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Rapid kinetics of endocytosis at rod photoreceptor synapses depends upon endocytic load and calcium

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Abstract

Release from rods is triggered by the opening of L-type Ca^{2+} channels that lie beneath synaptic ribbons. After exocytosis, vesicles are retrieved by compensatory endocytosis. Previous work showed that endocytosis is dynamin-dependent in rods but dynamin-independent in cones. We hypothesized that fast endocytosis in rods may also differ from cones in its dependence upon the amount of Ca^{2+} influx and/or endocytic load. We measured exocytosis and endocytosis from membrane capacitance (C_m) changes evoked by depolarizing steps in voltage clamped rods from tiger salamander retinal slices. Similar to cones, the time constant for endocytosis in rods was quite fast, averaging <200 ms. We manipulated Ca^{2+} influx and the amount of vesicle release by altering the duration and voltage of depolarizing steps. Unlike cones, endocytosis kinetics in rods slowed after increasing Ca^{2+} channel activation with longer step durations or more strongly depolarized voltage steps. Endocytosis kinetics also slowed as Ca^{2+} buffering was decreased by replacing BAPTA (10 or 1 mM) with the slower Ca^{2+} buffer EGTA (5 or 0.5 mM) in the pipette solution. These data provide further evidence that endocytosis mechanisms differ in rods and cones and suggest that endocytosis in rods is regulated by both endocytic load and local Ca^{2+} levels.

Keywords

Rod photoreceptors; Retina; Exocytosis; Endocytosis; Ribbon synapse

Introduction

To maintain signal transmission, neurons must sustain release of synaptic vesicles for long periods of time (Beutner et al., 2001; Wu et al., 2007; Alabi & Tsien, 2012). This process requires tight coupling between exocytosis and endocytosis (Rieke & Schwartz, 1996; Gundelfinger et al., 2003; Haucke et al., 2011), with vesicles retrieved *via* compensatory endocytosis after their release (Pysh & Wiley, 1974; Miller & Heuser, 1984; LoGiudice &

Matthews, 2007; Wu et al., 2007; Barg & Machado, 2008; Smith et al., 2008; Xue & Mei, 2011). Tight temporal coupling between exocytosis and endocytosis is especially important in rod photoreceptors. At conventional synapses, release occurs phasically in response to action potentials (Waites & Garner, 2011), but rods respond to changes in light and dark by graded changes in membrane potential that modulate the continuous release of vesicles (Dowling, 2012). In darkness, rods have a depolarized membrane potential of ca. -40 mV that promotes continued activation of L-type Ca^{2+} channels and sustained release of vesicles. To facilitate ongoing release, rods contain a specialized protein active zone structure known as the ribbon, which tethers releasable vesicles close to Ca^{2+} channels (Sterling & Matthews, 2005; Schmitz, 2009). Unlike mammalian rods that have a single ribbon per rod terminal (Sterling & Matthews, 2005), there are an average of 7 ribbons per terminal in salamander rods (Townes-Anderson et al., 1985). Measurements in both salamander and mouse retina suggest that synaptic vesicles are released from rods at a rate of 18 vesicles/second/ribbon in darkness (Berntson & Taylor, 2003; Sheng et al., 2007). At this rate, the entire cytoplasmic pool of vesicles would be depleted within 5–10 min after the onset of darkness without compensatory endocytosis (Sheng et al., 2007; Zampighi et al., 2011). Thus, even though a very large number of vesicles participate in release from rods (ca. 75,000 per rod in salamander retina; Sheng et al., 2007), endocytosis remains essential for maintaining ongoing release.

The properties of endocytosis have been studied directly in only a few neuronal cell types. Retinal bipolar cells are one of the most thoroughly characterized. Studies in bipolar cells show that multiple mechanisms of endocytosis with different kinetics are involved in retrieving vesicles after their release (von Gersdorff & Matthews, 1994b). A slow phase of endocytosis, with a time constant of approximately 10 s, was shown to be clathrin dependent (Jockusch et al., 2005). In clathrin-mediated endocytosis, a clathrin protein lattice coats the exterior of the vesicle prior to endocytosis. Another faster component of endocytosis, with a time constant of approximately 1 s, was not clathrin dependent (Jockusch et al., 2005), but did involve endophilin (Llobet et al., 2011). This fast component was not due to a kiss-and-run endocytosis mechanism, in which a transient pore connects the vesicle to the plasma membrane without full collapse fusion (Zenisek et al., 2002; Llobet et al., 2003). Both clathrin-dependent and clathrin-independent modes of endocytosis typically involve the GTPase dynamin (LoGiudice et al., 2009; Wan & Heidelberger, 2011). However, a GTP-independent mode of endocytosis has also been observed in bipolar cells (Heidelberger, 2001) as well as other neurons (Xu et al., 2008; Chung et al., 2010). A third mechanism, bulk endocytosis, was identified under conditions of strong and prolonged stimulation (Holt et al., 2003; Coggins et al., 2007). In this mechanism, portions of the synaptic membrane are retrieved less selectively forming large endosomes.

