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The Coupling between Synaptic Vesicles and Ca^{2+} Channels Determines Fast Neurotransmitter Release

Kristian Wadel,[1] Erwin Neher,[2] and Takeshi Sakaba[1,*]
[1] Research Group Biophysics of Synaptic Transmission, Max Planck Institute for Biophysical Chemistry, Göttingen, 37077, Germany
[2] Department of Membrane Biophysics, Max Planck Institute for Biophysical Chemistry, Göttingen, 37077, Germany
*Correspondence: tsakaba@gwdg.de
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SUMMARY

In order to release neurotransmitter synchronously in response to a presynaptic action potential, synaptic vesicles must be both release competent and located close to presynaptic Ca^{2+} channels. It has not been shown, however, which of the two is the more decisive factor. We tested this issue at the calyx of Held synapse by combining Ca^{2+} uncaging and electrophysiological measurements of postsynaptic responses. After depletion of the synaptic vesicles that are responsible for synchronous release during action potentials, uniform elevation of intracellular Ca^{2+} by Ca^{2+} uncaging could still elicit rapid release. The Ca^{2+} sensitivity of remaining vesicles was reduced no more than 2-fold, which is insufficient to explain the slow-down of the kinetics of release (10-fold) observed during a depolarizing pulse. We conclude that recruitment of synaptic vesicles to sites where Ca^{2+} channels cluster, rather than fusion competence, is a limiting step for rapid neurotransmitter release in response to presynaptic action potentials.

INTRODUCTION

Neurotransmitter is released within a millisecond in response to presynaptic action potentials (APs; Borst and Sakmann, 1996). The number of synaptic vesicles available for rapid release in response to APs determines the amplitude of postsynaptic responses (Zucker and Regehr, 2002; Stevens, 2003). If the releasable vesicles are depleted during a high-frequency train of presynaptic APs, synaptic responses will be depressed. On the contrary, if a large fraction of releasable vesicles still remains after a first AP, synaptic responses to subsequent ones will be facilitated due to accumulation of residual Ca^{2+} in the presynaptic terminal (Katz, 1969). The mechanisms regulating these forms of short-term synaptic plasticity, however, are under debate.

Recent studies on a number of glutamatergic synapses have demonstrated the existence of "reluctant" or slowly-releasing vesicles (Wu and Borst, 1999; Hallermann et al., 2003; Moulder and Mennerick, 2005). Typically, large postsynaptic responses can be elicited by strong stimuli (depolarizations of tens of milliseconds or tetanic stimulation) at a time when EPSCs in response to single APs are strongly depressed. At the calyx of Held, a glutamatergic synapse in the auditory pathway, the number of vesicles released during such strong depolarizations is about equal to the cumulative number of vesicles released during short, depressing trains of APs (Schneggenburger et al., 2002). We address the question of why these reluctant vesicles cannot be released by APs. Several alternatives can be considered as an explanation. First, they may not be fully release competent and may reach the "primed" state only during prolonged depolarization or during a sustained rise in presynaptic $[Ca^{2+}]$. Incomplete assembly of functional SNARE complexes or weak coupling of such complexes to the Ca^{2+} sensors may be the underlying molecular mechanism(s).

Second, APs elevate $[Ca^{2+}]$ only in microdomains surrounding Ca^{2+} channel clusters (Roberts et al., 1990; Adler et al., 1991; Llinas et al., 1992). They may be unable to trigger release of vesicles outside of these microdomains, such that reluctant vesicles are activated only during sustained depolarization, when the $[Ca^{2+}]$ is globally elevated.

Third, presynaptic Ca^{2+} influx may be decreased during repetitive stimulation. This last possibility can be excluded for the case of the calyx of Held, since measurements of Ca^{2+} currents during high-frequency trains, which lead to synaptic depression, show Ca^{2+} current facilitation, rather than inactivation (von Gersdorff and Borst, 2002). Also, in the presence of botulinum neurotoxin A, which decreases Ca^{2+} sensitivity of the release apparatus, a strong facilitation of release is observed during high-frequency trains (Sakaba et al., 2005), signaling a buildup of $[Ca^{2+}]$ at the release sites. We test the remaining two alternative possibilities at the calyx of Held in the present study, taking advantage of the unique experimental tools available (Borst and Sakmann, 1996; Takahashi et al., 1996).

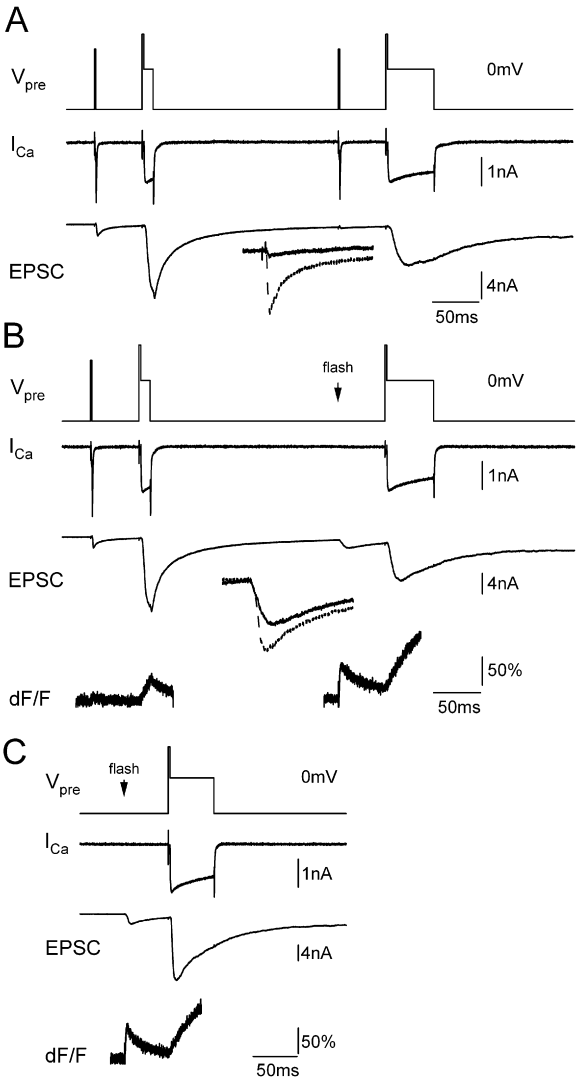

Figure 1. A Prepulse, which Depletes the Rapidly-Releasing Vesicles, Blocks Responses Evoked by Short Depolarizing Pulses but Leaves Flash-Evoked Responses Intact

(A) The presynaptic and postsynaptic compartments were whole-cell clamped at −80 mV. Traces shown are from top to bottom: presynaptic membrane potential, presynaptic current, and postsynaptic currents. An AP-like stimulus (depolarization to +40 mV for 1.5 ms) was applied to the presynaptic terminal, which was followed by a step depolarization (to 0 mV for 10 ms following the prepulse to +70 mV for 2 ms) with an intention of depleting the rapidly-releasing vesicles. After an interval of 200 ms, a second AP-like stimulus was applied, followed by a second step depolarization (to 0 mV for 50 ms following a short episode at +70 mV for 2 ms) to release all the releasable vesicles. The inset shows EPSCs evoked by the first (dotted trace) and the second (solid trace) AP-like stimulations.

(B) An experiment similar to that of (A), but laser photolysis was applied instead of the second AP-like stimulus. At the time point indicated by an arrow, a laser flash was applied to the cells, photolyzing DM-Nitrophen and eliciting a transient increase in presynaptic $[Ca^{2+}]$ (shown in dF/F, the fourth trace from the top). The same cell pair as shown in (A) and inset shows the EPSC evoked by flash in control (dotted trace) and after depletion of the rapidly-releasing vesicles (solid trace).

Comparing release induced by a depolarization with that elicited by spatially uniform $[Ca^{2+}]$ elevation after caged Ca^{2+} photolysis, we will show that the coupling between Ca^{2+} channels and synaptic vesicles is most critical for rapid release of synaptic vesicles.

RESULTS

Intrinsic Ca^{2+} Sensitivity of Synaptic Vesicles Is High at Times when AP-Evoked Release Is Depressed

The calyx of Held, a large synapse in the auditory brainstem of mammals, allows one to voltage-clamp both pre- and postsynaptic compartments (Borst and Sakmann, 1996; Takahashi et al., 1996) and to measure the intrinsic kinetics of vesicle fusion using Ca^{2+} uncaging (Bollmann et al., 2000; Schneggenburger and Neher, 2000). When a calyx of Held synapse is stimulated at a high frequency, it undergoes rapid depression, which has a strong presynaptic component (von Gersdorff and Borst, 2002; Schneggenburger et al., 2002). Wu and Borst (1999) showed that the synapse is still able to produce a large EPSC in the depressed state when stimulated by a 50 ms depolarization.

