Capacitance Measurements in the Mouse Rod Bipolar Cell Identify a Pool of Releasable Synaptic Vesicles

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Zhou, Zhen-Yu, Qun-Fang Wan, Pratima Thakur, and Ruth Heidelberger. Capacitance measurements in the mouse rod bipolar cell identify a pool of releasable synaptic vesicles. J Neurophysiol 96: 2539–2548, 2006. First published August 16, 2006; doi:10.1152/jn.00688.2006. The mouse is an important model system for understanding the molecular basis of neuronal signaling and diseases of synaptic communication. However, the best-characterized retinal ribbon-style synapses are those of nonmammalian vertebrates. To remedy this situation, we asked whether it would be feasible to track synaptic vesicle dynamics in the isolated mouse rod bipolar cell using time-resolved capacitance measurements. The results demonstrate that membrane depolarization triggered an increase in membrane capacitance that was Ca2+ dependent and restricted to the synaptic compartment, consistent with exocytosis. The amplitude of the capacitance response recorded from the easily accessible soma of an intact mouse rod bipolar cell was identical to that recorded directly from the small synaptic terminal, suggesting that in the carefully selected cohort of cells presented here, axonal resistance was not a significant barrier to current flow. This supposition was supported by the analysis of passive membrane properties and a comparison of membrane capacitance measurements in cells with and without synaptic terminals and reinforced by the lack of an effect of sine-wave frequency (200–1,600 Hz) on the measured capacitance increase. The magnitude of the capacitance response increased with Ca2+ entry until a plateau was reached at a spatially averaged intraterminal calcium of about 600 nM. We interpret this plateau, nominally 30 fF, as corresponding to a releasable pool of synaptic vesicles. The robustness of this measure suggests that capacitance measurements may be used in the mouse rod bipolar cell to compare pool size across treatment conditions.

INTRODUCTION

The rod-dominant bipolar cell of the goldfish (Mb1 bipolar cell), with its large, accessible synaptic terminal, has become one of the best characterized model systems in the vertebrate CNS for the study of synaptic vesicle dynamics (e.g., Heidelberger 2001; Sterling and Matthews 2005; Heidelberger et al. 2005). However, despite the many great advantages it provides, this preparation is poorly suited for the biochemical and molecular dissection of neurotransmitter release and vesicle retrieval mechanisms. Genetic and molecular approaches commonly used to study the molecular mechanisms of release are difficult to implement in the goldfish because of gene duplication (Nordstrom et al. 2004; Risunger and Larhammar 1993). In addition, differences in protein sequence between fish and mammals may preclude the use of available molecular tools designed for probing release mechanisms in mammalian systems.

Photoreceptors and bipolar cells respond to changes in illumination with graded changes in membrane potential, whereas some third-order retinal neurons, such as the AII amacrine cell of the mammalian retina, exhibit a light response characterized by both transient and sustained components (Bloomfield and Xin 2000; Dacheux and Raviola 1986; Nelson 1982; Trexler et al. 2005). The conversion from transient to sustained is thought to happen at the level of the synapse between bipolar cells and third-order neurons, although the mechanism is not well understood. Factors extrinsic to the bipolar cell, such as inhibitory feedback (Eggers and Lukasiewicz 2006; Heidelberger and Matthews 1991; Maguire et al. 1989; Maple and Wu 1998; Tachibana and Kaneko 1987), postsynaptic receptor desensitization, and glutamate clearance (Higgs and Lukasiewicz 1999; Tran et al. 1999) clearly influence the shape of the light response, although they may not generate it (Bieda and Copenhagen 2000; but see Dong and Werblin 1998). Factors intrinsic to the bipolar cell remain an intriguing possibility (Awatramani and Slaughter 2000; Pan et al. 2001).

It was recently suggested that a decrease in the rate of synaptic vesicle fusion in rod bipolar cells gives rise to the transient aspect of the light response (Trexler et al. 2005). This could occur if there were a limited number of glutamatergic synaptic vesicles available for immediate or rapid release and the replacement of these vesicles proceeded at a rate slower than the vesicle fusion rate. There is now compelling evidence that the transient component of the light response of All amacrine cells results from the fusion of a small number of vesicles at each ribbon-style active zone (Singer and Diamond 2003, 2006). These vesicles may be analogous to the rapidly releasing pool or ultrafast vesicle pool of the Mb1 bipolar cell (Mennerick and Matthews 1996; Neves and Lagnado 1999; Sakaba et al. 1997). The anatomical correlate of the latter is believed to be the subset of vesicles on the synaptic ribbons that contact the plasma membrane (von Gersdorff et al. 1996). The total releasable pool of synaptic vesicles, thought to constitute the rapidly releasing vesicles and those vesicles that feed into the rapidly releasing pool, has not yet been characterized for a mammalian rod bipolar cell.

In the present study, we assessed the feasibility of performing capacitance and calcium measurements in rod bipolar cells...
of the mouse. Membrane capacitance measurements allow changes in membrane surface area associated with synaptic vesicle fusion and retrieval to be monitored with high temporal resolution (Gillis 1995) and provide a complementary approach to the use of postsynaptic receptors for the detection of neurotransmitter release; the latter may be confounded by failure to detect all the released neurotransmitter, saturation of postsynaptic receptors, and changes in the activity or number of functional receptors (reviewed in Heidelberger 2001). We demonstrate that under carefully controlled and vetted conditions, capacitance measurements can be used to monitor exocytosis. We used this combination of approaches to define the releasable pool of synaptic vesicles in the mouse rod bipolar cell. The kinetics with which this pool is released suggests that it may be an early contributor to the sustained component of the third-order neuron light response.

