Two Actions of Calcium Regulate the Supply of Releasable Vesicles at the Ribbon Synapse of Retinal Bipolar Cells

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Ribbon synapses of sensory neurons are able to sustain high rates of exocytosis in response to maintained depolarization, but it is not known how this is achieved. Using the capacitance technique, we have found that Ca^{2+} regulates the supply of releasable vesicles at the ribbon synapse of depolarizing bipolar cells from the retina of goldfish. Ca^{2+} had two actions that could be differentiated by introduction of the Ca^{2+} chelator EGTA; one action stimulated refilling of the rapidly releasable pool of vesicles from a reserve pool, and a second action stimulated recruitment of vesicles to the reserve pool. The capacity of the reserve pool was ~3500 vesicles, which is similar to the number that can attach to the ribbons. These results suggest that continuous exocytosis at ribbon synapses is maintained by the Ca^{2+}-dependent translocation of vesicles from the cytoplasm, through the ribbon, to release sites on the plasma membrane.

Key words: synapse; vesicle; calcium; exocytosis; refilling; synaptic depression; retina; depolarizing bipolar cell; ribbon synapse; EGTA

The processes that make synaptic vesicles available for exocytosis play an important role in determining the efficiency of synaptic transmission during ongoing activity. For instance, many synapses exhibit short-term depression after a period of activity, and this is at least partly because of a decrease in the number of rapidly releasable vesicles docked at the plasma membrane (Del Castillo and Katz, 1954; Betz, 1970; Zucker, 1989; Rosenmund and Stevens, 1996; Dittman and Regehr, 1998). The question of how vesicles become available for exocytosis is of particular importance at ribbon synapses, which can support continuous high rates of exocytosis in response to maintained depolarization (Dowling and Ripps, 1973; Parsons et al., 1994; Lagnado et al., 1996; Rieke and Schwartz, 1996). Ribbon synapses are found in sensory neurons that generate graded voltage signals rather than action potentials, including photoreceptors and bipolar cells in the retina (Gray and Pease, 1971; Rao-Mirotznik et al., 1995; von Gersdorff et al., 1996) and auditory and vestibular hair cells in the ear (Jacobs and Hudspeth, 1990; Lenzi et al., 1999). Electron micrographs of these neurons show that the regions in which synaptic vesicles dock to the plasma membrane are associated with an electron-dense structure, called a ribbon or synaptic body, to which vesicles attach by short filaments. This arrangement suggests that vesicles attached to the ribbon form a reserve pool that supplies the active zone on the plasma membrane (Gray and Pease, 1971), although it has been difficult to test this idea experimentally (Burns and Augustine, 1995; Lenzi et al., 1999). Recently, our knowledge of the structure of ribbon synapses has been complemented by functional studies of exocytosis using capacitance and optical techniques. In particular, we have a relatively large amount of information about vesicle cycling at the ribbon synapse of depolarizing bipolar cells from the goldfish retina (Tachibana et al., 1993; Heidelberger et al., 1994; von Gersdorff and Matthews, 1994; Lagnado et al., 1996). An important feature of this synapse is that the size of the rapidly releasable pool of vesicles (RRP) can be directly measured using the capacitance technique (Mennerick and Matthews, 1996; Neves and Lagnado, 1999).

We have investigated the refilling of the RRP in goldfish bipolar cells during different patterns of stimulation and after introduction of the Ca^{2+} chelator EGTA. Our results indicate that Ca^{2+} entering through Ca^{2+} channels has two effects that stimulate the supply of vesicles to the RRP. Over the short-term, Ca^{2+} stimulated refilling of the RRP from a reserve pool, and over the long-term, Ca^{2+} stimulated the recruitment of vesicles to the reserve pool. The ability of residual Ca^{2+} to stimulate refilling of the RRP for periods of 5–10 sec indicates that this action occurs at free Ca^{2+} concentrations in the range of 1 μM or below. The capacity of the reserve pool was estimated to be ~3500 vesicles, which is similar to the total number attached to the ribbons, calculated from serial EM reconstructions (von Gersdorff et al., 1996). These results therefore provide functional evidence for the idea that the ribbon acts to supply vesicles to the active zone on the plasma membrane. Ca^{2+}-dependent trafficking of vesicles through the ribbon to the RRP may act to maintain high rates of exocytosis that occur during maintained depolarizing responses (Lagnado et al., 1996; Rouze and Schwartz, 1998; Neves and Lagnado, 1999).

MATERIALS AND METHODS

Isolated synaptic terminals of depolarizing bipolar cells from the goldfish retina were obtained by enzymatic dissociation (Burrone and Lagnado, 1997). Terminals were voltage-clamped using the perforated patch technique, and capacitance measurements were made using the piecewise linear method (Neher and Marty, 1982). Our implementation of this method is described by Neves and Lagnado (1999). Briefly, a sinusoidal command voltage (50 mV peak-to-peak, 2 kHz) was superimposed onto a holding potential of −70 mV. The output of the patch-clamp amplifier was analyzed by a two-phase lock-in amplifier, the phase of which was set using the "capacitance dither" technique, allowing independent measurement of changes in membrane capacitance and conductance (Gillis,

1995). The capacitance signal was calibrated by dithering the capacitance by 100 fF at the beginning of the recording episode. The change in capacitance elicited by the stimulus (ΔC_m) was measured by subtracting the capacitance signal before the stimulus from that measured after. The "before" measurement was averaged over a period of 50–300 msec, ending 20 msec before depolarization. The "after" measurement was averaged over a period of 50–300 msec, beginning 20 msec after repolarization.

The capacity of the RRP was measured after various stimulation patterns by measuring the capacitance increase elicited by a 20 msec depolarization to 0 mV. At this potential, the Ca^{2+} current is maximally activated. A 20 msec stimulus was sufficient to ensure that the whole of the RRP was exhausted, because it is normally released with a time constant of 2–4 msec (see Results). This duration stimulus also provided a safety margin that ensured depletion of the RRP when the Ca^{2+} current was partially inactivated, as sometimes occurred immediately after the introduction of a large Ca^{2+} load by prolonged depolarization.

The standard Ringer's solution contained (in mM): 120 NaCl, 2.5 $CaCl_2$, 2.5 KCl, 1 $MgCl_2$, 10 glucose, and 10 HEPES, pH 7.3. The solution in the patch pipette contained (in mM): 110 caesium gluconate, 4 $MgCl_2$, 3 Na_2ATP, 1 Na_2GTP, 10 TEACl, 0.4 BAPTA, and 20 HEPES, with 250 $\mu g/ml$ nystatin (260 mOsm/l, pH 7.2).

