Mechanisms Contributing to Tonic Release at the Cone Photoreceptor Ribbon Synapse

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Innocenti B, Heidelberger R. Mechanisms contributing to tonic release at the cone photoreceptor ribbon synapse. J Neurophysiol 99: 25–36, 2008. First published November 7, 2007; doi:10.1152/jn.00737.2007. Time-resolved capacitance measurements in combination with fluorescence measurements of internal calcium suggested three kinetic components of release in acutely isolated cone photoreceptors of the tiger salamander. A 45-fF releasable pool, corresponding to about 1,000 vesicles, was identified. This pool could be depleted with a time constant of a few hundred milliseconds and its recovery from depletion was quite rapid (τ = 1 s). The fusion of vesicles in this pool was blocked by low-millimolar EGTA. Endocytosis was sufficiently slow that it is likely that refilling of the releasable pool occurred from preformed vesicles. A second, slower component of release (τ_depletion ≈ 3 s) was identified that was approximately twice the size of the releasable pool. This pool may serve as a first reserve pool that replenishes the releasable pool. Computer simulations indicate that the properties of the releasable and first reserve pools are sufficient to maintain synaptic signaling for several seconds in the face of near-maximal stimulations and in the absence of other sources of vesicles. Along with lower rates of depletion, additional mechanisms, such as replenishment from distal reserve pools and the fast recycling of vesicles, may further contribute to the maintenance of graded, tonic release from cone photoreceptors.

INTRODUCTION

Vertebrate photoreceptors continuously release the excitatory neurotransmitter glutamate in the dark (Ashmore and Copenhagen 1983; Baylor and Fuortes 1970; Baylor et al. 1971; Fuortes et al. 1973). How such release is supported at the vesicular level is not well understood. Until recently, much of the information regarding tonic release from photoreceptors has been inferred from an examination of postsynaptic responses. Although such studies are critical for understanding retinal signal processing, they do not readily allow for the extrapolation of precise information about photoreceptor synaptic vesicle dynamics. This is due, in part, to nonlinearities inherent in postsynaptic responses. Second, activity-dependent changes in ion concentration in the synaptic cleft and local circuit interactions all modulate photoreceptor neurotransmitter release (Heidelberger et al. 2005). Finally, the extensive electrical coupling of photoreceptors (Attwell et al. 1984, 1987; Gold and Dowling 1979; O’Brien et al. 2004; Raviola and Gilula 1973) may confound the ability to selectively measure release from an individual photoreceptor (Yang and Wu 1996).

To provide critical information about the mechanisms underlying maintained release in vertebrate photoreceptors, we studied exocytosis in freshly dissociated cone photoreceptors from the tiger salamander using a combination of time-resolved capacitance measurements and fluorescence measurements of intracellular calcium. Our results indicate that at least two pools of synaptic vesicles in cone photoreceptors can be enlisted to support maintained release: a releasable pool and a first reserve pool. The presence of a small, rapidly releasing pool was also suggested. The kinetics of depletion and refilling of the releasable pool, if refilled from the first reserve pool, are sufficient to maintain release in the face of near-maximal stimulation for several seconds. Model simulations indicate that if the depletion rate of the releasable pool were made slower, the period over which exocytosis was graded with depolarization length could be significantly extended. This period of graded release would presumably be extended for even longer periods, provided that additional mechanisms, such as the recruitment of vesicles from the large cytoplasmic reserve or from newly recycled vesicles, were used.

METHODS

Cells

Larval tiger salamanders (Ambystoma tigrinum) were handled and used according to the guidelines approved by the Center for Laboratory Animal Medicine and Care at the University of Texas Health Science Center at Houston. The animals were kept in a temperature-controlled aquarium at 5°C, under a 12-h light–dark cycle. Retinal photoreceptors were acutely dissociated by enzymatic digestion and mechanical trituration as previously described (Thoreson et al. 2004). Briefly, light-adapted larval tiger salamander were killed by decapitation and rapidly pithed. After enucleation, the retinae were isolated in chilled low-calcium solution consisting of (in mM): 110 NaCl, 2.5 KCl, 1 MgCl2, 0.5 CaCl2, 10 HEPES, and 11-glucose (pH 7.6). One retina was stored for ≤4 h, in the same solution, at 4°C, for later use. The other retina was chopped with a razor blade into four to seven pieces and then transferred to the enzymatic digestion solution containing (in mM): 110 NaCl, 2.5 KCl, 1 MgCl2, 0.5 CaCl2, 10 PIPES, and 11 glucose (pH 7.4), supplemented with papain 1 mg/ml + cysteine, 1 mg/ml. The retina was incubated for 12 min at 20°C and then washed with the digestion solution containing 0.2 mg/ml albumin, followed by a low-calcium solution wash. The digested retinal pieces were stored at 4°C until use. Pieces were allowed to recover for 30 min after enzymatic digestion treatment. Before an experiment, a piece of retina was gently mechanically triturated with a fire-polished Pasteur pipette in the experimental saline (see following text) and

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plated on coverslips previously coated with a salamander-specific antibody, Sal-1 (MacLeish et al. 1983). Isolated cones and rods were identified on the basis of their distinctive morphology (Fig. 1A). Most of the isolated cones lost their outer segment during trituration, facilitating voltage-clamp control, reducing noise in the capacitance measurements, and preventing light responses related to fura-2 emissions. Large single and double cones were selected because their synaptic pedicles were better preserved after the isolation procedure than those of smaller cones. Large single- and double-cone subtypes are the most abundant in the retina of the tiger salamander (Mariani 1986) and most of them (~80%) have spectral sensitivity in the red (Sherry et al. 1998). Because both subtypes behaved similarly, the data were pooled together.

