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Rapid Reuse of Readily Releasable Pool Vesicles at Hippocampal Synapses

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Summary

Functional presynaptic vesicles have been subdivided into readily releasable (RRP) and reserve (RP) pools. We studied recycling properties of RRP vesicles through differential retention of FM1-43 and FM2-10 and by varying the time window for FM dye uptake. Both approaches indicated that vesicles residing in the RRP underwent rapid endocytosis ($\tau \approx 1$ s), whereas newly recruited RP vesicles were recycled slowly ($\tau \approx 30$ s). With repeated challenges (hypertonic or electrical stimuli), the ability to release neurotransmitter recovered 10-fold more rapidly than restoration of FM2-10 destaining. Finding neurotransmission in the absence of destaining implied that rapidly endocytosed RRP vesicles were capable of reuse, a process distinct from repopulation from the RP. Reuse would greatly expand the functional capabilities of a limited number of vesicles in CNS terminals, particularly during intermittent bursts of activity.

Introduction

The readily releasable pool (RRP) has been defined physiologically as a set of secretory vesicles immediately capable of transmitter release upon stimulation by elevated intracellular Ca^{2+} (Heinemann et al., 1994; Schneggenburger et al., 1999) or by a hypertonic challenge (Stevens and Tsujimoto, 1995; Rosenmund and Stevens, 1996). At the small nerve terminals of CNS neurons, the focus of this paper, the RRP consists of a subset of vesicles that are primed for fusion, probably corresponding to the coterie of morphologically docked vesicles (Schikorski and Stevens, 1997). There is compelling evidence that the size of the RRP governs the probability of transmitter release during presynaptic activation (Rosenmund and Stevens, 1996; Dobrunz and Stevens, 1997). How rapidly the RRP is replenished after previous release events helps determine the extent to which transmitter release can be maintained in the face of repeated Ca^{2+} entry (Stevens and Wesseling, 1999; but see also Wu and Borst, 1999).

In the prevailing picture of vesicular cycling (e.g., Südhof, 1995), transmitter release is mediated by fusion and collapse of synaptic vesicles into the presynaptic plasma membrane. After a vesicle releases its contents, its place at the active zone is taken up by another transmitter-containing vesicle. Thus, "refilling" of the RRP has been equated with repopulation, the replacement of spent vesicles with fresh ones. The source of the fresh vesicles is the reserve pool (RP), defined as another population of vesicles that are not morphologically docked and thus are not primed for fusion. As the RP supplies the RRP, its contents are restored in turn by endocytosis and vesicle recycling (Heuser and Reese, 1973).

There are at least two reasons for skepticism about the classical picture as a complete explanation of vesicle recycling at small central nerve terminals. The first concerns the limitations that this scenario imposes on their functional capabilities. During continual presynaptic spiking, the rate of repopulation will limit neurotransmission under steady-state conditions. Put simply, if the number of recycling vesicles were <40 (Murthy and Stevens, 1999), and full transit through the vesicle cycle took $\sim$40 s (Ryan et al., 1993; Liu and Tsien, 1995), then the average steady-state frequency of quantal transmission would be severely limited to $\sim$1 Hz. The second issue concerns the empirical underpinnings of the notion of repopulation. Selective depletion of the RRP is followed by a rapid exponential recovery ("refilling"; Stevens and Tsujimoto, 1995; Rosenmund and Stevens, 1996; Dittman and Regehr, 1998). However, upon exocytosis of a majority of the releasable vesicles, recovery is significantly slower (Ryan et al., 1993; Liu and Tsien, 1995; Ryan and Smith, 1995) and traces a biexponential time course, with time constants of $\sim$10 s and >30 s (Stevens and Wesseling, 1999). These and other kinetic features are inconsistent with a simple two-pool model whereby RRP and RP are connected by kinetically well-defined rate constants (Stevens and Wesseling, 1999).

One source of difficulty is that the RRP has been operationally defined by electrical recordings and not by imaging of fluorescent dyes. The electrophysiological data is inherently difficult to interpret on its own, because quantal excitatory postsynaptic currents (EPSCs) could in principle arise from either first-time exocytosis or repeated release events (von Schwarzenfeld, 1979; Zimmermann et al., 1993). Thus, repopulation by fresh vesicles could not be distinguished from repeated deployment of the same vesicles that have recently undergone fusion (Ceccarelli et al., 1973; Ceccarelli and Hurlbut, 1980; Neher, 1993; Neher and Zucker, 1993; Artalejo et al., 1995). Vesicular reuse would be of potential importance for sustaining secretory activity, given the limited number of vesicles within a presynaptic bouton (Harris and Sultan, 1995; Schikorski and Stevens, 1997) and the long time that may be required to retrieve fully collapsed vesicles or to construct vesicles de novo (Heuser and Reese, 1973; Jahn and Südhof, 1994; De Camilli, 1995). However, reuse has not attracted much attention due to the lack of firm experimental evidence. The styryl dye FM1-43 has been extremely valuable for studies of exocytosis and endocytosis (Betz et al., 1996), but is not useful for studying the RRP: stimuli that completely exhaust the RRP (for example, hypertonic challenges) fail to cause appreciable loss of FM1-43 fluorescence. To circumvent this problem, we have taken advantage of

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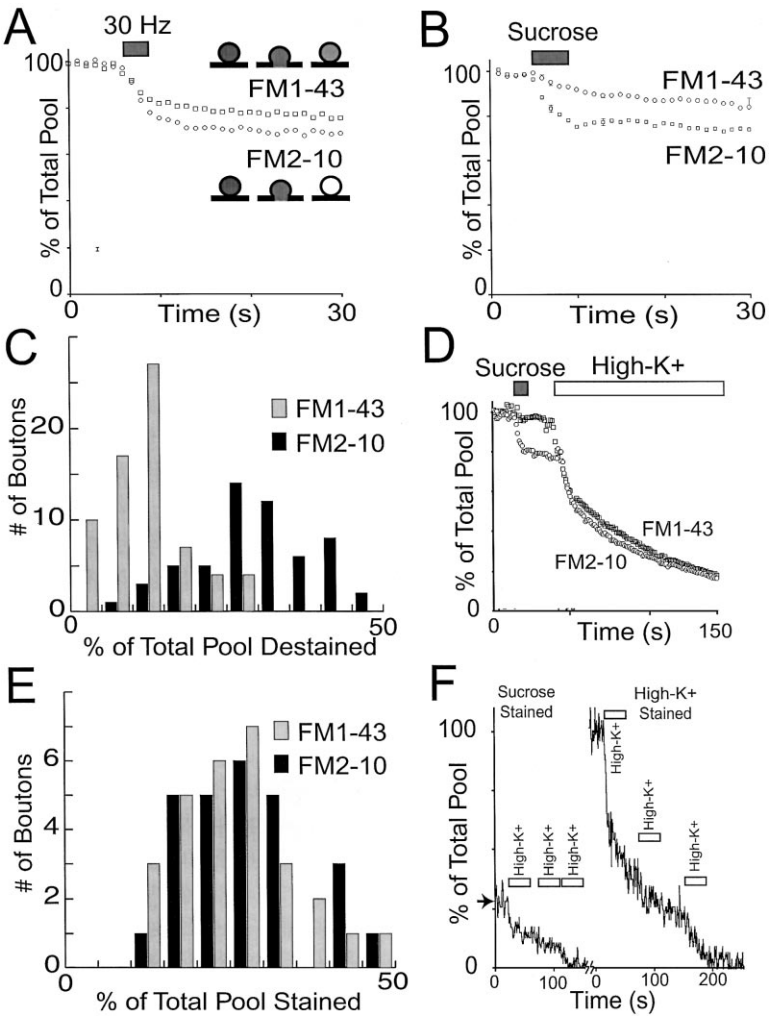

Figure 1. The Early Onset of Styryl Dye Destaining Differences Implicates the RRP

(A) A brief electrical stimulus (30 Hz/3 s) released vesicles of the RRP and resulted in differential destaining of FM1-43 and FM2-10 ($n = 75$ [3] and $n = 81$ [3], respectively). Insets depict the partial retention of the slower departitioning dye, FM1-43, in contrast to the near-complete escape of FM2-10 during the same fusion-retrieval events. Here and elsewhere, all n values represent the number of boutons, the number of separate experiments is indicated in brackets, and symbols show mean values ± SEM. Error bars for SEM were smaller than symbol size (largest error indicated by error bar shown).

(B) Hypertonic solution (800 mOsm) releases vesicles of the RRP in the absence of Ca^{2+}. Similar to the results shown in (A), the rapid release of readily releasable vesicles results in differential destaining between FM1-43 and FM2-10 ($n = 165$ [7] and $n = 188$ [7], respectively).

