Calcium-Dependent Synaptic Vesicle Trafficking Underlies Indefatigable Release at the Hair Cell Afferent Fiber Synapse

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SUMMARY

Sensory hair cell ribbon synapses respond to graded stimulation in a linear, indefatigable manner, requiring that vesicle trafficking to synapses be rapid and nonrate-limiting. Real-time monitoring of vesicle fusion identified two release components. The first was saturable with both release rate and magnitude varying linearly with Ca2+, however the magnitude was too small to account for sustained afferent firing rates. A second superlinear release component required recruitment, in a Ca2+-dependent manner, of vesicles not in the immediate vicinity of the synapse. The superlinear component had a constant rate with its onset varying with Ca2+ load. High-speed Ca2+ imaging revealed a nonlinear increase in internal Ca2+ correlating with the superlinear capacitance change, implicating release of stored Ca2+ in driving vesicle recruitment. These data, supported by a mass action model, suggest sustained release at hair cell afferent fiber synapse is dictated by Ca2+-dependent vesicle recruitment from a reserve pool.

INTRODUCTION

The hair cell afferent fiber synapse maintains a high level of tonic vesicle release and responds to graded input with linear changes in release across a wide range of stimulus frequencies (Furukawa et al., 1978). Information regarding frequency, intensity, and phase of stimulation are transferred across this synapse with high fidelity (Rose et al., 1967, 1971) and mechanisms by which this occurs are the focus of much work (Eisen et al., 2004; Meyer et al., 2009; Neef et al., 2007; Nouvian et al., 2006; Parsons et al., 1994; Schnee et al., 2005). Hair cells have a presynaptic dense body (DB) or synaptic ribbon, as do other sensory cells requiring graded and tonic release (Lagnado, 2003; Parsons and Sterling, 2003; Schnee et al., 2005; von Gersdorff and Matthews, 1997). The functional significance of the DB is unclear but synapses with DBs have common features including linear release with increasing Ca2+ load, high release rates, and limited fatigue. At conventional synapses, vesicle populations are classified based on location and release kinetics, with a readily releasable pool (RRP) of vesicles near the membrane, a more distal recycling pool that communicates with the RRP, and a larger reserve pool whose role varies with synapse type (Rizzoli and Betz, 2005). Physiological investigations with either capacitance measurements or optical techniques find that pools do not strictly adhere to these distributions and that the ability to move between pools varies with synapse type (Rizzoli and Betz, 2004, 2005).

At ribbon synapses, vesicle pools have been classified by position relative to the ribbon and plasma membrane (Nouvian et al., 2006). The locations of vesicles around the ribbons have been correlated with capacitance measurements that identify pools based on release kinetics and saturation (Gomis et al., 1999; Gray and Pease, 1971; Mennerick and Matthews, 1996; Moser and Beutner, 2000; Schnee et al., 2005). Data establishing a direct link between vesicle location and release pools are limited. Furthermore, vesicle populations are often more difficult to observe in auditory hair cells because saturation is less evident and rapid vesicle trafficking appears to create overlap between pools (Schnee et al., 2005).

The role of the DB in regulating synaptic transmission remains unclear. In hair cells lacking DBs because of knockout of the anchoring protein bassoon, sustained exocytosis is maintained but synchronous vesicle release is lost (Khimich et al., 2005). DBs may tether vesicles, clustering them near presynaptic membranes, a hypothesis supported by morphological data (Lenzi et al., 1999; Wittig and Parsons, 2008). The DB may also act as a reservoir for vesicles that are waiting to be released (Schnee et al., 2005). The functional significance of the DB is unclear but synapses with DBs have common features including linear release with increasing Ca2+ load, high release rates, and limited fatigue. At conventional synapses, vesicle populations are classified based on location and release kinetics, with a readily releasable pool (RRP) of vesicles near the membrane, a more distal recycling pool that communicates with the RRP, and a larger reserve pool whose role varies with synapse type (Rizzoli and Betz, 2005). Physiological investigations with either capacitance measurements or optical techniques find that pools do not strictly adhere to these distributions and that the ability to move between pools varies with synapse type (Rizzoli and Betz, 2004, 2005).

How vesicles reach synaptic regions is also contentious. In the visual system, vesicles may freely diffuse within the cytosol until affixing to DBs (Holt et al., 2004; LoGiudice and Matthews, 2009). Brownian motion can provide enough DB-vesicle encounters to maintain vesicle availability during long release paradigms (Beaumont et al., 2005). Data from hair cells suggest vesicles are present in a gradient; density is highest near the synapse and lower.
away from the synapse (Lenzi et al., 1999; Schnee et al., 2005), intimating a more structured system. Calcium dependence of vesicle trafficking has also been suggested (Spassova et al., 2004).

In this study, we assess synaptic vesicle trafficking and fusion at the hair cell afferent synapse in real time. Our approach has allowed us to separate release into a linear component that does not require recruitment of vesicles and a superlinear component dependent upon vesicle trafficking. We are able to clearly identify pools of depletable vesicles that correspond in size to those vesicles near the DB. Data presented here implicate strong interactions between the RRP and the recycling pool, which together account for the observed linear release component and also demonstrate an ability to rapidly recruit from the reserve pool. Vesicle trafficking is calcium dependent and release of stored calcium may be critical for recruitment of vesicles to the release site from the distant reserve pool.

RESULTS

Ca\(^{2+}\) Dependence of Trafficking

At retinal ribbon synapses, paired-pulse experiments identified an RRP that could be depleted (Coggins and Zenisek, 2009). For experiments with turtle auditory hair cells, we designed a protocol to elicit a capacitance change roughly equivalent to release of all vesicles associated with the DB (300 ms pulse to −20 mV, based on previous estimates of vesicle distribution, Schnee et al., 2005) and the interval between pulses was varied from 1 s to 10 ms (Figure 1A). Surprisingly, we did not observe depletion or reduction of release during the second pulse at any interpulse duration (Figure 1B). Rather, as the interpulse interval was reduced, capacitance increased (Figure 1). The increase in release from the second pulse approached that equivalent to a single 600 ms pulse (data not shown). These data suggest that vesicles can be rapidly recruited to release sites faster than they are depleted. To test whether a depletable pool could be observed by altering stimulus duration, we held the interpulse interval at 30 ms and varied stimulus duration between 10 and 300 ms (Figure 1C). Depletion was never observed and again, as stimulus duration was increased, the second response was greater than the first, indicative of rapid vesicle recruitment. Assuming capacitance reflects synaptic vesicle fusion, a change of 400 fF equates to 8000 vesicles (assuming 50 aF per vesicle) or 186 vesicles per synapse (see Figure 4 for synapse counts), more vesicles than previously identified to be near the synapse (Schnee et al., 2005), indicating that rapid vesicle recruitment is required.

