Readily releasable vesicles recycle at the active zone of hippocampal synapses

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Edited by Richard W. Tsien, New York University Neuroscience Institute, New York, NY, and approved March 4, 2014 (received for review November 18, 2013)

During the synaptic vesicle cycle, synaptic vesicles fuse with the plasma membrane and recycle for repeated exo/endocytic events. By using activity-dependent N-(3-triethylammoniumpropyl)-4-(4-(dibutylamino) styryl) pyridinium dibromide dye uptake combined with fast (<1 s) microwave-assisted fixation followed by photoconversion and ultrastructural 3D analysis, we tracked endocytic vesicles over time, "frame by frame." The first retrieved synaptic vesicles appeared 4 s after stimulation, and these endocytic vesicles were located just above the active zone. Second, the retrieved vesicles did not show any sign of a protein coat, and coated pits were not detected. Between 10 and 30 s, large labeled vesicles appeared that had up to 5 times the size of an individual synaptic vesicle. Starting at around 20 s, these large labeled vesicles decreased in number in favor of labeled synaptic vesicles, and after 30 s, labeled vesicles redocked at the active zone. The data suggest that readily releasable vesicles are retrieved as noncoated vesicles at the active zone.

synaptic vesicle retrieval | coated vesicles | hippocampus | endosome | electron microscopy

The mechanisms that govern synaptic vesicle (SV) retrieval have been debated since the discovery of the SV cycle (1, 2). Currently the two original mechanisms via coated vesicles and via "kiss and run" are proposed for mammalian excitatory central synapses (3–6). The proposed clathrin-mediated mechanism that retrieves the membrane via coated vesicles has comparable slow kinetics (7) (15–40 s until the endocytic vesicle separates from the plasma membrane) and a retrieval site outside the active zone (8, 9). First, the SV fully collapses into the release site and diffuses outside the active zone either as an entity or by its parts. At regions outside the active zone, coated pits form that sort SV proteins, and eventually a coated endocytic vesicle pinches off the plasma membrane. It is generally believed that coated vesicles shed their coat and fuse with early endosomes from which SVs bud off that join the SV cluster (8). This endosomal budding step is also believed to be mediated via coated vesicles. In contrast, SV retrieval via kiss and run has faster kinetics (10, 11) (<1 s), and SVs are retrieved at the active zone. During kiss and run, the SV is thought to maintain its identity and SVs are available for redocking and rapid reuse (12–14). Because SVs maintain their identity, fusion steps with potential endosomal compartments after endocytosis are not believed to occur after kiss and run.

There is overwhelming evidence that SV retrieval at mammalian central synapses depends on the major coat protein clathrin, but the visualization of coated vesicles shortly after physiological stimulation has only been shown for lower vertebrate synapses (8, 15, 16). Kiss and run, on the other hand, is not generally accepted as a retrieval mechanism at mammalian central synapses. Several fluorescent imaging techniques (e.g., pHluorin-based SV protein chimeras and nanoparticles) recently provided unique insights into kiss and run (6), but many open questions remain. The visualization of a labeled endocytic vesicle at or near the active zone right after a physiological stimulus would provide elegant additional proof for kiss and run. More so, if one could follow such a labeled vesicle until "redocking," it would greatly facilitate the investigation of the various steps SVs pass through on their way through the SV cycle.

Here, we introduce a technique that is based on activity-dependent labeling of SV retrieval with N-(3-triethylammoniumpropyl)-4-(4-(dibutylamino) styryl) pyridinium dibromide (FM1-43) followed by photoconversion and electron microscopic 3D analysis. This technique is combined with fast microwave-assisted fixation, ensuring a high time resolution that allows tracking of endocytic vesicles "frame by frame"—that is, at distinct time points (0, 4, 10, 20, 30, and 40 s) after stimulation.