As a first step toward characterization of the Ca^{2+} dependence of "depressed" vesicles, we performed an experiment similar to that of Wu and Borst (1999). Presynaptic and postsynaptic compartments were simultaneously whole-cell clamped and held at −80 mV. Postsynaptic AMPA receptor-mediated currents were used for monitoring transmitter release, and cyclothiazide (CTZ) and γ-D-glutamylglycine (γDGG) were present in the extracellular solution to block AMPA receptor desensitization and saturation. We applied a short depolarization, mimicking an AP, followed by a 10 ms depolarization (Figure 1A). The AP-like stimulus evoked an EPSC of 2 nA in this example. The EPSC amplitude was similar to that evoked by fiber stimulation (Borst and Sakmann, 1996; Schneggenburger et al., 1999). The duration of the subsequent pulse was chosen such that it would be long enough to cause complete depression of a second AP-like stimulus (Wu and Borst, 1999) applied 200 ms later. The elevation of global $[Ca^{2+}]$ by the predepolarizing pulse may cause facilitation (Wu and Borst, 1999; Sakaba and Neher, 2001a); however, at the time when the second AP-like stimulus was applied, global $[Ca^{2+}]$ had returned to the basal level, as confirmed by $[Ca^{2+}]$ measurements (Figure 1B). The second AP-like stimulus elicited only a small EPSC (120 pA in the case of Figure 1A). Finally, a 50 ms pulse was applied to deplete all releasable vesicles. As reported by Wu and Borst (1999), this second long depolarization elicited a large EPSC, although the preceding AP-like stimulus was largely depressed.

To address the question of whether this EPSC is mediated by vesicles with reduced Ca^{2+} sensitivity, we repeated this experiment, except that we replaced the

(C) An experiment similar to that of (B), but the first AP-like stimulus and a 10 ms predepleting pulse was omitted in order to obtain the control flash response. Same cell pair as that shown in (A) and (B).

second AP-like stimulus by a brief Ca^{2+} transient in the presynaptic terminal. Brief transients can be evoked by laser flash photolysis, as has been demonstrated by Bollmann and Sakmann (2005). In this technique, some extra nonphotolyzable buffer is included in the presynaptic pipette solution, which takes up Ca^{2+} rapidly after it has been released by the caged compound. We used a mix of the caged Ca^{2+} compound DM-Nitrophen (1–2 mM) together with EGTA (1 mM). Photolysis of DM-Nitrophen with a UV laser elevated [Ca^{2+}], which subsequently decayed with a time constant of 10–20 ms, as measured by the rapid Ca^{2+} indicator Oregon-Green-BAPTA-5N (OGB-5N) (Figure 1B; Bollmann and Sakmann, 2005). Because the indicator is not ratiometric, dF/F was used to indicate relative changes in [Ca^{2+}]. The time course of the [Ca^{2+}] transient was not as short as that elicited by a presynaptic AP. However, unlike a step-like change in [Ca^{2+}], which causes massive transmitter release, the amount of release caused by this transient stimulus is small and sensitive to slight differences in amplitude or time course. Unlike real APs, flashes under this condition cause spatially homogeneous Ca^{2+} transients. Laser intensity was adjusted so that the amplitude of the postsynaptic current of a nondepressed synapse was comparable to that of an AP-evoked EPSC (2 nA in the case of Figure 1C).

Figure 1B shows such a flash response given at the time of the second AP-like stimulus in a protocol that was otherwise exactly the same as that of Figure 1C. Unlike the AP-like stimulus, the Ca^{2+} transient evoked a large EPSC of 1.5 nA in the depressed synapse (insert in Figure 1B), indicating that vesicles remaining after the 10 ms predepleting depolarization are release competent, although they are reluctant to be released by an AP-like stimulus. Responses like those in Figure 1 were analyzed in seven cell pairs. In each case flashes were given both in isolation as well as within the protocol of Figure 1B. The dF/F signals were very similar for each cell pair with a ratio (after predepolarization over control) of 1.04 ± 0.03. The same ratio of EPSC amplitudes for flashes was 0.78 ± 0.21, while the ratio of EPSCs evoked by AP-like depolarization was 0.13 ± 0.05. The ratio of flash responses is slightly more than what one would expect, if ~50% of the remaining vesicles had the same intrinsic Ca^{2+} sensitivity as the entire population. The ratio for short AP-like stimuli, on the other hand, is much smaller. The discrepancies in sensitivities suggests that short stimuli are inefficient for sufficiently raising [Ca^{2+}] near the remaining vesicles. This is exactly what would be expected if these vesicles were located at larger distances from Ca^{2+} channels.

In the following experiments, the deconvolution method (Neher and Sakaba, 2001a) was used to estimate transmitter release rates from EPSCs in a more quantitative way. We estimated a quantity termed "release rate per vesicle," which we calculated by dividing the peak release rate, as obtained by deconvolution, by the number of remaining releasable vesicles. The latter was calculated from the cumulative release (see Experimental Procedures). On average (n = 7 cell pairs), the peak release rate per vesicle of the flash response in isolation was estimated to be 0.035 ± 0.008 ms^{-1}. In comparison, the same quantity of a flash response after a predepleting stimulus was 0.022 ± 0.004 ms^{-1}. When these values were compared within given cell pairs, the peak release rate per vesicle was 81% ± 16% of that observed during the control. The peak release rates per vesicle during the first and the second AP-like stimulations were 0.093 ± 0.04 ms^{-1} and 0.028 ± 0.019 ms^{-1}, respectively. The peak release rate per vesicle of the second AP-like stimulation was 29% ± 9% of the first one, when comparisons were made within given cell pairs. These data confirm that the effectiveness of Ca^{2+} influx for release was strongly reduced without losing intracellular Ca^{2+} sensitivity for release significantly (see also Figure S1 in the Supplemental Data). In other words, intrinsic fusion competence of remaining synaptic vesicles was reduced much less than the release evoked by depolarization.

Kinetics of Transmitter Release during a Step-Like Elevation of [Ca^{2+}]

In the above set of experiments, we had to use a fast nonratiometric Ca^{2+} indicator dye (Escobar et al., 1997). This Ca^{2+} measurement is not as precise as that achieved during a step-like elevation of [Ca^{2+}] using the ratiometric indicator Fura 2FF. We therefore probed the intracellular Ca^{2+} sensitivity of transmitter release with this indicator in a wider range (2–30 μM) by adjusting flash intensity and DM-Nitrophen concentration (1–2 mM). We did so for both stand-alone flashes and flashes following predepleting stimuli.

Figure 2A shows results for a stand-alone flash. [Ca^{2+}] was elevated to 8 μM after photolysis of DM-Nitrophen, which evoked an EPSC of 14 nA in amplitude. The EPSC decayed while the [Ca^{2+}] remained elevated, and a subsequent depolarizing pulse (to 0 mV for 50 ms) evoked only a small EPSC (Schneggenburger and Neher, 2000). The deconvolution method was used to estimate the time-dependent release rate and the cumulative release was calculated by integrating release rates (Figure 2B). The cumulative release was fitted by a double exponential, which in this example had time constants of 1.83 ms (65%) and 80 ms (dotted trace in Figure 2B). The second component is very slow, and its exact time constant cannot be estimated accurately, because it also includes synaptic vesicles newly recruited after depletion of the releasable vesicles (Wu and Borst, 1999; Sakaba and Neher, 2001b). When the effect of recruitment was corrected for (Sakaba and Neher, 2001a), the late component reached a plateau (solid line in Figure 2B), and the cumulative release could be fitted with a double exponential with time constants of 1.56 ms (78% of the total fit) and 41 ms. In 30 similar experiments, the first component constituted 87% ± 3% of the total fit (Figure 4). When a step depolarizing pulse was applied for 50 ms in the same condition (in the presence of DM-Nitrophen/Ca^{2+} mixture), the fraction of the first component was 48% ± 4% of the total fit (n = 13 cell pairs) after correction for vesicle recruitment. The