In some experiments terminals were loaded with the Ca^{2+} chelator EGTA by incubation in 0.1 or 0.2 mM EGTA-AM (0.1% DMSO; Molecular Probes, Eugene, OR) for 15–20 min.

All measurements are given as mean $\pm$ SEM.

RESULTS

Measuring the rapidly releasable pool of vesicles

In depolarizing bipolar cells, there is a clear kinetic distinction between the first and second phases of exocytosis elicited by strongly activating the Ca^{2+} current (Mennerick and Matthews, 1996; Neves and Lagnado, 1999). Here, we define the pool of vesicles involved in the first phase as the RRP. The approach we used to measure the size of the RRP is derived from the behavior shown in Figure 1. Figure 1*A* shows the capacitance response to two pairs of depolarizing stimuli to −10 mV, a potential at which the Ca^{2+} current is maximally activated. The interval between the stimuli was 100 msec. The duration of the first stimulus was varied between 0.5 and 20 msec so as to release a varying proportion of the RRP. These responses are plotted as the *filled circles* in Figure 1*B*. In this example, the maximum amplitude of the first phase of exocytosis was 45 fF, equivalent to ~1700 vesicles, assuming the capacitance of a single vesicle is 26 aF (Neves and Lagnado, 1999). The RRP was exhausted with a time constant of 4 msec, so a 20 msec depolarization was more than sufficient to release all of it. Here, we term a 20 msec depolarization to a potential that maximally activates the Ca^{2+} current (−10 or 0 mV) the "emptying" stimulus, and we used it to probe the size of the RRP after various patterns of stimulation.

Evidence that this approach does indeed measure changes in the size of the RRP is shown in Figure 1*B* in which the *open circles* plot the response to the emptying stimulus applied after variable amounts of the RRP were released. The decline in the measured size of the RRP mirrored the amount of rapid exocytosis elicited by the first stimulus. The *dashed line* shows the time course over which the RRP was expected to decline, assuming that the RRP was equivalent to 45 fF at the start of each trial and that there was negligible refilling in the 100 msec period between stimuli. The decline in the measured size of the RRP mirrored the amount of rapid exocytosis elicited by the first stimulus in similar experiments on eight other cells.

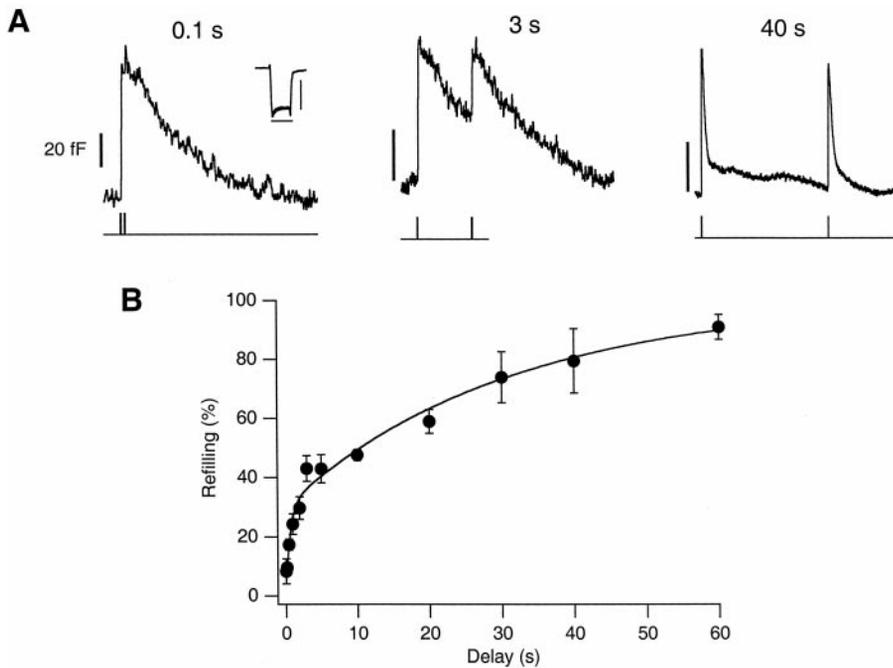### Refilling of the RRP after a brief stimulus

To assess how the RRP was refilled after a brief stimulus, two emptying stimuli were applied with varying delays, as shown in Figure 2*A*. After a delay of 100 msec, there was only a small
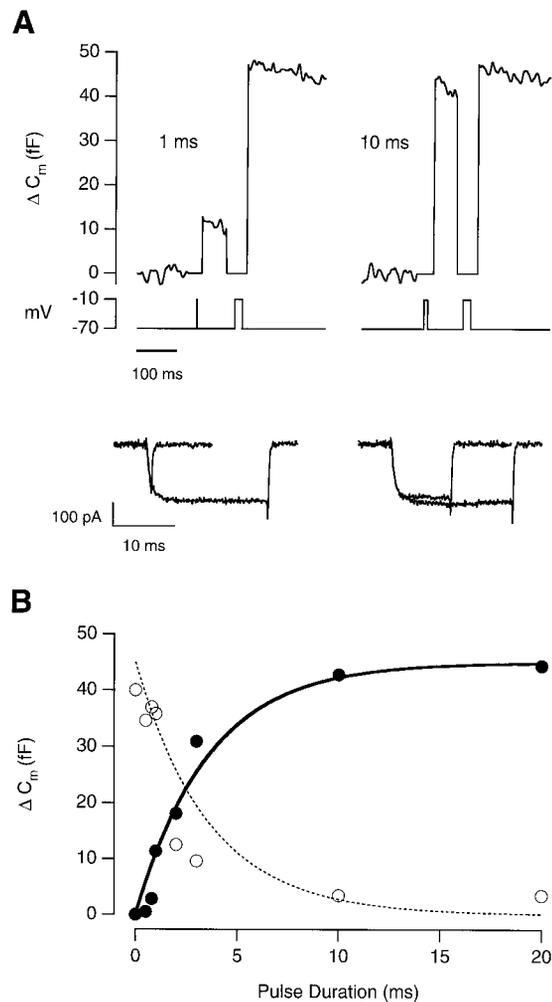
Figure 1. Measuring the rapidly releasable pool of vesicles. *A*, Capacitance increases elicited by a double-pulse protocol. The first depolarization from −70 to −10 mV lasted 1 msec in the example on the *left* and 10 msec in the example on the *right*. In each case, the second stimulus was a 20 msec depolarization, delivered after a delay of 100 msec, to release the remainder of the RRP (the emptying stimulus). The rest period between stimulation episodes was 1–3 min. The size of the RRP at the beginning of each stimulus episode was equivalent to a total response of ~45 fF. The Ca^{2+} currents are shown below. *B*, The capacitance increase elicited by the first stimulus plotted as a function of its duration (*filled circles*). The *bold line* through the *points* is a saturating exponential with a time constant of 3.6 msec and a maximum amplitude of 45 fF. The *open circles* plot the capacitance increase elicited by the emptying stimulus. The *dashed line* is a mirror image of the *bold line* (see Results).