Results

For the determination of when and where SVs are retrieved and to track these endocytic vesicles through the SV cycle, we used the following experimental procedure (Fig. 1*A*). Primary hippocampal cultures were stimulated in the presence of FM1–43 and fixed at various short (seconds) time intervals after stimulation. The resulting fluorescence was photoconverted, which resulted in a dark precipitate in the lumen of endocytic vesicles (17, 18). The occurrence and morphology of labeled vesicles and the synaptic structure of individual synapses were analyzed by electron microscopy (EM) of serial sections. The same procedure without fast fixation has been applied previously (17). Neurons were stimulated 40 times within 2 s by using electrical field stimulation. The stimulus was chosen because it has several advantages: (*i*) A 20 Hz stimulus for 2 s has been shown to release the readily releasable pool (RRP) at hippocampal synapses independent of its size and the release probability of the synapse (19). (*ii*) The size of the RRP coincides with the number of docked vesicles at individual synapses after electron microscopic 3D reconstruction (17). The fraction of labeled vesicles over docked vesicles thus can be used to monitor SV retrieval at the various time points. The 3D analysis on the ultrastructural level allows, in addition, the determination of the morphology and location of endocytic labeled vesicles.

The presented experiments relied on a fixation technique that accurately stops a distinct functional and structural stage of a biological process while optimally preserving the fine structure.

Significance

Nerve cells communicate via the release of a neurotransmitter that is stored in synaptic vesicles. The availability of synaptic vesicles is thus crucial for synaptic function. Availability of synaptic vesicles is ensured by vesicle retrieval after fusion with the plasma membrane; however, the underlying mechanisms of membrane retrieval at hippocampal synapses are still debated. Here we show that synaptic vesicles are retrieved at the active zone without the assistance of coated vesicles. Such a mechanism is similar to "kiss and run" but contradicts the classical pathway via clathrin-coated vesicles.

Author contributions: T.S. designed research, performed research, contributed new reagents/analytic tools, analyzed data, and wrote the paper.

The author declares no conflict of interest.

This article is a PNAS Direct Submission.

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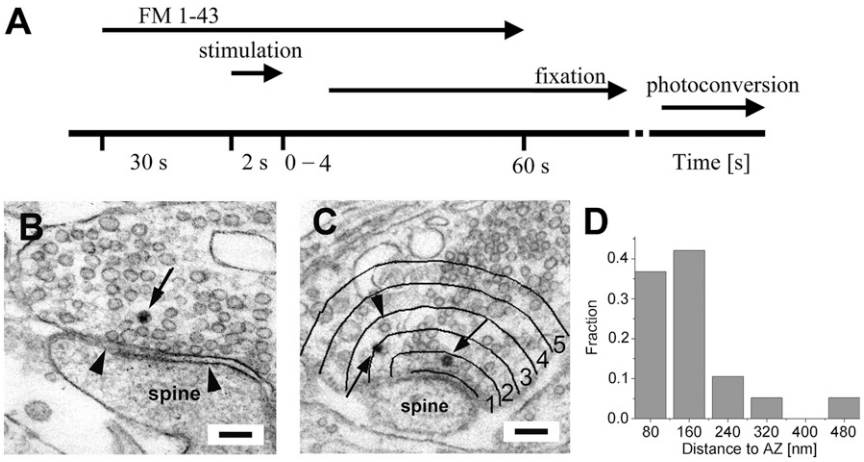

Fig. 1. Experimental rationale and the first labeled endocytic vesicles 4 s after stimulation. (*A*) The timeline of experiments. Dye is presented 30 s before electrical field stimulation for 2 s. Samples were rapidly fixed by using microwave-assisted fixation at various time points (0–60 s). The fluorescence trapped in endocytic vesicles after membrane retrieval was photoconverted, and the specimens were processed for 3D EM. (*B*) A synapse of a cultured hippocampal neuron that was labeled with FM1–43 and fixed 4 s after stimulation. The arrowheads indicate the postsynaptic density inside a spine. The arrow points at one labeled vesicle that still hoovers above the active zone. (Scale bar: 100 nm.) (*C*) An example of the compartments drawn to quantify the location of the earliest labeled vesicles. Compartments have a width of 80 nm. Two labeled vesicles (arrows) are shown that are still very close to the active zone. The arrowhead points at an unlabeled coated vesicle. (Scale bar: 150 nm.) (*D*) Relative frequency histogram of labeled vesicles in each compartment. The actual distance from the active zone (AZ) is indicated on the *x* axis for each compartment: 80 nm for the first compartment, 160 for the second, and so on. Almost 80% of all labeled vesicles were found within the two compartments closest to the active zone.