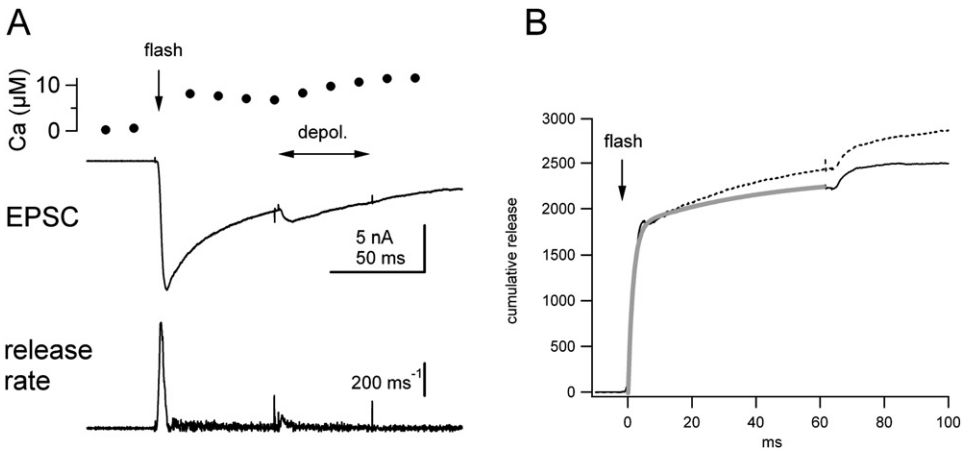

Figure 2. Transmitter Release Evoked by a Step-Like Elevation of [Ca^{2+}]

(A) Flash photolysis was applied at the time point indicated by the arrow. From top: [Ca^{2+}] measured by Fura 2FF, postsynaptic EPSCs, release rates estimated from the deconvolution method. 60 ms after the flash, a depolarizing pulse (to 0 mV for 50 ms after the prepulse to 70 mV for 2 ms) was applied.

(B) Cumulative release calculated from the control flash response. The dotted trace was obtained by integrating the release rate trace shown in (A). The solid trace shows the same curve after correction for the recruitment of new vesicles. A double exponential fit is superimposed on the corrected trace (gray).

time constants of release were 3.22 ± 0.39 ms and 26.9 ± 3.0 ms (n = 13 cell pairs). The appearance of two components of about equal amplitude, with one rapid while the other one is about 10-fold slower, is very common in such experiments on the calyx of Held. Under a condition studied most extensively, which is 0.5 mM EGTA in the presynaptic patch pipette, time constants were reported to be 2–3 ms (50% of the total fit) and 20–30 ms (Sakaba and Neher, 2001b; see also Figure 3A).

The deconvolution method is not highly accurate during the late phase of a prolonged period of transmitter release (Neher and Sakaba, 2001a, see Experimental Procedures). Nevertheless, in the flash response, only a small fraction of synapses had slow components of amplitudes similar to those regularly observed during depolarization (Figure 4C). The majority, particularly at higher [Ca^{2+}] levels, had a clearly dominating fast component, which suggests that spatially uniform [Ca^{2+}] elevation displays a more homogeneous vesicle population with respect to its release probability compared with that assayed by depolarization and localized Ca^{2+} influx.

In Figure 3A, a predepleting pulse (10–20 ms in duration) was applied to deplete the rapidly-releasing vesicle pool. A test pulse (50 ms in duration) was applied 200 ms after the prepulse. This interval was selected such that it was long enough to allow for the decay of overlapping facilitation of the slowly-releasing vesicles due to accumulation of global [Ca^{2+}] (Wu and Borst, 1999; Sakaba and Neher, 2001a). However, it is short enough such that the number of rapidly-releasing vesicles recovering within this interval is small (Sakaba and Neher, 2001b). Nevertheless, in order to block any recruitment of rapidly-releasing vesicles during the interval of 200 ms, calmodulin binding domain peptide (20 μM) was applied via the presynaptic patch pipette in the experiment of Figures 3A–3C. The peptide has been shown to retard the recruitment process (Sakaba and Neher, 2001b). As expected, the test pulse evoked a slowly rising EPSC (see solid trace in the inset of Figure 3B), which presumably reflected release of the slowly-releasing vesicles. When the cumulative release during the test pulse was analyzed (in analogy to the case of Figure 2B), it could be fitted by a single exponential with a time constant of 18.2 ± 1.2 ms in 27 out of 28 cells, confirming depletion of the rapidly-releasing vesicles that have a time constant of 2–3 ms. In one cell pair, 30% of the fast component remained.

In a second protocol applied to each pair, flash photolysis was applied instead of the test pulse (Figure 3B). The flash elicited a step-like elevation of [Ca^{2+}] to 10 μM and evoked a rapidly rising EPSC. The inset of Figure 3B compares the rising phases of depolarization-evoked (solid trace) and flash-evoked (dotted trace) EPSCs. The slower rise of the EPSC evoked by depolarization suggests that [Ca^{2+}] around the slowly-releasing vesicles must be lower during the depolarizing pulse than that during the flash, which is 10 μM (see below for detailed analysis). Following the flash a long-lasting depolarizing pulse was applied, which evoked little EPSC, suggesting that all vesicles, including the slowly-releasing ones, had been released by the preceding stimulation. Figure 3C shows the time course of cumulative release during the test depolarizing pulse and after flash photolysis. Cumulative release during the depolarizing pulse could be fitted by a single exponential with a time constant of 14.6 ms, whereas cumulative release evoked by the flash showed a much faster time course, and could be fitted by a double exponential with time constants of 1.54 ms (89% of the total fit) and 15.3 ms (gray trace). In this example, the amount of

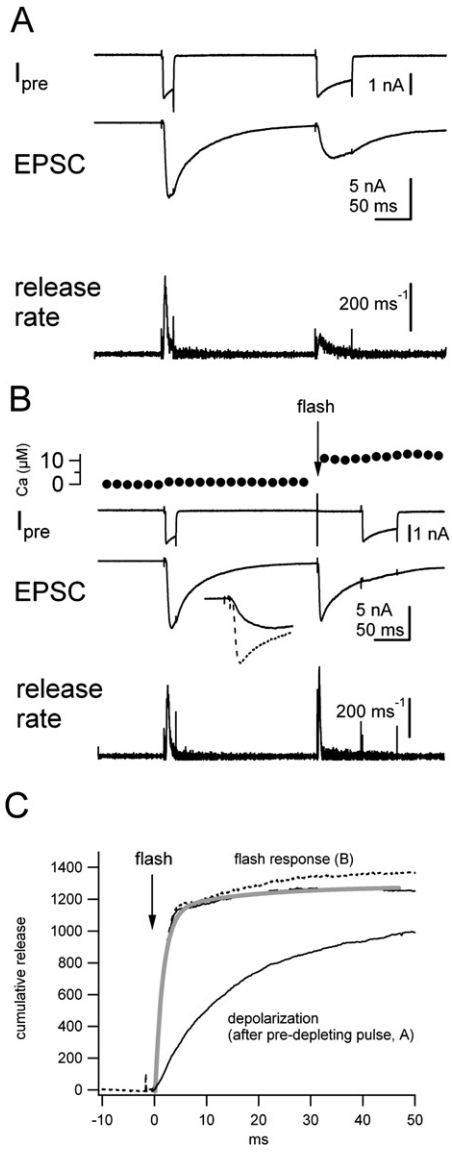

Figure 3. The Effect of Prepulses, which Deplete the Rapidly-Releasing Vesicles, on the Subsequent Release Evoked by either a Step Elevation of [Ca^{2+}] or Depolarization

(A) Traces are displayed like those in Figure 2. The presynaptic terminal was depolarized to 0 mV for 15 ms (after depolarization to +70 mV for 2 ms) to deplete the rapidly-releasing vesicles. 200 ms after the prepolarization, a test pulse (to 0 mV for 50 ms after a short episode at +70 mV for 2 ms) was applied to release remaining slowly-releasing vesicles. Note that the rising phase of the EPSC is much slower than that of the prepulse. The release rate during the test pulse decays more slowly than that during the prepulse. Calmodulin binding domain peptide was included in the presynaptic patch pipette. In the presence of the peptide, recovery of the rapidly-releasing vesicles was reduced to almost zero (Sakaba and Neher, 2001b). Therefore, only slowly-releasing vesicles are present to be released by the test pulse. This is reflected in the slow rise of the EPSC. Same cell pair as in Figure 2. (B) Instead of the test pulse, a flash was applied to elicit a step-like elevation of [Ca^{2+}] to approximately 10 μM. The inset shows the EPSCs evoked by the test pulse (solid trace) and flash (dotted trace), showing a faster rise after flash photolysis.

release during the test depolarizing pulse is less than that evoked by the flash, indicating that 50 ms of depolarization was not enough to release all releasable vesicles. Nevertheless, the fast time course of release evoked by the flash suggests that the slowly-releasing vesicles are capable of releasing rapidly when [Ca^{2+}] is elevated into the range typically obtained during an AP. The intracellular Ca^{2+} dependence of transmitter release both in control conditions and after application of the predepleting pulse will be shown over a wide [Ca^{2+}] range below (Figure 4).

