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Enhancement of the endosomal endocytic pathway increases quantal size

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Abstract

We combined recordings of spontaneous quantal events with electron microscopy analysis of synaptic ultrastructure to demonstrate that the size of a neurosecretory quantum increases following an activation of the endosomal endocytic pathway. We reversibly activated the endosomal endocytic pathway in *Drosophila* motor boutons by application of high K^+ solution. This treatment produced the formation of numerous cisternae, vacuoles and enlarged vesicles. Spontaneous quantal events recorded immediately after the cessation of high K^+ application were significantly enlarged, and this increase in quantal size was reversed after a 10 minute resting period. Actin depolymerization produced by latrunculin B pretreatment inhibited both the formation of endosome-like structures and the increase in quantal size. Loading the preparations with the dye FM1–43 followed by photoconversion of the dye combined with electron microscopy analysis revealed that the observed cisternae are likely to be the product of both bulk membrane retrieval and vesicle fusion.

Keywords

Synaptic vesicle; mEPSP; Electron microscopy; Latrunculin B

Introduction

Neurotransmitters are packed in synaptic vesicles and released by the fusion of a vesicle with the presynaptic membrane. A current generated by a postsynaptic cell in response to a single vesicle fusion is termed the quantal current. The efficacy of neuronal transmission can be regulated either via the number of vesicles releasing transmitters or via the magnitude of the quantal current (the quantal size).

Regulation of quantal size may underlie various forms of synaptic plasticity (Edwards, 2007) and can be determined by postsynaptic (Malinow and Malenka, 2002) or presynaptic factors (Liu, 2003). Presynaptic mechanisms that may determine the quantal size are the vesicular transmitter concentration (Wilson et al., 2005; Wu and Wu, 2007), fusion pore opening time (Lindau and varez de, 2003), or synaptic vesicle size (Zhang et al., 1998; Karunanithi et al., 2002).

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After releasing their content into the synaptic gap, vesicles are internalized and recycled. It has been demonstrated that vesicles are formed via clathrin-mediated endocytosis (De and Takei, 1996), which involves formation of a clathrin coat, pinching off from the plasma membrane and clathrin removal. The regulation of vesicle size by a clathrin adaptor (Zhang et al., 1998) suggested that vesicle sizes are “tailored” by the clathrin coat assembly (Zhang et al., 1999).

Vesicles may be reused directly after their reuptake (Koenig and Ikeda, 1996; Murthy and Stevens, 1998) or regenerated from endosomal intermediates or cisternae (Heuser and Reese, 1973; Takei et al., 1996; Leenders et al., 2002; de Lange et al., 2003). The question of whether the size of a vesicle and a neurosecretory quantum might be affected by the vesicle recycling pathway has not yet been explored. In the present study we investigated how the quantal size is affected at *Drosophila* motor boutons when the endosomal recycling pathway is activated.

Results

It has been demonstrated previously that endosomal recycling pathway can be activated by maintaining a prolonged membrane depolarization by employing either continuous stimulation by action potentials (Heuser and Reese, 1973; Teng and Wilkinson, 2000) or high K^+ applications (Marxen et al., 1999; Holt et al., 2003; de Lange et al., 2003; Coggins et al., 2007). To test whether this can be achieved in *Drosophila* motor boutons, we stimulated preparations by 5 minute application of 90 mM KCl and fixed them for the electron microscopy (EM) analysis immediately following the stimulation. Type Ib boutons were identified on the micrographs by their large size (boutons of at least 2 μ m diameter were selected) and small clear vesicles (Karunanithi et al., 2002). Numerous endosome-like vacuole and cisternae have been observed in the stimulated boutons (Fig. 1A), suggesting that the endosomal pathway has been activated. The preparations fixed with a 10 minute delay after high K^+ stimulation were not distinguishable from control preparations (Fig. 1A).

