

Rapid endocytosis does not recycle vesicles within the readily releasable pool

Xin-Sheng Wu and Ling-Gang Wu

Synaptic Transmission Unit, National Institute of Neurological Disorders and Stroke, 35 Convent Dr., Bldg. 35, Rm. 2B-1012, Bethesda, Maryland 20892

Supplemental Material

I. Supplementary Methods: measurement of the RRP size and the rate of the RRP replenishment

We measured the RRP size by capacitance measurements at the calyx, which avoids problems associated with postsynaptic receptor saturation and desensitization (Sakaba and Neher, 2001; Sun and Wu, 2001). As obtained from capacitance measurements, the RRP contains 3300 – 5500 vesicles (Sun and Wu, 2001), which is in the same order as that (1500 – 4000) obtained by measurements of AMPA receptor-mediated EPSCs in conditions that reduce receptor saturation and desensitization (Sakaba and Neher, 2001). The slightly different estimates are likely due to differences in the techniques as discussed previously (Schneggenburger et al., 2002).

In the presence of BoNT/C that abolished exocytosis, a 20 ms depolarization induced a ΔC_m of 50 ± 15 fF ($n = 4$), as measured 10 ms after stimulation (Wu et al., 2005) (see also (Yamashita et al., 2005)). This capacitance jump decayed with a time constant of 233 ± 76 ms ($n = 4$) (Wu et al., 2005; Yamashita et al., 2005)). Thus, for the measurement of the RRP, the ΔC_m evoked by a 20 ms depolarization, we neglected the first 250 ms of the capacitance after a single 20 ms depolarization, or after each 20 ms depolarization during a train (Wu et al., 2005). Endocytosis during this interval was negligible after a single 20 ms depolarization, because the time constant of endocytosis was $\sim 10 - 20$ s (Wu et al., 2005; Yamashita et al., 2005).

When a pair of 20 ms depolarization was applied at Δt of 0.1 or 0.2 s, ΔC_m induced by the first pulse could only be measured at 0.1 or 0.2 s after the stimulus. In this condition, the mean capacitance artifact would be 33 or 21 fF at Δt of 0.1 or 0.2 s. These estimated values were subtracted from the measured ΔC_m to correct for the capacitance artifact. This correction did not significantly affect the estimate of the RRP size, considering that the RRP size was 459 ± 29 fF ($n = 28$) at rest. Furthermore, we often confirmed our estimate of the RRP size with a single 20 ms depolarization applied without another depolarization within 250 ms.

When the test pulse, a 20 ms depolarization, was applied at various times after a conditioning stimulus to measure the RRP recovery, the capacitance value decayed slightly within the first 250 ms after the conditioning stimulus, owing to endocytosis (e.g., Figs. 2A, 2C, S1). In this condition, neglecting the first 250 ms of the capacitance after the test pulse would lead to a slight underestimate of the RRP size. To correct for this small error, the capacitance between 250 ms and 1000 ms after the 20 ms test pulse was fit with a linear regression line. This line was extrapolated back to the time

immediately after the test pulse. The capacitance value at this extrapolated time point, subtracted by the capacitance value before the test pulse (measured as the mean capacitance within 20 ms before the test pulse), was taken as the capacitance jump evoked by the test pulse (Fig. S1). This method was also used to estimate the ΔC_m induced by each 20 ms depolarization during a 10 pulse train at 1 Hz (see also Fig. 1 in Wu et al., 2005).

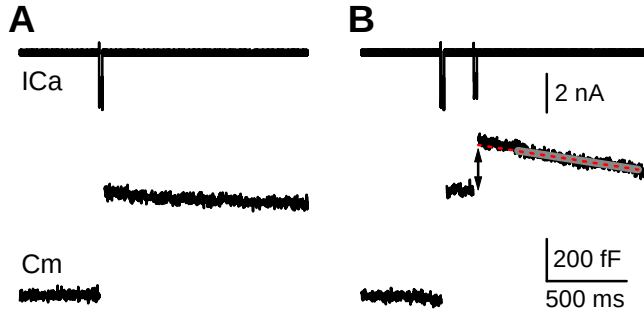


Figure S1. Measurement of the ΔC_m

(A) ICa and C_m induced by a single 20 ms depolarization

(B) ICa and C_m induced by a pair of 20 ms depolarization with an interval of 200 ms. The capacitance trace between 250 and 1000 ms after the 2nd pulse was fit with a linear regression line (gray line) and extrapolated (red) to the time the 2nd stimulus was applied. The capacitance value at the extrapolated time point, subtracted by the capacitance value before the 2nd stimulus (double arrow), was the ΔC_m induced by the 2nd stimulus.

