The Optically Determined Size of Exo/Endo Cycling Vesicle Pool Correlates with the Quantal Content at the Neuromuscular Junction of Drosophila Larvae

Hiroshi Kuromi and Yoshiaki Kidokoro

Institute for Behavioral Sciences, Gunma University School of Medicine, 3-39-22, Showa-machi, Maebashi, 371-8511, Japan

According to the current theory of synaptic transmission, the amplitude of evoked synaptic potentials correlates with the number of synaptic vesicles released at the presynaptic terminals. Synaptic vesicles in presynaptic boutons constitute two distinct pools, namely, exo/endo cycling and reserve pools (Kuromi and Kidokoro, 1998a). We defined the vesicles that were endocytosed and exocytosed during high K⁺ stimulation as the exo/endo cycling vesicle pool. To determine the role of exo/endo cycling vesicle pool in synaptic transmission, we estimated the quantal content electrophysiologically, whereas the pool size was determined optically using fluorescent dye FM1–43. We then manipulated the size of the pool with following treatments. First, to change the state of boutons of nerve terminals, motoneuronal axons were severed. With this treatment, the size of exo/endo cycling vesicle pool decreased together with the quantal content. Second, we promoted the FM1–43 uptake using cyclosporin A, which inhibits calcineurin activities and enhances endocytosis. Cyclosporin A increased the total uptake of FM1–43, but neither the size of exo/endo cycling vesicle pool nor the quantal content changed. Third, we increased the size of exo/endo cycling vesicle pool by forskolin, which enhances synaptic transmission. The forskolin treatment increased both the size of exo/endo cycling vesicle pool and the quantal content. Thus, we found that the quantal content was closely correlated with the size of exo/endo cycling vesicle pool but not necessarily with the total uptake of FM1–43 fluorescence by boutons. The results suggest that vesicles in the exo/endo cycling pool primarily participate in evoked exocytosis of vesicles.

Key words: synaptic vesicle pools; FM1–43; quantal content; neuromuscular junction; Drosophila larva; forskolin; cyclosporin A

Signal transmission at the synapse is mediated by the neurotransmitter packaged in synaptic vesicles at the nerve terminal. Transmitter release occurs in discrete quantal units as the result of exocytosis of these vesicles. According to the current theory of neurotransmission, the magnitude of evoked release depends on the number of quanta released after arrival of an action potential. To test this prediction, Koenig et al. (1989) compared the number of synaptic vesicles in the electron micrograph of nerve terminals with the amplitude of synaptic potentials at the neuromuscular junction of a temperature-sensitive Drosophila mutant, sibirets1 (shiets1). In shts1 at nonpermissive temperature, vesicle recycling is blocked, and the amplitude of evoked synaptic potentials declines during repetitive stimulation. Koenig et al. (1989) found that the fewer the vesicles in the close vicinity of active sites, the smaller were the amplitudes of synaptic potentials, and they concluded that the extent of transmitter release was indeed closely related to the number of vesicles. In this case, they counted synaptic vesicles clustered at release sites that may belong to one homogeneous pool. Many studies, however, have suggested that there are subpopulations of synaptic vesicles in the nerve terminal (Zimmermann and Whittaker, 1977; Ewing et al., 1983; Pieribone et al., 1995). Furthermore, in shts1, we have demonstrated two topographically and functionally distinct pools of synaptic vesicles, namely exo/endo cycling and reserve pools (Kuromi and Kidokoro, 1998a). The question then arises: which of the pool is directly correlated with synaptic transmission?

When the shts1 preparation was treated with cytochalasin D, the reserve pool size was greatly reduced, whereas the exo/endo cycling pool was unchanged. In this preparation, the amplitude of synaptic potentials did not change at 0.3 Hz stimulation but decreased rapidly at 10 Hz (Kuromi and Kidokoro, 1998a). We suggested that the vesicles in the reserve pool play a crucial role for sustaining high-frequency transmission but are not directly involved in the immediate exocytosis at low stimulation rates (Kuromi and Kidokoro, 1998a), whereas vesicles in the exo/endo cycling pool are primarily involved in synaptic transmission at low-frequency stimulation.