To identify discrete pools, it might be necessary to reduce calcium entry as a potential means of slowing release and possibly vesicle trafficking. Additionally, pool populations might be masked by priming of synapses such that the second stimulation might not provide similar information to the initial one. This can be a significant issue when multiple stimulations are required to assess release across a broad time frame. Additionally, both intra- and intercellular variability may make it more difficult to identify discrete vesicle pools.

Two-Sine Measurements

To address these concerns and more directly investigate vesicle trafficking, we developed a method for continually monitoring cell capacitance that simultaneously tracks capacitance and conductance changes. This two-sine technique requires accurate compensation of electrode capacitance and calibration of the recording system (for detailed description of technique see Santos-Sacchi, 2004 and Supplemental Information, Figures S1–S3). With this approach, we could resolve all components of release from a single cell with a single pulse. Importantly,
continuous monitoring of capacitance allowed the use of protocols eliciting submaximal $I_{\text{Ca}}$, thereby slowing Ca$^{2+}$ influx, with the goal of creating separation between individual components of trafficking and release.

Figure 2 provides an example of a cell probed with a depolarization eliciting either 75% or 35% of the maximal Ca$^{2+}$ current. As predicted, strong depolarization compressed release components so that saturable pools were difficult to observe (Figures 2B and 2C, left panel). Surprisingly though, the rate of release increased during the stimulation (Figure 2). We commonly observed a slight delay in release after the stimulus onset that varied with intensity and repetition making it difficult to quantify (Figure 2C). Probably this delay relates to strong calcium clearance mechanisms at the synapse and results from nonphysiological stimulus protocols where cells are held at very hyperpolarized potentials (see Figure 5). Slowing Ca$^{2+}$ entry separated release into at least two clearly identifiable components, an initial shallow component that showed depletion followed by a large, rapid, superlinear component (Figure 2B). These results are in contrast to those from photoreceptors where the initial release was fast, followed by longer but slower release components (Innocenti and Heidelberger, 2008). With slower Ca$^{2+}$ accumulation, the depletable pool size increased from 24 vesicles/synapse to 60 vesicles/synapse (based on 50 aF/vesicle and synapse numbers presented in Figure 4). Therefore, slowing Ca$^{2+}$ entry unmasked a saturable pool of vesicles whose pool size varied with Ca$^{2+}$ load. Depending on stimulus intensity this additional pool could be recruited into the depletable first component (Figure 2D).

Plotting the Ca$^{2+}$ load against capacitance changes corroborated the superlinear nature of the second release component (Figure 2E). Interestingly, the dramatic difference in Ca$^{2+}$ load required to elicit the secondary larger capacitance change depended on the rate of Ca$^{2+}$ entry. Depolarizations closer to the peak elicited the superlinear component with less than 200 pC of Ca$^{2+}$ entry as compared to 600 pC when Ca$^{2+}$ entry was slowed. This may reflect the presence of strong Ca$^{2+}$ clearance mechanisms at the synapse that were overwhelmed with rapid Ca$^{2+}$ entry. Fitting the data in Figure 2E with a Hill equation by using previously determined maximal release values (Schnee et al., 2005) yielded a Hill coefficient of 3.6 ± 0.4 for the high-frequency cells ($n = 14$). The coefficient was not dependent upon level of depolarization as the shift in the capacitance versus Ca$^{2+}$ load with depolarization was parallel (Figure 2E). This value is similar to that obtained when photolysis of caged calcium was used to stimulate release and to that observed in mouse IHCs (Beurg et al., 2010; Beutner et al., 2001). Interestingly, this value is predominated by the superlinear component of release that is at least in part a reflection of vesicle trafficking and not only of release.

The nonlinearity in release differs from previous measurements (Schnee et al., 2005). However, a limitation to those experiments was the use of the single-sine method, which provided no direct kinetic information; rather, kinetics were inferred from responses measured after the pulse by combining responses from multiple cells and/or multiple pulses to individual cells. A comparison of data collected by using the two-sine wave technique to that previously obtained by using the single-sine technique revealed a dramatic difference in Ca$^{2+}$ load required to elicit the secondary larger capacitance change.

Figure 2. Two Ca$^{2+}$-Dependent Components of Vesicle Release Were Observed during Hair Cell Depolarization

(A) $I_{\text{Ca}}$ elicited in response to membrane depolarization from −85 mV to −30 mV (75% peak, left) or −43 mV (35% of peak, right).

(B) Capacitance change elicited in response to $I_{\text{Ca}}$ in (A); arrow indicates superlinear onset. Colors show regions where rates are different.

(C) Expansion of the axis of (B). Red identifies a saturable pool of vesicles that differs in amplitude between stimulus protocols. Blue indicates an intermediary component that also is saturable depending on Ca$^{2+}$ load and purple represents the superlinear component.

(D) Expansion of plots in (B) to illustrate the intermediate component of release where depletion has ended but superlinear response has not started. The dashed line is the exponential fit showing depletion.

(E) Capacitance against Ca load (integral of current in A), demonstrating both linear and nonlinear (indicated by arrow) responses. Dashed lines are fit to Hill equation: $C_m = C_{m(max)} \times x^n / (k^n + x^n)$ where $n$ is Hill coefficient ($r^2 = 0.99$, see text for details).

(F) Plot of capacitance change after stimulation from high-frequency (1 mM EGTA internal) cells included in this study for varying intensities and durations of depolarization as compared to previous data obtained with single-sine wave method (Schnee et al., 2005).
technique confirmed that variability between and within cells may have masked the superlinear behavior of individual cells (Figure 2F). These data point out the limitations of using a technique that requires multiple sampling to intuit kinetic information as compared to direct measurements of kinetics.