We measured all the vesicles and endosome-like cisternae in stimulated and non-stimulated preparations and constructed frequency distributions (Figs. 1B–F). The distribution of vesicle size was found to be bell-shaped and rather narrow for control preparations, reflecting the fact that cisternae are very infrequent and vesicles are rather uniform in size (Fig. 1D top). In stimulated preparations, the bell-shaped Gaussian distribution had a long “tail”, which corresponds to the enlarged vesicles and endosome-like cisternae (Fig. 1D bottom). It is notable that we do not see a distinct border between the size of a vesicle and the size of an endosome-like structure. In addition to some endosome-like cisternae which are three–four times larger than vesicles and have a prolonged shape, numerous vesicle-like vacuoles appeared, which were only slightly larger than a typical vesicle.

Thus, the size of a typical vesicle can be only determined from control preparations. We define it as a range between 20 and 44 nm where the distribution is well described by a Gaussian with a mode in the middle of the range (at 32 nm). We define all the membranous structures exceeding 44 nm in their major cross-line as “cisternae”, independently of their shape (Fig. 1D). To test whether the size of a vesicle remained unchanged after the

treatment, we constructed cumulative distributions of the sizes of vesicles (those with the diameter of 44 nm or less) for control and stimulated preparations (Fig. 1F). Remarkably, the vesicle distribution was shifted towards larger values for stimulated preparations, and the size of a vesicle was significantly increased upon stimulation ($p < 0.01$ according to Kolmogorov–Smirnov test).

To test whether the appearance of cisternae and enlarged synaptic vesicles would affect quantal size, we recorded spontaneous synaptic activity from control and high K^+ stimulated preparations. Using a focal macropatch electrode, we selectively recorded activity of Ib type boutons (Fig. 2A). After control recording of spontaneous activity, the preparation were stimulated by high K^+ for 5 min, briefly washed, and the recording was repeated immediately following the stimulation. Stimulated preparations had an increased frequency of spontaneous quantal events (Figs. 2B, C), and increased quantal size (Figs. 2D–G), which was assessed either as the amplitude (Fig. 2D) or the area (Figs. 2E–G) of the recorded miniature postsynaptic potentials (mEPSPs). Both frequency and size of spontaneous events was reversed back to the control level within 10 min after high K^+ application (Figs. 2C–E).

Thus, we observed that high K^+ stimulation transiently activates the endosomal endocytic pathway and produces a formation of enlarged synaptic vesicles and an increase in the size of the neurosecretory quanta. To prove conclusively that the observed increase in quantal size is a consequence of the enhanced endosomal pathway, we employed actin depolymerization, which was demonstrated to block the formation of endosome-like structures (Holt et al., 2003; Richards et al., 2004). Treatment of the preparations with an actin depolymerizing agent latrunculin B (20 mM added to the bath 5 min prior to high K^+ stimulation) strongly inhibited the formation of endosome-like cisternae (Figs. 3A, B). Furthermore, quantal size in high K^+ stimulated and latrunculin treated preparations was not significantly increased (Figs. 3C, D). This result suggests that the observed increase in quantal size after high K^+ stimulation is a direct consequence of the activation of the endosomal pathway.

What is the mechanism of the increase in quantal size? It is possible that the observed increase in the amplitude/area of miniature postsynaptic responses is due to the shift in the size distribution of synaptic vesicles towards larger diameters (Fig. 1F). Since quantal size could be expected to correlate with vesicle volume (Bekkers et al., 1990; Bekkers and Stevens, 1995; Karunanithi et al., 2002), a small shift in the distribution of vesicle diameter could conceivably produce a noticeable enlargement of vesicle volume and, consequentially, a significant increase in the area of postsynaptic responses. To test whether this might be the case, we compared the standardized distributions of quantal size and vesicle volume (Bekkers et al., 1990) in high K^+ treated and control preparations (Fig. 4A). In control preparations, a good agreement was observed between the variability of quantal size and vesicle volume (Fig. 4A, open and solid squares, respectively). A similar observation has been made at *Drosophila* motor boutons by Atwood's lab (Karunanithi et al., 2002). In contrast, in high K^+ treated preparations the variability of quantal size substantially exceeded the variability in vesicle volume, and the cumulative probability distributions of quantal size and vesicle volume drastically differed (Fig. 4A, open and solid triangle, respectively). This result demonstrates that the observed increase in quantal size cannot be

accounted for by the slight enlargement of synaptic vesicles which we observe after high K^+ application (Fig. 1F). We conclude, therefore, that some of the endosome-like cisternae formed during high K^+ application are capable of exocytosis, thus producing enlarged postsynaptic responses.

