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Calmodulin Mediates Rapid Recruitment of Fast-Releasing Synaptic Vesicles at a Calyx-Type Synapse

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Summary

In many synapses, depletion and recruitment of releasable synaptic vesicles contribute to use-dependent synaptic depression and recovery. Recently it has been shown that high-frequency presynaptic stimulation enhances recovery from depression, which may be mediated by Ca^{2+}. We addressed this issue by measuring quantal release rates at the calyx of Held synapse and found that transmission is mediated by a heterogeneous population of vesicles, with one subset releasing rapidly and recovering slowly and another one releasing reluctantly and recovering rapidly. Ca^{2+} promotes refilling of the rapidly releasing synaptic vesicle pool and calmodulin inhibitors block this effect. We propose that calmodulin-dependent refilling supports recovery from synaptic depression during high-frequency trains in concert with rapid recovery of the slowly releasing vesicles.

Introduction

Depletion of a release-ready transmitter store has been recognized as a major cause of short-term synaptic depression already in some of the very early studies on synaptic transmission (Liley and North, 1953; Del Castillo and Katz, 1954; Elmqvist and Quastel, 1965). Depressed synapses can recover within several seconds by recruitment of quanta to a releasable pool (Betz, 1970; Rosenmund and Stevens, 1996; von Gersdorff and Matthews, 1997, von Gersdorff et al., 1997). The refilling process is also considered to contribute to the maintenance of a relatively high steady-state release achieved during continuous stimulation (Elmqvist and Quastel, 1965; Kusano and Landau, 1975). Such dynamic behavior of synaptic depression and recovery may enrich the computational ability of neuronal networks (Abbott et al., 1997; Tsodyks et al., 2000; Hopfield and Brody, 2001).

Recently it has been shown that recovery from synaptic depression was accelerated by high-frequency stimulation of the presynaptic terminal (Dittman and Regehr, 1998; Stevens and Wesseling, 1998; Wang and Kaczmarek, 1998; Gomis et al., 1999; Dittman et al., 2000). Many of the features of the enhanced recovery are in line with the idea that Ca^{2+}, accumulating during repetitive activity, is an essential regulator of the replenishment process, as suggested by these reports. However, detailed studies at the calyx of Held, a synapse in which pre- and postsynaptic compartments can be voltage clamped, suggest that enhanced recovery during repetitive trains may be due to a subpool of rapidly replenished vesicles, which has low release probability (P_r) at resting [Ca^{2+}]. It becomes available for release during repetitive activity by Ca^{2+}-dependent facilitation (Wu and Borst, 1999). In addition, experiments employing Ca^{2+} buffer injection indicated that submicromolar [Ca^{2+}] did not influence recovery from 10 Hz depression (Weis et al., 1999; see also Swandulla et al., 1991). Thus, it was suggested that the Ca^{2+}-dependent recruitment of vesicles might be an apparent one, not due to an acceleration in replenishment of vesicles, but due to the fact that a population of vesicles, which has intrinsically fast recruitment, becomes available for release in a Ca^{2+}-dependent manner. In fact, it was questioned whether the recruitment process has any Ca^{2+} dependence at all, such that the problem of Ca^{2+}-dependent recruitment remains controversial (Stevens and Wesseling, 1998, 1999; Wu and Borst, 1999; Weis et al., 1999).

Here, we propose a resolution of this issue by a detailed analysis of depletion and recovery of release-ready vesicles. The use of action potential-evoked EPSCs for assaying recovery from depletion is compromised by the fact that EPSC amplitude is a product of release probability P_r and the vesicle pool size, both of which may change with time (Stevens and Wesseling, 1999; Wu and Borst, 1999). Thus, we adopted an experimental design to separate the time course of release (P_r) and vesicle pool size by taking advantage of simultaneous whole-cell voltage clamp. In order to estimate release rates reliably, we used the deconvolution method adapted to the special condition of large glutamatergic synapses (Neher and Sakaba, 2001). In the experiments reported here, we confirmed that release probability is heterogeneous and that the releasable pool can be separated into fast and a slow releasing components (Sakaba and Neher, 2001). Such heterogeneity of release probabilities has been described also in other synapses (Walmsley et al., 1988; Hessler et al., 1993; Rosenmund et al., 1993; Dobrunz and Stevens, 1997; Huang and Stevens, 1997; Isaacson and Hille, 1997; Murthy et al., 1997). We further analyzed the recovery of the two components after full depletion. We found that slow vesicles recover rapidly, in agreement with Wu and Borst (1999). In addition, however, we found a Ca^{2+}-dependent acceleration of the recruitment of rapidly releasing vesicles, which is consistent with the mechanism suggested by Dittman and Regehr (1998), Stevens and Wesseling (1998), and Wang and Kaczmarek (1998). Furthermore, we showed that the Ca^{2+} sensor for this process is calmodulin. Thus, we can assign a distinct role in synaptic transmission to this important regulator of cellular mechanisms.

Results

Releasable Pool at the Calyx of Held

The ability to relate a given synaptic response to the number of vesicles available for release at the time of the stimulus is essential for the interpretation of the

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influences of experimental manipulations on synaptic mechanisms (Stevens and Wesseling, 1998; Schneggenburger et al., 1999; Wu and Borst, 1999). We, therefore, performed our studies at the calyx of Held synapse where pre- and postsynaptic compartments can be simultaneously whole-cell voltage clamped and the releasable pool of vesicles (RP) can be measured by applying presynaptic depolarizations strong enough to release the entire pool. AMPA receptor-mediated EPSCs were used to monitor transmitter release. We used the deconvolution method, which provides vesicle release rates by deconvolving EPSCs with mEPSCs (Figure 1). We have shown previously that the deconvolution method is valid when desensitization and saturation of postsynaptic AMPA receptors are blocked with cyclothiazide (CTZ; 100 μM) and kynurenic acid (1 mM) (Neher and Sakaba, 2001). It has been documented that CTZ has presynaptic effects on transmitter release (Diamond and Jahr, 1995; Bellingham and Walmsley, 1999; Ishikawa and Takahashi, 2001), but the effects have been shown to be negligible under our experimental conditions (Sakaba and Neher, 2001).

When a low concentration of EGTA (0.05 mM) was included in the presynaptic patch pipette and the presynaptic compartment was depolarized to −10 mV (or 0 mV) for 50 ms, two components of transmitter release were observed with time constants of 2.7 ± 1.2 ms (fast component, 66% ± 10% of the total RP, n = 5) and 10.7 ± 1.9 ms (slow component, Figure 1A). The total pool size was estimated to be 2409 ± 415 quanta (1623–3779 quanta). The pool size thus covered the range of previous estimates using the deconvolution method (Schneggenburger and Neher, 2000; Sakaba and Neher, 2001) and is at the lower limit of estimates from capacitance measurements (Sun and Wu, 2001). Separation of the two components was not always clear, however, and the data could sometimes be fitted with a single exponential. Thus, the separation of the response into a fast and a second slow component remained ambiguous under this condition, most likely because facilitation was developing in the course of the long-lasting depolarization. The time course of release was comparable to that reported previously using 0.05 mM BAPTA (Sakaba and Neher, 2001). In agreement with previous results (Borst and Sakmann, 1998, 1999; Cuttle et al., 1998; Forsythe et al., 1998), inactivation and facilitation of presynaptic Ca^{2+} currents was minor during this pulse protocol (Figure 1A).