In photoreceptors, both coated vesicles (Gray & Pease, 1971; Schaeffer & Raviola, 1978) and clathrin (Sherry & Heidelberger, 2005; Wahl et al., 2013; Fuchs et al., 2014) are found in perisynaptic regions adjacent to ribbons. Other endocytic proteins including dynamin, syndapin, amphiphysin, endophilin, and calcineurin are also found in this region of rod terminals (Ullrich & Südhof, 1994; Sherry & Heidelberger, 2005; Wahl et al., 2013; Fuchs et al., 2014). Rods and cones exhibit both fast and slow components of endocytosis (Rieke

& Schwartz, 1996; Rabl et al., 2005; Innocenti & Heidelberger, 2008; Van Hook & Thoreson, 2012). Cones have an ultrafast component of endocytosis, with a time constant of ~250 ms, much more rapid than the fast component in bipolar cells (Van Hook & Thoreson, 2012). Endocytosis in cones was not slowed by inhibition of dynamin with dynasore or GTP γ S (Van Hook & Thoreson, 2012), whereas endocytosis in rods was decreased by these same inhibitors (Van Hook & Thoreson, 2012). This result indicates that different mechanisms mediate endocytosis in rods and cones.

In the present study, we characterized endocytosis in rods further and observed an ultrafast component of retrieval with an average time constant of <200 ms following a brief depolarizing test step. Unlike cones, the kinetics of endocytosis in rods slowed with increasing release and decreasing Ca²⁺ buffering suggesting that endocytosis in rods is regulated by both endocytic load and local Ca²⁺ levels.

Materials and methods

Retinal slice

Slices were prepared from the retina of aquatic tiger salamanders (*Ambystoma tigrinum*; both sexes, 18–25 cm length; Charles Sullivan, Nashville, TN) using methods described by Van Hook and Thoreson (2013). Experimental procedures were approved by the University of Nebraska Medical Center's Institutional Animal Care and Use Committee. Animals were maintained at 4–8 °C on a 12:12 h light-dark cycle. Salamanders were sacrificed 1–2 h after the beginning of subjective night by rapid decapitation and hemisection of the head with heavy shears followed immediately by pithing. Then, the eyes were enucleated and the anterior segment of the eye and the lens were removed. The resultant eyecup was placed vitreal side down onto a nitro-cellulose membrane (5 × 10 mm; type AAWP, 0.8 μ m pores; Millipore). The tissue was cut into 125 μ m slices using a razor blade tissue slicer (Stoelting Co., Wood Dale, IL). Slices were rotated 90° and anchored to the recording chamber using vacuum grease. The recording chamber was placed on an upright fixed stage microscope (E600FN; Nikon, Tokyo, Japan) equipped with a long working distance, 60X, 1.0 NA water-immersion objective. Tissue was superfused at ~1 ml/min with an oxygenated amphibian saline solution containing the following (in mM): 111 NaCl, 2.5 KCl, 1.8 CaCl₂, 0.5 MgCl₂, 5 glucose, and 10 HEPES (pH 7.8; 240–245 mOsm).

Electrophysiology

Recording pipettes were fabricated from borosilicate glass pipettes (1.2 mm outer diameter, 0.9 mm inner diameter, with internal filament; World Precisions Instruments) using a Narishige PP-830 vertical puller to produce tips of ~2 μ m in diameter and resistance values between 12–20 M Ω . The shaft of each recording pipette was coated with dental wax to reduce stray capacitance. Pipettes were positioned with Huxley–Wall micromanipulators, so that the tip of the pipette contacted the rod cell body, which was identified morphologically. After establishing a giga-ohm seal, the patch was ruptured with gentle suction. The pipette solution contained (in mM): 40 Cs glutamate, 50 Cs gluconate, 10 TEACl, 3.5 NaCl, 1 CaCl₂, 1 MgCl₂, 10 MgATP, 0.5 GTP, 1 BAPTA, and 10 HEPES (pH 7.2; 235–240 mOsm). BAPTA was included to rapidly buffer Ca²⁺ and minimize Ca²⁺-dependent

conductances. In some experiments, we used 10 mM BAPTA, 5 mM EGTA, or 0.5 mM EGTA in place of the 1 mM BAPTA. The 0.5 mM EGTA solution was made without the 1 mM CaCl_2 . TEA and Cs are included to block K^+ channels, improving voltage clamp and Ca^{2+} current (I_{Ca}) measurements. Rods were voltage clamped with an Optopatch patch clamp amplifier (Cairn Research), which was controlled using an Axon Instruments Digidata 1322A interface (Molecular Devices) and pClamp 9.2 software (Axon/Molecular Devices). We excluded rods that required holding currents >200 pA in order to maintain a membrane potential of -70 mV. Membrane resistance, series resistance, and membrane capacitance (C_m) averaged 223.0 ± 16.8 M Ω , 34.5 ± 1.8 M Ω , and 31.2 ± 1.4 pF, respectively ($N = 21$).