To capture the various steps within the SV cycle, we have chosen microwave-assisted aldehyde fixation. Microwave fixation has been shown and widely used for fixation up to the millisecond range (20). Later on we describe the fine structure of labeled vesicles for the various time points.

Fixation After 0–5 s. In experiments, when we left 3 s or less between the last pulse of the stimulus train and the onset of the microwave-assisted fixation, we were not able to detect any labeled vesicles in electron micrographs (three experiments, 35 synapses). Rapid fixation 4 s after stimulation, however, revealed the earliest labeled vesicles (Fig. 1*B*). After serial sectioning and 3D reconstruction, we counted the number of labeled vesicles and compared the number with the number of docked vesicles. In 28 fully 3D reconstructed presynaptic boutons (four experiments), we found that, on average, an equivalent of 50% of the docked vesicle pool was retrieved.

First Retrieved Vesicles Are Close to the Active Zone. One may expect that the first retrieved SVs are still close to the retrieval site at the plasma membrane. We measured the distance of labeled SVs to the active zone by using a previously published method (17). Compartments with a width of two SV diameters were drawn using the active zone as a baseline. The second compartment was drawn two SV diameters wide using the first compartment as the baseline and so on (Fig. 1*C*). We counted the number of labeled vesicles in each compartment of synaptic profiles of three experiments. 36% of the labeled vesicles were found within the first and 42% within the second compartment; thus, 78% of the labeled SVs are less than 160 nm away from the active zone 4 s after stimulation (Fig. 1*D*). The remaining labeled vesicles were found in various compartments.

First Retrieved Vesicles Do Not Show Any Sign of a Protein Coat. No coated labeled vesicles were found at synapses fixed 4 s after stimulation or at any later time points. However, we found coated vesicles in dendrites, in axonal segments, and outside the main SV cluster (Figs. 1*C* and 2*B*). The presence of unlabeled coated vesicles suggests that the coated endocytic vesicle pinched off the membrane before FM1–43 was present. The very few labeled coated vesicles indicate that the photoconversion polymer did not occlude the structure of the protein coat in our EM graphs and that coated labeled vesicles were readily detectable in our samples.

Fixation After 10 s. In the next experiments, samples were fixed 10 s after stimulation (three experiments, 35 synapses). After photoconversion and 3D reconstruction, we did not find a significant increase in the fraction of labeled vesicles over docked SVs, suggesting that membrane retrieval came to an intermittent hold after the initial retrieval period at 4 s. However, there was a noticeable change in labeled vesicle morphology. About one-third of all labeled vesicles were clearly larger than typical electron lucent vesicles (Fig. 3*A*). To quantitatively address the size of labeled vesicles, we measured the area of typical electron lucent SVs and calculated the surface of these vesicles assuming they are spheres. As in previous reports (21, 22), we found that SVs have an average diameter of 40 nm and a surface of 5,000 nm^2. Next, we measured the area of labeled vesicles in synaptic profiles and found that labeled vesicle surfaces ranged from 5,000 to 20,000 nm^2. Because SV size varies among synapses

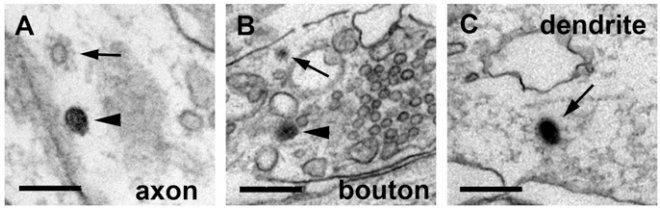

Fig. 2. Examples of unlabeled and labeled coated vesicles. (*A*) An unlabeled coated vesicle (arrow) inside an axon of a specimen that was fixed 10 s after stimulation. The arrowhead points at a noncoated labeled vesicle. (*B*) A labeled coated vesicle (arrow) and a labeled large vesicle (arrowhead) inside a presynaptic bouton fixed 20 s after stimulation. Note that both labeled vesicles are outside the main SV cluster to the left. (*C*) A labeled coated vesicle (arrow) inside a dendrite. (Scale bars: 100 nm in *A*, 300 nm in *B*, and 200 nm in *C*.)