To investigate this possibility further, we compared the distribution of quantal size with the distribution of volume of all the observed membranous structures, including vesicles and cisternae, in high K^+ treated preparations (Fig. 4B). This comparison demonstrated that the variability in volume of vesicles/cisternae significantly exceeded the variability in quantal size. Thus, it is very unlikely that the largest of the observed cisternae are capable for exocytosis, since the largest cisternae would produce a noticeable proportion of giant postsynaptic responses, which is not the case (Fig. 4B).

It is likely, therefore that only vesicles and intermediate-size vacuoles/cisternae are capable of exocytosis, but not the largest cisternae. The best fit between distributions of quantal size and vesicle/cisternae volume was obtained under an assumption that only the membranous structure of up to 80 nm diameter are capable of exocytosis (Fig. 4C). Of course, in reality the cutoff size of exocytosis-competent cisternae is unlikely to be rigid, and this would explain why the distributions of vesicle/cisternae volume and quantal size did not match perfectly when a rigid cutoff was used (Fig. 4C). Thus, the results of our analysis suggest that the observed increase in quantal size produced by high K^+ stimulation results from formation and exocytosis of endosome-like structures. Furthermore, our analysis suggests that the endosome-like structures which are 2–3 time larger than a typical vesicle are exocytosis-competent, while exocytosis of larger cisternae is very unlikely.

Next, we investigated the mechanism of cisternae formation. We questioned whether the observed vacuoles and cisternae are produced by bulk membrane retrieval, as was demonstrated at other synapses (Holt et al., 2003; de Lange et al., 2003), or represent an endosomal intermediate, which was observed in *Drosophila* motor boutons (Wucherpfennig et al., 2003). To elucidate these mechanisms, we loaded preparations with the fluorescent dye FM1–43 (10 μ M added to the bath) during brief (1.5 min) high K^+ stimulation and fixed them immediately following the stimulation. Then the preparations were fixed, treated with DAB and illuminated to produce photo-conversion of the dye into electron dense product and processed for electron microscopy analysis. Although many endosome-like cisternae and vesicles did take up the dye (Fig. 4A), the pattern of the dye uptake varied greatly among the cisternae (Fig. 4B). Some endosome-like cisternae were labeled almost completely and evenly (Fig. 4B1). These cisternae were often observed in the close proximity to the synaptic membrane, suggesting that they could be uptaken by the bulk membrane retrieval. In some cisternae, however, the black labeling was rather uneven (Fig. 4B2), suggesting that these structures might have been a product of a fusion of labeled and non-labeled vesicles. Furthermore, sometimes we observed structures which were likely to represent a fusion of a non-labeled cisterna with one or several vesicles, some of which were labeled (Fig. 4B3). These observations compelled us to suggest that besides bulk membrane retrieval the cisternae can be formed by vesicle fusion. To test this hypothesis, we loaded the preparations with FM1–43 dye during a more prolonged high K^+ stimulation (5 min) and performed photo-conversion of the dye. In these preparations the vast majority of vesicles

were labeled (Fig. 5A). Although overall number of cisternae did not increase after the more prolonged high K^+ stimulation, the number of labeled cisternae significantly increased (Fig. 5B). These results demonstrate that 1) all the observed endosome-like cisternae were formed after a relatively brief (1.5 min) stimulation, and a more prolonged stimulation did not significantly increase their number, and 2) only partial labeling of the cisternae was achieved after the brief stimulation, and a more prolonged stimulation significantly enhanced the labeling. This would not be the case if all the endosome-like cisternae were produced by the bulk endocytosis, since in the latter case the cisternae formation would be tightly correlated with their labeling. Thus, our results suggest that the vesicle fusion, in addition to the bulk membrane retrieval, is likely to be a prominent mechanism in the formation of the observed vacuoles and cisternae.