The two components of transmitter release were more clearly separated when overlapping facilitation was blocked with 0.5 mM EGTA (Figure 1B) (Sakaba and Neher, 2001). Then, the time constants were 3.1 ± 0.2 ms (fast component, n = 11) and 30.5 ± 2.0 ms (slow component). The fast component was 50% ± 3% of the total RP, and the total pool size was estimated to be 3547 ± 341 quanta, while the pool size varied among different cell pairs (2040–5804 quanta). The two components are illustrated in Figure 1B as a biexponential rise in the plot of cumulative release versus time. We interpret them as representing two populations of vesicles with high and low P_r, respectively (Sakaba and Neher, 2001).

When a higher concentration of EGTA (5 mM) was introduced into the presynaptic terminal, the time course

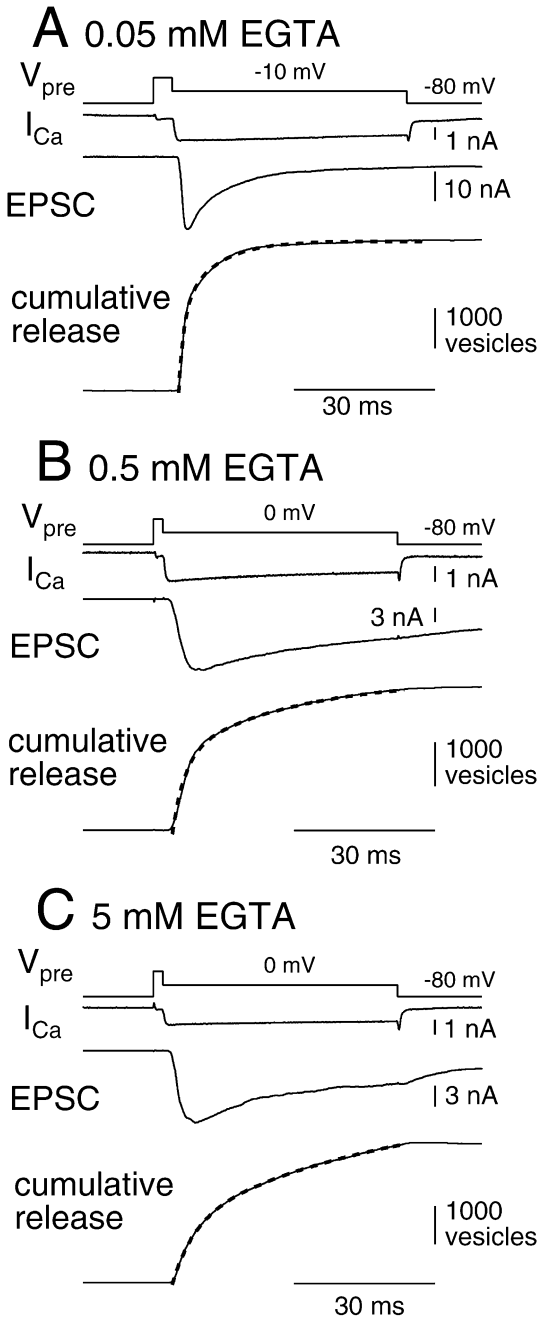

Figure 1. Time Course of Quantal Release at the Calyx of Held

The presynaptic terminal was depolarized from −80 to +70 mV for 2–4 ms and was then repolarized to −10 mV (or 0 mV) for 50 ms (V_{pre}) in order to elicit presynaptic Ca^{2+} current. This way an EPSC was evoked at the postsynaptic MNTB neuron, which was held at −80 mV. The extracelluar solution contained CTZ (100 μM) and Kyn (1 mM). Experiments using different concentrations of EGTA ([A], 0.05 mM; [B], 0.5 mM; and [C], 5 mM) in the presynaptic patch pipette are displayed. Cumulative release was fitted with a double exponential and was superimposed on the cumulative release (dotted line). In some cell pairs with 5 mM EGTA, the releasable pool could not be depleted and the data were fitted with a single exponential and a straight line. Time constants were 1.10 ms (44%) and 7.24 ms (A), 2.62 ms (52%) and 25 ms (B). In (C), the fast component could be fitted with τ = 4.63 ms.

of release of the fast component was also slowed (τ = 5.7 $\pm$ 1.0 ms, Figure 1C), consistent with a previously described effect of EGTA on evoked EPSCs (Borst and Sakmann, 1996). Five millimolar EGTA also slowed the release of the slow vesicle population, which, therefore, could not be fully depleted during a 50 ms depolarization (Figure 1C).

The experiments described here were mainly performed with 0.5 mM EGTA because under this condition fast and slow components can be well defined and the size of RP can be reliably estimated. These experiments will be referred to as the control condition.

Recovery from Depletion of the Releasable Pool of Synaptic Vesicles

We next examined the recovery of vesicle pool after depletion at the calyx synapse. Especially we were interested in whether the recovery of the two components differed. Recovery from vesicle depletion was monitored by applying pulse pairs at different interstimulus intervals (ISI), which is defined as an interval between the end of the first sequence to the beginning of the second sequence (Figure 2A). Enough time (45–60 s) was allowed between pairs to guarantee complete recovery. The fraction of recovered RP size and the relative contributions of fast and slow components, all normalized to the control RP size, were plotted against ISI in Figures 2B–2D. The time constants of the two components of the cumulative release, which reflect P_r, did not change significantly during the recovery phase (Figure 2A). The RP size recovered biexponentially (τ_1 = 196 ms [49%] and τ_2 = 4.5 s from the pooled data), confirming a previous report (Wu and Borst, 1999) and suggesting that recovery is mediated by at least two processes.

The previous study concluded that release probability decreased during replenishment (Wu and Borst, 1999). However, this conclusion was based on the assumption of a homogeneous pool of vesicles. Given our finding that P_r is heterogeneous at the calyx synapse (Figure 1), the apparent decrease in P_r described previously is likely to reflect a rapid recovery of the slowly releasing vesicles. Consistent with this expectation, a large fraction of the slow component was restored within 100–200 ms (Figure 2D). However, under the conditions of our measurement, the fast component also recovered quite rapidly (Figure 2C), 68% of the fast component being restored with τ = 360 ms and the rest with τ = 6.3 s (Figure 2C). This is in conflict with the expectation that it is this subpool of vesicles that is responsible for the slowly recovering EPSC following short trains of action potential-like stimuli (Wu and Borst, 1999; Weis et al., 1999). Therefore, an additional process has to be postulated in order to explain the rapid recovery of the fast vesicles seen here.

Smaller Ca^{2+} Influx Retards the Recovery of the Fast Component

A possible mechanism for the rapid recovery is presynaptic Ca^{2+} accumulation (Dittman and Regehr, 1998; Stevens and Wesseling, 1998; Wang and Kaczmarek, 1998) due to massive Ca^{2+} influx during the long-lasting stimulus. In that case, we have to postulate that Ca^{2+} entering the cell during the first pulse in a pair was sufficient to saturate most of the EGTA present in the presynaptic terminal, leading to a long-lasting rise in intraterminal [Ca^{2+}]. According to Helmchen et al. (1997), an action potential increases average [Ca^{2+}] in the terminal by 400 nM at an endogeneous Ca^{2+} binding ratio of 40. Thus, an equivalent of 16 μM of Ca^{2+} enters the terminal, which is the result of a Ca^{2+} charge, displaced during an action potential (AP), of 1 pC. The depolarization of Figure 2A induced a Ca^{2+} influx of approximately 60 pC, which can saturate accordingly 960 μM of high-affinity buffer. Therefore, 0.5 mM EGTA, which is present in the terminal, will be saturated, and [Ca^{2+}] will be significantly higher than the dissociation constant of EGTA at the end of a 50 ms depolarization in Figure 2. In the case of Figure 2A the data of Helmchen et al. (1997) would predict that [Ca^{2+}] overshoots and returns to values around 0.5 μM within several hundred milliseconds. During that time, the fast-releasing pool would recover rapidly. If this was the case, less Ca^{2+} influx should result in slower recovery.

