Release from the cone ribbon synapse under bright light conditions can be controlled by the opening of only a few Ca\textsuperscript{2+} channels

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Bartoletti TM, Jackman SL, Babai N, Mercer AJ, Kramer RH, Thoreson WB. Release from the cone ribbon synapse under bright light conditions can be controlled by the opening of only a few Ca\textsuperscript{2+} channels. J Neurophysiol 106: 2922–2935, 2011. First published August 31, 2011; doi:10.1152/jn.00634.2011.—Light hyperpolarizes cone photoreceptors, causing synaptic voltage-gated Ca\textsuperscript{2+} channels to open infrequently. To understand neurotransmission under these conditions, we determined the number of L-type Ca\textsuperscript{2+} channel openings necessary for vesicle fusion at the cone ribbon synapse. Ca\textsuperscript{2+} currents (I\textsubscript{Ca}) were activated in voltage-clamped cones, and excitatory post-synaptic currents (EPSCs) were recorded from horizontal cells in the salamander retina slice preparation. Ca\textsuperscript{2+} channel number and single-channel current amplitude were calculated by mean-variance analysis of I\textsubscript{Ca}. Two different comparisons—one comparing average numbers of release events to average I\textsubscript{Ca} amplitude and the other involving deconvolution of both EPSCs and simultaneously recorded cone I\textsubscript{Ca}—suggested that fewer than three Ca\textsuperscript{2+} channel openings accompanied fusion of each vesicle at the peak of release during the first few milliseconds of stimulation. Opening fewer Ca\textsuperscript{2+} channels did not enhance fusion efficiency, suggesting that few unnecessary channel openings occurred during strong depolarization. We simulated release at the cone synapse, using empirically determined synaptic dimensions, vesicle pool size, Ca\textsuperscript{2+} dependence of release, Ca\textsuperscript{2+} channel number, and Ca\textsuperscript{2+} channel properties. The model replicated observations when a barrier was added to slow Ca\textsuperscript{2+} diffusion. Consistent with the presence of a diffusion barrier, dialyzing cones with diffusible Ca\textsuperscript{2+} buffers did not affect release efficiency. The tight clustering of Ca\textsuperscript{2+} channels, along with a high-Ca\textsuperscript{2+} affinity mechanism and diffusion barrier, promotes a linear coupling between the opening of Ca\textsuperscript{2+} channels and vesicle fusion events.

To better understand how exocytosis is controlled in the photoreceptor terminal, we determined the number of Ca\textsuperscript{2+} channel openings required to trigger release of a single vesicle. Previous studies at the neuromuscular junction (Shahrezaei et al. 2006), mammalian rod bipolar cell ribbon synapse (Jarsky et al. 2010), and calyeal synapse in the ciliary ganglion (Stanley 1993) indicate that a single Ca\textsuperscript{2+} channel opening can be sufficient to stimulate release. Several coincident Ca\textsuperscript{2+} channel openings are needed to stimulate vesicle fusion at the squid giant synapse (Augustine et al. 1991), GABAergic basket cell-granule cell synapse (Bucurenciu et al. 2010), mature calyx of Held (Fedchyshyn and Wang 2005), and hair cell ribbon synapse (Brandt et al. 2005). An even larger number of coincident channel openings (>5) are required for fusion at CA3–CA1 hippocampal synapses (Wheel er et al. 1994), goldfish bipolar cell (Coggin and Zenisek 2009; von Gersdorff et al. 1998), granule cell-Purkinje cell synapses (Mintz et al. 1995), immature calyx of Held (Fedchyshyn and Wang 2005), and adrenal chromafmin cells (Wu et al. 2009). The number of Ca\textsuperscript{2+} channel openings required for release at a given synapse depends on both the molecular makeup of the release machinery and the architecture of the synapse, including the distance between Ca\textsuperscript{2+} channels and vesicles.

Photoreceptors have evolved specific mechanisms that allow them to efficiently transmit graded signals about light intensity. For example, release from cones occurs principally at the synaptic ribbon (Snellman et al. 2011). In photoreceptors, the ribbon is a platelike, electron-dense structure that appears to tether the releasable pool of vesicles (Bartoletti et al. 2010; Gray and Pease 1971; Heidelberger et al. 2005; Lasansky 1973; Ravola and Gilula 1975; Usukura and Yamada 1987). The ribbon is anchored to the membrane by a troughlike structure lying at its base known as the arciform density. Ribbons in other sensory neurons, including retinal bipolar cells and hair cells, generally lack an arciform density (reviewed by Heidelberger et al. 2005; LoGuidice and Matthews...
Another unusual property of photoreceptors is the relatively depolarized resting membrane potential in darkness (approximately ~40 mV). This depolarized membrane potential keeps a fraction of the voltage-gated Ca\textsuperscript{2+} channels tonically open and raises the Ca\textsuperscript{2+} concentration at the base of the ribbon to ~1 μM (Choi et al. 2008). As a consequence, the readily releasable pool of synaptic vesicles tethered at the bottom of the ribbon is depleted in darkness (Jackman et al. 2009). Under these conditions, the rate of release is limited by the rate of vesicle replenishment and not by the rate of Ca\textsuperscript{2+}-dependent fusion. However, because vesicle replenishment itself is accelerated by raising Ca\textsuperscript{2+}, fluctuations in intracellular Ca\textsuperscript{2+} levels can still exert control over the release rate in darkness (Babai et al. 2010). The protein that speeds replenishment is unknown, but Ca\textsuperscript{2+}-sensitive sites of replenishment are located >200 nm from the base of the ribbon, too far to sense the “plume” of Ca\textsuperscript{2+} entering through an individual Ca\textsuperscript{2+} channel (Babai et al. 2010).

In bright light, cones are hyperpolarized and voltage-gated Ca\textsuperscript{2+} channels are closed, allowing the releasable pool of vesicles at the base of the ribbon to be replenished. Under these conditions, vesicles anchored at the base of the ribbon close to voltage-gated Ca\textsuperscript{2+} channels could fuse in response to individual channel openings. To investigate the coupling between Ca\textsuperscript{2+} channel opening and vesicle fusion at membrane potentials representative of the transition from light to dark, we carried out voltage-clamp experiments and computer simulations. Our results indicate that in bright light there is a close coupling between Ca\textsuperscript{2+} channel opening and vesicle fusion so that the opening of only two or three Ca\textsuperscript{2+} channels is needed to trigger release. This relationship appears to be partly ensured by diffusion barriers that restrict the spread of Ca\textsuperscript{2+} away from open Ca\textsuperscript{2+} channels. Restricting the spread of Ca\textsuperscript{2+} prevents promiscuous fusion of multiple vesicles by a single channel opening. The close coupling between Ca\textsuperscript{2+} channel opening and vesicle fusion in bright light contrasts with release in darkness, which involves synaptic ribbon-mediated replenishment regulated by changes in cytoplasmic Ca\textsuperscript{2+} levels in the plasma membrane Ca\textsuperscript{2+} channels (Babai et al. 2010; Jackman et al. 2009). The tight coupling of Ca\textsuperscript{2+} channel opening and fusion events in bright light conditions ensures precise timing of release, crucial for synchronizing synaptic signaling to decrements in light intensity.