**METHODS**

**Cell preparation**

Mouse bipolar cells were isolated by mechanical trituration after enzymatic digestion using methods similar to those used to isolate bipolar cells of the goldfish retina (Heidelberger and Matthews 1992); all animal procedures conformed to National Institutes of Health guidelines and were approved by the appropriate Institutional Animal Care and Use Committee. In brief, adult C57BL/6J mice (Simonsen Laboratories, Gilroy, CA), 2–7 mo of age, were killed in a CO₂ chamber, and the eyes were opened in cold, oxygenated, low-calcium saline solution containing (in mM): 153 NaCl, 2.6 KCl, 1 MgCl₂, 0.5 CaCl₂, 10 glucose, and 10 HEPES (pH = 7.4, 310–315 mOsm). The lens and vitreous humor were removed and the retinas were detached from the epithelium and cut into small pieces (about 1 mm²). Retinal pieces were incubated for 25–30 min at room temperature in the low-calcium saline supplemented with 2.7 mM l-cysteine (Sigma) and 30 U/ml papain (Fluka). After being rinsed in low-calcium saline, pieces were then stored in an oxygenated environment at 10°C for ≤8 h before being mechanically triturated with a fire-polished Pasteur pipette and plated onto glass coverslips for recordings.

Rod bipolar cells were identified on the basis of their characteristic shape: a spheroid soma of 5–8 μm in diameter and a relatively stout axon (diameter ≥0.5 μm) and a relatively large lobulated terminal (diameter ≥2 μm) (Ghosh et al. 2004; Havérkamp and Wässle 2000; Vaquero and de la Villa 1999). Typical examples are shown in Figs. 1 and 2A. Roughly 54% of the isolated mouse bipolar cells (109/202) were double-labeled with an antibody against protein kinase C (PKC, green) and the synaptic ribbon protein ribeye (red). To better visualize the ribeye immunoreactivity, the green channel is rendered transparent. Scale bar = 10 μm. Right: enlarged view of the synaptic terminal of the cell shown on the left. Scale bar = 1 μm. Total of 202 ribeye-positive bipolar cells were counted and, of these, 109 were also PKC-positive, indicating that roughly 54% of the isolated bipolar cells were rod bipolar cells, consistent with a previous estimate in the rat (Euler and Wässle 1995). In addition, of the 112 bipolar cells that had morphological features suggestive of rod bipolar cells (large terminals, thick axons), 93 (roughly 83%) double-labeled for PKC, confirming their identity as rod bipolar cells.

**Electrophysiological and Ca²⁺ measurements**

Whole cell recordings from the cell soma were performed using 5- to 6-MΩ patch pipettes pulled from 1.5-mm thin-walled filamented borosilicate capillary glass. For whole-terminal recordings (whole cell recordings made with the pipette positioned on the terminal), 11- to 12-MΩ pipettes were typically pulled from 1.2-mm filamented capillary glass (Schott 8250). Pipettes were coated with Sylgard to minimize stray capacitance. Recordings were made at room temperature (21–24°C). The intracellular recording solution contained (in mM): 125 Cs-gluconate, 10 TEA-Cl, 2 Na₂ATP, 0.5 GTP, 0.5 EGTA, 0.3 fura-2, and 35 HEPES (pH = 7.4, 310–315 mOsm). In some experiments, fura-2 was replaced with 100 μM bis-fura-2, and equivalent results were obtained. The external recording solution typically contained (in mM): 127 NaCl, 5 CsCl, 20 TEA-Cl, 1 MgCl₂, 2 CaCl₂, 10 glucose, and 10 HEPES (pH = 7.4, 315–320 mOsm). When recording in whole cell mode, pipette pressure was controlled as previously described (Heidelberger et al. 2002). For perforated-patch recordings, amphoteratin B was prepared as a 1 mg/ml stock solution in DMSO and diluted into the patch-pipette solution every 3 h to achieve a final concentration of 500 μg/ml (Zhou and Neher 1993).

Electrophysiological and capacitance measurements were made with the use of an EPC-9 patch-clamp amplifier controlled by Pulse software (HEKA Electronic, Lambrecht, Germany; see also Heidelberger 1998; Heidelberger et al. 2002). For all recordings, the holding potential was set at −60 mV, the rod bipolar cell resting potential in darkness (Wu et al. 2004). To measure membrane capacitance, a sinusaloidal stimulus with a 30-mV peak-to-peak amplitude was applied about the holding potential and the resultant signal processed using the Lindau–Neher technique (Gillis 1995; Lindau and Neher 1988) to yield estimates of \( C_m \), \( G_m \), and \( G_r \). Whole cell recordings with a leak current <40 pA or an access resistance >35 MΩ were excluded from the data pool. Because of the difficulties inherent in obtaining stable whole-terminal recordings, access resistances ≤40 MΩ were tolerated in whole terminal recordings used for analysis of capacitative transients and ≥90 MΩ for whole-terminal and perforated-patch terminal recordings used to monitor capacitance jumps. In experiments in which the capacitative transients to hyperpolarizing voltage pulses were examined, the signal was filtered using either a 15-, 30-, or 100-kHz Bessel filter. For all other experiments, the filter setting was 3.2 kHz. Experiments examining bipolar-cell passive membrane properties used a 10-mV hyperpolarizing voltage pulse 3 ms in duration. Stable electrical recordings were relatively easy to achieve and the

