Ca^{2+} and calmodulin initiate all forms of endocytosis during depolarization at a nerve terminal

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Supplementary Information

I. Supplementary results and discussion for Figures 1 – 2

1. A small $Rate_{endo}$ after prolonged depolarization that induces no detectable calcium currents

In 2 mM $[Ca^{2+}]_o$, a low $Rate_{endo}$ of 6.9 ± 0.2 fF/s (n = 4 calyces) was observed after 2 pulses of 40 depolarization to a voltage between -25 and -35 mV with an interval of 2 s (e.g., Fig. S1). These depolarizations did not induce a detectable ICa (e.g., Fig. S1).

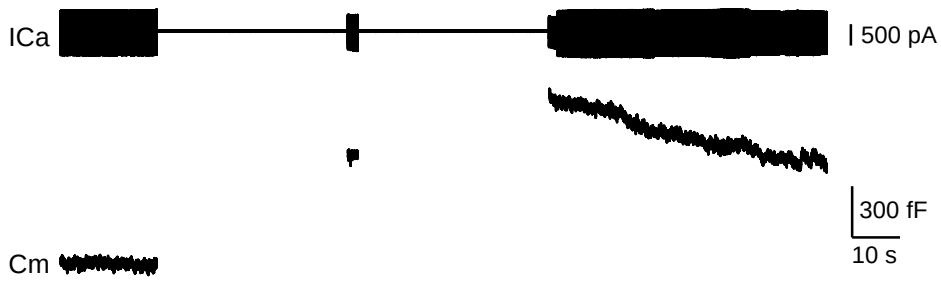

Figure S1. ICa (upper) and Cm change (lower) induced by 2 pulses of 40 s depolarization to –30 mV with an interval of 2 s. Note that ICa was too low to be detected. Sine wave voltage commands (1000 Hz) were applied before, in-between, and after depolarization to detect capacitance changes, which also induced sine wave currents (black bar). Traces are the averages of 4 responses from a calyx.

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2. Discussion of the endocytosis time course after a single vesicle fusion

By averaging ~0.32 million individual vesicle fusion events at the calyx, we reported a fast capacitance decay when the $[Ca^{2+}]_i$ was 0.5 μM[1]. However, this rapid decay was obtained by assuming a linear capacitance baseline drift, which was found to be incorrect in our recent study[2]. By averaging 2.7 million fusion events and by using a correct baseline correction method, our recent study did not observe any significant decay within 250 ms after a single vesicle fusion (Figure S2E in the supplementary Material of Ref. [2]). This observation[2] is consistent with the finding of the present work that endocytosis after exocytosis evoked at a $[Ca^{2+}]_i$ of 0.5 - 0.75 μM was very slow.

3. Estimate of the time constant of endocytosis at 0.5 - 0.75 μM of intracellular calcium

We assumed that during calcium dialysis, every fused vesicle was retrieved with a mean time constant τ. Based on this assumption, we calculated the number of fused vesicles, the vesicles being retrieved, and the vesicles that remained un-retrieved during every 10 s of calcium dialysis. For simplicity, we assume that all vesicles released within the 10 s window were synchronously released at the end of the 10 s window. This assumption would not significantly affect our calculation, because τ was much longer than 10 s (see results below). At any 10 s time window, the release amount was counted from the mean of N_{mEPSC} (Fig. 2c, f in the main text; Fig. S2a, b, circles), which is the accumulated number of mEPSCs in that 10 s window. This number of fused vesicles was retrieved exponentially with a time constant of τ. Accordingly, in the next 10 s, the number (of vesicles fused in the previous 10 s) being retrieved and the number that remained un-retrieved were calculated, and the additional vesicle fusion was counted again from the mean of N_{mEPSC}. We repeated these procedures from the first 10 s throughout the whole time course of calcium dialysis and calculated a predicted amount of membrane remained un-retrieved at any time during calcium dialysis. By varying the value of τ, we determined a τ value that gave the best match between the predicted ($N_{Cm\text{-}predicted}$, Fig. S2a, b, black curves) and the observed (N_{Cm}, Fig. S2a, b) net number of vesicles remained un-retrieved at the plasma membrane during calcium dialysis of 0.5 - 0.75 μM calcium. This τ value was 600 s, confirming that endocytosis at 0.5 - 0.75 μM calcium was very slow, resulting in the accumulation of fused vesicle membrane at the plasma membrane. Taking the standard error of the mean (s.e.m.) into account, a time constant of 400 – 800 s could fit the mean ± s.e.m. of the observed N_{Cm} during dialysis of 0.75 μM calcium, as judged by eye (Fig. S2a, b, blue curves).