MATERIALS AND METHODS

Retinal Slice Preparation

Male and female aquatic tiger salamanders (Ambystoma tigrinum, Kons Scientific, Germantown, WI and Charles Sullivan, Nashville, TN) 18–25 cm in length were handled humanely according to protocols approved by the Institutional Animal Care and Use Committee at the University of Nebraska Medical Center. Salamanders were maintained on a 12:12-h day-night cycle and were killed 1–2 h after the beginning of subjective night by decapitation with heavy shears followed by immediate pithing.

Retinal slices were prepared as described previously (Bartoletti et al. 2010; Thoreson et al. 1997). Briefly, retina was isolated under an amphibian saline solution on 5 × 10-mm pieces of nitrocellulose filter paper (type AAWP, 0.8-μm pores; Millipore, Billerica, MA). The retina was then cut into 125-μm-wide slices with a razor blade tissue chopper (Stoelting, Wood Dale, IL). Retinal slices were rotated 90° to see the retinal layers on an upright fixed-stage microscope (Olympus BHWI or Nikon E600FN, Tokyo, Japan) using a water immersion objective (×40, 0.7 numerical aperture (NA) or ×60, 1.0 NA).

Electrophysiology

Cones were voltage clamped simultaneously with adjacent postsynaptic horizontal cells with a Multiclamp 700A amplifier (Molecular Devices, Sunnyvale, CA). Some experiments were performed with an Otopatch (Cairn, Faversham, UK) amplifier for cones and Axopatch 200B (Molecular Devices) for second-order neurons. Cones and horizontal cells were identified by their morphology and response characteristics (Thoreson et al. 1997). Both recording pipettes were positioned with Huxley-Wall micromanipulators (Sutter Instruments, Novato, CA) and visualized through the eyepieces or with a video camera (Watec 502H, Orangeburg, NY) mounted on the microscope. Currents were low-pass filtered at 2 kHz and acquired at 0.1-ms intervals with a Digidata 1322 interface and pCLAMP 9.2 or 10.2 software (Molecular Devices). Acceptable access resistances for voltage clamp recordings were <50 MΩ. Photoreceptors were voltage clamped at ~70 mV and horizontal cells at ~60 mV.

Patch pipettes were pulled on a PP-830 vertical puller (Narishige USA, East Meadow, NY) from borosilicate glass pipettes (1.2-mm OD, 0.9-mm ID, with internal filament; World Precision Instruments, Sarasota, FL) and had tips of ~1-μm OD with resistance values of 10–17 MΩ.

For variance-mean analysis of single-channel current amplitudes, we applied 100 test pulses (2 ms) from ~70 to +50 mV. Currents were filtered at 5 kHz, and access resistance was compensated 80–90% with the Axopatch 200B amplifier. Retinal slices were superfused with a solution containing (in mM) 111 NaCl, 2.5 KCl, 2 BaCl\textsubscript{2}, 0.5 MgCl\textsubscript{2}, 10 N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), 5 glucose, and 10 TEACl with 100 μM niflumic acid and 5 μM BayK8644 (pH 7.8). The pipette solution contained (in mM) 42 CsCl, 48 Cs gluconate, 1.9 MgCl\textsubscript{2}, 32.9 HEPES, 9.4 TEACl, 9.4 MgATP, 0.5 GTP, and 5 BAIPA (pH 7.2). Most studies find that BayK8644 enhances the open probability of L-type Ca\textsuperscript{2+} channels with no effect on conductance (Brown et al. 1984; Hess et al. 1984; Kokubun and Reuter 1984; McDonald et al. 1994). Use of short test pulses, niflumic acid, TEA, BAIPA, and Ba\textsuperscript{2+} minimized activation of Ca\textsuperscript{2+}-activated Cl\textsuperscript{−} and K\textsuperscript{+} currents. Trials were excluded if baseline currents changed significantly during the repetitive application of test pulses. We calculated the variance between subsequent pairs of trials to minimize effects of rundown or potentiation of the current. To measure current amplitude, we subtracted passive and capacitative currents, using a P/200 protocol in which we summed two trials involving 100 test pulses of 1.2-mV amplitude recorded immediately before and after the test pulse series. The relationship between mean tail current amplitude and intertrial variance was determined at each time point and fit with a parabolic function:

\[
V = iI - \bar{I}/N + A
\]  

(1)

In this equation, \(i\) = mean current amplitude, \(iI\) = single-channel current amplitude, \(N\) = channel number, \(A\) = offset, and \(V\) = variance.

For deconvolution experiments, we obtained paired simultaneous whole cell recordings from cones and postsynaptic horizontal cells. The presynaptic recording pipette was filled with a solution containing (in mM) 40 Cs glutamate, 50 Cs gluconate, 9.4 TEACl, 3.5 NaCl, 1 CaCl\textsubscript{2}, 1 MgCl\textsubscript{2}, 9.4 MgATP, 0.5 GTP, 5 EGTA, and 10 HEPES (pH 7.2). Postsynaptic pipettes were filled with a solution containing (in mM) 48 Cs gluconate, 42 CsCl, 9.4 TEACl, 1.9 MgCl\textsubscript{2}, 9.4 MgATP, 0.5 GTP, 5 EGTA, and 32.9 HEPES (pH 7.2). The osmolarity of pipette solutions was adjusted, if necessary, to ~240 mosM. The liquid junction potential (LJP) was estimated from the calculator in pCLAMP 9.2 to be ~13 mV for the cone pipette solution and ~16

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mV for the horizontal cell pipette solution. Retinal slices were superfused with a solution containing (in mM) 116 NaCl, 2.5 KCl, 1.8 CaCl$_2$, 0.5 MgCl$_2$, 10 HEPES, and 5 glucose (pH 7.8). Use of HEPES as a pH buffer limited effects of proton feedback (DeVries 2001; Hirasawa and Kaneko 2003; Hosoi et al. 2005). The osmolarity measured with a vapor pressure osmometer (Wescor, Logan, UT) was 242 ± 5 mosM. Solutions were bubbled continuously with 100% O$_2$.

Deconvolution was performed with OriginPro 8 software (Northampton, MA). For deconvolution, we used P/2 leak-subtracted I$_{Ca}$ instead of the P/200 leak subtraction used for variance-mean experiments. To deconvolve single-channel events during I$_{Ca}$ evoked by a step to −10 mV, we used a single Ca$^{2+}$ channel probability density function with a peak amplitude of 0.31 pA and a decay time constant of 1.1 ms to represent variable channel open times. The channel open time constant of 1.1 ms was derived from single-channel measurements in photoreceptors (Thoreson et al. 2000) and is similar to open times for L-type Ca$^{2+}$ channels in many other preparations (Fenwick et al. 1982; Fox et al. 1987; Hagiwara and Ohmori 1983; Lux and Brown 1984; Zampini et al. 2006). Single-channel current amplitude was determined from results of variance-mean experiments described below and is similar to single-channel currents measured directly from individual L-type Ca$^{2+}$ channels in other preparations with physiological Ca$^{2+}$ levels (Church and Stanley 1996; Rodriguez-Contreras et al. 1982; Fox et al. 1987; Hagiwara and Ohmori 1983; Lux and Brown 1984; Zampini et al. 2006). Near-identical results were obtained by deconvolving I$_{Ca}$ with a square function with amplitude of 0.31 pA and duration of 1.1 ms (not shown). For steps to −30 mV, we used a mean amplitude of 0.37 pA to account for the greater driving force. For experiments using a ramp voltage protocol (−90 to +60 mV, 0.5 mV/ms), we adjusted single-channel amplitude for driving force throughout the ramp. The number of channel openings was scaled by an average of 13 ribbons per cone (Bartoletti et al. 2010; Pang et al. 2008) to estimate the number of openings per ribbon.