Intracellular Ca^{2+} Sensitivity of Transmitter Release Measured by a Step-Like Elevation of [Ca^{2+}]

Figure 4 shows pooled data from 30 cell pairs gathered from performing the type of experiment shown in Figure 2 and Figure 3. Figure 4A shows the relationship between [Ca^{2+}] and the release time constant of the first component of a double exponential fit. Open and filled circles present the data from control flashes and flashes after depletion of the rapidly-releasing vesicles, respectively. In this series of experiments, predepleting pulses released 52% ± 2% of all releasable vesicles. For example, in the range of 10–15 μM [Ca^{2+}], the time constants of release in control flashes (10.4 ± 0.4 μM; n = 7) and in flashes after depletion of rapidly-releasing vesicles (10.5 ± 0.3 μM; n = 6) were 1.3 ± 0.2 ms and 2.2 ± 0.4 ms, respectively. The fraction of the fast component in the flash response was more than 80% in both cases (87% ± 4% and 84% ± 4% in control flashes and flashes after predepolarization, respectively, Figure 4C). Considering data with and without predepleting pulses, one can state that there is no major difference in the relative contribution of the rapid component, and there is at most a 1.5- to 2-fold difference in the time course of release. The difference is much smaller than the 10-fold difference between the rapidly- and slowly-releasing vesicles (2–3 ms versus 20–30 ms, see also Figure 3A), which is typically observed during depolarizing pulses.

Our results regarding the Ca^{2+} sensitivity of flash-evoked release agree with previously published studies. Comparing our data with the predictions of a 5-site secretion model, as has been described by Schneggenburger and Neher (2000), we obtained a good fit (Figure 4A). In the [Ca^{2+}] range of our study, the recently developed allosteric model and the 5-site model agree relatively closely in their predictions (Lou et al., 2005; but see Sara et al., 2005). Both data sets in Figure 4A could be fitted by only changing the on-rate constant of Ca^{2+} binding from 1.2×10^8 (control) to 0.9×10^8 $M^{-1}s^{-1}$ (after predepleting pulse) and leaving other parameters unchanged. A very shallow [Ca^{2+}] dependence of the time constant of the second, slow component was observed (Figure 4B). Occasionally, we observed a higher fraction of the slowly-releasing component with a Ca^{2+} step below 10 μM. However, separation

(C) The cumulative release evoked by the flash (after a prepulse, [B]) is shown. For comparison, the cumulative release during the test pulse after a prepulse (A) is also shown. Its time course is considerably slower.

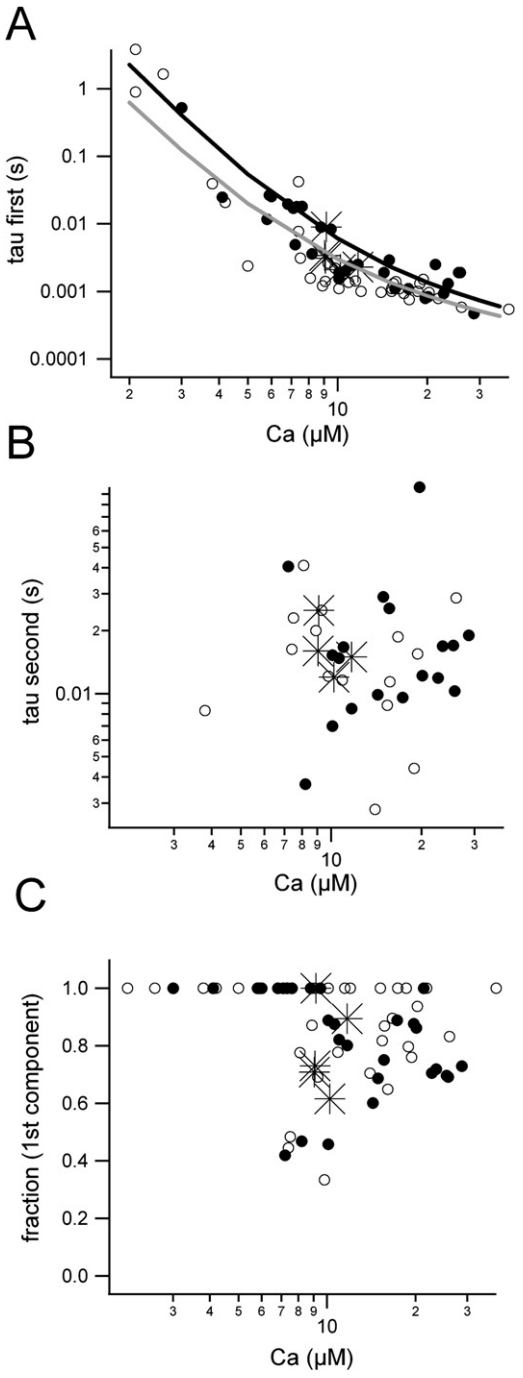

Figure 4. Relationship between $[Ca^{2+}]$ and the Release Time Constants

The fast (A) and the slow (B) time constants as well as the fractions of the fast components (C) evoked by flashes are plotted against the plateau $[Ca^{2+}]$. Open and filled circles show the data from control flash (Figure 2) and from flash after application of the predepleting pulse (Figure 3), respectively. Data from Figure 5 are included and marked as asterisks. The gray (control) and black (after prepulse) curves in (A) show the expectation of the 5-site model. The following parameters were found: $k_{on} = 1.2 \times 10^8\ M^{-1}s^{-1}$ (control) or $0.9 \times 10^8\ M^{-1}s^{-1}$ (after prepulse); $k_{off} = 9000\ s^{-1}$, b = 0.25; $\gamma = 8000\ s^{-1}$.

of components is difficult under these conditions. When the release time course becomes slow at lower $[Ca^{2+}]$, vesicle recruitment, occurring at a rate of about 10 vesicles ms^{-1}, and vesicle release compensate each other partially. This makes it difficult to separate the two effects. In addition, a slow decay in $[Ca^{2+}]$, which is often observed, may cause the time course of release to slow down markedly due to the highly nonlinear dependence of release on $[Ca^{2+}]$ in the low range. Although we cannot exclude the possibility that there may be a small fraction of intrinsically slow synaptic vesicles, the fraction of the fast component was much higher than that observed during a step depolarizing pulse (50%). Also, we cannot distinguish whether the slight slow-down of release in the flash data following partial depletion represents the contribution of a heterogeneous population of slower vesicles or a small decrease in the kinetics of the dominating fast population. Importantly, plotting the peak release rate per vesicle, which reflects instantaneous release kinetics and is not as dependent on fits of cumulative release, gave similar results. Also, in this type of analysis the release kinetics was affected less than 2-fold after application of predepleting pulses (Figure S2).

From the relationship between $[Ca^{2+}]$ and the time constant, as shown in Figure 4A, we can estimate the $[Ca^{2+}]$, which is sensed by the rapidly- and slowly-releasing vesicles during depolarization. Assuming that the release machineries are in equilibrium with Ca^{2+}, and comparing the time constants of release during a step depolarization (2–3 ms and 20–30 ms) for the fast and slow components, we found that $[Ca^{2+}]$ will be 10–12 μM and 6–7 μM around the rapidly- and slowly-releasing vesicles, respectively. The difference in $[Ca^{2+}]$ of only 2-fold is sufficient due to the highly nonlinear dependence of release on $[Ca^{2+}]$.

Rapid EPSCs Can Be Evoked by Flashes after Synaptic Depression

In order to test whether flashes can evoke rapid responses after bursts of AP-like stimuli, we also applied such trains (300 Hz × 10, Figure 5). To ensure synaptic depression after the tenth stimulus, external Ca^{2+} concentration was elevated to 5 mM, because the synaptic response often shows initial facilitation in 2 mM extracellular Ca^{2+} (Schneggenburger et al., 1999). The trains of AP-like stimuli released 66% ± 4% of the total releasable vesicles. While synchronous EPSCs were depressed during and after repetitive stimulations, a subsequent flash-evoked $[Ca^{2+}]$ step to 10 μM elicited rapid release (Figure 5). Similar observations were found in five cell pairs. A detailed kinetic analysis of cumulative release (data included in Figure 4 as asterisks) showed similar time courses of the flash responses irrespective of whether predepleting pulses or trains of AP-like stimuli were used.