Changes in C_m were used to measure exocytosis and endocytosis. Vesicle exocytosis was evoked by applying a depolarizing voltage step to the rod. Measurements of C_m were made using the phase-tracking circuitry of the lock-in amplifier integrated into the Optopatch patch clamp amplifier (Johnson et al., 2002; Rabl et al., 2006; Bartoletti et al., 2010; Van Hook & Thoreson, 2012). The membrane potential was varied sinusoidally (500–600 Hz, 30 mV peak-to-peak) about a holding potential of -70 mV. Series resistance and membrane capacitance controls were adjusted to cancel the sinusoidal output current of the lock-in amplifier. To limit possible C_m measurement artifacts that can be caused by conductance changes and allow time for the phase angle feedback circuitry to settle, capacitance recordings were suspended during the voltage step and for 30 ms afterward. Recordings that showed pronounced poststimulus changes in access resistance were excluded. The net change in C_m is due to exocytosis of vesicles minus any compensatory endocytic retrieval. The kinetics of endocytosis were characterized by the time constant for a single exponential fit to the decline in C_m after stimulation (τ_{endo}) or for the time required for the increase in C_m to decline by 50% (t_{50}).

In a few experiments, we obtained simultaneous paired whole cell recordings from rods and horizontal cells in retinal slices. Rod recordings were obtained as described for capacitance experiments. Rods were voltage clamped at -70 mV and stimulated with 200 ms voltage steps to -10 mV. Horizontal cells were voltage clamped at -60 mV with an Axopatch 200B amplifier. Horizontal cell pipettes were filled with a solution containing the following (in mM): 90 Cs gluconate, 10 TEACl, 3.5 NaCl, 1 CaCl_2 , 1 MgCl_2 , 10 MgATP, 0.5 GTP, 5 EGTA, and 10 HEPES (pH 7.2; 235–240 mOsm). Paired recording techniques are described in detail by Van Hook and Thoreson (2013).

Analysis

Capacitance traces were analyzed with pClamp 10 software. Data were analyzed using GraphPad Prism 4 to determine the mean $\pm$ SEM. Statistical significance of differences between experimental conditions was determined using two-tailed independent or paired Student's t tests with $P < 0.05$.

Results

Fast endocytosis in rods

To study endocytosis in rods, we used capacitance recording techniques. Membrane capacitance provides a direct, high temporal resolution method for measuring changes in membrane surface area that result from the exocytosis and endocytosis of vesicles. Depolarizing test steps applied to voltage clamped rods typically evoked rapid endocytic retrieval of membrane. Fig. 1 illustrates the response of a rod to a 100 ms step from -70 to -10 mV. This strong stimulus maximally triggers opening of the L-type Ca^{2+} channels beneath the ribbon, allowing an influx of Ca^{2+} that drives vesicle release (Thoreson et al., 2004; Rabl et al., 2006). The step evoked an inward I_m followed by an outward tail current after the step (Fig. 1: I_m). An increase in membrane capacitance due to exocytosis was observed when measurements were resumed 30 ms after the step. Capacitance declined thereafter due to endocytosis (Fig. 1: C_m).

We measured the kinetics of endocytosis in two ways. First, we determined τ_{endo} from a single exponential fit to the decline in C_m (gray line, Fig. 1). Fitting the records with two exponentials did not improve the fit. For 5 out of the 6 step durations tested, fitting the average trace with a two-exponential function produced a negative value for the amplitude and/or the time constant. When fits were constrained to positive values for amplitude and time constant, fits were not improved significantly by addition of a second exponential component (paired comparison of the sum of squared errors for fits to average waveforms with 5–200 ms steps, $P = 0.38$, $N = 6$). Although there is evidence that slower endocytic components also contribute to retrieval with longer steps (Rieke & Schwartz, 1996; Thoreson et al., 2004), these results suggest that a single kinetic process dominated endocytosis with the relatively short steps used in our experiments.

We often observed a brief plateau before capacitance began to decline. Because of this plateau, we also characterized endocytosis by the time required for the amplitude of the capacitance increase to decline by 50% (t_{50}). The t_{50} values were typically longer than τ_{endo} , because they included this initial plateau (Fig. 1).