EPSCs with peak amplitude >50 pA were deconvolved with an empirically determined average miniature EPSC (mEPSC) waveform (Bartoletti et al. 2010; Cadetti et al. 2008). The mEPSC waveform averaged from 16 horizontal cells had an amplitude of 5.7 pA and a total charge transfer of 15.5 pC. It had relatively slow kinetics, as expected from the large volume of the photoreceptor synaptic cleft (10–90% rise time of 780 μs and decay time constant of 2.4 ms; Cadetti et al. 2008). A single cone can contact a single horizontal cell at more than one ribbon, and paired cone-horizontal cell recordings showed that each ribbon contributes ~46 pA to the peak amplitude of the PSC evoked by a step to −10 mV (Bartoletti et al. 2010). The number of ribbon contacts was therefore calculated by dividing the peak amplitude of the EPSC evoked by a step to −10 mV by 46 pA/ribbon (Bartoletti et al. 2010). Release events were shifted forward by 300 μs to compensate for the latency from the rise in Ca$^{2+}$ concentration to the beginning of the EPSC (Duncan et al. 2010; LISMAN et al. 2007). This was the latency to the beginning of a detectable PSC (i.e., the time when the first molecules of glutamate reach postsynaptic glutamate receptors) measured after instantaneous elevation of Ca$^{2+}$ by flash photolysis of caged Ca$^{2+}$ at the cone ribbon synapse (Duncan et al. 2010). The additional spread of glutamate through the volume of the synaptic cleft was captured by the mEPSC waveform. Results of deconvolution were low-pass filtered with a fast Fourier transform filter with 5–10 points of smoothing. The rate of Ca$^{2+}$ channel openings was divided by the rate of release events to calculate the number of vesicle fusion events per channel opening at each time point.

Vesicle release was modeled with Mathematica (Wolfram Research, Champaign, IL). The immediately releasable pool of 20 vesicles was arranged in a square grid with 50-mm center-to-center spacing on the surface of a 250-mm-long ribbon in two rows of 5 vesicles on either side. Fifty-six Ca$^{2+}$ channels were placed randomly on the plasma membrane evagination, an arc with 100-mm radius centered at the ribbon apex. A simulated step to −10 mV opened Ca$^{2+}$ channels to a maximal open probability of 0.35, opening in time according to the equation $P_o = 0.35 \times \exp(t + 5 \text{ ms})$. Randomly chosen Ca$^{2+}$ channels opened for 1.1 ms, supporting a local Ca$^{2+}$ gradient as described by the linear approximation of nanodomains created by a point source of Ca$^{2+}$ in the presence of a diffusible buffer (NEHER 1998). Time-dependent Ca$^{2+}$ gradients in the presence of a diffusion barrier (Ait-Haddou et al. 2010) were modeled in increments of 100 μs, with release binned into 1.1-ms intervals to reflect the opening of Ca$^{2+}$ channels. The domains of all open channels were summed linearly. The Ca$^{2+}$ dependence of release was $R = 3.634 \times [\text{Ca}^{2+}]^{-1} (2 \mu M + [\text{Ca}^{2+}]^{-2})$ (Duncan et al. 2010), except when modeling release with the bipolar cell Ca$^{2+}$ dependence (Heidelberger et al. 1994). The $K_d$ for BAPTA and EGTA was 170 mM, with $K_{app}$ for BAPTA of 6 × 10$^{-6}$ M/s and $K_{app}$ for EGTA of 9 × 10$^{-6}$ M/s (Burrone et al. 2002). The diffusion coefficient for Ca$^{2+}$ was 220 μm$^2$/s, and the diffusion coefficient for Ca$^{2+}$ buffers was 20 μm$^2$/s. Each simulation was repeated for 1,000 trials.

Unless otherwise stated, chemicals were obtained from Sigma-Aldrich (St. Louis, MO). The criterion for statistical significance was chosen to be $P < 0.05$ and evaluated with GraphPad Prism 4.0. Variability is reported as ±SE.

RESULTS

Variance-Mean Analysis of I$_{Ca}$

Changes in the variance and mean amplitude of I$_{Ca}$ can be related to one another by a parabolic function involving single-channel number and amplitude (Eq. 1). We determined trial-to-trial variance and mean amplitude of I$_{Ba}$ tail currents recorded in the presence of 5 mM BayK8644. I$_{Ba}$ was activated by a series of 100 brief (2 ms) test steps from −70 to +50 mV, and passive membrane properties were subtracted with a P/200 protocol (Fig. 1A). The relationship between mean and intertrial variance at different time points was fit with Eq. 1. In the example shown in Fig. 1B, the best fit parabolic function to the variance-mean relationship indicated that the tail current resulted from 1,164 ± 100 channels with a single-channel current averaging −1.29 ± 0.10 pA. On average, the results showed the presence of 1,034 ± 93 (n = 23) Ca$^{2+}$ channels per cone with a single-channel current of 0.70 ± 0.08 pA. Given 13 ribbons per salamander cone (Bartoletti et al. 2010; Pang et al. 2008), this suggests up to 80 Ca$^{2+}$ channels per ribbon if every channel is located at the ribbon. If, as suggested for hair cells (Brandt et al. 2005; Zampini et al. 2010), only 70% of channels are at the ribbon, ~56 Ca$^{2+}$ channels will be located beneath each ribbon.

To estimate the open probability attained in control conditions when I$_{Ca}$ was fully activated, I$_{Ba}$ was measured with a ramp voltage protocol (−90 to +60 mV, 300 ms) without BayK8644. From the ohmic change in I$_{Ba}$ between 0 and +20 mV, we extrapolated a reversal potential of +89 mV. With a reversal potential of +89 mV, the single-channel amplitude of 0.7 pA measured at −70 mV would be predicted to diminish to 0.44 pA at −10 mV. The amplitude of whole cell I$_{Ba}$ measured at −10 mV averaged 162.6 ± 6.6 pA (n = 5), suggesting that 370 of the total of 1,034 Ca$^{2+}$ channels were open when I$_{Ba}$ was fully activated, yielding a peak mean open probability of 0.36. This is similar to the value found by single-channel measurements of L-type channels in chick hair cells from the semicircular canal (Zampini et al. 2006).

Application of a depolarizing step to −10 mV was found to stimulate release of the rapidly releasable pool of ~20 vesicles (range 15–24; Bartoletti et al. 2010) with a time constant of 2.7
ms (Rabl et al. 2005). This suggests that 12.6 (9.5–15) vesicles (63%) fuse within that brief period. Each Ca2+ channel has a 36% chance of opening and remains open for an average of 1.1 ms (Thoreson et al. 2000). If every channel is located at the ribbon, then 68 Ca2+ channel openings should occur at each ribbon within 2.7 ms (0.36 × 77 × 2.7/1.1). This in turn implies ~5.4 (4.5–7) channel openings per vesicle fusion event. If only 70% of the channels are found at the ribbon, then this suggests that ~3.8 (3.2–4.9) channel openings per vesicle fusion event should occur during the first 2.7 ms.