In this study we used the following three treatments to manipulate the size of the vesicle pool, and we examined the effects on the quantal content of synaptic transmission: (1) the axon severance, which might affect the state of nerve terminals and change the pool size, (2) the treatment with cyclosporin A, which enhances the endocytosis of synaptic vesicles by inhibiting calcineurin activities (Kuromi et al., 1997), and (3) the forskolin treatment. Forskolin has been shown to facilitate synaptic transmission at the crayfish (Dixon and Atwood, 1989) and Drosophila larval neuromuscular junctions (Zhang et al., 1998). Thus, we expected forskolin to change the size of exo/endo cycling vesicle pool.

Here we report that the optically determined size of exo/endo cycling vesicle pool is closely correlated with the quantal content.
of synaptic potentials at the neuromuscular junction of *Drosophila* larvae.

A preliminary report of these results has appeared elsewhere (Kuromi and Kidokoro, 1998b).

**MATERIALS AND METHODS**

*Fly stocks.* A wild-type strain of *Drosophila melanogaster*, Canton S, and a single-gene mutant of *Drosophila*, *shi* mutant, were used in this study. The stock flies were bred at 24°C on the standard cornmeal medium supplemented with yeast under a 12 hr light/dark cycle.

*Preparations.* Larval body wall neuromuscular preparations were made from third instar larvae. For dissection, a larva was placed prone on a thin layer of silgard resin (Dow Corning, Tokyo, Japan) in a plastic dish with the HL3 medium (Stewart et al., 1994, see below). The larval rostral and caudal ends were fixed to the silgard using insect pins. The larva was cut open along the dorsal midline using microdissecting scissors, and the digestive system and other internal organs were removed. In the completed preparations, the ventral longitudinal muscles (muscles 6 and 7) were easily identified.

*Experiments.* All experiments were performed on synapses on muscles 6 and 7 of abdominal segments A-2 or A-3.

**Solutions.** Preparations with intact or severed axons were incubated for 1–3 hr in HL3 medium, which was suitable for maintaining preparations for a long period (Stewart et al., 1994). The composition of the HL3 medium is (in mM): NaCl, 70; sucrose, 115; KCl, 5; MgCl2, 20; CaCl2, 1.5; NaHCO3, 10; trehalose, 5; and HEPES, 5, pH 7.3. For FM1–43 loading and unloading experiments and electrophysiological studies, the standard *Drosophila* medium (medium) containing (in mM): 130 NaCl, 36 sucrose, 5 KCl, 2 CaCl2, 2 MgCl2, and 5 HEPES, pH 7.3, was used for a long period (Stewart et al., 1994). The composition of HL3 medium (Stewart et al., 1994) was used for FM1–43 loading and unloading. For freshly isolated preparations, the HL3 medium was used.

**Chemicals.** Chemicals used in these experiments and their sources are as follows: FM1–43 (Molecular Probes, Eugene, OR), cytochalasin B and cyclosporin A, forskolin, and dibutyryl cAMP sodium salt (db-cAMP) (Wako Chemicals, Tokyo, Japan).

**Statistical analysis.** Statistical analysis was performed by Student’s t test.

**RESULTS**

**Effects of axon severance on FM1–43 loading and unloading in the nerve terminals**

In an attempt to affect the state of presynaptic boutons of nerve terminals, we severed the motoneuronal axons and examined the FM1–43 fluorescence uptake.

**Acute effects of axon severance**

We loaded and unloaded nerve terminals with FM1–43 in freshly isolated preparations with the intact CNS or after sectioning nerves near their entry to the ganglion. The staining patterns of boutons with FM1–43 were the same in both preparations, with staining predominantly in the periphery of boutons (Fig. 1A, top panel). Between these fresh preparations, there were no differences in FM1–43 fluorescence intensities before and after high K+ stimulation (Fig. 2A,B). The height of each column represents the total fluorescence intensity after loading, and that of the dotted portion indicates the fluorescence intensity after unloading. Thus, the blot portion of each column depicts the fraction of vesicles that are endocytosed and exocytosed in the normal condition and is called the exo/endo cycling vesicle pool. No effect of axon severance was found in the vesicle pool. However, it is possible that the effect of axon severance could become apparent after a certain period of time.