**Capacitance measurements**

Patch-clamp experiments were performed in the tight-seal, whole cell configuration in a standard external solution designed to eliminate all ionic conductances other than those through voltage-gated calcium channels. The composition was (in mM): 96 NaCl or N-methyl-D-glucamine (NMDG), 2.5 KCl, 0.5 MgCl₂, 1.8 CaCl₂, 5 CsCl, 10 tetraethylammonium chloride (TEA), 10 HEPES, and 11 glucose (pH 7.6). NMDG was used to reduce cyclic guanosine 3’,5’-monophosphate (cGMP)-activated currents and to block the activity of a presynaptic glutamate transporter that is activated by released glutamate (Picaud et al. 1995a). Activation of the transporter is associated with a large inward Cl⁻ current (Picaud et al. 1995b) that could potentially interfere with accurate measurements of membrane capacitance. In addition to reducing the magnitude of the Cl⁻ current (Taylor and Morgans 1998), the use of NMDG prevented a shift in the reversal potential of the cone. We avoided the use of niflumic acid to block Cl⁻ currents because it had adverse affects on cell health. In addition, niflumic acid has been suggested to activate cGMP-gated channels (Flynn et al. 2006), which may allow calcium entry, thereby stimulating exocytosis (Rieke and Schwartz 1994; Savchenko et al. 1997). CsCl and TEA were used to block voltage-dependent K⁺ currents (a delayed rectifier and a calcium-dependent K⁺ current) and to minimize the contribution of an inward current (Iᵢ) activated by membrane hyperpolarization (Bader and Bertrand 1984; Bader et al. 1982; Barnes and Hille 1989; Marić and Korenbrot 1988).

Patch pipettes with resistances between 4 and 6 MΩ were pulled from thin-walled 1.5-mm filamented borosilicate glass and coated with Sylgard to reduce stray capacitance. The intracellular pipette solution contained (in mM): 100 Cs methane-sulfonate (CH₃CsSO₃), 5 TEA, 3 MgCl₂, 0.5 EGTA, 35 HEPES, 2 or 5 Na₂ATP, 0.5 GTP, and 0.2 fura-2 (pH 7.2). Methane-sulfonate replaced Cl⁻ with Sylgard to reduce stray capacitance. The intracellular pipette solution contained (in mM): 100 Cs methane-sulfonate (CH₃CsSO₃), 5 TEA, 3 MgCl₂, 0.5 EGTA, 35 HEPES, 2 or 5 Na₂ATP, 0.5 GTP, and 0.2 fura-2 (pH 7.2). Methane-sulfonate replaced Cl⁻ with Sylgard to reduce stray capacitance.

Capacitance recordings were acquired by a computer-based patch-clamp amplifier system (EPC-9/2, HEKA, Lambrecht, Germany) and controlled by Pulse software (HEKA). All voltages were corrected for a liquid-junction potential of 10 mV between external and internal solutions. Fast capacitative transients were canceled before rupture of the gigaseal (“break-in”) using the automatic capacitance compensation of the EPC-9 amplifier. Cells were voltage-clamped at a holding potential of −80 mV, and an 800-Hz sinusoidal stimulus of 30 mV (peak-to-peak) was applied around the holding potential. The resultant electrical response, filtered at 3.2 kHz, was processed using the Lindau–Neher technique, to yield estimates of membrane capacitance (Cₘ) and access conductance (Gₐ) for each sine-wave cycle (Lindau and Neher 1988), assuming a reversal potential of 0 mV at the holding potential. The phase angle setting was calculated by Pulse software based on the EPC-9 amplifier circuitry and verified using a model cell. To make time-resolved measurements, 100-ms sweeps were given continuously and one averaged data point per sweep for each parameter was delivered to the X-Chart extension of Pulse. Capacitance measurements were halted during the membrane depolarization due to the time- and voltage-dependent changes in Gₘ associated with calcium channel activation.

**[Ca²⁺]** measurements

For measurement of spatially averaged intracellular calcium ([Ca²⁺]ᵢ), alternating excitation at 345 and 388 nm was provided by a computer-controlled monochrometer-based photometry system (ASI/TILL Photonics; Messler et al. 1996) controlled by Pulse soft-
ware. To minimize photobleaching and UV-induced phototoxicity, a 10% neutral density filter (Omega Optical, Brattleboro, VT) was placed in the light path. In addition, during each 100-ms sweep, the specimen was illuminated at 345 and 388 nm for 30 and 35 ms, respectively, followed by 35 ms of darkness. Excitation light was reflected by a dichroic mirror (400dxru, Chroma Technology, Rockingham, VT) into a water-immersion objective (Achromplan 63×, Carl Zeiss MicroImaging, Thornwood, NY). An adjustable aperture was used to position the collection field for emitted fluorescence over the soma and the nerve terminal. Emitted light was directed through a 455 long-pass filter (Chroma Technology, Brattleboro, VT) to a photomultiplier tube (Hamamatsu H5784-03), by a viewfinder assembly (TILL Photonics). Intracellular calcium was calculated from the ratio of the emitted light at the two wavelengths (Grynkiewicz et al. 1985) using calibration constants determined by dialyzing cells with highly buffered, known concentrations of calcium (Heidelberger and Matthews 1992). All measurements were performed at room temperature (22–24°C).

Immunohistochemistry

Isolated retinal photoreceptors were fixed for 20 min in 4% (wt/vol) formaldehyde (Ladd Research, Williston, VT) in phosphate-buffered saline (PBS), pH 7.4, at room temperature (RT). After two washes in PBS, the cells were permeabilized with 0.3% Triton X-100 in PBS for 5 min. After two washes in PBS, the coverslips were incubated for 30 min, at RT, with the blocking solution made of 3% normal goat serum and 0.1% Triton X-100 in PBS, to reduce background staining. The primary antibodies were diluted (1:1,000) in blocking solution and 0.1% Triton X-100 in PBS, to reduce background staining. The primary antibodies were diluted (1:1,000) in blocking solution and incubated overnight at 4°C. After three washes with PBS, the samples were incubated for 1 h, at RT, with secondary antibodies labeled either with AlexaFluor 488 or AlexaFluor 568 (dilution 1:1,000; Molecular Probes, Carlsbad, CA) in blocking solution. After washes in PBS, the coverslips were mounted using antifade medium (Molecular Probes). Fluorescence images were acquired with a confocal microscope with a krypton–argon laser (Zeiss LSM 410).