Note that at 0.5 μM calcium, we only performed calculations for the group of synapses showing a mEPSC frequency of > 50 Hz, because the signal-to-noise ratio in this group is higher than in the other two groups.

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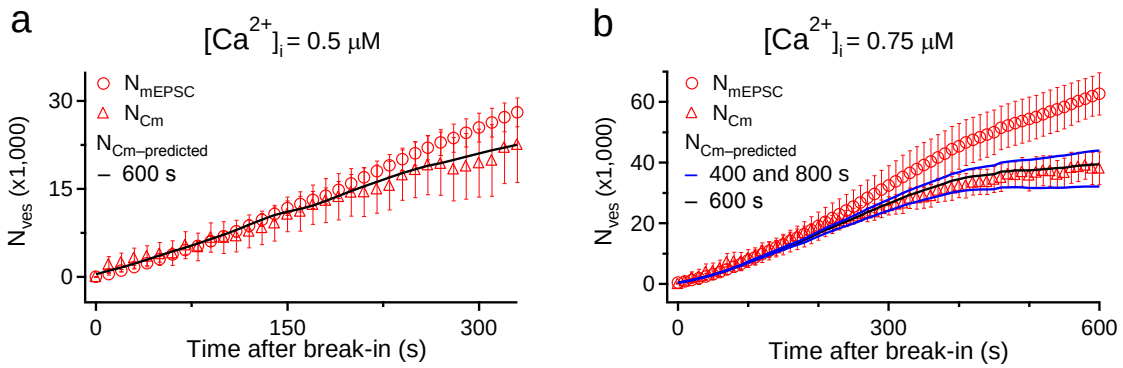

Figure S2. Estimate of the time constant of endocytosis at 0.5 - 0.75 μM of $[Ca^{2+}]_i$

a, The N_{mEPSC} (circles), N_{Cm}, (triangles) and the $N_{Cm\text{-}predicted}$ (black curve) with a τ of 600 s after presynaptic break in (time 0) with a pipette containing 0.5 μM calcium. N_{mEPSC} and N_{Cm} are the same as those in Fig. 2c (red) for the group of synapses with a mEPSC frequency > 50 Hz. $N_{Cm\text{-}predicted}$ was calculated as described in the text of this section.

b, Same as in panel a, except that the calcium concentration was 0.75 μM. N_{mEPSC} and N_{Cm} are the same as those in Fig. 2f (red). $N_{Cm\text{-}predicted}$ with a τ of 400 s (blue curve, lower), 600 s (black curve), and 800 s (blue curve, upper) matched the observed N_{Cm} at mean - s.e.m., mean, and mean + s.e.m., respectively, as judged by eye.

4. Very slow endocytosis can balance exocytosis occurring at various firing rates

We performed simulations similar to the ones shown above (Supplementary Information I-3), except that instead of the experimentally measured mEPSCs, we used arbitrarily fixed mEPSC frequencies of 1, 10, and 100 Hz. Considering that a calyx contains ~550 active zones, these frequencies (1, 10, and 100 Hz) correspond to release of 0.18, 1.8 and 18 vesicles per active zone per 100 s, respectively, which covers a wide range of rates of spontaneous release. Our simulation showed that endocytosis with a time constant of 600 seconds was able to balance out exocytosis at all of these frequencies (Fig. S3). After initiation of exocytosis, the balance was approximately reached within 1200 - 1800 s, or 2 - 3 times the endocytosis time constant (Fig. 3). The time course to reach the balance was independent of the mEPSC frequency. Thus, the very slow endocytosis is capable of achieving a net balance with exocytosis, regardless of the spontaneous firing rate, which can prevent the unlimited expansion of the terminal due to spontaneous fusion.

The time to reach the balance between exocytosis and endocytosis was longer for the arbitrarily fixed mEPSC frequency (Fig. S3, ~1200 - 1800 s) than for the experimentally measured mEPSCs (Fig. S2b, ~600 s). This is because the measured mEPSC frequency was higher within the first 400 s of calcium dialysis, but decreased after 400 s of calcium dialysis (Fig. S2b), allowing for endocytosis with a constant τ to balance exocytosis at an earlier time.