The kinetics of endocytosis following a 100 ms step were quite rapid ($\tau_{\text{endo}} = 174.4 \pm 33.54$ ms, $N = 13$; $t_{50} = 192.9 \pm 23.77$ ms, $N = 14$; 100 ms; -70 to -10 mV). Cones often exhibited an endocytic overshoot, in which C_m went below baseline after stimulation before returning to the pre-stimulation level (Van Hook & Thoreson, 2012). By contrast, in rods we did not see convincing evidence for such an overshoot even with 200 ms steps. Some individual records appeared to show endocytic overshoot, but when we averaged multiple records to reduce the influence of baseline noise, we observed no evidence for an endocytic overshoot in rods. A similar method of analysis in cones using averaged records persisted in demonstrating endocytic overshoot (Van Hook & Thoreson, 2012).

A 100 ms step to -10 mV evoked a capacitance increase averaging 76 fF. The diameter of a single vesicle in rods averages 45 nm, equivalent to a C_m of 57 aF (Thoreson et al., 2004), suggesting 1333 vesicles were released during the step. Salamander rods possess an average of 7 ribbons/terminal (Townes-Anderson et al., 1985) and each ribbon has an average of 710

vesicles, yielding a total ribbon pool of 4970 vesicles (Thoreson et al., 2004). In mammalian rods, 17% of the ribbon pool is docked in contact with the plasma membrane (Sterling & Matthews, 2005). Assuming a similar proportion in salamander, this suggests an immediately releasable pool of 845 vesicles. Many vesicles were also probably released from non-ribbon sites (Chen et al., 2013).

We performed a number of control experiments to confirm that capacitance increases represented synaptic exocytosis. 1) Depletion of the releasable pool of vesicles by a 1 s pulse train (25 ms steps to -10 mV at 13.3 Hz) abolished the capacitance increase evoked by a subsequent depolarizing test pulse. The left trace in Fig. 2A shows a capacitance increase evoked by a depolarizing test step (25 ms to -10 mV) prior to the train. Immediately after the train, the response was abolished (center trace, Fig. 2A). The depolarization-evoked capacitance increase subsequently recovered after waiting >1 min for replenishment of the releasable pool of vesicles (right trace, Fig. 2A). 2) Capacitance increases were not consistently correlated with the presence or absence of tail currents following depolarizing steps. Fig. 2B (left traces) shows a recording in which depolarization (25 ms to -10 mV) evoked both a capacitance increase and a brief inward tail current. Application of the same depolarizing step later in the same cell evoked a similar tail current but no capacitance increase (Fig. 2B, center traces). Subsequent application of a 200 ms step evoked a large inward tail current but did not evoke a capacitance increase (Fig. 2B, right traces). This indicates that the small tail current evoked by the 25 ms step applied earlier in the same cell was not responsible for the observed capacitance increase (Fig. 2B, left traces). The absence of capacitance increases in the two later traces was presumably due to rundown of exocytosis (Linton et al., 2010). In some cells, we observed outward rather than inward tail currents. These were also not consistently correlated with capacitance increases. Together these experiments showed that, in the absence of any significant change in the resistance trace, tail currents were not responsible for capacitance increases. However, if tail currents were accompanied by large changes in the resistance trace (e.g., the 200 ms record in Fig. 2B, right trace), we excluded those records out of concern that such large changes in resistance might induce artifacts that could influence capacitance measurements. 3) As expected for exocytosis, there was rundown in the amplitudes of capacitance responses over time (average slope significantly nonzero, $P = 0.0015$), averaging -5.5 ± 1.3 fF/min ($N = 13$) for cells stimulated with 10 ms steps (-70 to -10 mV). 4) Depolarization-evoked capacitance increases were unchanged after blocking $I_{Cl(Ca)}$ with niflumic acid (10 ms steps: 0.1 mM $t_{50} = 142.7 \pm 24.02$ ms, $N = 8$; control $t_{50} = 115.6 \pm 6.85$ ms, $N = 58$; $P = 0.19$). Two results from earlier studies provide further evidence that depolarization-evoked capacitance increases in rods are due to exocytosis: 1) The amount of exocytosis measured from capacitance increases was linearly correlated with the amount of release measured by postsynaptic currents in paired recordings from rods and horizontal cells (Rabl et al., 2005). 2) In a model photoreceptor cell, large changes in membrane resistance did not produce a capacitance increase (Rabl et al., 2005).

