Frequency selectivity of synaptic exocytosis in frog saccular hair cells

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The ability to respond selectively to particular frequency components of sensory inputs is fundamental to signal processing in the ear. The frog (Rana pipiens) saccus, which is used for social communication and escape behaviors, is an exquisitely sensitive detector of sounds and ground-borne vibrations in the 5- to 200-Hz range, with most afferent axons having best frequencies between 40 and 60 Hz. We monitored the synaptic output of saccular sensory receptors (hair cells) by measuring the increase in membrane capacitance (∆C_m) that occurs when synaptic vesicles fuse with the plasmalemma. Strong stepwise depolarization evoked an exocytic burst that lasted 10 ms and corresponded to the predicted capacitance of all docked vesicles at synapses, followed by a 20-ms delay before additional vesicle fusion. Experiments using weak stimuli, within the normal physiological range for these cells, revealed a sensitivity to the temporal pattern of membrane potential changes. Interrupting a weak depolarization with a properly timed hyperpolarization increased ∆C_m. Small sinusoidal voltage oscillations (±5 mV centered at −60 mV) evoked a ∆C_m that corresponded to 95 vesicles per s at each synapse at 50 Hz but only 26 vesicles per s at 5 Hz and 27 vesicles per s at 200 Hz (perforated patch recordings). This frequency selectivity was absent for larger sinusoidal oscillations (±10 mV centered at −55 mV) and was largest for hair cells with the smallest sinusoidal-stimuli-evoked Ca²⁺ currents. We conclude that frog saccular hair cells possess an intrinsic synaptic frequency selectivity that is saturated by strong stimuli.

The auditory and vestibular systems in the ear and the related mechanosensory and electrosensory organs of the lateral line employ a remarkable variety of mechanical and neural mechanisms to distinguish frequency components of sensory signals from <10 Hz to nearly 100 kHz (1, 2). In each of these organs, a sensory stimulus passes through one or more stages of mechanical or electrical filtering (3), leading to graded changes in the sensory receptor cell’s membrane potential, V_m. Oscillatory sensory stimuli usually produce oscillations in V_m, with the greatest amplitude occurring at a preferred frequency. Hair cells in the frog saccus possess a broadly tuned electrical filter that causes V_m to oscillate preferentially at frequencies between 35 and 75 Hz (4), as well as spontaneous oscillations of the mechanosensory apparatus at frequencies between 5 and 50 Hz (5).

At the hair cells’ output synapses, the information contained in V_m is transmitted by a chemical neurotransmitter (glutamate) to postsynaptic terminals and encoded as a train of action potentials that travel to the brain. Each of these afferent synapses contains a presynaptic dense body [also known as the synaptic body (SB) or synaptic ribbon] (Fig. 1a) similar to ribbon synapses in the retina. As at other chemical synapses, V_m controls calcium influx through voltage-gated calcium channels, which in turn controls neurotransmitter release. Ribbon-class synapses differ from conventional chemical synapses in that transmitter release is controlled by small, graded changes in V_m rather than large action potentials.

We now demonstrate that the afferent synapses in frog saccular hair cells possess an intrinsic frequency selectivity that enhances exocytosis of neurotransmitter in the middle of the saccus preferred frequency range (50 Hz). Similar to many tuning mechanisms (6), this frequency selectivity is saturated by large stimuli. Because the SB is intimately associated with synaptic vesicles (SVs) and is likely to be involved in timing their availability for release, our results raise the possibility that the SB plays a previously unknown role in frequency selectivity.

Results

We used whole-cell and perforated-patch voltage clamp recordings from frog saccular hair cells to measure the ∆C_m associated with the increase in cell surface area that accompanies fusion of SVs with the plasmalemma. To avoid using proteases, which are known to shift the voltage dependence of the Ca²⁺ current and alter other ion channels in these cells, we used a semintact epithelial preparation (4) rather than dissociated cells. Stepwise depolarizations from −80 mV caused rapid exocytosis followed by compensatory endocytosis (Fig. 1b and c). In contrast with a previous study (7) that did not include GTP or glutathione in the pipette solution, we consistently observed 50–100% compensatory endocytosis within 30 s after stimuli that evoked ∆C_m > 50 fF; noise and slow baseline drift obscured the extent of the ∆C_m recovery after smaller responses.

