G1 is 12 on each side of the fiber.

By doing thin serial sectioning of the TTM and observing by electron microscopy, it was determined that every sarcostyle (contractile unit of the muscle fiber composed of a bundle of contractile proteins ensheathed by surrounding sarcoplasmic reticulum) receives input from approximately one active zone. An example of this is shown in Fig. 3 (active zones indicated by arrows). To determine the average number of active zones per fine branch, the average length of a fine branch was determined to be 24 ± 3 μm (10 flies) and the average width of a sarcostyle was determined to be 0.8 ± 0.1 μm (13 sections each from four flies). It was then calculated that the average number of sarcostyles along one fine branch of the G1 fiber was 30. Therefore G1 receives input from 30 active zones for each branch. With 24 branches innervating this fiber (12 per side), this means that 720 active zones release transmitter onto fiber G1. Previously, it was determined that each active zone possesses an average of about 10 vesicles per plane of sectioning and one active zone spans about five EM sections (Koenig et al. 1993). Therefore each active zone is estimated to possess about 50 vesicles and approximately 36,000 (720 × 50) vesicles exist at the active zones to the G1 fiber at any given moment in a condition of rest. An example of these active zones (arrows) at higher magnification is shown in Fig. 3.

**Number of quanta**

As mentioned in the introduction, in the *shi* mutant the process of endocytosis (recycling) is normal at 19°C, but is blocked at 29°C. The process of exocytosis, on the other hand, is normal in this mutant, so that vesicle depletion occurs gradually with stimulation as vesicular release of transmitter proceeds without recycling to replenish the vesicular population. The total number of releasable quanta onto TTM fiber G1 was calculated by recording intracellularly from this fiber while stimulating to depletion under conditions of blocked recycling using the *shi* mutant. For this experiment, *shi* mosaic flies were used that possessed heads and abdomens composed of wild-type tissue, but thoraces composed of *shi* tissue (see methods for how these mosaics are constructed). Mosaics were used for this experiment because the flies were exposed to 29°C for about 25 min and respiration is impaired if the abdomen is composed of *shi* tissue. For intracellular recording, a glass pipette was inserted through the dorsal cuticle of the *shi* fly into the G1 fiber where the TTM attaches to the tergum. The location of this fiber could be identified with appropriate lighting through the cuticle and could be further identified by the particular cuticular bristles above it. The fiber was also filled with dye after the experiment to be sure of its proper identification. (For examples of the intracellularly recorded TTM response to stimulation, see Koenig and Ikeda 2005.)

After insertion of the recording electrode, the temperature was raised to 29°C to block synaptic vesicle recycling. Then the giant interneuron, the axon of which runs from the brain to the thorax and innervates the TTM giant motor axon, was stimulated at the neck. We previously determined (unpublished data) that synaptic depression occurs at frequencies ≥ 3 Hz in the TTM. Therefore to eliminate a possible complication by frequency-dependent depression, we chose to stimulate at 1 Hz (a frequency at which no depression occurs) until the excitatory junction potential (EJP) reached the failure level, i.e., until complete depletion occurred. This occurred after about 1,500 ± 120 stimuli (five flies). After the EJP reached the failure level, the preparation was allowed to rest at 29°C for 1 min and stimulated again to verify that no recycling had occurred and no residual vesicles were present. No response was observed after the rest period. The fly was then fixed for.

**Fig. 1.** Light microscopic longitudinal section through the tergotrochanteral muscle (TTM). Note regularly spaced lateral branches from the giant motor axon (arrows) that innervate the muscle fibers G1–G19. Anterior to the left. Terg, tergum; Tro, trochanter; C, coxa. Bar: 100 μm.
EM to verify that no vesicles remained in the terminals. An example of depleted TTM synapses is shown in Fig. 4A.