To determine whether the superlinear release component was an artifact of whole-cell recording, we performed perforated-patch experiments to maintain endogenous buffering. We observed two release components in both perforated-patch recordings and whole-cell recordings by using 1 mM EGTA (Figure 3), indicating that the observed release properties are not due to the whole-cell recording technique. To ensure that the superlinear release component is not unique to turtle, we recorded from rat and mouse inner hair cells (ages postnatal day P7–P15) and observed two components of release in these preparations (Figure 3C). Previous work in chick auditory hair cells also documented two release components (Eisen et al., 2004), suggesting multiple release components may be a ubiquitous feature of vesicle release in hair cells.

Comparisons between Hair Cells of Different Frequency Locations
Quantitative comparison of release properties between frequency positions requires knowing the number of synapses present. Whole-mount papillae were double labeled with Ctbp2+PSD-95 or Ribeye+PSD-95 antibodies to count the number of functional ribbon synapses at the same tonotopic positions used in the electrophysiological analysis (n = 6). Examples from a high-frequency position are shown in Figures 4A–4F. Ribbon synapses were localized in hair cell basolateral regions (Figures 4D and 4E), were scarce above the nucleus and were typically present in series, probably corresponding to the finger-like projections of the afferent fiber (Figures 4D and 4F, inset). No synapses were included that did not positively label adjacent pre- and postsynaptic markers (Figure 4F, inset). PSD-95 puncta that did not have a corresponding Ribeye component accounted for less than 5% of the observed puncta. Frequency histograms are plotted to show the distribution of synapses for both frequency positions (Figure 4G). The low-frequency distribution was best fit with a normal distribution having a center value of 19.5 ± 0.4 synapses/cell and a full-width at half-maximum value of 9 (r² = 0.92; n = 38 cells). The high-frequency distribution was best fit by the sum of two Gaussians with center peaks of 46 ± 1 and 75 ± 4, and full-width at half-maximum values of 21 and 9, respectively (r² = 0.92; n = 90 cells). Previous morphological work suggested that at higher frequencies some hair cells are dually innervated (Sneary, 1988). Hypothesizing that dual innervations might account for the bimodal distribution in synapse number and further correlate with I_{Ca}, we plotted the frequency distribution of peak I_{Ca} (Figure 4H), revealing a bimodal distribution. The second population of cells with larger I_{Ca} (~3% of the total) and those with larger synapse number (~5% of the total) may represent dual innervations and skew the absolute mean values (Sneary, 1988). Therefore, we used the major peak value in all analyses, rather than the mean of the total population, to ensure similar cell populations were compared between high- and low-frequency cells. I_{Ca} (peak of fit) increased from 313 pA to 586 pA between frequency locations; similarly, synapse number increased from 20 to 46 from low to high frequency such that the Ca²⁺ load per synapse was 16 pA/synapse for low frequency compared to 13 pA/synapse for high frequency. Calcium channels are considered clustered at release sites based on previous measurements in turtle (Tucker and Fettiplace, 1995) and frog (Roberts et al., 1990).

First Release Component: Pool Size
As discussed above, depolarizations elicited two distinct components of release, the first corresponding to a saturable pool whose release rate varied with Ca²⁺ entry and a second component in which the release rate was increased relative to the first component. Sixty-four percent of high-frequency cells and 80% of low-frequency cells had a clearly identifiable saturable vesicle pool. The smallest saturable pool observed (Figures 4K and 4L) had asymptotic capacitance measurements of 48 ± 20 fF (n = 12) and 90 ± 35 fF (n = 9) for low- and high-frequency cells, respectively. This pool size agrees reasonably well with vesicle numbers under the ribbon closest to the plasma membrane and might represent the RRP (Schnee et al., 2005; Rizzoli and Betz, 2005). The largest saturable pools identified (Figures 4K and 4L) were 145 ± 71 (n = 11) for low- and
328 ± 187 fF (n = 12) for high-frequency cells. These values are not statistically different from previous morphological measurements estimating vesicles associated with the DB and the total pool may correspond to the recycling pool and the RRP (Rizzoli and Betz, 2005; Schnee et al., 2005). Movement of vesicles within this pool was dependent on the rate of Ca\(^{2+}\) entry, suggesting that trafficking of vesicles to release sites is fast and Ca\(^{2+}\) dependent and that the ability of vesicles to move between pools is enhanced at auditory ribbon synapses.

**First Release Component: Release Rates**

Release rates varied linearly with Ca\(^{2+}\) load (Figures 4M and 4N). To compare high- and low-frequency cells, we selected stimuli where the Ca\(^{2+}\) load was comparable when normalized to synapse number. Rates were estimated by fitting lines to the initial portions of the release plots prior to depletion. The release rate at low-frequency synapses was significantly faster (530 ± 10 vesicles/s/synapse, n = 14) than at high-frequency synapses (191 ± 60 vesicles/s/synapse, n = 11) (p < 0.05, see Figure S6A). We also compared the Ca\(^{2+}\) dependence between frequency positions (Figures 4M and 4N). Release varied linearly with Ca\(^{2+}\) for the initial release component but the relationship often appeared more exponential in low-frequency cells (Figure 4M), as has been described for mammalian low-frequency cells (Johnson et al., 2008). However, careful inspection reveals encroachment of the superlinear release component (Figures 4K and 4L). No superlinear component is seen in high-frequency cells at these stimulus levels (Figure 4L). The presence of this superlinear component may account for the exponential appearance, suggesting perhaps that vesicle trafficking and not intrinsic

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**Figure 4. Low-Frequency Cells Release Faster per Synapse Than High-Frequency Cells**

(A–C) Maximal-intensity projections of confocal Z-stacks from a whole-mount preparation (high frequency). Immunofluorescence portrays the presence of ribbons (Cbtp2, red) adjacent to postsynaptic densities (PSD95, green).

(D) A three-dimensional (3D) reconstruction of a single hair cell with red being Cbtp2, green being PSD95, and blue being DAPI.

(E) A 3D confocal reconstruction showing the presence of ribbons (Ribeye, red) in the basolateral region of hair cells. Hair bundles can be localized with phalloidin staining (green).

(F) A maximum-intensity projection by using Ribeye (red) and PSD95 (green) to colocalize synapses. Insets in F represent regions outlined to better illustrate the coupling of label. Scale bars: C and E, 5 μm; D and F, 1 μm. Note that the Ribeye antiserum also labels the kinocilium nonspecifically.

