Activity-dependent modulation of endocytosis by calmodulin at a large central synapse

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Although Ca^{2+}/calmodulin has been suggested to play a role during endocytosis, it remains unknown if binding of Ca^{2+} to calmodulin is essential for initiating endocytosis or if this interaction only has a modulatory effect on endocytosis. In this study, using time-resolved capacitance measurements at the rat calyx of Held synapse, the role of calmodulin in endocytosis was examined. Our results demonstrate that blocking calmodulin with an inhibitory peptide, which interfers with the binding of calmodulin to downstream targets, slowed the rate of endocytosis, but only when accompanied by high Ca^{2+} influx. In response to a short train of action potential-like stimulation, blocking calmodulin had no effect on endocytosis. Furthermore, we have identified conditions in which inhibition of calmodulin fails to affect the rate of endocytosis, but nevertheless retards recruitment of synaptic vesicles to the fast-releasing vesicle pool responsible for synchronous release. The results indicate that calmodulin facilitates endocytosis in an activity-dependent manner but is not mandatory for endocytosis, and suggest that calmodulin modulates an endocytotic intermediate process, which in turn affects synaptic vesicle recruitment and membrane fission.

protein kinase A | synaptic transmission | calcium

At the presynaptic terminal, the role of Ca^{2+} in endocytosis remains unclear. Depending on the preparation, Ca^{2+} either accelerates the rate of endocytosis, has no apparent effects (1), or can inhibit (2). In addition, it is unknown whether the increase in intraterminal [Ca^{2+}] initiates endocytosis in an all-or-none manner or has only a modulatory role. The uncertainty may partially arise from the different kinetic components of endocytosis that have been observed both within a given cell type and among different preparations (3, 4).

The calyx of Held synapse has a large presynaptic terminal where direct recordings can be performed (5). In this preparation, presynaptic capacitance measurements allow for the monitoring of membrane retrieval with high temporal resolution (6). At the calyx synapse, it has recently been shown that both rapid and slow forms of endocytosis are Ca^{2+}-dependent and are sensitive to calmodulin inhibitors (7). Based on these results, it was subsequently postulated that Ca^{2+}/calmodulin is the major Ca^{2+} sensor for endocytosis. However, the stimulation protocols used in this previous study were not representative of physiological conditions. Thus, it remains unclear whether calmodulin triggers endocytosis in response to milder, more physiologically relevant stimulation. Finally, although Wu et al. (7) concluded that Ca^{2+}/calmodulin is the major sensor for endocytosis, recent studies have demonstrated that other Ca^{2+} sensors can mediate various forms of endocytosis in this (8, but see ref. 7) and other preparations (9).

Because of the unanswered questions surrounding the role of calmodulin and other Ca^{2+} sensors during endocytosis, we applied more physiologically relevant stimulation to the calyx of Held. In the present study we demonstrate that blocking calmodulin with an inhibitory peptide does not affect endocytosis in response to a short train of action potential (AP)-like stimuli that is representative of more physiological conditions. Previously we found (10, 11) that interfering with the function of both calmodulin and several proteins of the endocytic pathway retard the recovery from short-term synaptic depression. We therefore asked whether it is obligatory that calmodulin's effect on endocytosis is coupled to its effect on depression. We found stimulation conditions in which calmodulin blockers leave endocytosis untouched but retard the recruitment of fast-releasing synaptic vesicles that are relevant for synchronous release in response to APs at the calyx of Held synapse (12). These results suggest that recruitment of synaptic vesicles does not directly depend on the rate of membrane fission and that it may be regulated by an intermediate step in between exocytosis and membrane fission.

Results

Calmodulin Facilitates Endocytosis in an Activity-Dependent Manner. To test the role of calmodulin in endocytosis, capacitance measurements were performed to monitor exo- and endocytosis at the calyx of Held synapse in postnatal day (P) 9 to P11 rats (6, 13) in the presence or absence of a calmodulin inhibitory peptide, which interferes with binding of calmodulin to downstream targets. In addition, 0.05 to 0.1 mM EGTA was included in the presynaptic patch pipette because it has been experimentally shown to be similar in buffering capacity to an unperturbed synapse (14), First, using an identical simulation protocol as Wu et al. (7), a depolarizing pulse was applied for 20 ms (from −80 mV to 0 mV) to the presynaptic terminal. In response to stimulation, a capacitance increase was observed, which reflects the amount of exocytosis (6, 13). Following the capacitance increase, the capacitance decreased with a time constant of tens of seconds, which reflects endocytosis (13) (Fig. 1*A*). Consistent with Wu et al. (7), intracellular application of calmodulin inhibitory peptide (20 μM) slowed endocytosis ($n = 5$), with no effect on exocytosis. In the presence of calmodulin inhibitory peptide, the average capacitance jump was 400 ± 35 fF vs. 386 ± 19 fF (control; P = not significant), but the rate of endocytosis was dramatically affected and slowed (~10-fold 1.9 ± 0.7 fF/s vs. 21.0 ± 2.9 fF/s control), respectively ($P < 0.01$, $n = 5$) (Fig. 1*A*). To rule out any potential nonspecific effects, a control peptide (calmodulin inhibitory peptide with one Trp replaced by Glu; see *Materials and Methods*) was introduced as a negative control (Fig. 1*A*, *Right*, and Fig. S1). In these cases, the capacitance decay was similar to that of control (no peptide), consistent with Wu et al. (7).