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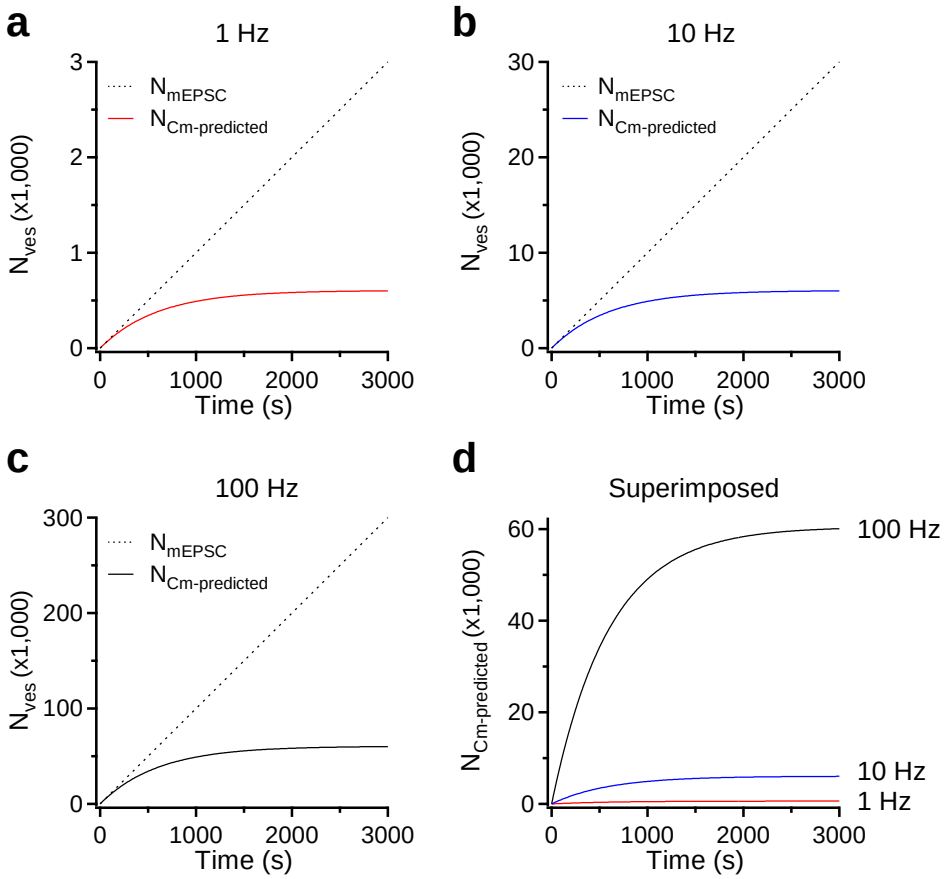

Figure S3. Very slow endocytosis can balance exocytosis occurring at a wide range of firing frequencies

a – c, The number of vesicles (N_{ves}), including N_{mEPSC} and $N_{Cm\text{-}predicted}$, are plotted as a function of time after exocytosis starts. N_{mEPSC} is calculated based on the arbitrarily set spontaneous fusion rates (dotted line: a, 1 Hz; b, 10 Hz; c, 100 Hz). $N_{Cm\text{-}predicted}$ (a, red; b, blue; c, black) is predicted from the corresponding N_{mEPSC} with a very slow endocytosis time constant of 600 s.

d, $N_{Cm\text{-}predicted}$ in panels a-c are superimposed. The color codes in panel a-c apply to panel d.

5. Reduction of the $Rate_{endo}$ is independent of the capacitance jump

We showed that reducing the $[Ca^{2+}]_o$ or the $[Ca^{2+}]_i$, or buffering calcium influx reduced the initial rate of endocytosis ($Rate_{endo}$) to nearly 0 (Figs. 1b, 1d, 2, S1). There are two reasons why this reduction was independent of the exocytosis amount. First, our current data did not reveal any correlation between the capacitance jump and the $Rate_{endo}$. For example, a similar $Rate_{endo}$ was induced by a 20 ms depolarization with 10 mM EGTA in the pipette (8.0 ± 1.2 fF/s, n = 18, Fig. 1c) and by 2 pulses of 40 s depolarization to a voltage between -25 and -35 mV with an interval of 2 s (6.9 ± 0.2 fF/s, n = 4, Fig. S1). However, the capacitance jump in these two conditions differed by ~5 - 6 times (20 ms depolarization with 10 mM EGTA: 161 ± 13 fF, n = 4, e.g., Fig. 1c; 2 pulses of 40 s depolarization: 910 ± 74 fF, n = 4, e.g., Fig. S1; $p < 0.01$).