FIG. 1. An acutely isolated rod bipolar cell of the mouse retina retains its synaptic ribbons in the terminal compartment. Left: 3-dimensional reconstruction of a confocal stack (0.2-μm step size) obtained from a mouse bipolar cell double-labeled with an antibody against protein kinase C (PKC, green) and the synaptic ribbon protein ribeye (red). To better visualize the ribeye immunoreactivity, the green channel is rendered transparent. Scale bar = 10 μm. Right: enlarged view of the synaptic terminal of the cell shown on the left. Scale bar = 1 μm. Total of 202 ribeye-positive bipolar cells were counted and, of these, 109 were also PKC-positive, indicating that roughly 54% of the isolated bipolar cells were rod bipolar cells, consistent with a previous estimate in the rat (Euler and Wässle 1995). In addition, of the 112 bipolar cells that had morphological features suggestive of rod bipolar cells (large terminals, thick axons), 93 (roughly 83%) double-labeled for PKC, confirming their identity as rod bipolar cells.
terminal Ca\textsuperscript{2+}eter-based system (ASI/TILL Photonics; Messler et al. 1996). Intra-
345 and 388 nm was provided by a computer-controlled monochrom-
tance were adequately separated. Time base is the same for

Coverslips coated with 0.1 mg/ml poly-D-lysine, allowed to settle for
are expressed as means
Excel and SAS software (SAS Institute, Cary, NC). All pooled data
thesda, MD). Statistical analyses were performed with Microsoft
length, images of bipolar cells and a calibration graticule were taken
squared values and confirmed by eye.
individual fit was assessed with the use of residual plots and chi-
in response to hyperpolarizing voltage steps were fit with either a
monoeponential function or a biexponential function with the use of
intron terminal 

duced from further analysis because such oscillations presum-
izations of Ca\textsuperscript{2+} evoked (Ma and Pan 2003; Protti et

C: corresponding conductance traces, G\textsubscript{m}(top) and G\textsubscript{c}(bottom), indicate that capacitance and conduc-
tance were adequately separated. Time base is the same for B and C.

measured input resistance of the isolated cells was quite high (2.6 ±
0.7 G\textsubscript{\Omega}; n = 8).

For measurement of intraterminal Ca\textsuperscript{2+}, alternating excitation at
345 and 388 nm was provided by a computer-controlled monochrom-
eter-based system (ASI/TILL Photonics; Messler et al. 1996). Intrater-
mental Ca\textsuperscript{2+} 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 concentra-
tions of Ca\textsuperscript{2+} (Heidelberger and Matthews 1992).

Analysis

Capacitance, conductance, fluorescence, calcium, and capacitative
records acquired with Pulse were exported into Igor (Wavemetrics;
Lake Oswego, OR) for analysis. Analyses of capacitative transients
were performed on an average of 50–100 traces. The current decays
in response to hyperpolarizing voltage steps were fit with either a
monoeponential function or a biexponential function with the use of
an iterative sum-of-squares minimization algorithm. The quality of an
individual fit was assessed with the use of residual plots and chi-
squared values and confirmed by eye.

To estimate the diameter of a soma or synaptic terminal or the axon
length, images of bipolar cells and a calibration graticule were taken
with an USB AlphaData video TurboAdapter AD-VDO1 Video Cap-
ture (Alpha Data) and software AVRE Capture Tool (Nogatech) and
analyzed in software ImageJ 1.26t (Wayne Rasband, NIMH, Be-
thesda, MD). Statistical analyses were performed with Microsoft
Excel and SAS software (SAS Institute, Cary, NC). All pooled data
are expressed as means ± SE.

Immunocytochemistry

Cells isolated for immunocytochemistry were plated onto glass
coverslips coated with 0.1 mg/ml poly-D-lysine, allowed to settle for
15–30 min, and then fixed with 4% formaldehyde in 0.1 M PBS or
sodium cacodylate buffer (pH 7.4), for 15–30 min. After rinsing
with PBS, the cells were incubated in a blocker buffer containing 10% goat
serum, 5% BSA, and 0.1% Triton in PBS for 30 min. Cells were
incubated with primary antibodies overnight at 4°C. Primary antibod-
ies used were 1) mouse anti-protein kinase C (Transduction Labora-
tories, San Diego, CA) at dilution 1:500, as a marker of rod bipolar
cells (Wang et al. 2003) and 2) ribbon-specific rabbit anti-ribeye
antibody (Schmitz et al. 2000), dilution 1:2,000, provided as a gen-
erous gift by Dr. Thomas Südhof. Cells were then rinsed and blocked
again. For fluorescence labeling, cells were incubated with a mixture of
Alexa-tagged goat anti-mouse (480 nM) and goat anti-rabbit (540
nM) secondary antibodies, for 45 min at room temperature in a
light-protected environment. Cells were rinsed, mounted on a slide
with anti-fade mounting medium (Molecular Probes), and scanned
with a 0.2-µm step size on a Zeiss LSM 510 META confocal
microscope. Image data were processed for three-dimensional recon-
struction using Zeiss 3-D for LSM.