**Effects of axonal severance after 1–3 hr of incubation**

When preparations with intact axons were incubated for 2 hr in HL3 medium and then loaded with FM1–43 in high K+ saline, the FM1–43 staining pattern of boutons and the fluorescence intensity did not differ from those in freshly isolated preparations (Fig. 2A, column marked with “2 h” at the bottom). The similar results were obtained when preparations were incubated in Ca2+-free saline to prevent spontaneous activities evoked by action potentials generated in the CNS (Fig. 2A, column marked with “−Ca2+, 2 h”). Approximately 90% of the FM1–43 fluorescence in boutons disappeared after high K+ stimulation. Thus, the exo/endo cycling vesicle pool in these preparations was similar to that of freshly isolated ones.

When preparations with severed axons were loaded with FM1–43 at 1, 2, and 3 hr after incubation in HL3 medium, the
boutons showed staining patterns and the total fluorescence intensities similar to those in freshly isolated preparations (Fig. 1C, top panel; Fig. 2B, columns marked with “1 h”, “2 h”, and “3 h”). Similar results were obtained when preparations were incubated in Ca\(^{2+}\)-free saline (Fig. 2B, column marked with “–Ca\(^{2+}\), 2 hr”). However, in preparations with axons severed and incubated for 2–3 hr, only ~30% of the fluorescence disappeared after high K\(^+\) stimulation (Fig. 1C, bottom panel; Fig. 2B, note larger dotted portions in columns marked with “2 h” and “3 h”), indicating that the exo/endo cycling vesicle pool was smaller in these preparations.

These results suggest either that a part of previously releasable fraction became unreleasable or that a new unreleasable fraction appeared during the incubation period. To distinguish these alternatives, the following experiment was performed in which synaptic vesicles were first loaded with FM1–43 and then incubated for 2 hr.

FM1–43 was loaded in freshly isolated preparations with intact or severed axons, and then the preparations were incubated for 2 hr in Ca\(^{2+}\)-free saline. The fluorescence intensity was measured before and after high K\(^+\) stimulation to assess a releasable portion of the fluorescence intensity. The total FM1–43 fluorescence intensities in boutons were slightly decreased after 2 hr incubation compared with those observed in freshly isolated preparations, but the difference was not significant (Fig. 2C, D). In preparations with intact axons, ~85% of FM1–43 fluorescence in boutons was destained by high K\(^+\) stimulation (Fig. 2C, the blank portion of column marked with “–Ca\(^{2+}\), 2 h”). In contrast, when FM1–43-loaded preparations with severed axons were incubated for 2 hr and then stimulated by high K\(^+\) saline, only 25% of fluorescence was destained (Fig. 2D, the blank portion of the column marked with “–Ca\(^{2+}\), 2 hr”). Thus, in nerve terminals of axons severed, FM1–43 fluorescence became mostly unreleasable during the 2 hr incubation period. We further examined the mechanism of this unexpected effect of axon severance.

**Effects of cytochalasin B and D on the FM1–43 fluorescence fractions**

It is plausible that synaptic vesicles became unreleasable as the result of binding to actin filaments (Hirokawa et al., 1989). We thus tested the effect of cytochalasin B and D, which are known to disrupt actin polymerization (Cooper, 1987; Smith, 1988). Preparations with severed axons were incubated for 2 hr in the HL3 medium containing cytochalasin B (10 μM) or cytochalasin D (10 μM) and then loaded with FM1–43. The total FM1–43 fluorescence intensities in boutons of these preparations were not significantly different from those of nontreated preparations (Fig. 2B, compare the height of columns marked with “CB, 2 h” and “CD, 2 h” with that of column marked with “2 h”). However, after high K\(^+\) stimulation, ~80% of the FM1–43 fluorescence was destained in cytochalasin B- or cytochalasin D-treated preparations (Fig. 2B, the blank portion of columns marked with “CB, 2 h” and “CD, 2 h”), whereas only ~30% of the fluorescence was destained in nontreated preparations (Fig. 2B, the column marked with “2 h”).

To further confirm the effect of actin polymerization disrupting agents, preparations with severed axons were first loaded with FM1–43 and then incubated for 2 hr in Ca\(^{2+}\)-free saline containing cytochalasin D (10 μM). The same effects of the drug were observed as described above (Fig. 2D, the blank portion of column marked with “–Ca\(^{2+}\), CD”). Thus, it appears that after 2 hr incubation synaptic vesicles became unreleasable with high K\(^+\) stimulation as the result of binding to actin filaments in preparations with severed axons. However, there was the possibility that the dye became compartmentalized and was no longer releasable. To test this possibility, we used shi\(^{ts1}\) mutants as described below.