Another example was that the capacitance jump induced by 20 pulses of 20 ms depolarization with 10 mM BAPTA in the pipette (288 ± 32 fF, n = 10, e.g., Fig. 1d) was

Nature Neuroscience: doi:10.1038/nn.2355

in the same order as that induced by a 20 ms depolarization in 0.5 mM $[Ca^{2+}]_o$ (205 ± 18 fF, n = 18, e.g, Fig. 1a). However, the $Rate_{endo}$ with 10 mM BAPTA in the pipette was only 0.2 ± 0.5 fF/s (n = 10, Fig. 1d), which was much (~70 - 80 times) smaller than that observed in 0.5 mM $[Ca^{2+}]_o$ (15.4 ± 2.2 fF/s, n = 18; Fig. 1a). These examples indicated that the change in the $Rate_{endo}$ as shown in Fig. 1 was not related to the difference in the capacitance jump.

Second, three studies at the calyx (including our previous studies) revealed that a decrease of the capacitance jump from ~450 fF to ~50 fF at the same $[Ca^{2+}]_o$ (2 mM) was linearly proportional to the decrease of the endocytosis τ[1,3,4]. This linear relation indicates that the $Rate_{endo}$ (= capacitance jump/τ) does not change as a function of the capacitance jump. The constant $Rate_{esndo}$ may reflect saturation of the endocytic machinery, as suggested by a study at cultured hippocampal synapses[5]. These published results[1,3,4] indicated that the decrease of the capacitance jump to as low as ~50 fF in normal calcium conditions would not cause a reduction of the $Rate_{endo}$.

II. Supplementary results and discussion for figures 3, 4, and 8

1. Slow endocytosis during repetitive stimulation

When the calyx was subjected to 10 pulses of 2 - 50 ms depolarization at 10 Hz (at 2 mM $[Ca^{2+}]_o$) or 10 pulses of 50 ms depolarization at 5.5 mM $[Ca^{2+}]_o$, endocytosis was bi-exponential with a rapid (τ: ~1 - 4 s) and a slow component (τ: ~8 - 20 s) (Fig. 3a-b). As QICa increased, $Rate_{endo}$ increased mostly because of an increase of the rapid component of endocytosis (Fig. 3a, b). However, $Rate_{endo}$ of the slow component of endocytosis ($Rate_{endo\_slow}$) did also increase (Fig. S4a). For example, $Rate_{endo\_slow}$ was 36 ± 7 fF/s (n = 10) after 10 pulses of 2 ms depolarization, but significantly increased to 86 ± 9 fF/s (n = 6, $p < 0.01$, Fig. S4a) after 10 pulses of 50 ms depolarization at 5.5 mM $[Ca^{2+}]_o$. These results are consistent with our finding that calcium influx triggers slow endocytosis.

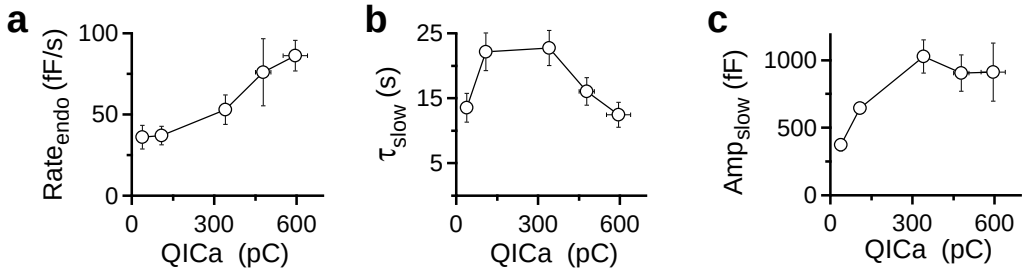

Figure S4. The slow component of endocytosis

a, The $Rate_{endo}$ for the slow component of endocytosis is plotted versus the ICa charge (QICa). The stimulation included 10 pulses of 2, 5, 20 and 50 ms depolarization at 10 Hz (in 2 mM $[Ca^{2+}]_o$), and 10 pulses of 50 ms depolarization at 10 Hz in 5.5 mM $[Ca^{2+}]_o$. QICa increased in the order listed (applied to a-c)

b, The time constant of slow endocytosis (τ_{slow}) is plotted versus QICa.

c, The amplitude of the slow component of endocytosis is plotted versus QICa.

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As QICa increased, the time constant of slow endocytosis (τ_{slow}) increased slightly first, then decreased (Fig. S4b). As shown in hippocampal synapses, the time constant of endocytosis may not necessarily reflect the rate of endocytosis[5,6]. When the capacity of endocytosis is saturated by a large amount of exocytosis, the apparent time constant would increase due to saturation, even though the $Rate_{endo}$ did not change[1,3,5-7]. The initial slight increase of τ_{slow} as QICa increased in Fig. S4b may thus reflect saturation of the endocytic capacity (owing to a small increase in $Rate_{endo\_slow}$). Consistent with this suggestion, the increase in τ_{slow} was accompanied by an increase of the amplitude of the slow component of endocytosis (Fig. S4c). This is further demonstration that the $Rate_{endo}$ is more accurate in reflecting the rate of endocytosis[5,6].