For variance-mean experiments, we used Ba2+ as the charge carrier to minimize activation of Ca2+-activated K+ and Cl− channels. For deconvolution experiments described in the next section, we used 1.8 mM Ca2+ as the charge carrier. We scaled single-channel Ba2+ currents for these Ca2+ levels by comparing the amplitude of the whole cell current measured with 2 mM Ba2+ as the charge carrier ($I_{\text{Ba}^2+}$: 162.6 ± 6.6 pA) to the amplitude measured with 1.8 mM Ca2+ as the charge carrier ($I_{\text{Ca}^2+}$: 116 ± 6.5 pA, $n = 60$). The smaller current amplitude observed with 1.8 mM Ca2+ suggests a single-channel amplitude of 0.31 pA at −10 mV, close to the amplitude of single-channel currents measured with physiological Ca2+ levels in L-type channels from other preparations (Church and Stanley 1996; Gollasch et al. 1992; Rodriguez-Contreras et al. 2002).

**Simultaneous Measurements of $I_{Ca}$ and Synaptic Release**

The comparisons described above suggested that an average of 3–5 Ca2+ channel openings accompanied each vesicle fusion event during the first few milliseconds of release. However, fewer channel openings may be needed to stimulate fusion at the instant of peak release efficiency. We therefore compared the numbers of presynaptic Ca2+ channel openings and postsynaptic vesicle fusion events in individual pairs of cells by deconvolving individual channel openings from cone $I_{Ca}$ and individual quantal release events from horizontal cell EPSCs. To stimulate release of the entire rapidly releasable pool, we applied a strong step depolarization to the voltage-clamped cone (~70 mV to −10 mV, 100 ms). For these experiments, cone $I_{Ca}$ was leak subtracted with a P/100 protocol. Ca2+ channel opening rates were deconvolved from $I_{Ca}$ by assuming a single-channel current amplitude of 0.31 pA and τ_{open} = 1.1 ms (Thoreson et al. 2000) and scaling for 13 ribbons/cone (Bartoletti et al. 2010; Pang et al. 2008) (Fig. 2). Vesicle fusion rates were deconvolved from the simultaneously recorded EPSC with a waveform averaged from mEPSCs recorded in 16 horizontal cells (Cadetti et al. 2008). The number of ribbon contacts at each cone-horizontal cell pair was estimated from the size of the EPSC by assuming that each ribbon contributed 46 ± 23 pA to the peak amplitude of the EPSC (Bartoletti et al. 2010). Finally, the rate of fusion at each ribbon (Fig. 2B) was divided by the rate of Ca2+ channel openings per ribbon (Fig. 2B) to give the number of vesicles per channel opening (0.3 at the peak of release in Fig. 2C). Release and release efficiency quickly rose to a peak and then declined. Peak release efficiency (Fig. 2C) was attained 5.27 ± 0.54 ms ($n = 18$) after the beginning of the test step, before the actual peak of the PSC (Fig. 2A) that was attained at 9.78 ± 0.64 ms. The peak efficiency averaged 0.33 ± 0.040 ($n = 18$) vesicle fusion events per channel opening. The reciprocal value of 3.0 provides the number of channel openings per fusion event. If only 70% of the channels reside at the ribbon, then this suggests a peak efficiency of 2.1 channel openings per fusion event. As expected, the peak efficiency exceeded the average efficiency over the first few milliseconds of release.

Application of a depolarizing test step rapidly activated $I_{Ca}$ and thus generated fast EPSCs in horizontal cells. We were concerned about the possibility that small kinetic differences between the rise times for $I_{Ca}$ and EPSCs might have a significant impact on calculations of release efficiency. To lessen the possibility of kinetic differences, we activated $I_{Ca}$ more slowly by using voltage ramps (0.5 mV/ms, −90 mV to +60 mV). Ramp-evoked EPSCs were smaller in amplitude but broader than step-evoked EPSCs. $I_{Ca}$ and EPSC from one cell pair are overlaid in Fig. 3A. The number of fusion events per channel opening is shown in Fig. 3B. By examining small $I_{Ca}$ evoked during stimulation with voltage ramps, Jarosy et al. (2010) showed that single Ca2+ channel openings were capable of driving fusion. Deconvolution of ramp-evoked responses at the cone synapse sometimes suggested apparent release efficiency exceeding one vesicle per channel opening. However, abrupt increases in the apparent efficiency were also sometimes
observed even without stimulation as a result of small baseline fluctuations in the cone membrane current and the occurrence of spontaneous mEPSCs derived from release by neighboring unclamped photoreceptors. Similar abrupt jumps in efficiency were sometimes seen at strongly hyperpolarized potentials during the voltage ramp, but efficiency typically rose steadily with increasing membrane depolarization during the ramp (Fig. 3). The peak efficiency was attained when the cone membrane potential was between −30 and −40 mV (uncorrected for LJP), well below the peak of $I_{Ca}$. The peak number of openings per fusion event averaged $0.44 \pm 0.064$ ($n = 9$) during a ramp voltage protocol. This was only slightly higher than ratios found with steps to −10 mV ($P = 0.16$, unpaired t-test). Thus differences in the activation kinetics of $I_{Ca}$ and EPSCs did not appear to significantly influence the conclusion that ~2 channel openings are needed to initiate fusion of each vesicle.

We examined the possibility that activation of $I_{Ca}$ with a strong test step may have stimulated unnecessary and redundant Ca$^{2+}$ channel openings. We therefore compared the number of Ca$^{2+}$ channel openings and vesicle release events during submaximal activation of $I_{Ca}$ using depolarizing steps to −30 mV rather than −10 mV. We scaled for the number of ribbon contacts, using the size of the EPSC evoked by steps to −30 mV. To account for the change in driving force with steps to −30 mV, we used the single-channel current amplitude of 0.37 pA for deconvolution of $I_{Ca}$. In the example shown in Fig. 4, the peak rate attained was 0.43 vesicles per Ca$^{2+}$ channel opening. On average, steps to −30 mV stimulated $0.40 \pm 0.06$ ($n = 8$) vesicle fusion events per Ca$^{2+}$ channel opening. This was not significantly different from the peak efficiency attained with ramp protocols ($P = 0.66$, unpaired t-test) or with steps to −10 mV ($P = 0.39$), suggesting that there are few unnecessary channel openings.

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**Fig. 2.** Deconvolution of Ca$^{2+}$ channel openings and release rates from the presynaptic $I_{Ca}$ and excitatory postsynaptic current (EPSC) of a simultaneously voltage-clamped cone and horizontal cell provides an estimate of Ca$^{2+}$ channel cooperativity. In these experiments, we used 1.8 mM Ca$^{2+}$ without BayK8644. A: cone $I_{Ca}$ overlaid on the EPSC recorded simultaneously from a horizontal cell. B: rate of Ca$^{2+}$ channel openings per millisecond per ribbon obtained from deconvolution of $I_{Ca}$ (see MATERIALS AND METHODS). Deconvolution of the EPSC with the average miniature EPSC (mEPSC) waveform gives the release rate per ribbon. In this example, release rate was normalized for 6 ribbon contacts. C: dividing the release rate per ribbon by the rate of Ca$^{2+}$ channel openings per ribbon gives the number of release events per opening. The reciprocal value provides the number of channel openings per fusion event. In this example, peak efficiency of 0.3 vesicle fusion events per channel opening (3.3 channel openings/fusion event) was reached at 20.9 ms, 4.7 ms after the beginning of the test step.