Materials

Fura-2 K+-salt was obtained from Molecular Probes; GTPNa and ATPNa were from Boehringer; other chemicals were from Sigma. SV2 antibody was a generous gift from Dr. R. Janz and ribeye antibody from Dr. T. C. Sudhof (Buckley and Kelly 1985; Schmitz et al. 2000).

Analysis

Results are reported as means ± SE. Statistical significance was evaluated using a Student’s t-test in Microsoft Excel. Ensemble analyses were performed in IgorPro 4.0 (WaveMetrics, Portland, OR). For this purpose, capacitance records in response to pulses of the same length were aligned according to the peak of the capacitance response, baseline subtracted, and normalized to create ensemble averages.

Model simulations of synaptic vesicle pool dynamics in response to a single depolarization were based on the following kinetic scheme

\[
A \rightarrow \text{B} \rightarrow \text{fused}
\]

The differential equations used to describe the transitions between the states were solved using the Integrate ODE function of IgorPro 4.0. The starting magnitudes of the vesicle pools were taken from the data shown in Figs. 2B and 5B. The A pool was set to an initial value of 75 fF, the B pool was set to an initial value of 45 fF, and the number of fused vesicles was initially set to 0. The forward rate constant \(k_1\) was set to 1 s\(^{-1}\), consistent with the rate of recovery from paired-pulse depression (Fig. 6). The forward rate constant \(k_2\) was set to the reciprocal of the experimentally derived time constant for pool depletion (e.g., 1/420 ms; Fig. 2B). The transition from B back to A was not included in this model because no information is currently available about this process; only the overall forward rates have been experimentally determined. Furthermore, experiments in the ribbon synapses of bipolar cells have indicated that loss of vesicles from the releasable pool (i.e., the B pool) in the absence of refilling is negligible for at least several minutes (Heidelberger et al. 2002a). Finally, inclusion of a hypothetical backward rate constant adds a free parameter to the model, whereas one of the intents of this model is to relate the measured depletion and refilling rates to the overall secretory response evoked by a single stimulus, starting from the position of fully occupied A and B pools.

**FIG. 2.** Extent of exocytosis grows with pulse duration, revealing 2 kinetic components of release. A: a 0.5-s voltage step (−80 to −10 mV) evoked a 50-nm increase in intracellular calcium ([Ca\(^{2+}\)]\(i\)) (bottom, open triangles) and a 34-nm increase in \(C_{m}\) (top, filled circles). In the same cone, a 5-s depolarizing step given about 100 s later evoked a 340-nm rise in [Ca\(^{2+}\)]\(i\) and 115 fF in \(C_{m}\). Arrows mark the onset of each depolarization. B: relationship between \(\Delta C_{m}\) and the duration of membrane depolarization has 2 phases. Evoked \(\Delta C_{m}\) increased with pulse duration until it reached a plateau at approximately 50 fF. Rising phase is described by a single-exponential function (dotted line) given by: \(y(t) = 49.4 - 27.8e^{-0.33t}\). With longer depolarizations (>2 s), the evoked \(\Delta C_{m}\) jump increased further, such that the mean increase in \(\Delta C_{m}\) was 138 ± 31 fF (n = 4) at 10 s. This second component of release is described by the single-exponential function (solid line): \(y(t) = 144.9 - 188.3e^{-0.34t}\). Data points at each pulse duration are expressed as the average ± SE, for 3–15 trials performed in 26 cones.
RESULTS

Vertebrate photoreceptors are neurons that are specialized for the tonic release of the excitatory neurotransmitter glutamate by the process of exocytosis. At the vesicular level, the ability to release neurotransmitter continuously could rely on the existence of a virtually limitless releasable pool and/or of a highly efficient mechanism for replenishing the supply of releasable synaptic vesicles. To examine the strategies used by a vertebrate photoreceptor for supporting tonic release, we studied synaptic vesicle dynamics in acutely dissociated cone photoreceptors lacking outer segments from the tiger salamander using time-resolved membrane capacitance measurements.

Several features of the tiger salamander cone photoreceptor make it a suitable choice for this endeavor. First, isolated cone photoreceptors can easily be distinguished from other retinal neurons, including rod photoreceptors, by their characteristic morphology. Figure 1A (top) shows a typical isolated cone photoreceptor lacking an outer segment. Note the cone-shaped inner segment and the absence of a long axon. The relatively large soma of the tiger salamander cone photoreceptor is readily accessible for patch clamping, and the close proximity of the terminal and soma minimizes concerns about multiple electrical compartments that could confound capacitance measurements (Gillis 1995; Rabl et al. 2005; Taylor and Morgans 1998; Zhou et al. 2006). In addition, key synaptic proteins such as ribeye, a ribbon synapse active zone protein (Schmitz et al. 2000), and SV2, an intrinsic synaptic vesicle protein (Buckley and Kelly 1985), remain correctly localized to the synaptic terminal after isolation (Fig. 1A).

Freshly dissociated cone photoreceptors that had lost their outer segments during dissociation were patch clamped in the whole cell configuration within 3 h of dissociation. Exocytosis was triggered by a depolarizing voltage step (−80 to −10 mV) that activated calcium influx through voltage-gated channels (Fig. 1B, inset) and triggered the fusion of synaptic vesicles with the plasma membrane. The associated increase in membrane surface area was detected by monitoring changes in membrane capacitance (C_m). Figure 1B shows a typical experiment. A 0.5-s depolarizing pulse given at the time marked by the arrow evoked a 92-fF increase in membrane capacitance. There were no significant correlated changes in the membrane (G_m) or access (G_a) conductances, indicating that capacitance and conductance were adequately separated. On average, a 0.5-s voltage step triggered a 48.6 ± 10.6-fF increase in C_m (n = 10). Depolarization-evoked increases in C_m were not observed in cones that lost their synaptic terminal during the isolation procedure, indicating that increases in C_m arose from the terminal compartment. Consistent with a calcium-triggered process, the addition of CdCl2 (0.2 mM), a calcium channel blocker, to the bath (n = 5) or the elevation of the concentration of the calcium buffer EGTA in the internal recording solution from 0.5 to 5 mM (n = 7) completely abolished the response to a 0.5-s depolarization.