As QICa further increased by 10 pulses of 50 ms depolarization at 5.5 mM $[Ca^{2+}]_o$, $Rate_{endo\_slow}$ increased and τ_{slow} decreased (Fig. S4a, b), consistent with a calcium-dependent speeding up of the slow component of endocytosis as shown in Fig. 1.

2. Additional discussion and results of bulk endocytosis

A brief summary of our previous study on bulk endocytosis. In our previous study of bulk endocytosis[8], we found that the instant of bulk membrane fission was reflected as a brief downward capacitance shift (DCS) of ~20 - 500 fF with 10 - 90% decay time of ~30 - 500 ms. DCSs could be induced by 20 ms depolarization, a train of 20 ms depolarization or action potential-like stimuli. The frequency of DCSs increased as the stimulation intensity increased, consistent with the hypothesis that the increase of calcium influx increases DCS frequency. Block of exocytosis by botulinum neurotoxin C also led to the block of DCSs, indicating that DCSs retrieve fused vesicle membrane. During a DCS, a decrease in the fission pore conductance was detected, from which we found that the fission pore diameter decreased at ~39 nm/s. This allowed us to determine whether calcium modulates the fission rate in the present study.

The DCS frequency before stimulation was very low. The reason for this baseline activity was unclear. It could be a response to the whole-cell break in, which might trigger release at the instant of break in.

Rapid DCS reflects rapid rate of fission. We found that the rate of the G_p decrease within 10 s after 10 pulses of 50 ms depolarization at 10 Hz in 5.5 mM $[Ca^{2+}]_o$ was much faster than that before stimulation (Fig. 4). This was not an artifact for two reasons. First, this rate was significantly reduced by addition of 70 mM EGTA (Fig. 4). Second, large DCSs with a rapid time course were often accompanied by a reversible decrease in the series conductance (Fig. S5, Gs), an indication of bulk endocytosis[8].

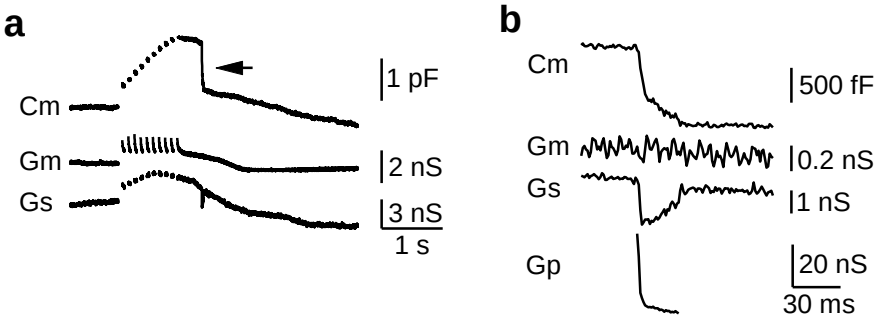

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Figure S5. Rapid DCS is accompanied by a reversible change in the series conductance (Gs)

a, Sampled trace showing the capacitance change induced by 10 pulses of 50 ms depolarization at 10 Hz in 5.5 mM $[Ca^{2+}]_o$. The membrane conductance (Gm) and the series conductance (Gs) are also shown. The arrow indicates a DCS.

b, The DCS in panel a and the corresponding Gm and Gs changes during the DCS are shown in larger scales. The fission pore conductance (Gp, bottom) was also calculated from the Cm trace using equation 1. This recording was obtained in the control condition with a pipette containing 0.1% DMSO.

3. The effect of calmodulin inhibitors on the presynaptic calcium current

We found that calmodulin inhibitors, including CBD, MLCK and calmidazolium, inhibited endocytosis (Fig. 5, main text). This inhibitory effect was independent of their effects on calcium currents (ICa), as described below.

The three calmodulin inhibitors mentioned above significantly inhibited slow endocytosis (Fig. 5a, main text), but did not significantly affect QICa induced by a 20 ms depolarization (Fig. S6a-c). Thus, the block of slow endocytosis by calmodulin inhibitors was not due to a block of ICa.