II. Additional discussion on the definition of the RRP in the calyx

The readily releasable pool depleted by a 20 ms depolarization was composed of two kinetically different sub-pools of vesicles, a dominant fast component and a minor slow component (Fig. 1A, B). This result, obtained in the presence of 50 μM BAPTA in the pipette, was consistent with an earlier finding that the slow release component is minor or even absent when the calyx was dialyzed with 50 μM EGTA, but become more prominent in the presence of 500 μM EGTA (Sakaba and Neher, 2001). We chose to include 50 μM BAPTA in the pipette solution, because 50 μM BAPTA (or EGTA) was close to the endogenous calcium buffer capacity in the 8 - 10 day old calyx (Borst et al., 1995; Helmchen et al., 1997). The slow release component could be due to a sub-population of the RRP (Sakaba and Neher, 2001), rapid replenishment and release of vesicles during prolonged depolarization (see also Fig. 1C-D for rapid RRP replenishment) (Sakaba and Neher, 2001; Taschenberger et al., 2002; Wu and Borst, 1999), and/or adaptation of the calcium sensor to vesicle fusion during prolonged depolarization (Wolfel et al., 2007).

The ΔC_m induced by 1 - 30 ms depolarization, when normalized to that evoked by a 20 ms depolarization at the same calyx, could be fitted well with a bi-exponential function (equation 1 in the main text, Fig. 1B). With a 10 - 20 ms depolarization, the slow release component was only ~14 - 22% of total release (equation 1, main text). The RRP was replenished with a bi-exponential time course with parameters described in the main text (equation 1, Fig. 1C-D), which predicted ~5% replenishment for a period of 20 ms.

This prediction was likely an underestimate during 20 ms depolarization, during which large calcium influx may facilitate the RRP replenishment (Hosoi et al., 2007). Thus, if we subtract the vesicle replenishment during a 20 ms depolarization, the slow release component was likely less than 17%. We concluded that the majority of vesicles in the RRP belong to the fast release component.

III. Inactivation of calcium currents does not affect the measurement of the RRP replenishment

1. Measurements after a 20 ms depolarization

When a pair of 20 ms depolarization (from -80 to +10 mV, if not mentioned) were applied to the calyx at various intervals (Δt), the ΔC_m induced by the 2nd depolarizing pulse recovered with a bi-exponential function (Fig. S2A, B, see also Fig. 1C, D). The charge of the calcium current (QICa) induced by the 2nd depolarizing pulse was $77 \pm 4\%$ ($n = 6$) of the 1st one at $\Delta t = 0.05$ s. It recovered to $88\% \pm 4\%$ ($n = 6$) at $\Delta t = 0.1$ s, and mostly recovered at 0.5 - 1 s (Fig. S2A, B).

We used two approaches to determine whether the decreased QICa was still sufficient to deplete the RRP. First, in a control condition (without a conditioning pulse), we applied to the calyx a 20 ms depolarization from -80 to +25 mV. Compared to the 20 ms depolarization to +10 mV, this depolarization induced a similar ΔC_m ($95 \pm 4\%$, $n = 6$, $p = 0.24$), but only $79 \pm 2\%$ of QICa ($n = 6$, $p < 0.001$, Fig. S2C). Thus, a $21 \pm 2\%$ decrease in QICa, similar to that observed at 0.05 s after a 20 ms depolarization, would still be able to deplete the RRP and thus would not affect the measurement of the RRP. Second, we replaced the conditioning pulse (20 ms depolarization to +10 mV) with a 20 ms depolarization to +25 mV, and applied the test pulse, a 20 ms depolarization to +10 mV, at Δt of 0.2 s. The QICa induced by the test pulse was slightly larger than that of the first pulse ($106 \pm 4\%$, $n = 5$, $p = 0.17$), whereas the RRP recovered to $47 \pm 4\%$ ($n = 5$, Fig. S2D, E), which was similar to that (41 ± 3 , $n = 10$, $p = 0.27$) measured with a pair of identical 20 ms depolarization (Fig. S2E). We concluded that the slight QICa reduction after a conditioning pulse did not affect the measurement of the RRP replenishment.

