Vesicle Pool Size at the Salamander Cone Ribbon Synapse

Theodore M. Bartoletti,1,2 Norbert Babai,1 and Wallace B. Thoreson1,2

1Departments of Ophthalmology and Visual Sciences and 2Pharmacology and Experimental Neuroscience, University of Nebraska Medical Center, Omaha, Nebraska

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Bartoletti TM, Babai N, Thoreson TM. Vesicle pool size at the salamander cone ribbon synapse. J Neurophysiol 103: 419–428, 2010. First published November 18, 2009; doi:10.1152/jn.00718.2009. Cone light responses are transmitted to postsynaptic neurons by changes in the rate of synaptic vesicle release. Vesicle pool size at the cone synapse constrains the amount of release and can thus shape contrast detection. We measured the number of vesicles in the rapidly releasable and reserve pools at cone ribbon synapses by performing simultaneous whole cell recording from cones and horizontal or off bipolar cells in the salamander retinal slice preparation. We found that properties of spontaneously occurring miniature excitatory postsynaptic currents (mEPSCs) are representative of mEPSCs evoked by depolarizing presynaptic stimulation. Strong, brief depolarization of the cone stimulated release of the entire rapidly releasable pool (RRP) of vesicles. Comparing charge transfer of the EPSC with mEPSC charge transfer, we determined that the fast component of the EPSC reflects release of ~40 vesicles. Comparing EPSCs with simultaneous presynaptic capacitance measurements, we found that horizontal cell EPSCs constitute 14% of the total number of vesicles released from a cone terminal. Using a fluorescent ribeye-binding peptide, we counted ~13 ribbons per cone. Together, these results suggest each cone contacts a single horizontal cell at ~2 ribbons. The size of discrete components in the EPSC amplitude histogram also suggested ~2 ribbon contacts per cell pair. We therefore conclude there are ~20 vesicles per ribbon in the RRP, similar to the number of vesicles contacting the plasma membrane at the ribbon base. EPSCs evoked by lengthy depolarization suggest a reserve pool of ~90 vesicles per ribbon, similar to the number of additional docking sites further up the ribbon.

INTRODUCTION

Photoreceptors transmit their responses to second-order neurons at a specialized structure known as the synaptic ribbon. Ribbons are formed largely from the protein, ribeye, and tether synaptic vesicles at the active zone of the synapse (Dowling and Werblin 1969; Lasansky 1973; Schmitz et al. 2000). Synaptic ribbons are also present in the terminals of other sensory neurons including hair cells, retinal bipolar cells, and electoreceptors. The shape of the ribbon differs in various cell types (Lenzi and von Gersdorff 2001; Parsons and Sterling 2003). For example, the ribbon in cone photoreceptors is a surfboard-shaped structure, whereas it is spherical in some vestibular hair cells. The role of the ribbon in regulating release may also differ among cell types. In bipolar cells, depolarization stimulates rapid release of a pool of vesicles that is equal to or greater than the number of vesicles tethered to the plasma membrane at the base of the ribbon and maintained depolarization results in an exocytotic plateau comparable to the number of vesicles lining the entire ribbon (Coggins and Zenizek 2009; Mennerick and Matthews 1996; Neves and Lagnado 1999; Sterling and Matthews 2005; von Gersdorff et al. 1996). By contrast, the fastest release component in hair cells can exceed the number of vesicles lining the bottom row almost 10-fold (Edmonds et al. 2004), and the total releasable pool is more than fivefold greater than the number of vesicles lining the entire ribbon (Eisen et al. 2004; Griesinger et al. 2005; Lenzi et al. 1999; Parsons et al. 1994). In rod photoreceptors, capacitance measurements indicate that the size of the entire releasable pool is comparable to the number of vesicles tethered to each ribbon (Thoreson et al. 2004). The RRP in cone photoreceptors has not been measured, but the total releasable pool measured with capacitance techniques constitutes 50–100 vesicles per ribbon (Innocenti and Heidelberger 2008), less than or equal to the number of vesicles tethered to the ribbon (Sterling and Matthews 2005).

In paired recordings from cones and horizontal cells, exocytosis of the RRP generates a fast, transient EPSC component that is kinetically distinct from later, more sustained components (Rabl et al. 2005). Fast EPSCs at cone synapses can be constructed from a linear sum of individual quantal mEPSCs where each mEPSC is thought to represent fusion of a single vesicle (Cadetti et al. 2005, 2008). Evidence for independence and linear summation of quantal mEPSCs at cone synapses include 1) convolution of mEPSCs with the presynaptic cone release function reproduces EPSC waveforms, 2) the broadening of mEPSCs produced by blocking AMPA receptor desensitization causes a proportional broadening of EPSCs, and 3) the low-affinity glutamate receptor antagonist, kynurenic acid, blocks large and small EPSCs equally well, indicating that similar levels of glutamate are achieved at postsynaptic receptors, suggesting that vesicles act independently from one another (Cadetti et al. 2005, 2008). At the cone/off bipolar cell synapse, quantal independence is achieved partly because postsynaptic glutamate receptors recover quite rapidly, allowing them to recover from desensitization before the next release event (Pang et al. 2008). The high density of glutamate receptors at ribbon synapses also allows them to avoid receptor saturation (Li et al. 2009; Raghavachari and Lisman 2004).