Calmodulin-dependent components of endocytosis may depend on the extent of Ca^{2+} accumulation in the terminal. To test the effect of the calmodulin inhibitory peptide at more physiological conditions, trains of (10–50 pulses) AP-like stimuli (1-ms

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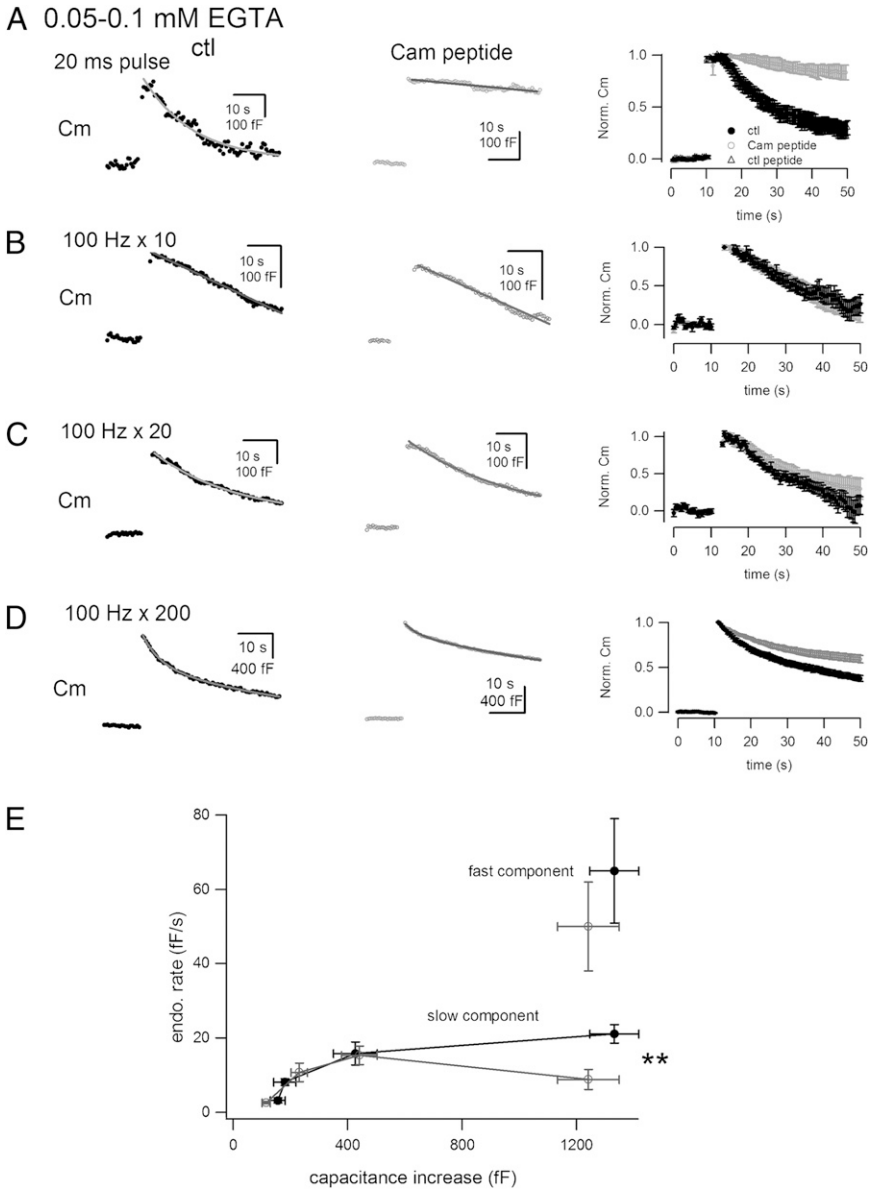

