Calcium Dependence of Exo- and Endocytotic Coupling at a Glutamatergic Synapse

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SUMMARY

The mechanism coupling exocytosis and endocytosis remains to be elucidated at central synapses. Here, we show that the mechanism linking these two processes is dependent on microdomain-$[Ca^{2+}]_i$ similar to that which triggers exocytosis, as well as the exocytotic protein synaptobrevin/VAMP. Furthermore, block of endocytosis has a limited, retrograde action on exocytosis, delaying recruitment of release-ready vesicles and enhancing short-term depression. This effect sets in so rapidly that it cannot be explained by the nonavailability of recycled vesicles. Rather, we postulate that perturbation of a step linking exocytosis and endocytosis temporarily prevents new vesicles from docking at specialized sites for exocytosis.

INTRODUCTION

Synaptic vesicles fuse with the plasma membrane upon presynaptic Ca^{2+} influx through Ca^{2+} channels. After exocytosis, membrane is retrieved by endocytosis, and the endocytosed vesicles are recycled for reuse (Stevens, 2003). The time course of endocytosis varies depending on the stimulation patterns and among cell types but is often classified into fast and slow modes based upon time scale (Wu, 2004; Wu et al., 2007; but see Fernandez-Alfonso and Ryan, 2006). The fast mode of endocytosis has a time scale of 100 ms to a few seconds, whereas the slow mode has a time scale of seconds to minutes. The fast mode of endocytosis is often thought to be responsible for the "kiss-and-run" type of vesicle cycling and rapid reuse of synaptic vesicles, although fast endocytosis can be caused by rapid membrane retrieval after full collapse of synaptic vesicles without formation of a clathrin coat (Klyachko and Jackson, 2002; Sun et al., 2002; Gandhi and Stevens, 2003; Zhang et al., 2009; Zhu et al., 2009). Blocking the fast mode of endocytosis affects transmitter release by preventing the rapid reuse of synaptic vesicles (Deak et al., 2004; Ertunc et al., 2007). The slow mode is mediated by classical, clathrin-mediated pathways. Also, it has been suggested that the sites of endocytosis are separated from that of exocytosis and that endocytosis occurs in a peripheral area surrounding the active zone (Heuser and Reese, 1973;

Ringstad et al., 1999; Roos and Kelly, 1999). Thus, exocytosis and endocytosis are assumed to be functionally independent of each other and, consistent with this idea, blockade of endocytosis does not immediately affect subsequent exocytosis (Shupliakov et al., 1997; Newton et al., 2006). Only a slow, secondary effect due to a reduction in the reserve pool of synaptic vesicles has been noticed (Yamashita et al., 2005; Newton et al., 2006).

While the roles of Ca^{2+} in transmitter release are quite well established (Zucker and Regehr, 2002; Neher and Sakaba, 2008), its role in endocytosis remains unclear. The release machinery has a low affinity for Ca^{2+} and requires the binding of multiple Ca^{2+} ions. It has been also shown that synaptic vesicles and Ca^{2+} channels are tightly coupled and that local Ca^{2+} levels at the transmitter release sites rise to tens of μM and fall immediately upon the opening and closure of Ca^{2+} channels. It is this change in the Ca^{2+} levels that is responsible for rapid and phasic transmitter release. In contrast, the possible roles of Ca^{2+} in endocytosis are unclear, largely because the results are diverse among different preparations (Wu et al., 2007). Nevertheless, a unifying scheme was proposed as follows (Wu, 2004). The fast mode of endocytosis is regulated by local Ca^{2+} achieved by microdomain Ca^{2+} at the active zone (Beutner et al., 2001; Neves et al., 2001). The slow mode of endocytosis is either modulated by the bulk Ca^{2+} (von Gersdorff and Matthews, 1994; Marks and McMahon, 1998; Teng and Wilkinson, 2005; Balaji et al., 2008) or is Ca^{2+}-independent (Ryan et al., 1996; Wu and Betz, 1996; Sun et al., 2002).

The calyx of Held synapse in the auditory brainstem has a very large presynaptic terminal, which is accessible to electrophysiological techniques, as well as optical methods (Forsythe, 1994). In the calyx terminal, both fast and slow modes of membrane retrieval have been reported. The fast mode (the time constant of ~1 s) was seen after intense stimulation of the presynaptic terminal and was Ca^{2+}-dependent (Wu et al., 2005). In contrast, under low to mild stimulation, a slow mode of endocytosis was found to predominate, which is characterized by a much slower time course (several to tens of seconds; Yamashita et al., 2005; Lou et al., 2008) and which is insensitive to changes in the bulk Ca^{2+} up to concentrations of a few μM (Sun et al., 2002). Here, we examined the Ca^{2+} dependence of the slow mode of endocytosis at the calyx of Held synapse quantitatively, using presynaptic capacitance measurements to measure the time course of membrane retrieval, combined with Ca^{2+} uncaging and Ca^{2+} measurement techniques. In contrast to the previous study (Sun et al., 2002), we find that Ca^{2+} regulates the rate of slow

membrane retrieval at the calyx of Held. Sensitivity to Ca^{2+} buffers also suggests that a local Ca^{2+} microdomain created by multiple Ca^{2+} channels is responsible for slow endocytosis (Bollmann et al., 2000; Schneggenburger and Neher, 2000). In addition, we provide a role for the SNARE protein synaptobrevin in endocytosis. Furthermore, we extend our findings to provide evidence for an interaction between exo- and endocytosis by blocking endocytic proteins and examining the consequence on exocytosis. Interestingly, the interaction is limited to a slowing in the rate of synaptic vesicle recruitment for up to 1 s following exocytosis. We suggest that exocytosis and endocytosis interact with each other at the calyx of Held, although the kinetics of membrane retrieval are slow.

RESULTS

Membrane Retrieval at the Calyx of Held

First, we examined the basic features of endocytosis at the calyx of Held terminal by using capacitance measurements, similar to those carried out in previous studies (Sun et al., 2002). The presynaptic terminal of the calyx of Held (postnatal day 8–11) was whole-cell voltage clamped at −80 mV and the membrane capacitance was measured using the sine + DC technique (Lindau and Neher, 1988). In Figure 1A, a train of action potential (AP)-like stimuli (20 depolarizing pulses to +40 mV for 1 ms, with a frequency of 100 Hz) was applied to the presynaptic terminal. In response to the train, a capacitance increase of 200 fF was observed, which reflected the fusion of between 2000 and 3000 vesicles (Sakaba, 2006). On cessation of stimulation, the capacitance returned to baseline within 30 s, reflecting membrane retrieval. The rate of membrane retrieval was 10 fF/ sec, similar to previous studies (Sun et al., 2002; Yamashita et al., 2005; Renden and von Gersdorff, 2007; Lou et al., 2008). In Figure 1B, we varied the amount of exocytosis by changing the number of AP-like stimulations (n = 16 cells). In some cases, a step depolarization was also applied to elicit larger amounts of exocytosis (>300 fF). As shown in Figure 1B, the endocytic time increased monotonically as the amount of exocytosis increased, although the slope was relatively shallow. This is consistent with previous studies and indicates that the membrane retrieval has a limited capacity (Sun et al., 2002; Fernandez-Alfonso and Ryan, 2004). The kinetics of endocytosis measured here correspond to that termed slow endocytosis (more than several seconds) in previous studies, since induction of fast endocytosis requires massive stimulation (more than 200 APs at 100 Hz; Wu et al., 2005).

The Role of Dynamin and AP2 in Slow Membrane Retrieval at the Calyx of Held

Slow endocytosis is usually thought of as being mediated by a clathrin-dependant pathway; which involves the complex, multistep assembly of clathrin cages on the plasma membrane and their eventual retrieval. In brief, clathrin coat formation is initiated using the adaptor protein AP2. AP2 not only recruits clathrin to the plasma membrane by binding it to plasma membrane cargo but it is also responsible for recruiting the accessory protein amphiphysin to the site of endocytosis. In turn, amphiphysin recruits dynamin, which is responsible for mediating
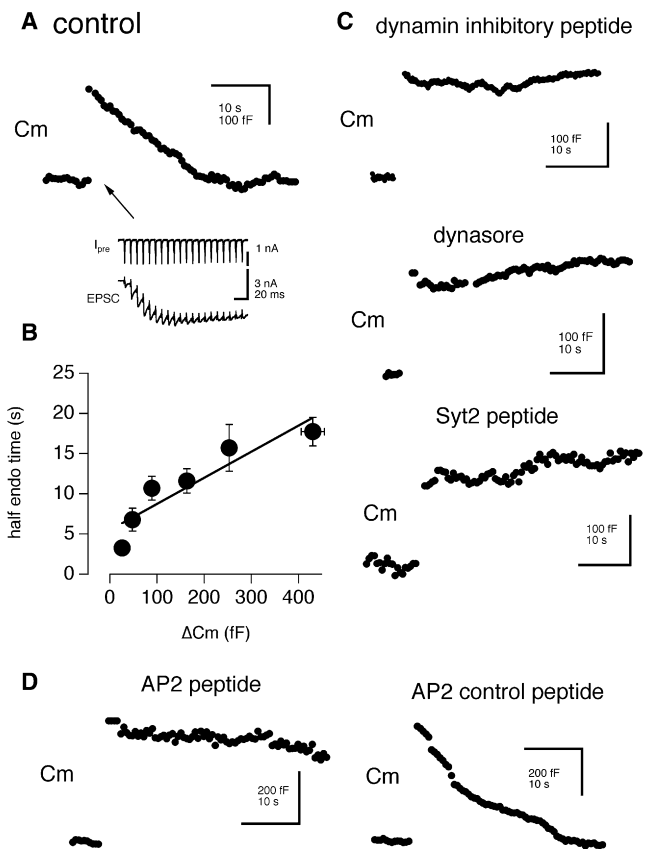