In the present study, we determined the size of the rapidly releasable and reserve pools of vesicles at individual cone ribbon synapses by measuring EPSCs in horizontal cells evoked by the application of depolarizing steps to simultaneously recorded presynaptic cones. Because mEPSCs sum linearly, an evoked EPSC at the cone synapse can be described as a product of the number of quanta (N), the probability that quanta will be released (P), and quantal amplitude (Q) (Cadetti et al. 2005, 2008; del Castillo and Katz 1954; Sakaba et al. 2002; Schneeggenburger et al. 1999, 2002). We therefore began...
by determining release probability and quantal amplitude. To determine how much release occurs at a single ribbon contact, we also calculated the number of contacts made by an individual cone onto one horizontal cell. Combining these measures, we find that the RRP at each cone ribbon consists of ∼20 vesicles with a reserve pool of ∼90 vesicles. Thus much like bipolar cell ribbon synapses, the number of vesicles in the RRP at the cone ribbon synapse is similar to the number of vesicles contacting the plasma membrane at the base of the ribbon and the size of the reserve pool is similar to the number of reserve docking sites available on the remainder of the ribbon (Sterling and Matthews 2005).

METHODS
Preparations

RETINAL SLICE PREPARATION. Aquatic tiger salamanders (Ambystoma tigrinum, Kons Scientific, Germantown, WI) 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. The salamander was decapitated with heavy shears, the cranium hemisected, and the spinal cord rapidly removed. The resulting hemibrain was placed into a modified Ringer solution (121–6, Ted Pella, Redding, CA) tissue chopper (Stoelting, Wood Dale, IL). Retinal slices were rotated 90° to permit viewing of the retinal layers when placed under a water-immersion objective (×40, 0.7 NA or ×60, 1.0 NA) and viewed on an upright fixed stage microscope (Olympus BHWI or Nikon E600FN, Tokyo, Japan).

Solutions were applied by a single-pass, gravity-feed perfusion system, which delivered superfusate to the slice chamber at a rate of ∼1 ml/min. The normal amphibian superfusate contained (in mM) 111 NaCl, 2.5 KCl, 2 CaCl₂, 0.5 MgCl₂, 10 N-2-hydroxyethylpipera- zine-N’-2-ethanesulfonic acid (HEPES), 5 glucose (pH 7.8). Use of HEPES as a pH buffer limited effects of proton feedback (DeVries et al. 2005). The holding potential was varied sinusoidally (500–600 Hz, 30-mV peak-to-peak) about a holding potential of −70 mV. For pool size comparisons, we used the first record obtained during simultaneous capacitance and EPSC measurements, typically within 2–3 min after patch rupture.

mEPSCs were detected and analyzed using MiniAnalysis 6.0.7 (Synaptosoft, Decatur, GA). Events were first detected by the software using an amplitude threshold of 1 pA. Each event was then evaluated individually by eye. If necessary, the preceding baseline period was adjusted to improve the amplitude measurement. Double peaks were analyzed using an algorithm within Synaptosoft that extrapolates the exponential decay of the first peak.

Confocal images were acquired using a laser confocal scanhead (Perkin Elmer Ultraview LCI) equipped with a cooled CCD camera (Orca ER) and mounted to a fixed stage upright microscope (Nikon E600 FN). Excitation and emission were controlled by a Sutter Lambda 10–2 filter wheel and controller. Images were acquired and analyzed using UltraView Imaging Suite software. The objective (×60; 1.0 NA) was used with a z-axis controller (E662 Physik Instrumente) to acquire a series of z slices (0.5-μm steps) at each time point. Image brightness and contrast adjustments were made using Adobe Photoshop software. For some experiments, fluorescence was deconvolved using a blind deconvolution algorithm (AutoDeBlur, Media Cybernetics, Bethesda, MD) and confocal stacks reconstructed in 3 dimensions using Velocity software (Improvision, Waltham, MA).

RESULTS
Quantal amplitude (Q)

To measure the amount of synaptic release from cones, we recorded EPSCs evoked in horizontal cells by applying depolarizing steps to simultaneously voltage-clamped presynaptic
vesicles. The initial fast component of the EPSC recorded in a horizontal cell by cone stimulation has been shown to arise from a linear sum of quantal mEPSCs (Cadetti et al. 2005, 2008). This indicates that we can determine the size of the releasable pool (N) from measurements of the EPSC if we know the quantal amplitude of individual mEPSCs (Q) and the probability that a vesicle will be released (P).