Fig. 1. Stimulus-dependent modulation of endocytosis by calmodulin. The presynaptic pipette contained 0.05–0.1 mM EGTA. (*Left* and *Center*) Individual capacitance traces under control conditions and in the presence of 20 μM calmodulin inhibitory peptide in the pipette. Either a step depolarizing pulse to 0 mV for 20 ms (*A*) or a train of AP-like stimuli [depolarization to +40 mV for 1 ms at 100 Hz, either 10 stimuli (*B*), 20 stimuli (*C*), or 200 stimuli (*D*)] was applied to the presynaptic terminal at the calyx of Held synapse. Gray lines indicate either an exponential or a line fit to the data, which were used for calculating the rate of endocytosis. (*Right*) Average capacitance traces are shown. Each capacitance trace was normalized to the peak capacitance jump, before the traces were averaged across cells. Filled black circles represent the control data, whereas open gray circles represent the data in the presence of calmodulin inhibitory peptide. In *A*, the average capacitance trace from control peptide is superimposed, which is indistinguishable from control condition (Fig. S1). (*E*) The capacitance decay was fitted with a line (100 Hz × 10 stimuli), a single exponential (100 Hz × 20 or 50 stimuli) or a double exponential (100 Hz × 200 stimuli). Filled black circles and open gray circles are the control condition and the condition in the presence of calmodulin inhibitory peptide, respectively. From the fit, endocytosis rates were calculated by dividing the amplitude of capacitance jump by the time constant. **Statistical significance of $P < 0.01$.

square wave pulse from −80 mV to +40 mV) were applied at a frequency of 100 Hz (15). Subsequently, the resultant capacitance jumps and decays of capacitance were analyzed. We found that 10 and 20 AP-like stimuli elicited capacitance increases smaller than those of 20-ms depolarizations (10 APs: 156 ± 26 fF; 20 APs: 180 ± 39 fF). More importantly, there was no significant effect of calmodulin inhibitory peptide on the rate of endocytosis in response to 10 and 20 AP-like stimuli (Fig. 1 *B* and *C*). Capacitance jumps were comparable to those under control conditions (10 APs: 115 ± 14 fF; 20 APs: 231 ± 28 fF). However, when stronger stimulation was applied (200 pulses), endocytosis was slowed by calmodulin inhibitory peptide ($P < 0.01$) (Fig. 1 *D* and *E*). The inhibitory peptide did not modify the presynaptic Ca^{2+} current amplitudes elicited by AP-like stimuli [1.41 ± 0.11 pC (control) and 1.49 ± 0.11 pC (calmodulin inhibitory peptide, ns, $n = 7$ in each condition]. Based on these results, we conclude that calmodulin enhances the rate of endocytosis following strong (200 APs at 100 Hz), but not weak stimulation (10–20 APs at 100 Hz).

Wu et al. (7) suggested that calmodulin could enhance the rate of synaptic vesicle recruitment to the readily releasable vesicle pool by increasing the rate of endocytosis and possibly clearing

released materials from the release site more effectively. To examine whether there is a direct link between calmodulin-dependent endocytosis and synaptic vesicle recruitment, 50-ms depolarizing pulses were used to completely deplete the readily releasable pool of synaptic vesicles. In this set of experiments, 0.5 mM EGTA was included in the patch pipette, allowing one to clearly separate the fast and slow components of release during depolarizing pulses (10). The fast component of release has been shown to be responsible for synchronous release at the calyx synapse, whereas the slow component is responsible for asynchronous release during bursts of APs (12). In addition, compared with 0.05 mM EGTA, we expected that 0.5 mM EGTA might prevent accumulation of bulk calcium at the site of a potential sensor for endocytosis. We found that calmodulin inhibitory peptide (20 μM) did not change the rate of endocytosis following a 50-ms depolarizing pulse under these conditions (Fig. 2 *A* and *B*, and Table S1). Presynaptic Ca^{2+} currents were not affected by the inhibitory peptide (total influx of 53.8 ± 3.3 pC vs. 61.2 ± 5.4 pC for a 50-ms pulse, control vs. peptide). The same result was also obtained by another peptide, calmodulin binding domain (20 μM). On the other hand, a dynamin inhibitory peptide, which is known to block dynamin-dependent components of endocytosis (16, 17), completely abolished endocytosis (Fig. S2 and Table S2). This finding indicates that the blocking of endocytosis by inhibitors could be reliably detected under our recording conditions.