response to the second stimulus (Fig. 2*A*, *top left*), averaging 6% of the first. The Ca^{2+} currents flowing during each stimulus were very similar (Fig. 2*A*, *inset*), so depression of the second response was not a result of inactivation of the Ca^{2+} current. As the interval between stimuli was increased, the second response recovered, reflecting refilling of the RRP (Fig. 2*A*, *middle*, *right*). Results from this type of experiment are collected in Figure 2*B*, which plots the refilling of the RRP as a function of the interval between stimuli. There were two phases of refilling: 30% of the RRP was refilled with a time constant of 0.64 sec, and the remainder with a time constant of 31 sec.

Behavior qualitatively similar to that shown in Figure 2 has also been observed at synapses from the CNS of mammals in which there are fast and slow phases of recovery from synaptic depres-

Figure 2. Refilling of the RRP after complete depletion by a 20 msec stimulus. *A*, The capacitance response to two 20 msec depolarizations is shown for intervals of 100 msec (*left*), 3 sec (*middle*), and 40 sec (*right*). In each case, the stimulus was a depolarization from -70 to 0 mV. The *inset* in the *left panel* is a superimposition of the Ca^{2+} currents elicited by two stimuli (calibration: 20 msec, 200 pA). Currents were leak subtracted using a single leak pulse (a 20 mV depolarization from -70 mV). *B*, The time course of refilling of the RRP measured from the type of experiment shown in *A*. The amplitude of the second response is expressed as a percentage of the first for various intervals. The *line* fitted through the *points* is a double-exponential, with 30% of sites refilled with a time constant of 0.64 sec and the remainder with a time constant of 31 sec. The number of observations for each interval are as follows: 100 msec, 7; 250 msec, 1; 500 msec, 24; 1 sec, 14; 2 sec, 13; 5 sec, 20; 10 sec, 10; 20 sec, 4; 30 sec, 3; 40 sec, 3; 60 sec, 3.

sion. The fast phase can be inhibited by the Ca^{2+} chelator EGTA, indicating that it reflects the transient acceleration of refilling by residual Ca^{2+} (Dittman and Regehr, 1998; Stevens and Wesseling, 1998; Wang and Kaczmarek, 1998). Below, we present evidence that residual Ca^{2+} also stimulates refilling of the RRP in the synaptic terminal of bipolar cells. However, at this ribbon synapse, Ca^{2+} appears to stimulate refilling of the RRP by two actions, one of which is more sensitive to EGTA.

Refilling of the RRP was stimulated by calcium

When the duration of the depolarizing stimulus was increased, refilling of the RRP was accelerated above the basal rate for a longer period. The protocol used to measure refilling after a longer stimulus is shown in Figure 3*A*, *Test*. First, the emptying stimulus was applied to measure the initial size of the RRP. Next, a 500 msec depolarization to 0 mV was delivered after a delay of 200 msec. Finally, the emptying stimulus was applied again to measure the RRP after a variable delay. In the example shown in Figure 3*A*, 91% of the RRP was refilled 10 sec after a 500 msec depolarization (*Test*), but only ~44% was refilled 10 sec after the emptying stimulus delivered alone (*Control*). The time course of refilling after depolarizations lasting 20 (*filled circles*) and 500 (*open circles*) msec is compared in Figure 3*C*. The basal rate constant of refilling, measured from the slow phase after a brief stimulus, was 0.03/sec (Fig. 2*B*). Figure 3*C* shows that after a 500 msec depolarization, refilling was accelerated above the basal rate for 5–10 sec until the RRP was almost completely replenished. In comparison, it took ~60 sec to refill 90% of the RRP after a 20 msec depolarization (Fig. 2*B*).

Although the net effect of a longer stimulus was to potentiate refilling of the RRP, at short delays the RRP was significantly smaller (Fig. 3*C*). For instance, in the example shown in Figure 3*B* (same terminal as Fig. 3*A*), there did not appear to be any refilling of the RRP 1 sec after a 500 msec depolarization, whereas ~19% was refilled 1 sec after a 20 msec depolarization. The delayed growth in the size of the RRP after a 500 msec stimulus is probably because residual Ca^{2+} continued to stimulate exocytosis after closure of Ca^{2+} channels. Capacitance and

optical measurements in bipolar cell terminals have demonstrated that residual Ca^{2+} can continue to drive exocytosis for periods of 1 sec or more, at rates of 400–800 vesicles/sec (Neves and Lagnado, 1999). We would expect that vesicles accumulate in the RRP only after their removal by exocytosis has ceased, which can take ~1 sec after a 500 msec depolarization to 0 mV. Consistent with the results in Figure 3*C*, exocytosis does not continue after a 20 msec stimulus that introduces a smaller amount of Ca^{2+} (Neves and Lagnado, 1999).

An idea of the changes in free cytoplasmic $[Ca^{2+}]$ that occurred after stimulation could be obtained using Ca^{2+}-activated conductances in the plasma membrane that are activated by residual Ca^{2+} and generate a slowly decaying tail current (Okada et al., 1995). Measurements of cytoplasmic $[Ca^{2+}]$ have demonstrated that the amplitude of this current is correlated with the spatially averaged $[Ca^{2+}]$, and it declines with a time constant that is similar to the fall in $[Ca^{2+}]$ (Neves and Lagnado, 1999). Figure 4*A* shows that the Ca^{2+}-activated tail current was much larger and longer after a 500 msec depolarization than after a 20 msec depolarization.

We tested whether residual Ca^{2+} might be the stimulus that accelerated refilling after a longer stimulus by loading terminals with the Ca^{2+} chelator EGTA so as to reduce the rise in cytoplasmic $[Ca^{2+}]$. Terminals were loaded by incubation in the AM ester (see Materials and Methods). Buffering the rise in $[Ca^{2+}]$ greatly reduced the Ca^{2+}-activated tail current (Okada et al., 1995). This was most clearly observed after a 500 msec depolarization (Fig. 4*A*,*C*), but it was also apparent after a 20 msec depolarization that caused a small activation of the tail current (Fig. 4*B*). The tail current averaged 50 ± 6 pA ($n = 20$) immediately after a 500 msec depolarization in a control terminal but only 10 ± 2 pA ($n = 12$) after loading with EGTA. Experiments in which we have altered the internal concentration of Ca^{2+} buffers through a patch pipette indicate that at least 2 mM EGTA is required to cause a similar inhibition of the tail current (J. Burrone and L. Lagnado, unpublished observations; Okada et al., 1995).