Previous measurements of spontaneously occurring mEPSCs recorded from horizontal cells showed a quantal amplitude averaging 5.7 pA or 16 pC of charge transfer per mEPSC (Cadetti et al. 2008). However, many of the spontaneously occurring mEPSCs observed in horizontal cells when a presynaptic cone is voltage clamped at -70 mV are likely to arise from release by neighboring photoreceptors and not from the voltage-clamped cone. In addition, properties of spontaneous mEPSCs can differ from those arising from calcium-dependent evoked release (Sara et al. 2005). We therefore tested whether the properties of spontaneously occurring mEPSCs in horizontal cells are representative of mEPSCs evoked by direct depolarization of a voltage-clamped cone.

In one approach, we measured mEPSCs evoked by weakly depolarizing a presynaptic cone while simultaneously inhibiting release from neighboring photoreceptors using a bright saturating background light. Bright background illumination caused a sustained reduction in baseline release of 30 ± 14.5% (n = 5). Depolarizing a cone from -70 to -30 mV stimulated a small increase in the number of mEPSCs (+13%, P = 0.049, n = 6; Fig. 1B). This increase was not seen after killing the presynaptic cone (n = 3). If the amplitude of mEPSCs released by depolarization stimulation differs from that of spontaneously released mEPSCs, then this additional release would be expected to cause a shift in the cumulative amplitude histogram. As shown in Fig. 1C, there was no significant difference between the average of cumulative amplitude histograms obtained during spontaneous release and during depolarizing stimulation (P = 0.42, Kolmogorov-Smirnov test, 222 ± 48 events during spontaneous release, 255 ± 50 events during depolarization-evoked release, n = 6 cone/horizontal cell pairs).

In a second approach to test for possible differences in the properties of spontaneously occurring and depolarization-evoked mEPSCs, we used variance-mean analysis techniques to measure the size of quantal events contributing to EPSCs during depolarizing stimulation of voltage-clamped cones. The quantal amplitude of individual mEPSCs contributing to the EPSC can be determined from the slope of the initial rise in the relationship between the variance and mean amplitude of depolarization-evoked EPSCs (Clements 2003; Meyer et al. 2001; Sakaba et al. 2002). Variance-mean analysis can provide a useful estimate of quantal amplitude from as few as five synaptic amplitude measurements per test potential (Clements 2003).

We measured the mean amplitude and variance in EPSCs evoked in horizontal cells by repeated application of a series of depolarizing steps to the presynaptic cone (-40, -30, and -20 mV). We determined the quantal amplitude for each cell pair from the mean and variance of EPSCs evoked by the two test steps showing the steepest initial rise in the variance-mean relationship (typically with steps to -40 and -30 mV, Fig. 1D). We obtained an average of 8.4 ± 2.2 synaptic responses at each test potential for each cell pair. The mEPSC amplitude calculated from the initial rise in the variance-mean relationship averaged 5.8 ± 0.8 pA (n = 6), similar to the amplitude of spontaneous mEPSCs (P = 0.76). Thus two different experimental approaches indicate that the quantal amplitude of spontaneously released mEPSCs in horizontal cells does not differ significantly from the amplitude of mEPSCs that contribute to depolarization-evoked EPSCs in paired cone/horizontal cell recordings. This contrasts with hair cell ribbon synapses in which depolarizing stimulation evokes large multiquantal synaptic currents (Li et al. 2009).

If spontaneously occurring mEPSC at cone synapses, like depolarization-evoked release at other ribbon synapses (Glowatzki and Fuchs 2002; Li et al. 2009; Matthews and Sterling 2008; Singer et al. 2004; Suryanaryanan and Slaughter 2006), results from the simultaneous release of more than one vesicle, then this would lead to an overestimate of the postsynaptic impact of a single vesicle. We tested for contributions from multivesicular fusion during spontaneous release by measuring mEPSCs after reducing [Ca2+]o to reduce release probability and minimize the likelihood of multivesicular events (Li et al. 2009; Singer et al. 2004). Lowering [Ca2+]o to 0.2 mM reduced release rates (143.7 ± 14.30 to 123.0 ± 10.80 Hz; n = 6, P = 0.01) but did not significantly affect quantal amplitude (4.9 ± 1.0 pA in control vs. 5.2 ± 1.2 pA in low Ca2+; paired t-test, n = 6, P = 0.53). This suggests that few multivesicular events contribute to spontaneously occurring mEPSCs. To-
brane current using a P/8 leak subtraction protocol. A rapidly releasable pool is released during the 25-ms test pulse. The size of the was originally available for release (Fig. 2). Extrapolating a straight line to the final 3 data points when vesicle replenishment balances release (Sakaba et al. 2002). Extrapolating this line back to reveals the size of the vesicle pool that was obtained within 8.9 ms (Rabl et al. 2005), and the peak of the resulting fast EPSC was 4.1 pA (Rabl et al. 2005). Exocytosis of the RRP from slow sustained release, we number vesicles (N)