**Fig. 3.** Efficiency of release evoked by activation of $I_{Ca}$ with a ramp voltage protocol. A: overlay of cone $I_{Ca}$ evoked by a ramp voltage protocol (−90 to +60 mV, 0.5 mV/ms) with the EPSC recorded from a simultaneously voltage-clamped horizontal cell. B: deconvolution of $I_{Ca}$ and the EPSC showed a peak cooperativity of 0.56 release events per opening (1.8 openings/simultaneous fusion event). Traces are plotted as a function of voltage applied during the ramp protocol ($V_{m}$).
We tested the effects of lowering Ca\(^{2+}\)/H\(_{11001}\) channel open probability by inhibition of I\(_{\text{Ca}}\) with nifedipine (Brown et al. 1986; Hess et al. 1984; Worley and Kotlikoff 1990). In the presence of a concentration of nifedipine that partially blocked I\(_{\text{Ca}}\) (3 \(\mu M\)), steps to −10 mV evoked release with a peak efficiency of 0.19 \(\pm 0.038\) (\(N = 15\)) fusion events per channel opening, significantly lower than control release efficiency \((P = 0.003;\) unpaired \(t\)-test). The inhibition of L-type Ca\(^{2+}\) channels by nifedipine can be rapidly unblocked with a bright ultraviolet (UV) light flash (Sanguinetti and Kass 1984). At the midpoint of a test step to −10 mV, we applied a bright UV flash to abruptly reduce nifedipine inhibition and thereby increase the number of open Ca\(^{2+}\) channels. Figure 5A shows the cone I\(_{\text{Ca}}\) and horizontal cell EPSC from one pair. Release efficiency immediately following the step is shown in Fig. 5B. The change in release efficiency following the UV flash is shown in Fig. 5C. I\(_{\text{Ca}}\) increased from 90.7 \(\pm 9.3\) pA to 120.0 \(\pm 11.2\) pA \((n = 15)\) following the flash. The additional release stimulated by the increase in I\(_{\text{Ca}}\) exhibited an efficiency of 0.36 \(\pm 0.056\) vesicle fusion events per channel opening, significantly greater than release efficiency measured prior to the flash \((P = 0.022;\) paired \(t\)-test). Application of a UV flash without nifedipine did not increase release \((n = 3)\). If vesicle release could be consistently triggered by the opening of only a single Ca\(^{2+}\) channel, then reducing the probability of channel opening with nifedipine should cause a linearly proportional reduction in the number of vesicles released. The finding that release efficiency increased slightly with Ca\(^{2+}\) channel open probability suggests a weak cooperativity among Ca\(^{2+}\) channels during release. These data also provide further evidence that there are few unnecessary channel openings when the releasable pool is full.

Because the spread of Ca\(^{2+}\) from open Ca\(^{2+}\) channels to release sites influences the efficiency of release, we examined the role of Ca\(^{2+}\) buffers in shaping release. To do so, we compared efficiencies obtained with 5 mM EGTA, 0.5 mM EGTA, and 1 mM BAPTA. Reducing Ca\(^{2+}\) buffering from 5...
mM to 0.5 mM EGTA would be expected to allow Ca²⁺ to spread further from the mouth of an open channel and thereby increase release efficiency. However, as illustrated in Fig. 6, A and B, use of 0.5 mM EGTA did not significantly change release efficiency (0.30 ± 0.069 vesicle fusion events/channel opening, \( n = 6 \)). compared with 5 mM EGTA (\( P = 0.67 \), unpaired \( t \)-test). BAPTA has a much faster ON rate than EGTA and therefore constrains the spread of Ca²⁺ to more narrow nanodomains. However, release efficiency was also unchanged by use of 1 mM BAPTA as the Ca²⁺ chelator (0.39 ± 0.066 vesicle fusion events/channel opening, \( n = 6 \); \( P = 0.50 \), unpaired \( t \)-test compared with 5 mM EGTA; Fig. 6, C and D). The latency from the beginning of the test step to the instant of peak efficiency also did not differ significantly in recordings obtained with 0.5 mM EGTA (6.30 ± 0.67 ms), 5 mM EGTA (5.27 ± 0.54 ms), or 1 mM BAPTA (5.77 ± 0.49 ms; \( P = 0.55 \), 1-way ANOVA). We assessed release efficiency in the presence of endogenous Ca²⁺ buffers by obtaining gramicidin-perforated patch recordings from cones (Thoreson and Bryson 2004). Release efficiency with intact endogenous buffering in cones (0.32 ± 0.050 vesicle fusion events/channel opening, \( n = 5 \); Fig. 6, E–G) was nearly identical to that observed with 5 mM EGTA (\( P = 0.86 \), unpaired \( t \)-test). Release efficiency measured with ramp voltage protocols was also not changed significantly by use of perforated patch techniques or different buffers (Fig. 6H). Ca²⁺ imaging experiments have established that chelators introduced through the patch pipette can successfully reach the synaptic terminal (Mercer et al. 2011a). We therefore interpret the resistance of synaptic release to effects of exogenous buffers as evidence that there may be diffusion barriers that limit access of these buffers to the base of the cone ribbon.

**Simulations of Release at the Cone Synapse**

To assess our understanding of the synapse, we simulated release at the cone ribbon synapse with a model that employed empirically determined values for Ca²⁺ channel number (present study), Ca²⁺ channel current amplitude (present study), and influx rates of Ca²⁺ into the synaptic terminal (Bermingham et al. 2006). The details of the model are described in the Methods section. We used this model to predict the release efficiency observed in our experiments with different concentrations of EGTA and BAPTA. As shown in Fig. 6, the predicted release efficiencies were consistent with our experimental results. The model also allowed us to explore the effects of different Ca²⁺ chelators on release efficiency and to test the hypothesis that there are diffusion barriers that limit the access of buffering agents to the base of the cone ribbon.

Fig. 6. Effects of Ca²⁺ buffering on release efficiency. A, C, and E: cone I_{Ca} evoked by a step from −70 mV to −10 mV (100 ms) overlaid on the simultaneously recorded horizontal cell EPSC. B, D, and F: number of vesicle fusion events per channel opening obtained by deconvolution of the EPSC and I_{Ca}, respectively. In A and B, the cone pipette solution contained 0.5 mM EGTA. In C and D, the cone pipette solution contained 1 mM BAPTA. In E and F, we used a gramicidin-perforated patch recording technique to maintain endogenous Ca²⁺ buffering. G: average peak efficiency of release obtained when using a step depolarization to −10 mV and cone Ca²⁺ buffering provided by 5 mM EGTA (\( n = 18 \)), 0.5 mM EGTA (\( n = 6 \)), 1 mM BAPTA (\( n = 6 \)), or endogenous Ca²⁺ buffers (perforated patch, \( n = 5 \)). H: average peak efficiency of release with these same buffers obtained with a ramp voltage protocol (0.5 mV/ms; 5 mM EGTA, \( n = 9 \); 0.5 mM EGTA, \( n = 6 \); 1 mM BAPTA, \( n = 6 \); perforated patch, \( n = 5 \)).