To further elucidate the mechanisms by which high K^+ induced depolarization enhances the endosomal endocytic pathway, we tested whether cisternae formation is activated by the influx of Ca^{2+} . Preparations were stimulated (high K^+ applied for 5 min) in low Ca^{2+} (0.2 mM) solution and fixed for electron microscopy. Although a slight increase in the number of cisternae was observed compared to non-stimulated preparations (Fig. 6), this increase was not statistically significant and not as prominent as at normal Ca^{2+} conditions. Thus, we conclude that the observed endosome formation is produced by a Ca^{2+} dependent mechanism, which is a consequence of membrane depolarization.

Interestingly, prolonged stimulation by repetitive action potentials failed to produce a similar effect, independently of the stimulation frequency (Fig. 6). Although a 15 minute stimulation at 50 Hz frequency produced a slight increase in the number of cisternae, this increase was far not as prominent as after high K^+ application. Apparently, in our preparation high K^+ stimulation produces a stronger depolarization than repetitive action potentials. This result contrasts to the findings reported in the seminal study at the frog neuromuscular junction by Heuser and Reese (1973), where prolonged continuous stimulation by action potentials produces marked cisternae formation.

Thus, we conclude that the cisternae formation is produced by massive nerve depolarization, while specific stimulation paradigm required to induce this formation may vary among different experimental preparations. Our study demonstrated the formation of endosome-like cisternae is Ca^{2+} dependent and actin dependent, that it is produced both by bulk membrane retrieval and by fusion of synaptic vesicles, and that it results in an increase in quantal size, which is due to exocytosis of small and intermediate size cisternae. This mechanism may enable a synaptic terminal to increase quantal size and, consequentially, the release efficacy, in response to prolonged intense stimulation.

Discussion

The main finding of our study is that quantal size may depend on the vesicle recycling pathway. We have demonstrated this by activation of the endosomal endocytic pathway employing high K^+ applications. Subsequent recordings of spontaneous synaptic activity demonstrated that the quantal size was reversibly increased. The conclusion about the dependence of quantal size on the endocytic pathway was further strengthened by our

experiments demonstrating that pretreatment with the actin depolymerizing agent latrunculin B abolishes both the activation of the endosomal pathway and the increase in quantal size.

What is the mechanism producing the increase in the size of the neurosecretory quantum? One possibility is that some of the endosome-like cisternae are exocytosis-competent. This suggestion is supported by an earlier study at cerebellar granule cells (Marxen et al., 1999) demonstrating that vesicles and endosome-like vacuoles produced by the bulk membrane retrieval share membrane and membrane-associated proteins and, thus, may share major functional properties. Furthermore, a recent optical study (Coggins et al., 2007) directly demonstrated exocytosis of endosome-like structures in goldfish bipolar neurons. Exocytosis of the endosome-like vacuoles would produce enlarged neurosecretory quanta.

Another possibility is that the size of a vesicle is somewhat differently “tailored” depending on whether it is retrieved from the plasma membrane or from a cisterna. Interestingly, we observed that a size of a typical vesicle (a membranous structure of less than 44 nm diameter) was slightly enlarged in the preparations where the endosomal endocytic pathway has been activated. Molecular studies of clathrin-mediated endocytosis at *Drosophila* motor boutons suggested that vesicle sizes are “tailored” during the assembly of the clathrin coat (Zhang et al., 1998, 1999) and that all the vesicles in *Drosophila* motor boutons, including those formed by pinching off from endosomes, are formed via a clathrin-dependent mechanism (Heerssen et al., 2008). Thus, if the clathrin coat assembly is universal in vesicle formation and if it is a determinant of vesicle size, it should not be expected that a typical size of a vesicle formed by the pinching off from an endosome would substantially differ from the size of a vesicle produced by membrane retrieval. Indeed, we observe a very minor although significant enlargement of the vesicle in the preparations where the endosomal endocytic pathway has been activated. Our observation that vesicle size may depend on the vesicle recycling pathway may account for the variability in vesicle size observed among individual synaptic terminals (Hu et al., 2008).