Figure 1. Block of Endocytosis by Dynamin Inhibitors and an AP2 Blocking Peptide in the Calyx of Held Nerve Terminal

(A) A 100 Hz train of AP-like stimuli (+40 mV for 1 ms, 20 times) was applied to the presynaptic terminal and the time course of membrane retrieval was determined using capacitance measurements. Presynaptic Ca^{2+} currents and evoked EPSCs are shown in the inset.

(B) The amount of exocytosis was systematically changed to examine the consequences on the time course of endocytosis (as measured by presynaptic capacitance recordings), either by varying the number of AP-like stimuli or by applying a long step depolarizing pulse (0 mV for 50 ms). In (B), the half decay time of endocytosis is plotted against the amount of exocytosis (ΔCm) (n = 16 cells).

(C) The same experiment as in (A), but dynamin inhibitory peptide (top), dynasore (middle), and C2B domain peptide of synaptotagmin 2 (bottom) were applied to block endocytosis. See Figure S1 for summary plots.

(D) The same as (C), but AP2 blocking peptide and the control peptide were applied to the terminal (see Figure S1 for summary).

membrane scission via GTP hydrolysis (Schmid and McMahon, 2007). Thus, we decided to investigate the role of clathrin in mediating slow endocytosis at the calyx of Held synapse, by applying four compounds known to block clathrin-mediated endocytosis at these discrete stages; dynasore (100 μM) which is known to block the GTPase activity of dynamin (Macia et al., 2006; Newton et al., 2006), a dynamin inhibitory peptide (1 mM), which interferes with the binding of dynamin to the SH3 domain of amphiphysin (Shupliakov et al., 1997), C2B domain peptide of synaptotagmin 2 (10–100 μM), which sequesters AP2 and prevents it participating in clathrin cargo formation (Grass et al., 2004), and an AP2 blocking peptide (100 μM), which

disrupts the interaction between AP2 and amphiphysin (Jock-usch et al., 2005). The peptides were applied directly to the presynaptic terminal via a patch pipette, whereas dynasore was applied extracellularly (with a preincubation of between 15 and 60 min). Both dynasore and the dynamin inhibitory peptide blocked membrane retrieval as shown in Figure 1C (n = 4 cells each, see Figure S1, available online, for a summary plot), consistent with previous results at the calyx of Held (Yamashita et al., 2005; Xu et al., 2008). The C2B domain peptide of synap-totagmin 2 also blocked endocytosis at the calyx of Held (Fig-ure 1C; n = 6). Furthermore, an AP2 blocking peptide also abol-ished endocytosis (Figure 1D, left panel; n = 4). In contrast, a point-mutated AP2 blocking peptide, which has a 50 times lower affinity for AP2 (Olesen et al., 2008), was ineffective in blocking endocytosis at an equivalent concentration (Figure 1D, right panel; n = 6 cells). These results suggest that slow endocytosis at the calyx of Held synapse is dependent on dynamin and AP2.

Slow Mode of Membrane Retrieval at the Calyx of Held is Ca^{2+} Dependent

It has been previously reported that slow endocytosis at the calyx of Held terminal is Ca^{2+} independent, because modulation of internal Ca^{2+} concentration to a low micromolar level did not change the rate of endocytosis (Sun et al., 2002). Here, we rein-vestigated the role of calcium in endocytosis at higher intracel-lular concentrations. Two classical manipulations were used to lower intracellular Ca^{2+} concentration during neuronal stimula-tion. First, lowering the extracellular Ca^{2+} reduces Ca^{2+} influx through Ca^{2+} channels and hence both the local Ca^{2+}, close to the channels, and also the bulk Ca^{2+} level. We examined the Ca^{2+} dependence of endocytosis by changing the external Ca^{2+} concentration from 2 mM to 0.8 mM. Lowering the external Ca^{2+} reduced the presynaptic Ca^{2+} currents, EPSC amplitudes and the cumulative release estimated using the deconvolution method (Figure 2A). At the same time, it slowed the rate of membrane retrieval dramatically when a train of AP-like stimuli was applied (Figure 2B; n = 5 cells, see Figure S2 for a summary). In Figure 2B, the capacitance traces crossed 15 s after cessation of the stimulus.

Previously, it was reported that extracellular Ca^{2+} is necessary for endocytosis at the frog neuromuscular junctions (Zefirov et al., 2006). In order to confirm whether membrane retrieval at the calyx of Held is sensitive to presynaptic intracellular Ca^{2+}, the Ca^{2+} chelators BAPTA or EGTA (both at 1 mM, K_d of 200 nM) were loaded into the presynaptic terminal via a recording patch pipette, while the extracellular Ca^{2+} concentration was re-turned to normal levels (2 mM). Capacitance increases in response to stimulation were much smaller under 1 mM BAPTA (70 ± 17 fF) and 1 mM EGTA (55 ± 9 fF), compared with control (237 ± 29 fF), consistent with an effect on exocytosis. However, as can be seen in Figures 3A and 3C; 1 mM BAPTA also blocked membrane retrieval (n = 5 cells). In the presence of 1 mM EGTA, endocytosis was still observed although the time course was somewhat slower than under control conditions (Figure 3B; n = 6 cells). In Figure 3C, the normalized time course of membrane retrieval under control conditions, as well as in the presence of BAPTA and EGTA, is plotted. It is clear that BAPTA blocks membrane retrieval, whereas EGTA merely slows the rate of
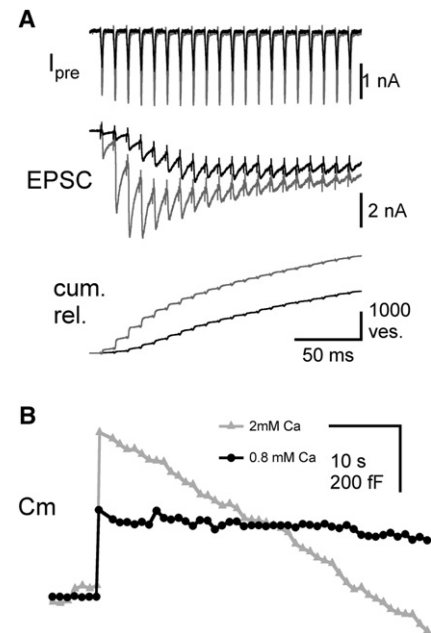

Figure 2. Lowering the External Ca^{2+} Concentration Slows Endocy-tosis

A train of AP-like stimuli (100 Hz x 20) was applied to the presynaptic terminal and capacitance changes were monitored. External Ca^{2+} was lowered from 2 mM Ca^{2+} to 0.8 mM during the recording. In (A), presynaptic currents, EPSCs and cumulative release are shown (gray, 2 mM Ca^{2+}; black, 0.8 mM Ca^{2+}). In (B), capacitance changes in response to the train are shown (left 2mM Ca^{2+}, right 0.8 mM Ca^{2+}). See Figure S2 for summary plots.

membrane retrieval. The latter result contrasts with Sun et al. (2002), who observed little effect of EGTA. Rates of membrane retrieval were obtained using a line fit to the capacitance decay. By dividing by the amplitude of the peak capacitance increase, rate constants were obtained. Compared with the control (0.043 ± 0.005 s^{-1}), the endocytotic rate constants were smaller in the presence of 1 mM BAPTA (0.002 ± 0.003 s^{-1}), whereas with 1 mM EGTA the slow-down was less pronounced (0.017 ± 0.005 s^{-1}). The difference between BAPTA and EGTA was statis-tically significant (p < 0.05). Importantly, a nonspecific effect on endocytosis resulting from a block in exocytosis could be excluded, as the rate constant under control conditions was maintained (0.041 ± 0.008 s^{-1}), even when the number of AP-like stimuli applied to the presynaptic terminal was reduced (capacitance increase of 64 ± 7 fF). Rather, these results suggest a role for intracellular Ca^{2+} in modulating endocytosis at the calyx of Held.