Results

Membrane depolarization evokes an increase in
intraterminal calcium and membrane capacitance in rod
bipolar neurons

To study exocytosis in the mammalian rod bipolar cell,
bipolar neurons were acutely dissociated from the adult mouse
retina by enzymatic digestion followed by mechanical trituration
(Heidelberger and Matthews 1992; Kaneko et al. 1989;
Karschin and Wässle 1990). Presumed rod bipolar cells were
identified according to the criteria listed in METHODS. A typical
isolated rod bipolar cell is shown in Fig. 1. Note the charac-
teristic morphology: bushy main dendrites and a thick axon
ending in a relatively large, lobulated bouton (Ghosh et al.
2004). For simplicity, we will refer to this as an “intact bipolar
cell,” although some processes, particularly those in the den-
dritic tree, are likely to have been lost during dissociation.
To facilitate voltage-clamp control of the synaptic terminal from a
pipette placed on the cell soma, we focused our attention on
presumed rod bipolar cells that had axons lengths ≤40 µm in
length (mean axon length = 36 ± 2 µm; n = 19). Thus we
may have preferentially selected for rod bipolar cells of the
Group 2 type (Pang et al. 2004; Wu et al. 2004). Calcium-
dependent changes in membrane surface area were monitored
using membrane capacitance measurements. Intraterminal
Ca\textsuperscript{2+} was measured ratiometrically using a fluorescein indica-
tor dye. The concentration of indicator dye in the synaptic
terminal reached equilibrium within 1–2 min of achieving the
whole cell recording configuration. In nearly 5% of the record-
ings, spontaneous calcium oscillations suggestive of regenera-
tive calcium spikes were observed (Ma and Pan 2003; Protti et
al. 2000; Zenisek and Matthews 1998). These data were ex-
cluded from further analysis because such oscillations presum-
ably indicate escape from voltage-clamp control (Mnenrick et
al. 1997).

Figure 2 shows a representative recording from a bipolar
neuron that met selection criteria. At the arrow (Fig. 2B), the
membrane voltage was stepped from a holding potential of
−60 to 0 mV for 500 ms. This triggered an increase in
intraterminal calcium and a 23-fA increase in membrane cap-
citance (C\textsubscript{m}). After calcium channel closure, both internal
calcium and membrane capacitance recovered to baseline. The
membrane (G\textsubscript{m}) and series conductance (G\textsubscript{s}) traces did not
show changes that correlated with the change in $C_m$. Thus the observed jump in membrane capacitance most likely represents a true increase in membrane capacitance. The mean jump in membrane capacitance evoked by a 500-ms depolarization ($-60$ to 0 mV) was $21.6 \pm 2.8 \, \text{fF} \, (n = 15)$.

If the depolarization-evoked capacitance jump indicates exocytosis, this jump should be both calcium-dependent and restricted to the synaptic compartment. Accordingly, capacitance jumps were not evoked in the absence of external calcium ($n = 5$) or in the presence of 200 $\mu$M external Cd$^{2+}$ ($n = 5$), consistent with a requirement for calcium entry through voltage-gated channels. In addition, bipolar cells that lost their axon terminals during dissociation did not exhibit an increase in membrane capacitance in response to membrane depolarization or an increase intracellular calcium in the cell soma ($n = 12$). The latter is consistent with the absence of a high-threshold calcium channel in the rod bipolar cell soma (de la Villa et al. 1998). To rule out the possibility that calcium ions entering a terminal bouton diffuse to the soma where they trigger an increase in the surface area of the cell body, we measured the intracellular free calcium in the somatic compartment of neurons that both retained their terminals and exhibited a depolarization-evoked capacitance jump. In five such recordings, membrane depolarization failed to increase the intracellular calcium in the cell body. Together, the data indicate that the observed calcium-triggered increases in membrane capacitance reflect a calcium-dependent increase in the membrane surface area of the terminal compartment.

**Accuracy of capacitance measurements made from the soma**

The electrical recordings in the preceding paragraphs were made with the patch pipette positioned on the cell soma. However, the capacitance jumps arise exclusively from the terminal cluster, which is separated from the soma by an axon. Although the rod bipolar cell may be specialized for passive propagation, the interposed axonal resistance could confound the capacitance measurements, which in the standard mode assumes that the recorded cell is electrically equivalent to a single electrical compartment. However, Mennerick et al. (1997) showed that the goldfish Mb1 bipolar cell is better modeled as two electrical compartments, representing the soma and terminal, separated by a resistor, representing the axon. The presence of an additional electrical compartment places constraints on the mathematical equations used to extract capacitance information and also on the frequency of the sine-wave voltage command (Gillis 1995; Hallermann et al. 2003).