Given that a 25-ms depolarizing pulse to -10 mV stimulates release from cones with nearly 100% probability and that cone-driven EPSCs in horizontal cells reflect a linear sum of individual mEPSCs (Cadetti et al. 2005, 2008), we can calculate the size of the releasable pool from the measurements of the EPSC. Like other neurons, release of vesicles from the cone synapse involves both fast and slow components (Li et al. 2009; Rabl et al. 2005; Singer and Diamond 2003). To separate fast release of the RRP from slow sustained release, we integrated charge transfer during EPSCs evoked by 100-ms test pulses to -10 mV and fit the resulting integral with a double-exponential function (Fig. 3). Dividing charge transfer of the number of vesicles released (N)

Consistent with a release probability of nearly 100%, we also found that increasing Ca\(^{2+}\) influx by elevating extracellular Ca\(^{2+}\) to 5 mM did not enhance EPSCs (Fig. 2C). In fact, EPSCs were reduced by 19% (129.3 ± 43.0 to 104.6 ± 39.2 pA, n = 8, P = 0.006) despite a robust 81.1 ± 27.8% (n = 4; P = 0.01) increase in \(I_{\text{Ca}}\). The reduction in EPSC amplitude may be due to inhibition of AMPA receptors by elevated Ca\(^{2+}\) because increasing Ca\(^{2+}\) to 5 mM caused a 17% (n = 9, P = 0.044) reduction in the amplitude of individual mEPSCs. The inhibition of mEPSCs by elevated Ca\(^{2+}\) is consistent with the possible presence of Ca\(^{2+}\)-permeable AMPA receptors as found in salamander OFF bipolar cells (Gilbertson et al. 1991). The finding that EPSCs did not increase in amplitude despite large increases in \(I_{\text{Ca}}\) provides further evidence that all available vesicles are released by application of a depolarizing step to -10 mV with normal Ca\(^{2+}\) levels.

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ENTIRE FAST COMPONENT BY AN AVERAGE CHARGE TRANSFER OF 16 pC/mEPSC, WE FOUND THE NUMBER OF VESICLES IN THE ENTIRE FAST COMPONENT OF RELEASE AVERAGED 42.5 ± 2.0 VESICLES (n = 217). THE NUMBER OF VESICLES IN THE RRP DETERMINED FROM EPSC CHARGE TRANSFER MEASUREMENTS AVERAGED 2.4 TIMES MORE THAN THE NUMBER OF VESICLES CALCULATED BY DIVIDING PEAK EPSC AMPLITUDE BY THE AVERAGE mEPSC AMPLITUDE OF 5.7 pA, INDICATING THAT NOT ALL VESICLES IN THE RRP ARE RELEASED SYNCHRONOUSLY.

AN INDIVIDUAL HORIZONTAL CELL CAN CONTACT A SINGLE CONE AT MORE THAN ONE RIBBON (AHNELT AND KOLB 1994). WE PLOTTED THE AMPLITUDE HISTOGRAM OF EPSC AMPLITUDES FROM 217 CONE/HORIZONTAL CELL PAIRS AND FOUND THAT IT COULD BE DESCRIBED BY A MULTIPLE GAUSSIAN FUNCTION WITH INDIVIDUAL COMPONENTS OF 46.3 pA A PIECE (FIG. 4A). WE POSTULATE THAT EACH GAUSSIAN COMPONENT REPRESENTS RELEASE AT A SINGLE RIBBON. THE MULTICOMPONENT GAUSSIAN DISTRIBUTION SUGGESTS THAT SOME HORIZONTAL CELLS RECEIVE ONLY A SINGLE RIBBON CONTACT FROM THE VOLTAGE-CLAMPED CONE WHEREAS OTHER HORIZONTAL CELLS MAY BE CONTACTED BY SIX OR MORE RIBBONS IN A SINGLE CONE. THE WEIGHTED AVERAGE OF INDIVIDUAL GAUSSIAN COMPONENTS SUGGESTS THAT EACH CONE CONTACTS A SINGLE HORIZONTAL CELL AT AN AVERAGE OF 2.1 RIBBONS. IF WE ASSUME THAT THE EPSCs ARISE FROM RELEASE AT 2.1 RIBBON CONTACTS, THEN 42.5 VESICLES IN THE RRP REPRESENTS RELEASE OF 20 VESICLES PER RIBBON.