The Pool of Docked SVs Accounts for the Fastest Exocytic Component.

Our initial experiments were designed to test the hypothesis that there are two kinetically distinct “pools” of SVs tethered to the SB (Fig. 1a); a small pool of docked SVs in contact with the cell plasmalemma that are immediately available for exocytosis, and a larger pool of nondocked SVs that become available more slowly (8, 9). Steps to −20 mV were used to maximally stimulate exocytosis for durations between 0.5 and 500 ms. The resulting multicomponent ∆C_m waveform (Fig. 1d) is strikingly different from the linear relationship between stimulus duration and total Ca²⁺ influx (Fig. 1e). A positive ∆C_m was apparent by 2 ms, reached a plateau at 10 ms, and did not increase further for steps lasting up to 30 ms. A second rise began after 30 ms and reached 160 fF at 100 ms. This rise was followed by a third, sustained secretory component that included, which raises the count to 66 docked SVs per synapse (Fig. 1f).

We estimated the size of the first kinetic component by averaging ∆C_m over all steps lasting between 10 and 30 ms (n = 25). The result (44 ± 8 fF) corresponds to 59 SVs per synapse [calculated by assuming 20 synapses per hair cell (10) and 37 aF per SV (11)], comparable with the results of a previous electron tomographic study (12) that reported 43 docked SVs associated with the SB at inhibited synapses in these hair cells. The agreement is improved if all docked SVs within 300 nm of the center of the active zone are included, which raises the count to 66 docked SVs per synapse.

The correspondence between the second kinetic component and the rest of the SVs tethered to the SB is less clear. The total pool

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Abbreviations: RRP, readily releasable pool; SB, synaptic dense body; SV, synaptic vesicle.

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of 400 docked and nondocked SVs on the SB (12) can contribute $\sim 300 \text{ pF}$, sufficient to account for $\Delta C_m$ during steps lasting up to 500 ms (Fig. 1d), but stimuli of longer duration elicit much larger responses (see Fig. 7b) (7). The lack of a plateau in $\Delta C_m$ for long depolarizations is consistent with the morphological result that the pool of nondocked SVs on the SB is depleted by $< 50\%$ during maintained stimulation (12). The simplest explanation is that the SB is continuously resupplied with SVs from the cytoplasm. Alternatively, prolonged depolarization may evoke exocytosis of extrasynaptic, outlying docked SVs (12).

**Interrupting a Depolarization Can Increase Exocytosis.** When the frog saccus is stimulated in vivo by sounds or substrate vibrations at frequencies $\leq 50 \text{ Hz}$, many postsynaptic axons fire at most one phase-locked action potential during each stimulus cycle (13, 14).

To account for this observation, we hypothesized that the depolarizing phase of each cycle triggers a brief burst of transmitter release and that the hyperpolarizing phase reenables the synapse to release transmitter during the next depolarization. This hypothesis predicts that periodic weak stimuli in which depolarizations are interrupted by hyperpolarizations can cause more exocytosis than a maintained depolarization.

To test this hypothesis, we used four different patterns of depolarization (Fig. 2) that were weak compared with the stimuli commonly used in voltage-clamp experiments but probably larger than the receptor potentials that modulate synaptic transmission in vivo.

Fig. 1. SV pools and $\Delta C_m$ in whole-cell recordings from frog saccular hair cells. In these and all other whole-cell experiments, the intracellular Ca$^{2+}$ buffer was 1 mM EGTA. (a) The SB is surrounded by docked (green) and nondocked (purple) SVs. “Afferent” labels a postsynaptic terminal. (Scale bar, 200 nm.) (b) Representative $C_m$ traces for depolarizations to $-20 \text{ mV}$ lasting 10 ms (small response) or 200 ms (large response). We did not attempt to interpret $C_m$ measurements during the depolarization-induced changes in membrane conductance, which have been blanked during the interval from the onset of the depolarization until 30 ms after $V_m$ was returned to $-80 \text{ mV}$ (dashed lines). (c) Boxed region from b. $C_m(t)$ was averaged in 100-ms windows (red) surrounding the blanked interval, and the difference was used to compute $\Delta C_m$. (d) $\Delta C_m$ plotted as a function of step duration. Each point is the average $\Delta C_m$ for the first depolarization applied to each cell (mean $\pm$ SEM; $n$ shown in parentheses; each cell contributed one $\Delta C_m$ value at one duration only; total, $n = 66$ cells). There was no significant difference for any pairwise comparison of means at 10, 25, 30, and 50 ms (see Table 2, which is published as supporting information on the PNAS web site). Green and purple dashed lines indicate estimated numbers of docked SVs and docked plus undocked SVs, respectively, that are associated with synaptic ribbons. (e) Mean Ca$^{2+}$ influx during the stimuli in d. Data are for whole-cell, voltage-clamp recordings. Evidence for the Ca$^{2+}$ dependence of $\Delta C_m$ and the ensemble-averaged $C_m(t)$ traces for the data in d are shown in Figs. 8, 9b, and 10, which are published as supporting information on the PNAS web site.