(C) Pooled data from experiments in which the total recycling pool was labeled with high-K^+ depolarization and then destained with hypertonic solution (800 mOsm). Presynaptic terminals labeled with FM2-10 lost 26.1% ± 1.3% of the total pool fluorescence, whereas terminals labeled with FM1-43 lost only 7.2% ± 0.8%. The differential destaining resulted from rapid recycling events that retained FM1-43 to a greater extent than FM2-10.

(D) A hypertonic prepulse separates vesicles of the RRP from a subsequent release of the total vesicle pool by high-K^+ depolarization. Once vesicles of the RRP are released, the remaining vesicles of the total recycling pool show little difference in destaining, suggesting that rapid recycling may be an exclusive property of vesicles that were held in the RRP prior to stimulation ($n = 57$ [2], FM1-43; $n = 54$[2], FM2-10).

(E) Pooled data from experiments in which synaptic terminals were stained by application of hypertonic solution (800 mOsm), with the degree of staining calibrated to the entire recycling pool by a subsequent staining with high-K^+ depolarization. Hypertonic staining of the RRP labels a similar percentage of vesicles with both styryl dyes: 23.2% ± 2.4% for FM1-43 ($n = 30$ [3]) and 22.1% ± 1.7% for FM2-10 ($n = 26$ [3]). Note, in comparison with (C), that both destaining and staining experiments using FM2-10 quantify the RRP as ~25% of the total recycling pool (closed bars).

(F) FM1-43 fluorescence profile of a single hippocampal bouton after hypertonicity-induced staining. The fluorescence loss from readily releasable vesicles labeled by hypertonic stimulation was referenced to the fluorescence loss from the total recycling pool after a subsequent staining with high-K^+ depolarization. To accurately quantify the amount of activity-induced staining, multiple rounds of high-K depolarization were used to completely destain the terminal following each of the staining protocols.

another FM dye, FM2-10, that dissociates from neuronal membranes much more quickly than FM1-43 (Ryan et al., 1996; Klingauf et al., 1998; Schote and Seelig, 1998). Using this probe, we were able to define the behavior of the RRP with optical measurements and to distinguish between first-time exocytosis and repeated transmitter release due to vesicular reuse. The results call for a revision of the prevailing conception of vesicle recycling.

Results

During brief, intense electrical stimulation, hippocampal nerve terminals released a smaller proportion of the more slowly dissociating FM1-43 than the more rapidly dissociating FM2-10 (Figure 1A), as expected if vesicles were only briefly connected to the external milieu due to rapid endocytosis following exocytosis (Klingauf et al., 1998; Kavalali et al., 1999). The disparity in destaining was established within seconds after the onset of stimulation, indicating that rapid retrieval was operative for the first few vesicles that fused with the plasma membrane. The vesicles that underwent exocytosis during the first several seconds of strong stimuli belonged to a subpopulation of vesicles defined as the RRP (Neher and Zucker, 1993; Stevens and Tsujimoto, 1995; Rosenmund and Stevens, 1996; Schneggenburger et al., 1999; Wu and Borst, 1999). Since the differential destaining of the FM dyes and the liberation of the RRP both took place over the same interval, we wondered whether rapid retrieval might be a specialized property of this subset of vesicles. To test this idea, we imposed challenges known to selectively release the RRP and looked

for differences in destaining of FM1-43 and FM2-10 (Figure 1A). Three seconds of 30 Hz stimulation produced differential destaining similar in magnitude and time course to that seen with a longer stimulus train or high-K^+ depolarization (Klingauf et al., 1998). The same disparity in fluorescence loss was maintained whether the stimulation was stopped (Figure 1A) or continued (Klingauf et al., 1998), implying that the extra release elicited by prolonged stimulation did not give rise to additional differential destaining.

Exocytosis Triggered by Hypertonicity Is Associated with Rapid Vesicular Retrieval

Exposure to hypertonic solution is known as a reliable means of causing fusion of the RRP (Stevens and Tsujimoto, 1995) and acts in a Ca^{2+}-independent manner (Rosenmund and Stevens, 1996). Application of a sucrose-containing solution (800 mOsm) caused a similar degree of release of FM2-10 as the brief action potential stimulation (Figure 1B). The hypertonic stimulus was also associated with a marked difference in the degree of destaining of FM1-43 and FM2-10. This can be seen in pooled data from six experiments in which presynaptic boutons were stained by high-K^+ depolarization and then destained with hypertonic sucrose (Figure 1C). There was a consistent retention of the more slowly dissociating dye, indicating that the vesicles in the RRP had undergone rapid vesicular retrieval.

Additional experiments tested whether actively cycling vesicles not initially part of the RRP were equally capable of rapid endocytosis and differential FM destaining (Figure 1D). After applying a 15 s pulse of hypertonic sucrose to deplete the RRP, further exocytosis was evoked by high-K^+ depolarization. Although the second stimulus released the majority of remaining presynaptic vesicles, signaled by the large degree of destaining of both FM1-43 and FM2-10, the differential retention of the fluorescent indicators was barely detectable. These experiments suggest that the fast endocytosis that gave rise to differences in styryl dye destaining was largely restricted to vesicles held in the RRP.

Styryl Dyes Label the Same Fraction of Vesicles during Hypertonic Stimulation

Our interpretation of destaining experiments with different styryl dyes depended on their ability to label the same population of synaptic vesicles (Richards et al., 2000). Accordingly, we carried out staining experiments to find out whether FM1-43 and FM2-10 labeled the RRP to equal extents (Figures 1D and 1E). Presynaptic terminals were stained with either dye during hypertonic stimulation and then destained by repeated applications of high K^+ (Figure 1E). The resulting decrease in fluorescence, a measure of the hypertonicity-induced staining, was referenced to subsequent staining by high-K^+ depolarization, which efficiently labels the total recycling pool. Figure 1F shows a specific example, a bouton in which the hypertonic challenge stained 26% of the total recycling pool. In pooled data from many terminals (Figure 1E), the same proportion of the total recycling pool was stained with either FM1-43 (23.2% $\pm$ 2.4%, mean $\pm$ SEM) or FM2-10 (22.1% $\pm$ 1.7%). These data are consistent with the idea that both FM1-43 and FM2-10 were able to gain full access to the vesicles of the RRP. The similar extent of staining (Figure 1E) stands in contrast to the disparate degree of destaining with the same mode of stimulation (Figure 1C). This makes sense because FM dye equilibration during staining (Schote and Seelig, 1998; Neves and Lagnado, 1999) should be much faster than dye departitioning during destaining (Henkel and Betz, 1995; Klingauf et al., 1998).

For FM2-10, unlike FM1-43, the mean fluorescence changes induced by hypertonic stimulation were similar, whether dye was taken up (22.1% $\pm$ 1.6%) or released (24.7% $\pm$ 1.2%). Thus, both types of experiments using FM2-10 as a vesicular marker gave equivalent estimates of the relative size of the RRP. The close agreement implies that vesicles which fused under hypertonic stimulation released their content of FM2-10 almost completely. This makes FM2-10 a suitable probe for optical monitoring of exocytosis from the RRP.

Brief Stimulation Labels Vesicles by Way of Rapid Endocytosis

The preceding results suggested that there was a strong relationship between the amount of exocytosis and the mode of endocytosis. To test this idea, we carried out additional staining experiments to examine the relative contributions of fast and slow endocytosis (Figure 2). Slow endocytosis was assessed by measuring the uptake of FM1-43 when the dye remained present for 30 s after the stimulus epoch (delayed wash) and comparing it to the uptake when dye was removed immediately at the end of the stimulation (immediate wash). Figure 2A illustrates effects of a long stimulus train (30 s at 20 Hz) that liberated most of the total recycling pool. When FM1-43 was present for 30 s beyond the stimulus period, 75.5% $\pm$ 1.6% of the total recycling pool was labeled, in contrast to only 27.8% $\pm$ 1.1% labeling when dye was present during the stimulation only ($p < 0.01$). The $\sim$50% difference reflects slow endocytosis that took place within the additional 30 s of dye exposure. Delayed endocytosis after a period of stimulated exocytosis has been previously reported for stimuli that mobilize the great majority of vesicles (Ryan and Smith, 1995). These results may be compared to the consequences of a much milder stimulus (2 s at 20 Hz; Figure 2B), which would be expected to trigger exocytosis of only readily releasable vesicles (Rosenmund and Stevens, 1996). In this case, the same labeling was observed whether or not FM1-43 remained present for an extra 30 s after the cessation of the stimulus. The mean percentage of the total recycling pool stained was 20.7% $\pm$ 1.2% for the delayed wash and 19.5% $\pm$ 1.2% for the immediate wash ($p > 0.5$). The lack of difference indicated that the dye uptake was largely completed during the 2 s stimulation, with little or no additional uptake if dye exposure was extended for another 30 s. These results provided direct evidence that fusion of readily releasable vesicles was followed by fast endocytosis rather than slow vesicle recycling.