The N-Terminal of Synaptobrevin Is Important for Recruitment of the Rapidly-Releasable Vesicles

At the calyx of Held, cleavage of synaptobrevin during prolonged exposure to tetanus toxin blocked all releasable

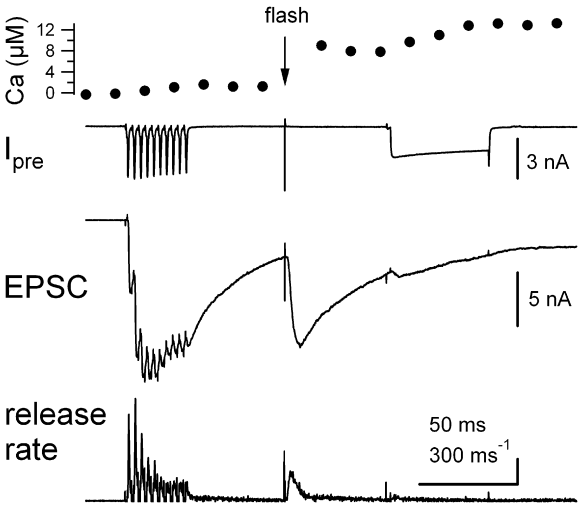

Figure 5. The Flash Response after Application of a 300 Hz Train of AP-Like Stimuli

Traces are presented in the same order as in Figure 3. A 300 Hz train of AP-like stimuli was applied (10 × depolarization to +40 mV for 1.5 ms). To ensure synaptic depression, external Ca^{2+} was elevated to 5 mM. In this example, synaptic responses are depressed at the end of the stimulus train. Fifty milliseconds later, flash photolysis of Ca^{2+} was applied, which still evoked a rapid EPSC. Subsequently, a step depolarization (to 0 mV for 50 ms following a predepolarization to 70 mV for 2 ms) was applied.

vesicles. However, short-term exposure preferentially blocked the rapidly-releasing component and slowed down the recruitment of the rapidly-releasing vesicles (Sakaba et al., 2005). These findings suggest that the action of tetanus toxin interferes with vesicle recruitment. Tetanus toxin cleaves part of the SNARE motif and the proline-rich N-terminal domain off the rest of the synaptobrevin molecule. To further learn how synaptobrevin acts in the recruitment of synaptic vesicles, we infused the proline-rich domain of synaptobrevin (1-26, 100 μM) into the calyx terminal via a recording patch pipette (Figure 6A). One-half millimolar EGTA was also included in the patch pipette for a more precise separation of the rapidly- and slowly-releasing components (Sakaba and Neher, 2001b). Pairs of pool-depleting pulses (depolarization to 0 mV for 50 ms following a prepulse to +70 mV for 2 ms) with varying interstimulus intervals (ISI; Sakaba and Neher, 2001b) were used to monitor vesicle recruitment. As described previously, first depolarizing pulses within pairs evoked two components of release under control conditions. They could be fitted by double exponentials with time constants of 2.25 ± 0.23 ms (54% ± 2.3% of the total fit) and 27.2 ± 1.68 ms (n = 8 cell pairs, Figure 6A, left panel, gray traces). This time course of release was not altered by the synaptobrevin peptide (2.36 ± 0.36 ms; 55% ± 3.1% of the total fit, and 25.6 ± 3.1 ms, n = 7 cell pairs, Figure 6A, right panel, gray traces). Neither did the peptide reduce the total amount of release over the recording period, in contrast to the action of tetanus toxin (Sakaba et al., 2005). However, recruitment of the rapidly-releasing component was slowed down by the peptide as monitored by the second depolarizing pulse (Figure 6A, right panel, black traces). When the recovered fraction of the fast component was plotted against ISI (Figure 6B), the time course could be fitted by a single exponential with a time constant of 2.7 s in the presence of peptide, while under control conditions a double exponential fit was required with time constants of 454 ms (58%) and 8.5 s. A slow-down of the recovery was also seen by blocking the proline-rich domain of endogenous synaptobrevin with Fab fragments of an antibody raised against residues 2–17 of synaptobrevin (2 μM, Figure 6B). In the presence of the fragment, the recovery time course could again be fitted by a single exponential with a time constant of 5.5 s (pooled data from five cell pairs). Neither the peptide nor the antibody affected the recruitment of the slowly-releasing vesicles (Figure 6B, right panel). We conclude that the proline-rich domain of synaptobrevin is important for recruiting rapidly-releasing synaptic vesicles, while cleavage of the SNARE motif by the toxin may be responsible for the reduction of release, which occurs over a period of 10 min following infusion of the toxin.

When the second pulse was applied 1 s after the first pulse, the slowly-releasing vesicles were almost fully recovered, whereas there were only few newly recruited rapidly-releasing ones in the presence of synaptobrevin peptide (Figure 6B). This situation allowed us to examine the intracellular Ca^{2+} sensitivity of the newly recruited slowly-releasing vesicles by replacing the second depolarizing pulse with flash photolysis; the experiment is shown in Figure 7A. A step-like elevation of $[Ca^{2+}]$ to 12 μM was applied at 1 s following the first depolarizing pulse, which evoked a rapidly rising EPSC (inset of Figure 7A). In Figures 7B–7D, the intracellular Ca^{2+} sensitivity of transmitter release during the recovery period was compared with that of control (stand-alone flash). For quantitative analysis, cumulative release was fitted by a single or a double exponential. Figure 7B shows the relationship between the fast time constant and $[Ca^{2+}]$. Compared with stand-alone flashes (open circles), intracellular Ca^{2+} sensitivity of the newly recruited slowly-releasing vesicles was reduced at most 2-fold. The two superimposed curves in Figure 7B, which are fits to the data obtained under control conditions and during the recovery phase, are exactly the same as those of Figure 4. It should be noted that data in Figure 7 represent vesicles that have been newly recruited after a 50 ms, fully depleting stimulus, while those in Figure 3 and Figure 4 are from slowly-releasing vesicles that had been spared during a preceding 10 ms depolarization.

Figures 7C and 7D show the Ca^{2+} dependence of the second time constant and of the fraction of the first component of the double exponential fits, respectively. Again, similarly to Figure 4, the slow time constant had no systematic relationship to intracellular Ca^{2+}, and ~80% of the total release was mediated by the first component of the double exponential fit.

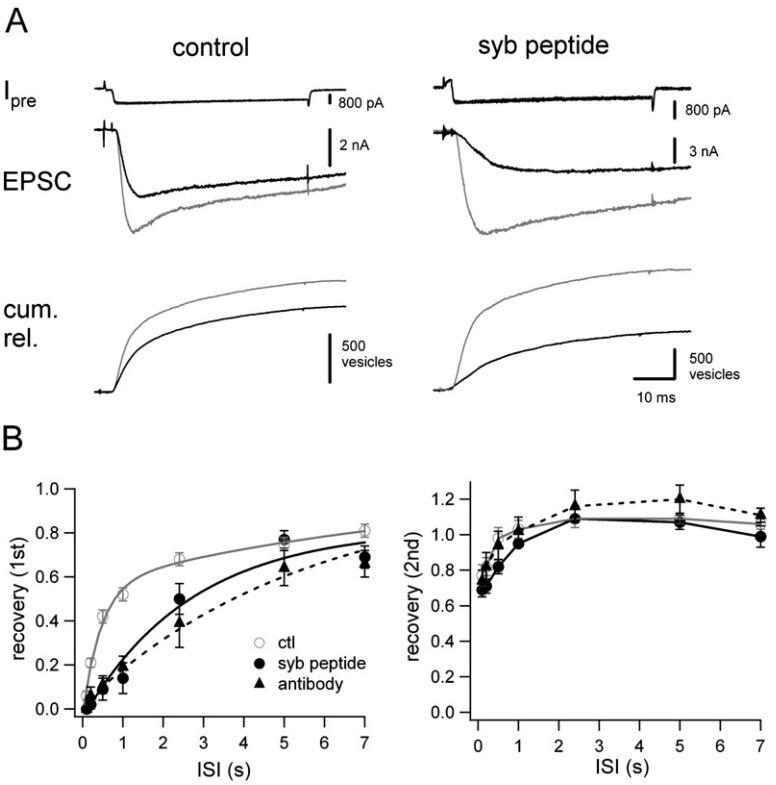

Figure 6. The Proline-Rich Domain of Synaptobrevin Is Important for Recruiting Rapidly-Releasing Synaptic Vesicles

(A) A pair of depolarizing pulses (0 mV for 50 ms following a prepulse to +70 mV for 2 ms) was applied to the presynaptic terminal. ISI was 1 s in this example. Gray and black traces represent data from the first and the second depolarizing pulse, respectively. Left and right panels are from control and in the presence of proline-rich domain of synaptobrevin (1-26, 100 μM), respectively. From top: presynaptic current, EPSC, and cumulative release estimated by the deconvolution method are shown.