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Ca$^{2+}$ channel mean open time (Thoreson et al. 2000), synaptic dimensions (Lasansky 1973; Pang et al. 2008; Raviola and Gilula 1975), vesicle pool size (Bartoletti et al. 2010), and Ca$^{2+}$ dependence of release (Duncan et al. 2010). We simulated a step to $-10$ mV by assuming the stochastic opening of Ca$^{2+}$ channels with a probability of 0.35. Each channel opened for 1.1 ms, establishing a local Ca$^{2+}$ nanodomain with the gradient shaped by a diffusible buffer (Neher 1998). Overlapping domains of neighboring channels were summed linearly. As shown in Fig. 7, simulations with this model using 5 mM EGTA as the diffusible buffer predicted a transient burst of release that achieved a maximum release efficiency of 3.7 vesicle fusion events per channel opening. This is $\sim$10-fold higher than the experimentally observed peak efficiency of 0.32 fusion events per opening (or 0.4 vesicles/channel if only 70% of channels are located at the ribbon). Reducing EGTA to 0.5 mM produced a transient burst of release with an even greater peak release efficiency of 5.3 vesicle fusion events per channel opening (not shown). Simulations with 1 mM BAPTA produced a peak efficiency of 0.13 fusion events per channel opening (Fig. 7D) that was lower than the value observed in actual recordings. In addition, simulations with BAPTA predicted a relatively slow release profile, with peak efficiency attained 3.3 ms later than the peak predicted for 5 mM EGTA. By contrast, the time to peak efficiency in actual recordings using 1 mM BAPTA or 5 mM EGTA did not differ significantly (unpaired t-test, $P = 0.47$).

Simulations predicted peak release efficiency with EGTA higher than the measured efficiency and peak efficiency with BAPTA lower than the measured efficiency. We examined various elements of the model to see which might account for these differences.

**Properties of the exocytotic calcium sensor.** Cones utilize a Ca$^{2+}$ sensor with an unusually low cooperativity ($n = 2$) and high Ca$^{2+}$ sensitivity (release threshold of $\sim$400 nM; Duncan et al. 2010) compared with other CNS neurons. To test the impact of sensor properties, we simulated release by using properties of the more conventional goldfish Mb1 bipolar cell Ca$^{2+}$ sensor, which exhibits 5 Ca$^{2+}$ binding sites and a threshold of $\sim$20 $\mu$M (Heidelberger et al. 1994). With this sensor, maximal release efficiency declined to 0.02 vesicle fusion events per channel opening with 5 mM EGTA and <0.0002 fusion events per opening with 1 mM BAPTA (Fig. 8, A–C). Thus the high affinity of the photoreceptor Ca$^{2+}$ sensor appears to be important for maintaining high release efficiency at the cone synapse.

Combining a bipolar cell Ca$^{2+}$ sensor with photoreceptor architecture, the model predicted lower release efficiency than has been observed empirically in bipolar cells (Coggins and Zenisek 2009; Jarsky et al. 2010; von Gersdorff et al. 1998). As a test of the model, we simulated release from bipolar cells by combining bipolar cell Ca$^{2+}$ dependence with bipolar cell-like architecture. Because bipolar cells typically lack an arciform density (Raviola and Raviola 1982), we modeled release at this synapse by placing Ca$^{2+}$ channels along a 20-nm strip immediately adjacent to release sites. The combination of this architecture with bipolar cell Ca$^{2+}$ dependence yielded a peak efficiency of 0.44 fusion events per channel opening with 5 mM EGTA and 0.27 fusion events per opening with 1 mM BAPTA (not shown). This is similar to results obtained in goldfish retinal bipolar cells by von Gersdorff et al. (1998; 0.14–0.27 fusion events/opening with 0.2 mM BAPTA). These results suggest that Ca$^{2+}$ sensor properties and architecture of the synapse both help to shape release at ribbon synapses.

**Single Ca$^{2+}$ channel properties.** Both variance-mean analysis (present study) and single-channel recordings (Thoreson et al. 2000) indicate that the properties of photoreceptor Ca$^{2+}$ channels are similar to those of other L-type channels (Church and Stanley 1996; Fenwick et al. 1982; Fox et al. 1987; Hagiwara and Ohmori 1983; Lux and Brown 1984; Rodriguez-Contras et al. 2002; Zampini et al. 2006). However, recordings of heterologously expressed Ca$_{1.4}$ channels showed 5-fold lower conductance and >10-fold lower open probability
We simulated release with the use of these latter channel properties and obtained a peak release efficiency of 1.04 fusion events per channel opening with 5 mM EGTA and 0.26 fusion events per opening with 1 mM BAPTA (Fig. 8, D–F). While the efficiency predicted for BAPTA was similar to the peak efficiency observed empirically, the kinetics of release predicted by the model differed considerably from the kinetics observed in real recordings: the model predicted that release with 1 mM BAPTA was maintained at a nearly steady rate throughout the depolarizing step (Fig. 8E), whereas actual recordings showed an initial phasic burst of release followed by only a small amount of tonic release (Fig. 6C).

We tested the impact of increasing the number of Ca\(^2+\) channels at the synapse 10-fold. This is equivalent to reducing peak open probability 10-fold to 0.035. With 5 mM EGTA, simulations using a maximal \(P_o\) of 0.035 yielded a peak release efficiency of 4.7 fusion events per channel opening, far above observed values (Fig. 8, G–I). With 1 mM BAPTA, efficiency declined to 0.24 fusion events per opening, but as described above, the model predicted continuous sustained release (Fig. 8H) rather than the transient release kinetics observed in actual recordings.

Proximity of Ca\(^2+\) channels to release sites. Physiological experiments have shown that Ca\(^2+\) channels are located within 50–100 nm of release sites (Mercer et al. 2011a). However, in principle, release efficiency could be reduced by placing channels further away from release sites. In model 1, channels were placed 25–100 nm from release sites. Expanding the active zone by an additional 50 nm so that channels could be as far as 150 nm from release sites reduced peak release efficiency to

(P\(_o\) < 0.015; Doering et al. 2005). We simulated release with the use of these latter channel properties and obtained a peak release efficiency of 1.04 fusion events per channel opening with 5 mM EGTA and 0.26 fusion events per opening with 1 mM BAPTA (Fig. 8, D–F). While the efficiency predicted for BAPTA was similar to the peak efficiency observed empirically, the kinetics of release predicted by the model differed considerably from the kinetics observed in real recordings: the model predicted that release with 1 mM BAPTA was maintained at a nearly steady rate throughout the depolarizing step (Fig. 8E), whereas actual recordings showed an initial phasic burst of release followed by only a small amount of tonic release (Fig. 6C).

Ca\(^2+\) channel number or open probability. We tested the impact of increasing the number of Ca\(^2+\) channels at the synapse 10-fold. This is equivalent to reducing peak open probability 10-fold to 0.035. With 5 mM EGTA, simulations using a maximal \(P_o\) of 0.035 yielded a peak release efficiency of 4.7 fusion events per channel opening, far above observed values (Fig. 8, G–I). With 1 mM BAPTA, efficiency declined to 0.24 fusion events per opening, but as described above, the model predicted continuous sustained release (Fig. 8H) rather than the transient release kinetics observed in actual recordings.

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2.2 fusion events per channel opening with 5 mM EGTA and 0.018 vesicles per channel opening with 1 mM BAPTA (Fig. 8L). Increasing the size of the active zone even further to 500 nm yielded a peak efficiency of 0.018 fusion events per channel opening with both 5 mM EGTA and 1 mM BAPTA. The release profiles with BAPTA or an expanded active zone were also much more sustained than that of actual recordings (Fig. 8K).