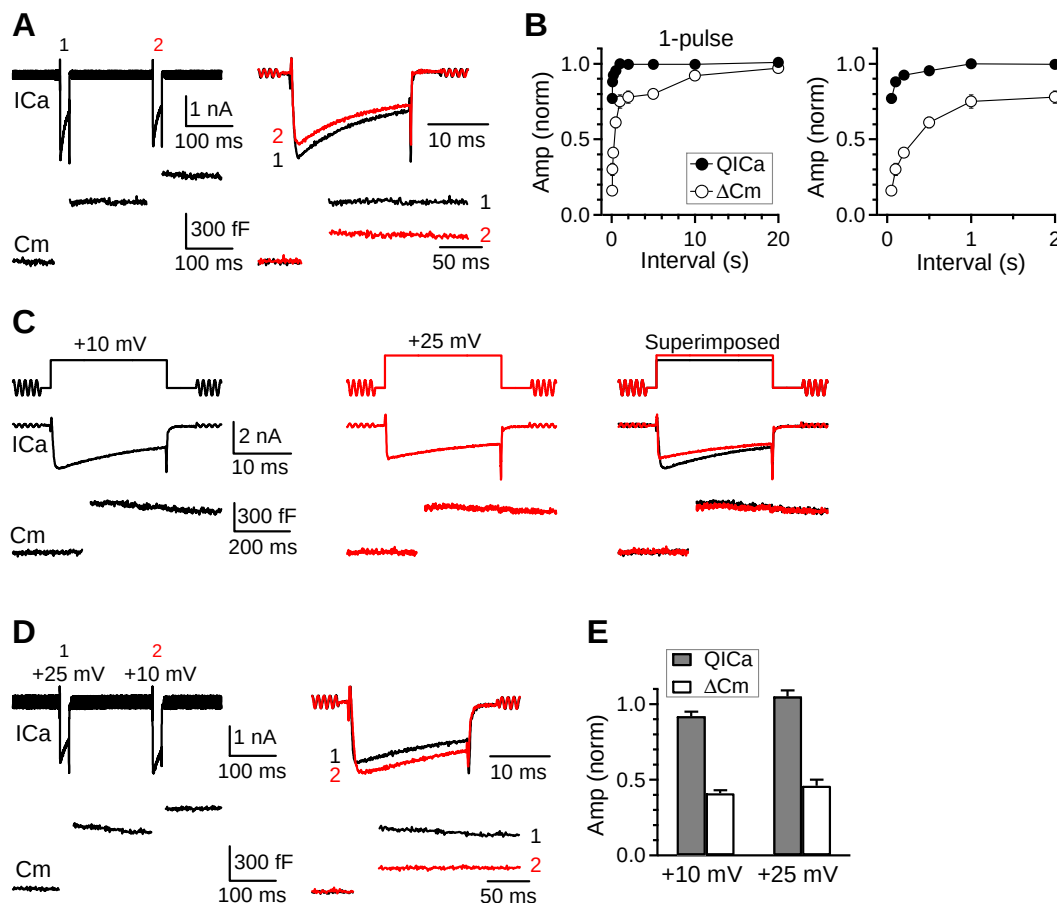


Figure S2. ICa inactivation does not affect the measurement of the RRP replenishment after a 20 ms depolarization