(B) Recovered fraction of the fast (left) and the slow (right) components of release during the second depolarizing pulse is plotted against ISI. Open circles, filled circles, and filled triangles are data from control, in the presence of proline-rich domain peptide, and Fab fragments of a synaptobrevin antibody raised against the proline-rich domain of synaptobrevin (2 μM), respectively.

DISCUSSION

Rapidly-releasing vesicles are responsible for synchronous release during presynaptic APs, and their depletion during trains of APs or during long-lasting depolarizations is the main cause for short-term synaptic depression at glutamatergic synapses (Zucker and Regehr, 2002; Sakaba, 2006). Here, we show that $[Ca^{2+}]$ elevation to a level of about 10 μM by photolytic Ca^{2+} uncaging still elicited rapid transmitter release after such depletion, when the AP-evoked responses were largely depressed. These data suggest that lack of fusion competence of vesicles remaining after a "depressing" stimulus is not a cause for depression, and that acquisition of fusion competence is not a rate-limiting step for restoration of rapid transmitter release. Rather, our results indicate that during short depolarizations slowly-releasing vesicles experience $[Ca^{2+}]$ levels lower than about 10 μM. Otherwise, they should be released by physiological stimulation, since 10 μM is the Ca^{2+} concentration which the rapidly-releasing vesicles experience during a step depolarization or during an AP (Bollmann et al., 2000; Schneggenburger and Neher, 2000). We conclude that a process of recruiting synaptic vesicles to presynaptic Ca^{2+} channel clusters is a limiting step for rendering them competent for rapid release, and that at rest only about half of all vesicles are located at these preferred positions.

The present study also indicates that intrinsic release rates of vesicles after depletion of the rapidly-releasing pool are reduced at most 2-fold (Figure 4 and Figure S2). This constitutes only a minor contribution to the heterogeneity of release probability observed during a long-lasting depolarizing pulse. We cannot exclude that a small fraction of vesicles (less than 20% from the cumulative release analysis; Figure 4C) becomes intrinsically slow by mechanisms which may develop during stimulation, such as mutual inhibition among releasing vesicles or adaptation of the release machinery (Hsu et al., 1996; Zucker, 1996; Dobrunz et al., 1997; M. Wölfel, X.L. Lou, and R. Schneggenburger, personal communication). Such intrinsically slow vesicles, however, are unlikely to participate in release during transient $[Ca^{2+}]$ signals elicited by APs, because otherwise we should have observed a larger decrease in the peak release rate per vesicle when we applied transient $[Ca^{2+}]$ stimuli after depletion of the rapidly-releasing vesicles (Figure 1).

Recruitment of Synaptic Vesicles to Ca^{2+} Channel Clusters

We have previously shown that two components of release (time constants of 2–3 ms and 20–30 ms) could be observed during a prolonged depolarization followed by rapid recruitment of new vesicles (time constant of 200 ms to 1 s; Wu and Borst, 1999; Sakaba and Neher, 2001b; see also Hallermann et al., 2003). Initially the rapidly-*recruited* vesicles are released only as slowly-releasing ones, while recovery of the rapidly-*releasing* vesicles takes several seconds. Fusion competence of synaptic vesicles is a critical factor for rapid fusion. It has been shown that loss of vesicle priming factors eliminated or reduced the size of the readily releasable pool of synaptic

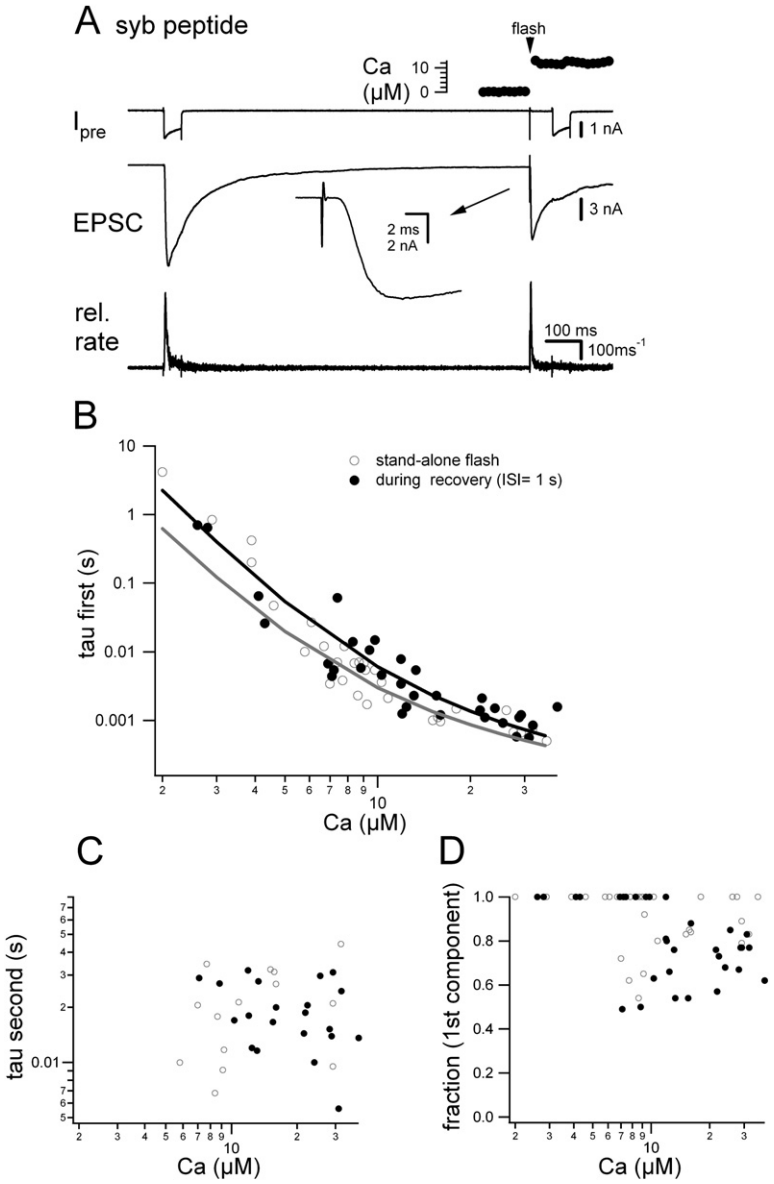

Figure 7. Flash Photolysis during Recovery from Vesicle Depletion

(A) The presynaptic patch pipette contained proline-rich domain of synaptobrevin to slow down recovery of the rapidly-releasing vesicles. With an interval of 1 s following the pool-depleting pulse (0 mV for 50 ms following a prepulse to +70 mV for 2 ms), flash was applied to the presynaptic terminal (timing indicated by an arrow). From top: presynaptic current, EPSC, and release rate estimated by the deconvolution method. The inset magnifies the flash-evoked EPSC.

(B–D). The fast (B) and the slow (C) time constants as well as the fraction of the fast component (D) evoked by flashes are plotted against [Ca^{2+}]. Open and filled circles show the data from control flashes and from flashes during recovery from depletion, respectively. All data are obtained in the presence of proline-rich domain peptide. The gray and black curves in (B) show the expectation of the 5-site model, the parameters of which are exactly the same as those shown in Figure 4.

vesicles (Augustin et al., 1999; Verhage et al., 2000). Sucrose application has often been used for determining this pool and for calculating vesicular release probability during an AP (Rosenmund and Stevens, 1996). However, it has been shown that APs could not release all releasable vesicles determined by sucrose application (Moulder and Mennerick, 2005), suggesting the presence of mechanisms causing heterogeneity of release during short depolarizations other than vesicle priming.