To determine whether the isolated mouse bipolar cell can be modeled as a single electrical compartment, we examined its passive membrane properties. Passive current transients were induced by a 10-mV hyperpolarizing pulse from a holding potential of $-60$ mV. Figure 3A shows a representative example of a current transient measured with the patch pipette sealed to the soma of an intact bipolar cell. The relaxation phase of the capacitative transient was fitted with monoexponential (solid black line) and biexponential (dotted black line) functions. The quality of the fit was assessed with the use of residual plots, $\Psi^2$ values, and confirmed by eye. In the example shown, a single exponential fit provided a satisfactory description of the data. Similar results were observed in a total of 12 intact neurons. A comparison of single- and double-exponential fits to the data did not substantiate the hypothesis of two electrical compartments with different relaxation properties. We then analyzed passive membrane properties measured from the terminal end of intact bipolar cells. To facilitate patching of such small structures and achieving acceptable access resistance ($R_a < 40 \, \text{M} \Omega$), pipettes were fashioned from 1.2-mm glass (see also Glowatzki and Fuchs 2002). To decrease noise and increase the resolving power, the average of

![Figure 3](http://jn.physiology.org/doi/10.1152/jn.01531.2006)
50–100 transients, filtered at 100 kHz, was analyzed. An example is shown in Fig. 3B. The relaxation phase of the capacitative transient was fitted with monoexponential (solid black line) and biexponential (dotted black line) functions. In contrast to what has been reported for the Mb1 bipolar cell, the single- and double- exponential fits were almost superimposable. Similar results were observed in seven additional terminal-end recordings of bipolar cells with an intact somatic compartment. The mean relaxation time constant of the intact cell measured from the terminal was 70 ± 7 μS (n = 8). The mean relaxation time constant of intact cells obtained from somatic recordings under similar conditions (100-kHz filter, average of 50–100 sweeps) was 72 ± 7 μS (n = 6). Thus for the majority of intact cells that met our selection criteria, the experimental evidence did not support the supposition of multiple electrical compartments.

If the axon poses a significant barrier to current flow, then with higher-frequency stimuli, the ability to successfully follow the command voltage would be attenuated, resulting in a greater underestimation of membrane capacitance. To address this concern, we compared the resting capacitance and evoked capacitance jump at three different sine-wave frequencies (200, 800, and 1,600 Hz). The resting capacitance averaged 4.25 ± 0.20 pF (n = 9) in recordings with a 200-Hz sine-wave frequency, 3.96 ± 0.12 pF (n = 16) in recordings with an 800-Hz sine-wave frequency, and 3.81 ± 0.13 pF (n = 8) in recordings with a 1,600-Hz sine-wave frequency (Fig. 4A). Statistical analysis of the means indicated that sine-wave frequency does not significantly affect the estimate of the average resting membrane capacitance (P > 0.18; one-way ANOVA followed by Duncan multiple comparison). However, within a given cell, there was a trend for the higher-frequency sine wave to report a lower resting capacitance (Fig. 4B). Furthermore, when viewed within a cell, the difference in resting capacitance as a function of sine-wave frequency was significant (P < 0.005; mixed effect model with original scaling). Overall, the difference in the estimate of resting capacitance measured at 200 and 1,600 Hz was about 440 fF. However, a similar difference (460 fF) was measured with an MC-9 model cell (HEKA). Thus some, if not all, of the apparent frequency dependency is inherent to the capacitance calculation performed by the PULSE software rather than properties of the bipolar cell.

We next asked whether the magnitude of the capacitance jump, recorded with a pipette on the soma, was sensitive to the sine-wave frequency. Importantly, the mean capacitance jump evoked by a 2-s depolarizing voltage-step failed to reveal an effect of sine-wave frequency (one-way ANOVA followed by Duncan multiple comparison: P > 0.63; or mixed effect model with original scaling: P > 0.47). The average capacitance jump evoked by a 2-s depolarization was 32.3 ± 13.1 fF (n = 5), when measured with a 200-Hz sine wave, 19.1 ± 2.7 fF (n = 9) with an 800-Hz sine wave, and 20.6 ± 5.9 fF (n = 6) in recordings using a 1,600-Hz sine-wave stimulus (Fig. 4C). Further inspection of the potential affect of sine-wave frequency within a given cell also failed to yield a significant difference in the amplitude of the capacitance jump for most cells (Fig. 4D). Within a cell, the magnitude of the capacitance jumps measured with 200- and 800-Hz sine waves was not significantly different (n = 5; P > 0.15). Similarly, magnitudes of the capacitance jumps measured with an 800- or 1,600-Hz sine wave were not significantly different (n = 4; P > 0.57). However, to err on the conservative side and reduce noise (Gillis 1995), we performed the remainder of the experiments using an 800-Hz sine-wave stimulus.

Empirical tests of capacitance measurements

To experimentally assess the validity of capacitance measurements in isolated mouse rod bipolar cells, we first compared the resting membrane capacitance of bipolar cells that retained their terminals with the resting membrane capacitance of bipolar cells in which the axon and terminals were severed during the dissociation procedure. Results are summarized in Fig. 5A. The mean resting capacitance of the intact cell (3.9 ± 0.12 pF; n = 17) was significantly larger than the mean resting capacitance of bipolar cells without terminals (2.6 ± 0.4 pF; n = 6; P < 0.001), consistent with the hypothesis that terminal surface area is detected by a patch pipette placed on the cell soma.

Next, we made whole cell membrane capacitance measurements directly from the synaptic terminal of isolated, intact bipolar cells. Such recordings were difficult to hold for more than a few minutes. Figure 6 shows the capacitance and calcium records from a rare, whole-terminal recording. The average resting capacitance measured from the terminal compartment of intact bipolar cells was 3.3 ± 0.3 pF (n = 3) and
indistinguishable from that obtained from somatic recordings (Fig. 5B). These data, along with those in the preceding paragraph, provide strong experimental evidence that axonal resistance does not prevent detection of the distal compartment.