Kinetics of Repopulation of the RRP by Vesicles from the RP

If fusion of the RRP is followed immediately by rapid retrieval, one might expect the newly-retrieved vesicles

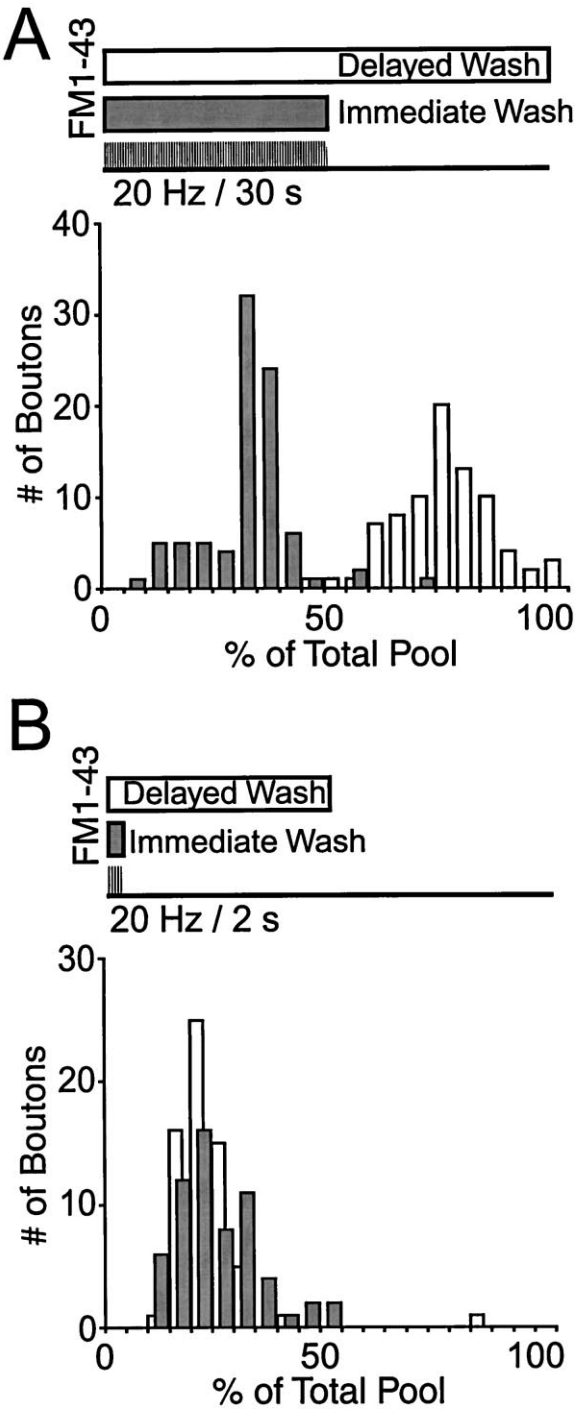

Figure 2. FM1-43 Rapidly Labels Presynaptic Terminals during the Selective Exocytosis of Readily Releasable Vesicles

(A) Presynaptic terminals were labeled by field stimulation (20 Hz, 30 s) in the presence of FM1-43. Of the total recycling pool, 75.5% ± 1.6% was labeled by 600 action potentials when the dye was allowed to remain for an additional 30s following the stimulus (delayed wash). In contrast, only 27.8% ± 1.1% of the total pool was labeled when FM1-43 was washed out immediately at the end of the stimulus (immediate wash). The large difference in staining was attributed to significant endocytosis that continued to retrieve presynaptic vesicles long after exocytosis had ceased. Percentages of the total pool size were determined by subsequent staining with high-K^+ depolarization at the end of each experiment.

(B) Presynaptic terminals labeled by FM1-43 during a stimulus intended to release only readily releasable vesicles (20 Hz, 3 s). When FM1-43 remained for 30 s following the 60 action potentials, the fraction of the total recycling pool labeled was 20.7% ± 1.2%, slightly less than the percentage obtained by labeling terminals with hypertonic stimulation (Figure 1E). When FM1-43 was immediately removed at the end of the same stimulus, 19.5% ± 1.2% of the total pool was labeled. Since the labeling of readily releasable vesicles did not increase significantly with prolongation of the exposure to FM1-43, endocytosis of RRP vesicles appears to occur primarily during the 2 s stimulation.

to hamper the repopulation of the RRP from the RP. We examined the rate of RRP repopulation by comparing the destaining responses evoked by two successive hypertonic challenges separated by a variable interval (Figure 3). Most of the experiments relied on the rapidly departitioning FM2-10; high-K^+ depolarization was used to achieve a high degree of initial staining. In contrast to the substantial loss of fluorescence produced by the first sucrose application, a second challenge following a 15 s interval elicited a much smaller fluorescence drop (Figure 3A, single bouton; Figure 3C, pooled data from 278 boutons, nine experiments). However, when the time between pulses was increased to 150 s, the second destaining response was considerably larger, approaching the size of the first (Figure 3B, single bouton; Figure 3C, pooled data from 164 boutons, six experiments). As the time interval between hypertonic challenges was systematically lengthened (Figure 3D), the second response recovered with a smooth time course that could be fitted with an exponential curve ($\tau \approx 100$ s). As we have shown that a single round of hypertonic stimulation caused near-complete loss of FM2-10 fluorescence from the RRP, we attributed the second hypertonicity-induced release of FM2-10 to the fusion of dye-containing vesicles that had moved to the RRP from the RP. The exponential time course of this repopulation agreed reasonably well with the kinetics of spontaneous vesicle undocking ($\tau \approx 140$ s; Murthy and Stevens, 1999).

The slow recovery of FM2-10 destaining suggested that following hypertonic stimulation, repopulation of the RRP with RP vesicles must await the undocking of the vesicles that have already lost their styryl dye. The next question was whether the still-docked vesicles could be reused. Our first approach used FM1-43 as a vesicular marker, since its loss from individual vesicles in response to a single round of stimulation was only partial (Figure 1C). If the same vesicles were reused, an observable loss of fluorescence would be expected even after a brief recovery interval. This prediction was tested in two-pulse experiments (Figure 3E). Following a 15 s interval, the FM1-43 destaining during the second challenge was ~77% of the size of the first response, a much larger degree of recovery than that observed for FM2-10 destaining (second response, 9% as large the first).

Recovery of the Ability to Release Neurotransmitter and Reuse of RRP Vesicles

As an independent approach to detect vesicle reuse, simultaneous recordings of postsynaptic currents and FM dye destaining were carried out (Figure 4A). After

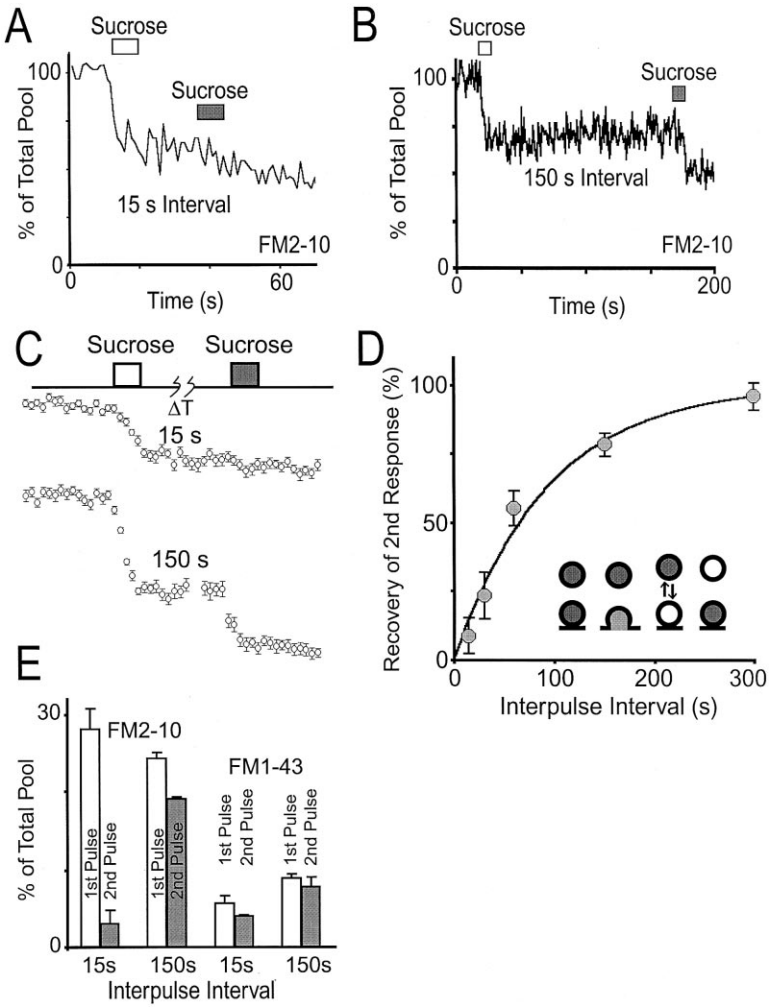

Figure 3. Fluorescence Loss during Consecutive Applications of Hypertonic Solution Measures the Rate at which the RRP Is Repopulated by Vesicles of the RP

(A) A single synaptic bouton stained with FM2-10 releases dye in response to mobilization of the RRP by hypertonic solution (800 mOsm). A second hypertonic challenge 15 s later releases significantly less FM2-10 than the first.