To test this expectation, a shorter pulse (10 ms), which is long enough to deplete the fast component only, was applied, and a test pulse (50 ms) was applied to monitor recovery from partial depletion (Figure 3A). With only 10 ms of depolarization ($\approx$12 pC of Ca^{2+} influx in Figure 3A), free [Ca^{2+}] should be relatively well or only partially buffered. In the case of Figure 3A we expect [Ca^{2+}] to be in the range of the dissociation constant K_D of EGTA ($\approx$176 nM) or lower at the end of the depolarizing pulse. Recovery of the fast component was much slower than in the case of Figure 2 (a single exponential with τ = 6.7 s was obtained; pooled data from six cell pairs, Figure 3B). The amplitude of the slow component was almost constant during the recovery from partial depletion (Figure 3B) after an initial very rapid recovery.

In a similar experiment on the fusion of large, dense core vesicles at chromaffin cells, a decrease in the amplitude of a slow release component was observed during the recovery of the fast component (Voets et al., 1999). This was taken as an indication that the fast pool draws upon the slow one during recovery. The absence of such a decrease in the data reported here may suggest that recovery of the two vesicle populations is mediated by parallel mechanisms. In fact, constancy of the pool of slowly releasing vesicles during large changes in the pool of rapidly releasing ones (Figure 3), as reported here, indicates that the two pools do not share a common limiting resource, such as "docking" sites.

High Concentration of EGTA Slows the Recovery of the Fast Component

In order to confirm that presynaptic Ca^{2+} mediates rapid refilling of the fast component, we drastically increased the concentration of EGTA, a slow Ca^{2+} chelator, by infusion through the presynaptic patch pipette (n = 9 cell pairs, Figure 4A). As we show in Figure 1C, 5 mM EGTA, which is high enough to prevent a rise in [Ca^{2+}], slowed the time course of release of the fast component and also slowed the release of the slow vesicle population, which, therefore, could not be fully depleted during a 50 ms depolarization (Figure 4A). In order to study the recovery of the fast component (which was fully

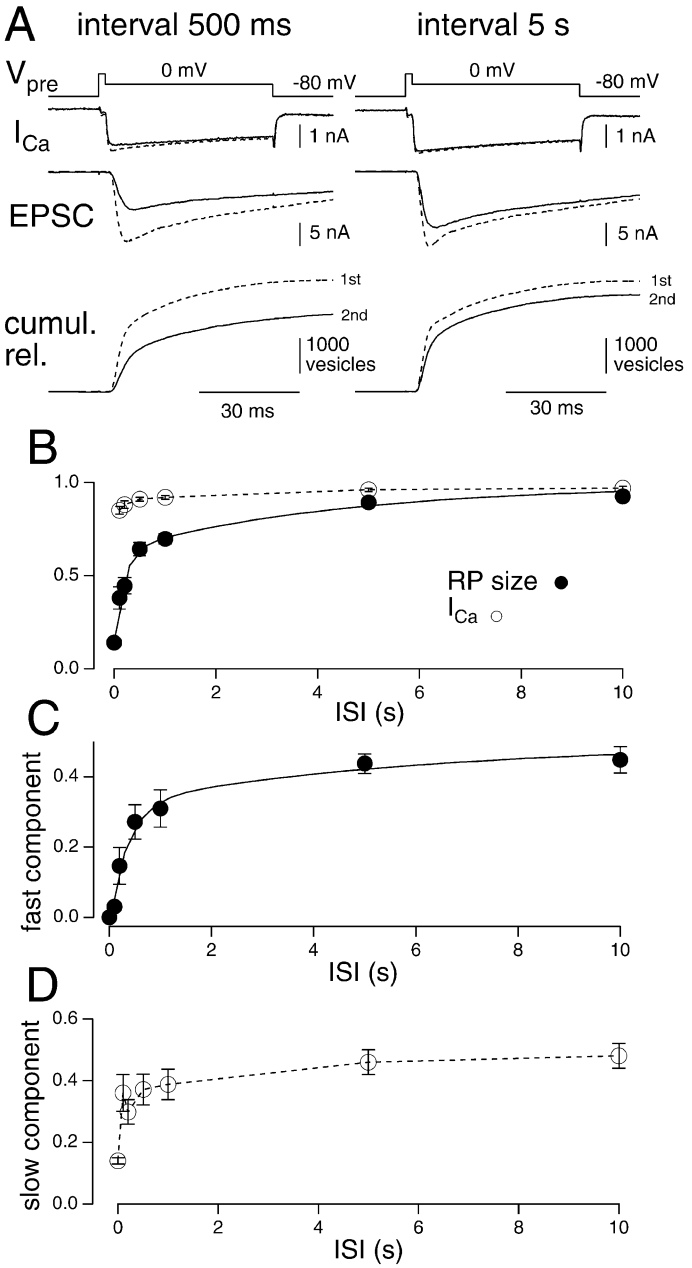

Figure 2. Recovery from Depletion of the Releasable Pool of Vesicles at the Calyx of Held

(A) The dual pulse (0 mV for 50 ms after predepolarization to +70 mV for 2 ms) was applied at different interpulse intervals (left, 500 ms; right, 5 s), and responses evoked by the first and the second pulses were shown as dotted and continuous traces, respectively. From EPSCs, quantal release rates were estimated by the deconvolution method (Neher and Sakaba, 2001), and cumulative release was calculated by integrating release rates. Intervals between pairs of pulses were 45–60 s.

(B) Dual pulses with different intervals, as shown in (A), were applied and the fraction of the RP during the second pulse (relative to that of the first pulse) was plotted against the ISI (filled circles, relative units). Also, the ratio of the presynaptic Ca^{2+} current amplitudes was plotted (open circles, relative units). The recovery time course of the RP was fitted with a double exponential.

(C) The amount of the fast component during the second pulse, which was estimated by fitting cumulative release curves as shown in (A) with a double exponential. Values are normalized to the total RP size during the first pulse and are plotted against the ISI. The data was fitted with a double exponential.

(D) The same as (C), but recovery of the slow component was plotted. The first four points in parts (B), (C), and (D) were taken at 0, 0.1, 0.2, and 0.5 s.

depleted at the end of a 50 ms depolarization), we calculated the ratio between its amplitude during the second and the first pulse in a given pair. In the control condition (0.5 mM EGTA), this ratio could be fitted with a double exponential with $\tau = 379$ ms (69%) and 7.14 s (Figure 4B). Five millimolar EGTA slowed the recovery of the fast component, and the recovery could be fitted with a single exponential with $\tau = 9.1$ s. Thus, clamping presynaptic [Ca^{2+}] to very low levels slows down the recovery of the fast component and yields time constants of recovery slower than those obtained during short trains of nerve stimulation (von Gersdorff et al., 1997; Weis et al., 1999). Slowing of the recovery could be reversed by eliciting larger presynaptic Ca^{2+} influx with a longer prepulse (>100 ms, data not shown).