Kinetics of release

As a step toward determining the mechanisms that allow a cone photoreceptor to sustain neurotransmitter release, we examined the magnitude of the exocytotic response evoked by fixed-amplitude depolarizing voltage steps (−80 to −10 mV) of different lengths. This type of pulse-duration protocol is typically used to identify the different kinetic components of release that contribute to the secretory response (Horrigan and Bookman 1994; Moser and Beutner 2000; Thoreson et al. 2004; von Gersdorff and Matthews 1994a). Figure 2A shows a typical experiment, in which the calcium (bottom, triangles) and secretory responses (top, circles) of the same cone photoreceptor to depolarizations of 0.5 and 5 s are compared. In response to the 0.5-s depolarization, [Ca^{2+}]_i increased by 50 nM, evoking a 34-fF increase in C_m. The longer depolarization evoked a 340-nM rise in [Ca^{2+}]_i and a C_m increase of 115 fF.

In a similar manner, we recorded the secretory responses of 49 cells to depolarizations that ranged from 0.1 to 10 s in duration. To compare responses within and across cones, we designed a set of minimum standards. First, data were not included in this analysis if G_m at −80 mV was >1 nS. The rationale was that with increasing G_m, resting calcium also increased, and elevated calcium is known to affect several aspects of synaptic vesicle dynamics (Gomis et al. 1999; Rouze and Schwartz 1998; Smith et al. 1998; von Gersdorff and Matthews 1994b; von Ruden and Neher 1993). Second, the interval between depolarizations given to a cell was ≥30 s. This allowed the intracellular calcium levels to return to prestimulus levels between pulses, minimizing potential short-term facilitation, and allowed sufficient time for the releasable pool to be replenished (see following text). Third, a test stimulus, usually a 0.5-s depolarizing step, was randomly interspersed among the other stimuli. The amplitude of the response to this test stimulus was used to control for the potential rundown of calcium entry and/or the secretory capability of a cell. Cells that exhibited a change in response amplitude ≥10% were excluded from further study. Fourth, ΔC_m responses accompanied by changes in G_m >0.5 nS were excluded from the analysis even when it was apparent that the changes in C_m and G_m had different kinetics.

Figure 2B shows the pooled data from the 26 cone photoreceptors that met all criteria. The evoked change in C_m increased with pulse duration until a brief plateau was reached at about 50 fF. This component of release could be described by a single-exponential function with a time constant of 420 ms (Fig. 2B, dotted line). With even longer depolarizations, a second phase of release was revealed. The average increase in membrane capacitance for a 10-s depolarization was 138.5 ± 11.4 fF (n = 4). An exponential fit to the latter portion of the ΔC_m–pulse duration relationship suggests that this second component of release was about 95 fF in size and was depleted with a time constant of 3.0 s (Fig. 2B, solid line).

Origin of the two phases of the secretory response

A plateau in the secretory response has typically been interpreted to represent the depletion of a distinct pool of vesicles, with the resumption of exocytosis attributed to refilling of the releasable pool or the fusion of a second vesicle pool (Heidelberger 2001a). However, several mechanisms that are distinct from pool depletion could also potentially contribute to a brief plateau in the relationship between the amplitude of the secretory response and stimulus duration.

First, we considered whether there were changes in G_m that could potentially confound the measurement of exocytosis (Gillis 1995; Horrigan and Bookman 1994; Joshi and Fernandez...
significant change in evoked by a 5-s depolarization that is not accompanied by a 
flow through all ionic channels with the exception of (Cadetti et al. 2005; Taylor and Morgans 1998). Furthermore, 
reported previously for isolated cones (Rieke and Schwartz 1994), and higher than that reported for cones in retinal slices 
capacitance records demonstrate that the rate of \( C_m \) recovery after exocytosis is comparable for 0.5- and 5-s pulses. 
the capacitance record. In all experiments that met criteria for inclusion (i.e., Figs. 2–6), stimulus-associated changes in \( G_m \) were minimal (\( \Delta G_m < 0.5 \text{nS} \)). This was true even after a long depolarization. Figure 3 shows a representative example of a large (\( \sim 140 \text{fF} \)) capacitance response evoked by a 5-s depolarization that is not accompanied by a significant change in \( G_m \) (\( \Delta G_m = 0.07 \text{nS}; \text{bottom trace} \)). Thus the data do not support a role for significant changes in \( G_m \), either at rest or after a stimulus, that would affect the ability to measure changes in \( C_m \).

Next, we considered the potential contributions of membrane retrieval to the generation of an apparent secretory plateau. After the termination of a stimulus, \( C_m \) and \([\text{Ca}^{2+}]_i\) typically declined slowly back to baseline (i.e., Figs. 2A and 5A). The relaxation in \( C_m \) after calcium channel closure presumably represents endocytosis, although small contributions from ongoing exocytosis cannot be excluded (Cadetti et al. 2005; Rabl et al. 2005). If fast endocytosis were inhibited by longer, but not by short, depolarizations (e.g., Heidelberger 2001b; Neves and Lagnado 1999; von Gersdorff and Matthews 1994b), then the secretory response to long pulses might appear larger than predicted from the shorter pulses, thereby contributing to a discontinuity in the relationship between response amplitude and pulse duration. To test for this potential artifact, we compared the time course of recovery of \( C_m \) after exocytosis evoked by 0.5- and 5-s depolarizing pulses. These durations were chosen because they span the secretory plateau. Figure 4A shows a typical experiment. Exocytosis was evoked first by a 0.5-s depolarization (\(-80 \text{ to } -10 \text{ mV}\)) and then by a 5-s depolarization (\(-80 \text{ to } -10 \text{ mV}\)) in the same cone. As predicted from Fig. 2, the longer depolarization triggered a larger exocytotic response relative to the shorter depolarization (191 vs. 43 fF). The right panel shows the same responses normalized by the amplitude of the increase in \( C_m \). Note that in the first few seconds after closure of calcium channels, a period when endocytosis might dominate the capacitive record, no difference in the kinetics of recovery to basal \( C_m \) was observed.