(G) A frequency histogram (blue being low frequency and red high frequency throughout) for the number of synapses per cell with solid lines as Gaussian fits to plots (see text for details).

(H) A plot of the frequency distribution for peak IC\(_{\text{Ca}}\), again with Gaussian fits to data as solid lines; arrow indicates small peak corresponding to high-frequency cells with twice the IC\(_{\text{Ca}}\).

(I–N) IC\(_{\text{Ca}}\) (I and J) and capacitance measurements (K and L) in response to different voltage steps (-44, -41, -38, -35, -43 mV from -80 mV) for low (blue) and in response to different voltage steps (-50, -47, -44, -41, -38 mV from -80 mV) for high-frequency (red) cells. Both high-frequency (J, L, and N) and low-frequency (I, K, and M) hair cells show linear, incremental increases in release with increased Ca\(^{2+}\) load. Expanding the smallest capacitance response from (K and L) reveals a saturating component (inset, scale bars are 200 ms and 20 fF). Black lines indicate linearity of release for larger depolarizations. (M and N) plot the data from (K and L) against the integral of the corresponding IC\(_{\text{Ca}}\), illustrating the linear relationship between release and Ca\(^{2+}\) entry for the first release component. Note that the low-frequency response appears nonlinear because of encroachment of the superlinear component. Encroachment is depicted by horizontal solid lines above traces derived from the point where the capacitance response diverges from linearity (L). Solid black lines in (M and N) point out linearity of response.
differences in Ca$^{2+}$ dependence of release may be responsible for the observed results (Figure 4M). We consistently observed that the superlinear component required less Ca$^{2+}$ influx in low-frequency cells than high-frequency cells, which could create an apparent exponential appearance to the Ca$^{2+}$ dependence.

**Superlinear Release Components**

The larger superlinear release component was observed in all cells when the Ca$^{2+}$ load was high (Figure 5). The superlinear nature of the response is denoted by a sharp increase in release rate during constant stimulation. As in Figures 3 and 4, capacitance traces elicited by smaller ICa showed a linear response until reaching a point where release rate dramatically increased. Additional depolarization did not further increase the release rate but rather shortened the onset time of this faster component (Figure 5B). Maximal rates, obtained by fitting a linear equation to the slope of the superlinear component, were 0.9 ± 0.5 pF/s (n = 13) and 1.0 ± 0.8 pF/s (n = 17) for low- and high-frequency cells, respectively, corresponding to 20,000 vesicles/s and 18,000 vesicles/s/synapse and 434 vesicles/s/synapse for low- and high-frequency cells, respectively. As with the first release component, low-frequency synapses operated faster than high-frequency synapses, though release rates per cell were comparable. Plotting the change in capacitance against Ca$^{2+}$ load (Figure 5C) shows that the inflection point where the superlinear component began was at the same Ca$^{2+}$ load for the two responses, suggesting the temporal difference in Figure 5B was due to the difference in rate of Ca$^{2+}$ entry. As seen in Figure 2, this onset time for the superlinear component could be varied by altering the Ca$^{2+}$ load. Further inspection of the Ca$^{2+}$ load driving capacitance change (Figure 5D) shows that first component release plateaus just prior to the onset of the superlinear response. Whether initial vesicle pool depletion provides a signal for rapid vesicle recruitment to the synapse remains to be explored.

**Functional Relevance of Superlinear Component**

The superlinear capacitance changes are paradoxical in that postsynaptic recordings have indicated that release at the hair cell afferent fiber synapse is linear (Keen and Hudspeth, 2006; Li et al., 2009). No physiological experimental data exist that correspond to the superlinear release kinetics, yet large numbers of vesicles must be released continually in order to account for afferent firing properties (Taberner and Liberman, 2005). To address this question, we voltage clamped hair cells at $-45$ mV, near the expected resting potential (Farris et al., 2006) and then depolarized the cells to the peak ICa. The response was compared to the conventional experimental protocol in which the cell is held at $-85$ mV (Figures 5E–5G). At the hair cell’s resting potential, where Ca$^{2+}$ channel open probability is nonzero (Figure 5E), the ICa in response to the depolarization was minimally reduced, yet the capacitance change was dramatically increased (Figure 5F). The capacitance response from $-85$ mV was small and saturating, indicating that release was depelled and the superlinear component not recruited, while the response at the resting potential was almost linear, more in line with what is expected based on afferent fiber recordings. These data suggest that vesicle release and trafficking kinetics are strongly dependent on calcium homeostasis such that altering homeostasis by hyperpolarizing the cell results in the recruitment of an apparent superlinear process, whereas under physiological conditions release could be maintained for much longer periods of time by the merging of linear and superlinear processes. The magnitude of the release observed with the prepulse requires recruitment of vesicles to release sites (i.e., a superlinear process), suggesting that the prepulse results in the temporal merging of the two release components. Biophysically, it is possible to distinguish...
between release and trafficking, but physiologically, the process is created to provide rapid and continual release. The linearity obtained by incorporating the superlinear component is clearly demonstrated by plotting the Ca\(^{2+}\) load against capacitance (Figure 5G). This is not unlike arguments made previously when investigating Ca\(^{2+}\) dependence of release in hair cells by using caged Ca\(^{2+}\) (Beutner et al., 2001). These data suggest multiple sequential first-order processes could account for trafficking and release in the hair cell.