At ~4-6 min after dialysis of CBD, QICa induced by 10 pulses of 20 ms depolarization at 10 Hz was not significantly reduced (Fig. S6d), whereas the $Rate_{endo}$ after this 10-pulse stimulus, which reflected mostly rapid endocytosis (Fig. 3b), was significantly reduced (Fig. 5b). Thus, inhibition of rapid endocytosis by CBD was not due to a block of QICa. A small reduction of QICa was more clearly observed at 4 - 5 min after dialysis of MLCK and calmidazolium. The QICa induced by the 10-pulse train in the presence of MLCK was 78 ± 8% (n = 12) of that (100 ± 8% n = 7) in the presence of a mutant MLCK peptide (Fig. S6e, $p < 0.05$). The QICa induced by the 10-pulse train in the presence of calmidazolium was 84 ± 5% (n = 8) of that (100 ± 4%, n = 12) in control (Fig. S6f, $p < 0.05$).

To determine whether a small decrease in QICa affects the $Rate_{endo}$, we mimicked a small QICa reduction by changing the depolarization voltage of each pulse during the 10-pulse stimulus from +10 mV (from the holding potential of -80 mV) to +30 mV (from the holding potential of -80 mV). Such a change reduced QICa induced by the 10-pulse train to 79 ± 11% (n = 8, Fig. S6g-h, $p < 0.01$, paired t test), but did not significantly affect the $Rate_{endo}$ induced by the 10-pulse stimulus (Fig. S6g-h, $p = 0.17$). Consistent with this result, the relation between the $Rate_{endo}$ and the QICa shown in Fig. 3b (left) predicted a minor decrease of the $Rate_{endo}$ that may be too small to be detected consistently when the QICa was reduced by only 21%. We concluded that inhibition of endocytosis by as large as more than 75% by calmodulin inhibitors was independent of their minor effects on QICa.

Calmodulin has been shown to be involved in both ICa facilitation and inactivation[9]. The ICa induced by each 20 ms depolarization during the 10-pulse train gradually decreased from the 1st to the 10th pulse. This decrease might reflect the summed effect of ICa facilitation and inactivation. The QICa reduction by calmodulin inhibitors during the 10-pulse train could thus be due to a more potent block of ICa facilitation than

Nature Neuroscience: doi:10.1038/nn.2355

ICa inactivation by calmodulin inhibitors during the 10-pulse stimulus. We suggest that ICa facilitation also occurred during the 10-pulse train in control, despite the concurrence of a stronger ICa inactivation that resulted in a net ICa inactivation (e.g., Fig. 3a, see Ref. [41]).