Because no calmodulin-dependent component of endocytosis was observed after a 50-ms pulse with 0.5 mM EGTA, we expected that increasing the pulse duration might overcome intracellular Ca^{2+} buffering and stimulate such a component. In response to a 500-ms pulse, capacitance decays could be fitted by a double exponential (Fig. 2*A*). The rate of fast endocytosis (obtained by dividing the amplitude of the fast component by the time constant) was not significantly affected by calmodulin inhibitory peptide [145 ± 23 fF/s for control (n = 9) and 125 ± 31 fF/s for calmodulin inhibitory peptide (n = 6), $P > 0.05$] (Table S3), whereas the slow component of endocytosis was significantly slowed by the peptide [the endocytotic rate was 36.0 ± 7.9 fF/s for control (n = 9) and 10.0 ± 0.9 fF/s for calmodulin inhibitory peptide (n = 6), $P < 0.01$] (Tables S3 and S4). Together with Fig. 1, the results suggest that calmodulin, rather than being mandatory for initiating endocytosis, modulates endocytotic rates when bulk [Ca^{2+}] is increased in the presynaptic terminal to levels >> 1 μM, as estimated from [Ca^{2+}] measurements in previous studies (18). Our findings are similar to those regarding Ca^{2+}-control of endocytotic capacity in cultured hippocampal neurons (19). However, such strong stimulation causing massive Ca influx does not usually happen under physiological conditions.

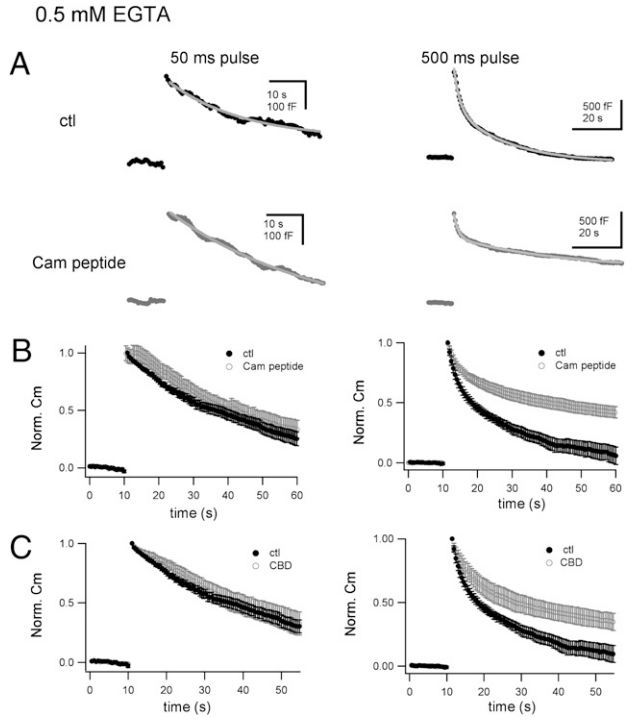

Fig. 2. Calmodulin-dependent component of endocytosis in response to a step depolarizing pulse under 0.5 mM EGTA. (*A*) A depolarizing pulse (from −80 mV to 0 mV for 50 ms) was applied to the presynaptic terminal in the control condition (*Upper*) and in the presence of calmodulin inhibitory peptide (*Lower*) in the presynaptic patch pipette. The pipette solution also contained 0.5 mM EGTA. The pulse duration was either 50 (*Left*) or 500 ms (*Right*). A single (50-ms pulse) or a double (500-ms pulse) exponential fit was used to calculate the endocytosis rates (gray lines). (*B* and *C*) The normalized time courses of endocytosis are plotted. The control (filled black circles) condition and the condition in the presence of calmodulin inhibitory peptide (*B*) or calmodulin-binding domain (*C*) are superimposed (open gray circles).

cAMP/PKA Signaling Pathway Modulates Endocytosis in Response to Strong Stimulation. It has been shown that cAMP/Epac (exchange protein activated by cAMP), but not protein kinase A (PKA), is involved in recruitment of synaptic vesicles to the fast-releasing pool (20–22). Therefore, if it can be shown that PKA is involved in endocytosis, one may conclude that endocytosis and synaptic vesicle recruitment must be modulated by different signaling pathways. To test for the role of cAMP in endocytosis at the calyx of Held, brain slices were incubated with MDL 12330A (50 μM), an inhibitor of adenylate cyclase, for 30–60 min. Similar to Fig. 2, 0.5 mM EGTA was included in the patch pipette, and a 50- or 500-ms pulse was used to elicit exo- and endocytosis. Endocytosis following a 50-ms pulse was only moderately slowed by MDL 12330A (Fig. 3*A*). In contrast, endocytosis following a 500-ms depolarizing pulse was significantly slowed by MDL 12330A (Fig. 3*B*). The data suggest that a basal cAMP level is required for full-fledged endocytosis following a 500-ms—but not a 50-ms—pulse at the calyx of Held terminal. cAMP acts through both PKA and Epac (20). We wished to determine if the cAMP effect on endocytosis, unlike that on synaptic vesicle recruitment, was dependent on PKA. We therefore incubated brainstem slices with KT5720 (2 μM), an inhibitor of PKA, for 30–60 min at room temperature. Similar to MDL 12330A, endocytosis following a 500-ms pulse was slowed down significantly under KT 5720 (Fig. 3*B*), but the effect was only moderate in response to a 50-ms pulse (Fig. 3*A*). Therefore, cAMP/PKA is required for intact endocytosis following a 500-ms, but not a 50-ms pulse in the presence of 0.5 mM EGTA in the patch pipette. The effect is similar to that of perturbing calmodulin action. In conclusion, unlike synaptic vesicle recruitment, endocytosis is modulated by activation of PKA.