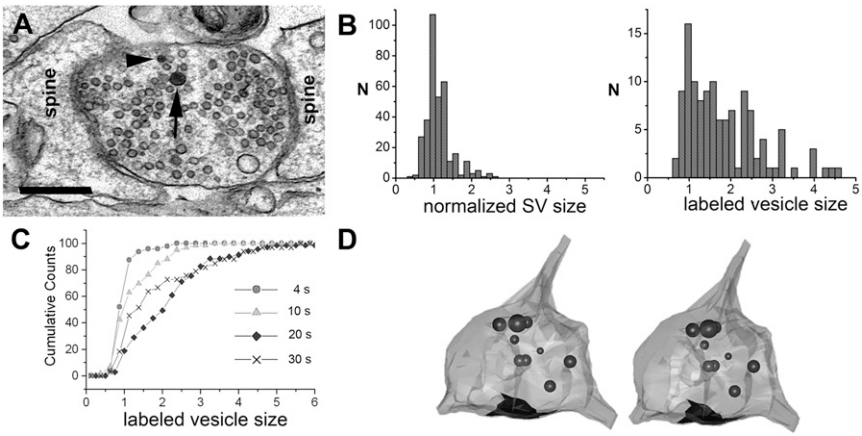

Fig. 3. Large labeled vesicles appear 10 s after stimulation. (*A*) A synaptic profile that was fixed 10 s after stimulation. The presynaptic bouton is contacted by two spines. The arrowhead points at a labeled small endocytic vesicle. The arrow indicates a large labeled vesicle. (Scale bar: 400 nm.) (*B*) Frequency histogram of electron lucent SV (*Left*) and labeled (*Right*) vesicle size 10 s after stimulation. The *x* axis indicates SV size normalized to the average size of SVs for each synapse (for details, see *Fixation After 10 s*). Labeled vesicle size (*Left*) is up to four times the size of SVs within the same bouton. (*C*) Cumulative frequency histogram of labeled vesicle sizes at different fixation times. At 4 s, all labeled vesicles have the same size as typical SVs. The number of large vesicles increases until 20 s after stimulation. At later time points (30 s) large vesicles decrease and small vesicles increase proportionally. The histograms are significantly different from each other (Kolmogorov–Smirnov test, $P < 0.01$ for 4 s compared with 10 s and 10 s to 20 s; $P < 0.05$ for 20 s to 30 s). (*D*) Localization of large labeled vesicle 20 s after stimulation, and a pair of images for stereoviewing of a 3D rendering of a presynaptic bouton 20 s after stimulation. Labeled vesicles are shown as spheres with a diameter corresponding to the area measurements in EM graphs. The black area at the bouton indicates the active zone. Note that large vesicles are closer to the plasma membrane surrounding the SV cluster in the center. The SV cluster is omitted for clarity.

(21), we normalized the size of labeled vesicles by the average size of electron lucent SVs for each synapse. The frequency histogram in Fig. 3*B* shows that 10 s after fixation labeled vesicles consisted of sufficient membrane for up to 4 SVs.

Fixation After 20–40 s. Next, we investigated labeled vesicles at 20 s (two experiments), 30 s (two experiments), and 40 s (two experiments) after stimulation. At 20 s after stimulation, the fraction of labeled large vesicles to total labeled vesicles reached a maximum of 66% (Fig. 3*C*). At this time point, not only is the number of large vesicles at a maximum, but we also found the largest labeled vesicles (up to 5 SV sizes). Fig. 3*D* shows a 3D rendering of large labeled vesicles within a presynaptic bouton fixed 20 s after stimulation. All labeled vesicles surrounded the main SV cluster. At later time points, the fraction of labeled large vesicles decreased in favor of small vesicles until up to 90% of all labeled vesicles were single sized 40 s after stimulation and later. Labeled SVs mix with the SV cluster without showing any apparent preference. Starting 30 s after stimulation, when the number of small SVs increases, we found the first yet very few labeled docked vesicles at active zones.