(B) Extending the interval between the hypertonic challenges to 150 s allows marked recovery of the bouton's ability to release FM2-10.

(C) Pooled data from many synapses stained with FM2-10 and destained by paired hypertonic challenges (n = 278 [9] for 15 s interval, n = 164 [6] for 150 s interval; mean ± SEM).

(D) Synapses stained with FM2-10 and destained by hypertonic challenge recovered their ability to release dye during a second hypertonic challenge. Percent recovery represents ratio of the fluorescence drops during the second and first responses (n = 278 [9] for 15 s, n = 79 [3] for 30 s, n = 121 [5] for 60 s, n = 164 [6] for 150 s, n = 65 [3] for 300 s). The smooth curve is a single exponential with $\tau \approx 100$ s. The inset depicts our interpretation of this recovery: (1) both reserve and readily releasable vesicles contained dye, (2) the first hypertonic challenge destained vesicles of the RRP, (3) the RRP was repopulated by exchange with dye-containing vesicles of the RP, and (4) the RRP became capable of releasing additional dye during a second hypertonic challenge.

(E) A comparison between the release of FM2-10 and FM1-43 provided evidence of vesicular reuse. During the first pulse of hypertonic solution, FM2-10 was nearly completely released while FM1-43 was not. Therefore, a second hypertonic challenge that reused the same vesicles as the first should elicit virtually no further release of FM2-10 but significant further release of FM1-43. For pairs of hypertonic challenges separated by 15 s, the ratio of second and first destaining signals was ~9% for FM2-10 and ~77% for FM1-43. With an interval of 150 s, the ratio for both dyes became more similar as vesicles of the RP repopulated the RRP.

staining hippocampal nerve terminals with FM2-10, whole-cell recordings were obtained from cell bodies of pyramidal neurons with prominent fluorescent puncta on their apical dendrites. Two pulses of hypertonic solution separated by a variable interval were delivered to a small number of dye-labeled synapses. With an interval of 15 s, FM dye destaining was clear-cut during the first stimulus but hardly detectable during the second, just as in Figure 3. However, recordings of postsynaptic membrane currents showed prominent inward current transients during both hypertonic challenges, each reflecting the release of hundreds of neurotransmitter quanta. Thus, during the second challenge, vesicular release took place in the virtual absence of any loss of FM dye fluorescence. When the interval between hypertonic stimuli was varied, the ability to release transmitter recovered with a rapid exponential time course (Figure 4B). The time constant, $\tau \approx 11$ s, was in good agreement with previous time constant estimates using a similar protocol (7–12 s; Stevens and Tsujimoto, 1995; Rosenmund and Stevens, 1996; Stevens and Wesseling, 1998). This rapid recovery stood in striking contrast to the much slower recovery of the ability to release additional FM2-10 (Figure 3E).

Evidence for Vesicular Reuse Following Exocytosis Triggered by Action Potentials

It was of considerable interest to look for similar discordance between recovery of neurotransmitter release and dye destaining when exocytosis was triggered by action potentials. Compelling evidence has been provided that several aspects of vesicular trafficking and recovery from depression are strongly Ca^{2+} dependent (Neher and Zucker, 1993; Heinemann et al., 1994; Dittman and Regehr, 1998; Wang and Kaczmarek, 1998; Wu and Borst, 1999). Figure 5 shows changes in postsynaptic currents and FM2-10 fluorescence, monitored separately during the application of the same patterns of extracellular stimulation (single 3 s bursts of stimuli at 30 Hz, or pairs of such bursts separated by a variable interval). In FM fluorescence experiments, stimuli applied through field electrodes evoked widespread destaining of fluorescent puncta within areas of study; in electrophysiological experiments, focal extracellular

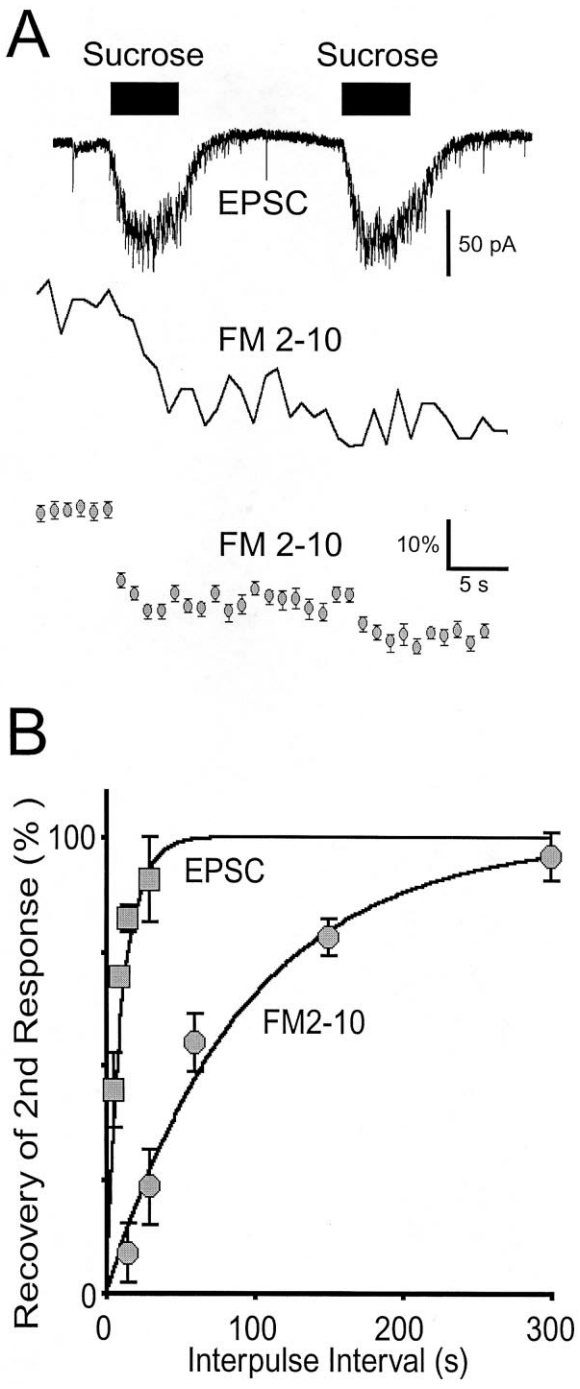

Figure 4. Vesicles of the RRP Are Capable of Multiple Release Events by Means of Rapid Endocytosis and Recovery of Release Competence

(A) Simultaneous recordings of postsynaptic whole-cell currents and FM2-10 fluorescence loss at single boutons during pairs of hypertonic challenges. The initial pulse of hypertonic solution elicited vigorous neurotransmitter release (upper trace) with a corresponding drop in FM2-10 fluorescence (lower trace). A second pulse of hypertonic solution 15 s later showed near complete recovery of neurotransmitter release; however, virtually no FM2-10 release was detected.

(B) Following depletion by hypertonic challenge, the rate at which vesicles become available to release neurotransmitter greatly exceeded the rate at which the RP repopulates the RRP. The percent reduction of neurotransmitter release was calculated as a ratio of inward current areas, with the second response over the first (5 s, n = 3; 10 s, n = 3; 15 s, n = 5; 30 s, n = 2; 60 s, n = 3; mean ± SEM). The recovery of neurotransmitter release was well fit by a single exponential ($\tau \approx 11$ s, $R^2 = 0.99$), with kinetics nearly 10-fold faster than recovery of FM2-10 fluorescence loss ($\tau \approx 100$ s, $R^2 = 0.85$). The n values for electrophysiology experiments correspond to the number of cells contributing to the pooled data.

stimulation of a single presynaptic neuron selectively activated its synaptic connections onto a nearby target cell chosen for whole-cell recording.

As the interval between the bursts of electrical stimulation was progressively lengthened, we observed a gradual recovery of the size of the second response as a percentage of the first. The time course of recovery was widely different for FM2-10 destaining (Figure 5A) and postsynaptic current (Figure 5B). With an interpulse interval of 5 s, secretion of neurotransmitter was just as great during the second stimulation as during the first, whereas the release of FM2-10 had only recovered halfway (Figure 5B). These observations were qualitatively similar to results obtained with hypertonic stimuli (Figure 4), although the time courses of recovery were quantitatively different. Recovery of EPSCs and FM dye destaining were both considerably faster in the wake of Ca^{2+}-induced secretion. When fitted with a single exponential, the time constant of recovery of neurotransmission decreased from ~11 s after Ca^{2+}-independent activity to ~1 s following spike-driven Ca^{2+} entry—a >14-fold rate increase. The time constant of recovery of FM2-10 destaining decreased in a similar manner, from ~100 s to ~7 s. Thus, the kinetics of both forms of recovery were accelerated to the same degree, leaving their relative relationship to one another essentially unchanged.