**Ca\(^{2+}\) Buffering Alters the Two Release Components Differently**

The effect of Ca\(^{2+}\) buffering on release properties was investigated with EGTA, BAPTA, and perforated patch at stimulations that elicited about 60% of the maximal I\(_{\text{Ca}}\) (Figures 6A–6C). First, component rates varied with stimulus intensity so rates were compared for a similar Ca\(^{2+}\) load (Figure 6D). BAPTA significantly slowed release as compared to EGTA or perforated patch. The perforated-patch recordings suggest an endogenous buffer capacity less than 1 mM BAPTA, but more than 1 mM EGTA, similar to that suggested previously (Moser and Beutner, 2000) and consistent with a release mechanism located near the source of Ca\(^{2+}\) influx. A comparison of initial release rates against Ca\(^{2+}\) load for individual cells is presented in Figure S6B illustrating how BAPTA reduces release rates. As compared to EGTA, BAPTA also increased the duration of the plateau between the first release component and the onset of the superlinear component, represented by the Ca\(^{2+}\) load required for the onset of the superlinear release (Figure 6G). In this instance perforated-patch responses suggest endogenous buffering was stronger than either BAPTA or EGTA. This might indicate that the site of action is further removed from the Ca\(^{2+}\) source where concentration rather than kinetics is more relevant (Naraghi and Neher, 1997). In contrast, changes in the rate of the superlinear component (Figure 6E) suggest the perforated-patch response was less efficacious than EGTA or BAPTA, supporting the contention that endogenous buffer kinetics are slow. Finally, the difference in Ca\(^{2+}\) load required to initiate the superlinear component of release led to an increase in the magnitude of the first component plateau (Figure 6F), supporting the conclusion that vesicle movement to release sites was rapid and Ca\(^{2+}\) dependent. The delay between release components also

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**Figure 6. Fast Ca\(^{2+}\) Buffers Slow Release Rates and Onset of Release but Pool Size Does Not Change**

(A–C) EGTA (tan) at 1 mM (A), BAPTA (green) at 1 mM (B), and perforated patch (purple) (C) with I\(_{\text{Ca}}\) (upper panels) and capacitance (lower panels) in response to a depolarization eliciting about 60% of the maximal I\(_{\text{Ca}}\). (D) A plot of the Ca\(^{2+}\) load (hatched bars) and the release rate for the first component under different internal Ca\(^{2+}\) buffering. Asterisks indicate significance at the p < 0.05 level against the recording condition indicated by color. (E–G) Similarly, (E) plots the rate of the superlinear component, (F) plots the capacitance change for the depleted pool, and (G) plots the Ca\(^{2+}\) load at the onset of the superlinear response. The number of cells is given in parentheses above bars in (E). (H) I\(_{\text{Ca}}\) recorded (upper panel) and capacitance change (lower panel) with 10 mM BAPTA internally in the absence (brown) or presence (tan) of 10 \(\mu\)M Bay K.
supports this conclusion, demonstrating that despite the presence of Ca$^{2+}$ to drive release, depletion persisted for longer periods of time when Ca$^{2+}$ buffering was increased because trafficking was slowed.

An alternative possibility is that vesicle position at the synapse is Ca$^{2+}$ dependent and that greater buffer efficacy leads to diffusion of vesicles away from the synapse. We tested this hypothesis by recording cells with 10 mM BAPTA internally, blocking release at all but maximal stimulus levels, and then increasing Ca$^{2+}$ load by using Bay K (10$^{2}$ M), which prolongs Ca$^{2+}$ channel open time (Figure 6H). Capacitance changes evoked by Bay K treatment included both linear and superlinear components supporting the hypothesis that vesicle movement toward the synapse is Ca$^{2+}$ dependent and largely unidirectional.

Calcium Imaging Demonstrates Multiple Release Components
It is possible that additional sources of Ca$^{2+}$, for example Ca$^{2+}$ stores, enhance release during longer stimulations (Lelli et al., 2003). We tested this hypothesis with high-speed confocal Ca$^{2+}$ imaging. Labeling ribbons with a rhodamine-tagged ctbp2-terminal binding peptide in the patch electrode (Zenisek et al., 2003) allowed synapses to be localized during simultaneous Ca$^{2+}$ imaging and capacitance measurements (Figure 7A). The capacitance changes show a typical response with both release components (Figures 7C–7E). The fluorescent signal, however, is quite complex and not simply the integral of the current, as might be predicted. Rather, the initial response (Figures 7F–7H), representing mostly first component release, shows a rapid increase at the synapse followed by a plateau, similar to that observed in mouse (Frank et al., 2009), indicative of strong Ca$^{2+}$ clearance followed by an integrating increase in signal. Sites distal to the synapse show little change initially, consistent with the source of Ca$^{2+}$ being at a distance, followed by a slow increase. The later signals (Figures 7I–7K), corresponding to the onset of the superlinear response, present a different picture. Ca$^{2+}$ at the synapse shows an abrupt increase in signal, followed by a plateau and decrease in signal even in the face of constant Ca$^{2+}$ entry (Figures 7D and 7J). Ca$^{2+}$ signals away from the synapse show a slower integrating signal followed by a sudden increase in signal whose rate is faster and peak greater than that observed at the synaptic region (Figure 7J). The change in kinetics at these distant sites suggests a secondary source of Ca$^{2+}$. Similar results were obtained in five cells where the superlinear release component was observed. Smaller depolarizations revealed simple integrating responses that diminished away from the synapse while larger depolarizations yielded similar complex responses (Figure S7). Together these data suggest that Ca$^{2+}$ dynamics are complex, clearance near the synapse is strong, and a second source of Ca$^{2+}$ may play a role in vesicle

Figure 7. High-Speed Ca$^{2+}$ Imaging Coupled with Continuous Capacitance Measurements Reveals a Nonlinear Increase in Ca$^{2+}$ during Responses with Superlinear Release Properties
(A and B) Fluorescent image (A) of Ctbp2 binding peptide-labeled DBs (scale bar: 1 µm) with schematic in (B) showing regions selected for Ca$^{2+}$ imaging. (C–E) (C) shows stimulus above which the $I_{Ca}$ response elicits local changes in Ca$^{2+}$ (D) as well as capacitance changes (E). (F–H) An expansion of the plots from (C–E) in (F–H) focuses on the initial changes in Ca$^{2+}$ current (F), local Ca$^{2+}$ changes (G), and first component of capacitance (H). (I–K) A focus on the onset of the superlinear component again with the Ca$^{2+}$ current (I), local Ca$^{2+}$ signaling (J), and capacitance (K) signals.
trafficking. Also, although the second component of release appears to be superlinear when compared to the Ca\(^{2+}\) integral, indicating more release per Ca\(^{2+}\) for the second component, when compared to the Ca\(^{2+}\) fluorescent signal the opposite is true. By using fluorescence changes at the synapse, the ratio (Cap/Fluor) of the first component divided by the second component provided an indicator of relative efficiency of release and was 1.5 ± 0.4 (n = 3), indicating that release was more efficient at lower values of Ca\(^{2+}\).