A depression curve (average of five flies) illustrating TTM vesicle depletion in a state of complete recycling blockage is shown in Fig. 4B (for depression curves at higher frequencies of stimulation, see Koenig and Ikeda 2005). To calculate the number of quanta released until complete depletion occurred, we used Martin’s correction to determine the number of quanta constituting large synaptic potentials (see METHODS). By this calculation, the average total number of quanta released by the TTM synapses of TTM fiber G1 was 31,800 (area under the curve).

According to the vesicle hypothesis, if all vesicles have the potential to become releasable, then the total number of quanta should correspond to the total number of vesicles that existed in the terminals when stimulation commenced (recycling blocked). As can be seen, the total average number of quanta released to fiber G1 as a result of stimulation (i.e., evoked release) in five flies was 31,800, which is about 10% less than the estimate of the total average number of vesicles in synapses to fiber G1 from five flies (36,000). However, considering that spontaneous release also occurs during the 25 min of stimulation, it would be expected that the number of quanta from evoked release alone would be less than the total number of vesicles. An accurate estimate of the number of quanta released spontaneously during the experiment cannot be made because the frequency first increases as the temperature is raised and then decreases as depletion begins to occur at high temperature, although it is probable that 4,000 quanta could be released spontaneously during this time (spontaneous release to the G1 fiber is subsequently described).

Recycling rate of readily releasable population

In the preceding section, we showed the number of available quanta to a G1 fiber when recycling is blocked. Here we look at the number of available quanta to the G1 fiber when recycling is present, to determine the contribution of recycling to release. For this experiment, the TTM synapses of a shibire fly were recorded intracellularly while stimulating at 1 Hz for the same number of stimuli as the experiment described above, i.e., the number of stimuli that completely depleted all the vesicles in the synapses to fiber G1. However, this experiment was performed at 19°C, so that recycling was occurring. No depression was observed while stimulating at 1 Hz, i.e., every evoked response elicited a full response. The difference between the number of quanta released with and without recycling represents the additional contribution of the recycled vesicles to release for a certain number of stimuli. The average number of quanta released after 1,500 stimuli (the number of stimuli that will deplete all the already existing quanta in shi flies at 29°C) at 1 Hz while recycling is going on was found to be 286,500 ± 9,680 (five flies). Because a full complement of vesicles exists in the terminal after this stimulation regimen (i.e., no depletion occurs), the recycling rate represents the difference between the number of quanta released after 1,500 stimuli with and without recycling (286,500 – 31,800) or 254,700 vesicles in 1,500 s, i.e., 170 vesicles/s. With 720 active zones, this means that 24% of the active zones recycle a vesicle each second during 1 Hz stimulation.

Relationship between number of docked vesicles and the number of quanta released

To determine what percentage of morphologically docked vesicles are primed and readily releasable, we compared the average number of docked vesicles determined by electron microscopic sections at all of the active zones onto G1 muscle fiber in 10 flies with the estimated number of quanta released by one action potential as determined by intracellular recording.

To determine the average number of morphologically docked vesicles in all of the active zones presynaptic to the G1 fiber, five wild-type and five shi flies (at 19°C) were dissected.
to expose the TTM, fixed, and observed by electron microscopy. The active zone includes the presynaptic dense body and specialized pre- and postsynaptic membranes (see Koenig and Ikeda 1999 for diagrammatic representation and electron micrographs defining this structure). The TTM nerve was cut to prevent any activity at the moment of fixation. To verify this, the G1 fiber being fixed was also recorded intracellularly as the fixative was poured directly onto it. As observed with other muscle fibers, no activity was observed in the G1 fiber. After fixation and processing for EM (see METHODS for details), the fiber was sectioned and the number of docked vesicles at 200 randomly selected active zones (one plane of sectioning through the active zone) was counted. A docked vesicle was defined as a vesicle that is located up against the presynaptic membrane under the dense body plate. An example is shown in Fig. 5 (for other examples of docking, see Koenig et al. 1993). It was observed that an average of 19 ± 2 active zone sections of the 200 observed per fly (~10%) possessed a docked vesicle. Furthermore, these active zones typically possessed only one docked vesicle per active zone. To verify this, 50 active zones were serially sectioned and none possessed more than one docked vesicle (for an example of a serially sectioned active zone, see Koenig et al. 1993). Because an active zone spans five electron microscopic thin sections (Koenig et al. 1993), the total number of sections through a G1 fiber that could possess an image of an active zone is 720 (total number of active zones) × 5 = 3,600. Therefore the total number of docked vesicles onto a G1 fiber at any given moment can be