Interestingly, these observations are similar to the effects of BAPTA and EGTA on transmitter release at the calyx of Held synapse (Borst and Sakmann, 1996), where exocytosis is also much reduced in the presence of Ca^{2+} chelators, except that the influence on endocytosis manifests itself much later than the local Ca^{2+} signal is created. As BAPTA is known to bind Ca^{2+} ions faster than EGTA (Adler et al., 1991), the stronger effect of BAPTA suggests that it is local Ca^{2+} signals, close to Ca^{2+} channels, that regulate endocytosis—although EGTA also affected endocytosis, albeit less effectively. This suggests that
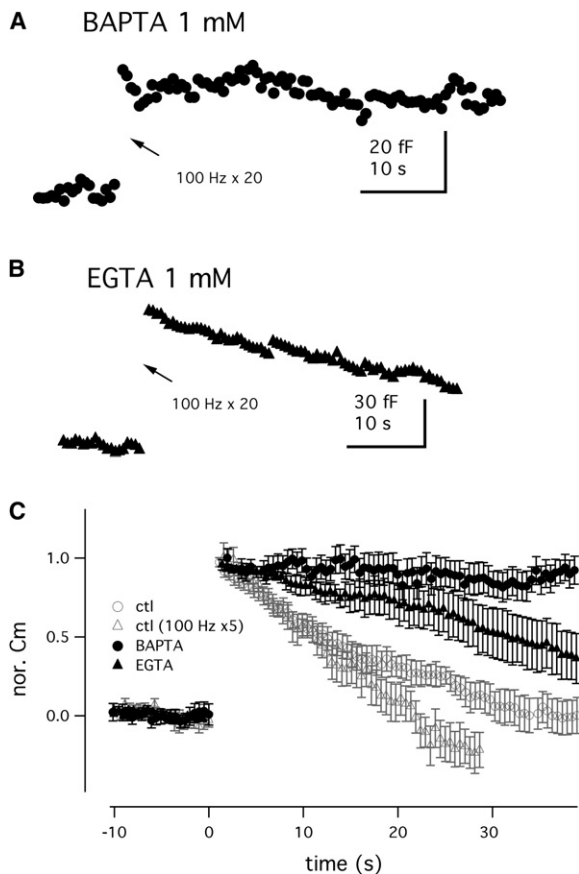
Figure 3. Sensitivity of Endocytosis to BAPTA and EGTA

(A and B) The presynaptic terminal was infused with 1 mM BAPTA (A) or 1 mM EGTA (B), and a 100 Hz train of AP-like stimuli (×20) was applied. (B) is the average of two traces in a single cell.

(C) The effect of 1 mM BAPTA and 1 mM EGTA on the time course of membrane retrieval. The normalized time course of capacitance decay under control conditions (n = 6 cells; open circles, 100 Hz × 20 pulses; open triangles, 100 Hz × 5 pulses), in the presence of 1 mM BAPTA (n = 5 cells, filled circles), and in the presence of 1 mM EGTA (n = 6 cells, filled triangles). The data were obtained by applying a 100 Hz train of APs (20–50 pulses).

the process of triggering endocytosis at the calyx synapse also experiences similar microdomain Ca^{2+}, formed by multiple Ca^{2+} channels close to the release machinery. 5-Nitro BAPTA (1 mM), a low-affinity BAPTA-type Ca^{2+} chelator (K_d of 40 μM), also slowed down membrane retrieval, consistent with endocytosis being dependent on local Ca^{2+} (Figure S3).

The Ca^{2+} Requirement for Triggering Endocytosis

The above experiments indicate that endocytosis is Ca^{2+} dependent (Figures 2 and 3) and suggests that it is the local increase in Ca^{2+}, created by the synchronous opening of multiple Ca^{2+} channels, that is responsible (Figure 3). In order to determine the Ca^{2+} dependence of the rates of membrane retrieval quantitatively, the Ca^{2+} uncaging technique was used to elevate Ca^{2+} concentration uniformly in the presynaptic terminal (Figure 4). Because the influence of Ca^{2+} on endocytosis manifests itself much slower than the time course of local Ca^{2+} signals (Figure 3),
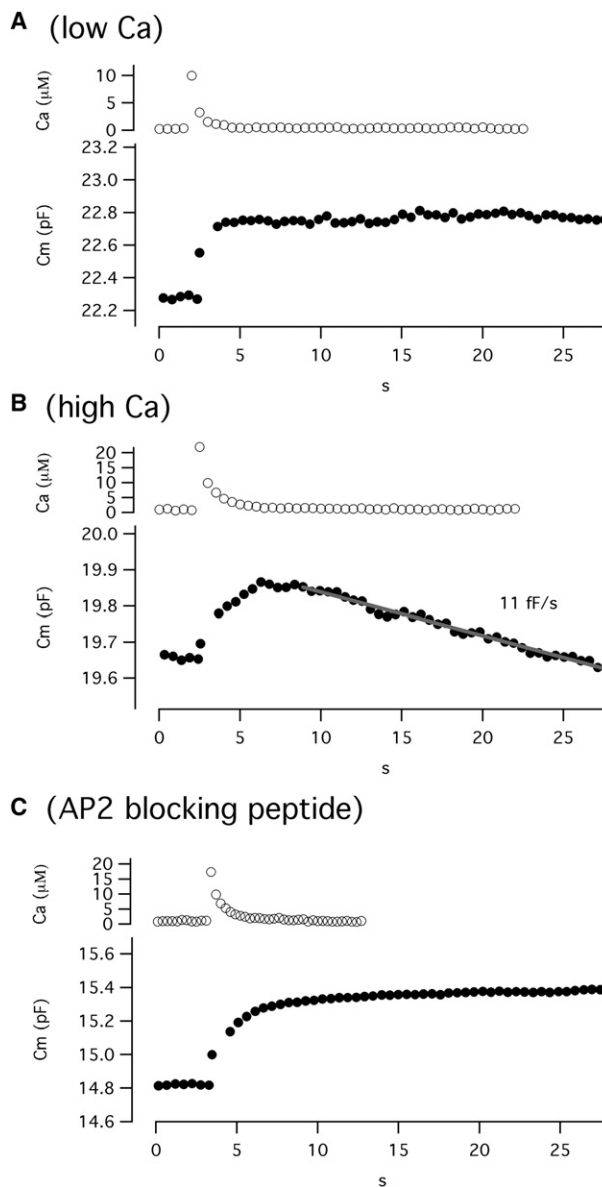

Figure 4. Ca Uncaging Evokes Endocytosis in the Calyx of Held Presynaptic Terminal

(A and B) Presynaptic terminals were loaded with a DM-nitrophen (1 to 3 mM)/ Ca^{2+} mixture. Flash photolysis elevated the presynaptic Ca^{2+} concentration from its basal level to 10 μM (A) and 22 μM (B). The level of free Ca^{2+} then returned to the basal level within several seconds, concomitant with the cessation of exocytosis. Following the initial capacitance increase evoked by Ca^{2+} uncaging, a decrease in capacitance (which reflects endocytosis) was seen in (B) but not in (A). The slope of the capacitance decay, obtained from the line fit, was used to estimate the rate of membrane retrieval.

(C) A similar experiment to that shown in (B) but using the AP2 blocking peptide to specifically inhibit endocytosis (illustrated by the sustained increase in membrane capacitance) following the transient elevation in Ca^{2+}.

the rates of membrane retrieval can be measured after the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) decays to the basal level. This also avoids a possible balance between exo- and endocytosis during the $[Ca^{2+}]_i$ elevation complicating interpretation of
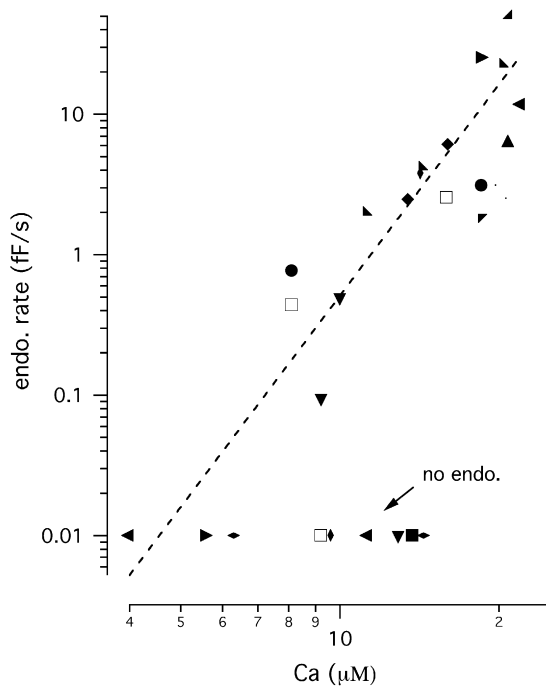
Figure 5. Intracellular Ca^{2+} Dependence of the Rate of Endocytosis
The rate of endocytosis (expressed as fF/s) was plotted against the peak Ca^{2+} concentration following flash photolysis. Below 15 μM, no endocytosis was observed in nine trials. The dotted line is a fit of the data using a 5th power function. The data were obtained from 13 cells. Different symbols indicate the data from different cells. Open symbols represent the data obtained when NP-EGTA was used instead of DM-Nitrophen (three cells).

the capacitance recordings, and is in agreement with results obtained in the giant reticulospinal synapse in the lamprey (Gad et al., 1998), where it was possible to temporally dissociate the calcium influx that occurs during stimulation and exocytosis from endocytic recovery.