**DISCUSSION**

Utilization of a two-sine technique for real-time tracking of vesicle fusion has allowed for more detailed investigation of presynaptic release components at the auditory hair cell-afferent fiber synapse. By using stimuli that did not elicit maximal I\(_{\text{Ca}}\), saturable pools were clearly identified, whereas variability between and within cells made this impossible (in turtle) with the single-sine technique (Schnee et al., 2005). A superlinear release component whose onset varied with Ca\(^{2+}\) load and correlated with release of an additional source of Ca\(^{2+}\) was also revealed. The superlinear component of release is postulated to reflect the ability of hair cells to rapidly recruit vesicles from regions distant from the synapse, which may underlie the inability to deplete vesicle pools and the ability of hair cell synapses to operate at high rates for sustained periods of time.

The first component of vesicle release had a pool size consistent with vesicles within the vicinity of the DB and thus did not require significant vesicle recruitment, though movement of vesicles to release sites on the plasma membrane would be required. The release rate and amplitude of the first component varied linearly with Ca\(^{2+}\) entry. Saturable vesicle pools could be observed within this first release component. The size of the saturable pool varied both with frequency position and Ca\(^{2+}\) load and could increase significantly if Ca\(^{2+}\) entry were slowed, suggesting that additional vesicles could be recruited to release sites faster than the existing pool of vesicles could be released. This vesicle pool may be as small as the vesicle population associated with the plasma membrane and DB to as large as all the vesicles associated with the DB (Figure 8A). This first component of release is very similar to what has been described for ribbon synapses of hair cells and other sensory cells (Beutner et al., 2001; Eisen et al., 2004; Moser and Beutner, 2000; Neef et al., 2007; Neves and Lagnado, 1999; Parsons et al., 1994; Schnee et al., 2005; Thoreson et al., 2004; von Gersdorff and Matthews, 1997).

The second, superlinear component represented a much larger pool of vesicles, requiring trafficking of distant vesicles to the synapse, likely equating to the reserve pool (Figure 8A). It also behaved as if there were a threshold Ca\(^{2+}\) load required for onset that was sensitive to factors affecting homeostasis, such as Ca\(^{2+}\) buffering and the rate of Ca\(^{2+}\) entry. Ca\(^{2+}\) imaging experiments suggest a correlation with release of an internal pool of Ca\(^{2+}\), though further studies are needed. Third, the release rate did not increase with Ca\(^{2+}\) load; rather, the onset time decreased. These results suggest the superlinear component is more a reflection of vesicle trafficking than vesicle fusion.

One possible mechanism for the increased yet insensitive release rate for the superlinear component is that release sites are not maximally filled at stimulus onset, in a similar manner to the DB being only 50% occupied (Lenzi et al., 1999; Schnee et al., 2005).
et al., 2005), but during stimulation the percentage of occupied release sites increases such that the measured release rate increases (vesicle trafficking is faster than vesicle fusion). This would explain the variability in size of depletable vesicle pool with stimulus intensity. The assumption is that the measured release rate is a reflection of the sum of filled and unfilled release sites and the machinery controlling release is operating maximally during stimulus conditions where Ca\(^{2+}\) at the release site is saturating. As more sites are filled, the measured release rate increases. When all release sites are full, the release rate will be constant, with the time to achieving this condition varying with Ca\(^{2+}\) load.

An alternative hypothesis is that additional synapses are recruited during the stimulation as the Ca\(^{2+}\) signal spreads. This seems unlikely, as synapses without Ca\(^{2+}\) channels have not been identified in mature hair cells (Frank et al., 2009; Issa and Hudspeth, 1996; Schnee et al., 2005).

Another possibility is that superlinear release does not represent synaptic vesicle fusion, but rather, endosomal fusion or fusion of vesicles at some distant site (Coggins et al., 2007; Zenisek et al., 2000). Direct tests of this possibility are lacking; however, the ability of afferent fibers to operate spontaneously at rates of more than 100 spikes/s and to sustain release in the face of stimulation at rates more than 400 spikes/s argue for the requirement of rapid vesicle replenishment (Liberman and Brown, 1986; Taberner and Liberman, 2005). The maximal release rate reported here for mammalian inner hair cells when the superlinear component is included is about 307 vesicles/s/synapse (assuming 15 synapses and 50 aF/vesicle)(Meyer et al., 2009), probably underestimating the release required to sustain these large firing rates. Prepulse experiments further illustrate that under more physiological stimulation conditions, release is linear and sustained; neither of these properties would occur without the superlinear component summing with the first release component. Finally, previous experiments have imaged vesicle release in mammalian hair cells at rates higher than reported here for superlinear component of release and also suggested trafficking must be rapid (Griesinger et al., 2005).

Data suggest that low-frequency cells release at faster rates per synapse than high-frequency cells, though the release rate per cell was similar for both components. In turtle, largely one fiber innervates one hair cell, but with multiple synapses it may be the overall release rate that is more significant than release per synapse, in contrast to the mammalian system in which one fiber innervates one synapse. The underlying mechanisms responsible for differences in release per synapse remain to be determined. In contrast, work in mammalian systems (Johnson et al., 2008) has shown a difference in the Ca\(^{2+}\) dependence of release. In turtle there was an apparent difference in Ca\(^{2+}\) dependence associated with the ability of low-frequency cells to recruit superlinear release with less Ca\(^{2+}\) than high-frequency cells. Comparable experiments are needed to test this in mammalian hair cells.

Our data are consistent with the existence of multiple vesicle pools, with the first linear saturable release component including both the RRP and recycling vesicle pools and the superlinear release component corresponding to the reserve pool (Figure 8A). Based on release measurements, we estimated vesicle pool sizes of 600 vesicles in the RRP (0.6%), 8000 vesicles in the recycling pool (7.4%), and 100,000 vesicles in the reserve pool (92%) (Figure 8A). These sizes are consistent with data from other synapse types (Rizzoli and Betz, 2005), the major difference being the ability of vesicles to be recruited for release from each pool. The rate and extent of Ca\(^{2+}\) entry appear to finely regulate the first two pools, while release of intracellular stored Ca\(^{2+}\) may be involved in recruitment of the reserve pool of vesicles. At other synapses recruitment of the reserve pool vesicles appears to be limited (Rizzoli and Betz, 2005).