Fig. 2. Interposed hyperpolarization can increase $\Delta C_m$. (a) Representative $I_{Ca}$ during the four stimulus patterns shown in b. Each cell received patterns A and B, plus one or more of the other patterns. (b) Stimuli A–D are steps to $-55 \text{ mV}$, except in a few cells in which the $\Delta C_m$ responses to stimuli A and B were too small to measure, in which case the depolarization was increased to $-50 \text{ mV}$. Step timing: stimulus A, 30 ms; stimulus B, 2 $\times$ 10 ms separated by 10 ms; stimulus C, 2 $\times$ 14 ms separated by 2 ms; stimulus D, 2 $\times$ 2 ms separated by 26 ms. (c) Mean $\Delta C_m$ responses ($\pm$SEM) to the four stimulus patterns. The response to stimulus B was significantly larger than all others (see text). An alternative analysis in which the $\Delta C_m$ responses in each cell were first normalized by the $\Delta C_m$ response to stimulus waveform B gave similar results. (d) Individual $\Delta C_m$ from the 19 cells receiving stimulus patterns A and B. Responses to the first stimulus presentation are shown for waveforms A (solid gray bars) and B (striped bars).
vivo (15). Continuous depolarization for 30 ms (stimulus A) evoked a ΔC_m of 14 ± 3 fF, whereas two 10-ms depolarizations separated by 10 ms of hyperpolarization (stimulus B) delivered to the same 19 cells evoked a significantly larger ΔC_m of 25 ± 4 fF (P = 0.018), although stimulus A caused more total Ca^{2+} influx than stimulus B (Fig. 2a). Stimulus B can be thought of as a 30-ms depolarization that has been interrupted in the middle by a 10-ms hyperpolarization to -80 mV. A briefer (2 ms) interruption (stimulus C) evoked a significantly smaller ΔC_m than stimulus B (12 ± 5 fF, n = 7, P = 0.03), which was not significantly different from the response to stimulus A. This result is particularly intriguing because stimuli B and C produce identical Ca^{2+} tail currents. The large difference in ΔC_m evoked by stimuli B and C argues against the possibility that the additional tail current alone was responsible for the increased ΔC_m response to stimulus B. The response to stimulus C also shows that 2 ms of hyperpolarization to -80 mV is insufficient to have the facilitating effect caused by 10 ms of interposed hyperpolarization (stimulus B). A pair of brief (2 ms) depolarizations separated by 26 ms (stimulus D) evoked no measurable ΔC_m (-1 ± 5 fF). The lack of ΔC_m response to stimulus D provides further evidence that Ca^{2+} tail currents generated by these weak depolarizations did not cause exocytosis, although the tails in stimulus D are expected to be slightly smaller than in stimuli A–C because I_{Ca} had not reached steady-state activation (Fig. 2a). When we applied stimulus patterns with the same timing as stimuli A and B but used depolarization to -20 mV, we found no significant effect of the interposed hyperpolarization on ΔC_m (data not shown).