In order to test whether the observed vesicle enlargement may account for the enlargement in quantal size, we performed statistical analysis of the distribution of quantal size and vesicle volume. The results of this analysis demonstrated that the minor enlargement of synaptic vesicles after high K^+ stimulation cannot explain a prominent increase in quantal size. We concluded, therefore, that the observed enlargement in quantal size, most likely, results from exocytosis of membranous structures which are larger than a typical vesicle. It is important to note that the diameter distribution of all the vesicles and cisternae demonstrated that there is no distinct border between these two classes of membranous structures. The membranous structures of intermediate diameters cannot be unambiguously classified in the electron microscopy analysis as a vesicle or a cisterna. Our statistical analysis demonstrated that exocytosis-competent endosomes are likely to be only 2–3 times larger than a typical vesicle, while exocytosis of larger cisternae is very unlikely, since corresponding giant quanta were not observed.

The activation of the endosomal endocytic pathway employed here was achieved by maintained prolonged depolarization of the presynaptic membrane employing high K^+ , and this was observed previously at the Torpedo electrical organ (Fox and Kriebel, 1997),

cerebellar granule cells (Marxen et al., 1999), calyx of held (de Lange et al., 2003) and in retinal bipolar cells (Holt et al., 2003; Coggins et al., 2007). Appearance of the endosome-like structures has been also observed after continuous action potential stimulation (Heuser and Reese, 1973; Teng and Wilkinson, 2000). Several studies (Teng and Wilkinson, 2000; de Lange et al., 2003; Holt et al., 2003) demonstrated that the observed endosome-like vacuoles and cisternae are produced by bulk membrane retrieval. The results of FM1–43 photoconversion experiments performed here suggest that in *Drosophila* motor boutons bulk membrane retrieval accounts for the cisternae formation only partially, and that some of the cisternae are likely to be produced via vesicle fusion and endosome formation. This suggestion is in line with an earlier study (Wucherpennig et al., 2003) which stressed the role of the endosomal compartment in endocytosis in *Drosophila* motor boutons.

In summary, we demonstrated that maintained depolarization of the presynaptic membrane produces massive formation of endosome-like structures, which is due to both bulk membrane retrieval and vesicles fusion. This endosomal formation is Ca^{2+} -dependent and actin-dependent (Fig. 7). The small endosome-like vacuoles and cisternae are exocytosis-competent, and this mechanism enables the nerve terminal to enhance the synaptic efficacy by increasing the quantal size.

Experimental methods

Preparations and chemicals

Canton S strain of *Drosophila melanogaster* was used in this study. Experiments were performed on Ib boutons (Lnenicka and Keshishian, 2000; Dasari and Cooper, 2004) of the muscles 6 and 7 of abdominal segments 2, 3 or 4 of the third instar larvae. Preparations were dissected in physiological solution containing (in mM) 130 NaCl, 36 sucrose, 5 KCl, 2 CaCl_2 , 2 MgCl_2 , and 5 HEPES, pH 7.3. (Jan and Jan 1976), pinned to sylgard, cut open along the dorsal midline, and internal organs were removed to expose the nerves and the muscles. In high K^+ stimulated preparations, KCl concentration was increased to 90 mM and balanced by the decrease in NaCl to 45 mM. At low Ca^{2+} conditions, the Ca^{2+} concentration was decreased (to 0.2 mM) 5 min prior to stimulation and left at 0.2 mM during high K^+ application. In latrunculin B pretreated preparations, latrunculin B (20 μM , A.G. Scientific Laboratories) was applied to the bath 5 min prior to stimulation and also added to the high K^+ solution during the stimulation.