Ca$^{2+}$ diffusion kinetics. As described above, substituting alternative parameters for the empirically determined values used in the model tended to worsen the agreement between actual and predicted results. We therefore examined a key assumption of the model: that Ca$^{2+}$ diffuses freely away from open channels, so that Ca$^{2+}$ domains develop and collapse instantaneously with the opening and closing of Ca$^{2+}$ channels (Simon and Llinás 1985). However, our experiments showed that introducing different exogenous diffusible buffers did not affect release (Fig. 6), suggesting that there may be a diffusion barrier that restricts access of buffers to release sites. Such a barrier could also slow the diffusion of Ca$^{2+}$ away from channels and thereby slow the rise in intracellular [Ca$^{2+}$]. The cone ribbon synapse possesses an arciform density, a dense proteinaceous structure situated between vesicles and Ca$^{2+}$ channels, which provides a plausible anatomical basis for such a barrier. To test the impact of diffusion barriers on the spread of Ca$^{2+}$ at the cone ribbon synapse, we developed a kinetic model that incorporated analytical, time-dependent solutions for the diffusion of Ca$^{2+}$ away from open Ca$^{2+}$ channels (Ait-Haddou et al. 2010). To simulate a diffusion barrier, we slowed the rate of Ca$^{2+}$ diffusion and limited the concentration of diffusible buffer by the same factor. A diffusion barrier is mathematically equivalent to fixed endogenous buffers that act to lower the effective diffusion constant of Ca$^{2+}$ (e.g., from a diffusion barrier) may be important for shaping release efficiency at the cone synapse.

**DISCUSSION**

**How Many Ca$^{2+}$ Channels Are Present at Each Ribbon?**

Variance-mean analysis of $I_{Ca}$ tail currents indicated that there are $\sim$1,000 Ca$^{2+}$ channels per salamander cone. Ca$^{2+}$ channels are clustered beneath synaptic ribbons in photoreceptors (Morgans 2001; Morgans et al. 2005; Nachman-Clewner et al. 1999; Specht et al. 2009; Steele et al. 2005; tom Dieck et al. 2005; Xu and Slaughter 2005). Ca$^{2+}$ imaging studies suggest as few as 70% of photoreceptor Ca$^{2+}$ channels may be in the terminal (Steele et al. 2005; Szikra and Krizaj 2006). Excision of the rod terminal reduced rod $I_{Ca}$ by 95%, suggesting that almost all of the Ca$^{2+}$ channels are located in the terminal (Xu and Slaughter 2005) but not every channel in the terminal may be located at a ribbon. In adult hair cells $\sim$70% of Ca$^{2+}$ channels appear to be localized with ribbons (Brandt et al. 2005; Zampini et al. 2010), and in immature hair cells only 27% of Ca$^{1.3}$ channels localize with synaptic ribbons (Zampini et al. 2010). If every Ca$^{2+}$ channel is located beneath a synaptic ribbon, then the average of 13 ribbons per cone (Bartoletti et al. 2010; Pang et al. 2008) suggests that there are $\sim$80 Ca$^{2+}$ channels per ribbon. If only 70% of Ca$^{2+}$ channels are located at the cone ribbon, then there may be as few as $\sim$56 channels/ribbon.

Freeze-fracture electron micrographs of macaque cone pedicles show $\sim$400 particles along the synaptic ridge that are thought to be Ca$^{2+}$ channels (Raviola and Gilula 1975). Macaque cone ribbons are 700–1,000 nm long, whereas salamander cone ribbons are 150–350 nm long (Pang et al. 2008; Raviola and Gilula 1975). Scaling for the smaller size of salamander cone ribbons yields 80–140 particles per ribbon, somewhat greater than the number of Ca$^{2+}$ channels suggested from variance-mean measurements.

We calculated a peak mean open probability for photoreceptor Ca$^{2+}$ channels of 0.36. This is similar to values obtained...
from single-channel recordings in hair cells (Zampini et al. 2010) but higher than the value of 0.1 estimated from single-channel recordings in rods (Thoreson et al. 2000). However, measurements of maximal open probability by Thoreson et al. (2000) were limited by the small size of single-channel currents at strongly depolarized membrane potentials. The value of 0.36 is much higher than the mean open probability found for heterologously expressed Ca\textsubscript{v}1.4 channels (~0.015; Doering et al. 2005). However, the small single-channel open probability and conductance values found in this expression system predict an unrealistically large number of Ca\textsuperscript{2+} channels (~6,700 channels/ribbon), suggesting that these properties may not be retained in situ.

**How Many Ca\textsuperscript{2+} Channel Openings Are Associated with Release of Each Vesicle?**

Measurements of the size of the immediately releasable pool, release kinetics, and Ca\textsuperscript{2+} channel properties suggested that an average of 3–5 Ca\textsuperscript{2+} channel openings are associated with fusion of each vesicle during the first few milliseconds of release. However, the peak efficiency is likely to be higher than this average value, so we also determined release efficiency by deconvolving individual Ca\textsuperscript{2+} channel openings and quantal synaptic currents from simultaneously recorded cone \(I_{Ca}\) and horizontal cell EPSCs. The only shared assumption between deconvolution analysis and comparisons between the average pool size and number of channels was the Ca\textsuperscript{2+} channel mean open time of 1.1 ms (Thoreson et al. 2000), a value that is consistent with single-channel measurements from L-type channels in many preparations (Fenwick et al. 1982; Fox et al. 1987; Hagiwara and Ohmori 1983; Lux and Brown 1984; Zampini et al. 2006). Deconvolution analysis of paired simultaneous recordings showed that at the peak of release ~3 Ca\textsuperscript{2+} channel openings accompanied fusion of each vesicle. If only 70% of the channels are located at ribbons, then an average of ~2 channel openings per ribbon may be sufficient to stimulate fusion of a single vesicle. Thus two different analytical approaches utilizing largely independent measurements suggest that only a small number of Ca\textsuperscript{2+} channel openings are capable of driving fusion of an individual vesicle. These results are consistent with those in hair cells and bipolar cells showing that a very small number of channel openings are necessary for fusion of each vesicle (Brandt et al. 2005; Jarsky et al. 2010).

Using measurements of channel properties, vesicle pools, calcium dependence of release, and active zone architecture, we developed a biophysically realistic simulation of release at the cone photoreceptor ribbon. The original version of our model assumed that Ca\textsuperscript{2+} gradients developed instantaneously upon Ca\textsuperscript{2+} channel opening and collapsed instantaneously after channel closure. However, that simulation predicted that with EGTA as the Ca\textsuperscript{2+} buffer Ca\textsuperscript{2+} entering through an individual channel should diffuse far enough beneath the arciform density to stimulate fusion of multiple vesicles. This prediction is a consequence of the high sensitivity of the photoreceptor release mechanism to Ca\textsuperscript{2+} (Duncan et al. 2010; Rieke and Schwartz 1996; Thoreson et al. 2004). The original version of the model also predicted that with BAPTA as the Ca\textsuperscript{2+} buffer Ca\textsuperscript{2+} would not spread far enough from individual channels to reliably stimulate vesicle fusion. By contrast with these predictions, our electrophysiological recordings showed that release efficiency was not changed by use of different exogenous Ca\textsuperscript{2+} buffers in the cone patch pipette. The insensitivity of release to changes in Ca\textsuperscript{2+} buffering (Fig. 6) suggested the possibility of a diffusion barrier at the cone synapse that limits access of diffusible buffers to release sites and may therefore also limit the spread of Ca\textsuperscript{2+} away from open Ca\textsuperscript{2+} channels. To examine the impact of a diffusion barrier, we modified our model to incorporate a time-dependent rise and fall of Ca\textsuperscript{2+} gradients (Ait-Haddou et al. 2010). We simulated a diffusion barrier by slowing the rate of Ca\textsuperscript{2+} diffusion away from open Ca\textsuperscript{2+} channels by 1/200. With this slower rate of diffusion, the model predicted release efficiencies with both BAPTA and EGTA of 2.3–3.6 channel openings per vesicle fusion event, similar to the efficiency observed empirically. Although our results do not allow us to identify the site of this diffusion barrier, one appealing possibility is the arciform density that lies just beneath the ribbon. Interestingly, this structure is absent from ribbon synapses in retinal bipolar cells as well as hair cells. Changes in the rate of diffusion improved the model’s predictions, but changes in other model parameters worsened the agreement between predicted and actual results, suggesting that channel properties, properties of the release mechanism, and architecture of the synapse are all important for establishing efficient release at the cone ribbon synapse.