Time Course of Membrane Retrieval. The time course of membrane retrieval after the release of the RRP can be measured in two ways in our experiments: first, as the number of labeled vesicles independent of their size, and second, as the sum of the membrane surface of all labeled vesicles (which includes the size of labeled vesicles). When labeled vesicles were counted as multiple vesicles (surface area of vesicles divided by average surface area of typical SVs), membrane retrieval completed around 20 s after stimulation (Fig. 4). When counting every labeled vesicle as a single event, the number of labeled vesicles equaled the number of docked vesicles 40 s after stimulation.

Discussion

By using fast microwave-assisted fixation and photoconversion, we were able to follow endocytic vesicles labeled via FM1–43 uptake on the ultrastructural level at a high time resolution, frame by frame. In an effort to quantify our data, we have chosen electrical field stimulation of 40 pulses in 2 s, as this stimulus releases the functional RRP and labels an amount of vesicles in photoconversion experiments that is equal to the number of docked SVs at the active zone (17).

The reported results rely on the accuracy of fixation times. To determine the accuracy, we can compare the time course of the SV cycle in the present study with previous in vitro studies on living cells. First, studies that have used pH-sensitive fluorescence protein to probe membrane retrieval detected the first reuptake after a delay of 4 s (23–25), which is consistent with the data in this report. Second, live imaging of labeled endocytic vesicles has shown that an entire cycle of an SV takes about 35–40 s (26–29). This is consistent with our data, as we find the first labeled vesicles docked at the active zone starting at 30 s

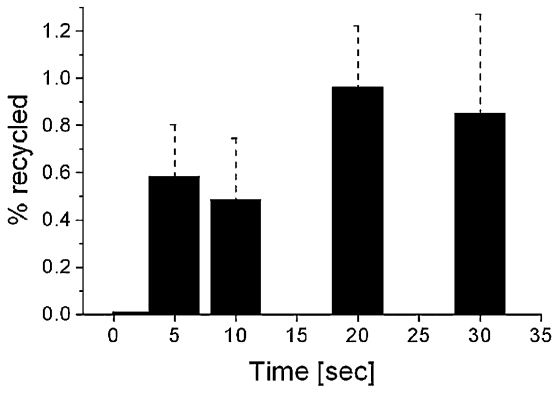

Fig. 4. Time course of SV retrieval. The bar histogram shows the percentage of recycled membrane at different time points. The percentage of recycled vesicle membrane was calculated as the percentage of labeled vesicle surface over the number of docked vesicles for each 3D reconstructed synapse at each time point. It appears that the membrane is labeled in two steps. The initial retrieval is at 4 s after stimulation. Between 10 and 20 s, labeled membrane doubles to the final amount of labeled vesicle membrane that equals the docked vesicle pool. Mean and SD are shown for each time point: 4 s, 58 ± 22%; 10 s, 49 ± 26%; 20 s, 96 ± 26%; 30 s, 85 ± 42%. The means between 10 and 20 s are statistically different ($P < 0.01$).

after stimulation. In addition, we compared microwave-assisted aldehyde fixation with fixation by immersion in the same fixative. In immersion fixation experiments, the fixative was added with the exact end of the 2-s-long stimulus. After photoconversion and EM processing, we found stained vesicles of various sizes and the frequency histogram of labeled vesicle sizes resembled those fixed microwave-assisted 10 s after stimulation. The existence of labeled large vesicles after immersion fixation indicates that microwave-assisted fixation is at least 10 times faster. Finally, a previous study (24) arrested the SV cycle by using ice-cold fixation and found labeled vesicles at 4 s and large labeled vesicles at 10 s, matching our data. The comparison with previous approaches suggests that microwave-assisted fixation is accurate to the second in capturing the various functional states of the SV cycle while resulting in excellent preservation of the fine structure of synapses.