EGTA shortened the accelerated phase of refilling that fol-

A   10 s delay

B   1 s delay
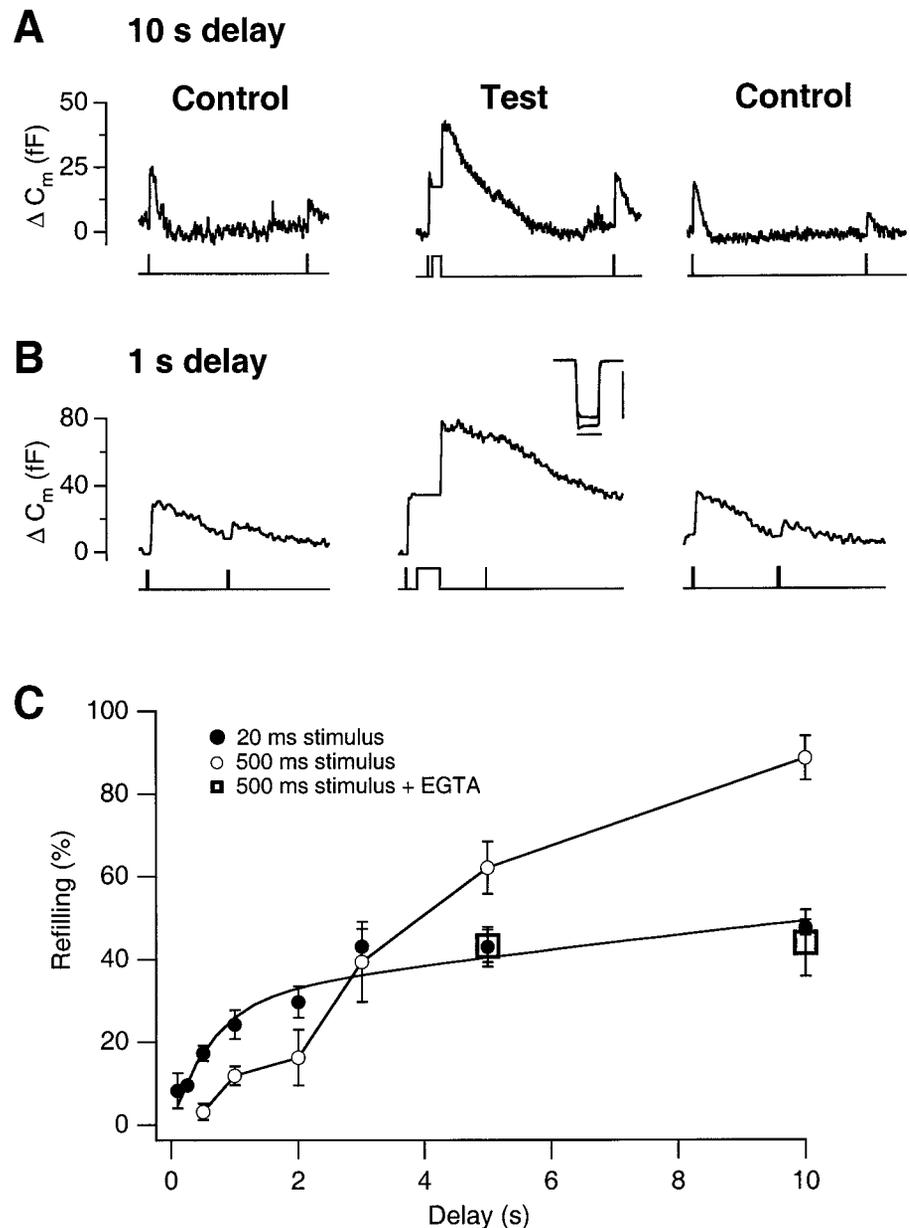
Figure 3. Refilling of the RRP after a long stimulus. *A*, Recovery of the capacitance response 10 sec after a 20 msec depolarization (*Control*) is compared with that measured 10 sec after a 500 msec depolarization (*Test*). Note that in the *Test* protocol, the 500 msec stimulus was preceded by a 20 msec depolarization to measure the initial size of the RRP. In this terminal, the RRP recovered 91% of its initial size 10 sec after a 500 msec depolarization but only 44% in the controls immediately before and after. *B*, Recovery of the capacitance response 1 sec after a 20 msec depolarization is compared with that measured 1 sec after a 500 msec depolarization. In this terminal, the RRP did not exhibit any measurable recovery 1 sec after the 500 msec stimulus but recovered 17% in the control immediately before and 21% in the one after. The *inset* in the *middle panel* is a superimposition of the Ca^{2+} currents elicited by the two emptying stimuli (calibration: 20 msec, 200 pA). *C*, The time course of refilling of the RRP measured from the type of experiment shown in *A* and *B*. The *open circles* show refilling measured after a 500 msec depolarization; the amplitude of the response to the second depleting stimulus is expressed as a percentage of the first for various intervals. The *open squares* show results obtained in terminals loaded with EGTA (see Results and Fig. 5). The *closed circles* provide a comparison with the results in Figure 2*B*, showing refilling measured after a 20 msec depolarization.

lowed a long stimulus. Examples of these measurements are shown in Figure 5*A*, and averaged results for delays of 10 and 5 sec are plotted as the *open squares* in Figure 3*C* to allow a comparison with cells that did not contain any exogenous Ca^{2+} buffers. After a 500 msec depolarization, the acceleration of refilling above its basal rate normally continued for ~10 sec (*open circles*), but buffering the rise in cytoplasmic $[Ca^{2+}]$ blocked refilling at delays longer than 5 sec (*open squares*). EGTA did not, however, block the initial accelerated phase of refilling. Figure 3*C* shows that 43 ± 4% of the RRP was refilled over the first 5 sec, although there was no further refilling over the next 5 sec. The refilling of 43% of the RRP in the first 5 sec indicates an average rate constant of 0.09/sec over this period. In comparison, the basal rate constant of refilling was 0.03/sec in control terminals, as measured from the second phase of the double-exponential fit in Figure 2*B*. The initial rate constant of refilling in EGTA-loaded terminals was therefore at least three times that measured in control terminals at resting levels of Ca^{2+}.