We next compared the time course of endocytosis in a larger cohort of cones by creating ensemble averages of the \( C_m \) responses evoked by 0.5- and 5-s pulse durations (Fig. 4B). Despite the 10-fold difference in the length of the stimuli, there was no significant difference in the poststimulus return of \( C_m \) to baseline. In addition, the first seconds of \( C_m \) recovery after a 10-s depolarization (\( n = 4 \)) also exhibited kinetics similar to that of 0.5 s (data not shown). Thus these data argue against the possibility that a large, stimulus-dependent change in the rate of endocytosis gives rise to the two phases of the secretory response. Furthermore, the slow rate of recovery of \( C_m \) to resting levels suggests that endocytosis was unlikely to appreciably interfere with the measurement of exocytosis (Rabl et al. 2005).

A cessation in the magnitude of the secretory response with respect to increasing stimulus duration is commonly interpreted as representing the depletion of a discrete pool of releasable vesicles, although this interpretation relies on the assumption that calcium entry scales linearly with pulse duration. This is a reasonable assumption for brief depolarizations.

FIG. 4. Time course of \( C_m \) recovery is similar after a 0.5- or 5-s depolarization. A, right: capacitance response of a cone to a 0.5-s (black-filled circles) and 5-s (gray, open circles) depolarization are overlaid and aligned according to the peak of the response. Left: same \( C_m \) traces are shown after normalization by the increase in \( C_m \) and alignment according to the peak of the \( C_m \) change. B: ensemble averages of normalized capacitance records demonstrate that the rate of \( C_m \) recovery after exocytosis is comparable for 0.5- and 5-s pulses. Black-filled circles are normalized ensemble capacitance responses evoked by a 0.5-s depolarization (data are from 5 trials conducted in 5 cones). Gray open circles are normalized ensemble capacitance responses evoked by a 5-s depolarization (data are from 6 trials conducted in 3 cones). Traces were aligned so that time 0 represents the peak capacitance response after membrane depolarization. Error bars represent SE.

FIG. 3. Changes in \( C_m \) evoked by long depolarizations are not confounded by correlated changes in \( G_m \). A representative recording demonstrates that although changes in \( C_m \) may be large (140 fF; left), there is little change in \( G_m \) (right). Arrow marks the onset of a 5-s depolarization.
in photoreceptors because the presynaptic calcium channel exhibits little in the way of voltage-dependent inactivation and undergoes relatively slow calcium-dependent calcium channel inactivation (Bader et al. 1982; Corey et al. 1984; Heidelberger et al. 2005; Rabl and Thoreson 2002). However, with longer depolarizations, the extent of calcium entry may be sufficient to trigger calcium-dependent calcium channel inactivation (Rabl and Thoreson 2002), changing the amount of calcium entering the cell per unit time as a function of time. Also with longer depolarizations, contributions from calcium-induced calcium release might become more prominent (Cadetti et al. 2006; Suryanarayanan and Slaughter 2006).

Therefore to investigate whether there were unexpected nonlinearity in calcium signaling that could potentially contribute to the 45-fF plateau, we reexamined the data of Fig. 2B with respect to the magnitude of the stimulus-evoked increase in the spatially averaged calcium concentration ([Ca\(^{2+}\)]\(_{i}\)). In the cone shown in Fig. 5A, a 1-s depolarization evoked an approximately 200-nM rise in the spatially averaged internal calcium that triggered an approximately 57-fF increase in \(C_m\). Figure 5B shows a summary of data obtained from 11 cones. The magnitude of the capacitance increase grew with increasing calcium elevation until the spatially averaged calcium increase reached about 55 nM. Between 55 and 225 nM, no further increases in the mean capacitance response were detected. Beyond 225 nM [Ca\(^{2+}\)]\(_{i}\), a second increase in \(C_m\) was observed. This analysis indicates that changes in calcium entry and handling did not generate the two components of release. Taken together, the data suggest that the first component of release most likely represents a discrete, 45-fF pool of vesicles. The second component, which reached a plateau value of about 120 fF (Fig. 5B), suggests that the second component may represent the fusion of vesicles from a pool of about 75 fF.

**Refilling kinetics of the 45-fF pool**

The 45-fF pool is depleted either when the spatially averaged calcium is transiently elevated by 55 nM (Fig. 5B) or in response to 1-s depolarization (Fig. 2B). To examine the refilling rate of this releasable vesicle pool, we first depleted the pool using a stimulus that met the above-cited criteria and then probed the state of pool recovery after a variable time using the identical stimulus. Figure 6, A–C shows a representative experiment. In Fig. 6A, two identical depolarizations, separated in time by 5 s, were given. These stimuli evoked comparable elevations in intracellular calcium (Δ\([Ca^{2+}]_i\) ≈ 140 nM; Fig. 6B, right) that were of sufficient magnitude to deplete the first vesicle pool (Fig. 5B). Inspection of the corresponding capacitance trace reveals that the exocytotic responses were virtually identical (Δ\(C_{m1}\) = 46 fF; Δ\(C_{m2}\) = 44 fF; Fig. 6A, left) and corresponded in size to the first pool (Figs. 2B and 5B). When the two identical pulses were separated by 2 s, comparable elevations in calcium were again seen (~140 nM; Fig. 6B, right), but the amplitude of the second capacitance was reduced by 24% relative to the first (Δ\(C_{m1}\) = 46 fF; Δ\(C_{m2}\) = 35 fF; Fig. 6B, left). With a further decrease in the interval between pulses (interpulse interval = 1 s), the amplitude of the second capacitance was reduced by 41% relative to the first (Δ\(C_{m1}\) = 44 fF; Δ\(C_{m2}\) = 26 fF; Fig. 6C, left). This depression occurred despite a slightly larger increase in calcium with the second stimulus (~123 vs. ~146 nM; Fig. 5C, right). These results suggest that in the cone depicted, the releasable pool could be completely refilled within 2–5 s.