Because EGTA binds to Ca^{2+} with a slow on-rate, it has been assumed that EGTA affects only global [Ca^{2+}] without affecting domain [Ca^{2+}] close to Ca^{2+} channels, which triggers phasic release (Adler et al., 1991; Delaney et al., 1991; Swandulla et al., 1991). However, high concentrations of EGTA affect transmitter release at the calyx of Held and at other CNS synapses (Borst and Sakmann, 1996; Ohana and Sakmann, 1998; Rozov et al., 2001) where Ca^{2+} channels and release sites are not strictly colocalized (Bollmann et al., 2000; Schneggenburger and Neher, 2000). In order to discriminate whether domain Ca^{2+} or global Ca^{2+} is responsible for rapid recruitment, we set global [Ca^{2+}] to 500 nM by introducing 20 mM EGTA/15 mM Ca^{2+} (free EGTA = 5 mM) into the terminal (Figure 4B). As has been shown before (Borst and Sakmann, 1996), a mixture of EGTA and Ca^{2+} did not change the kinetics of evoked release,

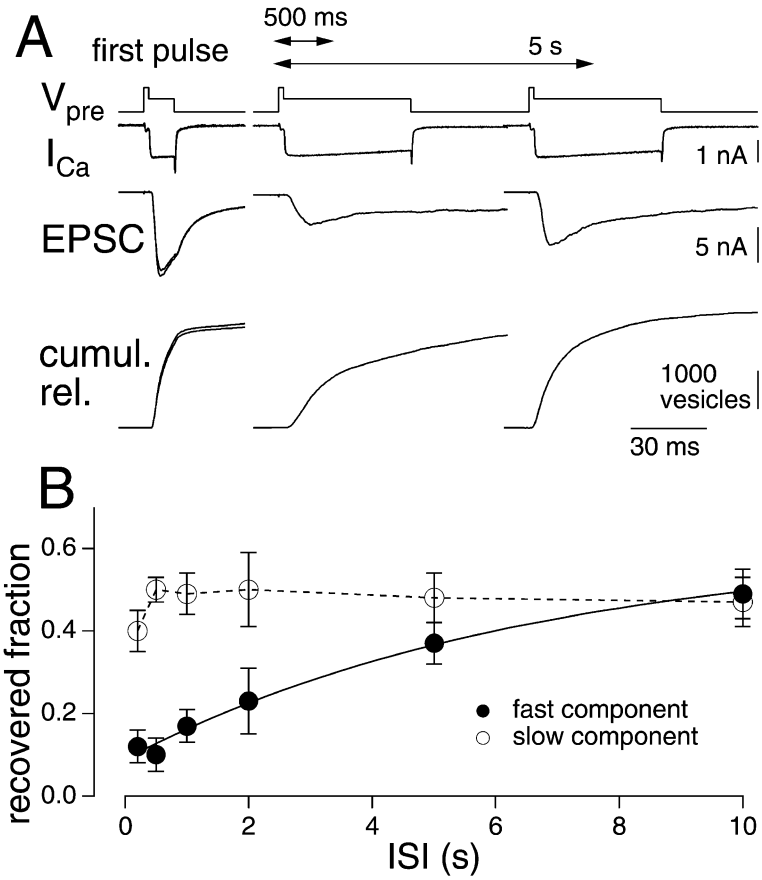

Figure 3. Recovery of the Fast Vesicle Pool after Partial Depletion of Vesicles

(A) A short depolarizing pulse (to 0 mV for 10 ms after 2 ms pulse to +70 mV) was applied and a test pulse (50 ms pulse to 0 mV) was applied at different intervals (500 ms and 5 s). The duration of the prepulse (10 ms) was chosen to deplete the fast vesicle pool (τ = 3 ms) without releasing the majority of slow vesicles.

(B) The amplitudes of the fast (filled circle) and the slow components (open circle) during the recovery phase, normalized to the control RP size, are plotted. Recovery of the fast component is fitted with a single exponential.

as compared to 5 mM EGTA alone (the fast component released with τ = 5.94 ± 1.74 ms, n = 10 cell pairs). In contrast, recovery of the fast component of release was accelerated, and the relative amplitude of the fast component could be fitted with a single exponential with τ = 3.4 s (Figure 4B). Therefore, the result shows that global Ca^{2+} of ≈ 0.5 μM, which is much lower than domain Ca^{2+} (10–25 μM) (Bollmann et al., 2000; Schneggenburger and Neher, 2000), can enhance the recovery process.

Calmodulin Mediates the Fast Recovery of the Fast-Releasing Vesicles

One of the known proteins that can be activated by submicromolar [Ca^{2+}] levels is calmodulin (100 nM to 10 μM) (Chin and Means, 2000). To examine whether calmodulin is involved in rapid refilling of the fast component, we introduced a calmodulin binding domain (CAM kinase II 290–309, 200–400 μM) (Yazawa et al., 1992), a potent peptide calmodulin inhibitor, into the terminal (Figure 5A, n = 8 cell pairs). Otherwise, conditions were the same as those of Figure 2 (0.5 mM EGTA), in which the fast component recovered rapidly. The presynaptic Ca^{2+} current amplitudes seemed unchanged; I_{Ca} was 1454 ± 70 pA under control and 1490 ± 53 pA in the presence of the calmodulin binding domain peptide. More importantly, the ratio of the presynaptic Ca^{2+} current amplitudes was unaffected (Figure 5B). Ca^{2+} channels are known to be modulated by calmodulin, but tight and constitutive interaction of Ca^{2+} channels with calmodulin probably makes them insensitive to pharmacological calmodulin inhibitors (Lee et al., 1999; Peterson et al., 1999; Zühlke et al., 1999; DeMaria et al., 2001). While recovery of the slow component was not affected (Figure 5E), recovery of the fast component was strongly retarded (Figure 5D) with a slight slowing of recovery of the total RP size (Figure 5C). The rapid recovery phase of the fast component (several hundred milliseconds) was eliminated, and the recovery could be fitted with a single exponential with τ = 4.6 s (Figure 5D), whereas the kinetics of the fast component during the first pulse was unchanged (τ = 2.8 ± 0.3 ms versus 3.1 ± 0.2 ms in the control). Similar effects were also observed with MLCK (myosin light chain kinase) peptide (20 μM), another peptide inhibitor (Török and Trentham, 1994). In that case, the recovery of the fast component could be fitted with a single exponential with a time constant of 5 s (n = 6, Figure 5D). Extracellular application of calmidazolium (20 μM), an organic blocker of calmodulin, reduced the recovery. At a 1 s interpulse interval, the fast component was 13% ± 3% of the normal total RP size (n = 5, Figure 5). Although this drug is less effective than peptide, the result is consistent with the notion that calmodulin mediates the rapid recovery.

Activation of CAM kinase II augments transmitter release and is involved in presynaptic plasticity (Llinas et al., 1991; Silva et al., 1992; Augustine et al., 1994; Salin et al., 1996). We tested the effects of CAM kinase II on recovery by applying its potent inhibitor AIP (10 μM), but rapid refilling of the fast component was not altered (n = 8 cell pairs).

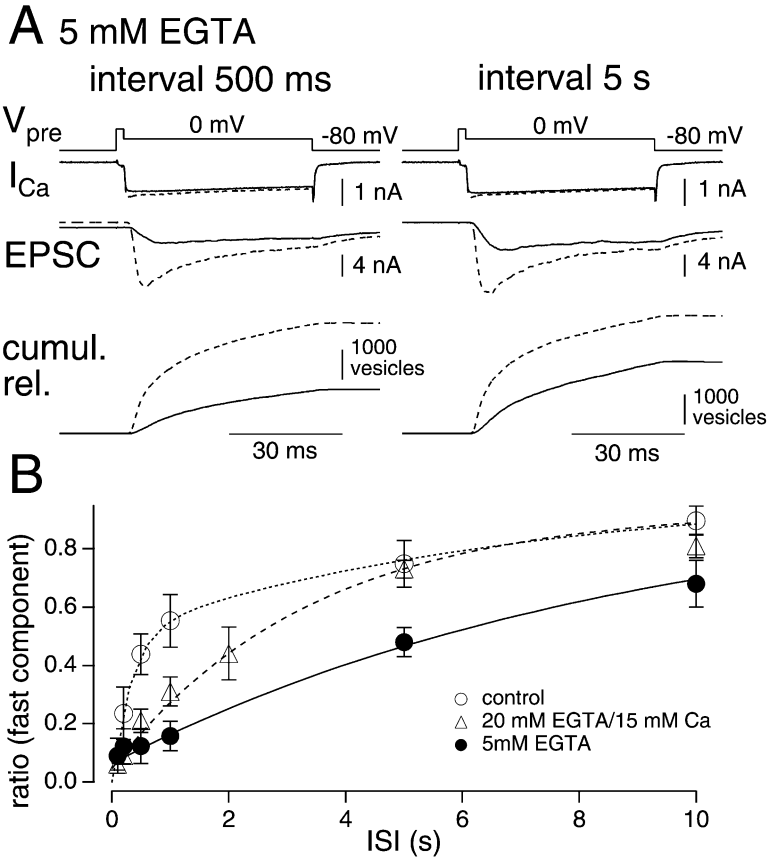

Figure 4. Effect of High EGTA and EGTA/Ca^{2+} on the Recovery of the Fast Vesicle Pool