Finally, we tested the validity of capacitance measurements made from the soma by comparing the average magnitude of the exocytotic jump with that measured from a pipette positioned directly on a synaptic terminal, in either the whole-terminal or perforated-patch recording configuration. Figure 6 shows the capacitance and calcium records of a rare, whole-terminal recording. At the arrow, a 2-s depolarization evoked a transient increase in intraterminal calcium and a 30-fF increase in membrane capacitance. As with the somatic recordings, there were no correlated changes in $G_m$ and $G_s$. Little or no endocytosis was observed in whole-terminal recordings. The loss of endocytosis may be a result of the unfavorable deformation of the plasma membrane by the patch pipette (Heidelberger et al. 2002) and/or the rapid dialysis of soluble cytosolic factors required for endocytosis out of the terminal compartment (Parsons et al. 1994). In support of the latter, endocytosis was routinely observed in the perforated-patch recording configuration. Importantly, both recording methods gave similar mean capacitance jumps (see following text), indicating that, as in other retinal ribbon synapses (e.g., Thoreson et al. 2004; von Gersdorff et al. 1998), concurrent endocytosis does not confound the estimate of exocytosis under the conditions tested.

On average, the membrane capacitance jump evoked by a 2-s depolarization measured in the whole-terminal recording configuration was $25 \pm 6$ fF ($n = 3$). In perforated-patch recordings made from the terminal, the mean capacitance jump evoked by a 2-s stimulus was $32 \pm 11$ fF ($n = 30$). These values are virtually identical to the mean capacitance jump evoked by the identical stimulus in somatic whole cell recordings ($28 \pm 3$ fF; $n = 18$). Thus there was no difference in the magnitude of the capacitance jump estimated by a patch pipette placed on the soma versus a patch pipette placed directly on the terminal (Fig. 7).

The releasable pool in the mouse rod bipolar cell

To determine the extent of the releasable pool of synaptic vesicles in the mouse rod bipolar cell, exocytosis was evoked...
by a voltage step from −60 to 0 mV and monitored by capacitance measurements using an 800-Hz sine wave. The associated increase in membrane capacitance was then plotted as a function of the increase in the peak spatially averaged intraterminal calcium elicited by the voltage step. As shown in Fig. 8, for small increases in intraterminal calcium (<500 nM), the magnitude of the capacitance jump grew with an increase in the spatially averaged calcium concentration, as expected for a calcium-dependent process. Above approximately 600 nM, the magnitude of the capacitance jump achieved a plateau value of about 32 fF. A pulse-duration plot (Horrigan and Bookman 1994; Moser and Beutner 2000; Thoreson et al. 2004; von Gersdorff and Matthews 1994) yielded a similar plateau of the capacitance jump at about 28 fF (data not shown). Before the plateau, the rise in capacitance with respect to pulse duration could be described by a single exponential with a time constant of about 200 ms, similar to that of goldfish bipolar cells and rod photoreceptors (Mennerick and Matthews 1996; Thoreson et al. 2004; von Gersdorff and Matthews 1994). Neither raising the spatially averaged intraterminal calcium from about 600 to 2,000 nM nor increasing the duration of the fixed amplitude voltage step from about 500 ms to 2 s evoked an additional increase in the average size of the capacitance response. A similar relationship between the magnitude of the exocytotic response and intraterminal calcium was also evident in the three recordings in which capacitance and calcium measurements were successfully made in the whole-terminal recording configuration (Fig. 8, open circles). In agreement with the somatic recordings, the terminal data also suggest a plateau at about 31 fF (see also Fig. 7).

**Figure 8.** Mouse bipolar cells contain a finite pool of releasable vesicles. Mean capacitance increase is plotted as a function of the average peak of the corresponding spatially averaged calcium transient. Filled circles represent somatic whole cell recordings. Data are binned according to the peak of the calcium transient elicited by a depolarization from −60 to 0 mV (0–99 nM: n = 2; 100–299 nM: n = 10; 300–499 nM: n = 10; 500–750 nM: n = 8; 750–999 nM: n = 7; 100–1,400 nM: n = 10; 1,545–2,800 nM: n = 9). Open circles indicate nonbinned data from the 3 whole-terminal recordings. Fits to the data are through the binned data using the equation $Y = Y_0 + Ae^{-Bx}$, where $Y_0 = 32 ± 2$, $A = −31 ± 3$, $B = 0.0051 ± 0.002$.

**DISCUSSION**

The ability to track changes in membrane surface by membrane capacitance measurements has proven to be a powerful approach for understanding mechanisms of neurotransmitter release. Traditionally, capacitance measurements were relegated to neuroendocrine cells because of their approximately spherical morphology or to the unusually large and round synaptic terminal of the goldfish Mb1 bipolar cell. Gradually, capacitance measurements have been extended to a few neuronal preparations with varying degrees of morphological complexity. These include electrically compact neurons, such as photoreceptors (Thoreson et al. 2004) and hair cells (Moser and Beutner 2000; Parsons et al. 1994), and nerve terminals with a stub of attached axon from the rat calyx of Held (Sun et al. 2004), the rat bipolar cell (Pan et al. 2001), the posterior pituitary (Hsu and Jackson 1996), and mossy fiber terminals (Hallermann et al. 2003). The mouse rod bipolar cell is exceptionally amenable to genetic and molecular manipulation and therefore we asked whether it too might prove suitable for the study of synaptic mechanisms using membrane capacitance measurements. We show that this approach is successful provided that several selection criteria are met. The use of these criteria does not place an excessive burden on the experimenter, but rather opens the potential for applying molecular biological tools to the study of retinal ribbon synapses in the mammalian CNS.