Although our results suggest an average peak efficiency of release of 2–3 Ca\textsuperscript{2+} channel openings per vesicle fusion event at each ribbon, it is possible that the opening of only a single channel may be sufficient to drive release. A depolarizing test step to ~10 mV stimulates release of the entire immediately releasable pool of ~20 vesicles per ribbon (Bartoletti et al. 2010). However, the findings that there are ~56 Ca\textsuperscript{2+} channels at each ribbon that can open with a maximum probability of 0.36 suggest that a step to ~10 mV stimulates the opening of only ~20 channels per ribbon at any instant or 1 channel per vesicle in the immediately releasable pool. Assuming that the immediately releasable pool represents docked vesicles, not every channel may always be close enough to a docked vesicle to trigger release (Shahrezaei et al. 2006). The presence of channel openings that contribute little or no Ca\textsuperscript{2+} to release would reduce the average peak efficiency. For example, the small Ca\textsuperscript{2+} nanodomains predicted by the kinetic model suggest that the opening of a single channel located at the very edge of the active zone may not elevate Ca\textsuperscript{2+} sufficiently to stimulate vesicle release. Lower efficiency could also result from partial depletion of the ribbon allowing some Ca\textsuperscript{2+} channels to open beneath empty release sites.

In calculations of release efficiency, we did not include a delay for Ca\textsuperscript{2+} to diffuse from the open channel to the Ca\textsuperscript{2+} sensor. With a distance (\(x\)) of 100 nm and diffusion coefficient (\(D\)) of 0.22 \(\mu\text{m}^2/\text{ms}\), the relationship \(x^2 = 2Dt\) predicts a diffusional delay of only 23 \(\mu\text{s}\). However, slowing diffusion (e.g., with a barrier) might increase this delay. An additional diffusional delay would mean that the true efficiency is higher than the efficiency estimated from a step depolarization, since vesicle fusion was actually triggered by Ca\textsuperscript{2+} channel openings that occurred earlier when \(I_{Ca}\) was just beginning to be activated. However, if the true efficiency was substantially higher, this should have been revealed by use of the ramp voltage protocol, which causes slower changes in \(I_{Ca}\) than those produced by a step protocol. But the ramp produced only a slightly higher efficiency (0.44 vesicles/channel opening).
The finding that release efficiency improved slightly when open channel probability was increased suggests that although the opening of one channel may sometimes be sufficient to stimulate release, the simultaneous opening of more than one Ca$^{2+}$ channel is needed for consistent vesicle fusion. While some channels may be close enough to a docked vesicle to trigger release, other release events may require the opening of two or more adjacent channels, each contributing a portion of the Ca$^{2+}$ needed to stimulate release. This appears similar to the situation at rod bipolar cell synapses, where single channel openings can trigger vesicle fusion but the average peak release efficiency is closer to two channel openings per vesicle fusion event (Jarsky et al. 2010).

Release Modes Differ in Light and Dark

In the present study, cones were voltage-clamped at a potential of $-70$ mV between test pulses. This is close to the membrane potential evoked in a cone by a saturating bright light. When the cone is strongly hyperpolarized, the releasable pool of vesicles tethered at the base of the ribbon is likely to be fully replenished (Jackman et al. 2009) and Ca$^{2+}$ channel openings are tightly synchronized to depolarizing membrane potential changes. The ability of only 1 or 2 Ca$^{2+}$ channel openings to trigger vesicle fusion can thus improve the ability of the cone synapse to signal the occurrence of a depolarizing decremental light stimulus with high precision.

Release from bipolar cells has also been reported to show a high efficiency (Jarsky et al. 2010; von Gersdorff et al. 1998). Although mouse rod bipolar cells may have a high-affinity Ca$^{2+}$ sensor (Jarsky et al. 2010), goldfish Mb1 bipolar cells use an exocytotic Ca$^{2+}$ sensor with a much lower affinity for Ca$^{2+}$ (Heidelberger et al. 1994). Using the bipolar cell Ca$^{2+}$ sensor and bipolar cell-like architecture without a diffusion barrier, the model predicted high release efficiency at the bipolar cell synapse. Why, then, do cones employ an unconventional high-affinity Ca$^{2+}$ sensor? One answer may be that use of a high-affinity sensor promotes depletion of the releasable pool when a cone remains depolarized in continued darkness (Jackman et al. 2009). The present results indicate that the efficiency of release increases quickly when depolarization is initiated but then diminishes to very low levels as the releasable pool of vesicles is depleted. This low efficiency reflects the fact that, after depletion of the releasable pool, the sustained rate of vesicle release is not determined by individual Ca$^{2+}$ channel openings but by the rate at which the releasable pool can be replenished (Babai et al. 2010; Jackman et al. 2009). This replenishment process has been shown to be Ca$^{2+}$ dependent, and thus changes in intracellular Ca$^{2+}$ levels driven by changes in cone membrane potential control the rate of release (Babai et al. 2010). However, the Ca$^{2+}$-dependent sites involved in replenishment are further from Ca$^{2+}$ channels ($>200$ nm) than are release sites ($<50–100$ nm). This links the sustained rate of release to average intraterminal Ca$^{2+}$ levels rather than individual channel openings. This, in turn, may help to make sustained release in darkness less noisy by reducing synaptic noise introduced by the stochastic probability of individual channel opening.

The clustering of Ca$^{2+}$ channels beneath the ribbon, the presence of diffusion barriers that limit the spread of Ca$^{2+}$ to localized nanodomains, and the use of a high-Ca$^{2+}$ affinity release mechanism at the photoreceptor synapse allow the cone synapse to shift between two signaling modes as the cone moves from bright light to darkness. In bright light, cones are hyperpolarized and synaptic output is tightly coupled to the opening of individual Ca$^{2+}$ channels stimulated by decremental light stimuli. This enhances information transmission by improving the timing precision of release events and reducing the occurrence of redundant events. In darkness, cones are depolarized and release is governed by replenishment rather than individual channel openings. This enhances information transmission by reducing synaptic noise associated with the stochastic opening of individual Ca$^{2+}$ channels in darkness.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


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FeW Ca\textsuperscript{2+} CHANNELS STIMULATE VESICLE RELEASE IN CONES


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