The effects of EGTA on the first and second phases of exocytosis

We compared the effects of EGTA on refilling of the RRP with its effects on the different kinetic components of exocytosis. EGTA had a small effect on exocytosis from the RRP. The first response to a 20 msec depolarization averaged 55.5 ± 4.7 fF ($n = 58$) in control terminals and 40.6 ± 3.6 fF ($n = 38$) in terminals loaded with EGTA (Fig. 5*B*). The somewhat smaller response in the presence of EGTA might reflect a slowing of exocytosis from the RRP and/or a decrease in the initial size of the pool. The latter possibility seems more likely because the kinetics of fast exocytosis are little affected by EGTA (Mennerick and Matthews, 1996), whereas EGTA inhibited refilling of the RRP over long time scales (see below). Our estimate of the initial size of the RRP in EGTA-loaded terminals is therefore likely to be a lower limit, especially in view of the fact that isolated bipolar cell terminals can spontaneously depolarize, triggering exocytosis

A

B

C
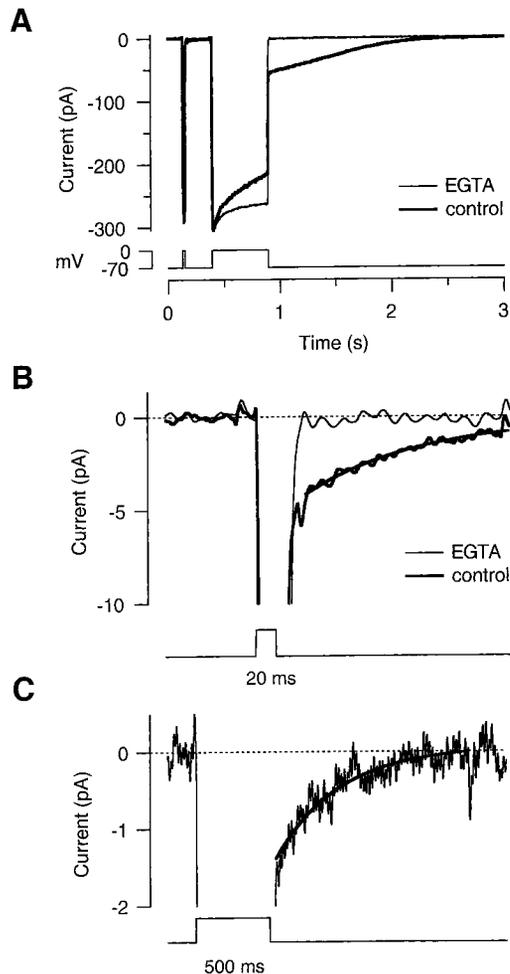
Figure 4. The rise in cytoplasmic $[Ca^{2+}]$ was reduced in terminals loaded with EGTA. *A*, A comparison of the currents elicited by a 20 and 500 msec depolarization from -70 to 0 mV in a terminal with endogenous Ca^{2+} buffers (*bold line*) and a terminal additionally loaded with EGTA (*thin line*). These terminals were used for this comparison because the Ca^{2+} currents were of similar amplitude. EGTA blocked the Ca^{2+}-activated tail current that decayed slowly after the 500 msec stimulus. The latter part of the decay in the tail current occurred with a time constant of 0.51 sec in the control. *B*, The tail currents after the 20 msec stimuli shown on expanded scales. The *bold line* fitted to the control *trace* has a time constant of 0.11 sec. *C*, EGTA did not completely block the Ca^{2+}-activated tail current after a 500 msec stimulus; expansion of *trace* in *A*. The *bold line* fitted to the *trace* has a time constant of 0.43 sec.

(Burrone and Lagnado, 1997). In an effort to ensure that estimates of the size of the RRP were made before exocytosis had been stimulated to any appreciable extent, cells were loaded with EGTA in the absence of external Ca^{2+}, and Ca^{2+} was replaced only after Ca^{2+} channels were held closed by voltage-clamping the terminal.

EGTA had a stronger inhibitory effect on the second, slower, phase of exocytosis. Figure 5*A* compares responses to a 20 and 500 msec stimulus, first under control conditions and then in a terminal loaded with EGTA. Considering only the first stimulus episode in a given terminal, a 500 msec depolarization normally released 1.56 ± 0.24 ($n = 22$) times as many vesicles as the preceding emptying stimulus. However, after loading with EGTA, this ratio was reduced to 0.73 ± 0.17 ($n = 10$) (Fig. 5*C*). We propose that these two actions of EGTA, inhibition of the second phase of exocytosis and reduced refilling after a long

stimulus, reflects the involvement of the same set of vesicles, which undergo Ca^{2+}-dependent transfer to the RRP (see Discussion).

Recruitment to a reserve pool was stimulated by calcium

To investigate refilling over longer time scales, the RRP was repeatedly exhausted by delivering trains of emptying stimuli at a frequency of 0.1 Hz for durations of 3 min or more. The second response in a train normally released 40–50% as many vesicles as the first (Fig. 6*A,B*), as would be expected from the results obtained with a double-pulse protocol (Fig. 2*B*). The amplitude of the capacitance response elicited by the emptying stimulus then remained constant, indicating that the same fraction of the RRP was refilled after each emptying stimulus and that the average rate of refilling was fixed (Fig. 6*A*). The cumulative response during these stimulus trains, relative to the initial size of the RRP, is plotted as the *filled circles* in Figure 6*C*. The first response averaged 50 ± 15 fF ($n = 18$). The RRP could be refilled at least ten times over, equivalent to the transfer of at least 20,000 vesicles, without any apparent depletion of the reservoir from which it was supplied.

When terminals were loaded with EGTA, the rate of refilling was not affected at first, because the second response was still $\sim50\%$ of the first (Fig. 6*A,B*). Thus, EGTA did not directly affect the transfer of vesicles to the RRP after a brief stimulus. Over the long term, however, the rate of refilling gradually declined during a train, until no more vesicles could be transferred to the RRP and the capacitance response was abolished (Fig. 6*A,B*). The cumulative response during these stimulus trains is plotted as the *open circles* in Figure 6*C*. In the presence of EGTA, the maximum number of vesicles transferred to the RRP averaged 2.7 times its initial size, or $\sim3500 \pm 400$ vesicles (the amplitude of the first capacitance response in this set of EGTA-loaded terminals was 33.4 ± 3.5 fF; $n = 16$). In other words, only ~3500 vesicles could be rapidly transferred from a reserve pool to the RRP in a terminal loaded with EGTA. A simple explanation for this result is that refilling of the RRP occurs from a reserve pool, which has an initial capacity of ~3500 vesicles, and EGTA blocks the transfer of vesicles to this pool, causing it to gradually empty (see Discussion).