Electron microscopy

Preparations were fixed in 1% glutaraldehyde/4% paraformaldehyde in 0.1 M cocodylate buffer for 2 h at room temperature and then incubated at 4°C overnight. After washing in 0.1 M cocodylate buffer with 0.1 M sucrose added, samples were post-fixed for 1 h in 1% osmium tetroxide, dehydrated through a graded series of ethanol and acetone, and embedded in Embed 812 epoxy resin (Electron Microscopy Sciences). Thin sections (70–90 nm) were collected on Formvar/carbon coated copper slot grids, and contrasted with lead citrate. Samples were examined on a Phillips 420 transmission electron microscope at 100 kV. To ascertain that only Ib type boutons were analyzed, we selected micrographs showing boutons of at least 2 μm diameter with small (approximately 30 nm diameter) clear vesicles

(Atwood et al., 1993; Jia et al., 1993). In action potential stimulated preparations, we labeled the stimulated segment by a cactus needle, which is always clearly seen in the tissue fixed for EM, and thus the stimulated segment was readily identified in the semi-thin slices and in the micrographs. The outer diameters of well defined vesicles and cisternae were measured employing ImageJ 1.36b software (NIH).

Photoconversion of the dye FM1–43

Photoconversion procedure was adopted from (Teng and Wilkinson, 2000; Harata et al., 2001; Schikorski and Stevens, 2001). After the dye loading (10 μ M FM1–43 added to the bath during stimulation), preparations were briefly washed in Ca^{2+} free solution containing 75 mM Advasep-7 (Biotium) and then rinsed in physiological saline without Advasep-7. Application of Advasep-7 (Kay et al., 1999) reduced background fluorescence and sharpened the borders of the boutons. Then preparations were fixed for 15 min in a regular EM fixative, washed for 30 min in physiological saline, preincubated in DAB (1.5 mg/ml in physiological saline) for 10 min, and illuminated for 20 min under $\times 63$ water immersion objective using a mercury lamp with 485 ± 10 bandpass excitation filter. At this point fluorescence staining was completely bleached, and DAB reaction product was visible. After a brief superfusion with a physiological saline, the illuminated site was marked by a cactus needle to be identified in subsequent EM analysis. Then the preparations were left overnight in a regular EM fixative and processed as for conventional EM.

Recordings and quantal analysis of postsynaptic responses

Synaptic activity was recorded from preparations bathed in the physiological solution containing in mM: 130 NaCl, 36 sucrose, 5 KCl, 2 CaCl_2 , 2 MgCl_2 , and 5 HEPES, pH 7.3. (Jan and Jan 1976). Spontaneous quantal events were recorded focally from the boutons visualized with DIC optics using macropatch electrodes of 5–10 μ m tip diameter. The electrodes were manually bent to enable recordings under $\times 63$ magnification water immersion objective (Zeiss) with 1.8 mm working distance. Recordings were digitized with Digidata A/D board and Axoscope software (Axon Instruments) and analyzed off-line. Area and amplitude of spontaneous synaptic potentials were measured using in-house software (Bykhovskaia, 2008). Spontaneous activity was recorded for 5 min at each recording site.

Statistical analysis

Datasets were compared employing two-sided *t*-test, one-way ANOVA, and Kolmogorov–Smirnov test.

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Fig. 1. High K⁺ stimulation reversibly activates the endosomal endocytic pathway

(A) Representative electron micrographs of Ib boutons at rest (left), immediately after high K⁺ stimulation (center), and after a 10 minute delay following high K⁺ stimulation (right). Scale bar: 200 nm. (B) The number of endosome-like cisternae significantly and reversibly increases after high K⁺ stimulation. (C) The number of vesicles significantly and reversibly decreases after high K⁺ stimulation. Asterisks indicate significant difference ($p < 0.05$). (D) Distributions of the diameters of all the membranous structures, including vesicles and cisternae, for non stimulated (top) and high K⁺ stimulated (bottom) boutons. (E) Cumulative

frequency distributions of the diameters of all the membranous structures, including vesicles and cisternae, for non stimulated (solid line) and high K^+ stimulated (dotted line) boutons.

(F) Cumulative frequency distributions of the diameters of vesicles (cisternae excluded) for non stimulated and high K^+ stimulated boutons. The distributions are significantly different ($p < 0.01$ according to Kolmogorov–Smirnov test).