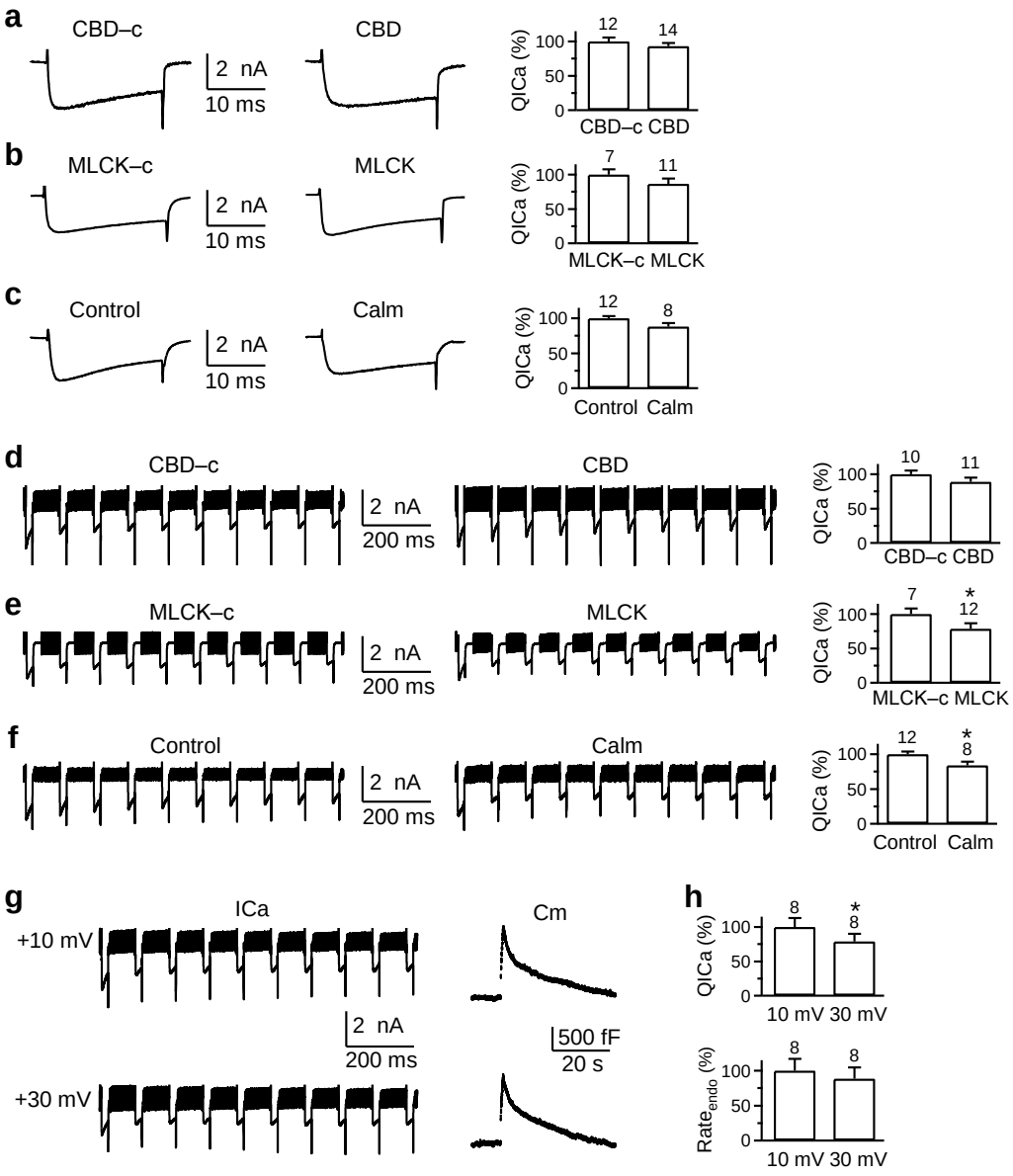

Figure S6. The effects of calmodulin inhibitors on ICa at the calyx

a, Sampled ICa induced by a 20 ms depolarization in the presence of scrambled CBD (CBD-c, 500 μM, left) or CBD (500 μM, middle). The QICa (mean ± S.E, n in labels, applies to all panels) in these two conditions, normalized to the mean QICa in control (CBD-c), is shown on the right ($p > 0.05$).

b, Similar to a, but with a mutant MLCK (MLCK-c, 20 μM) or MLCK peptide (20 μM).

c, Similar to a,, but with 0.1% DMSO (Control) or calmidazolium (Calm, 10 μM with 0.1% DMSO).

Nature Neuroscience: doi:10.1038/nn.2355

d, QICa induced by 10 pulses of 20 ms depolarization in the presence of CBD-c (500 μM, left) or CBD (500 μM, middle). The QICa (mean ± S.E, n in labels) in these two conditions, normalized to the mean QICa in control (CBD-c), is shown on the right ($p > 0.05$).

e, Similar to d, but with MLCK-c (20 μM) or MLCK (20 μM). The * indicates $p<0.05$ (applies to all figures).

f, Similar to e, but with 0.1% DMSO (Control) or calmidazolium (Calm, 10 μM with 0.1% DMSO).

g, Sampled ICa (left) and the capacitance change (right) induced by 10 pulses of 20 ms depolarization from a holding potential of -80 mV to +10 mV (upper) or to +30 mV (lower) at 10 Hz.

h, QICa (upper) and $Rate_{endo}$ (lower) induced by 10 pulses of 20 ms depolarization to +10 mV or +30 mV (from -80 mV) at 10 Hz. Data were normalized to the mean value obtained with the 10-pulse depolarization to +10 mV.

4. Measurements of the vesicle mobilization

The readily releasable pool (RRP) could be depleted by a 20 ms depolarization[10-12]. CBD, calmidazolium and dynasore did not affect the capacitance jump induced by the 20 ms depolarization, indicating that these drugs do not affect the RRP size[11,13].

To measure the replenishment of the RRP after depletion, we measured the capacitance jump evoked by each 20 ms depolarization during a train of 10 such depolarizing pulses at 10 Hz (Fig. 7). A 20 ms depolarization induced a capacitance artifact of ~50 fF that decayed with a time constant of 233 ms[4]. As measured 100 ms after stimulation, the artifact would be ~30 fF, ~6% of the capacitance jump induced by a 20 ms depolarization. For two reasons, this artifact did not affect our conclusion that calmidazolium, CBD, and dynasore reduced the capacitance jump and thus the rate of the RRP replenishment during 10 pulses of 20 ms depolarization at 10 Hz (Fig. 7).