We tested whether properties of the phase lock amplifier might influence the measured kinetics of endocytosis. Changing the gain of the phase tracking feedback circuitry (by changing the amplifier gain) changed the rise time of depolarization-evoked capacitance

increases, quickening it with higher gains, but did not significantly alter endocytosis kinetics (10 ms steps: gain 2 $t_{50} = 83.44 \pm 5.02$ ms, $N = 8$; gain 5 $t_{50} = 86.43 \pm 16.68$ ms, $N = 8$; $P = 0.88$). Slowing the frequency of the sine wave used for capacitance measurements from 500 to 300 Hz also did not significantly alter endocytosis kinetics or amplitude (25 ms steps: 500 Hz $t_{50} = 126.6 \pm 22.28$ ms, amplitude = 100.6 ± 21.11 fF, $N = 5$; 300 Hz $t_{50} = 137.4 \pm 25.23$ ms, amplitude = 79.9 ± 12.18 fF, $N = 5$; $t_{50} P = 0.62$; amplitude $P = 0.42$). Finally, we obtained similar time constants for endocytosis following 50 ms steps whether we used the phase tracking hardware of the Optopatch amplifier ($\tau_{\text{endo}} = 169.5 \pm 28.82$ ms, $N = 15$) or the software-based, dual sine-wave protocol incorporated into JClamp (Scisoft) (Santos-Sacchi, 2004) and implemented with a Multiclamp 700A amplifier (Molecular Devices) ($\tau_{\text{endo}} = 176 \pm 37$ ms, $N = 4$).

Role of calcium and endocytic load

Next, we tested two factors that are commonly found to regulate the kinetics of endocytosis: the amount of Ca^{2+} influx and the endocytic load (the amount of membrane that needs to be retrieved) (Wu et al., 2014). To do so, we varied Ca^{2+} influx and endocytic load by changing the duration and strength of voltage steps. Longer durations and larger voltage steps promote greater influx of Ca^{2+} and also trigger more exocytosis, and thus a greater endocytic load. The single cell and average traces illustrate that as the step duration increased, the time for the C_m to return to baseline after the step also increased (Fig. 3). Both τ_{endo} and t_{50} values increased for steps from 5 to 50 ms and then remained about the same with 100 and 200 ms steps (Fig. 4A and 4B). Steps longer than 200 ms stimulated large tail currents that were accompanied by large resistance changes made capacitance measurements of the endocytosis kinetics unreliable.

In addition to longer values for τ_{endo} , slowing of the kinetics of endocytosis also involved a lengthening of the plateau in the capacitance trace prior to endocytic retrieval. This plateau tended to increase in length with increasing test step duration (5 ms = 56.1 ± 6.27 ms, $N = 14$; 10 ms = 54.4 ± 3.12 ms, $N = 49$; 25 ms = 58.9 ± 8.25 ms, $N = 20$; 50 ms = 88.8 ± 8.58 ms, $N = 14$; 100 ms = 106.8 ± 14.72 ms, $N = 13$; 200 ms = 103.5 ± 15.99 ms, $N = 11$). Because Ca^{2+} influx increases with longer duration steps, these results are consistent with evidence that endocytosis can be inhibited by high Ca^{2+} concentrations and thereby delay the capacitance decline (von Gersdorff & Matthews, 1994a; Wan et al., 2008; Leitz & Kavalali, 2011). However, another possible explanation for lengthening of the plateau is that endocytosis may be balanced by release that continues after the end of the voltage step (e.g., Singer and Diamond, 2006; von Gersdorff et al., 1998). To examine that possibility we obtained paired whole cell recordings from rods and horizontal cells. We stimulated the rod with a depolarizing voltage step to -10 mV for 200 ms and recorded the excitatory postsynaptic current (EPSC) evoked in the postsynaptic horizontal cell. Fig. 3C shows an EPSC from a single rod/horizontal cell pair as well as the average EPSC from 22 cell pairs. The EPSC persisted for an average of 72.7 ± 13.3 ms ($N = 22$) after the end of the step. This is slightly shorter than the average plateau duration in the capacitance response evoked by 200 ms steps measured with the same pipette solution (5 mM EGTA; 109.4 ± 19.2 ms, $N = 7$; $P = 0.17$), suggesting that continued release may also contribute to lengthening of the plateau with increasing step duration.

Ca^{2+} influx and endocytic load were also varied by changing the strength of 50 ms depolarizing steps. The voltage step was increased from -40 mV, which is near the threshold for opening of L-type Ca^{2+} channels in rods, up to -10 mV. The voltage needed for maximal Ca^{2+} channel activation is near -20 mV. As the probability of Ca^{2+} channel openings increased, the Ca^{2+} influx and endocytic load also increased. As with increasing step duration, increasing the strength of the voltage step resulted in slowing of the kinetics of endocytosis as represented by increasing t_{50} values (Fig. 4C).