A Simple Model for RRP Reuse

We constructed a quantitative model to see if measurements of the disparate recovery of FM2-10 destaining and the recovery of the ability to release neurotransmitter could be reconciled (Figure 6). In this scheme, recovery of neurotransmission can take place by the reuse of vesicles of the RRP (γ), following their exocytosis (α) and their retrieval by rapid endocytosis (β). This reuse can be observed even in the absence of repopulation by other active vesicles, which normally takes place at a slower rate (ϵ). As in an earlier theoretical treatment (Klingauf et al., 1998), the degree of dye retention is determined by the time constant of vesicle availability for the release of styryl dye ($1/\beta$), relative to the time constants of FM2-10 or FM1-43 dissociation ($1/\delta$), which were experimentally determined (Ryan et al., 1996; Klingauf et al., 1998). In the fitting procedure, experimentally determined values were used to assign the initial pool fractions (0.25 in the RRP; 0.75 in the RP) and the redocking time constant ($\tau = 11$ s and 1 s for hypertonicity-induced and action potential–induced release, respectively). The theoretical parameters, α, β, and ϵ, were then generated by standard error minimization procedures. Excellent fits were obtained to experimental data for destaining evoked by both hypertonic challenges and action potential stimulation (smooth curves in Figure

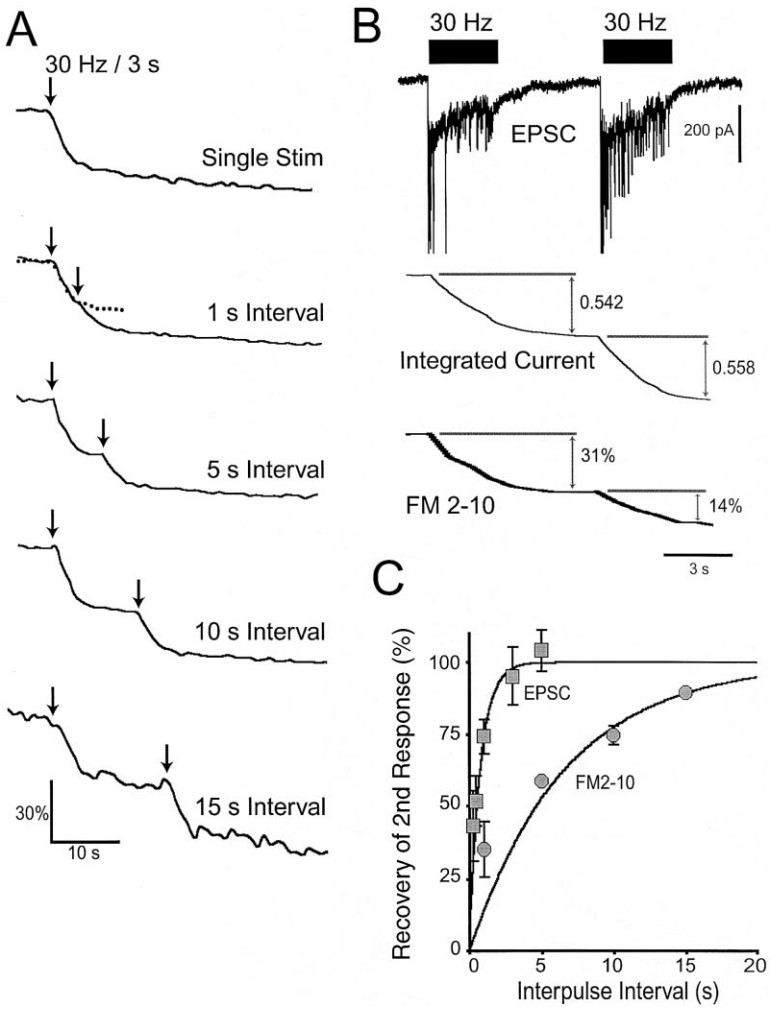

Figure 5. Vesicles of the RRP Are Capable of Multiple Release Events during Ca^{2+}-Dependent Stimulation

(A) Destaining of FM2-10 associated with bursts of activity driven by electrical stimulation (30 Hz for 3 s, onset indicated by downward arrow). A single burst, chosen to cause near-complete release of RRP vesicles, caused FM2-10 destaining corresponding to ~30% of the total recycling pool (n = 72 [3]). A second identical burst, following 1 s after the first one, gave rise to much less FM2-10 fluorescence loss (10% of the total pool, n = 82 [3]). For reference, the dotted line gives the time course of fluorescence loss during a single burst. Additional traces show the effect of increasing the interval between the bursts of stimuli. Expressed as a percentage of the total pool, the release of FM2-10 during a second stimulus recovers to 13% after 5 s (n = 123 [5]), 18% at 10 s (n = 131 [5]), and 27% at 15 s (n = 122 [5]).

(B) Comparison between the recovery of postsynaptic whole-cell currents and FM2-10 fluorescence loss during the interval between bursts of electrical stimulation. Ca^{2+}-dependent recovery of the ability to support neurotransmitter release was monitored by recording the postsynaptic response in a follower cell while focally applying bursts of extracellular stimuli to the cell body of a nearby neuron. The initial volley of action potentials elicited vigorous neurotransmitter release (EPSC; upper trace). As seen in the running integral of current signal (middle trace), the initial response (0.542 pC) was closely matched by the second response (0.558 pC). In contrast, the corresponding drop in FM2-10 fluorescence (lower trace) was 31% of the total recycling pool for the first stimulus but only 14% for the second.

(C) Following depletion by electrical stimulation, the rate of recovery of the availability of vesicles to release neurotransmitter greatly exceeded the rate of repopulation of RRP from the RP. The percent recovery of neurotransmitter release was calculated as the ratio of integrated currents from the second and first responses (for intervals of 250 ms, n = 2; 500 ms, n = 3; 1 s, n = 5; 3 s, n = 5; 5 s, n = 3; 10 s, n = 3; n values indicate the number of cells contributing to the pooled electrophysiological data). The percent recovery of FM2-10 destaining was obtained from traces where double challenges were applied by calculating the ratio of the second and first fluorescence changes, with appropriate correction for the decline in FM2-10 fluorescence that would have occurred after only a single challenge (Figure 5A; for intervals of 1 s, n = 82 [3]; 5 s, n = 123 [5]; 10 s, n = 131 [5]; 15 s, n = 122 [5]. The recovery of neurotransmitter release, fit by a single exponential ($\tau \approx 1$ s, $R^2 = 0.96$), was considerably more rapid than the recovery seen in Ca^{2+}-independent conditions.

6B). The only difference between the theoretical curves for FM1-43 and FM2-10 was the empirically determined rate of dye departitioning, δ. The best fits yielded independent estimates of $1/\beta$ as a measure of the rapidity of endocytotic retrieval. The estimated $1/\beta$ was close to 1.1 s for both hypertonic and action potential stimulation.

We used the parameters generated by these fits to predict how the release of FM2-10 and neurotransmitter would respond to a second hypertonic challenge, delivered 15 s after a prior conditioning stimulus (see Figure 4 for experimental results). The model was successful in reconstructing the experimental observation without additional adjustable parameters (Figure 6C): only a small amount of simulated destaining occurred during the second stimulation, even though the amount of exocytosis recovered to ~85% of its original size.

The estimated rate of endocytosis (0.9 s^{-1}) was far slower than the time constant for dye equilibration at the loading concentration of FM1-43 (8 μM) (Henkel and Betz, 1995; Schote and Seelig, 1998; Neves and Lagnado, 1999). Thus, the model was also consistent with experimental results showing that stimulation of readily releasable vesicles led to virtually complete FM1-43 uptake within 2 s (Figure 2B).