The Morphology of Endocytic Vesicles. In contrast to previous fluorescent approaches that followed SV retrieval at hippocampal synapses, our electron microscopic approach allowed us to also describe the position and morphology of endocytic vesicles in fully 3D reconstructed synapses. This advantage led to several new observations about the various steps of the SV cycle at hippocampal synapses. One noteworthy observation is that we did not encounter coated vesicles in our experiments. Neither the very first labeled vesicles detectable in our experiments nor labeled vesicles at any later stage in the SV cycle exhibited protein coats. However, we were able to detect unlabeled and very few labeled coated vesicles at axonal regions outside the presynaptic bouton, suggesting that coated vesicles are stable during the time span of our experiment and are not lost during microwave-assisted fixation. Using the timeline of our experiments, we can estimate the minimum age of the detected unlabeled coated vesicles. Because most coated vesicles did not contain the photoconversion product, they must have pinched off the plasma membrane before FM1–43 was present. Because we added FM1–43 30 s before stimulation, unlabeled coated vesicles were 36 s or older (30 s dye exposure before stimulation + 2 s stimulation + 4 s delay to fixation). For example, the coated "synaptic" vesicle in Fig. 3*C* was at least 36 s old. Previous live imaging studies have reported similar life spans of clathrin-coated vesicles (7, 9). Because we could not detect a significant amount of coated vesicles that could support membrane retrieval after the release of the RRP, it seems likely that SV retrieval via coated pits and coated vesicles does not play a major role at hippocampal synapses at least not under our experimental conditions. On the other hand, there is overwhelming evidence that clathrin plays a central role in SV retrieval at hippocampal synapses (6, 9, 22). The lack of coated vesicles in our samples may suggest a function of clathrin in noncoated vesicle endocytosis.

Kiss and Run. The second important observation is that the very first labeled endocytic vesicles were close to the active zone. Among the several retrieval mechanisms that have been proposed for hippocampal synapses, only kiss and run retrieves noncoated vesicles at the active zone. The close location of new endocytic vesicles to the active zone may suggest kiss and run as the main retrieval mechanism for readily releasable vesicles in our experiments.

Kiss and run is usually defined as a form of SV retrieval faster than 1 s (6), even as fast as 100 ms (11). Membrane retrieval at active zones in our experiments, however, appears to be about 4–10 times slower. We may have missed fast kiss and run due to our experimental design. Kiss and run was first detected at hippocampal synapses by using a high concentration of FM2–10, a variant of FM1–43 (10). However, FM2–10 is difficult to photoconvert and thus not suitable for our experiments. Alternatively, we may have not missed kiss and run, but vesicles may stay at the active zone after kiss and run in an intermediate state between fusion pore closure and undocking. Fixation may render these vesicles leaky, and they may have lost their dye content before photoconversion.

Kiss and run is unique because it preserves the identity of the SV during exo-endocytosis cycles. That the first labeled vesicles were found close to the active zone and were of the same size as SVs may indicate that these vesicles indeed maintained their identity. Once retrieved, however, labeled vesicles move away from the active zone and become part of large vesicles. Similar results have been reported for readily releasable vesicles at the calyx of Held (30). Together, these results contradict common beliefs that SVs retrieved via kiss and run maintain their identity throughout the SV cycle and may suggest an unknown step after endocytosis.

The Time Course of SV Retrieval. In our experiments, no labeled vesicles were apparent with fixation at less than 4 s. A very recent report using fast freezing (31, 32) suggested endocytosis of large vesicles outside the active zone as early as 50 ms after stimulation. Because the membrane was retrieved via pits and invaginations, we should have detected this form of ultrafast endocytosis in our experiments. One explanation may be the lower temperature in our experiments (21 °C versus 34 °C) that may have slowed this retrieval mechanism until 10 s after stimulation, when we also observed large vesicles close to the plasma membrane. Another reason may be the different stimulation protocols used that may trigger different endocytic mechanisms.