Previous work developed a simple mass action model for vesicle release accounting for observed release properties (Schnee et al., 2005). No specific role for the DB was included and the model did not incorporate Ca\(^{2+}\) dependence of release or vesicle trafficking. Alone, this model cannot reproduce superlinear release. Modification of this simple model to include both first-order Ca\(^{2+}\)-dependent release and Ca\(^{2+}\)-dependent vesicle trafficking reproduced all of the basic release properties reported (Figures 8A–8D, see Supplemental Information for more detailed description). Simulations show both saturable linear release components and a superlinear release component of invariant rate (Figures 8B–8D). Saturating levels correspond well with anticipated pool sizes. Models that did not include Ca\(^{2+}\) dependence of vesicle trafficking could not reproduce the superlinear component of release unless higher order release functions were incorporated and even here the superlinear component did not correspond well with available vesicles (data not shown). The model varied from physiological measurements in that the separation between vesicle pools was more sharply defined, probably reflecting the artificial nature of threshold Ca\(^{2+}\) levels to recruit vesicle pools. Perhaps vesicle trafficking is uniformly Ca\(^{2+}\) dependent and the recruitment depends on the location of vesicles with respect to Ca\(^{2+}\) influx, with the Ca\(^{2+}\) gradient into the cell dictating the pool size and rate of movement more than the location or specialization of the vesicle. This possibility is consistent with data demonstrating that vesicle movements are similar between different regions of the cell (Zenisek et al., 2000), but is unusual in that it suggests vesicles are tethered in some manner, whether directly associated with the ribbon or not. It is in contrast with arguments that vesicle movement is diffusion based (Holt et al., 2004; LoGiudice and Matthews, 2009), unless diffusion can be regulated via Ca\(^{2+}\) levels, but is consistent with recent cryo-electron tomography arguing that all vesicles are tethered by the cytoskeleton (Fernández-Busnadiego et al., 2010). Perhaps the differences in release properties between ribbon synapses in the visual and auditory system, mainly being that release in hair cells is much less defatigable, have more to do with trafficking than with mechanisms of release. Hair cells are required to maintain continual and rapid release in order to maintain high spontaneous activity in the afferent fiber but this is much less of a requirement in the visual system.

Afferent fibers show a pronounced neural adaptation in which firing rates can be reduced by more than 50% during the initial phase of stimulation (Liberman and Brown, 1986). Data presented here may provide insight into possible mechanisms by which this may happen. We suggest the initial decrease in firing represents the time when vesicles are being released faster than...
replenished, so that vesicle trafficking is not maximized. That firing rates do not adapt to zero but rather to a relatively high rate indicates that trafficking (superlinear component) is rapidly accessible under physiological conditions. Similar to the response described in Figure 5, under physiological conditions the processes tend to merge but vesicle release shows a reduction in slope initially that becomes sustained. The level of neural adaptation may in part be determined by how rapidly each synapse is capable of recruiting vesicles between pools—the faster the recruitment, the less adaptation is observed. In fact, it may be argued that steady-state firing requires recruitment of vesicles such that the rate of release at any given synapse may be dictated by access to the reserve pool of vesicles. Thus it may be that spontaneous firing rates are regulated by resting calcium currents and vesicles in the RRP and recycling pool, while stimulated release is more dependent upon vesicle recruitment from the reserve pool and the ability to modulate release of stored calcium (Guth et al., 1991).

In summary, we used real-time capacitance measurements to identify saturable pools of vesicles and discovered a superlinear release component requiring recruitment of vesicles to release sites. We suggest that Ca2+-dependent vesicle trafficking is responsible for this movement, which is required for hair cell synapses to maintain high rates of sustained vesicle fusion. We postulate that the superlinear release component reflects synapses operating at maximal rates of release and trafficking and that release of an as yet undefined internal pool of Ca2+ may be required. These characteristics of synaptic vesicle recruitment and release make hair cell ribbon synapses quite unique as compared to other synapses.

EXPERIMENTAL PROCEDURES

Tissue Preparation

The auditory papilla from red-eared sliders (Trachemys scripta elegans) were prepared as previously described (Schnee et al., 2005) by using methods approved by the IACUC committee at Stanford University and following standards established by NIH guidelines. Tectoral membranes were removed as previously described by using a hypertonic and hypercalcemic (10 mM Ca2+) solution (Farris et al., 2006). The external recording solution contained 125 mM NaCl, 0.5 mM KCl, 2.8 mM CaCl2, 2.2 mM MgCl2, 2 mM pyruvate, 2 mM creatine, 2 mM ascorbate, 6 mM glucose, and 10 mM N-(2-hydroxyethyl)piperazine-N’-(2-ethanesulfonic acid) (HEPES) with pH adjusted to 7.6 and osmolality maintained at 275 mosmol/kg. One hundred nanometers epinephrine was included in the external solution to block SK potassium currents (Tucker and Fettiplace, 1996). Cells were imaged with a BX51 fixed-stage upright microscope (Olympus) with bright-field optics. Conventional epifluorescence was used for the Ca2+ imaging.

Cochlea from rat pups (ages P7–P10) were dissected into an external solution containing 135 mM NaCl, 5.8 mM KCl, 1.3 mM CaCl2, 0.9 mM MgCl2, 0.7 mM NaH2PO4, 10 mM HEPES, 5.6 mM glucose, 2 mM pyruvate, and 2 mM creatine. Both stria and spiral ganglia were peeled from the organ of Corti and the remaining epithelium was placed into a glass-bottomed recording chamber. The tectoral membrane was removed and the organ of Corti held in place with single strands of dental floss. Apamin was included at 100 nM to block small conductance calcium-activated potassium currents.