To assess whether the kinetics of endocytosis depend upon the amount of Ca^{2+} influx, we changed intracellular Ca^{2+} buffering to vary the local Ca^{2+} levels near release sites. In our standard pipette solution, we used 1 mM BAPTA, which is a relatively rapid chelator of Ca^{2+} and confines Ca^{2+} to within 100 nm of open Ca^{2+} channels (Van Hook & Thoreson, 2012). We compared this to stronger buffering with 10 mM BAPTA, which abolished endocytosis entirely in mature calyx of Held neurons (Yamashita et al., 2010). We also used the slower Ca^{2+} chelator EGTA at 5 and 0.5 mM concentrations, which allows Ca^{2+} to spread further away from sites of Ca^{2+} entry. These different buffering conditions should produce significant differences in Ca^{2+} concentration at more distant regions of the terminal where endocytosis occurs (LoGiudice et al., 2009; Wahl et al., 2013; Fuchs et al., 2014).

For a given Ca^{2+} buffer, the amount of release increased with longer step durations up to 200 ms (Fig. 5A). In addition, at a given step duration, decreasing the amount of Ca^{2+} buffering from 10 mM BAPTA to 0.5 EGTA tended to increase the t_{50} value of endocytosis (Fig. 5B). Fast endocytosis persisted with use of 10 mM BAPTA, although the amount of exocytosis was diminished. With weak intracellular Ca^{2+} buffering using 0.5 mM EGTA, test steps longer than 50 ms evoked large tail currents that prevented accurate capacitance measurements. The lengthening of t_{50} values with the shift from a high concentration of the fast buffer BAPTA to a low concentration of the slow buffer EGTA suggests that elevation of cytosolic Ca^{2+} slows endocytosis.

Decreasing Ca^{2+} buffering also increased the amplitude of exocytotic capacitance responses (Fig. 5A), perhaps by stimulating greater release at sites relatively far from the ribbon (Chen et al., 2013, 2014). We therefore tested whether the kinetics of membrane retrieval might also be influenced by endocytic load by plotting t_{50} values against the amount of release (endocytic load) for the different test conditions described above: different durations, voltage steps, and Ca^{2+} chelators. As shown in Fig. 5C, there was a linear correlation between the slowing of endocytosis and the increase in endocytic load ($r^2 = 0.66$; Fig. 5C) over a range of experimental conditions. This correlation between endocytic load and kinetics held even under conditions where perisynaptic Ca^{2+} levels should be quite different due to the use of different Ca^{2+} buffers. This suggests that endocytic load also regulates the kinetics of endocytosis in rods.

Discussion

Our results indicate that endocytosis in rods involves “ultrafast” retrieval with time constants of less than 300 ms and an onset delay that lengthened with greater release. The rates of retrieval observed in rods were much faster than the fast endocytic component

observed in many neurons including bipolar cells (Neves & Lagnado, 1999; Wan & Heidelberger, 2011), the calyx of Held (Sun & Wu, 2001), and frog auditory hair cells (Cho et al., 2011). However, ultrafast endocytosis has been observed previously in cones (Van Hook & Thoreson, 2013), mammalian inner hair cells (Beutner et al., 2001), hippocampal neurons (Gandhi & Stevens, 2003; Watanabe et al., 2013b), neuromuscular junction (Watanabe et al., 2013a), and neuroendocrine cells (Thomas et al., 1994; Artalejo et al., 1995; Hsu & Jackson, 1996). Kiss-and-run fusion is one potential mechanism for ultrafast endocytosis, but unlike ultrafast endocytosis in rods (Van Hook and Thoreson, 2012) kiss-and-run fusion is not thought to be sensitive to dynamin inhibitors. Furthermore, imaging of single vesicle fusion events suggested that most fusion events in rods involve full collapse of vesicles (Chen et al., 2013).

Most cell types, including bipolar cells and the calyx of Held, exhibit at least two kinetic components to membrane retrieval (von Gersdorff & Matthews, 1994b; Neves & Lagnado, 1999; Wu et al., 2005; Dittman & Ryan, 2009). We found that endocytosis in rods following steps up to 200 ms in duration was better fit by a single exponential. Nevertheless, previous studies in enzymatically isolated rods and with longer test steps have shown evidence for a second slower component of retrieval (Rieke & Schwartz, 1996; Thoreson et al., 2004). The presence of clathrin and related endocytic proteins in rod terminals is also consistent with the presence of a slower component of retrieval by clathrin-mediated endocytosis (Wahl et al., 2013; Fuchs et al., 2014). It is possible that small, slow components of endocytosis may have been obscured by unrelated slow baseline capacitance changes in our recordings. In addition, slow retrieval is typically more prominent after longer steps that stimulate greater Ca^{2+} influx and greater release. In our experiments, such long steps stimulated large Ca^{2+} -activated tail currents and large resistance changes that made measurements of endocytosis unreliable.