Calmodulin Modulates Synaptic Vesicle Recruitment and Endocytosis Differently. Previously, we have shown that two components of transmitter release can be observed during a 50-ms pulse when 0.5 mM EGTA is included in the patch pipette (10). We also showed that calmodulin inhibitors (calmlodulin inhibitory peptide, calmodulin binding domain, and calmidazolium) slow recovery of the fast-releasing component following depletion. Recently, Wu et al. (7) have proposed that calmodulin accelerates synaptic vesicle recruitment by clearing release sites through endocytosis, although it is not known mechanistically how this is accomplished. To examine this issue, we looked for conditions in which blocking calmodulin would slow down the recruitment of synaptic vesicles, but not influence endocytosis (Fig. 2). In this set of experiments, simultaneous pre- and postsynaptic recordings were performed, as described previously (10). To examine whether calmodulin

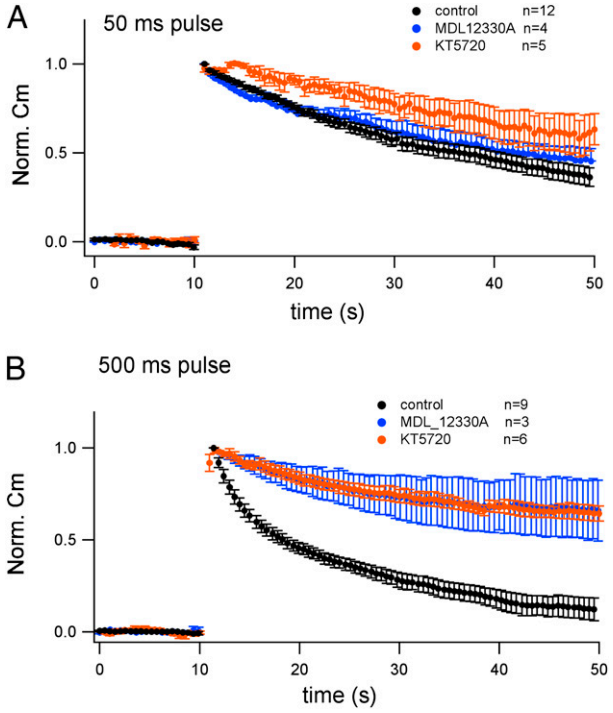

Fig. 3. Modulation of endocytosis by cAMP. Average capacitance traces in response to 50-ms (*A*) and 500-ms (*B*) pulses are shown. Presynaptic pipettes contained 0.5 mM EGTA. Each capacitance trace was normalized to the peak capacitance jump, before the traces were averaged across cells, similar to Fig. 1. The data were obtained either under control conditions (black circles) or in the presence of 50 μM MDL 12330A (blue circles), or KT5720 (red circles).

inhibitors influence vesicle recruitment under this same set of conditions, we applied pairs of 50-ms pulses at a 500-ms interval and measured the subsequent recovery of both the fast and slow pool (11, 16) using the deconvolution method (23) (*Materials and Methods*). Release rates during depolarizing pulses were estimated from the evoked excitatory postsynaptic currents (EPSCs) (Fig. 4*A*). Subsequently, cumulative transmitter release during the pulse could be fitted by double exponential functions, with time constants of 2–3 ms (about 50% of the total readily releasable pool, fast-releasing vesicles) and 10–30 ms (slowly releasing vesicles) (Table S5). For a 500-ms interpulse interval, the fast component of release recovered to 60 ± 4% of its baseline value, whereas the slowly releasing component recovered almost fully. Intracellular application of calmodulin inhibitory peptide (20 μM) significantly blocked the recovery of the fast-releasing component (15 ± 5%, $P < 0.01$) without affecting that of the slowly releasing component (Fig. 4 *B* and *D*), consistent with the work of Sakaba and Neher (10). Because endocytosis was not altered by calmodulin inhibitors under this condition (Fig. 2), we conclude that the recovery of the fast-releasing component is not directly connected to the calmodulin-dependent component of endocytosis.