**Figure 3.** ΔC_m responses to sinusoidal stimulation at 5 (gray), 50 (red), and 200 (blue) Hz. Each cell received either weak (±5 mV centered at -60 mV, solid bars) or strong (±10 mV centered at -55 mV, striped bars) stimulation for 1 s at each frequency with 30 s between stimuli. Presentation order was randomized among cells from the six possibilities. The strong stimuli were delivered around a more depolarized baseline than the weak stimuli to mimic the asymmetric transduction current in these cells (45). To avoid possible rundown effects, only responses to the first presentation of the three frequencies are included in this figure. (a) V_m and leak-subtracted I_m from one representative whole-cell recording. (b) Total Ca^{2+} influx did not differ across frequencies (same cells as d). (c) Ensemble-averaged R_0 (Upper) and C_m (Lower) for all weak stimuli (same cells as d). (d) For weak stimuli (whole-cell, n = 11 cells), ΔC_m at 50 Hz was significantly greater (55 ± 12 fF) than at 5 Hz (20 ± 10 fF, P = 0.0001) or 200 Hz (18 ± 12 fF, P = 0.008). No significant differences between frequencies were observed for strong stimuli (whole-cell, n = 5 cells). (e and f) Similar results were obtained from perforated-patch recordings (n = 11 cells; only the weak stimuli were used). ΔC_m at 50-Hz stimuli (76 ± 17 fF) was significantly greater than ΔC_m at 5 Hz (26 ± 14 fF, P = 0.014) or 200 Hz (20 ± 13 fF, P < 0.006).

**Frequency Dependence of Synaptic Exocytosis.** We measured the ΔC_m caused by small, sinusoidal voltage changes (±5 mV centered at -60 mV) delivered for 1 s at 5, 50, and 200 Hz. We hypothesized that ΔC_m would be largest at 50 Hz, the frequency where the sacculus is most sensitive to sound and seismic stimuli (13, 14). The sinusoidal stimuli evoked periodic Ca^{2+} currents that varied among cells (50–200 pA peak-to-peak and 0 to -100 pA offset), but the peak I_{Ca} amplitude (Fig. 3a) and the integrated Ca^{2+} influx (Fig. 3b) in each cell were nearly the same across the three frequencies. Nevertheless, ΔC_m was significantly greater at 50 Hz than at 5 or 200 Hz (Fig. 3d). ΔC_m was not accompanied by any apparent change in series resistance (Fig. 3c). The preference for 50 Hz was absent when stronger stimuli (±10 mV centered at -55 mV) were applied to other cells (Fig. 3d). A similar preference for 50 Hz was seen in perforated-patch experiments (Figs. 3 e and f), which maintain the cell's endogenous Ca^{2+} buffering conditions.

We observed a large coefficient of variation (cv) between cells in the amplitude of the Ca^{2+} currents evoked by the small sinusoidal stimuli in both whole-cell (cv = 0.9; mean peak I_{Ca} = 99 pA) and perforated-patch (cv = 1.0; mean peak I_{Ca} = 65 pA) experiments (Fig. 4). Much of this variability can be explained by small errors in V_m which have large effects on I_{Ca} near -60 mV, where I_{Ca} is steeply voltage-dependent. We used this variability to test the hypothesis that frequency selectivity was most pronounced when I_{Ca} was small. Fig. 4b shows the predicted negative correlation (r = -0.4), although not all cells with small Ca^{2+} currents had a strong preference for 50 Hz.
Data from the 13 cells in which we were able to present two or more repetitions of the weak stimulus at each frequency revealed a range of responses. The cell shown in Fig. 5a had no significant preference for 50 Hz. The means of within-cell means (Fig. 5b) showed a clear preference for 50 Hz, and 12 of the 13 cells had the preference for 50 Hz. The means of within-cell means (Fig. 5c) represent this measure with the mean integrated Ca2+ influx across frequencies for perforated-patch (gray inverted triangle) and whole-cell (black inverted triangle) recordings. The regression line and correlation coefficient are shown.

**Steep Voltage Sensitivity of Im and ΔCm Suggests a Narrow Operational Range.** Large or prolonged depolarizations (Fig. 6) evoked much larger ΔCm responses that were qualitatively different from responses to weaker stimuli. Sinusoidal stimuli (10 s duration) centered at −70 and −65 mV evoked small Ca2+ currents and capacitance increases that either remained constant or returned toward baseline after the stimulus, whereas stimuli centered at −60 mV and −55 mV evoked large Ca2+ currents and capacitance increases that continued to rise for several seconds after Vm was returned to −80 mV.