**High K\(^+\) stimulation at 22 and 34°C in shi\(^{ts1}\) mutants**

Previously we have shown in shi\(^{ts1}\) mutants that vesicles in the reserve pool are not releasable with high K\(^+\) stimulation at room temperature but can be released at nonpermissive temperature (Kuromi and Kidokoro, 1998a). Thus, we tested whether those
area of columns marked with “34°C” in shibire). These results suggest that the FM1–43 fluorescence that remained after high K⁺ stimulation at 22°C in preparations with severed axons and incubated for 2 hr was not caused by dye taken up nonspecifically in the boutons, but rather represented dye in the releasable vesicles.

These results were not simply the effect of temperature, because 60% of FM1–43 fluorescence of wild-type larvae with axons severed and incubated in HL3 medium for 2 hr remained after high K⁺ stimulation both at 22°C and at 34°C (Fig. 3A, B, dotted and filled areas of columns marked with “22°C” and “34°C” in wild-type).

**Effects of cyclosporin A on FM1–43 loading and unloading in the nerve terminals**

Previously we have shown that pretreatment with cyclosporin A increases the number of nerve terminals visibly stained with FM1–43 by high K⁺ stimulation in Drosophila larvae and suggested that cyclosporin A enhances endocytosis of synaptic vesicles in the nerve terminals (Kuromi et al., 1997). To determine whether one vesicle pool is affected selectively by cyclosporin A, we performed the following experiment.

Preparations with intact axons were incubated for 20 min in Drosophila medium containing 10 μM cyclosporin A and then loaded with FM1–43 by high K⁺ stimulation. In these cyclosporin A-pretreated preparations, entire boutons were stained, as shown in Figure 1D (top panel). The FM1–43 fluorescence intensity in boutons of cyclosporin A-pretreated preparations was significantly higher than that in nontreated ones (Fig. 4, compare the height of column marked with “10 μM, cyclosporin A” with that of column marked with “control”). In cyclosporin A-pretreated preparations some amounts of FM1–43 fluorescence remained in the central part of boutons after high K⁺ stimulation (Fig. 4, the dotted portion of column marked with “10 μM cyclosporin A”).

When the preparations with intact axons were first loaded with FM1–43 and then treated with cyclosporin A, no change was observed in the total intensity of FM1–43 fluorescence and in its sensitivity to high K⁺ stimulation (Fig. 4, the column marked with “loaded with FM1–43 and then treated with cyclosporin A”),

**Figure 2.** FM1–43 fluorescence intensities in boutons of preparations that were incubated from various lengths of time in different media before (A, B) or after (C, D) loading with FM1–43. Preparations with intact axons (A, C) and with severed axons (B, D) were incubated in HL3 medium (not labeled) or in Ca²⁺-free saline (labeled with “Ca²⁺”). Some preparations were pretreated for 20 min with cytochalasin B (10 μM, CB) or cytochalasin D (10 μM, CD), which were also present in the medium during incubation. The ordinates show FM1–43 fluorescence intensities (pixel values) in boutons before (heights of columns) and after (top ends of dotted portion of columns) high K⁺ stimulation. For measurements of the intensities of fluorescence of boutons, 12-bit images were acquired with the CCD camera, and pixel values were compressed to 8 bits. Average intensities were computed for each bouton. Five to six boutons were examined in each preparation. Mean fluorescence values of boutons were determined in each preparation and summarized (mean and SEM). The numbers in columns are the number of preparations examined. Vertical bar of each column is SEM. The time below columns shows the incubation time.

unreleasable fluorescence in boutons of axons severed and incubated for 2 hr can be released in shi-ts1 mutants at nonpermissive temperature.

As was observed in wild-type larvae, 60% of FM1–43 fluorescence remained after high K⁺ stimulation at 22°C in shi-ts1 terminals of axons severed and incubated in HL3 medium for 2 hr (Fig. 3A, preparations incubated and then loaded with FM1–43; Fig. 3B, preparations loaded with FM1–43 and then incubated, the dotted area of column marked with “22°C” in shibire). On the other hand, after high K⁺ stimulation at 34°C, only ~10% of FM1–43 fluorescence remained in shi-ts1 (Fig. 3A, B, the filled
Effects of forskolin and db-cAMP on FM1–43 loading and unloading in the nerve terminals