(A) The presynaptic terminal was infused with 5 mM EGTA, and dual pulses at different intervals (left, 500 ms; right, 5 s) were applied as shown in Figure 1. Dotted and continuous traces show responses during first and second pulses, respectively.

(B) The amplitude of the fast component during the second pulse was divided by that during the first pulse, and this ratio was plotted against the stimulus interval. Open circles, filled circles, and triangles show the control case (0.5 mM EGTA), 5 mM EGTA, and 20 mM EGTA/15 mM Ca, respectively. Recovery was fitted with a single (5 mM EGTA, 20 mM EGTA/15 mM Ca) or a double (control) exponential.

Role of Calmodulin on Recovery from Synaptic Depression Induced by Action Potential-like Stimulation

We have shown that two kinetically distinct refilling processes exist at the calyx of Held synapse: (1) slow recovery of a fast vesicle pool, which can be accelerated in a Ca^{2+}/calmodulin-dependent manner, and (2) rapid recovery of a slow vesicle pool. In order to learn how the calmodulin-mediated process contributes to the recovery from synaptic depression (von Gersdorff et al., 1997; Wang and Kaczmarek, 1998; Wu and Borst, 1999; Weis et al., 1999), we applied pairs of trains of short depolarizations (+40 mV for 1 ms, 300 Hz for 100 ms) to the presynaptic terminal at different intervals between trains in a given pair (Figure 6A). In order to mimic more realistic stimulus conditions, the presynaptic patch pipette was filled with 0.05 mM EGTA, and CTZ and Kyn were omitted from the extracellular solution. A single depolarization elicited Ca^{2+} influx of 1.5–2 pC (control, 1.52 ± 0.11 pC; calmodulin binding domain, 1.99 ± 0.41 pC), which is 50 to 100% larger than that elicited by a single action potential (0.9–1 pC) (Borst and Sakmann, 1996; Helmchen et al., 1997). The EPSC size varied (380 pA to 14.5 nA, mainly 1.5–6 nA). Variability of the synaptic response has been documented at this synapse (von Gersdorff et al., 1997; Taschenberger and von Gersdorff, 2000). The average EPSC size (3.52 ± 0.75 nA) was within the range of the synaptic responses that have been reported in the rat calyx of Held (5.3 nA, Borst and Sakmann [1996]; 3 nA, von Gersdorff et al. [1997]; 1.5 nA, Chuhma and Ohmori [1998]; and 10 nA, Taschenberger and von Gersdorff [2000]).

Synaptic responses displayed strong depression during the first train (Figure 6A, control; see dotted line in the right panel for an expanded trace), due to depletion of the vesicle pool (Wu and Borst, 1999) and desensitization of postsynaptic receptors (Neher and Sakaba, 2001). The depression resembled that induced by fiber stimulation (Taschenberger and von Gersdorff, 2000). With an interval of 500 ms, half of the response was recovered (Figure 6A, control, continuous line). This amount is comparable with that observed by Wang and Kaczmarek (1998) in the mouse calyx of Held. Addition of calmodulin binding domain peptide to the presynaptic patch pipette reduced the response during the second stimulation without affecting the response during the first stimulation. For analysis we integrated EPSCs during each stimulation period. Ratios of the response during the second over the first trains were plotted against the stimulation interval in Figure 6B (control, $n = 7$ cell pairs; calmodulin binding domain, $n = 8$ cell pairs). The recovery of the response was more variable among different cell pairs as compared to experiments using the pulse protocol of Figures 2–5. Probably, not only did the variability of the refilling process contribute to the synaptic response but so did that of other processes, such as the degree of desensitization of postsynaptic receptors. Nevertheless, in the control condition, recovery of the response could be fitted by double exponentials with $\tau = 316$ ms (62%) and 6.6 s on average (Figure

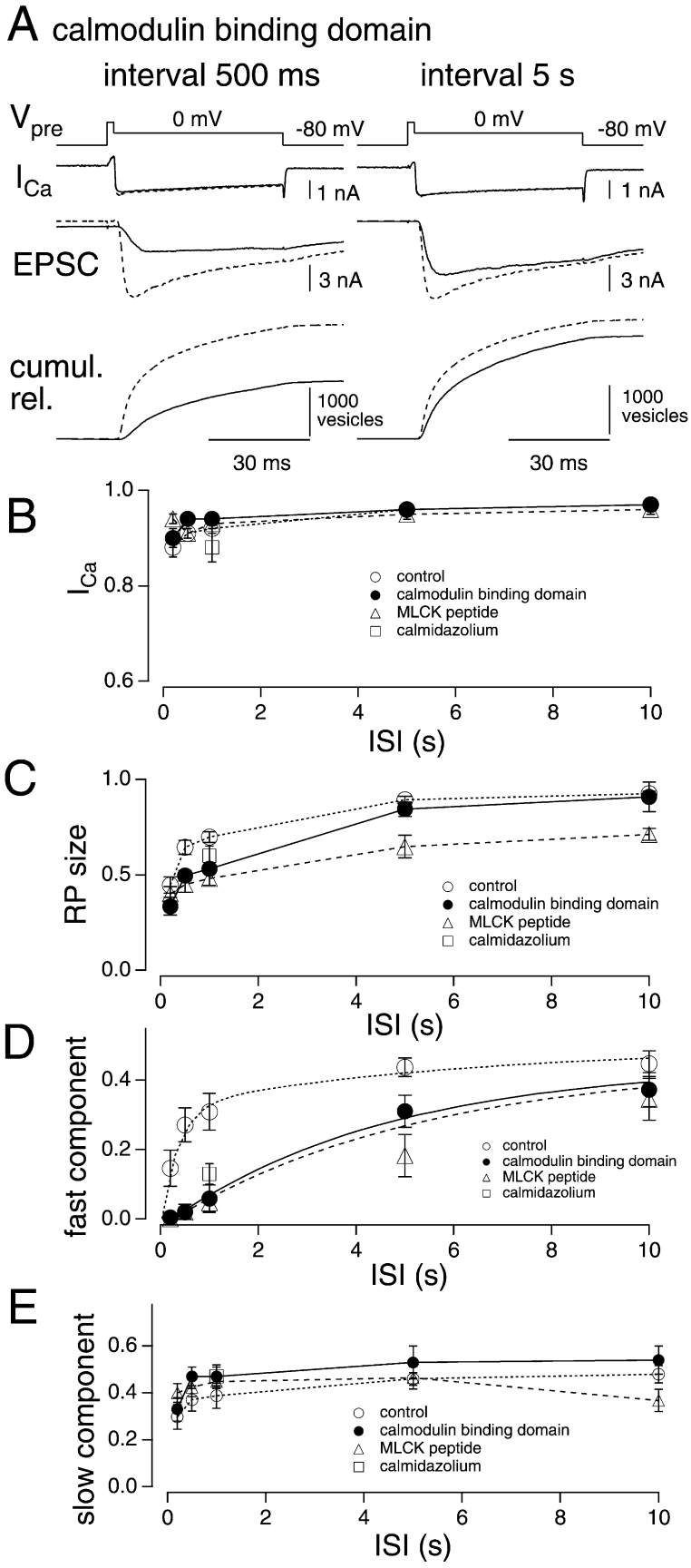

Figure 5. Calmodulin Inhibitors Block Rapid Refilling of the Fast Vesicle Pool

(A) A calmodulin binding domain peptide was introduced via a presynaptic patch pipette, and the same protocol, as shown in Figure 1 was applied. Dotted and continuous traces show responses during first and second pulses, respectively.