We show that a critical step for rapid release is the recruitment of synaptic vesicles to sites where Ca^{2+} channels are densely packed and Ca^{2+} influx can cause [Ca^{2+}] transients of 10–25 μM amplitude with a half-width of 0.5 ms, as are generated during an AP (Bollmann et al., 2000; Schneggenburger and Neher, 2000; Meinrenken et al., 2002; Bollmann and Sakmann, 2005). Such sites must be relatively fixed in number, because (1) modulators such as Ca^{2+} or second messengers, which influence the rate of recovery, do not change the number of rapidly-releasing vesicles significantly (Sakaba and Neher, 2001b; 2003), in contrast to the case of hormone release from neuroendocrine cells (Smith et al., 1998), and (2) fluctuation analysis based on the assumption of a fixed number of release sites well describes synaptic responses during short-tem plasticity (Silver et al., 1998; Scheuss et al., 2002, but see Foster and Regehr, 2004). Such sites are postulated to exist also for presynaptic Ca^{2+} channels (Cao et al., 2005). It is plausible that the sites are located at the presynaptic active zones where presynaptic proteins and Ca^{2+} channels are aggregated. The number of vesicles docked at the active zones (Sätzler et al., 2002) matches approximately with the number of rapidly-releasing

vesicles at the calyx of Held (three rapidly-releasing vesicles and three slowly-releasing vesicles per active zone). Active zone geometry may well be heterogenous, and this may be especially the case at immature synapses, which we study here (Fedchyshyn and Wang, 2005). Nevertheless, elucidating active zone organization in molecular terms will be important for future studies. It has been shown that some active zone/adhesion proteins such as α-neurexin (Missler et al., 2003) or Bruchpilot (Kittel et al., 2006) are important for coupling Ca^{2+} channels and synaptic vesicle exocytosis. In addition, vesicle targeting to active zone Ca^{2+} channels by SNARE proteins has been demonstrated (Mochida et al., 1996; Rettig et al., 1997).

We have previously shown that cleavage of synaptobrevin by tetanus toxin preferentially blocked rapid release at an early stage of toxin action. We interpreted this as an effect of the toxin on vesicle recruitment in addition to its well-known effect on exocytosis (Sakaba et al., 2005). We also showed that the slowly-releasing vesicles remaining after synaptic depression behave exactly the same (Figure 3 and Figure 4) as vesicles remaining during the early phase of toxin action. We now have identified the proline-rich domain of synaptobrevin as an important element for recruiting rapidly-releasing vesicles (Figure 6 and Figure 7) since it retards recruitment if infused into the nerve terminal. The domain has been shown to inhibit transmitter release in *Aplysia* synapses (Cornille et al., 1995). It is unclear at present how it is involved in vesicle recruitment. Although the domain interacts with synaptophysin (Calakos and Scheller, 1994), synaptophysin KO mice have no apparent phenotype (McMahon et al., 1996). Future biochemical analysis is necessary to identify other binding partners, which are presumably more relevant for vesicle recruitment.

Since we have shown that slowly-releasing vesicles are fully release competent, it follows that vesicle docking and priming—i.e., the buildup of the release machinery—can be a very rapid process. Slow vesicles achieve release competence within 100 ms after the end of a depleting stimulus. Therefore, the rate-limiting second step for the generation of rapidly-releasing vesicles, which typically takes several seconds, must be their placement in an environment which is rich in Ca^{2+} channels. This maturation accelerates depolarization-induced release by about a factor of ten while it increases the intrinsic rate of release (at a given $[Ca^{2+}]$) by at most a factor of two—possibly by additional interactions with active zone components (superpriming). It is interesting to note that this process is a highly modulated one. We have previously shown that it can be accelerated by the simultaneous presence of elevated global $[Ca^{2+}]$ and cAMP (Sakaba and Neher, 2003). Ca^{2+}-dependent acceleration of vesicle recruitment has been observed at several synapses (Dittman and Regehr, 1998; Stevens and Wesseling, 1998; Wang and Kaczmarek, 1998). Recovery from short-term depression has usually been interpreted to reflect either vesicle recycling and docking or the buildup of the release machinery. We would suggest, however, that all of these processes are rapid under normal physiological conditions and that recovery, as well as the level of depression during sustained activity, reflects an interaction of fully release-competent vesicles with the active zone, which brings them into the vicinity of Ca^{2+} channels. Future research on synaptic depression and other aspects of short-term plasticity should pay attention to such a scenario.

Physiological Roles of Rapidly- and Slowly-Releasing Vesicles

Asynchronous release distinct from release in synchrony with APs has been described for many types of synapses (Xu-Friedmann and Regehr, 2000; Lu and Trussell, 2000; Zucker and Regehr, 2002). A high-affinity slow Ca^{2+} sensor for asynchronous release has been postulated on the basis of differential sensitivities of the two release components to divalent cations (Goda and Stevens, 1994) and the analysis of synaptotagmin-deficient mice (Geppert et al., 1994).

At the calyx of Held, synchronous release during APs is mediated by the rapidly-releasing vesicles (Schneggenburger et al., 1999; Wu and Borst, 1999; Bollmann et al., 2000). The slowly-releasing vesicles contribute relatively little and are released mainly in an asynchronous way during a high-frequency train of APs (Sakaba, 2006). Our finding that the intrinsic release kinetics of most of the slowly-releasing synaptic vesicles is fast (Figure 3, Figure 4, and Figure 5) may provide an alternative interpretation. In this view, the distinction between the two types of vesicles relates mainly to their localization with respect to Ca^{2+} channels. During AP-induced Ca^{2+} inflow, local $[Ca^{2+}]$ domains would only release appropriately positioned vesicles. Following the AP, $[Ca^{2+}]$ equilibrates spatially and activates more remote vesicles. Finally, all release, including that of both rapidly-releasing and slowly-releasing vesicles, stops while $[Ca^{2+}]$ decays (Figure 1 and Figure S1). It follows that the time course of asynchronous release is not determined by the intrinsic kinetics of release machineries, but rather by the time course of $[Ca^{2+}]$. In other words, the slowly-releasing vesicles are spared for release initially, but they release asynchronously during a high-frequency train of APs. This is a straightforward explanation for the mechanism of asynchronous release and is consistent with some of the previous studies (Xu-Friedmann and Regehr, 2000). It should be emphasized, however, that the calyx synapse shows little asynchronous release in response to a single AP (Borst and Sakmann, 1996) compared with some other types of synapses, where asynchronous release can be easily observed during a single or low-frequency stimulus (Lu and Trussell, 2000, Hefft and Jonas, 2005). Therefore, additional mechanisms that do not exist at the calyx of Held may be responsible for asynchronous release in other synapses.

The difference in estimated $[Ca^{2+}]$ between the rapidly- and the slowly-releasing vesicles during a prolonged depolarization was only about 2-fold (10–12 versus 6–7 μM, see Figure S1). Nevertheless, this small difference

in [Ca^{2+}] readily caused a 10-fold difference in kinetics of release due to the 4^{th}-5^{th}-power dependence of release on [Ca^{2+}]. Differences in [Ca^{2+}] are likely to be larger during short stimuli such as APs when only a small amount of presynaptic Ca^{2+} influx takes place. Global presynaptic [Ca^{2+}] is kept low during such short episodes of influx because of Ca^{2+} buffering, which might be at least partially saturated during a long-lasting step depolarization. Expression of Ca^{2+} buffers is known to change with synaptic development (Kandler and Friauf, 1993; Felmy and Schneggenburger, 2004) and to differ among synapses. It is therefore possible that asynchronous release under physiological conditions may be regulated depending on the expression and the degree of saturation of endogenous Ca^{2+} buffers (Rozov et al., 2001). Our findings therefore imply that both the structural organization of the active zone and changes in Ca^{2+} dynamics are important regulators of short-term synaptic plasticity.

EXPERIMENTAL PROCEDURES

Electrophysiology and Flash Photolysis

Presynaptic and postsynaptic recordings at the calyx of Held were performed using the acute slice preparation from P8–P11 rat brainstem (Borst and Sakmann, 1996). The experiments were carried out according to the guidelines of German laws on animal protection. Slices were transferred to the recording chamber and perfused continuously with normal saline containing 125 mM NaCl, 2.5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 25 mM glucose, 25 mM $NaHCO_3$, 1.25 mM Na_2Po_4, 0.4 mM L-ascorbic acid, 3 mM myo-inositol, and 2 mM Na-Pyruvate (pH 7.3–7.4), 320 mOsm, bubbled continuously with 95% O_2 and 5% CO_2. During the recordings, the solution was supplemented with TTX (1 μM), TEA (10 mM), D-AP5 (50 μM), CTZ (100 μM), and γDGG (2 mM). The first three were added to block presynaptic Na currents, K currents, and postsynaptic NMDA receptors, respectively. CTZ was used to block postsynaptic receptor desensitization (Koike-Tani et al., 2005), but when applied alone quantal sizes were still reduced by 20%–30%, possibly due to AMPA receptor saturation and voltage-clamp errors (Ishikawa et al., 2002; Neher and Sakaba 2001a, 2001b). We therefore also added γDGG, which is a low-affinity antagonist that prevents receptor saturation (Wadiche and Jahr, 2001). Using fluctuation analysis, we have confirmed that quantal sizes stay constant in a wide range of stimulation conditions (Figure S3) when both CTZ and γDGG are present. In the experiment shown in Figure 6, another low-affinity antagonist, kynurenic acid (1 mM), was used instead of γDGG (Neher and Sakaba, 2001a).