An elevation of cAMP in the presynaptic terminal has been shown to enhance synaptic transmission (Dixon and Atwood, 1989; Zhong and Wu, 1990; Zhang et al., 1998). It is possible that cAMP enhances synaptic transmission by increasing the size of vesicle pools. To test this possibility, we examined the effect of treatments that increase cAMP in the terminal on the vesicle pools. When preparations with intact axons were treated with forskolin (5 and 20 μM) for 7 min and then exposed for 5 min to high K⁺ saline containing 10 μM FM1–43, the FM1–43 fluorescence intensity in boutons was significantly higher than that in nontreated preparations (Fig. 4, the height of columns labeled with “5 μM forskolin” and “20 μM forskolin”). The staining patterns of boutons in forskolin-pretreated preparations were essentially the same as those in nontreated preparations, i.e., the periphery of boutons was predominantly stained, but the boutons in forskolin-pretreated preparations were more brightly stained than in nontreated preparations (Fig. 1B, top panel). More than 90% of FM1–43 fluorescence was destained by high K⁺ stimulation in forskolin-pretreated preparations (Fig. 1B, bottom panel; Fig. 4, the blank portion of columns labeled with “5 μM forskolin” and “20 μM forskolin”), as observed in nontreated preparations. Thus, the forskolin treatment increases the exo/endo cycling vesicle pool.

Pretreatment with db-cAMP (150 μM for 15 min) had essentially the same effects on FM1–43 loading and unloading as pretreatment with forskolin (Fig. 4, the column labeled with “150 μM db-cAMP”)  

Relationships between the FM1–43 loading time and the size of exo/endo cycling vesicle pool in variously treated preparations

As shown above, in forskolin-treated preparations, the size of exo/endo cycling vesicle pool increased, whereas it decreased in preparations with axons severed and incubated for 2 hr. Because we loaded boutons with FM1–43 for 5 min in experiments so far described, the possibility remains that the 5 min loading time might not be long enough to estimate the size of the vesicle pool.

To test this possibility, we varied the loading time of FM1–43 and measured the amounts of FM1–43 fluorescence released by high K⁺ stimulation. The amount of the fluorescence increased as the loading time was prolonged, and the steady state was achieved within 1 min in nontreated preparations (Fig. 5, open circles). As shown in Figure 5, although the loading rate of the exo/endo cycling vesicle pool was similar, the magnitude of the steady-state levels was significantly enhanced in forskolin-pretreated preparations (Fig. 5, filled circles) and significantly reduced in preparations with axons severed and incubated for 2 hr (Fig. 5, open triangles), compared with nontreated preparations. There were no significant differences in the loading rate of the exo/endo cycling vesicle pool and the magnitude of steady-state levels between cyclosporin A-pretreated and nontreated preparations (Fig. 5, filled triangles, open circles).
Quantal contents in forskolin- and cyclosporin A-pretreated preparations

In saline containing 2 mM Ca$^{2+}$, the quantal content was larger in the forskolin-treated preparations and unchanged in the cyclosporin A-treated preparations. To assess the Ca$^{2+}$ dependence of these effects, we measured EJPs and MEJPs at different extracellular Ca$^{2+}$ concentrations. The ratio of mean EJP amplitude to mean MEJP amplitude [corrected for nonlinear summation, (Martin, 1955), using a reversal potential of 0 mV; see Jan and Jan, 1976; Chang et al., 1994] was used as an estimate of quantal content. Forskolin (20 μM) increased the quantal content in the whole range of Ca$^{2+}$ concentrations between 0.1 and 0.8 mM (Fig. 6F, filled circles). Cyclosporin A (10 μM) had no effect (Fig. 6F, filled triangles). Although the slopes were unaffected, there was a considerable shift in the concentration of Ca$^{2+}$ needed to evoke a given level of quantal release in the forskolin-treated versus nontreated preparations. Thus, these effects of forskolin and cyclosporin A were observed in all Ca$^{2+}$ concentrations tested in this experiment.