Data from seven such experiments are summarized in Fig. 6, D and E. To determine the time course of refilling of the releasable pool after its complete depletion, the ratio of the second capacitance increase relative to the first was plotted as a function of the interpulse interval (Fig. 6D). The relationship could be described by an exponential function with a time constant of about 1 s (Fig. 6D, curve), consistent with the observation that pool refilling was complete within a few seconds. Analysis of the calcium records demonstrated that with the exception of the shortest interpulse interval (0.5 s), the second calcium transient was virtually identical to the first and of sufficient magnitude to tap the entire releasable pool (Fig. 6E). Thus depression of the second calcium transient relative to the first did not underlie the depression and subsequent recovery of the capacitance response.

**Secretory behavior of the cone photoreceptor**

In a previous study in the salamander cone photoreceptor, the 45-fF pool described in detail in the present study was not reported (Rabl et al. 2005). One difference between the previous study and this one is that our selection criteria were specifically designed to reveal the potential for a small, discrete pool. To determine whether this pool might also be observed under conditions more similar to those of Rabl et al. (2005), we added to the data set of Fig. 2B cones that had resting \(G_m\) values as high as 3.9 nS (mean: 0.73 ± 0.14 nS, \(n = 49\)) and cones that had changes in \(G_m\) after the depolarization that exceeded 0.5 nS (mean: 0.17 ± 0.05 nS, \(n = 49\)), provided that...
vesicle pools—a releasable pool and a first reserve pool—that are in series such that exocytosis proceeds only from the releasable pool and the reserve pool refills the releasable pool (Fig. 7B; see also Schnee et al. 2005; Sikora et al. 2005). No free parameters were included in the model; every parameter was taken directly from the experimental data (see METHODS). The solid line depicts the simulated secretory response (Fig. 7A). Although this simple model does not predict a plateau in the relationship between the magnitude of the secretory response and pulse duration at 2 s, it does a good job of predicting the average secretory response for other pulse durations ≤ 5 s. The model undershoots the secretory response at 10 s because by this time point the first reserve pool also becomes depleted, and we chose not to incorporate a mechanism for the replenishment of this pool due to a lack of

the kinetics of $G_m$ and $C_m$ were distinct. The relationship between the magnitude of exocytosis and pulse duration for the expanded data set is shown in Fig. 7A. There is the suggestion of a plateau in the 0.5- to 2-s pulse-duration range. The amplitude, estimated by a single-exponential fit through the data (dotted line), is about 80 fF. The time constant for this apparent component is 811 ms, which is nearly two times slower than that of the 45-fF component. In addition, the increase in exocytosis with respect to pulse duration on the expanded data set is less dramatic in the 2- to 5-s range than that in the selected data set. The larger amplitude and slower time course of the apparent first component raise the possibility that, rather than represent a true vesicle pool, this apparent component may reflect a composite of the smaller, faster 45-fF pool and some amount of pool refilling. An earlier blending of refilling and release might also lead to the shallower rise in the amount of exocytosis between 2 and 5 s.

To compare the data sets in a quantitative manner, we used the data obtained from the selected data set to build a simple computational model of exocytosis in the cone photoreceptor. This model is not designed to capture the full complexity of synaptic vesicle dynamics (see also Hsu et al. 1996; Stevens and Wesseling 1999), but models of the form used here generally provide a reasonable description of many of the salient features of exocytosis (Neher 1998). We assumed two
information about this particular process. The good correspondence between the simulation and the expanded data set supports the identification of the 45-fF pool as a discrete vesicle pool and verifies the measured depletion and refilling rates.

Both the data and the simulation agree that there is an initial period of release in which the relationship between exocytosis and pulse duration is quite steep and approximately linear before reaching a plateau. Given the nature of glutamate release from cone photoreceptors, we manipulated the model parameters to see whether we could prolong this period of graded release. In the example shown (Fig. 7A, dashed line), we decreased the pool depletion rate, while holding the other parameters constant. This would be analogous to reducing the probability of release or stepping to a more modest membrane potential. With a 10-fold reduction in the depletion rate, the period over which release is graded with stimulus duration was extended from about 2 to nearly 10 s. Thus the two pools described in this study are sufficient to support graded and tonic release over a period of seconds.

**DISCUSSION**

To gain a more complete understanding of the vesicular mechanisms that support tonic neurotransmitter release from cone photoreceptors, we performed a detailed, time-resolved analysis of synaptic vesicle dynamics in the isolated salamander cone photoreceptor sans outer segment. Results of this investigation extend earlier work performed on release from intact cone photoreceptor measured in retinal slices. An advantage of the present study is that the measurements of neurotransmitter exocytosis are not confounded by electrical coupling between photoreceptors, and there are no concerns about network effects or changes in conductance or capacitance related to the membrane-rich outer segment.