Membrane depolarization triggers calcium entry into the synaptic terminal of a mouse rod bipolar cell and an increase in membrane capacitance. Several lines of evidence indicate that this increase in capacitance reflects exocytosis. First, blockade of voltage-gated calcium channels or removal of external calcium abolished the depolarization-evoked increase in membrane capacitance. Second, the increase in membrane capacitance required the presence of a synaptic terminal. Third, as observed at other ribbon synapses (Moser and Beutner 2000; Thoreson et al. 2004; von Gersdorff and Matthews 1994), the magnitude of the capacitance jump increased with the magnitude of calcium entry until a plateau was reached (Fig. 8). Fourth, similar depolarization protocols trigger glutamate release from retinal bipolar cells of several vertebrate species [goldfish (von Gersdorff et al. 1998) and rat (Singer and Diamond 2003)]. Fifth, immunolabeling for the ribbon protein ribeye and the integral synaptic vesicle protein SV2 (see also Wang et al. 2003) indicates that these proteins do not redistribute after dissociation, but remain properly localized to the synaptic compartment. For all of these reasons, the increase in membrane capacitance evoked by membrane depolarization most likely reflects the fusion of synaptic vesicles with the plasma membrane during the process of exocytosis.

As a result of difficulties inherent in measuring exocytosis directly from a small synaptic terminal, the majority of our capacitance measurements were made with a patch pipette positioned on the bipolar cell soma. However, in the Mb1 bipolar cell of the goldfish, the axial resistance of the axon that links the soma and terminal may introduce errors into the capacitance measurements, the magnitude of which will depend on the circuit parameters, position of the recording electrode, and sine-wave frequency (Mennerick et al. 1997). This raises the important question of whether the data should be considered qualitative and thus useful for comparative purposes or quantitative and used for extracting specific information about the secretory process. Indeed, whenever a new preparation is subjected to capacitance measurements, it is prudent to assess its suitability and the extent to which the data may be interpreted.

From our data, it is clear that the axon does not pose an absolute barrier to current flow between the soma and terminal.
The average resting capacitance for the isolated rod bipolar cell of about 3.9 pF corresponds to a surface area of 433 μm², assuming a specific capacitance of 0.9 μF/cm² (Gentet et al. 2000). This value is in excellent agreement with the estimate of 400 μm² obtained from the morphological analysis of rod bipolar cells in situ (de la Villa et al. 1998). Consistent with the ability to detect the terminal from a pipette on the soma, loss of the synaptic terminal cluster and axon segment reduced the mean resting capacitance by about 1.3 pF. The calculated capacitance of three boutons, each with a diameter of 3 μm, and a 10-μm segment of a 1-μm-diameter axon is about 1.1 pF. Furthermore, depolarization-evoked increases in membrane capacitance were observed only in the somatic recordings of neurons that retained their axon terminals. These findings are in line with the estimated length constant of 700 μm for the mouse rod bipolar cell (de la Villa et al. 1998; Vaquero et al. 1999). Length constants of several hundred microns have also been recently reported for mammalian hippocampal and cortical neurons (see Marder 2006). Thus the nearly 40-μm distance separating the soma and terminal of selected rod bipolar cells falls easily within a single length constant by an order of magnitude, consistent with the presumed behavior of an electrically compact, nonspiking neuron.

Although the above convincingly argues that there is not an absolute barrier to current flow, the intervening axon could potentially alter a rapidly changing voltage command imposed on the distal compartment. To minimize this complication, we selected for presumed rod bipolar cells with relatively short, stout axons. In addition, we experimentally addressed this concern by comparing capacitance measurements obtained using sine-wave stimuli of different frequencies. The responses to a pool-depleting stimulus were virtually identical, regardless of sine-wave frequency or position of the recording electrode (soma vs. terminal). Thus the experimental evidence does not support the hypothesis that the axon represents an unacceptably large barrier to current flow for sine-wave stimuli ~1,600 Hz. However, it is conceivable that such an effect might be revealed with shorter depolarizations that do not produce saturation of the exocytotic response or at frequencies higher than those tested here.

The passive membrane properties of intact rod bipolar cells and rod bipolar cells without terminals also failed to indicate that the mouse rod bipolar cell functions other than as a single electrical compartment. One reason may be that the intact bipolar cell is quite small (about 3.9 pF) and therefore the entire membrane might be expected to charge very quickly. Indeed, the membrane surface area of the entire mouse rod bipolar cell is comparable to the surface area of the isolated terminal of the goldfish Mb1 bipolar cell (2–4 pF; Heidelberger 1998; Mennerick et al. 1997). However, it may be that the soma and the lobulated terminal cluster of the mouse rod bipolar cell are difficult to separate on the basis of their time constants because the sizes of these potential compartments are small and differ by only slightly more than a picofarad, thereby yielding somewhat similar time constants. By comparison, the resting membrane capacitance of the intact goldfish Mb1 bipolar cell can be as high as 10–15 pF, with the terminal compartment accounting for ≈4 pF (Mennerick et al. 1997). The difference in size between the two compartments in the Mb1 bipolar cell could yield as much as a threefold difference in the time constant attributed to each compartment (Mennerick et al. 1997). Thus the large sizes of the goldfish bipolar cell compartments may more readily reveal the barrier to current flow posed by the axonal resistance. Modeling of the equivalent electrical circuit may be required to ascertain whether there are more subtle effects of the intervening axon in the mouse rod bipolar cell that cannot be experimentally demonstrated.