**DISCUSSION**

To study the functional role of the exo/endo cycling vesicle pool in synaptic transmission, the size of vesicle pool in single boutons was optically determined using fluorescent dye FM1–43, and the quantal content was estimated by the ratio of the EJP amplitude to that of MEJP at the neuromuscular junction of *Drosophila* larvae. Among the FM1–43 fractions taken up in boutons in high K$^+$ saline, the fraction that was released by high K$^+$ stimulation in the absence of the dye is considered the exo/endo cycling vesicle pool because these vesicles are involved in both endocytosis and exocytosis processes. In the following three types of preparations, the size of the exo/endo cycling vesicle pool and the quantal content of synaptic transmission were found to change in parallel. (1) In preparations with axons severed and incubated for 2–3 hr, the total amount of FM1–43 fluorescence taken up by these boutons was in the normal range, but the fraction released by high K$^+$ stimulation was sharply decreased, compared with that in freshly isolated control preparations. In these preparations, the quantal content was significantly smaller than controls. Furthermore, the treatment of these preparations with cytochalasin B or D during the incubation period prevented the decrease in the FM1–43 fraction released by high K$^+$ stimulation and, at the same time, blocked the reduction in the quantal content. (2) The cyclosporin A treatment increased the total amount of FM1–43 fluorescence taken up by boutons, but the fraction releasable by high K$^+$ stimulation did not change. The quantal content in these preparations was not different from controls. (3) Forskolin increased the total amount of FM1–43 fluorescence taken up by boutons, and the exo/endo cycling vesicle pool was enlarged. The quantal content in these preparations was also increased. Thus, the present study showed that changes in the exo/endo cycling vesicle pool paralleled those in the quantal content and that there is not necessarily a tight relationship between the total FM1–43 fluorescence taken up by boutons and the quantal content. These results suggest that synaptic vesicles in the exo/endo cycling vesicle pool participate primarily in impulse-evoked release of synaptic vesicles from nerve terminals.

In preparations with axons severed and incubated and also in those pretreated with cyclosporin A, there was a difference between FM1–43 loading and unloading efficiency. If exocytosis of vesicles caused the same extent of endocytosis of vesicles, we cannot account for this discrepancy. It appears that although endocytosis is closely coupled to exocytosis, two processes are differentially controlled. For example, exocytosis of vesicles occurs at the active sites of nerve terminals, whereas endocytosis is also observed in other sites (Miller and Heuser, 1984).

---

**Table 1. Membrane potentials and quantal contents of synaptic potentials in variously treated preparations**

<table>
<thead>
<tr>
<th>Preparation</th>
<th>N</th>
<th>Rp (mV)</th>
<th>MEJP (mV)</th>
<th>EJP (mV)</th>
<th>M</th>
</tr>
</thead>
<tbody>
<tr>
<td>Freshly isolated</td>
<td>9</td>
<td>51 ± 2.8</td>
<td>0.93 ± 0.06</td>
<td>33 ± 1.7</td>
<td>100 ± 9.3</td>
</tr>
<tr>
<td>FSK-treated</td>
<td>9</td>
<td>51 ± 2.6</td>
<td>0.93 ± 0.07</td>
<td>38 ± 1.8</td>
<td>191 ± 28*</td>
</tr>
<tr>
<td>Cyc. A-treated</td>
<td>8</td>
<td>50 ± 2.6</td>
<td>0.96 ± 0.06</td>
<td>34 ± 1.7</td>
<td>130 ± 26</td>
</tr>
<tr>
<td>Intact, 2 hr</td>
<td>7</td>
<td>49 ± 0.6</td>
<td>0.89 ± 0.05</td>
<td>30 ± 1.6</td>
<td>90 ± 9.5</td>
</tr>
<tr>
<td>Severed, 2 hr</td>
<td>8</td>
<td>49 ± 1.0</td>
<td>1.07 ± 0.08</td>
<td>16 ± 1.5*</td>
<td>22 ± 2.6*</td>
</tr>
<tr>
<td>Severed, 2 hr, Cyt. D.</td>
<td>7</td>
<td>49 ± 1.9</td>
<td>1.08 ± 0.10</td>
<td>33 ± 0.9</td>
<td>102 ± 14</td>
</tr>
</tbody>
</table>

Values represent the mean ± SEM. N, Number of preparations examined; Rp, resting membrane potential; MEJP, amplitude of spontaneous junctional potential; EJP, amplitude of nerve-evoked junctional potential; M, quantal content of synaptic potential, which was determined by dividing the mean EJP amplitude of each fiber by its mean MEJP amplitude and correcting for the nonlinearity of EJP amplitude; Freshly isolated, freshly isolated nontreated preparations with intact axons. The ratio of mean EJP amplitude to mean MEJP amplitude was significantly enhanced in forskolin-pretreated (20 μM) preparations and significantly reduced in preparations with axons severed and incubated for 2 hr, compared with that in freshly isolated nontreated preparations with intact axons (Table 1). There were no significant differences in the parameters examined in the present study and also in the frequency of MEJPs (data not shown) between cyclosporin A-pretreated (10 μM) and nontreated preparations (Fig. 6B, Table 1), in accord with the result reported in our previous paper (Kuromi et al., 1997).
more, Ca\textsuperscript{2+} is necessary for exocytosis of vesicles, but endocytosis occurs independently of Ca\textsuperscript{2+} (Ryan et al., 1993).