Both the amount of preceding exocytosis (endocytic load) and cytosolic Ca^{2+} levels have been shown to promote endocytosis (Yamashita, 2012; Wu et al., 2014). Increases in Ca^{2+} speed endocytosis in many cell types (Wu et al., 2014), but increasing Ca^{2+} influx can also slow endocytosis (Hsu & Jackson, 1996; Wu & Betz, 1996; Sun & Wu, 2001; Sun et al., 2002; Olstedal & Hartveit, 2010; Cho et al., 2011). Slowing of endocytosis may be caused by global elevation of cytosolic Ca^{2+} , whereas enhancement of endocytosis may be triggered by localized elevation of synaptic Ca^{2+} (Wu & Wu, 2014). In retinal bipolar cells, Ca^{2+} slows the time constant of the slow mode of endocytosis (von Gersdorff & Matthews, 1994a; Wan et al., 2008), but can also increase contributions from fast endocytosis without altering the fast time constant (Neves et al., 2001). Mammalian inner hair cells show a similar enhanced contribution from fast endocytosis with increasing Ca^{2+} influx (Beutner et al., 2001; Neef et al., 2014). We found that shifting from high concentrations of the fast Ca^{2+} buffer BAPTA to low concentrations of the slower Ca^{2+} buffer EGTA, which promotes greater increases in perisynaptic Ca^{2+} , was accompanied by a slowing of endocytosis in rods. While this suggests that Ca^{2+} regulates endocytosis kinetics in rods, greater Ca^{2+} influx also stimulates greater release. Unlike other neurons that show a marked cooperativity between Ca^{2+} influx and release, the amount of exocytosis in rods increases linearly with the amount of Ca^{2+} influx (Thoreson et al., 2004). This makes it especially

difficult to distinguish the contributions of endocytic load and intracellular Ca^{2+} in shaping endocytosis kinetics in rods. However, we found that the slowing of endocytosis paralleled increases in endocytic load over a variety of different conditions, including conditions where perisynaptic Ca^{2+} levels should differ considerably (Fig. 5). This is similar to data from other cell types showing that slowing of endocytosis is linearly related to the amount of preceding exocytosis (Sankaranarayanan & Ryan, 2000; Wu et al., 2005; Renden & von Gersdorff, 2007). Slowing of endocytosis with greater release may be due to saturation of endocytic capacity (Wu et al., 2014). Taken together, our results suggest that both Ca^{2+} and endocytic load play a role in regulating endocytosis in rods, similar to other systems (Wu et al., 2014).

The present results provide further evidence that endocytosis mechanisms differ in rod and cone photoreceptors. Endocytosis in both cell types involves ultrafast retrieval. However, ultrafast endocytosis in rods is dynamin-dependent, whereas it is dynamin-independent in cones (Van Hook & Thoreson, 2012). Furthermore, we found that endocytosis in rods slowed with greater Ca^{2+} -triggered release, whereas the time constant of membrane retrieval in cones was independent of both Ca^{2+} and endocytic load. Cones also showed an endocytic overshoot that appeared to be absent from rods (Van Hook & Thoreson, 2012). The molecular mechanisms responsible for rod/cone differences in endocytosis remain to be elucidated. It is possible that rod-cone differences in endocytosis may relate to rod-cone differences in the sites at which release occurs. Release from cones occurs almost exclusively at ribbons (Snellman et al., 2011), whereas a significant amount of release from rods can occur at non-ribbon locations (Chen et al., 2013, 2014). It is plausible that vesicles released at ribbon sites may be retrieved by different mechanisms than vesicles released at non-ribbon sites. Differences in retrieval at ribbon and non-ribbon release sites might also contribute to differences in kinetics observed within rods under different experimental conditions. In rods, shorter steps and Ca^{2+} increases that remain confined near the ribbon favor more ribbon release, whereas longer steps and greater spread of intraterminal Ca^{2+} favor more non-ribbon release (Chen et al., 2013, 2014). Thus, the faster kinetics of endocytosis observed in rods with shorter steps and stronger Ca^{2+} buffering might reflect a faster retrieval mechanism for ribbon release compared to non-ribbon release.

Exocytosis in rods is continually balanced by endocytosis (Rieke & Schwartz, 1996). Ongoing endocytosis is essential at the rod synapse in order to maintain synaptic signaling, especially during darkness when there is continuous vesicle release for hours at a time. The fast rate of endocytosis may facilitate this balance by helping to rapidly clear previously exocytosed proteins from release sites, readying them for a subsequent round of release (Neher, 2010).

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Fig. 1.