(B–E) Recovery time courses of the presynaptic Ca^{2+} current amplitudes (B), the total RP size (C), the fast component (D), and the slow component (E) were plotted against the stimulus interval. All data were normalized to the value (Ca^{2+} current or total RP size) obtained during the first pulse. Open circles, filled circles, triangles, and a square are from control, calmodulin binding domain, MLCK peptide, and calmidazolium (ISI = 1 s), respectively. The time courses were fitted with a single (when blockers were present) or double exponential (control).

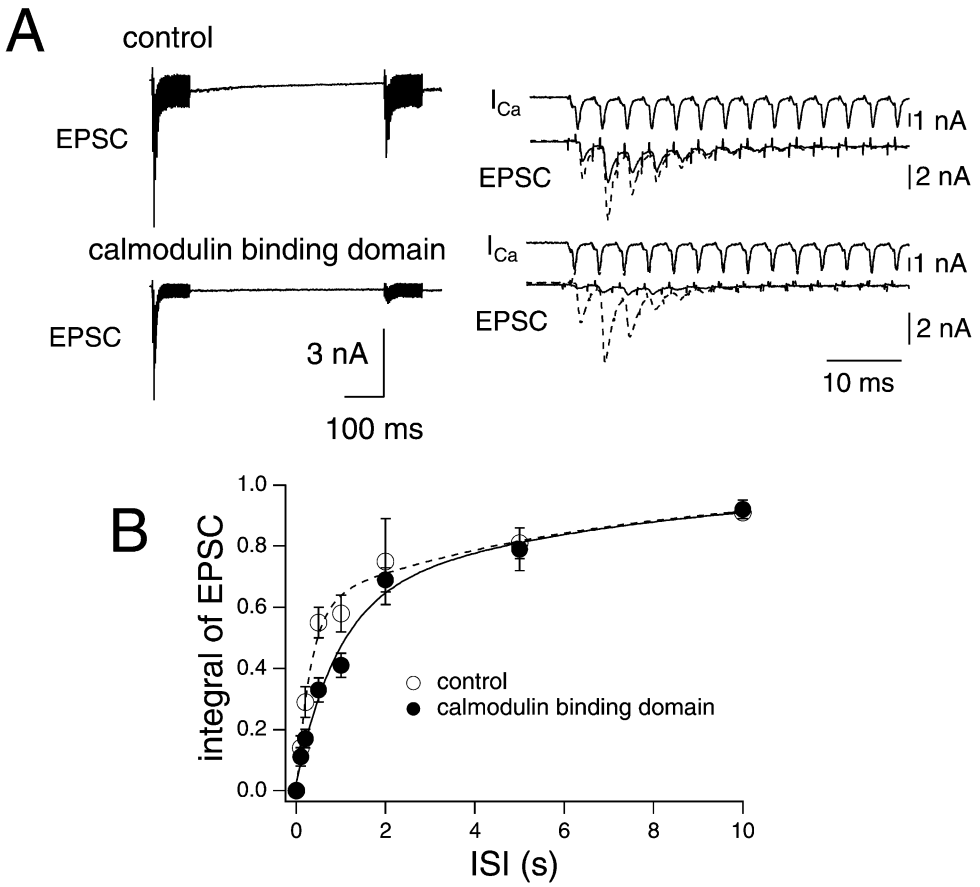

Figure 6. Recovery from Synaptic Depression and the Effect of a Calmodulin Blocker

(A) A short depolarizating pulse (+40 mV for 1 ms) was applied repetitively (300 Hz for 100 ms). After an interval of 500 ms, the same train was applied once more (left panel). Experiments were done without CTZ or Kyn, and examples in the control condition (upper traces) and in the presence of calmodulin binding domain (lower trace) are shown. In right panel, presynaptic calcium currents (I_{Ca}) and postsynaptic EPSCs are shown at expanded time scale. Dotted and straight lines show traces obtained from the first and the second trains, respectively. Note that I_{Ca} during the first and second trains were hardly distinguishable.

(B) Integral of EPSCs during the first and the second trains were calculated, and the ratio between the two was plotted against the stimulus interval. Dotted and filled circles represent the data from the control (from seven cell pairs) and in the presence of the calmodulin binding domain (from eight cell pairs). Data were fitted with a double exponential.

6B, open symbols). In the presence of the calmodulin binding domain peptide, the recovery was fitted by a double exponential with τ = 901 ms (59%) and 6.9 s (Figure 6B, filled symbols). Thus, a calmodulin-dependent component of recovery does contribute to recovery from synaptic depression, at least in the condition we examined. The remaining rapid phase of recovery is likely to be mediated by rapid recovery of slow vesicles, which are facilitated during high-frequency stimulations (Wu and Borst, 1999). It should be noted, however, that recovery from desensitization of postsynaptic AMPA receptors might also contribute to recovery from synaptic depression examined under the experimental conditions employed in Figure 6 (Trussell et al., 1993; Otis et al., 1996; Neher and Sakaba, 2001).

Discussion

We have carried out a detailed analysis of vesicle depletion and its recovery at the calyx of Held. Consistent with previous results (Sakaba and Neher, 2001), we found a releasable pool consisting of thousands of vesicles (1600–5800) and showed that release probability is heterogeneous: close to 50% of the total vesicle pool can be released rapidly (within a few milliseconds), whereas the rest is released more slowly (Figure 1). Recovery of the slow vesicles is very fast (τ = 200 ms) irrespective of the stimulus patterns we used (Figure 2). Recovery of the fast vesicles, however, is accelerated by the amount of Ca^{2+} accumulation (Figures 2–4), an effect that can be blocked by calmodulin inhibitors (Figure 5). This calmodulin-dependent component contributes to recovery from synaptic depression, in concert with other mechanisms (Figure 6).

The Releasable Pool at the Calyx of Held

Consistent with previous results using the deconvolution approach (Schneggenburger and Neher, 2000; Sakaba and Neher, 2001), we confirmed that there is a large vesicle pool (2000–4000 vesicles) at the calyx of Held (Figure 1). Recent estimates from capacitance measurements at this synapse gave estimates of 5000

vesicles (Sun and Wu, 2001). Reasons for differences between the two estimates have not been identified yet, but both methods have limitations. The deconvolution method relies on the exact estimation of the residual current (mediated by delayed clearance of glutamate), and errors are most serious at the end of depleting pulses where release rates are low and residual currents are large (Neher and Sakaba, 2001). An increase in capacitance may arise not only from glutamate release but also by other mechanisms (Xu et al., 1998a), and the calibration of the capacitance method relies on synaptic vesicle diameters that are compromised by possible shrinkage effects during fixation. Sun and Wu (2001) also correlated capacitance jumps with the peak EPSC amplitude and concluded that the EPSC is a reliable measure of release only in the presence of CTZ and Kyn. Although this conclusion is consistent with our previous results from fluctuation analysis (Neher and Sakaba, 2001), we have to stress that the EPSC amplitude is a good measure for exocytosis only if the release time course is much faster than the mEPSC decay ($<$1 ms without CTZ, and $<$3 ms with CTZ). Otherwise, the peak amplitude is determined by both the release rates over a narrow time window and the mEPSC decay (van der Kloot, 1988; Neher and Sakaba, 2001). It should also be noted that the pool size varies considerably among different cell pairs (1600–5800 quanta in this study and 800–4500 in Schneggenburger and Neher, 2000; Sakaba and Neher, 2001). This may be due to intrinsic variability between synapses or due to developmental changes in pool size (Taschenberger and von Gersdorff, 2000; Iwasaki and Takahashi, 2001).