Because PKA inhibitors and calmodulin inhibitors blocked endocytosis similarly (Fig. 3), we examined whether PKA inhibitors affected synaptic vesicle recruitment similar to calmodulin inhibitors. Incubating brainstem slices with KT5720, which affected endocytosis in a manner similar to calmodulin inhibitors, did not affect the recovery of synaptic responses during the second pulse (Fig. 4 *C* and *D*). Therefore, blocking PKA slowed endocytosis without affecting the rate of synaptic vesicle recruitment. Although inhibition of calmodulin and PKA had similar effects on endocytosis, only inhibition of calmodulin slowed the rate of synaptic vesicle recruitment to the fast-releasing pool. Taking these data together, we conclude that membrane retrieval and synaptic vesicle recruitment do not necessarily have a causal relationship.

Discussion

In this study, the roles of calmodulin and cAMP in endocytosis were examined using capacitance measurements. Based on our results we can conclude: (*i*) Block of Ca^{2+}/calmodulin inhibits the rate of endocytosis following a depolarizing pulse associated with large Ca^{2+} influx but has no effect on responses to weaker stimulation; (*ii*) PKA antagonists slow endocytosis in response to strong stimulation, similar to calmodulin inhibitors, but unlike calmodulin inhibitors, have no effect on recruitment of synaptic vesicles to the fast-releasing pool; and (*iii*) In the presence of

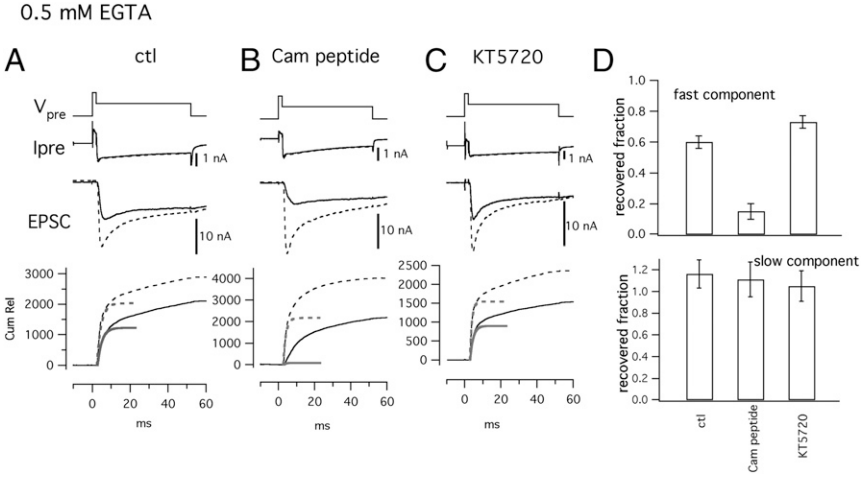

Fig. 4. Dissociation between the calmodulin-dependent component of synaptic vesicle recruitment and calmodulin-dependent endocytosis. A pair of the step-depolarizing pulses (prepulse of +70 mV for 2 ms followed by repolarization to 0 mV for 50 ms) was applied with an interval of 500 ms. Presynaptic pipettes contained 0.5 mM EGTA. Dotted and solid lines indicate the data from the first and the second pulse, respectively. From the top, voltage protocols, presynaptic currents, EPSCs, and cumulative amounts of release estimated by the deconvolution method are shown. In the cumulative release plot, the fast component of release is shown in gray, which was estimated by the double-exponential fitting. Control data (*A*), the data obtained in the presence of calmodulin inhibitory peptide (*B*), or KT5720 (*C*) are shown. (*D*) Recovery of the fast (*Upper*) and the slow component (*Lower*) of transmitter release are plotted. The data were obtained by comparing the values during the first and the second pulses.

0.5 mM EGTA in the patch pipette, calmodulin is essential for fast vesicle recruitment following a 50-ms pulse without influencing the rate of endocytosis. This latter finding may indicate that calmodulin acts on synaptic vesicle recruitment and endocytosis independently.