Several other differences between the study by Watanabe et al. (31) and this report are noteworthy. Their study describes morphological changes in synaptic profiles after the release of a single SV triggered by a single action potential. The single stimulus would not have triggered release in all synapses, and it is thus not clear whether the observed endocytosis of large vesicles is in response to a release event at the same synapse. It may have been triggered by calcium or the presence of a foreign cation channel. Also in their experiments, endocytic vesicles are identified by their location close to the membrane, but recycling of individual vesicles at the active zone is not detectable by location. Watanabe et al.'s (31) study, however, is likely to provide more accurate "fixation" time points than in the present study, and the observation of an increased number of open vesicles is a stunning result that supports vesicle fusion and transmitter release at active zones.

SV retrieval started 4 s after stimulation, when on average about 50% of the RRP was endocytosed, although the amount of membrane retrieval varied from around 20–70%. This perhaps sudden onset of endocytosis may be reflected in that vesicles are retrieved independently of each other. Every vesicle has to undergo the same number of steps before membrane fission, which could result in an apparent synchrony of retrieval. Starting around 10 s after stimulation, the amount of labeled membrane began to double. A second membrane retrieval mechanism outside the active zone via large vesicles may explain the doubling of labeled membrane between 10 and 20 s. Two retrieval mechanisms that match the time course of SV endocytosis in our experiments have been reported after the release of individual SVs at hippocampal synapses (23). In our EM graphs, however, the detection of a second retrieval mechanism was hampered, as we can identify the location of membrane retrieval only by the proximity of labeled vesicles to the plasma membrane or by the detection of protein structures that assist membrane retrieval (e.g., coated vesicles). Unfortunately, the localization of labeled large vesicles at the margin of the SV cluster and as such close to the plasma membrane convolutes our analysis; we simply cannot differentiate between newly retrieved vesicles and labeled vesicles that traveled from the active zone to the margins of the SV cluster at time points of 10 s and later.

Are Large Labeled Vesicles Endosomes? Alternatively, the doubling of labeled membrane 10 s after stimulation may occur through fusion of labeled endocytic vesicles (originating at the active zone) with unlabeled membrane compartments inside the presynaptic bouton. In this scenario, the dye of the endocytic vesicles would spread to unlabeled membrane and thus result in the doubling of labeled vesicles. Because we used a relative high concentration of FM1–43, photoconversion was capable of labeling all membrane compartments even though the dye was diluted across membranes.

At least two fusion partners for endocytic vesicles come to mind: latent endosomes and homotypic fusion with other SVs. An endosomal sorting step has been proposed for the SV cycle early on. Originally, vesicle budding-off endosomes were believed to be the origin of coated vesicles in EM graphs (2). The large labeled vesicles detected in our study may represent endosomes (24). The observation that large labeled vesicles decreased in size and almost disappear 40 s after stimulation in favor of small SVs may suggest that labeled SVs originate from these large labeled vesicles via a budding step. Because we also did not encounter significant numbers of coated vesicles at these time points, such a budding step also appears to be independent of coated vesicles.

In addition, a step where endocytic labeled vesicles originating from two different retrieval mechanisms fuse "homotypically" with each other may also explain the presented data.

We also were able to roughly follow the position of labeled vesicles. At the very early stages of the SV cycle, small labeled vesicles were close to the active zone. Large vesicles seem to preferentially locate outside the SV cluster, and at 20 s, when the large labeled vesicles peak, almost all labeled vesicles surround the SV cluster like a shell. Similar localizations of large endocytic vesicles were observed at the neuromuscular junction of frogs (15). Because large vesicles did not mix with the SV cluster, it is likely that they possess different trafficking signals than SVs and thus may be indeed "endosome-like" structures. On the other hand, if there were endosomes, it may be expected that such latent endosomes remain outside the main SV cluster, as labeled membrane compartments. In this case, two groups of labeled vesicles would exist 40 s after stimulation and later: one that redistributes within the SV cluster and another group that surrounds the cluster. However, the present study and previous studies (17, 18) have shown that all labeled SVs rejoin the SV cluster with no preference to the active zone or the margin of the SV cluster.