FIG. 3. Electron microscopic cross section through the TTM demonstrating the active zones (arrows) presynaptic to the G1 muscle fiber along a branch of the giant motor axon running between G1 and G2. Note the regular spacing of these active zones, one at every sarcostyle. Bar: 1 μm.

FIG. 4. A: electron micrograph demonstrating typical TTM synapses completely depleted after stimulating at 1 Hz until the failure level. Arrows: active zones; bar: 1 μm. B: graph demonstrating the depression of quantal content in G1 fibers after successively applied stimulation at 1 Hz in a condition of blocked recycling at 29°C using the shi mutant. Recordings from 5 flies were averaged (maximum SD: 18. y-axis: number of quanta determined using Martin’s correction; x-axis: successive stimuli. Area under the curve (31,800 quanta) represents the total number of releasable quanta to the G1 fiber from the TTM motor neuron. Total number of vesicles in TTM terminals to the G1 fiber (36,000 vesicles) determined morphologically is displayed for comparison.

FIG. 5. Electron microscopic thin section through an active zone presynaptic to the G1 fiber demonstrating a vesicle docked at the presynaptic membrane. Long arrow: docked vesicle; short arrow: dense body. Bar: 200 nm.
calculated as follows: 3,600 × 0.1 = 360. This suggests that around 360 of the 720 active zones possess a docked vesicle at any given moment while in a resting state. The amplitude of the EJP of the TTM G1 fiber in response to a single stimulus was estimated to be 45 mV using 4 mM Na-L-glutamate to block the electrogenic response (Koenig and Ikeda 2005). With the amplitude of a single MEJP being about 0.5 mV (see following text), the number of quanta released to cause a 45 mV EJP (when a single action potential invades the terminals onto muscle fiber G1 without prior activity) was estimated using Martin’s correction (see Methods) to be 191 quanta. These quanta must represent the RRP ( docked and primed).

Because about 191 quanta are released with a single stimulus and 360 morphologically docked vesicles were observed, these data suggest that approximately half (53%) of the vesicles that appear morphologically to be docked are released when an action potential invades the terminal. Because of the large diameter of the TTM motor axon and the similarity of the innervation pattern and diameter of the branches possessing active zones, it is expected that an action potential will invade all branches equally. Therefore these data suggest that only about half of the morphologically docked vesicles are in a readily releasable state at any given moment.

We next looked at the number of docked vesicles at active zones that were partially depleted in 10 shi flies at high temperature. For this experiment, we cut the TTM nerve where it leaves the ganglion and sucked it into a suction electrode for stimulation. Then we raised the temperature to 29°C and, while recording intracellularly, stimulated the TTM motor neuron until the EJP of the G1 fiber reached an amplitude of 10 mV. We then fixed the fiber and processed it for EM as described earlier. In this condition, it was observed that 1,360 of 2,000 active zones observed in 10 flies (68% in the particular plane of sectioning being observed) did not possess vesicles, whereas the rest of the active zones possessed at least one vesicle. Of the 640 sections through active zones that did possess vesicles, 61 (~10%) possessed a docked vesicle, which suggests that partial depletion does not appear to influence the percentage of active zones possessing a docked vesicle within the subpopulation of active zones possessing vesicles. Interestingly, if one is to repeat the above calculation for total number of docked vesicles [3,600 × 0.32 (number of active zones possessing vesicles) × 0.1], it would be predicted that 115 docked vesicles exist at the active zones and 63 (55%) of them should be releasable. However, using Martin’s correction, only about 22 quanta would be necessary to produce the 10 mV EJP used in this experiment. Thus it appears that there is an additional factor reducing the probability of release under conditions of depletion in addition to the reduction in the number of docked vesicles. Apparently, the process between morphological docking and priming for immediate release is altered in the depleted state caused by blocking endocytosis.