If EGTA inhibits refilling of the reserve pool by buffering Ca^{2+} transients, then it should be possible to overcome this effect by introducing a larger Ca^{2+} load into the terminal. A demonstration that this is the case is shown in Figure 7. In this experiment, both the RRP and reserve pool were completely exhausted by applying a train of emptying stimuli, as in Figure 6. After a 150 sec period of rest, application of the emptying stimulus elicited a response of only 2 fF, confirming that the RRP was refilled very slowly at resting levels of Ca^{2+}. A larger number of vesicles were released by a 500 msec depolarization, and refilling of the RRP was stimulated, as demonstrated by the much larger response to the emptying stimulus applied after a delay of 10 sec. Similar behavior was observed in four other terminals. These results provide further support for the idea that Ca^{2+} stimulates the transfer of vesicles to the reserve pool and then to the RRP.

DISCUSSION

Two basic observations indicate that Ca^{2+} stimulates refilling of the RRP in the synaptic terminal of depolarizing bipolar cells. First, accelerated refilling of the RRP was prolonged by introducing a larger Ca^{2+} load into the terminal (Fig. 3*C*), and this

Figure 5. EGTA inhibited the second phase of exocytosis and the acceleration of refilling caused by a prolonged stimulus. *A*, Recovery of the capacitance response after a 500 msec depolarization is compared in a terminal with endogenous Ca^{2+} buffers (*left*) and a terminal additionally loaded with EGTA (*right*). In each case, the size of the RRP was measured after a delay of 10 sec. EGTA had two effects: a 500 msec depolarization caused less exocytosis, and recovery of the RRP was reduced. *B*, EGTA caused a small reduction in the capacitance response to the emptying stimulus. Only the first response in each terminal was used for this comparison because EGTA inhibited refilling of the RRP over the long term (see Results). *C*, EGTA inhibited exocytosis from the second pool of vesicles. The capacitance response to a 500 msec depolarization is shown relative to the response to the emptying stimulus delivered 200 msec before. All measurements were made from terminals in which these were the first stimuli.
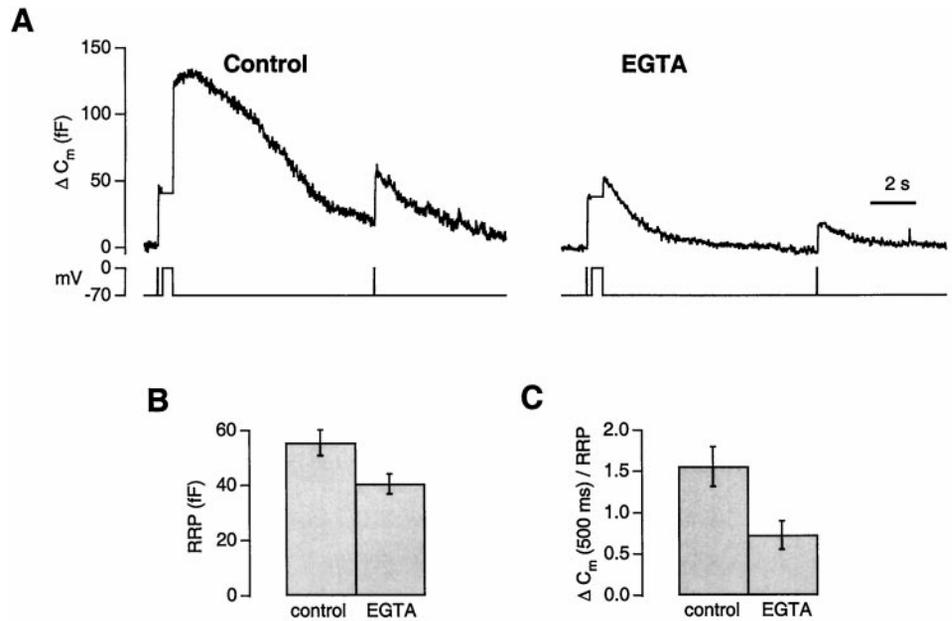
effect could be inhibited by buffering the rise in cytoplasmic $[Ca^{2+}]$ with EGTA (Fig. 5). Second, EGTA limited the number of vesicles that could be released by repetitive brief stimuli (Fig. 6), and this effect could be counteracted by introducing a larger Ca^{2+} load into the terminal (Fig. 7).

## Vesicle pools in the bipolar cell terminal

After brief depolarizations, EGTA blocked the Ca^{2+}-dependent transfer of all but a small fraction of available vesicles, although it had no immediate effect on refilling of the RRP (Fig. 6). The differential action of EGTA allows us to provide a functional definition for two pools of vesicles involved in refilling of the RRP: a reserve pool of ~3500 vesicles that refills the RRP over the short term, and a much larger reservoir that maintains the supply of vesicles over the long term. Figure 8 shows a simple model of vesicle transfer in the bipolar cell terminal that seeks to

integrate these results with previous anatomical and physiological work. Features of this model are discussed below.

It is possible to define at least three pools of vesicles in the bipolar cell terminal. When the Ca^{2+} current is activated maximally, the most rapid phase of exocytosis involves the release of 1200–1800 vesicles within ~10 msec (Heidelberger et al., 1994; Mennerick and Matthews, 1996; Neves and Lagnado, 1999). A similar number of vesicles are docked to the plasma membrane beneath the ribbon, suggesting that these correspond to the RRP (von Gersdorff et al., 1996). The second phase of exocytosis involves the release of ~4400 vesicles over 1 sec (Neves and Lagnado, 1999). Two pieces of evidence indicate that these vesicles constitute the reserve pool that is used to refill the RRP after closure of Ca^{2+} channels. First, the reserve pool contained a similar number of vesicles, ~3500 (Fig. 6). Second, EGTA