**Discussion**

Ribbon synapses, which have been described only in the retina, pineal body, and acousticolateralis sensory organs of vertebrate animals are defined by the presence of a prominent presynaptic structure (the SB or ribbon) at each active zone. Numerous physiological investigations have sought to understand the function of the SB (7–9, 11, 12, 16–22). The predominant hypothesis is that the SB facilitates high rates of sustained transmission by capturing and/or transporting SVs to release sites, although other functions have been proposed (23), including frequency selectivity (24).

**Functional and Anatomical Vesicle Pools.** Only a small fraction (1–2%) of SVs at most chemical synapses are immediately available to undergo exocytosis (i.e., within a few milliseconds after the onset of a depolarization that maximally activates the presynaptic Ca2+ current). This SV population is often called the “readily releasable pool” (RRP) and has been proposed to correspond anatomically to the “docked” SVs, defined as those in contact with the plasma-lemma at the synapse (25). To estimate the size of the RRP in frog saccular hair cells, we held Vm at −80 mV to inhibit synaptic transmission and then stepped to −20 mV to maximally stimulate exocytosis. We found a clear temporal separation between the fastest exocytic component, which is complete in 10 ms (Fig. 1d), and the slower component(s) that begin ∼20 ms later, similar to the 25-ms recovery time for postsynaptic potentials in the goldfish saccular afferents (26). These results agree with more extensive studies of SV pools at ribbon synapses in mouse inner hair cells (8), chick cochlear hair cells (27), and retinal bipolar cells (16, 28), in which paired-pulse paradigms have shown that the most rapidly available SV pool can be depleted faster than it can be refilled. The fastest component, which we define as the RRP at these synapses, corresponds to 59 SVs per active zone, similar to the 43 docked SB-associated SVs (66 docked SVs within 300 nm of the active zone center) that were counted in cells in which transmission had been inhibited before and during fixation (12).

In contrast, the RRP estimate from ΔCm measurements in mouse inner hair cells is ≈53–64 SVs per synapse, whereas the morphologically docked SV pool contains only 16–30 SVs (29). Some of this difference may be due to the fact that synaptic exocytosis had not been inhibited before fixation, which in frog saccular hair cells reduced the number of docked SVs per synapse from 43 to 32 (11). Another difference arises from the 25% smaller capacitance per SV (28 aF) used in their calculations compared with ours (37 aF). Both...
we report in whole-cell recordings from the semiintact sacculus 2902 ically dissociated saccular hair cells from 2902 corresponding to the docked SV pool would be difficult to see in 2902 responses to depolarizations 2902 calculations assumed the same rough estimate of specific mem- 2902 brane capacitance (1 μF/cm²), but different estimates of SV 2902 diameter (30.0 nm in mouse inner hair cells vs. 34.3 nm in frog 2902 sacculus, both of which were measured in electron micrographs of 2902 glutaraldehyde-fixed tissue at the middle of the apparent mem- 2902 brane thickness) (11, 29). Such measurements are subject to 2902 significant errors due to shrinkage and ovoid SV shapes. For 2902 example, electron tomographic reconstruction of SVs in three 2902 dimensions (11) gave a mean volume of 12,500 nm³, which corre- 2902 sponds to a spherical diameter of 28.8 nm (estimated capacitance, 2902 26 aF), significantly smaller than the value calculated from the same 2902 data as the diameter of the circle having the same area as the largest 2902 cross section through the SV. We favor the larger estimate to 2902 compensate for overall shrinkage, but clearly these are all rough 2902 estimates of SV capacitance. Multivesicular fusion of nondocked 2902 ribbon-associated SVs (22, 30) could also contribute to discrepancies 2902 between the sizes of RRP and docked SV pools in Δm 2902 measurements at ribbon synapses. Given the many sources of error, 2902 comparisons of pool sizes should be interpreted cautiously (29).

Experiments in chick cochlear hair cells (31) did not investigate responses to depolarizations <50 ms, but we expect that the Δm 2902 corresponding to the docked SV pool would be difficult to see in 2902 these cells because this pool is very small (18).