First, calmidazolium, CBD, and dynasore should not affect this artifact. Thus, if we could remove this artifact, the inhibition of the rate of the RRP replenishment should be larger. This means that we might have underestimated the inhibition of these drugs on the rate of the RRP replenishment. Second, when a pair of 20 depolarizations was applied with different intervals in control and in the presence of CBD (300 - 500 μM), CBD reduced the rate of the RRP replenishment at intervals of 0.5 - 5 s (n = 7 - 9, data not shown), at which the capacitance artifact had disappeared[4]. Thus, inhibition of the RRP replenishment by CBD was independent of the capacitance artifact. Furthermore, our results are consistent with a previous study at calyces which, by using a method (EPSC recording) not related to capacitance measurements, concluded that the calmodulin inhibitor CBD inhibits the RRP replenishment[11].

III. Supplementary discussion: early experiments regarding the role of extracellular calcium in endocytosis at synaptosomes

In 1998, two studies in synaptosomes using FM dyes proposed a triggering role for calcium in endocytosis[14,15]. As discussed below, the research was insightful, but incomplete, and the evidence was insufficient to support this hypothesis, as the studies lacked techniques for direct and quantitative measurements of endocytosis and manipulation of the intracellular calcium concentration. Consistent with this notion, the

Nature Neuroscience: doi:10.1038/nn.2355

authors of one of these two studies proposed an opposite hypothesis (calcium inhibits endocytosis) in a publication two years later[16].

In these two early studies, the entire vesicle recycling process was measured, not endocytosis, yet recycling defects in their experimental conditions were inferred to be due exclusively to defects in endocytosis. In the studies, FM dye was applied during a strong stimulation (often with high KCl solution) that evoked exocytosis and subsequent endocytosis, causing internalization of the dye. After washout of external dye and a rest period, a second KCl stimulation was applied to measure the amount of dye release. It was this amount that was quantified, not the amount of internalized dye, as the amount that could be released was deemed to be what had been internalized through specific endocytosis of synaptic vesicles. A large component of dye release (> 50%) was independent of the extracellular calcium for an unknown reason. This large fraction was assumed the same for the first and the second stimulus and thus was subtracted. When the extracellular calcium was replaced with barium during the first stimulus, dye release during the second stimulus (in calcium) was found to decrease significantly. Mainly based on this result, the hypothesis that calcium influx triggers endocytosis was proposed. The evidence was insufficient to support the hypothesis for five reasons.

First, endocytosis was not measured as the amount of dye uptake, but the amount of dye release by the second stimulus. This reflects the entire recycling process: first, exocytosis and subsequent endocytosis induced by the first stimulus, then mobilization of the retrieved vesicles to the readily releasable pool, priming of these retrieved vesicles before release, and release. These different steps were not distinguished[14,15]. Calcium-dependent mobilization of vesicles to the readily releasable pool has been well documented[17,18], and may thus contribute to the observed phenomenon. Thus, these two studies examined the effect of the extracellular calcium on vesicle reuse after a round of exocytosis, rather than on endocytosis itself.

Second, a large component (> 50%) of dye release was independent of calcium for an unknown reason, which calls into question whether the mechanism of dye release was physiological, as well as the more obvious issue that most release was not in fact related to calcium levels.

Third, it could not be determined from the experiments whether extracellular calcium had a triggering role or simply regulated vesicle recycling. This is because 1) barium did not fully block dye release by the second stimulus; 2) vesicle reuse, not endocytosis, was measured; and 3) a slowing down in the endocytic process during dye application would also reduce dye uptake and release.

Fourth, the primary stimulation was a prolonged (~100 - 200 s) high potassium application that was four to five orders of magnitude longer than physiological action potential stimulation. In one of the papers[15], application of barium itself was the stimulus, which presumably depolarized the synaptosomal membrane via inhibition of a potassium channel, though this was not tested in the study. Also, the preparation was synaptosomes, at which the damage is likely much more severe than the nerve terminal in brain slice conditions. Thus, whether the results found in this condition apply to physiological conditions is unclear.

Fifth, it is unclear whether the observed effect is caused by the extracellular calcium acting on an extracellular calcium sensor or acting on the intracellular calcium sensor via calcium influx.

Nature Neuroscience: doi:10.1038/nn.2355

In summary, based on these studies[14,15], the proposal that calcium influx triggers endocytosis after physiological stimulation at synapses was very much a speculation. To prove or disapprove this speculation requires direct and quantitative measurements of endocytosis with the ability to manipulate the intracellular calcium concentration, the ability to detect endocytosis under very low calcium conditions that may separate exocytosis from endocytosis, and the ability to monitor endocytosis after brief depolarization in the order of milliseconds in near physiological conditions. These requirements were met in the present study.

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