Figure 6. EGTA inhibited refilling of a reserve pool of vesicles. *A*, Capacitance responses during a train of emptying stimuli (20 msec depolarizations to 0 mV) delivered at a frequency of 0.1 Hz. The *top* is from a control terminal, and the *bottom* is from a terminal loaded with EGTA. The figures below the responses indicate the stimulus number in the train. *B*, Averaged results from the type of experiment shown in *A*. The capacitance responses during the stimulus train are plotted relative to the first response, which averaged 50 fF in control terminals (*n* = 18; *closed symbols*) and 33 fF in terminals loaded with EGTA (*n* = 16; *open symbols*). In control terminals, the second response was 45% of the first, and subsequent responses were not significantly different. In terminals loaded with EGTA, the response to the emptying stimulus gradually declined to zero. *C*, The accumulated amount of exocytosis averaged from the type of experiment shown in *A*. In control terminals, refilling of the RRP occurred at a constant average rate, as indicated by the *solid line*. In terminals loaded with EGTA, the maximum number of vesicles that could be transferred to the RRP was 2.7 times its initial size, as indicated by the *dashed line*.
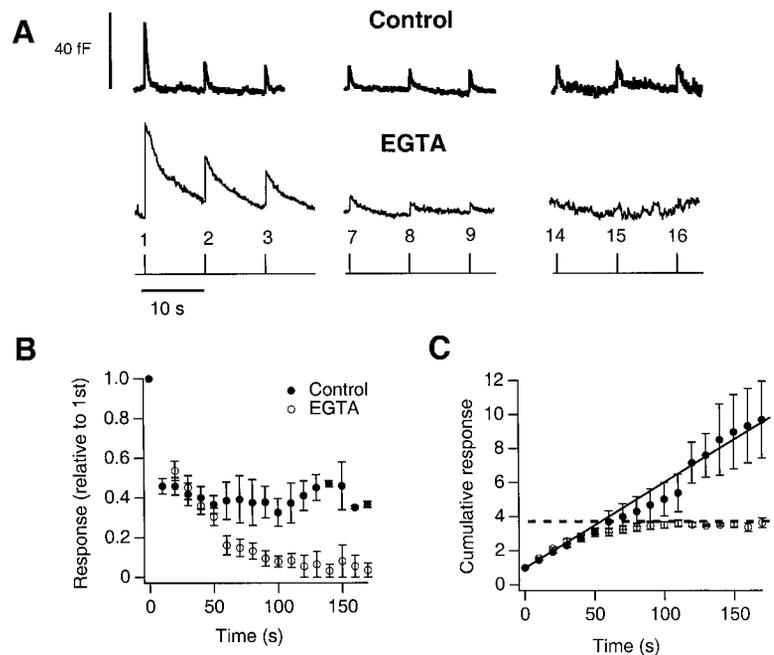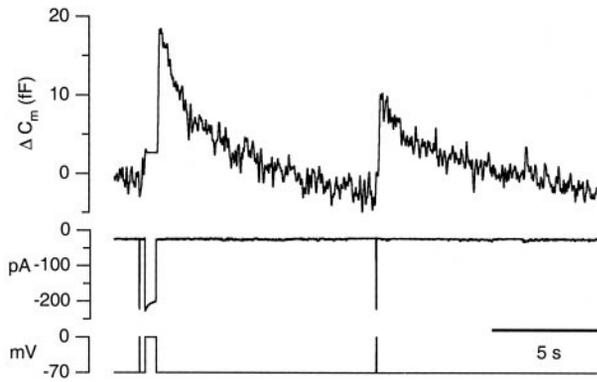
Figure 7. The effects of EGTA were partially overcome by introduction of a large Ca^{2+} load. Capacitance response from a terminal loaded with EGTA, after complete depletion of both the RRP and reserve pool by a train of stimuli. Before this stimulation episode, the terminal was at rest for 150 sec. The first emptying stimulus elicited a negligible response. A 500 msec depolarization stimulated exocytosis and caused the RRP to refill, as demonstrated by the much larger response to the second emptying stimulus.
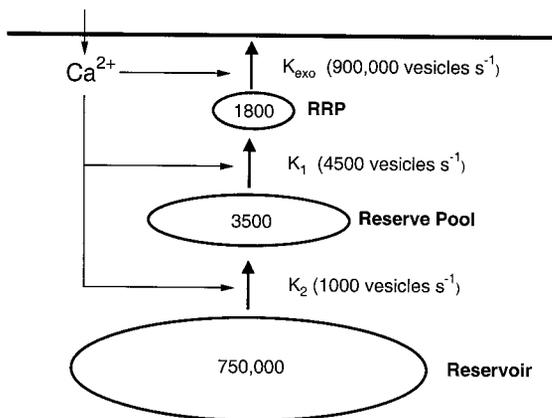
Figure 8. Vesicle pools in the synaptic terminal of depolarizing bipolar cells. Evidence for this model is summarized in Discussion. The *numbers* in *parentheses* indicate the rates of the vesicle transport when the Ca^{2+} current is maximally activated.

inhibited the second phase of exocytosis and also reduced refilling of the RRP stimulated by residual Ca^{2+} (Fig. 5). The anatomy of the bipolar cell terminal immediately suggests that the reserve pool of vesicles is attached to the ribbon behind the plasma membrane, and this idea is supported by EM measurements indicating that there are ~4800 vesicles attached to the ribbons (von Gersdorff et al., 1996). The third component of exocytosis observed during maintained depolarization occurs at a rate of ~1000 vesicles/sec but does not fatigue for periods of many tens of seconds (Lagnado et al., 1996; Rouze and Schwartz, 1998; Neves and Lagnado, 1999). Measurements made with FM1–43 indicate that this continuous process of exocytosis is supported by a reservoir of ~750,000 vesicles that is efficiently replenished by endocytosis (Lagnado et al., 1996). EM measurements indicate that there are a similar number of vesicles in the cytoplasm, free of the ribbons (von Gersdorff et al., 1996).

In the model shown in Figure 8, the rate constants K_1 and K_2 are both increased by Ca^{2+}, but during brief periods of Ca^{2+} influx, EGTA is more effective at preventing the increase in K_2. When the Ca^{2+} current is activated maximally, the second phase of exocytosis occurs at a rate of ~4500 vesicles/sec (Neves and

Lagnado, 1999), so the maximum rate constant for the transfer of vesicles from the reserve pool to the RRP, K_1, is ~1.3/sec (assuming the capacity of the reserve pool is 3500 vesicles). As might be expected, the rate of refilling that we measured after closure of Ca^{2+} channels was somewhat slower, occurring with a rate constant of ~0.5/sec in the few hundred milliseconds after a 20 msec depolarization (Fig. 2*B*). It appears that Ca^{2+} could modulate the rate of refilling over a range of ~40-fold, because the basal value of K_1 at resting levels of Ca^{2+} was ~0.03/sec (Fig. 2*B*). After the first two pools of vesicles have been exhausted, continuous exocytosis occurs at a rate of ~1000 vesicles/sec (Neves and Lagnado, 1999), indicating that the maximum rate constant for the transfer of vesicles from the reservoir to the reserve pool, K_2, is ~1.3×10^{-3}/sec.