**The releasable pool**

The first vesicle pool was identified by the plateau in Fig. 2B and confirmed as a true plateau in the secretory response by the data shown in Fig. 5. The magnitude of this pool was about 45 fF. This corresponds to a population of 700–1,200 vesicles, assuming a vesicle diameter of 35–45 nm (Lasansky 1973) and a specific capacitance of 1 μF/cm². Cone photoreceptors in several vertebrate species, including the tiger salamander, have been suggested to contain an average of 10–12 ribbons/terminal (Sterling and Matthews 2005; SM Wu, personal communication). Thus if evenly distributed, this pool would be predicted to be on the order of 50–100 vesicles per ribbon-style active zone. This is comparable to the magnitude of the releasable pool per active zone in retinal bipolar cells (Sterling and Matthews 2005; Zhou et al. 2006). However, it is ten times smaller that of the salamander rod photoreceptor (Thoreson et al. 2004), consistent with the use of numerous, small ribbons in cones relative to rods (Sterling and Matthews 2005). The cone pool is larger per active zone than the releasable pools of several conventional synapses (Fernandez-Alfonso and Ryan 2006; Stevens and Tsujimoto 1995; Taschenberger et al. 2002; but see Satzler et al. 2002), the high-output cerebellar mossy fiber terminal being an important exception (Saviane and Silver 2006). These comparisons support the hypothesis that neurons with a greater synaptic demand use larger releasable pools.

However, although the cone pool is relatively large, at a dark rate of about 20–80 vesicles·ribbon⁻¹·s⁻¹ (Ashmore and Copenhagen 1983), it would become quickly depleted within a few seconds in the absence of a mechanism for replenishment.

The time course of depletion for the cone releasable pool and the type of stimulus that triggers its depletion are reminiscent of the releasable pools found at other retinal ribbon synapses (Mennerick and Matthews 1996; Thoreson et al. 2004; von Gersdorff and Matthews 1997; Zhou et al. 2006). Also like the releasable pool of other well-characterized secretory cells and neurons, fusion of vesicles in this pool was completely blocked by the addition of 5 mM EGTA to the patch pipette (Heidelberg 2001). This suggests that, although vesicles in the cone releasable pool are located near the source of calcium that triggers release, the average vesicle in this releasable pool and the calcium channels that trigger its fusion are unlikely to be molecularly coupled. Thus this pool of vesicles is distinct from the pool that has been termed the “rapidly releasing” or “ultrafast” pool that is thought to represent a subset of releasable vesicles that are docked at the plasma membrane and thus nearest the sites of calcium entry and relatively resistant to blockade by millimolar EGTA (Heidelberger 2001a; Mennerick and Matthews 1996; Neher 1998).

A small, rapidly releasing pool has been identified in cone photoreceptors using paired recordings in retinal slices (DeVries 2000; Rabl and Thoreson 2007). This rapid pool is not blocked by 5–10 mM internal EGTA or BAPTA and therefore is unlikely to correspond to the releasable pool studied here. Rather, it may correspond to the small docked pool of rapidly releasing vesicles. Although we did not directly observe such a pool in our study, its presence is implied by the positive y-intercept of Fig. 2B. Because this pool is inferred by the presence of the releasable pool, rather than being directly measured, we do not know whether this pool is sensitive to millimolar EGTA. This implied small, rapid pool would most likely contribute to the fast activation of a concerted, postsynaptic current at light offset and, as suggested for bipolar cells (von Gersdorff et al. 1998), the releasable and first reserve pools would contribute to later components of release.

**Refilling of the releasable pool**

The fast rate at which the releasable pool refills contributes to the ability of a cone photoreceptor to maintain a continuous output. Under our experimental conditions, the releasable pool refilled with a time constant of about 1 s, achieving >95% refilling in about 3 s. By contrast, it takes nearly 20 s to refill the entire releasable pool at the goldfish Mb1 bipolar cell ribbon-style synapse (von Gersdorff and Matthews 1997) and at some conventional synapses (Stevens and Tsujimoto 1995; Stevens and Wesseling 1999; von Gersdorff et al. 1997). If the time constant for replenishment of the cone releasable pool were lengthened from 1 to 10 s, simulations indicate that the predicted secretory response would be about 34% smaller on average than the actual observed responses for stimuli 0.5–10 s in duration. Thus the speed of refilling determines not only the duration of maintained release, but also the magnitude of the response. Comparably fast components of pool refilling have been observed in another sensory-transducing neuron specialized for tonic release, the cochlear hair cell (Beutner et al. 2001), in a downstream synapse of the auditory pathway.
required for sound localization, the calyx of Held (Kushmerick et al. 2006), and in cerebellar mossy fiber terminals (Saviane and Silver 2006).

The fast refilling rate of the cone releasable pool reported here is reminiscent of the rate of recovery from paired-pulse depression reported for cones in retinal slices, although the latter is approximately ten times faster (DeVries 2000; Rabl et al. 2006). These studies, as noted earlier, may have focused on a subset of releasable vesicles (i.e., the rapidly releasing pool) rather than the entire releasable pool (DeVries 2000). Consistent with this interpretation, the time course of recovery from cone paired-pulse depression reported in slice recordings is comparable to the fast component of refilling attributed to the replenishment of the rapidly releasing pool at another ribbon synapse (Moser and Beutner 2000; Spassova et al. 2004). This interpretation is also consistent with the observation that the refilling of a release site after a stimulus that does not fully deplete the entire releasable pool is substantially faster than if the entire releasable pool had been depleted (Rosenmund and Stevens 1996; Stevens and Tsujimoto 1995; Stevens and Wang 1995). Another concern for recordings made in slices is that incompletely blocked circuit effects and postsynaptic receptor saturation may contribute to a form of synaptic depression that precedes pool depletion (Sun and Wu 2001). In addition, synaptic depression that precedes pool depletion can occur if a given stimulus does not evoke the required increase in intraterminal calcium (Xu and Wu 2005). In the present study, we circumvented these concerns by working in isolated cells, by carefully defining the releasable pool and the stimuli required to deplete it, and by monitoring intracellular calcium.