- (A) Sampled presynaptic ICa and Cm induced by a pair of 20 ms depolarization with an interval of 200 ms (left). The 1st (black) and the 2nd (red) response were also superimposed and shown in a larger time scale for comparison (right). Note that ICa and Cm traces are plotted in different time scales in the right panel (applies to panel D).
- (B) The calcium current charge (QICa) and ΔC_m induced by a 20 ms depolarization applied at various intervals (0.05 - 20 s) after a conditioning 20 ms depolarization ($n = 5 - 11$). Data were normalized to the response induced by the conditioning pulse. Left and right panels are the same data plotted in different time scales.
- (C) Sampled ICa and Cm induced by a 20 ms depolarization to +10 mV (left) or to +25 mV (middle) at the same calyx(from the holding potential of -80 mV). These traces are also superimposed for comparison (right).
- (D) Sampled ICa and Cm induced by a 20 ms depolarization to +25 mV (from the holding potential of -80 mV), followed 200 ms later by a 20 ms depolarization to +10 mV. The 1st and the 2nd responses were also superimposed (right).
- (E) QICa and ΔC_m induced by a 20 ms depolarization to +10 mV applied at 200 ms after a conditioning pulse of 20 ms depolarization to +10 mV or to +25 mV. Data were normalized to the response induced by the conditioning pulse.

2. Measurements after 10 pulses of 20 ms depolarization at 10 Hz

As compared to the RRP replenishment after a 20 ms depolarization (Fig. 1D, main text), the RRP replenishment was slower after 10 pulses of 20 ms depolarization at 10 or 1 Hz (Fig. 2D, F, main text). Although after 10 pulses of stimuli, QICa induced by the 20 ms depolarization was transiently decreased, as discussed below, this decrease would not affect our conclusion that the RRP replenishment was slower.

After 10 depolarizing pulses at 1 Hz, the QICa decrease measured at Δt of 0.1 - 20 s was less than 13% (Fig. S3A-B). Based on results shown in Fig. S2C, such a slight decrease of QICa would not affect the measurement of the RRP. Similarly, the QICa decrease at 0.5 s or longer after 10 pulses of depolarization at 10 Hz was less than 15% (Fig. S3C-D), suggesting that in most conditions, the RRP measurement was not affected by the slight QICa decrease.

At 0.1 - 0.2 s after 10 pulses of depolarization at 10 Hz, QICa decreased by ~30% (Fig. S3D). This decrease did not significantly affect the RRP measurement for two reasons. First, when we increased the test pulse duration from 20 ms to 25 ms, the ΔC_m did not increase significantly (data not shown). Second, at 0.1 - 1 s after 10 depolarizing pulses at 1 and 10 Hz, the RRP recovery was similar, whereas the QICa recovery was different (Fig. S3B, D), indicating that the RRP recovery was not controlled by the QICa decrease.

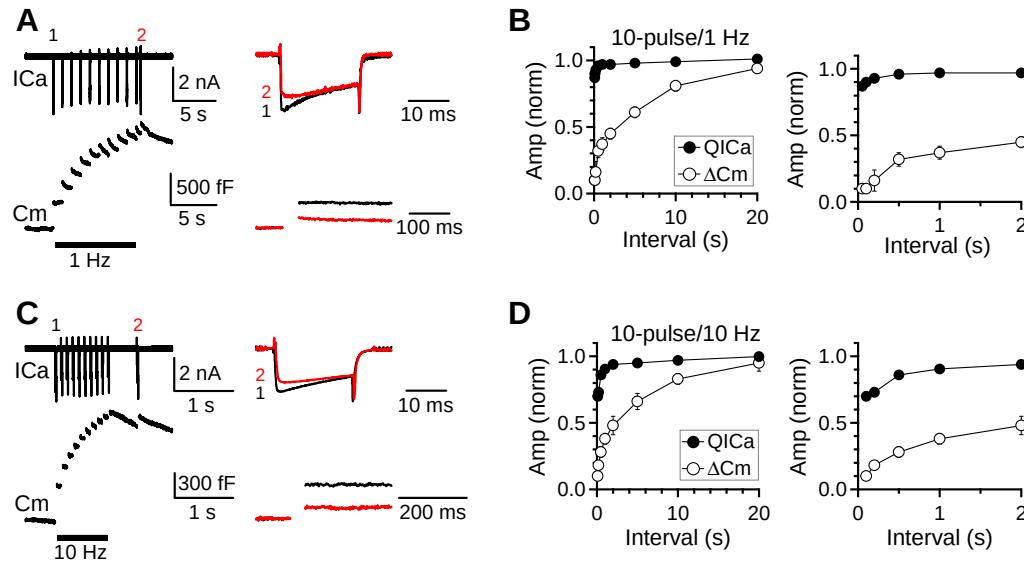


Figure S3. ICa inactivation does not affect the measurement of the RRP replenishment after 10 pulses of 20 ms depolarization at 1 - 10 Hz