Other recent perforated-patch experiments (32) using enzymat- 2902 ically dissociated saccular hair cells from Rana pipiens reported 2902 nearly 5-fold larger Δm responses to 10-ms depolarizing steps than we 2902 report in whole-cell recordings from the semintact sacculus 2902 (Fig. 1d) and a mean Ic 2902 that was twice as large as what we found 2902 in either perforated-patch or whole-cell recordings. We do not have 2902 a compelling explanation for these differences, but they could be 2902 due to the different preparations used (isolated cells vs. semintact 2902 sacculus), selection of different subpopulations of hair cells, or 2902 other methodological differences.

As in previous studies, we interpret Δm measurements as purely 2902 exocytotic because the time constant for endocytosis stimu- 2902 lated by depolarization (7.5–14 s) (7, 8) appears to be too slow for 2902 endocytosis to significantly contribute to Δm measurements over times ≤1 s, although faster membrane retrieval (τ = 300 2902 ms) was reported in response to global elevation of intracellular 2902 Ca²⁺ (33). Nevertheless, we cannot rule out the alternative 2902 explanation that some stimulus frequencies favor a mode of SV 2902 cycling, such as kiss-and-run (34), in which fast endocytosis 2902 predominates. Experiments using fluorescent membrane tracers are needed to test this possibility.

**Frequency Selectivity.** The frequency selectivity of exocytosis that we observed is expected to contribute to the overall tuning of the 2902 sacculus (35), which matches the power spectrum of seismic signals 2902 associated with frog mating calls (peak power between 20 and 70 2902 Hz) (36) and other sources. Recordings from saccular afferent 2902 fibers in bullfrogs, white-lipped frogs, and the northern leopard frog (R. pipiens) have shown that weak seismic and auditory stimuli 2902 evoke maximum spike rates for frequencies near 50 Hz (13, 14, 37, 2902 38), and that the low-frequency roll-off is due to the phase- 2902 locked firing of a single spike in the postsynaptic cell per stimulus 2902 cycle (13, 14). The saccular stimuli used here (±5 mV centered 2902 at −60 mV) were similar compared with the stimuli used in most 2902 voltage-clamp studies but were larger than the Δm oscillations 2902 expected to occur at sensory threshold (15). In both whole-cell and 2902 perforated-patch experiments, the exocytic rate during 1 s of 50-Hz 2902 stimulation was two to three times the rate for 5- or 200-Hz 2902 stimulation, but, when expressed as vesicles per stimulus cycle, the 2902 rate was greatest at 5 Hz (5.1 SV per cycle, compared with 1.9 SV 2902 per cycle at 50 Hz and 0.14 SV per cycle at 200 Hz) (Fig. 5b, 2902 perforated patch). Therefore, the presynaptic frequency selectivity that we observed may not by itself be sufficient to account for the 2902 low-frequency roll-off in firing rate (Hz) of the postsynaptic cell. 2902 It is difficult to invoke mechanisms involving depletion of the 2902 RRP to explain our results using interrupted steps (Fig. 2) and 2902 sinusoids (Figs. 3–5), in which the Δm responses are smaller than 2902 the RRP and the effects are largest for weak stimuli. For example, 2902 the response to 30 ms of weak depolarization (Fig. 2, pattern A) 2902 caused only a 32% depletion of the RRP; interrupting this depo- 2902 larization by 10 ms of hyperpolarization (pattern B) nearly doubled 2902 the response but still caused only 57% depletion of the RRP. 2902 Similarly, the Δm responses to 1 s of sinusoidal stimulation at 5 and 200 2902 Hz caused only ~45% depletion of the RRP (Fig. 5b, perfor- 2902 rated patch). Therefore, if SV depletion is involved, the deplet- 2902 ed pool must be smaller than the RRP measured using strong depo- 2902 larizations (Fig. 1). Such a model was proposed by Furukawa et al. 2902 (26, 39) to explain the “adaptive rundown” in excitatory postsyn- 2902 aptic potential amplitude that they observed in recordings from 2902 goldfish saccular afferents. The authors proposed that weak stimuli 2902 cause exocytosis only at the center of the active zone, causing 2902 depletion of this subset of docked SVs, which must be replenished 2902 by migration of SVs from the surrounding region. If this migration 2902 were inhibited while Ca²⁺ channels are open, depletion of this small 2902 pool might explain the small Δm responses that we observed 2902 during 5-Hz stimulation and during 30 ms of maintained weak 2902 depolarization. A separate mechanism would be needed to explain 2902 the small Δm response to 200-Hz stimulation. It is also important 2902 to note that many other possible mechanisms involving a delayed 2902 inhibitory effect of Ca²⁺ (or even Vm by itself), could account for 2902 our results. It has been proposed that at some central synapses, an 2902 unexplained mechanism prohibits exocytosis of more than one SV 2902 per action potential (40). There is no straightforward way to apply 2902 this type of rule to explain the Δm responses to small sinusoids 2902 that we observed, which correspond to much more than 1 SV per cycle 2902 at 5 Hz and much less than 1 SV per cycle at 200 Hz.