Two Components of Quantal Release

In our experiments employing 0.5 mM EGTA, two components with characteristic release rates were observed, which were the same in control and during recovery (Figure 1, see also Sakaba and Neher, 2001). The most parsimonious explanation for this finding is that vesicles can exist in two states, with different, but constant Ca^{2+} sensitivities and that the presence of EGTA assures that [Ca^{2+}] at the release sites stays constant over most of the stimulation interval. Nevertheless, we find that refilling is accelerated following a 50 ms stimulation, which suggests that [Ca^{2+}] escapes control by EGTA toward the end of such long pulses. This notion is consistent with estimates regarding Ca^{2+} buffer capacity and Ca^{2+} fluxes (see Results). It suggests that the rapid phase of refilling of fast vesicles under the control condition (Figure 2C) is curtailed by the return of [Ca^{2+}] toward baseline. When experiments are performed with low EGTA and facilitation is allowed to develop, the two components of release fuse, although it is still possible to fit the overall time course with a double exponential in many cases. Recent capacitance measurement (Sun and Wu, 2001) only resolved one component, but this is probably due to overlapping facilitation, since very low Ca^{2+} buffering was used in this study. In many synapses, some heterogeneity of release probabilities have been described (Walmsley et al., 1988; Hessler et al., 1993; Rosenmund et al., 1993; Dobrunz and Stevens, 1997; Huang and Stevens, 1997; Isaacson and Hille, 1997; Murthy et al., 1997), and such heterogeneity may be mediated by common mechanisms, such as different biochemical states of synaptic vesicles (Xu et al., 1998a) or the degree of colocalization of Ca^{2+} channels and synaptic vesicles (Voets et al., 1999; Rozov et al., 2001). This issue should be further resolved by biochemical and genetic manipulations of the refilling processes.

Calcium-Dependent Recovery from Vesicle Depletion

Recently, it has been suggested that Ca^{2+} stimulates recruitment of synaptic vesicles to the releasable pool (Dittman and Regehr, 1998; Stevens and Wesseling, 1998; Wang and Kaczmarek, 1998). These three reports used different preparations and different experimental protocols; Dittman and Regehr (1998) observed rapid recovery (order of 100 ms) from synaptic depression in the climbing fiber to Purkinje cell synapse. Stevens and Wesseling (1998) determined the releasable pool in the hippocampal culture preparation by application of high concentrations of sucrose. They found that refilling of synaptic vesicles to the readily releasable pool (order of seconds) was accelerated by electrical stimulation. At the calyx of Held synapse, Wang and Kaczmarek (1998) observed enhanced recovery (order of 100 ms or less) from synaptic depression induced by high-frequency nerve stimulation (300 Hz). All three studies further showed that EGTA, a slow Ca^{2+} buffer, blocked the enhanced recovery.

Subsequently, the mechanism of Ca^{2+}-dependent recovery was questioned for the calyx of Held by Wu and Borst (1999), who suggested that vesicles recover rapidly after depletion but initially have very low release probabilities. Slow maturation from the slowly to a rapidly releasing type would determine the time course of responses to action potential-like stimuli. The analysis of Wu and Borst (1999), however, was based on the measurement of EPSC amplitudes and did not quantify a late component of transmitter release, which can only be resolved by deconvolution of postsynaptic currents. Our analysis with deconvolution confirms their suggestions and further demonstrates that rapidly recovered vesicles are those that intrinsically have low release probability (rapid recovery of the slow component). Thus, we showed that release probability does not increase uniformly for all synaptic vesicles during the recovery phase. Rather, the apparent maturation of vesicles is due to a slow recovery of rapidly releasing vesicles (Figure 2) or else a slow conversion of slowly releasing vesicles to rapidly releasing ones as discussed by Wu and Borst (1999). In addition, we found that an elevation of global [Ca^{2+}] stimulates this process (Figures 3–5), in contrast to Wu and Borst (1999). In this respect, the result is consistent with the mechanism suggested by Dittman and Regehr (1998), Stevens and Wesseling (1998), and Wang and Kaczmarek (1998).

The present results also bear on the conclusions of Weis et al. (1999), who studied synaptic depression during 10 Hz fiber stimulation at the calyx of Held synapse. They found that recovery from depression was hardly influenced by manipulation of the Ca^{2+} signal with buffers and concluded that this was inconsistent with a simple model of Ca^{2+}-dependent vesicle recruitment,

although such a model faithfully represented both time course and steady-state responses during 10 Hz stimulation. However, in the case of Weis et al. (1999), [Ca^{2+}] decayed from a level of 400 nM with a time constant of about 0.7 s. According to the data of Figure 4 this should result in only little Ca^{2+}-dependent recovery of the fast vesicles. Rather, the main component of fast release is expected to recover with a time constant of several seconds, as found experimentally (Weis et al., 1999). In fact, strong stimulation (for example 300 Hz in the case of mouse calyx of Held [Wang and Kaczmarek, 1998]) is necessary to induce Ca^{2+}-dependent rapid recovery. The discrepancies between the expectations of the model and experimental data, pointed out by Weis et al. (1999), are most likely a consequence of the fact that the model considered only a single class of vesicles.

Although we exclude the possibility that P_r of all vesicles increases uniformly during the recovery phase, it is possible that fast-releasing vesicles have to transit through the slow-releasing vesicle pool during the recovery phase (serial pool scheme). In order to demonstrate such a maturation process, partial depletion experiments, as shown in Figure 3, might provide some clues. Similar types of experiments in adrenal chromaffin cells showed a decrease in a slow component of release while a fast component recovered from depletion, and this was taken as evidence that granules of the slow variety get converted into fast ones (Voets et al., 1999). However, at the calyx of Held, this could not be observed, and the slow component stayed constant while the fast one recovered. This rather suggests that refilling of the fast and the slow components are two processes proceeding in parallel, and it is possible that they represent "different synapses" located in the same large presynaptic terminal. It is also possible that a maturation step from slow to fast vesicles exists but does not influence the slow pool to any measurable extent because the latter is in rapid equilibrium with a reserve pool.