THE AMPLITUDE HISTOGRAMS IN FIG. 4 SUGGEST EACH CONE CONTACTS A SINGLE HORIZONTAL CELL AT AN AVERAGE OF APPROXIMATELY TWO RIBBONS. WE ADDRESSED THIS QUESTIONS MORE DIRECTLY BY COUNTING THE NUMBER OF RIBBON SYNAPSES IN EACH CONE TERMINAL AND COMPARE EPSC AMPLITUDES WITH THE TOTAL RELEASE FROM EACH CONE MEASURED USING CAPACITANCE TECHNIQUES. TO COUNT SYNAPTIC RIBBONS, THEY WERE STAINED WITH A HYLITE488-CONJUGATED FLUORESCENT RIBEYE-BINDING PEPTIDE INTRODUCED INTO THE CONE THROUGH A PATCH PIPETTE. RIBBONS STAINED BY THIS FLUORESCENT PEPTIDE WERE COUNTED BY EXAMINING A SERIES OF INDIVIDUAL CONFOCAL SECTIONS OBTAINED AT 0.5-µM INTERVALS. Figure 5 SHOWS AN EXAMPLE OF 14 RIBBONS IN 20 CONFOCAL SECTIONS FROM A SINGLE CONE. RIBBONS EXTENDED THROUGH MULTIPLE SECTIONS; NEWLY DISTINGUISHABLE RIBBONS ARE DENOTED BY ARROWS. ON AVERAGE, WE COUNTED 12.4 ± 0.8 (n = 8) RIBBONS PER CONE. IN TWO CELLS, WE DECONVOLVED THE FLUORESCENCE AND THEN RECONSTRUCTED THE CONES IN THREE DIMENSIONS. WE COUNTED THE SAME NUMBER OF RIBBONS USING THIS PROCEDURE AS BY EXAMINING THE SERIES OF INDIVIDUAL CONFOCAL SECTIONS. CONFOCAL MICROSCOPY DOES NOT PROVIDE THE ABILITY TO DISTINGUISH RIBBONS <300 NM FROM ONE ANOTHER DUE TO THE DIFFRACTION LIMIT, BUT OUR RESULTS WERE NONETHLESS QUITE SIMILAR TO THOSE OBTAINED FROM SERIAL SECTION ELECTRON MICROGRAPHS WHICH SHOWED AN AVERAGE OF 13.4 RIBBONS PER SALAMANDER CONE (PANG ET AL. 2008). FOR CALCULATIONS IN THE FOLLOWING TEXT, WE CHOSE A VALUE OF 13 RIBBONS PER CONE.

WE DETERMINED THE AMOUNT OF RELEASE OCCURRING AT THE SYNAPSES BETWEEN A SINGLE CONE AND A SINGLE HORIZONTAL CELL FROM THE SIZE OF THE EPSC AND THEN COMPARED THIS WITH ЕХОТОСИΣ FROM THE ENTIRE CONE TERMINAL MEASURED USING CAPACITANCE TECHNIQUES. SIMULTANEOUSLY RECORDED HORIZONTAL CELL EPSCS AND CONE CAPACITANCE RESPONSES WERE EVOKED BY A 25-MS DEPOLARIZING TEST STEP TO ~10 mV (FIG. 6). THE AMPLITUDE OF THE CONE CAPACITANCE RESPONSE WAS MEASURED 30 MS AFTER THE END OF THE TEST STEP TO AVOID GATING CHARGES AND ALLOW TIME FOR THE PHASE ANGLE FEEDBACK CIRCUITRY TO SETTLE. ENDOCYTOSIS IS MINIMAL DURING THIS BRIEF TIME (INNOCENTI AND HEIDELBERGER 2008; Rabl et al. 2005; Rieke and Schwartz 1996). THE CAPACITANCE RESPONSE AVERAGED 51.0 ± 8.4 fF (n = 9). DIVIDING THE TOTAL CAPACITANCE RESPONSE BY A SINGLE VESICLE CAPACITANCE OF 57 aF (Thoreson et al. 2004) INDICATES THAT AN AVERAGE OF 895 VESICLES WERE RELEASED FROM THE ENTIRE COLLECTION OF RIBBONS IN EACH CONE TERMINAL. INTEGRATING CHARGE TRANSFER OF THE SIMULTANEOUSLY RECORDED HORIZONTAL CELL EPSC DURING THIS SAME TIME PERIOD YIELDED AN AVERAGE OF 1,730 ± 266 pC (n = 9). DIVIDING THIS VALUE BY A SINGLE QUANTAL CHARGE TRANSFER OF 16 pC INDICATES THAT THE EPSC RESULTED FROM RELEASE OF 108 VESICLES IN THESE CELL PAIRS. PAIRED COMPARISONS OF THESE TWO MEASUREMENTS INDICATE THAT RELEASE FROM A SINGLE CONE TO A SINGLE HORIZONTAL CELL INVOLVED 13.7 ± 3.2% (n = 9) OF THE NUMBER OF VESICLES RELEASED BY THE ENTIRE CONE. GIVEN 13 RIBBONS PER CONE, THIS INDICATES THAT A SINGLE CONE CONTACTS AN INDIVIDUAL HORIZONTAL CELL
bipolar cell pairs with large EPSCs. The best-fit parameters for the two exponential components did not differ significantly between these two groups of cells ($P > 0.33$), so we combined the samples. The amplitude of the slow exponential component, which we take to represent the reserve pool, was $4.6 \pm 0.5$ (n = 20) times as big as the fast component (RRP). Given an RRP of ~20 (15–24) vesicles per ribbon, these results suggest that the reserve pool consists of ~90 (69–110) vesicles per ribbon and the entire releasable pool of ~110 (84–134) vesicles per ribbon. If there is an accumulation of glutamate in the cleft during sustained depolarization, then this could produce glutamate receptor desensitization that would lead to an underestimate of release. While we cannot rule this out, Pang et al. (2008) show that the ability of AMPA receptors to recover rapidly from desensitization allows them to evade effects of desensitization at cone synapses.