Discussion

Rapid Retrieval and Reuse of Vesicles in the RRP

Our experiments revealed unexpected distinctions between the recycling properties of vesicles that had been held in the RRP and vesicles newly recruited from the RP. Two findings indicated that RRP vesicles were preferentially retrieved by rapid endocytosis ($\tau \approx 1$ s): their strikingly different retention of slowly and rapidly departitioning FM dyes (Figure 1) and their narrowly de-

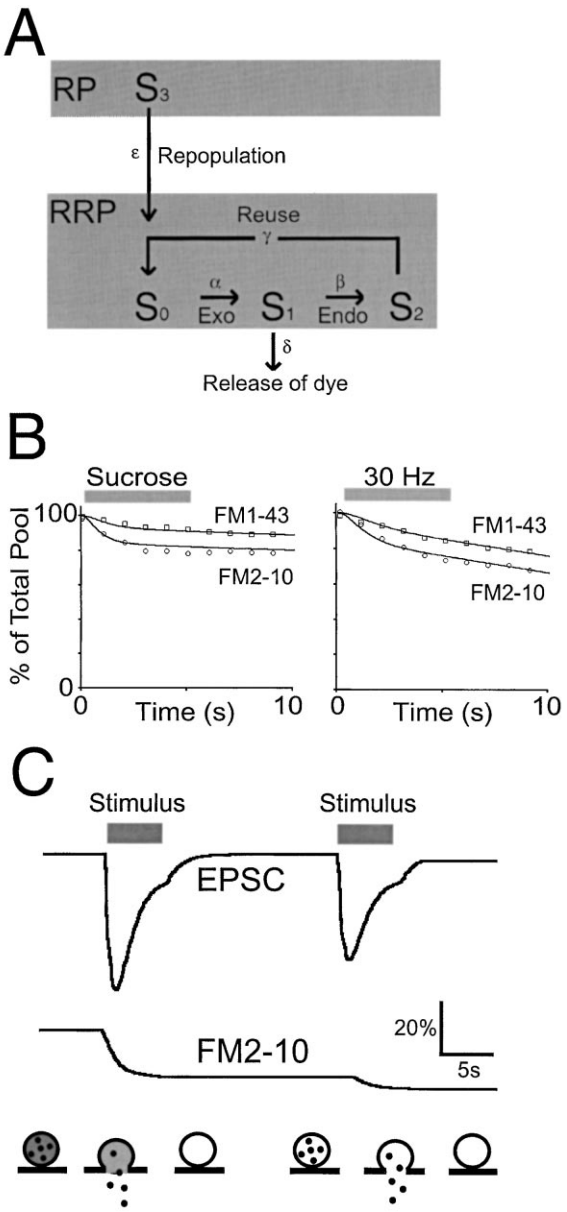

Figure 6. A Simple Kinetic Model Describes the Differences between the Release of Neurotransmitter and the Release of FM Dyes

(A) Topology of a kinetic model that incorporates reuse of the RRP. The RRP can be replenished either by reuse of vesicles (γ) following exocytosis (α) and rapid retrieval (β) or by repopulation by vesicles of the RP (ϵ). States following the exocytosis of vesicles (S_1 and S_2) were initially assigned zero content. Experiments that stained or destained the RRP with styryl dyes (Figure 1) set the fractional content of the RRP relative to the total pool as 0.25 ($= S_0/[S_0 + S_3]$). The rate of RRP reuse (γ) was assigned on the basis of recovery of the capability of neurotransmission as determined electrophysiologically (0.091 s^{-1} with a preceding hypertonic challenge, 1 s^{-1} with preceding action potentials; Figures 4 and 5). The departitioning rates of FM dyes from surface membranes of neuronal cells (δ) were empirically determined as 0.38 s^{-1} (FM1-43) and 1.67 s^{-1} (FM2-10) (Klingauf et al., 1998).

(B) Destaining experiments with different styryl dyes (Figure 1) were used to constrain values for three rate constants: exocytosis (α), endocytosis (β), and repopulation (ϵ). The fluorescence signal was given by $S_0 + S_1 + S_2 + S_3$. Good fits were obtained for kinetics of destaining elicited by hypertonic stimulation (rates in s^{-1} during stimulus pulse: $\alpha = 1.79$, $\beta = 0.92$, $\epsilon = 0.00011$) or bursts of action potentials ($\alpha = 1.45$, $\beta = 0.94$, $\epsilon = 0.135$). Similar rates for β were obtained for both methods of stimulation (but see Ales et al., 1999). The curve-fitting procedure yielded a low estimate of the repopulation rate of the RRP (ϵ) upon destaining with hypertonic sucrose.

(C) Simplified modeling of a two-pulse experiment in which neurotransmitter release and loss of styryl dye were simultaneously measured during two hypertonic challenges (Figure 4). Whereas the fraction of vesicles undergoing exocytosis during the second stimulation relative to the first was 80%, the fraction of dye released in the second stimulation relative to the first was 20% (cf. Figure 4A). For this simulation, the model parameters were constrained as in (A) and (B), without further adjustment. To simulate ideal vesicular filling and release of neurotransmitter, the EPSC signal was calculated as the instantaneous flux from S_0 to S_1 with the same set of parameter values for all rates except δ, which was set at zero. The two-pulse experiments and simulation were consistent with the scenario depicted at the bottom of the panel: (1) prior to the onset of the first stimulus, vesicles of the RRP contained both neurotransmitter and dye; (2) during the first stimulation, both NT and dye were released; (3) vesicles were rapidly retrieved following exocytosis; (4) vesicles became available for reuse following refilling with NT; and (5) the second stimulation caused reuse of vesicles that had been replenished with NT but had lost their content of FM dye during the previous round of exocytosis.

fined time window for FM dye uptake (Figure 2). In contrast to vesicles resident in the RRP, vesicles freshly mobilized from the RP were recycled by conventional slow endocytosis ($\tau \approx 30$ s).

The functional implications of rapid retrieval were clarified by recording FM2-10 destaining and postsynaptic currents. Because FM2-10 dissociates rapidly from neuronal membranes ($\tau \approx 0.6$ s; Ryan et al. 1996; Klingauf et al., 1998), it served as a one-time marker, akin to a radioactive tracer. This was essential for revealing vesicular reuse, as near-complete destaining minimized the confounding effects of repeated fusion of individual vesicles. Repeated neurotransmission by RRP vesicles was uncovered by the application of pairs of secretory stimuli, either hypertonic challenges (Figure 4) or short trains of electrical stimulation (Figure 5). The first challenge evoked a crisp drop in FM2-10 fluorescence along with a burst of postsynaptic events, indicating fusion of vesicles containing both FM dye and glutamate. In contrast, an appropriately delayed second challenge caused significant release of glutamate with little or no loss of FM2-10 fluorescence, reflecting the exocytosis of vesicles containing transmitter but not dye. Multiple lines of evidence (next paragraph) indicated that these vesicles were the same as those that had previously destained during the first stimulus. This led us to conclude that the second exocytotic response arose from vesicles that had recently released neurotransmitter and dye and had undergone rapid retrieval and repeated neurotransmission—what we have termed "reuse."

The second round of transmitter release could not be attributed to a fresh group of vesicles that had never filled with FM dye for three reasons. First, staining was performed by depolarization with K^+-rich solution, the most powerful protocol available for loading the entire pool of recycling vesicles; high-K^+ depolarization was more effective than either action potentials (Figure 2A) or hypertonic challenges (Figure 1E) in allowing dye access to vesicular pools. Second, long washout periods

were used to allow ample time for random mixing of stained synaptic vesicles so that any residually unstained vesicles would be evenly distributed among the general population (Murthy and Stevens, 1999; Harata and Tsien, 1999, Soc. Neurosci., abstract) and thus incapable of introducing systematic biases in the tracking of exocytosis. Third, vesicular labeling with FM1-43 demonstrated that the second trial in two-pulse experiments was highly effective in producing FM dye destaining (Figure 3E). FM1-43-labeled vesicles remained partially stained after the first trial and thus retained the ability to display a relatively large fluorescence loss after a second challenge ($\sim$77% as large as the first destaining).

The notion of vesicular reuse is a significant addition to the prevailing picture of vesicular turnover at central synapses, which owes much to the pioneering work of Heuser, Reese, and colleagues (e.g., Heuser et al., 1974). Here, we introduce a distinction between reuse and "repopulation" and avoid the ambiguous term "refilling." Repopulation of the RRP occurs by recruitment of vesicles from the RP and is often portrayed as the terminal stage of a continuous loop that begins with vesicular exocytosis by full fusional collapse, slow endocytosis, and recycling (Jahn and Südhof, 1994; De Camilli, 1995). This route may or may not involve membrane trafficking through an endosome-like compartment (Takei et al., 1996; Murthy and Stevens, 1998). We propose that repopulation of docked vesicles can be supplemented by rapid vesicular retrieval in readiness for reuse. Rapid retrieval can be thought of as a shunt pathway within the longer loop of conventional vesicle trafficking that allows efficient reutilization of the RRP.

Compatibility with Earlier Kinetic Data at Hippocampal Synapses

Our revised view of vesicle recycling is based on several novel experimental findings but is fully compatible with previous kinetic studies at hippocampal synapses, for which literature comparisons are most pertinent (Ryan et al., 1993; Liu and Tsien, 1995; Ryan and Smith, 1995; Stevens and Tsujimoto, 1995; Rosenmund and Stevens, 1996; Klingauf et al., 1998; Stevens and Wesseling, 1998, 1999). Several puzzling aspects of the earlier data can now be interpreted.