**Materials and Methods**

Frogs (R. pipiens) were maintained at 17°C on a 12 h:12 h light/dark cycle. All procedures were in compliance with the University of Oregon Institutional Animal Care and Use Com- 2902 mittee. The semiintact saccular preparation (4) was visualized at 2902 ×40 magnification with differential interference contrast micro- 2902 roscopy optics under an upright microscope (Zeiss). Dissection 2902 and recording were performed in an extracellular solution (112 2902 mM Na⁺/119 mM Cl⁻/5 mM Hepes/3 mM d-glucose/2 mM 2902 K⁺/1.8 mM Ca²⁺) with 0.01 mM d-tubocurarine to block Ca²⁺ 2902 entry through mechanoelectrical transduction channels (41).
Electrophysiology. All experiments were performed at 20°C within 3 h of dissection. We only used cells that showed no evidence of swelling or internal Brownian motion. Recording pipettes (2–6 MΩ; tip diameter, 1–2 μm) were pulled from borosilicate glass and shaped by heating under pressure (42). The pipette solution contained 94 mM glutamate, 93 mM Cs⁺, 10 mM Hepes, 10 mM tetraethylammonium, 2 mM Mg²⁺, 1 mM EGTA, 0.274 mM Ca²⁺ ([Ca²⁺]p = 50 nM), 14 mM Cl⁻, 8 mM Na⁺, 5 mM glutathione, 3 mM ATP, 1 mM GTP. Dry glutathione, ATP, and GTP were stored 94 mM glutamate, 93 mM Cs⁺, 10 mM Hepes, 10 mM tetraethylammonium, 2 mM Mg²⁺, 1 mM EGTA, 0.274 mM Ca²⁺ ([Ca²⁺]p = 50 nM), 14 mM Cl⁻, 8 mM Na⁺, 5 mM glutathione, 3 mM ATP, 1 mM GTP. Dry glutathione, ATP, and GTP were added immediately before the recording session. For perforated-patch experiments, glutamate was substituted for glutamate and the back ends of filament-glass electrodes were dipped into pipette solution for 5–10 s to fill the tip; the electrodes were then filled with pipette solution supplemented with 500 μg/ml solubilized amphoteracin. All reagents were purchased from Sigma.

Pipette current was zeroed in the bath at −13 mV to account for the liquid junction potential (4). Perfusion of the extracellular tericin. All reagents were purchased from Sigma.

Membrane Impedance Measurements. We used an Optopatch amplifier (Cairn, Kent, U.K.), in whole-cell voltage-clamp mode with passive RC compensation and feedback RC tracking, using the amplifier’s internally generated sinusoidal probe stimulus (44). The sinusoidal probe input (±23 mV at 1.5 kHz, centered at −80 mV) was on continuously except during depolarizations. The probe did not activate Ca²⁺ channels. For example, the current in Fig. 3b (gray trace, 5 Hz) was the same for t < 0 (probe on) and at t = 0.15 s (probe off; I_m = −65 mV). For additional details, see Supporting Materials and Methods, which is published as supporting information on the PNAS web site.

Statistics. Population measures of central tendency and variance are expressed as mean ± SEM. We used independent and paired Student’s t tests for comparisons of means between groups when applicable. P < 0.05 was considered significant. Data groups were confirmed normal with Shapiro–Wilk and Kolmogorov–Smirnov tests. One-tailed t tests were used for tests of a priori hypotheses in Figs. 2, 3, and 5 (see Supporting Materials and Methods). Alpha was adjusted to the familywise error rate for multiple comparisons.

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