Spontaneous release of the active zone population

In our previous publication (Koenig and Ikeda 1999), we demonstrated in the dorsal longitudinal muscle (DLM) that when the reserve population was eliminated, leaving only the active zone population, the frequency of spontaneous release was very much attenuated and the MEJPs had unusual release characteristics (multiquantal and clustered MEJPs). Creating DLM synapses that possessed only the active zone population was accomplished by depleting both populations of vesicles by exposing shi DLM synapses to high temperature while stimulating and then allowing the active zone population to recover at 19°C before the recovery of the reserve population. With the discovery of the TTM preparation, we can now directly characterize the spontaneous release characteristics of the active zone population, to determine whether the unusual release characteristics represent some kind of side effect of depletion or are truly characteristic of the active zone population.

The TTM G1 fibers of five wild-type flies were recorded intracellularly and spontaneous release was observed. The frequency of release at 19°C averaged 7 ± 3/s, and increased appreciably as the temperature was raised. Thus by 21°C, there were so many events that they overlapped continuously, making a determination of frequency impossible. By 26°C, a considerable summation of events occurred, causing baseline fluctuations of several millivolts. Although the initial frequency of the TTM was lower than that of the DLM (7 vs. 40/s), the increase in frequency with increased temperature was indistinguishable from that previously demonstrated for the DLM (for an example of temperature effect on release frequency, see Fig. 5 of Koenig and Ikeda 1999).

The amplitude of TTM MEJPs, as mentioned earlier, was about 0.5 mV, as is demonstrated by the typical example of a TTM amplitude histogram of MEJP amplitudes in Fig. 6 (solid line). As this figure shows, no suggestion of multiquantal release is observed. Also, no clustering was apparent in this recording. Therefore multiquantum and clustered release are not characteristics of spontaneous release from the active zone population, but rather, must be the result of some other condition brought on by subjecting the terminal to complete depletion using shi. To verify that multiquantum and clustered release is induced by the depletion regimen using shi, the TTM G1 fiber of a shi fly was recorded intracellularly, exposed to 29°C, and stimulated until complete depletion occurred, then brought to 19°C and allowed to recover. During the time at 29°C, it was observed that as the amplitude of the evoked
response decreased, the MEJP frequency also decreased, which was previously observed for the DLM as well (Ikeda and Koenig 1988). During the initial recovery period, spontaneous multiquantal release and clustered release similar to that observed in the DLM were observed as shown in Fig. 6 (dashed line). This clustered and multiquantal release gradually disappeared after about 5 min at 19°C, after which release became indistinguishable from that occurring before depletion.

One unusual difference in spontaneous release between the TTM, which is exclusively from the active zone population, and the DLM, which is predominantly from the reserve population, is the response to a high-frequency burst of activity. It is commonly known in various preparations including the DLM that after a high-frequency burst of spikes, the frequency of spontaneous release increases transiently, which is believed to be in response to the transient increase in $\text{Ca}^{2+}$ concentration inside the terminal resulting from the burst (Zengel and Sosa 1994). With the TTM preparation (wild-type), after a high-frequency burst, the frequency of spontaneous release was observed to decrease rather than increase (Fig. 7). This suggests that in the TTM motor neuron terminals, the number of vesicles available for spontaneous release (from the active zone population) decreases after the burst, whereas in the DLM, the reserve population apparently supplies enough vesicles to actually allow an increase in frequency. A few seconds after the burst, the MEJP frequency of the TTM appeared to return to preburst levels, as determined by the amplitude of MEJP summation fluctuations.