It has been hypothesized that among synaptic vesicles in nerve terminals, only few vesicles at active sites of synapse participate in the immediate release of transmitter (Von Gersdorff et al., 1996; Schikorski and Stevens, 1997; Neher, 1998) and contribute to the quantal content. In the present study, when quantal contents were plotted against optically determined sizes of exo/endo cycling vesicle pool in various preparations, there was a linear relationship between the two parameters (Fig. 7). The size of exo/endo cycling vesicle pool changes in parallel with the quantal content. Thus, the present study suggests that an equilibrium may exist between the exo/endo cycling vesicle pool and immediately releasable vesicles. This idea is supported by the results obtained by electrophysiological analysis that once depleted, the immediately releasable vesicle pool was refilled by vesicles located nearby. In addition, it has been shown that a reduction in the number of synaptic vesicles at the synapse results in the decrease in transmitter release. In the Drosophila mutant, shi\textsuperscript{ts1}, a temperature block in endocytotic retrieval of synaptic vesicles was used to systematically reduce the number of synaptic vesicles. The amplitude of nerve-evoked synaptic potentials reduced in parallel with the vesicle number in the immediate vicinity of release sites (Koenig et al., 1989). Furthermore, in okadaic acid-treated frog nerve–muscle preparations, the FM1–43 vesicle fraction releasable by high K\textsuperscript{+} stimulation decreased in parallel with the amplitude of evoked potential (Betz and Henkel, 1994). The total releasable cycling vesicle pool was significantly reduced at synapses in hippocampal cultures derived from synapsin I knock-out mice compared to their wild-type counterparts, and the number of vesicles released during brief trains of action potentials also decreased (Ryan et al., 1996). Taken together, results of these reports and our present results it seems reasonable to conclude that the number of vesicles released after arrival of an action potential is causally related to the size of exo/endo cycling vesicle pool in the presynaptic terminal.

When axons were severed and incubated for 2–3 hr, FM1–43 staining patterns of boutons were essentially the same as those

![Figure 6](image-url)  
Figure 6. EJPs and MEJPs in variously treated preparations (A–E) and Ca\textsuperscript{2+} dependence of evoked release (F). A–E, Representative traces of synaptic potential in freshly isolated preparation (A), cyclosporin A-treated (10 \mu M for 20 min) preparation (B), forskolin-treated (20 \mu M for 7 min) preparation (C), preparation with axons severed and incubated for 2 hr in HL3 medium (D), and preparation with axons severed and incubated for 2 hr in the presence of cytochalasin D (E, 10 \mu M). The topmost record in each panel shows evoked junctional potentials, and the bottom four traces show spontaneous events recorded in the same muscle fibers. MEJP records were selected to show representative amplitudes. Calibration: 8 mV, 0.1 sec (for EJPs); 1 mV, 0.2 sec (for MEJP). F, Quantal content is plotted as a function of extracellular Ca\textsuperscript{2+} concentration for forskolin-treated (20 \mu M, filled circles), nontreated (open circles), and cyclosporin A-treated (10 \mu M, filled triangles) preparations. The Ca\textsuperscript{2+} concentration of the recording solution was adjusted to 0.1, 0.2, 0.4, and 0.8 mM with fixed 5 mM MgCl\textsubscript{2}.