Fast endocytosis in rods. Whole-cell capacitance recordings from a rod in a salamander retinal slice preparation. Changes in membrane current (I_m), membrane capacitance (C_m), and access resistance (R_a) evoked by a 100 ms depolarizing step to -10 mV from a mean holding potential of -70 mV. The increase in C_m caused by fusion of synaptic vesicles was followed, after a brief delay, by a decline in capacitance due to compensatory endocytosis. C_m and R_a measurements were suspended during the step and for 30 ms afterward. The light gray trace shows an exponential fit (τ_{endo}) to the decline with a time constant of 142 ms. Endocytosis kinetics were also measured by determining the time at which the capacitance increase had declined by 50% (t_{50}), which was 213 ms in this example.

Fig. 2.

Capacitance changes were not due to conductance or amplifier artifacts. **(A)** Emptying the releasable pool of vesicles with a 1 s pulse train (25 ms steps to -10 mV at 13.3 Hz) abolished the depolarization-evoked capacitance increase. Three responses recorded in the same cell show the membrane capacitance (C_m) and access resistance (R_a) evoked by a test step (25 ms to -10 mV) prior to the pulse train (left traces), immediately after the pulse train when the vesicle pool was depleted (center traces), and following subsequent recovery of the releasable vesicle pool (right traces). This result is consistent with capacitance increases being due to vesicle exocytosis. **(B)** Capacitance responses are not due to tail currents. A step depolarization (25 ms step to -10 mV) induced a capacitance increase and brief inward tail current (left traces). The same test step applied later in the same cell evoked a similar tail current but did not evoke a capacitance increase (center traces). Subsequent application of a 200 ms test step evoked a large tail current but no capacitance increase (right traces). The absence of a capacitance increase in later records was presumably due to rundown of exocytosis.

Fig. 3.

The kinetics of endocytosis slowed with longer duration test steps. Representative traces from an individual cell (**A**) (all responses from the same cell) and average traces from multiple cells (**B**) with different duration steps: 5, 25, 100, and 200 ms (-70 to 10 mV). Average traces show the mean in black and SEM in gray (5 ms, $N = 15$; 25 ms, $N = 21$; 100 ms, $N = 14$; 200 ms, $N = 13$). Endocytosis kinetics slowed with longer steps. (**C**) EPSCs evoked in a horizontal cell by stimulation of rods with a depolarizing step (200 ms, -70 to -10 mV). The trace at the left shows an example from a single cell pair and the trace at the right shows the postsynaptic response averaged from 22 cell pairs.

Fig. 4.

Effects of test step duration and amplitude on endocytosis kinetics. With longer step durations (5, 10, 25, 50, 100, 200 ms), there was a similar increase in both t_{50} (**A**) and τ_{endo} (**B**) values (t_{50} : 5 ms = 102.4 ± 10.33 ms, $N = 15$; 10 ms = 115.6 ± 6.85 ms, $N = 58$; 25 ms = 117.9 ± 17.97 ms, $N = 20$; 50 ms = 157.0 ± 20.19 ms, $N = 14$; 100 ms = 179.5 ± 21.72 ms, $N = 13$; 200 ms = 177.4 ± 23.33 ms, $N = 11$ and τ_{endo} : 5 ms = 79.20 ± 12.39 ms, $N = 15$; 10 ms = 96.89 ± 9.036 ms, $N = 54$; 25 ms = 134.4 ± 20.72 ms, $N = 21$; 50 ms = 169.5 ± 28.82 ms, $N = 15$; 100 ms = 174.4 ± 33.54 ms, $N = 13$; 200 ms = 170.3 ± 45.96 ms, $N = 12$). (**C**) With increasing voltage steps (-70 to -40, -30, -20, and -10 mV; 50 ms) there was an increase in the t_{50} of endocytosis (-40 mV = 80.50 ± 12.17 ms, $N = 8$; -30 mV = 145.0 ± 38.62 ms, $N = 8$; -20 mV = 170.5 ± 47.32 ms, $N = 11$; -10 mV = 172.9 ± 22.03 ms, $N = 28$; -40 vs. -10 mV, $P < 0.05$).

Fig. 5.

Changes in step duration and Ca^{2+} buffering altered the kinetics of endocytosis. **(A)** The amplitude of capacitance increases observed with different test step durations and intracellular Ca^{2+} buffers (10 mM BAPTA, 1 mM BAPTA, 5 mM EGTA, and 0.5 mM EGTA). **(B)** Average t_{50} for steps of increasing duration measured using the same intracellular Ca^{2+} buffers. **(C)** There was a linear correlation between t_{50} and the amplitude of exocytosis, which is proportional to endocytic load ($r^2 = 0.66$; slope significantly nonzero, $P < 0.0001$).