**DISCUSSION**

Because the G1 fiber of the TTM does not possess a reserve vesicle population, it offers an unparalleled opportunity to observe transmission and recycling events of the readily releasable and recycling vesicle populations without the complication of contributions from the reserve population. Furthermore, because of its small size, exceptional regularity of structure, and uncomplicated innervation pattern, an remarkably accurate estimate of the number of active zones to the G1 fiber and number of vesicles at these active zones can be made. With these advantages, it was determined by electron microscopy that the average number of active zones to the G1 is 720, that the average number of vesicles to the G1 fiber is 36,000, and that the total number of vesicles that appear morphologically docked is 360. By intracellular recording, it was determined that about 191 quanta are released with a single stimulus, 31,800 quanta are released to achieve total depletion when recycling is blocked, and 286,500 quanta are released under the same stimulus conditions when recycling is present. Also, spontaneous MEJP release was characterized.

By comparing the morphological data with the electrophysiological data, a wealth of information can be obtained.

1) **Vesicles versus quanta.** These data demonstrate that a good correlation exists between the number of vesicles in the terminal and the number of releasable quanta, as would be predicted by the vesicle hypothesis (del Castillo and Katz 1955). As mentioned in RESULTS, the total number of released evoked quanta (31,800) is about 4,000 quanta less than that predicted by the total number of vesicles (36,000). One explanation for this difference is that quanta are being released spontaneously during the 25 min of the experiment monitoring evoked release.

2) **Docked versus docked/primed (RRP).** The data demonstrate that only about half of the active zones possess a docked vesicle at any point in time. Furthermore, not all morphologically “docked” vesicles are readily releasable, as is evidenced by comparing the number of quanta released by a single evoked response (191) with the number of vesicles that appear docked by morphological criteria (360). Two assumptions can be made from these data: 1) immediately after a single stimulus, about 170 (360 – 191) docked vesicles must remain at the active zones, presumably becoming the next readily releasable population; and 2) these 170 remaining docked vesicles are located at other active zones from the ones that released during the single stimulus because it was shown that only one docked vesicle exists at any given active zone. If these assumptions are correct, then with each stimulus to the giant axon, a different subset of active zones, each possessing a single readily releasable vesicle, will exocytose. Furthermore, it is assumed that a new population of docked vesicles will be continuously moving into place. Possibly, the probability that a vesicle will dock at an active zone will be lowest at those active zones that have just released, so that a different subset of active zones will release with each subsequent stimulus. Considering the various molecular events that are involved in the docking/priming process, it is not surprising that differences in level of release readiness of morphologically docked vesicles exist (see review by Rettig and Neher 2002).

In synapses of the calyx of Held (Satzler et al. 2002) and also in hippocampal synapses (Schikorski and Stevens 2001), it was reported that the number of morphologically docked vesicles fits well with the number of vesicles in the RRP. However, these reports define the RRP based on fluorescent dye uptake rather than actual number of quanta released and therefore may not be comparable to the present data.

When the synapses are in a state of partial depletion as a result of blocking recycling while exocytosis proceeds using the shi mutant, it was observed that the ratio of readily
releasable vesicles to the number of morphologically docked vesicles decreased from about one half (0.55) to about one third (0.35). This suggests that the state of partial depletion may cause a condition within the terminal that slows the priming process. Interestingly, it was previously reported that long-term, mild physiological activity appears to depress vesicle priming (but not docking) in hippocampal pyramidal neurons (Molder et al. 2006). Perhaps there is a correlation between the condition resulting from partial depletion described here and that resulting from prolonged activity.