- (A) Sampled presynaptic ICa and Cm induced by 10 pulses of 20 ms depolarization at 1 Hz, followed 500 ms later by a testing pulse, a 20 ms depolarization. The responses to the 1st 20 ms depolarization of the 10-pulse train (1, black) and to the testing pulse (2, red) are superimposed in the right. The baseline drift for the capacitance jump induced by the testing pulse was corrected by fitting the baseline with a linear regression line and subtracting this regression line. Note that ICa and Cm traces in the right are plotted in different time scales.
- (B) QICa and ΔC_m induced by a 20 ms depolarization applied at various intervals (0.1 - 20 s) after 10 pulses of 20 ms depolarization at 1 Hz ($n = 5 - 8$). Data were normalized to the response induced by the conditioning pulse. Left and right panels are the same data plotted in different time scales.

(C-D) Similar to A-B, except that the conditioning stimulus was 10 pulses of 20 ms depolarization at 10 Hz ($n = 7 - 12$).

IV. Additional discussion and control experiments for the measurement of the RRP in the presence of GTP γ S

In the presence of GTP γ S, a GTP- and dynamin-independent endocytosis developed after massive release as the capacitance jump reached $\sim 3 - 4$ pF (Xu et al., 2008). Thus, we limited our analysis to capacitance traces showing no endocytosis in the first few minutes of recordings after break in (e.g., Figs. 1E, 2E).

GTP γ S slowed the rise time of the presynaptic I_{Ca} (Fig. S4) (Yamashita et al., 2005). However, in the presence of GTP γ S (0.3 mM), a 20 ms depolarization induced a ΔC_m of 467 ± 30 ($n = 9$), which was similar to the control (459 ± 29 fF, $n = 28$, $p > 0.2$). Thus, in the presence of GTP γ S, the RRP could still be depleted by a 20 ms depolarization.

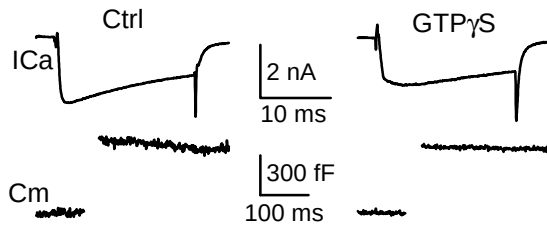


Figure S4. Sampled I_{Ca} and C_m induced by a 20 ms depolarization in control (left, 0.3 mM GTP) and in the presence of 0.3 mM GTP γ S in the pipette solution (right).