Fig. 2. Quantal size increases following high K⁺ application

(A) A string of Ib boutons (left). An arrow points to the bouton from which recordings were taken (right). (B) Representative recordings of mEPSPs before (top) and after (bottom) high K⁺ stimulation. (C–E) mEPSP frequency (C) amplitude (D) and area (E) transiently increases after high K⁺ stimulation. Asterisks indicate significant difference ($p < 0.05$). (F) Distribution of mEPSP areas demonstrates a large proportion of enlarged mEPSPs after high K⁺ stimulation. (G) Cumulative frequency distributions of mEPSP areas for non-stimulated

(solid line) and high K^+ stimulated (dotted line) synapses. Frequency distributions are significantly different ($p < 0.01$, Kolmogorov–Smirnov test).

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Fig. 3. Disruption of actin polymerization by latrunculin B inhibits the formation of cisternae and the increase in quantal size

(A) A representative micrograph of latrunculin B treated and high K⁺ stimulated bouton. Scale bar: 200 nm. (B) Latrunculin B treatment inhibited cisternae formation in high K⁺ stimulated boutons. An asterisk indicates that significantly ($p < 0.05$) less cisternae were observed in latrunculin B treated high K⁺ stimulated treated preparations (black) compared to untreated high K⁺ stimulated preparations (gray). (C, D) No significant changes in mEPSP area (C) or amplitude (D) were observed in latrunculin B treated high K⁺ stimulated preparations, compared to untreated and non-stimulated preparations.

Fig. 4. Statistical analysis of the distributions of quantal size and vesicle volume

(A) The slight enlargement of synaptic after high K^+ application (solid squares — control, solid triangles — after high K^+) cannot account for the noticeable enlargement in quantal size (open squares — control, open triangles — after high K^+). Size unit corresponds to the whole range of the control vesicle distribution. (B) After high K^+ application, the volume of all the membranous structures, including vesicles and cisternae, has higher variability than the quantal size. The distributions of vesicle/cisternae volume and quantal size do not match. The distribution of vesicle/cisternae volume predicts a noticeable proportion of giant

quantal, which are not observed. (C) After high K^+ application, the distribution of the volume of vesicles and cisternae of up to 80 nm diameter, provides a reasonably good match to the distribution of quantal size.

Fig. 5. FM1-43 photoconversion reveals different patterns of cisternae formation

(A) A representative micrograph of a bouton loaded with the dye FM1-43 by a 1.5 minute high K^+ stimulation after the dye was photoconverted into an electron dense product. Both dark (stained with the dyes) and light translucent vesicles and cisternae are observed. Scale bar: 200 nm. (B) Gallery of representative micrographs demonstrating different staining patterns in cisternae after the dye photoconversion. Cisternae are marked with arrows. (1) Cisternae labeled completely and evenly located close to the presynaptic membrane are likely to be produced by the bulk membrane uptake. (2) Cisternae unevenly labeled are

likely to be produced by the fusion of labeled and unlabeled vesicles. (3) Fusion of evenly labeled vesicles with unlabeled or unevenly labeled cisternae.

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Fig. 6. A proportion of the labeled cisternae increases upon a more prolonged stimulation
 (A) A representative micrograph of a bouton loaded with the dye FM1-43 by a 5 minute high K^+ stimulation after the dye was photoconverted into an electron dense product. The majority of the vesicles and cisternae are labeled. Scale bar: 200 nm. (B) A proportion of labeled (gray portions of the bars) cisternae significantly ($p < 0.05$) increased as the stimulation time increased, while the total number of cisternae remained unchanged. Asterisks indicate significant difference ($p < 0.05$).

Fig. 7. The endosomal endocytic pathway is Ca^{2+} -dependent, but action potential stimulation is not as efficient in its activation as high K^+ applications

(A) A representative micrograph of the bouton which was stimulated by the application of high K^+ and low Ca^{2+} (0.2 mM) solution (top); a representative micrograph of the bouton which was stimulated by repetitive action potentials (50 Hz frequency for 15 min, bottom). Some cisternae are observed, but they are not abundant. (B) The stimulation by high K^+ and low Ca^{2+} solution or prolonged stimulation by repetitive action potentials (at 10 Hz or 50 Hz frequency) fails to produce a marked increase in the number of cisternae. The increase

observed at the 50 Hz stimulation is statistically significant ($p < 0.05$ indicated by an asterisk), although modest.