In Figure 4A, $[Ca^{2+}]_i$ was elevated to 10 μM, and it decayed back to the basal level within several seconds. The capacitance jump observed upon flash photolysis was 400 fF. Sometimes we saw a slow increase in capacitance for several seconds after flash photolysis as long as the $[Ca^{2+}]_i$ was elevated (for example, in Figure 4B, $[Ca^{2+}]_i$ decays more slowly compared with Figure 4A). This presumably reflects the release of newly recruited vesicles, which is also Ca^{2+} dependent. After the $[Ca^{2+}]_i$ decay, no capacitance decay was found in Figure 4A, indicating no membrane retrieval. In contrast, when $[Ca^{2+}]_i$ was elevated over 15 μM, a detectable capacitance decay was observed after the $[Ca^{2+}]_i$ decayed to the basal level (Figure 4B). In the presence of AP2 blocking peptide (100 μM), membrane retrieval was not seen (Figure 4C; n = 6 cells).

Figure 5 summarizes a set of the experiments shown in Figure 4, plotting the rate of membrane retrieval against the peak post-flash Ca^{2+}-level (rate constants are given in Figure S4). Below 10 μM $[Ca^{2+}]_i$, almost no membrane retrieval was found. Above this level, the rate of membrane retrieval depends nonlinearly on $[Ca^{2+}]_i$, although below 15 μM $[Ca^{2+}]_i$ no membrane retrieval was seen in four cases. To achieve the rate of 10–20

fF/s comparable to the one observed during a train of action potentials (Figures 1 and 3), we estimate that a peak $[Ca^{2+}]_i$ elevation of 20 μM would be required. We were unable to study endocytosis at Ca^{2+} concentrations higher than 20 μM, because the $[Ca^{2+}]_i$ decay is too slow when a such a high concentration of DM-Nitrophen is loaded into the presynaptic terminal (Wölfel et al., 2007), making it hard to isolate endocytosis, as exo- and endocytosis superimpose in the capacitance traces. We conclude that the local Ca^{2+} close to the Ca^{2+} channel cluster regulates endocytosis at the calyx of Held. In fact, such a geometrical arrangement is similar to that of exocytosis.

Disruption of Synaptobrevin Blocks Membrane Retrieval
Next, we tested whether exo- and endocytic proteins interact with each other during clathrin-mediated (slow) endocytosis (Zhang et al., 1994; Poskanzer et al., 2003). We first tested the involvement of synaptic vesicle proteins in membrane retrieval. A particularly attractive candidate is the integral membrane protein synaptobrevin. Synaptobrevin is the vesicular SNARE protein and plays an essential role in vesicle fusion because of its interaction with the plasma membrane SNAREs syntaxin 1 and SNAP-25. Genetic ablation of this protein leads to a severe phenotype in mice, with the fast mode of endocytosis being absent (Deak et al., 2004).

We tested for a role of synaptobrevin in slow endocytosis at the calyx by acutely interfering with its function using a highly specific protease—tetanus toxin (1–6 μM). Tetanus toxin cuts synaptobrevin close to the transmembrane region, removing most of the N-terminal part of the molecule, including the so-called SNARE motif (which is essential for interactions with syntaxin and SNAP-25) and the proline-rich domain which may mediate intermolecular associations (Schiavo et al., 2000). As expected, no capacitance change was usually seen with or without stimulation within 5–6 min of whole-cell dialysis of the toxin, indicating effective proteolysis and a complete block of exocytosis (data not shown). We then took advantage of the partial action of the toxin by using a shorter dialysis time, resulting in digestion of only a fraction of the total synaptobrevin pool. In this case, the remaining fractions of intact synaptobrevin can elicit exocytosis, whereas cleavage of synaptobrevin may affect the interactions between synaptobrevin and its binding partners, for example the soluble synaptobrevin domain may sequester cytosolic binding partners. In four cells, exocytosis was not completely blocked (capacitance jump of 50–150 fF), making it possible to study the effects, if any, of the toxin on endocytosis. In Figure 6A, a 100 Hz train was applied to the calyx of Held within 3 min after whole-cell configuration was achieved. While exocytosis could be still elicited, no membrane retrieval was observed (Figure 6C shows a summary plot).

Previously, the N-terminal proline-rich domain of synaptobrevin was shown to inhibit neurotransmitter release (Cornille et al., 1995), and this was later linked to a role in synaptic vesicle recruitment (Wadel et al., 2007). Here we present evidence that further elucidates the role of the proline-rich domain. Infusion of this peptide also blocked membrane retrieval at the calyx (Figure 6B, n = 5 cells and Figure 6C for the summary plot). However, no block of exocytosis was observed in this case. Therefore, interference with synaptobrevin and/or its interacting
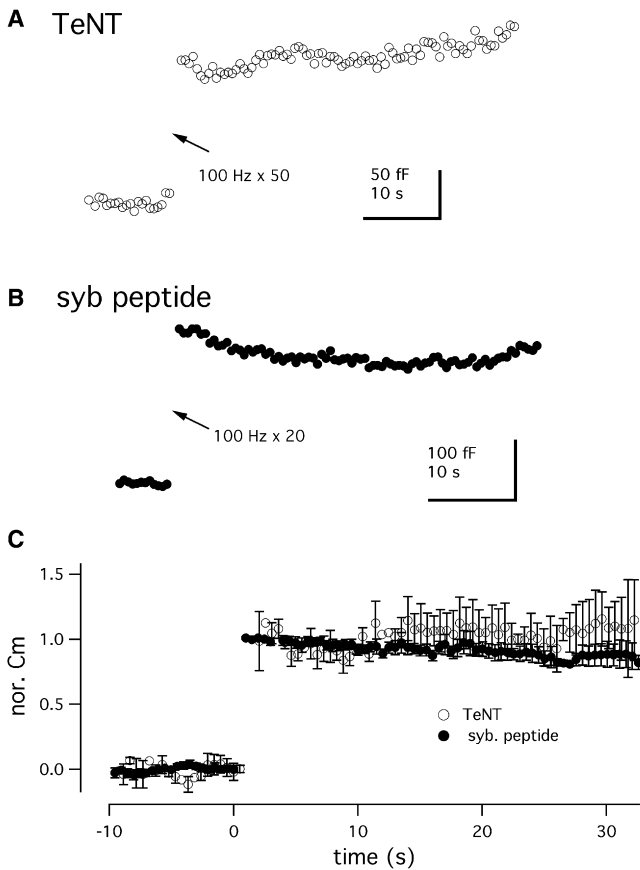
Figure 6. Synaptobrevin Influences Endocytosis

In (A) tetanus toxin light chain (TeNT; 1–6 μM) was infused into the presynaptic terminal. Following a 5–6 min incubation, a 100 Hz train of AP-like stimuli was applied to the presynaptic terminal and the resulting capacitance changes were monitored. In (B), the proline-rich domain of synaptobrevin (syb peptide, 100 μM) was dialyzed into the presynaptic terminal.

(C) The average time course of capacitance changes in the presence of TeNT and proline-rich domain peptide. The values were normalized to the peak capacitance change in each trace.

partners affects not only exocytosis but also some processes that lead to endocytosis after vesicle fusion.