The reserve pool could be depleted with a time constant of $396 \pm 80$ ms. After depletion of this second pool, release was maintained at a slow rate resulting in a linear increase in EPSC charge transfer. After subtracting the linear rate of basal release present before the depolarizing test step, we found that the slope of the linear component of the EPSC charge transfer function averaged $-4.339 \pm 1.669$ pC/s in cone/horizontal cell pairs (n = 4) and $-2.023 \pm 298$ pC/s in cone/Off bipolar pairs (n = 16). Assuming an RRP of ~20 vesicles per ribbon, the size of the fast component of charge transfer suggests that horizontal cell EPSCs in this sample arose from an average of 3 ribbon contacts apiece. This in turn suggests that sustained release can be maintained at a rate of ~90 vesicle·s$^{-1}$·ribbon$^{-1}$. A similar calculation for Off bipolar cell pairs suggests that sustained release can be maintained at ~40 vesicle·s$^{-1}$·ribbon$^{-1}$ (Fig. 7). Measurements of these small sustained currents were quite sensitive to accurate subtraction of basal release prior to the test step. Furthermore, if there is significant glutamate receptor desensitization during lengthy depolarizing stimulation, then these may be underestimates of release rates. Nonetheless, it is clear that strongly depolarized...
The quantal amplitude of mEPSCs that occur spontaneously in horizontal cells and OFF bipolar cells in darkness has been previously shown to average 5.7 pA or 16 pC/mEPSC (Cadetti et al. 2005, 2008). However, many of the spontaneously occurring mEPSCs observed during paired recordings are not due to release from the voltage-clamped cone but are instead due to release from neighboring presynaptic photoreceptors that are likely to have a more depolarized resting membrane potential, even under light-adapted conditions. Spontaneous mEPSCs can also arise from calcium-independent release involving a different population of vesicles than calcium-dependent evoked release (Sara et al. 2005). However, we found that the amplitude of spontaneously occurring mEPSCs recorded from horizontal cells in bright light is indistinguishable from the amplitude of mEPSCs evoked by direct depolarizing stimulation of cones under conditions where spontaneous release is minimized. Furthermore, we obtained a similar quantal amplitude from the relationship between variance and mean amplitude of the EPSC. The amplitude of mEPSCs was not significantly diminished in low-Ca²⁺ conditions, indicating that spontaneously occurring mEPSCs primarily reflect the fusion of individual vesicles and not the simultaneous compound fusion of multiple vesicles. From these results, we conclude that the quantal amplitude of mEPSCs at the cone-synapse represents the postsynaptic effects of single glutamate-filled vesicles. This contrasts with results at hair cell ribbon synapses where the unitary events underlying evoked and spontaneous EPSCs involve the simultaneous fusion of multiple vesicles (Li et al. 2009).

Our results showed that application of a 25-ms test pulse from ~70 to ~10 mV to fully activate the L-type I_{Ca} in cone terminals stimulated exocytosis from the RRP with 100% probability. This conclusion was confirmed by the finding that EPSCs were not enhanced by elevating extracellular calcium despite a substantial enhancement of I_{Ca}. Because the amplitude of the EPSC is a product of the quantal content and release probability, the finding of a release probability near 100% indicates that we can calculate the number of vesicles contributing to the EPSC by simply dividing the EPSC by the size of the individual quanta. In the photoreceptor, we demonstrate that fully activating I_{Ca} stimulated release with 100% probability, but stimuli that trigger less calcium influx would naturally be expected to evoke release with lower probability. Thus for example, release evoked by an action potential in the calyx of Held exhibits a much lower release probability largely due to the brief duration of Ca²⁺ influx accompanying an individual action potential (Sakaba et al. 2002; Schneggenburger et al. 2002).

We find that depolarization-evoked synaptic release from cones involves three phases of release, similar to results in retinal bipolar cells (Neves and Lagnado 1999; von Gersdorff and Matthews 1997). There is a RRP of ~20 vesicles per ribbon and a more slowly releasable reserve pool of ~90 vesicles per ribbon. After depletion of these two pools, cones can sustain release at a steady rate of ~40–90 vesicles·s⁻¹/ribbon⁻¹. We calculated these numbers by measuring EPSCs evoked in horizontal or OFF bipolar cells by depolarizing pulses applied to simultaneously recorded cones. We converted EPSC measurements into the number of vesicles per ribbon using measurements of quantal amplitude, release probability, number of ribbons per cone, and number of ribbon contacts from a single cone to a single horizontal cell. As discussed in the following text, we confirmed these various measurements using multiple approaches.