![Figure 7](image-url)  
Figure 7. Relationship between the size of exo/endo cycling vesicle pool and the quantal content. The size of exo/endo cycling vesicle pool (abscissa) and the quantal content (ordinate) were determined in various preparations in the medium containing 2 mM Ca\textsuperscript{2+} as described in Results. Vertical and horizontal bars of each circle are SEM. FSK, Forskolin-treated preparations; Cyc.A, cyclosporin A-treated preparations; Fresh, freshly isolated nontreated preparations; Severed, Cyt.D. 2h, preparations with axons severed and incubated for 2 hr in HL3 medium containing cytochalasin D; Intact, 2 h, preparations with intact axons incubated for 2 hr in HL3 medium; Severed, 2 h, preparations with axons severed and incubated for 2 hr in HL3 medium.
observed in the freshly isolated preparation with severed axons. Nevertheless, some of the fluorescence was not released by high K⁺ stimulation. Furthermore, when nerve terminals with axons severed were first loaded with FM1–43 and then incubated, the fluorescence was only partially released by high K⁺ stimulation. These results suggest that some changes occurred in the exo/endo cycling vesicle pool during incubation in nerve terminals disconnected from the cell bodies. When cytochalasin B or D was present during the incubation period, however, the appearance of an FM1–43 fraction unreleased by high K⁺ stimulation was prevented. Cytochalasin B and D are known to disrupt actin polymerization (Cooper, 1987; Smith, 1988), and it has been proposed that polymerization and depolymerization of actin molecules occur in intact nerve terminals (Bernstein and Bamburg, 1989; Furukawa et al., 1997). The FM1–43 fraction unreleased by high K⁺ stimulation, which was observed in nerve terminals with axons severed and incubated for 2–3 hr, had the following features, which are the same as those of the reserve pool revealed in shi(ts1) (Kuromi and Kidokoro, 1998a). (1) The unreleasable FM1–43 fraction by high K⁺ stimulation at room temperature was released at nonpermissive temperature in shi(ts1). (2) The unreleasable FM1–43 fraction was not detected after the treatment with cytochalasin D. Therefore, to explain our present results, we suggest that when an axon is severed, the depolymerization is somehow impaired, and filamentous actin becomes dominant, resulting in a decrease in the size of exo/endo cycling vesicle pool and a concomitant increase in the size of reserve pool.

Previously using the shi(ts1) mutant, we have demonstrated that the exo/endo cycling pool of synaptic vesicles is localized in the periphery of boutons and is released after high K⁺ stimulation at permissive temperature (Kuromi and Kidokoro, 1998a). On the other hand, the reserve pool of synaptic vesicles, predominantly located in the central part of boutons, was not released by high K⁺ stimulation at permissive temperature, but released at nonpermissive temperature. In the present study in wild-type Drosophila larvae, the total amounts of FM1–43 taken up by boutons were increased by treatment with forskolin or cyclosporin A. Predominantly, the peripheral region of boutons was stained after the forskolin treatment, whereas the whole bouton was labeled with FM1–43 after the cyclosporin A treatment. After high K⁺ stimulation, the fluorescence virtually disappeared in forskolin-treated preparations. On the other hand, in cyclosporin A-treated preparations, the fluorescence in the center of boutons remained after the stimulation, although the fluorescence in the periphery disappeared. These results obtained in wild-type Drosophila further support the conclusion in shibire that vesicles in the periphery of boutons are readily releasable, and those in the center are not.

Both forskolin, a specific activator of adenyl cyclase (Seamon and Daly, 1983), and dibutylryl cAMP, an analog of cAMP, increased the FM1–43 fraction released by high K⁺ stimulation, suggesting that the increase in the size of exo/endo cycling vesicle pool may be mediated by an elevation of cAMP in the terminal. The cAMP cascade enhances synaptic transmission in various preparations (Goldberg and Singer, 1969; Dixon and Atwood, 1989) and also increased the quantal content at the Drosophila larval neuromuscular junction (Zhong and Wu, 1990; Davis et al., 1998). Although we cannot exclude the modulation of ion channels or the direct action on the exocytotic machinery by cAMP (Kandel and Schwartz, 1982; Trudeau et al., 1996), the increase in the size of exo/endo cycling vesicle pool may be one mechanism for the observed facilitation of synaptic transmission induced by cAMP, as pointed out by Gingrich and Byrne (1985).

In conclusion, previous (Kuromi and Kidokoro, 1998a) and present studies support the hypothesis that there are at least two pools of synaptic vesicles in presynaptic boutons of Drosophila larvae. Vesicles in the exo/endo cycling vesicle pool are primarily responsible for evoked release, and the size of the pool determines the quantal content of synaptic potentials. The reserve pool is not directly involved in exocytosis of synaptic vesicles but responsible for sustaining release at high-frequency stimulation.

REFERENCES