Block of Endocytic Proteins Slows Down the Rate of Synaptic Vesicle Recruitment for a Short Time Span

While we have now shown that interference of synaptobrevin blocks endocytosis at the calyx of Held (Figure 6), we have previously shown that such disruption also slows the rate of synaptic vesicle recruitment (Sakaba et al., 2005). Thus, the observed block in endocytosis may be linked to a slowing in vesicle recruitment. If so, it may be expected that blocking endocytic proteins with other reagents may also affect synaptic vesicle recruitment. To examine whether the block of endocytosis interferes with vesicle pool dynamics, we studied the depletion and the recruitment of vesicle pools by applying pairs of long-lasting depolarizations and measuring the subsequent recovery in synaptic response (Sakaba and Neher, 2001a). Simultaneous pre- and postsynaptic patch-clamp recordings were carried out at the calyx of Held. A step depolarizing pulse (0 mV for 50 ms following a prepulse to +70 mV for 2 ms) was applied to the presynaptic terminal in Figure 7A, which was long enough to completely deplete the readily releasable pool (RRP) of 2000–3000 synaptic vesicles (Sakaba and Neher, 2001a). The time course of neurotransmitter release during a depolarizing pulse was estimated from the evoked EPSCs (Figure 7A; middle panel) using the deconvolution method (see Experimental Procedures). When 0.5 mM EGTA was included in the presynaptic pipette to block overlapping facilitation of transmitter release (Sakaba and Neher, 2001a), release could be fitted by a double exponential function, with time constants of 2–3 ms (~50% of the total RRP) and 10–30 ms, respectively (Figure 7A, bottom panel). Thus, the RRP consists of two sets of synaptic vesicles with distinct release probabilities, the fast-releasing vesicles (~50% of the RRP) and the slowly releasing vesicles, although it should be noted the amounts and the time constants of the slowly releasing vesicles are variable from study to study (Sakaba and Neher, 2001a; Sakaba et al., 2005; Wadel et al., 2007; Wölfel et al., 2007). The fast component of release presumably corresponds to synaptic vesicles tightly coupled to the Ca^{2+} channel cluster (Sätzler et al., 2002), whereas the slow component corresponds to vesicles more loosely coupled (Wadel et al., 2007).

In order to block endocytosis, we used the four inhibitors characterized in Figure 1 (dynasore, dynamin inhibitory peptide, AP2 blocking peptide, and the C2B domain of synaptotagmin 2). In addition, we also applied an antibody against dynamin, which has previously been shown to inhibit endocytosis in the calyx (100 ng/ml; Xu et al., 2008). Presynaptic Ca^{2+} currents were within the normal range (1–2 nA) in all five conditions (Table S1). More importantly, both the time constants of the fast and slow components of release (2–3 ms and 15–30 ms, respectively) and the fraction of the fast component to the total release were within the normal range in the presence of the inhibitors (Table S1). This suggests that the inhibitors do not affect the transmitter release machinery.

The time course of recovery from vesicle pool depletion was monitored by applying a second depolarizing pulse at varying interstimulus intervals (ISI).

All five endocytosis inhibitors affected recovery of the fast component of release, without significantly affecting that of the slow release component (Figures 7D and 7E; see Figure S5 for recovery of the RRP). Extracellular application of dynasore did not change the amplitude nor the time course of miniature EPSCs (Figure S6), excluding possible postsynaptic effects. In addition, the point-mutated AP2 blocking peptide (control for the AP2 blocking peptide) was ineffective in blocking recovery of the fast-releasing vesicles (n = 4 cell pairs). Under control conditions, the recovery of the fast-releasing component has rapid and slow phases (Figure 7D, dotted line) with a half time of a few hundred ms. In the presence of our five endocytosis inhibitors, the recovery followed a much slower time course (with a time constant of ~5 s; Figure 7D, solid lines). Recovery of the slowly releasing component was not affected (80% recovery within 200 ms), irrespective of the presence of endocytosis inhibitors (Figure 7E). It is important to note that recovery of the slowly releasing component was more variable in previous studies (approximately 20%, Sakaba and Neher, 2001a; Wadel et al.,
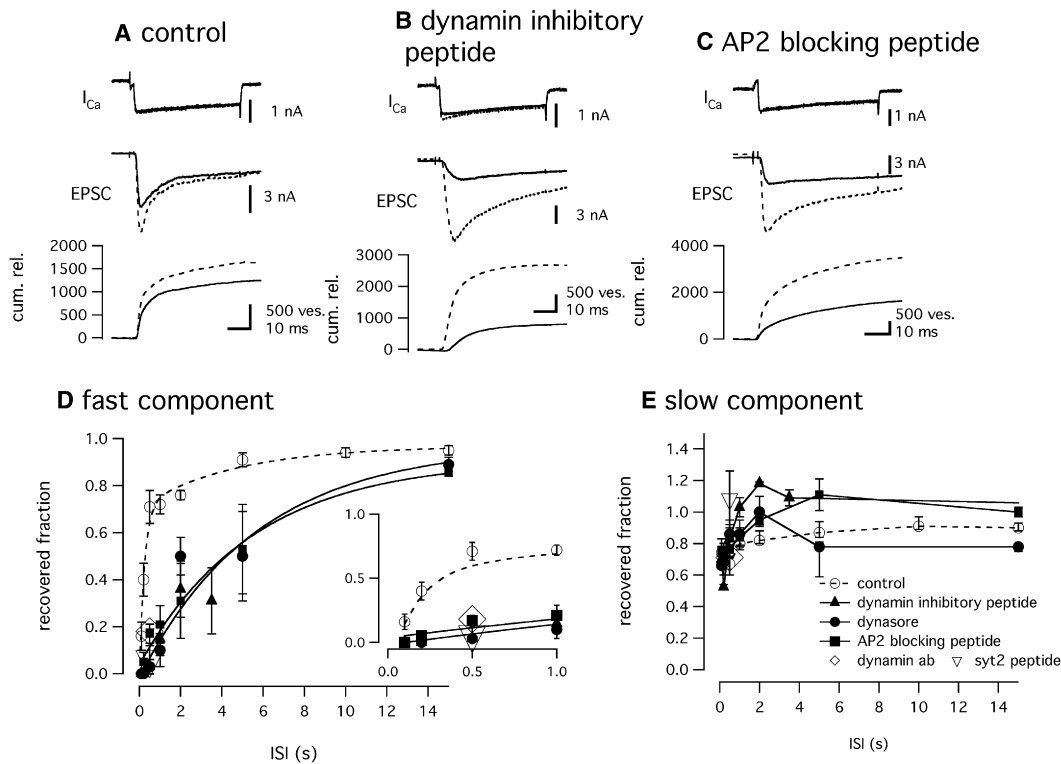
Figure 7. Blocking Endocytosis Affects the Recruitment to the Pool of Readily Releasable Synaptic Vesicles

(A, B, and C) A pair of step depolarizing pulses (+70 mV for 2 ms followed by repolarization to 0 mV for 50 ms) was applied with an interval of 500 ms. The prepulse (to +70 mV) was applied to activate Ca channels maximally without causing presynaptic Ca influx. Dotted and solid traces show data obtained from the first and the second pulses, respectively. From the top, traces show the presynaptic Ca currents, EPSCs and cumulative release estimated from the deconvolution method. (A), (B), and (C) illustrate responses from control cells and cells treated either with dynamin inhibitory peptide or AP2 blocking peptide.

(D and E) The time course of recovery for the pool of fast-releasing (D) and slowly releasing vesicles (E) monitored by varying the interval (interstimulus interval, ISI) between pulses. Open circles, filled triangles, filled circles, and filled squares represent data obtained under the respective conditions: control (n = 5 cell pairs), dynamin inhibitory peptide (n = 5 cell pairs), dynasore (n = 6 cell pairs), and AP2 blocking peptide (n = 5 cell pairs). Data obtained from experiments using an anti-dynamin antibody (dynamin ab; n = 3 cell pairs) and peptide derived from the C2B domain of synaptotagmin 2 (n = 4 cell pairs) are superimposed (for ISI = 500 ms). In (D), control data are fitted by a double exponential with time constants of 242 ms (74%) and 4.6 s. In the presence of dynasore and dynamin inhibitory peptide, the pooled data are fitted by a single exponential with a time constant of 5.7 s. In the presence of AP2 blocking peptide, the data are fitted by a single exponential with a time constant of 5.5 s.

2007). In conclusion, the effect of the endocytosis inhibitors was specific to the recovery of the fast component of release.

It is possible that the slow recovery of the fast component of release may be due to a secondary effect on the block of endocytosis. Blocking endocytosis may reduce the size of the reserve pool by consumption of synaptic vesicles through exocytosis and slowed vesicle recycling rates (Dickman et al., 2005; Newton et al., 2006). If so, the RRP size under resting conditions should decrease with time as a result of vesicle consumption through exocytosis. However, as shown in Figure S7, this is not the case, at least at the calyx of Held synapse. By waiting for a sufficiently long time period (>10 s), the amount of cumulative release was restored to the initial value, even in the presence of the inhibitors (Figures 7D and 7E). Indeed, when the pulse interval was >1 min, the RRP size (fast + slow components of release) during a step depolarizing pulse (50 ms in duration) consistently showed only a minor decrease over the measurement period, both in the presence and absence of inhibitors (Figure S7). This indicates that the slowed recovery was not caused by a reduction in the

size of the reserve pool that supplies vesicles to the RRP. In contrast, such a block of vesicle recycling and concomitant reduction of RRP size over time has been observed under the influence of GTP-γS in one previous study (Yamashita et al., 2005). GTP-γS is a general nonhydrolysable GTPase activator. As such, it affects not only dynamin but also all other G proteins and their signaling pathways. Thus, the effect of GTP-γS observed previously may be, at least partially, independent of the block in endocytosis itself. It is important to note that we blocked endocytosis acutely, and a more chronic block of endocytosis (for example in knock out mice) may actually lead to depletion of the reserve pool of synaptic vesicles (Dickman et al., 2005).