Electrophysiology

Fire-polished borosilicate patch electrodes of resistance 3–5 MΩ were used for all recordings. Unless otherwise stated, the internal solution for turtle contained 110 mM CsCl, 5 mM MgATP, 5 mM creatine phosphate, 1 mM ethylene glycol-bis (N,N,N’,N’-tetraacetic acid (EGTA), 10 mM HEPES, 2 mM ascorbate (pH 7.2). Osmolality was maintained at 255 mosmols by adjusting CsCl levels; pH was 7.2. For perforated-patch recordings the internal solution contained 110 mM Cs aspartate, 15 mM CsCl, 3 mM NaATP, 3 mM MgCl2, 1 mM BaATP, and 10 mM HEPES. Amphotericin dissolved in dry DMSO was used as the perforating agent. In several experiments Alexa 488 was included in the recoding pipette to verify the whole-cell mode was not obtained. For rat hair cells the internal solution contained 90 mM Cs methanesulfonate, 20 mM TEA, 1 mM EGTA, 5 mM MgATP, 5 mM creatine phosphate, 3 mM ascorbate, 3 mM MgCl2, and 10 mM HEPES. Stimulus protocols were applied 10 min after achieving whole-cell mode to allow equilibration of internal solution and run up of ICa (Schnee and Ricci, 2003). Hair cells were voltage clamped with a lock-in amplifier (Caim) allowing for capacitance measurements as initially described by Neher and Marty (1982) and later used for hair cell recordings (Johnson et al., 2002; Schnee et al., 2005). A ± 40 mV sine wave at 1.5 kHz was imposed onto the membrane holding potential, blanked during depolarization that elicited ICa, and resumed so that capacitance measurements before and after stimulus were obtained. Capacitance data were amplified and filtered at 100 Hz offline. This amplifier was also used initially for validation of the two-sine wave method (see below). The multi-clamp amplifier (Axon Instruments) was also used for capacitance measurements. All data were sampled with a Daq/3000 (IOTech) driven by jClamp software (Scisoft).

Two-Sine Method

Vesicle release was determined by measuring membrane capacitance correlates of surface area change. Capacitance was measured with a dual sinusoidal, FFT-based method (Santos-Sacchi, 2004; Santos-Sacchi et al., 1998) relying on component solutions of a simple model of the patched cell (electrode resistance, Rs, in series with a parallel combination of membrane capacitance, Cm, and membrane resistance, Rm; see Figure 1 in Santos-Sacchi, 2004). In this RC analysis method, two voltage frequencies, f1 and f2 (twice the f1 frequency) are summed and the real and imaginary components of the current response are used to determine the magnitudes of the three model components. The time resolution of the Cm measurement is the period of f1, which we varied from 5.12 to 0.32 ms, corresponding to 195 to 3125 Hz. Comparisons between single-sine and dual-sine-wave methods showed no differences for membrane responses to 100 ms depolarizations to −20 mV. Single-sine values of 90 ± 15 fF (n = 12) were obtained as compared to 110 ± 24 fF for dual-sine wave measurements from the same cell population. The same two-sine wave technique (implemented in jClamp) has been used previously, but only as a before and after measurement which gave similar results to the single-sine method (Edmonds et al., 2004; Thoreson et al., 2004). Details of methods and control data are presented in Figures S1–S3. The time between stimuli was varied based on the previous stimulus but was never less than 2 min and typically varied between 5 and 10 min to ensure appropriate time for reaching equilibrium.

Calcium Imaging

Swept-field confocal high-speed (SFC) calcium imaging was performed as previously described (Beurg et al., 2009). The SFC (Prairie Technologies, Middleton WI) was coupled to a Redshirt camera. Fluor 4ff was used as the indicator, chosen to both limit effects on release properties and serve to localize the calcium source. Images were captured at 125 fps with a 35 mm silt. A 100 x dipping lens with an added 1.25 magnification gave a final pixel size of ~350 nm. Ribbons were identified by using the Ctbp2 peptide tagged with rhodamine (Zenisek et al., 2003). Data was analyzed by selecting 3 × 3 pixel regions uniformly encompassing Ctbp2-labeled regions. Image planes were selected to help isolate individual synapses to ensure individual synapses were being investigated.

Data Analysis

Data were included based on several parameters. Leak currents needed to be less than 50 pA at ~85 mV and series resistance (uncompensated) needed to be stable and below 15 MΩ. As electrode capacitive compensation is critical for the accurate use of the two-sine wave methodology, bath height and electrode filling were kept low to limit stray capacitance. Unless otherwise stated, data are presented as mean ± standard deviation with number of samples (n)
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given. Where appropriate Student’s t tests, two-tailed, were performed to assess significance; p values and correlation coefficients ($r^2$) are listed with data.

Immunofluorescence and Quantification of Synapse Number
Isolated papillae were incubated for 1 hr at 4°C in external solution containing 4% formaldehyde and 0.1% Triton X-100. After washing four times for 10 min at room temperature in external solution containing 0.1% Triton X-100, papillae were incubated for 30 min in the same solution supplemented with 5% bovine serum albumin. Specimens were then incubated with primary anti-sera diluted at 1:250 to 1:500 in the same solution overnight at 4°C. Antiseria included those against Ctbp2 (rabbit polyclonal, #1869), Ribeye (rabbit polyclonal, #1848), and PSD-95 (mouse monoclonal, Abcam #2723, concentrated at 3.3 μg/ml with UPSPA-Protein Concentrate Kit, #662120, Calbiochem). After washing five times for 10 min at room temperature, samples were incubated with FITC or TRITC-conjugated secondary antibodies (Invitrogen, Carlsbad, CA) at a dilution of 1:500. Where mentioned, FITC-conjugated phalloidin (#77415, Sigma) was added during the incubation with the secondary antibodies. After washing five times for 10 min at room temperature, specimens were mounted between two coverslips with Vectashield (H1200, Vector Laboratories, Burlingame, CA) and images were acquired with a Zeiss LSM5 Exciter confocal microscope with a 63 x 1.4 NA oil-immersion objective or with a spinning disc confocal by using 100 x 1.4 NA oil-immersion lens. Background noise and contrast enhancement were adjusted with Velocity software [Improvision]. Confoocal Z-stacks taken with 0.1 μm steps were analyzed by using Velocity software. Ribeye- and PSD95-positive fluorescent objects with 25%–100% intensity were identified on independent channels. Intersecting objects were subsequently selected as potential synaptic ribbons (Figure S5A) and manually confirmed by using the point tool of Velocity (Figure S5B). When two synaptic ribbons were in close proximity, objects were analyzed in the XZ-YZ planes (Figures S5C and S5D) and line intensity profiles were performed (Figure S5E) to identify individual synaptic terminals. The number of peaks, typically one, identified the number of synapses (see Movie S1 and Figure S5).

SUPPLEMENTAL INFORMATION
Supplemental Information includes six figures, Supplemental Experimental Procedures, one table, and one movie and can be found with this article online at doi:10.1016/j.neuron.2011.01.031.

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REFERENCES


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