Summary

Our ultrastructural approach to monitor SV retrieval and track endocytic vesicles may draw the following picture of the SV cycle at hippocampal synapses: About 50% of the RRP recycle as individual SVs at the active zone after the release of the RRP via a mechanism independent of coated vesicles. The retrieved vesicles move away from the active zone toward the margin of the SV cluster. Starting at around 10s after stimulation, the remaining vesicles of the released RRP appear in the form of large vesicles either via a second retrieval mechanism, fusion with unlabeled membrane compartments, or both. At 20 s after stimulation, large labeled vesicles may give rise to individual labeled SVs that join the main SV cluster. The first redocked labeled vesicle can be found 30 s after release, however only very few of the recycled SVs redock at the active zone (about 5%)—that is, become part of the RPP. The majority of labeled SVs distribute with no apparent preference toward the active zone within the SV cluster as part of the reserve pool (18).

Methods

Cell Culture. Cell cultures were prepared according to standard protocols (33). Briefly, purified astrocytes were plated on poly-D-lysine/collagen–coated coverslips and grown to confluence. Hippocampal neurons were collected from newborn rat pups, dissociated, and plated on top of the astrocyte feeding layer at a density of 30,000 cells per mL. Neurons were grown in Neurobasal Medium supplemented with B27 for 14–21 d. All animal experiments were approved by the Animal Care and Use Committee of Universidad Central del Caribe and conformed to National Institutes of Health guidelines.

Stimulation and Rapid Fixation. The stimulation chamber containing neurons on a coverslip was placed inside a microwave oven (Biowave, Ted Pella) at room temperature. Electrical field stimulation was applied by using two platinum wires (pulse duration, 1 ms; amplitude, 16–20 V at a frequency of 20 Hz). After short time intervals of 0, 4, 10, 20, 30, and 40 s, fixative (4 g/100mL paraformaldehyde, 0.5% glutaraldehyde, 0.2 mM $CaCl_2$, in 100 mM cacodylate buffer, pH 7.4) was added to the stimulation chamber, and microwave irradiation (750 W) started immediately. A temperature probe automatically stopped microwave irradiation when the fixative reached 40–42 °C. Fixation continued in fresh fixative at room temperature for 20 min to 2 h. After a thorough wash in cacodylate buffer, neurons were incubated in 0.15% diaminobenzidine in cacodylate buffer (pH 7.6) for about 5 min, placed under a fluorescence microscope, and photoconverted for 8–12 min. Specimens were washed in cacodylate buffer and postfixed in Karnovsky fixative (4% paraformaldehyde, 5% glutaraldehyde, 0.2 mM $CaCl_2$, 80 mM sodium cacodylate, pH 7.4) at 20 °C overnight.

EM. Neurons were embedded according to a previously published protocol (34).

Image Acquisition and Analysis. Electron micrographs (magnification between 14,000× and 75,000×) were taken with a Jeol 100CX equipped with a digital camera and analyzed with the AnalySIS software package (Olympus Softimaging Solutions GmbH). Only excitatory synapses were included in our datasets. In brain, excitatory synapses were identified by their asymmetric junction. In cell culture, postsynaptic densities are less pronounced than in the brain (35), and excitatory synapses were identified by the presence of round SVs. Inhibitory synapses are rare in culture as revealed by immunocytochemical staining against GABA and can be identified by their flattened SVs in the electron microscope. The areas of vesicles were determined by outlining the vesicle membrane using AnalySIS software tools (arbitrary area). EM graphs are presented without significant processing except for correcting shading by using Photoshop's dodging and burning tools. The 3D rendering in Fig. 3 was created by using the Reconstruct software package (http://synapses.clm.utexas.edu/tools/index.stm).

ACKNOWLEDGMENTS. This work was supported by National Institutes of Health Grants 1SC1NS077776, 5G12RR003035-27, and 8G12MD007583-27.

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