Given that we have only seen a slow form of endocytosis following stimulation (Figures 1 and S8), we think it is highly unlikely that rapid recycling of synaptic vesicles is responsible for rapid synaptic vesicle recruitment at the calyx of Held.

Finally, we demonstrate that the slowed vesicle recruitment observed here has an impact under more physiological
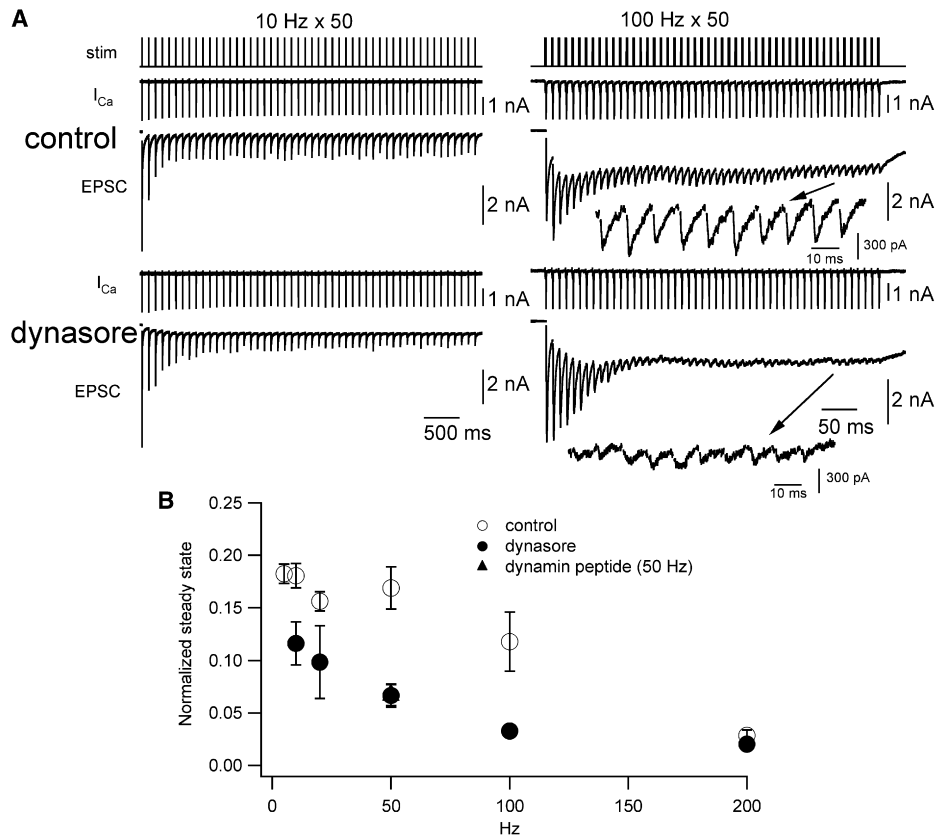
Figure 8. Blocking the Action of Dynamin Modulates Short-Term Synaptic Depression

(A) A train of AP-like stimuli (+40 mV for 1.5 ms, 50 stimuli) was applied with a frequency of 10 Hz or 100 Hz. In each panel, the presynaptic voltage, presynaptic Ca currents, and the postsynaptic EPSCs are displayed. The inset on the right shows magnified EPSC traces taken from a period occurring later during the train. Control data (top) and the data obtained in the presence of 100 μM dynasore (bottom) are shown. Data were obtained from different cell pairs.

(B) The steady-state EPSC amplitudes (mean amplitudes of last 10 EPSCs) relative to the first EPSC are plotted against stimulus frequency. Open circles were obtained from the control, whereas filled circles and the filled triangle were obtained in the presence of dynasore and dynamin inhibitory peptide, respectively. Note that the stimulus train applied in the presence of dynamin inhibitory peptide had a frequency of 50 Hz.

conditions, such as during a train of APs. When a train of AP-like stimuli (+40 mV for 1.5 ms) was applied to the presynaptic terminal, the postsynaptic EPSCs showed a pronounced depression at frequencies higher than 5 Hz (Figure 8A). However, the steady-state EPSC amplitudes do not decrease proportionally when the stimulus frequency is further increased to levels between 5 and 100 Hz (Figure 8B, control). Interestingly, we found a more pronounced depression and a steep dependence on the stimulus frequency in the presence of dynamin inhibitors (Figures 8A and 8B). With dynasore, the steady-state EPSC amplitudes relative to the first EPSC in a train were remarkably reduced (Figure 8B; summary from n = 7 pairs) and depression was more steeply dependent on the stimulus frequency. Note that during a 100 Hz train synaptic responses are markedly diminished after the 10th−20th stimuli (Figure 8A, right). This is not due to unspecific interference with release, since the first EPSC amplitudes were similar between the two conditions (control versus dynasore, 5.4 ± 1.5 nA and 8.0 ± 2.3 nA respectively, p = 0.36). The steady-state EPSC amplitudes were reduced in a very similar manner by the dynamin inhibitory peptide (Figure 8B; 50 Hz). This effect is similar to that seen

when blocking Ca^{2+}-dependent recruitment of synaptic vesicles by the use of calmodulin blockers (Hosoi et al., 2007) or GTP analogs (Takahashi et al., 2000). Figure 7 showed that vesicle replenishment was slowed when endocytosis was blocked, without other parameters of transmitter release being affected. Given that vesicle replenishment determines the steady-state EPSC amplitudes (Hosoi et al., 2007), the observed reduction in the steady state is most likely due to a slowed replenishment rate, while other mechanisms, in addition to vesicle replenishment, may also determine the steady-state EPSC amplitudes during a high-frequency train of APs (Wölfel et al., 2007).

DISCUSSION

We have shown that Ca^{2+} regulates the slow mode of endocytosis in the calyx of Held presynaptic terminal. While membrane retrieval at the calyx of Held is slow with a time scale of several seconds (Figure 1), the endocytic process itself is actually linked to increases in the local intracellular Ca^{2+} concentration, which rises and falls very rapidly during an AP (Figures 2 to 5). Furthermore, we show that disruption of endocytosis also has a role in

synaptic vesicle recruitment, which can also be mimicked by disrupting an integral membrane protein on the synaptic vesicle. Cleavage of the vesicular SNARE protein synaptobrevin, or infusion of its N-terminal proline-rich domain, blocked slow membrane retrieval (Figure 6), in addition to its previously reported role in synaptic vesicle recruitment (Sakaba et al., 2005). Inhibitors of clathrin-mediated endocytosis also retarded synaptic vesicle recruitment (Figures 7 and 8). Therefore, the processes of exocytosis and endocytosis are interacting with each other, although the kinetics of endocytosis are slow compared to exocytosis. However, the impact on exocytosis is limited to the replenishment of the fast-releasing vesicles within a short time window (several seconds) after vesicle depletion (Figure 7), and other parameters of exocytosis were unchanged (Table S1).

Ca²⁺ Dependence of Endocytosis at the Calyx of Held Synapse

At the calyx of Held synapse, fast and slow modes of endocytosis have previously been reported (Wu et al., 2007; Smith et al., 2008), although the fast mode is only seen when the stimulation is very strong (Wu et al., 2005). The slow mode of membrane retrieval has a time constant of several seconds, and the time constant increases progressively with increasing amounts of exocytosis (Sun et al., 2002; Yamashita et al., 2005; Lou et al., 2008). We have shown that slow endocytosis is Ca^{2+}-dependent and driven by increases in the local Ca^{2+} concentration, which rises much higher than the bulk Ca^{2+}. Previously, Sun et al. (2002) reported that the rate of slow endocytosis is dependent on the amount of exocytosis, but not on $[Ca^{2+}]_i$. However, their experimental manipulation of $[Ca^{2+}]_i$ was limited to increases of up to ~1 μM, which is too low to change the rate of membrane retrieval in our data (Figure 5). In this sense, the results of Sun et al. (2002) are compatible with our results. It is also unlikely that the rate of slow endocytosis is controlled only by the amount of membrane added by exocytosis since, in Figures 3A and 3B, the amount of exocytosis was comparable, but the rate of endocytosis was significantly different between BAPTA, EGTA, and control conditions (under weak stimulation).