Two actions of Ca^{2+}

The differential effect of EGTA on vesicle transfer to the RRP and reserve pool (Figs. 6, 7) might be explained if the Ca^{2+}-binding molecule regulating K_2 was further from Ca^{2+} channels than the Ca^{2+}-binding molecule regulating K_1. During brief periods of Ca^{2+} influx, the molecule regulating K_2 would be subject to smaller rises in $[Ca^{2+}]$ that are more effectively buffered by EGTA (Neher, 1998).

The EGTA concentrations we introduced into the terminals were sufficient to strongly buffer Ca^{2+} at the Ca^{2+}-dependent conductances in the plasma membrane (Fig. 4). Two factors might explain why they were not sufficient to inhibit refilling of the RRP after a brief stimulus (Fig. 6). First, the Ca^{2+}-sensor for refilling may be closer to Ca^{2+} channels than the Ca^{2+}-dependent conductances, so that it senses a larger rise in $[Ca^{2+}]$ while Ca^{2+} channels are open. Second, the Ca^{2+}-sensor for refilling may have a higher affinity for Ca^{2+} than the Ca^{2+}-dependent conductances. The latter idea is supported by the observation that residual Ca^{2+} stimulated refilling for ~10 sec after a 500 msec depolarization (Fig. 3*C*), whereas the Ca^{2+}-activated tail current had decayed completely after 2 sec (Fig. 4). Neves and Lagnado (1999) used furaptra, a Ca^{2+}-indicator of relatively low affinity, to measure the cytoplasmic $[Ca^{2+}]$ in the bipolar cell terminal. Strongly activating the Ca^{2+} current for 1 sec caused the spatially averaged $[Ca^{2+}]$ to reach ~25 μM. After repolarization, the $[Ca^{2+}]$ fell to undetectably low levels with a time constant of ~0.7 sec, causing the Ca^{2+}-activated tail current to decay similarly (Neves and Lagnado, 1999, their Fig. 10). Together, these measurements indicate that residual Ca^{2+} stimulated the transfer of vesicles at concentrations that did not activate Ca^{2+}-dependent conductances at the plasma membrane and that were close to the limit of detection with furaptra. In the experiments of Neves and Lagnado (1999), it was quite possible to detect Ca^{2+} levels of 1.5 μM with furaptra, so this figure provides an upper limit for the $[Ca^{2+}]$ sufficient to stimulate the transfer of vesicles to the RRP.

What is the relationship between transient and continuous exocytosis?

Lagnado et al. (1996) and Rouze and Schwartz (1998) have demonstrated that continuous exocytosis at the bipolar cell synapse can be driven by Ca^{2+} concentrations of the order of 1 μM. Refilling of the RRP probably occurs at similar low levels of Ca^{2+}, suggesting that continuous exocytosis might occur by the trafficking of vesicles from the bulk cytoplasm, through the reserve pool, to the RRP (Fig. 8). The initial rapid phase of exocytosis requires much higher levels of Ca^{2+}; it is half-

maximal at $\sim$200 μM Ca^{2+} (Heidelberger et al., 1994). So, if the model presented in Figure 8 is correct, the final Ca^{2+}-dependent step during the first phase of release is probably different from that controlling the later phases. This might occur if vesicles docked at release sites could undergo exocytosis from one of two states: state 1 in which fusion requires Ca^{2+} to bind to a sensor of high affinity (or does not require Ca^{2+} at all), and state 2 in which fusion requires Ca^{2+} to bind a sensor of low affinity. If all docked vesicles were in state 2 after a period of rest, the first phase of exocytosis measured experimentally would have a requirement for high levels of Ca^{2+}. But if vesicles arrived at release sites in state 1, the continuous component of exocytosis would occur at lower Ca^{2+} concentrations. The idea of exocytosis from two different states is not new. In other neurons in which the kinetics of exocytosis have been characterized, the fast component of exocytosis synchronous with the action potential is followed by a slower asynchronous component that appears to be driven by residual Ca^{2+} (Zucker, 1989; Delaney and Tank, 1994; Goda and Stevens, 1994). Geppert et al. (1994) found that the synchronous component of exocytosis was blocked in hippocampal neurons cultured from mice in which synaptotagmin I had been knocked out, although the asynchronous component appeared intact.

At the moment, we do not have any information about the proteins that are involved in these vesicle movements, although Heidelberger (1998) has demonstrated that the hydrolysis of ATP is required for the refilling of the RRP in the terminal of bipolar cells.

A comparison with other synapses

The ribbon synapses of hair cells and photoreceptors also support continuous exocytosis at rates that indicate that release sites are efficiently replenished (Parsons et al., 1994; Rieke and Schwartz, 1996). Recently, Lenzi et al. (1999) used electron tomography to investigate the structure of ribbon synapses in saccular hair cells and found that it was not possible to reduce the number of ribbon-associated vesicles by continuous depolarization, leading to the conclusion that the transfer of vesicles to the ribbon was not rate-limiting during continuous exocytosis. It may therefore be that the Ca^{2+}-dependent transport of vesicles through ribbons also occurs in hair cells.

In neurons that fire action potentials, residual Ca^{2+} accelerates recovery from synaptic depression after repetitive high-frequency stimulation (Dittman and Regehr, 1998; Stevens and Wesseling, 1998; Wang and Kaczmarek, 1998). This effect can be completely blocked by EGTA, which, as pointed out by Wang and Kaczmarek (1998), suggests that the reserve pool of vesicles is some way from the RRP. In contrast, EGTA did not inhibit the transfer of vesicles to the RRP at the ribbon synapse of bipolar cells (Figs. 6, 7). This functional difference might reflect the introduction of lower concentrations of EGTA into the bipolar cell terminal, or it might be caused by the different anatomical arrangement of the reserve pool of vesicles in "classical" synapses and ribbon synapses. At a typical synapse in the CNS, there may be 100–200 vesicles per active zone, of which 10–20 may be docked, while the remainder form a cluster extending $\sim$500 nm from the presynaptic membrane in which they are linked to each other and the cytoskeleton (Burns and Augustine, 1995). At ribbon synapses, the reserve pool of vesicles appears to be more organized (Gray and Pease, 1971; Jacobs and Hudspeth, 1990; Rao-Mirotznik et

al., 1995; Lenzi et al., 1999). In goldfish bipolar cells, vesicles dock to the plasma membrane in two rows of 11, on either side of the ribbon, and each face of the ribbon has approximately five rows of 11 vesicles attached by short filaments (von Gersdorff et al., 1996). The ribbon is only one vesicle diameter from the presynaptic membrane, and extends $\sim$150 nm. This arrangement may hold vesicles of the reserve pool closer to the RRP and Ca^{2+} channels than is the case at other synapses.

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