**DISCUSSION**

Anatomical studies show that a single horizontal cell can make multiple contacts with a single cone (Ahnelt and Kolb 1994; Kolb 1974; Lasansky 1973). To determine the number of ribbon contacts, we counted the number of ribbons per cone observed after infusing a fluorescent ribeye-binding peptide into the cell. Our finding of 12.4 ribbons per cone agrees closely with serial section EM results of 13.4 ribbons per salamander cone (Pang et al. 2008). We then calculated the fraction of the cone’s total release that is responsible for the EPSC in an individual horizontal cell by comparing the amount of release measured from the EPSC with the total amount of release from the cone measured using capacitance techniques. Combining these two results, we calculate that each salamander
cone contacts an individual horizontal cell at an average of ~1.8 synaptic ribbons. If release from cones involves kiss-and-run fusion events that do not produce a maintained increase in membrane capacitance, then capacitance measurements would underestimate the amount of release and this, in turn, would mean that we overestimated the number of contacts made from a cone onto a single horizontal cell. We cannot exclude this possibility, but there is no evidence for kiss-and-run fusion at ribbon synapses (Prescott and Zenisek 2005). Additionally, the weighted average of individual components from the multiple Gaussian function fit to the EPSC amplitude histogram also suggested that each cone contacts a single horizontal cell at an average of 2.1 synaptic ribbons. The large amplitude of EPSCs in some paired cone/horizontal cell recordings suggests that in some cases, there may be more than six ribbon contacts between a single voltage-clamped cone and a postsynaptic horizontal cell. If we assume that each cone contacts a single horizontal cell at an average of 1.8 ribbons, then the average of 42.5 vesicles released in the initial exocytotic burst suggests an RRP of 24 vesicles per ribbon. If we use the number of ribbon contacts estimated from the amplitude histogram (2.1), then this suggests an RRP of 20 vesicles per ribbon. The size of individual components of the multiple Gaussian fit to charge transfer measurements of the RRP suggest a slightly smaller RRP of 15 vesicles per ribbon. We therefore conclude that the RRP at the cone synapse consists of ~20 (15–24) vesicles per ribbon.

In addition to synaptic ribbon contacts, photoreceptors make flat or basal contacts onto bipolar cell dendrites (Kolb 1974; Lasansky 1973). Flat contacts exhibit pre- and postsynaptic paramembranous densities but lack the vesicle clusters found at conventional synapses (Lasansky 1973). There is evidence that nonribbon sites can participate in release from hair cells and retinal bipolar cells (Khimich et al. 2005; Midorikawa et al. 2007; Zenisek et al. 2003; Zenisek 2008), but this has not been shown for photoreceptors. Glutamate released from cone ribbons can rapidly diffuse throughout the cone synapse, and thus release from nonribbon sites is not required to evoke responses at flat contacts of off bipolar cells (DeVries et al. 2006). Nonetheless, if nonribbon sites contribute to release from cones, they are likely to contribute to slow components of release as found for bipolar and hair cells (Khimich et al. 2005; Midorikawa et al. 2007; Zenisek et al. 2003, 2008). If so, this would imply that some of the vesicles in the reserve pool may represent release at nonribbon sites.

Vesicles on the bottom two to three rows of the ribbon can contact the plasma membrane along the cone synaptic ridge (Jackman et al. 2009; Lasansky 1978). Pang et al. (2008) reported that ribbons of salamander cones are 150–350 nm long, suggesting that each row along one side of the ribbon can accommodate three to seven vesicles with a diameter of 35–45 nm (Lasansky 1978; Thoreson et al. 2004). This suggests that 12–28 vesicles can contact the plasma membrane along the bottom two rows of the ribbon. Thus the finding of ~20 (15–24) vesicles in the RRP of each cone ribbon is consistent with the hypothesis that this pool consists of vesicles contacting the plasma membrane along the bottom two rows of the ribbon. Ultrastructural studies of foveal cones from primate retina show ~36 vesicles docked at the base of each synaptic ribbon (Sterling and Matthews 2005). The conclusion that the RRP is similar to the number of vesicles docked at the base of the ribbon is consistent with findings at bipolar cell ribbons (Coggins and Zenisek 2009; Sterling and Matthews 2005; von Gersdorff et al. 1996) but differs from results in hair cells where the RRP greatly exceeds the number of docked vesicles (Edmonds et al. 2005). This latter finding led to the proposal that one function of the ribbon may be to facilitate compound fusion by allowing more distant vesicles to fuse with neighboring vesicles on lower rows of the ribbon during depolarization (Parsons and Sterling 2003). While our results do not preclude the possibility of compound fusion, they suggest that compound fusion events are not sufficiently common or large enough to deplete a substantially larger population of vesicles further up the ribbon during the initial burst of release. Furthermore, evidence for compound fusion also indicates that it is more prominent with stronger depolarization (Li et al. 2009).