A role for local high Ca^{2+} on endocytosis has been postulated in retinal bipolar cells (Neves et al., 2001), hair cells (Beutner et al., 2001), and chromaffin cells (Ales et al., 1999), but in these studies it mainly acts on the rapid form of endocytosis, which has a time course of less than a second. It has been considered that the slower form of endocytosis, as observed in this study, is either Ca^{2+} independent (Sun et al., 2002), or modulated by the bulk Ca^{2+} (von Gersdorff and Matthews, 1994; Marks and McMahon, 1998; Teng and Wilkinson, 2005; Balaji et al., 2008; but see Cousin and Robinson, 2000). In order to explain a local Ca^{2+} dependence of endocytosis, one has to postulate that Ca^{2+} binding to a relatively low-affinity sensor is the first step in a multistep pathway (Figure 5). Following "activation" of the sensor, subsequent events are most likely Ca^{2+} independent (and irreversible) because endocytosis itself manifests itself long after the microdomain collapses. This is consistent with the well-established concept that endocytosis consists of several enzymatic reaction steps prior to membrane fission (Ryan, 2006; Schmid and McMahon, 2007), with a possible

Ca^{2+}-dependent intermediate step before fission also postulated (Ramaswami et al., 1994).

The local Ca^{2+} dependence of endocytosis may not be a general mechanism for endocytosis in the nerve terminal. For example, spontaneous fusion events are associated with endocytosis when the Ca^{2+} concentration is at the basal level (Balaji and Ryan, 2007). Also sucrose, which stimulates exocytosis at synapses through a Ca^{2+}-independent mechanism (albeit on the time-scale of seconds) (Rosenmund and Stevens, 1996) keeps synaptic vesicle recycling relatively intact (Stevens and Wesseling, 1998, 1999; Deak et al., 2004). We have not studied the endocytotic rate at or near basal Ca^{2+} levels. It is possible that endocytosis may operate at a low rate at such levels (He et al., 2006), and Ca^{2+} may have a critical role only when the amount of exocytosis exceeds a certain level. In other words, the capacity of endocytosis may be regulated by Ca^{2+}, as in hippocampal synapses (Balaji et al., 2008), although in the calyx of Held it is the local Ca^{2+} concentration that is the scaling factor. It remains to be seen why the Ca^{2+} sensitivity is diverse among different synapses (Neves et al., 2001; Wu et al., 2007). It is also important to note that other forms of endocytosis, such as bulk retrieval and dynamin-independent endocytosis (Xu et al., 2008), might operate in parallel with the slow form of endocytosis described here to keep vesicle recycling under physiological conditions.

The Ca^{2+} sensor for triggering slow endocytosis at the calyx of Held synapse remains to be identified. It has been known for several years that a number of endocytic proteins, referred to collectively as dephosphins (and which includes dynamin and amphiphysin), become dephosphorylated at nerve terminals during activity. However, it is hard to integrate this pathway into our present findings. First, the precise role of these phosphorylation events has not been elucidated in most cases, and second, these molecules are usually thought to be substrates for the calcium-dependent phosphatase calcineurin. Thus, the lack of an endocytic response when intracellular $Ca^{2+}$was uniformly raised to levels of ~10 μM means that a high-affinity sensor such as calcineurin (Marks and McMahon, 1998) is an unlikely candidate. Synaptophysin interacts with dynamin at high Ca^{2+} concentrations (~150 μM) (Daly and Ziff, 2002), although this Ca^{2+} level is too high for the calyx synapse, and synaptophysin actually seems to regulate clathrin-independent endocytosis (Daly et al., 2000). However, synaptotagmin 2, remains an attractive potential candidate for the Ca^{2+}-sensor in slow endocytosis. This is because synaptotagmin 2 is the major Ca^{2+}-sensor for exocytosis at the calyx of Held (Sun et al., 2007), and there is increasingly strong evidence that synaptotagmin may also be involved in endocytosis (Zhang et al., 1994; Poskanzer et al., 2003; Nicholson-Tomishima and Ryan, 2004), thus linking the processes of exo- and endocytosis together.

Interaction between the Exo- and Endocytic Processes

Disruption of synaptobrevin by toxin cleavage, as well as dialysis of the proline rich domain of synaptobrevin into the presynaptic terminal, blocked membrane retrieval at the calyx of Held (Figure 6). This suggests that synaptobrevin has an important role in endocytosis. This is reinforced by the fact that infusion of the proline-rich domain into the calyx did not affect release

itself, in contrast to tetanus toxin, suggesting that the proline-rich domain of synaptobrevin may be responsible for mediating molecular interactions that are necessary for endocytosis. Conceptually, it is not difficult to imagine that disruption of one of the proteins on the synaptic vesicle may result in a block in the release process at any point from synaptic vesicle fusion till membrane fission, because synaptic vesicle proteins are essentially cargo molecules that have to be endocytosed and recycled, to allow further rounds of release (Voglmaier et al., 2006). For example, a dead *cis*-SNARE complex may sit at the active zone, preventing further reactions of synaptobrevin with endocytic proteins and thus inhibit recycling, until the complex is dissociated into the individual SNARE components by NSF. It has been previously proposed that synaptobrevin is responsible for rapid endocytosis (Deak et al., 2004), whereas synaptotagmin (another vesicular protein) mediates slow endocytosis (Poskanzer et al., 2003). In contrast, this study indicates that synaptobrevin influences slow endocytosis at the calyx synapse.

We have previously shown that disruption of synaptobrevin slowed recruitment of the fast-releasing synaptic vesicles for release (Sakaba et al., 2005; Wadel et al., 2007), in addition to blocking endocytosis. This effect could also be mimicked by blocking one of the discrete molecular steps that must occur between vesicle fusion and final membrane fission, as shown in Figure 7. Therefore, endocytosis directly influences exocytosis, and it is likely that the slowed recruitment seen in the presence of tetanus toxin is also mediated by a block in endocytosis, probably an early step such as recruitment of adaptor proteins (AP2) to the membrane. Previously, stronger synaptic depression has been observed after block or deletion of endocytic proteins (Shupliakov et al., 1997; Kawasaki et al., 2000; Ferguson et al., 2007), and this has been often attributed to the disruption of a "kiss-and-run" type vesicle cycling that utilizes a rapid form of endocytosis; rapid reuse of synaptic vesicles through rapid vesicle cycling could counteract synaptic depression observed during a high frequency train of APs (Kawasaki et al., 2000; Verstreken et al., 2002; Ertunc et al., 2007). However, this was questioned by more recent studies, which suggested that strong synaptic depression was actually mediated by a secondary effect—a reduction in the size of the reserve pool of synaptic vesicles that occurs after endocytosis is blocked (Dickman et al., 2005). Accordingly, it is more common not to assume a limiting step between exo- and endocytosis, especially when endocytosis is slow (Granseth and Lagnado, 2008; Wu and Betz, 1996). In contrast, we have not seen a reduction in the RRP size over time after acute block of endocytosis (Figure S7), suggesting that a reduction in the reserve pool of synaptic vesicles is not responsible for the slowed vesicle replenishment and synaptic depression observed in this study.

At the calyx of Held, two components of release could be seen when a step-depolarizing pulse was applied to the presynaptic terminal (Sakaba and Neher, 2001a). We propose that the fast-releasing component represents synaptic vesicles that occupy a limited number of release sites at the active zones, close to the Ca^{2+} channels (Wadel et al., 2007). They mediate synchronous release during an AP (Sakaba, 2006). The slowly-releasing synaptic vesicles, on the other hand, represent vesicles located further away from Ca channels and it is these vesicles that

mediate asynchronous transmitter release during an AP train (Sakaba, 2006; Wadel et al., 2007). The number of release sites for the fast-releasing vesicles appears relatively fixed, because variance analysis, assuming a fixed number of release sites, explains synaptic responses at the calyx of Held very well (Scheuss et al., 2002), and also because the number of fast-releasing synaptic vesicles is relatively insensitive to modulation by second messengers (Sakaba and Neher, 2001b; Sakaba and Neher, 2003; Awatramani et al., 2005; Srinivasan et al., 2008).

It has been shown that the complex geometric association between synaptic vesicles and Ca channels ("positional priming"), rather than molecular priming of synaptic vesicles (Neher and Sakaba, 2008), is rate limiting for fast transmitter release at the calyx of Held (Wadel et al., 2007). To some degree, this association process must reflect the recruitment of synaptic vesicles to the release sites, as has been previously assumed (Zucker and Regehr, 2002; Wadel et al., 2007). However, if there is only a fixed number of release sites responsible for fast transmitter release (Zenisek et al., 2000), then not only recruitment of vesicles to release sites but also the availability of release sites may be rate limiting during repetitive stimulation, because the release sites must be cleared of used vesicular proteins and the release machinery must be reset before further synaptic vesicle can fuse at the same sites (Neher and Sakaba, 2008). So, if exo- and endocytic sites are identical, the RRP size should decrease with time when endocytosis is blocked because used vesicular proteins would be expected to accumulate at the release sites blocking further rounds of fusion. However, our results are not compatible with this. Rather, it appears that sites of exo- and endocytosis are close to each other, perhaps even overlapping, but not identical, and it is most likely that clearance of exocytosed materials from these exocytic sites becomes rate limiting for a short period (less than 5 s) following exocytosis (Figure 7). It should be noted that the fast-releasing pool recovers completely, even in the presence of endocytosis blockers, when left for more than 15 s after initial depletion. This also indicates that the clearance of exocytosed proteins from release sites can occur not only by endocytosis but also by other mechanisms, such as diffusion of vesicular proteins through the plasma membrane, which has been reported in some studies (Li and Murthy, 2001; Fernandez-Alfonso et al., 2006; Wienisch and Klingauf, 2006). In contrast, the slowly releasing vesicles do not seem to have this constraint of "release-site clearance" (Figure 7). This may be because the slow vesicles are outliers from Ca^{2+} channels clusters (Zenisek et al., 2000), and they do not need highly specialized fixed sites for release, consistent with the distribution of the SNARE proteins syntaxin and SNAP-25 all over the presynaptic membrane, rather than being restricted to discrete sites. This is certainly the case with chromaffin cells, in which exocytosis can occur over the whole cell surface (Neher, 2006) and a "traffic jam" of exocytosed proteins at the release site should not occur. For slowly releasing vesicles, "molecular priming" for release may matter, rather than "positional priming" (Wadel et al., 2007).