Although the empirical evidence in the mouse rod bipolar cell does not favor a problem with space clamp or in the ability to follow a sine-wave stimulus, this conclusion applies only to cells in which the access resistance was <35 MΩ. With high access resistances (i.e., 60 MΩ), there was increasing evidence of escape from voltage clamp when patching from the soma and a second, slow component became apparent in the passive capacitative transient (i.e., τ2 ≈ 500 ms; n = 6). We also noted that bipolar cells with unusually long, thin axons were more difficult to clamp. Thus when attempting to voltage clamp a bipolar cell terminal from a pipette positioned on the cell soma, as was done in this study and is often done in slice recordings, careful attention to cell selection and recording conditions is imperative. In addition, changes in membrane capacitance should be interpreted with caution if the goal is to extract quantitative information. Features such as absolute pool size should be verified by either direct terminal measurements as done here or by an independent method.

The results presented here allow several important conclusions about exocytosis to be drawn. The first is that there is a discrete pool of vesicles available for release. The entirety of this pool was discharged when the spatially averaged intraterminal calcium rose to >600 nM; further elevations in intraterminal calcium ≤2 nM did not trigger additional release. Thus the plateau in the magnitude of the exocytotic response is not explained by a plateau in the calcium signaling, but by the saturation of a step in the secretory process that is downstream of calcium entry. The common interpretation is that the plateau represents the exhaustion of a releasable pool of vesicles (Horrigan and Bookman 1994; Moser and Beutner 2000; Thoreson et al. 2004; von Gersdorff and Matthews 1994). Consistent with this interpretation, the magnitude of this pool was preserved in whole-terminals recordings, which did not display endocytosis. In addition, it matched the magnitude of the exocytotic response to a 2-s depolarization in perforated patch recordings made from the terminal, suggesting that the plateau value is preserved across recording configurations. Thus the plateau value may be a useful point of comparison between different treatment conditions.

Two discrete pools containing fully primed, fusion-competent vesicles have been identified at ribbon-style synapses (Heidelberger 2001; Heidelberger et al. 2005; Sterling and Matthews 2005). The first is typically small, releases rapidly (~1 ms), and is thought to represent those vesicles at the base of the ribbon that are docked at the plasma membrane near calcium channels. The second pool is larger, has slower release kinetics, and is thought to represent the remainder of the ribbon-associated vesicles. The pool identified in this study is likely to represent the latter group. Similar to previously described releasable pools, it is released over a period of several hundred milliseconds. It is interesting to note that a vesicle pool with similar release kinetics was proposed by Singer and Diamond (2006) for the rat rod bipolar cell, based on the release rate of the sustained component of the AII.
amacraine cell excitatory postsynaptic current (EPSC) measured in paired voltage-clamp recordings.

Both the terminal and somatic recordings suggest that the size of the releasable pool is about 30 fF. This corresponds to nearly 1,200 vesicles, assuming a specific capacitance per vesicle of 0.9 μF/cm² (Gentet et al. 2000) and vesicle diameter of 30 nm. Each mouse rod bipolar cell has 30–40 synaptic ribbons (Sterling and Matthews 2005; Tsukamoto et al. 2001). If the releasable pool corresponds to those vesicles tethered to the synaptic ribbons and no boutons were lost during the dissociation process, this would imply that there are 40–60 fusion-competent vesicles available for release at each ribbon-style synapse. This is somewhat smaller than the estimate obtained for the releasable pool at the Mb1 bipolar cell (80–110 vesicles; Sterling and Matthews 2005; von Gersdorff et al. 1996) but similar to that predicted for the rat rod bipolar cell (≈35 vesicles; Singer and Diamond 2006). We note that identification of the anatomical correlate of the releasable pool will minimally require a detailed ultrastructural analysis of mouse rod bipolar cell active zones. Such information is not currently available for any mammalian rod bipolar cell.

The ratio between the total number of vesicles tethered to a synaptic ribbon and tethered vesicles docked at the plasma membrane in retinal ribbon synapses has been put at 5:1 (Sterling and Matthews 2005). Using this relation, the projected magnitude of the rapidly releasing pool would be on the order of 240 vesicles or 8 to 12 vesicles per ribbon-style synapse. We did not look for this small component of release in this study (≈6 fF). However, careful analysis of paired rod bipolar cell/amacraine cell recordings in the rat retina revealed that a similarly sized vesicle pool, about seven vesicles/synapse, generates the transient component of the AII amacraine cell EPSC (Singer and Diamond 2006). The rapid burst of transmitter release associated with depletion of the rapidly releasing pool, followed by a slower rate of release associated with the releasable pool characterized in this study may constitute the intrinsic mechanisms that underlie the transient and sustained phases of third-order neuron light responses.

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