Wu et al. suggested that calmodulin is the major Ca^{2+} sensor for endocytosis and triggers large part of endocytosis (7). Consistent with that study, we found that calmodulin inhibitors slowed the rate of endocytosis following a 20-ms depolarizing pulse under low Ca^{2+} buffering conditions. However, the inhibitors did not change the rate of endocytosis following short trains of AP-like stimuli (100 Hz × 10 stimuli; 100 Hz × 20 stimuli), eliciting less Ca^{2+} influx and evoking less amounts of exocytosis (compared with 200 APs). We ruled out artifacts caused by off-target effects of the reagents because a control peptide for calmodulin inhibitory peptide did not affect the rate of endocytosis at all (Fig. S1 and Fig. 1*A*). In addition, two calmodulin inhibitors that are supposed to act on the same or related targets were used in this study, and both had similar effects on endocytosis. In the studies of Wu et al. (7) and Yamashita et al. (24), inhibition of endocytosis by blockers was not complete in all of the experiments shown, perhaps indicating that a calmodulin-insensitive component may have been present (7). Taken together, our data are consistent with previous studies and further demonstrate that calmodulin enhances the rate of endocytosis in an activity-dependent manner, but not being mandatory for endocytosis. The results are also consistent with the idea that Ca^{2+}/calmodulin modulates the endocytotic capacity, similar to the role of Ca^{2+} in cultured hippocampal neurons (19). Given that endocytosis is not totally eliminated by blocking calmodulin, other Ca^{2+} sensors, which remain to be identified, must be involved. It is not clear at present how much these findings at the calyx synapse can be generalized, because the roles of Ca^{2+} in endocytosis seem to be different among different types of preparations (1–4).

After exocytosis, vesicular materials associated with synaptic vesicle fusion have to be rapidly cleared from release sites through endocytosis to allow new synaptic vesicles to dock at such sites and become readily releasable (25, 26). In contrast to synaptic vesicle priming (molecular priming), which makes synaptic vesicles biochemically ready for fusion, site clearance is a post-fusion event, which may affect upstream events (25, 27). The hypothesis of release-site clearance came from the findings that the inhibition of endocytotic proteins not only blocks endocytosis, but also subsequent rounds of synchronous release (7, 11, 27). This hypothesis is based on the idea that the number of release sites is limited and that the availability of the sites becomes rate-limiting during repetitive activity (25, 28). Wu et al. (7) showed that calmodulin inhibitors block both the recruitment of synaptic vesicles to the fast-releasing pool and membrane retrieval under the conditions of their experiments. However, the connection between the two processes remains unclear: Might Ca^{2+}/calmodulin facilitate the structure recovery (of release sites), clearing the release sites by endocytosis and enhancing synaptic vesicle recruitment?

We identified several aspects, which discriminate the mechanisms of vesicle recruitment to the fast-releasing pool from endocytosis. The former process is readily shown to be calmodulin-dependent after a 50-ms depolarizing pulse (10), but the rate of endocytosis was not affected by blocking peptide under identical conditions (Fig. 4). In addition, PKA inhibitors and calmodulin inhibitors similarly slowed endocytosis following strong—but not weak—stimulation; however, only calmodulin inhibitors, but not PKA inhibitors, slowed the rate of synaptic vesicle replenishment. Furthermore, it should be pointed out that Ca^{2+} requirements for vesicle recruitment and endocytosis are different, which argue that the two processes are predominantly regulated by different mechanisms (7, 11, 18). Taking these data together, one may therefore conclude that recruitment of synaptic vesicles and endocytosis (at least membrane fission) do not have a direct relationship with each other.

The data presented here suggest two possibilities for the role of calmodulin in synaptic vesicle recruitment. First, calmodulin might act at a step before exocytosis, increasing the rate of synaptic vesicle priming (molecular priming) and thereby enhancing recruitment of synaptic vesicles to the fast-releasing pool. The present findings at first glance seem to support this view because we describe a stimulation protocol for which the blocking of calmodulin retarded vesicle recruitment without affecting endocytosis. However, under different conditions (for example in the presence of dynamin inhibitors), endocytosis and vesicle recruitment were inhibited in parallel (7, 11) and both effects were observed under conditions, in which availability of vesicles should not be limiting. Furthermore, it is unlikely that molecular priming is the most important rate-limiting step for recruitment of fast-releasing synaptic vesicles, because evidence has been provided that recruitment of synaptic vesicles to the sites where Ca^{2+} channels cluster (positional priming) is a requirement for fast release (29). To reconcile these findings, we favor a second scenario, in which calmodulin acts at a step in between exo- and endocytosis. Capacitance measurements detect membrane fission, but fission is only the final step among the processes leading to endocytosis (28). Before fission, intermediate steps, such as the clearance of released materials from the release sites, their transport to the endocytotic zone, and the formation of clathrin-coated pits may have to take place. Clearance of release sites has been postulated to be required for positional priming of synaptic vesicles (7, 11, 27, 30). Calmodulin might modulate one of these intermediate steps, and thereby affect synaptic vesicle recruitment, without its effects being detected by capacitance measurements. The consequences of retarding intermediate steps before membrane fission may develop slowly and may only be apparent after massive presynaptic stimulation, which will obstruct the endocytotic processes; this would be plausible, if the kinetics of membrane fission were much slower than intermediate steps leading to endocytosis. This scenario is indeed the case becuase the time course of endocytosis is on the order of tens of seconds, which is quite different from that regulating synaptic vesicle recruitment, which takes place within hundreds of milliseconds.