Calmodulin-Dependent Component of Recovery from Vesicle Depletion

It has been shown that calmodulin mediates facilitation and inactivation of Ca^{2+} channels (Lee et al., 1999; Peterson et al., 1999; Zühlke et al., 1999, DeMaria et al., 2001), which may be involved in short-term synaptic plasticity (Borst and Sakmann, 1998, 1999; Cuttle et al., 1998; Forsythe et al., 1998). However, the role of calmodulin on short-term plasticity mediated by exocytotic processes has not yet been fully understood (Augustine et al., 1994; Kamiya and Zucker, 1994; Salin et al., 1996). We did not find a significant change in Ca^{2+} currents upon application of calmodulin inhibitor peptides (Figure 5). Ca^{2+} channels are known to be modulated by calmodulin, but phrmacological calmodulin inhibitors may not be effective because of tight and constitutive interaction of Ca^{2+} channels with calmodulin (Lee et al., 1999; Peterson et al., 1999; Zühlke et al., 1999; DeMaria et al., 2001). It is also possible that the presynaptic Ca^{2+} channels from P8–P11 rats (N, P/Q, and R types) (Iwasaki and Takahashi, 1998; Wu et al., 1998, 1999) might be calmodulin insensitive. Instead of modulating Ca^{2+} channels, we found that an increase in global [Ca^{2+}], as it occurs during more intense stimulation (Figures 2–6), accelerates refilling of fast-releasing vesicles (Wang and Kaczmarek, 1998), which is mediated by calmodulin. This may be similar to the case of hippocampal cultures (Stevens and Wesseling, 1998), while the recovery time course is faster at the calyx synapse (hundreds of milliceconds versus the order of seconds). It is unlikely that the effect on recovery is a consequence of some remaining facilitation (in spite of the presence of EGTA), because the amount of release, but not its kinetics, is changed during the rapid refilling. This is opposite to the case of facilitation (Zucker, 1973; Kamiya and Zucker, 1994; Sakaba and Neher, 2001). Also, we did not see any effect of calmodulin blockers on facilitation (our unpublished data; see also Figure 6) (Kamiya and Zucker, 1994) and on the time constants of individual components (Figure 5). Thus, calmodulin has a well-defined role in short-term synaptic plasticity, which is distinct from the Ca^{2+}-triggering step in the release process. In this sense, synaptic release seems to be quite different from Ca^{2+}-dependent vacuole fusion (Peters and Mayer, 1998; Peters et al., 2001), where calmodulin is postulated to constitute the sensor for the triggering of fusion. Molecular targets of calmodulin are diverse and contain for example, Rab3A, Munc13-1, Pollux, CRAG, and synaptobrevin (Park et al., 1997; Xu et al., 1998b; Quetglas et al., 2000; Betz, A. and Brose, N., personal communication), proteins that are thought to be involved in vesicle cycling and release. In addition, calmodulin may interact with other cellular signaling pathways, such as adenylate cyclase and myosin light chain kinase (Weisskopf et al., 1994; Huang et al., 1994; Ryan, 1999).

Because residual Ca^{2+} elevates to about 1 μM during high-frequency stimulation (Helmchen et al., 1997), it is likely that the rapid refilling contributes to recovery from high-frequency stimulations. Figure 6 confirms this expectation. While the time constant of rapid recovery slowed down about 3-fold in the presence of calmodulin blockers, a considerable amount still recovered rapidly; half of the response recovered within 1–2 s. Most likely, this component is mediated by the rapid recovery of slow vesicles, which becomes available through facilitation (Figure 2) (Wu and Borst, 1999). The relative contributions of the two mechanisms of rapid recovery are likely to change depending on the stimulus pattern; it seems that high-frequency stimulation (for example, 300 Hz) is necessary to induce a significant amount of calmodulin-dependent recovery at the calyx of Held (Wang and Kaczmarek, 1998; Weis et al., 1999). It is also possible that recovery from desensitization of postsynaptic AMPA receptors mediates part of the rapid recovery from synaptic depression. Although the time constant of this recovery process is much faster ($<$100 ms) (Raman and Trussell, 1995), one has to take into account the delayed clearance of glutamate from the synaptic cleft (Otis et al., 1996; Neher and Sakaba, 2001). Nevertheless, the rapid refilling, in combination with facilitation (Wu and Borst, 1999) and a large vesicle pool, provide for the maintenance of synaptic transmission during high-frequency action potential trains. Clarifying the relative contribution of these pre- and postsynaptic mechanisms needs further study (Dittman et al., 2000) since precise knowledge of the mechanisms underlying short-term plasticity is an indispensable prerequisite for un-

derstanding the dynamic behavior of neuronal assemblies (Abbott et al., 1997; Tsodyks et al., 2000; Hopfield and Brody, 2001).

Experimental Procedures

Electrophysiology

Transverse brainstem slices (150–200 μm thick) were prepared from 8- to 11-day-old wistar rats (Sakaba and Neher, 2001). The standard extracellular solution contained (in mM): 125 NaCl, 2.5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 25 glucose, 25 $NaHCO_3$, 1.25 NaH_2PO_4, 0.4 ascorbic acid, 3 myoinositol, and 2 Na-pyruvate (pH 7.4) gassed with 95% O_2, 5% CO_2. During recordings, 0.5 μM TTX, 10 mM TEA-Cl, and 50 μM D-AP5 were added to isolate presynaptic Ca^{2+} currents and postsynaptic AMPA receptor-mediated EPSCs, which were used to assay transmitter release rates. In most experiments (except for Figure 6), cyclothiazide (100 μM) and Kyn (1 mM) were added to block desensitization and saturation of AMPA receptors. A calyx of Held and the postsynaptic MNTB principal neuron were whole-cell clamped at −80 mV with an EPC9/2 amplifier (HEKA, Germany). The presynaptic patch pipette (3–5 MΩ) solution contained (in mM): 135 Cs-gluconate, 20 TEA-Cl, 10 HEPES, 5 Na_2-phosphocreatine, 4 MgATP, 0.3 GTP, and 0.5 EGTA (pH 7.2); 0.5 mM EGTA was used in order to block facilitation. In some experiments, the concentration of EGTA was changed (Figures 1, 4, and 6). When 20 mM EGTA and 15 Ca^{2+} were introduced, HEPES was increased to 40 mM and Cs-gluconate was reduced. The presynaptic series resistance (5–20 MΩ) was compensated by 30%–90%. The postsynaptic pipette (2–3.5 MΩ) contained the same solution as the presynaptic pipette, except that EGTA was increased to 5 mM. The postsynaptic series resistance (3–8 MΩ) was compensated by the amplifier so that the uncompensated resistance was <3 MΩ. The remaining resistance was further compensated off-line. CTZ (cyclothiazide), D-AP5 (D(-)-2-Amino-5-phosphonopentanoic acid) were obtained from Tocris. Calmodulin binding domain (CAM kinase II 290-309), MLCK (myosin light chain kinase) peptide, and calmidazolium were from Calbiochem. CTZ was dissolved in DMSO, and the final concentration in the extracellular solution was 0.1%.

The Deconvolution Method

Quantal release rates were estimated by the deconvolution method adapted for the calyx of Held (Neher and Sakaba, 2001). This method assumes that the total EPSC can be separated into a residual current due to the delayed clearance of glutamate in the synaptic cleft and a current component evoked by quantal release events. By combining deconvolution with fluctuation analysis, we have shown that this method is valid in the presence of cyclothiazide and kynurenic acid, which block desensitization and saturation of the postsynaptic AMPA receptors (Neher and Sakaba, 2001). Therefore, most experiments (except Figure 6) were carried out in the presence of these drugs. Furthermore, 0.5 μM TTX, 10 mM TEA-Cl, and 50 μM AP-5 were added to the extracellular solution in order to isolate presynaptic Ca^{2+} currents and to block postsynaptic NMDA receptors. Release rates, as determined by deconvolution, were integrated to obtain cumulative release, as displayed in the figures. All values are expressed as mean ± SEM. Cumulative release was fitted with a double exponential. In the double-pulse protocol, the second pulse was fitted with a double exponential either by allowing for two time constants as free parameters, or by fixing the time constants to the same values as used in the first pulse. Both methods gave satisfactory fits. In high-EGTA conditions, we fitted the cumulative release with a single exponential and a straight line, because the slow component could not be fully depleted. A double exponential fit, however, did not result in major qualitative differences.

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