The presynaptic and postsynaptic compartments were simultaneously clamped at −80 mV using an EPC9/2 or EPC10/2 amplifier controlled by the Pulse or Patchmaster software (HEKA, Lambrecht, Germany). For postsynaptic recordings, the pipette (2–4.5 MΩ) contained the following: 130–145 mM Cs-gluconate, 20 mM TEA-Cl, 10 mM HEPES, 5 mM Na_2-phosphocreatine, 4 mM MgATP, 0.3 mM NaGTP, and 5 mM EGTA (pH7.2), 320 mOsm. Series resistance (4–10 MΩ) was compensated for by the amplifier such that the residual resistance became 1.5–3 MΩ. The presynaptic pipette (4–6 MΩ) was filled with different solutions depending on the types of experiments as described below. Presynaptic series resistance was typically 8–30 MΩ, and was compensated for electrically by 20%–80%.

A frequency-tripled Nd:YAG laser (5 ns pulse width; Continuum, Santa Clara, CA) was used to create 355 nm pulses to photolyze DM-Nitrophen. Intracellular Ca^{2+} changes were measured by perfusing the fluorescent Ca indicator dye OGB-5N into the presynaptic terminal via the patch pipette. Dye excitation (at 480 nm) was performed with a Polychrome V monochromator (TILL Photonics, Gräfelfing, Germany) coupled into the microscope via the epifluorescent port. In order to minimize photobleaching, the duration of the excitation was minimized to 100 ms at any one time. Fluorescent light was collected in a rectangular window that was adjusted using a PIN photodiode to cover the area of the pre- and postsynaptic sites (Hamamatsu, Japan). The photodiode signal was amplified with an Axopatch 200B amplifier (Axon Instruments, Sunnyvale, CA) and then fed into an EPC9/2 patch-clamp amplifier for data acquisition. All traces were corrected for both dark current of the photodiode and background fluorescence, which was acquired after patching was terminated by moving both pipettes (excised patch) and the window to a neighboring location on the slice. Fluorescence changes were expressed as dF/F. The presynaptic patch pipette contained the following: 135 mM Cs-gluconate, 20 mM TEA-Cl, 20 mM HEPES, 0.5 mM $MgCl_2$, 5 mM Na_2ATP, 0.5 mM NaGTP, 1 mM EGTA, 2 mM DM-Nitrophen, 1.7 mM $CaCl_2$, and 0.5 mM Oregon-Green-BAPTA-5N (pH 7.2), 320 mOsm. We added EGTA in order to accelerate the decay of [Ca^{2+}], evoking a transient EPSC (Bollmann and Sakmann, 2005). Addition of higher concentrations of EGTA or of the faster Ca^{2+} buffer BAPTA accelerates the decay of [Ca^{2+}] even faster, approaching that of an AP, which we confirmed in vitro. However, addition of a high concentration of exogenous buffer slows the time course of release during depolarizations (Borst and Sakmann, 1996) and makes depletion of releasable vesicles by a presynaptic depolarization impossible. Therefore, we used 1 mM EGTA as a compromise, which allowed us both to elicit [Ca^{2+}] transients and to deplete the vesicle pool with a presynaptic depolarization. The time constants of decay of [Ca^{2+}] transients were about 10–20 ms under these conditions.

A UV flash lamp (photolysis duration of 1 ms, Rapp, Hamburg, Germany) was used to elicit step-like elevations of [Ca^{2+}] in the presynaptic terminal as has been described in Schneggenburger and Neher (2000). In this second type of experiment, presynaptic [Ca^{2+}] was monitored by Fura 2FF, which was excited at 350/380 nm by a monochromator (Polychrome 4, TILL Photonics, Gräfelfing). Fluorescence was collected by a CCD camera (VGA, TILL Photonics, Gräfelfing, Germany). Monochromator and CCD camera were controlled by the TILLvisION software (TILL Photonics, Gräfelfing, Germany). Presynaptic patch pipettes contained 130 mM Cs-gluconate, 20 mM TEA-Cl, 20 mM HEPES, 0.5 mM $MgCl_2$, 5 mM Na_2ATP, 0.5 mM NaGTP, 1–3 mM DM-Nitrophen, 0.8–2.7 mM $CaCl_2$, and 0.2 mM Fura 2FF (pH 7.2), 320 mOsm. Presynaptic [Ca^{2+}] was derived from the ratiometric measurements of Fura 2FF. Calibration of Fura 2FF was performed in vitro, and R_{min} and $R_{intermediate}$ were confirmed by in vivo measurements (Schneggenburger, 2005).

In the experiment of Figure 6, presynaptic patch pipette contained the same solution as the postsynaptic one, except that EGTA was reduced to 0.5 mM. Proline-rich domain of synaptobrevin (NH_2-SATAATVPPA APAGEGGPPA PPPNLT-amide) was synthesized by Biosyntan (Berlin, Germany). Fab fragments of the antibody raised against the N-terminal of synaptobrevin (2-17) were kindly supplied by Alexander Stein, in the Department of Neurobiology of the Max Planck Institute for Biophysical Chemistry.

Deconvolution

In order to estimate the transmitter release rates, EPSCs were deconvolved with mEPSCs, as has been described by Neher and Sakaba (2001a). The deconvolution method also takes into account the residual current due to delayed clearance of glutamate in the synaptic cleft. We have shown that release rate estimates from the deconvolution method agree relatively well with those from fluctuation analysis (Neher and Sakaba, 2001a, 2001b) and presynaptic capacitance measurements (Sakaba, 2006). As has been described by Neher and Sakaba (2001a), the release rate estimate is accurate for short, transient episode of release, as long as the derivative of the EPSC is large. On the other hand, when release rates are low, systematic errors, which might originate from an inaccurate estimation of the residual current component, will create a bias in the estimation of release rates. We

prevented our conclusions from depending on such inaccuracies by designing appropriate experimental protocols. In Figure 1, a flash or an AP-like stimulation only evoked a small amount of transient release, and analyzing the EPSC amplitudes instead of release rates led to the same conclusions as those obtained by deconvolution. Both types of analysis showed that flashes, but not AP-like stimuli, evoked release after depletion of the rapidly-releasing vesicles.

We designed most of the pulse protocols such that each test episode was followed by a depleting pulse, which was strong enough to deplete the remaining vesicle pool. Thus, we could calculate the number of remaining vesicles in the releasable pool for time points preceding such depleting pulses. We calculated peak release rate per vesicle of a given response by dividing peak release rate by the amount of remaining vesicles at the onset of the stimulation protocol. This calculation avoids fitting of exponentials to cumulative release traces (Figure 1). For Figure 2 to Figure 5, cumulative release was fitted with double exponentials after correction for the contribution of vesicle recruitment, which has been described by Sakaba and Neher (2001a).

Release Model

The relationships between [Ca^{2+}] and release rate per vesicle, time constants, and delays of transmitter release (defined as the interval between the onset of flash and the time point when 15 vesicles have been released) were modeled by the 5-site model developed by Schneggenburger and Neher (2000) as described below.

$$\mathrm{V} \underset{k_{\mathrm{off}} b^{0}}{\overset{\mathrm{Ca}^{2+}\ 5k_{\mathrm{on}}}{\rightleftharpoons}} \mathrm{V}_{\mathrm{Ca}} \cdots \mathrm{V}_{4\mathrm{Ca}} \underset{5k_{\mathrm{off}} b^{4}}{\overset{\mathrm{Ca}^{2+}\ k_{\mathrm{on}}}{\rightleftharpoons}} \mathrm{V}_{5\mathrm{Ca}} \xrightarrow{\gamma} \text{fused}$$

The forward rate k_{on}, the backward rate k_{off}, cooperativity factor b, and the final rate γ were found by trial and error using Euler integration. The parameters are described in the figure legends. The recently developed allosteric model (Lou et al., 2005) predicts time courses very similar to those of the 5-site model in the Ca^{2+} range we studied.

Supplemental Data

The Supplemental Data for this article can be found online at http://www.neuron.org/cgi/content/full/53/4/563/DC1/.

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