V. Block of endocytosis by GDP β S does not affect the RRP replenishment

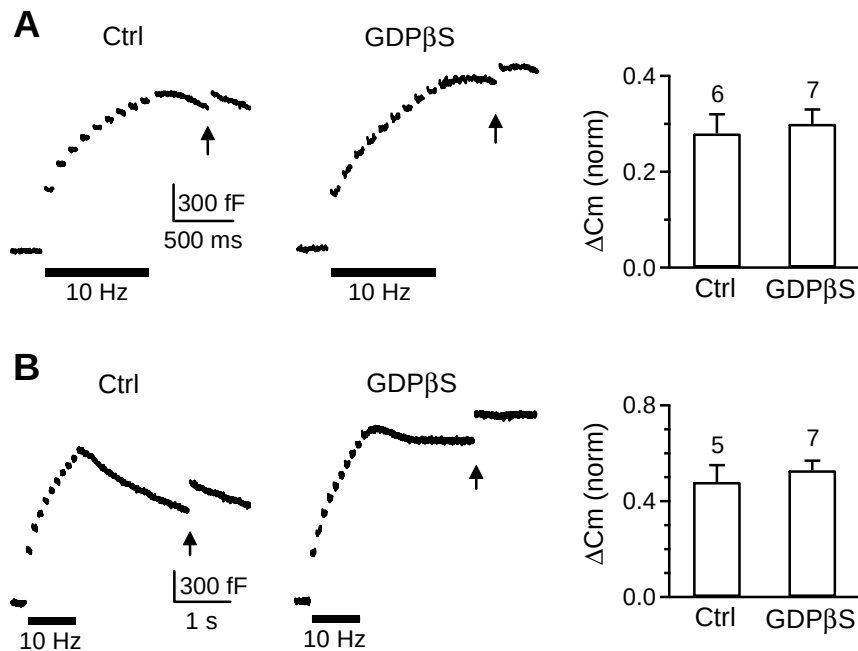


Figure S5. The RRP replenishment is similar in the presence or absence of GDPβS

- (A) Sampled C_m induced by a 20 ms depolarization (arrow) applied at 0.5 s (right) after a conditioning stimulus composed of 10 pulses of 20 ms depolarization at 10 Hz in control (0.3 mM GTP, left) and in the presence of GDPβS (0.3 mM GDPβS, 0 mM GTP, middle). The right panel shows the RRP recovery, measured as the capacitance jump (ΔC_m) induced by a 20 ms depolarization applied at 0.5 s after the 10 pulse train, in control (Ctrl, $n = 6$) and in the presence of GDPβS (0.3 mM, $n = 7$). The ΔC_m was normalized (norm) to that induced by a 20 ms depolarization applied more than 20 s after the 10-pulse train.
- (B) Similar to panel A, but at 2 s after the 10 pulse train (control, $n = 5$; GDPβS, $n = 7$).

Reference List

- Borst,J.G.G., Helmchen,F., and Sakmann,B. (1995). Pre- and postsynaptic whole-cell recordings in the medial nucleus of the trapezoid body of the rat. *J.Physiol.* 489, 825-840.
- Helmchen,F., Borst,J.G.G., and Sakmann,B. (1997). Calcium dynamics associated with a single action potential in a CNS presynaptic terminal. *Biophys.J.* 72, 1458-1471.
- Hosoi,N., Sakaba,T., and Neher,E. (2007). Quantitative analysis of calcium-dependent vesicle recruitment and its functional role at the calyx of Held synapse. *The Journal of Neuroscience* 27, 14286-14298.

Sakaba,T. and Neher,E. (2001). Calmodulin mediates rapid recruitment of fast-releasing synaptic vesicles at a calyx-type synapse. *Neuron* 32, 1119-1131.

Schneggenburger,R., Sakaba,T., and Neher,E. (2002). Vesicle pools and short-term synaptic depression: lessons from a large synapse. *Trends in Neurosciences* 25, 206-212.

Sun,J.Y. and Wu,L.G. (2001). Fast kinetics of exocytosis revealed by simultaneous measurements of presynaptic capacitance and postsynaptic currents at a central synapse. *Neuron* 30, 171-182.

Taschenberger,H., Leao,R.M., Rowland,K.C., Spirou,G.A., and Von Gersdorff,H. (2002). Optimizing synaptic architecture and efficiency for high-frequency transmission. *Neuron* 36, 1127-1143.

Wolfel,M., Lou,X., and Schneggenburger,R. (2007). A mechanism intrinsic to the vesicle fusion machinery determines fast and slow transmitter release at a large CNS synapse. *The Journal of Neuroscience* 27, 3198-3210.

Wu,L.G. and Borst,J.G.G. (1999). The reduced release probability of releasable vesicles during recovery from short-term synaptic depression. *Neuron* 23, 821-832.

Wu,W., Xu,J., Wu,X.S., and Wu,L.G. (2005). Activity-dependent acceleration of endocytosis at a central synapse. *J Neurosci* 25, 11676-11683.

Xu,J., McNeil,B., Wu,W., Nees,D., Bai,L., and Wu,L.G. (2008). GTP-independent rapid and slow endocytosis at a central synapse. *Nature Neuroscience* 11, 45-53.

Yamashita,T., Hige,T., and Takahashi,T. (2005). Vesicle endocytosis requires dynamin-dependent GTP hydrolysis at a fast CNS synapse. *Science* 307, 124-127.