3) RRP versus active zones. The data demonstrate that only about a quarter (191 of 720) of the active zones to the TTM G1 fiber releases a single vesicle to produce a full-sized evoked response in the postsynaptic cell, the G1 fiber. They also suggest that another distinct quarter of active zones (360 – 191) possesses a docked vesicle that is not readily releasable (presumably not primed) at the time of the first evoked response. These observations suggest that to achieve a full-sized postsynaptic response in the TTM, it is not necessary for any particular active zone to bring a vesicle to immediate readiness for release each time an evoked response occurs. Thus other active zones are available to respond to a second (or more) evoked response(s), allowing an active zone that has just released a vesicle, time to dock and prime another vesicle. This scenario most likely exists at other *Drosophila* neuromuscular junctions that we have observed (coxal, cervical, DLM) because the number of active zones onto a single fiber of these muscles also greatly exceeds the number of quanta that would be needed to evoke a full-sized EJP.

4) Recycling rate. In the present study, we observed the recycling rate with 1 Hz stimulation, a rate that does not create any depression. It was determined that depression, which is thought to arise primarily from the unavailability of vesicles when the recycling rate cannot keep up with the release rate, occurs in the TTM at stimulation rates ≥3 Hz. At 3 Hz stimulation, if only a quarter of the active zones release a vesicle with any given response, then this means that after an active zone releases a vesicle, there should be three quarters of the active zones left to provide the vesicles for the next three evoked responses, each 333 ms apart. This would suggest that any particular active zone would need to recycle a vesicle within about 1 s to keep up with 3 Hz stimulation. However, because spontaneous release is also occurring at these active zones, the recycling rate may be somewhat <1 s from exocytosis to subsequent exocytosis at any particular active zone.

Other authors previously made estimates of recycling rate in other preparations, but these were observed with high-frequency stimulation. In hippocampal synapses, with 30 Hz stimulation, vesicles were shown using styryl dye destaining to be reused after 1–3 s (Sara et al. 2002). Our estimate, under conditions of low-frequency stimulation of 1 s, falls within that range. In the *Drosophila* larval preparation, it was shown that with 100 s of tetanic stimulation (10 Hz), recycling reached an estimated maximum rate of fewer than two vesicles/s at each active zone (Delgado et al. 2000). Under different ionic conditions, a maximum rate of 0.65 vesicles/s per active zone has been reported at this same synapse (Dickman et al. 2005). Hansen and Gustafsson (2001) estimated that about two vesicles per active zone were released in hippocampal slices with a short 50 Hz stimulation, whereas Fernandez-Alfonso and Ryan (2004) estimated maximal rates of up to one vesicle/s. Our estimate of 0.25 vesicle/s at each active zone expresses the amount of recycling required with a frequency of stimulation that is probably higher than would be experienced by the muscle during normal activity (jump response). Furthermore, our estimate relates to the active zone (readily releasable/recycling) population of vesicles only. Because it was previously shown that the reserve population contributes to release during high-frequency evoked release, it is possible that there may be a contribution from this population in the estimates from synapses mentioned earlier. Also, Delgado et al. (2000) showed that the recycling rate increases with increased frequency. Therefore the discrepancies in these estimates may be a result of differences in the stimulus frequency applied, a possible contribution by the reserve population, differences in experimental methods, or simply a difference in recycling rates of different types of preparations.

The recycling rate of the TTM synapse with a stimulus frequency that does not cause depression (1 Hz) is determined to be 170 vesicles/s for all the active zones to the G1. Because no depression or recognizable depletion occurs at 1-Hz stimulation, the recycling rate should be approximately the same as the number of quanta released per evoked response. As mentioned earlier, 191 quanta are released with a single stimulus, which is about 10% less than the total number of vesicles that are estimated to be recycled at 1-Hz stimulation. However, considering the fact that the synapses to this muscle fiber possess in total about 36,000 vesicles that are already formed, this difference between exocytosis rate and endocytosis rate would be undetectable over thousands of evoked responses.