Materials and Methods

Brainstem slices were prepared from P 9–11 Wistar rats, as previously described, using a vibratome under a low Ca^{2+} extracellular solution (5). The animal research was approved by the review board of Max Planck Institute for Biophysical Chemistry. Slices were incubated in a normal extracellular solution for at least 30 min at 36 °C. The experiments were carried out at room temperature. The extracellular solution contained: 125 mM NaCl, 2.5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 25 mM glucose, 25 mM $NaHCO_3$, 1.25 mM NaH_2PO_4, 0.4 mM ascorbic acid, 3 mM myo-inositol, and 2 mM Na-pyruvate (pH 7.4, gassed with 95% O_2, 5% CO_2). The low Ca^{2+} solution was the same as the extracellular solution, except that 0.1 mM $CaCl_2$ and 3 mM $MgCl_2$ were included. During the measurements, 0.5–1 μM TTX and 10 mM TEA were added to the extracellular solution. Presynaptic terminals of the calyx of Held synapse were patch clamped with a patch pipette (4–6 MΩ) containing: 140 mM Cs-gluconate, 20 mM TEACl, 10 mM Hepes, 5 mM Na-phosphocreatine, 4 mM MgATP, 0.3 mM GTP, and 0.05–0.5 mM EGTA. Postsynaptic pipettes (2–4 MΩ) contained the same solution as presynaptic one, except that EGTA was increased to 5 mM. In some experiments, either calmodulin inhibitory peptide, a control peptide, calmodulin-binding domain, or cAMP (purchased from Calbiochem) were included in the presynaptic patch pipette. The concentration of calmodulin inhibitory peptide was the same as that used in Wu et al. (7) (20 μM). In some experiments, the concentration was increased to 100 μM to ensure that the concentration of the peptide was sufficient, but the results were the same. The sequence of calmodulin inhibitory peptide is Ac-Arg-Arg-Lys-Trp-Gln-Lys-Thr-Gly-His-Ala-Val-Arg-Ala-Ile-Gly-Arg-Leu-NH_2. The sequence of a control peptide is Ac-Arg-Arg-Lys-Glu-Gln-Lys-Thr-Gly-His-Ala-Val-Arg-Ala-Ile-Gly-Arg-Glu-NH_2. MDL 12330A (Calbiochem) was applied extracellularly during the recording. In some experiments, slices were incubated in the extracellular solution

containing KT5720 (Calbiochem) for 30–60 min. MDL12330A and KT5720 were dissolved in DMSO and final concentrations of DMSO were 0.5% and 0.1%, respectively. DMSO (0.5%) did not affect the rate of endocytosis.

Capacitance measurements were carried out using the Patch Master Program, similar to previous studies (6, 11). The terminal was voltage-clamped at −80 mV and sine waves (± 30 mV at 1,000 Hz) were superimposed to measure presynaptic capacitance, which is proportional to the membrane surface area. Capacitance traces were accepted for further analysis only when the series resistance was less than 20 MΩ (usually 10–15 MΩ). A perforated patch clamp may be a better way to prevent the washout of endocytosis, but it is difficult to keep a low series resistance at the calyx of Held using that method. Endocytosis was measured as capacitance decay following the presynaptic stimulation. The decay was fitted with a single or a double exponential in some experiments. The rates of endocytosis were calculated according to Wu et al. (7). The rates were calculated as the capacitance jump divided by the endocytosis time constant, which was similar to the rate of capacitance decay in the first few seconds after stimulation. Similarly, the rates of endocytosis for the rapid and slow components of endocytosis were calculated as the amplitude of the fast and the slow component divided by the respective time constant. When endocytosis could not be fitted by an exponential or blocked, the rate of endocytosis was measured as the mean capacitance decay within 10–40 s after stimulation. Mean capacitance decays were also calculated for the data, which could be fit by an exponential to confirm that the main results were not biased by the method of fitting. In some figures, average data are presented, which were normalized to the peak capacitance jump.

The deconvolution method adapted to the calyx synapse was described by Neher and Sakaba (23). The method assumes that EPSCs are composed of direct release events and residual currents mediated by the delayed clearance of glutamate. Residual currents were estimated by a model incorporating the diffusion of glutamate in the synaptic cleft, and the model parameters were determined in each cell by fitting the EPSC traces evoked by short successive pulses. After subtracting the residual current component, the EPSCs were deconvolved with the mEPSC waveform. Next, 100 μM clothiazide and 1 mM kynurenic acid were added to the extracellular solution to block postsynaptic receptor desensitization and saturation.

The data are presented as mean ± SEM. Statistical tests were done using *t* tests.

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