In the first report of the different destaining kinetics of FM1-43 and FM2-10 (Klingauf et al., 1998), rapid vesicular retrieval was depicted as a homogeneous feature of the entire recycling pool in the absence of evidence to the contrary. To fit the experimental data, rapid endocytosis was modeled with $\tau \approx 6$ s, which is much slower than other estimates, and the proportion of vesicles in the RRP was set at 60%, which is considerably higher than in other secretory systems. These features of the model can now be revised in light of new observations. First, rapid endocytosis was largely restricted to the RRP and was not detected upon additional recruitment of reserve vesicles (Figure 1D). Second, endocytosis of the RRP was almost exclusively rapid, with no detectable increment in retrieval over an additional 30 s (Figure 2B). Thus, the differential retention of the rapidly and slowly departitioning dyes can now be ascribed to the specific and near-uniform behavior of a minority fraction of vesicles. In a revised but still oversimplified model (Figure 6), the time constant of rapid retrieval was estimated as $\sim$1.1 s, which is within the range of values of 1–2 s found in pituitary melanotrophs (Thomas et al., 1994), chromaffin cells (Artalejo et al., 1995; Smith and Betz, 1996; Smith and Neher, 1997; Engisch and Nowycky, 1998), and bipolar nerve terminals (von Gersdorff and Matthews, 1994; Neves and Lagnado, 1999). More sophisticated simulations are underway to track vesicles as discrete units and to allow for escape of vesicles from the RRP and restocking of the RP (Pyle and Tsien, 2000, Juan March Meeting, abstract).

The proposed combination of fast recycling of vesicles within the RRP and slow exchange of vesicles between the RRP and the RP (Figure 3D) may help explain discrepancies in measured rates of vesicular recovery following exocytosis. Following selective depletion of the RRP, electrophysiological responsiveness recovers with $\tau \approx 10$ s under Ca^{2+}-independent conditions (Figure 4; Stevens and Tsujimoto, 1995) and $\tau \approx 1$–3 s in Ca^{2+}-dependent conditions (Figure 5; Stevens and Wesseling, 1998). But recovery was much slower ($t_{1/2} \approx$ 20–30 s) following near-complete depletion of the total recycling pool (Ryan et al., 1993; Liu and Tsien, 1995; Stevens and Wesseling, 1999). Systematic increases in the extent of exocytosis engendered a progressive slowing of vesicular recovery, described as the sum of two exponential recovery functions ($\tau_{fast} = 10$ s, $\tau_{slow} = 60$ s; Stevens and Wesseling, 1999). The greater the evoked release, the further the balance was tilted toward the slow exponential; such kinetic behavior was inconsistent with a simple two-pool model. Our results supported the two-component analysis and suggested that the fast component originated from vesicular reuse and the slow component arose from repopulation of the RRP. The gradual growth of the slow component suggested that rapid retrieval was neither all encompassing nor perfectly efficient. As more vesicles fuse, the RRP would suffer a gradual loss of vesicles to a slower endocytotic process, thus leaving room for repopulation from the RP.

The slow recovery of the FM2-10 destaining signal (Figure 3) provided a way to determine the rate of exchange between vesicles in the RP and the RRP under resting conditions. The approach was complementary to that of Murthy and Stevens (1999). Because the RP was prelabeled with FM2-10 in our experiments, there were no restrictions on waiting times to allow washout of extracellular dye and reduction of background staining. We found that recovery of the FM2-10 destaining signal followed an exponential time course with $\tau \approx 100$ s, not remarkably different than Murthy and Stevens' estimate of undocking rate ($\tau \approx 141$ s).

Cell Biological and Molecular Implications

The capability to undergo fast endocytosis was not distributed evenly across the entire set of vesicles but seemed to be limited to the RRP (Figures 1 and 2). This provided hints about the possible mechanism of fast endocytosis and its relationship to fusion. Our working hypothesis is that rapid endocytosis was restricted to vesicles in the RRP because it required a state of readiness: only vesicles docked for a sufficient time can undergo rapid endocytosis and reuse. For example, fast

retrieval might require a high-order organization of SNARE complexes or recruitment of additional protein components to already assembled SNARE core complexes. Vesicles that lacked such preparation could undergo a single round of exocytosis, but full membrane-to-membrane collapse would not be prevented.

Rapid reuse of RRP vesicles would carry interesting implications for the cell biology of transmitter reaccumulation. The time constant of recovery must encompass vesicular retrieval, reacidification, and transport of neurotransmitter by proton and membrane potential–dependent mechanisms. Endocytosis and recovery is fast according to our data ($\tau \approx 1$ s). Reacidification is also rapid, based on studies of vesicles containing a pH-sensitive green fluorescent protein (GFP) reporter (Miesenbock et al., 1998; Sankaranarayanan and Ryan, 2000). Neurotransmitter uptake shows extraordinary capabilities (Zhou et al., 2000); quantal size can be maintained despite continuous and intense synaptic stimulation (4000 pulses at 50 Hz). A clearer understanding of transmitter reaccumulation by vesicles may be gained once molecular clones of the transport proteins and functional assays become generally available.

Functional Implications for Central Synapses

The rapid reuse of RRP vesicles would offer significant functional advantages for small nerve terminals with a limited number of recycling vesicles. The kinetics of recovery that support reuse give it a 4- to 6-fold advantage in speed over replenishment of the RRP by repopulation from the RP. Reuse has the effect of sharply increasing the number of quantal events that the nerve terminal can support over a period of heightened impulse activity, expanding this well beyond the actual number of recycling vesicles. Indeed, previous measures of pool size that relied on intermittent K^+ depolarization (Liu and Tsien, 1995) should be interpreted as functional estimates of the maximal number of quantal events during repetitive activity rather than a census of the active vesicle pool.

The impact of reuse in expanding the capabilities of the vesicle pool may be greatest when the presynaptic terminal is subjected to bursts of activity separated by intervals of relative quiescence, as often occurs during physiological neurotransmission. Under such conditions, a small number of rapidly recycling vesicles in the RRP may be adequate to meet the pulsatile demand for transmitter release. Vesicles of the RP would remain largely unused during short episodes of rapid firing separated by pauses of several seconds. RP vesicles would stay available to augment the RRP when secretory requirements exceeded the RRP's capabilities. However, because the capability of rapid retrieval was restricted to the RRP (Figure 1D), neurotransmission by newly recruited reserve vesicles seems to involve complete fusion with the plasma membrane and to incur the additional burden of classic endocytosis and recycling.

In conclusion, our experiments may resolve an apparent paradox first pointed out by Betz et al. (1996): central nerve terminals that could in principle capitalize on existing mechanisms for speedy retrieval and vesicle recycling did not appear to use them. On the contrary, our results suggest that rapid endocytosis is not only well expressed in hippocampal presynaptic terminals but leads directly to vesicular reuse, an efficient strategy for maintaining secretory competence during brief periods of high demand.

Experimental Procedures

Cell Cultures

Hippocampal neurons of 1-day-old Sprague-Dawley rats were prepared in sparse culture according to previous protocols (Liu and Tsien, 1995), with minor modifications. Cultures were used after 10–14 days in vitro.

Dye Loading and Destaining

Presynaptic terminals were labeled by exposure to styryl dye (8 μM FM1-43 or 400 μM FM2-10) during high-K^+ depolarization (modified Tyrode, 45 mM KCl), by field stimulation (platinum bath electrodes delivering 30 mA/1 ms/pulse), or by hypertonic challenge (modified Tyrode, 500 mM sucrose). Dye was allowed to remain in the extracellular solution for 30 s following a staining protocol, except for the experiments shown in Figure 2, in which field stimulation was followed by an immediate wash, accomplished by the brisk perfusion of 10 volumes of bathing solution (2 ml/s for 2.5 s) at the conclusion of the stimulation, followed by a constant wash (2 ml/min). All staining and washing protocols were performed with modified Tyrode containing 10 μM CNQX to prevent recurrent activity. Images were taken after 10–15 min washes in dye-free solution. Destaining of hippocampal terminals with hypertonic challenge was achieved by direct perfusion of modified Tyrode (500 mM sucrose added) onto the field of interest with either gravity-fed perfusion or by use of a picospritzer (General Valve Corporation). Fluorescence images were obtained by a cooled, intensified CCD camera (Stanford Photonics) during repetitive arc lamp illumination (480 nm) via an optical switch (Solamere Technology Group). Images were digitized and processed with Image Lightning and Imaging Workbench (Axon Instruments) and later were analyzed with custom software.

Electrophysiology

Postsynaptic responses of hippocampal synapses to electrically evoked action potentials and picospritzer-delivered pulses of modified Tyrode (500 mM sucrose) were obtained by whole-cell recording using an Axopatch 200B amplifier with Clampex 8.0 software (Axon Instruments). Patch pipettes and stimulating pipettes were pulled from borosilicate glass to a tip diameter corresponding to a resistance of 2–4 MΩ. Internal solution included 135 mM Cs gluconate, 10 mM EGTA, 2 mM $MgCl_2$, 5 mM Mg-ATP, and 10 mM HEPES (pH 7.35). Series resistance and cell capacitance were compensated and the data were filtered at 5 kHz by an 8-pole Bessel filter.