From responses to long depolarizing steps, we estimated a reserve pool of ~90 vesicles per ribbon that can be depleted with a time constant of ~400 ms. Combining the rapidly releasable and reserve pools yields a pool size of ~110 (84–134) vesicles per ribbon. Measurements of EPSCs can overestimate release if some of the postsynaptic current arises from release by neighboring gap-junctionally coupled photoreceptors or underestimate release if there is substantial desensitization of the postsynaptic glutamate receptors during sustained release. However, in good agreement with EPSC measurements, capacitance measurements of exocytosis in dissociated cones suggest a releasable pool of 50–100 vesicles per ribbon that can be depleted with a time constant of 420 ms (Innocenti and Heidelberger 2008). Evidence that the reserve pool is four to five times larger than the RRP is consistent with anatomical observations from primate foveal cones showing four times as many reserve sites on the ribbon as docking sites at the base (Sterling and Matthews 2005).

Cell-attached patch recordings of single Ca2+ channels in rod photoreceptors show single channel conductance, open states, and closed states similar to other L-type Ca2+ channels (Thoreson et al. 2000). At physiological Ca2+ levels, L-type channels have a single channel conductance of ~2 pS (Church and Stanley 1996; Rodriguez-Contreras et al. 2002). The peak amplitude of ICa in salamander cones measured at ~25 mV averages ~90 pA (L. Cadetti and W. B. Thoreson, unpublished observations). If we assume a driving force of 100 mV, then this yields a whole cell conductance of 900 pS, suggesting that 110 channels (35/ribbon) are open at any moment when ICa is fully activated. The time constant for release of the RRP in cones is 2.7 ms (Rabl et al. 2005), indicating that 63% of the RRP (i.e., ~10–15 vesicles) fuse at each ribbon within that brief period. Assuming that each Ca2+ channel remains open for an average of 1.1 ms (Thoreson et al. 2000), this suggests that ~85 channel openings are likely to occur within the first 2.7 ms. In other words, five to nine channel openings accompany the fusion of each vesicle during the first few milliseconds of release. However, not every cone Ca2+ channel is located at the ribbon. It has been estimated that 70–95% of Ca2+ channels are located in the terminal with most of these clustered at the ribbon (Nachtman-Clewner et al. 1999; Steele et al. 2005; Szikra and Krizaj 2006; Xu and Slaughter 2005). If only 70% of the channels are clustered at the cone ribbon, then this suggests that approximately three to six channel openings accompany each vesicle fusion event. The test pulses used for our experiments fully activated ICa and it is possible that more
Ca\(^{2+}\) channel openings occurred than necessary. Thus we may have overestimated the minimum number of channel openings required for release of a single vesicle. At hair cell ribbon synapses, it is estimated that 70% of the Ca\(^{2+}\) channels are located at the ribbon and two to three channel openings accompany fusion of each vesicle (Brandt et al. 2005). Measurements of release at the Mb1 bipolar cell synapse suggest a somewhat greater number of channel openings (>5) accompany each fusion event (Coggins and Zenisek 2009; von Gersdorff et al. 1998). With sustained depolarization, the coupling between Ca\(^{2+}\) channel opening and release inevitably weakens as release sites become depopulated with vesicles during maintained stimulation. Because fewer vesicles are available for release, the probability that opening of an individual Ca\(^{2+}\) channel will stimulate release necessarily declines. Thus with maintained depolarization, the rate of release depends less on the rate of Ca\(^{2+}\) channel opening and more on the rate at which release sites can be replenished with releasable vesicles.

It has been proposed that delivery and docking of vesicles at the base of the ribbon is the rate-limiting step in sustained release (Jackman et al. 2009). Assuming that sustained release involves release from the same ribbon sites as rapid release, the size of the RRP (i.e., the number of available release sites) constrains the rate of sustained release. The time constant for vesicle replenishment of 250 ms determined from paired-pulse depression measurements (Rabl et al. 2006) implies that 63% of the RRP can be replenished every 250 ms. This rate of replenishment would be capable of sustaining a steady release rate of \(\sim 50\) vesicle/s/ribbon\(^{-1}\), consistent with sustained release rates of 40–90 vesicle/s/ribbon\(^{-1}\) determined from responses to long depolarizing steps.

In addition to limiting sustained release rates, the size of the RRP also constrains the size of transient OFF responses in second-order bipolar and horizontal cells. The RRP in cone terminals is emptied in darkness and replenished when cones are hyperpolarized in bright light (Jackman et al. 2009). Release of the RRP on depolarization of cones at light offset generates transient OFF responses in postsynaptic bipolar and horizontal cells that may be used to help encode light decrement (Jackman et al. 2009).

The present results suggest that cone ribbons are similar to bipolar cell ribbons in that the RRP corresponds to the number of vesicles tethered to the plasma membrane at the base of the ribbon, the reserve pool corresponds to the population of vesicles tethered further up the ribbon, and a slow steady rate of release can be maintained after depletion of these two pools. The sizes of these vesicle pools constrain the dynamic range of changes in synaptic output that can be produced by changes in cone membrane potential during light and dark.

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