At first glance, the fast refilling rate observed in paired-pulse experiments appears incongruous with the ability to detect the depletion of the releasable pool in Fig. 2B. However, many neurosecretory cells, including retinal ribbon synapses (Burron et al. 2002; Mennerick and Matthews 1997; von Ruden and Neher 1993), exhibit calcium-accelerated refilling of the releasable pool. In the paired-pulse experiments, refilling of the depleted pool occurs during the interpulse interval, when calcium is still elevated from the first stimulus (Fig. 6), potentially accelerating pool refilling. By contrast, in the pulse-duration protocol of Fig. 2B, the cone is sitting at basal calcium before stimulation. Under these conditions, not only might pool refilling be somewhat slower, but potentially may occur after a short delay, as indicated by the fit through the data (Fig. 2B, solid line). Furthermore, when including data from cells with higher $G_{\text{in}}$, which were often associated with higher resting calcium concentrations, the secretory plateau occurred at a higher value (Fig. 7A), suggesting that more vesicles were recruited. Whether these additional vesicles represent the recruitment of new vesicles or the ability of elevated calcium to tap more distant, fusion-competent vesicles is unknown. The effect of calcium on pool refilling and the extent of exocytosis is a complex topic that also may be related to the amplitude and duration of the calcium signal (e.g., Bollmann and Sackmann 2005) and thus is beyond the scope of the present study.

Source of vesicles for pool refilling

At conventional synapses, the retrieval of vesicles is intimately linked to the ability of a synapse to maintain signaling, such that inhibition of endocytosis can lead to profound synaptic fatigue (Daly et al. 2000; De Camilli et al. 1995; Shupliakov et al. 1997). By contrast, at the ribbon synapses of retinal bipolar cells and hair cells, the evidence suggests that pool replenishment can occur from sources other than newly retrieved vesicles (Heidelberger et al. 2002b; Holt et al. 2004; Parsons et al. 1994; von Gersdorff and Matthews 1997). In the cone photoreceptor, the return of membrane capacitance to baseline after a stimulus is on the order of tens of seconds over a wide range of stimulus durations (Fig. 4). This is significantly slower than the time course of pool refilling (Fig. 6). Although we cannot rule out the possibility that contributions from ongoing exocytosis after the closure of calcium channels contribute to the slow recovery, components of endocytosis with time constants on the order of 10–40 s are commonly reported in neurons (Matthews 1996). Thus it is not unreasonable to propose that, similar to other ribbon synapses, the cone releasable pool may be refilled on a fast timescale from preformed vesicles.

The second component of release

The present study identified a second component of synaptic release activated when the spatially averaged change in internal calcium rose 300 nM above resting levels. The size of this component was extrapolated to be about 100 fF, a value roughly twice the size of the releasable pool. A major unanswered question is the mechanism by which this pool contributes to the maintenance of release. One possibility is that this second pool could represent the recruitment and subsequent fusion of vesicles that are located further from sites of calcium entry than vesicles in the first pool (Fig. 7B; see also Beaumont et al. 2005). The slower kinetics of release might then reflect the diffusion time of calcium to the distant vesicles, provided that the vesicles are poised for release at the plasma membrane at these distant locations. Suggestions of such ectopic release have been raised at several synapses, including retinal bipolar cells (Beutner et al. 2001; Lenzi et al. 2002; Matsui and Jahr 2003; Midorikawa et al. 2007; Zenisek et al. 2000, 2003). Although definitive evidence of ectopic release that exceeds the magnitude of active zone release has yet to be established, given the many contacts that they receive outside of ribbon-style active zones (Heidelberger et al. 2005), the possibility of ectopic release should be formally addressed in photoreceptors. If the two pools operate in series, the second pool (Fig. 7B, stippled circles) could represent vesicles that are recruited to refill empty sites at the active zone, in which case the slower kinetics of release might reflect the combination of calcium diffusion and vesicle movement, in addition to docking, priming, and fusion. Under some conditions, the second wave of release might also represent a form of compound fusion in which distant vesicles are recruited to the active zones, where they fuse with already docked or fused vesicles (e.g., Beutner et al. 2001; Edmonds et al. 2004; Fig. 7B, black vesicle). Additional experiments will be needed to distinguish between these different possibilities and determine where, with respect to the synaptic ribbon, the vesicles in this different pool reside and undergo fusion.
Experimental versus physiological stimuli

The stimuli used in this study are typical of those used to probe synaptic capabilities and define synaptic vesicle pools in a host of secretory cells, including ribbon synapses. They allow for invaluable comparisons across cell types and reveal insights into the different strategies used by photoreceptors for maintaining release. They can be used for the formulation of model simulations of exocytosis that drive further experimentation. However, one question that arises is how these types of stimuli compare with natural stimuli. Photoreceptors, for example, have a resting membrane potential in the dark of about −35 to −40 mV. In response to light, they hyperpolarize to −65 to −70 mV. An argument could be raised that photoreceptors in vivo do not experience the types of stimuli we used. However, although a cone hyperpolarizes when illuminated, it will de- polarize when its surround is illuminated (Baylor et al. 1971; O’Bryan 1973). These depolarizations can reach 0 mV and last several seconds (Barnes and Deschenes 1992), similar to our O’Bryan (1973). These depolarizations can reach 0 mV and last several seconds (Barnes and Deschenes 1992), similar to our stimuli. In addition, although the half-activation potential of cone $I_{Ca^{2+}}$ is often reported to be about −20 to −10 mV (Bader et al. 1982; Barnes and Hille 1989; Corey et al. 1984; Maricq and Korenbrot 1988), which is more positive than the cone dark potential, the calcium activation curve may be shifted to more negative potentials in intact tissue as a consequence of horizontal cell feedback and circuit interactions (Verweij et al. 1996). Thus certain physiological events may approximate the stimuli used here, and our data demonstrate that the two vesicle pools described here could meet the needs of such events. Moreover, our computational model predicts that more modest stimulus intensities will extend the period of time over which the relationship between the magnitude of release and stimulus duration is approximately linear, to further expand the dynamic range of the cone photoreceptor.

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