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References

Ales, E., Tabares, L., Poyato, J.M., Valero, V., Lindau, M., and Alvarez de Toledo, G. (1999). High calcium concentrations shift the mode of exocytosis to the kiss-and-run mechanism. Nat. Cell Biol. *1*, 40–44.

Artalejo, C.R., Henley, J.R., McNiven, M.A., and Palfrey, H.C. (1995). Rapid endocytosis coupled to exocytosis in adrenal chromaffin cells involves Ca^{2+}, GTP, and dynamin but not clathrin. Proc. Natl. Acad. Sci. USA *92*, 8328–8332.

Betz, W.J., Mao, F., and Smith, C.B. (1996). Imaging exocytosis and endocytosis. Curr. Opin. Neurobiol. *6*, 365–371.

Ceccarelli, B., and Hurlbut, W.P. (1980). Ca^{2+}-dependent recycling

of synaptic vesicles at the frog neuromuscular junction. J. Cell Biol. *87*, 297–303.

Ceccarelli, B., Hurlbut, W.P., and Mauro, A. (1973). Turnover of transmitter and synaptic vesicles at the frog neuromuscular junction. J. Cell Biol. *57*, 499–524.

De Camilli, P. (1995). The eighth Datta Lecture. Molecular mechanisms in synaptic vesicle recycling. FEBS Lett. *369*, 3–12.

Dittman, J.S., and Regehr, W.G. (1998). Calcium dependence and recovery kinetics of presynaptic depression at the climbing fiber to Purkinje cell synapse. J. Neurosci. *18*, 6147–6162.

Dobrunz, L.E., and Stevens, C.F. (1997). Heterogeneity of release probability, facilitation, and depletion at central synapses. Neuron *18*, 995–1008.

Engisch, K.L., and Nowycky, M. (1998). Compensatory and excess retrieval: two types of endocytosis following single step depolarizations in bovine adrenal chromaffin cells. J. Physiol. *506*, 591–608.

Harris, K.M., and Sultan, P. (1995). Variation in the number, location and size of synaptic vesicles provides an anatomical basis for the nonuniform probability of release at hippocampal CA1 synapses. Neuropharmacology *34*, 1387–1395.

Heinemann, C., Chow, R.H., Neher, E., and Zucker, R.S. (1994). Kinetics of the secretory response in bovine chromaffin cells following flash photolysis of caged Ca^{2+}. Biophys. J. *67*, 2546–2557.

Henkel, A.W., and Betz, W.J. (1995). Staurosporine blocks evoked release of FM1-43 but not acetylcholine from frog motor nerve terminals. J. Neurosci. *15*, 8246–8258.

Heuser, J.E., and Reese, T.S. (1973). Evidence for recycling of synaptic vesicle membrane during transmitter release at the frog neuromuscular junction. J. Cell Biol. *57*, 315–344.

Heuser, J.E., Reese, T.S., and Landis, D.M. (1974). Functional changes in frog neuromuscular junctions studied with freeze-fracture. J. Neurocytol. *3*, 109–131.

Jahn, R., and Südhof, T.C. (1994). Synaptic vesicles and exocytosis. Annu. Rev. Neurosci. *17*, 219–246.

Kavalali, E.T., Klingauf, J., and Tsien, R.W. (1999). Properties of fast endocytosis at hippocampal synapses. Philos. Trans. R. Soc. Lond. B Biol. Sci. *354*, 337–346.

Klingauf, J., Kavalali, E.T., and Tsien, R.W. (1998). Kinetics and regulation of fast endocytosis at hippocampal synapses. Nature *394*, 581–585.

Liu, G., and Tsien, R.W. (1995). Properties of synaptic transmission at single hippocampal synaptic boutons. Nature *375*, 404–408.

Miesenböck, G., DeAngelis, D.A., and Rothman, J.E. (1998). Visualizing secretion and synaptic transmission with pH-sensitive green fluorescent proteins. Nature *394*, 192–195.

Murthy, V.N., and Stevens, C.F. (1998). Synaptic vesicles retain their identity through the endocytotic cycle. Nature *392*, 497–501.

Murthy, V.N., and Stevens, C.F. (1999). Reversal of synaptic vesicle docking at central synapses. Nat. Neurosci. *2*, 503–507.

Neher, E. (1993). Cell physiology. Secretion without full fusion. Nature *363*, 497–498.

Neher, E., and Zucker, R.S. (1993). Multiple calcium-dependent processes related to secretion in bovine chromaffin cells. Neuron *10*, 21–30.

Neves, G., and Lagnado, L. (1999). The kinetics of exocytosis and endocytosis in the synaptic terminal of goldfish retinal bipolar cells. J. Physiol. *515*, 181–202.

Richards, D.A., Guatimosim, C., and Betz, W.J. (2000). Two endocytic recycling routes selectively fill two vesicle pools in frog motor nerve terminals. Neuron *27*, 551–559.

Rosenmund, C., and Stevens, C.F. (1996). Definition of the readily releasable pool of vesicles at hippocampal synapses. Neuron *16*, 1197–1207.

Ryan, T.A., and Smith, S.J. (1995). Vesicle pool mobilization during action potential firing at hippocampal synapses. Neuron *14*, 983–989.

Ryan, T.A., Reuter, H., Wendland, B., Schweizer, F.E., Tsien, R.W., and Smith, S.J. (1993). The kinetics of synaptic vesicle recycling measured at single presynaptic boutons. Neuron *11*, 713–724.

Ryan, T.A., Smith, S.J., and Reuter, H. (1996). The timing of synaptic vesicle endocytosis. Proc. Natl. Acad. Sci. USA *93*, 5567–5571.

Sankaranarayanan, S., and Ryan, T.A. (2000). Real-time measurements of vesicle-SNARE recycling in synapses of the central nervous system. Nat. Cell Biol. *2*, 197–204.

Schikorski, T., and Stevens, C.F. (1997). Quantitative ultrastructural analysis of hippocampal excitatory synapses. J. Neurosci. *17*, 5858–5867.

Schneggenburger, R., Meyer, A.C., and Neher, E. (1999). Released fraction and total size of a pool of immediately available transmitter quanta at a calyx synapse. Neuron *23*, 399–409.

Schote, U., and Seelig, J. (1998). Interaction of the neuronal marker dye FM1-43 with lipid membranes. Thermodynamics and lipid ordering. Biochim. Biophys. Acta *1415*, 135–146.

Smith, C.B., and Betz, W.J. (1996). Simultaneous independent measurement of endocytosis and exocytosis. Nature *380*, 531–534.

Smith, C., and Neher, E. (1997). Multiple forms of endocytosis in bovine adrenal chromaffin cells. J. Cell Biol. *139*, 885–894.

Stevens, C.F., and Tsujimoto, T. (1995). Estimates for the pool size of releasable quanta at a single central synapse and for the time required to refill the pool. Proc. Natl. Acad. Sci. USA *92*, 846–849.

Stevens, C.F., and Wesseling, J.F. (1998). Activity-dependent modulation of the rate at which synaptic vesicles become available to undergo exocytosis. Neuron *21*, 415–424.

Stevens, C.F., and Wesseling, J.F. (1999). Identification of a novel process limiting the rate of synaptic vesicle cycling at hippocampal synapses. Neuron *24*, 1017–1028.

Südhof, T.C. (1995). The synaptic vesicle cycle: a cascade of protein-protein interactions. Nature *375*, 645–653.

Takei, K., Mundigl, O., Daniell, L., and De Camilli, P. (1996). The synaptic vesicle cycle: a single vesicle budding step involving clathrin and dynamin. J. Cell Biol. *133*, 1237–1250.

Thomas, P., Lee, A.K., Wong, J.G., and Almers, W. (1994). A triggered mechanism retrieves membrane in seconds after Ca^{2+}-stimulated exocytosis in single pituitary cells. J. Cell Biol. *124*, 667–675.

von Gersdorff, H., and Matthews, G. (1994). Inhibition of endocytosis by elevated internal calcium in a synaptic terminal. Nature *370*, 652–655.

von Schwarzenfeld, I. (1979). Origin of transmitters released by electrical stimulation from a small metabolically very active vesicular pool of cholinergic synapses in guinea-pig cerebral cortex. Neuroscience *4*, 477–493.

Wang, L.Y., and Kaczmarek, L.K. (1998). High-frequency firing helps replenish the readily releasable pool of synaptic vesicles. Nature *394*, 384–388.

Wu, L.G., and Borst, G.G. (1999). The reduced release probability of releasable vesicles during recovery from short-term depression. Neuron *23*, 821–832.

Zimmermann, H., Volknandt, W., Wittich, B., and Hausinger, A. (1993). Synaptic vesicle life cycle and synaptic turnover. J. Physiol. (Paris) *87*, 159–170.