5) Spontaneous release from RRP. With the TTM preparation, we determined that normal spontaneous release occurs from the active zone population in wild-type or shi at low temperature and that, similar to the DLM, the release characteristics after total depletion resulting from blocking recycling become nonrandom. This suggests that the nonrandom release characteristics previously observed in the DLM, when only the active zone population was present, arise from some condition caused by first depleting the synapse. As suggested in the previous publication, one possible cause of the nonrandom spontaneous release characteristics might be increased cytosolic Ca$^{2+}$ levels because such abnormal release characteristics can be induced by exposure to high Ca$^{2+}$ saline (Koenig et al. 1993).

After a high-frequency burst of spikes to the G1 fiber, it was observed that spontaneous release was greatly attenuated, which, as mentioned in RESULTS, is the opposite of what is commonly observed in synapses possessing both readily releasable/recycling and reserve populations. This result suggests that the population of vesicles that produces spontaneous release may be the same as that which produces evoked release, i.e., the RRP, and with high-frequency firing, the RRP becomes partially depleted. It further suggests that in synapses that possess a reserve population, the transient increase in cytosolic Ca$^{2+}$ created by the high-frequency burst induces this population to contribute to the RRP, actually causing an increase in spontaneous MEJP frequency.

In hippocampal synapses, Sara et al. (2005) showed that vesicles that release spontaneously originate from a pool distinct from the pool that responds to an action potential. This pool might represent a contribution from the reserve pool, which we previously showed contributes the vast majority of...
spontaneous release in DLM synapses (Koenig and Ikeda 1999). At any rate, the observations presented here demonstrate that at least some spontaneous release also occurs from the readily releasable/recycling pool and high-frequency evoked release reduces the frequency of spontaneous release from that pool.

6) “Fast” recycling at the active zone. Recycling at the active zone was previously referred to as “fast” recycling (see reviews by Galli and Haucke 2004; Sudhof 2004), as opposed to the slower recycling pathway of the reserve population. At the present time, various possible pathways were suggested for how fast recycling at the active zone operates. One possibility is the “kiss and run” pathway, in which the vesicle ejects transmitter through a transient fusion pore, after which it pinches back off from the plasma membrane, thereby retaining its structural integrity rather than collapsing fully into the plasma membrane (Ceccarelli et al. 1973; Fesce et al. 1994). However, our results demonstrate a fast recycling pathway at the active zone that operates by invagination of the plasma membrane to form a pit, followed by pinch-off to form a new vesicle, i.e., not “kiss and run” (retinula cell: Koenig and Ikeda 1996; DLM: Koenig and Ikeda 1999). Another observation that argues against “kiss and run” in Drosophila is the fact that the shibire (dynamin) mutant, which blocks pinch-off from the plasma membrane at 29°C, causes complete depletion of the active zone population when recycling is blocked at the restrictive temperature while exocytosis proceeds normally. Dynamin was previously implicated in fusion pore closure (Graham et al. 2002; Hinshaw 2000; Hinshaw and Schmid 1995; Holroyd et al. 2002) and if “kiss and run” were occurring at the active zone, the vesicles involved in a “kiss and run” pathway should remain trapped on the plasma membrane at the restrictive temperature. Also, Dickman et al. (2005), observing null mutants of synaptojanin and endophilin in Drosophila, observed that after exocytosis, vesicles undergo full fusion and reformation, and saw no evidence for “kiss and run.” Therefore the results presented here probably do not apply to a “kiss and run” recycling mechanism.

In conclusion, these results demonstrate the release and recycling characteristics of the RRP/recycling population exclusively. In the DLM, which possesses both RRP/recycling (active zone) and reserve populations, we have observed the active zone population without the reserve using the shi mutant to preferentially deplete the latter (Koenig and Ikeda 1999). Comparing those results with these suggests that the characteristics described here represent those of the RRP/recycling population, even in synapses that possess a reserve population.

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References