As an alternative to the "clearance" hypothesis, endocytic proteins may control synaptic vesicle recruitment independently of endocytosis, perhaps through the mutual control of exocytic proteins, as has been postulated for other systems (Peters

et al., 2004), an idea which sits well with the effect of synaptobrevin disruption on endocytosis and the proximity between exocytic and endocytic sites, and reinforces the idea that exo- and endocytic processes interact to a certain degree even when endocytosis is slow.

The slow mode of endocytosis has often been thought to occur at sites remote from active zones (Heuser and Reese, 1973; Ringstad et al., 1999; Roos and Kelly, 1999). The present study shows that the slow mode of endocytosis at the calyx synapse shares some features commonly attributed to the fast mode of endocytosis, such as (1) local Ca^{2+}-dependence, (2) synaptobrevin dependence, and (3) a limited influence on the exocytic process. We have used young rats in this study, and the features described here may change with development (Taschenberger and von Gersdorff, 2000; Iwasaki and Takahashi, 2001; Joshi and Wang, 2002; Renden and von Gersdorff. 2007). It also remains to be seen whether this results from the specialization of this auditory synapse or else is a general feature in other synapses (Hayashi et al., 2008).

EXPERIMENTAL PROCEDURES

Transverse brainstem slices (200 μm thick) were prepared from 8- to 11-day-old wistar rats (Forsythe, 1994). 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). Experiments were performed at room temperature. During recordings, 0.5 μM TTX and 10 mM TEA-Cl were always included in the external solution to block Na^+ and K^+ channels, allowing the isolation of presynaptic Ca^{2+} currents. In paired recordings of pre- and postsynaptic cells, 50 μM D-AP5, 100 μM cyclothiazide (CTZ), and kynurenic acid 1 mM (Kyn) were added to isolate postsynaptic AMPA-receptor mediated EPSCs and to block desensitization and possible saturation of AMPA receptors. This manipulation also avoids introducing voltage-clamp errors into EPSC measurements (Neher and Sakaba, 2001). For flash experiments, 2 mM γDGG was used instead of Kyn (Wadel et al., 2007; Wölfel et al., 2007). For recording miniature EPSCs from postsynaptic cells, Kyn was omitted from the recording solution and 10–80 μM bicuculline and 10–20 μM strychnine were added to block spontaneous inhibitory inputs. A calyx of Held and its postsynaptic MNTB principal neuron were whole-cell voltage clamped at −80 mV using an EPC9/2 or EPC10/2 amplifier (HEKA, Germany). The presynaptic patch pipette (3 to 5 MΩ) solution contained (in mM) 135 Cs-gluconate, 20 TEA-Cl, 10 HEPES, 5 Na_2-phosphocreatine, 4 MgATP, 0.3 GTP, and 0.05–0.2 EGTA (pH 7.2). In experiments using low extracellular Ca^{2+} (Figure 2), 0.8 mM Ca^{2+} was substituted for 2 mM Ca^{2+} while the concentration of the other divalent cation (Mg^{2+}) in the extracellular solution was either kept constant at 1 mM or raised to 2.2 mM. For the experiments in Figure 7, the final concentration of EGTA was increased to 0.5 mM, in order to separate the fast and slow components of transmitter release (Figure 7). Dynasore (100 μM) was included in the extracellular solution, and slices were incubated for 15–60 min before experiments to ensure that this reagent permeated into the presynaptic terminal. This approach was taken because preliminary attempts to include dynasore in the patch pipette solution prevented gigaohm seal formation between the patch pipette and the presynaptic terminal. As a precaution against tissue deterioration, control slices were exchanged every 60 min. The presynaptic series resistance (5 to 20 MΩ) was compensated by 30% to 90%. The postsynaptic pipette (2 to 3.5 MΩ) contained the same solution as the presynaptic pipette, except that the EGTA concentration was increased to 5 mM. The postsynaptic series resistance (3 to 8 MΩ) was compensated by the amplifier so that the uncompensated resistance was below 3 MΩ. The remaining resistance was further compensated off-line.

Presynaptic capacitance measurements were carried out using an EPC9/2 or EPC10/2 amplifier in the sine + DC configuration (Lindau and Neher, 1988). A

sine wave (30 mV in amplitude, 1000 Hz) was superimposed on a holding potential of −80 mV. Measurements were included in the data set if the presynaptic series resistance was below 20 MΩ. The osmolarity of the intracellular solution was routinely checked in order to obtain relatively stable baseline recordings.

CTZ, D-AP5, Kyn, and γDGG were obtained from Tocris. Dynasore was obtained from Sigma-Aldrich. The dynamin inhibitory peptide was from Calbiochem. CTZ and dynasore were dissolved in DMSO, and the final concentration in the extracellular solution was 0.2%. The N-terminal peptide from synaptobrevin contained the first 18 amino acids, including the proline rich domain (NH2-SATAATVPPAAPAGEFFPPAPPPNLT-amide). The AP2 blocking peptide (DNF-12-mer, INFFEDNFVPEI) contains the DNF motif from amphyphysin-1 and binds the α-subunit of AP2 with a K_d of 2.5 μM (Olesen et al., 2008), while the point-mutated blocking peptide (DPF-12-mer, INFFEDPFVPEI) has a 50-fold lower affinity (Olesen et al., 2008) and was used as a control (Jockusch et al., 2005). In a series of separate experiments, the C2B domain from synaptotagmin 2 (KRLKKKKTTVKK, 10-100 μM) was used to sequester AP2 and interfere with endocytosis (Grass et al., 2004). The peptides were supplied by Biosyntan (Berlin, see Supplemental Data for information on the dialysis of peptides into internal solution). Antibodies against dynamin were purchased from Santa Cruz and were purified as described in supplementary information.

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 CTZ and Kyn (or γDGG, Neher and Sakaba, 2001; Wadel et al., 2007). Quantal release rates, as determined by deconvolution, were integrated to obtain the cumulative release, displayed in the figures. Cumulative release was fitted by a double exponential after correction for synaptic vesicle replenishment (Sakaba and Neher, 2001a). In the paired-pulse protocol (Figure 7), the time constants for the second pulse were derived in the same manner as those for the first pulse, because a small fraction of the fast-releasing component might have been missed by using a single exponential with free parameters, which fit the data equally well in some cases. Capacitance decay was quantified as the rate of membrane retrieval. When the capacitance decays to the baseline, a half decay time was obtained, and the rate was obtained using the Cm jump and half decay time. When the capacitance decay was too slow to return to base line level in our measurement period (40 s), a line fit was used to estimate the rate. For capacitance records, baseline correction was not made.

For Ca-flash experiments, the presynaptic patch pipettes contained (in mM): Cs-gluconate 125–130, TEA-Cl 20, HEPES 20–30, NaATP 5, NaGTP 0.5, $MgCl_2$ 0.5, DM-nitrophen 1–3, $CaCl_2$ 0.85–1.7, and Fura-2FF 0.2 (pH 7.2). As ATP is present in excess to Mg, most of the Mg binds to ATP rather than the DM-Nitrophen (Schneggenburger and Neher, 2000). In three cells, NP-EGTA (5 mM) was used instead of DM-Nitrophen. Flashes of UV light (duration of 1 ms) were applied using a flash-lamp (Rapp, Germany) and the fluorescence signal from Fura-2FF (excitation at 350 and 380 nm) was measured with a CCD camera (with an exposure time of 5 ms for each wavelength and a cycle time of 15 ms. Till Vision, Till Photonics, Germany). Background fluorescence was measured in a region neighboring the calyx simultaneously with the test measurements. Calibration of Fura-2FF was carried out in vitro.

Data are expressed as the mean ± SEM, except when indicated otherwise.

SUPPLEMENTAL DATA

Supplemental Data include eight figures, one table, and supplemental text and can be found with this article online at http://www.cell.com/neuron/supplemental/S0896-6273(09)00463-2.

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